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SUPLEMENTO COMEMORATIVO

SIMPÓSIO INTERNACIONAL SÔBRE VENENOS ANIMAIS

INTERNATIONAL SYMPOSIUM ON ANIMAL VENOMS

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INTERNATIONAL SYMPOSIUM ON ANIMAL VENOMS

INSTITUTO BUTANTAN

17 a 23 de julho de 1966

Sob os auspícios do GOVÊRNO DO ESTADO DE SÃO PAULO, da FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DE SÃO PAULO e do FUNDO DE PESQUISAS DO INSTITUTO BUTANTAN

SUPLEMENTO COMEMORATIVO

CENTENÁRIO DE NASCIMENTO DE VITAL BRAZIL

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Busto de VITAL BRAZIL



Vista aérea do Instituto Butantan



INSTITUTO BUTANTAN — Exposição de Animais Venenosos



INSTITUTO BUTANTAN — Inauguração do S.I.V.A.

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I.

BIOGRAPHICAL DATA OF VITAL BRAZIL

Vital Brazil Mineiro da Campanha was born on April 28, 1865 in Campanha, State of Minas Gerais. He studied at the Faculty of Medicine in Rio de Janeiro from 1886-1891, and received his doctor's degree based on a thesis on "The function of the spleen".

As sanitary inspector of the Public Health Service in the State of São Paulo, he organized campaigns against typhoid fever, plague, smallpox, diphteria, cholera-morbus and mainly tetanus, from 1893-1895.

From 1895-1897 he practiced medicine in Botucatu and performed his first experiments with the venoms of poisonous snakes, principally rattlesnakes and jararacas.

From 1897-1900 he became a member of the Instituto Bacteriológico in São Paulo, directed by Adolpho Lutz, where besides his bacteriological studies, he succeeded in immunizing dogs and goats against rattlesnake and jararaca venom, and experimentally prepared an anti-venom sera. On November 8, 1899 he was made head of the State laboratories at the Butantan Farm, in order to prepare sera against the plague. Finally the Instituto Serumtherapico was officially inaugurated under the direction of Vital Brazil, in the farm, on February 23, 1901.

Following his retirement in 1919, he established the Instituto Vital Brazil in Niterói, returning in 1924 to the Instituto where he commissioned as Director until 1927.

On May 8, 1950 he died in Rio de Janeiro, 85 years old.

Vital Brazil was an excellent organizer, an exceptional and well-known researcher. He surrounded himself with a well-chosen staff and planned guiding principles for the Instituto. Together with the Federal Government he worked out the free transportation system of poisonous snakes. In 1914 he obtained, from the State Government the permission to erect new buildings, which are used up to present.

Some hundred original papers, monographs and lectures on his research work were published.

As the first one he proved the specificity of the anti-venom sera; observed the parcial immunization against jararaca venom by immunization through the yellow fever virus; he made intense studies on immunology, improved the methods for venom and serum dosage; discovered that serum, even expired, may and should be used, however in higher than prescribed doses.

II.

SESSÃO INAUGURAL — INAUGURAL MEETING

DISCURSO INTRODUTÓRIO

MÁRIO MACHADO DE LEMOS

Secretário de Estado da Saúde Pública e da Assistência Social
Representante do Govêrno do Estado de São Paulo

Nesta solenidade, em que se inaugura o "Simpósio Internacional Sôbre Venenos Animais", é oportuno expressar, com a maior ênfase, o integral apoio e a grata satisfação do Govêrno do Estado de São Paulo, por dois aspectos fundamentais: 1) pelas suas perspectivas científicas de alto interêsse para todos os povos; 2) pela honrosa deferência que hoje, aqui, se concretiza na escolha dêste local para a sua realização, isto é, no Estado de São Paulo e exatamente neste Órgão da Secretaria da Saúde, com a presença de cêrca de 200 cientistas de quase todos os continentes, alguns detentores de Prêmio Nobel.

E foi nesta mesma Casa que Vital Brazil desenvolveu, neste campo específico, as suas atividades de pesquisa, autêntico galardão de benemerência para nosso país. E hoje, quando se comemora o seu centenário, por uma feliz coincidência, o Instituto Butantan, convertido em Fundação, inicia uma nova etapa de profundas transformações estruturais e administrativas, a fim de que possa desenvolver-se adequadamente, na plenitude de seus objetivos. E terá, para isto, todo o apoio do Chefe do Executivo, ora empenhado na reformulação, em têrmos mais racionais e decisivos, de todos os problemas fundamentais de saúde pública do Estado.

Êste Simpósio, pelo alcance e magnitude de seus propósitos, inclui-se entre as recomendações do Plano Decenal de Saúde Pública da Aliança para o Progresso da Carta de Punta del Leste, referendada pelo Grupo de Estudos dos Ministros da Saúde que se reuniu em Washington, sob os auspícios da Organização Sanitária Pan-Americana.

Em meu nome, na qualidade de Secretário de Estado da Saúde Pública e da Assistência Social e, sobretudo, em nome do Governador Laudo Natel, a quem tenho a honra de representar, formulo, com o maior aprêço, o nosso agradecimento à Comissão Organizadora do conclave e as nossas boas-vindas a todos os congressistas nacionais e estrangeiros, neste espetáculo soberbo de humanitarismo e de confraternização científica de todos os países em proveito de todos os povos.

Mem. Inst. Butantan
Simp. Internac.
33(1):XIII-XVI, 1966

TRANSCENDENCE OF VITAL BRAZIL'S WORK

B. A. HOUSSAY

Instituto de Biologia y Medicina Experimental, Buenos Aires, Argentina

This First International Symposium on Venomous Animals justly takes place in São Paulo, as it is organized in honour of the outstanding work and illustrious personality of Vital Brazil, founder of the Instituto Butantan, which has been and still is an example in its field in the world. It was the first in South America to introduce the specific treatment and prophylaxis of accidents in man and domestic animals by snake, scorpion and spider venom. His life should be an example for the future generations and his work rendered it a glory to Brazil and South America.

The great human achievements are obtained by scientific studies and their application. There is no pure and applied science, but only science and application of sciences. The basic investigation is the fountain of all progress of the applied science and of the technology which unrelentlessly transforms the world. In the past century a movement of nationalist affirmation and of faith in its destiny was observed in Brazil. Numerous Brazilians changed their family names to names of regions and rivers, such as Americano, Brazil, Amazonas and Tocantins, etc., or else to those of ancient tribal chiefs, such as Tibiriçá, Juracy, Tamandaré, etc. Therefore, Mr. Santos Pereira gave his firstborn the name of Vital Brazil Mineiro da Campanha. Vital, for his strengh of life, Brazil, for his country, Mineiro because he came of the State of Minas Gerais, Campanha because this was the city where he was born. His life honoured these names, due to his own enduring and intelligent endeavour.

Born on April 28, 1865 and deceased on May 8, 1950, with 85 years of age. This way, in such a large life span, he could look back on the success of the work he began.

He studied humanities in São Paulo (1880 to 1885), then enrolled in medicine in Rio de Janeiro, where he studied from 1886 to 1891, working to pay for his studies; by contest he won the job of technician of physiology and presented a final theses on "Physiology of the spleen".

Back in São Paulo, he hold the job of physician of the police force, sanitary inspector (1892 to 1895), worked in epidemics of cholera, yellow fever, cow-pox, typhoid fever and diphtheria. He also worked successfully as a general practitioner during the years (1895-1896) in Botucatu and was well aware of the medical and sanitary problems of his country.

Those were times of great medical progress and useful application of bacteriology and serumtherapy. This promising field awoke the enthusiasm of the young serious and hard working physicians, wherefore Vital Brazil, in 1897, enrolled as a technician in the Instituto Bacteriológico de São Paulo, under the

direction of Adolfo Lutz, one of the most eminent scientists Brazil ever had. There Vital Brazil made his first attempts at the immunization against snake venoms. In 1889 an epidemic of bubonic plague erupted in São Paulo. Vital Brazil was sent to study it and prepare an anti-plague serum in an improvised laboratory in Butantan. He fell gravely ill with plague, but fortunately recovered to take charge of his job.

With him in this epidemic worked also Oswaldo Cruz, and it is interesting to point out that from there on Vital Brazil founded Butantan and Oswaldo Cruz founded Manguinhos.

In 1901, on advice of Adolfo Lutz, the Institute of Serumtherapy of the State of São Paulo was created in Butantan, under the direction of Vital Brazil, who directed it from 1901 to 1909, and later from 1924 to 1927.

Called by the government of the State of Rio de Janeiro, he founded in 1919, in Niterói, the Institute of Hygiene, Serumtherapy and Veterinary, which bears his name and where his sons worked with him.

Under his direction were undertaken the prophylaxis and treatment of plague, typhoid fever, cow-pox, tetanus, diphtheria and zoonoses or diseases of domestic animals.

However, his most important work was the fight against ophidism and his studies on the snake, scorpion and spider venoms, the preparation of specific sera against the snake (1901), scorpion (1918) and spider (1925) venoms. He also studied the cutaneous batrachian venoms (1925).

The most original and successful work of Vital Brazil was the fight against ophidism and animal venoms, where he set a model treatment for all America and one of the best of the world.

Vital Brazil proved the specificity of the antivenomous sera, as he showed that the antitoxic sera prepared against the Asiatic poisons were inefficient against the poisons of the South American snakes. He obtained specific sera against the latter and showed that there are three types of clinical poisoning by the venoms of *Bothrops*, *Crotalus* and *Elaps*, the symptoms of which can be treated with specific sera. There are some common ones to the poisons of the *Bothrops* and the antipoisonous serum against one species has paraspecific effect up to a certain point on the poisons of other *Bothrops*. The serum against the venom of our South American *Crotalus* has a certain paraspecific effect against the venom of the North American *Crotalus*.

These knowledges made Butantan prepare several sera: monovalent antibothropic, polyvalent antibothropic, antiophidic for Central America, anticrotalic, antielapidic. The more commonly used sera are two: anticrotalic and antiophidic prepared against the venoms of *Bothrops* and *Crotalus durissus terrificus*.

To obtain the venom necessary to prepare these sera a great number of serpents were needed. The Institute established an exchange system, delivering one ampule of serum for each snake specimen sent by the farmers. These were given also a snare to capture the snakes and a wooden box to ship them. The railrooads carry them for free. This method was also tried in Argentine, however, at present an additional amount is paid in money for each snake received.

The serumtherapy is responsible for a decrease in mortality. Of 20,000 men bitten each year, the mortality decreased from 25-30% to a mere 2%, and these due to late or insufficient treatment.

The snake pit at the Instituto Butantan, at the outside, constitutes a highly visited attraction for the tourists, that arrive by land, sea or air in São Paulo or Santos. Distinguished visitors of all classes of activities have expressed their interest and admiration for this spectacle.

The work of Vital Brazil was continued by his collaborators and followers. The zoological classification of the serpents initiated by J. F. Gomes was accomplished, principally by Afranio do Amaral. The chemical identification of the active components by Slotta, Fraenkel Conrat, Moura Gonçalves, Diniz and Klobusitzky; the physiological action was studied by Vellard, Oswaldo Vital Brazil, Rosenberg and others.

Vital Brazil and his collaborators have studied several actions of the venoms, coagulant, anticoagulant, hemolytic, agglutinant, cytologic, proteolytic, etc. The liberation of bradikinin was shown by Rocha e Silva, Beraldo and Rosenberg.

The poisons contain numerous enzymes which have been isolated and studied with interest in all parts, since they explain many of the symptoms and constitute interesting biochemical reactives. Vital Brazil diffused the prophylactic measures and studied the ophiophage serpents, such as the "muçurana", the ophiophage mammals, such as the skunk-like *Conepatus chilensis* and others, the ophiophage birds and certain spiders.

The scorpion venom was studied since 1907 by Maurano, Magalhães and Diniz. The antiscorpionic serum is being prepared in Butantan since 1916.

The action of the spider venoms were studied by Vital Brazil, Vellard and Bücherl. Since 1925 are being prepared in Butantan antilicosic and antictenidic sera against the venom of *Ctenus*, today *Phoneutria*. The action of the poison of *Latrodectus* has been studied in Argentine by Troise, Sampayo and, under the influence of Abalos, the antilatrodectic serum was prepared by Pirosky. The venom of *Loxosceles* and its antiserum has been investigated mainly by Machiavello, Gajardo-Tobar and the staff of Butantan. The action of the cutaneous frog and other batrachian venoms has been studied by Vital Brazil, Jayme Pereira and Vellard.

Vital Brazil was a modest, persistant and endeavouring with great faith in the immunological methods, large capacity of simplifying and finding practical, endeavouring and simple solutions.

Vital Brazil undertook two travels to Europe (1904 and 1914) in order to get acquainted with progresses in his speciality. In 1915 he was invited by the Carnegie Foundation for the improvement of peace, to visit the United States. There he was summoned to treat an employee of the Bronx Zoo, who had been bitten by a *Crotalus atrox* and presented grave symptoms; Vital Brazil injected anticrotalic serum of Butantan, with which a swift recovery was secured, thus showing the paraspecific action of this serum.

He published many papers and gave numerous lectures to diffuse knowledge on serumtherapy against snake poisons and to organize collaboration by the people for the prophylaxis and to secure shipment of snakes and distribution of sera. He also published scientific papers; his book "La défense contre l'ophidisme", published in French in 1914, attained international repercussion with three editions.

Vital Brazil was distinguished with several honours. The President of his country has his name inscribed in the Book of Merits of Brazil. The Argentine Society of Biology appointed him, on November 9, 1922, a correspondent member and later an honorary member.

In 1943, I had the privilege to join in a tribute justly paid to Vital Brazil when a new building was inaugurated in the Instituto Vital Brazil, in Niterói, and I today rejoice to repeat textually:

"Vital Brazil is a glory to South America and his name should be remembered as that of Oswaldo Cruz, among those who initiated the true immunological science in Latin America".

"My studies have allowed me to measure the great value of the extensive work of Vital Brazil on venoms. His demonstration of antitoxic specificity of the antipoisonous sera obliges to consider him, with justice, as a founder of the South American antiophidic serumtherapy, while high authorities wrongly assured the efficiency of antitoxic sera prepared against venoms in India".

"It gives me a great satisfaction and a true honour to express in public all appreciation and respect his work and example inspire me, and cordially join the intended tribute.

Great and eminent persons are found today in the science of Brazil, however, their way was largely paved by the initial work of Oswaldo Cruz and Vital Brazil".

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33(1):XVII-XX, 1966

CONFERÊNCIA INAUGURAL

A. VALLEJO-FREIRE

Diretor do Instituto Butantan

Presidente do Simpósio Internacional Sôbre Venenos Animais

Autoridades,

Presidente de Honra,

Meus senhores, minhas senhoras:

O Instituto Butantan sente-se orgulhoso e agradecido de poder encerrar as comemorações do centenário do nascimento de seu fundador, Vital Brazil, com a especial homenagem que representa a presença de cientistas, vindos dos mais longínquos recantos para participar do Simpósio Internacional Sôbre Venenos Animais, que se inicia com esta cerimônia.

Coube ao grupo de pesquisadores desta instituição a sugestão de reunir nesta oportunidade especialistas em animais peçonhentos e peçonhas animais.

Ao constituir-se a comissão organizadora, que temos o privilégio de presidir e ao estabelecer os primeiros contactos com cientistas do exterior, foi do Prof. Bernado Houssay, nosso presidente de honra, que recebemos o mais efusivo apoio, insistindo mesmo em participar das homenagens a Vital Brazil, não só pelo respeito à obra por êle realizada, mas também pela amizade pessoal que a êle dedicava.

Senhores simposistas, o programa estabelecido é o resultado da consulta feita a cada um de vós. Nêle certamente encontrareis incluídas muitas das vossas sugestões.

A aceitação e o interêsse manifestados ultrapassaram as nossas previsões de tal modo, que o número de participantes interessados permitiria a organização de uma reunião mais ampla, com caráter de congresso, o que coloca em destaque a importância e a atualidade do assunto a ser tratado. É provável que, por êste motivo, o tempo colocado à vossa disposição para exposição e debate seja exíguo, levando-se em conta a importância dos trabalhos inscritos no temário, mas estamos certos de que o convívio amistoso entre os especialistas durante tôda a semana do Simpósio será compensador e proveitoso.

Não é nossa intenção, nestas palavras de saudação, em que vos damos as boas-vindas em nome do Instituto Butantan, discorrer demoradamente sôbre a vida de Vital Brazil, que, em seus aspectos marcantes, foi focalizada pelo Prof. Houssay. Parece-nos, no entanto, apropriado traçar um nítido perfil do passado vivido por Vital Brazil que, a nosso ver, poderá nesta oportunidade servir para

u'a melhor análise da importância presente dos estudos sôbre os animais peçonhentos e de suas peçonhas e antever de forma mais adequada as perspectivas do futuro.

Vários fatôres, dos quais dois parecem ter sido do maior significado, contribuíram para alterar profundamente o equilíbrio demográfico no Estado de São Paulo no fim do século passado e nos primeiros anos do século XX: o término da escravatura, em 1888, desorganizando o trabalho do campo e o grande e rápido desenvolvimento da lavoura do café nas férteis terras do planalto paulista. Em pràticamente dez anos, êste Estado, que não contava com uma população muito superior a um milhão de habitantes, recebeu um milhão adicional de pessoas, sendo que não menos de 800.000 europeus. Em tão reduzido espaço de tempo deve ter sido esta uma das maiores migrações para as proximidades do trópico. Esta avalanche humana invadiu principalmente as zonas rurais e provocou o início de grande desenvolvimento das zonas urbanas. São Paulo, a cidade que hoje vos hospeda, contava então com 60.000 habitantes e, doze anos depois, atingia 240.000, isto é, 400% de aumento de população.

Não foi sem conseqüências para a saúde pública esta explosão demográfica. Pagou-se alto tributo em vidas humanas; às moléstias transmissíveis propagadas pela contínua chegada de navios abarrotados de emigrantes imediatamente encaminhados para as zonas rurais, juntou-se o recrudescimento de infecções e infestações de várias naturezas, endêmicas nesta região, que tomavam caráter epidêmico com a chegada de grande número de indivíduos suscetíveis, falhos de imunidade adquirida. A peste bubônica, a cólera, a varíola, a difteria, a escarlatina, a febre amarela, as febres tíficas, com pouca diferença de tempo incidiram intensa e gravemente sôbre a grande massa de população flutuante.

As medidas de ordem sanitária e profilática, devidas à aplicação de recursos práticos introduzidos na era pasteuriana, aliadas à imunidade progressivamente adquirida pela população, foram suficientes para restabelecer o equilíbrio sanitário.

É bem conhecida a ativa e destacada participação de Vital Brazil nestas campanhas de saúde pública e que o levaram à criação do Instituto Butantan.

Permanecia, entretanto, para os pioneiros, constante e incontrolável por quaisquer meios conhecidos ou recursos médicos, o risco de morte por acidentes devidos ao envenenamento por picada de animais venenosos, principalmente serpentes. A mortalidade por acidentes ofídicos atingia 3 por 1.000 da mortalidade geral no Estado de São Paulo.

As campanhas antiofídicas com o auxílio da soroterapia específica trouxeram, sem dúvida, a solução parcial do problema do ofidismo, mais tarde igualmente aplicada ao escorpionismo e ao araneísmo.

A utilização das terras para cultura e o progressivo extermínio dos ofídios, provocados pelo homem, a destruição das matas, a moderna mecanização da lavoura, enfim, a alteração da geografia provocada pelo homem, deveria teòricamente trazer como conseqüência a eliminação do problema nas áreas rurais mais intensamente cultivadas; porém, a experiência veio mostrar que é o contrário que se verifica: a ruptura do equilíbrio biológico em certas regiões — como é o caso de São Paulo — proporciona uma seletiva multiplicação, devida às suas características biológicas e conduz a uma ofio-fauna predominantemente constituída de espécies peçonhentas, principalmente daquelas que se nutrem de preferência de roedores e que se reproduzem mais intensamente nas vizinhanças de campos cultivados.

Não tem diminuído o envio de animais peçonhentos ao Instituto, principalmente ofídios. Apenas, como curiosidade, vos informamos que, até a presente data, um milhão de exemplares foram recebidos pelo Instituto Butantan.

Acreditamos, senhores simposistas, aproximar-se para a humanidade um momento de histórica importância, em que a experiência pioneira, a solução encontrada por Vital Brazil no planalto paulista, no limite do trópico, servirá como modêlo de organização de proteção às populações, que agora invadem com recursos e técnicas modernos, mas de forma maciça, o mundo tropical, para utilizá-lo em benefício da humanidade e afastar as preocupações da superpopulação de outras áreas.

Momentos semelhantes aos verificados no comêço do século, nesta região, estão se repetindo em vários países e regiões da terra, com os mesmos riscos e idênticos problemas. Sentimos no Instituto Butantan, os apelos que se renovam, vindos de novas regiões conquistadas pelo progresso, no caminho para o oeste, rumo ao centro e ao norte do país, assim como de outros países, do nosso e de outros continentes. Nos dois hemisférios, a moderna civilização, na contínua aventura do homem, segue a natural tendência para caminhar em direção ao Equador. Não há dúvida que, inexoràvelmente, a grande fusão da humanidade irá processar-se, em futuro próximo, nas zonas semitropicais e tropicais, até agora não densamente povoadas, mas em intenso desenvolvimento, inicialmente no continente americano e posteriormente no africano. Ora, nestas regiões, à medida que, do norte ou do sul das zonas subtropicais, se caminha para o trópico, rumo ao Equador, aumentam progressivamente o número e a variedade de espécies de animais peçonhentos, principalmente da ofio-fauna.

O estudo, pois, dos animais peçonhentos e dos venenos animais, passa a ser não mais de interêsse local ou regional, mas sim internacional. As soluções não serão ràpidamente encontradas, a não ser que se realize um esfôrço conjugado e que os métodos da moderna ciência, com a sua estreita aliança com a tecnologia, nos proporcionem novos conhecimentos básicos sôbre muitos aspectos, que são o motivo principal dêste Simpósio. Ressalta a urgência de, em extensas áreas, independente dos artificiais contornos políticos das nações, bem estudar tôdas as espécies de interêsse médico, para difundir a sua adequada identificação e conhecer, de forma global, a distribuição geográfica das espécies de maior importância. Muito há a ser esclarecido sôbre a biologia e ecologia de animais venenosos e até, paradoxalmente, sôbre a maneira de obter a sua multiplicação artificial controlada, visando à obtenção dos grandes volumes de veneno indispensáveis ao estudo das suas propriedades ou para utilização como antígenos na obtenção de antídotos.

É na fisiopatologia, na bioquímica e na farmacologia, principalmente, que a pesquisa pode ser especialmente proveitosa e altamente esclarecedora. É de todo interêsse intensificar os estudos sôbre a maneira de ação dos venenos, o conhecimento adequado da sua estrutura química, o isolamento dos princípios ativos, de ação direta ou indireta, que compõem a complexa natureza dos venenos animais e bem determinar as suas ações lesivas para o organismo.

Muito esfôrço ainda deve ser feito para aprofundar o conhecimento sôbre os venenos e chegar à obtenção de conceitos mais adequados, que permitam a padronização internacional daqueles de maior importância e dos sôros mono e polivalentes dotados de maior potencial de neutralização dos princípios realmente responsáveis pela ação letal dos venenos.

Nós nos consideraríamos imensamente satisfeitos se o esfôrço agora dispendido para a realização dêste Simpósio puder, de alguma maneira, contribuir para alcançar êste objetivo. Esperamos que possais tirar benefícios dêste convívio que hoje se inicia e que êste primeiro Simpósio Internacional seja apenas o comêço de uma série de outros encontros, semelhantes e periódicos, para revisão dos resultados alcançados e estímulo para o vosso dignificante trabalho.

III

ANIMAIS VENENOSOS

VENOMOUS ANIMALS

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1. VENOMOUS MARINE ANIMALS OF BRAZIL

BRUCE W. HALSTEAD

World Life Research Institute, Colton, California, U.S.A.

Our knowledge of the venomous marine animals of Brazil dates back to the Renaissance and the writings of Willem Piso and George Marcgrave, who published an excellent biological description of the venomous spotted eagle-ray *Aetobatus narinari* in their HISTORIA NATURALIS BRASILIAE in 1648. This particular description is noteworthy because it included a brief general description of the venom apparatus on the tail of the ray. Despite this early beginning of investigations on the venomous marine animals of Brazil, there was a gap of several centuries before these organisms received very much scientific attention. Some of the more comprehensive references by Brazilian workers that deal with the subject are by Diniz (1905) and Gonsalves (1907) on venomous fishes; Silvado (1911) on the noxious fishes of the Rio de Janeiro Bay; Fonseca (1917) on the venomous fishes of Brazil; Fróes (1932, 1933a, b) on venomous fishes and particularly the toadfish *Thalassophryne*; and various other workers, most of whose publications also deal with venomous fishes, viz: Carvalho, 1947; Costa, 1958; Fonseca, 1952; Santos, 1952. In view of this most auspicious occasion of the INTERNATIONAL SYMPOSIUM ON ANIMAL VENOMS in Commemoration of the Centennial of Vital Brazil here at the Instituto Butantan, it should be recognized that the Instituto Butantan has played a significant role in these studies on venomous marine animals.

In discussing the venomous marine fauna of Brazil, one must first take into consideration the vast coastal expanses of the country which extend from latitude 4°20'45" N. to latitude 33°45'09" S., or 38 degrees of latitude, a coastline of 4,603 miles. The river systems of Brazil are the most extensive in the world, and they exert a profound effect on the marine flora and fauna. The Brazilian marine fauna is comprised of tropical Atlantic and West Indian (or Caribbean) forms, as well as the temperate western Atlantic species. Caribbean species occur as far south as Bahia (Lagler, Bardach, and Miller, 1962).

It is the purpose of this paper to briefly review the phylogenetic distribution of the venomous marine animals of Brazil, the nature of their venom organs and venoms, the clinical effects which they produce, and the treatment of their stings. The term "venomous marine animal" is defined as a marine animal that is equipped with a venom apparatus, i.e., a traumagenic device associated with a poison or venom gland, capable of producing an envenomation. Organisms that are poisonous to eat have not been included in this discussion. The organisms have been arranged according to their phylogenetic position.

INVERTEBRATES

PHYLUM COELENTERATA: HYDROIDS, JELLYFISHES, SEA ANEMONES, CORALS.

Class **HYDROZOA**: Hydroids.

Family MILLEPORIDAE: *Millepora alcicornis* Linnaeus. Stinging coral or fire coral (USA). — Caribbean Sea, northern coast of Brazil.

Family OLINDIADIDAE: *Olindias sambaquiensis* Müller. Stinging medusa (USA), relojinho (Brazil). — Coast of tropical Brazil.

Family PENNARIIDAE: *Pennaria* cf. *tiarella* (Ayres). Stinging hydroid (USA). —Coast of Maine to Brazil.

Family PHYSALIIDAE: *Physalia physalis* Linnaeus. Portuguese man-o-war, bluebottle (USA), caravela (Brazil). — Tropical Atlantic.

Family PLUMULARIIDAE: *Lytocarpus philippinus* (Kirchenpauer). Feather hydroid (USA). — Circumtropical.

Family RHIZOPHYSIDAE: *Rhizophysa eysenhardti* Gegenbaur. Stinging hydroid (USA). — Warm waters of all oceans. *Rhizophysa filiformia* (Forskal). Stinging hydroid (USA). — Warm waters of all oceans.

Class **SCYPHOZOA**: Jellyfishes.

Family CARYBDEIDAE: *Carybdea alata* Reynaud. Sea wasp (USA). — Circumtropical. *Carybdea marsupialis* (Linnaeus). Sea wasp (USA). — Tropical Atlantic. *Tamoya haplonema* Müller. Sea wasp (USA). — Tropical Atlantic.

Family CHIRODROPIDAE: *Chiropsalmus quadrumanus* (Müller). Sea wasp (USA). — Tropical Atlantic.

Family LINUCHIDAE: *Linuche unguiculata* (Schwartz). Thimble jellyfish (USA). — Tropical Atlantic and Pacific Oceans.

Family LYCHNORHIZIDAE: *Lychnorhiza lucerna* Haeckel. Água viva (Brazil). — Coast of Brazil, French Guiana.

Family NAUSITHOIDAE: *Nausithoë punctata* Kölliker. Stinging alga (USA, juvenile form). — All warm seas.

Family PELAGIIDAE: *Chrysaora quinquecirrha* (Desor). Sea nettle (USA). Tropical Atlantic and Pacific Oceans.

Family ULMARIDAE: *Aurelia aurita* (Linnaeus). Moon Jelly (USA). — All warm seas.

Class **ANTHOZOA**: Corals and sea anemones. There are no reports of stinging anthozoans from Brazilian waters.

Biology — **HYDROZOA:** Included within the **HYDROZOA** are the hydroids (e.g., *Aglaophenia*), stinging or hydroid corals (e.g., *Millepora*), and the free-floating siphonophores (e.g., *Physalia*). The hydroids are generally found attached to a substratum in shallow waters, from low tide level down to depths of 1,000 meters or more. The ecological conditions in which hydroids are found are extremely variable and they fluctuate according to the species. Some species have a wide distribution, whereas others are restricted to definite latitudes. Generally, hydroids are more abundant in temperate and cold zones. The form of the colony may be radically altered by such environmental factors as wave shock, currents, and temperatures. Colonies usually are small or moderate in size, but some species may attain a length of 2 m. Because of the sessile habits of hydroids, commensalism with other animals is of frequent occurrence. Since hydroids attach themselves to pilings, rafts, shells, rocks, or algae, and display their fine moss-like growth, they are sometimes mistaken for seaweed. Colonial hydroids are comprised of two kinds of polyps or zooids: the feeding hydranth that takes in food for the colony, and the reproductive polyp or gonangium. The nutritive polyps have a crown of tentacles and a central mouth that leads into the stomach cavity to which all the other polyps are connected by the coenosarc which encloses the common enteron. The nematocysts nettle or stinging cells are restricted to the tentacles. Food is procured by the use of the nematocysts.

Hydroids reproduce by budding; the free-swimming, solitary medusae that separate from the reproductive polyp, produce ova that result in attached hydroids. Medusae are more difficult to obtain than are the plant-like hydroids, but they may be taken in fine-meshed plankton nets. The medusae may be likened to a tiny umbrella with a short handle, the manubrium, which contains the mouth. Tentacles provided with stinging cells hang from the velum or margin of the umbrella. Medusae swim in a jerky fashion by spasmodic contractions of the umbrella. In the medusae, the sexes are separate.

The order **HYDROCORALLINA** or hydroid corals of which *Millepora* is the best-known genus, is widely distributed throughout tropical seas in shallow water to the depth of 30 m. Hydroid corals are important in the development of coral reefs, for they form upright, clavate, blade-like, or branching calcareous growths, or encrustations over other corals and objects. They vary in color from white to yellow-green; because of their variable appearance, they are sometimes difficult to recognize. The order is characterized by a massive exoskeleton of lime carbonate, the surface of which is covered with numerous minute pores. There are two sizes of pores: the larger gastropores, which are 1 to 2 mm apart, and the smaller dactylopores, irregularly interspersed about the gastropores. The surface of the coral between the pores has a pitted appearance. The entire stony mass is traversed by a complex system of branched canals which communicate with the pores. From the gastropores protrude the feeding gastrozooids, equipped with a hypostome and capitate tentacles. Dactylozooids, extending from the dactylopores, are mouthless; however, they are provided with tentacles, which are believed to have a protective and tactile function. According to Hyman (1940), millepores have two or three types of powerful nematocysts located on the polyps, polyp bases, and in the general coenosarc. Apparently most of the *Millepora* have the ability to sting, but the venomousness of the sting varies from one species to the next (Boschma, 1948).

The order Siphonophores, of which *Physalia* is a pertinent example, is highly polymorphic free-swimming or floating colonies composed of several types of polypoid or medusoid individuals attached to a floating stem. Siphonophores

are pelagic animals, inhabiting the surface of the sea. They depend largely upon currents, wind, and tides for their movement. They are widely distributed as a group, but most abundant in warm waters. The float or pneumatophore of *Physalia* is greatly enlarged, and it is represented by an inverted, modified, medusan bell, whereas the remainder of the coenosarc is correspondingly reduced. *Physalia* may attain a large size with a float 10 to 30 cm in length. From the underside of the float hang gastrozooids, dactylozooids, and the reproductive gonodendra with their gonophores or budding medusoids. The female gonophores are medusoid and may swim free, but the male reproductive zooid remains attached to the float. The gastrozooids or feeding polyps are without tentacles. Some of the tentacled dactylozooids are small; however, several of the large dactylozooids are equipped with very elongate "fishing" tentacles.

The number of fishing tentacles varies with the species of *Physalia*. In the Pacific form, *P. utriculus*, there is a single fishing tentacle; but in the Atlantic species, *P. physalis*, there are multiple fishing tentacles. Extending along the entire length of the large dactylozooid, a band of specialized tissue covers diverticulae of the gastrovascular cavity of the tentacle. These fishing tentacles or large dactylozooids may be found in the water to a depth of more than 30 m and, because of their almost transparent appearance, constitute a definite hazard to the unsuspecting swimmer. Upon contraction, the remainder of the tentacle shortens more completely than does the superficial band, and this causes the band to be thrown into loops and folds that are known as "stinging batteries". The nematocysts are contained in cnidoblasts located in the superficial epithelium of the battery. The toxin, a structureless fluid within the nematocyst capsule, bathes the surface of the nematocyst tubule (Lane, 1960).

According to Parker (1932), scores of these fishing filaments may extend down into the water from a single *Physalia*. Parker has observed that the nematocyst heads occur at regular intervals along the side of the filament opposite the point from which the main muscle plate takes its origin. Each full-sized head contains about 500 large nematocysts and about 2,000 small ones. In one extended filament measuring 9 m in length, the nematocyst heads were distributed at intervals of approximately 3 cm apart. According to these figures, each fishing filament contained about 750,000 nematocysts. When one considers the large number of fishing filaments on each *Physalia*, he finds a formidable venom apparatus.

When the animal is moving through the water, the fishing tentacles undergo a continuous rhythmic movement, alternately contracting and relaxing. Thus there is a constant sampling of the water beneath the pneumatophore. If the tentacle brushes against a prey organism, the nematocysts are stimulated, and they trigger the immediate release of the coiled nematocyst thread. The fully uncoiled thread may be several hundred times as long as the diameter of the parent capsule. The extreme length of the tubule, together with its chitinous barbs and spines, constitute a highly effective entanglement. If the tip of the cnidal thread penetrates the victim, the toxin is conveyed directly into the body of the prey through the hollow thread. Lane (1960) found that the thread can penetrate even a surgical glove.

Lane further observed that the magnitude of the response to contact with the victim is proportional to the area of contact between tentacle and prey. A small copepod may elicit the discharge of 20 to 50 adjacent nematocysts, whereas contact with a larger animal might evoke a discharge of several hundred thousand nematocysts. Gentle stimulation of the nematocyst results in a rapid release of

the nematocyst thread, but does not dislodge the parent capsule from its position in the epithelium. Vigorous resistance by the prey results not only in greatly increasing the number of cnidae but also in disloding many of them from the epithelium. Dislodged nematocysts are replaced by cnidoblasts that differentiate outside the stinging battery but subsequently come to occupy a definitive position in the battery epithelium.

It is interesting that the loggerhead turtle, *Caretta caretta*, has been reported to feed on *Physalia*. The potency of the toxin and the ability of the *Physalia* nematocyst to penetrate even a surgical glove make this a gastronomic feat of no small accomplishment (Lane, 1960).

SCYPHOZOA: All Scyphozoans or jellyfishes are marine and the majority are pelagic. A few species are known to inhabit depths of 2,000 fathoms or more. In the adult stage, most jellyfishes are free swimming. Because their swimming ability is relatively weak, jellyfishes are greatly influenced in their movements by currents, tides, and wind. Scyphozoans are widely distributed throughout all seas. Many medusae reveal that they are affected by light intensity in that they surface during the morning and late afternoons and descend during the midday and in the darkness, whereas other react in just the opposite manner. A descension is usually made during periods of stormy weather. Swimming is accomplished by rhythmic pulsations of the bell, and this action determines the vertical rather than the horizontal progress of the animal. Jellyfishes display a remarkable ability to withstand considerable temperature and salinity changes. They are carnivorous, some of the larger species being capable of capturing and devouring large crustaceans and fishes. Jellyfishes display a wide variety of sizes, shapes, and colors; many of them are semitransparent or glassy in appearance and often have brilliantly colored gonads, tentacles, or radial canals. In some species, they may vary in size from a few millimeters to more than 2 m across the bell, with tentacles more than 36 m in length, as in *Cyanea capillata*.

Regardless of their size, jellyfishes are very fragile; many of them contain less than 5 percent of solid organic matter. Scyphomedusae have an eight-notch marginal bell, but lack a velum; the gonads are connected with the endoderm. Reproduction is by an alternation of generations, as in the hydroids, although the polyp stage is reduced. Jellyfishes have a complex system of branched radial canals, and numerous oral and marginal tentacles.

The cubomedusae are among the most venomous marine creatures known. The genera, *Chiropsalmus* and *Carybdea*, contain some of the more dangerous species of the group. They range in size from a small grape to that of a large pear. Cubomedusae are widely distributed throught all the warmer seas. They generally seem to prefer the quiet shallow waters of protected bays and estuaries and sandy bottoms, although some species have been found in the open ocean. During the summer months, the immature forms which stay on the bottom, reach maturity. The adults may then be found swimming at the surface. Light-sensitive cubomedusae, however, descend to deeper water during the bright sun of the middle of the day and come to the surface during early morning, late afternoon, and evening.

Morphology of the venom apparatus — The venom apparatus of coelenterates consists of the nematocysts or stinging cells which are largely located on their tentacles. These nematocysts are situated within the outer layer of tissue of the tentacle. Each of the capsule-like nematocysts is contained within an outer capsule-like device called the cnidoblast. Projecting at one point on the outer

surface of the cnidoblast is the trigger-like cnidocil. Contained within the fluid-filled capsular nematocyst is the hollow, coiled, thread tube. The opening through which the thread tube is everted is closed prior to discharge by a lid-like device called the operculum. The fluid within the capsule is the venom. Stimulation of the cnidocil appears to produce a change in the capsular wall of the nematocyst causing the operculum to spring open like a trap door, and the thread tube conveying the venom is everted. The sharp tip of the thread tube penetrates the skin of the victim and the venom is thereby injected. When a diver comes in contact with the tentacles of a coelenterate, he brushes up against the cnidocils of literally thousands of these minute stinging organs.

Clinical characteristics — The symptoms produced by coelenterate stings vary according to the species, the site of the sting, and the person. In general, those caused by hydroids and hydroid corals (*Millepora*), are primarily local skin irritations. *Physalia* stings may be very painful. Sea anemones and true corals produce a similar reaction, but may be accompanied by general symptoms. Symptoms resulting from scyphozoans vary greatly. The sting of most scyphozoans is too mild to be noticeable, whereas *Carybdea* and *Chiropsalmus* are capable of inflicting very painful local and generalized symptoms. *Chiropsalmus* is probably the most venomous marine organism known and may produce death within 3 to 8 minutes in humans.

Symptoms most commonly encountered vary from an immediate mild prickly, or stinging sensation like that of a nettle sting, to a burning, throbbing or shooting pain which may render the victim unconscious. In some cases, the pain is restricted to an area within the immediate vicinity of the contact, or it may radiate to the groin, abdomen, or armpit. The area coming in contact with the tentacles usually becomes reddened, followed by a severe inflammatory rash, blistering, swelling, and minute skin hemorrhages. In severe cases, in addition to shock, there may be muscular cramps, abdominal rigidity, diminished touch and temperature sensation, nausea, vomiting, severe backache, loss of speech, frothing at the mouth, sensation of constriction of the throat, respiratory difficulty, paralysis, delirium, convulsions, and death.

Treatment — Treatment must be directed toward accomplishing three objectives: relieving pain, alleviating effects of the poison, and controlling primary shock. Morphine is effective in relieving pain. Intravenous injections of calcium gluconate have been recommended for the control of muscular spasms. Oral histaminics and topical cream are useful in treating the rash. Dilute ammonium hydroxide, sodium bicarbonate, olive oil, sugar, ethyl alcohol, and other types of soothing lotions have been used with varying degrees of success. Artificial respiration, cardiac and respiratory stimulants, and other forms of supportive measures may be required. There are no known specific antidotes.

Pharmacology — See Chemistry section.

Chemistry — Coelenterate venom contains a number of quaternary ammonium compounds, of which tetramine is the most active. The venom also contains 5-hydroxytryptamine, histamine and histamine releasers, and several proteins of relatively low molecular weight. The paralyzing and lethal effects of the toxin appear to be caused largely by the proteins which may act directly on cholinergic neurons. The localized dermatological signs and symptoms may be attributable to 5-hydroxitryptamine, histamine and histamine-releasing substances (Russell, 1965; Halstead, 1965). The chemistry of most coelenterate venoms has not been studied.

PHYLUM ECHINODERMATA: STARFISHES, SEA URCHINS, etc.

Family ARBACIIDAE: *Arbacia lixula* (Linnaeus). Sea urchin (USA), ouriço do mar (Brazil). — Tropical Atlantic and Mediterranean Sea.

Family DIADEMATIDAE: *Diadema antillarum* Philippi. Black sea urchin, needle-spined urchin (USA). — Tropical Atlantic, West Indies.

Family TOXOPNEUSTIDAE: *Lytechinus variegatus* Lamarck. Sea urchin (USA), ouriço do mar (Brazil). — West Indies, North Caroline, south to Brazil.

Biology — Sea urchins are free-living echinoderms, having a globular, egg-shaped, or flattened body. The viscera are enclosed within a hard shell or test, formed by regularly arranged plates, carrying spines articulating with tubercles on the test. Between the spines are situated three-jawed pedicellariae, which are of interest to the venomologist. In some species of sea urchins, the spines are also venomous. Tube feet are arranged in 10 meridian series rather than in furrows. A double pore in the test corresponds to each tube foot. The intestine is long and coiled, and an anus is always present. The gonads are attached by mesenteries to the inner aboral surface of the test. The mouth, situated on the lower surface, turns downward, and is surrounded by five strong teeth incorporated in a complex structure termed "Aristotle's lantern". Their power of regeneration is great, but autotomy, as observed in the asteroids, does not occur. By means of spines on the oral side of the test, sea urchins move slowly in the water. The tube feet are utilized to climb vertical surfaces. Some forms have the ability to burrow into crevices in rocks, while others cover themselves with shells, sand, and bits of debris.

Some urchins are nocturnal, hiding under rocks during the day and coming out to feed at night. Echinoids tend to be omnivorous in their feeding habits, ingesting algae, mollusks, foraminifera, and various other types of benthic organisms.

Sea urchins are dioecious, hermaphroditism occurring only as a rare anomally. Sexual dimorphism is generally absent. Spawning usually takes place during the spring and summer in the Northern Hemisphere, but somewhat earlier in the more southern latitudes. The reproductive periods of echinoids have been discussed at great length by Hyman (1955). Several species of European and tropical echinoids serve as important sources of food to man. Only the gonads are eaten, either raw or cooked. The bathymetric range of echinoids is great, extending from the intertidal zone to great depths.

Morphology of the venom apparatus — The venom apparatus of sea urchins is believed to consist of their hollow venom-filled spines, and the globiferous pedicellariae. However, usually only one or the other is present within a single species of sea urchin.

The spines of sea urchins vary greatly from group to group. In most instances the spines are solid, have blunt, rounded tips, and do not constitute a venom organ. However, some species have long, slender, hollow, sharp spines, which are extremely dangerous to handle. The acute tips and the spinules permit ready entrance of the spines deep into the flesh, but because of their extreme brittleness, they break off readily in the wound and are very difficult to withdraw. The spines in *Diadema* may attain a length of a foot or more. It

is believed that the spines of some of these species secrete a venom, but this has not been experimentally demonstratep. The aboral spines of *Asthenosoma* are developed into special venom organs carrying a single large gland. The point is sharp and serves as a means of introducing the venom.

Pedicellariae are small, delicate, seizing organs which are found scattered among the spines of the shell. There are several different types of pedicellariae. One of these, because of its globe-shaped head, is called the globiferous pedicellariae, and serves as a venom organ. They are comprised of two parts, a terminal, swollen, conical head, which is armed with a set of calcareous pincer-like valves or jaws, and a supporting stalk. The head is attached to the stalk either directly by the muscles, or by a long flexible neck. On the inner side of each valve is found a small elevation provided with fine sensory hairs. Contact with these sensory hairs causes the valves to close instantly. The outer surface of each valve is covered by a large gland which in *Toxopneustes* has two ducts that empty in the vicinity of a small tooth-like projection on the terminal fang of the valve. A sensory bristle is located on the inside of each valve. Contact with these bristles causes the small muscles at the base of the valve to contract, thus closing the valves and injecting the venom into the skin of the victim.

One of the primary functions of pedicellariae is that of defense. When the sea urchin is at rest in calm water, the valves are generally extended, moving slowly about, awaiting prey. When a foreign body comes in contact with them, it is immediately seized. The pedicellariae do not release their hold as long as the object moves, and if it is too strong to be held, the pedicellariae are torn from the test, or shell, but continue to bite the object. Detached pedicellariae may remain alive for several hours after being removed from the sea urchin.

Clinical characteristics — Penetration of the needle-sharp sea urchin spines may produce an immediate and intense burning sensation. The pain is soon followed by redness, swelling, and an aching sensation. Numbness and muscular paralysis have been reported. Secondary infections are not uncommon.

The sting from sea urchin pedicellariae may produce an immediate, intense, radiating pain, faintness, numbness, generalized muscular paralysis, loss of speech, respiratory distress, and in severe cases, death. The pain may diminish after about 15 minutes and completely disappear within an hour, but paralysis may continue for six hours or longer.

Treatment — Insofar as the venom is concerned, sea urchin stings should be handled in a manner similar to any other venomous sting. However, attention is directed to the need for prompt removal of the pedicellariae from the wound. When pedicellariae are detached from the parent animal, they frequently continue to be active for several hours. During this time they will introduce venom into the wound.

The extreme brittleness and retrorse barbs of some sea urchin spines present an added mechanical problem. Nielly (1881) recommended that grease be applied, stating that this would allow the spines to be scraped off quite easily. Cleland (1912), Earle (1940), and others, are of the opinion that some sea urchin spines need not be removed, as they are readily absorbed. Absorption of the spines is said to be complete within 24 to 48 hours. However, Earle (1941) later pointed out that the spines of *Diadema setosum* are not readily absorbed, and months later roentgenological examination may reveal them in the wound. It is recommended that the spines of *Diadema* be removed surgically.

Pharmacology — The only attempt to evalute the general pharmacological properties of globiferous pedicellarial venom of sea urchins has been made by Mendes, Abbud, and Umiji (1963). Saline extracts were prepared from homogenates of globiferous pedicellariae of *Lytechinus variegatus* and tested on accepted cholinergic effector systems, viz, guinea pig ileum, rat uterus, amphibian heart, longitudinal muscle of a holothurian, the protractor muscle of a sea urchin lantern, and the blood pressure of dogs. The response obtained was consistent with that of a dialyzable acetycholinelike substance which the researchers concluded to be in pedicellarial venom.

Chemistry — Unknown.

PHYLUM MOLLUSCA: SNAILS, BIVALVES, OCTOPUSES, etc. — There are no reports of human encounters with venomous mollusks in Brazilian waters.

PHYLUM ANNELIDA: SEGMENTED WORMS.

Family AMPHINOMIDAE: *Chloeia viridis* Schmarda. Sea mouse, bristle worm (USA). — Tropical America, both sides. *Eurythoë brasiliensis* Hansen. Bristle worm (USA). — Coast of Brazil. *Eurythoë complanata* (Pallas). Bristle worm (USA). — Circumtropical.

Biology — The polychaetes are divided into two major groups: the *Errantia*, which includes most of the free-moving kinds, and the *Sedentaria* or tube-dwelling and burrow-inhabiting species. The polychaetes that have been incriminated as toxic are largely errant forms.

Polychaetes have cylindrical bodies and are metameric, having numerous somites — each bearing a fleshy paddle-like appendage, or parapodia, that bears many setae. The head region has tentacles. There is no clitellum. Sexes are usually separate. There are no permanent gonads and fertilization is commonly external. Polychaetes have a trochophore larval stage, and there is a sexual budding in some species.

Most polychaetes are free living; a few are ectoparasitic. They have a bathymetric range from the tide line to depths of more than 5,000 meters. A few species are pelagic. Several of the polychaetes inhabit freshwater. Polychaetes are largely carnivorous. Some of the burrowing worms feed on bottom detritus, whereas the tube dwellers subsist on plankton. Generally, polychaetes spend their existence crawling under rocks, burrowing in the sand or mud, in and around the base of algal growths; or they construct tubes, which they leave at periodic intervals in search of food. The majority of polychaetes range in size from 5 to 10 cm. However, some of the syllids are only 2 mm in length; whereas the giant Australian species *Onuphis teres* and *Eunice aphroditois* may attain a meter or more in length.

Morphology of the venom apparatus — *Setae*: The members of the polychaete genera *Chloeia*, *Eurythoë*, *Hermodice*, and others, possess elongate pungent chitinous bristles or setae which project from the parapodia. The parapodia are a pair of lateral appendages extending from each of the body segments. The structure appears as a more or less laterally compressed fleshy projection of the body wall. Each parapodium is biramous; it consists of a dorsal portion, the notopodium, and a ventral part, the neuropodium. Each division of the parapodium is supported internally by one or more chitinous rods,

termed acicula, to which are attached some of the parapodial muscles. Each of the distal ends of the two parapodial divisions are invaginated to form a setal sac or pocket in which the projecting setae are situated. Each seta is secreted by a single cell at the base of the setal sac. Generally the setae of polychaetes project some distance beyond the end of the parapodium. However, *Eurythoë* and *Hermodice* have the ability to retract or extend their setae to a remarkable degree. When the living worm is at rest, the setae appear to be quite short and barely in evidence; but when irritated, the setae are rapidly extended and the worm appears to be a mass of bristles.

The severity of symptoms reported in some of the clinical accounts lends credence to the belief that both *Eurythoë* and *Hermodice* possess venomous setae. The setae of both *E. complanata* and *H. carunculata* appear to be hollow, and at times seem to be filled with fluid. A seta of *E. complanata* has a series of retrorse spinules along the shaft, whereas the seta of *H. carunculata* is without spinules and has a needlelike appearance. The setae of *Chloiea* are said to be nonvenomous (Pope, 1963), but are listed as a "stinging" worm by others (Phillips and Brady, 1953; Steinbeck and Ricketts, 1941). Examination of histological sections of the parapodia of both species failed to reveal any glandular elements. However, the material examined was poorly preserved and it is quite possible that glandular structures might have been present and were not recognized. Final decision on this matter awaits further histological study.

Clinical characteristics — Bristle worm (*Chloeia*, *Eurythoë*, *Hermodice*) stings may result in an intense inflammatory reaction of the skin, consisting of redness, swelling, burning sensation, numbness, and itching. According to Mullin (1923), *Hermodice carunculata* is able to inflict a "paralyzing effect" with its setae. Contacts with the setae of bristle worms have been likened to handling the spines of prickly pear cactus or nettle stings. Severe complications may result in secondary infections, gangrene, and loss of the affected part. The clinical effects of bristle worm stings have also been discussed by Baird (1864), Paradice (1924), Levrat (1927), Roughley (1940, 1947), Pope (1947, 1963), LeMare (1952), Phillips and Brady (1953), Halstead (1956, 1959), and Gillett and McNeil (1962).

Treatment — The treatment of worm bites and bristle worm stings is largely symptomatic. There are no specific antidotes. Secondary infections may occur which require the use of antibiotic therapy. The setae of bristle worms can best be removed from the skin with adhesive tape, and the ammonia or alcohol should be applied to the area to alleviate the discomfort.

Pharmacology — Unknown.

Chemistry — Unknown.

VERTEBRATES

Venomous marine fishes are members of the phylum CHORDATA. The Brazilian marine fauna does not appear to be especially rich in venomous fishes. The families TRACHINIDAE, SIGANIDAE, SCATOPHAGIDAE, MONODACTYLIDAE, HISTIOPTERIDAE, and several of the more important genera of venomous SCORPAENIDAE are not represented in Brazilian waters.

Despite the fact that some of the earliest literature in biotoxicology deals with venomous fishes, the bulk of our knownledge on the morphology of the venom organs of fishes has been published during the past two decades. Unfortunately,

most of this literature deals with European, North American, or tropical Indo-Pacific species. The only endemic Brazilian species that have been studied to any extent are members of the genus *Thalassophryne* of the family BATRACHOIDIDAE. The field of piscine venomology offers a vast field of untappped opportunity to the researcher. There is urgent need for a thorough systematic investigation of the venomous fishes of Brazil in particular.

Class **CHONDRICHTHYES**: SHARKS, RAYS, etc.

HORNED SHARKS — Venomous sharks are limited to those species which possess dorsal fin spines, namely, members of the families HETERODONTIDAE, SQUALIDAE, and DALATIIDAE. Although a number of species within these three families are suspected of having venom organs, only two species, *Hetodontus facisci* and *Squalus acanthias*, have been studied to even a limited extent, and only the latter is found in Brazilian waters. Clinical reports from horned sharks are based on *S. acanthias*, and most of these reports are from Europe.

Family SQUALIDAE: *Squalus acanthias* Linnaeus. Spiny dogfish (USA), galhudo (Brazil). — Atlantic and Pacific Oceans. *Squalus fernandinus* Molina. * Spiny dogfish (USA), galhudo (Brazil). — Circumpolar and widespread throughout boreal and cool temperate latitudes of the Southern Hemisphere.

Biology — Squalids are widely distributed throughout subarctic, temperate, tropical, and subantarctic seas.

Most dogfish are somewhat sluggish in their movements, traveling singly or in schools, and somewhat erratic in their migrations. Their bathymetric range extends from the surface to depths of 100 fathoms or more. They are not pelagic, preferring relatively shallow protected bays. The migration of squalids seems to be governed by thermal changes, showing a preference for water temperature from 7° to 15°C. Squalids are viviparous, giving birth to their young from late summer through the winter in some regions but earlier in others. Dogfish are voracious and include a variety of fishes in their diet: capelin, herring, menhaden, mackerel, hake, cod, haddock. They also feed on coelenterates, mollusks, crustaceans, and worms. Squalids have been used to a considerable extent for fertilizer and as a source of vitamins A and D. Dogfish are of considerable economic importance because of the damage that they do to fishing gear.

Morphology of the venom apparatus — The venom apparatus of horned sharks is comprised of the dorsal fin spines and the associated glandular tissue. The dorsal stings are situated adjacent to the anterior margins of each of the two spines in most of the horned sharks. Some of the dalatiid sharks have only a single somewhat rudimentary fin spine or the spines are entirely absent. Therefore, there is some question as to whether a true venom organ is present in this latter group.

The anterior fin spine in *Squalus acanthias* is only slightly curved anteroposteriorly, whereas the posterior spine is more curved and in lateral view is somewhat sabreshaped. The two sides are slightly convex and the longitudinally grooved posterior aspect of the spine forms the base of the triangle. The spine

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* *Squalus fernandinus* is reported as venomous, but there is no information regarding the nature of its venom apparatus or venom.

is grooved only in its exposed portion, and the groove becomes more shallow toward the tip. The glandular tissue appears as a glistening white substance situated in the shallow posterior groove, or interdentate depression, of the spine.

Microscopic examination of the sting in cross section reveals it to be trigonal in shape and comprised of three principal layers: an outer layer of integument which covers a thick wall of hard vasodentine and an inner core of cartilage. Careful examination of the structure reveals that the glandular cells are situated in the epithelial portion of the integumentary layer in the area of the antero-lateral glandular grooves and in the interdentate depression. The glandular cells are sparsely scattered in the anteroglandular-groove area, but are heavily concentrated in the interdentate depression. The glandular cells are of two basic types. Some of these are polygonal-shaped, clear, finely granular cells having slightly pycnotic nuclei, which appear to be of the mucin type. However, the venom cells in hematoxylin and triosin preparations are oval-shaped, containing homogenous brown-staining material with accumulations of finely granular material. Venom production is apparently by a holocrine type of secretion. Morphological studies on *Squalus* have been conducted largely by Evans (1921, 1923, 1943).

Clinical characteristics — The symptoms consist of immediate intense, stabbing pain which may continue for a period of hours. The pain may be accompanied and followed by a generalized erythema and severe swelling of the affected part. Tenderness of the affected part may continue for several days. According to Coutière (1899), dogfish stings may be fatal. The only clinical reports are by Evans (1920, 1923, 1943).

Treatment — Wounds produced by spined sharks are usually of the puncture wound variety. Since shark spines do not have an enveloping integumentary sheat, and the bulk of the glandular tissue is located near the base of the spine, it would be a rare instance for the glandular tissue to become embedded in the wound of the victim. In the most instances, effects resulting directly from the action of the venom are of minor concern. Nevertheless it is advisable to irrigate the wound with saltwater and to do whatever debridement may be necessary if the tissues have been lacerated. If there is little or no bleeding, then moderate bleeding should be encouraged. The pain is usually mild in comparison with most stingray stings, but opiates may be needed. The extremity should be submerged in hot water for a period of 30 minutes or more at as high a temperature as the victim can tolerate without doing further injury. The addition of sodium chloride or magnesium sulfate to the water is optional. Suturing may be required. Antitetanus agents should be administered. Secondary infections from shark spines may sometimes occur, and antibiotic therapy may be needed. Elevation of the injured limb is recommended.

Pharmacology — Unknown.

Chemistry — Unknown.

STINGRAYS

Stingrays constitute an important group of venomous fishes in that they are probably the most common cause of fish stings. The Suborder MYLIOBATOIDEA includes the seven ray families: DASYATIDAE, stingrays or whiprays; POTAMO-

TRYGONIDAE, river rays; GYMNURIDAE, butterfly rays; UROLOPHIDAE, round stingrays; MYLIOBATIDAE, eagle or bat rays; RHINOPTERIDAE, cow-nosed rays; and MOBULIDAE, devil rays or mantas. (The caudal spines of the MOBULIDAE when present, are generally quite rudimentary and will not be considered further in this presentation). With the exception of the POTAMOTRYGONIDAE, which are confined to the rivers of South America, most stingrays are marine, inhabiting shallow coastal waters, bays, brakish water lagoons, but may enter river mouths and freshwater rivers. Most reports on stingray attacks and venom organs are based on either European or North American species. Very little is known regarding the venom organs of most of the stingray species of Brazil.

Family DASYATIDAE: *Dasyatis americana* Hildebrand and Schroeder. Southern stingray (USA), raia (Brazil.) — Western Atlantic, New Jersey to Rio de Janeiro, Gulf of Mexico. *Dasyatis centroura* (Mitchell). Roughtail stingray — Atlantic Ocean, Mediterranean Sea. *Dasyatis guttatus* Bloch and Schneider. Stingray (USA). — West Indies, Gulf of Mexico to southern Brazil. *Dasyatis sabina* (Lesueur). Atlantic stingray (USA). — Western Atlantic from Chesapeake Bay to Brazil, Gulf of Mexico. *Dasyatis sayi* (Lesueur). Bluntnose stingray. — Western Atlantic from New Jersey to southern Brazil.

Family GYMNURIDAE: *Gymnura altavela* (Linnaeus). Spiny butterfly ray (USA). — Tropical and temperate Atlantic Ocean. *Gymnura micrura* (Bloch and Schneider). Smooth butterfly ray (USA). — Western Atlantic from Chesapeake Bay to Brazil, Gulf of Mexico.

Family MYLIOBATIDAE: *Aetobatus narinari* (Euphrasen). Spotted eagle ray (USA). — Tropical and warm temperate waters of the Atlantic, Red Sea, Indo-Pacific. *Myliobatis freminvillei* Lesueur. Bullnose ray (USA). — Western Atlantic, from New York to Brazil.

Family RHINOPTERIDAE: *Rhinoptera bonasus* (Mitchell). Cownose ray (USA). — Western Atlantic, from southern New England to Brazil.

Family UROLOPHIDAE: *Urolophus jamaicensis* (Cuvier). Yellow stingray (USA). — Western tropical Atlantic.

Biology — Rays are common inhabitants of tropical, subtropical, and warm temperate seas. With the exception of the family POTAMOTRYGONIDAE, which is confined to freshwater, rays are essentially marine forms, some of which may enter brackish, of freshwaters, freely. Rays are swimmers of moderate depths, and are most common in shallow water. A deep sea species has recently been reported from the Central Pacific Ocean. Sheltered bays, shoal lagoons, river mouths, and sandy areas between patch reefs are favorite habitats of rays. They may be observed lying on top of the sand, or partially submerged, with only their eyes, spiracles, and a portion of the tail exposed. Rays burrow into the sand and mud, and excavate the bottom with the use of their pectoral fins, by which means they obtain the worms, molluscs, and crustaceans upon which they feed.

Morphology of the venom apparatus — The venom apparatus of stingrays is an integral part of the caudal appendage. The venom organs of stingrays have been divided into four anatomical types based upon their adaptability as a defense organ. This subject was discussed by Halstead and Bunker (1953).

Gymnurid type: This is the most weakly-developed type of stingray venom apparatus. The caudal appendage in gymnurid rays are cylindrical, tapering, and greatly reduced in size. The sting is small, seldom exceeding 2.5 cm in length, and usually situated in the middle or proximal third of the tail. The striking ability of the organ is relatively feeble.

Myliobatid type: The venom organs of myliobatid rays are better adapted as a striking organ than those found in gymnurid rays. The caudal appendage is cylindrical and tapers out to a long whip-like tail. The sting is generally situated on the proximal portion of the basal third of the tail and is moderate to large in size, ranging from about 5 to 12 cm or more in length. Although myliobatid rays can inflict serious wounds, the striking force of the sting is less than that of the dasyatid rays largely because of the proximal location of the sting on the caudal appendage.

Dasyatid type: The venom organs of dasyatid rays are better adapted as a striking organ than are those of myliobatid rays. The caudal appendage is cylindrical and tapers out to a long whip-like tail. The sting in some species may be very large, attaining 37 cm or more in length, and is located in the distal portion of the basal or middle third of the tail. The more distal location of the sting improves the striking force of the sting.

Urolophid type: The venom organs of urolophid rays are probably the most highly developed of any of the sting rays. The caudal appendage is relatively short, very muscular, and is not as a whip-like structure, but rather the tail becomes compressed distal to the sting and forms a more or less distinct caudal fin. The sting is usually located in the middle or distal third of the tail and is moderate in size, seldom exceeding 5 cm in length. The powerful muscular structure of the tail and the distal location of the sting make this a highly efficient defensive weapon.

Although there is considerable variation in the morphology of the venom organs of various stingray species there is a basic pattern which all of the species examined thus far appear to follow. For the purpose of this review only a general description of the stingray will be given.

The venom apparatus of stingrays consists of a bilaterally retroserrate spine and its enveloping integumentary sheath. The spine is an elongate tapering structure that ends in an acute sagitate tip. The spine is composed of an inner core of vasodentine which is covered by a thin layer of enamel. It is firmly anchored in a dense collagenous network of the dermis on the dorsum of the caudal appendage. The dorsal surface of the spine is marked by a number of shallow longitudinal furrows. These furrows are usually more pronounced on the basal portion of the spine and disappear distally. The serrate edges of the spine are termed the dentate margins. Medial to each dentate margin, on the ventral side, is a longitudinal groove, the ventro-lateral-glandular groove. The grooves are separated from each other by the median ventral ridge of the spine. Contained within the grooves of an "unsheathed" or traumatized sting is a strip of gray tissue. The tissue lying within the ventrolateral glandular grooves consists of glandular epithelium and blood vessels. This is the primary venom producing area of the sting. In most stingray species there is a thickened wedge-shaped portion of the integument on the dorsum of the caudal appendage ventral to the sting which is known as the cuneiform area. Toxicological studies of the cuneiform integument indicate that the glandular cells of this area also secrete venom.

Microscopic examination of histological cross sections of an intact sting reveals that it is roughly diamond shape and consists of a broad T-shaped dentinal structure completely enveloped by a layer of integument. The integument is comprised of two layers. The inner layer, the dermis, consists of areolar connective tissue and vascular channels. The outer layer, the epidermis, is composed of modified squamous epithelium containing many glandular cells. A cross section of the ventrolateral-glandular groove has been termed the glandular triangle. Glandular activity is generally most concentrated in the epidermis in the immediate vicinity of the ventrolateral-glandular grooves which is believed to be the principal site of venom production. There is no histological evidence of a venom duct. Venom production is by a holocrine type of lysis.

Clinical characteristics — Pain is the predominant symptom and usually develops immediately or within a period of ten minutes following the attack. The pain has been variously described as sharp, shooting, spasmodic or throbbing in character. The freshwater stingrays are reputed to cause extremely painful wounds. More generalized symptoms of fall in blood pressure, vomiting, diarrhea, sweating, rapid heart beat, muscular paralysis, and death have also been reported.

Stingray wounds are either of the laceration or puncture type. Penetration of the skin and underlying tissue is usually accomplished without serious damage to the surrounding structures, but withdrawal of the sting may result in extensive tissue damage due to the recurved spines. Swelling in the vicinity of the wound is a constant finding. The area about the wound at first has an ashy appearance, later becomes cyanotic and then reddened. Although stingray injuries occur most frequently about the ankle joint and foot as a result of stepping on the ray, instances have been reported in which the wounds were in the chest.

Treatment — The following recommendations are based on the clinical investigations of 1,725 cases of stingray attacks during the past 15 years by Russell (1953, 1965) and his associates, who have had more first-hand experience in this field than any other group.

Efforts of treatment should be promptly and vigorously instituted. The treatment is directed toward alleviating the pain, combating the effects of the venom, and preventing secondary infection. Successful results are in large measure dependent upon the rapidity with which treatment is instituted. The victim should immediately irrigate the wound with the cold saltwater at hand. This procedure facilitates removal of the venom, and the cold water tends to act as a vasoconstrictor thus reducing the amount of absorption of the poison, while serving as a mild anesthetic agent. A tourniquet may be applied immediately above the stab site but must be released every few minutes in order to preserve circulation. The wound should be explored carefully for evidence of pieces of the sting's integumentary sheath. All pieces of integumentary sheath must be completely removed or envenomation will continue and the results of the treatment will be greatly impaired. As soon as the wound has been thoroughly cleansed, the injured member should be soaked in hot water. The water should be maintained at as high a temperature as the patient can tolerate without producing further injury to the tissues. Soaking should continue for a period of 30 to 90 minutes. The addition of magnesium sulfate to the water is sometimes desirable because of its mild anesthetic properties. The addition of other anesthetic and antiseptic agents is optional. Following the soaking procedure, the wound should be debrided, cleansed, and closed with dermal sutures. The use of antitetanus agents is recom-

mended. Antibiotic agents may be required. The use of intramuscular or intravenous demerol has been found effective in controlling the pain. The primary shock so often seen immediately following the injury usually responds satisfactorily to routine supportive measures. However, the secondary shock resulting directly from the action of the venom on the cardiovascular system may require immediate and vigorous therapy. Treatment should be directed toward maintaining cardiovascular tone and the prevention of any further complicating factors. Elevation of the injured member is advisable.

The use of potassium permanganate, ammonia, and cryotherapy (Mullins, Wilson, and Best, 1957) is not only useless but may even have adverse effects. They are not recommended for the treatment of stingray stings. For further reading on the treatment of stingray attacks see Bayley (1940), Evans (1943), Halstead and Bunker (1953), Russell and Lewis (1956), Halstead (1959), Russell (1959), and Halstead and Mitchell (1963).

Pharmacology — The most complete studies on the pharmacological properties of stingray venom have been conducted by Russell and his associates working primarily with the venom of *Urolophus halleri*.

Stingray venom has a deleterious effect on the vertebrate cardiovascular apparatus. The action on the blood vessels appears to be diphasic. Low concentrations of the venom give rise to simple peripheral vasodilatation or vasoconstriction. With massive doses the venom causes vasoconstriction without a preliminary period of dilatation. The most obvious effect, and perhaps the more important one, is that of vasoconstriction. This effect has been observed in all the blood vessels examined. Some of the most serious effects were those directly upon the heart. The most consistent change seen in the electrocardiographic pattern of cats that were injected with small amounts of the venom was bradycardia with an increase in the PR interval giving a first, second, or third degree atrioventricular block. The second degree block was usually followed by sinus arrest. Reversal of the small dose effect occurred within 30 seconds following the end of the injection. When cats were given larger amounts of the venom, they showed in addition to the PR interval change, almost immediate ST, T wave change indicative of ischemia, and in some animals, true muscle injury. High concentrations of the venom caused marked vasoconstriction of the large arteries and veins as well as the arterioles. The direct effects on the heart muscle are quite drastic. The venom produces changes in the heart rate and amplitude of systole, and may cause complete, irreversible, cardiac standstill. It appears that stingray venom affects the normal pacemaker. The new rhythm evoked following cardiac standstill is frequently irregular and is believed to be elaborated outside the sino-atrial node (Russell and van Harreveld, 1954; Russell, Barritt, and Fairchild, 1957).

Stingray venom depresses respiration. Although part of the respiratory depression is secondary to the cardiovascular changes, the venom may have a direct effect on the respiratory centers of the medulla.

Stingray venom produces many changes in the behavior of animals. Some of these changes can be attributed to the direct effects of the venom on the central nervous system. In mammals the venom occasionally produces convulsive seizures, but the mechanism of these seizures is not apparent. They may be due in part to cardiovascular failure. Seizure patterns were not reported in electroencephalograms from anesthetized animals (Russell *et al.*, 1958). The venom does not seem to have a deleterious effect on neuromuscular transmission (Russell and Long, 1960; Russell and Bohr, 1962). When the venom is injected into the

lateral ventricles of mammals it produces transient apathy, astasia, and licking motions (Russell and Bohr, 1962). Mice injected with lethal doses of venom developed hyperkinesis, prostration, marked dyspnea, blanching of the ears and retina, and exophthalmos. These signs were followed by complete atonia, gasping respiratory movements, coma, and death. A similar syndrome was observed in cats and monkeys including ataxia, dilated pupils, increased salivation, micturation defecation, marked atonia, cyanosis, and hypoactive or absent deep and superficial reflexes. One monkey exhibited a conic-clonic generalized motor seizure accompanied by hypersalivation, twitching of the head, and marked dilatation of the pupils (Russell *et al.*, 1958; Russell, 1965).

Chemistry — There is very little information available regarding the chemistry of stingray venoms. The most specific data are those provided by Russell *et al.* (1958), Russell, Fairchild, and Michaelson (1958), and Russell (1965). The freshly prepared water extract of crude venom prepared from *Urobatis halleri* is described as clear, colorless, or faintly gray in color. The pH was 6.76. The crude extract loses its toxicity within 4 to 18 hours upon standing at room temperature but is more stable at lower temperatures or in 20 to 40 percent glycerol. The venom will not tolerate lyophilization. The total protein content was found to be approximately 30 percent, total nitrogen 3 percent, and total carbohydrate 3 percent. Ten amino acids have been found to be present. With the use of disc electrophoresis, they have identified 15 fractions in extracts from the venomous integumentary sheath of *U. halleri*. Extracts prepared from sponges that had been stabbed with fresh stings were found to contain 10 fractions. Further studies on these extracts using gel filtration (Sephadex G 100 and G 200) suggested that the toxic protein, or proteins, may have a molecular weight in excess of 100,000. The fraction of the toxin having the greatest lethality was found to have two or three distinct bands when subjected to disc electrophoresis. Crude venom extracts have been shown to contain serotonin, 5-nucleotidase, and phosphodiesterase. Protease and phospholipase activity were absent.

Class **OSTEICHTHYES**: Catfishes, Scorpionfishes, Toadfishes, etc.

Catfishes — The suborder SILUROIDEA includes a group of fishes having a wide variety of sizes and shapes. Their body shape may vary from short to greatly elongate, or even eel-like. The head is extremely variable, sometimes very large, wide or depressed, again very small. The mouth is not protractile but the lips are sometimes greatly developed, usually with long barbels, generally with at least one pair from rudimentary maxillaries, often one or more pairs about the chin, and sometimes one from each pair of nostrils. The skin of these fishes is thick and slimy, or with bony plates. There is an absence of true scales. About one thousand species are included within this group, most of which are found in the fresh-water streams of the tropics, but a few species are marine. Considering the large number of catfish species, amazingly little is known regarding the morphology of their venom organs or the nature of their venom. Most of the published literature on catfish venom organs deals with North American fresh-water species. Papers on these species have been published by Reed (1900, 1906, 1907, and 1924), and Halstead, Kuninobu, and Hebard (1953). There are no reports on the venom apparatus of any Brazilian catfish.

Family ARIIDAE: *Genidens genidens* (Cuvier and Valenciennes). Catfish (USA), mandi (Brazil). — Brazil. *Netuma barbus* (Lacépède). Sea catfish (USA), bagre marinho (Brazil). — East coast of South America, from Guianas to Argentina. *Scideichthys albicans* (Cuvier and Valenciennes). Catfish (USA), bagre marinho (Brazil). — Brazil.

Biology — The ariid catfishes are a large group of subtropical and tropical marine fishes which are worldwide in distribution. They are active fishes and unlike their freshwater counterparts in that they are constantly on the move, frequently in large schools. They resemble most other catfishes in appearance but differ from other species in that the anterior and posterior nostrils are close together, the latter covered by a valve. Sea catfish have an interesting habit in which the male catfish incubates their eggs by placing them in its mouth. The male places up to 50 eggs or more in his mouth for a period of two months. After the eggs are hatched out, the young fish remain in the mouth for an additional period of two weeks.

Morphology of the venom apparatus — The venom apparatus of catfishes consists of the dorsal and pectoral stings and the axillary venom glands. The dorsal and pectoral spines are comprised of modified or coalescent soft rays which have become ossified, and so constructed that they can be locked in the extended position at the will of the fish. The mature dorsal spine is a stoutly-elongate, compressed, tapered, slightly arched, osseous structure bearing a series of retrorse dentations along the anterior and posterior surfaces, and having an acute sagittate tip. The spine is generally enveloped by a thin layer of sparsely pigmented skin, the integumentary sheath, which is continuous with that of the softrayed portion of the fin. There is no external evidence of a venom gland. The shaft of the pectoral spine is similar to the dorsal spine in its general morphology.

Microscopic examination reveals that the level of the middle third, the sting may be divided into three distinct zones: a peripheral integumentary sheath, an intermediate osseous portion, and a central canal. The integumentary sheath is comprised of a relative thick outer layer of epidermis and a thin layer of dermis. The glandular cells which comprise the venom gland are most concentrated at the anterolateral and posterolateral margins of the sting where they are sometimes clumped two or three cells deep within the epidermal layer. The venom glands of most of the catfish species that have been studied appear as a cellular sheet wedged between the pigment layer and the stratified squamous epithelium of the epidermis. The microscopic anatomy of the dorsal and pectoral stings are similar in appearance. The axillary pore, which is the outlet of the axillary gland, is located just below the vertical center of the posthumeral process of the cleithrum. The gland is enclosed within a capsule of fibrous connective tissue, and is divided into three or four lobes which are further subdivided into a variable number of lobules. The lobules are composed of large secretory cells. It is believed that this gland may contribute to the venom supplied to the pectoral stings in those species of catfishes in which the axillary gland is present.

Clinical characteristics — The pain is generally described as an instantaneous stinging, throbbing, or scalding sensation which may be localized or may radiate up the affected limb. Some of the tropical species, such as *Plotosus*, are capable of producing violent pain, which may last for 48 hours or more. The area about the wound becomes pale immediately after being stung. The pallor is soon followed by a cyanotic appearance, and then by redness and swelling.

In some cases the swelling may be very severe, accompanied by numbness and gangrene of the area about the wound. Shock may be present. Improperly treated cases frequently result in secondary bacterial infections of the wound. Some species of catfishes may produce wounds which may take weeks to heal, but in most instances the wounds are of minor consequence. Deaths have been reported from the stings of some of the tropical catfishes.

Treatment — Symptomatic. There are no specific antidotes. Follow the same procedure as used in the treatment of stingray wounds.

Pharmacology — Unknown.

Chemistry — Unknown.

SCORPIONFISHES — The vast family of SCORPAENIDAE closely resemble the sea basses from which they are distinguished by a suborbital stay which is present in the scorpionfishes. The suborbital stay joins the other bones of the head to form a coat of mail which covers the whole head. The excessive number of spines about the head are characteristic of the members of this family. Scorpionfishes vary greatly in their form and coloration. A few species may attain large size and many are considered to be valuable food fishes. Representatives of the family are widely distributed throughout all tropical and temperate seas, and several species occur in Arctic waters. The family SCORPAENIDAE, as the name implies, includes some of the most dangerous species of venomous fishes known.

Family SCORPAENIDAE *: *Scorpaena brasiliensis* Cuvier. Barbfish (USA), peixe escorpião (Brazil). — Western Atlantic, from Virginia to Bahia (Brazil). *Scorpaena grandicornis* Cuvier and Valenciennes. Lionfish, long-horned scorpionfish (USA), peixe-escorpião (Brazil). — Western Atlantic, Florida to Brazil. *Scorpaena plumieri* Bloch. Spotted scorpionfish, sculpin (USA), peixe-escorpião (Brazil). — Western Atlantic, from Massachussetts to Brazil.

Biology — Members of the genus *Scorpaena* are for the most part shallow-water bottom dwellers, found in bays, along sandy beaches, rocky coast line, or coral reefs. Their habit of concealing themselves in crevices, among debris, under rocks, in seaweed, together with their protective coloration which blends them almost perfectly into their surrounding environment, makes them difficult to see. Scorpaenids are generally captured by hook and line, and in many regions they are a popular and important food fish. When they are removed from the water they have the defensive habit of erecting their spinous dorsal fin and flaring out their armed gill covers, pectoral, pelvic, and anal fins. The pectoral fins, although dangerous in appearance, are unarmed. Hinton (1962) and Breder (1963) have reported observations on the defensive behavior of *Scorpaena guttata* and *S. plumieri* respectively. Whenever an object comes in close proximity to *S. guttata*, the dorsal spines are immediately erected and the fish moves swiftly toward the object so as to deliver a sharp blow to the object with the side of its head or with its dorsal sting. In the case of *S. plumieri* it was observed that the fish was generally quiescent, but when touched with a stick, it would settle

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* Although only three species of scorpaenids are listed here, it is believed that any members of the genus *Scorpaena* inhabiting Brazilian waters are venomous. The species given in this list are the only three species listed in venomological literature.

down tightly on the sand and sometimes arch its back. The head of the fish was directed slightly downward and the opercula expanded. If the intrusion continued the fish would suddenly change its stance and expose large yellow patches on the pectoral fins. Prior to this, all of the exposed surface of the fish was somewhat drab, but with the change in stance the pectorals would be suddenly flipped over displaying the brightly colored undersurface. The pale dots near the base of the pectorals became a bright iridescent blue, and the interradial light-colored patches along the margin to mid-part of the fins were bright yellow. The dark area around the blue spots became an intense black and the fin margin gray. If the provocation continued, the fish would repeatedly ram or butt the intruding object.

Morphology of the venom apparatus — The venomous members of the family SCORPAENIDAE can be classified into three basic types on the basis of the morphology of their venom organs: (1) *Pterois* or zebrafish type; (2) *Scorpaena* or scorpionfish type; and (3) *Synancejа* or the stonefish type. Only the *Scorpaena* type are found among the Brazilian scorpionfishes. Unfortunately no one has described the venom apparatus of a Brazilian scorpionfish. Therefore one can only assume that the venom apparatus of *Scorpaena brasiliensis*, *S. grandicornis*, and *S. plumieri* resemble that of *S. guttata*, the California scorpionfish, which has been studied in detail. The following description is based on the studies of Halstead, Chitwood, and Modglin (1955). The venom apparatus includes 12 dorsal spines, 3 anal spines, 2 pelvic spines, their associated venom glands, and their enveloping integumentary sheaths. If the integumentary sheath is removed, a slender, elongate fusiform strand of grey or pinkish tissue can be observed lying within the glandular grooves on either side of the spine. Microscopic examination of cross sections of venom glands reveals a cluster a large polygonal glandular cells with pinkish-grey, finely granular cytoplasm located in the dermal layer within the anterolateral glandular grooves. The large venom-producing cells have a pinnate, heart-shaped arrangement, and vary greatly in size and morphology.

Clinical characteristics — Stings from scorpionfishes vary from one species to the next, but generally the introduction of scorpaenid venom immediately produces an intense throbbing pain. Within a few minutes the area about the wound becomes ischemic and then cyanotic. The pain becomes progressively more severe and may radiate to the groin or axilla. The intensity of the pain may be comparable to that produced by renal colic and may continue for several hours. Within a short period of time the affected part becomes swollen, erythematous, and indurated. Profuse perspiration, pallor, dyspnea, restlessness, nausea, vomiting, diarrhea, loss of consciousness, and extreme tachycardia are commonly present. Abscesses, necrosis, and sloughing of the tissues about the wound have been reported. Bayley (1940) and Colby (1943) state that a maculopapular or scarlatiniform rash over the body may occur. Cecca (1902) cites a case which resulted in peripheral neuritis, paralysis, and muscular atrophy due to a sting by *Scorpaena nera*. Secondary bacterial infections, tetanus, and primary shock are frequent complications which must be considered. According to Blanchard (1890), Coutiere (1899), Scott (1921), and Colby (1943), scorpionfish stings may result in death.

Treatment — Scorpionfish sting should be treated in the same manner as stingray envenomations.

Pharmacology — There are no reports available on the pharmacology of the venom of Brazilian scorpionfishes.

Chemistry — There are no reports available regarding the venom of Brazilian scorpionfishes.

TOADFISHES — The BATRACHOIDIDAE, or toadfishes, are a group of small bottom fishes which inhabit the warmer waters of the coasts of America, Europe, Africa, and India. Toadfishes are of little commercial value and are not generally considered as food fishes, although they are eaten in some countries. The flesh is said to be fine-flavored, but the fishes are small in size and bony. According to Taschenberg (1909), the liver of some of the batrachoids is poisonous to eat. However, the greatest interest of these fishes to biotoxicologists is their unique and highly developed venom organs.

Family BATRACHOIDIDAE: *Marcgravichthys cryptocentrus* (Valenciennes). Toadfish (USA), niquim-niquim, sapo (Brazil). — Brazil. *Thalassophryne amazonica* Steindachner. Brazilian toadfish (USA), pocomon, niquim-niquim, sapo (Brazil). — Mouth of the rivers Negro, Amazon and Xingu (Brazil). *Thalassophryne branneri* Starks. Toadfish (USA), niquim-niquim, sapo (Brazil). — Brazil. *Thalassophryne punctata* Steindachner. Toadfish (USA), niquim-niquim, sapo (Brazil). — Brazil.

Biology — Batrachoid fishes, with their broad, depressed heads and large mouths, are somewhat repulsive in appearance. Most toadfishes are marine, but some are estuarine or entirely fluviatile, ascending rivers for great distances. They appear to enjoy turbid water. Regardless of the type of water in which they are found, batrachoids are primarily bottom fishes. They hide in crevices, burrows, under rocks, debris, among seaweed, or lie almost completely buried under a few centimeters of sand or mud. Fróes (1932, 1933a) stated that the Brazilian species of *Thalassophryne* has the habit of covering itself with a thin layer of sand or mud, but with careful observation one can usually detect the outline and protruding eyes of the fish as one wades along in the clear shallow water of sandy beaches. Toadfishes are quite hardly and are able to live for several hours after being removed from the water. According to Goode (1884), the bottom temperature of the water frequented by these fishes would appear to range from 10°C to 32°C. Toadfishes tend to migrate to deeper water during the winter months where they remain in a somewhat torpid condition.

They also are experts at camouflage. Their ability to change their color to lighter or darker shades at will and their mottled pattern make these fishes difficult to see.

Most toadfishes tend to be somewhat sluggish in their movements, but when after food they can dart out with surprising rapidity. They are somewhat omnivorous in their eating habits but seem to prefer among other things, crabs, mollusks, worms, and small fishes. Toadfishes are said to be quite vicious and will snap at almost anything upon the slightest provocation. Although they are not capable of producing a severe wound, they can inflict a bite that is not readily forgotten. When they are disturbed or their dorsum touched, they immediately erect their dorsal spines and flare out their opercular spines in defiance. Toadfishes do not school, but they are gregarious and tend to congregate together. For additional information regarding the habits of these interesting fishes, the excellent works of Gill (1907) and Gudger (1910) are recommended.

Morphology of the venom apparatus — The venom apparatus of toadfishes consists of two dorsal fin spines, two opercular spines, and their associated venom glands. In the case of *Thalassophryne dowi*, which can be considered as typical of the group, there are two dorsal spines which are enclosed together within a single integumentary sheath. The dorsal spines are slender and hollow, slightly curved, and terminate in acute tips. At the base and tip of each spine is an opening through which the venom passes. The base of each dorsal spine is surrounded by a glandular mass from which the venom is produced. Each gland empties into the base of its respective spine. The operculum is also highly specialized as a defensive organ for the introduction of venom. The horizontal limb of the operculum is a slender hollow bone which curves slightly, and terminates in an acute tip. Openings are present at each end of the spine for the passage of venom. With the exception of the extreme distal tip, the entire opercular spine is encased within a glistening, whitish, puriform mass. The broad rounded portion of this mass is situated at the base of the spine, and tapers rapidly as the tips of the spine is approached. The pyriform mass consists of a tough sac-like outer covering of connective tissue in which is contained a soft, granular, gelatinous-like substance having the appearance of fine tapioca. This mass is the venom gland. The gland empties into the base of the hollow opercular spine which serves as a duct.

Microscopic examination of the venom glands shows strands of aerolar connective tissue, large distended polygonal cells filled with finely granular secretion, and vascular channels. In some instances the polygonal cells will appear to have undergone complete lysis and there remain only areas of amorphous secretion. The microscopic anatomy of the dorsal and opercular venom glands are essentially the same.

Clinical characteristics — The pain from toadfish wounds develops rapidly, is radiating and intense. Some have described the pain as being similar to that of a scorpion sting. The pain is soon followed by swelling, redness, and heat. No fatalities have been recorded in the literature. Little else is known about the effects of toadfish venom.

Treatment — Toadfish stings should be handled in a manner similar to stingray envenomations.

Pharmacology — The only published reports on toadfish venom are those by Fróes (1933). Injections of the venom into guinea pigs and chicks resulted in mydriasis, ascites, paralyses, necrosis about the injection site, convulsions and death. The author believes that the venom of *Thalassophryne* has both proteolytic and neurotoxic properties.

Chemistry — Unknown.

MISCELLANEOUS VENOMOUS FISHES — There does not appear to be any data available on other kinds of venomous fishes in Brazilian waters.

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2. TAXONOMIA DE *ANTHOZOA* (*COELENTERATA*) BRASILEIROS: DISTRIBUIÇÃO E FREQÜÊNCIA EM ÁGUAS BRASILEIRAS

DIVA DINIZ CORRÊA

Departamento de Zoologia, Universidade de São Paulo, São Paulo, Brasil

A classe **ANTHOZOA**, a maior classe do filo COELENTERATA, é composta de duas subclasses, **ALCYONARIA (OCTOCORALLIA)**, que abrange seis ordens e **ZOANTHARIA (HEXACORALLIA)**, com cinco ordens.

Entre as ordens maiores as melhores conhecidas para o Brasil são a ordem **GORGONACEA** (subclasse **ALCYONARIA**) com 17 espécies, a ordem **ACTINIARIA** (subclasse **ZOANTHARIA**) com 14 espécies e a ordem **MADREPORARIA** (subclasse **ZOANTHARIA**) com 14 espécies. Entre as ordens menores, que são ainda menos conhecidas, posso mencionar apenas uma espécie da ordem **TELESTACEA** (subclasse **ALCYONARIA**), *Telesto risei*, uma espécie da ordem **PENNATULACEA** (subclasse **ALCYONARIA**), *Renilla mülleri* e uma espécie da ordem **CERIANTHARIA** (subclasse **ZOANTHARIA**), *Ceriantheomorphe brasiliensis*. Isto significa um conhecimento ainda muito pequeno para a totalidade do litoral brasileiro. São no total 48 espécies numa classe que contém cêrca de 6.000 espécies.

De acôrdo com meu conhecimento, os livros mais modernos sôbre a sistemática da classe **ANTHOZOA**, nos quais material brasileiro foi considerado são o de Frederick M. Bayer (1961), "The shallow-water **OCTOCORALLLIA** of the West Indian region", e o de F. G. Walton Smith (1948), "Atlantic reef corals — a handbook of the common reef and shallow-water corals of Bermuda, West Indies and Brazil".

A inclusão de animais brasileiros nos dois livros é baseada em razões zoogeográficas. Faunìsticamente extensões das Índias Ocidentais penetram no Gôlfo do México e ao longo da costa leste da Flórida, para o sul, ao longo da costa nordeste da América do Sul até os recifes do Brasil. É impossível traçar um limite rígido entre a fauna do Gôlfo do México e da costa sudeste dos Estados Unidos com aquela das Índias Ocidentais, a qual inclui também quase tôda a informação disponível da fauna de **ANTHOZOA** do Brasil.

Com exceção de alguns relatórios fornecidos por algumas investigações pesqueiras recentes, a costa da América do Sul, ao sul e a leste de Trinidad, constitui uma lacuna nos nossos conhecimentos faunísticos neste grupo de animais. Algumas coleções mais antigas desta área foram relatadas, muito inadequadamente, por Stiasny (1951) e ainda mais antigo é o estudo de Verril (1912) sôbre **GORGONACEA** da costa brasileira. Coleta completa nesta região está ainda grandemente necessitada.

De particular interêsse é o material de **GORGONACEA** obtido, durante as explorações pesqueiras mencionadas, na costa das Guianas e do Brasil pelo navio pesqueiro 'Coquette' em 1957 e pelo 'Oregon' em 1957 e 1958. A presença nestas regiões de algumas espécies como *Iciligorgia schrammi, Diodogorgia nodulifera, Elisella barbadensis* e *Elisella elongata* mostra que a fauna fora da praia, mas em águas relativamente rasas, apresenta decididamente um sabor das Índias Ocidentais e as regiões citadas seriam apenas uma extensão faunística antilhana. Em profundidades maiores, de cêrca de 90 a 200 metros, a ocorrência de gêneros como *Thesea, Muricea, Elisella* e *Callogorgia* indica que as afinidades faunísticas com as Índias Ocidentais ainda persistem nesta indicação batimétrica.

A fauna de recife do Brasil contém elementos endêmicos como as espécies *Phyllogorgia dilatata* e *Plexaurella grandiflora* e elementos não endêmicos, do Atlântico Ocidental, como *Leptogorgia virgulata* e *Lophogorgia hebes*, os quais se estendem em direção ao norte até às vizinhanças do Cabo Hatteras na costa norte-americana. Os limites geográficos dos componentes endêmicos não são conhecidos. Aproximadamente as mesmas afirmações podem ser tomadas em consideração para os corais verdadeiros, ordem **MADREPORARIA.**

Com exceção das 14 espécies da ordem **ACTINIARIA**, as quais foram estudadas por especialista brasileiro, tôdas as outras espécies de **ANTHOZOA** mencionadas foram estudadas fora do Brasil por especialistas estrangeiros.

A fim de melhorar o conhecimento desta grande classe de animais marinhos foi iniciado recentemente pelo Departamento de Zoologia da Universidade de São Paulo, um levantamento das espécies ocorrentes na costa do Estado de São Paulo. Êste levantamento abrange no momento o estudo de três ordens apenas, a ordem **GORGONACEA**, as gorgônias, a ordem **ACTINIARIA**, as anêmonas do mar e a ordem **ZOANTHIDAE**, cujos representantes são chamados popularmente de palitoas, de acôrdo com o seu gênero maior e melhor conhecido, o gênero *Palythoa*.

O estudo das anêmonas do mar está mais adiantado que o das outras ordens, mas ainda encontra-se em sua fase inicial. Por enquanto estamos realizando um levantamento faunístico, sistemático, a fim de conhecermos os animais e podermos com isso fornecer material classificado para interessados em estudos de fisiologia, ecologia, genética, microscopia eletrônica e bioquímica. Como disse Cadet Hand (1961), estudioso americano dos Celenterados, o conhecimento dêste importante filo do reino animal ainda está na sua infância e dificilmente outro campo da Zoologia pode apresentar tantos problemas como o dos nematocistos, cápsulas urticantes, característica do filo.

A área escolhida situa-se entre Itanhaém e Ubatuba, abrangendo, portanto, uma parte do litoral sul de São Paulo, todo o litoral centro e todo o litoral norte. As espécies estudadas, com exceção de uma que é comensal com eremitas ou caranguejos, ocorrem na zona das marés, usando rochas, areia, lôdo e algas como substrato. Enumeradas em ordem sistemática, essas espécies são: *Actinia bermudensis* (McMurrich, 1889), *Anemonia sargassensis* Hargitt, 1908, *Bunodosoma caissarum* Corrêa, 1964, *Bunodosoma cangicum* Corrêa, 1964, *Anthopleura cascaia* Corrêa, 1964, *Phyllactis conchilega?* (Duch. & Mich., 1860), *Phymanthus canous* Corrêa, 1964, *Paranthus rapiformis* (Lesueur, 1817), *Calliactis tricolor* (Lesueur, 1817) e *Aiptasia pallida* (Verrill, 1864).

Pouco pode ser dito a respeito da distribuição e freqüência destas espécies para a totalidade do litoral brasileiro. A espécie comensal, *Calliactis tricolor*, ocorre desde o Ceará até o extremo sul do Estado de São Paulo, Ilha do Bom Abrigo,

Cananéia, sendo relativamente comum em profundidades de cêrca de 5 metros. Sete espécies são conhecidas desde Ubatuba até Itanhaém e apresentam, algumas delas, intensa freqüência em certas localidades como a Enseada do Flamengo e a Praia do Segrêdo, no litoral norte, a Ilha das Palmas, na Baía de Santos e a Praia do Sonho, em Itanhaém. Duas espécies apresentaram até agora uma ocorrência muito restrita em ambientes muito especiais e freqüência bastante baixa. São o *Phymanthus canous* de praia lodosa do Saco da Ribeira e o *Paranthus rapiformis* de areia da Praia de Baraqueçaba, ambos os locais situados no litoral norte do Estado de São Paulo.

Tendo estudado também 16 espécies de anêmonas do mar da Ilha de Curaçau, Antilhas Holandesas, sugeri a hipótese que algumas espécies que ocorrem nesta ilha em praias tìpicamente coralinas, ricas em corais vivos, deveriam também ser encontradas em ambientes coralinos do litoral brasileiro. Quatro confirmações já foram obtidas. *Lebrunia coralligens* (Wilson, 1890) e *Condylactis gigantea* (Weinland, 1860) ocorrem no Arquipélago dos Abrolhos, sul da Bahia. *Lebrunia danae* (Duch. & Mich., 1860) e *Stoichactis helianthus* (Ellis, 1767) foram verificadas em uma praia de Recife, Pernambuco.

Nenhum caso de queimadura ou de envenenamento é do meu conhecimento nas regiões onde tenho trabalhado, a Ilha de Curaçau e a costa de São Paulo. Afora a capacidade intensa de adesão tentacular que algumas espécies apresentam, não tive até agora nenhuma sensação de queimadura após o contato direto com as 23 espécies que estudei.

Consultando o livro de Keegan e MacFarlane, referente ao *Simpósio sôbre animais venenosos e plantas nocivas da região do Pacífico* (1963), no artigo de R. V. Southcott, sôbre Celenterados de importância médica, verifiquei que os 30 casos de envenenamento conhecidos ocorreram entre o norte da Austrália e o continente asiático até o norte das Filipinas. São mencionados casos de injúrias causadas pelo contato com anêmonas do mar, com corais verdadeiros e com corais falsos que pertencem a outra classe dos Celenterados.

A anêmona do mar *Sagartia elegans* (Dalyell, 1848), de acôrdo com Halstead (1959), é a causadora da "sponge diver's disease" ou "maladie des plongeurs" no Mediterrâneo. Estas anêmonas utilizam-se das esponjas como seu principal substrato. Os velhos pescadores de esponjas possuem numerosas cicatrizes nas mãos causadas pelas injúrias. Outras espécies como *Actinodendron plumosum*, *Actinodendron arboreum*, *Actinia equina*, *Adamsia palliata* e *Anemonia sulcata* são mencionadas como causadoras de severas queimaduras. Corais causam úlceras na região indo-pacífica assim como *Acropora palmata* das Índias Ocidentais.

Uma espécie conhecida como "matamalu" em Samoa, *Rhodactis howesii*, semelhante a uma anêmona do mar verdadeira, quando cozida, faz parte da alimentação dos nativos. São conhecidos casos de envenenamento após ingestão acidental ou intencional destas anêmonas cruas.

Summary

The present "mise au point" about the state of research on **ANTHOZOA** (COELENTERATA) from the Brazilian coast, including taxonomy, distribution, and frequency, shows that very little is known up to now of this large Class of marine invertebrates. Of the about 6,100 species in the group, only 17 **GORGONACEA**, 14 **ACTINIARIA**, 14 **MADREPORARIA**, 1 **TELESTACEA**, 1 **PENNATULACEA** and 1 **CERIANTHARIA**, were already described from Brazilian waters.

The Department of Zoology, University of São Paulo, Brazil, has recently started a survey of the species of two larger Orders, the **GORGONACEA** and the **ACTINIARIA**, occurring on the coast of São Paulo. The chosen area extends from Itanhaém, on the Southern, to Ubatuba, on the Northern part. Ten species of sea-anemones were already described and a few more are in process of study together with several species of **GORGONACEA**.

Some of the ten above mentioned species of sea-anemones have a large distribution and frequency in the Western Atlantic Ocean. A few others are as yet known only for the coast of São Paulo, one or 2 of which have a low frequency.

No severe injure to man, caused by these animals, has been noticed among the Brazilian species, as it is known for some sea-anemones and corals occurring in the others parts of the world.

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3. TOXIC MARINE INVERTEBRATES — VENOMOUS AND NOXIOUS FISHES OF FRESH WATER

PAULO SAWAYA

Departamento de Fisiologia Geral e Animal, Universidade de São Paulo, São Paulo, Brasil

TOXIC MARINE ANIMALS — Research on toxic marine animals has been done in Brazil by Faria (1914, p. 29) on PROTOZOA. *Pteridinium tochoideum*, which is associated with mass mortality of marine organisms, and *Prorocentrum sp.* were described. As it is well known, the first one is very common on the Brazilian coasts, but their appearance in mass is, fortunately, not very frequent. In the sound of São Sebastião, where the laboratory of the Marine Institute of Biology is located, the red tide has been observed in 10 years only once.

MARINE INVERTEBRATES — PORIFERA — De Laubenfels (1932, p. 85) says that *Tedania toxicalis* is toxic and its toxicity is one of its striking characters. The spicules are long and provoke a very strong irritation on the hands. Even other animals are sensitive to those spicules. According to De Laubenfels (l.c.) if a specimen of this sponge be placed in a bucket with other living sea animals, as for example, fish, molluscs, crabs, and worms, in an hour or less they are observed to die, while in controls lacking the sponge they survive.

It seems that not all species are sensitive to the toxin because the sponges live in very dense community with other animals, chiefly, Echinoderms (Ophiuroids), small crustaceans (Amphipods, Polychaetes), etc.

The zonation of these animals is very interesting. Their habitat is restricted to the middle and inferior littoral.

In the same place, on the beach of the São Sebastião sound, there are a number of Coelenterates. For example, *Palythoa sp.* are very common. The abundant mucus secreted by these animals, injected into the vascular system of some mammals, such as the Rat, seems to be toxic. The mechanism of secretion of this mucus, and the toxicity is unknown. Some experiments have been run at the laboratory of the Institute of Marine Biology at São Sebastião, 230 km to the north of São Paulo, and we expect to confirm or not its toxicity.

ECHINODERMS — It is known that several Echinoids are venomous. In the Institute of Marine Biology, Mendes, Abbud and Umiji (1963, p. 408) have studied a substance produced by the pedicellariae of the Sea Urchin *Lytechinus variegatus*. It has an acetylcholine-like behavior, according to the results obtained on the responses of the guinea pig illeum, rat uterus, blood pressure in the dog, heart-beating of toad, longitudinal muscle of holothurian, and the protractor muscle of the sea urchin lantern.

The famous venomous sea-urchin gen. *Diadema* does not occur on the Brazilian coast. The Sea Urchins common here are *Lytechinus variegatus, Echinometra lucunter* and *Arbacia lixula. Echinometra lucunter* is the most common sea-urchin on the Brazilian seashores. The spines are straight and pointed. It is not known if the spines of *Echinometra* have the so-called poison glands. They are borne in a tubercule of the thecal plate. When the spines of *Echinometra* perfurate the skin the wound is painful, and sometimes an inflammation occurs.

Diadema has been captured in the bay of Acapulco, Mexico. Some spines can exceed 50 cm in length, and injure the skin when touched. I know this by my own experience when I visited the Acapulco bay. Russell (1965, p. 285) says: "There does not appear to be any biochemical or toxicological evidence, at the present time, to indicate that these structures do indeed contain a poison". All I know is that when the spines are touched the skin shows an irritation and is painfull.

Holothurians — On the Brazilian sea-shore *Holothuria grisea* is very common. They have some toxin in the Cuvierian tubules. Some investigators think that the toxin secreted by the Cuverian tubules is related to the fact of evisceration, shown by this animal, when out of water, or when exposed to excessive changes of temperature, pH and oxygen tension. Nigrelli (1952, p. 89) named the substance of the Bahamian sea cucumber, *Actinopyra agassizi,* as holothurin. It is composed of 60% glycosides and 30% pigment salts, polypeptides and free aminoacids, 5 to 10% insoluble protein and 1% cholesterol. Holothurin has deleterious effects on *Hydra, Planorbis,* and *Tubifex.*

Worms — Among the Polychaetes several species are toxic. On the Brazilian coasts the AMPHINOMIDAE are very frequent. Hartmann (1951, p. 21) indicates that the common name, "fire-worm" alludes to the stinging sensation caused from handling specimens. The injury is mechanical, resulting from the penetration into the skin of numerous, fine, glass-like, harpoon-shaped setae that are difficult to remove. Inflammation and considerable discomfort result, but there is no permanent injury.

I have observed these worms under the stones in the intertidal zone, and the collectors must be careful, because they can irritate the hands. Several cases, that I observed indicate that the sensitiveness of people is different in degree, probably related to some allergic reaction.

The same Polychaetes (AMPHINOMIDAE) were considered long time ago as venomous, according to Baird (1864, p. 450) who says: "The specimens of this worm *Amphinome didymobranchiata* came from the Island of Ascención where they are collected by the boatmen and sold as curiosities. They pretend that they are of venomous nature, and are able to inflict serious wound upon those who incautiusly handle them. This idea no doubt takes its origin from numerous setae with which their feet are clothed, but which (to judge from their appearance...) in reality powerful weapons for offense and defense against these animals which prey upon or are fitted for food for them are in fact powerless for harm to human beings". Arndt (1930, p. 292) refers to noxious Polychaetes such as *Hermodice corunculata,* which is very toxic.

According to Russell (1964, p. 480) the composition of the venoms of marine animals varies considerable. Among the Coelenterates there are the following composition: several quaternary ammonium compounds, the most toxic of which

being the tetramethylammonium hydroxide our "tetramine", 5-hydroxytriptamine, histamine and histamine releasers, several proteins whose composition has not yet been determined.

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VENOMOUS AND NOXIOUS FRESHWATER FISHES — South American freshwaters are populated by several species of venomous fishes.

The so-called black rays or *Arraia arara* and *Arraia pintada* (*Trygon strongylopterus*, *Potamotrygon brachyurus* or *Partrygon motoro*), are small rays, found in the rivers Amazon and Paraguai. One or two stings are located in the tail. They are known also by the common name of stingray. The sting is bilaterally serrated. The venom and also the venom apparatus of Brazilian stingray have not been studied. Russell (l.c., p. 339) describe the marine stingray, *Urolophus halleri*, and says that the venom is known to exert a deleterious effect on the mammalian cardiovascular system. It causes bradycardia and increase the PR interval of the ECG.

Observations of some cases of attacks by the *Partrygon* from the Araguaia river, in the north of Brazil, indicates that the sting delivers a large quantity of mucus, and the venomous substances probably exist in it.

Another venomous fish is *Potamotrygon brachyurus* from Guyana, Venezuela and the Amazon region. Some information on the biology of this ray has been quoted by Dr. Hermann von Ihering. The fishermen say that this fish when attacked keep the fry in the vagina.

SILURIDAE — In Brazilian rivers several fishes of this family are very common. Most of them have the first ray of the pectoral and dorsal fins serrated. When caught they attack the men with this serrated ray. In the beginning the wound provokes only a strong pain, but afterwards as a result of the mucus left inside the skin, inflammation occurs, followed by headache and in some cases vomiting. It has not been demonstrated up to now if this serrated fin ray is venomous or not in spite of the information from Lagler, Bardach and Miller (1962, p. 132) who indicate that catfishes (*Clarias* and others) possess spines of dorsal and pectoral fins with glands beneath the skin, opening through poses at bases of spines.

The same authors (l.c., p. 184) inform that in the North American freshwater catfishes (ICTALURIDAE), the sharp, hardened ray of the leading edge of the pectoral fin has a locking structure, which enables the catfish to erect and hold it erect, presumably as an instrument of combat. In the madtonus (*Noturus*) this spinous ray has a special gland at its base. The secretion of the gland, injected by the spine, has a stinging, paralyzing effect on man.

In this family several fishes are edible and the fishermen cut out the first ray of the pectoral and dorsal fins to maintain the fishes in the hand. In the creeks and rivers of the outskirts of São Paulo, fishes of the family PIMELODIDAE (*Pimelodus sp.*, and *Clarias sp.*) and others can be easily found. When caught the fish spread out the rays of the dorsal and pectoral fins for defense. If the fisherman is wounded by the serrated first ray and inflammation usually follows.

Russell (1965, p. 480) informs that fish venoms are composed of 3 to 10 proteins and have little or no enzymatic activity.

Other fishes — The famous piranha (sub fam. SERRASALMONINAE) from tropical waters can be here included. These fishes usually damage cattle, other

fishes and men. The fishermen say that the piranhas smell blood, and by this attack the victim. Cannibalism also occurs.

Another curious fish noxious to man is the so-called Candiru (*Vandellia cirrhosa* or *Hemicetopsis candiru*). People say that this small fish of 3-5 cm in length penetrates the urethra of man and the vagina of woman when they are taking a bath. When the fish penetrates the urethra there is an copious bleeding. Eingenmann and Allen (1942, p. 142) give a long report on the "candiru" and say (p. 146-147) that "there is little clear-cut evidence by which we can definitely sort out cases of urinotropism from parasitism".

Santos (1962, p. 114) also describes this curious fish, but by the figure and the size of the animal it does not seem probable that it can enter into a man's urethra.

Within the TETRAODONTIDAE there is a species, *Colomesus psittacus*, named "baiacu" or "mamaiacu", which similar to its relative from the sea water, is believed to be venomous (Couto Magalhães 1931, p. 95). It lives in the rivers of the North of Brazil. The flesh is edible but the liver is very toxic. The fish is small (18 cm in length) and usually can be kept in aquarium as an ornamental fish.

The South American electrical — *Electrophorus electricus* — could be listed here. This fish is neither toxic nor venomous, but is noxious to the human being and other animals.

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4. LA FONCTION VENIMEUSE CHEZ LES ARAIGNÉES

J. VELLARD

Instituto Boliviano de Biología de Altura, La Paz, Bolivia

MORPHOLOGIE DES GLANDES — Deux familles à caractères archaiques bien marqués, les FILISTATIDAE et les SCYTODIDAE s'éloignent de toutes les autres araignées par la morphologie des glandes de leurs chélicères.

Celles des FILISTATIDAE sont multilobées et occupent une grande partie du céphalothorax.

Dans le genre *Scytodes*, des glandes volumineuses donnent à la région thoracique sa forme bombée, caractéristique du genre. Les travaux de Millot, et avant lui ceux de Monterosso, ont montré que ces glandes se divisent en deux parties histologiquement differentiées, séparées par un repli interne: une partie antérieure, souvent réduite, correspondant aux glandes venimeuses habituelles; et une partie postérieure beaucoup plus développée, à sécretion basophile, visqueuse, très voisine de celle des glandes sérigènes et servant à ces araignées à engluer leurs proiés. Cette double fonction rappelle celle des glandes des chélicères des pseudoscorpions.

Dans un second genre de SCYTODIDAE, le genre *Sicarius* (nous avons surtout étudié *S. peruviensis*), les glandes volumineuses, pouvant atteindre 6 mm de longueur, sont divisées extérieurement en nombreux lobes granuliformes, à paroi très mince.

Un troisième genre, *Loxosceles* (*L. laeta* et autres espèces sud-américaines) marque le terme de l'évolution de cette famille. A l'extérieur, les glandes sont divisées du côté interne par de profondes incisions correspondant a des cloisons intérieures séparant incomplètement la lumière de la glande en plusieurs lobes.

Des glandes en partie lobulées existent encore chez les PALPIMANIDAE: *Anisaedus stridulans* de la côte du Pérou.

Le type classique, en forme de sac, lisse extérieurment, sub-cylindrique ou plus ou moins arqué ou coudé, se trouve dans toutes les autres familles étudiées.

Les glandes des Mygalomorphes, sauf rares exceptions, sont logées dans la convexité des chélicères.

Chez les aranéomorphes, elles sont entièrement thoraciques ou partiellement engagés à la base des chélicères (CLUBIONIDAE, DRASSIDAE, SALTICIDAE, TETRAGNATHIDAE). Les glandes des PALPIMANIDAE très réduites, sont situées dans les chélicères (*Anisaedus*).

HISTOLOGIE DES GLANDES — Chez toutes les araignées les glandes sont constituées par une fine adventice conjonctive externe, qui parfois disparaît sur les coupes; une tunique musculaire d'épaisseur variable, formée de fibres striées disposées en spirale et une basale sur laquelle repose l'épithelium glandulaire.

L'adventice conjonctive envoie de fins prolongements à travers les fibres musculaires jusqu'à la basale.

La tunique musculaire, bien développée chez les Mygalomorphes et chez beaucoup d'Aranémorphes, peut être parfois très mince, comme chez *Ctenus medius* et les Dysdéridés; chez d'autres araignées elle atteint au contraire un développement considérable (*Nephila cruentata*).

Sur les coupes l'aspect de la glande varie suivant la phase de sécrétion du venin.

Au début, les cellules épithéliales, formées d'éléments prismatiques, à petit noyau basal, reposent sur la basale et sur les franges internes très développées formant un réseau remplissant la lumière de la glande.

A une période plus avancée, les cellules augmentent de volume de la base au sommet de ces franges qui disparaissent en grande partie; les cellules se remplissent de granulations acidophiles comprimant le noyau vers la périphèrie; puis elles se rompent et les noyaux déjà peu visibles sont mis en liberté au milieu de la sécrétion acidophile remplissant le centre de la glande.

En fin de sécrétion, les franges internes ont à peu près disparu. La sécrétion acidophile, presque sans vestiges de noyaux, occupe toute la lumière de la glande, comprimant contre la basale les cellules épithéliales. La glande n'étant plus soutenue par le réseau des mailles internes se déforme facilement à la coupe.

Dès que la glande a vidé son contenu, les cellules épithéliales entrent en prolifération active.

Le temps nécessaire pour remplir les glandes de venin dépend de nombreux facteurs et varie d'une espèce à l'autre: 5 ou 6 jours au moin pour *Phoneutria fera.*

Rôle du venin. Mécanisme d'inoculation — Les chélicères des araignées ont conservé leur rôle primitif d'organes de préhension. L'existence de la glande venimeuse accroît leur valeur d'armes de chasse.

L'injection du venin est volontaire, non subordonnée au mouvement des chélicères, et sous la dépendance de la tunique musculaire striée propre des glandes. L'action mécanique des chélicères suffit souvent pour immobiliser et tuer les petites proies, sans intervention du venin. Tenue avec une pince l'araignée peut mordre les animaux qui lui sont présentés sans innoculer de venin, faussant ainsi le résultat de beaucoup d'expériences. Le cas est fréquent avec des mygales à venin très actif, telle que *Trechona venosa*, dont l'action de la morsure ne peut être étudiée par à procédé.

Le venin des araignées est dépourvu d'action protéolytique et n'intervient pas dans la digestion; ce rôle est réservé aux glandes des maxilles dont l'activité digestive est très élevée.

Variation du venin — L'activité et les proprietés du venin varient de mode considérable d'un groupe à l'autre et il est possible de caractèriser des types de genres ou de familles, tels que le venin des DIPLURIDAE, celui des Aviculaires ou ceux de *Latrodectus* ou de *Phoneutria*. Les propriétés du venin s'ajoutent aux élements morphologiques pour identifier certains phylums.

Dans une famille ou dans un genre, quelques espèces peuvent se distinguer par une activité particulière, exaltation des propriétés communes à tout le groupe: parmi les SCYTODIDAE, les *Loxosceles* possèdent un venin de même nature

que celui des *Sicarius*, mais beaucoup plus actif. Seuls les Latrodectes noirs et rouges sont dangereux pour l'homme: le venin des Latrocdetes fauves ou gris (*L. geometricus*, par exemple), montre des propriétés identiques, mais bien moins accentuées, propriétés qui se retrouvent à un degré moindre chez de nombreux THERIDIIDAE.

Ces variations du venin, d'origine génètique, peuvent s'observer à l'intérieur d'une espèce, associées ou non à celles d'autres caractères morphologiques ou éthologiques peu apparents; certains auteurs ont conclu ainsi à l'existence d'espèces physiologiques ou cryptiques: le cas s'est produit pour *Latrodectus mactans*, de Santiago del Estero. Le venin de cette espèce offre d'ailleurs de grandes variations régionales dans son aire très vaste de dispersion.

Les différences individuelles ont beaucoup moins d'importance et il est toujours possible d'établir des moyennes d'activité pour une espèce dans une région ou dans des conditions saisonnières ou climatiques données.

L'influence du climat et de la température sont en effet considérables sur les araignées. Dans mes premiers travaux au Brésil j'avais déjà noté de variations sensibles de l'activité du venin de divers Ctenides des environs de Rio de Janeiro (*Ph. fera, Ct. medius, Ct. ornatus*) suivant l'époque de l'année, la température et le degré hygrométrique. Le maximum de toxicité s'observait par temps chaud et humide. Il m'avait même été possible en plaçant des araignées vivantes dans des chambres à températures différentes (+15° et +30°C) de contrôler expérimentalement ces observations. Les modifications n'affectent le venin que pendant la période de sécrétion et non le venin déjà élaboré remplissant la glande. En même temps que la toxicité augmente, le pH du venin s'abaisse.

J'ai eu l'occasion d'observer des faits identiques avec *Loxosceles laeta*. Le venin des exemplaires des environs de Lima est beaucoup plus toxique que celui fourni par des exemplaires du Chili.

Exemplaires de Lima: avec 0,25 glande, 100% de mortalité pour le cobaye; avec 0,10 glande, 50 à 70% de mortalité.

Exemplaires de Santiago: avec 0,40 glande, 50 à 70% de mortalité; beaucoup d'animaux résistent à l'injection d'une demie glande.

Il est par contre plus difficile d'obtenir chez le cobaye des lésions de nécrose avec le venin de Lima: la dose limite mortelle étant très proche de la dose nécrosante, ces deux seuils sont plus largement séparés avec le venin de Chili.

Des exemplaires recueillis non pas sur la côte, mais au-dessus de Lima, entre 2.000 et 2.500 mètres d'altitude, en climat plus froid, ont donné des résultats identiques à ceux du venin chilien.

SPÉCIALISATION DU VENIN — La toxicité plus marquée du venin pour les proies habituelles est un fait banal chez beaucoup d'animaux venimeux. Il se retrouve chez les araignées et pour juger de l'activité réelle de leur venin, il est nécessaire de l'étudier sur une série étendue d'animaux d'experience.

Tous ces venins sont généralement très actifs pour les insectes, mais un bon nombre d'entre eux présentent aussi une toxicité élevée pour des groupes très differents.

Le venin des *Latrodectus* possède une activité particulière pour les scorpions, mais est également dangereux pour les vertébrés et pour l'homme.

Le venin de *Trechalea* et d'autres PISAURIDAE aquatiques possède une action marquée sur les têtards et les petits poissons.

Les grands *Enoploctenus* qui chassent la nuit à l'entrée des grottes et sur les parois de rochers ont un venin beaucoup plus actif que celui des autres *Ctenus* pour les geckos qui abondent aux mêmes endroits.

Le venin de nombreuses THERAPHOSIDAE se montre curarisant pour les vertébrés; beaucoup de ces grosses mygales capturent des lézards, des petits rongeurs, ou même de jeunes oiseaux.

Un des cas les plus remarquables est celui de grandes *Grammostola* dont le venin est particulièrement actif pour des batraciens ou des petits reptiles, permettant à ces araignées de tues de jeunes serpents.

Je n'ai pu vérifier l'action du venins des ARCHEIDAE américaines sur les autres araignées, mais j'ai trouvé à plusieurs reprises *Mecysmauchenius segmentatus*, qui vit sous les écorces humides des *Notofagus* de la Terre de Feu, dévorant des araignées beaucoup plus grosses qu'elle: *Anyphaena* et *Homeomma*.

Caractères généraux du venin — Il existe trop peu de travaux sur l'analyse chromatographique du venin et de l'hémolymphe des araignéees pour en tirer des conclusions valables sur leur structure dans tout l'ordre.

Nous avons, par contre, de nombreuses études sur les propriétés pharmacologiques de ces venins dans les diverses familles d'araignées.

Ces venins, bien plus simples que ceux des serpents, n'ont pas l'action complexe de ces derniers.

Les venins d'araignées peuvent se ranger dans deux grandes catégories: les venins d'action neuro-musculaire et les venins cytotoxiques à propriétés protéasiques prépondérantes.

Les venins neuro-musculaires sont des curarisants, tels ceux de la plupart des THERAPHOSIDAE; ou des venins élevant le tonus musculaire et provoquant des contractures de la musculature striée et lisse et des convulsions de type tonique (Diplures, *Ctenus*) ou clonique (*Latrodectus*).

Certains venins cytotoxiques limitant leur action au derme sont responsables de lésions cutanées plus ou moins étendues, sans répercussion sur l'état général (Lycoses). D'autres déterminent la mise en liberté d'histamine, se traduisant par des lésions locales et un état de choc avec son cortège habituel de manifestations: hypotension, stases viscérales intenses, chûte du nombre des globules rouges, hémolyse, diminution des protéines sanguines (aranéisme cutanéo-hémolytique), suivies généralement de lésions hépatiques et rénales tardives; avec d'autres venins enfin predominent les altérations du foie et des reins.

Aucun des venins étudiées, même pas ceux responsables de l'aranéisme cutanéo-hémolytique (*Loxosceles*), n'ont montré d'action coagulante ni d'action lytique *in vitro* sur les globules rouges, seules ou en présence de lécithine ou de serum normal, soulignant le mécanisme indirect de leur puissante action hémolytique dans l'organisme. Leur action *in vitro* protéolytique, anti-coagulante ou sur le complément est très peu marquée et ne s'exerce, qu'avec des doses élevées de venin.

Résistance des araignées à leur propre venin — Le problème de l'existence d'une véritable immunité et de son mécanisme chez les invertébrés, a été

souvent posé sans recevoir de solution définitive. Cette immunité est généralement considérée comme différente de celle des vertébrés, et serait liée aux hémocytes, sans intervention de sensibilisatrice ni de complément.

La résistance des invertébrés venimeux à leur propre venin est un cas particulier de ce problème général. Son étude se heurte à de nombreuses difficultés techniques: sensibilité très grande de la plupart de ces animaux, des araignées en particulier, aux injections et aux hémorragies; toxicité de leur hémolymphe pour les animaux habituels de laboratoire, ne permettant pas le plus souvent d'employer des doses suffisantes pour étudier son pouvoir protecteur ou son action neutralisante sur le venin.

Les travaux de Metchnikof, de Catouillard et de Marie Phisalix ont cependant bien établi que les scorpions offrent une résistance élevée à leur propre venin et que leur hémolymphe possède contre ce venin un pouvoir neutralisateur et même préventif.

Peu de choses ont été publiées sur les araignées. R. Lévy, en 1916, a montré le premier que l'hémolymphe des Tégénaires et de quelques autres araignées exerce une légère action préventive contre le venin homologue.

Toute les araignées sont loin de se prêter à ces recherches.

La toxicité élevée pour le pigeon de l'hémolymphe de *Phoneutria fera* et de *Trechona venosa* (DIPLURIDAE) ne m'avait permis dans mes premières recherches que des résultats incomplets: l'injection endoveineuse d'une dose inframortelle d'hémolymphe (0,1 à 0,2 ml) additionée d'une dose mortelle de venin, avait évité la mort, mais non les symptômes graves avec la première espèce, et atténué les phénomènes convulsifs sans survie appréciable des animaux avec la seconde.

Ces recherches ont été reprises dans des conditions bien plus favorables avec une mygale de taille moyenne de la côte du Pérou, *Hapalopus limensis*. Son venin curarisant est très actif pour les vertébrés, de la souris au chien; souris, lapin et cobaye supportent sans accident l'injection intramusculaire, intrapéritonéale ou endoveineuse de doses élevées, 1 à 2 ml d'hémolymphe. La curarisation chez le lapin et le cobaye se produit en 10 à 15 minutes avec des doses de venin voisines de la minima mortelle.

0,50 ml d'hémolymphe injectés par voie intramusculaire en même temps que le venin protègent complètement le cobaye contre une dose mortelle de venin (0,5 gl.); 1,0 ml protège contre deux doses mortelles.

0,25 ml ont protégé 50% des animaux contre une dose mortelle de venin; les autres ont succombé en une vingtaine d'heures; mort des témoins en moins d'une heure.

L'hémolymphe a montré une action préventive nette: 0,5 ml ont protégé le cobaye contre l'injection postérieure, 12 à 30 minutes, d'une dose mortelle de venin; témoins curarrisés en 20 minutes.

La même dose, dans des conditions idéntiques, a retardé considérablement la mort avec 2 doses mortelles de venin: curarisation en 80 minutes, mort en 12 heures; contrôle, curarisation en 3 minutes, mort en 17 minutes.

L'hémolymphe d'une grande mygale du Haut-Amazone, *Pamphobeteus nigricolor*, a montré une action analogue vis à vis de son propre venin:

1,0 ml d'hémolymphe, en injection mixte par voie veineuse au lapin, a neutralisé complètement le venin d'une demie-glande; curarisation du contrôle en 15 minutes, mort en 48 heures.

Par contre, avec une grande mygale du nord de l'Argentine, *Acanthoscurria chacoana*, les résultats ont été négatifs. Le mélange d'hémolymphe et de venin injecté par voie veineuse au lapin a accentué l'effet de choc de ce venin. Le peu d'exemplaires dont nous disposions n'a pas permis de poursuivre ces expériences par d'autres voies:

1 ml d'hémolymphe plus le contenu d'une glande ont provoqué une chûte immédiate de la pression artérielle, tuant l'animal en 3 minutes. Des contrôles recevant 1 ml d'hémolymphe ou le venin d'une glande ont montré seulement une chûte passagère de la pression.

L'hémolymphe des araignées est donc capable, de proteger contre le venin homologue et possède même un pouvoir protecteur contre l'injection postérieure du venin; mais cette action neutralisante est souvent marquée par l'effet toxique ou hypotenseur de l'hémolymphe pour les vertébrés supérieurs.

SEROTHÉRAPIE CONTRE LES VENINS D'ARASGNÉES: La toxicité de l'hémolymphe ne garde aucun rapporte avec celle du venin et un serum préparé avec l'hémolymphe ou avec une macération totale du corps de l'araignée n'a aucine action sur le venin.

Les premières tentatives pour obtenir un serum actif contre le venin d'araignés remontent au début du siècle et sont dues à des auteurs russes (Schtscherbina et Konstausoff) qui utilisèrent le chameau pour préparer un serum contre le venin du Latrodecte russe, le *Karakurt*.

En 1928, avec Vital Brazil, nous avons préparé les premiers serums thérapeutiques contre le venin de *Lycosa*, contre celui de divers *Ctenus* et un polyvalent anti-ctenolycosique, avec comme animal producteur le mouton, afin d'économiser l'antigène. Le choix n'était pas très bon. Le mouton es un médiocre producteur d'anticorps et le rendement en serum est faible. Cet exemple a cependant été suivi aux Etats Unis (d'Amor, 1939; Smith Dorms, 1929) pour obtenir un serum anti-Latrodecte, et en Afrique du Sud (Finlayson, 1937) pour un polyvalent anti-Latrodectes.

En 1939, Maxianovich a utilisé, pour la première fois et en Russie, le cheval avec le venin du *Karakurt* et en 1942, à Buenos Aires, Pirosky et ses collaborateurs ont obtenu avec le cheval un serum très actif contre *Latrodectus mactans*.

En 1953, à Lima, avec l'âne et le cheval, j'ai préparé des serums très actifs contre le venin de *Loxosceles laeta*, toujours utilisés depuis au Pérou. Ces travaux ont été continués à Butantan, en 1961, par Reynaldo Schwindt Furlanetto, qui a préparé des serums actifs contre les venins de *Phoneutria*, *Lycosa*, *Loxosceles rufipes* et *rufescens* et de scorpions.

Les venins d'araignées sont des bons antigènes et la préparation de ces serums n'offre aucune difficulté.

L'action relativement simple de ces venins permet aussi de combattre les accidents avec une medication symptomatique, qui peut donner d'assez bons résultats, mais inférieurs à la sérothérapie spécifique.

Le glyconate de chaux a parfois une action spéctaculaire mais inconstante dans les accidents par *Latrodectus*. La cortisonne, la néo-stigmine, la chloropromazine ont donné quelques résultats favorables avec les *Latrodectus*. Les anti-histaminiques sont indiqués en cas de piqûre de *Loxosceles*.

L'association du traitement symptomatique avec la sérothérapie spécifique constitue la méthode de choix.

LE VENIN DES PRINCIPALES FAMILLES D'ARAIGNES

Nous ne pouvons ici entrer dans de longs details sur les propriétés du venin dans les principales familles d'araignées et nous nous limiterons à quelques considérations générales.

MYGALOMORPHES: Les propriétés du venin sont très voisines chez les ACTINOPODIDAE, les CTENIZIDAE et les DIPLURIDAE. Ce sont des venins neurotropes provoquant des tremblements, des contractures toniques et dans les cas graves des convulsions et la paralysie, avec exagération des sécrétions, sans réaction locale. Celui des deux premières familles est surtout actif pour les insectes et parfois pour les petits réptiles et batraciens. Aucune espèce n'est dangereuse pour l'homme.

La toxicité du venin des DIPLURIDAE est plus élevée. La géante de la famille, *Trechona venosa* peut tuer des petits mammifères et des oiseaux; 1/100 de glande suffit pour un pigeon. Vivant dans de profondes terriers, elle n'offre aucun danger pour l'homme. Un venin aussi actif, mais en faible quantité se trouve chez des petites espèces de cette famille, *Ischnothele*, *Diplura monticolens*. En Australie, deux représentants du genre *Atrax* peuvent être causes d'accidents graves.

Le venin des THERAPHOSIDAE et BARYCHELIDAE est de type curarisant. Les espèces étudiées d'AVICULARIIDAE et d'ISCHNOCOLINAE tuent rapidement des petits mammifères de la taille d'une souris au d'un cobaye et même des lapins ou de jeunes chiens; au-dessus d'une dizaine de kilos leur piqûre est sans effet.

Il en est de même pour les GRAMMOSTOLINAE; quelques unes de celles-ci présentent, nous l'avons vu, une activité particulière pour les reptiles et les batraciens.

Le venin de nombreuses THERAPHOSINAE, principalement des *Acanthoscurria*, *Pamphoboeteus*, *Phormictopus* et formes voisines possède en plus une action cytotorique marquée, provoquant une lésion locale pouvant aboutir à l'escharre, un état de choc plus ou moins accusé et des lésions hépatiques et rénales tardives, les rendant dangereuses pour l'homme.

ARANEOMORPHES: Nous avons étudié des représentants de presque toutes les familles américaines de ce groupe.

Seules les THERIDIIDAE, les CTENIDAE, les HETEROPODIDAE, les LYCOSIDAE et les SCYTODIDAE comptent des espèces ayant une réelle importance pratique.

Beaucoup d'autres araignées possèdent des venins très intéressants, mais dont nous ne pouvons nous occuper ici. Leur étude permet de comprendre que les espèces dangereuses ne représentent que l'exaltation d'un caractère existant dans tout un genre ou toute une famille.

THERIDIIDAE: Dans toutes les régions tropicales et tempérées. Les *Latrodectus* sont redoutés et causes d'accidents graves. Ce sont des araignées ubiquistes, s'adaptant aux conditions les plus diverses, apparaissant certaines années en grande abondance; elles peuvent alors occasioner de nombreux accidents, de véritables épidémies, comme en 1947 en Italie Centrale et en Yougoslavie.

Leur venin neurotoxique provoque des douleurs intenses, irradiantes, une hyperexcitabilité généralisée, des contractures musculaires cloniques, des convulsions, une élévation notable de la PA; certains cas peuvent faire penser à un abdomen

aigu. Leur action parait s'exercer directement sur le système nerveux central et le système nerveux végétatif.

Parmi les animaus d'expérience le cobaye est particulièrement sensible et succombe à un œdème aigu du poumon.

L'espèce américaine, *L. mactans*, s'etend des Etats Unis à l'Argentine et au Chili. Dans cette vaste aire de dispersion, elle présente de nombreuses variations de colorées. L'activité de son venin offre également d'une région à l'autre de sensibles différences, soit pour les conditions climatiques, soit pour les différentes modalités d'accidents, soit enfin pour des variations génétiques.

Les propriétés du venin de *L. mactans* se retrouvent, avec quelques differences, dans toutes les espèces du genre, parfois très accusées avec une action plus marquée sur la fibre musculaire lise (*L. indistinctus*), parfois très atténuées (*L. geometricus*).

Ces mêmes propriétés existent dans le venin de nombreuses autres THERIDIIDAE, mais beaucoup moins accentuées; les glandes de plusieurs exemplaires sont nécessaires pour produire des symptômes analogues. Nous avons beaucoup étudié, par exemple, le venin de diverses espèces de *Lithyphantes* du Pérou, *L. andinus, L. nigrofemoratus* et quelques autres. Tous ont un venin analogue à celui des *Latrodectus*, mais bien moins actif. Nous avons également vérifié que le venin des espèces de la côte péruvienne est 3 ou 4 fois plus toxiques que celui des mêmes espèces vivant à 3.000 mètres dans les Andes.

CTENIDAE: Nous nous sommes longuement étendus dans des travaux antérieurs sur le venin des *Ctenus*. Ce sont des venins neurotropes, provoquant des contractures et des convulsions toniques, et une douleur intense avec élévation de la PA, et des altérations profondes du rythme cardiaque. La mort avec les grandes espèces du sous-genre *Phoneutria* (*fera, nigriventer* du Brésil; *rufibarbis*, d'Argentine; *reidyi* et *andrewsi*, de l'Amazone; *boliviensis*, de Bolivie), peut survenir chez l'homme en 2 ou 3 heures.

Des propriétés identiques se retrouvent dans le venin des araignées de taille moyenne de cette famille. (*C. medius, ornatus, curvipes* et autres) qui ne possèdent pas une dose suffisante de venin pour être dangereuses pour l'homme. Le venin de grosses espèces voisines, les *Cupiennius* est en général bien moins toxique; cependant une espèce de ce genre, non déterminée du Mato Grosso — les exemplaires ont été perdus au cours d'un voyage accidenté — a montré un venin aussi actif que celui des *Phoneutria*, et très redouté des indiens Nambikwaras qui lui imputent des accidents mortels.

Les mêmes propriétés existent à un faible degré chez les espèces amoindries de cette famille, du genre *Odo*, qui ne disposent que de quantités réduites de venin.

LYCOSIDAE: Les Lycoses possèdent un venin d'action nécrosante limitée, sans action générale sur les vertébrés. Il est facile de reproduire ces lésions chez le cobaye ou par injection intradermique dans l'oreille du lapin.

Quelques grandes espèces, *Lycosa raptoria, L. erythrognatha*, du Brésil, occasionnent des accidents locaux, mais sérieux chez l'homme. D'autres espèces du Honduras, du Pérou, du Chili ne m'ont donné que des résultats insignificants. Il en est de même d'une grosse espèce de Bolivie, *L. rufimanoides*, des environs de La Paz.

Des araignées d'un genre voisin de la même famille, les *Porrima* (*P. diversa*, du Brésil; *P. harknessi*, du Pérou), provoquent également des petites lésions locales chez les animaux d'expérience.

HETEROPODIDAE: L'espèce type, la grande *Heteropoda venatoria*, est une des araignées domiciliaires les plus communes dans toutes les régions tropicales et tempérées chaudes.

Son venin, assez peu actif, de type histaminique, provoque un œdème local parfois assez important, accompagné de vésicules et suivi d'une petite escharre superficielle. Dans certains cas on observe une éruption scarlatiforme généralisée. Ces accidents n'ont aucune gravité, mais ont pu être confondus avec des manifestations allergiques.

Une espèce voisine, *H. meticulosa*, du Haut-Amazone, de plus petite taille, possède un venin du même type mais un peu plus actif. La morsure, ou l'injection du contenu de deux glandes, peut tuer le cobaye; l'autopsie montre, en dehors de l'œdème local, une stase viscérale généralisée.

D'autres espèces de la même famille, entre autres le gros *Polybetes maculatus* d'Argentine, ont un venin analogue, mais très peu actif.

SCYTODIDAE: Dans cette famille se trouvent les venins cytotoxiques, d'action histaminique, les plus typiques.

Les diverses espèces de *Loxosceles* que nous avons étudiées (*laeta*, *yura*, *taeniopalpus*, *rufipes*) présentent peu de différences dans l'activité de leur venin. La première est repasable de nombreux cas d'aranéisme classé sous le nom de cutanéo-ictéro-hémolytique et longtemps attribués à diverses araignées domiciliaires, *Filistata*, *Dysdera* et autres, avant que la véritable responsable ne fût déterminée en 1935, au Chili, par Escudero.

Les autres *Loxosceles* étudiés, bien que possèdant un venin aussi actif, étant moins domiciliaires, ne causent pas d'accidents.

Nous avons déjà indiqué que le venin des *Sicarius* est du même type, avec une activité bien moindre.

Les FILISTATIDAE (*Filistata hibernalis*, du Brésil; *F. brevipes*, du Pérou), n'offrent aucun danger, leur venin étant peu actif, mais cependant du même type que celui des *Loxosceles*.

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5. EL ARANEISMO EN EL MUNDO TROPICAL Y SUBTROPICAL

ROBERTO GAJARDO-TOBAR

Chile

Hace muchos años, siendo médico rural, presencié un hecho que me impresionó vivamente y que nunca podré olvidar.

Regresaba, en un mediodía de ardiente verano, al hospital del modesto pueblo donde ejercía mi profesión, cuando unos gritos despavoridos comenzaron a oirse desde una cuadra de distancia, proferidos por un mozo joven que transportaban dos camilleros sobre una improvisada parihuela. Consternaba escuchar la creciente intensidad de los quejidos a medida que el pequeño cortejo se acercaba. Un atleta de 18 años, convertido en una miseria, sudoroso, se retorcía gritando desesperado por terribles dolores en todo el cuerpo y por convulsiones que le hacían presagiar una muerte inminente. ¡Había sido picado por una araña, en un trigal! La araña del trigo o viuda negra. ¡El *Latrodectus mactans*!

Desde entonces comenzó mi interés por las arañas, sus costumbres y la acción de su veneno.

Desde tiempo inmemorial estos extraños seres han ocupado la atención del hombre. Unos les temieron, otros ensalzaron sus cualidades curativas, muchos les despreciaron y algunas tribus indígenas han empleado la maceración de sus cuerpos para envenenar sus armas defensivas. Han transcurrido siglos antes de que se les estudiara y les fuese asignada una justa posición médico-zoológica y un lugar entre los agentes determinantes de emponzoñamientos.

Aristóteles cita en sus escritos arañas venenosas y Plinio describe la "phalangia" y recomienda como tratamiento para su picadura el uso del cuerpo macerado de la araña.

Durante la Edad Media son empleadas las arañas en la composición de drogas y filtros misteriosos y su tela se convierte en el remedio más eficaz contra las hemorragias.

Surge después la historia fantástica de la tarántula, una araña de Tarento (*Lycosa tarentula*) a la que se atribuía una curiosa enfermedad, de carácter epidémico y de la que se dice se extendió desde Italia Meridional hasta Europa Central.

El tarantulismo describiose en las formas más extrañas y la gente llegó a temerle más que a Satanás. ¡El picado de araña caía presa de una agitación espantosa, reía y lloraba alternativamente, moviéndose desesperado, de un lado para otro, gritando y gesticulando, saltando y haciendo cosas absurdas! ¡Estaba picado de araña!

De este raro mal sobrevenían epidemias y muchas personas enfermaban a un mismo tiempo.

En vista de las dolencias y de los síntomas, pensaron los contemporáneos que lo más viable sería someterles a tratamiento colectivo y como habían observado que mejoraban mejor y más luego los que transpiraban más abundantemente, les animaron a danzar con aires musicales de movimientos muy vivos, horas de horas, hasta que caían al suelo totalmente agotados, bañados en sudor y se dormían. De ese sueño despertaban sanos. ¡La tarantela les había mejorado!

Ahora no hay epidemias. Se ha comprobado que lo que se llamó tarantulismo corresponde al envenenamiento producido por la picadura de otra araña: el *Latrodectus tredecimguttatus*. Sin embargo, en Italia siguieron por bastante tiempo, en muchas partes, asignando a la tarántula los mentados accidentes. Por otro lado, algunos taimados se hacían pasar por "picados de araña" para cometer sus picardías!

En mi país, a aquellos mozos jóvenes muy enamoradizos, de corazón ardiente, de ideas estrambóticas y de conducta extraña, se les tilda de "picados de araña".

Antes de la Conquista, ya los aborígenes y sobre todo los araucanos conocían muy bien los accidentes producidos por el *Latrodectus mactans*, al que llamaban "guina" y "pallú", araña cuya picadura experimentaron también los españoles y a la que llegaron a temer tanto como a la viruela (Padre Valdivia, 1606. Padre Febrés, 1765).

No corresponde ahora tratar de la anatomía, de la fisiología ni de la sistemática de los arácnidos. El tema sólo comprende los accidentes producidos, en el hombre, por el veneno.

Extraños habitantes de la tierra, las arañas disponen de un arma poderosa, los quelíceros, para coger y matar a las bestezuelas conque se alimentan o para defenderse cuando son agredidas.

Los quelíceros son apéndices quitinosos situados en la parte frontal del céfalo-tórax, por encima de la boca, de la que están separados por el rostrum. Son dos, uno al lado del otro. Cada uno consta de dos segmentos: uno basal, el tallo, de mayor volumen, cilindroídeo, rígido, muy firme y, otro terminal, la garra o gancho, móvil, puntudo, en la extremidad libre del tallo.

El quelícero es el órgano destinado a inyectar el veneno que preparan dos glándulas, alojadas dentro del tallo o en el interior del céfalo-tórax. La ponzoña sale de la glándula por un pequeño conducto que va a desembocar cerca del extremo puntudo del gancho.

Los quelíceros son antenas modificadas, homólogos con el segundo par de antenas de los crustáceos, pero no con las antenas de los insectos.

El gancho o garra de los quelíceros es móvil, pero sólo en un plano. En las arañas Mygalomorphas se mueven paralelamente uno al otro en sentido vertical, mientras que en las Arachnomorphas se cruzan yendo de fuera a adentro.

El veneno de las arañas es un líquido claro, que deja después de ser sometido a la desecación un polvo amarillento. Presenta los caracteres de los proteídos, pero de su íntima composición no es mucho lo que se sabe. La cantidad de veneno que son capaces de producir las glándulas no guarda relación con el tamaño de la araña. El total de veneno seco, por glándula oscila entre 0.05 mg. y 6.00 mg., según la especie. La ponzoña es alcalina en tiempo caluroso y se acidifica en la estación fría. Es más activa en su condición alcalina. El polvo, disuelto en concentraciones adecuadas en suero fisiológico tiene igual acción que el veneno fresco.

Las arañas tienen otras toxinas en su cuerpo, especialmente a nivel de los ovarios en el período del celo. Ya muchos años atrás Kobert y después Walbum dieron cuenta de ello, pero no intervienen en las actividades ofensivas ni defensivas de los arácnidos.

Los venenos de las glándulas ponzoñosas de las arañas están destinados a actuar sobre los seres que constituyen el alimento de ellas y naturalmente se encuentran acondicionados para tal evento. Son diferentes en su acción según cada especie, aun cuando hay algunas muy vecinas con efectos parecidos.

Las arañas pican al hombre cuando éste las irrita, apretándolas o lastimándolas intencional o accidentalmente. Jamás atacan en forma deliberada.

Aún cuando todas las arañas, con excepción del pequeño grupo de los ULOBORIDAE, poseen aparato venenoso, sólo algunas especies disponen de veneno suficientemente activo y en cantidad adecuada como para producir accidentes graves o mortales para el hombre.

Fuera del género *Latrodectus*, de una amplia repartición por el mundo, la mayor parte de las especies peligrosas son sudamericanas.

Los accidentes de arañeísmo son más o menos frecuentes. Hasta antes de las pruebas experimentales hubo entre los hombres de ciencia incredulidad, en tanto que en el público existía exuberancia imaginativa.

Han actuado en forma negativa en la exacta determinación de los agentes causantes de los emponzoñamientos varios hechos: Por una parte, ante una picadura, el afectado no siempre veía la araña, o si la advertía no era capaz de cogerla, y si la atrapaba la destruía, de manera que resultaba bien difícil poder juntar el efecto con la causa. Luego, la experimentación, cuando pudo hacerse, por errores de clasificación de las arañas o por fallas de técnica, muchas veces fracasó en sus resultados.

En otras circunstancias, los pacientes aseguraban haber sido picados por arañas cuando se trataba de insectos o atribuían a arañas forúnculos y abscesos sin ninguna relación con ellas.

Por fortuna, no siempre ha sido así y gracias a que en un buen número de veces fue posible pillar a la araña picando, la causa etiológica de la mayor parte de los emponzoñamientos se ha logrado establecer y más tarde con la reproducción experimental del accidente se ha probado.

En cualesquier caso, en materia de emponzoñamiento por veneno de arañas tienen importancia algunos factores: 1.° — Durante el verano las arañas están más vivaces y el calor, alcalinizando las ponzoñas, las hace más activas. 2.° — Una araña bien nutrida está fisiológicamente en mejores condiciones para actuar. 3.° — Si no ha usado de su veneno durante un tiempo, tendrá más ponzoña con que defenderse. 4.° — La piel fina o las regiones muy irrigadas son las que van a determinar los cuadros clínicos más serios. 5.° — El camino seguido por el veneno y su ubicación, en la piel, el paso al torrente circulatorio o la invasión del sistema nervioso condicionarán el proceso. 6.° — La resistencia humana y la edad de los afectados influirán a su vez. Los niños son los que hacen los más graves casos de arañeísmo. 7.° — Finalmente también importarán las variaciones de venenosidad que ocurren durante el período del celo.

Los accidentes causados por la picadura de las arañas, aracnidismo o arañeísmo, que se acostumbra a denominarlos según el nombre del género al cual pertenece la araña, determinan cuadros clínicos bien característicos, que pueden

agruparse en tres tipos: nerviosos, cutáneos y cutáneo-viscerales, dependientes, naturalmente, del caracter de la ponzoña, del tropismo de ella y de los tejidos sobre los que actúa.

Los accidentes más espectaculares son los producidos por venenos neurotóxicos, es decir aquellos que actúan fundamentalmente sobre el sistema nervioso. Las arañas, más importantes, que los provocan pertenecen a los géneros *Ctenus* o *Phoneutria*, *Latrodectus* y THERAPHOSIDAE.

El emponzoñamiento desencadenado por la toxina de los *Ctenus* o *Phoneutria* es de desarrollo rapidísimo y dramático.

Sobreviene el pinchazo de los ganchos de los quelíceros, en cualesquier parte de la piel, más frecuentemente en las partes descubiertas. El dolor es agudo, localizado en el sitio afectado al principio, irradiado más tarde y generalizado por fin. Sobrevienen calambres en las extremidades y contracturas musculares violentas. Surgen vértigos y transtornos de la visión. Hay rigidez toráxica y abdominal, dolores precordiales, angustia, malestar general, escalofríos y grandes sudores.

A este cortejo sintomatológico se suman formidable temblor permanente, hiperestesia marcada y convulsiones tónicas. Luego aumento sensacional de las secreciones salivales, nasales y bronquiales. Le acompañan también taquicardia, muchas veces arritmia, mientras el pulso se hace incontable y filiforme.

Cuando el proceso se agrava más se imponen la hipotermia, el aumento de las contracturas musculares llegando hasta la rigidez general en opistótono y con crisis convulsivas de tal manera imponentes que hacen pensar en tétanos.

No hay lesiones locales en el sitio de la picadura.

Hay a veces retención de orina. No se ha comprobado albuminuria ni hematuria. En general se produce estreñimiento rebelde.

En los casos mortales ha sobrevenido la muerte en las primeras cinco horas.

En los casos de evolución favorable, el pulso se va regularizando lentamente, la temperatura se normaliza, los dolores y calambres se aplacan y, cosa curiosa, son reemplazados por adormecimiento local, paresia y anestesia de las zonas circunvecinas al lugar picado. Esto acontece más o menos en una semana.

El período más agudo, de sintomatología más violenta y más grave dura entre seis y doce horas.

La casuística más importante y mejor estudiada corresponde a las regiones rurales de São Paulo, hacia el final de los veranos, siendo con mayor frecuencia responsable de los accidentes *Phoneutria nigriventer*. En cambio, en los aledaños de Río de Janeiro se deben, de preferencia, a *Phoneutria fera*. En Argentina se inculpa a *Phoneutria rufibarbis*.

Las Phoneutrias son arañas bien conformadas, de 4 a 5 cm, de 8 ojos, de patas robustas, peludas, de color gris amarillento, de caracter muy agresivo, nocturnas y errantes.

Son arañas tropicales, pero llegan con los cargamentos de bananas a los países subtropicales, donde también han producido accidentes, menos serios que en su tierra de origen.

Otro tipo de arañeísmo nervioso es el producido por el veneno del *Latrodectus*, que en América está representado por *Latrodectus mactans* y habita desde California hasta Chile, Argentina y Uruguay. Araña muy temida, cono-

cida bajo diversos nombres, como ser araña del trigo, del lino, rastrojera, araña brava, guiña, pallú, mico-mico, lucacha y black widow, según la zona donde viva.

Es una araña mediana, negra con el abdomen globuloso con manchas rojas, patas largas y firmes, vive en las grietas del terreno, en la base de las plantas, sobre todo en los trigales; hace su tela irregular y deposita una decena de capullos amarillentos.

El color y la disposición de las manchas coloradas del abdomen han inducido a muchos autores a crear especies que en verdad deben ser sólo variedades de *Latrodectus mactans*.

El veneno de *Latrodectus mactans*, esencialmente neurotropo, determina un emponzoñamiento violento, de rápida y patética evolución, grave y a veces mortal. A siete días de grandes padecimientos siguen profunda astenia y severa fatiga intelectual.

Al lancetazo de la picadura de la araña sigue un período mudo de una veintena de minutos. Después aparece dolor local que acrece, quema e irradia a todo el cuerpo, más marcado en la cintura y extremidades. Con angustia y temor, aniquílanse las fuerzas y abátese el espíritu. Contracciones musculares, temblores y convulsiones estremecen el cuerpo. Las paredes toráxicas y abdominales se ponen rígidas. Malestar, dolores, opresión al pecho y abdomen condicionan la impresión de muerte inminente.

Sudores extenuantes, sialorrea, lagrimeo, hipersensibilidad dérmica, exageración de los reflejos, disnea y superficialización de los movimientos respiratorios, taquicardia que va a bradicardia después, a veces arritmia (con alteraciones electrocardiográficas), fuerte crisis hipertensiva, parálisis vesical e intestinal, disuria, enuresis y anuria, priapismo, ocasionalmente poluciones, y por otro lado hiperglicemia fugaz, uremia marcada y albuminuria conforman los cuadros clínicos típicos.

Contrastando con el espectacular compromiso del sistema nervioso, las lesiones locales se reducen a una manchita rosada y a los pequeños orificios dejados por los quelíceros de la araña en la piel afectada.

Con exacerbaciones y atenuaciones el proceso evoluciona hacia la mejoría, en la mayor parte de los casos.

Cuando la gravedad es extrema, la muerte se produce por edema agudo del pulmón, entre las 30 y las 50 horas después del accidente.

En un centenar de casos, hemos tenido 4% de mortalidad.

El veneno ejerce su máxima actividad sobre los nucleos centrales del sistema nervioso vegetativo, en la médula, bulbo, protuberancia y cerebro.

La anatomía patológica enseña intenso edema pulmonar con acentuada hiperemia de las bases, hiperemia de la pia medular y cerebral, hiperemia y edema del cerebro, hiperemia del hígado y riñones, gastritis catarral, dilatación gastrointestinal y vesical.

Hay un bonito estudio, muy reciente, de los Drs. Lebez, Maretić y Kristan de Pula, en el que describen sus experimentos destinados a determinar la distribución del veneno del *Latrodectus tredecimguttatus*, marcado con P32 en conejillos de India emponzoñados. Mediante un procedimiento muy sencillo, dejando a las arañas sin agua una semana, les ofrecían después agua conteniendo 660 u. por ml. de Na2HP32O4. Cuatro días después hacían picar a los cobayos. Sacrificados éstos entre 3 y 120 minutos después de picados y algunos 2 y 3 horas

más tarde, enseñaron grandes cantidades de P32 en el sistema nervioso central y en los nervios periféricos y pequeñas cantidades en el hígado, bazo, pulmones, corazón, riñones, suprarenales, músculos y sangre.

Cabe recordar aquí un hecho histórico que pone de relieve el brutal efecto del veneno de *Latrodectus*. En la víspera de la batalla de Loncomilla (8-XII-1851), numerosos batallones aguardaban en una sementera, ocultos y listos para dar una sorpresa, cuando al anochecer, las arañas picaron a muchos soldados de tal manera que los gritos y las lamentaciones de los infelices habrían comprometido la posición del ejército de no haber mediado la medida extrema de tener que cloroformizarlos.

Por último, dentro de los accidentes de tipo nervioso, están los causados por las arañas **MYGALOMORPHAE**, que algunos médicos consideran básicamente como narcóticos y otros como curarizantes.

La picadura de algunas de estas arañas, causa, como las otras, el dolor de una clavadura. Luego sobreviene adormecimiento local, con anestesia y posteriormente paresia y parálisis de los músculos vecinos al sitio afectado.

En general son casos benignos. Otras veces producen lesiones locales, y en algunos comprometen el estado general.

Se ha hablado y han sido publicados trabajos sobre accidentes producidos por la picadura de algunas de las grandes arañas "pollito", señalando que la picadura es poco dolorosa, que aparecen edema, flictenas y eritema, acompañados de fiebre, que a las 24 a 48 horas habría ictericia, oliguria y albuminuria, e incluso hematuria. Otras veces se ha publicado de grandes ulceraciones. También se citan casos mortales entre el 4.º y el 5.º día. Se señala que este tipo de accidentes sería producido por los venenos de *Acanthoscurria*, *Phormictopus* y *Pamphobeteus*.

Experimentalmente no siempre se ha podido probar. Hoy existe la impresión que en Sud-América no hay arañas "pollito" (Mygalas) peligrosas. El veneno de ellas ha demostrado ser anestésico relajante de la musculatura.

Está bien probado que los pelos finos que cubren el cuerpo de estas arañas (THERAPHOSINAE) producen, en muchas personas, fuerte prurito cutáneo y erupción urticariforme.

Totalmente diferentes son los emponzoñamientos producidos por la picadura de arañas cuyo veneno tiene acción necrosante. Entre estos hay ponzoñas que sólo actúan sobre la piel y, otros que obran sobre la piel pero, también, y, a veces en forma mortal, sobre sangre y vísceras.

El veneno de las Lycosas produce dolor, más o menos acentuado, en el punto de la picadura, más tarde engendra una pápula blanquecina, con una zona sin sensibilidad, rodeada de una guarda rojiza, congestiva, dolorosa. Paulatinamente el rubor se va extendiendo a su alrededor, a veces con erupción generalizada. Sobrevienen edema voluminoso, manchas equimóticas y flictenas. Se produce la necrosis entre el 4.º y 5.º días, después de formarse una escara seca, que delimitada, cae al 15.º día, dejando una úlcera irregular que llega hasta las aponeurosis de los músculos.

La cicatrización es lenta y defectuosa. No hay compromiso del estado general. Muchas veces los afectados se agravan por infecciones secundarias. *Lycosa raptoria* ha sido inculpada en São Paulo y otras partes.

En el Perú, Escomel ha descrito una acción local parecida en casos de picadura de *Glyptocranium gastheracanthoides* pero, en los que habría además compromiso del estado general.

Por último, por muchos años, ya desde 1852, vienen dando cuenta los médicos de Chile, de la existencia de una enfermedad llamada "la mancha gangrenosa" que, siendo en la mayor parte de los casos un proceso local, había oportunidades en que, comprometiendo gravemente el estado general, llevaba a la muerte en pocas horas.

Habiendo sido este accidente muy bien descrito y muchísimas veces observado y estudiado, sólo en 1934, con los hallazgos en Antofagasta y la experimentación hechos por A. Macchiavello, pudo determinarse al agente causal: *Loxosceles laeta* (que al decir de otros investigadores debe llevar el nombre de *rufipes*).

Loxosceles produce dos cuadros clínicos diferentes, según como actúe su veneno, en razón al camino seguido en el organismo: En uno hay acción local de la ponzoña, con extensa o limitada acción gangrenosa de la piel, con nulo o escaso compromiso del estado general. En el otro, con fulminante y grave alteración del riñón y del hígado y una violenta hemólisis, acusadas por fiebre, postración, ictericia, hematuria y hemoglobinuria.

En la forma cutánea, al dolor de la clavadura de los quelíceros de la araña sigue un período sin síntomas de minutos a horas, al cabo del cual irrumpe fuerte dolor local intensamente quemante, a veces con un poco de prurito. La violencia del dolor crea impotencia, causa insomnio y desesperación creciente. Una mancha rojo-vinosa señala el sitio de la picadura. A la 24 horas se torna violácea y después negra. Se forma allí una placa de 1 a 30 y más centímetros de diámetro, muy característica, con zonas pálidas, blancas y moradas, como vetas irregulares, rodeada de un halo intensamente rojo. Es la lesión que nosotros hemos llamado "la placa marmórea".

Sobreviene edema que se extiende muchísimo y una gran infiltración dura bajo la placa. En la parte negra y en la placa marmórea en general desaparece la sensibilidad al dolor y térmica, mientras que en la periférica se exageran. Surgen desde el comienzo, sobre la placa en formación, grandes flictenas serohemáticas.

Al 6.º día se deslinda una escara apergaminada, seca, brillante y negra. A la 3.ª semana se desprende y cesa el dolor. A este proceso es al que se llama, en Chile, desde más de cien años, "la mancha gangrenosa".

La curación es muy lenta y a veces requiere de injertos. La cicatriz es irregular y azuleja.

El estado general no se compromete o lo hace en poca monta, con calofríos, fiebre, desasosiego, insomnio, etc. Con esto o sin ello, el dolor quemante es tan terrible, a veces, que algunos pacientes desesperados han deseado que les fuese amputado el miembro afectado para librarse de él, o han llegado hasta intentar suicidarse en su aflicción.

Cuando el veneno pasa la barrera de la piel y ya sea por la cantidad introducida o por su pasaje directo al torrente circulatorio e invasión de todo el organismo, se produce la forma cutáneo-visceral, en que la lesión dérmica se insinúa y no alcanza a desarrollarse porque el compromiso del estado general mata antes que aquella evolucione.

Se extiende el dolor local, surgen escalofríos, cefaleas, decaímiento y desasosiego. A las doce horas hay vómitos, a veces con sangre, luego ictericia, más tarde hematuria y hemoglobinuria. El proceso avanza con insomnio, taquicardia, hipertermia, hipotensión, disnea, cianosis y anemia. Por último vienen congestión y edema pulmonar.

La anemia es hemolítica, con gran destrucción de los glóbulos rojos, hemoglobinemia, con brusca leucocitosis de tipo leucemoíde, trombocitopenia variable y sedimentación alta.

Manifiéstanse hematuria, hemoglobinuria y cilindruria. Les acompañan gran alza de la uremia, hiperglicemia fugaz y luego hipoglicemia e hiperbilirubinemia.

La muerte acaece entre las 30 y las 40 horas después del accidente. Hay lesiones de necrosis incipiente de la piel, hiperemia y edema polivisceral, gran hemólisis, necrosis focal hepática, nefrosis hemoglobinúrica y hemorragias múltiples. Nosotros, en 200 casos de loxoscelismo hemos tenido 22 casos de grave forma cutáneo-visceral con 7 muertes, es decir 2.5% de los 200 y 31.81% de los 22 de tipo cutáneo-visceral.

La casuística humana de arañeísmo ha dependido, naturalmente, de la extensión del habitat de las especies peligrosas y, hoy día, se describen accidentes de loxoscelismo desde los Estados Unidos (*Loxosceles reclusa*), Chile, Perú, Bolivia, Argentina, hasta Uruguay (*Loxosceles laeta* o *rufipes*); y de latrodectismo, de muy antigua observación hay casos de casi todo el mundo, a saber, en Italia, Yugoslavia, España (*Latrodectus tredecimguttatus*), en Rusia del Sur, Turquestán, los países del Mar Negro y del Mar Caspio y Asia Menor (*Latrodectus erebus* o Karacurt), en Australia y Nueva Zelanda (*Latrodectus hasselti* o Katipo), en Madagascar (*Latrodectus menavodi*) y en África del Norte (*Latrodectus tredecimguttatus*), etc., etc.

Muchas otras arañas han sido inculpadas de producir accidentes importantes humanos de emponzoñamiento pero no hay al respecto demostración experimental irredarguible. Con todo, tenemos nosotros también accidentes por el veneno de otras arañas, de lo que daremos cuenta en otra oportunidad.

Con relación al diagnóstico, el antecedente de haber sorprendido a la araña picando es categórico pero, muchas veces los afectados no ven a la hechora y es entonces cuando hay que proceder con ingenio, paciencia y agudeza a analizar el cuadro clínico para hacer el diagnóstico.

Ya sabemos de la acción específica de muchos venenos, luego nos ayudarán la procedencia del enfermo, el tipo de su trabajo, la presencia de arácnidos en el lugar y por sobre todo el cuadro clínico.

Luego, según el tipo de la lesión debemos tener en cuenta todas las afecciones parecidas del país donde el accidente ha sucedido. Con cierta experiencia no es problema muy difícil.

En cuanto al tratamiento, es interesante recordar que ya los indios usaban muchas yerbas, agua de estiercol de guanaco e incluso un remedio que pasó al uso popular, la celebremente repugnante ulpada (deposiciones humanas diluídas con agua) empleadas para combater la parálisis intestinal del latrodectismo.

De todas maneras, para impedir la difusión del veneno no parece servir ningún procedimiento.

El ideal, en cualesquier arañeísmo, es la terapéutica específica o sea el empleo de sueros específicos.

Las arañas tienen venenos bien definidos para cada especie y los sueros deben ser entonces específicos. Sin embargo, las especies de un mismo género poseen ponzoñas muy afines, de manera que la preparación de sueros contra una especie del género dará un producto que puede ser usado y resultar bien para

el emponzoñamiento producido por otra del mismo género, como lo demostramos usando suero contra el veneno del *Latrodectus tredecimguttatus* en casos de latrodectismo por *Latrodectus mactans*, con excelente resultado.

Hemos empleado el tratamiento con sueros específicos, en latrodectismo y loxoscelismo, en los casos que estimamos más graves, en nuestro país, siempre con muy buenos resultados. El suero utilizado lo obtuvimos gracias a la gentileza de los Drs. Pirosky, Sampayo y colaboradores del Instituto Malbrán de Buenos Aires, en casos de latrodectismo y de los Drs. Stanić y Maretić del Instituto de Higiene de Pula, también para el latrodectismo. En loxoscelismo usamos suero específico del Instituto Butantan que nos proporcionó el Dr. Bücherl y otro preparado por el Dr. Vellard.

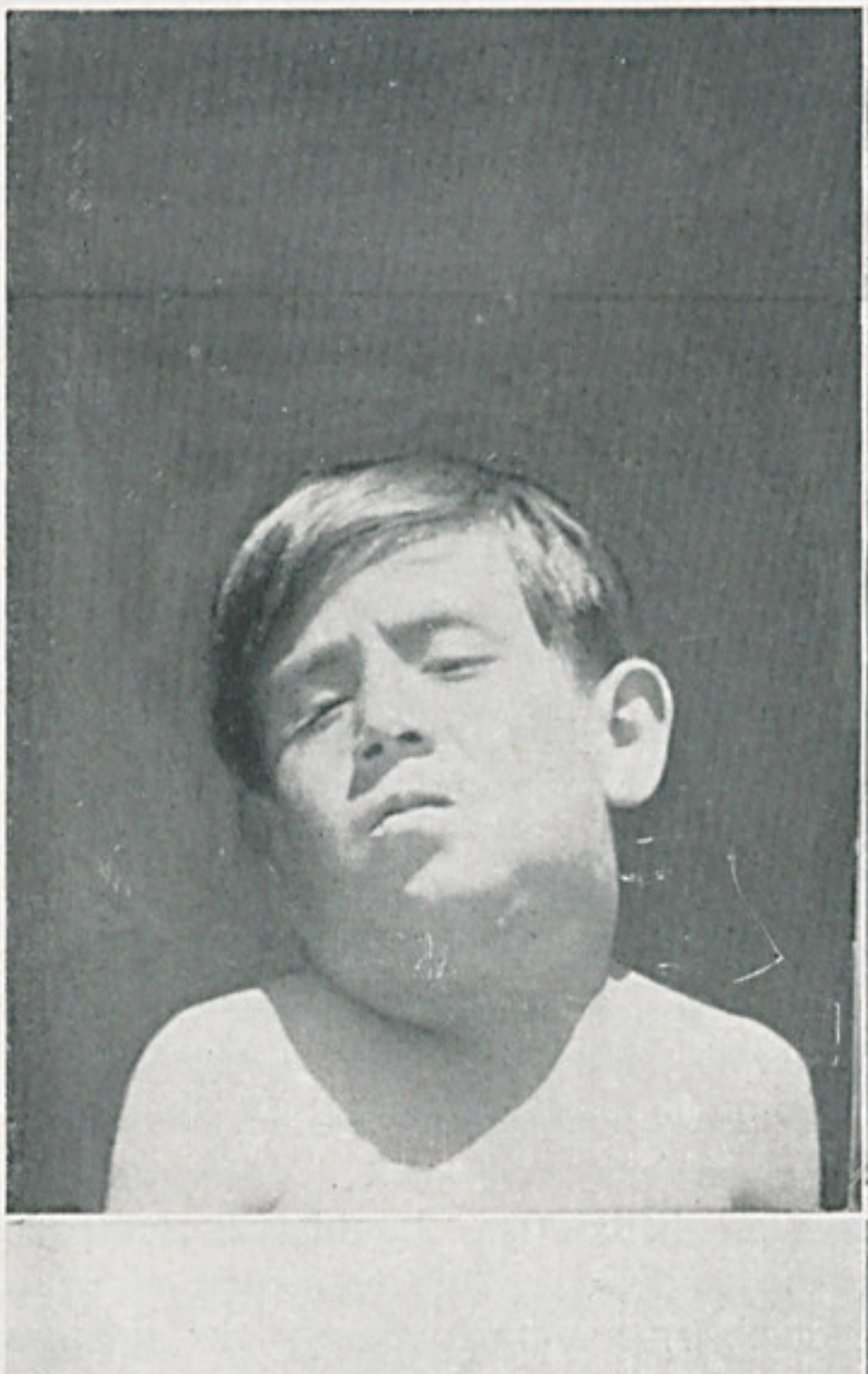
Hoy día el Instituto Butantan prepara excelentes sueros contra el veneno de *Phoneutria fera*, *Lycosa* y *Loxosceles*. El Instituto Malbrán obtiene suero contra el veneno del *Latrodectus*, pero en diversas partes del mundo se producen también, sobre todos en los Estados Unidos y en Yugoslavia.

Otra arma efectiva en el arañeísmo, sobre todo en la casuística visceral, está constituída por los corticosteroídes, empleados tempranamente y con generosidad.

También debemos seguir recurriendo al tratamiento sintomático, destinado primero que a nada a calmar las manifestaciones fundamentales. En cualesquier arañeísmo el dolor predomina, luego las crisis nerviosas, las convulsiones, los transtornos de las secreciones, las alteraciones digestivas, la deshidratación, la anemia y la postración deben juiciosa y rápidamente ser atendidas. Sueros glucosalinos, gluconato de calcio, sedantes, opiaceos, cardiotónicos, etc., encuentran su aplicación en el arañeísmo.

No creemos, a pesar de todo lo dicho, que habría que liquidar o tratar de hacer desaparecer a las arañas. ¡Por ningún motivo! Ellas mantienen el equilibrio biológico en la Naturaleza y destruyen un gran número de especies dañinas para la agricultura y para el hombre. Pero como, por otro lado, hemos visto de lo que son capaces mediante el empleo de su veneno, como arma defensiva, estimamos que frente a las arañas no hay que tener temor, pero tampoco ser confiados. Corremos el riesgo de que nos digan, como en nuestra tierra se acostumbra, con mucha picardía, que somos unos "picados de araña"!

LOXOSCELISMO CUTANEO



1



2



3



4

Fig. 1 — El edema. Fig. 2 — *Loxosceles sp.* Fig. 3 — Las flictenas. Fig. 4 — La mancha gangrenosa.

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6. BIOLOGICAL SIGNIFICANCE OF CUTANEOUS SECRETIONS IN TOADS AND FROGS

BERTHA LUTZ

Museu Nacional, Universidade Federal, Rio de Janeiro, Brasil

The skin plays a very important role in the biology of the **AMPHIBIA ANURA**, i.e., Toads and Frogs. It subserves the respiratory function, since pulmonary respiration is not quite adequate for their needs. It is a naked skin unprovided with scales. It is kept moist thus permitting gaseous exchanges through the superficial capillaries.

The skin of the **ANURA** receives a special blood-supply over a large area of the body through cutaneous arteries and returns it through musculocutaneous veins. The total respiratory capillary length of the skin varies from 20.5 to 65.1 in the anurans studied for this factor. It is greater in thin-skinned and in aquatic forms than in terrestrial toads with a glandular skin and better developed lungs. Classic and modern studies of certain species show that carbon dioxide is mostly excreted by the skin and varies with physiological condition and activity where as the intake of oxygen by the lungs, being dependent on outside tension, is more constant.

Two main types of multicellular glands occur in the skin of adult Anurans: mucous glands and granular glands. The mucous glands produce a rather fluid secretion which lubricates the skin. The granular glands produce a creamy granular secretion which contains poisonous substances. The skin glands may be disseminated over the body but they tend to become localized and form masses at certain points, especially the granular glands. Some frogs, such as the neotropical genus *Cyclorhamphus* and a number of small kinds of *Paludicola*, have gelatinous glandular disks on the flanks. In the genus *Bufo*, which comprises the true toads, the granular glands form large masses in the postocular region; they are called paratoids, from a false analogy with the parotoid glands of mammals. Some other frogs also have parotoids, f.i. the large species of neotropical tree-frogs belonging to the genera *Phyllomedusa* and *Pithecopus*, but their parotoids are long and thin and continue along the dorsolateral edges of the body. The skin glands of Anurans, especially the glandular glands of toads, are of interest to students of Venomous Animals on account of the poisonous substances contained in their secretions but they are well-known in only a few genera and species.

From a biological point of view the skin glands of the **ANURA** are a mechanism of defense which aids survival of the individual.

The slimy secretion of the mucous glands is greatly increased the moment a frog is seized and makes it difficult to maintain the hold, the more so as lubrication is accompanied by intense wriggling to get away. The secretion frequently

has a strong odor, for instance of pepper, garlic, crushed leaves, or musk. It is often sternutatory and sometimes induces tears. Some frogs, like the garlic toad, or "Knoblauchkroete", *Pelobates*, cause the death of other frogs put into the same container with them. Another European frog, *Bombina*, can be kept alive in a vivarium full of turtles because it is not attacked by them. One of the most interesting cutaneous secretions is that of the large, glandular species of tree-frog of the neotropical *Hyla venulosa* group. The secretion dries to a rubbery consistence. Often, but not invariably, it provokes a reaction after handling the frogs.

These substances are poorly known. Their virulence may vary from one form to another but possibly also in the same form at different seasons or in different physiological conditions. My former assistant Miss Kloss had a prolonged and violent headache on inadvertently rubbing her eyes soon after handling the Amazonian form of *Hyla venulosa*. Other specimens, from Belém do Pará, very much handled by me, caused no symptoms at all. A painful rash, lasting a few hours came out on my hand and arm after catching a *Hyla imitatrix*, of the same group, dropped by a bird scuffling with it. The other frogs put with it arrived dead and glued together. Another specimen of the same form left a painless weal, which lasted many days, on my hand by laying a leg across it. The bromealiad collectors Mr. and Mrs. Racine and Mulford Foster called the Chaco form, *Hyla hebes* Cope, the "india rubber frog", because the secretion was abundant enough to permit them to make small pellets with it. Neither they not I felt any effects from it, even when I rubbed it on the inside of my lip. Professor Mertens mentions a burning sensation on seizing the unrelated *Hyla vasta* from Santo Domingo.

The secretions of the parotoid glands of the true toads are easier to obtain and have been studied more consistently. A number of poisonous substances have been extracted from them and named. There seem to be specific differences. However, these glands also constitute a mechanism of defense. Their secretion is seldom released spontaneously and even then after a great deal of provocation has been endured. The author only saw it spurt out once, in a toad that hit the ground after being dropped from a height. Nor can the toads introduce their venom into the body of their enemies. For it to take effect, the glands have generally got to be bitten into. Small dogs have worried toads may die or become very ill as a result of their indiscretion but the toad is a passive element. Experiments with toad venom by injection may be of biochemical interest but biologically they are artificial and disregard natural conditions.

Cutaneous secretions are supplemented by a few other simple defense mechanisms. The first is derived from the integument and is the coloring, especially of the permanently visible dorsal aspect. There are two main types of protective coloration, procrypt and aposematic. In procrypt animals the color is concealing either by resemblance to the background, or by a disruptive pattern which conveys a false outline and breaks up the visual image of the surface. The tree-frogs *Phyllomedusa* and *Pithecopus* are protected by similarity to the background. They live on the vegetation and in day-time the dorsal aspect is uniformely green. The color is brusquely out off at the edges of the permanently visible surfaces and thus separates them abruptly from those concealed in repose, which may have bright spots of aposematic color on them. At night, when they move about, the large species become very dark, purple or chocolate-colored, almost black. The true toads, *Bufo*, may have a uniform or mottled dorsal

surface, with a sexual dichroism in some species; which is very unusual in Anurans. The color may blend into the background or the surface be disrupted by mottling. *Bufo typhonius* and other forest-forms may look like leaves from above; some of them, like *Bufo guttatus*, are so dark beneath that they seem flat. In aposematic frogs, the bright coloring is seldom general, though it is uniform in a few, f.i., the minute pumpkin-colored *Brachycephalus ephippium*. As a rule, the bright colors either serve to disrupt the visible surface or they are flash-colors, often on the thighs, which are only seen momentarily when the frog moves.

An additional means of defense derives from increase of ossification, in direct opposition to the evolutionary reduction of the skeleton from ancient to recent amphibians. Some have a bony shield on the back, such as the tiny *Brachycephalus ephippium* just mentioned and the huge horned toads. More often the increased ossification is on the top of the head, the part of the body most vulnerable to attack. Many species of *Bufo* have bony crests which stand out; they vary from species to species and are used in taxonomy. Their greatest development is attained in *Bufo typhonius*, old and large specimens of which may have veritable wings to the sides of the head. Other toads develop a more or less complete skull-cap or helmet; these are burrowing forms. The function of these adaptative structures evidently is to prevent seizure and crushing of the head.

Habits and patterns of behaviour also provide means of defense. Nocturnal life is the rule in frogs and toads. One way of avoiding their enemies is by hiding and sleeping in day-time in holes and burrows, both natural or artificial (ground-dwellers), in bromeliads or among leaves (tree-frogs). Only the most agile running-water frogs, f.i., *Elosia* and *Megalosia*, can afford the luxury of diurnal life.

Some anurans have evolved attitudes of defense. The best known is probably the "Unkenreflex", from the German popular name "Unke" for the genus *Bombina*. It consists in arching the body with the back uppermost and curving the limbs up over it and the head, bringing the flash-colors of the ventral aspect into evidence. Many neotropical tree-frogs "play possum", lying quietly on the ground as if dead, while they await the chance to turn rapidly and leap away. Toads inflate their lungs to the utmost. Some rear up on their hind legs and butt with their heads. Others lean to one side and present a lateral view to the predator. This greatly increases the surface that has to be bitten into or swallowed. *Holaden bradei*, a minute toad from the Itatiaia, which lays terrestrial eggs and guards its nest, also rises on its legs and leans forward, hissing, when its spawn is threatened.

The simple mechanisms enumerated above exhaust the modest arsenal of defense of the toads and frogs, leaving them often at the mercy of their enemies. Some are better protected than others, f.i. the Antillean, phragmotic, casque-headed, toads which live in the ground and plug the openning of the burrow with their heads. Small toads with insignificant crests, like *B. granulosus*, hiding in hollows in dunes are however sometimes yanked out of their mobile homes by the head by snakes, as observed by Gliesch in Rio Grande do Sul. The British naturalist Loveridge once saw a rat rip the skin off a toad down the middle of the back and start to devour its flesh. He also observed other small mammals avoid the parotoids by tumbling over the toad and attacking it from the belly. In nature the predator-pray relationship is apt to be less favorable to anurans than to their enemies and loss may have to be compensated for by excessive spawning or by protection of eggs and larvae; this is done in many ways, beginning with the unpalatable and perhaps poisonous eggs of toads. They are seldom interfered with.

Among their enemies the **AMPHIBIA** must reckon human beings.

Toads have been barbarously used in witchcraft and primitive medicine; to this day many are flayed alive for their skins and let loose to die or be devoured by fire ants. The Tucana Indians imprison the large *Phyllomedusa bicolor* in little cages like birds. After bouts of drinking and gorging they scarify the skin at the temples and wrists and rub the paratoids of the frog on the scratches to bring on vomiting and catharsis. Many beautiful little frogs of the genus *Dendrobates* are caught by Amazonian Indians and impaled alive on sticks to roast over the fire; the agonic gouts of poison gained are used on arrows for killing monkeys and other small game. Can one really consider Venomous Animals those that never attack and can barely defend themselves? In the interests of science one must study them but the study should be carried out in a logic and in a humane manner.

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Discussion

H. Edery: "During the last years Prof. Erspamer and his collaborators have found in the skin of a number of frogs and toads extremely active peptides, some them related to bradykinin. Have you any information how they are formed? Are they originated in precursors proteins? The second question: which are the natural enemies of these frogs and toads you mentioned? I suppose they should be particular sensitive to the venom. Have you any information on this point?"

B. Lutz: "First question: I am afraid that I do not know the answer, but I refer you to Dr. J. M. Cei of the University at Mendoza, Argentina, who will answer your question.

Second question: The main enemies are snakes, arboreal (tree-frogs), terrestrial and aquatic. They seize the frogs by the head. Those which are casgne-headed are better protected, especially if they are phragmotic, taking refuge in a cavity and plugging the lienem with a snake. I once observed a *Bothrops jararaca* trying to get one out of a bromealiad, unsuccessfully. Those with less perfect helmet, like small *Bufo granulus* with crests only, are less well protected. They live in holes in the dunes, are janked out of their mobile burrow by the head, as seen by Prof. Rudolph Gliesch in Rio Grande do Sul.

Tree frogs, both male and female, seized by snakes, give a loud cry of distress. Mammals also sometimes eat frogs and toads. I presume that some of the predators are insensitive to the venom or avoid biting directly into the parotid glands. Dr. Freiberg of Argentina saw a very large chulean frog, *Calyptocephalella goeji*, swallow toads.

A British naturalist, Loveridge, saw a rat attack a toad in the middle of the back, behind the glands. He also saw small mammals tumble over the toad and attack it on the belly. I mentioned a birth that released, in mid-air, a *Hyla imitatrix* with irritant secretion. Aquatic frogs may be attacked by leeches. Many neotropical ones, especially bromeliad-dwellers, have larval mites in the skin. Some mosquitoes bite frogs. They also have worms, f.i. *Trematodes*. Dr. Adolpho Lutz found an oligochaete worm *Schomardaella lutzi* Michaelson in the bladder of some tree-frogs."

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7. TAXONOMY AND DISTRIBUTION OF ARROW-POISON FROGS IN COLOMBIA

DORIS M. COCHRAN

Division of Reptiles and Amphibians, U.S. National Museum, Washington, D.C., U.S.A.

Some of the dendrobatid frogs are known to secrete from their skin a substance used by native tribes of Colombia for poisoning their arrows. The genus *Phyllobates*, to which the highly toxic "kokoi" belongs, is represented by 14 known forms in Colombia, while *Dendrobates*, also poisonous, has 11 kinds within the borders of that country.

These frogs are all rather small, the largest reaching a head-an-body length of less than two inches. They are often brilliantly colored with yellow or orange spots or stripes on a dark background in a certain pattern characteristic of each form. Unfortunately, these bright hues disappear in preservative, and the specimen becomes gray or bluish, sometimes with darker areas.

Arrow-poison frogs of the genera *Phyllobates* and *Dendrobates* are told apart by the presence or absence of teeth on the maxilla, members of *Phyllobates* possessing teeth that may be felt with the point of a pin along the inner maxillary border, while in *Dendrobates* this area is completely smooth and devoid of such teeth.

It was formerly believed that the presence or absence of a web between the toes of the hind foot further divided the genus *Phyllobates*, the frogs lacking such webs being placed in the genus *Prostherapis*. The variation in degree of webbing in some of the species is so great, however, that no valid reliance can be placed on it to justify such a separation on that characteristic, therefore since *Phyllobates* is the older name, all frogs since referred to as *Prostherapis* have been placed under *Phyllobates*.

While much collecting remains to be done in Colombia before we can have a complete picture of the distribution of the 25 forms there, at present the largest number of kinds is recorded from Antioquia and Chocó, each of these departments having four *Dendrobates* and five *Phyllobates*. Undoubtedly more intensive searching in other favourable regions will turn up as many or more for some of the other departments.

Other Central and South American countries also have a good population of arrow-poison frogs, although these at present appear to be more numerous in Colombia than in any of the adjoining regions.

These little frogs are terrestrial as a rule, living among the vegetation on the floor of the forest, or in heavy grass near some stream or pool. Their dappled golden spots or lines render them nearly invisible in such situations. They give

a gentle, whistling call, and this betrays their hiding place, although they are not readily seen even then. Presumably they feed upon small insects and other minute invertebrates. Very little has been recorded as to their actual feeding habits in the wild state.

Breeding occurs during the spring and summer. When the eggs have hatched, a male will present himself among the very young tadpoles, and they instinctively attach themselves to the skin of his back by their suction organs. He then carries them safely for several weeks, until they have grown sufficiently to fend for themselves. While the male is thus "incubating" the tadpoles, he frequently visits a shallow stream or a tree-hole containing rainwater, in which he immerses himself and the tadpoles for some time, thus keeping them from becoming too dry.

When using slivers of bamboo as blow-gun darts, the Indians had to employ a very quick-acting killing or paralyzing substance that would drop the bird or mammal they had hit before it could escape into the dense underbrush beside the jungle trail and so be lost to them as an item of food. For this purpose kokoi poison was in use long before the coming of the white man, and was singularly quick and effective once it had entered the bloodstream of the animal shot. According to Dr. Bernard Witkop of the National Institute of Health, whose analysis of it will be presented to you later in this symposium, it is one of the most powerful animal poisons yet known.

As a taxonomist, my chief interest in these frogs has been the identification of the Colombian forms, together with their aparent relationships. There follows a short summary of some of their characteristics, taken from a forthcoming paper on the frogs of Colombia by Dr. Coleman J. Goin and myself.

Both *Dendrobates* and *Phyllobates* are now considered to belong to the Subfamily DENDROBATINAE of the family RANIDAE. As I have already mentioned, members of the genus *Dendrobates* have no teeth on the maxillary bone, while *Phyllobates* possess such teeth, not readily visible except under a high-power lens, but easily felt by drawing a pin or other pointed object along the inside of the maxilla. Both genera have a longitudinal furrow along the top of each digit, forming two distinct platelets, unlike the single undivided disks found in most other frogs having enlarged toe and finger tips.

I shall consider first the 11 forms of *Dendrobates* now known from Colombia. In these the relative lengths of the first and second fingers are usually diagnostic, also the position reached by the adpressed heel in relation to the eye or tympanum. The color pattern is usually highly characteristic of the species. Due to the considerable amount of variation in the individuals of each species, the following statements as to the finger proportion, leg length and color pattern are only generally applicable, however, and each frog should be compared with the best available description and figure.

The largest Colombian *Dendrobates* known at present is *trivittatus*, reaching a head-and-body length of 46 mm. In alcohol it is mostly slatecolor, with a lighter stripe down each side of the back, and a lighter unspotted venter. It is one of the two species in Colombia in which the first finger is distinctly longer than the second. It is known from Brazil, British Guiana, Ecuador and Peru, as well as from Amazonas in Colombia.

The other *Dendrobates* with the first finger longer than the second represents a new species found in Caquetá, Colombia. Until its description has been published, I am not at liberty to discuss it further.

Two well-known *Dendrobates* with the first finger nearly as long as the second and with the heel reaching to the center of the eye when extended forward are *hahneli* and *lugubris*. A pale axillary spot, light narrow dorsolateral lines and reticulations on the posterior half of the belly distinguish *hahneli*, a small species reaching 23 mm in length and known from Meta and Putumayo in Colombia, as well as Peru. Lacking the axillary spot, having wider dorsolateral lines, and with light spots or reticulations scattered on the belly, *lugubris* in alcohol is a slate-colored frog of 34 mm., perhaps much brighter in life; it is widely distributed in Colombia and is known also from Panama.

The next large division of Colombian *Dendrobates* consists of those having the first finger much shorter than the second. The first subgroup contains very small frogs, not longer, than 18 mm. In *opisthomelas* and *minutus ventri maculatus* there is a large light spot on the under side of the upper arm reaching from the axilla nearly to the elbow. In the former the heel reaches the anterior border of the eye, and there are no definite dorsal stripes; it is known from Antioquia in Colombia. The latter, first described from Ecuador, has a shorter leg, with heel reaching only to the posterior corner of the eye; usually there is a wide middorsal and a pair of dorsolateral light stripes. It is found in Caldas and Caquetá, Colombia.

The nominate form of the last-named has no conspicuous light spot below the upper arm. It is known at present from Panama and from Antioquia in Colombia.

The last subgroup under those frogs with short first fingers are the "clown-frogs", so-called because of their brilliant colors and patterns. The subspecific name of one of these is *histrionicus*, meaning an "actor". This one is black above with a large light spot (yellow or orange in life) on the snout and another between the shoulders. Its lower surface is also dark, with a rectangular light spot covering throat and chest, and another on the posterior part of the belly. Its maximum known size is 38 mm., and it occurs in Antioquia, Caldas and Chocó in Colombia.

A second form of *tinctorius* is *wittei*, about the size but with many small rounded silvery-white (orange in life) spots on the anterior part of the back, these becoming much larger on the sacrum. The lower surface is pearl gray in alcohol, with an irregular black patch on the center of the throat. It is known only from Chocó in Colombia.

A third subspecies is *chocoensis*, light above with several irregular dark spots on the back and dark brown below. Possible intergrades between *chocoensis* and *histrionicus* have been taken at Playa de Oro on Rio San Juan, Chocó. This most interesting mountainous region gives rise to streams draining two watersheds, one pouring into the Caribbean, the other into the Pacific. Perhaps the early geological history of this area explains the occurrence here within a short distance of each other of the three subspecies of *tinctorius* just mentioned.

A fourth subspecies of *tinctorius*, *confluens*, occurs in Cauca and Nariño, Colombia. In alcohol it is olive-gray with many small irregular black spots, but was said to be scarlet in life. A great deal more remains to be done both in collecting and in analysing the characters of these little frogs before the final word as to their identity can be said.

The genus *Phyllobates* is as variable in some of its characteristics as is the foregoing *Dendrobates*. Some authorities have recognized Cope's genus *Prostherapis* for frogs with maxillary teeth having webbed toes,

reserving *Phyllobates* for those without webbing on the feet. I find that the variation in regard to degree of webbing is so great in some Colombian species that this distinction can no longer be applied, as some frogs may have no perceptible web, while others of the same species from the same locality may have a small but distinct vestigal web. A separation on the character of skin, — whether it tears easily or seems resistant to abrasion — coupled with large size, heavy build and lack of distinct light striped may yet be possible. Such a distinction using only preserved material has not proved feasible. At any rate, the 14 Colombian forms to be discussed are now considered to belong to the genus *Phyllobates*.

In the first section of these, in which the toes are free or nearly so, and the first finger is usually longer than the second, we find four species. In *bicolor* (the poisonous "kokoi" of which you will hear later), the back and posterior part of belly are uniform pearl-gray or slate color, although these parts are known to be bright red-orange in life, while the upper surfaces of the limbs, now gray, were once yelowish in tone. The anterior half of the belly, as well as the throat and chest, are cinereous in alcohol, but in life were straw yellow to cream color. A Peruvian example had numerous black spots on the throat and chest, with a dark triangle on the center of the belly. The "kokoi" is about 42 mm. in length when adult. In addition to Peru, this frog has been collected in Antioquia, Caldas, Cauca, Chocó and Valle.

A light dorsolateral stripe, sometimes faint, occurs in the remaining three species having the toes free. The first finger is longer than the second, and the heel reaches between the anterior and posterior corners of the eye in *boulengeri* and *femoralis*. The former has coarse dark reticulations on the belly which continue on the throat and form a pair of short parallel dark stripes on the chin. It is a small frog measuring only 22 mm. and is found in abundance on Gorgona Island, and rarely on the adjoing mainland in Nariño and Valle. Its close relative is *femoralis*, with the chin and anterior part of belly black, the posterior half of belly being marbled dark and light. It is not known to exceed 26 mm in length. We know it from British Guiana and Peru, and from Amazonas, Caldas, Caquetá, Mata, Putumayo and Valle in Colombia. A fourth species in this section is *mertensi*, with toes that may be barely to almost 1/8 webbed. Its legs are short, the heel reaching between the tympanum and the posterior corner of the eye. Its belly is drab with heavy dark reticulations, and it reaches a length of some 30 mm. At present it is known only from Cauca.

In the remaining Colombian *Phyllobates*, the toes are webbed at the base up to 2/3 webbed. Some have the first finger shorter than the second. Among this, nominate *subpunctatus* has the toes webbed only at the base, while its heel reaches the posterior corner of the tympanum. Its belly is pinkish buff often small scattered dark spots, and its maximum size is about 22 mm. It appears to be restricted to Cundinamarca. The next three have more extensive webs. In *vergeli* the heel reaches the tip of the snout, the belly is immaculate, and the size is about 22.5 mm. It likewise is unknown outside of Cundinamarca. In the two remaining in this section, both have dark spots or reticulations on the belly; in *chocoensis* the toes are 1/2 webbed, the maximum size is about 27.5 mm., and the range is Chocó and Antioquia. A new species not yet published falls near the three preceding.

In the next section the first and second fingers are subequal. *Palmatus* has the toes 1/3 to 1/2 webbed; the belly is immaculate drab, and the size is up to 36.5 mm. This is one of the species most notable for its easily abraded

skin. In the rather large number of preserved specimens examined, I found almost none in which the skin was not ragged and torn, even when obvious care had been taken in collecting and packing the specimens. In spite of its susceptibility to abrasion, it is one of the commonest *Phyllobates* in Colombia, being recorded from over half the states. A new subspecies of *subpunctatus* from Boyacá is likewise placed with frogs having the first and second fingers subequal.

The final species have the first finger longer than the second and possess distinct webs. In *brunneus* the heel reaches the anterior corner of the eye, the belly may be immaculate or finely spotted, while its size does not exceed 22.5 mm. It is found over a wide range, from Panama, Ecuador and Brazil, and including most of the states of Colombia. Two species have the heel reaching the center of the eye; one of these, *pratti*, has the belly immaculate and is very small, measuring only 18 mm. in length. It is known at present only from Chocó. The other, *latinasus*, has the lower surface immaculate except for a dark line around the lower jaw and a patch of dark dots below the shoulder. Its size may be up to 26 mm., and its range is Panama, Ecuador and through much of Colombia. The final species, *inguinalis*, has the toes 1/4 webbed, the heel reaching to the center of the eye, and the belly drab with a few darker spots below the femur and tibia. It attains a length of 29 mm., and occurs in Panama and in northern and western Colombia.

Needless to add, a great deal more collecting and comparison of species is needed before we can say the final word on the taxonomy and variation of these remarkable genera of arrow-poison frogs.

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8. MODES OF EVOLUTION IN NEW WORLD OPISTHOGLYPH SNAKES

JOSEPH R. BAILEY

Department of Zoology, Duke University, Durham, U.S.A.

The area I have chosen to discuss this afternoon is the general phenomenon of opisthoglyphy and its evolutionary implications with emphasis on New World forms. Opisthoglyphy is the condition in colubrid snakes marked by the presence of grooved teeth on the posterior expanded portion of the maxillary bone immediately below its articulation with the ectopterygoid. These teeth are usually in pairs on either side, with one of each pair shed alternately, so that at least one is functional on a side at any given time. The grooves are on the anterior or outer face and serve as channels for the flow of venom by ducts from glands in the temporal region of the head. These glands, their histology, homology and biochemistry are currently being studied by my colleague, Aaron Taub, at Pennsylvania State University.

A half century ago the possession of grooved posterior maxillary teeth by a snake was thought to indicate subfamilial status among the COLUBRIDAE; i.e., all snakes with such dentition were considered to have been derived from a common ancestor. Since that time there has been a growing consensus among ophiologists that this opisthoglyph dentition has arisen many times in the course of snake evolution, and today systematists consider the grooved rear teeth to be useful taxonomically only at the generic and specific levels, and at times to vary even within a species. While being discredited, or at least demoted, as a useful character in classification, the morphological condition has, at the same time, been grossly neglected as a biological phenomenon. The fact that a considerable number of species of opisthoglyphs on three continents are known to be dangerous to man, and at times even fatal, indicates that the rear fangs as functional mechanisms are worthy subjects of investigation in their own right.

This paper examines opisthoglyphy from evolutionary, phylogenetic, geographical and ecological aspects in an attempt to discover any generalizations which might pertain to the condition, and which indicate promising avenues for further research.

In some species the grooves become subjective since their degree of development is sufficiently variable that one specimen may show it and another may not. This has been shown by Stickel (1943) in *Sonora*, *Erythrolamprus mimus* by Dunn and Bailey (1939) and it occurs in *Rhadinaea guntheri* according to verbal information from Charles Meyers who is making an intensive study of that genus and its relatives. This latter species currently resides in the literature under two names in two different genera.

Apparently any teeth may show a grooved condition. I have examined a skull of *Oxyrhopus formosus* which bears distinct grooves on the outer faces of the mandibular teeth. The type of *Calamodontophis* has them on the

anterior maxillary teeth as well as the posterior, and a skull of *Tomodon dorsatus* has been examined in which the posterior fangs are deeply and doubly grooved. These rear fangs vary considerably from species to species as to their absolute length and as to their length relative to the more anterior teeth. In many these are scarcely differentiated. The most extreme condition I have observed is in *Tomodon dorsatus* in which the length of the fang may be nearly seventy per cent of the length of the maxillary bone. This species also illustrates what is probably the extreme of evolution towards functional dependence upon the fangs, since in some individuals the maxillary teeth are missing altogether or may be represented by one or two teeth only. In these individuals the dentigerous blade of the maxillary bone is thinned to much less than the basal diameter of a tooth. If only a single tooth is present the bone is thickened at that point only sufficiently to accommodate it, as if in development the tooth primordium had induced the deposition of the necessary bony base. Curiously and admittedly on an insufficiency of data, it appears that the fangs are relatively shorter when the anterior maxillary teeth are lacking. It is my belief that the anterior maxillary teeth are in the process of being lost and the fangs will take over as the whole maxillary dentition. This has perhaps reached the quantum stage (Simpson, 1953:389) and its evolutionary progression is currently very rapid. We know little of the functional aspects of this problem. No one to my knowledge has even described the feeding behavior of *Tomodon*, and until a careful analysis of the mechanical aspects of feeding in this species has been made I think it premature to speculate further.

How many different times the opisthoglyph condition has been derived in the course of evolution may never be determined. However its multiple origin would indicate that under certain ecological situations there is an adaptive advantage conferred on its possessors just as there is in the case of webbed feet in aquatic birds or mammals.

I am convinced from a long time intensive study of the relatives of *Drepanoides* that its flattened sabre-like posterior maxillary teeth, lacking grooves, have evolved from an opistoglyph *Clelia*-like ancestor. The only food record I have for it is reptile eggs which hardly require the injection of venom for subjugation.

Where, geographically, do we find opisthoglyphy? Table I makes it clear that the opisthoglyph condition is increasingly more prevalent as we move outward from a north polar center toward the tips of the three peninsulae of Austral-Asia, Africa and South America. Whether based on genera or species the percentages range from zero in the north to approximately fifty per cent in Australia, South Africa and Argentina. Fifty years ago it might have been tempting to invoke Mathew's (1915) then recently proposed thesis that the more primitive opisthoglyphous forms were forced out of more northern centers of origin by more progressive aglyphous forms. However, this interpretation would run counter to classical, and to my beliefs, of the true phylogenies. I think the answer is to be found in the ecological-behavioral area, possibly combined with recent invasions of northern geographical elements.

Habitudinally, without going into details and citing long lists of examples, opisthoglyps in the New World are well represented in arboreal, fossorial, and terrestrial habitats, and have only failed to establish themselves in the aquatic zone. In the Old World however, the opisthoglyph HOMOLOPSINAE are highly aquatic. A quantitative analysis of New World Snakes by habitats at this time is imprudent

due to the dearth of reliable field knowledge of the snakes preferences. Many genera include species of diverse habitat selection, as for instance *Philodryas*, an opisthoglyph, and *Thamnophis*, an aglyph. Tabulation then becomes necessary on a specific basis which is, of course, well beyond the scope of today's discussion.

Granted the supposition that an elliptical pupil indicates greater nocturnal activity, as interpreted from histological evidence by Gordon Walls (1932), we find a geographic analysis on this morphological basis revealing in the New World fauna. In Table 2 we see that among the aglyphous genera the round pupil greatly predominates in North and South America. Among ophisthoglyphous forms the elliptical pupil predominates when the continents are combined and they form 60% of the ophisthoglyph colubrid fauna of South America. The prevalence of nocturnal forms in the tropics is not all surprising among poekilotherms since cool nights of temperate regions are inhospitable to requisite nocturnal activity, but the hospitable tropic evening invites exploitation. The opisthoglyphs have apparently been better able to exploit nocturnal niches efficiently than the aglyphous groups. Whether this is due to the presence of a venom apparatus may be open to question. However, the more specialized dangerously venomous families are predominately night adapted. On the other hand the American opisthoglyph genera with elliptical pupils may be traced to not more than four phylogenetic stocks and two of these, comprising the relatives of *Pseudoboa* and those of *Tachymenis* contribute two thirds of the genera so that the success of these two lines in South America tips the balance in their favour. The phylogenetic stocks comprising the round pupil ophisthoglyph element are far more diverse.

In both groups the greater exploitation of the nocturnal environment in the tropics goes far to explain the greater diversity of colubrids in low latitudes.

Turning to the question of comparative food habits we are again confronted with a dearth of reliable knowledge of the animals in nature. Throughout my systematic studies of snake collections I have kept careful notes of food items, but these accumulate slowly, detailed literature reports are few and scattered, and the general ones are mostly repetitions of previous sketchy data.

Again, without going into detail, I suggest that on the average, and I emphasize average, the diet of opisthoglyphs is more narrowly circumscribed than that of aglyphous species. Perhaps it would be more accurate to say the diet of nocturnal forms is more restricted than that of diurnal species, because within my limited knowledge the nocturnal aglyphous species have as limited dietary selection as do the opisthoglyphs. I do not know any opisthoglyph which has the broad spectrum of food selection which is exhibited by *Coluber constrictor*, the elaphes, *Agkistrodon mokeson* and *A. piscivorous*. The mollusc eating DIPSADINAE are tropical, aglyphous, and nocturnal with very narrow dietary restriction. Most opisthoglyph species which are adequately known fit this pattern. For instance from my own work I find that adults of *Clelia* feed on other snakes and usually good sized venomous ones, perhaps, not because they are venomous but because they are more sedentary and available. *Erythrolamprus* is a snake feeder. Adults of *Oxyrhopus* and *Pseudoboa* are mammal eaters whereas *Rachidelus* is reputed to eat birds. *Phimophis* and *Siphlophis* are lizard feeders as are the juveniles of *Clelia*, *Oxyrhopus*, and *Pseudoboa*. *Tantilla*, with its curious short blunt maxillary teeth, is the only genus I know to specialize in centipedes, as indicated by the scattered

half dozen or so records I have plus literature reports (Hamilton, 1956; Force, 1935). Our knowledge of this area of snake biology is too embryonic to justify conclusion at this time. Not only do we lack information on which food items are taken, but just as important, we do not know to what extent choice enters the picture. Are items taken simply because they are available at the time and place of snake activity and are of a suitable size, or does real choice and biological selection influence the results?

In conclusion I would like to briefly summarize the points suggested above; and then to point out other areas of insufficient knowledge:

1. Grooves may appear irregularly on teeth in several parts of the mouth.
2. Opisthoglyphy is polyphyletic in origin.
3. In *Tomodon dorsatus* the anterior maxillary teeth are in the process of being lost entirely and the large fangs will remain as the only maxillary dentition.
4. Aglyphy may be secondarily derived from an opisthoglyph condition, as in *Drepanoides*.
5. Opisthoglyphy is absent in the most northern colubrid faunas but is at least equally numerous with the aglyphous in the most southern faunas.
6. Opisthoglyphy is found in all habitats (except the aquatic in the New World).
7. Opisthoglyph genera predominate among nocturnal colubrids.
8. Opisthoglyph snakes take a wide variety of foods, but the diet of each species (or genera) is rather narrowly circumscribed.

Areas of future research:

1. Little has been done of an experimental nature on the activity patterns of snake species; at what hours are they active, at what temperatures, light intensities, humidities, etc.... This subject could best be approached on snakes in captivity.
2. We know far too little of the detailed food habits of all snakes in nature. I suggest these be kept and recorded in the literature in detailed and quantitative fashion including the size of the snake. Records in captivity should be noted as such, and food items rejected noted along with those accepted.
3. We need careful observation and analysis of the feeding behaviour and mechanics of nearly all snakes. Such analysis should utilize high speed photography when possible.
4. Virtually nothing is known of the pharmacology, immunology and biochemistry of opisthoglyph venoms. Will these disciplines help us in understanding opisthoglyph phylogenies or explain restricted food habits?

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TABLE 1 — LATITUDINAL TRENDS IN OPISTHOGLYPHY IN THE COLUBRIDAE

New World

	Genera	Percent Opisthoglyph	Species	Percent Opisthoglyph
Canada	9	0	13	0
North Carolina	20	5.0	37	2.7
Mexico	66	22.7	264	31.4
Costa Rica	45	28.9	115	27.8
Ecuador	42	35.7	117	27.4
Argentina	34	50.0	70	47.1

Europe-Africa

	Genera	Percent Opisthoglyph	Species	Percent Opisthoglyph
England	2	0	2	0
Europe	8	37.5	21	14.3
Africa	73	45.2		
South Africa	26	53.8	54	46.3

Asia-Australia

	Genera	Percent Opisthoglyph	Species	Percent Opisthoglyph
Japan	15	26.6	36	14.0
China	27	22.2	90	11.1
Thailand	33	33.3	72	30.0
Malaya	30	40.0	53	34.0
Australia	9	55.5	15	46.7

TABLE 2 — CORRELATION OF DENTAL TYPE WITH PUPIL SHAPE IN AMERICAN COLUBRIDAE

	Pupil Shape	Americas		South America	
		Number Genera	% Genera	Number Genera	% Genera
Aglyphous	Round	73	89.0	28	84.8
	Elliptical	9	11.0	5	15.2
Opisthoglyphous	Round	16	47.1	11	39.3
	Elliptical	18	52.9	17	60.7

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9. POISONOUS SNAKES OF SURINAM

L. D. BRONGERSMA

Rijksmuseum van Natuurlijke Histoire, Leiden, Holland

Relatively little has been published about the poisonous snakes of Surinam. Scattered notes on isolated specimens or on small collections have been published in various journals. Moreover, some information may be obtained from comprehensive works, like Schlegel's (1837) "Essai", Boulenger's (1896) catalogue, Amaral's (1929, 1931) check lists, Klemmer's (1963) list of the poisonous snakes of the world, and as far as ELAPIDAE are concerned from Schmidt's (1936) preliminary account of South American coral snakes. It seems that in the last hundred years only two authors (Kappler, Van Lidth de Jeude) have dealt with the Surinam snake fauna as such. Van Lidth de Jeude (1914-1916) dealt with poisonous snakes in a series of articles in an encyclopaedia; he did not aim at completeness, and his notes have only a very limited value for our purpose. From 1842 to 1846 Kappler made it his business to collect zoological specimens in Surinam, and in two books he published notes on the snakes (Kappler, 1881:137-139, 166-167; 1887:128-137). Of most interest is the list of species (Kappler, 1881: 166-167) of which he sent specimens to the Stuttgart Museum; it is not known to me who was responsible for the identifications. The following poisonous snakes were recorded by Kappler (1881:166-167): *Elaps surinamensis, E. hemprichii, E. lemniscatus, E. collaris, Crotalus horridus, Lachesis mutus* (on pp. 33, 138, named: *Trigonocephalus rhombeatus*), *Bothrops bilineatus, B. atrox.*

Since 1881 only three species have been added to this list, viz., *Micrurus psyches, M. averyi,* and *Bothrops neglecta.*

Taking into account taxonomic and nomenclatorial changes, to-day a list of the poisonous snakes of Surinam reads as follows:

ELAPIDAE

Leptomicrurus collaris (Schlegel, 1837),

Micrurus averyi Schmidt, 1939,

Micrurus hemprichii hemprichii (Jan, 1858),

Micrurus lemniscatus lemniscatus (Linnaeus, 1758),

Micrurus psyches (Daudin, 1802-1803),

Micrurus surinamensis surinamensis (Cuvier, 1817)

CROTALIDAE

Bothrops atrox (Linnaeus, 1758),

Bothrops bilineatus (Wied, 1825),

Bothrops neglecta Amaral, 1923,

Crotalus durissus terrificus (Laurenti, 1768),

Lachesis mutus mutus (Linnaeus, 1766).

Leptomicrurus collaris (Schlegel)

The history of *Elaps collaris* Schlegel was discussed by Schmidt (1937; 1939: 45, note 1), but as his survey is incomplete, and erroneous as regards some details, a more complete account will be published by me elsewhere. For long years this species has been included in surveys in the Philippine fauna as *Hemibungarus collaris*. Schmidt (1937:361) believed that the specimen from (British) Guiana was the first that proved in which part of the world the species is to be found. This is incorrect, however. Both Schmidt (1937) and Thompson (1913) overlooked that the species had been recorded from Surinam already by Kappler (1881:167). This record is substantiated by a specimen, which Kappler sent in 1844 to the Stuttgart Museum (now: Staatliches Museum für Naturkunde, Ludwigsburg). Another specimen from Surinam, collected more than hundred years ago, was discovered recently in the collections of the Zoologisch Museum, Amsterdam. At one time it belonged to the Vrolik collection *, part of which was acquired by the Anatomical Institute of Amsterdam University; in 1943 it was passed on to Zoological Museum. Schmidt made *Elaps collaris* Schlegel the type of his new genus *Leptomicrurus*.

Leptomicrurus collaris is one of the Coral Snakes that lacks an annulate pattern. Except for a whitish collar, an indication of a whitish bar across the snout, and large whitish spots on the ventrals reaching laterally on to scales of the first two rows, it is uniformly dark brown.

Micrurus lemniscatus lemniscatus (L.)

The Surinam specimens examined by me agree with the typical subspecies both in the number of ventrals and in coloration. A specimen from Macasseema, (British) Guiana (BM 87.1.22.14), with 256 ventrals also agrees with this subspecies. A male from Cayenne (French Guiana) (ML 1422) has only 223 ventrals, and in this respect it would come within the range of variation of *M. l. diutius* Burger (1955), which, according to the original description, occurs in Trinidad, Venezuela, and part of Guiana; in coloration this Cayenne specimen agrees with the typical subspecies.

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* The Vrolik collection belonged to Prof. G. Vrolik (25-iv-1775 — 10-xi-1859), and later to his son Prof. W. Vrolik (29-iv-1801 — 22-xii-1863) (Engel, 1939:329-330).

Micrurus surinamensis surinamensis (Cuv.)

Although it is usually stated that one of the characters of Elapid snakes is the absence of a loreal, the (posterior) nasal being in contact with the preocular, and thus separating the prefrontal from the upper labials, there are some abnormal specimens of *M. s. surinamensis* in which a scale is present between the nasal and the preocular, and which hence show a loreal. In one Surinam specimen (ML no. 1398) such a loreal is present on either side; another Surinam specimen (ML no. 1417) has a loreal on the right side, and in a further specimen (ML no. 1419) two loreals, one behind the other, are present on the left side; in a specimen from (British) Guiana (BM, purchased of Mr. Leadbeater) a loreal is present on the left side.

Bothrops atrox (L.)

Bothrops atrox is a species with a very wide range of distribution in South-America, and it is not to be wondered that it is found also in Surinam. I have not used trinomials in this case, because I believe that more research is necessary on the variation of this species before one can safely divide the species into subspecies. Should the occasion arise, that Guiana specimens have to be recognized as a distinct subspecies, it must be borne in mind that at least three names are available, viz., *Bothrops subscutatus* Gray (1842:47), *Bothrops sabinii* Gray (1842:47), and *Bothrops affinis* Gray (1849:7).

Bothrops atrox is fairly common in the low coastal area, but it also occurs farther into the interior. In the Nassau Mountains it was found in a river valley at 464 m above sea level. As far as our information goes at present, it seems that it has a preference for damp areas near water.

Bothrops neglecta Amaral

This species was described by Amaral (1923:100-101) from two specimens, both males. The holotype came from Bahia, Brazil, the paratype from (British) Guiana. At the time, Amaral (1923:101) suggested that the locality record for the paratype might be erroneous. Amaral (1929:237; 1931:100, reprint: 8), mentions the species from Bahia only; Klemmer (1963:408) mentions it from Bahia and Venezuela. However, it seems to be unlikely that the paratype, which the British Museum (Natural History) received from the Demerara Museum came from anywhere else than Guiana. Moreover, Parker (1935:525) mentions four other Guiana specimens received by the British Museum (Natural History), and since that time still another specimen was added to the London collection. From Surinam I have examined eight specimens. The range of variation in the numbers of ventrals and subcaudals is small, and as far as the small number of specimens allows, of any tentative conclusions, there seems to be very little difference between the sexes; in eight males the number of ventrals varies from 156-162, that of subcaudals (pairs + one) from 45-52; three females show 156-162 ventrals, and 43-46 subcaudals.

A specimen, taken on the Upper Tapanohoni river, in the mountains on the Surinam-Brazil border, was referred by Hoge (1964:63) to *Bothrops brazili* Hoge (1953). After having examined this specimen, I identified it as being *Bothrops neglecta*, for the following reasons. The specimen, a male, has 159 ventrals, and

the subcaudals 8/7 + 4 + 26/26 + 1 + 6/6 + 1 + 0/1 + 1 (47 in all). With these counts it comes within the range of variation of *B. neglecta*, but it remains below the counts of the two type specimens of *B. brazili*, which have 175 and 179 ventrals, and 55 and 60 subcaudals respectively. It must be remembered that Hoge (1953:15) pointed out that there was a strong resemblance in colour pattern between *B. brazili* and *B. neglecta* (as well as with *B. pirajai* Amaral). The colour pattern of the Tapanahoni specimen agrees very well with that of the other Surinam and Guiana specimens of *B. neglecta*, but with reference to Hoge's (1953:15) remarks, this need not be decisive. However, *B. brazili* is stated to lack a nasal pore (Hoge, 1953:15), whilst in *B. neglecta* such a pore is present. After careful examination of the Tapanahoni specimen, I arrived at the conclusion that a nasal pore is present, and that it is of the same shape as that of *B. neglecta*. Taking all these features (ventral and subcaudal counts, colour pattern, nasal pore) into account I feel convinced that the specimen must be referred to *Bothrops neglecta* Amaral.

Whether the Guiana specimens (including the paratype) and the Surinam specimens of *B. neglecta* are conspecific with the holotype, which came from Bahia, is a question that can only be settled by direct comparison.

As far as our present knowldege goes, *B. neglecta* is not found in the coastal area of Surinam, but only on higher grounds more in the interior. It seems that it does not have the preference of *B. atrox* for the vicinity of water, but that it occurs in the forest on higher ground. The only specimen with a definite record of the altitude was taken in the Nassau Mountains at 406 m above sea level in the forest on the slope of a hill.

Bothrops bilineatus (Wied)

This species has been recorded from Surinam already by Kappler (1881:167; 1887:137); Schlegel (1837, II:540, *Trigonocephalus bilineatus*) mentioned its occurrence in Cayenne (French Guiana); Quelch (1899:407, *Lachesis bilineatus*), and Parker (1935:525, 529) recorded it from (British) Guiana. Therefore, it is rather astonishing that the occurrence of this species in the three Guianas is not mentioned in comprehensive works, like Klemmer's (1963:404) list.

Crotalus durissus terrificus (Laur.)

Allen & Neill (1957) have pointed to the possible existence of two ecological forms of *Crotalus durissus terrificus* in (British) Guiana. In Surinam too it is said that there are two different forms of rattlesnake, which differ in coloration, and which occur in different habitats. The material available to me is too small to form a definite opinion.

Comparing the snake fauna of the three Guianas, there seems to be no difference, at least as regards the poisonous snakes. It is true that *Bothrops neglecta* has not yet been recorded from French Guiana, but this will be only a matter of time. *Micrurus averyi* is known from a single specimen only, but I do no doubt that it will be found to occur in all three Guianas.

Very little is known about the distribution of snakes within Surinam. In the old times "Surinam" as a locality record was considered to be sufficient, and in any case most collecting was done fairly close to the coast. Gradually some

information is coming from the interior, as more collectors are penetrating farther to the south. *Bothrops neglecta* apparently is a species that prefers the higher parts of the country, and this may explain why it has been reported from Surinam only fairly recently. Coral snakes (genera *Micrurus* and *Leptomicrurus*) are not very often represented in collections, but this need not mean that they are exceedingly rare. More probably it is a matter of not knowing the habitats preferred by these species. *M. s. surinamensis* and *M. l. lemniscatus* are more often represented in collections than the other species, and this may also point to their being lowland species.

If little is known about the distribution of snakes within Surinam, still less is known about the frequency of snake bite. Recently, Kabaart (1962) reviewed the situation. Although military personnel often goes on patrol into the jungle, the data collected by Kabaart show that in the period 1925-1958 not a single case of snake bite by a poisonous snake occurred. In 1958 two civilians died from snake bite, but the species of snake is not mentioned. There have been a few cases of snake bite, apparently by non-poisonous snakes, no effects of poisoning being apparent. Earlier authors (reviewed by Kabaart, 1962:220-221, reprint: 3-4) also state that snake bite is very rare in Surinam.

Of course one does not know how many cases of snake bite occur in the interior, because these are not reported to the medical authorities.

As an inheritance of their African ancestors, the negro population of Surinam (and many other people as well) put great faith in "sneki koti", which may be used for inoculation, or as an antidote after snake bite has occurred. Its composition is not completely known, except that the main ingredient is the head of a poisonous snake, roasted and ground into powder. Opinions differ slightly as to what is added, but usually it is stated that roasted and ground leaves of various plants are added. Those, who know how to prepare "sneki-koti" are not allowed to tell what the ingredients are, because then the antidote would lose its power. Moreover, inoculated persons have to refrain from eating some kinds of food, e.g., deer or turtle, etc. Although it has repeatedly been shown that "sneki koti" is of no value at all, it is very difficult to eradicate this superstition. Only very rarely it is known which species of snake was responsible in a case of snake bite. "Sneki koti" will be applied to bites of harmless snakes too, and if the patients after this treatment do not show any signs of poisoning, this is ascribed to the effect of "sneki koti". If the patients dies, it is assumed that he has eaten of forbidden food.

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Discussion

A. do Amaral: "The generic name *Bothrops* being of feminine gender oblige us to say *Bothrops neglecta* and not *neglectus*. With regard to the variabilities of the markings and changes that occur during growth I have written a paper, in 1925, about the variations in colour pattern in other species."

B. Lutz: "The Guiana-specimens of *Bothrops bilineata* belong to the same subspecies as those occurring further to the south of Bahia?"

L. D. Brongersma: "Perhaps Dr. Hoge may answer the last question because he has recently distinguished between two subspecies of *B. bilineata.*"

A. R. Hoge: "I have only seen a few specimen from the Guianas and although very similar to *B. bilineata.* There are slight differences in colour and pattern but more material is needed to arrive to a conclusion."

After the meeting A. do Amaral, A. R. Hoge and L. D. Brongersma have examined and discussed the specimens of *Bothrops neglecta, B. pirajai* and *B. brazili* in the collection of the Instituto Butantan. It became clear to all that the Guiana- and Surinam-specimens, referred to *B. neglecta* by Brongersma, must be placed with *B. brazili,* of which this probably represents a subspecies. Brongersma and Hoge agree that *B. neglecta* is a synonym of *B. pirajai;* Amaral does not agree with this synonymy, in as much as the former comes from the subxerophytic section (N.E.) of Bahia and the latter from the S. wooded area. In addition A. R. Hoge informs: "The information about the origin of *Bothrops neglecta* type specimen is from Amaral, who never published it and there is no information in the snake-register of the Instituto Butantan."

Amaral's additional remarks: In view of the profound divergence existing among ophiologists concerning the real systematic status and nomenclatural situation of the various populations of *Bothrops atrox* and *atrox*-like forms (in their mutual relations as well as in their relation to *B. jararacussu*: *megaera, lanceolata, aspera, neglecta, pirajai, brazili* and others) as scattered from S. Mexico, Central America, some Antilles and S. America to N.C. Argentina, it seems to be high time for a thorough (preferably cooperative) revision to be undertaken of that complex group of serpents.

That revision should take into consideration, besides other possible bases of comparison, the following points: geographic, topographic (altitudinal, clinal and climatic) distribution; ontogenetic evolution of body markings; general pholidosis; body and head shape and relative size; head scutellation; nasal pore; hemi-penis formation; number and character of vertebrae; scale keel type, etc.

Whenever possible, that study should also include comparative observations of living specimens (behaviour and striking position; average number of young in a brood and venom characteristics: toxico-pharmacological, biochemical and physico-chemical peculiarities of active components; venom-antivenom reactions).

— Through the same scientific approach it would be advisable to try to clear the status of the various populations (morphologically too closely allied) of the Neotropic rattler, gen. *Crotalus*.

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10. ECOLOGY OF ROCK-VIPER (*VIPERA XANTHINA RADDEI* BOETTGER) IN THE NATURAL SURROUNDINGS OF ARMENIA

I. S. DAREVSKY

Zoological Institute, Science Academy, Leningrad, U.S.S.R.

The rock-viper (*Vipera xanthina raddei* Boettger) is one of the rare pretty poisonous snakes belonging to the Soviet Union Fauna; sparse reports concerning its biology and habits are to be found in literature. The following data on the biology of this snake have been collected by the author between 1951-1961 while doing field-work in the East Transcaucasus, particularly in the woods of mountainous Armenia.

Habits — The most characteristic habitats of the rock-viper in this country are the rocky, at times steepy and scraggy slopes within the belt at 1500-1800 m above sea level, overgrown with thin oak forests, where they live particularly abundant among dry rocks, thin oaks and bushes, and in piles of rock fragments at the wooded slopes. Less frequently they may be met among the thinned out xerophite vegetation upon hill-sides, among the sparse growth of juniper and in the wide open, rocky and xerophite steppe. In some places the viper spreads out into cultivated fields where it keeps to stone heaps along the bounderies. They shelter in cavities under stones, rock crannies, between roots or in holes of rodents. Their winter retreats are deep fissures of rocks, almost too narrow for entrance. Numbers of them, up to 20 mature specimens seek their winter retreat in the same hole. If those holes really are in connection one with another in the middle of the rocks, we may indeed be right in concluding that the vipers hibernate in great number together.

Frequency — Vipers varies in accordance with the different seasons of the year. After hibernation, between the end of April and the middle of May, they remain in the vicinity of their winter quarters and lie coiled up together en masse. In May 1953 the author could count up to 50-60 mature vipers, in groups of 4-6 specimens at an area less than 1 hectare, in the forested rocky surroundings of the Antharut village (Armenia). After coupling, about the end of June, higher up in the mountains even in its first week, the vipers disperse over adjacent areas, reassembling near winter quarters late in October, gradually increasing in number, not reaching however the amount of spring-time while the first comers already retire into their dens. There may be found perhaps 20-50 specimens per hectare in those areas.

Migration — Migration from the area near winter quarters takes place in daytime: the single specimen may be seen crawling from one tree to another to disappear finally in the thick underwood. The direction is indicated by pieces of shedded skin, which are seen all over the bushes, bordering the winter retreat,

from the middle of June on, whereas the snakes themselves are nowhere to be found. In order to elucidate the believes that the snakes really return to the same place for hibernating, the author caught and marked 63 vipers in May 1952 on the southern side of the Aragatz mountain (Armenia). On the end of October of the same year, 7 of those were caught within a radius of 100-200 m from the place of the first capture. Thus, it is confirmed that at least a part of snakes return to the same winter quarters.

SEASONAL AND DAILY ACTIVITIES — Awakening from hibernation depends on the altitude above sea level of the retreats and takes place in the middle of April to the beginning of May. Only when the rocks have been sufficiently heated by the sun, around 11-12 o'clock, the vipers leave their retreat to lie on the bare rocks until more or less 6-7 o'clock, the loose folds of skin on their sides giving them an emaciated appearance. Approximately at the beginning of July, the mature specimens adjust to crepuscular and nocturnal activity and are not to be seen in daytime at all. The knowledge of this fact helps the population of those areas to take every precaution during springtime, not even turning out cattle to grass; everyone becomes careless in summer-time, since the return of the vipers does not occur before the end of October. In case of some evident danger, for instance when suddenly faced by its human enemy, the viper tries to dive into the nearest cover producing characteristic jerky hissing sounds in quick succession. Having succeeded in intercepting its retreat, the snake takes up a peculiar threatening posture raising the for-part of its body almost to an upright position, making rapid thrusts with a wide open mouth into the direction of its enemy. Large full-grown males are particularly aggressive in such cases.

FEEDING HABITS — After emerging from hibernation the vipers don't feed at all or only on some insects, explaining the remainders of some orthoperans found in the stomach contents of vipers, caught and opened by Chernov in May of 1939. Later on the snakes feed on mouse-like rodents. Many vipers caught by the author at the beginning of June disgorged the swallowed rodents of the day before, mostly *Microtus arvalis*, less frequently *M. nivalis*. The consumed number of rodents is very large. It was checked that a single snake during the season consumes not less than one hundred small rodents co-existing with them in the same region. Less frequently the snakes feed on lizards and young birds which nest on the ground. The very young ones feed on insects and small lizards.

REPRODUCTION — The mating of vipers coincides with the first shedding of skin, in springtime and may be prolonged til the end of May or even until migration starts. While the snakes bask in the sunshine the males crawl restlessly around until finding a female, gliding around her agitating the tail at high speed trying to hook the female's tail which by this time also begins to stir. Now and than the male succeeds in his endeavor but the female frees her tail immediately forcing the male to start all over again. At least both snakes move convulsively with interlocked tails, this movement spreading gradually with violence over the whole body. Frequently the female breaks loose, being pursued closely by the male, which body twitches violently from time to time. After protracted chase during which the male or leaves or seeks the female, both snakes interlace the posterior parts of the body. Both, one third of the body erected in "S" form, start swaying, the male trying now and then to push the female's head to the ground by violent thrusts. After succeeding to force the head down for several times both drop abruptly, intertwine their bodies rope-like leaving free

a small part near the head. Some time later they begin coupling their cloacas tightly joined together. Copulation often occurs about 1-2 hours after the beginning of courtship and lasts 20-30 minutes, the departure of the snakes following soon. The young ones are born in general during the first days of September, but birth may be delayed until the end of the month. Depending on size the females may give birth to 3-9 youngs which break the egg membrane while still in the womb, dispersing immediately into all directions after birth. Females of 504-560 mm length give birth to 3, oftener 4 or 5 youngs, while those of 570 mm and longer bring forth 6 youngs. In one case there were recorded 9 youngs. Data of Groubant and Roudneva (1956) discovered 10-13 eggs in the oviducts of examined females. An investigation proved however, that besides the living youngs the female cast off 1-2 unfertilized eggs. The youngs are born large in size, about 204-214 mm length of which 11-19 mm consist of the tail. They are of dull color until the first shedding, about a week later, acquiring the most brilliant color with the sharpest outline of the pattern, normal to mature snakes.

Venom — Perfiliev (1941) made a number of experiments with *Vipera raddei* venom, applying it to various animals. His data show that bitten mice perish within few seconds or some minutes. A lizard *Lacerta agilis* lost its locomotion faculty after 2 minutes and died after 40 minutes. Rabbits died after 4 hours but it took 24 hours for a bitten dog to die. Records collected by the author during 1951-1953 in Armenia, show that the bite of those vipers are fatal in severe cases. One of those cases was a mature man, bitten on his right shoulder, death occurring within 12 hours. Two children aged 14, were bitten in the leg. There are no more data in literature on the action of rock-viper venom on humans.

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11. UNTERLAGEN ZUR ÖKOLOGIE, ETHOLOGIE UND EVOLUTION DER BAUMSCHLANGEN ARBORICOLOUS SNAKES: THEIR ECOLOGY, GEOGRAPHICAL DISTRIBUTION AND ECOLOGY

R. MERTENS

Natur-Museum und Forschungs-Institut Senckenberg, Frankfurt, Germany

Was mag eine Schlange veranlasst haben, ihr ursprüngliches Leben in oder auf der Erde aufzugeben und sich ins Geäst, ein paar Meter über dem Boden, zu begeben? Eine erfolgreiche Eroberung dieses Lebensraumes ist für ein beinloses Geschöpf wie eine Schlange eigentlich eine erstaunliche Leistung. Das umso mehr, als es unter den vielen schleichenförmigen Eidechsen mit rückgebildeten Gliedmassen keine bezeichnenden Baumtiere gibt, wenn man von einigen Bewohnern der Epiphytenballen in tropischen Wäldern absieht.

Sicherlich waren die ursprünglichen Schlangen, gleich vielen anderen in Entfaltung begriffenen Tiergruppen, von einem Ausbreitungstrieb beherrscht, einem Trieb, der sie in immer neue, von ihren Verwandten noch nicht besetzte Lebensräume geführt hat: so z.B. in die Gewässer verschiedenster Art — vom Bach bis zum Ozean —, in die Sand — und Steinwüsten und in die Höhen der Pflanzendecke, einschliesslich der Baumkronen. In einem üppigen Walde sind nämlich die Licht- und Wärmeverhältnisse in den höheren Regionen für eine Entfaltung des Kleintierlebens günstiger als auf dem Waldboden, und es mag daher verständlich sein, dass die Schlangen dorthin dem übrigen Getier gefolgt sind. In einer gewissen Höhe über dem Erdboden boten sich ihnen nicht nur neue Nahrungsquellen dar, sondern auch geeignete Wohnplätze.

Dass eine derartige Umstellung tatsächlich vorkommen kann, zeigen uns nicht wenige Bodenschlangen, wie z.B. *Elaphe*, die mehr oder weniger regelmässig den Erdboden verlassen und sich in luftiger Höhe aufhalten. Ja sogar Blindschlangen, wie die philippinischen *Typhlops dendrophis* und *longicauta* sind im Epiphytenhumus hoch über der Erde gefunden worden, wohin sie sicherlich den Bauntermiten oder Ameisen gefolgt sind. In Usbekistan ist *Vipera lebetina* an manchen Stellen zu einem häufigen Buschbewohner geworden. Auch auf der Cycladeninsel Milos verbirgt sich die Levanteotter, laut mündlicher Mitteilung des Herrn H. Kratzer (Zürich), sehr oft auf und in Sträuchern, zweifellos auf der Suche nach geeigneter Nahrung, die hauptsächlich aus Vögeln bestehen dürfte. Dasselbe trifft schliesslich für *Bothrops insularis* auf Queimada Grande zu, deren Neigung zum Leben ein paar Meter über dem Erdboden sogar seit langem bekannt ist. Das wurde neuerdings sogar für *Bitis nasicornis* in den Wäldern Ostafrikas festgestellt.

Das Leben auf Sträuchern und Bäumen hat nun, im Laufe von Jahrmillionen, der Erscheinungsform und den Lebensäusserungen vieler Schlangen bekanntlich einen einheitlichen Stempel aufgedrückt. Manche sind nähmlich untereinander so

ähnlich geworden, dass es zu weilen nicht leicht ist zu entscheiden, ob diese Ähnlichkeit auf einer Verwandschaft oder auf einer parallelen Entwicklung (Konvergens) beruht. Vergleicht man z.B. einen brasilianischen Hundskopfschlinger (*Corallus caninus*) mit einem papuanischen Baumpython (*Chondropython viridis*), so muss auch der Schlangenkenner sehr genau hinsehen, um beide auseinanderzuhalten: beide sind nähmlich laubgrün und haben auf der Rückenmitte einen kreideweissen, teilweise in Flecken übergehenden Längsstreifen; beide stimmen in der seitlichen zusammengedrückten Körperform überein und haben annähernd die gleiche Grösse. Ausserdem ist beiden eine ähnliche, rotbraune oder gelbe Jugendfärbung eigen, und beide pflegen sich in Ruhestellung auf einem Ast öfter in gleicher Weise zusammenzurollen, wobei der Kopf in der Mitte *auf* den Körperschlingen liegt (vgl. Mertens 1960, Taf 62). Und doch sind beide Schlangen nicht näher miteinander verwandt: sie gehören zwar zur gleichen Familie der Boiden, aber zu zwei verschiedenen, durch craniologische Merkmale gut unterschiedenen Unterfamilien: *Corallus* zu den BOINAE *Chondropython* zu den PYTHONINAE. Beide Arten, an zwei entgegengesetzten Punkten des Erdballs lebend, müssen daher völlig unabhängig voneinander ihre Ähnlichkeit erworben haben.

Hier liegt also ein sehr eindrucksvolles Beispiel einer Parallelentwicklung oder Konvergenz vor. Am auffälligsten wird davon die Erscheinungsform der Schlangen betroffen also etwa die Färbung, die bekanntlich sehr oft laubgrün oder rindenfarbig ist, während auffälig gefärbte — etwa schwarzgelbe wie *Spilotes pullatus* oder *Boiga dendrophila* — nur vereinzelt vorkommen. Ferner ist für eine Baumschlange bezeichnend eine Verlängerung des Körpers, der häufig seitlich zusammengedrückt ist. Seine Ausmasse und sein Gewicht sind trotzdem *gering*: über 2 m. lange Baumbewohner sind nicht allzu häufig, bei den meisten schwankt die Länge um 1-1,5 m. Im Gegensatz zur schlanken, zierlichen Körperform mit langem Schwanz der Baumnattern sind kurze und dicke Gestalten mit verkürztem Schwanz wie sie unter den Boiden und Baumottern vorkommen, seltener. Bei diesen hat der Schwanz die Funktion eines Greiforgans, während bei einigen Baumnattern der lange Schwanz zum Umklammern nicht befähigt ist. Der schlankeren Körperform entsprechen in vielen Fällen die Verminderung der Längereihen von Rückenschuppen (bei *Chironius* bis auf 10-12) und Erhöhung der Ventralia und Subcaudalia-Zahlen. Man findet diese Verschiebung der Schuppenzahlen fast immer wenn man eine Baumschlange mit ihren nächsten Verwandten unter den Bodenschlangen (z.B. *Pseudohaje* mit *Naja*) vergleicht. Auch die schmale Körperform trägt zu diesem Erscheinungsbilde bei; sie ist bei einigen Schlangen vorne stark verlängert und erheblich zugespitzt oder gar in einen Fortsatz ausgezogen (z.B. bei *Ahaetulla nasuta* und *pulverulenta*, bei *Rhynchophis boulengeri* und den *Langaha* — Arten). Auf Grund derartiger Merkmale kann man für manche Schlangen, deren Biotop unbekannt ist, mit einiger Sicherheit ein Baumleben annehmen.

Wenn es auch Baumschlangen mit glatten Rückenschuppen nicht allzuselten gibt, so fallen andere, wie z.B. die tropisch-afrikanischen Nattern *Gastropyxis smaragdina* und *Hapsidophrys lineata*, dann die Eierschlangen (*Dasypeltis*) und Baumvipern (*Atheris*) durch kräftige Kiele auf, die gewiss als Gleitschutz wirken. Dieselbe Bedeutung kommt auch den kräftigen Seitenkielen an den Ventral- und Subcaudal- Schildchen zu: letztere sind besonders ausgeprägt bei dem afrikanischen *Philothamnus semivariegatus* und den orientalischen *Chrysopelea* — Arten schwächer bei den gleichfalls orientalischen *Dendrela-*

p h i s — Arten und bei der *Gonyiphis margaritatus*. Bei manchen der erwähnten Nattern zeigt jedes Ventrale und Subcaudale ausser dem Kiel an den Seiten noch eine kleine, aber deutliche Kerbe.

Nicht wenigen Baumnattern vermögen mit einer geradezu unheimlichen Geschwindigkeit im Geäst dahinzugleiten, eine Fortbewegung die wie auf dem Erdboden auf einer lateralen Schlängelung des Körpers beruht. Um einen Nachbarzweig zu erreichen, kann eine schlanke Baumnatter ihren Vorderkörper gerade ausstrecken, wobei zu seiner Versteifung sowohl seine seitliche Kompression wie die zuweilen verlängerten, wenig beweglichen Wirbel und die geringe Zahl der dorsalen Schuppenreihen, deren mittelste häufig verbreitert ist, dienen. Peters (1960:18) hat auf diese Beziehungen hingewiesen, doch ist letzteres Merkmal nicht auf die Baumschlangen beschränkt. Es kommt nämlich auch bei den am Erdboden lebenden Schlangen (z.B. *B u n g a r u s*) vor, bei denen es aber auch im Zusammenhange mit einer erheblichen Verminderung der dorsalen Schuppenreihen auf eine Versteifung des Körpers ankommt. Manche Baumnattern (*C h r y s o p e l e a*, *D e n d r e l a p h i s*) sind imstande, durch das Vorwärtsschnellen einige Meter frei in der Luft zurückzulegen, wobei man etwas übertrieben von "fliegenden Schlangen" gesprochen hat.

Beim Erkennen der Beute muss die Zunge bei Baumberwohnern naturgemäss eine weit geringere Rolle spielen als bei den Bodenbewohnern: die Schlangen haben weniger Gelegenheit, mit ihrer Zunge ihre Beutetiere zu berühren und sie durch "Bezüngeln" als solche zu erkennen. An Stelle der Zunge treten bei ihnen die *Augen* in Funktion: es ist der Gesichtssinn, mit dessen Hilfe wohl die meisten Baumnattern (weniger die Baumottern) ihre Beute wahrnehmen. Das zeigen uns am anschaulichsten die indischen "Baumschnüffler" der Gattung *A h a e t u l l a*, welche das Bezüngeln ihrer Nahrungstiere völlig verlernt haben. Trotzdem pflegen diese langgestreckten, eindrucksvollen Geschöpfe lebhaft zu züngeln, eine Tätigkeit, die bei ihnen allenfalls zur Aufnahme von Duftstoffen dient oder eine ganz andere Bedeutung (vgl. weiter unten) bekommen hat.

Besonders grosse Augen sind also für Baumschlangen überaus zweckmässig. Das umso mehr, als ja in den meisten Lebensräumen dieser Tiere die Lichtverhältnisse nicht übermässig günstig sind. Es ist daher verständlich, dass nicht nur die Dämmerungstiere, sondern auch die Tagtiere unter den Baumnattern sich oft durch eine erhebliche Vergrösserung der Augen auszeichnen. Anschauliche Beispiele für diese Eigenart liefern unter den Tagnattern: *Dispholidus typus*, *Rhamnophis aethiopissa*, *Hapsidophrys lineata* und *Dendrelaphis formosus* sowie andere Arten dieser Gattung; unter den Nachtnattern: *Dipsadoboa duchesnii*, *Imantodes cenchoa*, *Aplopeltura boa*, *Dipsas indica* und ihre Verwandten. Auch bei den Baumnattern und Baumelapiden ist die relative Augengrösse in der Regel bedeutender als bei ihren auf dem Erdboden lebenden Stammformen, wie es sich z.B. aus einem Vergleich von *P s e u d o h a j e* mit *N a j a* ergibt. Oft sind die Augen nicht nur gross, sondern auch erheblich vorgewölbt, um das Gesichtsfeld zu erweitern, vor allem nach vorn und unten.

Die in der Dämmerung oder bei völliger Dunkelheit aktiven Baumschlangen sind in der Regel durch eine senkrechte, bei Lichtmangel stark erweiterungsfähige Pupille gekennzeichnet. Bei einigen Tagnattern mit verlängertem Vorderkopf hat sich bekanntlich eine eigenartige Pupillenform ausgebildet: sie ist nähmlich weder rund noch senkrecht, vielmehr waagerecht langgestreckt. Eine solche längsovale, zuweilen in der Mitte etwas eingeschnürte Pupille haben die indisch-sun-

daischen *Ahaetulla* und *Dryophios* sowie die äthiopische *Thelotornis*, indem sie sich bei schlafenden Tieren erheblich zusammenzieht. Die Bedeutung derartiger Pupille für die am Tage jagenden Nattern ist klar: die nach vorne ausgezogene Pupille ermöglicht den Tieren das für das Erkennen der Beutetiere wichtige binokulare Sehen.

Von vielen Baumschlangen wird die Beute schnell ergriffen und dann, soweit ihre Grösse ein bestimmtes Mass nicht überschreitet, ohne weiteres verschlungen. Ist aber ein Beutetier zu gross, so wird es entweder erdrosselt oder — bei Vorhandensein von Giftdrüsen — vergiftet. Im letzteren Falle kann es zwischen den Kiefern so lange festgehalten werden, bis sein Tod eintritt. Es ist verständlich, dass für baumbewohnende Schlangen ein Giftapparat von besonderer Bedeutung sein muss. So sind unter den Colubriden gerade bezeichnende Baumbewohner (z.B. *Thelotornis*, *Dispholidus*, *Boiga*, in Afrika; *Ahaetulla*, *Dryophiops*, *Boiga*, *Chrysopelea* in Asien; *Oxybelis*, *Philodryas*, *Imantodes* in Amerika) häufig opisthoglyph, auch alle arboricolen Nattern Madagaskars (*Mimophis*, *Lycodryas*, *Langaha*) gehören ebenfalls dazu. Bezeichnenderweise gibt es sogar unter baumbewohnenden Aglyphen Arten, deren Biss- auch eine für den Menschen spürbare — Giftwirkung hat: so *Leptophis ahaetulla* in Columbian und *Uromacer oxyrhynchus* auf Hispaniola.

Inbezug auf den Schutz vor dem Feinde zeigen die Baumschlangen eine Fülle von verschiedenartigen Verhaltensformen. Ein wesentliches Schutzmittel ist ihre Mimese. Manche Arten z.B. der Gattungen *Ahaetulla*, *Oxybelis* der *Imantodes* sind sehr schlank und heben sich von ihrer Umgebung in der Tat so wenig ab, dass sie übersehen werden sofern die Tiere regungslos verharren. Ich habe *Thelotornis kirtlandii* als Lianenschlange bezeichnet, so gross ist ihre Ähnlichkeit mit diesen Schlinggewächsen. Bei ihr sind übrigens ebenso wie bei *Langaha alluaudi*, und bis zu einem gewissen Grade bei *Ahaetulla pulverulenta*, die Internodien durch eine entsprechende Bänderung angedeutet. Die Wirkung der Mimese wird weiterhin unterstrichen durch die Kopfform, besonders durch die erwähnten Schnauzenfortsätze, die bei der madagassischen *Langaha* von höchster Eigenart sind und nicht nur als Geschlechtsmerkmale eine Bedeutung haben, sondern zweifellos auch zur Tarnung dienen. Einige Baumschlangen pflegen — übrigens ähnlich dem rein aquatilen *Erpeton* längere Zeit bewegungslos zu bleiben, auch mit emporgehobenem Vorderkörper, wie die madagassische *Mimophis mahfalensis*, was ebenfalls als Ausdruck einer Mimese zu werten ist. Von der Gattung *Atheris*, den äthiopischen Baumvipern, wird ebenfalls berichtet, dass sie ihren Vorderkörper aufrichten und in einen spitzen Winkel zum übrigen Körper bringen, wodurch ein abgeknickter oder halb abgebrochener Zweig vorgetäuscht wird. Dieser Eindruck wird noch verstärkt, wenn sie sich — wie *Amblycephalus* und *Aplopeltura* — herunterfallen lassen und sich dann "tot" stellen. Ein paar Baumnattern, z.B. *Leptophis ahaetulla*, die am Schwanze festgehalten werden, vermögen überraschend leicht und ohne grossen Blutverlust ihre Schwanzspitze einzubüssen, ohne sie allerdings regenerieren zu können.

Im Abwehrverhalten spielen bei den Baumschlangen chemische oder akustische Mittel offenbar keine allzugrosse Rolle. Von den ersteren sei auf das Erzeugen von Duftstoffen aus den Postanaldrüsen bei der südamerikanischen Gattung *Leptophis* und bei der indo-australischen Gattung *Dendrelapis* aufmerksam gemacht; in dieser Beziehung scheinen sich jedoch die einzelnen Arten innerhalb

dieser Gattung recht verschieden zu verhalten, indem die Duftstoffe z.B. bei *Dendrelaphis pictus* sehr intensiv, bei *tristis* dagegen kaum oder überhaupt nicht wahrnehmbar sind.

Als Ausdruck einer Erregung fasse ich das eigenartige Hin-und Herpendeln des frei von den Zweigen hinunterhängenden Vorderkörpers auf, das ich bei den *Ahaetulla* und *Oxybelis* — Arten am häufigsten beobachtet habe. Ähnlich ist es mit dem "Warn-Züngeln" (Mertens 1946:24). Dabei wird die Zunge für geraume Zeit ausgestreckt, wobei die Zungenspitzen sich langsam nach oben krümmen (*Dendrelaphis punctulatus, Dispholidus typus, Thelotornis kirtlandii*) oder gelegentlich sogar vibrieren (*Philothamnus semivariegatus, Ahaetulla prasina*). Besonders eigenartig nimmt sich bei den zuerst genannten Baumnattern die Färbung der Zunge aus, da sie einen scharfen Kontrast zur Gesamtfärbung bildet: sie ist tiefschwarz bei dem bläulichen *Dendrelaphis punctulatus*, ziegelrot mit schwarzen Spitzen bei der rindenfarbigen *Thelotornis*. In manchen Fällen streckt eine Baumnatter die Zunge gleich einer Sonde für mehrere Sekunden völlig bewegungslos heraus, wie ich es bei *Oxybelis seneus* und *Opheodrys aestivus*, beim Anblick anderer Lebenswesen, auch die Beutetiere, beobachtete.

In der Hauptsache bei Baumschlangen ist als recht bezeichnende Warnhandlung das Aufsperren der Kiefer verbreitet, wobei der Rachen durch grösstmögliche Auseinanderspreizen der Unterkieferäste erweitert wird. Dieses Abwehrverhalten, dem erst sehr viel später oder garnicht, ein Biss folgt, ist mir unter Baumnattern von *Leptophis* — Arten, *Dendrelaphis pictus, Oxybelis aeneus, Uromacer oxyrhyncus* und *Dasypeltis scabra loveridgei* bekannt und ausserdem von *Chlorophi irregularis, Chironius fuscus, Ahaetulla nasuta* und anderen beschrieben worden. Irgendwelche Schlüsse daraus auf etwaige verwandtschaftliche Beziehungen dieser Nattern zu ziehen, dürfte verfehlt sein, da sich die einzelnen Gattungsangehörigen recht verschieden verhalten: wohl ist das warnende Aufsperren der Kiefer z.B. für *Dendrelaphis pictus* charakteristisch, aber bei dem verwandten *Dendrelaphis tristis* habe ich es niemals festgestellt.

Eine recht häufige Abwehrhandlung stellt ferner das Aufblähen des Vorderkörpers dar, wie es z.B. von *Spilotes, Pseustes* und *Dispholidus* beschrieben worden ist. Dabei wird die oft sehr auffalende und mit der übrigen Färbung kontrastierenyde Zwischenschuppenhaut sichtbar: sie ist zum Biespiel schwarz-weiss bei der laubgrünen *Ahaetulla prasina*, blauviolett bei dem bronzefarbigen *Dendrelaphis tristis*. Indessen ist dieses Abwehrverhalten bekanntlich ebensowenig auf die Baumschlangen beschränkt, wie das Abplatten des Körpers oder seines vordersten Abschnittes (*Dendroaspis*) Immerhin verdient vermerkt zu werden, dass die extremsten Formen des Halsaufblähens, wobei tatsächlich der Eindruck eines mit Luft angefüllten Ballons entsteht, bezeichnenden Baumschlangen, wie der afrikanischen Lianennatter (*Thelotornis kirtlandii*), eigen ist.

Nur der Kenner kann in der Regel die Geschlechter bei den meisten Baumschlangen einigermassen sicher unterscheiden. Wie auch sonst bei den Schlangen, haben die Männchen meist weniger Bauchschildchen als die Weibchen und einen an der Wurzel gegenüber der präanalen Region kaum verschmälerten, relativ längeren Schwanz, was in der höheren Zahl der Unterschwanzschilder zum Ausdruck kommt. Bei *Ahaetulla prasina* und *nasuta* stellte ich fest, dass die Rüchenschuppen in der Analregion bei den Männchen kräftigere Kiele haben als bei den Weibchen. Weitere Beispiele dafür führt Kopstein (1941) von javanischen, auch bodenbewohnenden Arten an und bemerkt, dass diese Kiele das gegenseitige Festhalten während der Paarung erleichtern. Weitaus am stärksten — was im Zu-

sammenhange mit dem Baumleben verständlich ist — treten diese Kiele auf dem Rücken von männlichen *Leptophis ahaetulla* und einigen anderen Arten dieser Gattung und namentlich von *Chironius carinatus* hervor, dagegen können sie bei den weiblichen Tieren überhaupt fehlen.

Auf einen sehr merkwürdigen Geschlechtsunterschied stiess ich früher bei *Dendrelaphis pictus* und *calligaster* (Mertens 1937:173). Es zeigte sich, dass bei diesen in Südostasien und auf den indoaustralischen Inseln weitverbreiteten Strauchnattern die ausgewachsenen Männchen deutlich grössere Augen haben als die Weibchen. Obwohl die männlichen Nattern dieser Arten die Grösse der weiblichen nicht erreichen, beträgt der horizontale Augendurchmesser des javanischen *pictus* im Mittelwert 4,25 gegen 3,78 mm der Weibchen. Auch Kopstein (1941:164) bemerkt, dass die Männchen von *pictus* "an ihren wesentlich grösseren Augen leicht erkannt werden können." Ähnliche Verhältnisse fand ich neuerdings bei der äthiopischen Natter *Philothamus semivariegatus*, von der allerdings wenige Stücke zur Verfügung standen. Laut mündlicher Mitteilung von Dr. A. R. Hoge trifft dasselbe für den brasilianischen *Chironius bicarinatus* zu. In welcher Beziehung dieser Geschlechtsunterschied zur Lebensweise der Tiere steht, weiss man nicht. Da der Geruchsinn für Baumschlangen von untergeordneter Bedeutung sein dürfte, ist es nicht ausgeschlossen, dass die grösseren Augen der Männchen zum Erkennen der Geschlechter dienen. In anderer Weise erfüllen die gleiche Aufgabe verschiedene Formen der Schnauzenfortsätze bei den Männchen und Weibchen von *Langaha nasuta*. Kämpfende Männchen scheinen unter den Baumschlangen bisher nur bei Angehörigen der Gattung *Dendroaspis* beobachtet worden zu sein (Leloup 1964).

Über die Besonderheiten in der Fortpflanzung von Baumschlangen ist nichts bekannt. Dass bei Baumnattern die viviparen Formen (*Ahaetulla* Arten, *Lycodryas gaimardi*) gegenüber den eierlegenden überwiegen, wie man es aus naheliegenden Gründen erwarten würde, scheint sich nicht zu bestätigen. So bezeichnende Baumnattergattungen wie *Chrysopelea* und *Dendrelaphis* in Asien, *Philothamus*, *Dispholidus*, *Thelotornis* und *Dasypeltis* in Afrika, *Mimophis* und *Langaha* in Madagaskar, *Leptophis* und *Philodryas* in Amerika sind Eierleger. Hingegen bringen wohl alle Vertreter baumbewohnender Boiden und Ottern (*Atheris* in Afrika, *Trimeresurus* in Asien, *Bothrops* in Amerika) lebende Junge zur Welt.

Trotz des Eingangs erwähnten Beispiels *Corallus* — *Chondropython* kann es keinem Zweifel unterliegen, dass die Ähnlichkeit mancher Baumschlangen durchaus nicht immer auf Konvergenz zu beruhen braucht, sondern in vielen Fällen ganz einfach auf Blutverwandschaft zurückzuführen ist, indem sich aus einer baumbewohnender Art *unmittelbar* eine andere entwickelt hat. So dürften z.B. die gemeinsamen, mit dem Baumleben zusammenhängende Merkmale *aller* Arten der Gattung *Ahaetulla* sicher nicht durch eine parallele Entwicklung jedesmal von Neuem entstanden sein, sondern sind gewissermassen ein Ausdruck der verwandschaftlichen Beziehungen der Arten. Dabei können die einzelnen Anpassungsmerkmale selbst bei stark spezialisierten Arten eine recht verschiedene Entwicklungshöhe erreichen: *Ahaetulla pulverulenta* erscheint z.B. durch die graubraune Gesamtfärbung primitiver als die laubgrünen *prasina* und *nasuta*, aber durch den langen und feinbeschuppten Schnauzenfortsatz fortgeschrittener als *prasina* und sogar als *nasuta*. Ähnlich wie die *Ahaetulla* — Arten dürften die Glieder innerhalb der Gattungen *Leptophis*, *Uromacer*, *Oxybelis* oder *Langaha* zu beurteilen sein. Auch eine ganze verwandschaftliche Gruppe (Gattung) mit ihren strauch — oder baumbewohnenden Angehörigen braucht

nicht unbedingt von einer bodenbewohnenden Urform abzustammen, sondern kann als Vorfahren bereits einen bezeichnenden Strauch oder Baumbewchner aus einer anderen Gattung gehabt haben: *Ahaetulla* dürfte z.B. von einer *Dryophiops* — Form, *Chrysopeles* von einer *Dendrelaphis* — Form und *Philothamus* von einer *Chlorophis* — Form den Ausgang genommen haben.

Im Gegensatz dazu können jedoch nahe verwandte Arten völlig unabhängig voneinander ähnlich geworden sein Das zeigt uns das Beispiel gewisser Baumottern unter den Crotaliden: *Bothrops* in Amerika, *Trimeresurus* in Asien. Beide Gattungen stehen sich ganz nahe, so nahe, dass man sie verschiedentlich zu einer einzigen zusammengefasst hat, wie es zuletzt Parker (1965) getan hat. Und doch müssen die kleineren, meist grün gefärbten Arten trotz ihrer Ähnlichkeit von den entsprechenden Bodenottern als ihren Stammformen in beiden Kontinenten sich selbständig ausgebildet haben. Bei vielen anderen verwandschaftlichen Gruppen liegen indessen die Beziehungen weniger klar. Hat sich z.B. die hispaniolische Baumnatter-Gattung *Uromacer* von einer bodenbewohnenden Stammform ausgebildet oder ist sie ein Abkömmling einer anderen neotropischen Baumnatter? Es lässt sich nicht bestreiten, dass sie als ganzes am meisten an das Genus *Leptophis* erinnert, teilweise aber auch an die Nattern der Gattung *Oxybelis*. Dass letztere opisthoglyph sind, würde nicht gegen das angedeutete Verwandschaftsverhältniss sprechen, da ja auch zumindest der aglyphe *Uromacer oxyrhynchus* (ähnlich wie *Leptophis*) ein für seine Beutetiere wirksames Gift besitzt.

Eine weitere Frage: bilden die Gattungen *Ahaetulla* und Thelotornis engere verwandschaftliche Gruppe oder handelt es sich hier um einen Fall von Parallelentwicklung? Ihre äussere Ähnlichkeit ist unbestritten und kommt sowohl in der Körperform wie in der eigenartigen waagerechten Pupille und bis zu einem gewissen Grade im Abwehrverhalten zum Ausdruck. Was jedoch die Pupillenform betrifft, so kann sie im Laufe der Schlangen-Evolution mehrmals entstanden sein: bei *Dispholidus typus* einer ebenfalls opisthoglyphen Art mit Rundpupille, kann als eine individuelle Variation die Pupille nach vorne ausgezogen sein (Fitz Simon 1935:320), ein bemerkenswerter Befund, den ich bestätigen und auch bei *Philothamus semivariegatus* nachweisen konnte. Als noch nicht gesichert darf hingegen die früher angenommene Verwandtschaft zwischen den äthiopischen Eierschlangen (DASYPELTINAE) und dem indischen *Elachistodon westermanni* gelten (Gans & Williams 1954).

Reich an ungelösten Rätseln ist schliesslich die so überaus bemerkenswerte Schlangenfauna Madagaskars. Da zur Eidechsenfauna dieser Insel bekanntlich die sonst auf die Neue Welt und die Fidschi- und Tonga-Inseln beschränkte Familie der Leguane (IGUANIDAE) zählt und auch die Schildkröten durch eine Art der südamerikanischen Gattung *Podocnemis* vertreten sind, liegt der Gedanke nahe, dass sich auch bei den madagassischen Schlangen Beziehungen zur neotropischen Herpetofauna verbergen. Das ist in der Tat der Fall, und zwar im Hinblick auf die Riesenschlangen (BOIDAE), deren madagassische Vertreter (*Acrantophis*, *Sanzinia*) den amerikanischen, ebenfalls überwiegend baumbewohnenden Gattungen *Boa* und *Corallus* zumindest sehr nahe stehen, wie man es seit langem weiss. Unter den Baumnattern sind für Madagaskar 3 Gattungen bezeichnend, die ja alle zu den Opisthoglyphen zählen: *Mimophis*, *Lycodryas* (incl. *Stenophis*) und *Langaha*. Ihre verwandtschaftlichen Beziehungen sind noch ganz ungeklärt, man möchte sie aber ebenfalls unter

den Nattern des tropischen Amerika suchen: vielleicht unter den Angehörigen von *Leptodeira* für *Lycodryas* und *Oxybelis* für *Langaha*. Auf jeden Fall wäre die Klärung der stammesgeschichtlichen Beziehungen der Schlangen Madagaskar's für einen Morphologen eine sehr dankbare Aufgabe.

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12. BIOLOGY AND ECOLOGY OF VENOMOUS ANIMALS IN ISRAEL

AHARON SHULOV

The Hebrew University of Jerusalem, Israel

The variety of climatic conditions in the land of Israel ranging from 1,000 meter high hills rich in rainfall to dry deserts and the Dead Sea valley situated deep below the sea level provides different biotops for a very wide spectrum of animal life. In addition this tiny plot of land situated in the meeting point of Asia, Africa and Europe serves as a passageway for many migrating animals and birds. Nevertheless the zoogeographical analysis of the animal population of the land of Israel shows quite a high percentage of endemic species. Some of them are venomous.

The knowledge about the venomous animals of the land of Israel originates from the passages of the Old Testament in which references were made to venomous snakes, scorpions and spiders either describing the fatal results of their bites or as a warning.

Over the ages, information about the appearance and the way of life of these creatures and the methods of treatment of poisonous bites was accumulated in Talmudic and Jewish Medieval Literature. However these references were as a rule commentaries to the passages of the Bible without adding any new knowledge.

Although some of the dangerous creatures mentioned in the Bible became through the world wide use of the Bible, fabulous and mystical beings, nevertheless in zoologists living in and visiting the land of Israel succeed in identifying at least some of them.

This report deals only with terrestrial venomous creatures particularly with snakes, scorpions and spiders.

I. SNAKES:

Of seven poisonous snakes in Israel only one is of importance; this is the Palestine viper, *Vipera xanthina palestinae*. The others, *Echis colorata*, *Pseudocerastes fieldi*, *Aspis cerastes*, *Aspis vipera* as well as the Black cobra, *Walterinnesia aegyptia*, and Ein Gedi mole viper, *Atractaspis eingaddensis*, are comparatively rare and live only in remote and scarcely-populated areas of the Negev — the southern part of the land of Israel.

The Palestine viper which is the Biblical "Zerfa" is translated in the King James version as Basilisc. It is the most common Israeli poisonous snake, the distribution of which is connected with the Mediterranean zoolgeographical regions of Israel. It seems that the highest concentration of this snake is in the coastal plain of Sharon where the most intensive agricultural cultivation is taking place with consequent abundance of rural rodents on which this viper preys. Its

maximal length is 120 cm. This viper is frequent also around the fish ponds where it feeds on fish. This snake lives in burrows of rodents, and is as a rule nocturnal. However, in winter it can be met during the warm days when basting in the sun. It is sluggish in movements and does not strike as a rule when not provoked. Many cases are reported about these snakes being found and held in the hands by ignorant adults and children who were not bitten by the snake, as they treated it gently. However this snake is responsible for more than 99% of all ophidian bites in Israel. The Palestine viper is oviparous and, the hatching of the eggs takes slightly more than a month and a half at the temperature of the coastal plain. The young vipers have already developed a venomous apparatus and are able to kill their prey.

The second snake in order of importance is the saw-scaled sand viper, *Echis colorata*. It is smaller than the Palestine viper, not exceeding 40-50 cm. Its basic color varies from yellow to reddish rose with distinct oval markings in darker frames. It is found in and around the Dead Sea and along the Jordan and Arava valley, reaching the eastern slopes of the Judean and the Gilboa hills bordering the Jezreel valley. This snake inhabits stony areas with very scarce vegetation, being found under stones. As a rule, it does not live in burrows. It is very quick in its reactions, striking often instantly when approached and is therefore popularly believed more dangerous than the Palestine viper, although the comparison of the lethal dose of this snake venom with that of the Palestine viper shows that the venom of the Palestine viper is almost twice as strong as that of *Echis*. It preys on small rodents, reptiles, occasionally on young desert birds. Its diet consists also of some insects. It is oviparous, the development of eggs takes at 31°C, 43 days.

The *Pseudocerastes fieldi* vipers are yellowish-grey in colour. On each side of the body there is a row of light brown rhomboid blotches and the ventral side of it is pure white. The length of the body ranges between 60 to 90 cm and the weight of a well fed adult specimen may reach 500 gm. All specimens of this viper have "horns" consisting of small scales above the eyes and are easily recognized by the black tip of their tails.

Pseudocerastes vipers live in areas of the sandy soil with stretches of hard ground and a certain amount of vegetation. It seems that they prefer spacious hiding places under stones and rodents' burrows. These vipers are found quite often among vegetation during daylight although they live on small rodents, birds and lizards. It seems that they feed also on already dead animals and birds. In Israel, this snake is found in Jordan, Sinai, the central part of the Neger with the main population in the Nahel Ramon and northern Arabia.

Copulation has been observed in May and June. The number of eggs in a clutch ranges between 14 to 21. As the eggs are laid in more advanced stages than in *Echis colorata* and *Vipera xanthina palestina*, they develop relatively rapidly hatching in 30-31 days at 31°C. The duration of development until the adult stage is not yet known.

The venom of *Pseudocerastes* is quite a strong one and among the Israeli venomous snakes, it is second only to that of the black cobra *Walterinnesia*. There are no authentic reports regarding the influence of this snake's bite upon human beings.

The horned sand viper, *Aspis cerastes* is smaller than *Pseudocerastes*, being 50-70 cm long and 400 gms in weight. Its colour is yellow with brown

and grey markings. The specimens found in Israel are hornless in contrast to the horned ones found in North Africa, the Arab peninsula, Iraq, Sinai and Jordan.

It is found in sandy areas of the Arava valley. This snake burrows itself into the sand by sidewise movements of its ribs so that only its head and the nostrils remain above the surface. Sometimes the whole body is hidden in the sand. It seems that in this position it is ambushing its prey. However its permanent abode is usually the rodents' holes in which it exhibits the same burrowing habit.

The horned viper feeds on mice and lizards. It is oviparous. The female lays up to 20 eggs which hatch after more than two months in summer. The period of development until maturity lasts 1½ year for males and 2½ for females.

The venom of the horned viper seems to be quite strong. However, no authentic records of causalties have yet been reported in this land.

The *Aspis vipera* is the smallest of the sand vipers, reaching 30 cm in length and 50 gm in weight. It is the plumpest of all the venomous snakes of Israel. Its colour is similar to that of the horned viper, being yellowish with brown spots. It is possible to distinguish between males and females of this species by the black tip of the tail of the latter.

The distribution of the *Aspis vipera* is similar to that of the horned viper, In Israel, it has been found only in the sandy areas bordering Sinai, being sympatric with the horned viper.

This viper seems to be very well adapted to life in sand. Its ability to burrow itself in the sand is very well developed. When alarmed, instead of running away it burrows deeper into the sand. Like the horned viper, it digs itself vertically into the sand.

No authentic reports on bites in human beings have so far been published.

The black cobra (*Walterinnesia aegyptia*) is the only Elapid snake found in Israel. It is quite widely distributed in the south of Israel without reference of any type of soil or vegetation. It ranges from Egypt to Iran in the desert and steppe areas. As a rule its length doesn't exceed one meter. Its color is shiny black. This fact causes some trouble when the necessity arises to distinguish between the venomous black cobra and the harmless black colubrid snake (*Coluber jugularis*) in the border areas of their distribution. As a rule the black cobra does not appear farther north than a few miles north of the city of Beersheva, whereas *Coluber jugularis* does not occur to the south of this town. The other useful difference is in the body length — that of the black coluber is more than one meter.

The black cobra is a nocturnal animal and is a subterranean dweller, whose ability to burrow can easily be detected by the form of its head and the smoothness of its scales. Its sight is very poor and it locates its foods by smell only. It preys on a variety of creatures such as frogs, toads, mice, small birds and various reptiles. It also eats dead animals and even those already decomposing. It likes to drink water and it is found many times in humid places near or within settlements.

There are no authentic reports regarding reproduction in this snake. However, many papers have been devoted to the properties of its venom.

There are some lethal accidents attributed to Cobra bites in Sinai and Egypt but there have been no casualties in Israel, although several cases of bites were reported.

The Ein-Gedi Mole-Viper was discovered only in 1944 in the oasis of Ein-Gedi, situated on the western shore of the Dead Sea. It belongs to the Mole-Vipers of the genus *Atractaspis* living in several regions of tropical Africa. *Atractaspis engaddensis* has since been reported from other places in the Negev and in the Sinai peninsula.

It is a typical burrowing snake with a small head, without any marked constriction between the head and the body. Its color ranges from black to brown, the eyes being very small and the sight extremely poor.

The effect of the venom on human being and animals is neurotoxic. However, certain hemorrhagic effects exvasations have also been observed in autopsies of laboratory animals killed by this snake. No exact chemical and toxicological data regarding this venom have yet been published.

The relative toxicity of venom of 5 Israeli snakes is presented in the table I. The comparison was carried out by injecting dissolved venom subcutaneously into white mice 14-16 gr in weight. A similar investigation carried out with fresh pooled venom of the same snake gave similar results. However the differences in the strength of venom do not reflect the degree of potential danger of these snakes as the main factor in this respect would be the probability of human beings meeting with bearers of the different venoms. In this respect the Palestine Viper is important being responsible for almost all cases of ophidian bites in the population in Israel.

II. SCORPIONS:

In comparison to snakes, the scorpions are considered less dangerous. However, it appears that in Israel as well as in Mexico and in Algeria the number of casualties caused by scorpions is at least equal to the number of deaths caused by snakes. The lack of knowledge about the potential danger of the bite of a scorpion quite often causes delay in treatment and leads to lethal consequences.

There are some 12 species and subspecies of scorpions already described from Israel. Stings of at least two of which may cause death.

It is interesting to mention that the use of the poisonous sting by scorpions is not necessary connected with their feeding. As a rule a scorpion does not start using its sting when hunting. It does it only when the prey is too big and it cannot be crushed with pedipalps enabling sucking and feeding on it. Therefore the scorpion stings only when treaded on or otherwise seriously disturbed. Similarly to snakes there is a wide range of differences in the strength of scorpion's venoms with regard to their potential danger to human beings, ranging from almost harmless species to those whose sting might be fatal. The amount of venom injected at each sting varies considerably and its influence fluctuates according to the part of the body stung, to the proximity of the spot of sting to nerves or blood vessels, and many other conditions. Many other factors influencing the effect of the sting depend on the scorpion. Among these, secondary

only to the specific qualities of its venom is the fact that according to observations based on thousands of specimens, the pointed tip of the sting is frequently broken, thus enabling its bearer to sting the soft-skinned prey but rendering such a sting completely harmless to human beings.

The most dangerous of the scorpions of Israel is the common yellow scorpion *Leiurus quinquestriatus* H. et E. It is quite widely distributed, ranging from North Africa through the Arab peninsula and the countries of the Levant to Turkey and Persia. In Israel it is the most common species of scorpion, ranging throughout the country with the exception of the coastal plain where it is replaced by the black scorpion *Buthotus judaicus* E. Sim. In the Mediterranean part of the Judea and the Gallilee, both species appear to be sympatric. In the hilly region appears as a rule the biggest scorpion of the Near East *Nebo hierochonticus* E. Sim. which reaches 16 cm length. Two species of the genus *Prionurus crassicauda* Ol. and *bicolor* H. et E., are found in isolated groups mostly in the hills. In the Neger, the southern part of Israel, there they occur together with *Leiurus quinquestriatus*, the small black desert scorpion *Orthochirus Innesi* F. Sim. as well as with endemic *Buthus occitanus. Mardochei* var. *Israelis* Shulov et Amitai which is found only in few isolated localities.

Although as a result of intensive collection we now have considerable knowledge about the distribution of scorpions in Israel it would nevertheless be difficult to describe exactly the ecological habitats of each of the species mentioned above, especially in localities where as many as four species are found. It may be stated in general, that *Leiurus quinquestriatus* inhabits dry stony calcareous ground, often prefering the southern and eastern slopes of the hills. The Judean scorpion is found under stones in the Mediterranean region to the west of the watershed of the Jordan and Mediterranean water systems. Sometimes it is also found under the bark of trees. *Orthochirus Innesi* is found under small stones on light soils. The burrows of some of the scorpions are highly characteristic and can be easily identified especially those of *Scorpio maurus palmatus* Seurat and *Scorpio maurus fuscus* H. et E. as well as the typical entrance under a stone to the burrow of *Nebo hierochonticus* E. Sim.

All the scorpions of this country appear to be nocturnal, their activity being directed by a biological clock. Only sporadically, a scorpion may be found during the day apparently disturbed from its abode. They seem to be active through the whole year with the exception perhaps of the coldest days of the winter in the hill region. During the hottest part of the summer they burrow deep into their retreats or remain under large stones and in the crevices of rocks. The copulation of three species of scorpions observed in our laboratory almost simultaneously with the observations made in Germany, South Africa, Brazil, and Uruguay show quite an elaborate process of transfer of the spermatophore previously formed within a comparatively short period within the body of the male. The maturation of the eggs before oviposition within one body of the scorpion as described by Pavlovsky as early as 1923, revealed the possibility both of viviparity and ovoviparity in various groups of scorpions. According to our observations the period of development of local scorpions ranged between 6-7 years with the exception of the small *Orthochirus* where this period may be much shorter. The period of reproduction and appearance of the young scorpions on the back of their mothers is July-August. The experiments carried out in our laboratory showed quite a wide range of toxicity of various local scorpions. The results are presented in the table.

III. SPIDERS:

Unlike definite indications regarding potential danger of snake and scorpion bites is no direct reference of this kind in the Bible regarding the spiders. Only one problematic passage about the anger of an unidentifiable creature named Achshuv may bear some relation to spider venom.

It is remarkable that in such a tiny country as Israel three different species of *Latrodectus* spiders have been found, as well as a few specimens of *Latrodectus mactans* possibly recently introduced. The Karakurt palaearctic species of *Latrodectus tredecimguttatus* is found all over the country, reaching in several localities of the northern Negev and Arad, a density of almost one adult female spider per square meter. As a rule its retreat is situated under medium size stones from which a corridor-like shiny threads reveal the presence of a live spider. It preys mostly on beetles of the family TENEBRIONIDAE. Other insects as grass-hoppers, crickets and bugs are also found. It is worth mentioning the small **SOLIFUGA** and small and medium size scorpions that are also found in its snares. In spring these spiders are found together with numerous cocoons containing each up to 500 eggs or already hatched spiderlings. The cycle of development of the female spider depends on the supply of food and extends from one to two years. The development of a male spider is much shorter and takes a couple of months only. Despite its abundance, there are only a few records of this spider's bites and among them only one with a scientifically proved lethal result.

The second *Latrodectus* spider originally recorded from this country and later also from the Arab peninsula is *Latrodectus pallidus*. This spider was found in quite dense population in several localities in the valley of Jesreel south of the city of Beer-Sheva and along the coastal plain.

It feeds mainly on ants and for this purpose it builds its snare in a peculiar way, with threads extending as a rule between two or three shrubs at the height of 40-60 cm. This thread bridges over the path of the ants, and the spider catches them by descending on a thread from above, seizing its victim and lifting it through the bridge into its abode. The spider's retreat consists of a peculiar, very elaborate structure at the highest point of which is a small thumb like structure in which the spider sits awaiting its prey or digesting. The venom of this *Latrodectus* species is comparatively weak, although it may cause death to white mice as well as to field mice. Occasionally insects other than ants can be found in its snare.

Latrodectus revivensis is another species which up to present has been described only from the land of Israel. Its general appearance resembles that of the *Latrodectus tredecimguttatus* and it can be distinguished from it by the form of the hairs covering its abdomen and on close observation, by the general hue of the body which ranges from dull black to heavy brown with exceptionally occuring light coloured specimens. The markings and the colour of the young spiders are completely different from these of the other *Latrodectus* species. The adult male retains its peculiar markings, but the adult female loses all juvenile designs and becomes dark as described above. The snares of *Latrodectus revivensis* are similar to a certain extent to these of *Latrodectus pallidus* but the reatreat is much shorter and broader and the whole snare is situated obliquely in contrast to the almost vertical position of the snare of *Latrodectus pallidus*. Its height is 35-40 cm above the ground. The food of *Latrodectus revivensis* is

similar to that of the *Latrodectus tredecimguttatus* and all investigations carried out with dry and fresh venom of both species indicate that they are similar if not equal. No study both of *pallidus* and *revivensis* venom has yet been carried out.

Recently several specimens of *Latrodectus mactans* have been found along the coastal plain and it is suggested that they have been introduced by immigrants from the American continents.

Several cases of bite with quite severe symptoms have been reported as a result of the bite of the common house spider *Loxosceles rufescens* found frequently in houses, cellars and caves. Investigations carried out on the white mice showed symptoms of neurotoxic envenomation, which seem to be similar to those described for the bite of some spiders of *Loxosceles* genus described in South America but not showing any kind of histopathological effects as described for there.

Sporadic observations carried out on a specimen of *Hagna narbonnensis* lycosid spider showed a low degree of venom neurotoxic influence but clear histolytic and vasolytic effects around the location of bite.

Although it seems quite amazing that such a small land as Israel, harbours so many venomous creatures, it must however, be kept in mind that in addition to its geographical position as a meeting point of three continents the intensive zoological research carried out here by scientists both driven by an interest in the creatures mentioned in the Bible as well as fostered by secular scientific interest made the land of Israel one of the most studied countries of the entire globe.

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13. OBSERVATIONS ON THE BIOLOGY OF SEA SNAKES — *HYDROPHIIDAE* — WITH REMARKS ON THEIR SYSTEMATICS

K. KLEMMER

Natur-Museum und Forschungs-Institut Senckenberg, Frankfurt, Germany

Sea snakes are proteroglyphous snakes with vertically flattened tail. I gave this provisional definition because we have just heard from Dr. Roze this morning that he regards some genera, or at least one genus, of the sea snakes as belonging to the ELAPIDAE. Well, all sea snakes, we are sure, have ancestors among the old primitive stock within the Elapids. It is also sure that they have this stock in the south-eastern Aegean region. All the recent species within the sea snakes, nearly all of the 50 species known, can be found in the sea, only in the Indo-Pacific Ocean, not in the Atlantic. As you know some of the species often penetrate the mouth of the rivers, living in the brackish water, but only two have become inhabitants of fresh water: one species in the Tahal-sea on the Philippines and another one on the Renon Island on the Salomons. We know that the sea snakes are poisonous snakes and have a very effective venom. The only important observations we have had, however, in the last few years, were made by Saint Girons in New Caledonia, who is with us this evening. In the last seven years we had in Frankfurt the opportunity to import some specimens of living sea snakes, mainly of the genus *Laticauda laticauda laticaudata, Laticauda colubrina, Laticauda semifasciata* and *Lapemis hardwicki.* The main problem in keeping sea snakes in captivity is to find how to feed them. When the first sea snake reached us, we offered all smaller fish species available from the German sea, but none of these fishes arose the slightest interest on behalf of the sea snake. *Blenis*, *Dobis*, viviparous *Blenis*, for example, lived together in the aquarium with the sea snake for weeks without being swallowed or even bitten. This changed rapidly when we were able to offer eels, the common European eel, *Anguilla anguilla.* The first eel is put into the tank for the sea snake and immediately the snake begins searching movements and when the snake meets the eel, the fish is immediately bitten, but suddenly released. After the sting of the sea snake, it is quitened down. It was never possible to provoke another bite of the sea snake on the eel or on any other fish within the next ten to fifteen minutes. The eel becomes motionless within 3-10 minutes, depending on the weight of the eel and the place where the snake has bitten and then after a quarter of an hour or even later the snake begins seeking again for the eel and swallows it, head first usually. Sea snakes are highly specialized in their diet, we know this from new investigations on the stomach contents of the freshly captured sea snakes in the Indo-Pacific on the Javan coast, especially. But usually we cannot find any stomach content in sea snakes, although the specimens have been killed immediately after capturing. From about 450 sea snakes Bergmann has studied in Java, he only could find in three of more than 400 specimens,

rests of their food. Very recent studies made by Worries, showed us that sea snakes seem to be more specialized than we could imagine before. He found that sea snakes seem to be more specialized than we could imagine before. He found that sea snakes of the genus *Emydocephalus* feed exclusively on fish eggs, very small eggs. This species also has a very small head and a slender neck and then a relatively heavy body. We do not know what this species will do with its venom apparatus, as fangs are hardly used for protection against larger enemies. Usually sea snakes are very docile, can be handled without much danger, and it is only reported from some specimens of sea snakes from the Malayan coast that sometimes will bite when handled.

Sea snakes find their prey chemiotectedly, only they do not react on fast movements within the range of their heads, but they react quickly if chemical substances, let's say, the smell of the eel, in the *Laticauda* snakes, is brought into the aquarium and you can easily provoke lasting search movements of sea snakes by adding some water from the tank where the eels have been kept. We had the luck in finding out that *Laticauda* specimens and also *Lapemis* can be kept on a diet of eels exclusively. In their natural habitat, the European eel does not occur, but there are other species of genus *Anguilla* and probably other fish with similar smell. And thus we could keep *Laticauda* specimens for more than 5 years in captivity in relatively primitive aquariae, with only artificial sea water, which had to be changed very rapidly, and a plate of cork or wood above the sea water to give the snake the possibility to come out, and a brick hole within the water to give some protection to the snake. We could do also some observations on egg laying in *Laticauda* and on the skin shedding. *Laticauda* is an egg laying genus and in the one female, we kept for five and a half years, every year 3 eggs were laid, sometimes only 2 eggs, sometimes 4. The eggs are laid within two days, up to a week, and it was very curious to observe that when the snake was laying eggs, it always shed its skin. This was regularly combined after having laid the first one, sometimes the second egg; the skin shed and the sea snake was found very often outside the water, but it can also be found there when the skin is shed without laying eggs. The *Laticauda* egg is relatively large, very elongate. The eggs could not hatch, because we had at this time only a single female of *Laticauda laticauda laticauda*. The shedding of the skin takes place 4 to 6 times a year. This is compared with terrestrial snakes of similar size, which also show similar frequency in skin shedding. This is in strong contrast with observations done on the very advanced sea snake, *Pelamys platurus*, the only real open sea inhabitant species and the only one which has crossed the Pacific and is now inhabiting the western coast of the Americas. In *Pelamys* the skin sheds very often, more than 15 times a year, that means less than 4 weeks. Observations done by Shore at the San Diego Zoo showed this, and we can explain it also as an adaptation for living in the high sea. The skin of the sea snake is often infested by barnacles or other sessile marine invertebrates, and by shedding the skin regularly, the snake gets rid of barnacles and other such animals. All observations are done in adult specimens.

The sea snake family is usually divided into two subfamilies, the more primitive one with the genus *Laticauda*, often mentioned, and with *Aipysurus* and *Emydocephalus*. These are egg laying forms as far as we know. The supporting musculature is not reduced or hardly at all; they often come to the shore or are found on land since they have to go on land to deposit their eggs. All other genera and species, 37-40, this depends on the

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taxonomic decisions, belong to the more advanced subfamily: the HYDROPHIINAE, which are oviviparous. The more frequent shedding of the skin in the HYDROPHIINAE, data from the biology of the sea snakes, support this usual systematic arrangement which has been drawn from morphological data, especially from the morphology of the skull. I, for the moment at least, will not go so far as Dr. Roze has proposed this morning, to give the LATICAUDINAE the status of an own family or to incorporate them into the terrestrial ELAPIDAE. Within the ELAPIDAE we have also an aquatic genus, *Boulengerina*, in Central Africa, but in fresh water, living Elapids we can not see any adaptations towards all sea snakes living in the sea.

14. LE CYCLE SEXUEL DES SERPENTS VENIMEUX

H. SAINT GIRONS

Laboratoire d'Ecologie, Museum National d'Histoire Naturelle, Paris, France

Introduction

Il existe, éparses dans une littérature abondante, de nombreuses données occasionnelles sur les dates de ponte ou de gestation et de parturition des Serpents femelles, mais bien peu d'études donnent des renseignements complets sur le cycle sexuel d'une espèce particulière; mêmes les observations de dates d'accouplement sont généralement rares. La situation est encore plus mauvaise en ce qui concerne les mâles, puisque l'étude de la spermatogenèse et des caractères sexuels secondaires internes exige la mise en œuvre de techniques histologiques sur des pièces convenablement fixées au préalable.

D'autre part, on ne peut réellement différencier, du point de vue de la reproduction, les COLUBRIDAE des ELAPIDAE. Nous serons donc amené à faire état d'observations concernant le cycle sexuel de Couleuvres.

Le cycle sexuel dans les regions temperees, a latence hivernale due au froid

Viperinae:

Chez *Vipera berus* (Volsoe, 1944; Smith, 1951) et chez *Vipera aspis* (Rollinat, 1934), dans les conditions normales les femelles commencent à s'accoupler peu après la fin de l'hivernage, le plus souvent durant la deuxième quinzaine de mars et l'activité sexuelle se poursuit durant le mois d'avril. La plus grande partie de la vitellogenèse s'effectue au printemps et l'ovulation a lieu à la fin de mai ou au tout début de juin. La gestation est estivale et les dates de parturition s'étagent, selon l'espèce et la localité, entre le début d'août et la mi-septembre. Chez *Vipera aspis*, il existe une deuxième période d'accouplement à la fin de septembre, à laquelle toutes les femelles ne prennent pas part.

Dans les régions plus ou moins proches de la limite nord de l'aire de distribution de l'espèce, ou en montagne, un pourcentage croissant de femelles présente un cycle sexuel biennal, voir même tri- ou quadriennal (*Vipera berus*: Vainio, 1932; Volsoe, 1944; Saint Girons et Kramer, 1963. *Vipera aspis*: Saint Girons, 1957; Duguy, 1963). Les figures 1 et 2 donnent une idée de ce type de reproduction. Il convient de remarquer que la date de l'ovulation n'est pas modifiée. Au contraire, dans ces zones où les Vipères souffrent d'un déficit thermique, la parturition a lieu nettement plus tard. Dans quelques cas excep-

tionnels, la gestation peut se prolonger jusqu'au printemps suivant. La vitellogenèse a toujours lieu, pour la plus grande part, au printemps de l'année de reproduction.

Le cycle sexuel des mâles est toujours annuel. La spermatogenèse correspond au type dit "mixte", c'est à dire que la spermatocytogenèse a lieu en été et la spermiogenèse au début du printemps, juste avant l'accouplement; l'hiver correspond à une période d'arrêt du développement de la lignée séminale, la majorité des cellules étant au stade de jeunes spermatides. Toutefois, chez *Vipera aspis*, la spermatogenèse tend à être continue, avec deux périodes de vive activité, la plus longue de juillet à octobre, la plus courte en mars et avril. Dans les deux cas, il existe un stade de semi-repos en juin. Chez *Vipera berus*, les caractères sexuels secondaires et notamment le segment sexuel du rein présentent un maximum de développement au moment de l'accouplement vernal, mais ils ne sont atrophiés qu'en juillet. Chez *Vipera aspis*, le stade de grand développement est atteint dès le mois de septembre, lors de l'accouplement automnal.

CROTALINAE:

Aux Etats Unis, bon nombre de Crotales femelles semblent avoir un cycle annuel, analogue à celui qui a été décrit chez les Vipères européennes (voir Klauber, 1956, pour la bibliographie). Toutefois, les observations d'accouplement sont relativement rares et, surtout, difficilement interprétables car elles s'étendent sur la plus grande partie de la vie active, à l'exception du mois de juin. Il existe cependant deux périodes préférentielles, l'une en mars-avril, l'autre en août-septembre. Comme la gestation dure en général jusqu'en septembre, il est probable que beaucoup d'accouplements d'automne sont le fait de femelles ayant un cycle biennal.

En effet, chez plusieurs espèces (*Crotalus viridis viridis*, Rahn, 1942; *C. v. lutosus*, Glissmeyer, 1951; *Crotalus atrox*, Tinkle, 1962), l'étude de populations captureés dans les abris d'hivernage montre que les femelles peuvent se classer en deux catégories, l'une composée de post-parturientes à petits ovules, l'autre d'animaux pourvus de gros ovules et qui ne se sont pas reproduits l'année précédente. Chez *C. v. viridis*, ce dernier groupe a des spermatozoïdes dans le tube vaginal et semble s'être accouplé l'été précédent. Chez *C. v. oreganus* et *C. v. lutosus*, Fitch, 1949 et Glissmeyer, 1951 estiment que la copulation a lieu au printemps seulement. Il en est de même chez *Agkistrodon contortrix* (Fitch, 1960); de plus, l'étude de spécimens capturés durant la saison active prouve que, chez cette espèce comme chez les Vipères européennes, la majeure partie de la vitellogenèse a lieu au printemps qui précède l'ovulation, bien que la fin de l'hivernage soit tardive. Au contraire, chez *Crotalus viridis* et chez *C. atrox*, la vitellogenèse se situe l'été où les animaux ne se reproduisent pas (fig. 2).

Chez les Vipères, comme chez les Crotales, l'étude des corps gras montre que, dans les populations ayant des cycles biennaux, les femelles n'ont pas le temps de reconstituer leurs réserves au cours d'une seule année, sans doute en raison d'une température trop basse qui ralentit la vitesse de la digestion. Certes, l'activité des enzymes digestives à une température donnée est un caractère spécifique, mais à la limite septentrionale ou altitudinale de l'aire de répartition, un point critique est toujours atteint. La baisse de fécondité qui résulte d'une reproduction aussi espacée explique clairement l'un des mécanismes de la limitation de l'habitat d'une espèce.

Il n'existe aucune donnée précise sur l'évolution des caractères sexuels secondaires et de la lignée séminale chez les Crotales. Toutefois, Fitch (1960) signale la présence, durant toute l'année, de spermatozoïdes dans les canaux déférents de *Agkistrodon contortrix*. Il en est de même chez *Vipera aspis*, ce qui est un argument en faveur d'un cycle spermatogenétique voisin.

Autres Serpents :

Aucun ELAPIDAE ne vit dans les régions tempérées de l'hémisphère boréale. Chez plusieurs COLUBRIDAE d'Europe et des Etats Unis (voir, entre autres, Rollinat, 1934; Blanchard et Blanchard, 1940-41; Carpenter, 1952; Peter-Rousseaux, 1953; Fox, 1952 et 1954), le cycle des femelles et l'évolution des caractères sexuels secondaires des mâles sont analogues à ceux qui ont été décrits chez les Vipères. Mais la spermatogenèse est toujours estivale, de type post-nuptial, les spermatozoïdes étant stockés durant l'hivernage dans les canaux déférents. On connait également quelques exemples de cycles biennaux chez des espèces vivipares, notamment des *Thamnophis*.

Le problème des ELAPIDAE australiens sera évoqué plus loin.

Le cycle sexuel dans les regions subtropicales arides, a double latence et rares pluies de saison froide

Le cycle sexuel n'est connu de façon relativement complête que chez une seule espèce saharienne, ovipare, *Cerastes cerastes* (Saint Girons, 1962) et il présente une nette originalité. En effet, il n'existe qu'une seule période d'accouplement, à la fin du printemps (mi-mai à mi-juin) ; la vitellogenèse est vernale, l'ovulation a lieu fin juillet, la ponte début août, les éclosions en septembre. Les caractères sexuels secondaires des mâles ne sont développés qu'au printemps, d'avril à juillet et la spermatogenèse est de type pré-nuptial; la spermatocytogenèse commence en hiver et s'accélère de février à avril; la spermiogenèse se déroule de mars à juillet. L'été et l'automne correspondent à une période de repos sexuel complet pour les deux sexes, phénomène inconnu chez les autres Serpents.

Il semble bien que le cycle sexuel de *Crotalus cerastes*, hôte des déserts du Sud Ouest des Etats Unis dont l'écologie se rapproche beaucoup de celle de *Cerastes cerastes*, ne diffère guère de celui des Crotales des régions tempérées. Klauber, 1956, cite des accouplements en avril-mai et en septembre-octobre. En captivité, la parturition a lieu à la fin de septembre et en octobre. Le seul point qui pourrait rapprocher ces deux Serpents désertiques est représenté par les dates relativement tardives de l'accouplement vernal de *Crotalus cerastes*. Mais la présence d'une activité sexuelle automnale montre que le cycle des deux espèces est néanmoins très différent.

La plupart des ELAPIDAE australiens ont une vaste répartition incluant, à la fois, des zones tempérées chaudes, des régions subtropicales arides et des régions subtropicales plus ou moins humides. D'après les renseignements dispersés que l'on possède (voir Worrell, 1963), il semble que le cycle sexuel des femelles soit caracterisé par un accouplement et une vitellogenèse vernales et par une gestation ou une incubation estivales. Quelques prélèvements faits en hiver (observations personnelles inédites), suggèrent que la spermatogenèse peut être estivale

et post-nuptiale, comme chez les Couleuvres d'Europe et des Etats Unis (*Denisonia signata*, à Sydney; *Denisonia suta*, à Alice Spring), ou au contraire hivernale et pré-nuptiale, c'est à dire du même type que chez *Cerastes cerastes*, mais un peu plus précoce (*Pseudechis australis*, à Alice Spring; *Acanthophis antarticus*, dans le Nord Queensland).

Le cycle sexuel dans les regions subtropicales humides, a faible latence d'hiver et pluies de saison chaude

Dans ces régions (situées dans l'hémisphère nord sur le flanc oriental des continents, alors que les régions subtropicales arides sont localisées aux flancs occidentaux), les Lézards présentent des cycles sexuels particuliers, avec une longue période de reproduction au printemps et en été et une spermatogenèse prénuptiale. Aucun Serpent n'a été étudié de façon complête, mais des observations dispersées faites en Floride semblent montrer que la reproduction des Crotales y diffère peu de celle des espèces des zones tempérées voisines. Ce serait notamment le cas de *Crotalus adamanteus* (Klauber, 1956).

Regions tropicales et equatoriales

Selon toute vraisemblance, le cycle sexuel des Serpents diffère selon qu'il existe, ou non, une saison sèche accentuée et selon que les pluies tombent en saison froide ou en saison chaude. En Indes, chez *Natrix piscator* (aux environs de Bénares, par 25°12' Nord), la spermatocytogenèse débute en juillet, la spermiogenèse dès le mois de septembre et une spermatogenèse continue se poursuit jusqu'en janvier (Srivastava et Thapliyal, 1965). Le segment sexuel du rein ne subit pas des variations significatives, mais il y a tout lieu de penser que la reproduction est annuelle, avec un accouplement hivernal ou vernal et des naissances en été, saison des pluies. A Java, beaucoup plus près de l'équateur et où une saison sèche peu marquée dure de juillet à septembre, les observations de Kopstein (1938) et de Bergmann (1960), montrent que les espèces vivipares, comme *Agkistrodon rhodostoma* et *Trimeresurus gramineus*, gardent un cycle annuel, avec une vitellogenèse vernale de septembre à novembre et une gestation estivale, de décembre à mars. Au contraire, les espèces ovipares (des ELAPIDAE et la plupart des COLUBRIDAE), pondent plusieurs fois par an, le nombre des pontes variant de 3 à 10 selon la taille de l'animal. Bien qu'il n'y ait pas de rythme régulier, il existe une diminution très caractéristique du nombre des pontes durant la saison sèche.

Chez la Vipère africaine, *Causus rhombeatus*, dans le sud de la Nigéria, Woodward (1933) a décrit un cycle sexuel à peu près mensuel, lié d'ailleurs au cycle alimentaire et au cycle des mues. Les femelles muent, puis pondent 4 à 5 jours plus tard. Elles s'alimentent ensuite avidement pendant 10 à 12 jours, cessent de se nourrir pendant 11 à 18 jours et muent de nouveau. Ces femelles captives étant isolées des mâles, on ignore à quelle date de ce cycle très curieux se place l'accouplement. Il est assez probable que la spermatogenèse des mâles est continue dans les régions équatoriales sans saison sèche — bien que la longue conservation des spermatozoïdes, soit dans les canaux déférents des mâles, soit dans les voies génitales des femelles, rende ce phénomène non indispensable. Nous avons eu l'occasion d'examiner quelques testicules de Serpents provenant de forêts tropicales humides (Côte d'Ivoire et Madagascar) et tous les éléments de la lignée séminale étaient toujours abondamment représentés.

Le cycle sexuel des HYDROPHINAE présente un certain nombre de particularités (Bergmann, 1954 à 1962). Ces animaux passent toute leur vie dans la mer dont la température ne varie que de 18 à 25° environ; de plus, la haute chaleur spécifique de l'eau limite étroitement les possibilités de thermorégulation par insolation; enfin, la distribution de la plupart des espèces s'étend de part et d'autre de l'équateur. Ces conditions ont pour conséquence une viviparité obligatoire, une gestation de longue durée (puisque le développement des embryons est directement proportionnel à la température) et une indépendance relative par rapport aux saisons. Il semble toutefois que chaque population ait un cycle annuel plus ou moins régulier. Dans une région proche de Java, *Enhydra schistosa* (Bergmann, 1955) présente une vitellogenèse d'avril à juin, une ovulation en juillet et une parturition en novembre. Aux environs de Ceylan (Wall, 1921), la même espèce effectue sa vitellogenèse d'octobre à décembre, l'ovulation a lieu en juin ou juillet, la parturition entre février et mai. Avec d'assez nombreuses exceptions, à Java, chez *Thalassophis anomalus* (Bergmann, 1954), la vitellogenèse se situe d'avril à juin, l'ovulation en juin-juillet et la parturition en novembre. Chez *Hydrophis fasciatus*, dans la même région, Bergmann (1962) signale l'accouplement en juin, l'ovulation en juillet, la parturition en décembre. Il ressort de ces données que les HYDROPHINAE présentent un cycle reproductif annuel, avec d'importantes variations dans le temps en fonction de l'espèce et de l'habitat des différentes populations. Le cycle spermatogénétique des mâles est inconnu.

Chez les LATICAUDINAE (*Laticauda laticaudata* et *L. colubrina*) qui chassent dans l'eau mais passent une bonne partie de leur temps à terre et y pondent, l'hiver correspond à une période de repos sexuel des femelles et d'involution des caractères sexuels secondaires des mâles (Saint Girons, 1964). La vitellogénèse est vernale, la ponte a vraisemblablement lieu entre décembre et février, l'éclosion 2 mois plus tard. La spermatogenèse est soit continue, soit, plus probablement, hivernale et de type prénuptial, bien que d'assez longue durée.

FECONDATION RETARDEE ET CONSERVATION DES SPERMATOZOIDES

Le fait que, chez de très nombreuses espèces, il existe un délai de un à deux mois entre la fin de la période d'accouplement et l'ovulation — donc la fécondation — montre déjà que les spermatozoïdes peuvent survivre quelque temps dans les voies génitales femelles. D'autre part, de multiples observations ont montré que des femelles, séparées des mâles aussitôt après l'accouplement d'automne, peuvent se reproduire normalement l'année suivante. Enfin, les femelles isolées de *Causus rhombeatus*, étudiées par Woodward, ont pu donner jusqu'à 10 pontes fécondes successives, bien que le pourcentage d'œufs embryonnés décroisse régulièrement. Les spermatozoïdes sont stockés dans la lumière de la partie caudale de l'oviducte, ou tube vaginal (Rollinat, 1934; Rahn, 1942) et, peu avant l'ovulation, ils gagnent les glandes spéciales situées dans la trompe (Fox, 1956). Selon les espèces, ces spermatozoïdes disparaissent après la première ponte, ou survivent encore pendant un laps de temps variable. Leur présence dans les voies génitales femelles n'indique donc pas un accouplement récent, ce qui rend plus difficile encore l'étude des cycles sexuels.

Chez les mâles, les spermatozoïdes peuvent être stockés dans les canaux déférents pendant plusieurs mois. C'est ce qui se passe régulièrement chez les es-

pèces dont la spermatogenèse est post-nuptiale. Mais on ne connait aucun cas où des spermatozoïdes normaux puissent être trouvés en nombre important dans les canaux déférents plus de 9 mois après l'arrêt de la spermiogenèse.

Les facteurs extrinseques du cycle sexuel

La température

Dans les régions tempérées, l'étude comparative des différents cycles sexuels met en évidence l'influence primordiale de la température. Chez chaque espèce, celle-ci est directement responsable de la durée de la gestation. Elle règle aussi la vitesse de la digestion, donc la possibilité de reconstituer les réserves nécessaires à la vitellogenèse. Sans aucun doute, c'est à un déficit thermique qu'est due la présence de cycles reproductifs s'étendant sur plusieurs années, dans les régions les plus froides de l'aire de répartition de chaque espèce. Au contraire, la spermatogenèse, le développement des caractères sexuels secondaires dans les deux sexes et l'accouplement ne dépendent guère de la température — ou tout au moins celle-ci ne joue un rôle qu'à ses degrés extrèmes, alors que toute vie active est devenue impossible au Serpent.

Dans les régions subtropicales arides, tout au moins au Sahara, l'été correspond à une période de repos sexuel complet et, a priori, l'influence des températures élevées ne peut être écartée. On sait d'ailleurs que les hautes températures inhibent la spermatogenèse bien avant d'avoir un effet léthal sur les Reptiles. Chez *Cerastes cerastes*, le développement de la lignée séminale et des caractères sexuels secondaires commence dès le mois de décembre, c'est à dire au moment le plus froid de l'année et durant la courte latence hivernale.

Dans les régions subtropicales humides, l'hiver reste en général une période de repos sexuel, sauf sans doute en ce qui concerne la spermatogenèse. Dans les régions tropicales et équatoriales, l'influence de la température sur le cycle reproducteur semble faible, sauf dans le cas des Serpents marins.

Autres facteurs externes

La lumière ne joue vraisemblablement aucun rôle en tant que facteur quantitatif et les différentes phases du cycle sexuel peuvent se dérouler à n'importe quelle saison lorsque la température le permet. Mais la fréquence des cycles annuels suggère l'influence de la périodicité lumineuse en tant que facteur de "remise au jour".

L'humidité n'agit sans doute pas directement sur le cycle sexuel des Serpents dont on connaît les capacités de survie dans les zones arides. Mais, tout particulièrement dans les régions tropicales, elle joue un rôle important dans l'alimentation. Malgré le peu de données dont nous disposons, la rareté des éclosions ou des parturitions en saison sèche est un fait bien établi. De plus, la sécheresse est souvent associée à des températures élevées.

Les facteurs intrinseques du cycle sexuel

Il n'est pas possible de résumer ici, même brièvement, les données acquises en ce qui concerne la physiologie de la reproduction chez les Reptiles. Dans l'ensemble, les corrélations endocrines sont du même type que celles des Mammi-

fères, sauf dans le cas de la gestation; celle-ci s'assimile à une simple rétention des œufs dans les oviductes et seul le mécanisme de la parturition pose de véritables problèmes. Chez les rares espèces où elle été étudiée (voir Saint Girons et Duguy, 1962, pour la bibliographie), l'évolution des glandes endocrines au cours du cycle annuel suggère l'existence d'un rythme endogène spécifique. Dans les régions tempérées, la stimulation histologique du tissu adrénal et de diverses catégories cellulaires de l'adénohypophyse débute avant la fin de l'hivernage, donc à l'obscurité constante et à une température proche du minimum annuel. La fréquence d'une activité sexuelle automnale chez les Serpents exclut l'hypothèse d'un rôle déclencheur de la lumière, tel que celui qui a été mis en évidence chez beaucoup d'Oiseaux et de Mammifères (voir Benoit, 1953, pour la bibliographie). Il convient également d'insister sur la date très constante, d'une année à l'autre, de l'ovulation, alors que les dates des premières sorties et de la parturition sont sujettes à d'importantes variations. Lors des cycles tri- ou quadriennaux, on constate même que les ovules, s'ils n'ont pas eu le temps d'arriver à maturité au moment voulu, s'atrésient en masse à l'époque normale de l'ovulation.

La périodicité annuelle de la plupart des cycles sexuels, sensible même lorsque la reproduction ne s'effectue que tous les 2 ou 3 ans, indique clairement que des facteurs cosmiques interviennent au moins pour assurer une "remise au jour" qui interdit les décalages progressifs. Mais on ignore la nature de ce stimulus chez les Reptiles et la date à laquelle il agit. Chez *Vipera aspis*, un certain nombre de faits indiquent l'importance du mois d'octobre, plutôt que des premières sorties. Il est probable que des travaux ultérieures démontreront l'existence, chez les Reptiles comme chez d'autres Vertébrés, de périodes réfractaires des glandes endocrines et de leurs récepteurs, ainsi que de périodes neutres pendant lesquelles les facteurs externes (principalement la température et la lumière) peuvent accélérer ou ralentir l'évolution. C'est ce qui se passe, notamment, durant la deuxième partie de l'hivernage. Mais, dans les conditions naturelles, les grandes lignes du cycle sexuel (spermatogenèse, périodes d'accouplement, date de l'ovulation) sont sans doute déterminées par des facteurs endogènes, innés et spécifiques.

Summary

The sexual cycle of the COLUBROIDEA is only known in some species of the temperate regions. In these zones, the vittelogenesis is generally vernal, the ovulation takes place at the end of the spring and there is a second period of sexual activity in autumn. The secundary sexual characters of the males are developed the whole year, except for a short involution in July. There are two kinds of spermatogenesis, one estival for the COLUBRIDAE and probably among certain ELAPIDAE, and another mixted one among the VIPERINAE. The evolution of the spermatogenesis is unknown for the CROTALINAE.

As to the Snake of Sahara, the sexual cycle is characterized by a late reproduction period, at the end of spring, and by a complete sexual resting period in summer and autumn.

In the tropical regions, the sexual cycle of the viviparous snakes is generally yearly, but the differences between specimens and populations can be quite large. The oviparous species have generally several yearly laying periods, at irregular intervals, but rarely during the dry season. In the humid equatorial forests, *Causus rhombeatus* presents a very interesting monthly cycle, where reproduction, molting, and feeding are joined.

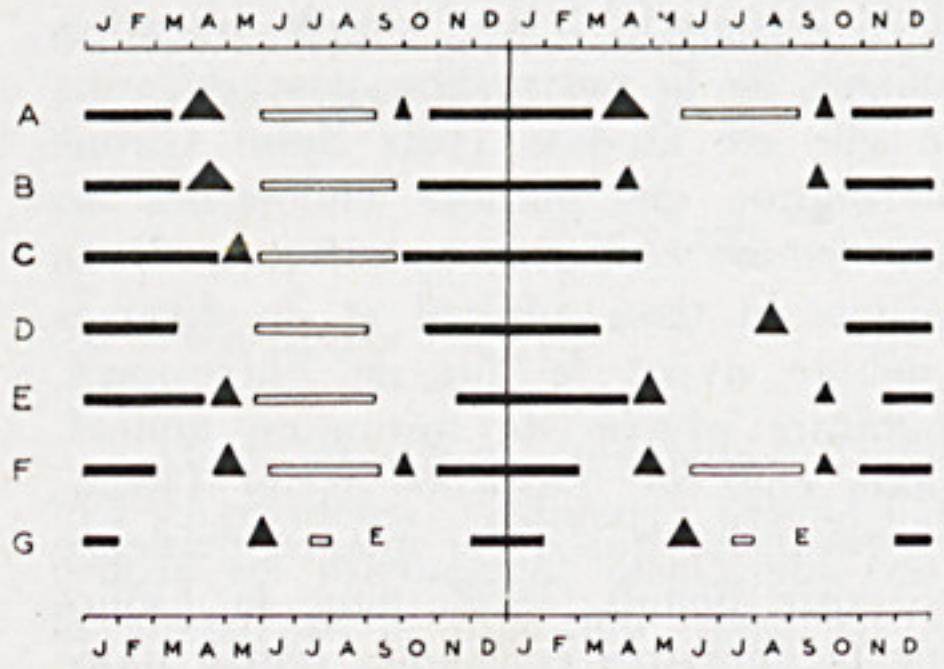


Fig. 1

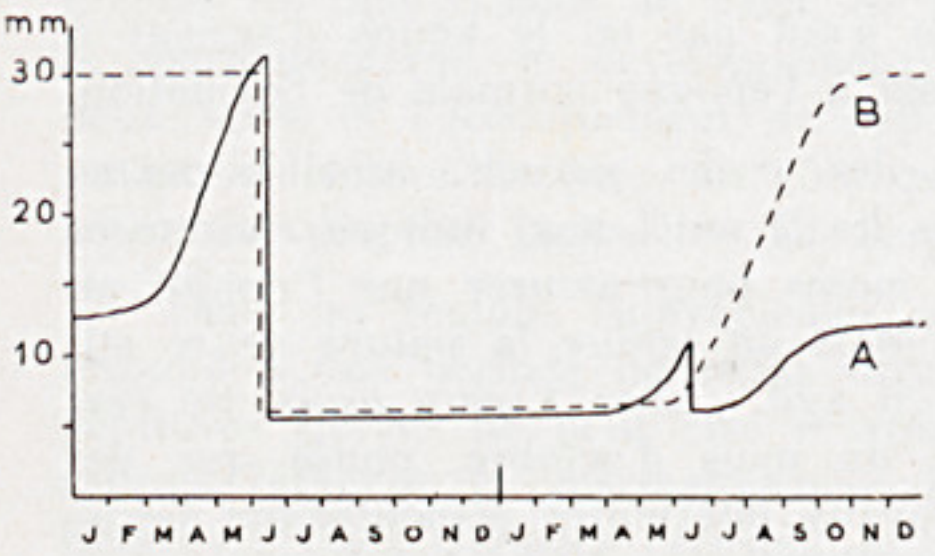


Fig. 2

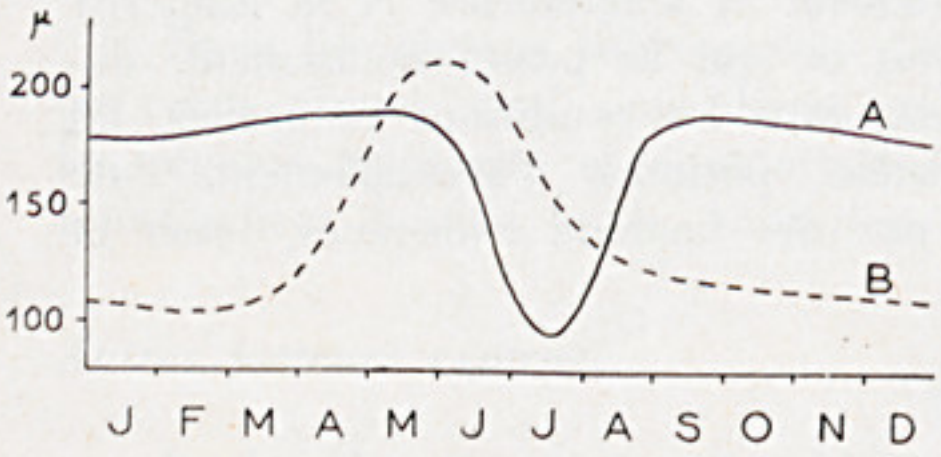


Fig. 3

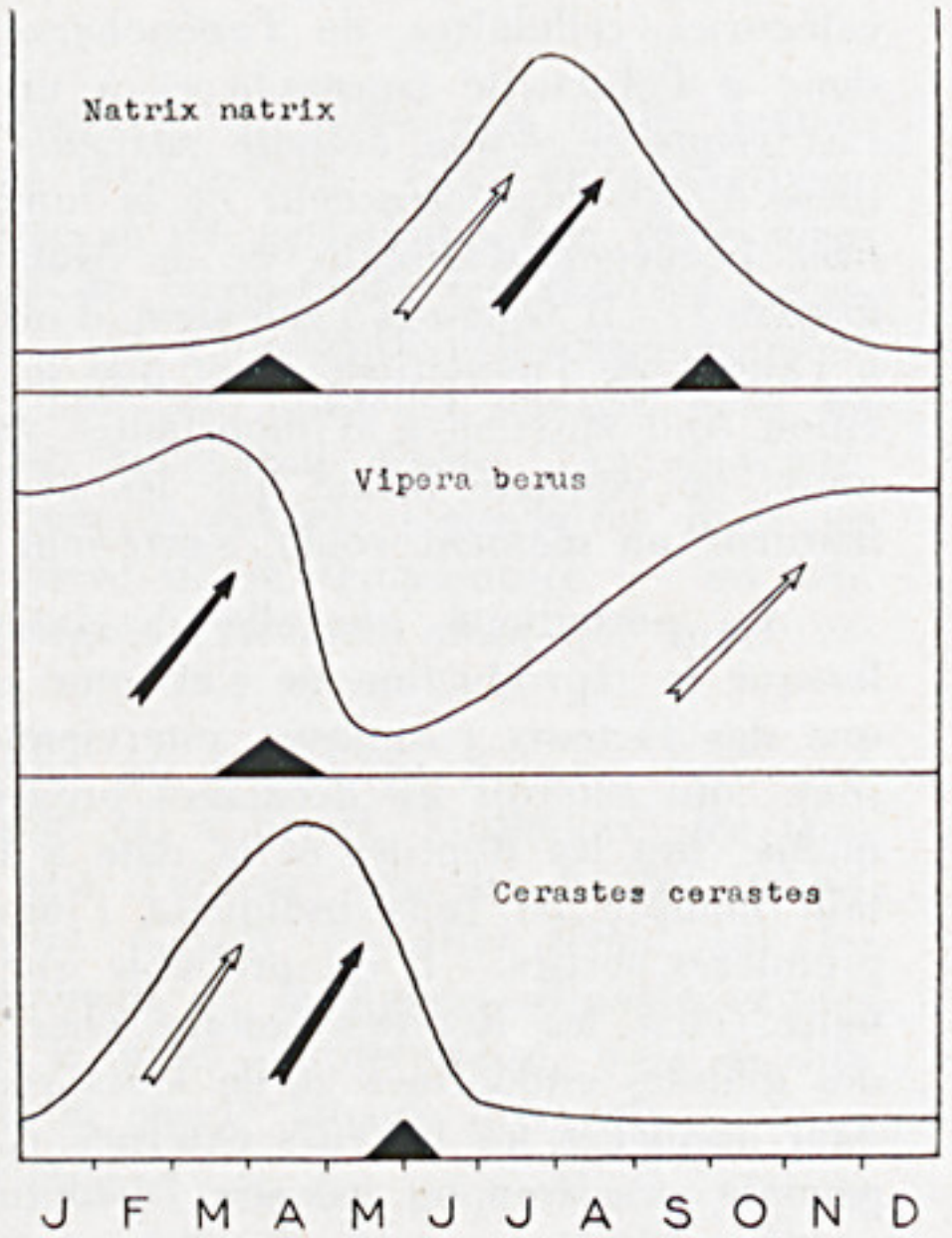


Fig. 4

Fig. 1 — Cycle sexuel des femelles chez divers Serpents venimeux.
A à E: espèces des régions tempérées. F et G: espèces des régions subtropicales arides.

A: *Vipera aspis.* Cycle annuel. C'est le type le plus répandu chez les Serpents vivipares, venimeux ou non, dans les régions tempérées.

B: *Vipera aspis.* Cycle biennal, à la limit Nord de 'laire de répartition de l'espèce.

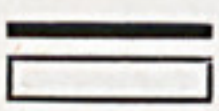
C: *Vipera berus.* Cycle biennal, en montagne.

D: *Crotalus viridis viridis.* Cycle biennal, d'après Rahn (1942).

E: *Agkistrodon contortrix.* Cycle biennal, d'après Fitch (1960).

F: *Crotalus cerastes.* Cycle annuel, en région subtropicale aride à hiver froid.

G: *Cerastes cerastes.* Cycle annuel en région subtropicale très aride (Sahara) et hiver doux.



Durée de l'hivernage.

Durée de la gestation.

E Date de l'éclosion chez les espèces ovipares.

Période d'accouplement principale.

Période d'accouplement secondaire.

Fig. 2 — Croissance des plus gros follicules ovariens au cours d'un cycle sexuel biennal, chez une Vipère et un Crotale.
A: *Vipera aspis*. Vitellogenèse vernale.
B: *Crotalus viridis*. Vitellogenèse estivale.
En abscisses: temps en mois.
En ordonnées: longueur du plus gros follicule, en millimètres.

Fig. 3 — Evolution des caractères sexuels secondaires (ici le segment sexuel du rein) au cours du cycle annuel.
A: *Vipera aspis* (région tempérée).
B: *Cerastes cerastes* (région subtropicale très aride).
En abscisses: temps en mois.
En ordonnées: diamètre du segment sexuel, en μ.

Fig. 4 — Différents types de cycles spermatogénétiques chez les Serpents. Type post-nuptial (*Natrix natrix*), mixte (*Vipera berus*) et pré-nuptial (*Cerastes cerastes*). La flèche blanche correspond à la spermatocytognenèse, la flèche noire à la spermiogenèse, les triangles à la période d'accouplement.
En abscisses: temps en mois.
En ordonnées: poids du testicule et diamètre des tubes séminifères.

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15. THE OVARIAN CYCLE OF *NATRIX RHOMBIFERA* — AN APPARENTLY GENERALIZED CYCLE OF SNAKES OF TEMPERATE LATITUDES

T. W. BETZ *

Department of Biology, Carleton University, Ottawa, Canada

Complete descriptions of ovarian morphology in reptiles are available for only a few species. However, acounts of gross ovarian morphology have been reported for *Vipera* (Loyez, 1906), *Bothrops*, *Crotalus*, *Xenodon* (Fraenkel and Martins, 1938; Fraenkel *et al.*, 1940), *Crotalus* (Tinkle, 1962), *Opheodrys* (Tinkle, 1960), *Thamnophis* (Bragdon, 1952; Cieslak, 1945; Tinkle, 1957), *Thyphlops* (Guibé, 1948), *Enhydrina* (Kasturirangan, 1951) and *Natrix* (Loyez, 1906); Bragdon, 1952; Tinkle, 1959; Betz, 1963a). Accounts have also been reported for *Anguis*, *Lacerta*, *Platydactylus*, *Terrapene*, *Testudo*, *Crocodylus* (Loyez, 1906), *Lacerta*, *Lygosoma*, *Mabuya*, *Tiliqua* (Weekes, 1934, 1935), *Lacerta* (Hett, 1924), *Hoplodactylus* (Boid, 1940), *Zootoca* (Panigel, 1956), *Xantusia* (Miller, 1958), *Cnemidophorus* (Carpenter, 1960) and *Holbrookia* (Johnson, 1960). Others appear in the reviews of Kehl and Combescot (1955) and of Matthews (1955).

Generally the criteria for grouping the animals in these kinds of studies depend upon the size of the ovarian follicles; the size and presence or absence of corpora lutea and embryos; and the condition of the uterus.

The ovaries of mature *Natrix rhombifera* (Betz, 1963a) are elongate, thin-walled, saccular structures with an irregular, lymph-filled central cavity. They consist of a loose, semitransparent stroma in which the oval, creamy-white, relatively avascular ovocytes are seen in contradistinction to the yellow, vascular atretic follicles and corpora lutea. Each ovary is suspended by a mesovarium in the pleuroperitoneal cavity between the dorsal mesentery and the mesotubarium of the oviduct. They generally extend from the level of the oviducal infundibulum to the posterior limits of the kidneys. The right ovary is typically heavier and more anteriorly placed than the left. The length of the ovary is positively correlated with the length of the animal (Fig. 1) and longer ovaries generally have more follicles than shorter ones. The positive correlation of the length of the ovary with body length has been reported for *Natrix sipedon* (Tinkle, 1959) and for some lizards (Carpenter, 1960). The smaller ovocytes of *Natrix* are usually restricted to the lateral aspect of the ovary. The larger mature follicles are pale yellow in color. The follicles occur in four size groups: one group (I) of follicles 0.1 mm long; another group (II) 5-10 mm long; a third (III) 10-20 mm long; and a fourth (IV) 20-46 mm long. The number

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of follicles decreases as they become mature. In Missouri ovulation occurs between 15 May and 15 June as specimens caught at this time, are immediately preovular. Postpartum females are caught between 15 August and 15 September. Consequently, gestation is approximately three months long. Deviations of several weeks from the above dates are probably not uncommon. The size-frequency distribution of follicles at various times of the reproductive season indicates that the production of mature ova probably requires 2.5 years, i.e., the neonatal late summer and early fall months, followed by two full years and the spring of the third year (Fig. 2). The ovary at the end of the first year (following the neonatal fall) would contain follicles of groups I and II. At the end of the second year the ovary would contain follicles of groups I, II, and III; group II follicles of the first year have become the group III follicles of the second year; group I follicles of the first year have become the group II follicles of the second year; and a new crop of group I follicles has emerged from the germinal epithelium. Immediately prior to ovulation, in the spring of the third year, group III follicles of the second year grow rapidly and become group IV follicles. During gestation the ovary, except for corpora lutea, appears identical to the two-year-old ovary in follicular sizes. Apparently follicular growth occurs gradually over a two-year period but most of the growth occurs rapidly in the spring of the third year. The ovarian cycle is apparently annual (Fig. 2). In other forms the time required for the production of mature ova may be two years as in *Xantusia* (Miller, 1948, 1958); three years as in *Thamnophis* (Bragdon, 1952; Cieslak, 1945) and *Crotalus* (Rahn, 1942; Tinkle, 1962). Although annual ovarian cycles are commonly reported, Weekes (1934) reported that *Amphibolurus* may have two ovarian cycles a year. Also nonseasonal cycles occur in some Javanese snakes (Kopstein, 1938), house geckos (Church, 1962), and in *Lygosoma* (Baker, 1929, *fide* Miller, 1958). Yolk deposition may occur gradually during most of the year, as in *Lacerta* (Regamey, 1935), or gradually during a long growth period with a final immediately preovulatory increased rate of deposition as in *Phrynosoma* (Blount, 1929), *Hoplodactylus* (Boyd, 1940), *Hemidactylus* (Dutta, 1944), *Xantusia* (Miller, 1958), *Natrix*, and *Thamnophis* (Bragdon, 1952).

Atretic follicles are hyperemic and more flaccid than developing follicles. The yolk is more fluid and paler than in developing follicles. The follicle wall is thin, fragile and easily broken. Follicles of all sizes in various stages of astresia are commonly present. As astresia proceeds (as seen in selected stages) the follicles become progressively smaller, more hyperemic, and the yolk more pale, more fluid, and diminished in amount. Eventually the corpus atreticum is replaced by stromal tissue leaving an indefinite, variously persistent scar. Atresia probably accounts for the decrease in the number of follicles as they mature. Also any follicles of group IV that are not ovulated undergo atresia. It is not uncommon to find ovaries during early pregnancy with large group IV atretic follicles. Follicular atresia is common in most reptiles and has been reported by Mingazini (1893), Dubisson (1905), Loyez (1906), Boyd (1940), Bragdon (1946, 1952), Bretschneider and Duyvene de Wit (1947), Miller (1948, 1958), and Altland (1951).

Occasionally irregularly shaped, creamy-white, viscous masses of ectopic yolk material occur both in the coelom and in the ovarian stroma. These masses are of the same consistency irrespective of their size. Apparently the ectopic yolk masses in the coelom or ovarian stroma are from burst atretic follicles. The shape and size would depend on the space available among the viscera or follicles

and the amount of yolk in the follicle at the time of bursting. The position of the masses would depend on the site of rupture of the follicle wall. Judging from the amount of yolk usually present in these masses, bursting atresia is more common in large follicles, probably because of the greater thinness and fragility of their walls. These ectopic yolk masses are apparently quickly reabsorbed since they do not occur in the preovulatory females but only in pregnant and early postpartum females. Bursting atresia has also been reported in *Thamnophis* (Bragdon, 1952). One factor which is a source of error in studies of reproductive cycles which are based only on gross morphology is that follicles which have just begun to undergo atresia could easily be miscounted as growing follicles. However, the two types of follicles are easily distinguished from each other on a histological basis (Betz, 1963b).

The corpus luteum of the first month of gestation is pale yellow. The surface is indented by a puckered umbilicus which may contain a transient ectopic blood mass. The gland is approximately 10-12 mm long. The shape is essentially oval but is variable and depends on the space available between the adjacent developing or atretic follicles. The gland is placed well within the ovarian stroma and does not appreciably project from the surface of the ovary. The gland is more vascular than the developing follicles but less so than well-advanced atretic follicles. There is a 1:1 correspondence between the number of corpora lutea and the number of embryos or yolk masses in the uterus. It is common to find a disparity between the number of corpora lutea in the ovary of one side and the number of conceptuses in the uterus of the same side which is probably due to the extrauterine migration of ova to the contralateral uterus as reported by Legler (1958).

During the second month of gestation the corpus luteum is darker yellow, smaller (5-7 mm) and the umbilicus and ectopic blood mass are not present. In the last month of gestation the corpus luteum is deep yellow and less than 5 mm long. The corpora lutea usually degenerate rapidly; in the two- or three-week postpartum animal they are small (1 mm), orange patches in the ovarian stroma. Usually by the following spring all traces of the glands have disappeared as evidenced by a general lack of these structures in the preovulatory females; however, occasionally the scars persist until the next spring. Corpora lutea have been described for oviparous, ovoviviparous, and viviparous species of reptiles (Lucien, 1903; Hett, 1924; Weekes, 1934, 1935; Fraenkel and Martins, 1938; Kasturirangan, 1951; Bertin, 1952; Amoroso, 1955; Miller, 1958). The length of time that the corpora lutea are maintained can be positively correlated with the egg-retaining habits of a species (Miller, 1958). In oviparous and ovoviviparous species, a corpus luteum develops which begins to regress before oviposition occurs (Weekes, 1934; Rahn, 1938; Harrison, 1948). In most viviparous forms the length of gestation is two to three months. Typically the corpora lutea begin to regress during the last third of gestation (Weekes, 1934; Cieslak, 1945; Miller, 1948; Bragdon, 1952), but in some species regression is not apparent until after parturition (Rahn, 1938, 1939, 1942).

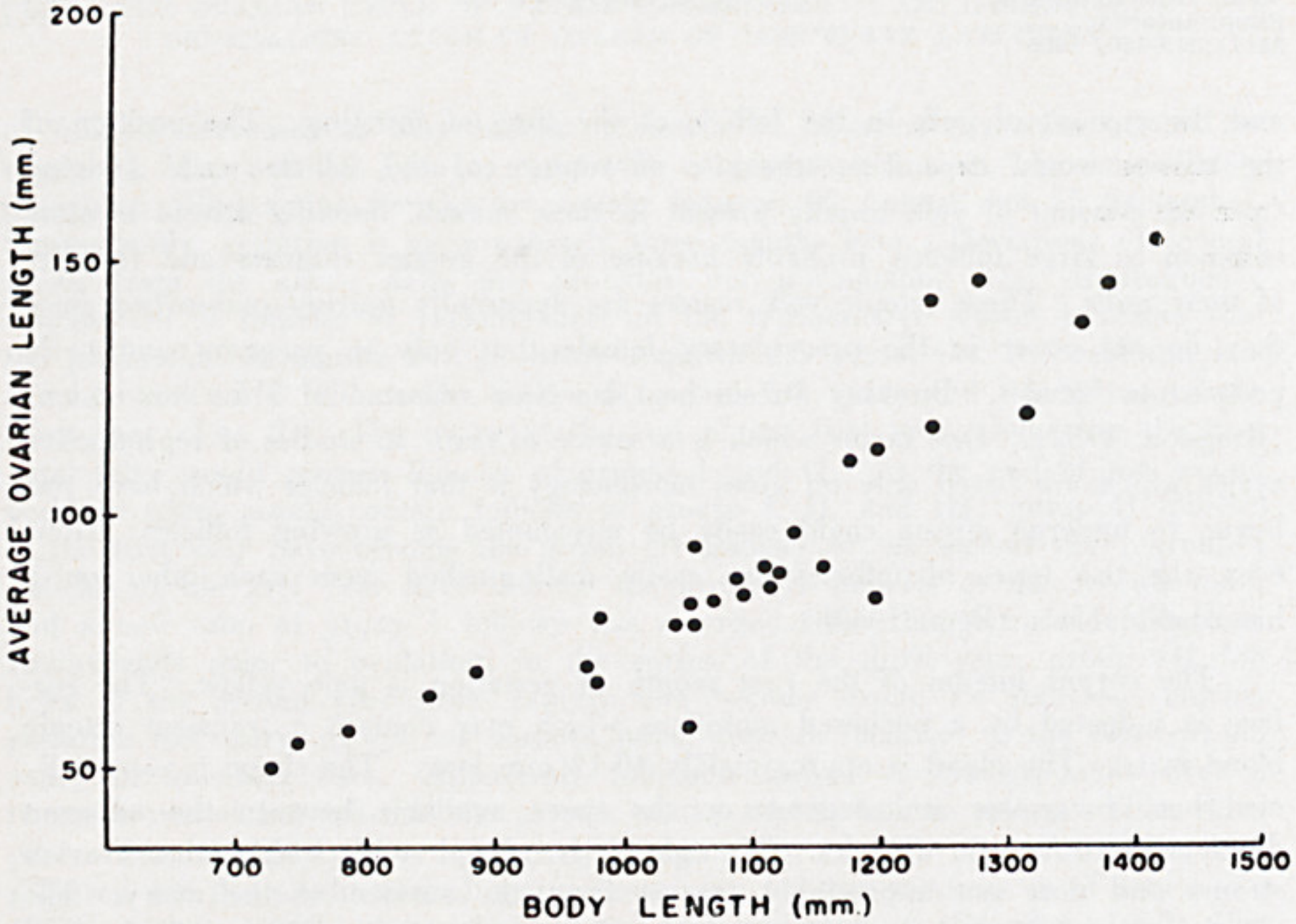


Fig. 1 — Scatter diagram of the correlation between average ovarian length with body length of *Natrix rhombifera*.

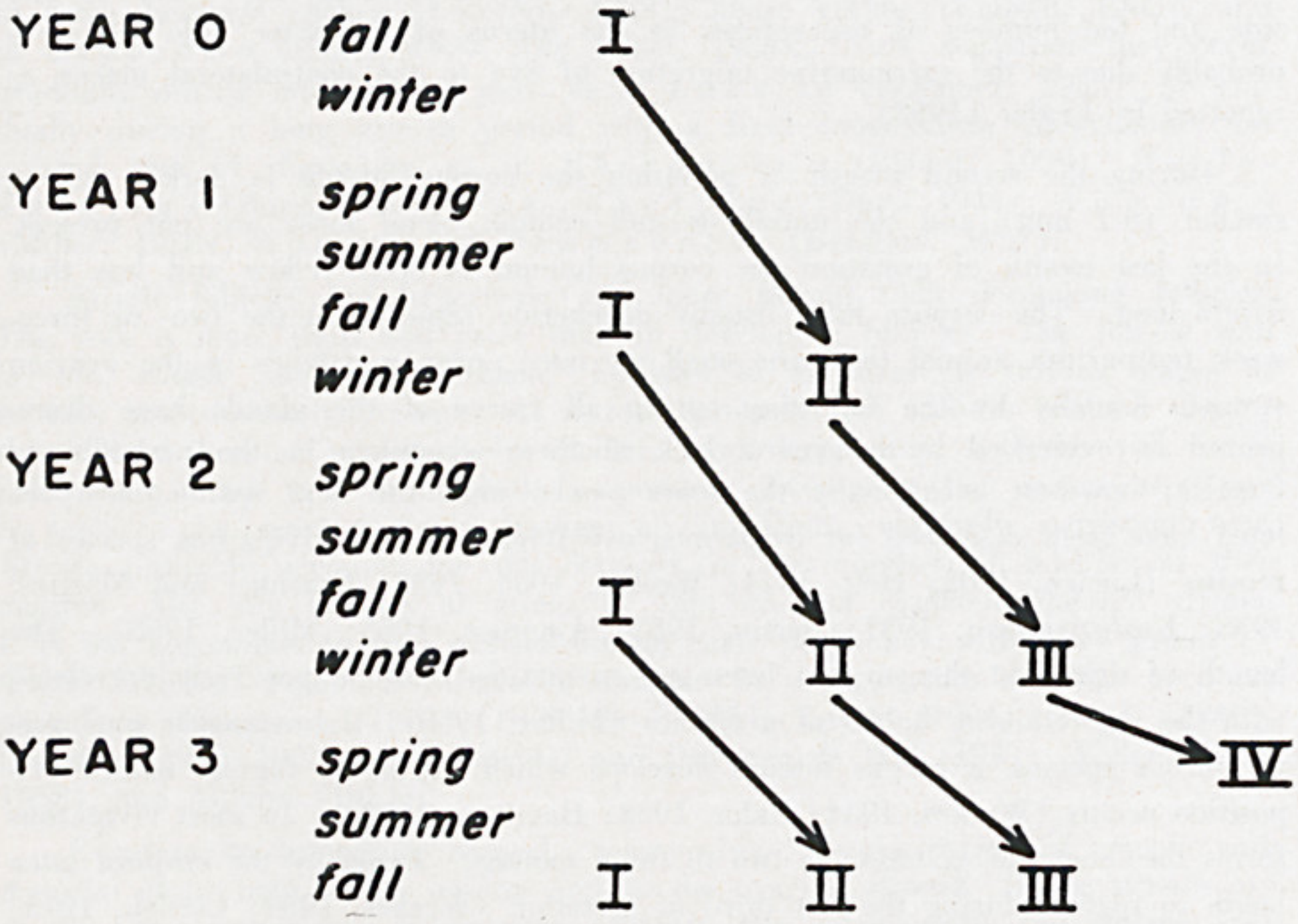


Fig. 2 — A diagram of the hypothetical sequence of follicular maturation in *Natrix rhombifera*. Roman numerals refer to follicle size groups.

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16. VERTEBRATE HORMONES AS DEFENCE SUBSTANCES IN DYTISCIDES

H. SCHILDKNECHT

Organisch-Chemisches Institut der Universität, Heidelberg, Germany

Introduction

As we have shown (1), many arthropods defend themselves against their enemies with special glandular substances. The existing research has concerned itself with secretions which serve to repulse small invertebrates, vertebrates and microorganisms. In continuation of this work we have examined for the first time, in the case of *Dytiscus marginalis*, a group of protective substances which react specifically against vertebrates. This glandular protective system is found in the breast section of the beetle (Fig. 1). Figure 2 shows one of the two secretion reservoirs, each about 3.5 mm long and 1.4 mm wide, on which lie a thick layer of glandular cells.

Blunck (2), (3) describes the formation of the milk-white secretion in the glandular cells and its transmission to the reservoir. Merely by holding or by a light pressure to the head, the beetle secretes the glandular fluid through muscular pressure.

It was of particular advantage, for the successful identification of the toxic factor in the secretion, that this crystallised out without further assistance after several weeks from the secretion, absorbed in a glass capillary.

Identification of the defence substance as an steroid

In agreement with the already published analytical data of the toxic component of the defence secretion we also found, at the onset of our recontinued work, in the UV-spectrum of the secretion taken in optically pure ethanol, an absorption maximum at 240-241 mμ typical of a conjugated chromophore.

According to Woodward, mono-alkylated enones absorb on an average at 224 mμ. By the calculation of similar chromophores one adds to this value 11 mμ for an additional alkyl group or a cyclic residue in the β-position, and a further 5 mμ for the exo-cyclic position of the C = C-double bond. Thereby, e.g. a maximum of 240 mμ is obtained for testosterone which is almost identical with the maximum of the hormone as found by us. This agreement between testo-

sterone and the hormone from *Dytiscus* is especially good when one compares the values of the absorption bands of both infra-red spectra (Table 1).

TABLE 1 — COMPARISON OF THE IR-ABSORPTION BANDS OF TESTOSTERONE AND THE HORMONE

Type of vibration	Position of the bands with testosterone in cm^{-1}	Position of the bands with the hormone in cm^{-1}
ν O−H	3520	3476
ν C=O		1693
ν C=O	1668	1668
ν C=C	1612	1613
ν C−C=O		1272
ν C−C=O	1230	1233
ν C=O	1063	1074
ν C=O	1053	1061
γ C−H	867	870

Alone, a superficial consideration of Table 1 indicates that the *Dytiscus* secretion constituent could be a steroid. Also the position of the γ_{C-H} vibrational band at 870 cm^{-1} and the $\nu_{C=C}$ vibrational band at 1613 cm^{-1} as well as the $\nu_{C=O}$ vibrational band at 1668 cm^{-1} indicates a Δ^4-3-keto-steroid. Moreover, as with testosterone, an OH-group could be detected by an IR-spectrum taken in carbon disulphide (Fig. 5).

The intensity of these bands is very low and from this one could infer that the hydroxyl group is hydrogen bonded to a carbonyl oxygen atom. It is possible of course to rule out bonding with the carbonyl group in position 3. There is, however, a further discernable band at 1693 cm^{-1} in the IR-spectrum of the *Dytiscus* hormone which does not appear in the IR-spectrum of testosterone and must be assigned to a saturated aliphatic ketone (Fig. 6 & 7).

In the UV-absorption spectrum, however, this carbonyl chromophore was not detectable. It should, on the other hand be detectable with the help of circular dichroism (CD), when the hypothesis that it is a steroidal ketone is correct i.e. a ketone with several optically active centres. In fact, the CD-spectrum taken showed two extrema at 283 mμ with the $\Delta\varepsilon_{max} = +3.076$ and at 321 mμ with the $\Delta\varepsilon_{max} = -1.16$ (Fig. 8).

According to Velluz and Legrand (4), the Δ^4-3-keto-steroids possess a minimum at 334 mμ with the $\Delta\varepsilon_{max} = -1.35 \pm 0.12$ (ind dioxan) which conforms with our interpretation of the minimum at 321 mμ (in ethanol) found by us. This finding confirms again that the *Dytiscus* hormone may be a Δ^4-3-keto-steroid. For the second maximum at 283 mμ (in ethanol), two possibilities are indicated in the literature (4):

a) 20-keto-steroids: 295 mμ (dioxan)

b) 17-keto-steroids: 303 mμ (dioxan)

An assignation was made more difficult in that the two relevant $\Delta\varepsilon_{max}$ values at 3.459 $\pm$ 0.12 and 3.290 $\pm$ 0.10 are also not so very different. We must at this point, however, introduce a correction for the use of different solvents. Probably our compound should have its maximum at (283 + 13 =) 296 mμ in dioxan. Since the corrected value of 296 mμ lies nearer to 295 than to 303, we concluded that the hormone is a 20-keto-steroid.

On considering that the ketone group at C_{20} can be bridged and that its accompanying absorption band in the IR-spectrum occurs, not as usual, at 1710 cm^{-1} but at 1693 cm^{-1}, one may suppose that the OH-group is bonded to the C_{21} atom.

On the basis of the partial structure just proposed, we can thus assume that the *Dytiscus* hormone is Δ^4-pregnen-3,20-dion-21-01 (11-desoxycorticosterone, cortexon).

We confirmed this postulation by a comparison of the CD of cortexone (Fig. 9) and the secretion constituent.

Cortexone shows the two extrema at 282 and 322 mμ with the corresponding $\Delta\varepsilon_{max}$ values of 3.275 and -1.16. Small differences in the $\Delta\varepsilon_{max}$ values are known to occur — according to the literature (5), the influence of temperature frequently plays a big role.

In 1959, Heller published a paper on the IR-spectra of steroids (6). His information on hydroxyprogesterone can be referred to for the further identification of our natural product. He was concerned with the wave numbers of the bands to be found between 1000 and 1150 cm^{-1}. The curves obtained for the spectrum of the secretion are shown in Fig. 10.

A simple comparison of these spectroscopic data with those which were found by us for the secretion constituent reveals that the latter is most probably identical with cortexone (Table 2).

TABLE 2 — COMPARISON OF THE IR-SPECTRAL BANDS IN THE REGION OF THE ν-C—O -VIBRATION BETWEEN 1000 AND 1150 cm-¹

Bands found for Cortexone (cm-¹)	Bands found for the Hormone (cm-¹)
1005 (w)	1009
1039 (w)	1040
1060 (st)	1061
1072 (st)	1074
1093 (w)	1093
1117 (w)	1117

Since cortexone is commercially available, it was possible to obtain an IR-spectrum of a synthetic sample from Fluka Ltd. for further information.

The spectrum of this sample of cortexone (Fig. 11) agreed so completely with that of our compound, obtained by thin-layer chromatography, that an identity was thereby suggested.

The UV-spectrum of cortexone (Fig. 13) agreed likewise with that of the secretion constituent (Fig. 14) isolated by us.

We obtained a further agreement between cortexone and our natural product by the comparison of the physical data of the corresponding 2,4-dinitrophenyl-hydrazones (2,4-DNP). The 2,4-DNP of cortexone was prepared following the method of Reich and Samuels (7). In the literature, different melting points are recorded for this product — Wettstein *et al.* (8) give a decomposition temperature of 278 to 284°C and Reich and Samuels (7) record that the 2,4-DNP of cortexone melts at 251°C to 254°C. The 2,4-DNP of cortexone prepared by ourselves decomposed between 267° and 269°C. The UV-spectra had, however, the same appearance.

For the preparation of the 2,4-DNP of the beetle secretion, the secretion was added to a solution of 2,4-DNP in 2N hydrochloric acid, left to stand for several hours and the crystals formed were then filtered off. By the thin-layer chromatographic separation we obtained two zones which were scratched apart, eluted and crystallised from aqueous ethanol.

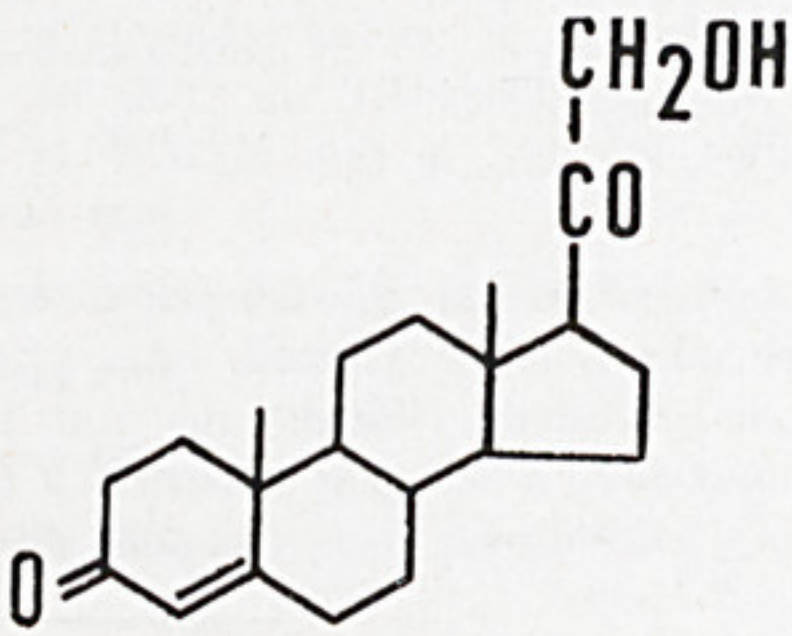
Reich and Samuels (7) record the UV — maxima and minima of the cortexone — 2,4-DNP as follows: maxima at 257 and 383 mμ and a minimum at 313 mμ. We found for the secretion — 2,4-DNP (decomp. 269°), maxima at 254 and 378 mμ and a minimum at 310 mμ. This is a satisfactory agreement.

Finally, there was still the agreement between the mass spectra of the synthetic cortexone and the material contained in the beetle secretion to be tested. In the mass spectrum of the beetle substance (Fig. 16), an additional mass peak at 316 was observed which could not be assigned. This may be caused by the contamination which also influenced the melting point. This was found at 135°C and not, as with cortexone, at 141°C.

The mass peak of 330 showed us the molecular weight of cortexone. Further, only the peak at 299 can be clearly assigned — the molecule having lost a CH_2OH group with mass 31. In then losses a carbonyl or an ethyl group of mass 28 resulting in a fragment of mass 271. An oxygen atom is removed as water leaving a residue of mass 253.

The mass spectrum of cortexone (Fig. 17) shows the same fragmentation pattern.

All these described results permit the conclusion that the non-volatile, ethanol-soluble compound from the water beetle's protective secretion is Δ^4-pregnen-3,20-dion-21-01.



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EXPERIMENTS ON THE PHYSIOLOGICAL EFFECT OF THE SECRETION AND ITS BIOLOGICAL IMPORTANCE

As previously mentioned in the introduction, the physiological effect of the beetle-secretion on various animal was investigated by Blunck (3). He found that it was a strong narcotic and poison effective in very small amounts, especially on cold-blooded vertebrates. On invertebrates, it produced only little or no effect. To begin with, we repeated some of Blunck's important experiments. In these, goldfish (*Carassius auratus*), **OLIGOCHAETA** (*Enchytreen*) and Dytiscides (*Colymbetes fuscus* and *Dytiscus marginalis*) were placed in an aqueous solution of the secretion. In agreement with Blunck's results, the goldfish were stupified whilst the worms and the beetles showed no reaction at all. The water beetle *Colymbetes* even ate meat, which had been treated with the poisonous substance, without harm.

Later, as it was found that the constituent of the beetle-secretion was cortexone, it was necessary to examine whether this substance had the same effect as the secretion. For this purpose we used cortexone from Fluka Ltd. The aqueous solution of cortexone is of 0.001% strenght and contains 10.39 mg of the substance per litre. From this, dilutions of 1:10 and 1:100 were prepared. Into these three solutions were placed the test fish (goldfish and young tench [*Finca vulgaris*]). The fish reacted to the concentrated solution exactly as to the natural secretion. After a short phase of excitement, the fish tired rapidly. After about eight minutes they could be turned onto their backs with a glass-rod and after twenty minutes they displayed severe equilibrium disturbances and lay immobile on their sides. Finally, they were completely motionless except for weak mouthing motions. When the stupified fish were placed in fresh water they recovered completely over a period of ten to twelve minutes and later could be used again in other experiments. Fish in the 1:10 dilution permitted themselves to be touched and turned over with a glass-rod without swimming away — Cortexone, in this instance, had no further action. In the case of the 1:100 dilution, the test fish showed no noticeable reaction.

Cortexone is a hormone of the cortex of mammalia. It possesses a mineralcorticoidal effect, i.e., it regulates the sodium-potassium content of the cells. On administration of an overdose, sodium is retained and potassium secreted, and so the ionic equilibrium is disturbed. This disturbance in the salt concentration corresponds to a disturbance in the sensitivity of the nerve and muscle cells by which, the physiological effect of the beetle-secretion and cortexone may be explained. This agreement of the biological effect of the secretion and cortexone again confirms the results of the chemical analysis.

In order to determine the biological importance of the secretion in nature, we carried out a series of further experiments. To this purpose we used synthetic cortexone as this is easier to administer and can be more easily obtained than the natural beetle-secretion.

There are two possibilities for the natural use of the poisonous secretion by the beetle. In the first instance, the predatory *Dytiscus* could stupify its prey with the cortexone containing secretion in order to overcome it more easily; one must then speak of an attacking-secretion. If, however, the beetle only delivers up the secretion when it is under attack in order to protect itself from an enemy, then it may be called a defence-secretion.

Initially we investigated the possibility of the *Dytiscus* secretion being used as an attacking weapon.

Experiment a: Water beetles were placed together with young tench and goldfish in small containers (100 ml) where the fish had no room to escape from the beetles. Soon after they were put together there was a fierce fight which ended in the death of the fish. In this experiment it was, however, impossible to observe the appearance of the white secretion. A following investigation of the water in order to detect cortexone by chemical means was also without success. In order to test whether the beetles had full reservoirs after all, they were so provoked after the experiment that they all gave up plentiful amounts of secretion.

Experiment b: In an experiment lasting several month, goldfish and water beetles were kept together in an aquarium (20 litre). Although the container was over-occupied by the water beetles and the beetles had not been fed for a long time, and already had begun to eat each other, the goldfish were not killed.

From these experiments it is concluded that the beetle-secretion is not used as an attacking weapon. It is therefore very probable that the beetle employs the secretion for protection from vertebrates. If a fish or an amphibian tries to eat a beetle, then the beetle immediately gives out a large quantity of secretion. This penetrates the gills of the fish and also, when the beetle has been swallowed, into the stomach-intestinal tract. The described experiments show that cortexone penetrates through the body surfaces and is effective.

In order to prove the effect, especially on the gills of the fish, we smeared 300 μg of cortexone on the gills of a large trout (*Salmo gairdneri*) of 750 g weight. After some time it was almost unable to move and could only with difficulty retain its balance. Only after more than six hours did it recover. It is interesting that this illness was immediately utilized by a small trout. Whilst before, this smaller fish had been continuously bitten by the larger one, now the order was reversed and the small fish attacked the larger continuously and pressed it into the very corner of the aquarium. Such examples could also be of importance in nature if a predatory fish ate a beetle.

Finally, we examined the effect of cortexone on stomach-intestinal tract of the fish. To this end, goldfish were fed with bread-crumbs in which 100 μg of cortexone was embedded. The same symptoms appeared as when cortexone penetrates through the body surfaces (skin and gills) of the organism. The effect takes place only a little later but on the other hand remains for a much longer time. Translated to the natural state, this means that a vertebrate that has eaten a water beetle temporarily suffers severe discomfort and probably will not capture further beetles in the future.

To sum up, it may be shown through the afore mentioned experiments that the prothoracic glandular secretion of the water beetle is a defence secretion that serves as protection against vertebrates. The physiological efficacy is caused by the contained cortexone.

Experimental Section

All IR-spectra quoted in the following work were taken on the Perkin-Elmer Spectralphotometer 221 and all UV-absorption spectra on the Beckman DB Spectrophotometer.

The concentration determination of cortexone in the beetle-secretion was carried out using a spectralphotometer PMQ II from Carl Zeiss Lts. Also, using this apparatus, the log ε value of cortexone was determined as 4.24.

A dichrograph from Roussel-Jouan Ltd., Paris was used for the circular dichroism measurements.

The mass spectra were taken with a mass spectrometer of the type CH 4 from the Atlas MAT Company, Bremen.

All melting points were determined on the Bock-monoscope.

The water beetle (*Dytiscus*) were compelled to give up the secretion by light pressure with the finger against the head. The secretion was taken up in a capillary and squirted into pure ethanol. After a few days the insoluble components collected on the bottom and could be centrifuged off using a microfuge. The insoluble components were dried in air and without preliminary treatment were examined spectroscopically in potassium bromide. The hormone was separated from the alcoholic solution and purified by the use of preparative thin-layer chromatography using plates coated with Kieselgel "G" and chloroform as the solvent. The plate was developed from a distance of 10 cm. Under a UV lamp of wave-length 366 mμ the compound is observable by fluorescence. Since the R_f-value is relatively low, the plate was redeveloped using chloroform. After this, the compound had moved 4 cm. The zone was scraped off and eluted with chloroform in a microcolumn. The compound so obtained was used without further preliminary treatment for spectral investigations.

Preparation of the 2,4-dinitrophenylhydrazones of the beetle-secretion and cortexone

The secretion of the *Dytiscus* was added to a solution of 2,4-DNP in 2N hydrochloric acid and allowed to stand for several days. The crystals formed were filtered off, dissolved in chloroform and the two 2,4-DNP derivatives preparatively separated on Kieselgel "G" thin-layer plates. As developing agent, chloroform was used and the plate was run for an hour. Although, after this time, the separation was noticeable we were unable to separate the zones by scraping them apart as they lay too closely together, and therefore the chromatography was carried out twice again each time for an hour. It was then easy to separate the two 2,4-DNP derivatives by scraping apart and eluting them separately on micro-columns. The products crystallised from aqueous ethanol with melting-points: 269°C.

The 2,4-DNP of cortexone was prepared following the method of Reich and Samuels (7). 10.7 mg of Cortexone and 15 mg of 2,4-DNP were dissolved in 1.8 ml of ethanol and 3 drops of concentrated hydrochloric acid were added. After 2.5 hours the crystals formed were filtered off, washed with ethanol and crystallised from chloroform/ethanol. Melting-point: 267-269°C.

Experiments to test the effects of the beetle secretion and cortexone

Experiments with the natural secretion were carried out on goldfish (*Carassius auratus*) and invertebrates. The secretion from seven beetles was dissolved in 100 ml of water and the goldfish was placed in the solution. After 30

minutes the excitation phase commenced. 75 minutes after the start of the experiment the reactions of the fish had been slowed down considerably, and after a further 90 minutes the fish was unable to react any more.

OLIGOCHAETA and *Dytiscus* exhibited no reaction. The effect of cortexone on fish was tested using young tench (*Finca vulgaris*).

Experiment a: A tench of 1.13 g weight was placed into a saturated solution of cortexone in water. As a blank-test, another fish was placed into tap-water. After 5 minutes the movements of the test-fish became unnatural. After a total of 9 minutes it swam on its back and had grown pale. 13 minutes after the start of the experiment it was unable to swim to the surface and lay on its side. After 18 minutes, it was unable to move and mouthed only seldom. After 24 minutes it lay, as if dead, on the bottom of the container. The control-fish was completely normal.

Experiment b: Young tench were tested in various concentrations of cortexone in water. A saturated solution of cortexone was made, and from this, dilutions of 1:10 and 1:100 were prepared.

250 ml of each of the three solutions of differing concentration were poured into separate containers and at the same time a young tench was placed into them. In container 1, with the concentrated cortexone solution, the fish swam immediately here and there in an agitated manner. After 5 minutes it reeled. It was possible after a period of 8 minutes to turn it onto its back with a glass-rod. After 11 minutes it lay down but now and then swam quite normally to the surface. 9 minutes later it had completely lost its sense of balance. The gills bled 28 minutes after the start of the experiment and after 60 minutes the fish lay unmoving on the bottom — it seldom made mouthing movements.

In container 2 with the dilution 1:10 the first reaction from the tench came after 11 minutes. It swam agitatedly, allowed itself to be touched with a glass-rod, and lay on its side. The influence of the cortexone did not proceed any further.

The fish in container 3 with the dilution 1:100 displayed no noticeable reaction throughout the duration of the experiment.

Experiment c: A young tench was placed in a saturated solution of cortexone and after some time was then placed into fresh water. 7 minutes after it was placed in the solution, the fish became disquitened. After a total of 16 minutes, it lay on its side and after a further 2 minutes it lay unmoving on the bottom. After 20 minutes it was mouthing only weakly and was then replaced in fresh water. Within 11 minutes the fish had recovered and then behaved again as normal. The experiment was repeated with the recovered-fish. In the cortexone solution it again displayed stupefaction symptoms and again recovered in fresh tap-water. This action may ostensibly be repeated as often as desired.

Experiment d: A young tench was kept for a longer time in a saturated cortexone solution.

After 18 minutes the fish tilted onto its side and after 26 minutes did not move any more. Soon it swam spontaneously (after 2 hours), but always lay down again. This lying-down occurred less and less frequently and the next day

the fish swam quite normally in its container. 26 hours after the commencement of the experiment a second fish was placed with the test-fish. This fish showed no reaction. After 10 minutes, both fish swam frequently to the surface probably due to an oxygen-shortage caused by the presence of two fish in the container.

Experiments to test the secretion as an attacking weapon

Experiment a: Eleven water beetles were placed singly into 100 ml of water together with goldfish and young tench. After a fierce battle the creatures were separated. The total amount of liquid (1100 ml) was reduced in a rotary evaporator and extracted with chloroform. The residue was treated with a solution of 2,4-DNP in 2N hydrochloric acid but a thin-layer chromatogram showed no evidence of the presence of cortexone-2,4-DNP.

Experiment b: Three goldfishes and up to 30 water beetles were kept in an aquarium, 39 by 25 by 22 cm with a capacity of ca. 20 litres. The fishes were fed continuously with dry forage and no beetle managed to kill a fish. If the fishes were not fed any more they soon became weak and unable to excape the beetles and were killed.

Experiments to test the secretion as a defence weapon

Experiment a: The effect of cortexone on the gills.

A trout (*Salmo gairdneri*) of 750 g weight was orally given 300 μg of cortexone in 10 ml water. The duration of the operation was one minute. After 9 minutes the fish began to pale and already after 13 minutes it succumbed in a battle with a smaller trout. After 85 minutes the trout stood inert on its head and it was possible to pull it out of the water by its tail.

Experiment b: The action of cortexone through the stomach-intestinal tract.

Different goldfishes were given cortexone embedded in bread by pushing it into their mouths with a glass-rod. A control fish was given only bread in the same amount.

In the case of the test-fishes they became weaker after 25 minutes. This weakening was so intense after 45 minutes that the fish was unable to turn back any more when one turned it over with a glass-rod. The fish was certainly not helpless but was constantly very slow to react. After 11 hours all the goldfishes behaved normally again.

The water beetles came from all parts of the Bundes-republik. They were kept in aquaria and were fed on meal-worms. As long as one changes the water frequently enough and give the beetles ample nourishment, also in form of meat and fish, they can be kept alive for a longer time. As previously stated, the beetles were "milked" by a light pressure on the head. They regenerated the secretion in about three weeks.

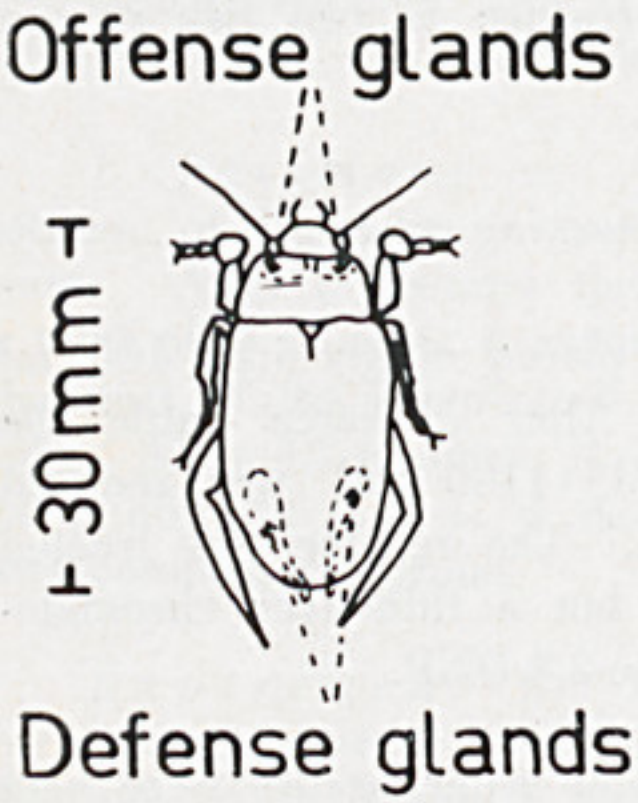


Fig. 1

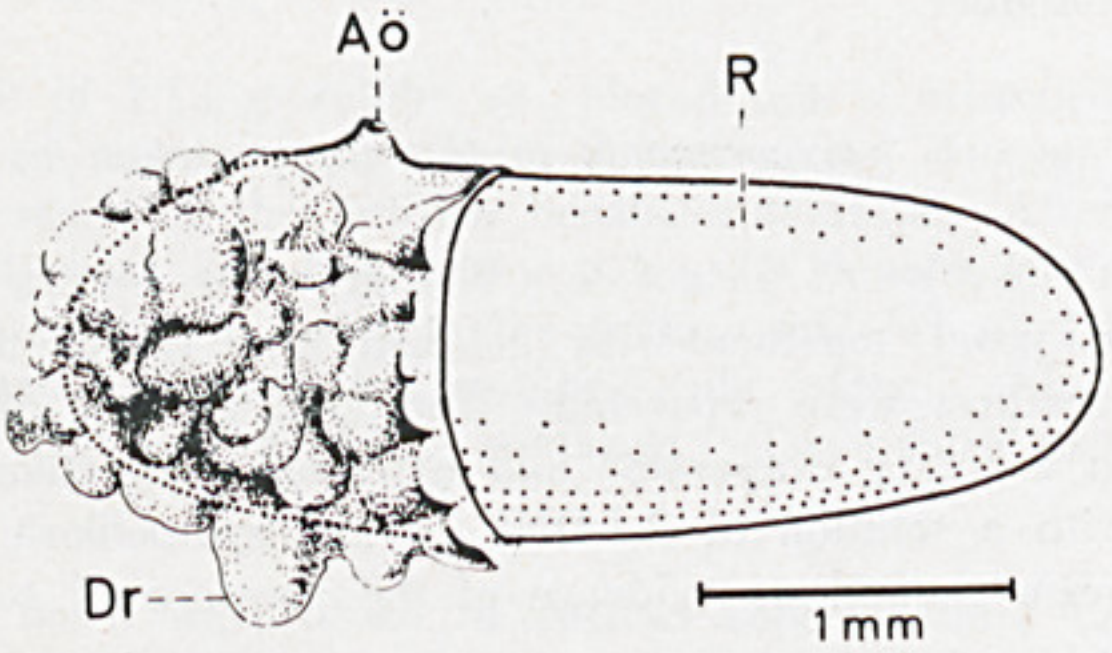


Fig. 2

Fig. 1 — Site of the protective glands of *Dytiscus*.

Fig. 2 — Secretion reservoirs with the overlying glandular cells

R = reservoir

Dr = glandular cells

Aö = secretary opening

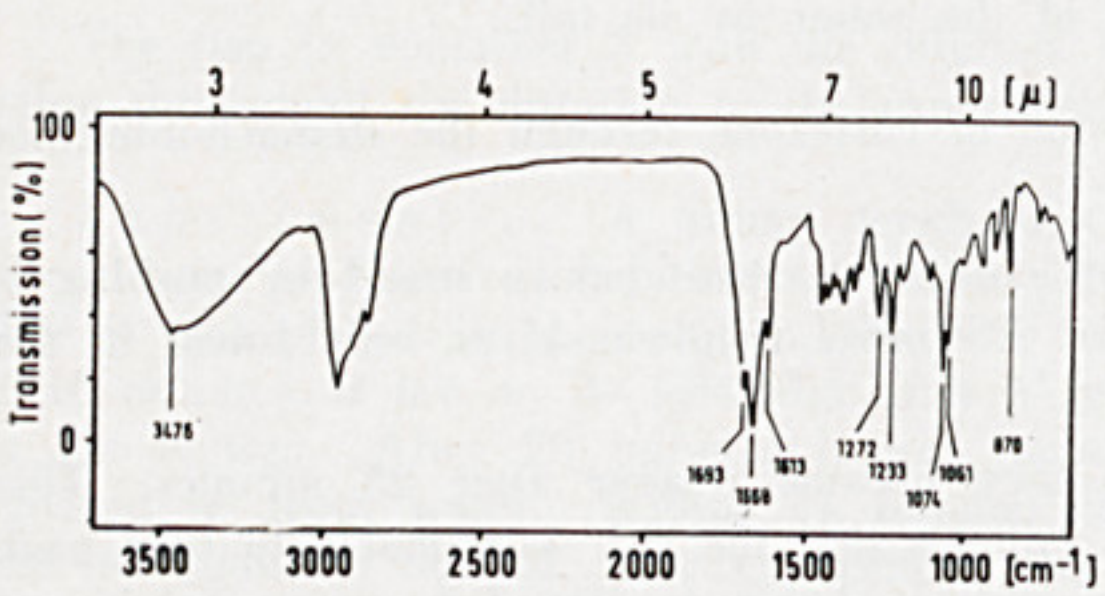


Fig. 3

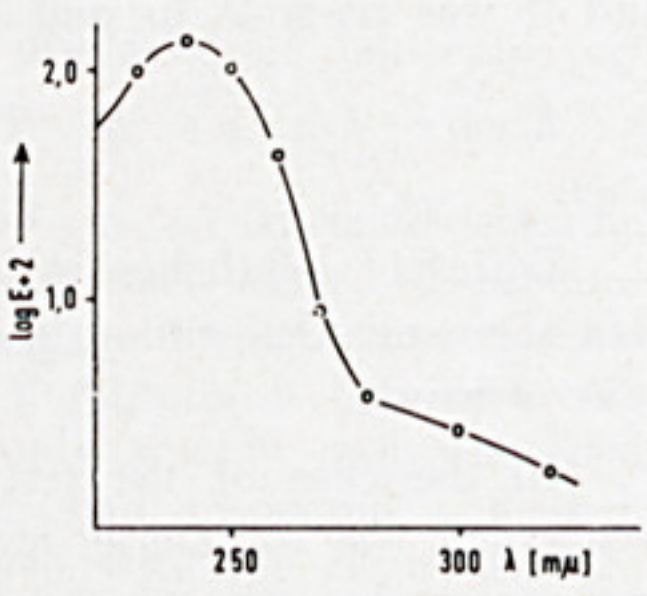


Fig. 4

Fig. 3 — IR-Spectrum of the crystalline crude secretion in KBr.

Fig. 4 — UV-Spectrum of the whole secretion in ethanol when taken immediately and after ¼ one years exposure to air.
λ_{max} 240-241 mμ

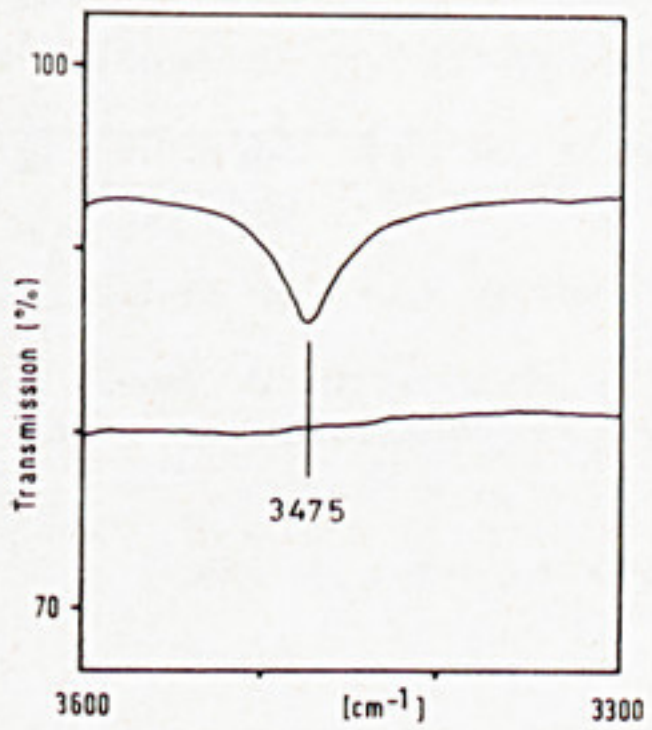


Fig. 5

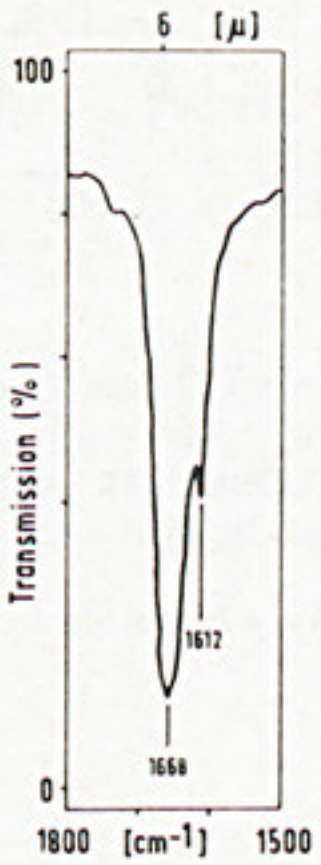


Fig. 6

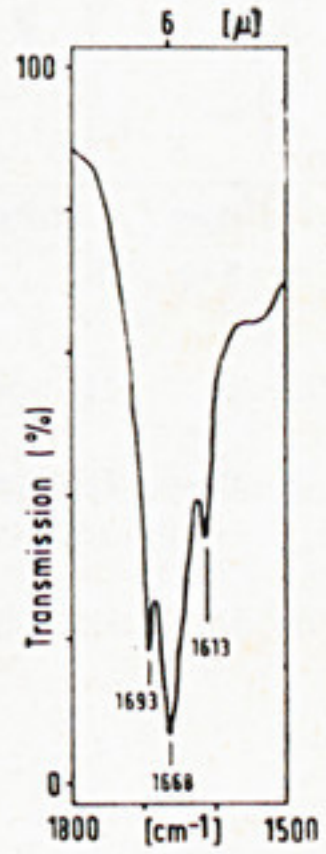


Fig. 7

Fig. 5 — IR-Absorption of the *Dytiscus* hormone in the region of the ν O—H vibration (upper curve in CS_2; lower curve pure CS_2).

Fig. 6 — IR-Absorption of testosterone in KBr.

Fig. 7 — IR-Absorption of the hormone in KBr.

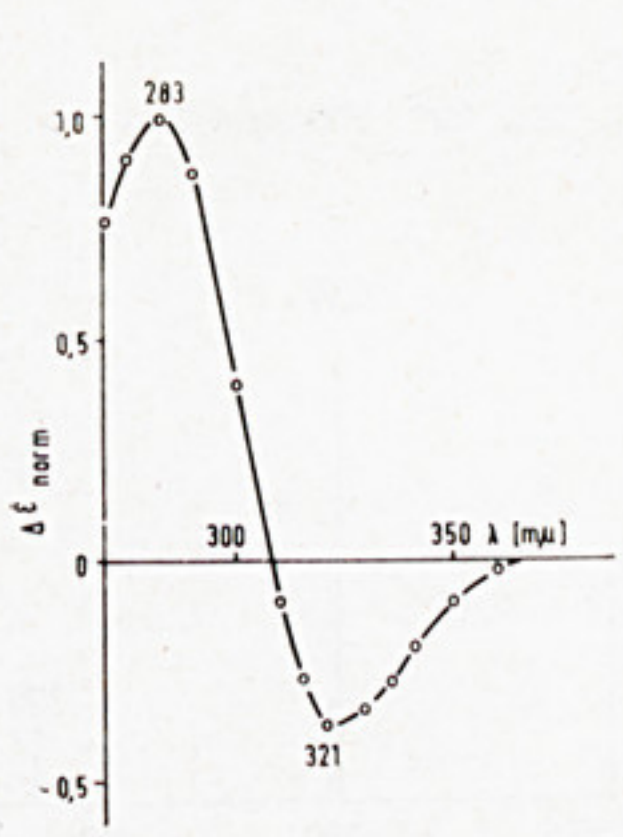


Fig. 8

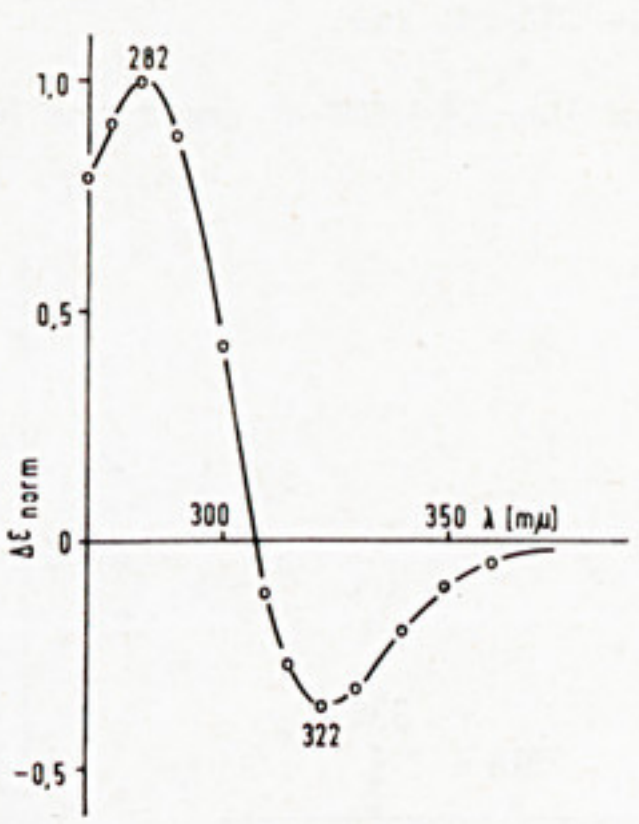


Fig. 9

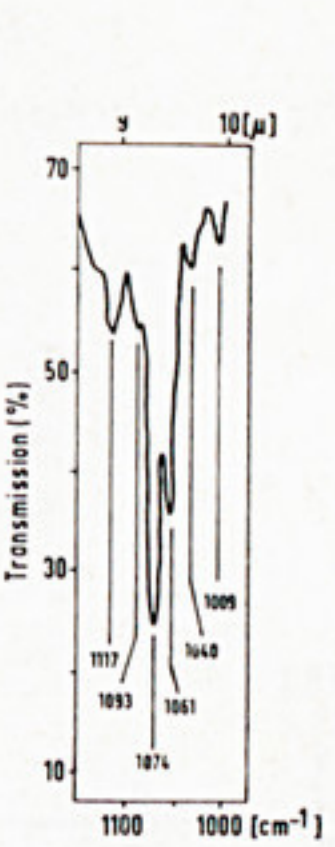


Fig. 10

Fig. 8 — The circular dichroism of the *Dytiscus* hormone.

Fig. 9 — The circular dichroism of cortexone.

Fig. 10 — IR-Absorption of the hormone in the region 1000 to 1150 cm-¹ taken in KBr.

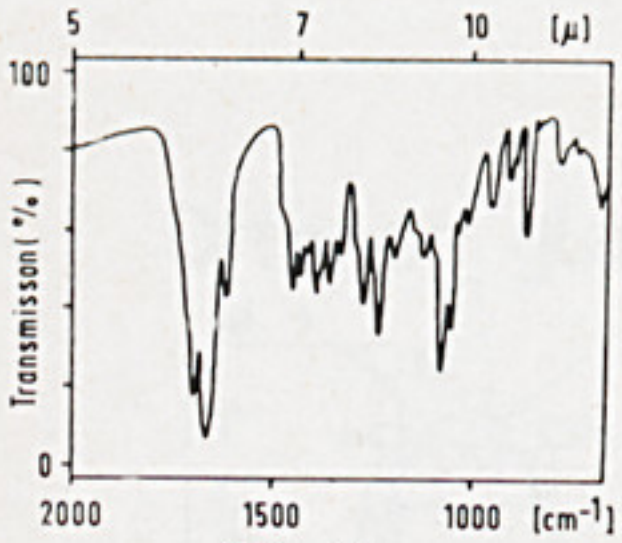


Fig. 11

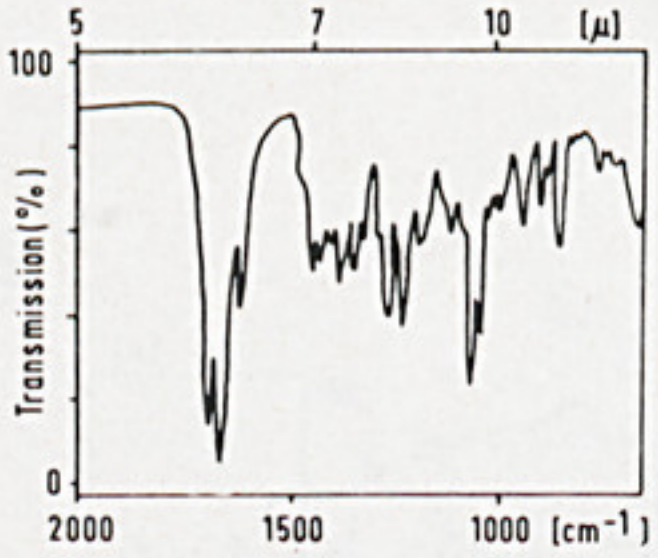


Fig. 12

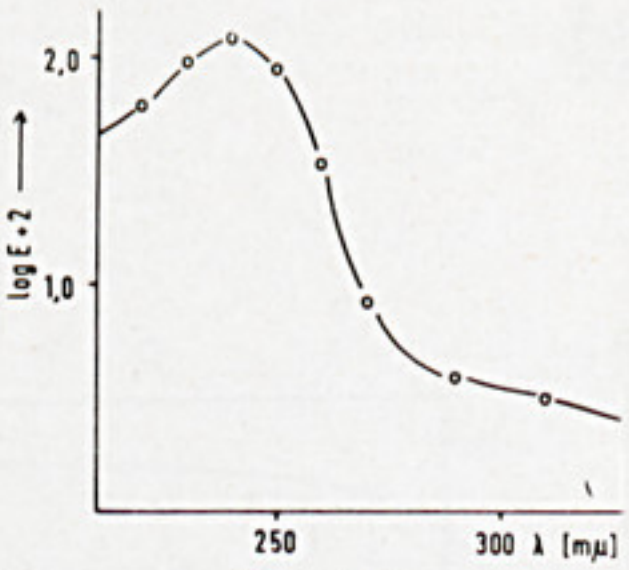


Fig. 13

Fig. 11 — IR-Spectrum of cortexone from Fluka Ltd., in KBr.

Fig. 12 — IR-Spectrum of the compound obtained by thin-layer chromatography using chloroform also taken in KBr.

Fig. 13 — UV-Spectrum of cortexone taken in ethanol λ_{max} 240 mμ, log ε = 4.24.

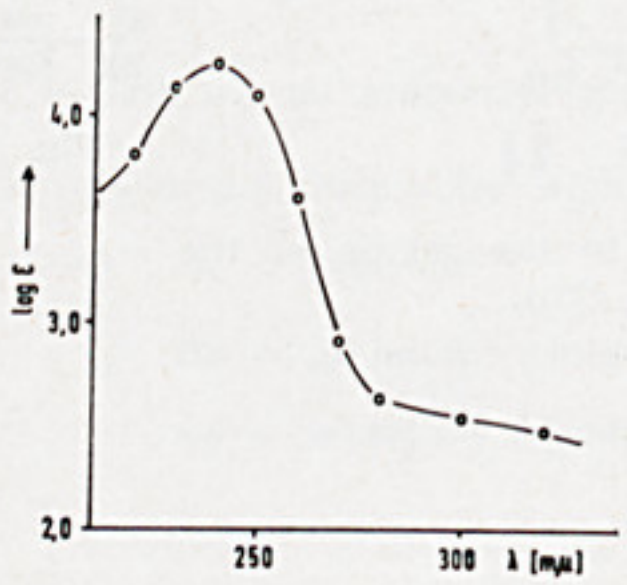


Fig. 14

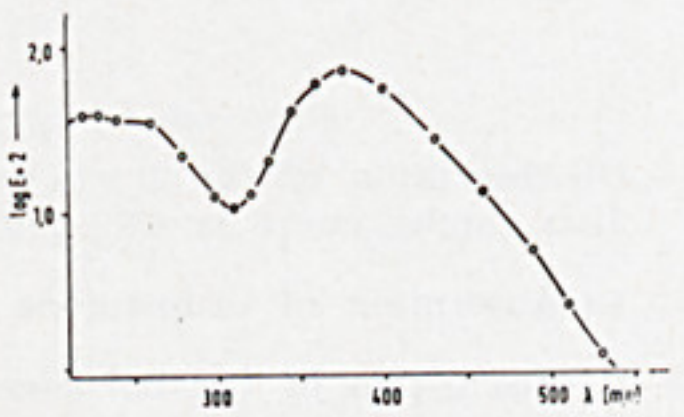


Fig. 15

Fig. 14 — UV-Spectrum of the compound obtained by thin-layer chromatography taken in ethanol λ_{max} 240-241 mμ.

Fig. 15 — UV-Spectrum of the 2.4-DNP of cortexone in ethanol λ_{max} 227, 256 and 376 mμ.

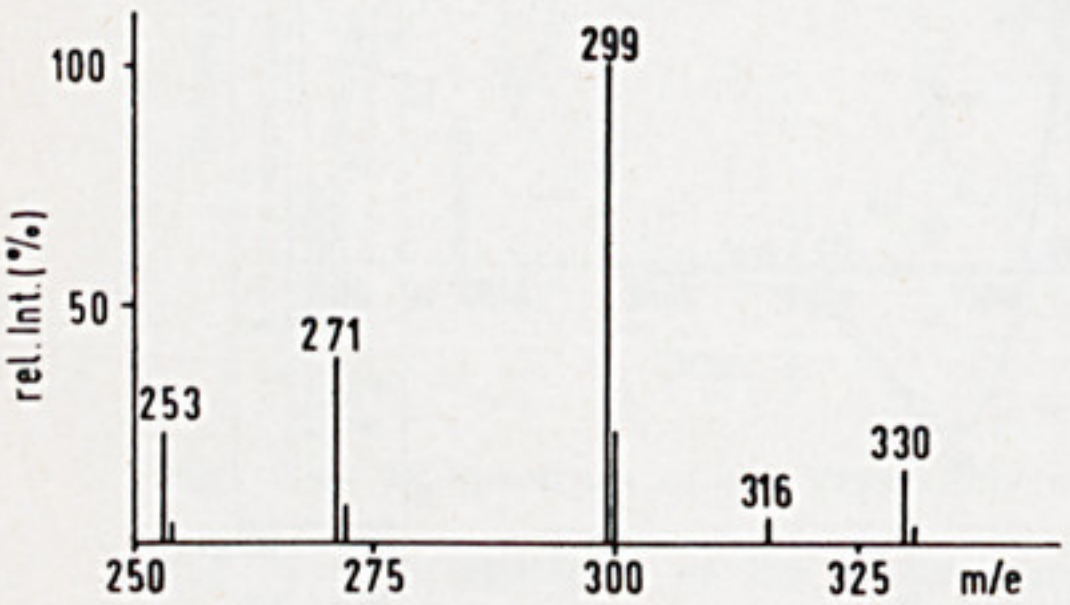


Fig. 16

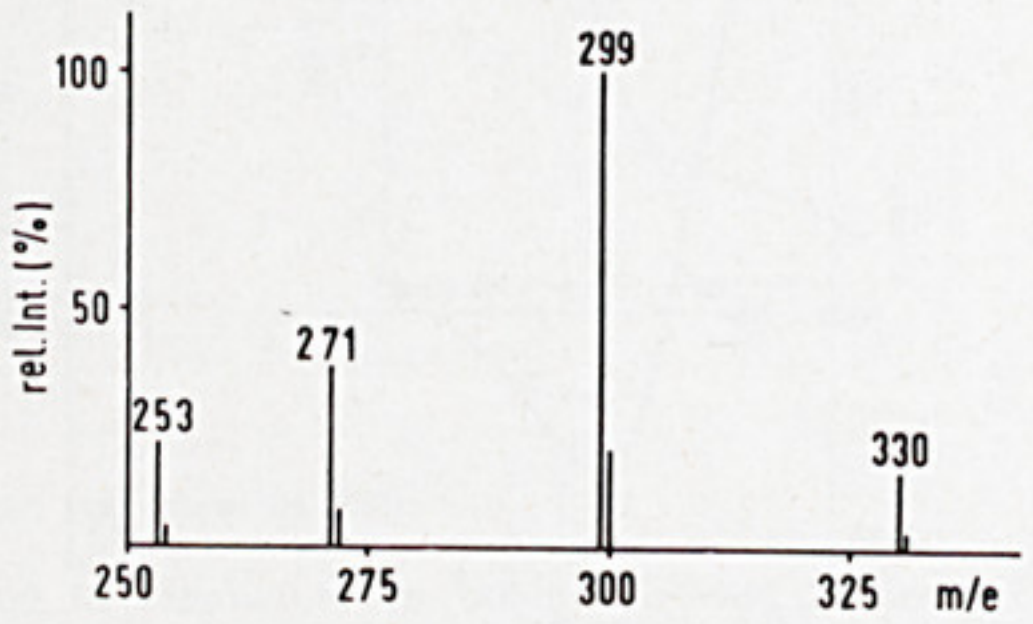


Fig. 17

Fig. 16 — Mass spectrum of the compound obtained by thin-layer chromatography.

Fig. 17 — Mass spectrum of cortexone.

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17. PESQUISAS DE CITOLOGIA QUANTITATIVA. XIX — DNA E VOLUME NUCLEAR NOS TECIDOS SOMÁTICOS DOS VERTEBRADOS

GIORGIO SCHREIBER *et al.*

Instituto de Biologia Geral, Faculdade de Filosofia da U.F.M.G., Belo Horizonte, Brasil

Introdução

O presente trabalho visa o estudo das relações quantitativas entre o conteúdo em DNA e o volume nuclear. O trabalho pioneiro de Walter Jacobj veio introduzir na citologia o conceito de "quantum". As velhas pesquisas sôbre a assim chamada "relação núcleo plasmática", tateavam sem uma diretriz teórica esclarecedora de um mecanismo determinante desta relação. A cariometria de Jacobj teve o valor de esclarecer que o crescimento da matéria viva se dá por valores descontínuos e com a regra de duplicação. As pesquisas de Jacobj, retomadas à luz da citogenética, que vinha se desenvolvendo então, levaram imediatamente a esclarecer que o "quantum" de reduplicação da matéria viva é o genoma. As variações do volume nuclear deveriam, portanto, ser paralelas às do DNA. Sucessivamente, porém, apareceram vários casos nos quais a relação entre genoma e volume nuclear não se mantém e núcleos com o mesmo DNA podem ter volumes diferentes. O primeiro caso de discrepância entre o teor em DNA e o volume nuclear foi descoberto por Schrader e Leuchtenberger (1956) na série espermatogenética de um hemíptero (*Arvelius*), onde os vários túbulos testiculares apresentam todos o mesmo valor de DNA nas fases correspondentes da meiose, porém, o volume nuclear é, em alguns túbulos, respectivamente, 2 ou 4 vêzes maior que os volumes dos correspondentes estádios dos túbulos normais. O teor de proteínas segue de perto as variações do volume nuclear independente do teor em DNA.

Variações da relação entre DNA e o volume nuclear, em condições fisiológicas normais ou experimentais, foram observadas por Bern, Alfert e colaboradores no estudo da ativação de determinados órgãos por hormônios específicos. Alfert e Bern (1951) verificaram que uma variação do volume nuclear (com módulo 2 ou às vêzes 1,5) se dá na tireóide ativada pelo hormônio hipofisário e nas células da parede uterina ativada pelos hormônios estrogênicos, sem que haja variação do conteúdo de DNA. Foram, prevalentemente, êstes fatos que levaram Bloch a considerar a existência dos dois tipos de interfase dos quais fala-

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Êste trabalho é apresentado em colaboração com Norma M. B. Melucci, Silvia E. Gerken, Yeda X. Sant'Ana, Luís Alexandre Fallieri e Flávia de O. Amorim. O trabalho é dedicado à memória da co-autora Norma M. B. Melucci, falecida em 1963. As pesquisas foram realizadas com o auxílio do Conselho Nacional de Pesquisas, CAPES e Fundação Rockefeller.

remos adiante. Outro caso de desvio das relações entre DNA e volume nuclear é aquêle descrito por Fautrez (1957-8) e chamado de "décalage", no qual o núcleo da classe diplóide do pâncreas nos roedores tem um volume igual à metade do correspondente volume do núcleo diplóide do fígado.

Na Escola de Lison (Valeri — 1962), foram evidenciados outros casos de independência do volume nuclear em relação ao conteúdo em DNA, nos tecidos patológicos. No estudo dos tumores do colo do útero, Valeri verificou que os núcleos podem ter volume maior ou menor em relação ao teor em DNA, e, pela primeira vez, foi empregado o têrmo "megetismo" para indicar o excesso (hipermegetismo) ou deficiência (hipomegetismo) do volume nuclear em relação ao tecido normal.

No estudo da ploidia somática das glândulas dos Moluscos, Schreiber e colaboradores (1964-1965) verificaram mais um caso de exceção à constância das relações entre DNA e volume nuclear. Os tecidos glandulares, especialmente o hepatopâncreas, e a glândula salivar, nos Gasterópodos, apresentam intensos fenômenos de endopoliploidismo (ou politenia), verificáveis por classes superiores (múltiplas de 2) no conteúdo em DNA (Schreiber e colaboradores — 1964-1965). Estas glândulas, portanto, representam um mosaico de células de valores múltiplos de ploidia. Schreiber e colaboradores demonstraram um fato nôvo neste tipo de fenômenos: a relação entre DNA e volume, na série dos meiocitos, é, respectivamente, 1:1, 2:2 e 4:4, ao passo que, nas células glandulares poliplóides dêstes tecidos, a relação DNA/volume é respectivamente 1/2, 2/4, 4/8, 8/16, etc. Isto quer dizer que as células dos tecidos especializados têm um conteúdo em proteínas não histônicas duas ou quatro vêzes maior do que as células germinais.

O estudo de todos êstes casos, nos quais a relação entre conteúdo em DNA e volume nuclear não fica constante, foi considerado por nós (Schreiber e colaboradores — 1965) de forma sintética, introduzindo o conceito de "relação plóidomegética". Estas considerações nos permitiram reconsiderar, sob nôvo aspecto, o trabalho dos antigos autores de cariometria.

A base bioquímica destas variações foi elaborada por Bloch (1958), que chegou à conclusão de que o núcleo pode seguir duas formas de crescimento interfásico. A primeira é a "autossintética", na qual duplicam todos os componentes bioquímicos e morfológicos do núcleo. Assim, duplicam o DNA, as proteínas histônicas que o acompanham em quantidade sempre constante, e as proteínas não histônicas, que constituem parte dos cromosomas e o suco nuclear. Êste tipo de interfase se verifica antes da divisão mitótica ou da endomitose. O segundo tipo de interfase, "heterossintética", verifica-se quando o DNA não duplica, mas trabalha na função de produzir o RNA e sucessivamente as proteínas citoplasmáticas específicas da função celular. Neste tipo de interfase, porém, duplicam as proteínas não histônicas e o volume nuclear segue êste ritmo de aumento. As proteínas não histônicas, que constituem o suco nuclear, determinam pela sua constituição físico-química o volume nuclear, que adquire geralmente valores múltiplos de duplicação (Schreiber — 1964). O volume do núcleo representa, portanto, uma "constante" celular que é característica de cada tipo de células. As variações do volume nuclear refletem fundamentalmente a função do núcleo, e, no presente trabalho, em falta de uso de métodos específicos para determinar as proteínas não histônicas, consideramos o volume nuclear como expressão morfológica embora grosseira do conteúdo destas proteínas. Deixamos aqui de considerar o problema das variações do conteúdo em RNA, que é um componente mais "móvel" e provàvelmente em estado de equilíbrio durante o

ciclo fisiológico ("steady state") e ligado às variações quantitativas do nucléolo. Do ponto de vista morfoquantitativo, a participação do DNA, histona e RNA, para a constituição do volume do núcleo, deve ser relativamente menos considerável do que a das proteínas não histônicas. Talvez os fenômenos de estado físico-químico (embebição, etc.) destas proteínas sejam os responsáveis pela determinação de um volume específico e constante nas diferentes fases da vida celular. O esquema apresentado por Alfert (1958, Fig. 3) esclarece a participação dos vários componentes na determinação do volume nuclear e as suas variações nas duas interfases, respectivamente, reprodutiva e funcional da célula. Pouco ou nada se conhece acêrca da origem destas proteínas, se endonuclear ou citoplasmática; o fato é que elas se encontram em quantidade e estado físico-químico constante após uma divisão e duplicam antes da divisão sucessiva (interfase autossintética), ou durante o ciclo funcional da interfase heterossintética. A quantidade destas proteínas não histônicas está de certa forma ligada ao genoma, mas elas podem variar independentemente dêle e a sua variação se dá, conforme a feliz expressão de Pollister, sob forma de "multiple sets of proteins". Neste fato está a base de todo o sistema de pesquisa cariométrica e do fenômeno fundamental que foi verificado por Jacobj sob a forma de "Verdoppelungsgesetz") do núcleo.

A função das proteínas não histônicas (proteínas acídicas, fosfoproteínas, etc.) parece ter uma importância fundamental, pois a estas proteínas parece estar conferida a função de desligar a união do DNA com as histonas. Esta ligação que parece ser a base de repressão da atividade do gen seria rompida pela nova ligação com as proteínas acídicas que agiriam como "depressors" do locus gênico, permitindo a sua atividade específica. Por esta razão nos parece interessante estudar quantitativamente a relação entre estas proteínas (provisòriamente expressas pelo volume nuclear) e o conteúdo em DNA nos diferentes tecidos especializados na série dos Vertebrados.

Uma comparação das variações de DNA e do volume nuclear entre vários grupos de tecidos e em vários grupos de animais nos levou a fazer uma classificação destas variações conforme a concepção de Bloch. O esquema aqui apresentado compara a relação entre DNA e volume nos tecidos de alguns Artrópodos, nos quais as variações entre tecidos se dão por politenia ou poliploidia e, portanto, as variações do DNA são paralelas às do volume nuclear (autossíntese), com a dos Vertebrados e Moluscos, nos quais o DNA é sempre diplóide na classe básica dos núcleos, mas o volume pode ter valores múltiplos nos diferentes tecidos (heterossíntese). A êste fenômeno se sobrepõe, em certos tecidos, como por exemplo o fígado, o poliploidismo somático que forma classes múltiplas nucleares do valor de DNA e do volume. O que varia entre os tecidos é, portanto, o valor da relação plóido-megética que será constante nas differentes classes de endopoliploidismo de cada tecido. Pode-se dizer que nos Artrópodos se dá um mosaico de ploidias entre os tecidos com relação plóido-megética 1:1, ao passo que nos Vertebrados se dá um mosaico de núcleos com relação plóido-megética diferente, em cada qual pode-se dar também endopoliploidismo (Fig. 1).

Podemos concluir, portanto, que do ponto de vista das relações entre DNA e volume nuclear (megetismo), os tecidos podem apresentar séries de volumes nucleares que classificamos como se segue:

I. *A série "eumegética"*, na qual o DNA e o volume variam paralelamente, devido ao mecanismo de interfase "autossintética". Esta série se verifica nos meiócitos (ide = 1, gônios e citos segundos = 2 e citos primeiros = 4), nos te-

cidos que apresentam poliploidia somática (polissomatismo), como em algumas glândulas nos Vertebrados e nos Moluscos, ou entre tecidos com ploidia característica como nos Artrópodos.

II. *A série "hipermegética" somática*, como se verifica na comparação de núcleos com igual grau de ploidia de órgãos diversos nos Vertebrados e Moluscos. Nestes, cada tecido tem um volume característico da classe diplóide e os volumes básicos dos vários tecidos formam uma série de duplicação (série "polimegética", "Verdoppellungs-gesetz" de Jacobj — 1925). Às vêzes, porém, podem ter valores múltiplos de 1,5 ("sesquifase" de Schreiber — 1960).

III. *A série "hipomegética"*, representada pelos linfócitos e núcleos de eritrócitos (Jacobj — 1925) que, embora tenham teor diplóide de DNA, podem apresentar volumes 1/2, 1/4 ou até 1/8 do volume básico do gônio.

Materiais e técnicas

O material foi sempre fixado, parte em Bouin e corado com hematoxilina-eosina para cariometria, e parte em formol neutro a 10% e corado com Feulgen sem contraste para citofotometria. Os cortes foram feitos com espessuras muito variáveis conforme o material (de 2 micra para os peixes até 20 micra para o *Siphonops*). Foram tomados como base os seguintes tecidos: testículo, fígado, pâncreas e baço. Nos Peixes foram feitas medições orientadoras no tecido nervoso e, quando possível, foi medida a série espermatogenética completa (gônios, citos I, citos II e espermátides), mas às vêzes foi difícil encontrar todos os estádios. Contudo foi fácil encontrar citos I e ides, com os quais foi feita a regressão DNA/volume. No baço foram medidos os linfócitos, e em Rato foram medidos os timócitos e linfócitos grandes. Do pâncreas, nesta pesquisa, foi considerado sòmente o tecido exócrino. Nos anamniotas foram medidos os eritrócitos nos vasos capilares, escolhendo os núcleos bem orientados para a medida. As medidas cariométricas foram feitas seja com o micrômetro filar do citofotômetro, seja com desenho à câmara.

As medidas de DNA foram feitas com citofotômetro, montado com Microscópio Ortholux Leitz, com objetiva 124 Nachet, imersão e ocular 10, montado num suporte Aristophot Leitz. Na cabeça binocular, uma das oculares é usada para a escolha do campo e a outra, substituída por um micrômetro filar de Zeiss com ocular K16, para a medição dos dois diâmetros, máximo e mínimo, do núcleo, e a centração do núcleo a ser fotometrado. No Aristophot foi adaptada a caixa do fototubo (RCA 931 A) com um disco de 4 diafragmas de superfície variável. No foco do visor lateral foi introduzida uma chapa diapositiva com as imagens concêntricas dos diafragmas para o "plug". O fole de Aristophot foi ajustado para obter uma imagem do núcleo a ser medido ampliado a 1.500 diâmetros. Utiliza-se uma área do "plug" com cêrca de 20% da área do núcleo. Foi feita uma medida de absorção de cada núcleo; para cada medição foi determinada a luz padrão em zona fora do núcleo e ajustado o valor do galvanômetro a 100 com pequenos movimentos do diafragma do condensador do microscópio. Com êste método cada leitura de absorção é dada em percentagem da intensidade da luz padrão usada. A iluminação foi dada por um monocromador Bausch & Lomb, a retículo, com lente frontal supletiva usando uma luz monocromática de 540 angstrons ou com filtro Wratten nº 58. Foi usado um micro-amperímetro "Norma", modêlo 251, de sensibilidade $2{,}10^{-7}$. Com as medidas de absorção de cada núcleo, e o diâmetro médio dois dois diâmetros cruzados, foi calculada a quantidade de DNA para cada núcleo, de acôrdo com a fórmula $DNA = D^2 (2 - \log In)$, sendo D^2 a superfície óptica do núcleo, In a medida de absorção e 2 o log 100. Dos dados de DNA assim obtidos foram calculados a média, desvio-padrão e êrro-padrão da média. Na elaboração estatística dos dados foi indicado sempre o valor da média e de $\pm S$ para o DNA. Nas medidas cariométricas geralmente foi indicada a moda, pois é a medida mais adequada nos casos de histogramas bimodais ou com modas encobertas.

Quando as medidas eram excessivamente dispersas, como em certos casos de volumes medidos ao micrômetro durante a citofotometria, foi indicado o valor mediano como o mais apto a dar uma indicação utiliâzável.

Nos diagramas de cascavel e parelheira aqui publicados utilizamos um nôvo tipo de gráfico, ou seja, as "áreas plóido-megéticas". Estas áreas (Figs. 4 e 8) resultam de quadriláteros, cujos lados correspondem ao valor total de dispersão dos dados respectivamente de DNA e volume. Embora se esteja ainda iniciando o estudo dêste tipo de representação, os diagramas exprimem com grande clareza a situação dos núcleos dos diferentes tecidos em relação ao DNA e ao volume, e a sua comparação entre os diferentes tecidos nos parece de grande utilidade.

Descrição dos resultados

Nas pesquisas precedentes desta série foram estudados vários tecidos de Vertebrados, determinando o volume nuclear e o DNA. Êstes trabalhos foram executados no decorrer de vários anos, cada um com banhos de Feulgen diferentes. Os resultados não podem ser confrontados quantitativamente entre as espécies, mas podem-se verificar as relações quantitativas entre estas duas variantes celulares sòmente entre os tecidos de cada espécie.

Os materiais estudados nas pesquisas anteriores foram os seguintes: *Peixes* (*Tilapia melanopleura*), por Yeda X. Sant'Ana (1965). Foram estudados o fígado, pâncreas, rim, eritrócitos e neurônios. O estudo foi feito não sòmente em adultos, mas também em fases de larvas recém-eclodidas e alevinos. Os dados desta pesquisa estão ainda em elaboração. Sòmente foi publicado o estudo da variação do tamanho do núcleo e seu conteúdo em DNA durante o desenvolvimento pós-embrionário. *Anfíbios*: (*Siphonops annulatus*), por N. Melucci (1962), no qual foram estudados o fígado, pâncreas, rim e espermatogônios. *Répteis*: (*Tupinambas sp.*), Melucci (não publicado), no qual foi estudado o fígado, rim e pâncreas. *Mamíferos*: (*Cricetus auratus* e *Mus rattus*). Em *Cricetus*, Gerken (1962) estudou o fígado, pâncreas, rim e glândulas salivares. Em *Rattus*, Yeda X. Sant'Ana estudou o fígado, pâncreas e os elementos do timus, relacionando estas medidas aos gônios (1964).

Ofídios: 1. *Crotalus durissus terrificus* (Laurenti) — Apresentamos na Fig. 2 os diagramas de regressão DNA/volume nuclear da série espermatogenética e na Fig. 3 os histogramas, respectivamente, do DNA e do volume nuclear com a tabela dos valores numéricos relativos. A Fig. 4 representa a regressão entre DNA e volume da linha germinal (linha contínua) e dos tecidos somáticos. Representamos de uma forma sintética os vários tecidos por meio da área ocupada pelos dados de cada tecido. Os retângulos abrangem a totalidade dos pontos do diagrama de regressão de cada tecido indicando assim a área de variabilidade. Chamamos êste diagrama de "áreas plóido-megéticas" por dar uma expressão geométrica da dispersão dos dados nos tecidos e indicar com grande evidência a situação de hiper e hipo megetismo em cada tecido. Dêstes diagramas aparece bem claro que os tecidos somáticos têm um teor em DNA geralmente na faixa diplóide. As variações dentro desta faixa provàvelmente são determinadas seja pela variação interfásica de uma certa percentagem de células em movimento mitótico ou endomitótico, seja por variações devidas ao estado fisiológico, como foi amplamente indicado na série de trabalhos da Escola de Fautrez (Roels-De Schrijver — 1961, Roels — 1954, Anteunis & Liu — 1960, Verwoerd-Verhoef & Verwoerd — 1962).

Se observarmos, agora, as variações do volume nuclear, resulta com grande evidência que os vários tecidos têm uma variação específica, dentro de determinados limites, e diferente em cada tecido. Êste é o fenômeno chamado de "décalage" por Fautrez na comparação dos volumes nucleares das classes diplóides do fígado e do pâncreas.

O pâncreas tem um volume mais ou menos duplo do espermatogônio e o fígado quatro vêzes maior. Às vêzes êstes volumes encontram-se na relação de 1:1,5. Mandamos os trabalhos anteriores desta série para o estudo dêste problema, que chamamos de "sesquifase" (Schreiber — 1960).

Nos Répteis, em geral, não se dá, como nos Mamíferos, a existência de classes múltiplas de endopoliploidia no fígado e às vêzes no pâncreas; por isto, a variabilidade dos núcleos é estritamente limitada à faixa diplóide.

2. *Philodryas schottii* (Schlegel) — A Fig. 5 representa a regressão DNA/volume na série meiocítica e as Figs. 6 e 7 representam os histogramas de DNA e do volume nos seguintes tecidos: fígado, pâncreas, supra-renal, tireóide, linfócitos e monócitos. A Fig. 8 representa a regressão entre DNA e volume nos tecidos somáticos com a representação da "área plóido-megética", como no caso precedente do *Crotalus*. A regressão entre DNA e volume dos valores nos meiócitos é perfeitamente regular. O DNA, como na cascavel, está na faixa diplóide para todos os tecidos, com exceção dos monócitos, nos quais tanto o DNA como o volume estão distribuídos na faixa 4c-6c, indicando um valor tetraplóide dos seus núcleos. Os volumes dos tecidos, como na cascavel, se encontram decididamente deslocados para uma situação de hipermegetismo, com exceção dos linfócitos, que se encontram com um valor 2c e 2, portanto exatamente como os espermatogônios. Os demais tecidos têm volumes nucleares altamente superiores ao volume da célula diplóide típica. Os dados numéricos dêstes volumes estão indicados na tabela II.

Muitas vêzes, devido à grande dispersão dos dados, os valores do volume não refletem o valor teórico da série de duplicação. As exceções a esta série, como já dissemos, se devem fundamentalmente a estados de "movimento" da população de núcleos, seja em atividade mitótica, seja em atividade fisiológica.

Na *Philodryas*, os valores da tireóide são um pouco discordantes do valor teórico, provàvelmente por serem os núcleos dos folículos tireoidianos deformados, dando um êrro na valutação do volume.

Análise geral dos resultados

Pelos dados das pesquisas precedentes e da nova série sôbre Ofídios, podemos verificar alguns fatos gerais que resumimos como segue:

a) o estudo comparativo do teor em DNA e do volume nuclear nos tecidos somáticos e na espermatogênese dos Vertebrados revelou o fato já constatado em trabalhos precedentes nos Moluscos que a relação plóido-megética é diferente na linha germinal masculina e nos tecidos adultos somáticos. A regressão entre DNA e volume na série dos meiócitos espermatogenéticos é uma regressão muito regular e quando as coordenadas são escolhidas com as mesmas unidades de medida, "b" = 1, isto é, as variações de DNA são iguais às do volume. Chamamos anteriormente a êste tipo de regressão de "série eumegética", conforme a nomenclatura de Valeri e Lison adotada com ligeiras modificações;

b) os vários tecidos somáticos aqui examinados apresentam todos a mesma forma de variação da relação plóido-megética, embora em grau quantitativo diferente. Pràticamente todos estão com um teor em DNA dentro do que chamamos de "faixa diplóide", isto é, com uma variabilidade de 2c-4c devida, em parte, aos fenômenos multiplicativos de uma certa percentagem de células e, em parte, aos desvios do teor em DNA que se verificam nos diferentes estados funcionais. Os volumes dos núcleos de cada tecido, pelo contrário, têm um campo de variação muito mais amplo e característico para cada um. Nas pesquisas precedentes, nos Mamíferos em geral, os valores modais dos histogramas de volume têm uma relação de duplicação entre si. Nos Ofídios esta regra, às vêzes, é mascarada por fatôres ainda não esclarecidos, mas que, por exemplo, para o fígado poderiam ser relacionados a condições fisiológicas excepcionais (jejum prolongado). Não podemos aqui examinar êstes desvios mais detalhadamente; limitamo-nos a constatar que os diferentes tecidos somáticos têm volumes que desviam, às vêzes, fortemente do valor que deveriam ter em base à ploidia. Isto significa que os núcleos se encontram naquela situação que Bloch define de interfase heterossintética.

O volume nuclear do pâncreas é geralmente o dôbro do volume da série eumegética (espermatogônio); o do rim, nos Répteis, maior e não excluímos que possa ser afetado por medidas feitas no assim chamado segmento sexual característico do rim dos Répteis.

O fígado se encontra mais ou menos com um volume quatro vêzes maior que o do correspondente estádio eumegético.

Os linfócitos e os eritrócitos, embora com ligeiros desvios no conteúdo em DNA, talvez devido a dificuldades técnicas da medição, têm nos Ofídios volume correspondente aos dos gônios e, portanto, têm uma relação eumegética. Nos trabalhos precedentes de Schreiber e Sant'Ana, nos timócitos do rato encontramos um valor do volume dos timócitos de 1/2 o valor da célula diplóide típica, confirmando quanto tinha encontrado Jacobj nas suas pesquisas cariométricas ("Unterklassen"). Nos monócitos de Ofídios, pelo contrário, se verifica um caso de endopoliploidismo, estando o DNA na faixa de 4c e os volumes correspondentes exatamente na linha de regressão eumegética.

c) O estudo da relação "plóido-megética" do tecido hepático durante o desenvolvimento embrionário e pós-embrionário nos Peixes e Anfíbios revelou um fenômeno nôvo. Pesquisas cariométricas precedentes, de Bruno Schreiber e Angeletti, nos Peixes, e de Giorgio Schreiber e Maria Romano Schreiber, nos Anfíbios, mostraram uma redução progressiva do volume nuclear por etapas de dimidiamento (chamado por G. Schreiber de "elaxis"). Nos Peixes (Sant'Ana) foi estudado, também, o teor em DNA e verificou-se estar a diminuição sòmente a cargo do volume, ficando constante o valor diplóide do DNA. Os núcleos embrionários e larvários, neste caso em situação polimegética, alcançam, por etapas de dimidiamento, o valor básico do núcleo no adulto. Êste fenômeno foi por nós comparado com o progressivo dimidiamento do volume nuclear dos blastômeros durante a segmentação, sendo, provàvelmente, a diminuição progressiva em certos tecidos pós-embrionários, um prolongamento do tipo de divisão característico dos blastômeros.

O megetismo dos blastômeros, porém, poderia ser devido a um tipo de proteínas diferente daquele que aparece na diferenciação histógena pós-embrionária. Devemos lembrar que o núcleo dos blastômeros em segmentação não possui ainda

genes ativados, salvo casos especiais, e, como demonstraram Bloch e Hew (1960), as histonas dêstes núcleos até à gastrulação pertencem a uma categoria diferente ("cleavage histones").

O caso do pâncreas, órgão altamente produtor de proteínas citoplasmáticas, e que geralmente apresenta hipermegetismo menor do que o fígado, deve-se talvez relacionar com um valor quantitativo do nucléolo, excepcionalmente superior aos demais tecidos (Schreiber e colaboradores). O caso do hipomegetismo dos linfócitos e eritrócitos deve ser ulteriormente estudado, estando a origem destas células ligada a processos característicos de maturação histógena permanente e não sòmente embrionária ou pós-embrionária.

O estudo de todos êstes fenômenos deve ser estendido aos estádios histogenéticos embrionários de cada órgão para se determinar o momento em que, para cada tecido, se estabelece uma dose múltipla de proteínas acídicas do suco nuclear ("multiple sets of proteins" de Pollister) e subseqüentemente um volume nuclear específico.

Conforme foi dito antes, a base bioquímica de todos os fenômenos aqui relatados (hiper e hipomegetismo) deve-se encontrar no conceito de interfase heterossintética de Bloch (1958), durante a qual, a célula está empenhada em trabalho de síntese de materiais não genômicos que constituem fundamentalmente o suco nuclear (RNA e proteínas não histônicas ou acídicas), cuja variação é geralmente proporcional à variação do volume nuclear. Êste tipo de proteínas acídicas são recentemente consideradas como os fatôres que deslocam a histona do DNA (depressores), permitindo a função heterossintética dos genes e, portanto, a produção de proteínas específicas da função de cada tecido. Markert recentemente (XVI Int. Cong. of Zoology, Washington) escreve textualmente: "If histones are indeed inhibitors of gene function, then the removal of this inhibitors through complexing with non histonic proteins (or RNA) may be the basic event in gene activation".

Opinião análoga é expressa por Moore (1966), em base às pesquisas de Langan, que mostram ter a cromatina ativa o dôbro de fosfoproteínas por unidade de DNA do que a cromatina repressa. Frenster (1966) também acha que as fosfoproteínas (não histônicas), que se encontram na cromatina expandida ativa em quantidade maior do que na cromatina condensada, têm a função de deslocar as ligações do DNA com as histonas que o reprimem.

Parece-nos, portanto, decididamente concordante o fato de que alguns tecidos somáticos (pelo menos nos Vertebrados e Moluscos) têm volume nuclear maior e, portanto, mais proteínas não histônicas do que a linha germinal. O fígado, de modo especial, se distingue nesse sentido; devemos lembrar que êste órgão está empenhado em um metabolismo extremamente mais complexo que as demais glândulas e, portanto, seu genoma deve ter um número maior de genes ativados em interfase heterossintética.

A êste propósito, uma observação na literatura sôbre a distribuição das enzimas nos diferentes órgãos (tabela à página 452 e seguintes de West e Todd) nos indica grosseiramente a quantidade de enzimas diferentes nos vários tecidos dos Vertebrados. Resumimos aqui muito superficialmente esta situação, indicando que existem 13 enzimas específicas do fígado, 8 do pâncreas e 3 do rim. Êstes valores estão na relação em que se encontram os volumes nucleares dêstes órgãos.

Tudo isto parece se dar nos Vertebrados. Nos Artrópodos, pelo contrário, como já mencionamos antes, o diferenciamento histógeno é acompanhado por um grau específico de poliploidia ou politenia, e as variações de volume dos núcleos entre os diferentes tecidos é paralela à variação do teor em DNA. Não queremos, portanto, generalizar demais o critério acima exposto da significação bioquímica do hipermegetismo no diferenciamento histógeno. Devemos, talvez, lembrar que os núcleos poliplóides dos Artrópodos têm uma grande parte de DNA sob forma de heterocromatina; é possível pensar que os genomas múltiplos não estejam todos funcionantes contemporâneamente, permitindo à totalidade das proteínas não histônicas correspondentes manifestar a sua ação ativadora sòmente sôbre os genomas funcionantes.

Podíamos traduzir a descrição dêstes fenômenos em têrmos da genética molecular, considerando a "relação plóido-megética" como a expressão morfoquantitativa dos fatôres de repressão e ativação dos genes durante a diferenciação dos tecidos somáticos.

Summary

In the precedent papers of this series, we have studied the DNA content per per nucleus in the nuclear volumes in different tissues of Vertebrates and Molluscs. The present paper deals with the same problems in Ophidia.

The theoretical basis of these researches consists in facts studied by Bloch in which appears the existence of two types of interphasic growth of the nucleus (autosynthetic and heterosynthetic). In the first one, the three fundamental constitutions of the nucleus, i.e., DNA, histonic and non histonic proteins, are reduplicated. The nuclear volume follows, always, the quantitative variations of the non histonic proteins. In the second type of interphase, there is a reduplication of the non histonic proteins, only, and not of the DNA and histones. In this case, the volume of the nucleus increases without the increase of the DNA content. The comparative study of the DNA and of the nuclear volume reveals, thus, the variations of the ratio "genome non histonic proteins". These proteins have been recently considered as the factors that free the genic DNA from its bound with the histone and, thus, acting as "de-repressors", allow the DNA to synthetize the RNA, specific for the genic action.

The present research considers the ratio DNA/Volume in comparison with the spermatogenetic stages (in which the DNA and nuclear volume are always in fixed ratio), and with the somatic tissues, in which the nuclear volume can vary independetly from the DNA content (heterosynthesis).

Thus, in *Crotalus* and *Philodryas*, we present here the regression line between the DNA and the nuclear volume in the spermatogenetic stages, and in some tissues (liver, pancreas, kidney, etc.). It is well evident, by these diagrams, that the somatic tissues have, all of them, characteristic nuclear volume greater than that of the spermatogonia (diploid typical cell). We call this DNA/Volume ratio, as the "ploido-megetic ratio".

We can correlate this excess of volume of the nucleus with the content in non histonic proteins, in each somatic tissue, that can explain the "de-repressing" action upon the specific gene loci.

Agradecimentos

Queremos, aqui, agradecer ao Dr. H. E. Belluomini, do Instituto Butantan, pelo fornecimento do material ofiológico.

Agradecemos, também, ao nosso colaborador Prof. Luis Alexandre Fallieri, pela revisão definitiva do texto.

TABELA I — CASCAVEL — *Crotalus durissus terrificus* (Laurenti)

Tecidos	Nº	DNA			VOLUME		
		$\overline{X} \pm S$	Mo	Md	$\overline{X} \pm S$	Mo	Md
Espermátides	20	4,0 ± 0,9	4,0	4,0	3,0 ± 0,8	3,0	3,0
Espermatócitos I	34	16,0 ± 1,6	16,0	16,0	11,0 ± 2,4	12,0	11,0
Fígado	35	8,0 ± 1,2	8,0	8,0	Bimodal (encoberto)	22,0	21,0
Pâncreas	20	8,0 ± 1,0	8,0	8,0	8,0 ± 1,8	8,0	8,0
Rim	20	8,0 ± 1,5	9,0	9,0	Polimodal	11,0 — 17,0 — 23,0	18,0
Hemácias	5	6,0 —	—	—	4,0	— — —	—

TABELA II — PARELHEIRA — *Philodryas schottii* (Schlegel)

Tecidos	Nº	DNA			VOLUME		
		$\overline{X} \pm S$	Mo	Md	$\overline{X} \pm S$	Mo	Md
Espermátides	31	7,0 ± 0,8	6,0	6,0	6,0 ± 0,9	6,0	6,0
Espermatogônios	31	13,0 ± 1,4	14,0	13,0	9,0 ± 1,1	9,0	9,0
Espermatócitos I	31	25,0 ± 1,9	25,0	25,0	21,0 ± 2,7	17,0	16,0
Fígado	30	14,0 ± 1,6	14,0	14,0	31,0 ± 5,0	30,0	31,0
Pâncreas	41	14,0 ± 1,9	13,0	13,0	16,0 ± 3,3	17,0	16,0
Tireóide	31	10,0 ± 1,5	10,0	10,0	—	—	12,0
Supra-renal	31	16,0 ± 2,0	16,0	17,0	26,0 ± 3,8	25,0	26,0
Linfócitos	31	15,0 ± 1,7	14,0	15,0	9,0 ± 1,4	8,0	9,0
Monócitos	31	31,0 ± 3,5	32,0	32,0	23,0 ± 4,4	23,0	22,0

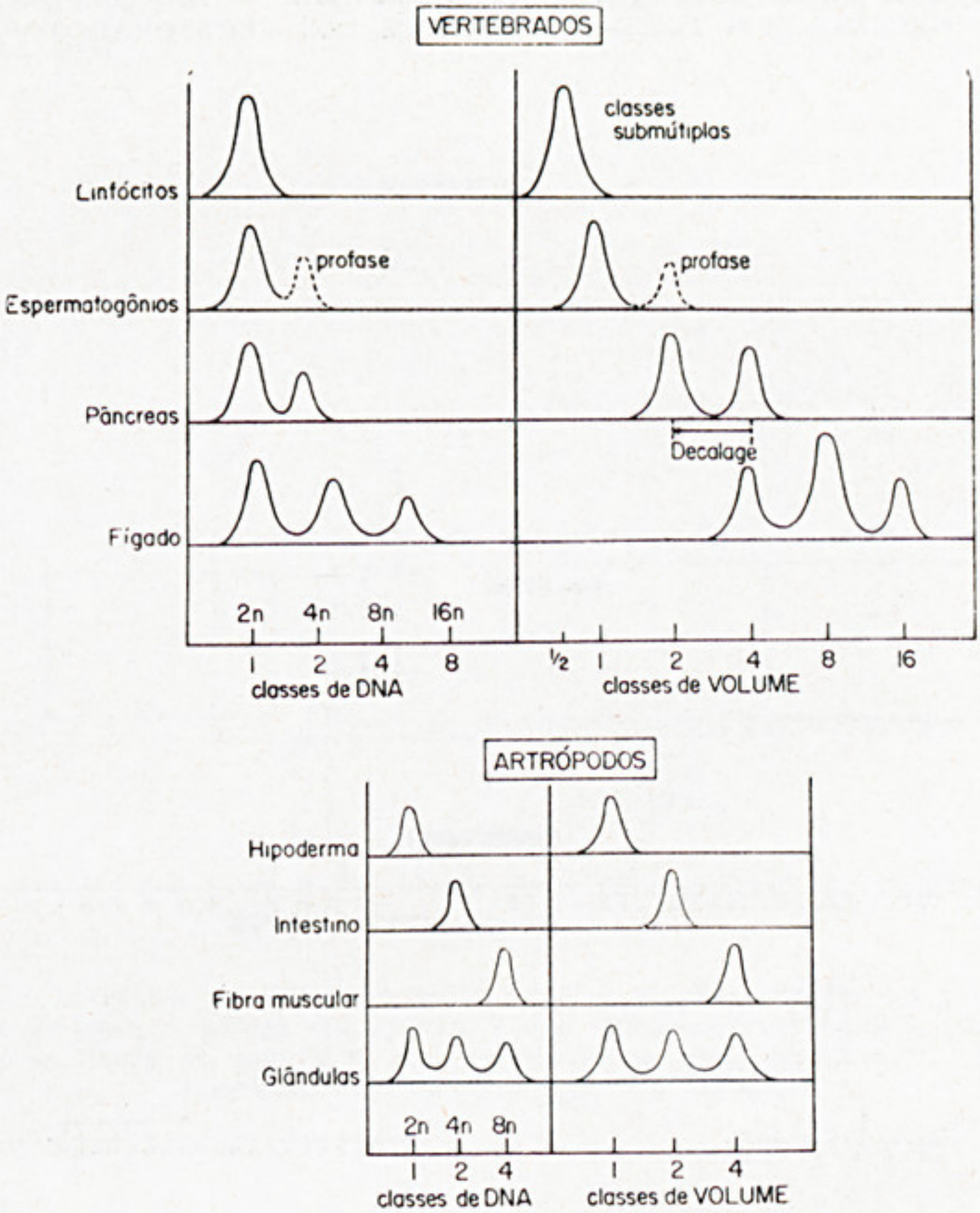


Fig. 1 — Curvas de freqüência do DNA e do volume nuclear em diferentes tecidos de Vertebrados e de Artrópodos.

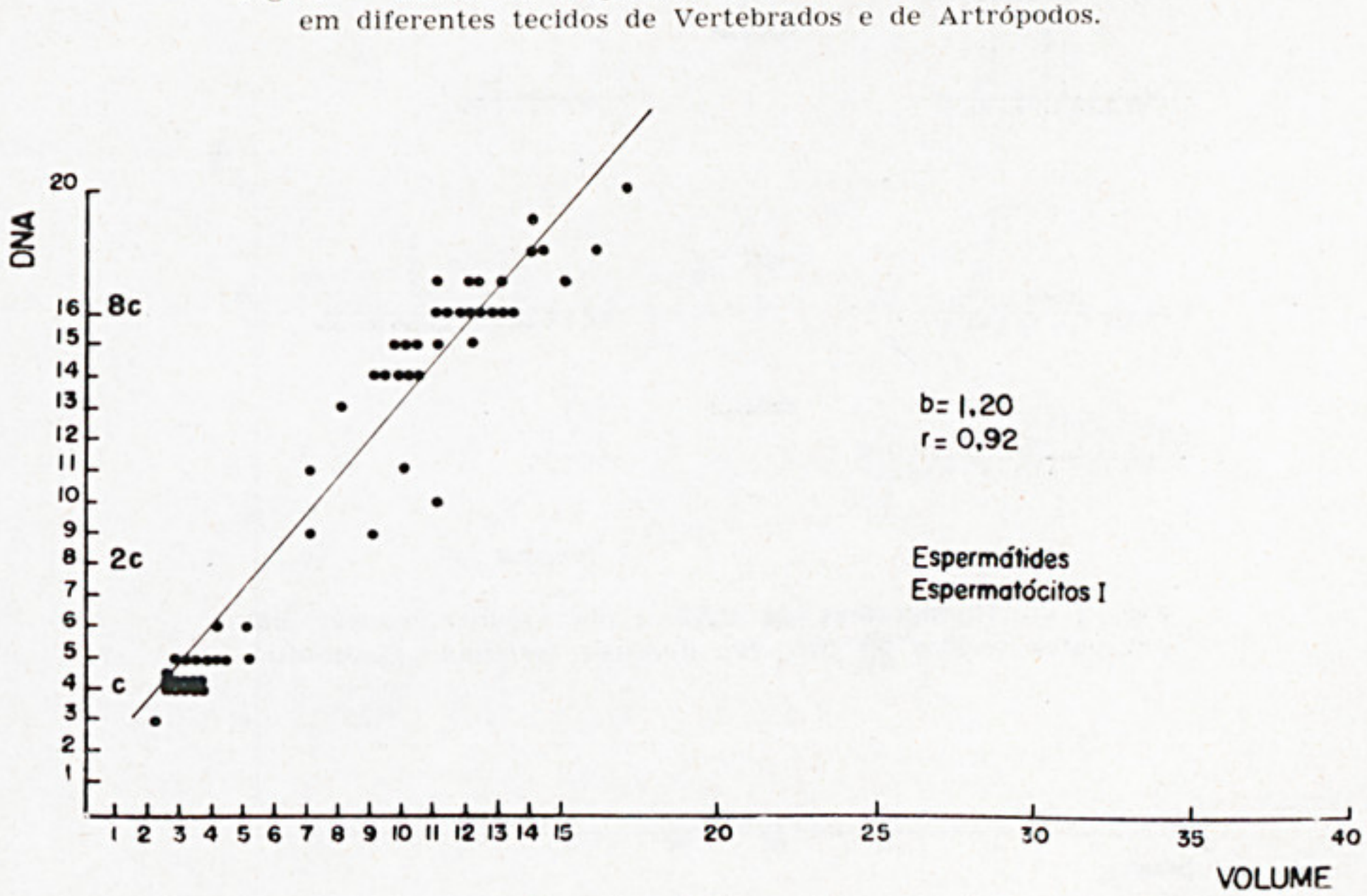


Fig. 2 — Diagrama de regressão entre DNA e volume nuclear na série espermatogenética de *Crotalus durissus terrificus* (Laurenti).

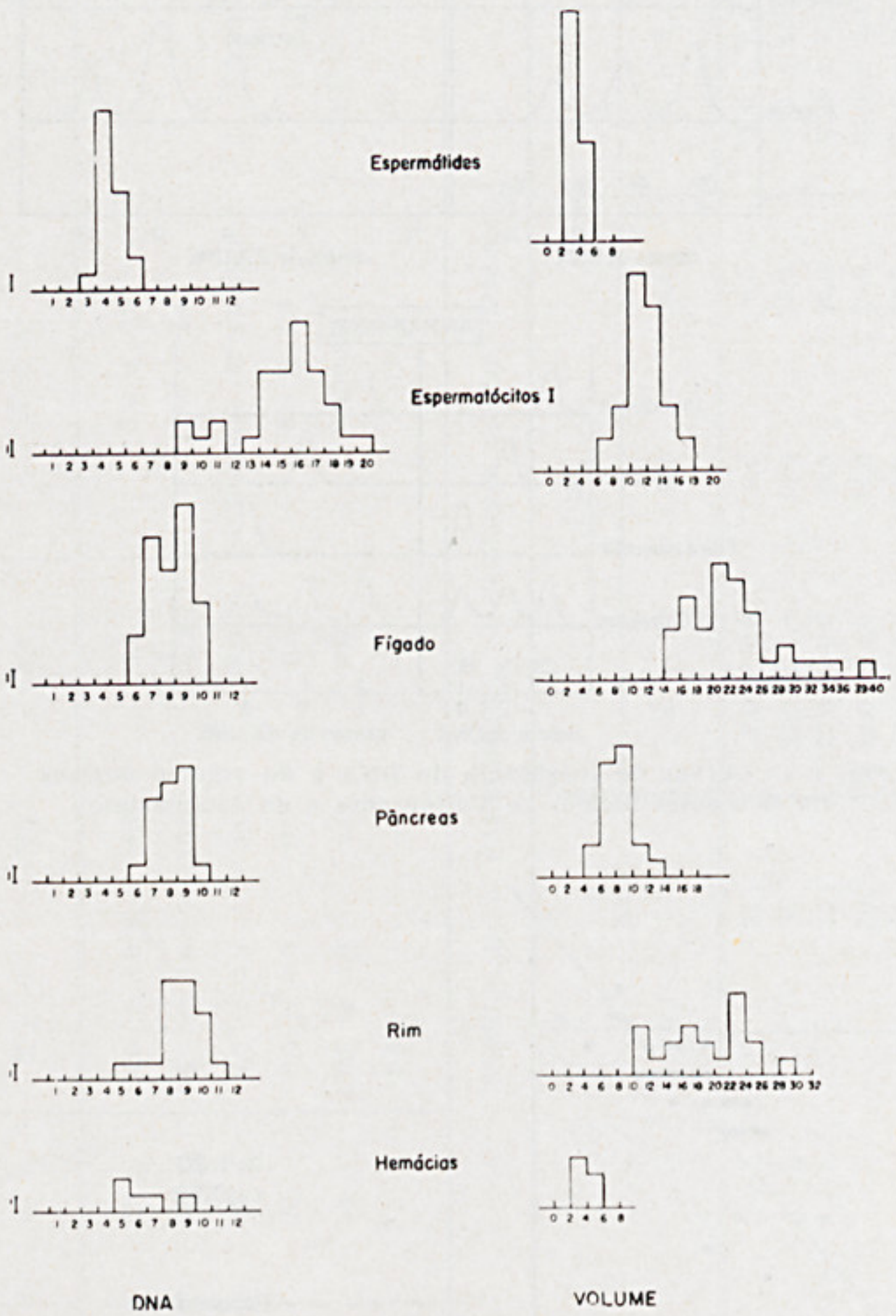


Fig. 3 — Histogramas do DNA e do volume nuclear nos diferentes tecidos de *Crotalus durissus terrificus* (Laurenti).

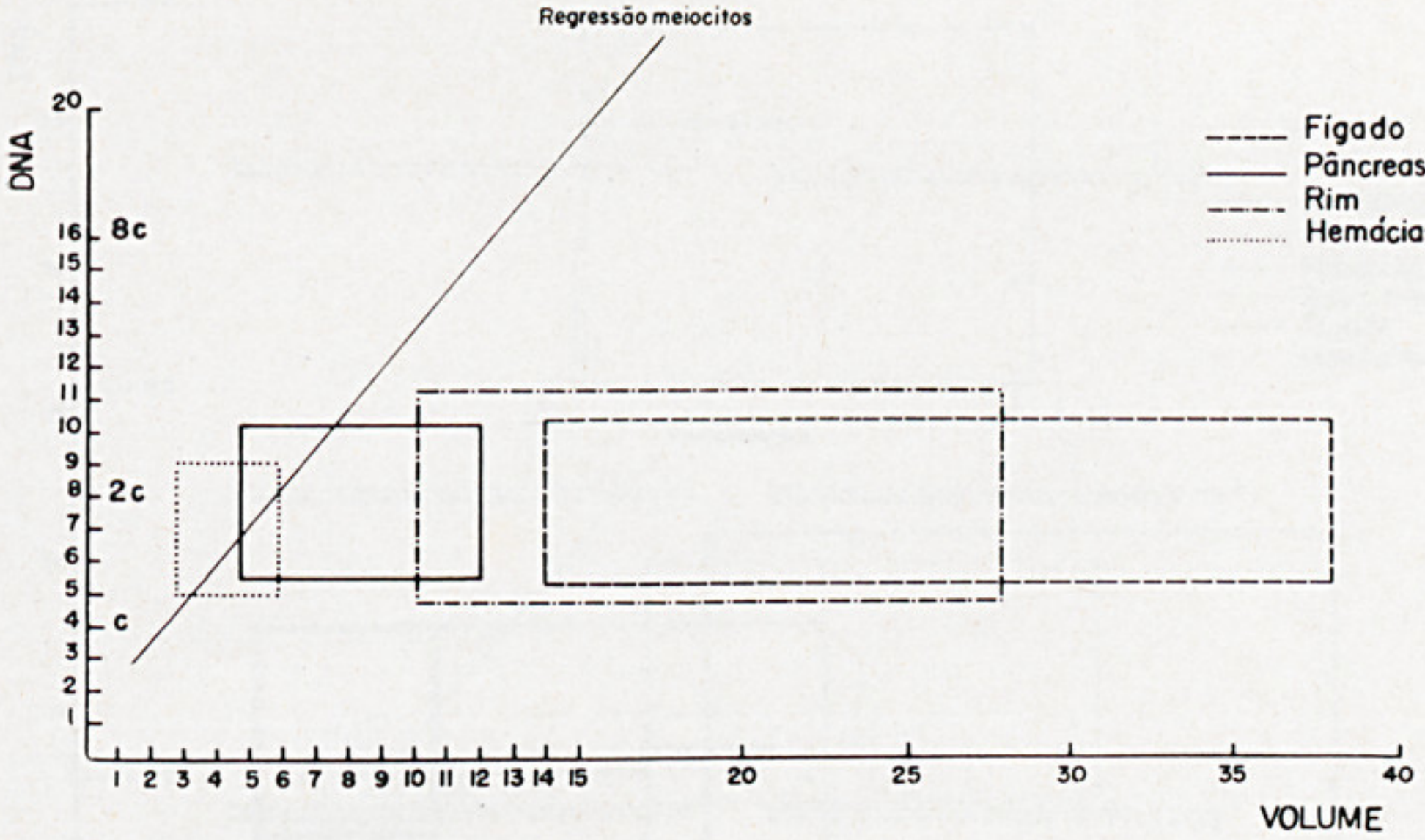


Fig. 4 — Diagrama de regressão entre DNA e volume nos tecidos somáticos de *Crotalus durissus terrificus* (Laurenti). Cada retângulo representa a área de distribuição de todos os valores de um tecido ("área ploido-megética"). A linha de regressão representa a série dos meiócitos.

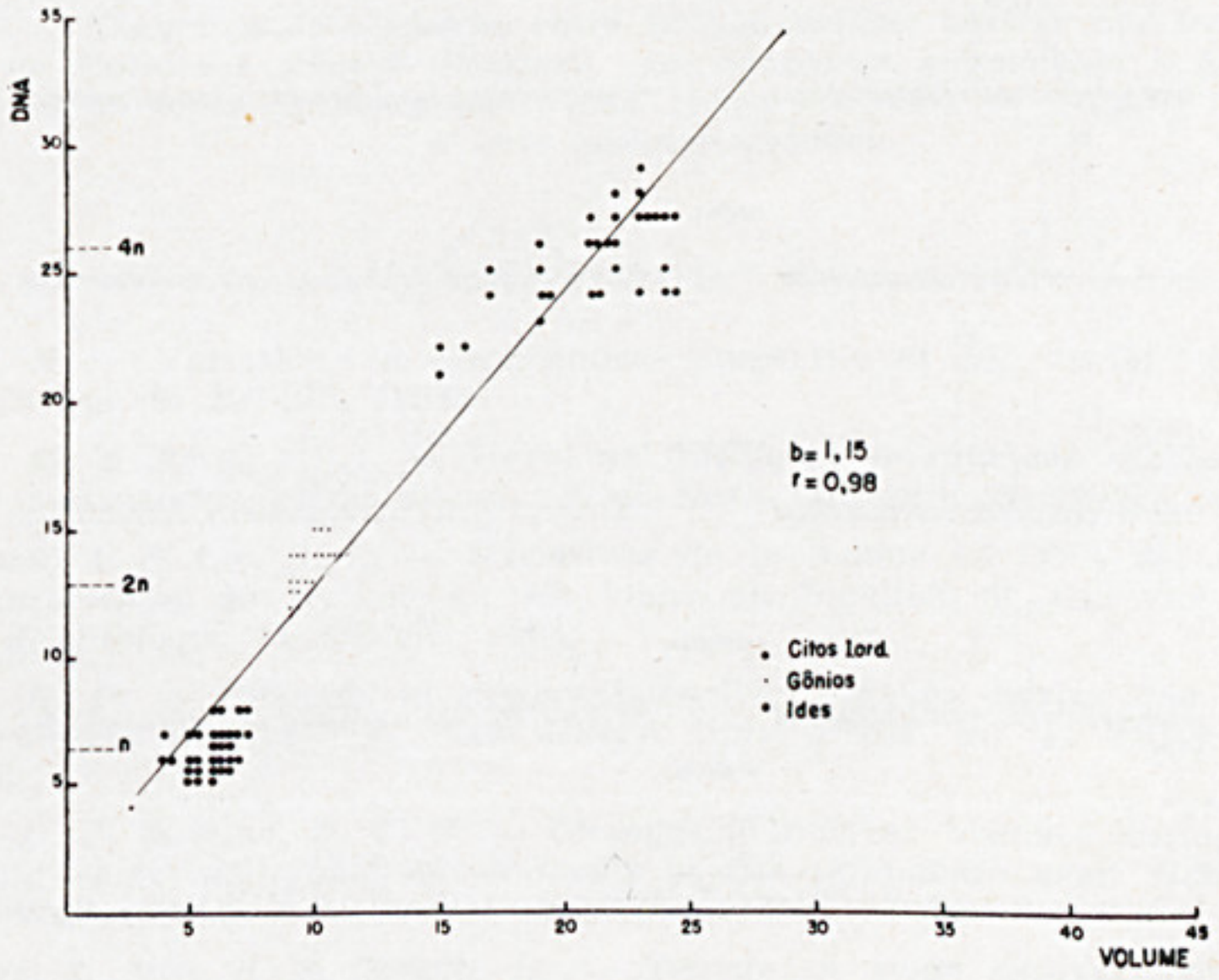


Fig. 5 — Diagrama de regressão entre DNA e volume nuclear da série espermatogenética em *Philodryas schottii* (Schlegel).

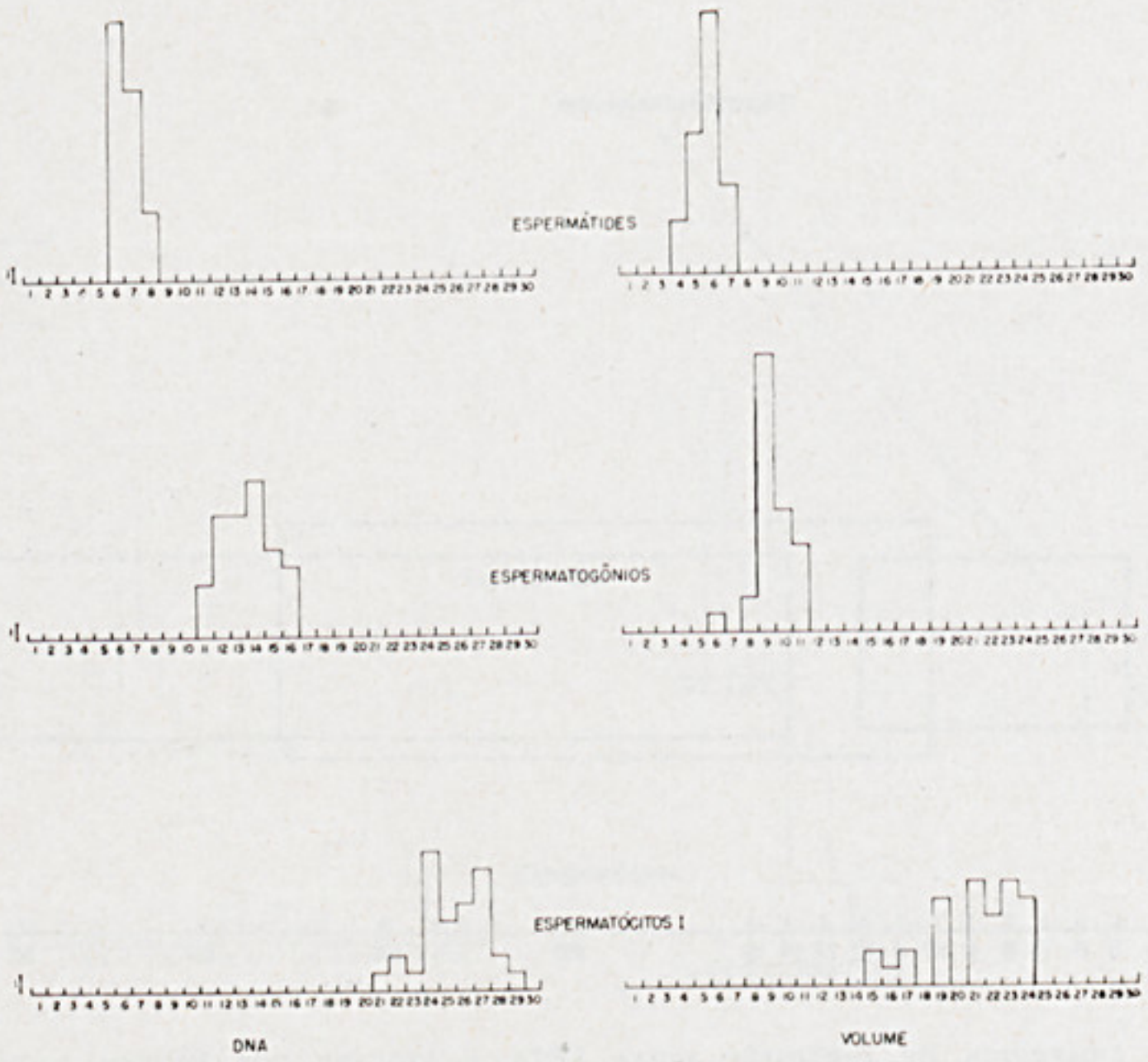


Fig. 6 — Histogramas do DNA e do volume nuclear na série espermatogenética de *Philodryas schottii* (Schlegel).

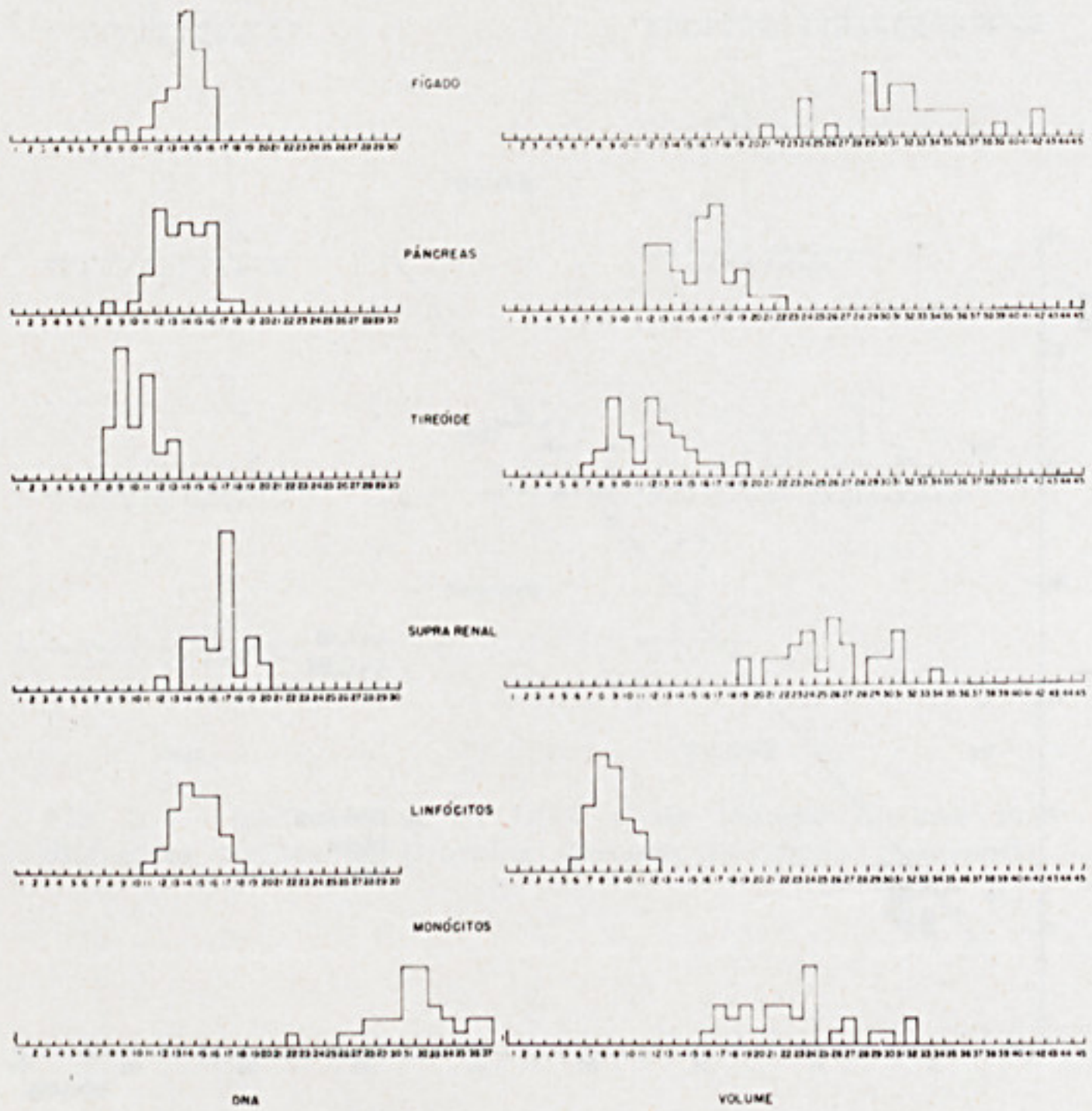


Fig. 7 — Histogramas do DNA e do volume nuclear nos tecidos somáticos de *Philodryas schottii* (Schlegel).

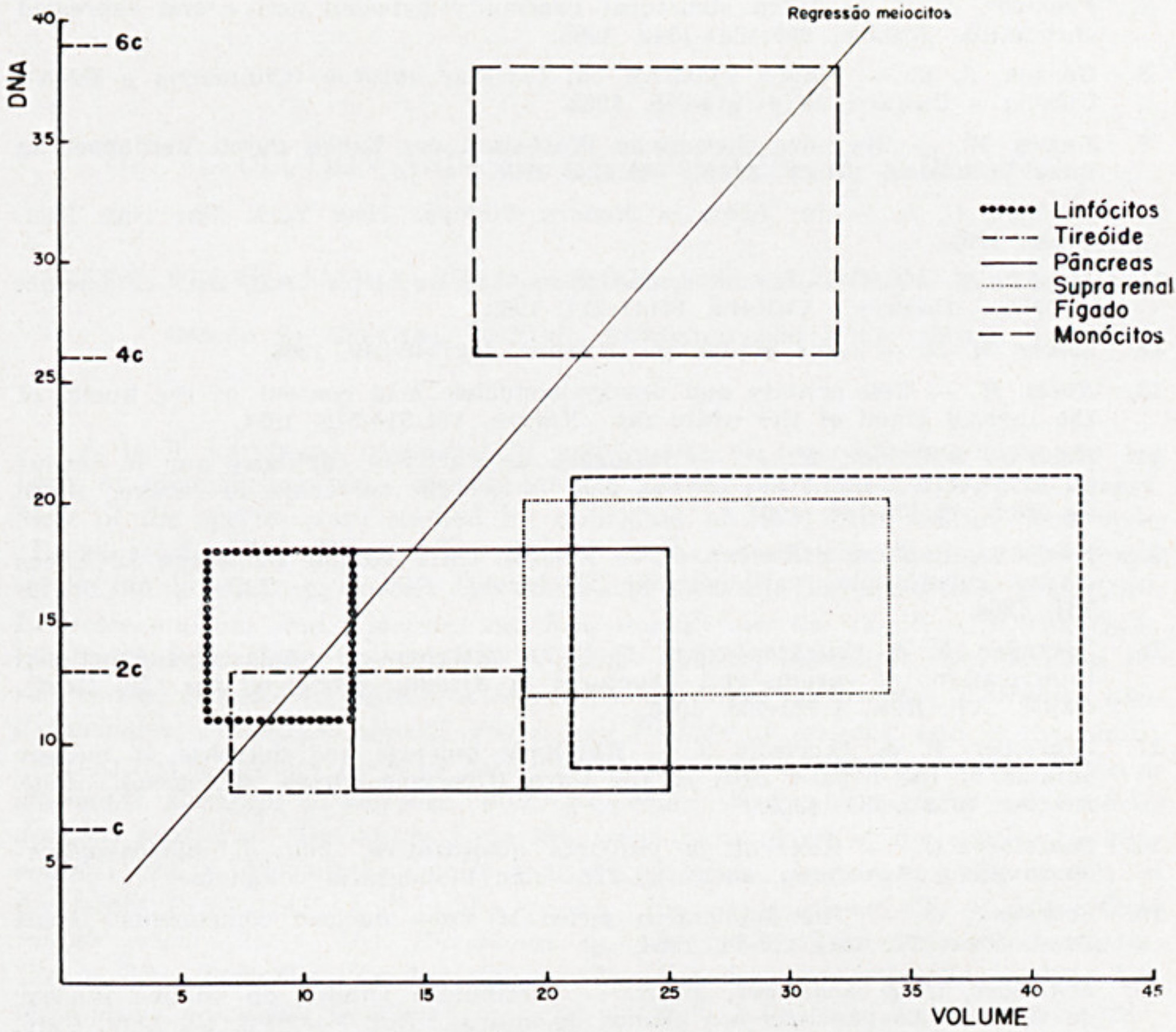


Fig. 8 — Diagrama de regressão entre DNA e volume nuclear nos tecidos somáticos de *Philodryas schottii* (Schlegel). Os retângulos representam a área de distribuição de todos os dados para cada tecido. A linha de regressão representa a série espermatogenética.

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18. EVOLUTION AND SEX CHROMOSOMES IN *SERPENTES*

WILLY BEÇAK, MARIA LUIZA BEÇAK and HELENEIDE NAZARETH

Secção de Genética, Instituto Butantan, São Paulo, Brasil

A basic karyotype consisting of eight pairs of macrochromosomes and ten pairs of microchromosomes appeared to be possessed by the majority of snakes. Most of the species were studied by utilization of short term culture techniques (Beçak *et al.*, 1963, 1964). The diploid number of 38 chromosomes was found in the family BOIDAE, species *Boa constrictor amarali, Boa constrictor constrictor, Eunectes murinus* and *Epicrates cenchria crassus;* in the family COLUBRIDAE, species *Spilotes pullatus anomalepis, Spilotes pullatus maculatus, Drymarchon corais corais, Dryadophis bifossatus bifossatus, Chironius bicarinatus, Chironius quadricarinatus, Philodryas olfersii olfersii* and *Philodryas aestivus,* and in the family CROTALIDAE, species *Lachesis muta, Bothrops jararaca, Bothrops atrox, Bothrops alternatus, Bothrops jararacussu, Bothrops pradoi, Bothrops insularis* and *Crotalus durissus terrificus.* Deviations from this were encountered in the species *Corallus caninus* (2n = 44) of the family BOIDAE, *Micrurus lemniscatus* (2n = 42) of the family ELAPIDAE, and in the species *Clelia occipitolutea* (2n = 50). *Oxyrhopus petola* (2n = 46), *Phrynonax* sp. (2n = 38, *Thamnodynastes strigatus* (2n = 32), *Tomodon dorsatus* (2n = 32), *Xenodon merremii* (2n = 30), *Erithrolampus aesculapii venustissimus* (2n = 28), *Liophis milliaris* (2n = 28), *Tropidodryas serra* (2n = 28), *Hydrodinastes bicinctus* (2n = 24) and *Lejosophis gigas* (2n = 24) of the family COLUBRIDAE (Beçak *et al.*, 1962; Beçak, 1965). The analysis of the diploid number and of the karyotypes of these snakes of the family COLUBRIDAE, indicates that, the more evoluted the species, according to systematic criteria, the smaller the diploid number. In those species the reductions in number are mainly due to reduction in the number of microchromosomes (Beçak *et al.*, 1965.

Uniformity of the suborder SERPENTES with regard to the total genetic content was established not only for those species possessing the basic karyotype but also for those with deviating numbers (Beçak *et al.*, 1964; Atkin *et al.*, 1965).

In the ophidians the fourth largest pair of the basic karyotype is generally the sex pair regardless of family. In the primitive BOIDAE the Z and W are still homomorphic to each other. Among the COLUBRIDAE initial steps towards the development of the heteromorphism between the male determining Z chromosome and the female determining W chromosome could be seen. The fourth pair is still represented by homomorphic chromosomes, in *Pseustes sulphureus.* Both members of the fourth largest pair in the female were still the same in absolute size, but a pericentric inversion appeared to have occurred in the W chromosome

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which is a subterminal element in the species *Spilotes pullatus, Drymarchon corais, Dryadophis bifossatus, Chironius bicarinatus, Chironius quadricarinatus, Philodryas olfersii, Philodryas aestivus* and *Tropidodryas serra.* Another approach toward heteromorphism was taken in *Clelia occipitolutea* in which the W is twice as large as the Z. In *Tomodon dorsatus* (2n = 32), *Thamnodynastes strigatus* (2n = 32). *Xenodon merremii* (2n = 30) and *Liophis milliaris* (2n = 28) of the family COLUBRIDAE and all species of the family CROTALIDAE the highly advanced poisonous snakes of the New World the W has become a distinctly smaller element comparable in degree of specialization to the minute W of birds (Beçak *et al.*, 1964).

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19. KARYOTYPES OF SOUTH-AMERICAN *ARANEIDA*

MANUEL O. DIAZ and FRANCISCO A. SAEZ

Departamento de Citogenética, Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay

The karyotype constitution of eleven species of **ARANEIDA** belonging to eight families was studied. The data about chromosome number, and sex determination system obtained from this study are summarized in Table I.

TABLE I

Species	2 n	n	Sex determ. system
Fam. DYSDERIDAE			
Dydera magna Keys	9	5	X-O
Fam. SEGESTRIDAE			
Ariadna mollis	9	5	X-O
Segestria ruficeps	14	8	XX-O
Fam. SICARIIDAE			
Scytodes maculata	14	8	XX-O
Loxosceles rufipes	20	11	XX-O
Fam. AMAUROBIIDAE			
Amaurobius simoni	40	21	XX-O
Fam. SPARASSIDAE			
Polybetes pitagorica	42	22	XX-O
Fam. LYCOSIDAE			
Lycosa erythrognata	22	12	XX-O
Lycosa nordenskölii	19	10	X-O
Fam. THERIIDIDAE			
Theridium tepidariorum	22	12	XX-O
Fam. ARGIOPIDAE			
Metepeira lathyrina	24	13	XX-O

In the karyotypes of *Scytodes maculata* and *Loxosceles rufipes* all the autosomes are metacentric and the Xs acrocentric. The location of the centromere in *Dysdera magna*, *Ariadna mollis* and *Segestria ruficeps* cannot be clearly determined because of the absence of centromeric constrictions or angulations during

gonial divisions. Meiotic bivalents are very contracted and their configuration suggest a metacentric nature. Nevertheless, this assumption does not explain the behaviour of the chromosomes in gonial divisions where there is no polarization of the centromeric regions during anaphases. For explaining these peculiarities it is necessary to assume they have a *diffuse centromere* of the type observed in *Tityus bahiensis* by Piza. (1)

In the other species studied all the chromosomes are acrocentric.

Multiple X sex determination system was observed in eight of the studied species and X-O systems only in three of them. The Xs are acrocentric in all species studied except in those belonging to DYSDERIDAE and SEGESTRIDAE where centromere position is uncertain. The Xs in *Scytodes maculata* show a strong heteropycnosis from diplotene until second anaphase and are paired by their proximal ends during all this period. In gonial divisions the Xs of *Scytodes* are free and isopyctonic.

The higher chromosome numbers were observed in *Amaurobius simoni* and *Polybetes pitagorica* with n = 21 and 22 respectively, but in account of chromosome arms number *Loxosceles rufipes* is close to the former with n = 20. *Lycosa nordenskölii* show a sharp reduction in the chromosome number relative to the modal number of the family n = 12 (Suzuki (2)) and have an aberrant X-o system for the family. The lower chromosome number was found in *Dysdera* and *Ariadna* with an n = 5. Suzuki (2) has described a karyotype with n = 4 for *Ariadna lateralis*, a related species. Evidently in the families DYSDERIDAE and SEGESTRIDAE there is a trend toward extreme chromosome reduction. This may well be the effect of a diffuse centromere system.

The chromosomes of *Loxosceles rufipes* have been studied previously by Beçak and Beçak (3), and our results confirm their findings. The chromosomes of *Theridium tepidariorum* have also been studied by other authors: Hackman (4), Montgomery (5) and Suzuki (2), and also in this case we do no more than confirming their descriptions.

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20. EVOLUTION OF VERTEBRATE GENOMES

SUSUMU OHNO

Department of Biology, City of Hope Medical Center, Duarte, U.S.A.

The Darwinian concept of evolution has revealed that speciation has been dependent upon the process of natural selection. Natural selection, in turn, can be effective only if there is hereditary variability among individuals comprising the population. The identical genetic constitution offers no chance for natural selection to operate; thus, evolution is quite obviously the consequence of genetic changes that have accumulated within the genome. The genome can be defined as a set of genes contained within the haploid set of chromosomes of an organism. In the case of speciation from an immediate ancestor, genetic changes are no doubt due mainly to allelic mutations at the already existing gene loci. What used to be a rare mutant allele of the old species would become the wild-type allele in a new species. For instance, a major component of adult hemoglobin of man and cattle is $\alpha_2 \beta_2$. Alpha- and beta-chains are different polypetides produced by two independent gene loci. The alpha-chain in man, however, has an amino acid sequence different from that of the cattle alpha-chain. Apparently, a common ancestor to diverse species of placental mammals already had the gene loci for two component polypeptides of adult hemoglobin. A series of mutations at each of these two gene loci finally gave rise to the genes for alpha- and beta-chains of today's human. Another series of mutations at the same gene loci, on the other hand, produced the gene for alpha- and beta-chains of the cattle.

When the scope is broadened to consider the evolution of the sub-phylum *VERTEBRATA* as a whole, allelic mutations of already existing genes cannot possibly account for all the genetic changes that occurred during the past 300 million years. There apparently were creations of new gene loci. Invertebrates are incapable of producing antibodies as such. The gene loci for light- and heavy-chains which comprise γ-globulin molecules were obviously created *de novo* at the beginning of the vertebrate evolution.

The creation of new genes now emerges as the most important single factor of evolution within the phylum. In the biological system, however, nothing is created anew out of the blue sky. The new material is produced by modification of the old which already existed.

IMPORTANCE OF GENE DUPLICATION IN VERTEBRATE EVOLUTION — The extensive study carried out by Margoliash (1963) on molecular structures of Cytochrome C revealed the extremely conservative nature of the gene. Cytochrome C is the heme-containing protein which engages in the intracellular transportation

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of oxygen. As such, it must have come to the existence soon after the cells made the first appearance on this earch as the unit of life. Yet, it was found that Cytochrome C of diverse organisms, from yeasts to man, not only have nearly the same molecular weight but also maintain similar amino acid sequences, in each instance about 104 amino acid residues making up a polypeptide chain. The clear implication is that the particular function assigned to the gene product imposes a severe limitation on that gene's freedom to mutate. If a change in the base sequence occurred at the wrong part of the DNA molecule, a new gene product would be unable to function as Cytochrome C. Such a mutation would quickly be eliminated.

Natural selection conserved only those mutations which were not deteriorative to the gene product's assigned function. The extremely conservative nature of the already existing genes indicate to us that the redundancy of genetic material was the prerequisite for the creation of new genes. By duplication, if the old gene had been represented twice within the genome, one of the duplicates was now free to mutate to an independent direction and acquire a new function.

In man and probably most other mammals, there are five independent gene loci for component polypeptides of hemoglobin. They are for α-, ε-, γ-, β-, and δ-chains.

It is the view of Ingram (1963) that the ancestry of all the five genes for five different components of hemoglobin can be traced back to a single ancestral gene. First, there was a duplication of this gene and by subsequent mutations to independent directions, one became a gene for myoglobin while the other became a gene for α-chain of hemoglobin. The ancient vertebrate when first emerged may have been able to produce only one type of a hemoglobin molecule which should be α_4. The genes for four other chains of hemoglobin are thought to have been derived from the multiplicates of the gene for α-chain.

Similarly, mammals and birds have three independent gene loci for component polypeptides of the enzyme, lactate dehydrogenase. They are known as A, B, and C (Markert, 1964; Blanco and Zinkham, 1962; Blanco *et al.*, 1964). Originally there may have been only one gene locus for LDH, and the other two may have been produced by duplication of the orginal one.

The gene duplication can be accomplished in two different ways. The longitudinal duplication of a small segment of an individual chromosome would accomplish the purpose for a small number of genes closely linked together. In fact, regional duplication of small chromosomal segments appears to be occurring among mammals of today. For instance, γ-globulin molecule is made of two different kinds of polypeptide chains: the heavy-chain (H) with a molecular weight of about 60,000 and the light-chain (L) of about 20,000. In man, it is becoming increasingly clear that, instead of having one gene locus each for the H- and L-chains, the so-called H-chain locus is actually made of several very closely linked but slightly different genes; the same can be said of the so-called L-chain locus. There apparently were longitudinal multiplication of one ancestral gene for the H-chain and the other ancestral gene for the L-chain. In man, the genes for β- and δ-chains of hemoglobin are also very closely linked. The δ-chain gene must have been derived by a regional duplication of the β-chain gene.

While regional duplication of a small number of genes might have played an important role in speciation from an immediate ancestor, more drastic changes must have occurred to the genomes during the course of vertebrate evolution.

Simultaneous duplication of the entire set of genes can be accomplished by polyploidization. It can be assumed with reasonable certainty that a series of polyploidization of the ancestral genome have taken place sometime in the history of vertebrates which is, after all, 300 million years old.

INCOMPATIBILITY BETWEEN POLYPLOIDY AND THE WELL ESTABLISHED CHROMOSOMAL SEX-DETERMINING MECHANISM — Polyploidy, however, is incompatible with the well established chromosal sex-determining mechanism. When the diploid organisms with the XY/XX-scheme of sex-determining mechanism become tetraploid, the male has to maintain the 4AXXYY-constitution and the female, 4AXXXX. During meiosis of the 4AXXYY-male, the four sex elements may pair off as the XX-bivalent and the YY-bivalent. If such occurs, every gamete would be of the 2AXY-constitution. Consequently, all the offspring resulting from the mating between a tetraploid male and a tetraploid female would emerge as the intersex of the 4AXXXY-constitution. Even if two XY-bivalents are formed in individual spermatocytes of the tetraploid male, in 50 per cent of the cases the X and the Y would move to the same division pole at first meiotic anaphase, again resulting in the production of the intersex of the 4AXXXY-constitution. Polyploidy invariably disturbs the chromosal sex-determining mechanism.

Indeed, among vertebrates viable and fertile polyploid individuals have been found among amphibians where the Z and the W are still largely homologous to each other, but not in birds and mammals where the W and the Y became a highly specialized determiner of the heterogametic sex. Furthermore, it was found that even in amphibians, polyploid individuals are, as a rule, incapable of perpetuating themselves as polyploid by bisexual mating.

Polyploid individuals of amphibians were most thoroughly studied by Humphrey and his colleague (Humphrey and Fankhauser, 1956; Fankhauser and Humphrey, 1959) on *Ambystoma mexicanum* and *A. tigrinum*.

In the case of triploidy, males were uniformly of the 3AZZZ-constitution, while three kinds of sex chromosome constitutions, ZZW, ZWW, and WWW were found among females. Triploids of both sexes were of very poor fertility, and males were more sterile than females; therefore, it was not possible to perpetuate the triploid race by mating of triploid males and triploid females. When mated to diploid males, triploid females produced many tetraploids, revealing that these females ovulate triploid eggs. This may account for the presence of a gynogenic all-female triploid race found in *Ambystoma jeffersonianum* (Uzzell, 1963). Tetraploid, pentaploid, hexaploid, and heptaploid individuals of *A. mexicanum* and *A. tigrinum* also showed very poor fertility. In short, it appears that even in amphibians with the undifferentiated sex chromosomes, the serious obstacle which prevents the emergence of a bisexual polyploid race exists.

From the above, it may be deduced that various degrees of polyploidization of the ancestral genome must have occurred very early in evolution of vertebrates before the emergence of terrestial forms.

Our study on DNA-contents of various vertebrates confirms the above prediction and reveals the polyphyletic origin of the genomes of various vertebrates.

UNIFORMITY OF THE DNA CONTENT OF VARIOUS PLACENTAL MAMMALS — All placental mammals of today descended from a common stock of protoinsectivores which emerged at the dawn of the Cezonoic era. In terms of geological time, the history of placental mammals is brief indeed. Reflecting this recent origin

is the sameness of DNA content. Diverse speciation appeared to be accomplished with little or no change in the total genetic content. Mandel and his colleagues (1950) were among the first to show that each diploid nucleus of man as well as cattle, sheep, pigs, and dogs contains about 7.0 mg $\times 10^{-9}$ of DNA, no more, no less. Recently, we restudied this matter of DNA constancy by means of microspectrophotometry. Six species representing four different orders were chosen: man (*Homo sapiens*, 2n = 46) representing the order **PRIMATES**, the dog (*Canis familiaris*, 2n = 78) representing the order **CARNIVORA**, the horse (*Equus caballus*, 2n = 64) of the order **PERISSODACTYLA**, the mouse (*Mus musculus*, 2n = 40), the golden hamster (*Mesocricetus auratus*, 2n = 44), and the creeping vole (*Microtus oregoni*, 2n = 17/18) of the order **RODENTIA**. There was no significant difference in DNA values between man, the horse, the dog, the golden hamster, and the mouse. A single exception was the creeping vole which had a DNA value 10% lower. This species (Ohno *et al.*, 1963) shares with the other member of the rodent subfamily MICROTINAE, *Ellobius lutescens*, 2n = 17 (Matthey, 1953) the distinction of having the lowest diploid chromosome number known among placental mammals. Such a drastic reduction in the number of chromosomes had to be accompanied by the loss of a number of centromeres with their adjacent heterochromatic chromosomal materials. This loss of genetically unimportant heterochromatin would account for the 10% lower DNA value found in the creeping vole (Atkin *et al.*, 1965).

It then follows that different species of mammals, by and large, share the same kinds of gene loci, even if they belong to different orders. Allelic mutations at each gene locus were mainly responsible for extensive diversification of placental mammals.

The sameness of total genetic content, however, does not exclude the possibility that duplications of a small number of genes may have occurred during speciation of mammals.

Regional gene duplications which occurred to a small number of genes of different mammals, however, do not change the over-all picture of mammalian evolution. Extensive speciation of placental mammals was accomplished without substantial change in the total genetic content. Yet, placental mammals of today display chromosome constitutions of infinite variety. The diploid number ranges from a high of 80 in the primitive primate, *Tarsius bancanus* (Klinger, 1963) to a low of 17 in two rodent species mentioned above. Nothing but acrocentric chromosomes are found in the mouse (*Mus musculus*, 2n = 40), and only metacentrics in the chinchilla (*Chincilla laniger*, 2n = 64) (Galton *et al.*, 1965). The enormous array of karyotypes reveals the extent to which the original autosomal linkage groups of a common ancestor has been shuffled around. An autosomal equivalent to the human chromosome 21 may only be found among his closest relatives, the chimpanzee (*Pan troglodytes*, 2n = 48) and the gorilla (*Gorilla gorilla*, 2n = 48) (Hammerton *et al.*, 1963).

UNIFORMITY OF THE DNA CONTENT OF VARIOUS AVIAN SPECIES — It is believed that ancestral forms of modern birds were already in existence near the end of the Jurassic period of the Mesozoic era. The fossil remains of the toothed bird, *Archeopteryx lithographia* found in slate deposits in Bavaria is said to be 150 million years old. Thus, it is clear that the avian lineage branched out from a reptilian lineage before the other reptilian lineage gave rise to a common ancestor to placental mammals.

Reflecting this independent evolution of the two classes of warm-blooded vertebrates is the fact that the male is the heterogametic sex in mammals, while in birds it is the female which is the heterogametic sex. The avian chromosome complements are also distinct from those of placental mammals in that they include numerous microchromosomes, each no larger than one micron in size.

In our experience, the diploid complements of present day birds belonging to the orders **PASSERIFORMES**, **COLUMBIFORMES**, **GALLIFORMES**, and **ANSERIFORMES** followed the common rule in that nine pairs of macrochromosomes or ordinary chromosomes and about 60 microchromosomes constituted each diploid complement. Members of the ordem **PSITTACIFORMES** were exceptional, having more macrochromosomes and fewer microchromosomes. For instance, twelve pairs of macrochromosomes and about 18 pairs of microchromosomes constituted the diploid complement of the Australian parakeet, *Melopsittacus undulatus* (Ohno *et al.*, 1964).

Relative DNA values were measured on the canary representing the order **PASSERIFORMES**, the chicken (*Gallus gallus domesticus*) representing the order **GALLIFORMES**, the pigeon (*Columba livia domestica*) of the order **COLUMBIFORMES**, and the Australian parakeet of the order **PSITTACIFORMES**. As the extreme similarity in their diploid chromosome complements already indicated the uniformity in the total genetic content of various avian species, the above four species representing four diverse orders were deemed sufficient.

As expected, four representatives of the class **AVES** gave the uniform DNA value. The value, however, was 44-59% that of placental mammals.

The above finding on the total genetic content of avian species, on one hand, reveals the fact that polyploidy played no role in extensive speciation within the class **AVES** and, on the other hand, shows that the genome lineage which gave rise to the class **AVES** has long been in separation from that which eventually gave rise to placental mammals.

THE COEXISTENCE OF THE TWO GENOME LINEAGES IN THE CLASS **REPTILIA** — Reptiles of today can be compared with the twigs of a great tree which flourished during the early Mesozoic era. About 95% of all the different kinds of living reptiles belong to the order **SQUAMATA**, yet fossil remains indicate that this order never held greater importance than today. On the contrary, fossil beds in many parts of the world are strewn with shells of many kinds of turtles. The orders **CROCODYLIA** and **CHELONIA** had seen better days.

We have no way of directly assessing the genomes of ancient reptiles which constituted the huge limbs of a great tree and which produced the direct ancestor to the placental mammals on one hand and that to the birds on the other. It was fortunate that the studies on relative DNA content and chromosome constitutions of a limited number of living reptiles enabled us to discern the presence of two different genome lineages, one showing close affinity to that of the class **AVES** and the other to that of the class **MAMMALIA**.

As stated earlier, the abundant presence of microchromosomes characterize the avian chromosome complements. It has been known for some time that microchromosomes are also possessed by lizards and snakes which constitute the ordem **SQUAMATA**. While the exact number of microchromosomes in the diploid complement of each avian species is nearly impossible to determine, the number of microchromosomes in each lizard or snake species can be determined with ease.

The diploid complement of the alligator lizard (*Gerrhonotus multicarinatus*, 2n = = 46-48) belonging to the family ANGUIDAE of the suborder SAURIA, contains 12 pairs of microchromosomes, while a great majority of snakes constituting the suborder SERPENTES, possess 10 pairs of microchromosomes.

Aside from the possession of microchromosomes, there is yet another common characteristic which reveals that the reptilian order **SQUAMATA**, suborder SERPENTES in particular, belong to the very same genome lineage which gave rise to the class **AVES**. The female heterogamety of the ZZ/ZW-type also operates in snakes (Beçak *et al.*, 1962; Kobel, 1962). Furthermore, the avian Z-chromosome and the ophidian Z-chromosome may have been derived from the same ancestral chromosome, as both constitute about 10% of the genome or haploid set (Beçak *et al.*, 1964).

DNA content was measured on six representatives of the order **SQUAMATA** which were: the chameleon lizard (*Anolis carolinensis*, 2n = 36) of the family IGUANIDAE and the alligator lizard (*Gerrhonotus multicarinatus*, 2n = 46) of the family ANGUIDAE of the suborder SAURIA. The suborder SERPENTES was represented by the *Boa constrictor* (*Boa constrictor amarali*, 2n = 36) of the family BOIDAE, the gopher snake (*Drymarchon corais couperi*, 2n = 36), and the South American *Xenodon* (*Xenodon merremii*, 2n = 30) of the family COLUBRIDAE, and the South American jararaca (*Bothrops jararaca*, 2n = = 36) of the family CROTALIDAE. These six representatives of the order **SQUAMATA** demonstrated a DNA value of 60-67% that of placental mammals. The value obtained was only slightly more than that obtained for various avian species which was 44-59% that of placental mammals (Atkin *et al.*, 1965).

While the above finding should not be interpreted to mean that lizards and snakes of today were directly ancestral to birds, it reveals that an ancestral reptile which evolved to toothed birds belonged to the same genome lineage which independently gave rise to ancestral forms of modern members of the order **SQUAMATA**; there was no further polyploidization of this genome lineage. Among members of the order **SQUAMATA** the well differentiated heteromorphic Z- and W-chromosomes are seen only in the poisonous family CROTALIDAE and certain members of the family COLUBRIDAE of the suborder SERPENTES; others possess the primitive homomorphic sex elements. Yet, there apparently exist the effective barrier to prevent the evolution of a bisxual polyploid species. Triploid species of the Teiid lizard of the genus *Cnemidophorus* were all females and apparently propagated by parthenogenesis (Pennock, 1965).

While present day members of the reptilian order **SQUAMATA** demonstrated the close kinship to the class **AVES** members of the order **CROCODYLIA** and **CHELONIA** appeared to represent the pre-mammalian genome lineage.

The South American alligator (*Caiman sclerops*, 2n = 42) representing the order **CROCODYLIA** gave the DNA value as 84% that of placental mammals. The diploid chromosome complement of this species is totally different in character from those of snakes and lizards. In fact, there is a striking resemblance between the diploid complement of *Caiman* and that of one species of mammals, the rat (*Rattus norvegicus*, 2n = 42). To be sure, this extreme similarity is a pure coincidence. Nevertheless, there is little doubt that among present day members of reptiles, those belonging to the order **CROCODYLIA** demonstrate the closest kinship with placental mammals, not only in DNA value but in karyological characteristics as well. Although the lower diploid chromosome number of 32 has been reported on the North American alligator (*Alligator mississippien-*

sis) and the African crocodile (*Crocodilus niloticus*), this reduction in chromosome number from 42 to 32 appears to be the result of simple Robertsonian translocations. The 10 largest pairs of acrocentrics of *Caiman* are represented as 5 pairs of metacentrics in *Alligator* and *Crocodilus* (Hollingsworth, 1957; van Brink, 1959).

DNA value similar to that of placental mammals was also obtained on representatives of the order **CHELONIA.** The fresh-water soft-shell turtle (*Amyda ferox*, 2n = 66) and the desert tortoise (*Gopherus agassizi*, 2n = 52) gave DNA value 80 and 89% that of placental mammals, respectively. While observing their metaphase figures, however, it was noted that their karyological characteristics are not at all similar to those of placental mammals. Many small members can be regarded as microchromosomes. It appears that members of the order **CHELONIA** demonstrate the closest karyological affinity to the infraclass **PROTOTHERIA**, rather than to either marsupials or placental mammals. A rather high diploid chromosome number of about 70 and 63 has been found in the duck-bill platypus (*Ornithorhynchus anatinus*) and the spiny anteater (*Tachyglossus aculeatus*) of the order **MONOTREMATA.** Many small members can be regarded as microchromosomes (Matthey, 1949; van Brink, 1959).

It would be of utmost interest to find out if the male heterogamety of the XY/XX-type operates in members of the orders **CROCODYLIA** and **CHELONIA** which represent the premammalian lineages. Unfortunately, the heteromorphic sex elements have not been found in these reptiles. No sex-linked gene is known, and the sex reversal experiments have not been performed on any of these species.

EXTREMELY HIGH DNA VALUES POSSESSED BY CERTAIN AMPHIBIANS WHICH SUGGEST THE POLYPHYLETIC ORIGIN OF TERRESTRIAL VERTEBRATES — It is known that birds, snakes, and lizards of today are the branches of one limb which originated from the ancestral reptile *Ornithosuchus* mammals emerged from the other limb which was started from *Lycaenops*. The fact that surviving members of the classe **REPTILIA** fell discretely into two groups (one group belonging to the preavian genome lineage and the other group belonging to the premammalian lineage) may be taken as an evidence that *Ornithosuchus* and *Lycaenops* of ancient times already belonged to the two different genome lineages.

Reptiles, in turn, were derived from ancient amphibians grouped together as Labyrinthodonts. It appears that Labyrinthodonts were of many kinds representing diverse genome lineages. Most, if not all, of the amphibians of today belong to the genome lineages independent from both the preavian and premammalian lineages.

The most comprehensive survey on DNA values of various amphibians was carried out by Joseph Gall of Yale University; his results are quoted here with his kind permission. All the amphibian species surveyed by him demonstrated higher DNA values than that of placental mammals. DNA values demonstrated by tailless amphibians constituting the order **SALIENTIA** were still not as fantastically high as those demonstrated by members of the order **CAUDATA.**

Within the order **SALIENTIA,** the American toad (*Bufo americanus*, 2n = 22) representing the suborder PROCOELA gave the DNA value 137% that of placental mammals. The DNA value of the Leopard frog (*Rana pipiens*, 2n = 26) and the bull frog (*Rana catesbiana*, 2n = 26) of the suborder DIPLASIOCOELA

was 200% of the DNA value of placental mammals. In terms of the absolute content, the family RANIDAE contained 14.6 mg $\times$ 10^{-9} DNA in each diploid nucleus. While these values are high, they show close enough affinity to the premammalian lineage. It is expected that if a truly extensive survey is done on tailless amphibians of today, the DNA value very similar to that of the premammalian lineage can be found in some of them.

On the contrary, members of the order **CAUDATA** showed absolutely no affinity to either the premammalian or the preavian lineages. Within this order, the lowest DNA value was found on the newt (*Triturus cristatus*, 2n = 24) of the suborder SALAMANDROIDEA. Yet, it was 830% that of placental mammals, and its close relative, *Triturus viridescens* (2n = 22) revealed even higher DNA value of 1300%. The Congo eel (*Amphiuma means*, 2n = 24) of the suborder PROTEIDEA, had the fantastically high DNA value of 2700% that of placental mammals.

Another interesting aspect of tailed amphibian genomes is that two closely related species belonging to the same family often demonstrated a remarkable difference in their DNA values. For instance, *Triturus cristatus* and *Triturus viridescens* belonged to the same family SALAMANDRIDAE, yet the DNA value of the latter was 50% greater than that of the former, despite the fact that both had the very similar diploid complements. Joseph Gall found that each lampbrush bivalent of the latter was longer and had more loops than its counterpart of the former. On this basis, he believes that the increase in DNA value is due to regional duplication of chromosomal segments that occurred to *Triturus viridescens*.

It has been shown that the Z and the W or the X and the Y of amphibians are in such a primitive state of differentiation. The W or the Y is still a genetical equivalent of the Z or the X. This primitive state of sex chromosomes may permit polyploid evolution to exceptional membres of present day amphibians.

One species might represent a newly arisen tetraploid state of the old diploid species. The pioneering study by Saez (1964) has indicated that polyploid evolution may have occurred to South American frogs belonging to the family CERATOPHRYDAE. Indeed, the tetraploid nature of *Odontophrynus americanus* has been proven beyond any doubt by M. L. Beçak and her colleagues at this symposium. The 44 chromosomes can be arranged to 11 different kinds of homologues, and 11 quadrivalents rather than 22 bivalents were seen in meiosis. Thus, among amphibians of today, the increase of DNA content by both regional duplication and polyploidization might still be occurring to some extent. Nevertheless, so far as members of the order **CAUDATA** are concerned, it is clear that they belong to the genome lineage or lineages altogether different from both the preavian and premammalian lineages.

DIVERSE GENOME LINEAGES FOUND AMONG FISHES — The inevitable conclusion to be drawn from the above survey on DNA values of the four classes of terrestial vertebrates is that the evolution from Crossopterygian fishes to Labyrinthodont amphibians was polyphyletic. Today, the subclass **CROSSOPTERYGII** is represented only by the lung fish of the order **DIPNOI** and the coelocanth of the order **ACTINISTIA.** These surviving members of the lobe-finned fish must merely represent a fraction of the diverse genome lineages which were possessed by ancient Crossopterygian fishes ancestral to terrestrial vertebrates. As much as we have no way of obtaning the information on genomes from the fossils, we

must turn to members of the ray-finned fish constituting the subclass **NEOPTERYGII** as the source of indirect information on ancient genome lineages.

Our study, although limited to eight species of the class **PISCES**, appeared to confirm the polyphyletic origin of terrestrial vertebrate genomes (Ohno and Atkin, 1966).

It was found that surviving members of the order **DIPNOI**, the subclass **CROSSOPTERYGII** show close kinship only to tailed amphibians (the order **CAUDATA**). The DNA value, 3540% that of mammals, was obtained on the South American lung fish (*Lepidosiren paradoxa*, 2n = 38). According to Alfrey *et al.* (1955), the absolute DNA value for the African lung fish (*Protopterus*, 2n = 34) was 100 mg $\times$ 10^{-9} which is about 1400% that of mammals. The relatively low diploid chromosomes number, the absence of acrocentrics, the enormous size of individual chromosomes, and the very high DNA value found in the lung fish are all precise characteristics of the genomes maintained by present-day membres of the order **CAUDATA** of the class **AMPHIBIA**. Although the chronology of evolution suggests that the lung fish could not have been the direct ancestor of the tailed amphibians, it is apparent that both belong to the same particular genome lineage. This lineage is not directly related to the main genome lineages which gave rise to tailless amphibians, reptiles, birds, and mammals of today.

The DNA values which demonstrated the close kinship to the premammalian and preavian genome lineages were found among members of the subclass **NEOPTERYGII**.

The rainbow trout (*Salmo irideus*, 2n = 58-64) is the anadromous species belonging to the family SALMONIDAE of the order **ISOSPONDYLI**. The DNA value, 80% that of mammals, corresponded well with the values possessed by the orders **CROCODYLIA** and **CHELONIA** of the class **REPTILIA**; thus, this species and other members of the family SALMONIDAE may be regarded as belonging to the premammalian lineage. It is not my intention to imply that trouts and mammals constitute one direct line of descent. My view is that crocodiles, turtles, and mammals of today descended from a particular group of ancient Crossopterygian fish which already possessed the DNA value similar to that of trouts.

The DNA value similar to that possessed by the class **AVES** as a whole and also by the order **SQUAMATA** of the class **REPTILIA** was found on the goldfish (*Carrasius auratus*, 2n = 96-104) of the family CYPRINIDAE, the order **OSTARIOPHYSI**. The DNA value obtained on this species was 52% that of mammals. Thus, members of the family CYPRINIDAE may be regarded as belonging to the preavian genome lineage.

Our study on various members of the subclass **NEOPTERYGII** further revealed the presence of DNA values much smaller than any of the values possessed by terrestial vertebrates. Our notion that a series of polyploidization of an ancestral vertebrate genome occurred while vertebrates were still in aquatic forms appeared to be confirmed.

The DNA value of only 30% that of mammals was obtained on two members of the order **PERCIFORMES**. The green sunfish (*Lepomis cyanellus*, 2n = 46-48) of the family CENTRARCHIDAE and the discus fish (*Synphysodon aequifasciata*, 2n = 60) of the family CICHLIDAE.

The lowest DNA value, only 20% that of mammals, was found among two diverse groups of fishes. This value was obtained on the swordtail (*Xyphophorus hellerii*, 2n = 48), hornyhead turbot (*Pleuronichthys verticalis*, 2n = 48), and fantail sole (*Xystreurys liolepis*, 2n = 48).

From the taxonomical point of view as well as from their natural habitats, the swordtail and the flatfish are as remotely related as they can be among members of the subclass **NEOPTERYGII**. The swordtail, a Central American fresh-water fish long bred in the aquarium, belongs to the order **MICROCYPRINI**, while two species of the flatfish belong to different families of the order **HETEROSOMATA**: the hornyhead turbot to the right-eyed flounder family, PLEURONECTIDAE, and the fantail sole to the left-eyed flounder family, BATHIDAE. Their natural habitat is the ocean bottom. The swordtail and the flatfish apparently had identical diploid complements made of 48 acrocentrics gradually declining in size and the lowest DNA value.

We propose to regard these ray-finned fishes as the retainers of the original diploid lineage of ancestral vertebrates. The original diploid lineage then had the DNA value, 20% that of mammals. In terms of the absolute value, this lineage contained 1.4 mg $\times$ 10^{-9} DNA in each nucleus.

It then follows that the green sunfish and the discus fish belong to the ancient triploid lineage, while the ancient pentaploid lineage is represented by the goldfish and among terrestial vertebrates, by lizards, snakes, and birds.

The rainbow trout, crocodiles, and turtles may be regarded as representing the octa- and nonaploid lineages, and placental mammals, the decaploid lineage.

All three constituent polypeptides A, B, and C of the mammalian lactate dehydrogenase have been found to exist in avian species as well as in many of the ray-finned fishes (Blanco *et al.*, 1964; Markert and Faulhaber, 1965). These findings on lactate dehydrogenase are in conformity with the view that in vertebrates, any DNA values above 20% that of placental mammals indicate polyploid lineages; therefore, sufficient gene duplication has occurred to these genomes. Flatfish of the order **HETEROSOMATA**, on the other hand, revealed the presence of the A-polypeptide only (Markert and Faulhaber, 1965).

Summary

It appears that gene duplication played a most important role in the evolution of vertebrates. A new gene with a new function arose from a duplicate of the old gene. When the same gene was represented twice within the genome, one redundant gene was allowed to mutate to an independent direction and acquire a new function, while the original function was maintained by the other.

Admittedly, regional duplication of a small number of genes might still be occurring to individual species of higher vertebrates. A series of polyploidization of the ancestral diploid lineage, however, appeared to have occurred while vertebrates were still in aquatic forms nearly 300 million years ago. Among fishes of today, some appear to retain the ancient diploid lineage which contain 1.4 mg $\times$ $\times$ 10^{-9} DNA per diploid nucleus. Placental mammals as a whole appear to belong to the ancient decaploid lineage, while birds represent the ancient pentaploid lineage. Once the chromosomal sex-determining mechanism is well established, no further polyploidization is possible.

As a result, diverse species of placental mammals contain the identical amount of DNA in the diploid complement, 7.0 mg $\times 10^{-9}$. Speciation within the infraclass **EUTHERIA** is accomplished almost exclusively by allelic mutations with little change in the total number of gene loci. The same can be said of various avian species. Among reptiles of today, snakes and lizards belong to the preavian pentaploid lineage. Crocodiles and turtles, on the other hand, show close kinship to the decaploid mammalian lineage.

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21. *PENTASTOMIDA* OF SNAKES — THEIR PARASITOLOGICAL ROLE IN MAN AND ANIMALS

A. FAIN

Institute of Médecine Tropicale, Antwerpen, Belgique

PENTASTOMIDA constitute an old and highly aberrant group of parasites. So far the systematic position of this group is not established with certainty. In spite of some affinities with both the arthropods and the annelids the **PENTASTOMIDA** cannot be attached exactly to neither of them nor to any other existing group of animals and it seems therefore preferable to establish for them an independant phylum.

PENTASTOMIDA are typically heteroxenous parasites. In the most evolved species the adults live in the respiratory tract of carnivorous animals, mainly snakes and carnivores, and the larvae develop in the tissues of various mammals.

The development in the intermediate hosts is very long and takes generally several months. It comprises a series of molts, the term of which is the tertiar larva of nymph which remains encysted in the tissues of the host, generally in the peritoneal cavity. It seems that in some cases this nymph is able to leave the cyst in which it is contained and to migrate through the tissues or the organs of the intermediate host. The nymphs may develop in different kinds of hosts. Many of these are accidental hosts that are not normally eaten by the definitive host. This polyxenism leads to an important waste of nymphs but it ensures to the species a very wide dispersal which is finally beneficient for its conservation. Some species of **PENTASTOMIDA,** mainly the most primitive ones, are probably able to undergo their complete life-cycle in the same host. This might be the case for the species that parasitize the insectivorous lizards. So far direct development in the **PENTASTOMIDA** has been established only for one species (*Sambonia lohrmanni*) that lives in the lungs of varans (Fain and Mortelmans, 1960). **PENTASTOMIDA** may produce lesions in man and in animals.

Lesions produced in man by Pentastomids of snakes

Parasitism of man by adult pentastomids is exceptional and it has been observed only for *Linguatula serrata*, a species that normally lives as an adult in the nasal cavity of dogs. Nymphal pentastomosis, on the contrary, has been reported on many occasions. It is particularly frequent in Central Africa but it is also known in other parts of the world. Man is not a normal host for the nymphs and human parasitism is therefore always accidental. Nymphal pentastomosis in man has been reported in connexion with several species of pentastomids. I am dealing here only with the species that live normally in snakes.

Genus *Armilifer* Sambon — The most important genus for man is *Armilifer*. It contains 3 species which all are able to produce nymphal pentastomosis in man:

1. *Armillifer armillatus* (Wyman): This species has been reported on many occasions in man. It is known only from tropical Africa but in these regions it is very frequent. The life-cycle has been elucidated by Broden and Rodhain (1908, 1909 and 1910) in Leopoldville. The adults live in the lungs of the large snakes such as the pythons and the vipers of the genus *Bitis*. The nymphs develop in all kinds of mammals including man. The degree of parasitism in the natural hosts may be very high. In a small antelope of Congo I found more than 5000 nymphs belonging to *A. armillatus*. All these nymphs were encysted in the peritoneal cavity. Monkeys may also be strongly parasitized. In man the nymphs are generally few in number but some heavy or very heavy infestations have been noted. The first human case has been related by Pruner in 1847. Since then many other authors have reported new cases. In some of the earlier papers the parasite was erroneously reported under the names *Porocephalus moniliformis* or *Armillifer moniliformis*. The nymphs are generally encysted in a thin and transparent cystic membrane. In some circumstances these nymphs escape from their envelope and become free. Some of them migrate through the tissues of the host. This nymphal migration seems to be frequent in animals but is rare in man. Chalmeras (1899) (in Sambon, 1922) reported a case in a negro who died in Accra: "Great number of parasites were observed moving freely in the abdominal cavity over the surface of the various organs, to which some were also observed to be attached." Mouchet (1914) found these non-encapsuled nymphs on different occasions in natives of Congo. He noted that two of these nymphs were attached by means of their hooks to the head of the pancreas, two others were free in the peritoneal cavity and one was free in a lymphatic vessel of the mesentery. So far it is not known with certainty if this escaping of the nymphs from their cystic membrane occurs during the life of the host or only after its death at the moment that, as a rule, the parasites pass from the intermediate to the definitive host. The distinction is very important for the migration through the tissues of the organs of the host may cause important lesions.

In man the encysted nymphs of *Armillifer armillatus* are commonly located in the peritoneal cavity. Most of them are encysted beneath the capsule of the liver or embedded in the superficial layers of this organ. They may also be found along the intestine, the mesentery or on other abdominal organs or tissues. More rarely they are encountered in other organs such as the lungs, the brain and under the ocular conjunctiva. In almost all the cases these nymphs were perfectly tolerated and pathological complications were very rare. In two cases they had produced important lesions that had finally produced the death of the patient. In the case described by Cannon (1942), the nymphs were extremely numerous and they had almost completely obstructed the large intestine. Bouckaert and Fain (1959) have observed a similar case in Congo but the nymphs were located along the hepatic angle of the large intestine and in addition there was a distinct inflammation of the peritoneum at the site of the nymphal masses.

2. *Armillifer moniliformis* (Diesing): The adults of that species are very common in the lung of Asiatic pythons. Nymphal parasitism in man has seldom been reported. This species has also been found once in the common *Python* (*P. sebae*) in Congo, but nymphs have never been found in that country.

3. *Armillifer grandis* Hett.: Nymphs of that species have been reported from the Water-Hen (*Porphyrio*).

Recently I have observed several cases of human pentastomosis produced by nymphs that I attribute to *Armillifer grandis*. So far it is the first-time that the nymphs of that species have been found in man. The nymphs were removed on the occasion of surgical operations from mesentery and the omentum of several natives in the Republic of Congo, province of Equateur (region of Flandria). They were encysted in these organs and apparently had produced no pathological lesions. These nymphs are distinctly smaller than those of *A. armillatus* and have more circular thickenings. In the female nymphs the anus is closer to the vulva than in *A. armillatus*, but however the two apertures do not open in the same depression as it is the case in the adults and probably also in the nymphs of the genus *Cubirea* (see Fain and Salvo, 1966).

Genus *Porocephalus* Humboldt — This genus is known only from America and Africa. Up to now human parasitism by either adults or nymphs of this genus has not observed with certainty. The cases reported by Sambon (1922) are doubtful and probably they were misidentifications.

Lesions produced in animals by Pentastomids of snakes

Little is known about the pathology caused by the pentastomids of snakes developing as larvae in the natural intermediate hosts other than man. I have never seen any inflammatory reactions in the animals parasitized by even very numerous nymphs of *Armillifer armillatus*. For instance I did not find any lesion in the antelope from Congo that was infested by more than 5000 nymphs. Pathological features have apparently not more been observed in intermediate hosts in relation with the other genera of pentastomids at least in natural conditions.

It seems that in some abnormal intermediate hosts, such as experimental hosts, pentastomids may produce inflammatory reactions. Esslinger (1962), studying the lesions produced in rats experimentally infected with *Porocephalus crotali*, found that the host reactions to the immature pentastomids follow patterns similar to those occurring with other agents of "*visceral larva migrans*". These lesions could be observed in the liver of the rats and they were strikingly similar to these which have been reported in human larval toxocariasis.

Lesions produced by Pentastomids in snakes

The lesions caused in snakes by the adult or the larval stages of pentastomids are not well known. The adult pentastomids are generally located in the lungs of the snakes but in some species (e.g. *Kiricephalus pattoni*) sexually mature specimens are regularly found in the dermis just under the scales. Studies of the tissues surrounding the parasites have shown no inflammatory reaction or tissue proliferation on the part of the host (Self and Kuntz, 1966). Some species of pentastomids are able to produce important lesions in the lungs of their hosts. In the genus *Cubirea* at least in the females the anterior part of the body, also called head, is distinctly separate from the rest of body by a thin neck. In all the specimens of *C. pomeroy* that I have collected this head was completely

enclosed in a fibrous pouch developed on the external wall of the lung. The opening of this pouch was very narrow and just large enough to give passage for the neck of the parasite. The rest of the body hanged freely in the lung cavity. The removal of the parasite was very difficult and needed a careful dissection of the fibrous pouch containing the head. It is well known that the infective nymphs arrive to the lung of the snake by direct penetration of the gut wall and lung tissue and not by migration up the oesophagus and passage into the lungs via the glottis and the trachea. This direct migration through the internal organs may cause perforation of blood vessels which may lead to the dead of the animal.

COMMENTED LIST OF **PENTASTOMIDA** PARASITIC SNAKES

PENTASTOMIDA are very common in snakes. This parasitism has been observed in all the continents but is particularly frequent in tropical regions. Not less than 26 species of **PENTASTOMIDA** grouped in 9 genera, have been found as adults in the lungs of snakes. They represent 5 families which belong to the two orders existing in the phylum (**CEPHALOBAENIDA** and **POROCEPHALIDA**).

Order **CEPHALOBAENIDA**: This order is the most primitive one. It comprises only one family (CEPHALOBAENIDAE), with two genera:

1. Genus *Cephalobaena* Heymons: This genus is represented by only one species *C. tetrapoda* Heymons, which parasitizes the lung of South-American CROTALIDAE (*Bothrops* and *Lachesis*) and COLUBRIDAE (*Leptophis*). The life-cycle is unknown.

2. Genus *Raillietiella* Sambon: This cosmopolitan genus is represented by about 20 species, half of them being parasitic in snakes, the other species living in lizards or varans.

In Africa 3 species have been described in snakes. The most common is *R. boulengeri* Vaney and Sambon. This species has been found as an adult in the lung of many kinds of snakes: BOIDAE, VIPERIDAE, ELAPIDAE and COLUBRIDAE. Completely developed nymphs have been found free in the lung of various snakes. They represent probably "migrating" nymphs coming from a prey swallowed some time before. Encysted nymphs, some of them being still in the moulting stage, have also been found but only in lizards. These hosts are probably the intermediate hosts for *R. boulengeri* (reported by Fain, 1964). The 2 other African species have been encountered only in one snake each. The first is *R. congolensis* Fain known from the lung of an undetermined snake in Congo, the other is *R. tetrapoda* (Gretillat, Brygoo and Domergue), described from a single male, apparently in the moulting stage, found in the lung of *Acrantophis dumerili* in Madagascar. In Southern Europe and in Asia there are 4 species of *Raillietiella* that parasitize snakes. The most common is *R. orientalis* (Hett). That species is closely related to *R. boulengeri* and it has also been found in different families of snakes: CROTALIDAE (genus *Ancistrodon*), ELAPIDAE (genus *Naja*) and COLUBRIDAE (genera *Coluber* and *Elaphe*). A nymph probably belonging to that species has been found free in the lung of an asiatic snake (*Tropidonotus maculatus*) (reported by Fain,

1964). The 3 other European or Asiatic species have been very seldom related they are: *R. mediterranea* (Hett) living as an adult in *Coluber*, *R. spiralis* Hett whose host is also a COLUBRIDAE (genus *Coelopeltis*) and *R. agcoi* Tub. and Masil, which parasitizes an ELAPIDAE of the genus *Naja*. In South America there is only one species *R. furococerca* Diesing. It has been reported from BOIDAE (genus *Boa*), from CROTALIDAE (genus *Lachesis*) and from numerous COLUBRIDAE (genera *Coluber*, *Elaphe*, *Phrynonax*, *Spilotes*, *Drymobius*, *Rhadinaea*). The life-cycle of *R. furcocerca* is still unknown. In North-America the only reported species is *R. bicaudata* Heymons and Vitzhum. It lives as an adult, in COLUBRIDAE of the genera *Elaphe* and *Ophibolus*.

Order **POROCEPHALIDA**:

1. Genus *Sebekia* Sambon: All the species of that genus (seven, in total) live as adults in the lungs of crocodiles. The nymphs of one species (*S. oxycephala* Diesing have been encountered frequently in fishes and in various snakes (genera *Bothrops*, *Dimades*, *Heterodon*, *Eunectes*) and occasionally in lizards.

2. Genus *Leiperia* Sambon: The adults live in crocodiles, the nymphs of the South American species of the genus (*L. gracilis* Diesing) have been found mainly in fishes, and once in a snake.

3. Genus *Sambonia* Noc and Giglioli: The adults of the only known species (*S. lohrmanni* Sambon) live in the lungs of varans in Africa, Asia and Australia. I have shown that species may perform its complete life-cycle in the same host (Fain and Mortelmans, 1960). Self and Kuntz (1957) have reported this species from the lung of a snake in Solomon Is., but this record seems doubtful and needs confirmation.

4. Genus *Waddycephalus* Sambon: The only one good species described in that genus is *W. teretiusculus* Baird. It has been found as an adult in the lung of several Australian elapid snakes. It has also been reported from a COLUBRIDAE (of the genus *Elaphe*) in Hong-Kong. Encysted nymphs attributed to that species have been reported from an Australian elapid snake (genus *Pseudechis*). The life-cycle of that species in unknown.

5. Genus *Porocephalus* Humboldt: This genus is represented by species all living, as adults, in the lung of snakes. The nymphs are encountered in mammals, in snakes and in amphibians. Three species are parasitic in American Snakes. The first is *P. crotali* (Humboldt) which as an adult, is very common in the CROTALIDAE but only in the genus *Crotalus*. Encysted nymphs have been reported from numerous mammals especially in Brazil but it is not sure if all nymphs belonged really to that species. Another American species is *P. clavatus* (Wyman). The adults are met only in the lungs of BOIDAE of different genera *Boa*, *Eunectes* and *Epicrates*. Nymphs attributed to that species have been reported from marsupials. The third species is *P. stilesi* Sambon, living as an adult in CROTALIDAE (genera *Lachesis* and *Bothrops*) and in a COLUBRIDAE (genus *Helicops*). Encysted nymphs are reported

from snakes and lizards. These three South-American species are morphologically very close each other and some authors consider that there is probably only one species *P. crotali*, and that the two other species are synonymous.

Stiles (1891) has worked out the life-cycle of *Pentastomum proboscideum* Rud., (which corresponds probably to *Porocephalus crotali*) from a *Boa constrictor*. He described 4 larval stages which were recovered from laboratory infected mice. The life-cycle of *P. crotali* has been studied again by Pen (1942) and Esslinger (1962). In Africa two species have been reported. The most frequent is *P. subulifer* (Leuckart). It has been encountered as an adult in several genera of snakes VIPERIDAE (*Causus*, *Bitis*), ELAPIDAE (*Naja*) and COLUBRIDAE (*Mehelya*). Curiously enough that species seems to be able to become completely adult only in snakes of the *Mehelya*. Encysted nymphs are common in snakes (*Causus*, *Neusterophis*, *Elapsoidea*, *Psammophis*) rare in mammals (monkeys and galagos). Another African species (*P. benoiti* Fain) is known from an undeterminate snake probably a *Naja*.

5. Genus *Kiricephalus* Sambon: This genus is represented by 3 species living, as adults, in the lung of snakes. The life-cycle of that genus in still unknown. Two species are known only from America. The first is *K. coarctatus* (Diesing). The adults are encountered in several genera of COLUBRIDAE (*Coluber*, *Elaphe*, *Drymobius*, *Thamnophis*, *Tropidonotus*, *Ophibolus*, *Herpetodryas*). Nymphs and young adults have been found encysted in the subcutaneous muscles of *Elaps fulvius* from Guatemala and of the North-American *Elaphe melanoleucus*. A young male has also been found in a mammal *Mephitis mephitis* (**CARNIVORA,** MUSTELIDAE). The second American species is *Kiricephalus tortus* (Shipley) described from a COLUBRIDAE (*Dipsadomorphus irregularis*) in North-America. The third species of that genus is *Kiricephalus pattoni* (Stephens) which is only known from Asia, Australia and Madagascar. The adults are found in the lung of COLUBRIDAE (in Asia and Madagascar) and of BOIDAE (in Madagascar and Australia). Nymphs and young adults have been found in the subcutaneous tissues or in the walls of the stomach of snakes. Encysted nymphs have also been reported from frogs in Java. Self and Kuntz (1966) have found that *K. pattoni* may inhabit tissues even in sexually mature stages.

7. Genus *Armillifer* Sambon: This genus is known from Asia, Africa and Australia. It comprises 3 species, living all as adults in the lungs of large snakes mainly BOIDAE but also in some VIPERIDAE: *Armillifer armillatus* (Wyman) is the most common species of the genus. It lives as an adult in African pythons and in the large VIPERIDAE mainly *Bitis*. *A. armillatus* may also develop in smaller snakes (*Boaedon*, *Bothrophthalmus*) but it seems that in these hosts the parasite is not able to reach its complete maturity. Nymphs, encysted or free, are very common in many kinds of mammals including man. They have also been found, but very rarely, in prey birds (*Bubo africanus* and *Pernis apivorus*). Another African and less common species is *Armillifer grandis* (Hett). It has been found only in large VIPERIDAE, particularly in the genus *Bitis*. The snake that is the most frequently parasitized, at least in Congo, is *Bitis nasicornis*. Other hosts less frequent are *Bitis gabonica* and *Cerastes cornutus*. Encysted nymphs have been found in a Water-Hen of a Zoological Garden (Fain, 1961). Similar nymphs have been found recently in man in Congo (Fain and Salvo, in press). The third species of the genus

is *Armillifer moniliformis* (Diesing). The adults are frequently found in Asiatic and Australian pythons. It has also been reported, but very rarely, from Central African pythons.

Encysted and free nymphs have been found in many kinds of mammals.

8. Genus *Cubirea* Kishida: The two species, known in that genus are found only in Africa. Self and Kuntz (1957) have reported the presence of immature specimens of *Cubirea pomeroy* from a snake in Solomon Is. This record cannot be accepted without confirmation for the young specimens of that genus are not well known and they are very difficult to identify with certainty. *Cubirea annulata* (Baird) lives, as an adult, in the lung of different species of *Naja*. It has also been recovered from *Bitis gabonica* and *Bitis nasicornis*. Nymphs or (?) adults of *C. annulata* have been found in a Demoiselle Crane (*Anthropoides virgo*) and encysted nymphs attributed to that species have been reported from a Water-Hen (*Phorphyrio*). The other species is *Cubirea pomeroyi* (Woodland). It is very close to the former. The hosts belong to the genus *Naja*. Nymphs are unknown.

9. Genus *Gigliolella* Chabaud and Choquet: This genus is very close to *Armillifer*. There is only one species, *G. brumpti* (Giglioli). It is parasitic in the lung of BOIDAE in Madagascar.

Nymphs have been found in lemur apes and in TENRECIDAE.

10. Genus *Ligamifer* Heymons: There is one species (*L. mazzai*) which lives in Asiatic snakes.

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Discussion

L. D. Brongersma: "Is *Armillifer moniliformis* also present in Central Africa?"

A. Fain: "Yes, I found it in an African python in Leopoldville, but it is possible that that species has been introduced in Africa by means of Asiatic pythons of Zoological Gardens."

IV

PATOLOGIA DO ENVENENAMENTO E PREVENÇÃO DE ACIDENTES

PATHOLOGY OF ENVENOMATION AND PREVENTION OF ACCIDENTS

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22. THE DIAGNOSIS, SYMPTOMS, TREATMENT AND SEQUELLA OF ENVENOMATION BY *CROTALUS ADAMANTEUS* AND GENUS *AGKISTRODON*

NEWTON C. McCOLLOUGH and JOSEPH G. GENNARO

(*U.S.A.*)

For the past ten years the authors have had an intense interest in the effect of the *Crotalus adamanteus* and the *Agkistrodon piscivorus* venom on the soft tissues of the extremities of patients bitten in Florida. Dr. Gennaro carried out a large amount of experimental work and I, interested in the clinical portion of the study, took careful case histories of all patients who were involved in loss of major tissue in either amputation or slough. Dr. Gennaro's studies, largely through the tagging of venom and antivenom, came to some conclusions long before I did in the clinical study. The clinical and laboratory findings were parallel to a rather remarkable degree. This involved not only the envenomation and the symptomatology but also the results of treatment. The number of amputations in the clinical series amounted to 39 and the serious sloughs which led to severe disability amounted to 20 additional cases. By far the majority of the bites treated were those of *Crotalus adamanteus* and only a few of the *Agkistrodon piscivorus* were seen.

Epidemiology of the situation in Florida was taken up very seriously January 1st, 1962, by the Department of Health and a Venomous Snake Bite Committee appointed by the Florida Medical Association. The Department then began a Registry of Venomous Bites by snakes and these have been tabulated statistically each year. At the present time, in Florida, we have registered annually approximately 270 to 300 snake bites, all of which have not been definitely identified as to the snake; but the large majority of them were definitely venomous according to fang mark description and sequelae. The number of deaths has averaged about three a year. We have been fortunate insofar as a number of treating physicians took colored photographs of the patient consecutively until either the extremity was lost or recovery of partial function occurred.

We have concluded of course that the character of the venom, i.e. $L.D._{50}$ or the amount of the venom injected into the patient by the bite of either one of these snakes can only be estimated by analizing the gravity of the clinical picture. Then, and only then, can the proper treatment be carried out. For instance, the bite of a snake which delivers a relatively small amount of venom in a small child may be an extremely serious matter when in an adult it would not. So, the weight of the victim has a great deal to do with the therapeutic considerations as does the number of fang marks. The most important, however, are those signs derived from the clinical picture of the patient and the rapidity in the sequence of symptoms which leads one to evaluate the amount of the envenomation. Wood, Hoback, & Green began a classification of envenomation in their original article: Grade I encompassing only local symptoms. Grade II, local symptoms plus some

mild systemic symptoms. Grade III, severe local symptoms with moderate systemic symptoms. These gradations were drawn from their observation of bites of *Crotalus horridus* of the eastern and northeastern United States.

Dr. H. M. Parrish, who has done a great deal of work with this subject, added a very important classification, namely Grade 0, in which the patient has the fang marks but exhibits no local or systemic symptoms of envenomation. This simply is applied to the patient bitten by a venomous snake which has fixed his fangs in the soft tissue but has not delivered any venom. We have found this in our statewide studies to be a not uncommon situation. The accuracy of the snake, as far as injecting the venom at the moment of fang penetration, is often questionable. Dr. Gennaro and this author, in consideration of the severe local symptoms and systemic collapse of individuals bitten by *Crotalus adamanteus* enlarged this classification to include Class IV. We felt this necessary insofar as the *Crotalus horridus* which Wood, Hoback & Green were studying does not, by any stretch of the imagination, except in very rare cases, provide the severe clinical picture which appears in the slides we will show you today of *Crotalus adamanteus* bites in man.

From the clinical standpoint there is a curious variation in the venoms of *Crotalus adamanteus* bites from individual to individual. Whether this is the reaction of the patient to the venom, or whether the venom of the snake varies in these regards, we do not understand. We have seen the hemorrhagic features develop to the point that they resulted in death from bowel, bladder, subcutaneous, intraperitoneal and intrathoracic hemorrhage and yet, in others, the neurotoxic features are dominant: i.e. fasciculation, immediate severe weakness which is usually generalized and marked painful muscular cramping. In these particular patients who exhibit these neurotoxic symptoms, there may be very little evidence of the hemolytic picture and even the local swelling may not be as marked. These are some of the characteristics of the *Crotalus adamanteus* which seem to be akin to those found in the *terrificus* and to a lesser extent in the *Crotalus durissus*. From reading case reports from the western part of the United States, and studying the number of amputees which have been reported in these areas, it is our feeling that there may be a tendency in the *Crotalus atrox* and others in the western and south western states for the neurotoxic factors to be more prominent than the hemolytic and proteolytic factors. The typical bite of the *Crotalus adamanteus* has a mixture of both, the locally destructive predominating. The onset is immediate and progression rapid. I will not attempt to discuss the fact that the secondary tissue products of the hemolytic and proteolytic factors magnify and add to both the systemic and local clinical situations.

In treatment of Grade III and IV bites, I will point out to you in the very beginning, that the intravenous route utilizing the polyvalent antivenin is the only way that antivenin should be administered. The four hours or more required for intramuscular absorption is a waste of time. Both experimentally and clinically we agree with Jackson, who did the original work, in 1926, that incision and suction for 30 minutes is beneficial if properly carried out. The use of a complete tourniquet we have never been able to prove is of any value unless you are intent on saving the patient at the risk of damaging the extremity. The incisions should be very small, one to two millimeters in length, and can be cruciate or single incisions across the orifice of the fang mark. They merely slightly enlarge the fang puncture, should only go through the skin itself, and can be classified as

percutaneous. They open the orifice of the fang mark to the point that suction is more effective. A larger or deeper incision will make suction less effective because of bleeding and may result in deeper spread of the venom.

If the local swelling is classified as a Grade I bite, there is a question as to whether antivenin is necessary at all. If no sensitivity is demonstrable, the local and systemic evidence of envenomation of Grades III and IV are best abolished by intravenous injection of antivenin in 250 to 500 c.c. of saline. If an anaphylactic reaction occurs it will be as prompt and severe as if serum is administered intramuscularly. In fact, if such a reaction occurs after one cubic centimeter is given by vein, it can be stopped and proper therapy administered by the same vein. To the contrary 10-15 c.c. buried in muscle cannot be restrieved and the treatment of anaphylactic shock rendered more difficult.

We have collected a series of cases, which does not exceed more than four or five, in which the intramuscular or local injection of antivenin apparently had no effect on the precipitous downward course in both the bitten extremity and the vital signs of the patient. When large amounts of antivenin are given intravenously, and we have used up twelve to sixteen ampules in a single instance over a period of two hours, with a total of twenty, the change in the patient is noted in a matter of two or three hours. The pulse settles to a reasonable level, the blood pressure rises, and the patient becomes alert and cooperative. No patients treated in this manner have failed to respond. We are firmly convinced that the amount of antivenin administered should be in excess of that needed to precipitate the venom injected by the snake and one can never estimate this until he recognizes the improvement previously mentioned. At this time there can be a reasonable waiting period before further antivenin is given. We have always administered rather massive doses of antivenin when confronted with what we considered to be a critical situation and we, apparently in all of the cases, have covered the venom injected to the point that there was never any sign of reversal of the clinical picture, i.e. dominance of the venom over the antivenin injected.

You will note the type of incision that we have recommended for the relief of tension which is blocking circulation to the distal portions of the extremity. These leave intact most of the skin and the subcutaneous fatty layer of fascia with its network of blood vessels, and permit it to act as a dressing over the deep longitudinal fascial incisions which release the inner tension of the muscles. It is the deep fascia which is the harmful constrictive agent in these instances, not the skin. If longitudinal incisions of the skin correspond anatomically to the longitudinal incisions of the deep fascia, the muscle would burst through the wound in a massive herniation and more tissue would be lost. There is some gentle compression which results from the skin as it holds back the herniation of the muscle in the method described. This is really beneficial control of edema and can't be controversial. We have also noted that there is an escape through these small transverse incisions, in the skin and subcutaneous fascia, after deep fascial release, of a profuse amount of serosanguinous fluid from the extremity, which certainly plays a major part also in dimishing the internal tension which is blocking the efferent circulation to the hand or foot digits. Supportive therapy i.e. blood etc. is understood.

In conclusion, I would like to report that the epidemiological studies by the Snake Bite Committee of the Florida Medical Association, and the Florida Public Health Department, have reported annually in the past three years 250 to 300 bites per year. Two-hundred of these bites have been due to venomous snakes.

By far the majority are due to the *Crotalus* snakes. About one per cent of these bites have proven fatal. There are usually eight to ten coral snake bites per year and the remainder of the venomous snake bites are due to the *Agkistrodon piscivorus* and *contortrix*. We had one death last year from *Agkistrodon piscivorus* envenomation. The reports which have accompanied the bites of this snake show rather marked hemolysis and marked fibrinolysis with the clotting mechanism of the blood almost reduced to zero in severe cases. Fewer and fewer doctors in Florida are using cryotherapy and more are using intravenous polyvalent antivenin, with rather astonishing success. There have been no cases of anaphylaxis reported. Last year an AK (above knee) and then later a hip disarticulation was done on one patient who was treated with cryotherapy for eighteen successive days due a bite of a *Crotalus adamanteus* in the lower extremity. It is our mutual conclusion that cooling or icing has no place in treatment. Six coral snake bites were treated with the antivenin from the Instituto Butantan with success. In each instance the antiserum was used intravenously. The reports of the program initiated by the Snake Bite Committee serves as a guidepost to the treatment and is also a source available to the practicing in management. Most of the bites in the past year reached hospital care within one-half hour of the time of the bite.

It has been my pleasure and a great honor to bring you this small bit of information from Florida and the southern part of the United States; but I must say that 98% of our statistics and case collections have originated within the State of Florida.

Discussion

A. Delgado: "Please, I would like to know which should be the precise indications and advantages for making transversal incisions in the arm or leg bitten, (if even doing so the patient will probably lose the member according to the evolution shown by the slides)."

N. C. McCollough: "The transverse skin incisions through which longitudinal deep fascial releases can be easily accomplished may readily save all or a large part of the limb. This is really vascular surgery in so far as it opens collapsed arteries."

H. Bicher: "Did the speaker observe cardiovascular failure in his patients? And if so, did the transversal section help to this situation?"

N. C. McCollough: "I did not understand this question fully, but stated we felt there was a definite cardiotoxin."

F. Kornalík: "Have you any evidence about the blood-coagulation changes in patients bitten by *Agkistrodon contortrix* or *A. piscivorus*."

N. C. McCollough: "Yes, in one or two cases the coagulations properties of the blood in patients bitten by this snake were markedly reduced."

P. J. Deoras: "It is necessary to make an incision at the site of bite when this kind of measure may not be useful as a first aid?"

N. C. McCollough: "Yes, we feel it increases the efficiency of suction. As described, it is not harmful."

A. do Amaral: "In your opinion (or Dr. Gennaro's) which is the maximum (record) amount of venom secured from a full-grown specimen of *C. adamanteus*?"

N. C. McCollough: "About 600 mg dried weight."

A. do Amaral: "Has Dr. Gennaro ever used enzymic medication (hyaluronidase) against the oedema producing effect of the venom hyaluronic acid, when used in connection with specific antivenin."

N. C. McCollough: "Not that I know of."

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23. THE INFLUENCE OF SNAKE VENOMS OF FIBRINOGEN CONVERSION AND FIBRINOLYSIS

F. KORNALÍK

University Karlovy, Praha, C.S.S.R.

The studies on the influence of snake venoms seem at the present time to be a special branch of the coagulation theme. Most of the work has been devoted to the procoagulant action of the venoms. Far less numerous are the papers dealing with anticoagulant properties of snake venoms.

In some of our previous works we were able to show, that the *in vitro* anticoagulant action of this second group is at least partially due to the destruction of prothrombin. However the role of the fibrinolytic properties of these venoms still remain to be elucidated. In this respect the most active proved to be those from *Agkistrodon piscivorus, Agkistrodon contortrix* and surprisingly also the venom of *Vipera lebetina.* The results obtained indicated that the fibrinolytic compound of these three toxins are mutually very similar as regards the quality of their action and differences are merely of quantitative nature, the venom of *Agkistrodon piscivorus* being the most potent.

The first approach to the estimation of fibrinolytic properties of any substance is the classical method of the native and heated fibrin plates, where plasminogen has been destroyed by the heat. We can see the lytic areas produced by 0.1% solution of the toxins (Fig. 1). *N = native plates, H = heated. In the center trypsin in 0.01% for comparison.*

At first one would conclude that the difference indicates the presence of the plasminogen activating system in the toxins. That was also our interpretation of these findings till we had the opportunity to work with purified plasminogen.

Plasminogen has been added both to the fibrinogen solution of which the plates have been made and/or it had been incubated with the venoms before dropping them on the plates. In neither case there was any significant difference in the lytic areas produced by venoms with or without plasminogen, i.e. the latter was not activated by the venoms (Fig. 2).

FBG + PLG = plasminogen added to the fibrinogen solution,

TOX + BLG = plasminogen incubated with toxins, N native plate.

If the bovine fibrinogen solution is incubated with venoms it gradually loses its ability to be converted into fibrin by thrombin, i.e. it is either denatured or split-off. There is no significant difference in the course of this action if plasminogen incubated for 10 min at 37°C either with toxin or toxin alone is added to the fibrinogen solution. From all these results it can be concluded, that the venoms have practically no plasminogen activating properties (Fig. 3).

By addition of 0.01% solution of epsilon amino kaproic acid (EAK) fibrin clot lysis from the test tube wall is prolonged from 5 to 8 min. Without EAK the venom acts synergically with plasmin. This is reflected in the shortening of the lysis time to 4 to 3 min. Toxins alone would cause the clot lysis only after a considerably longer period (over 20 min.), which corresponds to their own fibrinolytic activity. On the other hand toxins added to the test tube simultaneously with EAK are able, to a certain extent, to paralyse the inhibitory effect of a specific fibrinolysis inhibitor which EAK is known to be (Fig. 4).

Correlation between the venoms, plasmin and epsilon amino kaproic acid in lysis time of a fibrin clot

The dynamism of clot formation and its lysis can be very well observed through the method described by Gruedlinger in which the clot formation and lysis is assessed by ploting changes of the turbidity (measured photometrically at 350 μm) of the tested system against time. This method is definitely more accurate and therefore we have used it to ascertain the fibrinolytic properties of the venoms — plasmin mixtures and their blocking by various inhibitors of the proteolytic enzymes. We can observe that the venoms are able to paralyse the inhibitory effect of soya-bean inhibitor (SBI), Trasylol (TRA) and EAK (Fig. 5).

Synergic action of venom with plasmin and restriction of the inhibitory effect of various inhibitors by venom

To get an idea about the quantitative relations between the lytic activities of toxins, plasmin and trypsin and about the influence of the different inhibitors exerted on these enzymes an arbitrary unit has to be established. For this purpose the turbidimetric method was rather time consuming and therefore we have used the fibrinogenolytic properties of these active substances.

From the curves in Fig. 6 we can see that the fibrinogenolytic activity of 300 U/cc of plasmin can be compared in case of *Agkistrodon piscivorus* venom with 0.1 mg/cc and in case of *A. contortrix* venom with 0.25 mg/cc. Adhering to these quantitative relations, we have added to the tested system various inhibitors in different concentrations. Both plasmin and trypsin are inhibited by 0.01% EAK, 0.001 SBI, 250 U/TRA, whereas even hundred times stronger concentration of these inhibitors had practically no effect upon the fibrinogenolytic activity of venoms (Fig. 6).

Quantitative relation between plasmin & venoms and different inhibitors

We were further interested if the split products which result from the action of venoms upon fibrinogen are of a nature similar to those produced by the lytic action of plasmin, at least if there is an antithrombin VI activity which is attributed to the polypeptide D.

As can be seen in Fig. 7 fibrinogen split products of both lytic agents have been added to fibrinogen and thrombin solution and the increasing turbidity indicated the course of fibrin formation, i.e. the activity of thrombin. From the curves we can see that in case of snake venoms a considerable activity of antithrombin VI is present within split products of toxin fibrinogenolytic action (Fig. 7).

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Antithrombin VI activity of split products produced by action of snake venoms upon fibrinogen

Beside the fibrinolytic properties the tested venoms are known to possess a fair amount of proteases. It was of interest to compare the proteolytic activity of the venoms with the lytic activities of plasmin and trypsin and to find out how the toxin proteases are affected by inhibitors. Proteolysis has been assessed by a slightly modified method of Anson Mirsky using casein as substrate. We can observe that the caseinolytic proteases of the venoms are not inhibited by inhibitors, unlike trypsin, which is (Fig. 8).

Action of different inhibitors upon venom proteases and trypsin

To be at least partially sure that both the activities, fibrinolytic and proteolytic could be attributed to the same enzyme we had to isolate the active substances from the venom. After several attempts, using different separative procedures the most convenient method proved to be starch gel electrophoresis. From the curves showed in Fig. 9, we can see that both these activities always go along. The lytic fractions had no other enzymic activities. They were roughly 50 times less toxic than the whole venom and the lytic enzymes are 150 times more concentrated than in the whole venom, as could be computed from the protein content. These results are similar in all the three venoms. We were nevertheless not able to separate completely the haemorrhagins from these lytic fractions. Besides the proteolytic enzymes there is a different enzyme — the esterase spliting TAME — present in the venoms. This activity does not go parallel with the protease. Both these results are in full agreement with the findings obtained recently by Japanese authors for fractions of Habu snake venoms (Fig. 9).

Starch gel electrophoretic patterns of *Agkistrodon piscivorus* venom and the enzymic activities of the fractions

In the experiments *in vivo* we have injected sublethal doses of *Agkistrodon piscivorus* venom (400 μg/100 gr) subcutaneously into white rats. In these animals the routine blood coagulation check-up has been performed in time intervals of 30 min. 2 h. and 24 h., respectively. Except for a slight hypercoagulability of the whole blood in the first 30 min. there were no significant changes in cloting time of plasma in experimental animals compared with the control group.

The only pronounced difference was in the activity of euglobulin fraction in animals 30 min. after toxin application. Surprisingly the fibrinogen content was not altered in the sense of a decrease. On the contrary we could observe a significant rise in fibrinogen content in animals 24 h. after application. Both these findings can, in our opinion, be attributed to the stress reaction caused by toxin (Fig. 10 and 11).

By means of a 10% solution of formaldehyde in 60% alcohol applied in rats jugular vein an artificial thrombosis can be produced. 24 h. after operation on the average in 60% of so treated animals a thrombus can be found. The same amount of venom (400 μg/100 gr) has been injected in the animals 10 h., before or 10 h. after the operation. The toxin increased the amount of incidence

of thrombosis from 60% to 100%. This is probably caused by the action of the haemorrhagins exerted on the vessel wall rather than by the rise in the fibrinogen content.

It was surprising that venoms, which have *in vitro* a remarkable fibrinolytic activity are in animals unable to produce any change in their fibrinogen content, this being so even in amounts far exceeding those which have been used in experiments *in vitro*. It indicated the presence of a specific inhibitor in the blood which could inactivate the venom fibrinolysins. From the fig. 12 we can see that this seems to be the case. Fibrinogenolytic activity of both the most potent venoms i.e. *Agkistrodon piscivorus* and *A. contortrix* practically ceases in the presence of sera from different animals, human being included (Fig. 12).

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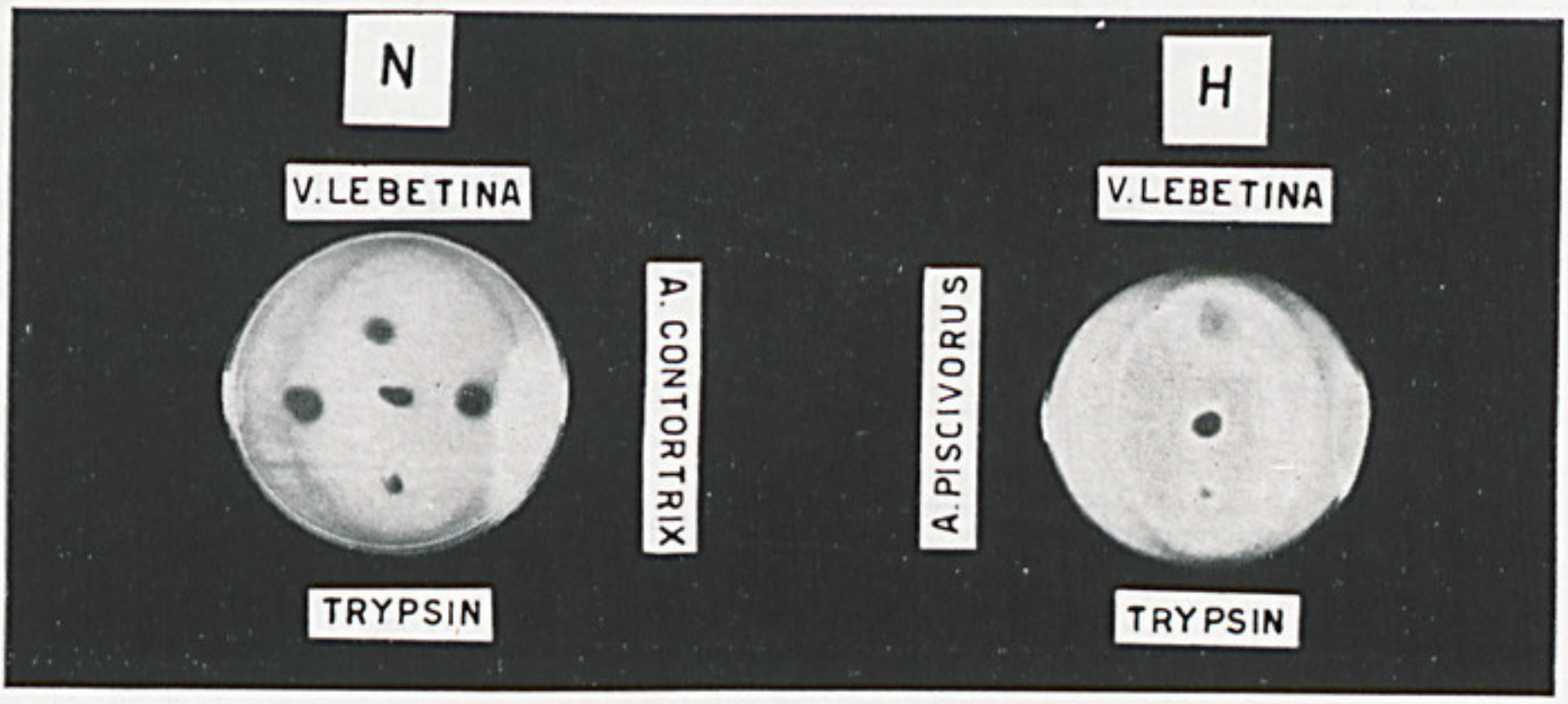


Fig. 1

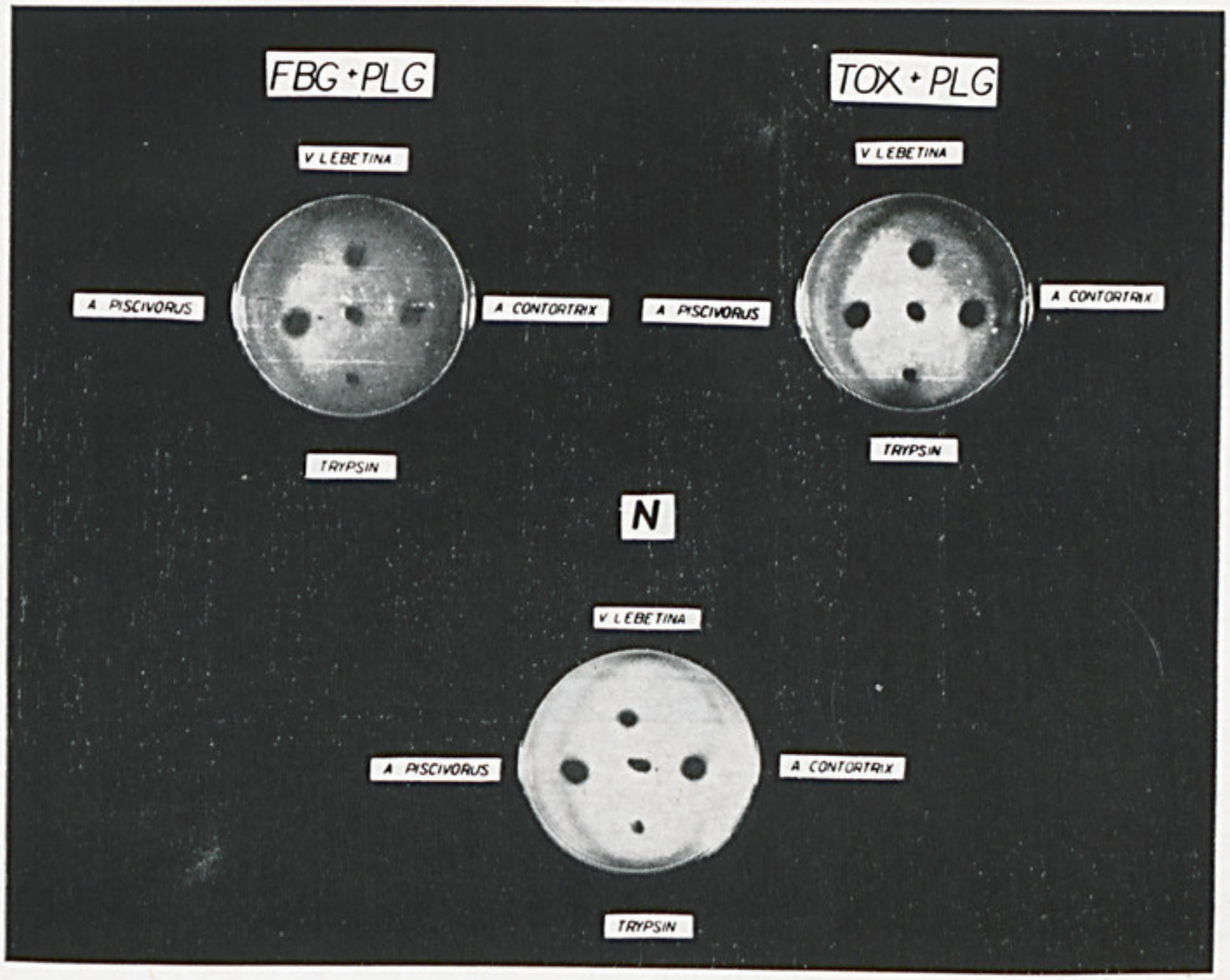


Fig. 2

Fibrinogenolysis by toxins and plasminogen

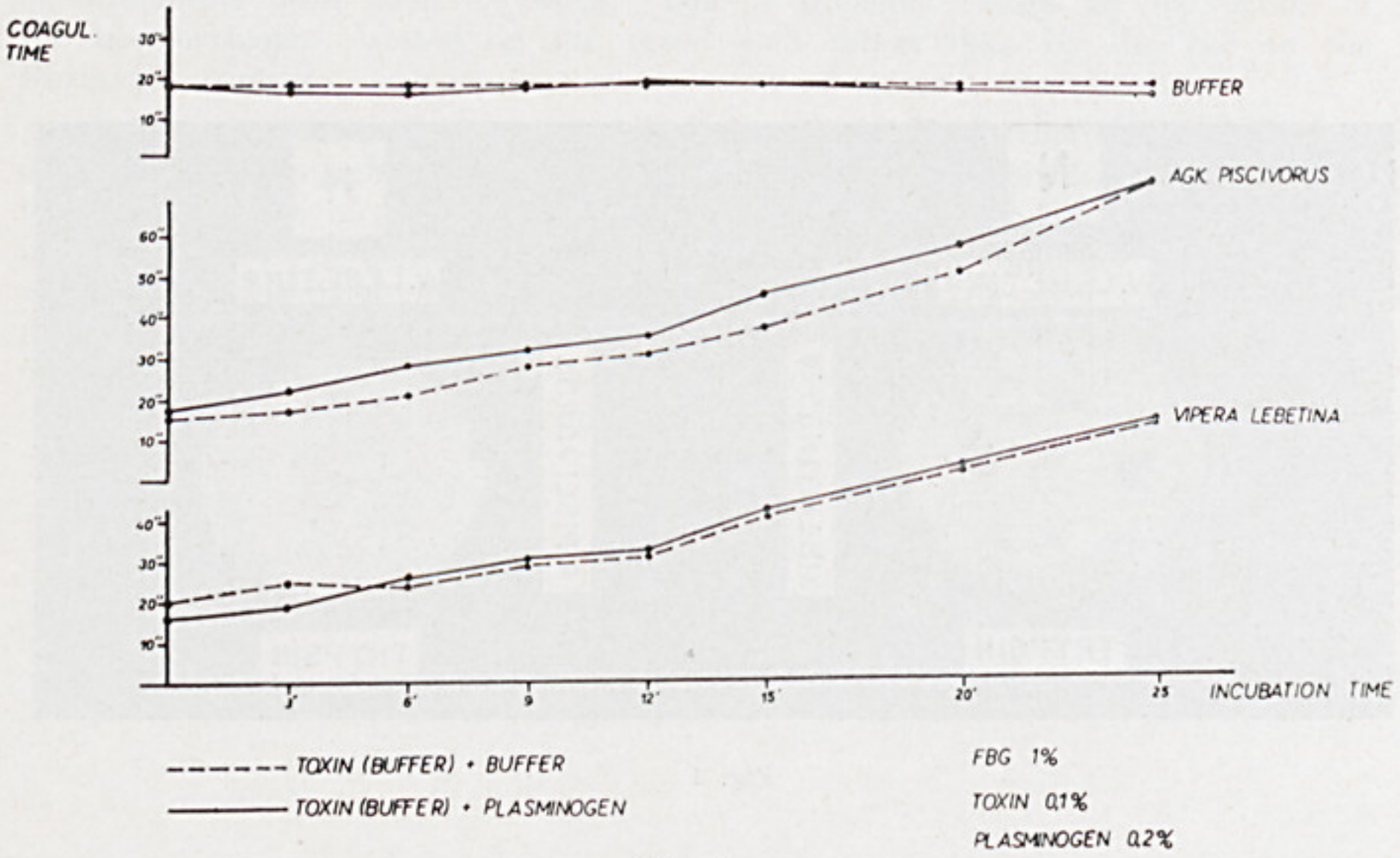
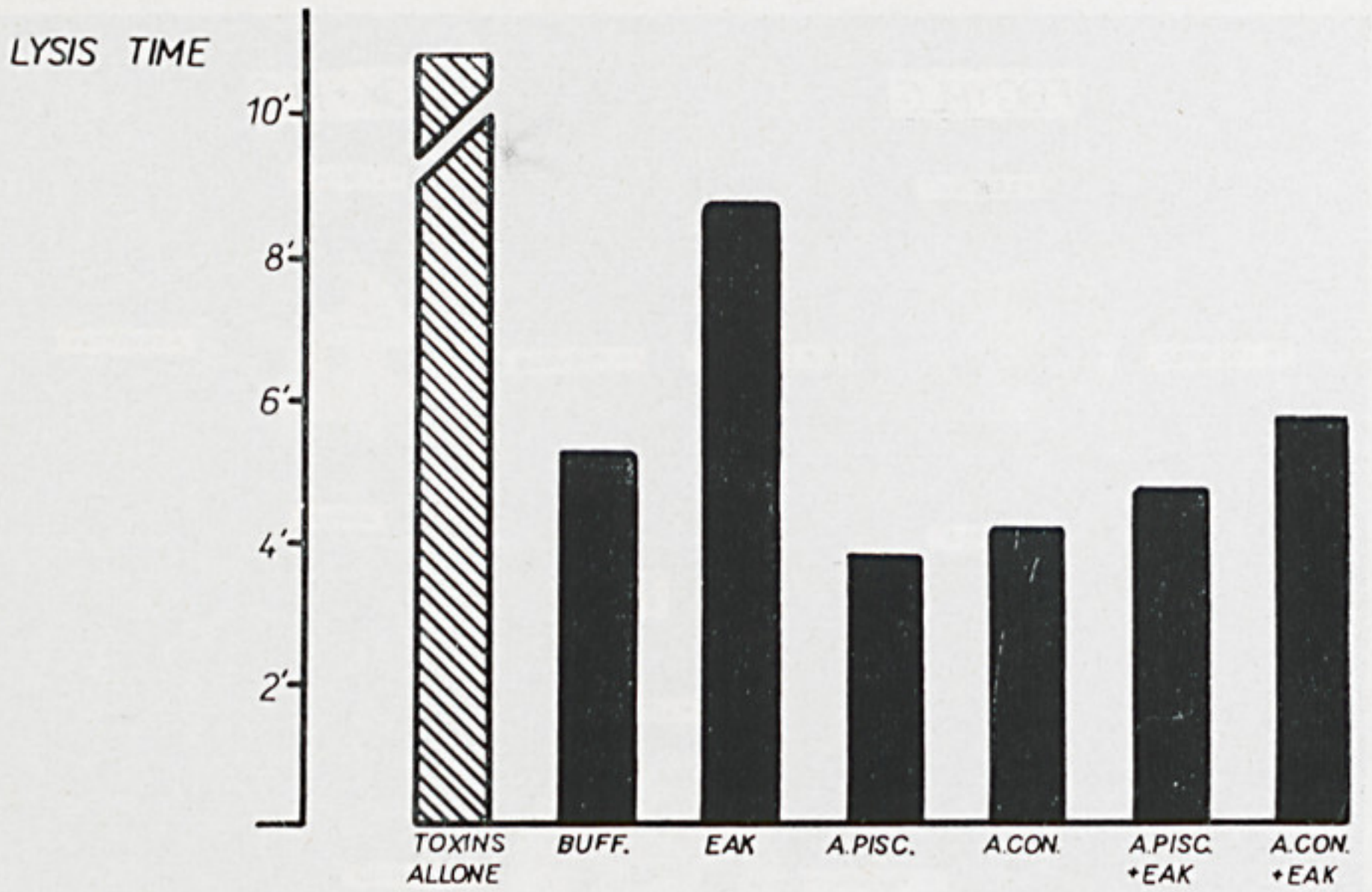


Fig. 3



2% FBG 0,2cc
0,2% PLM 0,1cc
100% cc STK 0,1cc
10% cc THRB 0,1cc

0,01% EAK 0,1cc
0,1% TOXIN 0,1cc

Fig. 4

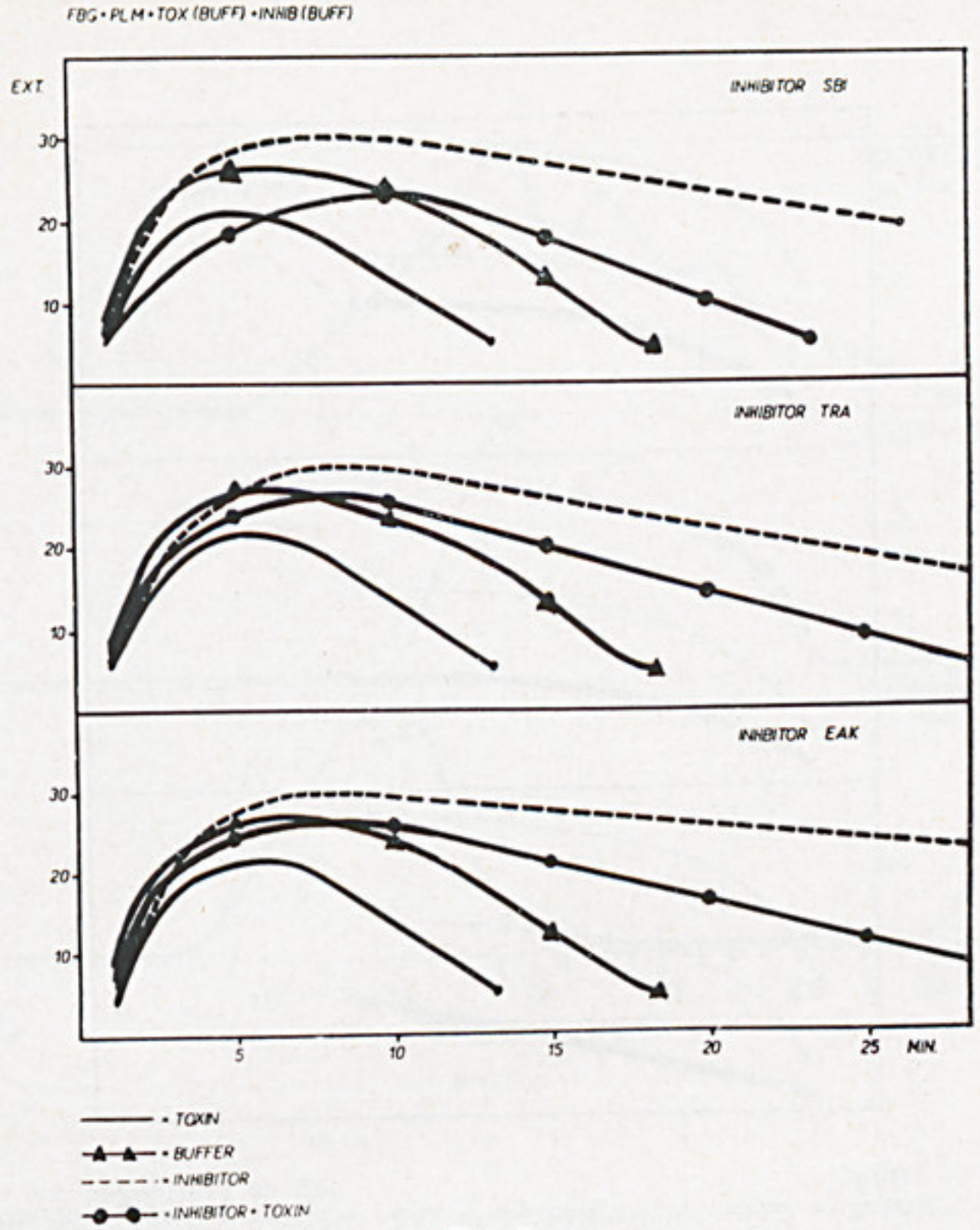

FBG • PLM • TOX (BUFF) • INHIB (BUFF)
EXT.
INHIBITOR SBI
INHIBITOR TRA
INHIBITOR EAK
30
20
10
5
10
15
20
25 MIN.
• TOXIN
• BUFFER
• INHIBITOR
• INHIBITOR • TOXIN

Fig. 5

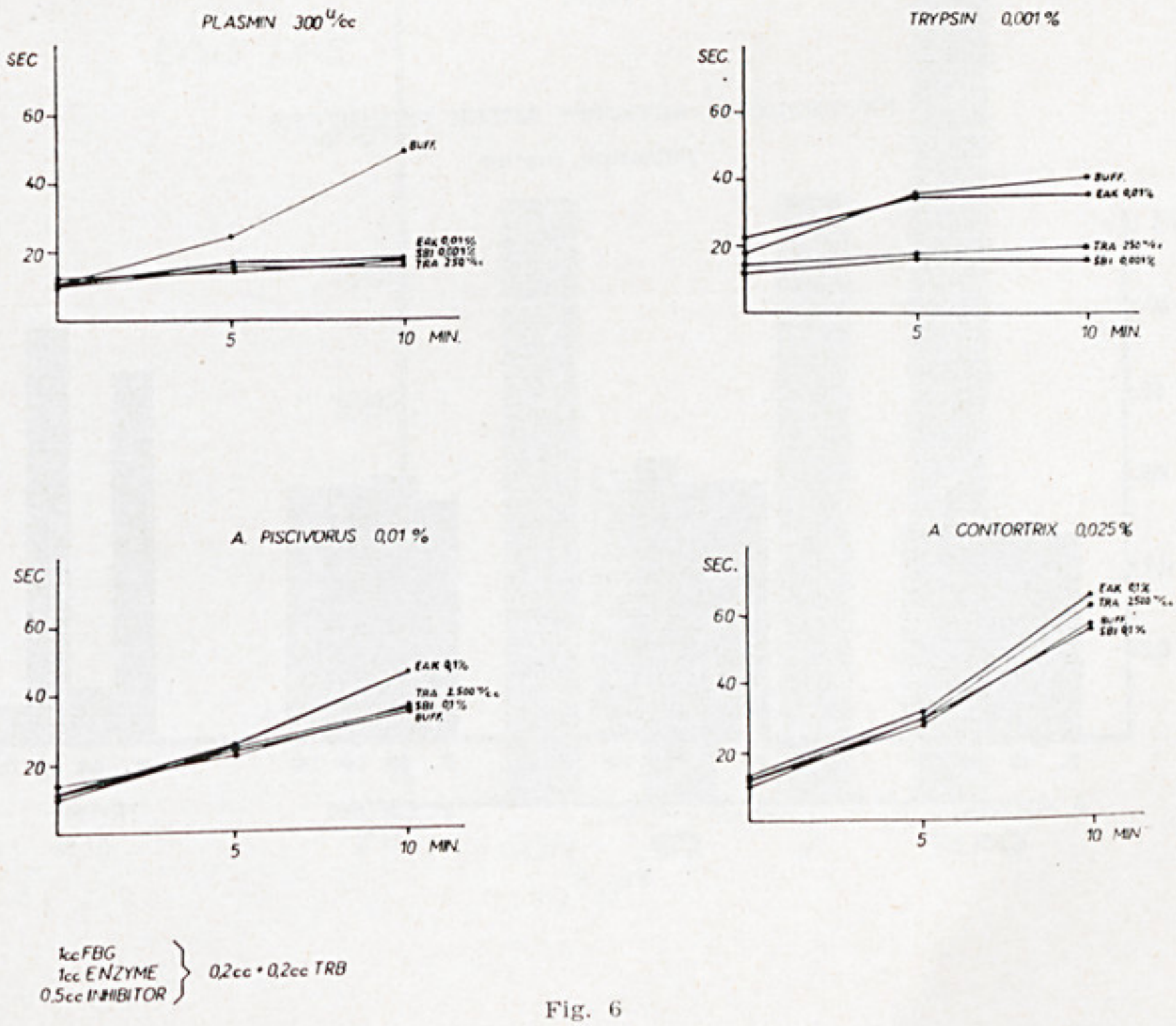
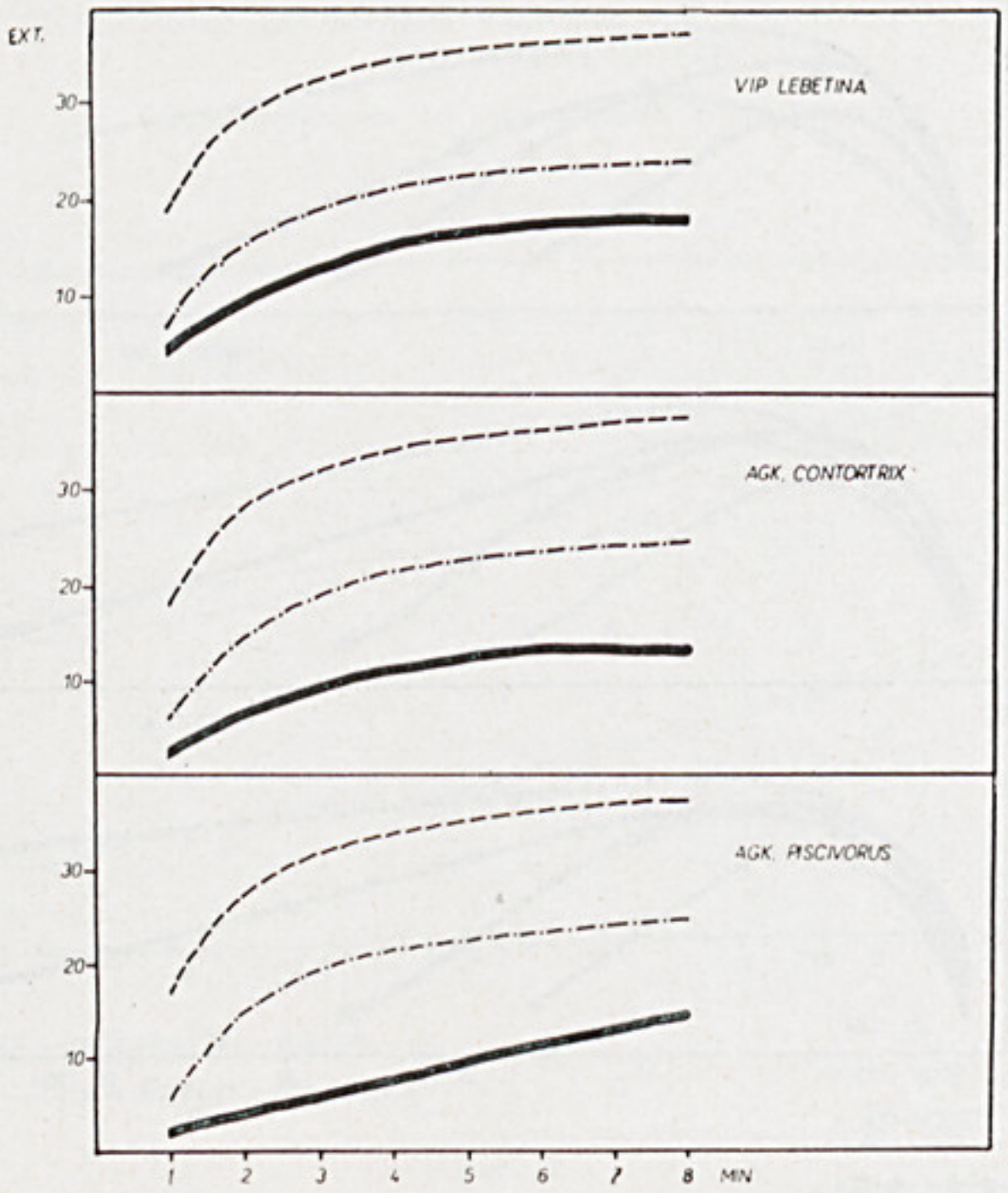

PLASMIN 300 U/cc
SEC
60
40
20
BUFF.
EAK 0,01%
SBI 0,001%
TRA 250%
5
10 MIN.
TRYPSIN 0,001 %
SEC.
60
40
20
BUFF.
EAK 0,01%
TRA 250%
SBI 0,001%
5
10 MIN.
A. PISCIVORUS 0,01 %
SEC
60
40
20
EAK 0,1%
TRA 2500%
SBI 0,1%
BUFF.
5
10 MIN.
A. CONTORTRIX 0,025 %
SEC.
60
40
20
EAK 0,1%
TRA 2500%
BUFF.
SBI 0,1%
5
10 MIN.
1cc FBG
1cc ENZYME
0,5cc INHIBITOR
0,2cc • 0,2cc TRB

Fig. 6



— — — Blank — 1,5 cc Fibrinogen 1%
—.—.— PLM + FBG (= Antithrombin VI) — 1,0 cc Antithrombin VI
▬▬▬ Toxin + FBG (=Antithrombin VI) — 0,2 cc Thrombin (10 μ/cc)

Fig. 7

PROTEOLYTIC ACTIVITY AFTER INHIBITORS

Substrate caseine

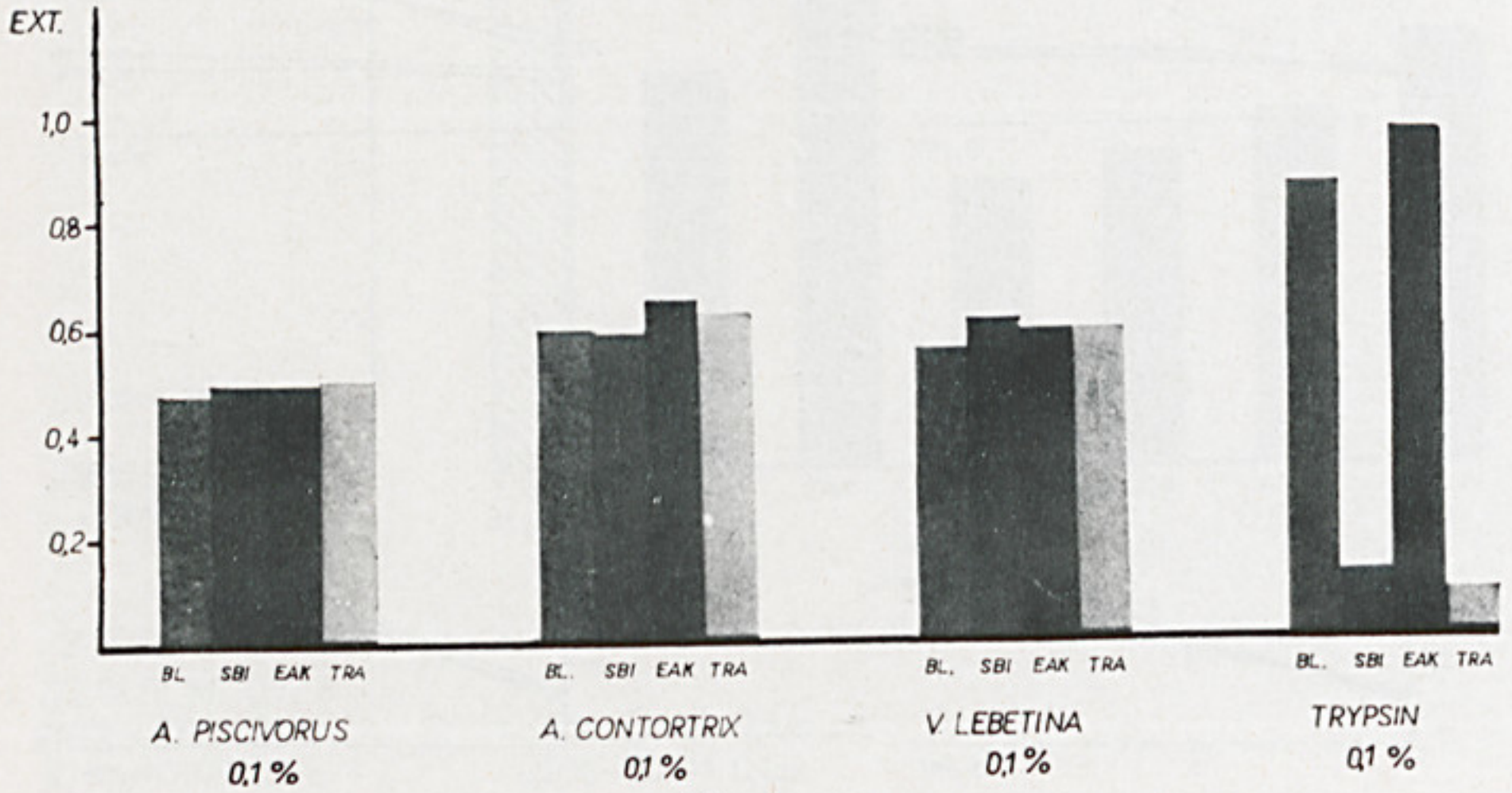


Fig. 8

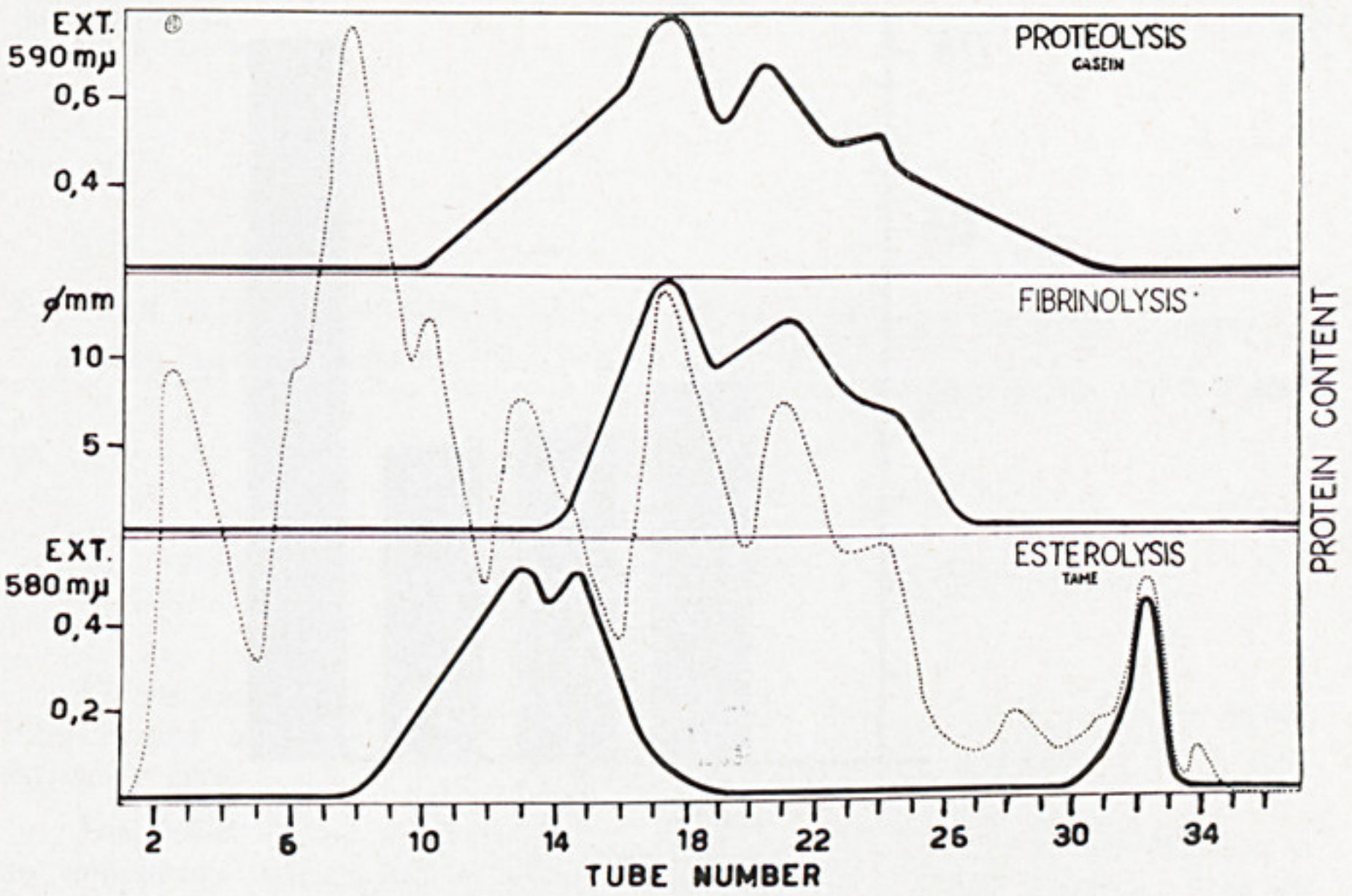


Fig. 9

Euglobulin fraction in rats after 4 mg/g of *Akgistrodon contortrix* venom

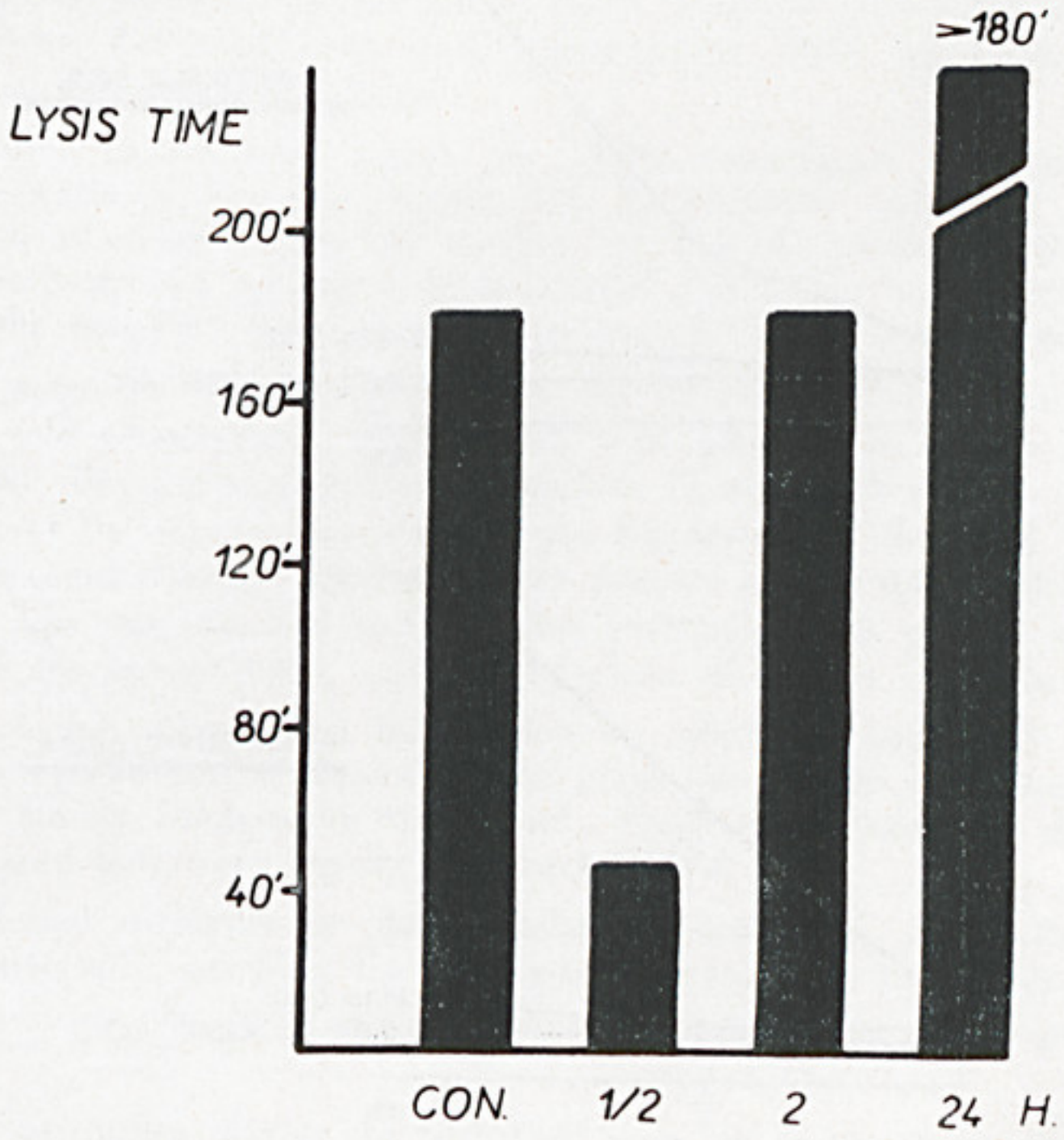


Fig. 10

FBG after *Agk. piscivorus* toxin

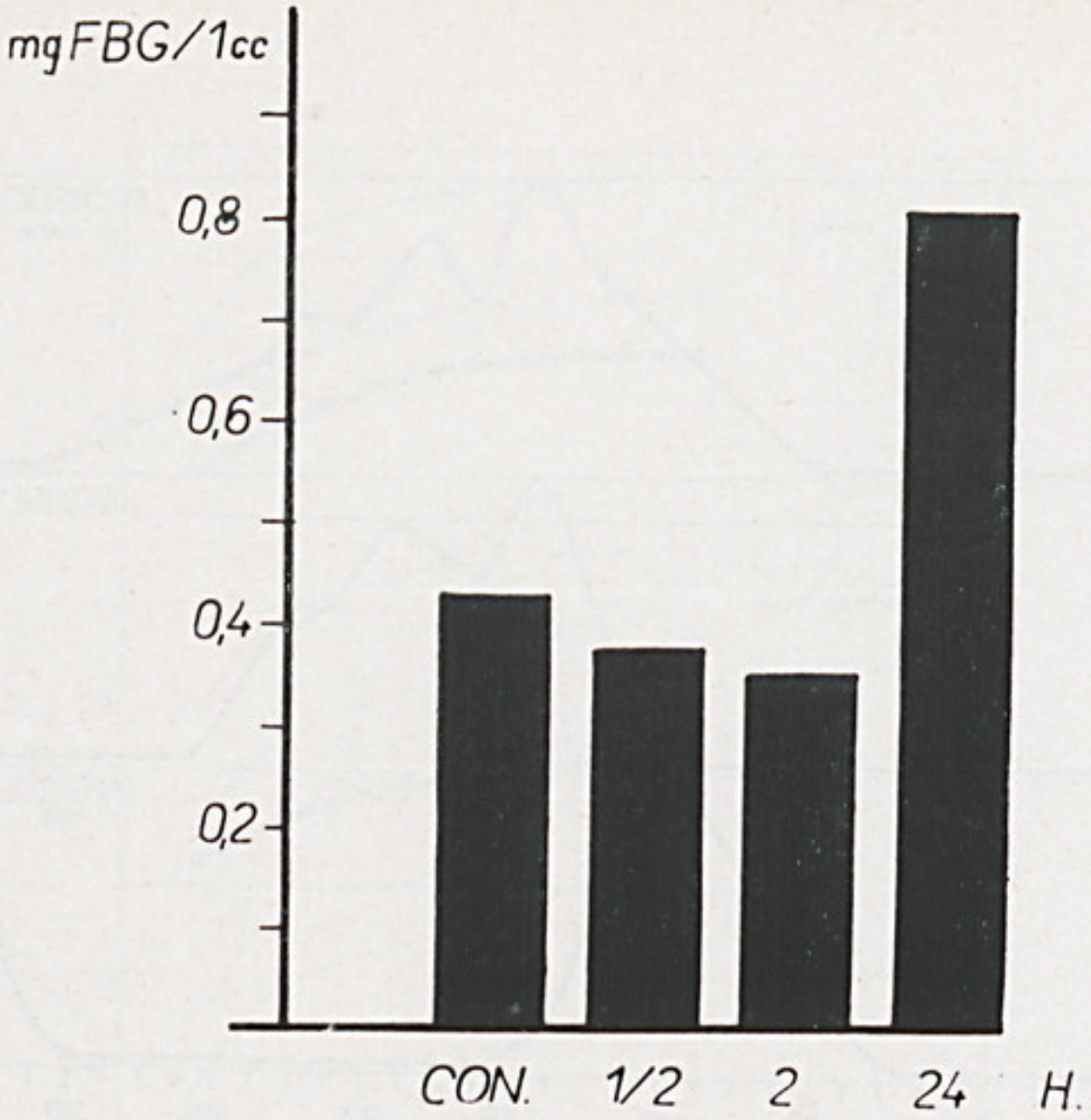


Fig. 11

Serum inhibitors

Fig. 12

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24. CLINICAL MANIFESTATIONS OF SNAKE BITE BY *VIPERA XANTHINA PALESTINAE* (WERNER) AND THEIR PATHOPHYSIOLOGICAL BASIS

P. EFRATI

Department of Medicine, Kaplan Hospital, Rehovot, Israel

Vipera xanthina palestinae is practically the only poisonous snake in Israel. Bites caused by other snakes, including *Echis colorata* (Guenther), have been extremely rare.

The actual annual incidence of snake bites in our country is not known, as no compulsory notification of cases is required. As a rough estimate, I would assume the incidence to be from 150 to 250 cases per year. For the last 25 years I has under my personal observation 21 cases of viper bite admitted to two hospitals. Of this material I have investigated 15 severe cases for the clinical manifestations which correspond, as a matter of fact, to the natural course of poisoning, as no specific antivenin was given. Specific antiserum produced by Institut Pasteur, Paris, has been on the market since 1956 only. The treatment used was mainly anti-shock treatment.

Soon after having been bitten, the victim experiences local pain (15)*. Shortly afterwards he becomes overwhelmed by weakness (11) and restlessness (10). 15 to 30 minutes after the accident the patients vomits repeatedly (14), perspires profusely and complains of abdominal pains (14) and diarrhoea (12). Sometimes the diarrhoea lasts several hours and the faeces becomes sanguinolent.

At this stage the victim usually reaches the hospital. He is pale, restless and covered by cold perspiration. Two patients were admitted in a state of clouded consciousness. Physical examination revealed in 11 of the 15 patients a conspicuous swelling of the tongue, regardless of the localization of the bite. Sometimes the speech becomes slurred. In 7 out of 15 patients angioneurotic oedema (Quincke) of the lips was observed and one (not included in the present series) lost consciousness for several hours, apparently because of oedema of the brain.

Seven of the patients had hypotension on admission and in 7 others the blood pressure could not be obtained. The pulse was usually weak, rapid or imperceptible. Rarely bradycardia was found. Fang marks could be detected in 11 patients, and they were usually not characteristic.

The affected extremity is often swollen on admission. Later the oedema advance centripetally, reaching the most proximal joint in 4 to 5 days. Often the oedema crosses the trunk and spreads up and down on the opposite side.

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* Number in parenthesis indicate the number of cases out of the 15 investigated ones, which manifest the symptoms.

Comparing the circumference of the affected extremity which that of the normal one, one can find differences of as much as 14 cm (at the proximal part of the thigh!).

Several hours after the bite had been inflicted, the swollen skin reddens in many places, representing subcutaneous haemorrhages and/or ascending lymphangitis. Later, blisters make their appearance differing in size and colour, containing plasma or blood. Usually they abound when the swelling becomes conspicuous. Some of them contain as much as 30-80 ml of fluid. On one occasion we removed 250 ml of blood from a single blister. Laboratory investigations often reveal signs of haemoconcentration, haemoglobin reaching 16-20 Gm% (11). Severe leucocytosis was found in 8 patients (15,000-34,000/cumm) and a slight one in others. In two cases of the series, thrombocytopenia (10,000; 90,000) was observed, and in two others fibrinolysis occurred. Half of the patients had albuminuria, disappearing subsequently.

Pathogenesis and pathological physiology

According to the literature, the viper venom, having a molecular weight higher than 20,000 is absorbed from the site of the bite through the lymph vessels into the circulation. The slow flow of the lymph enables the inactivation of a certain quantity of the venom "en route". On the other hand, a "spreading factor" possessing hyalonuridase-like activity favours the absorption of the venom. There is some clinical evidence supporting these assumptions. One can see for instance, lymphangitis and/or haemorrhages advancing along lymph to the regional lymph nodes. On post mortem examination there is sometimes a peculiar distribution of haemorrhages in the urinary bladder or in the uterus: they remain on one side of the mid-line.

Even small quantities of venom entering the circulation may cause generalized anaphylactoid reaction manifested by gastro-intestinal disturbances (vomiting, abdominal pains, diarrhoea), peripheral shock and angioneurotic oedema (type Quincke) of tongue, lips, glottis, brain, etc. Obviously, greater quantities of venom cause a more severe reaction. There is some experimental evidence suggesting that release of histamin from the tissues, initiated by the venom, might be the pathogenetic mechanism of this anaphylactoid reaction.

The primary anaphylactoid shock, is left untreated, passes readily over to secondary shock, owing to transsudation of plasma and bleeding into the tissues. Proteolytic enzymes — called haemorrhagins — in the venom increase the permeability of small vessels resulting in extravasation of fluid. Contraction of the blood volume perpetuates the failure of the circulation (shock). In addition, bleeding into vital organs (heart, lung, brain) may kill the patient.

The morbidity is also prolonged due to severe secondary anaemia. We have never encountered massive haemolysis causing anaemia. Complications in kidney function — shock kidney — occurred very rarely indeed. Because of the increased permeability of the small vessels, toxic substances may be resorbed from the intestines, causing liver damage.

No cases of disturbances in the function of the central nervous system were observed which could be ascribed to neurotoxic factors in the venom. Clouded consciousness, coma, restlessness, occurred together with manifestations of the anaphylactoid syndrome and cleared by treatment with gluco-corticoid hormones,

affirming — in this way — their anaphylactoid origin. In some cases there were disturbances in peripheral nerves located near to the site of the bite, apparently caused by direct local action of the venom. In a recent case transient aphasia occurred with positive Babinsky's sign, probably due to haemorrhage.

In a patient treated by us and in another one of a nearby hospital, radiculitis occurred as a part of a severe serum reaction.

Accordingly, we suggest the following essential treatment:

1. First-aid: complete immobilization of the affected extremity.
2. Adequate quantities of specific serum injected intravenously as early as possible.
3. Anti-shock treatment for shock and haemorrhages.
4. Hydrocortison for the anaphylactoid syndrome.

Treatment carried out as suggested has changed completely the clinical course, as well as the prognosis of viper bite.

Literature

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Discussion

E. R. Trethewie: "If the anaphylactoid shock is due to histamine one might expect this effect to be less in children, because the amount of histamine in tissue in children is less. Histamine release is proportional to the histamine content of tissue."

P. Efrati: "Nothing to answer."

H. I. Bicher: "Did the speaker consider a rôle for the neurotoxin, described in this venom, in the pathogenesis of his syndrome?"

P. Efrati: "The neurotoxic action of the venom was revealed only in mice poisoned by multiple lethal doses of venom and protected by antiserum. In human beings such a dose would cause death before neurotoxic symptoms could appear."

Mem. Inst. Butantan
Simp. Internac.
33(1):193-205, 1966

25. BIOCHEMICAL AND PATHOLOGICAL ASPECTS OF HEMORRHAGIC PRINCIPLES IN SNAKE VENOMS WITH SPECIAL REFERENCE TO HABU (*TRIMERESURUS FLAVOVIRIDIS*) VENOM

AKIRA OHSAKA, TAMOTSU OMORI-SATOH, HISASHI KONDO, SATORU KONDO and RYOSUKE MURATA

2nd Department of Bacteriology, National Institute of Health, Shinagawa-ku, Tokyo, Japan

Introduction

Hemorrhage is one of the most prominent symptoms following the bites by CROTALINAE or VIPERINAE snakes (1, 2). It was thought that certain proteolytic enzymes in snake venoms cause the hemorrhage (1-7).

We established a quantitative method for determining the hemorrhagic activity of the venom of *Trimeresurus flavoviridis* (8). We initiated systematic studies on the principles responsible for the hemorrhage by this method.

Evidences from our (9-12) and other (13-15) laboratories suggested the presence of more than one hemorrhagic principle in snake venoms. We fractionated the venom of *Trimeresurus flavoviridis* by zone electrophoresis and reported the presence of two hemorrhagic principles, that we designated as HR1 and HR2 (9). They are distinct immunologically from each other (16). Attempts were made to correlate the hemorrhagic activity with proteolytic activity, lethal toxicity, and other pathological activities (9-11, 17, 18).

The purpose of this presentation is to review our data and the data by others on the biochemical and pathological aspects of the hemorrhagic principles in the snake venoms.

Specification and quantitative determination of the hemorrhagic lesion

In order to approach biochemically the mechanism of the local actions caused by the venom it is essential (1) to specify the experimental conditions to reproduce a separate pathological change involved in the whole local pathological lesion and (2) to establish a method for determining quantitatively the specific principle responsible for each specified pathological change on the basis of the principle of bioassay.

We wish to express our gratitude to the Division of Public Health, Kagoshima Prefecture, Japan, for their generous gifts of Habu venom.

As for the hemorrhagic principles, these requirements were satisfied since we proposed the quantitative method for determination (8). The method consists of intracutaneous injection of 0.1 ml of venom into the depilated back skin of rabbits, measurement of size of the hemorrhagic spot after 24 hr from the inside of the removed skin and the estimation of the activity by the parallel line assay method.

As shown in Fig. 1, the hemorrhagic spot observed from the inner surface is sharply demarcated. A definite hemorrhagic spot visible from the inner surface was produced with such a small amount of Habu venom as 0.1 to 0.3 μg; while about 100 to 300 times as much dose was required to produce a feeble reaction, not clearly discernible from the outer surface (Fig. 2). The hemorrhage following the injection with such a large amount of venom as 300 μg spreads throughout the dermis and muscular layer, and necrosis of the muscle fibers was also observed (9). With such a large amount of venom, so-called "necrosis" (19) or "hemorrhagic necrosis" (20) was observable (8, 18).

When the results obtained by our procedures were analysed statistically, the log-dosage response curves obtained with a large number of crude venoms and their fractions were proved to be linear and parallel to each other (8). Some of the results are shown in Fig. 3. By measuring the potencies relative to that of a standard venom using the parallel line assay method, we succeeded in quantitative determinations of the hemorrhagic activities of various preparations of Habu venom. We concluded, therefore, that the size of the hemorrhagic spot in the skin as determined from the inside, but not from the outside, is an exact measure for the intensity of the hemorrhage under the specified conditions (8). We demonstrated that the method was applicable to venoms of different species of snakes.

Distribution of hemorrhagic, lethal and proteolytic activities in snakes venoms

We carried out comparative studies on hemorrhagic, lethal and proteolytic activities of venoms from various species. Lethal toxicity (9, 18) was assayed by intravenous injection into mice of an inbred strain weighing 14-17 g with four to five doses graded with 1.25-fold intervals. Death within 4 days was ascribed to lethal toxicity of the venom.

It is noted from Table 1 that hemorrhagic activity is distributed in all the venoms of both Crotalinae and Viperinae, although the ratios of LD_{50} to minimum hemorrhagic dose (MHD) especially for samples No. 5 (*Bothrops atrox*), No. 9 (*Crotalus durissus terrificus*) and No. 15 (*Vipera russellii*) are very small; almost none of the venoms of the Elapidae manifest hemorrhagic activity and therefore the ratios for such venoms are much smaller. The only exception is *Ophiophagus hannah* venom (sample No. 21) whose hemorrhagic activity is as high as that of Crotalinae or Viperinae venoms.

Macroscopic observation indicated the similarity of hemorrhagic lesions caused by venoms from different species of snakes. The common slope ($\bar{b}$) of log-dosage response curves for the hemorrhage is 4.72 with the venom of *Trimeresurus flavoviridis*. Values for b obtained with all the other venoms were approximately the same as that for *Trimeresurus flavoviridis*.

The results (Table 1) may be an indication that the hemorrhagic activity is not directly associated with the proteolytic activity or the lethal toxicity in the crude venoms.

TABLE 1 — DISTRIBUTION OF THE HEMORRHAGIC, LETHAL AND PROTEOLYTIC ACTIVITIES IN SNAKE VENOMS

Snake venom	Hemorrhagic activity MHD and its fiducial limits (μg)	Lethal activity LD_{50} and its fiducial limits (μg)	Proteolytic activity (units/mg)	LD_{50}/MHD
1. *A. contortrix contortrix*	1.90 (1.20-2.92)	200 (163-245)	33.7	105
2. *A. contortrix mokasen*	1.20 (0.76-1.90)	125 (102-153)	48.1	104
3. *A. piscivorus piscivorus*	0.80 (0.52-1.20)	60.0 (48.0-75.0)	41.5	75
4. *A. halys*	0.14 (0.09-0.22)	16.0 (13.0-20.0)	35.8	114
5. *Bothrops atrox*	2.11 (1.40-3.20)	5.6 (4.7-6.7)	44.5	2.7
6. *Bothrops jararaca*	0.75 (0.48-1.20)	18.5 (16.0-22.0)	74.0	25
7. *C. adamanteus*	0.04 (0.03-0.06)	18.5 (16.0-22.0)	9.76	462
8. *C. atrox*	0.43 (0.28-0.67)	45.0 (38.0-54.0)	83.6	105
9. *C. durissus terrificus*	18.0	3.6 (3.0-4.3)	39.2	0.2
10. *C. viridis viridis*	0.56 (0.37-0.85)	21.0 (17.0-26.0)	39.5	38
11. *T. flavoviridis*	0.20 (0.14-0.30)	54.0 (46.0-63.0)	33.0	270
12. *T. flavoviridis tokarensis*	1.15 (0.78-1.80)	160 (130-196)	37.8	139
13. *T. elegans*	0.30 (0.20-0.46)	71.0 (59.0-85.0)	13.0	237
14. *T. okinavensis*	1.38 (0.91-2.10)	140 (117-167)	69.0	102
15. *Vipera russellii*	21.0	2.2 (1.8-2.6)	5.56	0.1
16. *Vipera ammodytes*	0.47 (0.31-0.72)	7.4 (6.3-8.7)	41.7	16
17. *Vipera palestinae*	0.54 (0.34-0.92)	7.1 (5.9-8.5)	4.96	13
18. *Causus rhombeatus*	0.81 (0.53-1.30)	> 250	0.26	> 300
19. *Bitis arietans*	0.15 (0.10-0.22)	15.0 (13.0-18.0)	18.7	100
20. *Bitis gabonica*	0.04 (0.03-0.06)	13.5 (11.5-16.0)	11.7	338
21. *Ophiophagus hannah*	0.84	54.0 (46.0-63.0)	12.0	64
22. *Bungarus fasciatus*	» 100*	18.5 (16.0-21.0)	0.06	0.18
23. *Naja melanoleuca*	» 100	6.0 (4.8-7.5)	7.00	« 0.06
24. *Naja naja atra*	» 100*	8.0 (6.5-9.8)	0.12	« 0.08
25. *Naja naja*	» 100	5.6 (4.4-7.2)	2.85	« 0.06

A.: *Agkistrodon* C.: *Crotalus* T.: *Trimeresurus*

* A pinkish macule was observed at the site of injection.

Evidence for the presence of two hemorrhagic principles in certain snake venoms and for relationships of these principles to proteolytic, lethal and other pathological activities

The presence of more than one hemorrhagic principle in certain snake venoms has been suggested (9-16); attempts have been made to correlate the hemorrhagic activity to proteolytic activity (9-11, 14, 15, 18, 21).

We fractionated the venom of *Trimeresurus flavoviridis* by zone electrophoresis and demonstrated the presence of two hemorrhagic principles, HR1 and HR2 (9, 10), which are distinct immunologically from each other (16). Both of the hemorrhagic principles contained proteolytic activity (9, 10). CM-cellulose chromatography also indicated the presence of two hemorrhagic principles; one was separated from the main part of proteolytic activity, while the other associated with it (11, 14). Iwanaga and his associates (21) purified "proteinase b", one of the three proteinases present in the venom of *Agkistrodon halys* and stated that it is one of the two hemorrhagic principles in this venom.

It would be of much interest to clarify whether or not the principles responsible for hemorrhage also manifest lethal toxicity. We separated one of the hemorrhagic principles (HR2) from the main part of lethal toxicity but failed to separate the other principle (HR1) (9). Immunological studies of HR1 not yet published suggested that separate entities are responsible for the hemorrhagic activity and the main part of lethal toxicity. Separation of hemorrhagic activity from lethal toxicity was indicated also by Gitter and his associates (15) with the venom of *Walterinnesia aegyptia*. On the other hand, Omori and his associates (13) fractionated the venom of *Agkistrodon halys* by DEAE-cellulose chromatography and reported a close association of the major part of the lethal toxicity with the main hemorrhagic fraction.

It would be also of much interest to know whether or not one and the same principle is responsible for necrosis and hemorrhage (7, 18). Histological observations done by us (9) indicated that the muscle degeneration, which led to necrosis or death of the muscle fiber in its severer forms, did not run parallel to either hemorrhagic activity or proteolytic activity.

Further purification of the hemorrhagic principles is needed to correlate hemorrhagic activity with proteolytic, lethal and other pathological activities.

Purification of the hemorrhagic principles in the venom of *Trimeresurus flavoviridis*

We attempted purification of the hemorrhagic principles of *Trimeresurus flavoviridis* (Batch No. 64-A). The two hemorrhagic principles HR1 and HR2 (Step 1 in Table 2), separated by zone electrophoresis (9), were further purified by different procedures shown in Fig. 4.

Purification of HR1: Step 2 — A pooled fraction of HR1 (corresponding to 6 g of the crude venom) separated by zone electrophoresis was concentrated by lyophilization and dialysed against 0.005 M Tris-HCl buffer, pH 8.5. The solution at a concentration of 50 mg protein per ml was treated with solid ammonium sulfate to 60% saturation (369 g per liter) (22) and left to stand at 0° for several hours for partial settling. The precipitate was collected by centrifugation at 8,000 r.p.m. for 15 min. The precipitate was dissolved in 30 ml of 0.005 M Tris-HCl buffer, pH 8.5 at a concentration of approximately 65 mg protein per ml. Step 3 — The solution was passed through a Sephadex G-100 column (4 × 97 cm) previously equilibrated with the same buffer. Step 4 — The hemorrhagic fractions from Step 3 were combined and concentrated to about 14 ml by lyophilization. The solution was passed through a Sephadex G-200 column (5 × 115 cm) previously treated with the same buffer. Step 5 — The hemorrhagic fractions from Step 4 were combined, concentrated by lyophilization and

dialysed against 0.005 M Tris-HCl buffer, pH 8.5, the final volume being about 7 ml. The dialysate was placed on a CM-cellulose column (3 × 50 cm). The break-through fractions containing the hemorrhagic activity were combined. Step 6 — The combined fraction was concentrated by lyophilization and dialysed against 0.005 M Tris-HCl buffer pH 8.5. The dialysate of 7 ml containing 120 mg of protein was applied to a DEAE-cellulose column (1.5 × 30 cm). A linear gradient elution with 400 ml of the buffer and 400 ml of the buffer containing 0.5 M NaCl was started.

Purification of HR2: Step 2 — A pooled fraction of HR2 (corresponding to 6 g of the crude venom) separated by zone electrophoresis was concentrated by lyophilization and a final volume adjusted to 30 ml. The solution at a concentration of 37.5 mg protein per ml was passed through a Sephadex G-100 column (5 × 100 cm) previously equilibrated with 0.005 M Tris-HCl buffer, pH 8.5, containing 0.15 M NaCl. Step 3 — The hemorrhagic fractions from Step 2 were combined and concentrated to 40 ml by lyophilization. The concentrated solution containing about 15 mg protein per ml was passed through a Sephadex G-75 column (5 × 86 cm) previously equilibrated with 0.005 M Tris-HCl buffer, pH 8.5, containing 0.15 M NaCl. The hemorrhagic activity was eluted in the void volume. Step 4 — The hemorrhagic fractions were combined, concentrated by lyophilization and dialysed against 0.005 M Tris-HCl buffer, pH 8.5 for several hours. Twenty-six ml of the dialysate containing 467 mg of protein was applied to a DEAE-cellulose column (4.6 × 12 cm). After a break-through peak had been collected, elution was carried out with 500 ml of the buffer containing 0.3 M NaCl. The break-through fraction contained the hemorrhagic activity.

TABLE 2 — SUMMARY OF PURIFICATION PROCEDURES FOR HR1 AND HR2

Step	Protein (mg)	Hemorrhagic activity			Proteolytic activity	
		MHD (μg)	relative specific act.	recovery (%)	specific act. (units/mg of protein)	relative specific act.
Starting material	6,000	0.220	1.00	100	24.80	1.00
HR1						
1. Electrophoresis	3,670	0.255	0.94	55.0	6.55	0.26
2. Ammonium sulfate (0 - 60%)	1,890	0.259	0.86	27.2	8.26	0.33
3. Sephadex G-100	500	0.0825	2.69	22.2	14.41	0.58
4. Sephadex G-200	162	0.0367	5.95	16.3	16.20	0.65
5. CM-cellulose	120	—	—	—	—	—
6. DEAE-cellulose						
a fraction	14.4	0.0193	11.41	6.6	7.66	0.31
b fraction	22.5	0.0214	10.29		17.65	0.71
HR2						
1. Electrophoresis	1,125	0.220	1.00	18.8	74.10	2.99
2. Sephadex G-100	590	0.311	0.71	7.0	96.20	3.88
3. Sephadex G-75	467	0.312	0.71	5.5	129.00	5.20
4. DEAE-cellulose	333	0.294	0.75	4.2	128.00	5.17

The yield and the extent of purification at each step are summarized in Table 2. The hemorrhagic activity of HR1 was eluted from DEAE-cellulose into two peaks. The first peak of hemorrhagic activity (a-fraction in Step 6) containing less proteolytic activity than the second one served as the test sample of HR1 in the following experiment. The specific hemorrhagic activity of HR2 did not increase after Step 2 and the preparation in this step served as the test sample of HR2 in the following experiment.

The action of the hemorrhagic principles on animal cells cultivated *in vitro*

The crude venom of *Trimeresurus flavoviridis* and of the partially purified hemorrhagic principles, HR1 and HR2, were tested for toxic action on animal cells cultivated *in vitro* (17). The cell strains employed included HeLa cells, MLg cells originated from the lung of new-born mice of ddY strain and T5 cells originated from human embryonic fibroblasts.

The change of the cells observed in the earliest stage was rounding. When the rounded cells predominated, the cells became detached from the glass surface resulting in disruption of the cell monolayer. We designated the activity responsible for this change as the cell monolayer-disrupting activity (17).

Table 3 shows the effect of the crude venom and the partially purified hemorrhagic principles on the monolayer of T5 or HeLa cell. It is noted that HR2 with a high proteolytic activity was more potent in the cell monolayer-disrupting activity than HR1 with a low proteolytic activity.

As shown also in Table 3, HR1 at 30 μg protein per ml did not show any cell monolayer-disrupting effect within 24 hr but did show some effect after 48 hr. The cell monolayer-disrupting activity of HR1 on T5 cells is roughly one sixth that of the crude venom on the basis of protein content (Table 3). The specific hemorrhagic activity of HR1 is about 11 times higher than that of the crude venom (Table 3). Therefore, the cell monolayer-disrupting activity of HR1 per unit hemorrhagic activity is calculated as about one seventieth that of the crude venom. We conclude, therefore, that the hemorrhagic activity of HR1 is virtually independent of the cell monolayer-disrupting (or cytopathic) activity (17).

These results on one hand confirmed and on the other hand contradicted the suggestion made by Gaertner and her associates (23) that there is a close association of cytopathic with hemorrhagic and proteolytic activities.

Fig. 5 demonstrates the absence of parallelism between disruption of the MLg cell monolayer and cytocidal action by the venom preparations. The MLg cell monolayer was disrupted by the crude venom and by the preparation of HR2 but the disruption did not run parallel to the viability of the cells. It is, therefore, very likely that the venom preparations primarily act on the cell surface causing detachment of the cells from the glass surface but not causing serious damage to the vital function of the cells (17).

TABLE 3 — EFFECT OF THE CRUDE VENOM AND THE PARTIALLY PURIFIED HEMORRHAGIC PRINCIPLES ON THE MONOLAYER OF T5 OR HeLa CELL

Cell strain used	Incubation period(hr)	Venom preparations*								
		Crude (Hr=1.0 Pr=1.0)			HR 1 (Hr=11.4 Pr=0.3)			HR 2 (Hr=0.7 Pr=3.9)		
		30µg	10µg	5µg	30µg	10µg	5µg	30µg	10µg	5µg
T5	1.5	+++	-	-	-	-	-	+++	-	-
	3.0	+++	±	-	-	-	-	+++	+++	-
	4.5	+++	+	-	-	-	-	+++	+++	-
	24.0	+++	++	-	-	-	-	+++	+++	+
	48.0	+++	+++	++	++	-	-	+++	+++	+++
HeLa	1.5	+++	-	NO	-	-	NO	+++	-	NO
	3.0	+++	±	NO	-	-	NO	+++	+++	NO
	4.5	+++	±	NO	-	-	NO	+++	+++	NO
	24.0	+++	++	NO	-	-	NO	+++	+++	NO
	48.0	+++	+++	NO	+	-	NO	+++	+++	NO

−: No change (identical to the control cell culture)
±: Slight morphological changes of the cells without detachment from the glass surface
+: Rounding of the cells without detachment from the surface
++: Partial disruption of the cell monolayer
+++: Complete disruption of the cell monolayer
NO : Not tested
Hr : Specific hemorrhagic activity relative to the crude venom
Pr : Specific proteolytic activity on casein relative to the crude venom
* : Per ml of culture medium

Conclusion

We succeeded in reproducing experimentally the hemorrhage under our specified conditions. We also succeeded in establishing a quantitative method for determining the hemorrhagic activity. By this method we initiated systematic studies on the principles responsible for the hemorrhage.

We demonstrated that hemorrhagic activity is widely distributed in all the venoms of Crotalinae and Viperinae snakes. Evidences were accumulated to suggest the presence of at least two or more hemorrhagic principles in venoms of certain snakes including *Trimeresurus flavoriridis*. The venom of *Trimeresurus flavoriridis* was fractioned to correlate the hemorrhagic activity with proteolytic activity, lethal toxicity or other pathological activities.

However, further experiments will be necessary to characterize more precisely the principles responsibles for the hemorrhage.

ADDITIONAL LEGEND TO TABLE 1

Hemorrhagic activity was determined by the method previously described (8). The minimum hemorrhagic dose (MHD) was defined as the least quantity of venom causing a hemorrhagic spot of 10 mm in diameter 24 hr after the intracutaneous injection. Lethal activity was assayed by intravenous injection into mice of an inbred strain weighing 14-17 g with four to five doses graded with 1.25-fold intervals. The LD_{50} was calculated by the Reed-Muench method (24). The standard error of the LD_{50} was calculated according to Pizzi (25). Proteolytic activity was estimated at pH 8.5 with casein as substrate. One unit of the activity was defined as the amount of enzyme hydrolyzing casein at such an initial rate that the amount of TCA-soluble products formed per minute gives the same optical density as that of 1 μg of tyrosine with the Folin reagent.

The venom of *Crotalus adamanteus* was purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, U.S.A. The venom of *Bothrops atrox* was obtained from a supplier in Brazil. *Naja naja atra* venom of Formosan origin and *Bungarus fasciatus* venom were supplied by Dr. T. Suzuki of the Institute for Protein Research, Osaka University, Osaka, Japan and *Naja naja* venom by Dr. B. N. Ghosh of the University College of Science, Calcutta, India. Venoms of *Trimeresurus okinavensis* and *Agkistrodon halys* were obtained from a supplier in Tokyo. *Trimeresurus flavoviridis* venom was supplied by the Division of Public Health, Kagoshima Prefecture, Japan and *Trimeresurus elegans* venom by the Institute of Hygiene of the Ryukyu, Japan. The venom of *Trimeresurus flavoviridis tokarensis* was supplied by Dr. H. Fukushima of Kagoshima University, Kagoshima, Japan. All the other venoms were purchased from the California Corporation for Biochemical Research, Los Angeles, California, U.S.A.

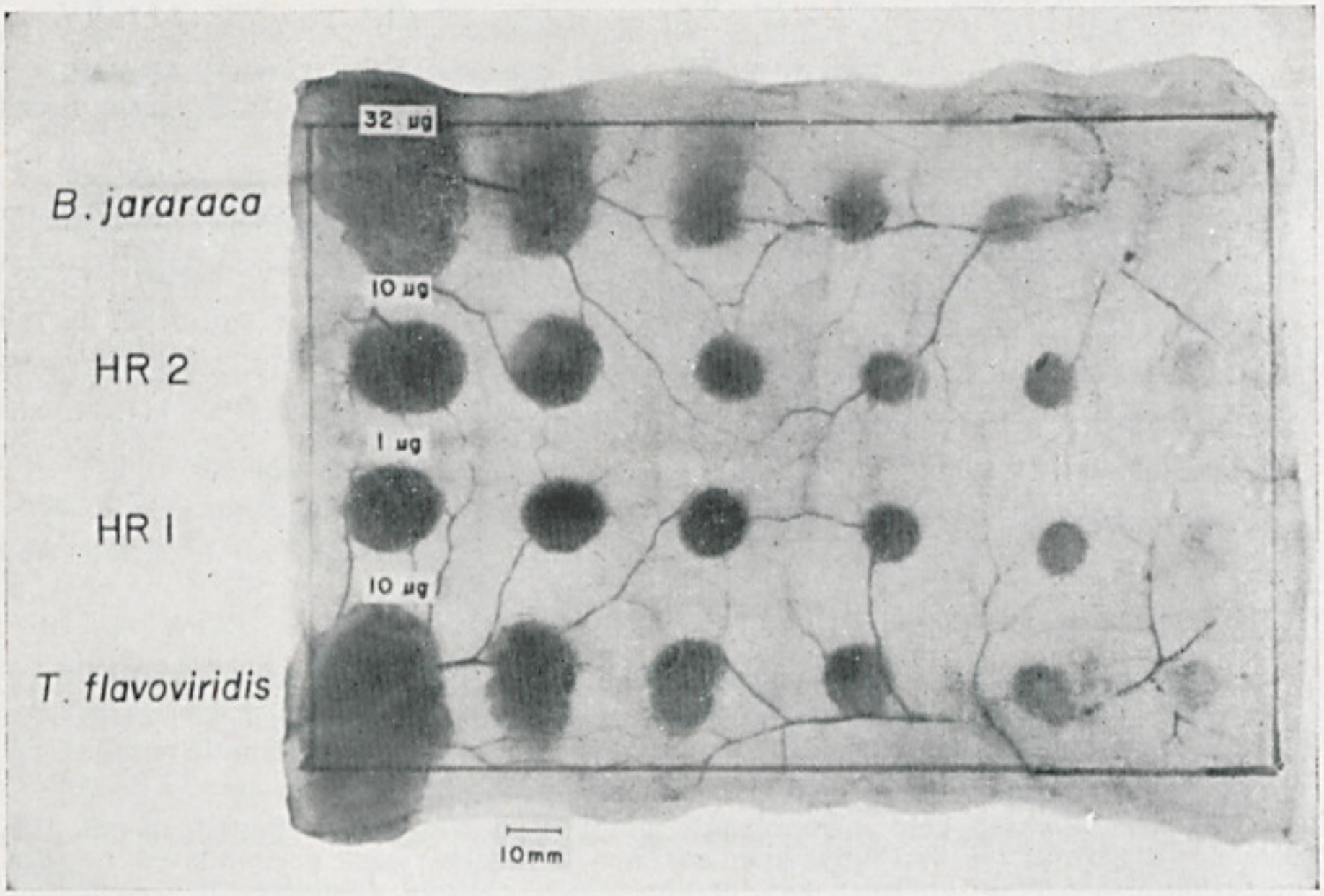


Fig. 1 — Patterns of hemorrhage observed from the inside of the removed skin. An aliquot (0.1 ml) from each of 3-fold dilutions of the venom of *Trimeresurus flavoviridis* or the partially purified hemorrhagic principles (HR1 and HR2) was injected intracutaneously into a rabbit and the reactions were observed after 24 hr. The venom of *Bothrops jararaca* was also injected for comparison.

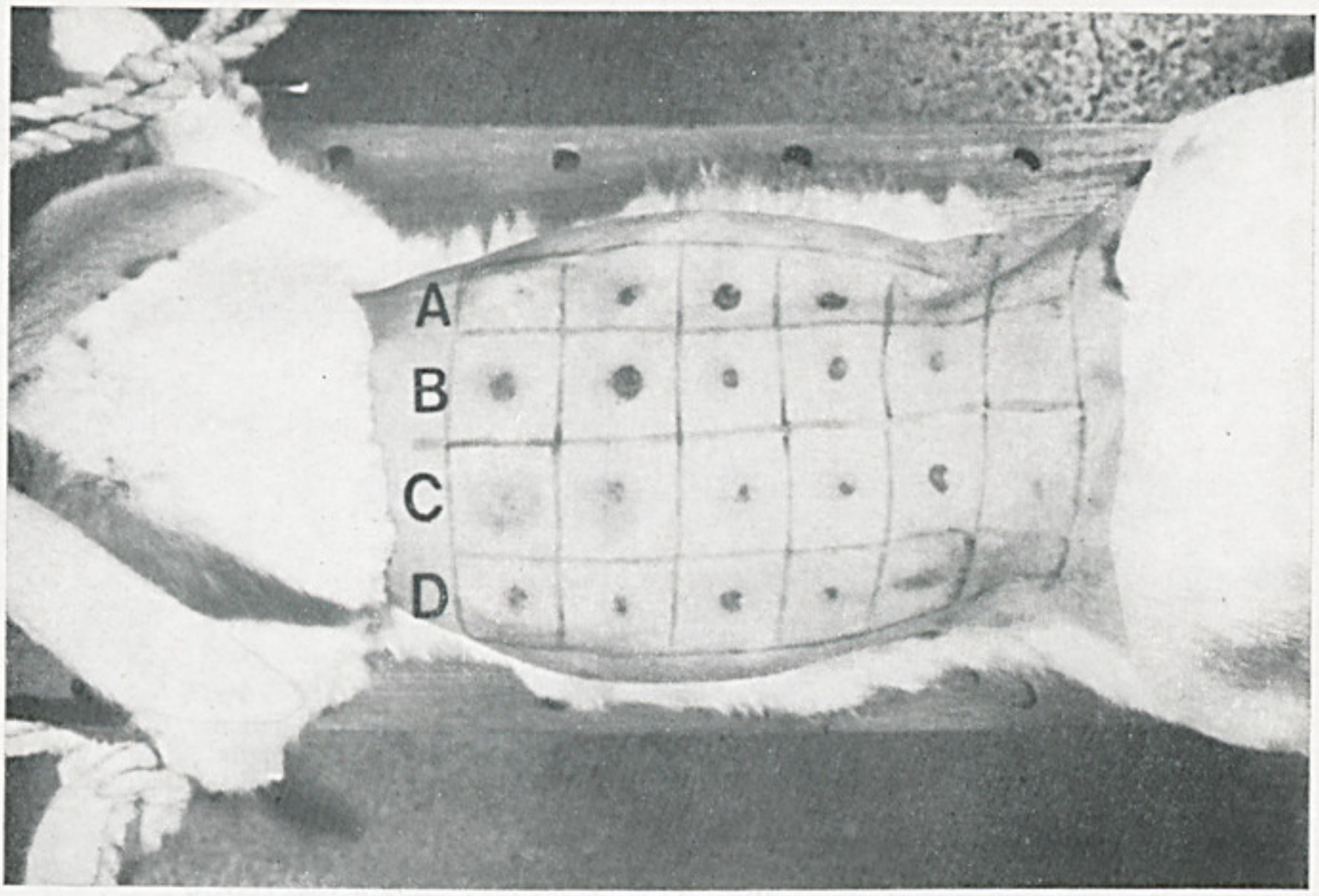


Fig. 2 — Patterns of hemorrhage observed from the outside. The skin is the same which is demonstrated in fig. 1. A: The venom of *Trimeresurus flavoviridis*; B: HR1; C: HR2; D: The venom of *Bothrops jararaca*.

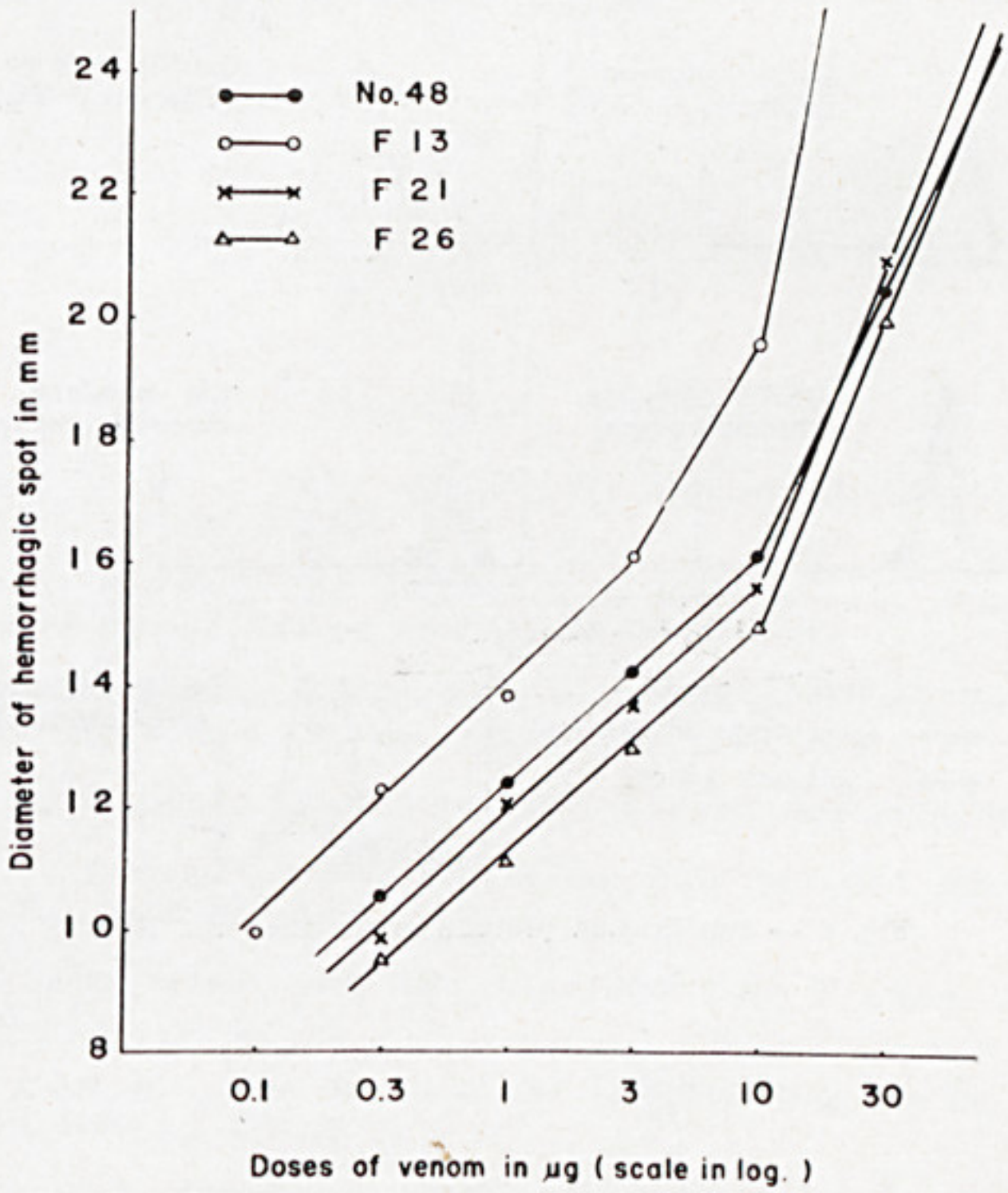


Fig. 3 — Dosage response curve for a crude venom (Batch nº 48) and its electrophoretic fractions (F 13 and F 26).

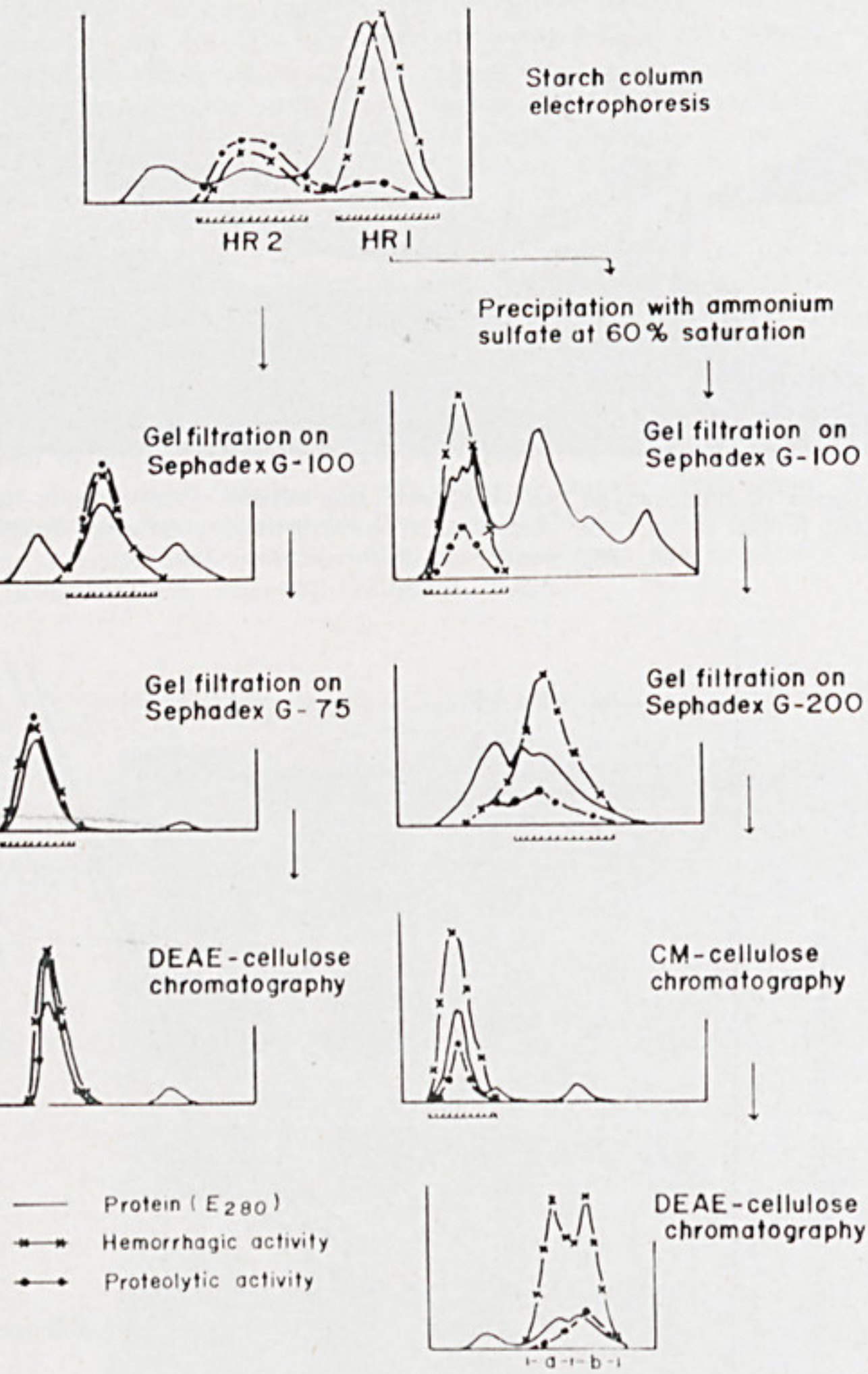


Fig. 4 — Purification procedures for HR1 and HR2.

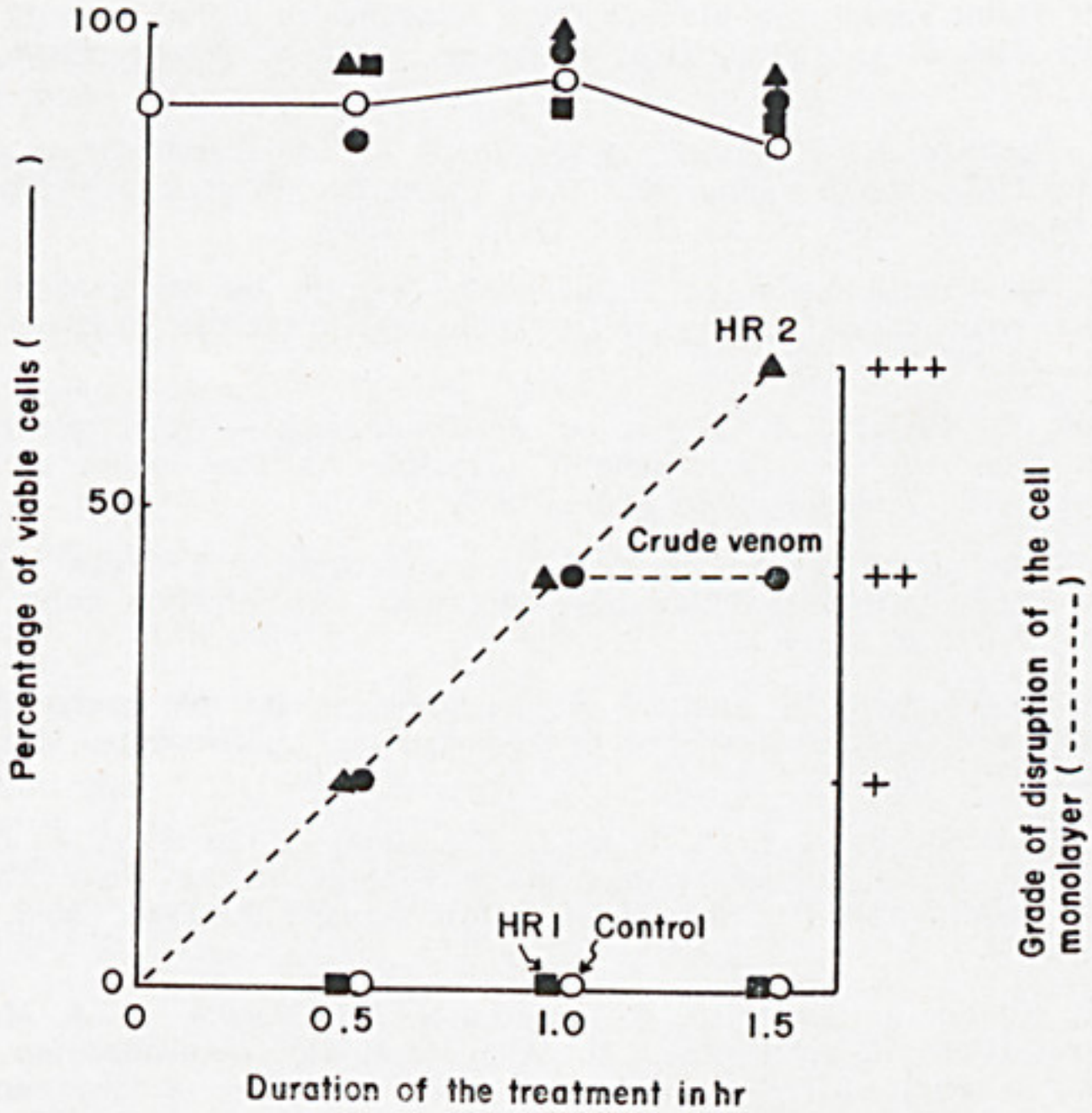


Fig. 5 — Disruption of the MLg cell monolayer without cytocidal action by the venom preparations. (The concentration of the venom preparations was 20 microgram/ml. See also the legend to Table 3 for the grades of disruption of the cell monolayer).

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Discussion

C. Purananda: "The haemorrhage principles HR_1 and HR_2 once introduced into the body, how long will they remain inside the body? Did you follow up?

A. Ohsaka: I don't know how long these principles remain in living animals because I haven't followed up."

P. Krag: "Were the neutralizing curves for HR_1 and HR_2 with same slope when treated quantitavely?"

A. Ohsaka: "Slopes of neutralization curve for HR_1 and HR_2 were statistically the same, showing a value around one."

A. do Amaral: "1. Have you done with the Mamushi venom the same large series of experiments (tests) as with the Habu venom? 2. Have you used, in the good series of venoms you tested, always and consistently the same process in: a) extraction; b) preparation; c) preservation — for every one of those venoms?"

A. Ohsaka: No, we haven't done any experiments with Mamushi venom. But Dr. Suzuki and his associates have worked on the separation of hemorrhagic principles from the crude Mamushi venom. 2. The venom of *Trimeresurus flavoviridis* was a pool of specimens collected throughout one year, being processed under the same conditions of extraction and drying and stored under the same conditions. All the other venoms we used were commercial preparations or gifts from other institutions."

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26. LATRODECTISMO Y LOXOSCELISMO EN CHILE. INCIDENCIA, CARACTERISTICAS CLINICAS, PRONOSTICO, TRATAMIENTO Y PREVENCION

H. SCHENONE

Departamento de Parasitología, Universidad de Chile, Santiago, Chile

El Latrodectismo es producido exclusivamente por *Latrodectus mactans*, araña de hábitos extradomésticos que se encuentra de preferencia en campos de cultivo de alfalfa y trigo. Sólo por excepción ha sido encontrada en la vivienda humana como resultado de un transporte pasivo junto con productos agrícolas. Los accidentes ocurren en su gran mayoría en las áreas rurales, durante el día en el período comprendido entre los meses de Diciembre y Abril inclusives (Verano y primera mitad del Otoño). Los individuos afectados son en su mayor parte obreros agrícolas, aunque en algunas oportunidades hemos tenido ocasión de observar casos de Latrodectismo en mujeres y niños (10).

El cuadro clínico, que ha sido descrito en Chile a partir de 1852 (1-2-9-11), se caracteriza por dolor urente en el sitio de inyección del veneno, seguido por dolores generalizados intensos, sensación de angustia, espasmos y temblores musculares, y aumento de las secreciones sudoral, salival, lagrimal y nasal. No se observa lesión local. Un signo clínico muy importante es el marcado aumento de la presión arterial, que en ocasiones puede ser de gran valor para orientar el diagnóstico (14). En algunos casos se presentan dolores abdominales intensos, que sumados a la rigidez de la pared del abdomen, pueden inducir al diagnóstico de abdomen agudo (2-14). En un grupo de 6 pacientes que en 1957 estudiamos desde el punto de vista electrocardiográfico, en 5 observamos alteraciones del segmento S-T y de la onda T que se normalizaron en un lapso aproximado de 2 semanas (16). Toda la sintomatología descrita va atenuándose en forma progresiva y termina por desaparecer al cabo de una semana, aunque el paciente queda muchas veces en una condición de adinamia y de hipersensibilidad músculo-cutánea que persiste por un tiempo más prolongado (2-11). De acuerdo con la literatura y con nuestra propia casuística, la mortalidad es de aproximadamente 2.4% (3-11-15).

Sin pretender desconocer el valor del tratamiento específico a base de suero anti-latrodectus (4), queremos destacar el éxito espectacular que se obtiene mediante el empleo de neostigmina (prostigmina) inyectable, opinión que es compartida por numerosos colegas que ejercen en las áreas rurales de Chile (14). La neostigmina, de la que se puede disponer fácilmente en cualquier servicio médico, por su acción parasimpático-mimética actuaría neutralizando los efectos farmacológicos del veneno de *Latrodectus*, cuya actividad estimuladora del sistema

simpático es particularmente acentuada a partir de la cuarta a sexta horas después de ocurrido el acidente, plazos mínimos en que el promedio de los pacientes acuden a solicitar atención médica.

La prevención de los accidentes producidos por la mordedura de *Latrodectus mactans* debe basarse en una adecuada información sanitária, aunque es del conocimiento popular el peligro que esta araña representa. En algunas oportunidades hemos obtenido buenos resultados en la eliminación de este aracnido en terrenos altamente infestados, mediante rociamientos aéreos con emulsión de Lindano al 0.25% en la proporción de 125 miligramos por metro cuadrado; o mediante la incineración de hierbas y malezas que habían sido invadidos.

El Loxoscelismo es causado por *Loxosceles rufipes*, araña predominantemente doméstica que se encuentra de preferencia tras de los cuadros y muebles. Al igual que *Latrodectus mactans* no es espontáneamente agresiva y sólo ataca inyectando su veneno, cuando se siente agredida al ser aplastada en forma inadvertida contra la superficie cutánea. *Loxosceles*, que desarrolla gran parte de su actividad durante la noche, ha sido encontrada en numerosas áreas urbanas del país comprendidas entre los paralelos 18 y 39 de latitud Sur. En un estudio realizado en la ciudad de Santiago en 1963, se pudo comprobar su existencia en el 38.4% de 395 viviendas escogidas al azar (12).

Lesiones necróticas causadas, al parecer, por arañas *Loxosceles* han sido descritas ya a fines del siglo pasado (11), aunque fué Macchiavello en 1937 quien demostró el papel etiológico de *Loxosceles* en lo que denominó aracnoidismo cutáneo o mancha gangrenosa de Chile (6-7).

La mayoría de los accidentes ocurre durante la época de calor, aunque también pueden presentarse en plena época invernal, no habiendo predominio por sexo ni por edad. En el 85% de los casos el accidente tuvo lugar en el ámbito doméstico, ya sea durante el sueño nocturno, o cuando el paciente estaba vistiéndose (12-17-21).

En líneas generales el cuadro clínico puede adoptar dos modalidades evolutivas: la cutánea o benigna y la cutáneo-visceral, grave o sistémica. En el momento inicial de ambas modalidades hay localmente sensación de clavadura quemante, la que es seguida posteriormente por dolor intenso que se irradía a las vecindades del sitio de penetración del veneno. Pocas horas más tarde, sobre una zona de edema duro, doloroso y a veces acentuadamente progresivo, aparece una placa violácea pálida al comienzo, que se transforma en una mancha equimótica que se va haciendo cada vez más oscura, la que puede alcanzar hasta 25 centímetros de diámetro. Esta mancha, que ha recibido el nombre de placa livedoide, suele aparecer rodeada de un halo eritematoso y presentar en su superficie ampollas de diferente tamaño y de contenido seroso o serohemorrágico. A medida que transcurre el tiempo prosigue el oscurecimiento de la placa, la que termina por transformarse en una escara negruzca, la que finalmente se desprende en un lapso aproximado de 4 semanas, dando lugar a una úlcera en algunas ocasiones. Esta lesión cutánea normalmente no se acompaña de adenopatía regional (17-20-21). Algunas veces hemos tenido oportunidad de observar casos de Loxoscelismo, en que la mordedura había ocurrido en la cara, y en los cuales el paciente presentó un edema gigante que comprometió todo el rosto, extendiéndose hasta el cuero cabelludo y el cuello, sin aparecer lesión equimótica ni necrótica en todo el transcurso de la evolución. Este es lo que constituye clínicamente la forma cutánea benigna de predominio edematoso (13).

Como ya lo hemos manifestado, la sintomatología que hemos descrito corresponde al Loxoscelismo cutáneo benigno, pero en el curso de las primeras 24 a 48 horas después de ocurrido el accidente, puede agregarsele una serie de manifestaciones tales como fiebre elevada, hematuria, hemoglubinuria, anemia e ictericia, que caracterizan a la forma cutáneo-visceral, que puede terminar en coma y muerte (8-12-17-20-21). No hemos observado ningun tipo de relación entre la aparición del compromiso visceral y la ubicación o tamaño de la lesión local, ya que hemos visto pacientes que se recuperaron y que habían presentado una lesión local mínima, a veces puntiforme, conjuntamente con un grave compromiso sistémico, al mismo tiempo que otros presentaron una extensa y severa lesión local que se acompañó también de grave compromiso visceral. Por otra parte en las formas cutáneas benignas, los pacientes han presentado lesiones de la piel que han ido desde las lesiones petequiales hasta extensas zonas de necrosis (8-13-17-20-21).

En el tratamiento de la forma cutánea de Loxoscelismo el empleo oportuno y mantenido de antihistamínicos inyectables ha dado buenos resultados, especialmente en lo que se refiere a la eliminación o reducción del dolor y del edema (12-13-17). En casos de necrosis cutánea extensa ha sido necesaria la reparación quirúrgica (12-17). En la forma cutáneo-visceral, que hasta 1952, época en que empezaron a usarse los corticoides para su tratamiento, era de pronóstico fatal, el uso de estas sustancias por vía inyectable ha dado resultados satisfactorios (5-8-12-17-20). Recomendamos la vía parenteral debido a que en los casos fatales, además de las lesiones renales y de otros parenquimas, se han observado fenómenos de congestión, edema y hemorragia en diferentes segmentos de la mucosa del tubo digestivo que impedirían la absorción adecuada de cualquier fármaco que se administre (20). Reconocemos el valor del uso oportuno y en dosis adecuadas de los sueros específicos antiloxosceles, y aunque los hemos usado en algunos pacientes, nuestra experiencia es limitada para poder dar un juicio definitivo al respecto.

En general, el 10% de los casos de Loxoscelismo corresponde a la forma cutáneo-visceral, de los cuales en la actualidad, sólo un 10 a 20% tiene una evolución fatal, siendo la mayoría de estos últimos, pacientes que solicitaron atención médica en forma muy tardía.

La prevención de los accidentes causados por las arañas *Loxosceles* se basa fundamentalmente en acciones educativas: conocimiento de la existencia de estas arañas, de sus hábitos y de su peligrosidad. Sobre estas bases se debe propiciar la adopción de medidas tendientes a evitar que ocurra el accidente, tales como mantener las camas alejadas de las paredes y examinar y sacudir las ropas que pueden servirles de refugio momentáneo, en el momento en que se las va a usar, y medidas de aseo cuidadoso y periódico de la vivienda con el objeto de destruir las arañas y sus telas, y eliminar las condiciones que hagan posibles su supervivencia y multiplicación (19).

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Discusion

H. Pesce: "*Latrodectismo*: En la fase vago-paralítica da excelente resultado en los casos dramáticos adrenalina — 1 ml." *Loxoscelismo*: Indicación de dialisis extra corporea (artificial) en los casos de nefrosis del nefrón distal por hemolisis y hemoglobinuria."

F. Saliba: "Suas observações sôbre inoculação em animais de veneno loxoscélico são iguais ao quadro humano?"

H. Schenone: "En animales susceptibles como el conejo, se puede reproducir un cuadro local y general muy similar al que se observa en el hombre."

Gajardo-Tobar: "1 — En chile no he observado casos de Latrodectismo por picadura de *Latrodectus curacaviensis.* Todos han sido por veneno de *Latrodectus mactans.*

2 — Sobre uso de suero: Excelente resultado tanto para Latrodectismo como para Loxoscelismo. Naturalmente, para cada caso, el suero específico debe emplearse precozmente y con cantidad adecuada.

3 — El nombre justo de *Loxosceles* es, como dice Dr. Bücherl, *L. rufipes,* pero todavía resulta un poco aventurado hacer cambiar a los médicos la nomenclatura porque recien han incluído en la patologia el Loxoscelismo."

H. I. Bicher: "Did the speaker find clinical correlation to the cardiotoxic action ascribed by Dr. Maretic to the *Latrodectus*?"

H. Schenone: "En un grupo de enfermos que presentaban un cuadro de Latrodectismo observamos alteraciones del electrocardiograma que eran similares a un trazado correspondiente a isquemia."

H. Pesce: 1º — Acerca del Latrodectismo, deseo poner de relieve que tanto en Perú como en Chile, raramente el paciente llega donde el médico en las primeras horas después del accidente. La primera fase es caracterizada por un síndrome simpático-tónico, que dura de 2 a 4 horas. En esta fase, el Dr. Couric, de Miami, obtuvo resultados espectaculares con el uso de ½ a 1 miligramo de cloruro de adrenalina, por via subcutanea, con desaparición pronta del dolor que no cedía ni a la morfina. En la segunda fase, la vagotónica, es conocido el buen resultado de la prostigmina (neostigmina).

2º — Acerca de los casos víscero-hemolíticos de Loxoscelismo, coincidimos con su menor frecuencia y su gravedad. En la serie de 31 casos peruanos de Izú, hubo 13 (42%) víscero-hemolíticos; dentro de estos hubo 5 fallecidos (16%) correspondiendo todos a niños menores de 5 años, en los cuales por lo tanto la mortalidad fue del 100%. Un estudio reciente de Maya (1963) en el Perú relata 3 casos de Loxoscelismo hemolítico grave en los que fue aplicada la hemodiálisis con riñón artificial. El primer caso fue en un obrero de 30 años, que trabajaba a 3900 metros de altitud, com gr. 4,7 de urea por litro; al 8º día, después de la diálisis, murió por bronco-neumonia bilateral. El segundo caso, 25 años, llegó a gr. 7,2 de urea por litro y estuvo 33 días en anuria; con 3 diálisis curó en 4 meses; controlado sano a los 2 años y medio. El tercer caso, 9 años, tuvo gr. 7 de urea por litro y estuvo en anuria 20 días; curó con 1 diálisis en 3 meses y medio.

Debemos recordar que, según varias estadísticas, la sobrevivencia máxima en anuria por nefrosis del nefrón distal, varía de 1 a 4 semanas; y según Baslow la sobrevivencia media en una serie de 500 casos fue de 9 días.

En cuanto al prognóstico, el mismo Baslow dice que con urea inferior a 3 gramos hay mortalidad del 34% y con urea mayor de 5 gramos la mortalidad llega al 78%.

Por lo tanto la indicación de la diálisis es importante: antes se hacia cuando el grado de uremia se iba elevando; en Lima se efectúa de inmediato, sin esperar la uremia alta.

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27. THE CLINICO-PATHOLOGY AND TREATMENT OF SNAKEBITE IN SOUTHERN AND CENTRAL AFRICA

D. S. CHAPMAN

University of Natal, Faculty of Medicine, Durban, South Africa

Introduction

If there are no fairies at the bottom of one's garden, in South Africa there may be a snake or two. Africa shares with other world areas a high attainment of its herpetologists and a paucity of detailed medical observations. Indeed, there is seldom opportunity to study the early and often urgent symptoms of snake bite as those exposed are mainly primitives and rural dwellers.

A local difficulty is apparent. Elsewhere, a certain family or even species is outstanding as the cause. In Southern Africa, by the many varieties available, these with a wide variation in colour and markings within the species (e.g. the cobras), by the frequent mimicking of venomous by harmless snakes, and by the fact that the snake usually flits after an attack, it is not surprising that snakes are rarely identified.

In our hospital experience also, it has not been possible to differentiate the elapine snakes on clinical grounds but from the scanty evidence previously available, the very rapidity and severity of a venom action has tended to point to a particular species, the black mamba. However, the puff adder has often been identified by its sluggish grossness and we have assumed that our most serious cases of local tissue death were caused by it when the snake was not identified. Similarly the night adder has been identified often enough to apportion blame. Elapine bite victims have tended to report early enough (Fig. 1) for the clinician to give a fair description of the clinical features but where adder bite victims have reported late (and only because of persistence of swelling or because of gangrene), there have been few competent early observations.

Though it is often claimed that amateurs can easily recognise our cobras and adders, most victims do not qualify, are bitten in their ignorance, often in the dark, and are often children to whom all snakes are large.

The common venomous snakes

Southern and Central Africa have the dubious distinction of harbouring some 137 venomous snakes comprised in three families, COLUBRIDAE, ELAPIDAE and VIPERIDAE but not more than 25 species however, are capable of causing death (Table 1). Of all areas Natal, my home province, has the greatest variety of snakes and highest incidence of bite.

TABLE I — COMMON POISONOUS SNAKES OF SOUTHERN AFRICA

COLUBRIDAE	ELAPIDAE	VIPERIDAE
Boomslang Bird snake Skaapstekers	True Cobras (4) Rinkals "Cobra" Mambas (3)	Puff Adder Night Adder Gaboon Viper Berg Adder * Mole Viper ** Carpet Viper *
Haemotoxic	Neurotoxic	Local cytotoxic

* and ** Also has special elapine effects.
* Has dominant colubrid effects.

The three COLUBRIDAE shown in Table 1 are all peculiar to Southern Africa. They are back-biters with a limited capacity for a successful strike but the boomslang and the bird snake have at least extremely potent venoms which have a marked effect on blood coagulation. Our peculiar experience is fortunately tempered by the docility of these snakes.

The ELAPIDAE have small permanently erect fangs and are all dangerously venomous with a serious neuro-toxic effect. Many are small inoffensive burrowers but included are 4 mambas and 4 true cobras. The Rinkals "cobra" and the black-necked cobra also spit accurately to cause conjunctivitis; but this can be adequately dealt with by simple lavage.

The vipers show the highest development of fangs which are mobile, large and hollow. Undoubtedly the Puff Adder is rightly feared for ubiquity, the potency of its cytotoxic venom and is productive of the majority of our serious bites. The Gaboon viper is our largest viper and very venomous. A fearsome creature but reputedly good natured, it is reluctant to strike except when trodden on. Found in rain forest areas, it is fortunately rare. There are two species of night adder which frequently cause the less serious bites. The berg adder has also a special elapine effect. Mole vipers abound and cause many of our lesser bites. The carpet viper, found in the more Northern areas, appears to have a special colubrid effect.

The highest incidence of snakes is in the more populous areas where land has been cleared for cultivation and where habitation has invited rodents.

Epidemiology

I present here evidence of a 7-year study of over 1,000 cases of snake bite, 892 of which were admitted to our hospital. Table II presents the cases according to each family, together with an analysis of those with serious effects, and the fatal cases. It is seen that overall there was a high incidence of morbidity and mortality though relative to the size of the population at risk the incidence of bites was low. Noted too is that in 484 instances the snake was not identified.

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TABLE II — SNAKE BITE — 7 years 1957-1963 — Natal

Species		Died	Severe effects
Total Cases	1068	21	80
COLUBRIDAE	5	0	0
Skaapsteker	3	0	0
Natal Black Snake	1	0	0
Herald Snake	1	0	0
ELAPIDAE	41	10	16
True cobra	18	3	9
Rinkals cobra	14	0	5 (4)
Black Mamba	7	7	—
Green Mamba	2	0	2
VIPERIDAE	538	11	64
Berg Adder	1	0	0
Puff Adder	210	11	56
Night Adder	8	0	1
Puff or Night Adder	319	0	7
Unknown			
venomous	314	0	0
harmless	170	0	0

Some human attitudes to snakes

Let us consider some important human attitudes which influence the incidence and severity of snake bite.

Whether as some would claim we are not born to fear snakes but are conditioned to it, fear is there and fascination too. Both no doubt lead to trouble. In our first aid we advise calm and immobilisation, yet the emotional distress is real and if only to get to a snake-bite outfit, the impulse is to run. In my series, some adults and many children were frightened long hours after the attack.

Only a few of us surely think snakes are beautiful but many are attracted and mainly perhaps by the possible danger.

So boys become amateur herpetologists and as they grow they become indifferent to the possible dangers. Ignorance reaches its zenith in the case of snake-handlers even to their ignoring bites when working in snake parks because they think they "know" their snakes and falsely believe in immunity from previous bites. Nor can snakes be tamed and an usually docile one can take offense at any time. Thus has one experienced herpetologist and at least 4 snake catchers lost their lives in Southern Africa. Snake catching and handling are obviously hazardous and it does not seem that enough care is taken.

Except when dangerously close, identification appears quite difficult. Our local snake park received its prize black mamba, sent in error as a rather handsome specimen of a mole snake.

The white population is snake conscious at least with regard to the facilities of protection and those such as nature lovers and fishermen carry snake-bite outfits, no doubt though, many with out-of-date antivenoms and dirty instruments. Unfortunately the main population at risk are the rural Bantu and Asiatics who in fact, do seem ready enough to seek help from mission hospitals, agricultural hospitals and farms, all of which are equipped.

Age incidence of bite

Children run the greatest risk in their play (usually barefooted) and through their curiosity of moving things (Fig. 2). Though the smaller children have no fear, they are bitten in large numbers. The older children also molest snakes. One, a Bantu herd-boy tried to strike a more agile mamba with his hand and died by his audacity.

Some snakes such as the rinkals "cobra" can sham death and some bite when they are dead. A Bantu male adult claimed the severed head of a black mamba which had just been shot for the purpose of fortifying a native remedy. He placed it in his pocket and taking it out a few minutes later, he was promptly bitten and was immediately very ill with neurotoxic symptoms. He survived.

A snake census is meritous but not entirely satisfactory. Numbers can be readily increased by the reward — conscious deliberately rearing less harmful snakes — even to enterprising schoolboys selling back to our snake park, snakes stolen from it the day before.

No doubt to allay our fears, herpetologists stress that only some 300 of the world's 3,000 species are dangerous to man and few will attack unless molested. This is cold comfort to the 1,000 victims of this series.

Seasonal incidence

Snakes may be more shy than those they bite but there is ample evidence of aggression in the breeding season when it is warmer and more humid (Fig. 3) and when they are cut off from their lairs. Whatever the basis for attack, snake bites do occur in large numbers wherever their paths cross with other animals. Yet the overall risk is small (Table III). In South Africa one has nine times the chance of injury travelling by car to a picnic than from the snakes there; and 40 times the chance of dying.

TABLE III

	Injury	Death
Snake bite	24	0.35
Road accidents	211 . 3	14 . 4
	×9	×40

Situation of the bite

The very great majority of bites (Fig. 4) occur on the feet and lower legs, 84%. The principal remainder are on the hand (7.7%). The risk is with terrestial snakes, often through treading on them, well camouflaged as they are, and most commonly with the more lazy adders which strike lower down than do the cobras (and especially the mambas) which rear to strike. We share with other areas, alarm over head and trunk bites and even those of the upper limb, situations where a tourniquet cannot be applied, and where better absorption is likely by more abundant vascular connections.

The moral is plain — when in the country to wear boots or at least shoes and never open sandals; also thick long trousers wherever the vegetation is abundant. To tread warily is not enough. Such precautions would reduce the risk by at least one half. One wonders whether snake catchers and herpetologists in their field work so protect themselves, and do they always wear gauntlets?

The clinical effects of bite

Table IV considers the general highlights of clinical action for both elapine and viperine snakes in our experience, and using the evidence of the fatal cases.

TABLE IV — SNAKE BITE DEATHS

1 — Long delay in treatment 2 — Profound effect in children
3 — Antivenom — too little, too late, unsuitable site, wrong type
4 — Negative *distant* necropsy findings

	ELAPINE (8)	VIPERINE (9)
Hospital admission	quite early	late
Clinical, onset	rapid	late
march	rapid	slow
Dramatic signs	consistent	lacking
Local, pain	little	often severe
swelling	little	rapid, massive extravasation

A. *Bites by Colubrid snakes*

Attack by these snakes is rare in any Southern African experience. All 5 cases in my series of such bite were almost symptom free and did not show the special haemotoxic effect.

B. *Bites by Elapine snakes* (Table V)

The essential effect is distant from the bite, neurotoxic and rapidly produced by venous spread. We relate our first aid to this — the tourniquet (the vital first step) and the antivenom given intravenously.

TABLE V — THE CLINICAL EFFECTS OF SOUTHERN AFRICAN ELAPINE SNAKES *

Clinical	No.	Fatal	Clinical	No.	Fatal
Severe pain	2	—	Respiratory distress	15	8
Swelling, none	14	4	Breathless	7	3
little	18	4	Shallow respiration	8	5
moderate	1	—	Low blood pressure	16	7
severe	2**	—	Cardiac arrest	1	1
Vertigo	7	—	Increased sweating	12	
Drowsy	1	—	Hyperpyrexia	3	
Restless	16	3	Nausea	1	
Unconscious	12	5	Severe vomiting	4	1
Convulsions	2	1	Pallor	3	1
Headache	1	—	Nose bleed	1	
Hallucinations	1	—			
Difficult swallowing ***	11	2			
Difficult speech	7	1			
Throat pain	2	—			
Increased salivation	14	8			
Ptosis	8	4			

* Hospital group of 35, of which 25 identified and 10 likely.

** Both associated with long applied tourniquet.

*** Excludes those unconscious.

Experimentally all South African cobras have more potent venoms than have the mambas but the mamba gains its rightful dreaded reputation on account of the greater volume of venom it can more rapidly inject.

Death from a cobra or a mamba bite is in anything from minutes to an average of 8 hours and the majority of those alive at 24 hours will survive and without ill effects.

There is a paralysis of motor action by a curare-like effect peripherally on motor nerve-endings or by a central brain-stem action, probably both. And there is plenty of evidence of widespread effects on the cerebrum — vertigo, convulsions and unconsciousness often occurring before respiratory failure is developed well enough to give cerebral anoxia. The noticeable eyelid ptosis, strabismus and speech inco-ordination are useful diagnostic features but not harmful.

The outstanding effect is a paralysis of respiration and of deglutition so that a victim embarrassed by the former can drown in his own saliva. Salivation is not increased but only seems to be; just as in other instances of interference of deglutition (e.g. oesophageal cancer, head injuries).

Once commenced, respiratory palsy is rapid in progress but there is probably no march of palsy from one muscle group to another. That diaphragmatic action appears to last the longest is probably because it is seen best and especially when

it is aided by vigorous struggles of abdominal muscles. In our cases it was not easy to test the tone of limb muscles but it was seen that these also share in the increasing flaccid palsy from lassitude to inertia. Muscles which need to overcome the effect of gravity are most easily seen to fail — in many cases the neck muscles tire and the head falls. Ptosis of the eyelids may well represent merely such a failure. Finally the basic sphincters fail, to give incontinence of urine and faeces.

Respiratory failure seems to take different forms depending on the venom dose. Most usually, the failure is quiet, breathing becomes increasingly shallow, the subject also lying quietly unconscious, as seen in many of our cases. But there can be more of a fight and an increased labour of respiration.

It has often been reported that for the less rapidly effective cobra venoms, the heart can continue to beat strongly long after respiration has ceased, which of course, is common to other forms of respiratory failure. Such, at least would mean that if respiration could be maintained artificially, the outlook need not be so gloomy especially if the venom could be neutralised, which for cobras we have proof that it can. We had one such case who in fact "died" 4 hours after a mamba bite (one hour after admission), was revived by exposed cardiac massage to what we thought was normal heart action and was maintained for two days on a respirator before he succumbed. Two others survived after 5 days of respiratory support and I can now report three other successes from the past two years.

More than half our cases had some disturbance of deglutition or speech and nearly all of these had profuse salivation. General cerebral effects were also common. One-third were unconscious on or after admission, 4 for very long periods and yet survived.

Restlessness was seen most often with conscious subjects and the usual demeanour to death was of quiet unconsciousness and shallow respiration.

Many had profuse sweating which, with a low blood pressure, constituted for some observers the picture of "shock", but most of these cases also had fever.

Eyelid ptosis was seen only in 8 cases. Pupillary changes were very variable or absent and were not considered of diagnostic importance.

Half the cases had respiratory distress, all severe, and half of these survived.

In this elapine series, the local effects of bite were minimal or not existent. Slight swelling was occasional and pain was rare.

Though all South African venoms are essentially anticoagulant *in vitro*, except in the case of the COLUBRIDAE, the haemorrhagic action is not important. No elapine cases ever had haemorrhages nor jaundice and in fact the haemorrhagic action in viperine bites was always local and not, I think, related to a coagulation defect.

The value of antivenom

I consider from my series that we now have abundant proof of the great value of the antivenom available (Table VI). Fourteen of 25 serious cases of elapid bite responded to antivenom, some very dramatically. One noted too the pleasant freedom from sensitivity reactions (1%) as contrasted with figures as high as 30% in some world areas.

TABLE VI — DETAILS OF ANTIVENOM THERAPY IN 892 CASES (Polyvalent *B. lachesis* — *H. haemachatus* — *N. nivea* antivenom. S.A.I.M.R.)

A.	Given	Not given *	No record
Antivenom	712	142	38
Not required **	186		
Required		6	
> 6 hours after bite	45		
30 mls or more	29		
Intravenous	14		

* Includes 26 reporting 24+ hours after bite and 1 Rinkal Cobra spitting.

** These cases had few or no signs. No observation period.

	Early	Late	Fatal
Sensitivity 7	5	2	0

B. ELAPINE SERIES 35

Severe 25

Died 9

Good response 14

Also other treatment 3

It is also seen that the doctors of Southern Africa seem shy of giving antivenoms intravenously, yet it is by this route that success will come in elapine bite cases. They also tend to prescribe doses which are low. We can see in Table VI how few were given 30 mls of the antivenom or more, yet this is the advised minimal dose for serious cases. Children also must be given at least an adult dose, a dose dependent on their size relative to the size of the snake.

I would never withhold this polyvalent serum from cases of puff adder bite but it is difficult to evaluate the benefit. It was problematic whether it was ever of any help in this series. Antivenom never appeared to resolve or indeed limit any moderate sized or severe swellings. Many injections were obviously of course, given too late.

In Southern Africa we now have a polyvalent anti-mamba serum, but it will be harder still to prove its value in the face of the very rapid fatal action of the mamba venoms. It was only used in one case in this series and did not alter the march of events.

Fig. 5, records in pictorial form, the march to death or recovery in severe cases in both the elapine and viperine series. Of 25 identified elapine bites, 8 died rapidly while 14 were resolved dramatically by the use of antivenom, 2 more succeeding by the aid of assisted respiration; and one, it was thought, by the exhibition of steroids.

C. *Bites by Viperine Snakes* (Table VII)

The dominant clinical effect of a viper venom is local, cytotoxic and self-limiting. There is no general bleeding diasthesis. General effects are uncommon, usually late and not neurotoxic (except in the case of 2 species). Spread of toxin is by lymphatics, which was shown well in 43 cases where lymphangitis was the main effect. This is a very important fact in management and immobilisation must be encouraged both of the part bitten and of the subject as a whole.

The effect is a destruction of all tissues especially blood vessels and their contents but the cytolysis with its tissue necrosis, the coagulation and thrombosis of the blood produces its own barrier to spread. Bleeding adds to the internal pressure to increase the ischaemia. So produced are the swelling, induration, haemorrhages as ecchymoses and blisters, and necrosis.

TABLE VII — LOCAL EFFECTS OF SNAKE BITE
(7 years: 1957-1963 — Natal)

Cases	892
Swelling	726 (slight, 290; moderate, 300; severe, 136)
Extravasation	67
Abcess	20 (initial, 12; later, 8)
Necrosis	23 (local, 11; extensive, 12)
Oligaemic shock	25 (died, 9; dramatic response, 9)
Lymphangitis	43
"Cellulitis"	104 (by snake, 100; by bacteria, 4)
Thrombophlebitis	4 (superficial, 1; deep, 3)

Tissue necrosis and oligaemic shock

Though an overwhelming envenomation is possible, for instance by an intravenous injection, with consequent convulsions and unconsciousness, each death in this series had a rapidly expanding extravasation of a limb. None had frank gangrene but one had marked ischaemia distal to the main blood collection.

Nearly all took some time to die as contrasted with the elapine series and in my opinion death was due to *decompensated oligaemic shock* caused by the rapid massive blood loss, either not treated at all or in those who came too late for help.

There is a strong inclination however, in cases who have apparently been doing well and who many hours after a bite have a sudden and fatal collapse,

for clinicians to invoke a new special factor of venom action now with a distant as against a previous local effect. This is unlikely and no evidence is in fact available. Three of the 9 fatal cases collapsed in hospital and others were rushed in when it had occurred at home.

If enough cases could be shown to develop collapse following removal of a long applied tourniquet, we might imply a release of breakdown products of muscle metabolism but in the many instances we released tourniquets, collapse did not follow.

It has seemed that the role of such breakdown products is no greater in the production of general effects in viperine bite than with other examples of infarction when, during the body's struggle to reject particularly frank gangrene, the patient can become very ill — but he does so gradually and does not die rapidly.

As I have indicated, if it occurs, any coagulation defect must be local, perhaps localised by the barriers of necrosis etc. and no cases had jaundice. At necropsy no distant effects were found and there was never evidence of pyogenic invasion. Yet many had severe anaemia when first reporting.

Consider a limb rapidly filling with blood from the general circulation while its own contents are immobilised by what amounts to an enclosed catastrophe of tissue death. If a surgeon acquainted with crush injuries was to witness this, he would not hesitate to replace the blood lost and he would do it quickly. It does not surprise that the volume increase of a part can represent fully half the original blood volume of the patient. In my series some extravasations reached well into the trunk after involving a whole limb.

The majority of victims are fortunately young adults who can adequately compensate for the sudden oligaemia and at first, will appear quite normal. But compensation cannot be kept up indefinitely and when it breaks, "shock" is then often irreversible. The picture of collapse in viperine bites is identical with such decompensation after crush injuries and extensive burns. The clinician should be aware that the *signs* usually described for oligaemic shock are either those of intense compensation or of decompensation — but shock is actually present from the onset of swelling, building as it goes. We should treat the situation and not wait for the signs. Those who survive on their own (many must hover on the brink), when they are admitted late, often appear toxic and their condition is not unlike the "illness of trauma" following crush injuries.

Except where the swelling is due to the inflammatory oedema consequent on the tissue insult by venom, when improvement will come from infusions of other fluids such as plasma, only blood will suffice. I have indicated that the infusions may have to be very large. Care of course is required with late arrivals — for these, packed cells should be used.

Vasopressor substances or steroids cannot restore blood volume depleted of blood and in fact the former are dangerous. They do not increase cardiac output but do increase the work of the heart and most important, produce profound renal ischaemia. Antivenom neither, can replace lost blood.

The swelling

This is two main types:

(1) Little or no surface extravasation with the limb soft or hard.

(2) Haemorrhages evident as ecchymoses when the swelling is usually more massive and solid.

Necrosis is induced in either type by the very concentration of venom but found more often in the second type. It is then often extensive, superficial or deep.

The lower limb has often been seen markedly flexed at the knee and by the hardness of the swelling, we feared serious muscle involvement by haemorrhage or coagulation but in most of these cases, residual induration was rare.

The resolution of swelling

Many of those admitted with severe and extensive swellings had persistence of these for over 10 days. The possible importance of venous occlusion as the basis of slow resolution is suggested by the outcome in 4 illustrative cases. One child had a thrombosis of the long saphenous vein of a lower limb as the solitary clinical feature. Three women who were bitten on the feet had swelling of the limbs which had not been considered severe at the onset, still present at the end of 3 months (Table VII). Solid swelling involved the greater part of a lower limb with a tendency to subside after rest and elevation. In all 3, venography showed irregularity of and evidence of recanalisation in the deep venous system (as though after thrombosis). Two of these women in the earlier phase of their venous oedema had severe pain and marked tenderness on palpation of the popliteal vein behind the knee. All 3 adults were back to normal after a further 4 months.

Fourteen cases had an extremely rapidly developed soft swelling of much of a limb, with almost as rapid a subsidence over some 12 hours. There were judged to have had lymphatic oedema caused by bites of the lesser adders.

Necrosis

Occlusion of a main vessel was only rarely the cause of the infarction seen, which even when large, tended to be patchy. The action is mainly on smaller vessels, sparing at least some tissue. Some of the deleterious effect is perhaps due to the explosive effects of disruption. Twelve cases of necrosis were extensive, 4 involving the whole lower limb below the knee.

The role of bacteria

Bacteria come no doubt on snake fangs and by dirty incisions but are they important? Though abscesses were found in 20 cases, only 8 had had an incision and some of the others almost certainly represented lysis of necrotic tissue.

Local temperature rise and redness were seen frequently. Diagnosed by some observers as "cellulitis" they occurred far too early after the snake attack to incriminate bacteria. Why not an inflammatory response to snake toxin?

Including those cases coming to necropsy, septicaemia or pyaemia were never encountered. Gas gangrene and tetanus never occurred either.

Only 9 cases of viperine snake bite had general effects separate from any distant influence of the local lesion. These were considered sensitivity reactions to the venom before any antivenom had been given. Six had vomiting, 2 had abdominal colic and there was one instance each of dizziness, headache, sweating, urticaria, hyperpyrexia, drowsiness.

Fig. 5 shows graphically the outcome of 18 severe viperine bites when the snake was identified. Though 9 died, some were admitted only after 24 hours when they had collapsed at home, presumably from the decompensation of blood loss. Nine others responded well to blood infusion.

Treatment

A summary of "A System of Management of Snake Bite in Southern and Central Africa", which includes a "Simple Regime of Early Treatment", is appended.

My criticisms of past and present modes of treatment is given in the light of my experience of snake habits, the probable routes of snake venom action, and the facilities available in our areas. From all the work, the simple regime was devised.

Discussion

A. Barrio: "In envenomation by ELAPIDAE neostigmine is useful, as demonstrated in neuro-muscular preparations, in 1949, in collaboration with O. Vital Brazil?"

D. Chapman: "I have no experience with *Micrurus*-envenomation. Prostigmine has been used in some of our cases of elapid snakes, but not with any measurable effect."

F. Kornalik: "Corroborate your opinion about the cause of death in viper-bites being oligaemic shock. We had the same results in experimental animals in which, even when a sublethal dose of venoms has been applied, anemia occurs with a rise of the haematocrit from 45% to even 80%."

D. Chapman: "The oligaemic shock is of great importance and is the primary cause of the illness and death which follows our African viper bites."

P. J. Deoras: "Have you any data about the areas the tourniquet has been applied by various workers?"

P. J. Deoras: "Have you any data about the areas the tourniquet has been aping our African viper bites."

Table legends

Table I — The common poisonous snakes of Southern and Central Africa.

Table II — Snake bite in Natal in a 7-year period showing the morbidity and mortality incidence, relative to each snake species.

Table III — Contrast the risk. A contrast of the chance of snake bite and its mortality with road accidents in South Africa.

Table IV — The outstanding events seen in those dying from elapine and viperine snake bites.

Table V — The clinical effects of Southern African elapine snake bite.

Table VI — How the available antivenom was used or abused. Its success in identified severe elapine bites is given. Note the low sensitivity incidence.

Table VII — The local effects of snake bite. Though this analysis includes all cases of bite, swelling was not a feature of elapid bite and the figures given here virtually represent the viper bite experience.

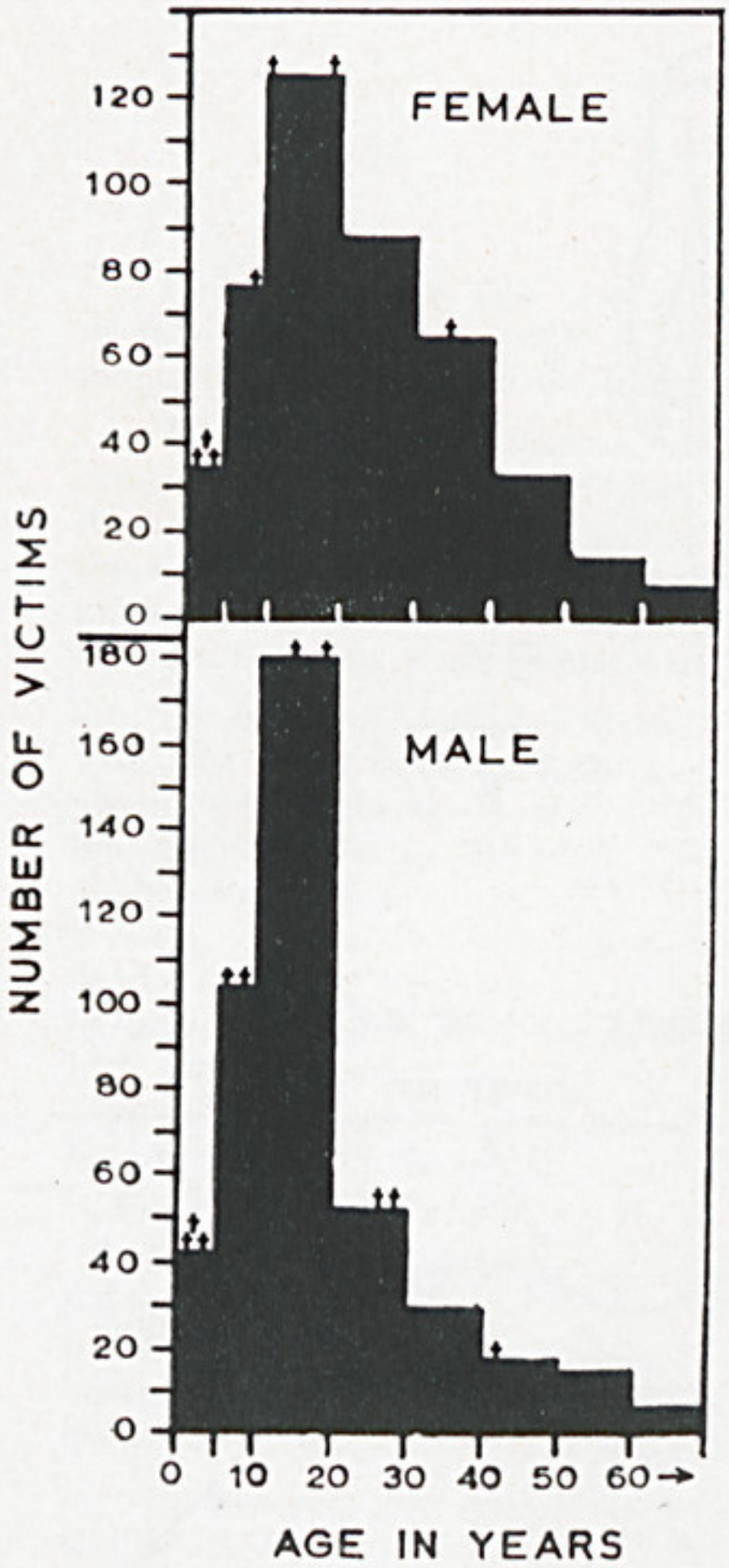


Fig. 2 — Age and Sex Incidence. Note that children of both sexes were the commonest victims and that most deaths occurred in young subjects.

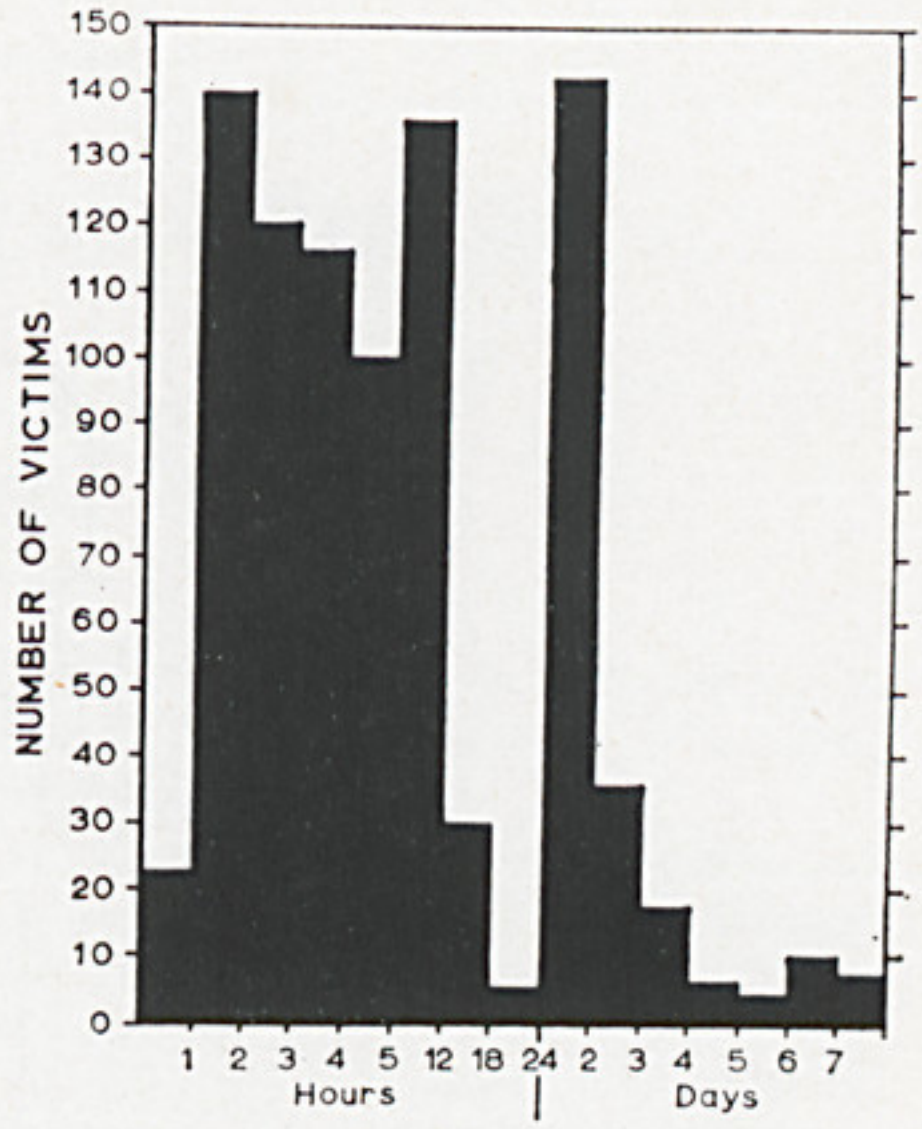


Fig. 1 — Bite: Admission Time. Elapine snakebite victims tended to report in the early hours whereas the very late admissions comprise mainly serious viper bites.

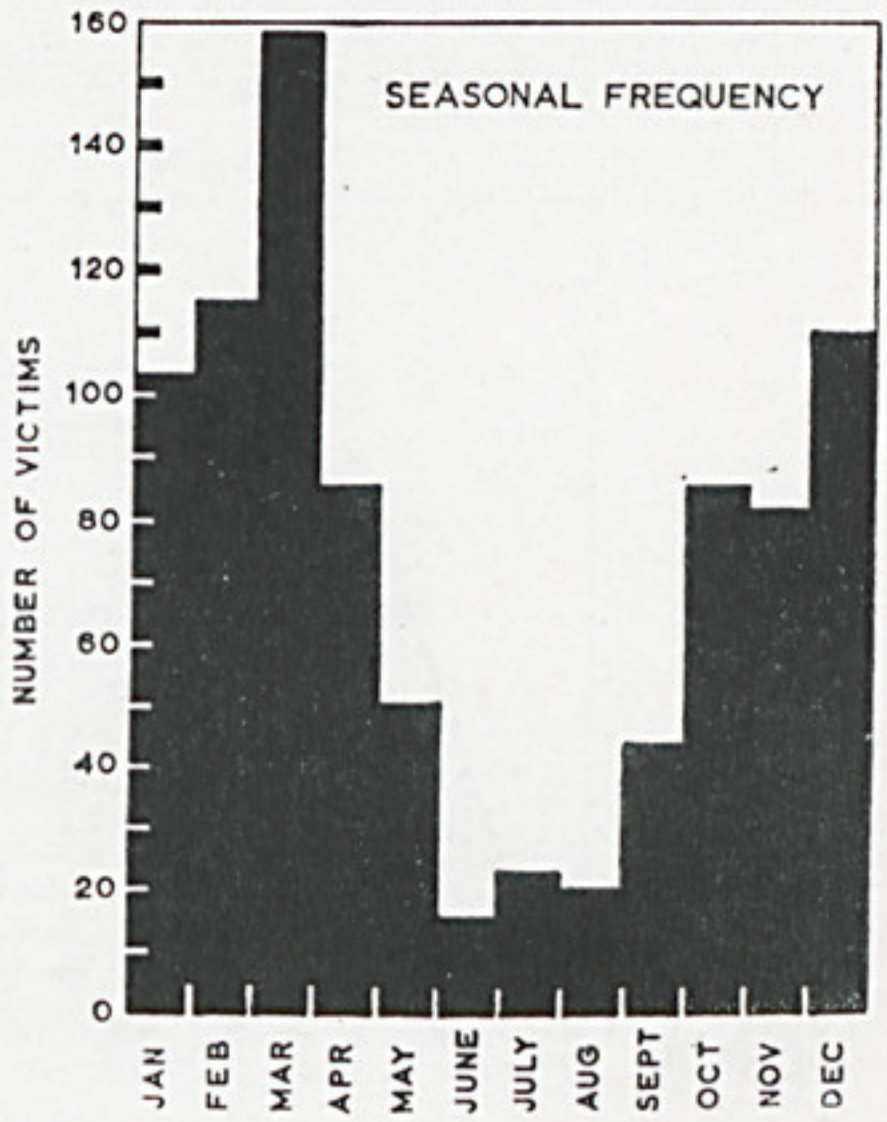


Fig. 3 — Seasonal Incidence of Snakebite. The highest incidence is in the hot humid months when snakes are breeding.

SITE OF SNAKE BITE

7 years 1957-63 Natal

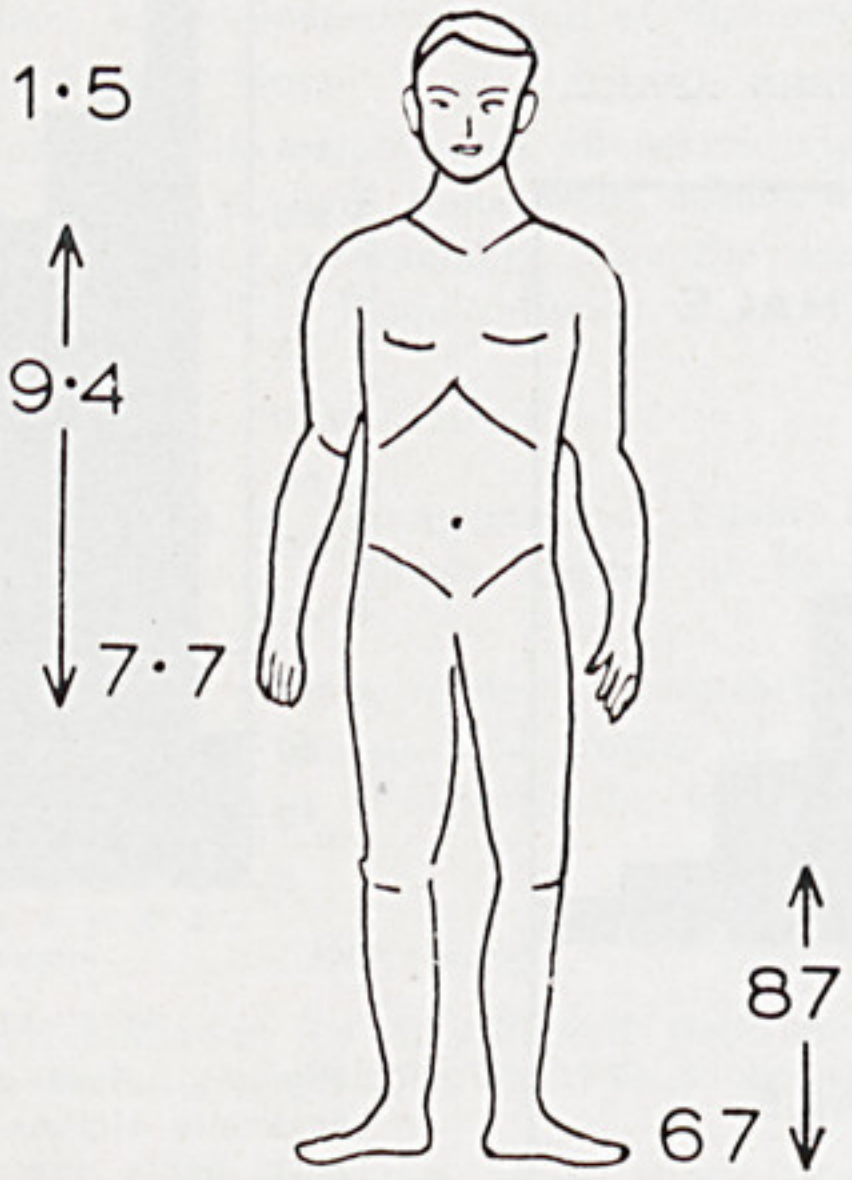


Fig. 4 — Situation of the Bite. The figures represent percentages.

SNAKE BITE — THE MARCH & RECOVERY OF SEVERE CASES

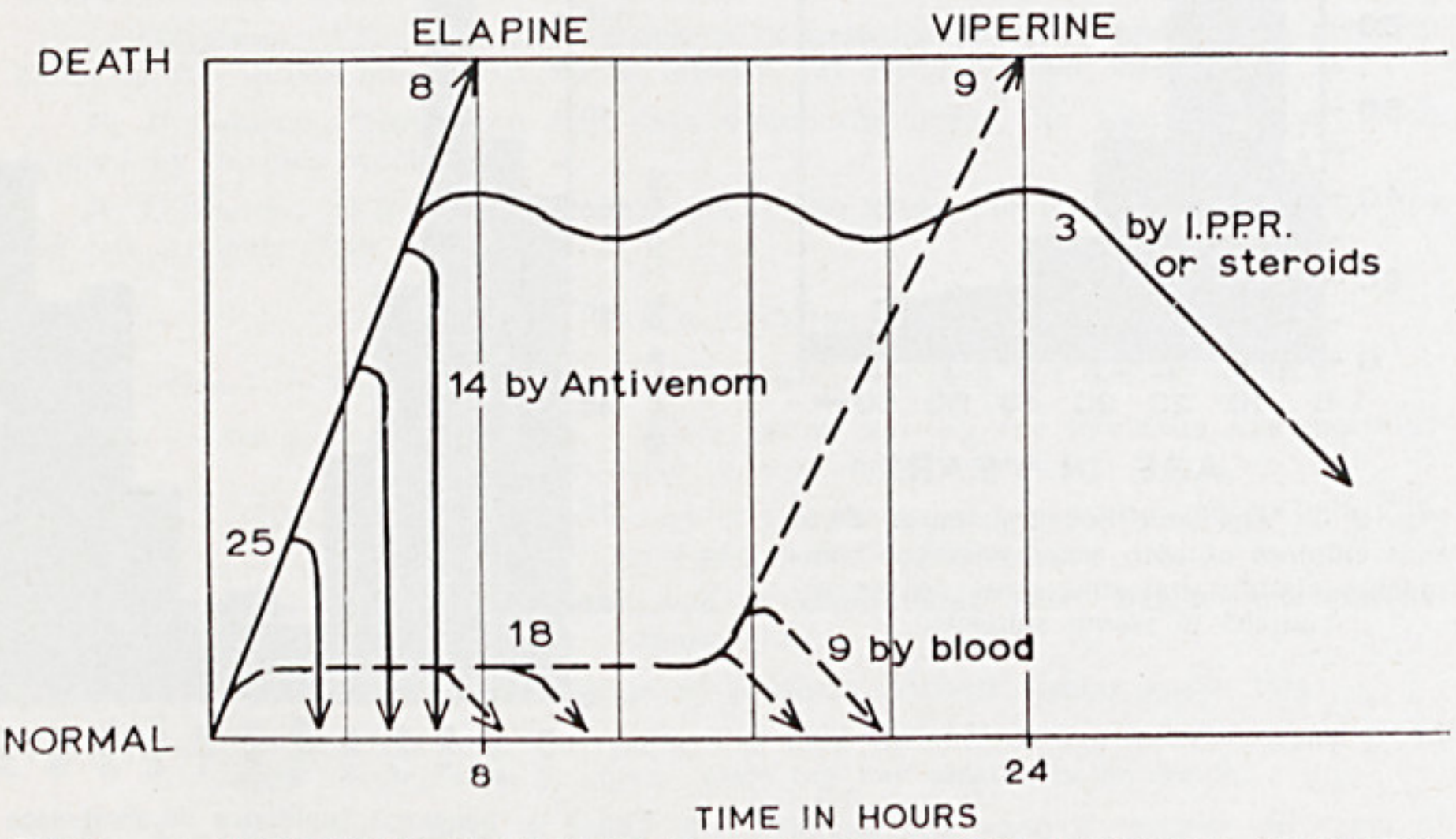


Fig. 5 — A graphic record of the march to death or recovery in severe cases of identified elapine and viperine snakebite.

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28. POISONOUS SNAKE BITES IN GERMANY

HERBERT LIESKE

(Germany)

Poisonous snake bites are only of little importance in Germany, thus most doctors are not familiar with a proper treatment, and there are only a very few publications dealing with the problem of snake bite in Germany.

The only native poisonous snake to be found in Germany is the adder or common viper (Kreuzotter, *Vipera berus*, as it is called in Latin) which belongs to the viper family, and in addition, we find the *Vipera aspis* (*Aspis viper*) in the southern part of the Black Forest (Schwarzwald). Bites caused by these two kinds of snakes do not produce grave symptoms and it is mostly the children who are bitten by snakes while strolling across the moors or through the meadows.

The board of statistics (Statistisches Bundesamt) at Wiesbaden cannot provide with any recent statistics concerning either adder — or poisonous snake bites in general or the deaths resulting from them. A partial statistical collection of snake bite accidents during the years 1883 and 1892 — this is before the introduction of the serumtherapy — indicates 14 deaths (that is 6.4%) out of 216 adder bites. Between 1907 and 1912, 265 adder bites were reported officially and only 6 deaths (that is 2.3%) were recorded. During the last 20 or 30 years the number of bites and deaths resulting from them has been decreasing, the reasons being both the introduction and the use of the serumtherapy and the housing development which naturally caused a further decrease in the number of the adder family. (Part of the decrease, by the way, is certainly due to offering rewards to people who caught adders.)

Notes on bite accidents are now only to be found from time to time in some publications. Between 1951 and 1956, for example, only 19 adder bites were treated in the hospital department of the Institute for Tropical Medicine in Hambourg, where snake bite accidents occurring in the northern part of Germany are looked after, and none of the patients died.

If, however, you compare the number of killed and injured persons due to traffic accidents in Hambourg, with its population of two million people, e.g. in April 1966 1,010 people were injured and 25 died, while in December 1965, 134 deaths caused by various accidents were registered, and now compare to these figures those of the snake bites, you will certainly realize the little importance of adder bites for the doctor in general.

Fatal adder bites are nowadays seldom heard of in Germany. In 1930, Fock reported the death of a girl harvest worker, who was bitten in an ankle vein and died 3 hours later. In 1952, Kirsch reported 2 deaths out of 20 cases treated for adder bites. Only lately it was made known to me that in 1959 a weak-hearted 60 year-old lady died of an adder bite. The case has never been published

officially. In comparison to the bites of the rather harmless German adders those of the imported tropical snakes are more serious and often prove fatal. Such accidents occasionally happen in the dock areas during unloading of imported fruits (e.g. bananas), when the poisonous snakes travelling as stowaways are disturbed. Furthermore similar accidents are reported from zoos, snake farms, pet shops, fairs, and snake fanciers. From 1951 until 1966, for example, 4 tropical snake bites were treated in the hospital department of the Institute for Tropical Medicine in Hambourg, 2 being from a *Bothrops schlegelii* which, concealed in a cluster of bananas, bit 2 stevedors, the other two being bites of a *Crotalus viridis* and a *Bitis arietans* which bit 2 pet handlers. In all cases the bites could be treated successfully.

The fatalities caused by tropical snakes in Germany are far more frequent than those resulting from adder bites. Since 1956 three fatal Cobra bites were reported, which, because of their rarity and dramatical singularity, were widely covered by the press, and in one case resulted in court proceedings: A showman in Königswinter on the Rhine suffered the first of these bites. In 1956 he was bitten in the tip of his right forefinger by a 4½ year-old *Naja naja* which he had reared from an egg. He made an incision himself and applied a tourniquet to his arm. It was 1½ hours later when he first called for a doctor and then was injected with a Cobra anti-serum (5 ml locally and 5 ml intramuscularily) which was already outdated for one year. Further anti-serum, which had been ordered immediately afterwards, was injected 3¾ hours after the bite occurred. However, although it was only a 10 ml polyvalent serum dose, produced by the Behring Werke for treatment of bites from European and Mediterranean vipers and not effective against Cobra bites, it was nevertheless injected. 6 hours and 10 minutes after the accident the patient died from respiratory paralysis. In December 1962 an experienced 55 year-old animal keeper was bitten by a 120 cm long Indian cobra in the tip of his right forefinger, while working in an animal compound in the Bremen Zoo. Hardly 10 minutes later the patient, a tourniquet already applied, was in hospital, where the specific antivenom not being available, he was injected locally and intravenously with 20 ml of an anti-serum for the treatment of European an Mediterranean snake bites. 1½ hours after the bite 30 ml of a polyvalent serum, produced by the Behring Werke for treatment of bites from North African vipers and some kinds of Cobra snakes, which had been ordered in the meanwhile, was injected. Only 5 hours after the bite 60 ml of a polyvalent serum for Cobra venom intoxication which had been flown in by helicopter could be injected intravenously. In addition, a Ringer drop infusion, blood transfusions, gammaglobulin, and a 4 hourly injected dose of 40 mg prednisolone (Urbason) were given, and, at intervals, artificial respiration was carried out. The patient at first responded well to the treatment and the situation seemed less grave, however he died suddenly 17 hours after the bite from respiratory paralysis. In April 1963 another bite from a Cobra (*Naja naja*) proved fatal. The 37 year-old owner of a small snake farm in the Danube region was bitten in the left forefinger while removing the poison from the snake's venom-tooth. He tied off his forefinger himself and only an hour later he went to the hospital where he brought with him 3 different ampules of a polyvalent anti-venom for the treatment of bites from Mediterranean vipers and the poisonous snakes of Africa, Central and South America. Before removing the tourniquet and after a thorough excision of the wound was carried out, he was injected intra-muscularily with a serum against poisonous snakes of the African continent. After this treatment the patient left the hospital against the strong advice of the

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doctor. But 2½ hours he was admitted to the hospital again because of an acute deterioration of his state. He was given a drop infusion and artificial respiration was carried out. After 12 and about 18 hours after the bite, 10 ml of African snake serum were injected again besides the daily injection of 5 mg Decortin H which was given intravenously. 28 hours after the bite the patient suddenly died from heart failure and respiratory paralysis.

The 3 cases reported have the following in common:

1. The patients bitten though handling poisonous snakes every day were not sufficiently informed about the danger of a snake bite.
2. They did not possess a specific antivenom.
3. Even the doctors who had to carry out the treatment could only apply the antivenom — if at all — too late.

After this exposition of the snake bite situation in Germany kindly allow me to give you some details about the treatment of snake bites usually carried out in Germany.

The treatment is divided into first aid measures and the definite therapy including the application of antivenom.

First aid measures are fairly well known to doctors and laypeople. The application of a tourniquet and absolute immobilization of the bitten limb is a measure everybody seems to know. Incision, suction, and sometimes cooling the bitten area is done in most cases. Sometimes bleeding from the bitten area is allowed. In order to stimulate the patient's blood circulation coffee or tea is administered to the bitten person. Whenever a person is bitten by an adder in Germany specific antivenom is readily available at many chemist's, usually as polyvalent serum of the "Europe"-type, produced by the Behring Werke. This antivenom may be injected by the doctor administering first aid when immediate transportation to a hospital is not thought necessary. It is interesting to note that surgeons usually make incisions in order to open the bitten area, whereas internal specialists commonly prefer the more conservative treatment without the surgical knife.

From my own experience with tropical snake bites as well as adder bites in Germany I must confess that I have never seen advantages of the incision therapy. On the contrary, there were more disadvantages because of delayed healing and heavy scars in the incision area.

Chemical neutralization of the snake venom or burning out the wounds is no longer part of the snake bite treatment in Germany. Unfortunately there is still the practice of spraying potassium permanganate solution into the fang marks, though this treatment is ineffective or even harmful. Cryotherapy is of no importance in Germany. Besides the nonspecific therapy the administration of antivenoms is the treatment generally chosen even in cases of adder bites. According to the seriousness of the case — concerning age and health of the patient, and taking the locality and the symptoms of the bite into consideration — 10 to 20 ml are injected, partly locally, partly intramuscularily or even intravenously. In order to avoid allergic reactions serum tests are always recommended. 20 up to 30 ml, at the most, of antivenom have proved to be sufficient in cases of adder bites. There is not one case report in the literature available to me, where larger doses were used. Since the first publications about using corticosteroids in the

treatment of snake bites (Metts, 1951; Hoback & Green, 1953) providing good results this treatment has found its way into Germany being tried increasingly.

Besides the specific therapy with antivenom and antihistamines treatment with corticosteroids is regarded to be the most effective one. In the German medical literature Haas, Hartmann & Wündisch, and Lieske have published successful treatment of snake bites using corticosteroids. These authors strongly suggest a combined treatment of snake bites administering antivenom and cortisteroids, although they have not had a chance, so far, to use the combined treatment with more than half a dozen patients. Haas has given a review of the literature dealing with the use of corticosteroids and after detailed research comes to the conclusion that the treatment with corticosteroids can only be efficient, if the drug is administered in time and in sufficient quantities. 100 up to 400 mg intravenously or intramuscularily injected seem to be helpful in the treatment of bites from Elapides, Viperides, and Crotalides.

While Benyajati and col. in Thailand observed, in 1961, that even after application of 100 mg of hydrocortisone only patients already seemed to be responding to the treatment, whereas patients who had been given little or no antivenom were in a deep coma with respiration difficulties, the injection of 5 mg Decortin H daily or 40 mg of Urbason four times a day did not rescue the patients in Germany, who were bitten by a cobra.

In Scandinavia Tallqvist and col., in 1961, out of 163 adder bites treated 11% with antivenom and cortisone, and 8% with cortisone only and even then saw favourable results.

The fatality of the bites from tropical snakes in Germany during the last few years only results from the total lack, or the delayed injection, of a sufficient dose of antivenom, as the 3 cases of fatal cobra bites clearly demonstrate. This is partly due to commercial reasons: The antivenoms are expensive, they have a limited durability, and bites are rare.

The fatal snake bite cases in mind it has been discussed in Germany that owners or keepers of exotic snakes should be forced by law to have the specific antivenom at hand, but the government has not reacted to these suggestions yet.

Nowadays there are only a few leaflets issued by the Behring Werke or notifications in the medical press from time to time making known to the public where snake bite antivenoms are available in Germany, when snake bite accidents occur.

Of course there is a nonspecific treatment besides the combined antivenom-corticosteroid therapy. Salt solutions, plasma and blood infusions form a helpful measure in fighting the collapse or shock syndrome. Prophylactic measures against secondary infection, such as tetanus and gas-gangrene, are usually carried out. To avoid the fatal respiratory paralysis in cases where neurotoxic venoms have been inoculated, internal specialists, surgeons, and specialists for anaesthesia should cooperate in applying artificial respiration if necessary. In Germany iron lung therapy has not been used yet.

May I draw these final conclusions:

1. Bites by the native German adder do not bring forth serious problems. Treatment is easy and even without antivenom there are hardly fatal cases.

2. Bites from imported exotic poisonous snakes are highly dangerous and should be treated immediately with specific antivenom and corticosteroids.

3. Lethality after exotic snake bites is significantly higher than after native adder bites, the reasons being both the ignorance about the dangerousness of exotic snakes and the impossibility to get hold of the specific antivenom in cases of emergency.

4. Increasing imports of exotic snakes and more frequent travelling to the tropics are compelling German doctors to look into the problems of poisonous snake bites and develop a keen interest in the best treament.

TREATMENT OF ADDER BITES IN GERMANY

Author		Kellner	Lieske	Tropenkranken-haus *
Number of cases		10	8	8
First aid measures	Tourniquet	4	4	1
	Suction	2	1	1
	Incision	0	2	0
Medical treatment and therapy during hospitalization	Incision	10	1	0
	Antivenom locally	3	2	3
	Antivenom intramusc.	6	7	7
	Antivenom intraven.	4	0	1
	Corticosteroids	0	1	1
	Antihistamines	0	3	4
	Antibiotics	5	1	1
	Tet.-prophyl.	2	2	3
	Additional therapy	cognac, splint	glucose sympatol, splint	Periston, splint, Stroph.

* Not yet published.

DISTRIBUTION OF ADDER BITES IN GERMANY

Author		Kellner	Lieske	Tropenkranken-haus *
Number of cases		10	8	8
Children up to 16 years of age		4	6	1
Localization of bite	hand	8	7	5
	foot	2	1	3
Time of bite (month)	May	?	3	3
	June	?	1	0
	July	?	4	1
	August	?	0	4

* Not yet published.

FATAL COBRA (*Naja naja*) BITES IN GERMANY

Year	1956	1962	1963
Profession	showman	animal keeper	snake farm owner
Localization of bite	right forefinger	right forefinger	left forefinger
Type and quantity of antivenom injected	1) outdate Cobra antivenom 10 ml i.m. loc. 2) poliv. Europ.-Medit. antivenom 10 ml i.m.	1) Europ.-Medit. antivenom 20 ml i.v. 2) poliv.-Afric. antivenom 30 ml i.v. 3) poliv. Cobra antivenom 60 ml i.v.	1) African antivenom 10 ml i.m. 2) African antivenom 10 ml i.m. 3) African antivenom 40 ml i.m.
Time between bite and antivenom treatment	1) 1½ h 2) 3¾ h —	1) 15 min. 2) 1½ h 3) 5 h	1) 1 h 2) 12 h 3) 18 h
Corticosteroids	No	every 4 h 40 mg = 160 mg	5 mg daily = 10 mg
Antihistamines	No	No	Yes
Additional therap. measures	Oxygen, vasopressors	artif. respiration, gamma globulin, Ringer sol., blood	artif. resp., infus.
Hospitalization	No	Yes	Yes
Time between bite and death	6 h 10 m	17 h	28 h
Cause of death	respirat. paralys	respirat. paralysis	respirat. paralysis

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Discussion

P. J. Deoras: "Können bei Bissverletzungen in Deutchland mit tropischen Giftschlangen nicht polyvalente Seren verwendet werden?"

H. Lieske: "Da es sich bei tropischen Giftschlangenbissen immer um dem Opfer bekannte Giftschlangen handelt, ist natürlich monovalentes dem polyvalenten Serum vorzuziehen, falls solches vorhanden ist. Hinsichtlich der deutschen Antiseren kann Ihnen auch Dr. Zwisler von den Behringswerken Auskunft geben."

O. Zwisler: "Antisera produced by the Behringswerke AG, Germany, are polyspecific and direct against *Crotalus* and *Bothrops* species."

H. Pesce: "En los casos en que no se dispone de suero antiofídico específico, se ha aplicado con éxito en Iran el "Periston-N" polivinílico de la Bayer, en perfusión intravenosa, 500 cm.? ¿Que experiencia hay en Alemania?"

H. Lieske: "Periston-N (Bayer) ist in Deutschland bei Giftschlangenbissen bisher wenig verwendet worden. Über gute Erfahrungen zur Behandlung haemolytischer Vorgänge berichtet jedoch Hentsch aus Indonesien, der bis zu 2 Mal tgl. 500 ml. intravenös als Schnelltropf (45-60 Tropfen pro Min.) gab."

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29. DIAGNOSIS OF SNAKE BITE

E. R. TRETHEWIE and P. RAWLINSON

Department of Physiology and Zoology, University of Melbourne, Australia

Introduction

Micro techniques are now available for estimating precipitin antibody (Crowle, 1961). This has been used for labelling lizard serum (Rawlinson, 1964) and antibody to albumin in guinea-pig anaphylaxis (Trethewie, 1964). In view of the difficulty often experienced concerning the diagnosis of snake bite this technique has been applied to simplify this.

Method

The technique employs slides coated with agar in which small wells are cut — one centrally and a number circularly around at a distance of 0.5 cm. This is illustrated in figure 1. The slides are coated with a preparation of agar 1% Ionagar and 1% NaCl to which is added a 1/10,000 solution of aqueous Merthiolate and distilled water to a volume of 100 ml. This is allowed to soak for 30 minutes and then heated to 98°C in a water bath. 12 ml of this solution is thoroughly mixed and poured into a 10 cm siliconized petri dish, to make a layer 2 to 3 mm thick. Once the agar is set, holes are punched to the desired pattern. This agar "skin" is removed from the petri dish under water with a spatula. It is placed on a slide where it is trimmed to size and lightly touched or covered with filter paper which draws off excess saline. It is left for 24 hours at room temperature to dry. It is sometimes necessary to replace the filter paper after the first seven minutes. When the agar on the slide is dry the filter paper is removed, using distilled water. The stain is prepared from 1.5 g Azocarmine B (red), 500 ml methanol, 100 ml acetic acid, and 400 ml distilled water. It is put in a large petri dish into which the slide is placed and left for about five minutes. Excess stain is wiped off the glass. A washing solution of 1,800 ml ethanol and 200 ml glacial acetic acid is placed in a second petri dish into which the slides are placed for destaining for about seven minutes. The use of tweezers makes the handling of the slides less difficult but care must be taken not to scratch the agar.

Principle

It appeared to one of us (E.R.T.) that in view of the difficulty in assessing:

1. Whether a child has been bitten by a snake at all (say in long grass) or

2. The type of snake biting which:

(i) may not have been seen

(ii) seen briefly and insufficiently to diagnose or

(iii) seen by someone unintiated in knowing the type when seen adequately;

that this technique could be applied to the diagnosis of snake bite.

Australian snakes present a well marked feature of cross and multi-antigenicity. This is so well developed that originally polyvalent tiger antivenene was regarded as the treatment of choice for snake bite by any snake in Australia. Most snakes in Australia are venomous. They are *Notechis scutatis* (Tiger snake), *Oxyuranus scutellatus* (Taipan), *Acanthopis antarcticus* (Death-adder), *Pseudechis porphyriacus* (Black snake), *Denisonia superba* (Copperhead), and *Demansia textilis* (Brown snake). Publishing recently on snake bite I referred to regional distribution in Australia as a guide to treatment (Trethewie, 1966) where the snake biting is not definitely known. This affords additional information as regards administering the specific antivenene.

As regards antivenene treatment and excluding for the moment other local and general measures, we recognize that sera from the Tiger snake, Taipan, Brown snake and Death adder are sufficiently distinct to make them individually useful for treatment rather than the universal use of tiger antivenene.

Experimental

When we place a known venom in the centre well of our preparation and differing antivenenes in the five circular wells we find the following distribution of precipitin reactions (Table 1). This we find is adequate to make a diagnosis.

TABLE 1

Snake or venom	Antivenene				
	N.S.	O.S.	P.P.	A.A.	D.T.
Notechis scutatus	111	11	11	11	11
Acanthopis antarcticus	11	1	1	111	0
Pseudechis porphyriacus	11	11	111	1	0
Demansia textilis	1	11	0	0	111

We have compared the use of saline extract of the bitten area, serum from whole blood, and the expression of serum from the bite or injection for diagnosis. Expressed serum is the best material.

In the case of Tiger snake venom placed in a well, or expressed serum from the "bite" (injected) area of the animal (in this case a guinea-pig), marked precipitin reaction occurs against the Tiger antivenene and moderate precipitation against the remaining four (Fig. 1).

In the case of Death adder venom injected into a guinea-pig leg (20 mgm) extract shows marked precipitation against Death adder antivenene, moderate against Tiger, slight against Taipan and Black snake and no significant reaction with Brown antivenene. These appearances are far clearer on the original glass slide preparation (Fig. 2).

With Black snake venom (5 mgm injected into a guinea-pig) likewise marked precipitation occurs with Black snake, moderate with Tiger and Taipan and slight reaction with Brown antivenene (Fig. 3).

In one instance a Brown snake was place against a guinea-pig and encouraged to bite and in this instance serum expressed from the bite on the guinea-pig's leg (Fig. 4) showed marked reaction with Brown antivenene, moderate with Taipan, slight with Tiger, and none with Black and Death adder. When live snakes are used, as is projected, reactions may be expected to be more clear cut because dried commercial serum may be faulty.

These findings are summarized in the Table 1 and Fig. 5. It can be seen that if one considers only the two latter columns there is an almost significant separation of the venom designation. When all five antivenenes are used the distinction is confirmed by maximal precipitant forms against the homologous antivenene.

In this way we are able to separate serum obtained from different types of bite or injection in the experimental animal. The reaction time is five or more hours except with Brown snake which is two hours and we are endeavouring to speed up this reaction. We suggest in the first instance to give antivenene according to the information of the snake if available and the geographic region bitten (with reference to the colour of the snake) and in the absence of this information to use Tiger antivenene in the first instance and subsequent injections to follow the diagnostic pattern. It is suggested that development of this technique may obviate the confusion arising from the two problems, 1. was the subject bitten at all? and 2. what was the nature of the snake? We are proceeding with these experiments.

Discussion

The above technique shows that material obtained from the site of the bite-either saline injected especially where there is severe thrombosis, or bled material from incision, affords satisfactory diagnosis of the snake-bite when set up in double diffusion.

The advantages of this procedure are obvious especially where specific antivenene is essential for treatment and this is preferable in the case of Australian snake bite as regards Tiger, Brown snake, Taipan and Death adder.

The time required for a positive test — 2 hours in the case of Brown snake and longer with the others, at room temperature — is a practical difficulty, but we have been able to obtain a positive result more quickly at 37°C and this technique is now being standardized. This should give an adequate answer in approximately one hour which is quite suitable for treatment. In Australia an initial injection of Tiger polyvalent antivenene is advised.

Summary

1. Diffusion techniques with agar plates give a set pattern of reaction for each Australian snake venom against the series of individual antivenenes.

2. Venom injected into a guinea-pig allows the collection of material from the region of the bite for similar analysis.

3. Material from the region of the actual bite of one snake on a guinea-pig also provided adequate diagnostic material.

4. It is considered this technique may be employed in hospitals to ensure accurate snake bite diagnosis.

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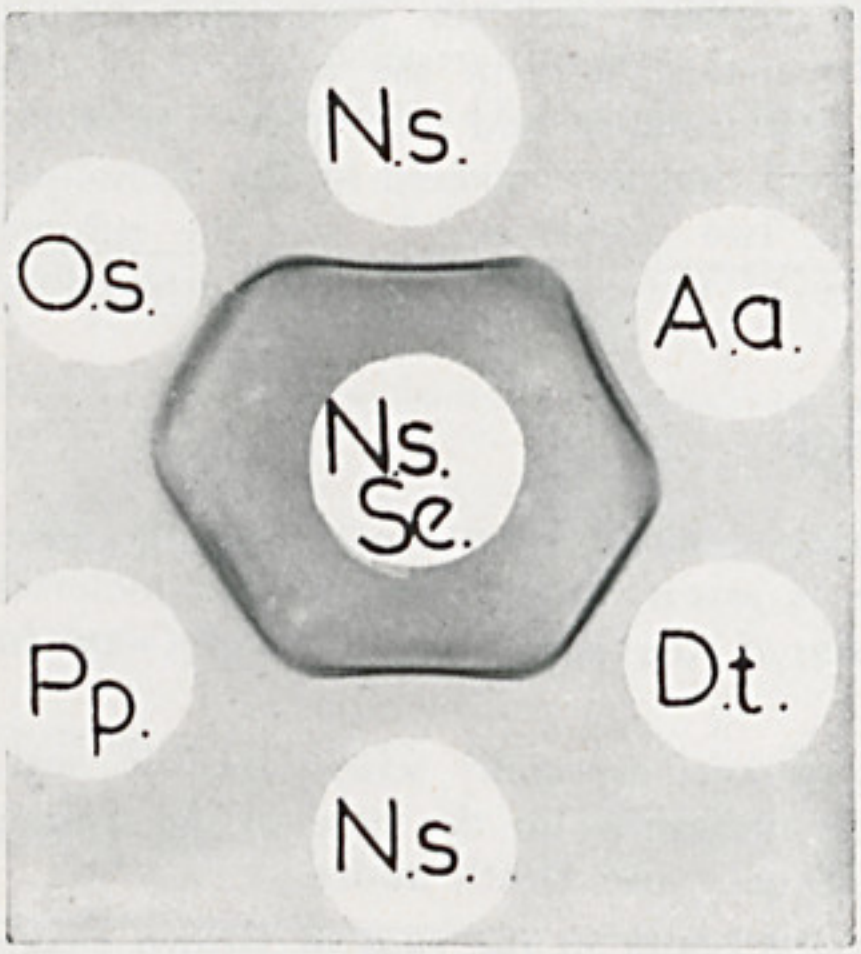


Fig. 1 — Reaction to Tiger venom (Centre well) against Death Adder (Aa), Brown Snake (Dt), Black Snake (Pp), and Taipan (Os) antivenene. (Room Temperature).

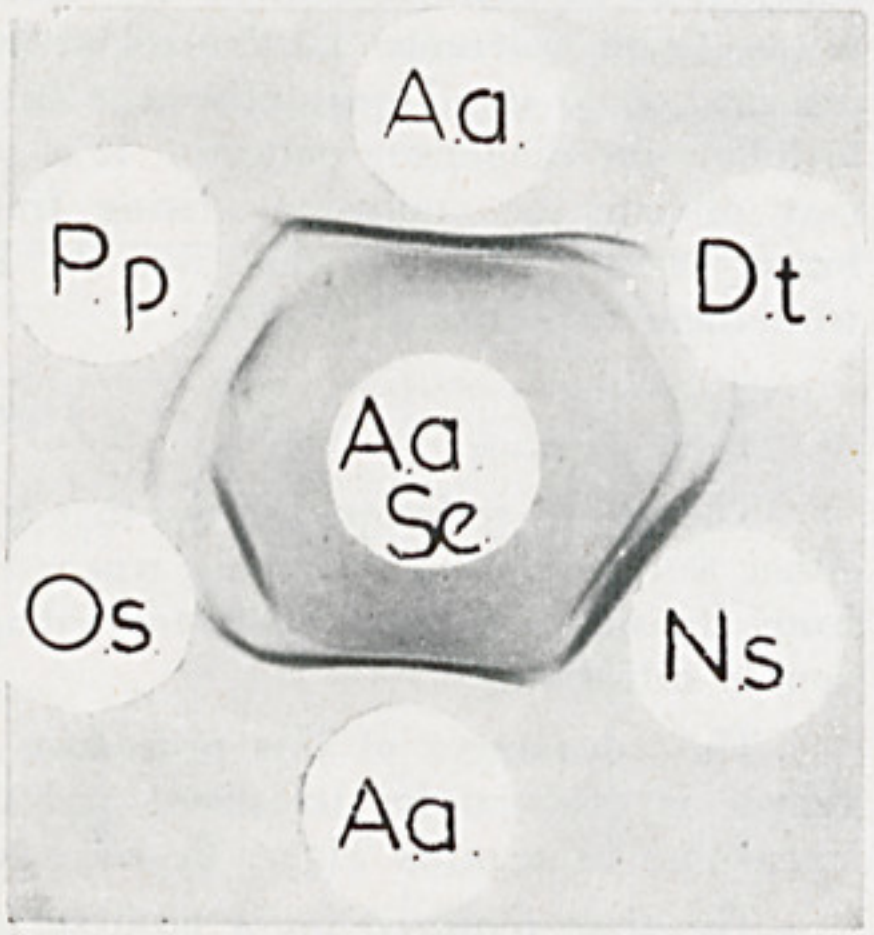


Fig. 2 — Reaction to material from the region of injected Death Adder venom from a guinea-pig (Centre Well). Outer wells contain Brown Snake (Dt), Tiger Snake (Ns), Taipan (Os), and Black Snake (Pp). (Room Temperature).

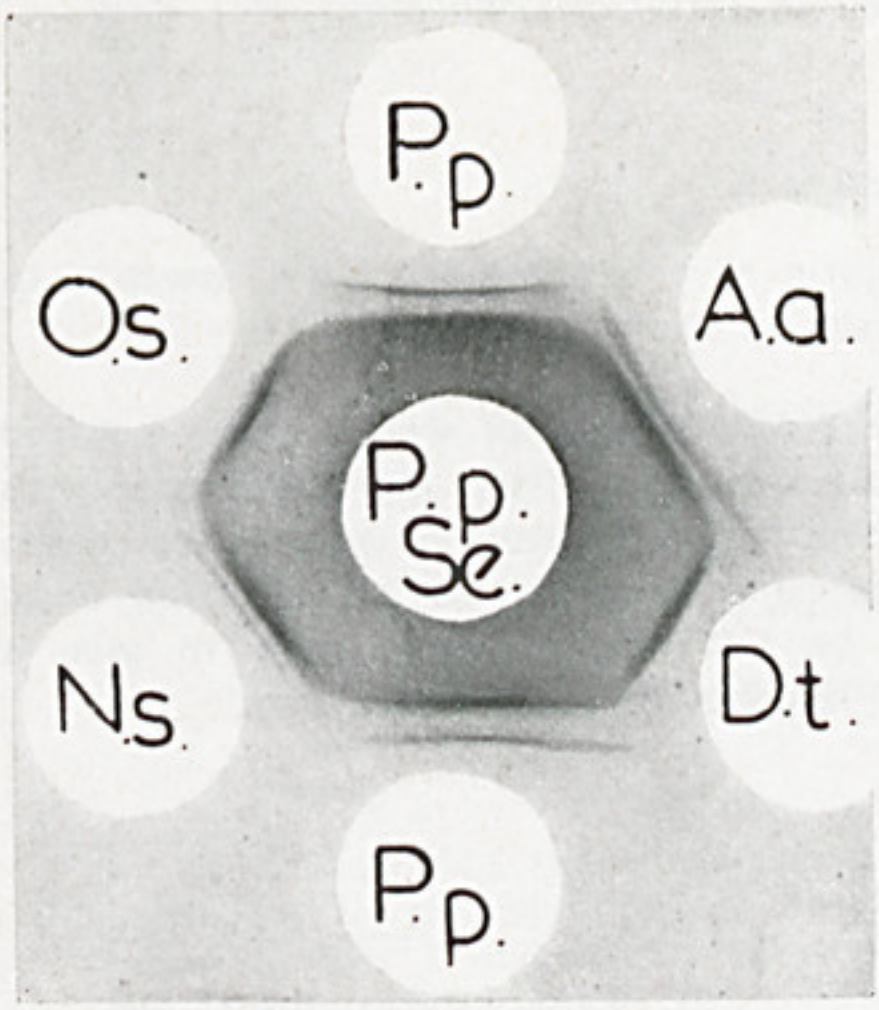


Fig. 3 — Reaction to material from the region of injected Black Snake venom from a guinea-pig (Centre well). Outer wells contains — Death Adder (Aa), Brown Snake (Dt), Tiger Snake (Ns), and Taipan (Os). (Room Temperature).

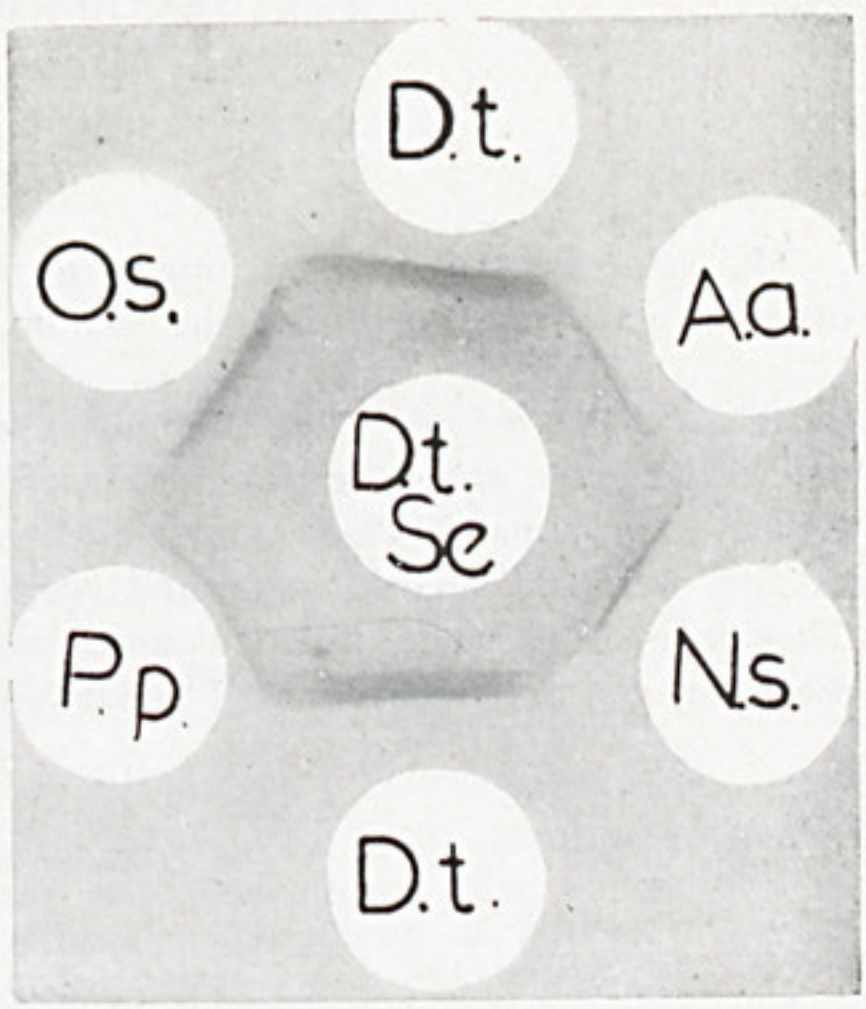


Fig. 4 — Reaction to material from the region of a bite of a Brown Snake on a guinea-pig (Centre well). Outer wells contain — Death Adder (Aa), Tiger (Ns), Black Snake (Pp), and Taipan (Os). (Room Temperature).

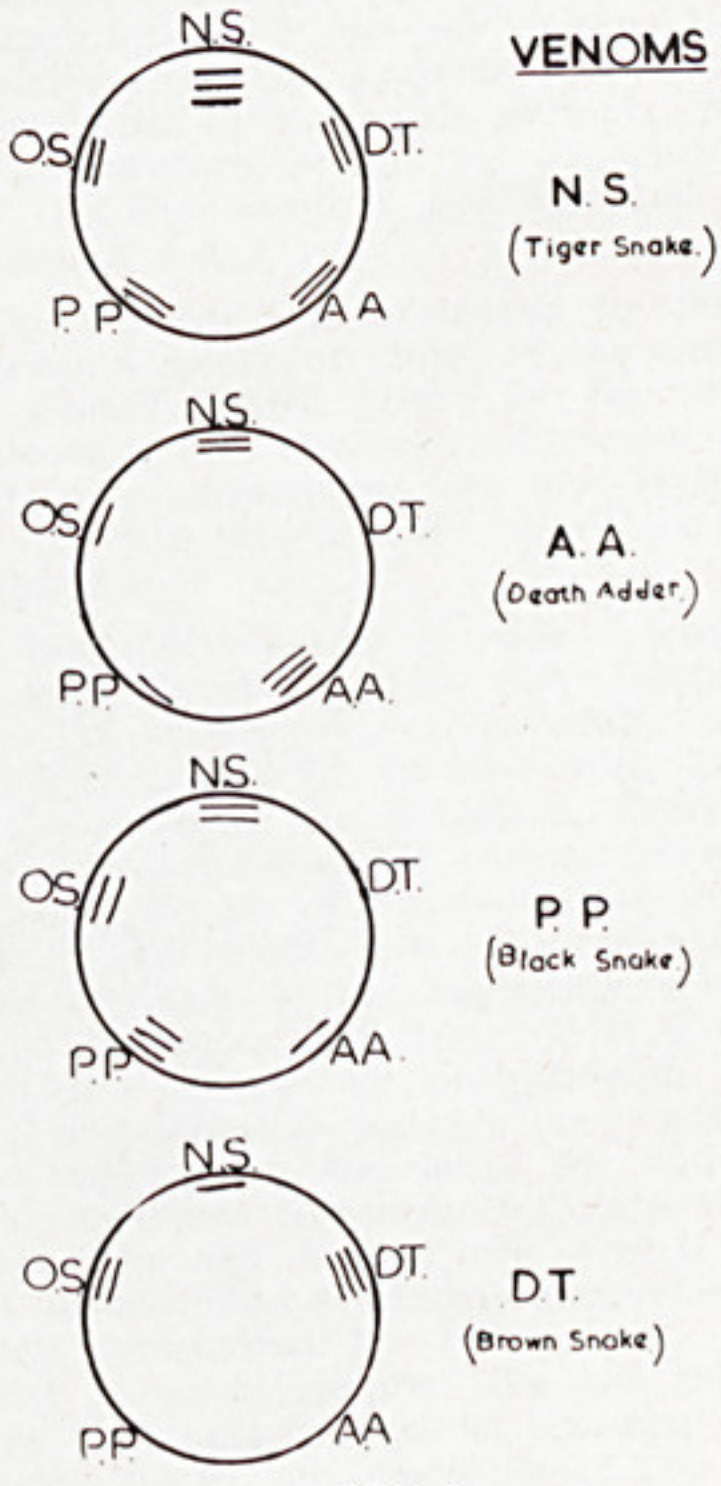


Fig. 5

Discussion

S. Minton: "How long might a precipitin test be obtained after death? In other words, how long does the venom persist in the body?"

E. R. Trethewie: "We had a case of Tiger snake bite where death ocurred after four days and at autopsy extract of the skin area bitten killed 100 mice with typical symptoms of Tiger snake envenomation. Allergic antigen stays in the tissue of the skin for several days and may produce a delayed reaction of seven days. Therefore I consider much venom would be left in the bitten area to give a diagnosis by this technique of medico-legal importance for several days."

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30. COMMENTS OF THE MODERATOR

GASTÃO ROSENFELD

Instituto Butantan, São Paulo, Brasil

It is a role of the moderator to comment and to make a summary of papers presented to the Symposium. I will not withdraw from this obligation, all the more so that I and the collaborators of the Department of Physiopathology and the Hospital Vital Brazil from Instituto Butantan were not active participants in this session, in view of the high number of foreign specialists who had kindly accepted to take part in this Symposium. It is obvious that with 13 foreign participants registered, each of them having 30 minutes for presentation and discussion of his paper, there would be a 6 hours and 30 minutes long session. Thus, there was material impossibility to register papers of our group, and we did not do it in order to leave the time for our foreign colleagues who had a long journey to attend this Symposium.

In 1954, Dr. Afrânio do Amaral, at that time Director of the Instituto Butantan, gave me the attribution to direct and reorganize the Hospital Vital Brazil, function which I carried out until March, this year. In this service and in the Department of Physiopathology, I and my collaborators have had the opportunity of raising some experience in the subjects discussed in this session. During this period, 15,709 patients bitten by poisonous animals came to our service to look for medical assistance. I, therefore, profit this opportunity to address the house as a Moderator of one of this Symposium's sessions. Let us then start to comment the different papers presented today.

The paper of Dr. Parrish was presented by Dr. McCollough and it gave us the opportunity to become aware of many of the epidemiologic aspects of poisonous snake bites in the United States. Here in the southern hemisphere, and with different climate, our data are, obviously, different. These aspects of accidents by poisonous animals are very interesting and are useful, not only for the evaluation of the problem, but for the informations which may guide to improve prevention of these accidents as well.

Dr. McCollough showed a series of cases bitten by snakes from the genera *Crotalus* and *Agkistrodon* from the United States. The frequency, intensity, and extension of necroses are impressing. In our opinion they are typical consequences of deficient treatment by antivenin. Deficiency of serumtherapy may be due to three factors: unspecific antivenin, delayed treatment, and unsufficient doses. The first and the second factor cannot be blamed in those cases, only the deficiency of antivenins' doses. What may have contributed to this fact is that the authors referred to the number of vials as a criterion for dosage evaluation. This is a mistake we all made at the beginning; it is why, at those times, we had similar cases to those we have seen now. It is a nonsense to say that a patient was treated by such or such number of antivenin vials. Antivenin's potency vary from one producing laboratory to another and, unfortunately, there are many of them which do not indicate on the label the neutralizing capacity of venom in miligrams. Even the Instituto Butantan fell into the same error with some antivenins up to a short time ago, and it still does it with arachnidic antivenins. It is essential that all laboratories producing antivenins should indicate on the label the number of "units" contained for the venom. "Units", as we propose, is the ability of neutralizing 1 mg of venom. We use this designation and definition in our works since some time ago, because we consider it of great practical importance, either to the physician or to the researcher. The reason is that, once the anti-

venin is an agent neutralizing the venom, it is necessary to inject, the earlier the better, such a number of "units" which will be able to neutralize all of the venom that eventually has been inoculated by the animal. By knowing from the specialists of each country or region the quantity of venom in mg contained in the different species' glands, the physician will be able to evaluate the amount of "units" to be injected in order to neutralize actually the whole venom which may have been inoculated. It is an elemental and a simple arithmetic question without any difficulty for anyone. This criterion is as obvious as in medicine's other fields, where nobody would give a drug unless its quantity could be referred to in relation to weight or unit. For instance, nobody prescribes a corticoid without indicating its number in mg of "units". Thus, we do not understand how one may have the courage to indicate serumtherapy measured in mililiters or vials without knowing how many mg of venom the antivenin is able to neutralize. We insist that this tradition is wrong and it should be corrected. It is interesting that I have already found, in a publication of Vital Brazil of about 1910, the statement about the necessity of indicating antivenins' potency in mg of venom which they are able to neutralize; unfortunately, this was forgotten.

From this reasoning comes our opinion that the results we have seen are only a consequence of insufficient serumtherapy and not of antivenin's ineffectiveness, as it may have seemed like. Obviously, the specific treatment is useless after the necrosis occurrence. There is only the symptomatic, clinical, and surgical treatment left, which, infering from Dr. McCollough's words, has been well conducted.

About this point, we would still like to present an information: on about 1,600 cases of *Bothrops* bitten patients treated in the Hospital Vital Brazil in the last 11 years, there was, practically, no need for amputations excepting those cases which came to the Hospital late after the bite, when dry necrosis had already occurred.

Another treatment presented by Dr. McCollough, deserving some comments, is the one of incisions done for elimination of venom. To us it lacks physiological basis. These incisions intersect blood vessels and through these intersections is going to flow the circulating blood which does not carry any venom. The venom is in the tissue and it penetrates by lymphatic way. Circulation stops, as a consequence of the solution of continuity and the venom will stay at the site, aggravating necrosis. Besides, it will not be in contact with the antivenin coming through the blood circulation. In envenomations with proteolytic venoms, which provoke necrosis, incisions will enhance this effect. Something that has physiological basis is, withdrawal of the venom from the site of the bite by suction of the site attained by the fangs, if it is bleeding. Otherwise, to prick with a needle around the site is an aid to the outflow of serosity. If this procedure is carried out within the first half hour after the accident, part of the venom will be eliminated; later it is useless.

One more comment about the ligature which has been advised. Dr. Deoras has already asked a rather "venomous" question during discussion, and we agree with him. In fact, ligature is of no reason. If the venom is proteolytic, the circulation retention keeps the venom at the site, helping to provoke necrosis. If the venom is neurotoxic, it penetrates with or without ligature, since it contains an appreciable amount of hyaluronidase which helps venom's dissemination. And still, if the ligature is perfectly done as to really prevent the venom from penetrating the circulation, there will not be any blood circulation. Then, depending on how long the ligature was kept, loosening it will provoke shock which may be fatal, since the general condition is aggravated by the envenomation. Here, at the Hospital Vital Brazil, nurses are afraid, by experience, when a patient arrives with a good ligature; they will not open it, leaving it to the physician, because at this very moment many patients have had a peripheric shock. By the way, in experiments we made with Dr. Schenberg, present at this session, we provoked many shocks in dogs using only ligature. A well done ligature is enough to provoke a fatal shock even in normals, there is no need for venom. As a matter of fact, this mechanism of shock was discovered by Trueta in the II World War, during the bombings of London.

Dr. Kornalik presented very interesting data on the problem of fibrinolysis and blood incoagulability as provoked by snake venom. The demonstrated facts are valuable but their interpretation may be another. In order to make it clear

to the audience, it would perhaps be better to explain (in our point of view) what happens with coagulant and proteolytic venoms. *In vitro,* a small amount of venom clots the blood due to its coagulant fraction, which is active even in very small concentrations. This is not so with the proteolytic fraction. In higher amounts the venom provokes clotting by its coagulant fraction, then the fibrin formed is lysed by the proteolytic fraction and its high concentration permits its action before coagulation would occur. *In vivo,* small amounts of venom provoke hypercoagulability at the first minutes, fibrinogen clotting, and then blood incoagulability, due to a gradual and massive defibrination. In the phase of hypercoagulability, a fugacious fibrinolytic activity appears which disappears when the blood becomes incoagulable by defibrination. *In vivo,* there is no fibrinolysis or fibrinogenolysis by the direct action of venom because such high amounts would be necessary for provoking these effects which are, practically, impossible to obtain. Besides, they would almost instantly provoke death.

The presentation of Dr. Efrati's paper was very clear and synthetic. The clinical picture presented is exactly the same as the one observed in accidents by *Bothrops* snakes and all snakes with coagulant and proteolytic venoms. His definition of some symptoms as being an "anaphylactoid picture" is very appropriate, since they are due to the proteins' decomposition by the venom and the consequent liberation of resulting substances in the circulation. Thus, it provokes the same kind of shock obtained by injecting proteins or their degradation products. Dr. Efrati pointed out that he did not observe hemolysis, in spite of the venom being hemolytic *in vitro.* The reason is that through the bite, the venom is inoculated in tissues and it penetrates slowly the circulation. It is very diluted in the blood, never reaching a high concentration. But, if the venom is injected intravenously, the hemolysis will appear. Dr. Efrati refered to a case in which neurological symptoms appeared. I would like very much to discuss this with him and to find out what kind of symptoms they were, for two reasons: first, that I did not know that snakes from the genus *Vipera* had neurotoxins; second, that I would like to know if these neurological symptoms manifest the presence of modifications which produce what we call "neurotoxic facies", and which exist in all cases of envenomation through snakes containing neurotoxins. Unfortunately, I am not able to show a demonstrative slide. I will do it in other opportunity. Dr. Efrati's recommendation about the antivenin intravenous injection, the earlier the better, is very exact and we have here the same principle. However, we do not agree with his reference to the number of vials for the serumtherapy, for reasons we have already mentioned. I would still like to utter my personal point of view: I consider as very peculiar the fear of the physicians to inject greater amounts of antivenin while not fearing to leave the patient exposed to the risk of death and necrosis as a consequence of insufficient treatment.

The work of Dr. Ohsaka is extremely good and of an edifying experimental perfection. The way of evaluating *in vivo* the hemorrhagic action of venoms, giving way to compare different venoms, is very nice and based on a well imagined experiment technic. Dr. Ohsaka presented an important fact which is, the separation of two hemorrhagic fractions which are not bound to the proteolytic factor. This is a new fact to us, since the general idea is that the proteolytic factor is responsible for hemorrhage by causing the rupture of capillary walls. During the discussion of Dr. Ohsaka's paper, Dr. Purananda asked about the time the active venom substances may stay in the circulation. Since there was not a clear answer, I may inform about what happens with bothropic venom which is of the same kind as the Habu. In a severe envenomation, while the blood is incoagulable, the venom's active substances are present in the circulation even 48 hours after the bite. They are rapidly neutralized when antivenin is injected intravenously.

Dr. Schenone's paper was very well presented and we already knew part of it. He advises antihistaminics administration and we agree with that for a long time ago. About the indication of corticoids, however, we disagree. We do not have any proof that it is useful. It seems more likely to be some kind of a crutch in case there is no antivenin. One has to do something, so one gives corticoid. It is a rest to the doctor's conscience to give the patient some medical treatment. What should be done in the countries facing the problem of loxoscelism is to produce a *Loxosceles* antivenin which really neutralizes the spider venom, as it was done by the Instituto Butantan, here in Brazil. We have experience with this antivenin which presented some interesting peculiarities for the physician. At

the beginning, even when serumtherapy was given in time, some cases showed, after the treatment, a hemolytic syndrome. At that time, 2 to 5 vials were injected. Later, in other patients we started to inject 10 antivenin vials and, after that, no more cases of hemolysis were observed after the treatment. We do not mention "units" but vials because we did not succeed when we suggested that this antivenin should also indicate the quantity of mg or *gama* which it is able to neutralize. In the discussion of Dr. Schenone's paper he was asked about the cardiotoxic activity of *Latrodectus* venom which is apparent by the arterial hypertension he has observed in these envenomations. We would also like to comment this topic. We have, with much frequency, accidents by the spider *Phoneutria fera* which, like the *Latrodectus*, has a neurotoxic venom (different from the ophidic one, since it acts on the peripheric nervous system). In these cases the symptoms are identical to those refered to by Dr. Schenone, including the arterial hypertension. However, we do not consider the hypertension as due to a direct or indirect cardiotoxic activity on the neuro-vegetative nervous system, because as long as the pain is suppressed by means of an hypnotic or an anesthetic, the arterial pressure gets normal, and the hypertension reappears when the pain returns. We consider it, by this clinical evidence, as a secondary symptoms to the pain and not a direct venom activity.

The paper of Dr. Chapmann is extremely interesting to us because we have very few literature and data on ophidic accidents occurring in Africa. It has been a great lesson the way he presented the well tabulated data, including symptomatology. Dr. Chapmann is against incision as therapeutics, since he thinks, like we do, that necrosis provoked by the venom is already sufficient damage. Dr. Chapmann is also against ligature for the same reasons we already discussed before. He referred to the use of ligatures as a psychological effect but it seems to me that even this should not be tolerated. In the medical and physiopathological point of view, it may only be unfavourable to the patient.

Dr. Lieske brought an interesting contribution to the problem of snake bites, showing that even in Germany accidents of this kind may occur, provoked by snakes imported with merchandise. It is a hard problem and it can only be solved, as said Dr. Deoras, providing these countries with antivenins suitable for the snakes of the countries with which they have trading. We had the opportunity to know one case in quite a peculiar way. Once, when I was in Valparaiso, Chile, talking about poisonous animals, a physician from the Chilean navy said that this was no problem to them because there were no poisonous snakes in his country. Some days after being back in Brazil, a radio appeal came to Butantan, asking urgently for Elapidic antivenin. A dock worker in Valparaiso had been bitten by a poisonous coral snake while unloading some banana bunches arrived from Equador. The antivenin was sent in a few hours and, fortunately, the patient was saved. Dr. Lieske advised the use of corticoid in the treatment of snake bites. We disagree of this point of view based on an experiment made in collaboration with Dr. Langlada, published in Memórias do Instituto Butantan, 1964. Neither Dexamethasone nor ACTH in small, medium, and high doses showed any usefulness. On the contrary, they increased mortality with some venoms in experimented animals. All the same, we use corticoid in ophidic envenomation, but only for treatment of shock when it occurs. Dr. Lieske related some fatal cases, in spite of serumtherapy given in time. But, as admitted by himself, the antivenin doses were not sufficient. So we will not discuss this point.

Thanks for all collaborators to this Symposium.

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31. THE PREPARATION AND PURIFICATION OF ANTIVENOMS

P. A. CHRISTENSEN

The South African Institute for Medical Research, Johannesburg, South Africa

This short talk on the preparation and purification of antivenoms must of necessity be influenced by the attitude adopted at the South African Institute for Medical Research, but knowing that there is room for improvements, and having noted the titles of the many papers to be presented today, this attitude may well have changed before the end of this Symposium.

My experience with the preparation of scorpion and spider antivenoms is rather limited, our main concern being the production of snake antivenoms, but the basic problems are the same, namely, 1, which animal to choose as serum producer, 2, which venoms to use as antigens, 3, how to use the venoms as antigens and 4, how to treat the resulting serum to make it suitable for use.

The use of sera from animals other than the horse may carry less risk of serum reactions in some persons, but the horse is the natural choice in climates where it thrives and can be obtained at a reasonable price. It is easy to handle, yields a large volume of serum, and methods of purification of horse antitoxins have been thoroughly studied and are technically more advanced than is the case with antitoxins derived from other animals.

With regard to which type of horse to choose, there can be no strict rule, and one's views are based on general impressions rather than controlled experimentation. It is natural to prefer big horses because they yield more serum, but young horses do not respond better than old, rather the opposite may be true, and leniently immunized, the life expectancy of antivenom producing horses is very long, though not always as long as that of a horse dying earlier this year at the age of 29 after 18½ years' continuous antivenom production.

Most laboratories making antivenoms prepare other antitoxins as well, and it is common practice to screen new horses for naturally induced antibodies to diphtheria and *Clostridium perfringens* toxin and to immunize them prophylactically against tetanus. Such horses can be allocated to the production of diphtheria, tetanus or gas gangrene antitoxin according to immediate requirement, and can be transferred from the production of one kind of antitoxin to that of another without much delay. The response of a horse to one antigen does not necessarily indicate its potential response to another, and horses previously immunized with bacterial toxins, but no longer required or discarded because of falling titre, are as good antivenom producers as any other horse. It can be argued that the presence of significant amounts of other antitoxins in the raw serum will reduce the ratio between antivenom potency and protein content of the final product because purification methods do not discriminate between antitoxins, but this is a minor objection.

Which venoms to use as antigens must obviously be determined by the frequency of bites by different snakes, the severity of the effects, and the availability of venom.

Availability of venom is essential for continued large scale serum production but goes usually hand in hand with the frequency of bites by different species and to some extent with the severity of the effects, because larger snakes delivering much venom tend to do most damage.

In the majority of snakebites the culprit is not seen or is not identified and this is one of the reasons why polyvalent sera are preferable to monovalent, except in areas where dangerous bites are almost exclusively due to a single species. The choice of venoms for the production of polyvalent serum must of course be influenced by the cross-neutralizing properties of monovalent sera prepared with the different venoms, but too much reliance should not be placed on paraspecific action. The immunological overlapping of protective antibodies, in which the earliest workers placed much faith, is very limited, as we know from the work of Dr. Vital Brazil and others who followed him. Not only is the paraspecific titre of a serum lower than the specific but a serum's therapeutic value must depend not only on titre but also on the firmness of the union between antigen and antibody, and paraspecific venom-antivenom complexes tend to dissociate.

Some laboratories prepare polyvalent sera by blending several monovalent sera, others, including ourselves, prefer to immunize the horses with all the antigens. The obvious argument against blending monovalent sera is that each serum is being diluted by the addition of the others. This must happen in the case of antibodies with strict specificity but not with antibodies to common antigens, just as antibodies to related antigens could show some additive effect. The dilution of antibodies could be counterbalanced if the horses receiving a single venom reached much higher titres than those immunized with several venoms. Horses given only one venom could respond better because larger doses could be injected or because a 'crowding' effect would suppress the response to important toxins in the horses receiving several venoms. However, horses given only one venom soon reach a state of immunity which does not improve unless the dose of venom is increased quite out of proportion to the rise in antibody titre. Furthermore, such monovalent horses usually maintain their titre to this particular venom after they have been transferred to the production of polyvalent serum.

Once immunized, a horse will tolerate large single venom doses, but new horses are easily killed. A horse immunized many years ago for the preparation of monovalent *Naja nivea* antivenom received single doses of up to 2 g of venom without symptoms, yet 15 mg of the same venom given in error killed a diphtheria antitoxin producing horse before the mistake was discovered and the outcome prevented with antivenom. To give new horses their first, basal, immunity with unmodified venom is a tedious process, and the initiation of basic immunity is therefore the first part of the next problem, how to use venoms as antigens.

To use venom-antivenom mixtures for this purpose is also tedious because such mixtures are dangerous when they are under-neutralized, and fully neutralized they are poor antigens. Many ways of rendering venoms atoxic without destroying their antigenicity have been suggested over the years, but only detoxification with formalin seems to have been used on any scale. But the loss in venom toxicity due to formalin is accompanied by a large loss in antigenicity, and the use of formol-toxoided venoms, or anavenoms, is wasteful of venom and can be the cause of much suffering of the horses. Actually no detoxification is neces-

sary if the venom is adsorbed on some inert carrier; the harmlessness of adsorbed venoms is presumably due to a decreased rate of absorption from the site of injection and the good response is probably due to prolonged stimulation by antigens held in subcutis, apart from any possible adjuvant effect of the adsorbant.

In principle this method originated with Calmette who in 1894 recorded that a small piece of chalk impregnated with venom and coated with collodion and inserted under the skin of a rabbit would serve as a continued stimulation from an artificial gland, a suggestion he credited to Dr. Roux, in whose laboratory he was working.

Criley (1956) had good results with venoms adsorbed on aluminium hydroxide, a method we found unsuccessful many years ago, possibly because our aluminium hydroxide gels were unsatisfactory, and I turned to the use of venoms adsorbed on bentonite, with good results during the last fifteen years.

The first basic immunity of the horses is achieved with a few injections of from 25 to 100 mg of venom adsorbed on a 2% suspension of bentonite in distilled water. The venom solutions are sterilized by filtration, the bentonite suspension by steam under pressure. This method saves time, venom and labour, but it would appear that not all preparations of bentonite are suitable. As soon as traces of circulating antibody are detectable by mouse protection tests, the immunization is continued with venom solutions sterilized by filtration and preserved with 0.25% cresol, but without the addition of bentonite. The injection of a suspension of bentonite does in some cases cause the formation of a small sterile abscess which requires incision, but the lesion heals in few days and does not upset the horses, and although some antigenic material must be evacuated through the incision, enough remains to stimulate antibody formation.

The greatest advantage of the use of plain solutions of unmodified venoms for the continued immunization is the lack of untoward reactions in basically immune horses. A healthy horse will not only live longer but will presumably in the long run respond better to antigenic stimulation than a horse in poor condition due to repeated injury, such as that caused by some adjuvants. The use of Freund's complete adjuvant gives amazing results in horses immunized with diphtheria or tetanus toxin (Mason, 1963), but tends to cause rather severe reactions. Unpublished experimental work by J. H. Mason has shown that the severity of the reactions is considerably reduced if antigen and Freund's adjuvant are incorporated in a multiple (water-in-oil-in-water) emulsion of the type described by Herbert (1965). As far as venoms are concerned, we have failed to observe any difference in the response of comparable groups of horses immunized with or without the addition of Freund's adjuvant in the form of simple or multiple emulsions.

South Africa's needs for scorpion and spider antivenoms are met by one or two immunized horses, too few to allow one to form any opinion on the value of different methods of immunization, but the methods in current use are briefly as follows.

Scorpions of the genus *Parabuthus*, regardless of species, are kept alive and the collected venom is dried under vacuum in a desiccator and dissolved in saline as required. Filtration through Seitz pads removes about 75% of the toxin, and the solution is therefore sterilized by the addition of phosphate buffer and beta-propiolactone to a concentration of 0.2%. Having stood overnight at

about 3°, the solution is mixed with procaine immediately before injection in order to make it painless. Sterilization by means of beta-propiolactone is very convenient even if it does reduce the toxicity by about 30%.

Latrodectus indistinctus antivenom is prepared with extracts of dried cephalothoraces. The tedious task of isolating the chelicerae with the venom glands for extraction and use as antigen was discontinued because extracts of the remaining cephalothoraces were toxic and sera prepared with either chelicera extract or cephalothorax extract gave complete cross-neutralization. This was more than likely due to incomplete removal of the venom glands but the practical implication was to save the effort of collecting chelicerae. One might add that a serum prepared against the extremely toxic extract of spider abdomens is ineffective against the true venom, just as the ordinary antivenom fails to neutralize the toxic material obtained from the abdomens. The extracts have hitherto been sterilized by filtration through Seitz pads or with beta-propiolectone but we intend to resort to membrane-filtration in order to minimize the loss of toxin which is quite considerable.

Irrespective or which kind of serum they produce, snake, scorpion or spider antivenom, the horses rest for about five weeks between courses of immunization lasting about two weeks. The crude serum, or rather plasma, obtained at the end of each course of immunization is improved for therapeutic use by purification, which removes inactive material, and by concentration, which reduces the volume to be injected.

Purification by means of salt fractionation came into general use after Dr. Vital Brazil had shown that the distribution of antibodies in antivenoms was similar to that in bacterial antitoxins, but, although they are still used, such earlier methods have been superseded by others involving treatment with proteolytic enzymes, pepsin in particular.

The interest in the use of pepsin for this purpose began in 1902 and was smouldering until Parfentjev (1936), Pope (1939a, 1939b) and Hansen (1941) published methods suitable for large-scale purification of bacterial antitoxins. In all the three methods, the serum is treated with pepsin at a controlled pH, but the methods of Parfentjev and Hansen rely on adsorption for the removal of inert material, whereas the unwanted protein is removed by heat-coagulation in Pope's method which was further developed by Harms (1948). *Latrodectus mactans* antivenom was successfully purified already in 1942 by Pirosky and co-workers in Argentina using Pope's technique, and this is the method used at Instituto Butantan (Höxter and Decoussau, 1949) and at the South African Institute for Medical Research (Grasset and Christensen, 1947).

The advantages of pepsin-treated sera are too well known to warrant lengthy discussion. Such sera are stable when stored at reasonable temperatures and, of more importance, the incidence of serum reactions is reduced to a low level. It may be of interest to note that the incidence of serum sickness in children treated with pepsin-refined diphtheria antitoxin has been found significantly lower in Bantu patients than in Whites (Mason & Christensen, unpublished), which is gratifying because the Bantu is more likely to need treatment with antivenom, which will behave as other equine antitoxins with regard to serum reactions.

The draw-back to the production of pepsin-refined antivenom is the cost. A contributing factor is a considerable loss of active material during the process. The well-known difficulties in assessing antivenom potency makes it difficult to

estimate the amount lost, but it is probably about 55%, which is not surprising as the antibodies are distributed in both the so-called pseudo- and eu-globulins, and even the water insoluble globulins of viper antivenom may contain some antibody (Christensen, 1955).

In retrospect, there have been no great forward strides in antivenom production apart from the introduction of better purification methods, and further developments in this direction will probably have to await improvements of bacterial antitoxins, which are easier to evaluate. The scope for improvement lies in the broadening of the specificity of polyvalent antivenoms and the use of purer antigens.

For reasons already stated, it is inadvisable to rely on paraspecific protection if specific sera can be produced, but taking the African region as an example, it might be necessary to immunize with ten or more venoms in order to cover the more important snakes. Each venom is likely to contain a dozen or more different antigens of which the most toxic is often the poorest antigen, and the solution to the problem may lie in the isolation of the important toxins from these venoms in quantities large enough for use as antigens, possibly after binding to a suitable carrier in order to enhance their antigenicity. Steps in this direction have already been taken by workers in Israel and France (Kochwa *et al.*, 1959; Moroz *et al.*, 1963), a lead we hope to follow in South Africa.

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Discussion

A. Shulov: "Whether you tried in your vast experience direct bites of snakes and direct stings of scorpions in order to increase the title of the antisera. In our laboratory we received good results in donkey and camels, *but not* in sheep, goats and rabbits."

P. A. Christensen: "No."

P. Cohen: "Have you observed any differences in the antibody titers of horses immunized with bentonite-absorbed venom compared to horses which have received unmodified venom."

P. A. Christensen: "Unmodified venom is not used for new horses. The basal immunity is achieved with venom adsorbed on bentonite, but continued with unmodified venom. No comparison has therefore been possible."

P. J. Deoras: "Does the speaker have any observation to make to the fact that venom when used with débris gives reactions?"

P. A. Christensen: "No."

C. Purananda: "When the speaker mentioned young horse, I want to know at what age?"

P. A. Christensen: "Difícil precisar a idade. Penso que é velho um que morreu aos 29 anos e moço um cavalo de 6 anos."

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32. ANTIVENIN TESTING AT DIFFERENT VENOM LEVELS

PETER KRAG and M. WEIS BENTZON

Internat. Lab. Biol. Standards, Copenhagen, Denmark

During the past few years the World Health Organizations has been interested in the standardization of antivenins. An international cooperation between laboratories in Europe, Africa and Asia has lead to a study of the *Naja* antivenins and the establishment of an International Standard for *Naja* Antivenin.

The usefulness of this new standard is for the time being under trial, and all producers of this antivenin have got samples of it with a request for a specialized study, to examine whether it is useful as a standard in comparison with *Naja* antivenins in test against *Naja* venoms of different geographical origin and from different species of snakes belonging to this group.

The evaluation of the *Naja* Antivenin Assay (WHO/BS/604 & 708) disclosed that although the relative potencies based on the values for the international standard were agreeing for *Naja nivea* and for several *Naja naja* venoms, some unexplained discrepancies remained for results obtained with the venom *Naja haje;* it was also noted that the quantities of venom neutralized per ml serum were not agreeing.

During this study a closer co-operation was established between the laboratories in Bangkok, Bombay, Johannesburg, Paris and Copenhagen. In this co-operation it was underlined from especially Dr. P. A. Christensen, that the composition of certain *Naja* venoms with two or more venom components may be an explanation for the discrepancies found between results from different laboratories, as it may have happened that some laboratories have been testing under conditions where a component number 1 was predominant, while other laboratories have had their testing level lying corresponding to the effect of venom component number 2.

Based on a renewed study of Dr. Christensen's publications and reports, a model for the titration curves was constructed.

The co-operation between these laboratories continued from the *Naja* antivenins to antivenins belonging to the *Echis/Bitis* group. It was agreed that a series of studies of the *Echis/Bitis* sera were needed, especially tests at several venom levels. Such studies were performed before June 1965. The studies have covered monovalent sera tested with the homologous venom at one level as well as polyvalent sera tested with several venoms at different LD-50 levels; the testing of same sera with venoms of different origin were also included.

The report I am giving you today will enclose facts from this co-operation where the major part of the testing work has been done by Dr. Christensen and Dr. P. Boquet, while the evaluation was made in Copenhagen by Mr. Weis Bentzon and me.

The evaluation

The logarithmic values for LD-50 and for ED-50 were estimated together with the slope at the 50 per cent point of the dose/response curves by means of a logit analysis (Finney, 1952)*. The results have been compiled in Tables I to IV, where the slopes and the log ED-50 values for each combination of serum and venom have been noted.

The survey on the results showed a wide variation of the slope of the dose/response curve. Those for the venom titrations (b_v) varied from 4 to 59, while those for the serum titrations (b_s) varied from 4 to 185.

The log ED-50 values show a clear increase with the testing level, but for the b_s values no simple relation to the levels could be detected.

The variation in b_s could be explained from the model constructed on the following assumptions.

A venom contains three components. The neutralization of the single component with corresponding antivenins in a serum takes place independently and in each case following the multiple proportions. The free quantity of venom could be expressed for any situation as the total dose minus the part of venom neutralized by the serum activity, and this expression could be applied for each of several venom components.

The quantity of serum needed to reduce a venom component (i) to its LD-50 level could thereafter be expressed as $y^{i}_{50} = \eta_i \times (v - a_i)$ where y is the serum quantity needed, η_i the amount of serum required to neutralize the component i in one μg of venom, v the quantity of venom used, and a_i the LD-50 of venom i expressed in terms of the whole venom.

Further we know that the ED-50 for the whole venom is larger than the serum quantities needed for this above partial neutralization of any component.

The verbal expression for the above conditions is that the serum quantity should be large enough to neutralize one of the components (i_o) at the same time as there is surplus of serum against the two other components.

From the study of diagrams showing the venom dose and the ED-50 values it has been possible to obtain graphic estimates of the LD-50 values for two, maybe three components of several of the venoms tested. Based upon this experience a calculation has been performed using the abovementioned expressions to estimate for a close series of venom doses the effect of each component and the summarized effect of all three components.

For a long series of venom titrations it has been found that the venom dose/response curve could be described by a logistic curve.

A neutralization curve is constructed under the assumption that 1) the log venom dose/response curve is logistic with the same slopes for all three venom components, and 2) the neutralization of each component follows the straight lines in Fig. 1. The resulting curve is shown in Fig. 1 as ... If the third component is absent, the neutralization curve will be as the curve indicated by ooo.

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* Finney, D. J. — *Statistical Method in Biological Assay*, London. Charles Griffin & Company Limited, 1952, p. 524.

For the calculation was chosen the following values for three components, a_i values 20, 50 and 80 and η_i values 0.2, 0.5 and 1.0. The calculation was based on an estimate of the probability of death for each of the three venom components.

The relation between logits to the per cent of death and the quantity of non-neutralized venom will be linear with β_v as the slope. Through a differentiation of the formula for the logit curve it was found, that the slope of the tangent to the usual dose/response curve would be $\frac{\beta_v}{4}$. As the logit curve is approximately linear between 30 and 70 per cent death the $\frac{\beta_v}{4}$ could be estimated as the increase in the probability of death per unit increase in the log dose (see Fig. 2).

Combining this expression with the formula for y_{50} we will arrive to $b_s = b_v \times (\frac{v}{a_i} - 1)$, that is the slope of the plain dose/response curve is equal to the product of the slope for the venom titration and the number of LD-50 neutralized for that particular component.

The dependency between the slope and the venom dose is illustrated in Fig. 3. An increase according to the formula is found for venom doses for which only the first component is the important one. The slope shows a decrease when another component "takes over" followed by an increase.

From the above example it is obvious that the relation between the venom dose and the results of the serum titrations, ED-50 and b_s, is rather complicated.

It has been possible to estimate a_i and η_i for two or three components, when sufficient number of observations have been available. In some cases the observations are fitting very well to the calculated values. An example of such a fitting is given below.

Bitis lachesis

Venom Dose µg	ED-50 µl	b_s	Component LD-50 a_i a_i = LD-50 = 9.7 µg	Serum quantity neutralizing 1 µg venom component η_i	Venom slope calculated b_v
100	3.5	8	a_2 76 µg	0.145	25
200	18.5	> 62			> 38
400	47.0	34	a_3 223 µg	0.265	43
600	100.0	39			23
800	155.0	38		0.275	15

In other cases the calculation gave for different venom doses, assumed to belong to the same linear part of the curve, clearly disagreeing values. This may indicate that more components are existing, but that we did not have suf-

ficient observations for disclosing this fact or it may indicate that other factors are influencing the outcome of the neutralization experiment.

A study of the relative potencies showed that in many cases a serum gave practically same relative potency for two or more testing levels and in some cases the relative potency was found the same for different venoms. It was realized that the random variation of b_s and ED-50 should be described thoroughly.

The stastical analysis

Through the logit analysis has been estimated the standard error of the logarithmic ED-50 value and of the slope of the dose/response curves (b_s for serum titrations and b_v for the venom titration). In some cases it has also been possible to estimate the common slope for all sera belonging to a group tested under similar conditions; these values are marked b_{sc}, in other cases are used the geometrical mean value for a group of sera $\overline{b}_s$ (Table VIII).

The above standard errors are only covering the part of variation corresponding to the variations in the sensitivity of the animals against the non-neutralized venom fractions.

The part of variation due to the dilution and pipetting errors will mostly influence the 50 per cent values, ED-50 as well as LD-50. Variance estimates covering also these error components were obtained by means of analysis of variances for log b_s and also for log ED-50 in all cases where at least two sera have been tested against the same venom in different doses (levels) (see Table IV).

For the slopes (b_s) it has been found that the variance estimates obtained are fitting very well to the expectations from the logit analysis.

A comparison of the slope b_s for the dose/response curve of the titration of antivenins at different testing levels and the standard error (SE_b) has shown that the standard error, estimated for each slope, is in close relation to the magnitude of the slope. For each venom and testing laboratory corresponding values of SE_b and b_s have been plotted against each other. In all cases per laboratory and venom these values are following a distinct slope varying from 0.3 to 0.4. The variance of log b, $0.1886 \times \frac{SE^2(b)}{2}$ thus varies between 0.0171 and 0.0304. The residual variances s^2_R are in accordance with the value 0.0304 except for two cases (0.12^+ and 0.18^{+++}).

If we accept 0.0304 as a general estimate of the variance of log b_s the standard error for average values of log b_s is for the three sera 0.10, for two sera 0.12. These values will correspond to the following limit factors for $\overline{b}_s$: three sera 1.60 and 0.63, two sera 1.76 and 0.58.

A difference between two log b_s average values will have SE values 0.14 and 0.17 for $n = 3$ and $n = 2$ respectively. This indicates that the ratio between two average b_s values has limits 1.90 and 0.53 or 2.10 and 0.48 respectively. In practice the ratio of average b_s values may vary with a 2-factor.

+, ++, and +++, indicate values passing the limits of significance at the 5, 1 and 0.1 per cent level respectively.

From Table VII it is seen that the slopes are not depending of the serum tested, but in some cases a dependance of the level is found.

The standard errors obtained by the logit analysis of *log ED-50 values* vary within an elevenfold range 139×10^{-6} to 1556×10^{-6}. The residual variances (s_R^2) in the analyses of variances are generally higher as will be apparent from Table VI. The ratio between the s_R^2 values and the average variances obtained by the logit analysis ($\overline{SE}^2$) show the following distribution:

5	about	0.9	
5	"	1.7	
3	"	3.0, 2 being significant at the 5 per cent level	
3	"	8.2	all significant at the 1 per cent level
2	"	40.7	all significant at the 1 per cent level

With the exception of the five highly significant ratios the variations of the residuals seem to be random. Therefore it seems reasonable to accept that the experimental variance is obtained by multiplying the value of $\overline{SE}^2$ by an average ratio for these cases, calculated to be 1.55.

The introduction of this increased variance leaves only five significant deviations: all three values from *Bitis nasicornis* (ratio 8.2 now reduced to 4.8) and two values from *Echis carinatus* (ratio about 41 reduced to 24).

The two *Echis carinatus* values correspond both to venom from Paris but tested in two different laboratories. The three *Bitis nasicornis* values were reported from one laboratory using two venoms of different origins.

The ED-50 showed usually significant variations between sera as well as between levels.

An interaction was found for *Bitis nasicornis* and *Echis carinatus* showing that the ranking of ED-50 values was different when same serum was tested by different venoms even from same species.

A study of the relative potencies for the sera mentioned above has shown that the increase of the ED-50 with the testing level is regular but differs in the rate for different sera in each of the groups with the extremely high variance. Therefore it is concluded that a higher variance factor than the above 1.55 is not indicated as the increased variance is due to real differences in the composition of the sera. The 13 cases with the lower variance are fully covered by the use of a variance factor 1.55 as no systematic changes in the relative potency is noted here.

During the preliminary survey was plotted the relation ED-50 to the number of LD-50 used per testing level. Mostly the plotting diagrams showed curves with one or more bends, where the use of a higher testing dose gave an unexpected increase in the ED-50 value. It became clear that the form of these curves could only be defined when many testing levels were examined and each "linear" part of the curve described by three observations. Some well described combinations of sera and venoms gave curves which had a gradual relative increase of ED-50 and no major linear parts at all.

It was in the following examined whether the observed curves fitted a linear relation between log ED-50 and log μg neutralized venom.

The relation is illustrated in Figures 4, 5, 6 & 7 using average logarithmic values of the ED-50 and the logarithm to the μg venom neutralized as given in Table IX.

This linear relation can be expressed by the following formula

$$\log \text{ED-50} = K_1 + K_2 \times \log (v - \text{LD-50})$$

where v is the venom dose in μg.

These diagrams are showing that for each venom these curves will be linear, having slopes, which with few exceptions, are characteristic for that particular venom.

Regression analyses performed on the results for each single serum showed that the variance due to variations around the line was only somewhat higher than the variance corresponding to the ED-50 variation previously described. Part of this additional variation is due to errors in the determinations of the LD-50 values and in the dilutions of serum.

The linear relation between log ED-50 and the log μg neutralized venom thus gives a reasonable fit.

Tests performed in different laboratories using different venoms are fitting in this system. The slopes of these curves are increasing from *Bitis nasicornis* 1.07, *Echis carinatus* 1.30 and *Bitis gabonica* 1.35 to *Bitis lachesis* 1.59. The *Echis coloratus* curve is different from this rule. The serum collection d showed in some cases higher slopes for *Bitis gabonica* and *Bitis nasicornis*.

On separate diagrams have been studied the overall trend for each particular venom and the individual curves for the combination of venoms and sera where significant differences between ED-50 values or between the slopes of the first mentioned curves have been observed.

This description of the neutralization curve seems rather different from the previous 3 component or 2 component curve. However by plotting the theoretical curve (Fig. 8) constructed as mentioned above in the same way, it was found that a straight line would fit the part where the second or the third component were the important ones. Since the above description is more simple (contains only two unknown values against six for the three component analysis) we prefer to use it for further description. The 3-component straight line is also given in Fig. 8.

It is seen that the overall fitting to the theoretical curve is good.

Table X shows for the relative potencies the influence of the use of venom of the same name but of different origin on the same sera. In nine cases were only seen random variations and in two (*Bitis lachesis* serum 504 and *Echis carinatus* serum 509) a major difference was found between venom from Johannesburg and from Paris. A similar difference was not seen for the other sera tested under the same conditions.

Table X gives also in some cases the variation in relative potency when the testing is performed with different venoms:

Serum 24, seven observations, only random variation; serum 12 and serum 17, *Bitis gabonica* and *lachesis*, are giving identical results, while *Echis carinatus* is giving a lower relative potency.

Sera 504 and 509 are different in their composition. In nine cases out of 18 combinations are seen relative potencies about −0.30. Higher potency values (about zero) are seen for serum 504 (*Echis carinatus* and *coloratus*) and for serum 509 (*Bitis nasicornis*). Low relative potency values, about −1.00, were observed in serum 509 (*Echis carinatus* and *coloratus*). This corresponds to the different program for the immunization of the two groups of horses, serum 509 not having included horses immunized with any *Echis* venom.

The additional immunization of horses for serum 504 with *Echis coloratus* venom has not given any remarkable difference in potency from serum 503.

The ED-50 values corresponding to a chosen common testing value 200 μg venom are given in Table XI (the ED-50 values were when needed obtained by interpolation).

When same serum was tested against two venoms of different origin but from same species, the difference in ED-50 exceeded only in 3 out of 16 cases a factor 1.3.

For venoms used in this study it seems more appropriate to use μg venom neutralized rather than number of LD-50 for such comparison.

It was noted that the neutralizing effect of the polyvalent sera tested varied from venom to venom examined, and that this variation not followed one common pattern.

Discussion

S. Schengerg: "Whenever making a venom standard, regional variations in venom composition were considered? This is an important point to be considered as accident results from the bite of a single snake."

P. Krag: "Venoms in my paper are mixtures as used in Johannesburg and Paris."

A. do Amaral: "1. To present compliments for his having followed a modern, scientific and more reliable process in his work on venom titration and antivenin standardization, both of which are quite serious and very complex questions."
"2. To ask what channel is used in the injection of venom and antivenin, for inoculating the animals (white mice)."

P. Krag: "Intravenous in white mice."

TABLE I — RESULTS FROM JOHANNESBURG IN TESTING 18 ANTIVENINS. NINE AGAINST ONE VENOM, NINE AGAINST ANOTHER VENOM

Challenge Venoms							
Name:	*Bitis gabonica*			*Bitis lachesis*			
LD-50:	14.2 µg			7.2 µg			
Slope b_v:	16			23			
Dose:	400 µg			400 µg			
Number of LD-50:	28			56			
Serum	Codes	log ED-50	b_s[z]	Serum	Codes	log ED-50	b_s
181	k	2.24	43	301[y]	a = 1	1.96	91[x]
301	1 = a	1.86	72[x]	425	b	1.73	31[x]
327	m	2.04	23	426[y]	c = n	1.83	15
426[y]	n = c	2.04	> 101[x]	463	d	1.93	20
576	o	2.04	51	498	e	1.95	33
655	q	2.10	30	555	g	1.80	12
816	r	1.90	32[x]	638	h	1.82	27
822	s	2.04	33	686	i	2.00	22
826	t	2.04	33	718	j	1.91	21
Range for nine results		0.38	23 to > 101			0.27	12 to 91
Common slope b_c			40				21

[x] Slope estimated through a Kärter evaluation.

[y] Sera a & c are identical to sera I & n respectively.

[z] The slope is expressed as the increase in mortality percent, when serum dose is (at 50% level) reduced with 10%.

b_s slope for the dose/response curve (mortality/serum dose)

b_c common slope

b_v slope for the dose/response curve (mortality/venom dose)

TABLE II — RESULTS FROM PARIS. CRUDE MONOVALENT SERA

SEVEN ANTI-*BITIS* SERA WITH ONE VENOM

SEVEN ANTI-*ECHIS* SERA WITH ANOTHER VENOM

	Challenge venoms and testing levels					
Name:	*Bitis gabonica* [o]				*Echis carinatus*	
LD-50:	31.0 µg				28.0 µg	
Slope b_v:	24				14	
Dose:	155	310	465 µg		140	280 µg
Number of LD-50:	5	10	15		5	10
Serum *140* b_s	44	63		Serum *161* b_s	35	y
log ED-50	1.85	2.31		log ED-50	2.09	2.52
Dev. of log ED-50	−0.46			Dev. of log ED-50	−0.43	
Serum *146* b_s	8	106		Serum *332* b_s	28	56
log ED-50	1.93	2.46		log ED-50	1.84	2.29
Dev. of log ED-50	−0.53			Dev. of log ED-50	−0.45	
Serum *387* b_s	20	34		Serum *1061* b_s	11	49
log ED-50	1.67	2.18		log ED-50	1.84	2.33
Dev. of log ED-50	−0.51			Dev. of log ED-50	−0.49	
Serum *945* b_s	14	23		Serum *1063* b_s	38	65[x]
log ED-50	1.87	2.31		log ED-50	1.83	2.20
Dev. of log ED-50	−0.44			Dev. of log ED-50	−0.37	
Serum *384* b_s	22	4		Serum *1066*[v] b_s	34[x]	185
log ED-50	1.95	2.49		log ED-50	1.91	2.38
Dev. of log ED-50	−0.54			Dev. of log ED-50	−0.47	
Serum *616* b_s	17	20		Serum 816 b_s	74	77
log ED-50	1.51	2.07		log ED-50	1.94	2.40
Dev. of log ED-50	−0.56			Dev. of log ED-50	−0.46	
Serum 617[z] b_s		8	16	Serum 781[z] b_s	14	26
log ED-50		1.85	2.33	log ED-50	1.77	2.20
Dev. of log ED-50			0.48	Dev. of log ED-50	−0.43	
Common slope b_c except 617 & 781	17.5	26.3		Common slope b_c except 781	29.2	70.7

Notes to Table II

[x] Slope estimated through a Kärber evaluation.

[v] Samples of serum 1066 also tested after a period of freezing (b_s reduced to 1/3, ED-50 unchanged; see document on the effect of freezing/thawing on sera).

[y] No estimate of slope (dose/response 0/6; 0/6; 2/6).

[z] Sera 617 & 781, strain of mice: Paris (all other sera HLA-mice).

[o] Additional information for *Bitis gabonica* venom:
Serum 781[z] showed at testing level 6 LD_{50} log ED-50 = 3.00 (ED-50 = 1000).

TABLE III — JOHANNESBURG AND BOMBAY

ECHIS/BITIS TESTING RESULTS OCTOBER 1964 TO JUNE 1965

Venoms and LD-50	Sera		Testing levels (Number of LD-50)			
			14	28	56	112
B. gab. 14.2 µg b_v = 16	k−o, q−t*	b_{sc} log ED-50		40 1.86 .. 2.24		
B. ariet. (lach.) 7.2 µg b_v = 23	a−e, g−j*	b_{sc} log ED-50			21 1.73 .. 2.00	
	VG[v]	b_s		>34[x]		62
		log ED-50		1.39		2.34
	Dev. of	log ED-50				0.95
	Trop[v]	b_s			22[x]	28
		log ED-50			1.78	2.14
	Dev. of	log ED-50				0.36
	353[v]	b_s	24	45[x]	15	
		log ED-50	1.20	1.63	2.13	
	Dev. of	log ED-50		0.43	0.93	

			Testing levels (Number of LD-50)				
			2.5	5	10	20	40
B. nas. 23.6 µg b_v = 15	840	b_s	10	20			
		log ED-50	1.56	2.14	18		
	Dev. of	log ED-50	−0.87	−0.29	2.43		
	504	b_s	37	24	45		
		log ED-50	1.53	1.89	2.20		
	Dev. of	log ED-50	−0.67	−0.31			
E. car. 21.9 µg b_v = 12	753	b_s		27	21	15	49[x]
		log ED-50		0.97	1.38	1.89	2.23
	Dev. of	log ED-50		−0.41		0.51	0.85
E. car. Bombay 44.0 µg b_v = 59		b_s		12			
		log ED-50		1.51			

* See also detailed list for these sera Table I.

[v] Test results for venom *N. nivea,* see table in report on *Naja* antivenin studies. Samples of sera VG, Trop & 353 were also tested after a period of freezing; b_s & log ED-50 showed no systematic changes, see document on the effect of freezing/thawing on sera.

[x] Slope estimated through a Kärber evaluation.

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TABLE IV — RESULTS FROM PARIS. FOUR ANTI-*BITIS/ECHIS* SERA* 12, 17, 23 & 24 ALL PURIFIED, AGAINST FOUR VENOMS. PARIS MICE

Origin:	J			P			
Name:	*Bitis gabonica*						
LD-50:	17.8 µg			28.2 µg			
Slope b_v:	18			39			
Dose:	70		340 µg	141	282	423	564 µg
Number of LD-50:	10		20	5	10	15	20
Serum *12* b_s				22	32		
log ED-50				1.86	2.31		
Dev. of log ED-50				−0.45			
Serum *17* b_s					19	27	37
log ED-50					2.11	2.38	2.49
Dev. of log ED-50						0.27	0.38
Serum *23* b_s	26		36		18		53
log ED-50	1.65		2.12		1.96		2.38
Dev. of log ED-50			0.47				0.42
Serum *24* b_s	40x		51x		22		38
log ED-50	1.69		2.08		1.90		2.32
Dev. of log ED-50			0.39				0.42

Name:	*Bitis lachesis*						
LD-50:	9.3 µg			12.6 µ			
Slope b_v:	15			47			
Dose:	93	186	372 µg	126		189	252 µg
Number of LD-50:	10	20	40	10		15	20
Serum *12* b_s					31	22	
log ED-50					2.11	2.35	
Dev. of log ED-50						0.24	
Serum *17* b_s					28		32
log ED-50					1.81		2.28
Dev. of log ED-50							0.47
Serum *23* b_s	8	15	29		19		45
log ED-50	1.41	1.97	2.40		1.75		2.22
Dev. of log ED-50		0.56	0.99				0.47
Serum *24* b_s	17	10	21		33		41
log ED-50	1.45	1.92	2.40		1.70		2.23
Dev. of log ED-50		0.47	0.95				0.53

TABLE IV (*continued*)

Origin:	J			P		
Name:	*Echis carinatus*					
LD-50:		30.9 μg			24.0 μg	
Slope b_v:		10			25	
Dose:	309	464	618 μg	240	360	480 μg
Number of LD-50:	10	15	20	10	15	20
Serum *12* b_s				54		28
log ED-50				1.89		2.37
Dev. of log ED-50						0.48
Serum *17* b_s	18	46	21[x]	43		106
log ED-50		2.34	2.52	1.87		2.20
Dev. of log ED-50	2.09	0.25	0.43			0.33
Serum *23* b_s				39	32	
log ED-50				2.12	2.32	
Dev. of log ED-50					0.20	
Serum *24* b_s				17	37	
log ED-50				2.07	2.33	
Dev. of log ED-50					0.26	

Notes to Table IV

Bitis nasicornis

	Johannesburg	Paris
	5 LD-50	
Dose:	151 μg	110 μg
Serum *23* b_s	3	4
log ED-50	2.22	1.64
Serum *24* b_s	0	6
log ED-50	2.23	1.54

The sera were tested with the venoms commonly used in Paris, LD-50 21.9; b_v 17, and with a sample received from Johannesburg, LD-50 30.2; b_v 4. The slope of the dose/response curve was exceptionally low also when compared with results from Johannesburg, Table V.

* Samples of these sera were also tested after a period of freezing. No systematic changes in b_s or in log ED-50 were noted, see the document on the freezing/thawing effect on sera.

[x] Slope estimated through a Kärber evaluation.

TABLE V — JOHANNESBURG. *BITIS/ECHIS* SEPTEMBER 1965. THREE SERA TESTED WITH FIVE VENOMS (FOUR OF WHICH OF TWO DIFFERENT ORIGINS)

Name and Origin:	*Bitis gabonica* — Jòhannesburg				
LD-50:			11.5 μg		
Slope b_v:	26	33	18	33	15
Dose:	80 μg	200 μg	400 μg	600 μg	800 μg
Number of LD-50:	7	17.5	35	52	70
Serum 503 b_s	38x	> 52x	15	52x	16
log ED-50	0.40	0.95	1.32	1.57	1.87
Deviation**	−0.55		0.37	0.62	0.92
Serum 504 b_s	23	26	15	20	18
log ED-50	0.61	1.23	1.58	1.87	2.13
Deviation	−0.62		0.35	0.64	0.90
Serum 509 b_s	19	25	35	30x	12
log ED-50	0.70	1.29	1.70	1.91	2.16
Deviation	−0.59		0.41	0.62	0.87

Name and Origin:	*Bitis gabonica* — Paris		
LD-50:		25.8 μg	
Slope b_v:			
Dose:	200 μg	400 μg	600 μg
Number of LD-50:	8	16	24
Serum 503 b_s	30x	20	10
log ED-50	0.89	1.52	1.82
Deviation	−0.63		0.30
Serum 504 b_s	33	12	10
log ED-50	1.22	1.82	2.17
Deviation	−0.60		0.35
Serum 509 b_s	27	13	7
log ED-50	1.07	1.79	2.02
Deviation	−0.72		0.23

Name and Origin:	*Bitis lachesis* — Johannesburg				
LD-50:			9.7 μg		
Slope b_v:	11	39	33	27	26
Dose:	100 μg	200 μg	400 μg	600 μg	800 μg
Number of LD-50:	10	20	40	60	80
Serum 503 b_s	8	> 62x	34	29	38
log ED-50	0.55	1.25	1.67	2.00	2.19
Deviation	−0.70		0.42	0.75	0.94
Serum 504 b_s	16	22	40	71x	25
log ED-50	0.95	1.54	2.03	2.28	2.50
Deviation	−0.59		0.49	0.74	0.96
Serum 509 b_s	10	40x	28	19	14
log ED-50	0.84	1.40	1.87	2.16	2.44
Deviation	−0.56		0.47	0.76	1.04

TABLE V (*continued*)

Name and Origin:	*Bitis lachesis* — Paris			
LD-50:	10.7 µg			
Slope b_v:	19		20	
Dose:	200 µg		600 µg	
Number of LD-50:	20		60	
Serum 503 b_s	22		18	
log ED-50	1.22		1.91	
Deviation			0.69	
Serum 504 b_s	14		< 42	
log ED-50	1.78		2.52	
Deviation			0.74	
Serum 509 b_s	31		20	
log ED-50	1.51		2.28	
Deviation			0.77	

Name and Origin:	*Bitis nasicornis* — Johannesburg			
LD-50:	25.2 µg			
Slope b_v:	27	55	54	30
Dose:	100 µg	200 µg	400 µg	800 µg
Number of LD-50:	4	8	16	32
Serum 503 b_s	53[x]	> 52[x]	57[x]	> 52[x]
log ED-50	1.23	1.65	2.02	2.35
Deviation	−0.79	−0.37		0.33
Serum 504 b_s	21	28	27	No test
log ED-50	1.47	2.02	2.46	
Deviation	−0.99	−0.44		
Serum 509 b_s	23	> 52[x]	> 63[x]	16
log ED-50	1.29	1.65	2.04	2.42
Deviation	−0.75	−0.39		0.38

Name and Origin:	*Bitis nasicornis* — Paris		
LD-50:	22.8 µg		
Slope b_v:	28	(14)	
Dose:	100 µg	400 µg	
Number of LD-50:	4.4	17.5	
Serum 503 b_s	34	28	
log ED-50	1.09	2.00*	
Deviation	−0.91		
Serum 504 b_s	20	7	
log ED-50	1.37	2.38*	
Deviation	−1.01		
Serum 509 b_s	36	38[x]	
log ED-50	1.24	1.98*	
Deviation	−0.74		

TABLE V (*continued*)

Name and Origin:			*Echis carinatus* — Johannesburg		
LD-50:			22.1 µg		
Slope b_v:	16	13	56	24	69x
Dose:	50 µg	100 µg	200 µg	400 µg	800 µg
Number of LD-50:	2.25	4.5	9	18	36
Serum 503 b_s	19	16	46	23	> 52x
log ED-50	0.46	0.97	1.51	1.93	2.25*
Deviation	−1.47	−0.96	−0.42		0.32
Serum 504 b_s	14	12	53x	24	34x
log ED-50	0.42	0.95	1.54	1.99	2.31*
Deviation	−1.57	−1.04	−0.45		0.32
		105 µg	134 µg		
		4.75	6.06		
Serum 509 b_s		8	11		
log ED-50		2.00	2.30		
Deviation		/	/		
Name and Origin:		*Echis carinatus* — Paris			
LD-50:			31.8 µg		
Slope b_v:	32	38	64	55x	
Dose:	100 µg	200 µg	300 µg	400 µg	
Number of LD-50:	3.1	6.3	9.4	12.6	
Serum 503 b_s	53x	> 52x	46	57x	
log ED-50	1.55	1.95	2.11	2.36*	
Deviation	−0.81	−0.41	−0.25		
Serum 504 b_s	22	24	102x	53x	
log ED-50	1.40	1.99	2.37	2.55*	
Deviation	−1.15	−0.56	−0.18		
Serum 509 b_s	57				
log ED-50	2.30				
Deviation	/				
Name and Origin:			*Echis coloratus*		
LD-50:			16.0 µg		
Slope b_v:	30	17	13		
Dose:	100 µg	200 µg	400 µg		
Number of LD-50:	6.3	12.5	25		
Serum 503 b_s	29	15	10		
log ED-50	1.18	1.37	2.00		
Deviation	−0.19		0.63		
Serum 504 b_s	32	19	21		
log ED-50	1.23	1.56	2.21		
Deviation	−0.33		0.65		

TABLE V (*continued*)

		108 µg 6.75
Serum 509	b_s	9
	log ED-50	2.30
	Deviation	/

x Slope estimated through a Kärber evaluation.

* ED-50 estimated using single b_s value, common b not accepted in () due to significant differences between the three single b_s values.

** The increase in dose and the deviations of ED-50 were estimated from values corresponding to 10 LD-50 or in several cases the value close to 10 LD-50.

Notes to Table VI

(Serum collection code a-i also used in Tables VII to IX)

The collection of sera* used for studies on ED-50 and testing level.

α	J	I	181, 301, 327, 426, 576, 655, 816, 822, 826
β	J	I	301, 425, 426, 463, 498, 555, 638, 686, 718

Collection	*Laboratory*	*Table*	*Sera*
a	P	II	140, 146, 387, 945, 384, 616
b	P	IV	23, 24
c	P	IV	17, 23, 23F^{xx}, 24
d^{z}	J	V	503, 504, 509
e	J	III	840, 504
f	P	II	161^{x}, 332, 1061, 1063, 1066, 1066F^{xx}, 816, 781^{y}
g	P	IV	12, 17
h	P	IV	23, 23F^{x}, 24
i^{z}	J	V	503, 504

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^{y} Table II, serum 781, not included in the estimate of common b_s.

^{x} Table II, serum 161, only used with its ED-50 value (b_s level 10, no estimate possible).

^{z} Number of b_s values are exceeding those of ED-50 values by one.

* Table I, serum collections α and β, tested at one level only, not used for study on ED-50 and testing levels.

^{xx} The sera marked F were tested among sera described in Tables II & IV, they are all tested after a period in frozen state.

×, ×× and ××× indicate values passing the limits of significance at the 5, 1 and 0.1 per cent level respectively.

(×) corresponds to cases where the degree of significance is reduced when the increased SE is used.

TABLE VI — ANALYSIS OF VARIANCES FOR RESULTS FROM TABLES II TO V

Venom	Laboratory	Sera	Table	log b_s $S^2_R \times 10^4$		$\overline{SE}^2 \times 10^6$		log ED-50 $S^2_R \times 10^6$ & ratio S^2_{R/SE^2}	
				P	J	P	J	P	J
				Origin of Venom					
B. gab.	P	a	II	$1765_5^{\times\times\times}$		600_6		1100_5 1.8	
	P	b/c	IV	116_3	1_1	271_4	577_2	250_3 0.9	1600_1 2.8
	J	d	V	55_4	278_8	1556_3	844_2	1275_4 0.8	638_8 0.8
B. lach.	P	b/c	IV	325_3	402_2	236_4	730_2	$700_3^{(\times)}$ 3.0	650_2 0.9
	J	d	V	22_3	408_8	1153_3	769_3	1600_1 1.3	1263_8 1.6

B. nas.	J	e	III		307_{2}		887_{2}		$7400_{2}^{\times\times(\times)}$ 8.3
	J	d	V	306_{2}	incompl. series	1192_{3}	524_{3}	$9300_{2}^{\times\times(\times)}$ 7.8	$4550_{4}^{\times\times(\times)}$ 8.6
E. car.	P	f	II	431_{6}		387_{7}		800_{6} 2.1	
	P	g	IV	$1156_{1}^{\times}$		139_{2}		$5600_{1}^{\times\times\times}$ 40.3	
	P	h	IV	253_{2}		243_{3}		450_{2} 1.9	
	J	i	V	570_{3}	57_{4}	402_{2}	1004_{2}	$16500_{3}^{\times\times\times}$ 41.0	1050_{4} 1.0
E. col.	J	i	V		103_{2}		1152_{2}		$3800_{2}^{(\times)}$ 3.3

The index indicates for S^{2}_{R} degrees of freedom (f)

for $\overline{SE^{2}}$ numbers of observations (n)

TABLE VII — SURVEY ON VARIATIONS DEMONSTRATED IN THE ANALYSIS OF VARIANCES ON RESULTS FROM TABLES II-V

Venom	Origin & testing level	Laboratory	Sera[2]	Table	Variation in b_s — Single results			Variation in ED-50		
					Sera	Level	Interaction[3]	Between Sera	Between Level	Interaction[3]
B. gab.	P 5-10	P	a,6	II	S non-syst.	S non-syst.	S $^{\times\times\times}$	S	S	
	J 10-20	P	b,2	IV				S	S	
	P 10-20	P	c,4	IV		S $b_{10} < b_{20}$		S	S	
	J 7..70	J	d	V		4)		S	S	
	P 8-16-24	J	d	V		$b_8 > b_{16}$ b_{24}		S	S	
B. lach.	J 10-20-40	P	b,2	IV	other $b_s < b_{40}$				S	
	P 10-20	P	c,4	IV	$b_{10} < b_{20}$			S		
	J 10..80 (5)	J	d	V	S $b_{10} <$ $b_{20}..b_{80}$			S	S	
	P 19-56	J	d	V				S	S	

B. nas.	J 2.5-5-10	J	e,2	III	$S^{\times}$ $b_{840} <$ b_{504}		S S	S $ED_{504} < ED_{840}$
	J 4..32 (4)	J	d	V	5)	5)	S S	ED_{501} & $ED_{509} <$ ED_{504}
	P 4.4-17.5	J	d	V	$b_{504} <$ b_{503} & b_{509}			S $ED_{509} < ED_{501} <$ ED_{504}
E. car.	P 5-10	P	f,7 (6 for b_8)	II	$S^{\times\times\times}$	$S^{\times\times\times}$ $b_5 < b_{10}$	S S	
	P 10-20	P	g,4	IV	S non-syst. level 20 $b_{12} < b_{17}$	S non-syst.	$S^{\times}$	S S S non-syst. non-syst. level 20 $ED_{17} < ED_{12}$
	P 10-15	P	h	IV			S S	
	J 2.25..36 (5)	J	i,2	V	$S^{\times}$ $b_{45} <$ b_9		S	
	P 3.1..12.6 (4)	J	i,2	V			S S	S $ED_{503} < ED_{504}$
E. col.	J 6.3-12.25-25	J	i,2	V			S S	S $ED_{503} < ED_{504}$

Notes to Table VII

1) Level indicated if 2 or 3; lowest and highest levels noted if 4 or 5 levels.

2) The code a ... i indicates the collection of sera used (the figure following the letter shows the number of sera, if not the usual number 3). Details given in Notes to Table VI.

3) The deviation from additivity, e.g. the non-parallelism of the log ED-50/log μg venom-neutralized curves.

The usual findings are random variations for b_s and significant differences for log ED-50; random variations correspond to open squares in the table, while significant deviations are marked with S and one to three $\times$-s indicating 5, 1 and 0.1 per cent level log significance.

The significant deviations are specified by additional notes showing the systematic differences:

$b_{10} < b_{20}$ e.g. b_s is smaller at testing level 10 LD-50 than at 20 LD-50.

$b_{840} < b_{504}$ e.g. b_s for serum 840 is smaller than that for serum 504.

$ED_{540} < ED_{840}$ e.g. the increase in ED-50 by increasing testing level for serum 504 is less pronounced than that for serum 840.

The lack of systematic and significant deviations of b_s or ED-50 values is noted by "non-syst.".

4) The successive differences between log b_s values are large (+0.10; −0.26; +0.26; −0.34).

The range of log b_s values 0.34 is however below the limits of significance with an SE value: 0.14.

5) No analysis of variances performed as b_s is often less well defined. Inspection has not revealed significant differences.

TABLE VIIIa — SURVEY OF SLOPES b_{sc}, $\overline{b_s}$ OR b_s AT DIFFERENT TESTING LEVELS

Venom Origin & b_v		Sera	Slope	5	10	20	40	80
B. gabonica								
J	16	α	b_{sc}				40_9	
P	24	a	b_{sc}*	18_6	26_6			
J	18	b	$\overline{b}_s$		32_2	43_2		
P	39	c	$\overline{b}_s$	22_1	26_5 27_1	47_4		
J	/	d	b_{sc}		26 33		18	33 15
P	/	d	b_{sc}		33 15		9	
B. lachesis								
J	23	β	b_{sc}					21_9
J	23	VG	b_{sc}				$>37_2$	57_2
		Trop	b_s					31_2 34_2
		353	b_{sc}		22_2		51_2	19_2
J	15	b	$\overline{b}_s$		12_2	12_2	25_2	
P	47	c	$\overline{b}_s$		23_5 22_1	42_4		
J		d	b_{sc}		11	39	33 27	26
P		d	b_{sc}			19	20	

The index at b_{sc} and b_s indicates the number of b_s values if different from 3.
J & P indicate origin of venom.

* High variance, see Table VI $s^2_R = 0.1765$.

TABLE VIIIb — SURVEY OF SLOPES b_{sc}, $\overline{b_s}$ OR b_s AT DIFFERENT TESTING LEVELS

Venom Origin & b_v		Sera	Slope	2½	5	10	20	40
B. nasicornis								
J	15	e	b_{sc}	15_2	22_2	26_2		
J		d	b_{sc}		27 55		54	30_2
P		d	b_{sc}		28		(14)	
E. carinatus								
P	14	f	b_{sc}		29_7	71_6		
J	12	753	b_s		27_1	21_1	15_1	$49\frac{x}{1}$
B	59	Bomb.	b_s		12_1			
J	10	17	$\overline{b}_s$			18_1 46_1	$21\frac{x}{1}$	
P	25	g+h	$\overline{b}_s$			34_3 41_3	55_2	
J		i	b_{sc}	16_2	13_2	56_2	24_2	$69\frac{x}{2}$
P		i	b_{sc}	32_2	38_2	64_2 55		
E. coloratus								
J		i	b_{sc}		30_2	17_2		13_2

x Slope estimated through a Kärber evaluation.
The letters α, β, and a ... i indicate the different collections of sera used.
J, P & B indicate origin of venom.

TABLE IX — ED-50 AND THE QUANTITY OF VENOM NEUTRALIZED AT DIFFERENT TESTING LEVELS
(log ED-50 and log μg venom for the average value)

Venom	Origin	Laboratory	Table	Sera		Numbers of LD-50						Slope for the full series of results (K_2)	Average slope
						≦2.5	3-5	6-10	11-20	21-40	41-80		
B. gab.	P	P	II	a	ED μg		1.80 2.09	2.30 2.45				1.41	1.35
	J	P	IV	b	ED μg			1.67 2.19	2.10 2.51			1.34	
	P	P	IV	c	ED μg			2.00 2.41	2.40 2.73			1.25	
	J	J	V	d	ED μg			0.57 1.84	1.16 2.28	1.53 2.59	1.78 2.05 2.77 2.90	1.36	
	P	J	V	d	ED μg			1.06 2.24	1.71 2.57	2.00 2.76		1.83	
B. lach.	J	P	IV	b	ED μg			1.43 1.92	1.95 2.25	2.40 2.56		1.52	1.59
	P	P	IV	c	ED μg			1.74 2.05	2.24 2.38			1.52	
	J	J	V	d	ED μg			0.78 1.96	1.40 2.28	1.86 2.59	2.15 2.38 2.77 2.90	1.66	
	P	J	V	d	ED μg				1.50 2.28		2.24 2.77	1.50	

TABLE IX (*continued*)

Venom	Origin	Laboratory	Table	Sera		Numbers of LD-50						Slope for the full series of results (K_2)	Average slope
						≦ 2.5	3-5	6-10	11-20	21-40	41-80		
B. nas.	J	J	III	e	ED μg	1.55 1.55	2.02 1.98	2.32 2.33				0.99 (1)	
	J	J	V	d	ED μg		1.33 1.87	1.77 2.24	2.17 2.57	2.50 2.89		1.16 (2)	
	P	J	V	d	ED μg		1.23 1.89		2.12 2.58			1.28 (3)	
E. car.	P	P	II	f	ED μg		1.90 2.05	2.34 2.40				1.26	1.30
	P	P	IV	g	ED μg			1.88 2.33	2.29 2.66			1.23	
	P	P	IV	h	ED μg			2.10 2.33	2.33 2.53			1.15	
	J	J	V	i	ED μg	0.44 1.44	0.96 1.89	1.53 2.25	1.96 2.58	2.28 2.89		1.31	
	P	J	V	i	ED μg		1.48 1.83	1.97 2.24 2.23 2.43	2.46 2.57			1.31	

ED indicates average log ED-50 for a collection of sera (sera mentioned in notes to Table VI).
μg indicates log μg neutralized at each testing lev, see Tables II-V for the exact testing levels.
1, 2 & 3: slopes for single sera show some discrepancies (1: 1.12 & 0.86; 2: 1.10, 1.42 & 1.11; 3: 1.32, 1.46 & 1.07).

TABLE X — AVERAGE RELATIVE POTENCIES (LOG VALUES) FOR SERA TESTED WITH DIFFERENT VENOMS

Venom	Origin	Laboratory and Sera				
		Paris			Johannesburg	
		12	17	24	504	509
B. gabonica	J			0.00_2	-0.26_5	-0.33_5
	P	-0.35_1	-0.13_2	0.05_2	-0.33_3	-0.22_3
B. lachesis	J			0.00_3	-0.33_5	-0.21_6
	P	-0.36_1	-0.06_2	0.02_2	-0.59_2	-0.33_2
B. nasicornis	J			-0.01_1	-0.35_3*	-0.04_4
	P			0.10_1	-0.33_2*	-0.07_2*
E. carinatus	J				-0.02_5	-1.03_2
	P	0.23_1	0.25_1	0.02_2	-0.09_4*	-0.59_1
E. coloratus	J				-0.15_3	-1.10_1

The letters J & P are used to indicate the laboratories in Johannesburg and Paris, and also to venom supplied from one of these laboratories for test in the others.

Indices denote the number of potencies averaged.

The estimate of relative potencies is based on log ED-50 for serum 23 and serum 503 in laboratories P & J respectively.

* High variance compare Table VI s^2_R

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33(1):251-280, 1966

TABLE XI — ED-50 ESTIMATED AT A COMMON LEVEL 200 μg NEUTRALIZED. SURVEY OF LD-50 VALUES FOR THE VENOMS USED

Sera	Laboratory	Name of Venom								
		B. gabonica		*B. lachesis*		*B. nasicornis*		*E. carinatus* (color.)[x]		
		J	P	J	P	J	P	J	B[y]	P
α, β,	a & f J & P	α 30-71	a 30-182	β 19-35						f 120-251
753	J							25		
VG				26						
Trop				21						
353				45						
840						251				
504						148				
Bombay	B								32[y]	
12	P		148		257					71
17			95		148			81		69
23		65	66	110	129					123
24		66	58	100	126					107
503	J	9	10	19	18	52	43	38	(28)	100
504		19	21	37	65	126[v]	93	41	(35)	132
509		21	16	27	35	52	48[v]			
LD-50:										
	J	14	26	8	11	24	23	22	(16)	32
	P	17	28	9	13			31		26
	B								44[y]	
K_2		1.35 1.83[u]		1.59		1.07 1.40[v]		1.30		

K_2: The slope of the curve log ED-50/log μg venom neutralized (see Figs. 4, 5, 6 & 7); no K_2 value for *E. coloratus* (curve not linear).

[x] *E. coloratus* results noted under B in ( ).

[y] Test in Bombay with a Bombay venom.

α, β: ED-50 values obtained by extrapolation using the slope K_2 1.35 & 1.59 resp.

[u] Sera 503, 504 & 509 Paris venom in laboratory J.

[v] Serum 504 venom J and serum 509 venom P showed high slope values (K_2 = 1.40).

J & P indicates origin of venom as well as testing laboratories.

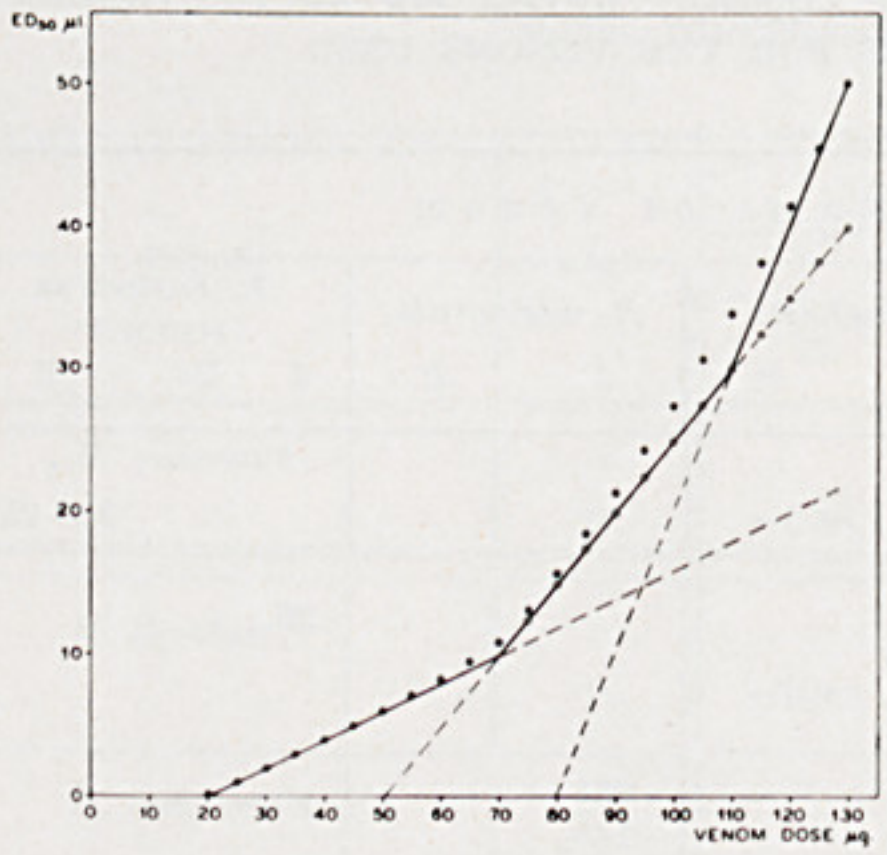


Fig. 1 — Relation between ED-50 and the venom level (dose in micrograms) constructed example with 3 venom components (...); with the third component absent use ... until venom dose 70 and .. for higher venom doses. The 3 straight lines represent the ED-50 values expected for each component separately. The full drawn parts of these lines represents for each level the component requiring maximum of ED-50.

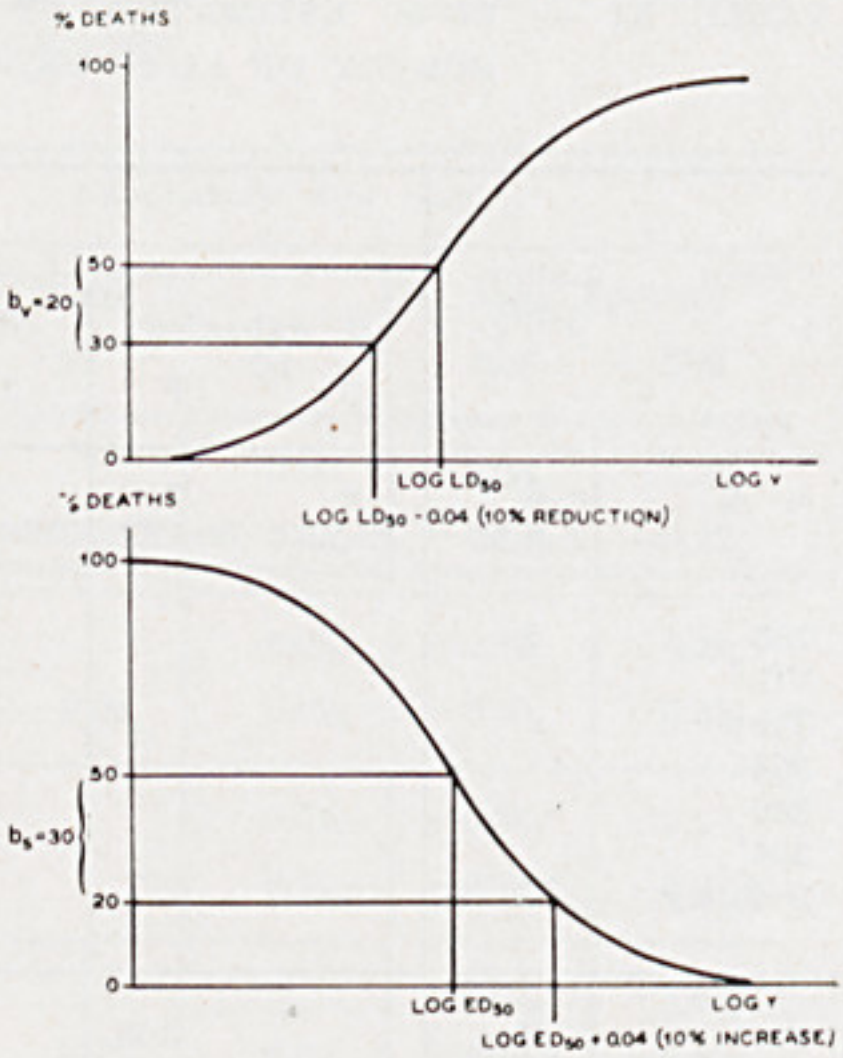


Fig. 2a — Dose/response curve for a venom titration.
v = venom dose
b_v = slope of dose/response curve
Fig. 2b — Dose/response curve for a serum titration.
y = serum dose
b_s = slope of the dose/response curve
testing level 2.5 × LD-50

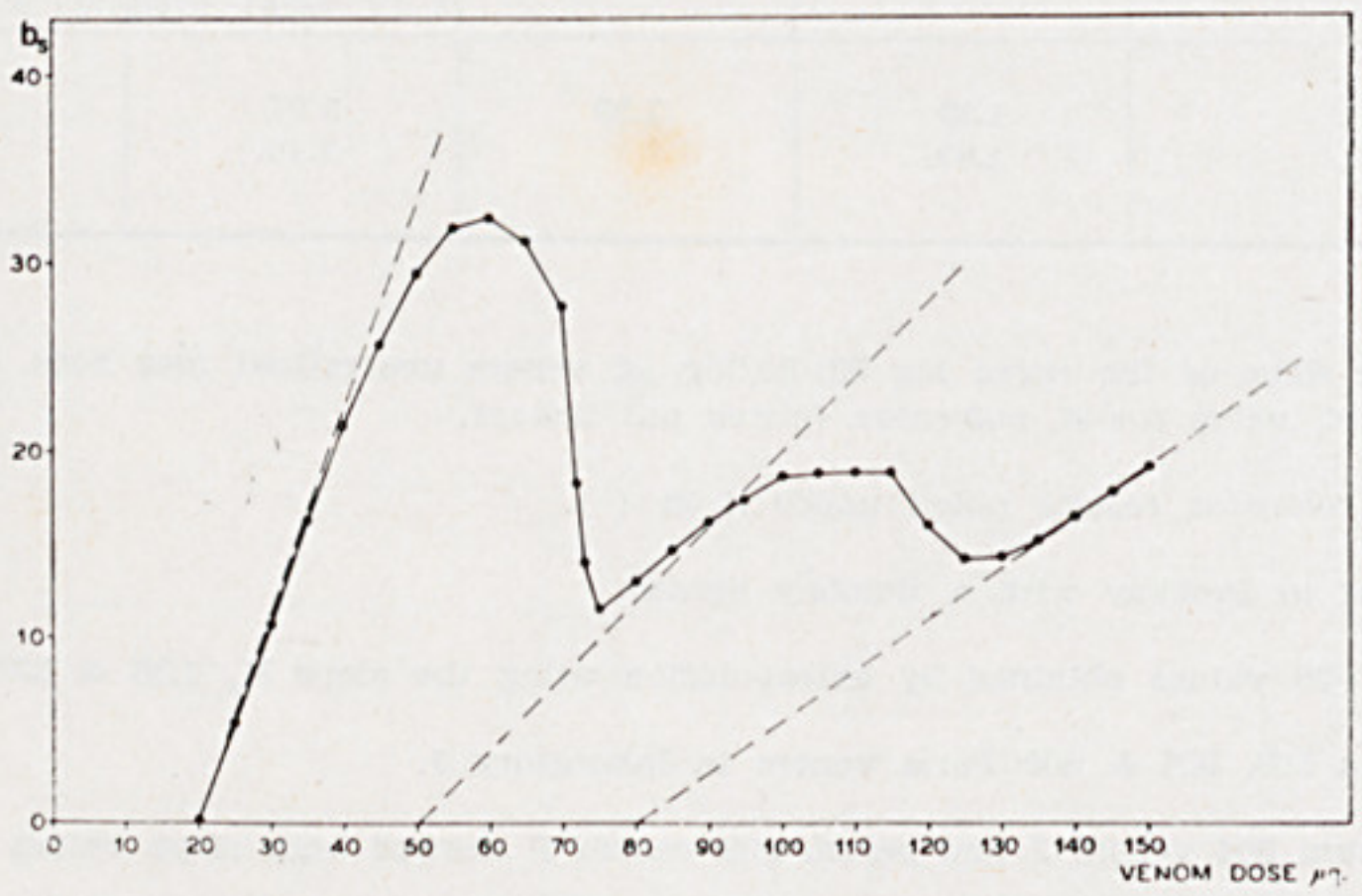


Fig. 3 — Relation between the slope (b_s) of serum titration curves and the venom level (dose in microgram). Constructed example with 3 venom components (.—.—.). The 3 straight lines represent values expected, if each component is considered separately (— — — — — — — —).

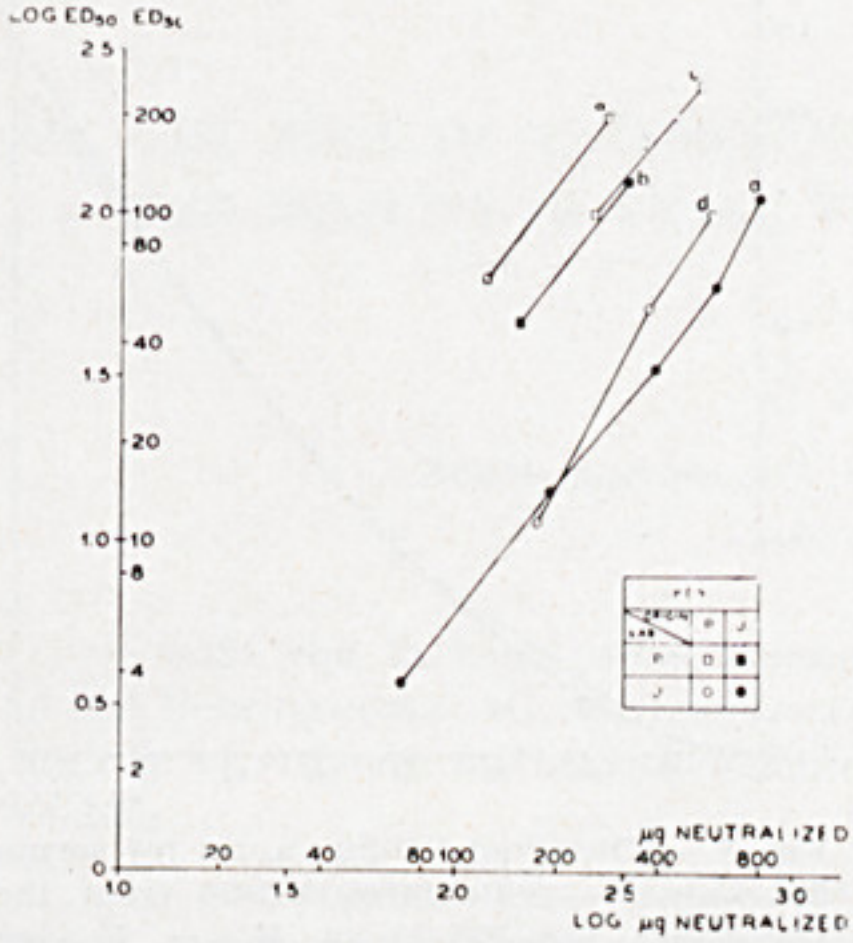


Fig. 4 — *Bitis gabonica.* Average values of log ED-50 plotted against log microgram venom neutralized. 4 different group of sera (a, b, c, d); one (d) tested against 2 venoms of different origin.

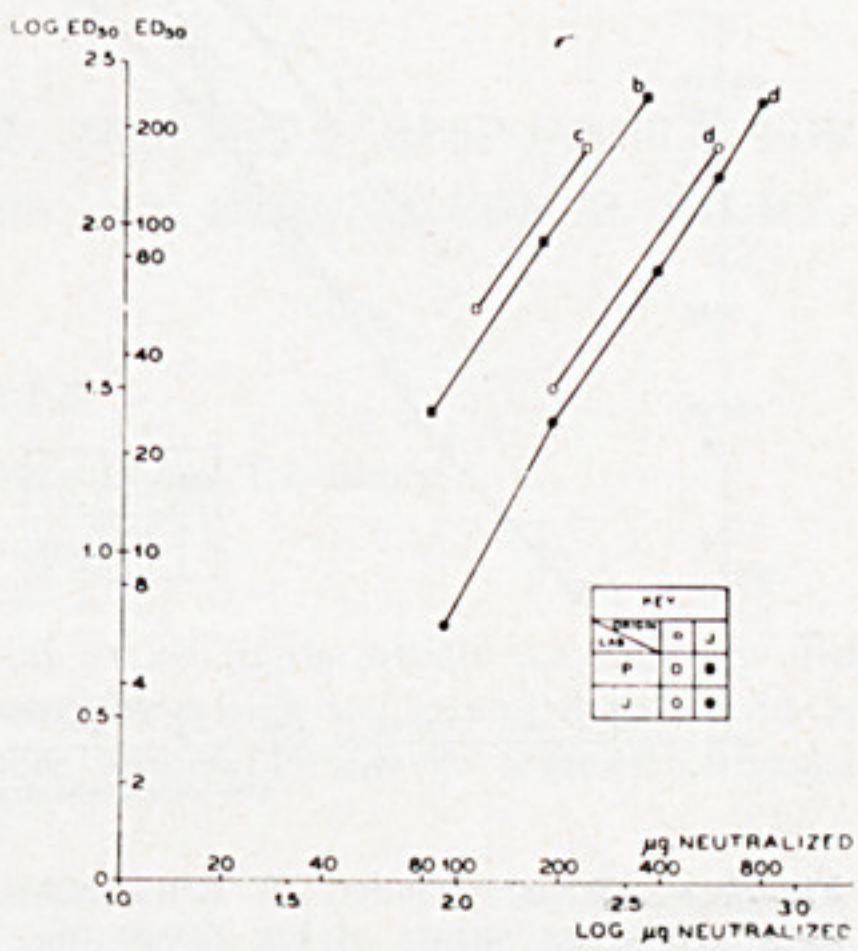


Fig. 5 — *Bitis lachesis.* Average values of log ED-50 plotted against log microgram venom neutralized. 3 different groups of sera (b, c, d); one (d) tested against 2 venoms of different origin.

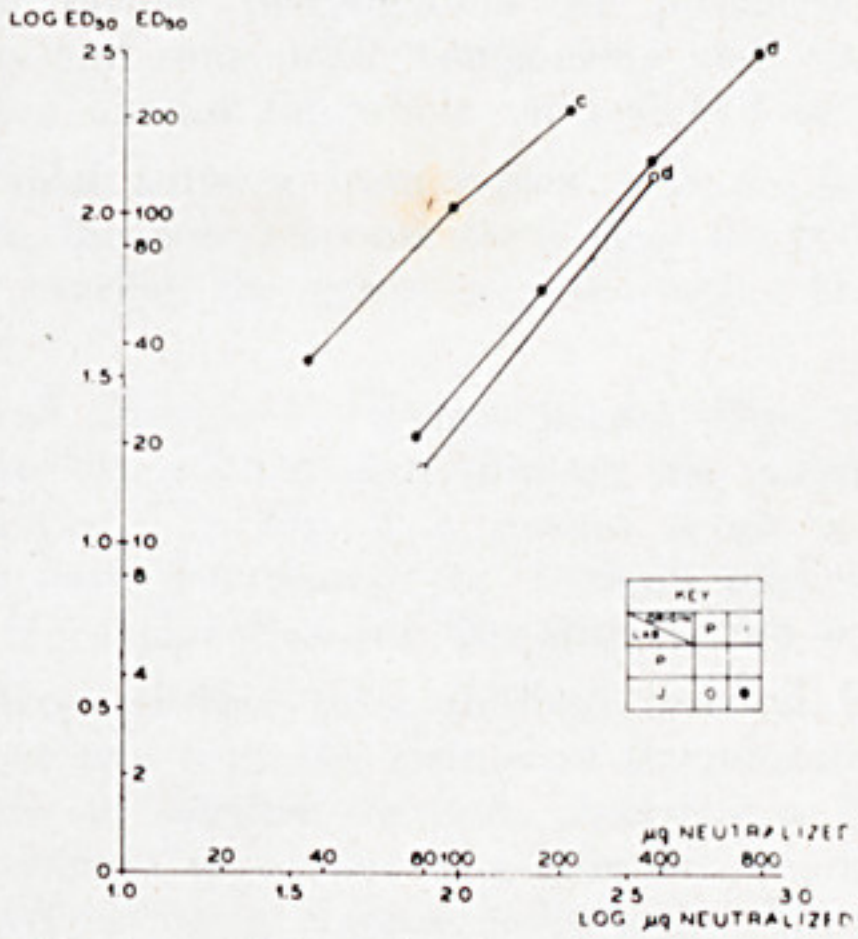


Fig. 6 — *Bitis nasicornis.* Average values of log ED-50 plotted against log microgram venom neutralized. 2 different groups of sera (d, e); one (d) tested against 2 venoms of different origin.

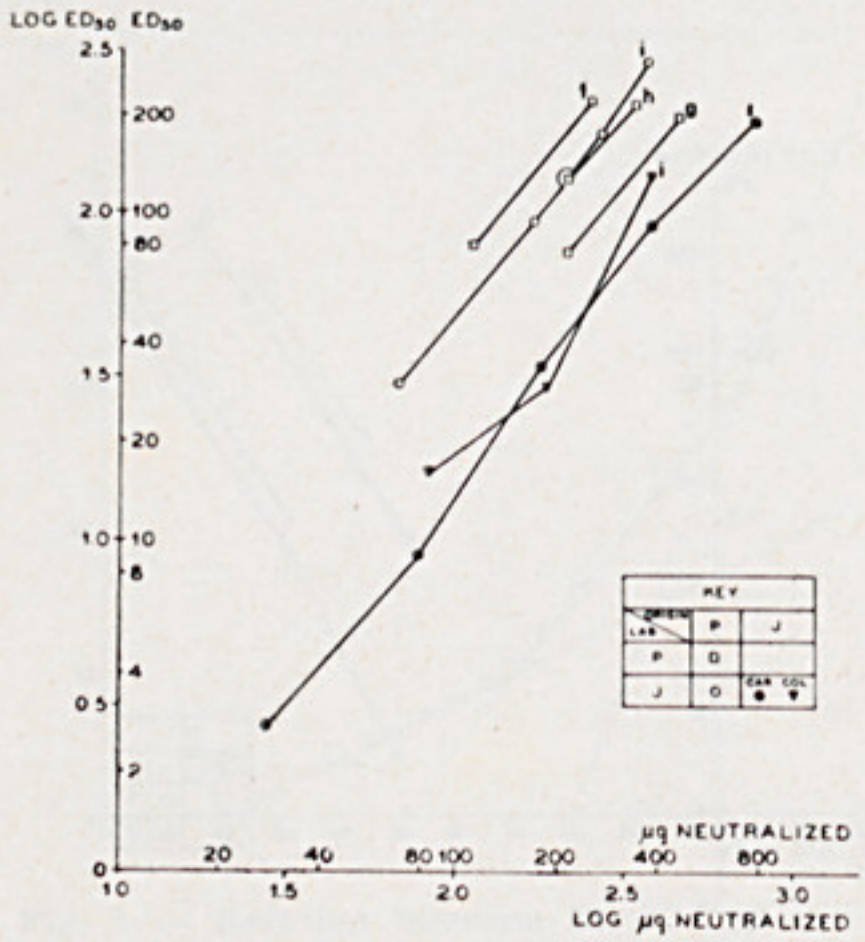


Fig. 7 — *Echis carinatus* & *Echis coloratus*. Average values of log ED-50 plotted against log microgram venom neutralized. 4 different groups of sera (f, g, h, i); one of these (i) tested against 2 *Echis carinatus* venoms of different origin and against one *Echis coloratus* venom.

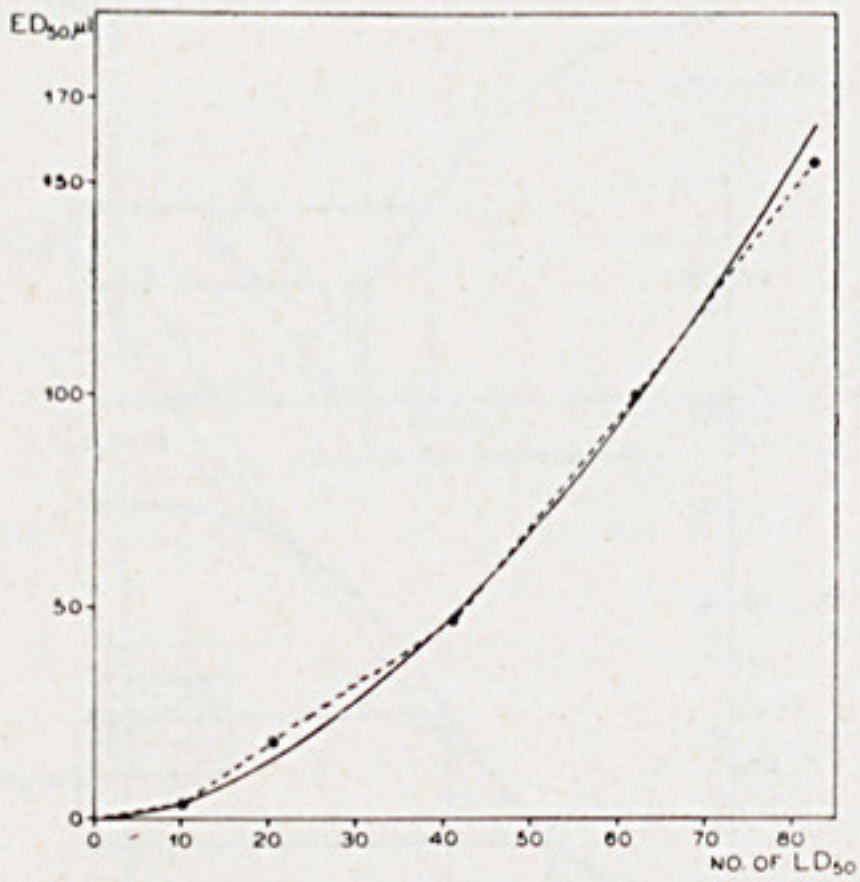


Fig. 8 — Observed ED-50 values for serum 503 — — — calculated ED-50 from the expression log ED-50 = K_1 + K_2 log (v − LD-50). K_1 a constant corresponding to potency of serum. K_2 the slope of the curve. log. ED-50/log microgram neutralized venom. (*Bitis lachesis*-serum 503— — K_2 =1.71).

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33. THE ROLE OF ENZYMES IN THE PROCESSES RESPONSIBLE FOR THE TOXICITY OF SNAKE VENOMS (AN IMMUNOLOGICAL STUDY)

O. ZWISLER

Behringwerke AG., Marburg-Lahn, Germany

I want you to know some immunological experiments which we recently did in the Behringwerke AG, Germany, experiments by which we intended to elucidate the role of enzymes in the *vitro* manifestations responsible for the toxicity of snake venoms.

As you know, snake venoms are a mixture of different pharmacologically active components. There are a number of papers in which these principles have been classified in accordance to their different mode of action (1-6), but I want to make a simple differentiation according to our knowledge of their substrates or receptors they act upon.

For an investigation whether the enzymes in snake venoms play an important role during the course of intoxication we have chosen different ways of experiments. In one series of experiment we compared the neutralization of lethal action and defined enzyme activity by polyspecific antisera from horses, in an other one we added an enzyme to venoms, which were either devoid of this enzyme or in which this enzyme was strengthened by the addition and tested the toxicity of this mixtures. In further investigations we produced monospecific antisera against some enzymes and some toxic components and determined the protective action of these antisera against the whole unfractioned venoms.

Titrating commercial antisera from horses with the venom, which has been used for immunization, the dose/response curve does not follow the law of multiple proportions and soon parallels the serum axis, showing a low content of antibodies against the toxins (7).

On the other hand, in case of elevated venom doses were there was *in vivo* no more protection by any volume of antiserum, the enzymes in this venom-dose were completely inhibited. In Fig. 1 a typical result is shown (8). There is only one enzyme, we have determined, the 1-amino acid oxidase, which was not at all inhibited, but the explanation for this special case is given later.

We asked ourselves, whether these findings resulted from the choice of the horse as antibody-donor and from the method of immunization used for production of antitoxic snake sera or whether we were observing a general feature of antivenomous sera. In attempt to answer this question we used other animals, i.e. rabbits, for immunization using various adjuvants. The venom we used was *Bitis gabonica* venom, which we know to be very good antigenic and against which generally animals produce potent antisera in a relatively short time. After two subsequent courses of immunization the sera were tested *in vivo* for their protection against the whole venom and *in vitro* for their enzyme-inhibiting

properties. In all cases the anti-enzyme titers were higher than that against toxic components (Fig. 2). Although these experimental arrangements can give insight into the contribution of enzymes to the killing power of venom, further refinements were needed for detailed analysis.

In order to examine the contribution of individual enzymes to the toxic efficiency of a snake venom, Kaiser and Michl have proposed the utilization of monospecific antisera, which are directed against only one of the many enzymes in the venom (9). This approach allows the selective inhibition of a single enzyme under physiological conditions. In this case a decrease in toxicity may be expected, provided the enzyme makes an essential contribution to the poisoning process. On the other hand, an antiserum against toxic components, but without any anti-enzyme action should give protection against the whole venom, if participation of enzymes in the process of intoxication can be neglected.

In order to obtain materials for immunization venom components were separated by electrophoresis in polyacrylamide gel (10). Figure 3 shows the electropherograms of some VIPERIDAE venoms. The various activities were localized in the gel slab whether by *in situ* methods or by cutting the gel into pieces, elution of the component contained herein and testing their activity *in vivo* and *in vitro* (Fig. 4). For immunization, the gel strip, containing the respective activity, was homogenized in an appropriate amount of saline and was injected subcutaneously into rabbits. The polyacrylamide acts as an adjuvant. By this method we have prepared monospecific antisera against l-aminoacid oxidase, phosphodiesterase and a fibrinolytic agent from the venom of *Vipera ammodytes*. We have similarly prepared monospecific antibodies to phospholipase A from venom of *Echis carinatus* and to acetylcholinesterase from *Naja haje* venom. The immunoelectrophoresis of these antisera, compared with the precipitation pattern of the respective polyspecific antisera from horses are shown in Figure 5.

Anti-toxic-sera were prepared against a toxic fraction from *Echis carinatus* venom. Work with this venom is convenient since its toxic components are not separated by electrophoresis (11), so that one small section of the gel contains practically the whole toxicity of the unfractionated venom. Further on we isolated crotactin from the venom of *Crotalus terrificus* (12) and prepared the respective antiserum. The immunoelectrophoresis of these antisera are shown in Figures 6 and 7. The protective potency of all these antisera was examined against whole venom.

As it is known, the phospholipase A can be completely inhibited by antiserum, but *in vivo* the monospecific antiserum even in a surplus necessary to neutralize the phospholipase A-activity in a small venom-dose is not capable to protect against the whole venom. So we cannot confirm the sentence of Kellaway, written 25 years ago (2): "It is by no means certain that part or all of the neurotoxic effect of some venoms may not be ultimately be found to be attributable to the phosphatidases", but our findings are in accordance with some recent zootoxic investigations made with purified phospholipase A, showing the low toxicity of this enzyme (13, 14).

Using phosphodiesterase-antiserum, there is also no inhibition of the toxicity of the whole venom, though this antiserum shows a sufficient inhibitory capacity for the respective enzyme, though the purified enzyme shows a certain toxicity (15).

The fibrinolysin-antiserum inhibits the fibrinolytic component of the *Vipera ammodytes*-venom *in vitro*. This can be shown by the inhibition of the lysis of thrombin induced fibrinclots and otherwise by comparing the clotting times

after injections of the fibrinolytic component or mixtures of the antiserum with whole venom into guinea-pigs. Intravenous injections of the fibrinolytic principle delay the clotting of guinea-pig blood up to about 600 seconds (determined in not treated animals to be about 300 seconds), while injections of a mixture of antiserum and whole venom accelerate the coagulation. In this case clot formation can be seen within 60 to 90 seconds, thus showing once again the dualistic action of snake venom containing a pro- and an anticoagulant principle upon coagulation. Although the fibrinolysin-antiserum did not neutralize the lethal action of unfractionated venom.

Two very interesting examples of an enzyme-antienzyme-system we found in the cases of the acetylcholinesterase and the 1-aminoacid oxidase.

In immunoelectrophoresis and applying other geldiffusion techniques, we found that a precipitation line retains acetylcholinesterase activity even after prolonged washing with buffers or saline, and which can be made visible by specific staining with indoxylacetate or acetylthiocholinchlorid (16) (Fig. 8). Inhibition of acetylcholinesterase by monospecific antiserum and also by two antisera from horses against *Naja haje*-venom could be determined quantitatively by *in vitro* assay, the inhibition was incomplete as it was to be expected from the activity in the precipitation lines. Even after addition of large volumes of concentrated antiserum to a constant amount of *Naja haje*-venom and varying the incubation time, temperature and buffer systems, the degree of inhibition was always 51%. We never obtained precipitates with acetylcholinesterase-activity, so that the activity could only be measured in the supernatants (Fig. 9). After geldiffusion of snake venom against the corresponding antiserum in most cases on precipitation line can be seen, which has 1-aminoacid oxidase activity. This phenomenon, indicating incomplete inhibition, was examined with monospecific antiserum, reacting with the venom of *Vipera ammodytes*. By estimating the residual activity in the well-washed and finely resuspended antigen-antibody-precipitates and its corresponding supernatants it was shown that the enzyme was completely precipitated by the antiserum and that 36% of the enzyme activity was inhibited. The antiserum crossreacted with the enzyme of other venoms of viperids but not with venoms of crotalides and elapides giving ultimately the same degree of inhibition (Fig. 10). However, more antibody was required in the heterologous than in the homologous system to lead to the given degree of inhibition (17). It may be that by this means the relationship of different species can be studied. We never got *in vitro* precipitates with antisera from horses, so that the lack of inhibition of the 1-aminoacid oxidase in the forementioned case titration of the antienzyme activity of *Vipera russelii*-antiserum can be explained in this way.

Tested *in vivo*, neither the acetylcholinesterase- nor 1-aminoacid oxidase-antiserum afforded protection against the lethal action of the corresponding venoms. Although the inhibition of these two enzymes *in vitro* by their respective antisera is incomplete, it can not be deduced, that this may be the reason for the lack of efficiency against unfractionated venom. Remember that antisera against urease, though there is only an inhibition of 20% of the enzyme activity, is capable to protect animals completely against the deleterious action of this enzyme (18). It seems to be sufficient for protection that the active agent reacts with the antibody. The complex which forms seems to be eliminated from the organism at a higher rate. These results are resumed in the next picture (Fig. 11).

Furthermore we were interested in the importance of the hyaluronidase during the course of intoxication. We never succeeded in isolating this enzyme, so

that we were not able to produce any antiserum. Since there is no proof that the hyaluronidase in snake venoms is completely different from that derived from testes, we added this latter enzyme to two different snake venoms and determined its influence on toxicity and death rate. The venoms we used were from *Naja nigricollis,* which was completely devoid of hyaluronidase and showed, injected subcutaneously, no haemorrhagic action, and from *Vipera ammodytes,* which showed considerable hyaluronate lyase activity and produced severe hemorrhages. The addition of various amounts of enzyme from testes did not increase the toxicity of both venoms applied s.c. or intramuscularly nor could we see a change of time between the moment of injection and death (Fig. 12). The only change which was to be detected was an increase of the local action of the *Vipera ammodytes*-venom, while the venom of *Naja nigricollis* though in mixture with hyaluronidase, remained without any local efficiency. This experimental arrangement allows to exclude the participation of hyaluronidase during intoxication by snake venoms.

Now let us regard the protection, which is achieved by toxin-antisera. Both were completely devoid of enzyme-antibody. Nevertheless these sera afforded protection against toxicity, that is they protect animals against the intoxication with whole venom, containing the not inhibited spectrum of all enzymes (Fig. 13).

Suming up these findings demonstrated here, give further support to the view that the toxins are the chief noxious principles in snake venoms, results which were shown by other immunological means by Kochwa and col., 1920. In order to obtain snake venom antitoxins of which the dose response curve is straightened, there must be a second immunization with enriched toxins or toxic components which are bound on high molecular carriers thus showing a better immunogenicity (21).

May be all these results are valuable for the production of potent antisera, for which, as you have seen, is advisable to use selected toxins with an especially high content of toxins.

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Discussion

A. do Amaral: "Just to compliment him for his splendid paper wherewith he has just delighted us, as specialists in this quite attractive field of investigation."

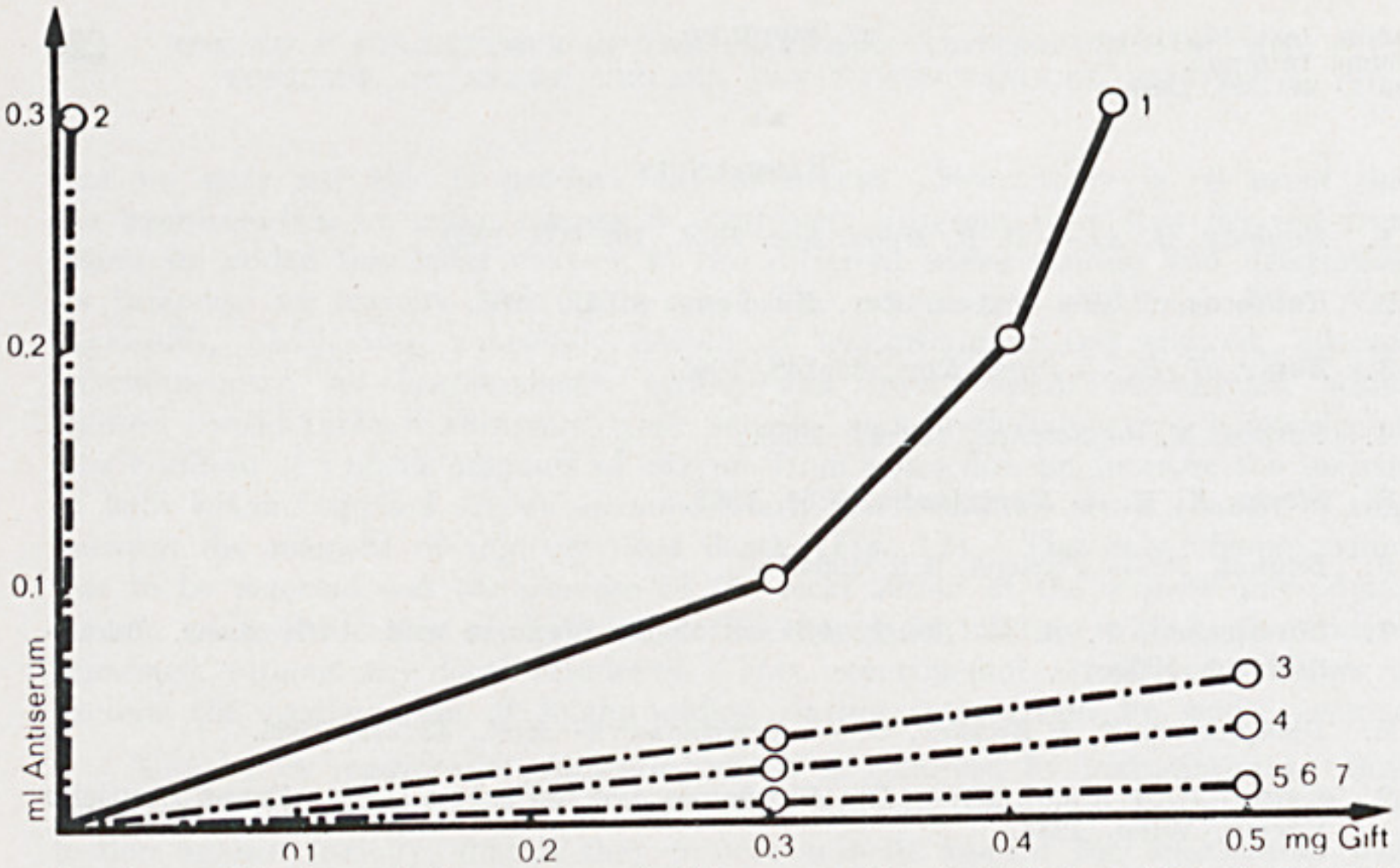


Fig. 1 — Titration of the venom of *Vipera russelii*. 1. Lethal action. 2. 1-amino-acidoxidase. 3. Phospholipase A. 4. Protease. 5. Alcaline phosphatase. 6. Phosphodiesterase. 7. Hyaluronidase.

ADJUVANS		dcl	ENZYME ACTIVITY OF dcl		
			Lecithinase A	Proteinase	Phospho-diesterase
Bajol/Arlacel	Rabbit Nr. 1	12	> 24	> 24	> 18
	2	12	> 24	> 24	> 18
	3	6	> 12	> 12	> 9
	4	6	> 12	> 12	> 9
	5	4	8	> 8	> 6
Bentonit	Rabbit Nr. 1	10	> 20	> 20	> 15
	2	8	> 16	> 16	> 12
	3	8	> 16	> 16	> 12
	4	6	> 12	> 12	> 9
	5	6	> 12	> 12	> 9
	6	4	> 8	> 8	> 6
$Al(OH)_3$	Rabbit Nr. 1	12	> 24	> 24	> 18
	2	12	> 24	> 24	> 18
	3	10	> 20	> 20	> 15
	4	10	> 20	> 20	> 15
	5	10	> 20	> 20	> 15
PAA	Rabbit Nr. 1	12	> 24	> 24	> 18
	2	12	> 24	> 24	> 18
	3	10	> 20	> 20	> 15
	4	2	> 4	> 4	> 4

Fig. 2 — Neutralization of toxicity and enzyme activity by antisera. Venom: *Bitis gabonica*. Antisera: rabbit. Neutralized by 1 ml antiserum.

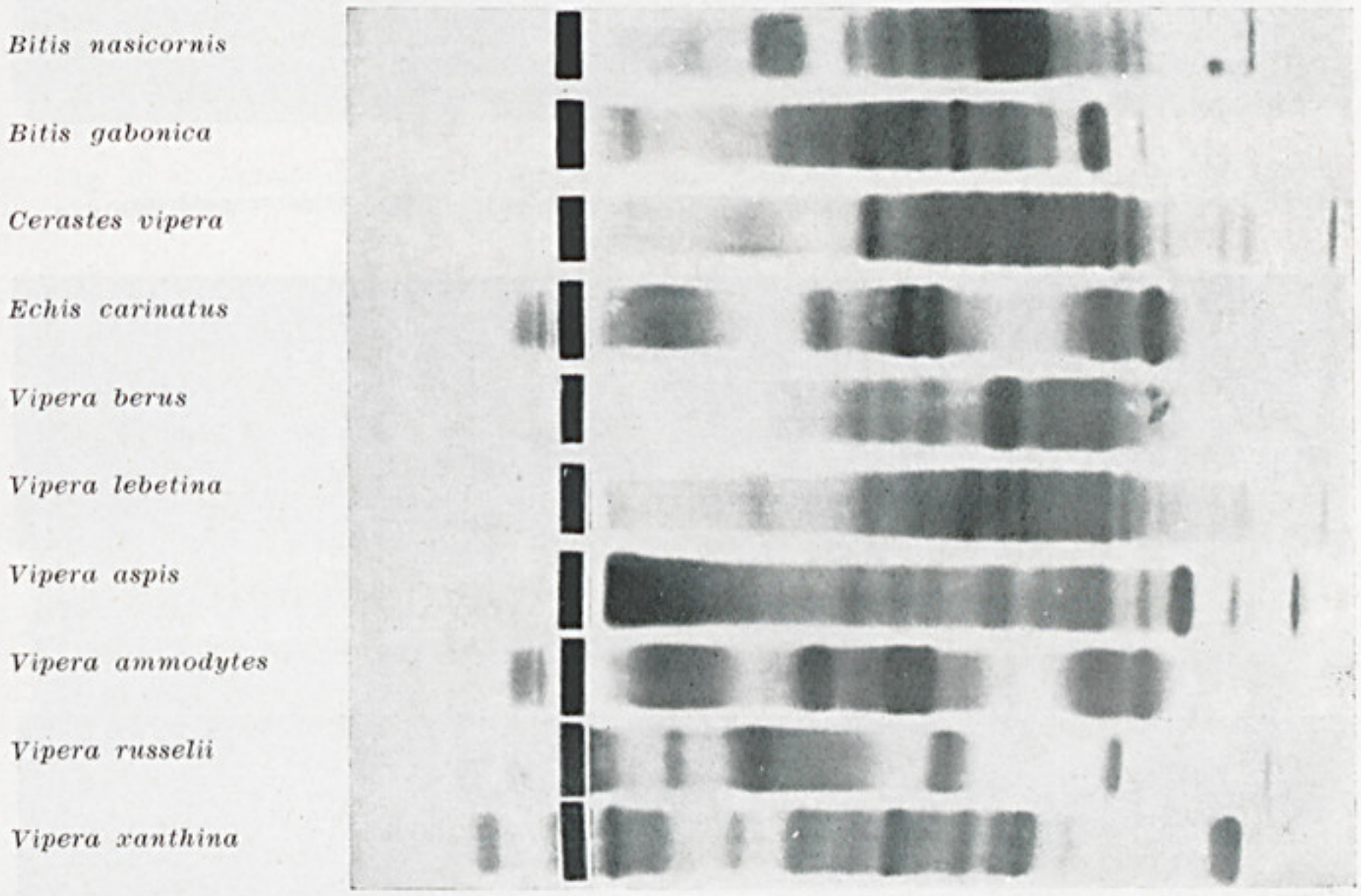


Fig. 3 — Polyacrylamide-electrophoresis of some VIPERIDAE venoms.

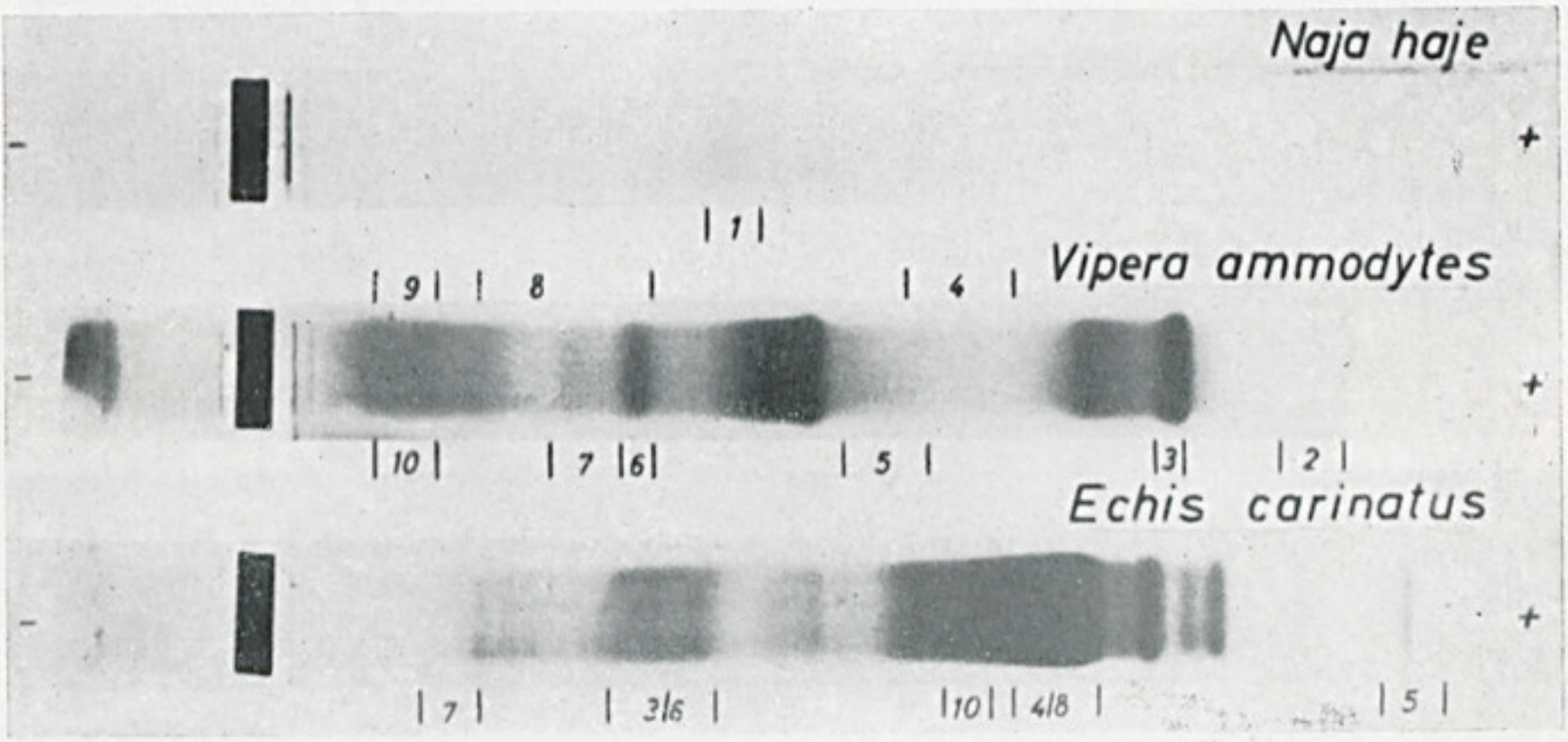
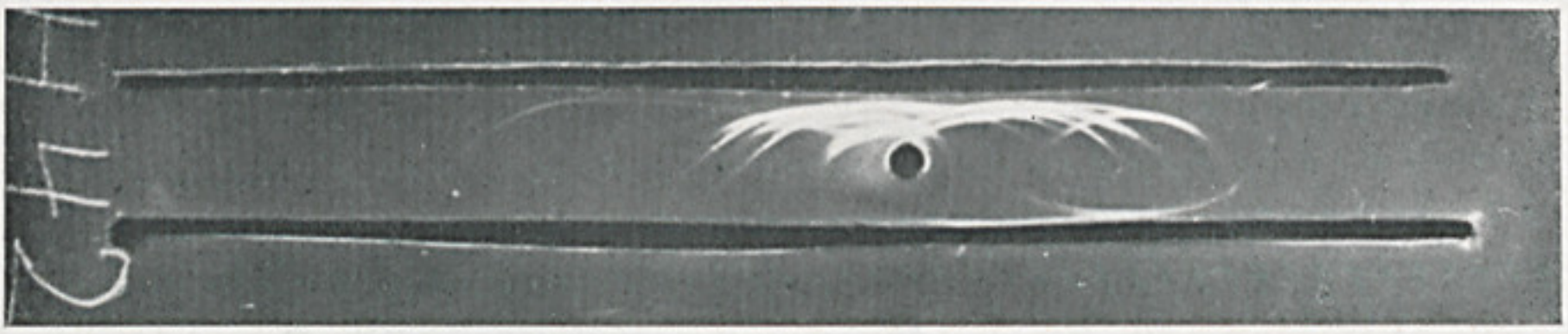
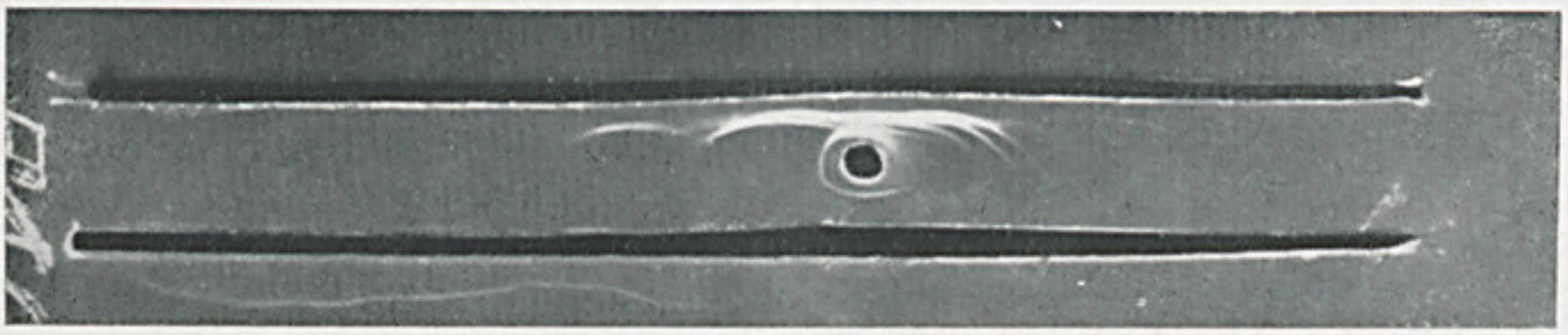


Fig. 4 — Localization of some venom components after PAA-gel-electrophoresis. 1. Acetylcholinesterase. 2. Fibrinolysine. 3. l-Aminoacidoxidase. 4. Haemorrhagine. 5. Phospholipase A. 6. Phosphodiesterase. 7. 5'-Nucleotidase. 8. Neurotoxin. 9. Protease. 10. Esterase (Tame).

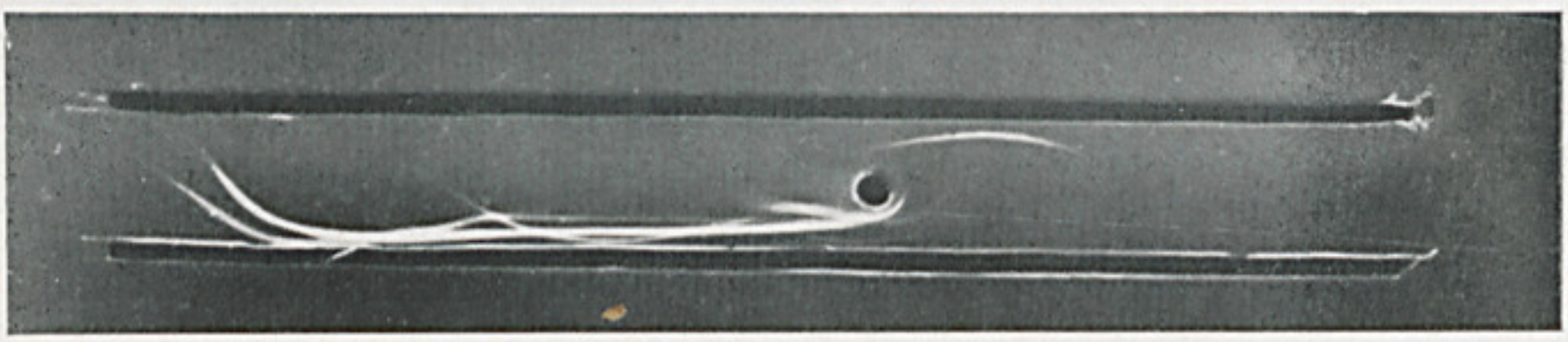
Vipera ammodytes — Above: antiserum. Under: anti-1-Aminoacidoxidaseserum.



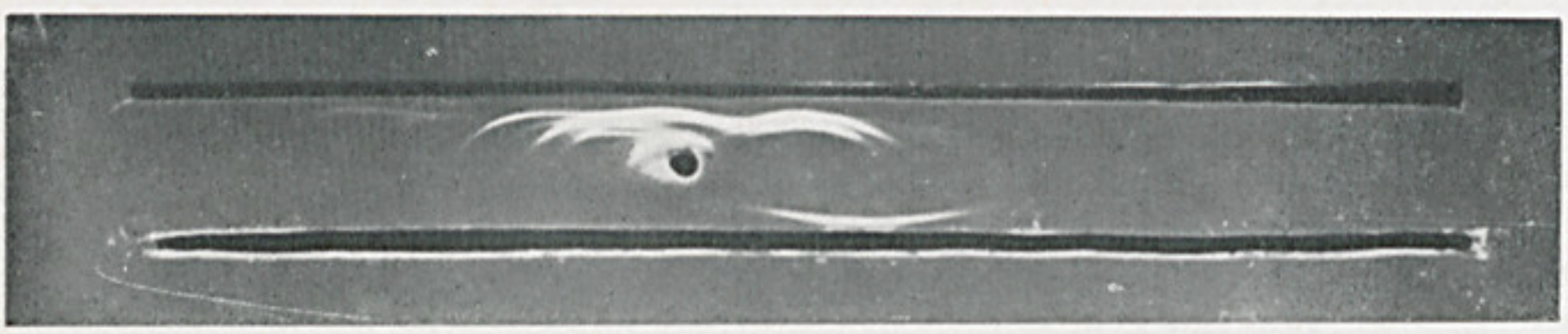
Vipera ammodytes — Above: antiserum. Under: anti-Phosphodiesteraseserum.



Naja haje — Above: antiserum. Under: anti-Cholinesteraseserum.



Vipera ammodytes — Above: antiserum. Under: anti-Fibrinolysinserum.



Echis carinatus — Above: antiserum. Under: anti-Phospholipase A serum.

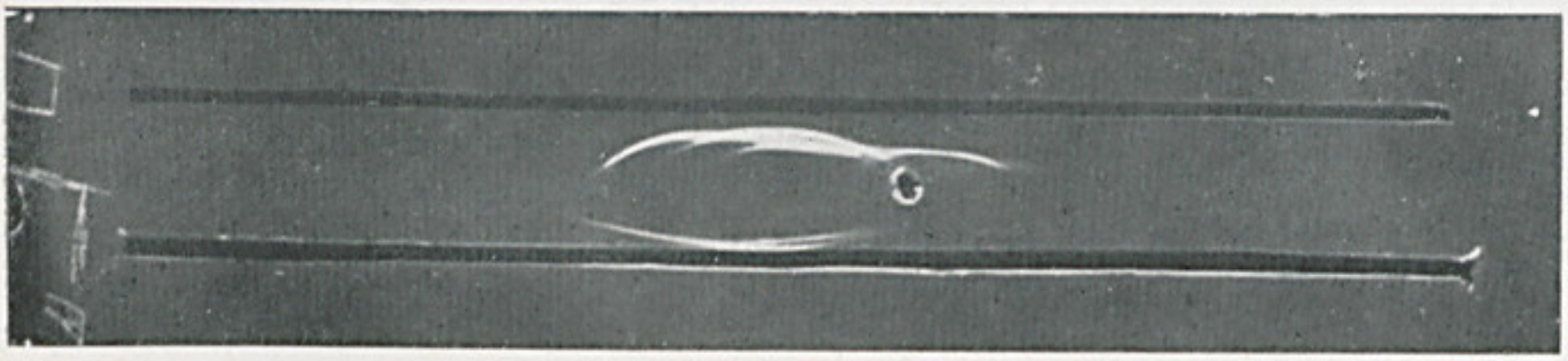


Fig. 5 — Immunoelectrophoresis of snake venoms with monospecific antisera.

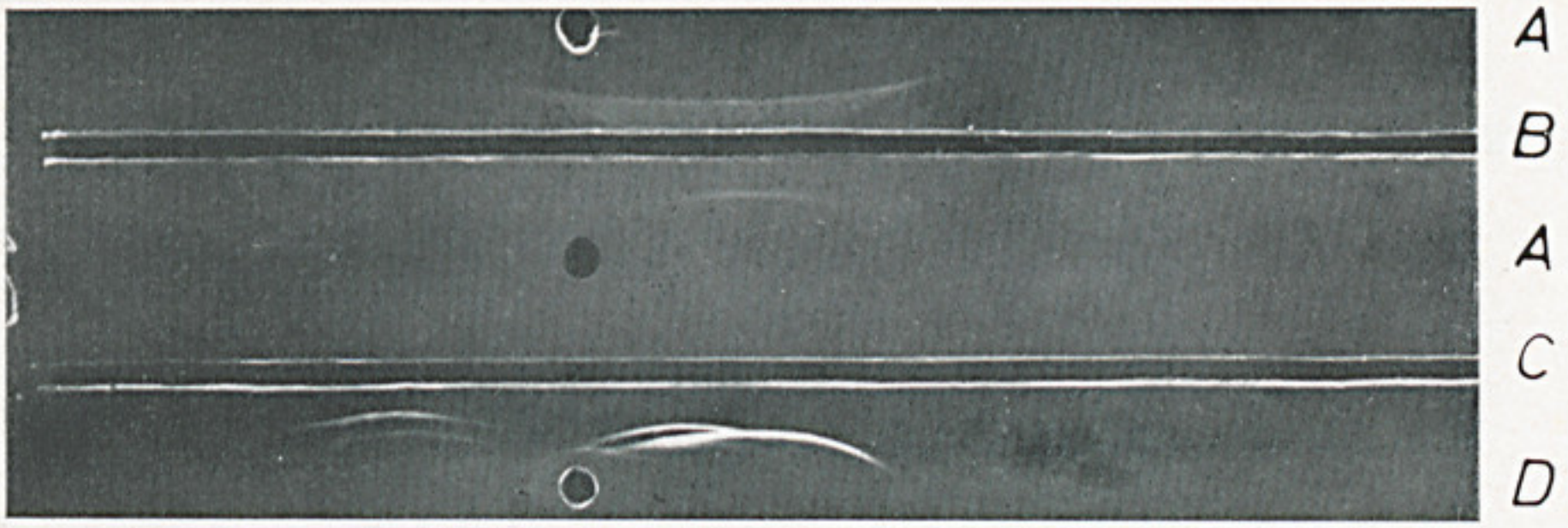


Fig. 6 — Antiserum against *E c h i s*-toxin. A. *E c h i s* — Toxin 1%. B. Antiserum against *E c h i s* — Toxin. C. Antiserum agains *Echis carinatus* from horse. D. *Echis carinatus* 2%.

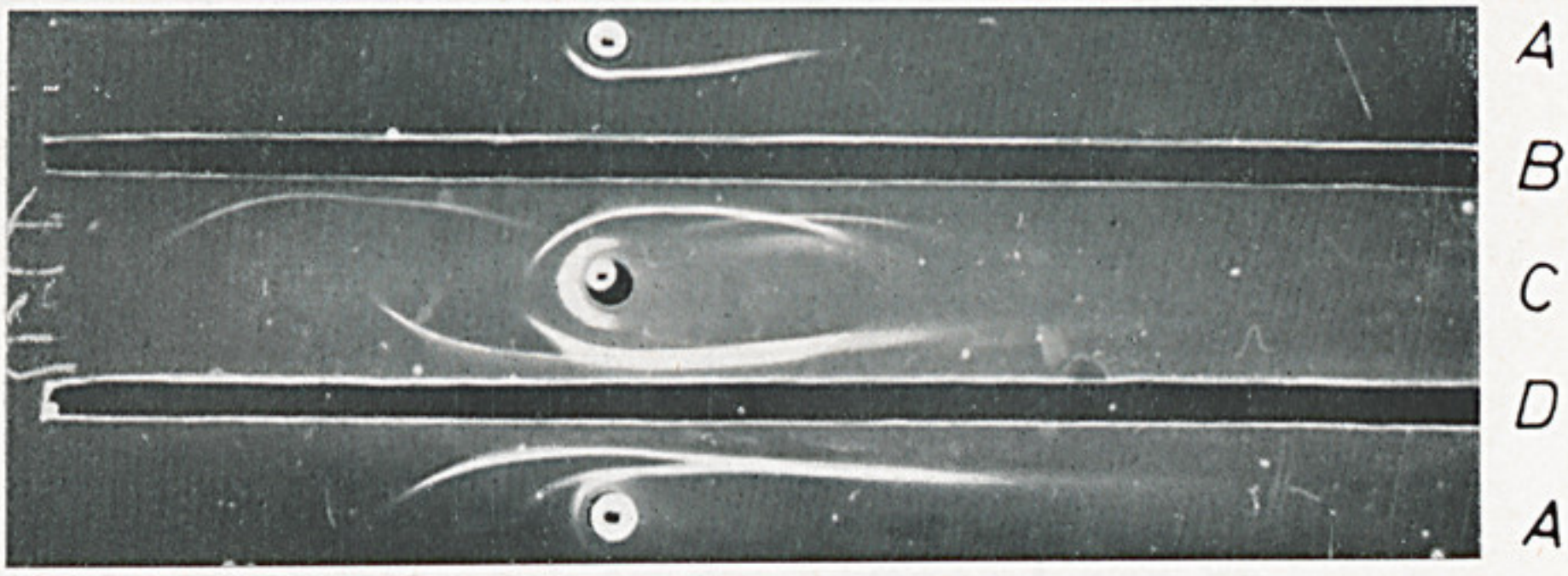


Fig. 7 — Antiserum against Crotactin. A. Crotactin 1%. B. Antiserum against *Crot. terr.* from horse. C. *Crotalus terrificus* 2%. D. Antiserum against Crotactin.

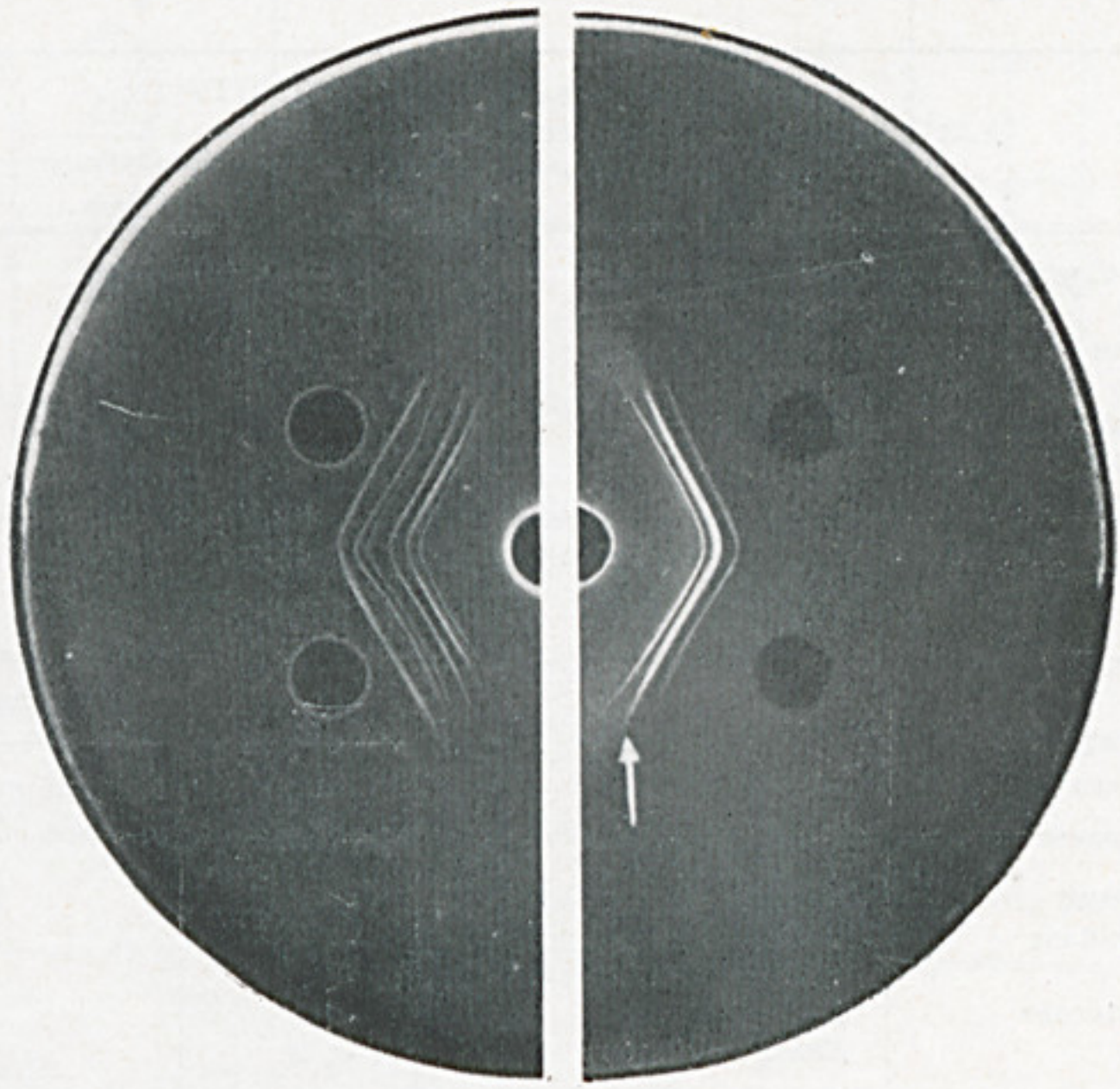


Fig. 8 — Incomplete inhibition of cholinesterase. Center well: 2% *Naja haje*. Outer wells:Anti-*Naja haje* serum from horse. Upper half: stained with indoxilacetate for acetylcholinesterase activity. Lower half: without staining.

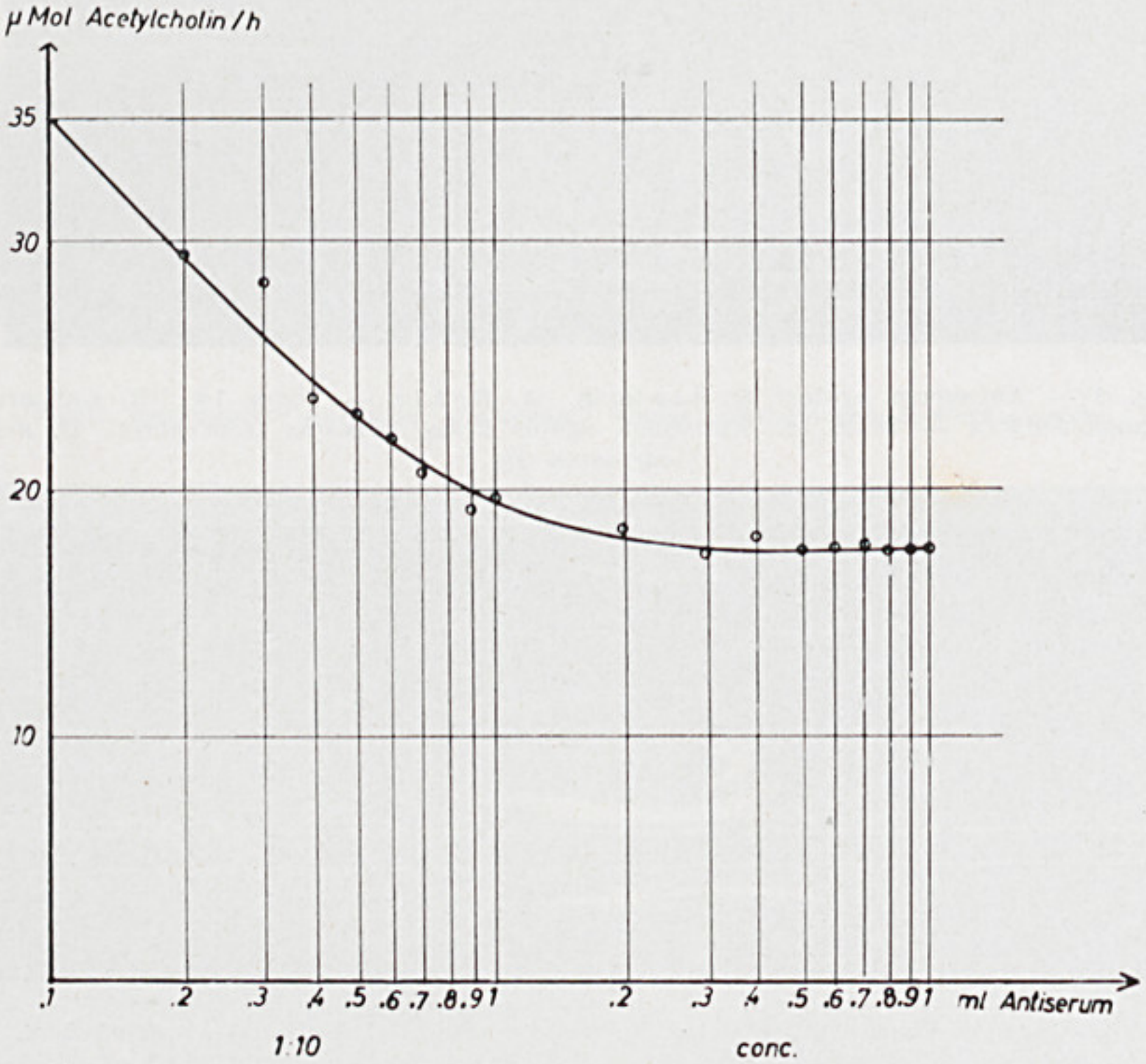


Fig. 9 — Inhibition of Acetylcholinesterase by antiserum.

VENOM	Activity (Q_{O_2})	Precipitation by antiserum (ml)	ACTIVITY IN		% Inhibition
			Supernatant (Q_{O_2})	Precipitate (Q_{O_2})	
Vipera ammodytes	0.1 mg = 44	0.35	0	28	36
Vipera ursinii	0.12 mg = 44	0.37	0	28	36
Vipera lebetina	0.08 mg = 44	0.41	0	28	36
Bitis gabonica	0.21 mg = 44	0.45	0	28	36
Cerastes cerastes	0.09 mg = 44	0.85	0	28	36
Echis carinatus	0.07 mg = 44	1.25	0	28	36

Fig. 10 — Inhibition of 1-Aminoacidoxidase by antiserum.

Serum against	Species	1 ml antiserum neutralized	
		dcl	Enzyme activity of dcl
Phosphodiesterase	*Vipera ammodytes*	< 2	10
1-Aminoacidoxidase	*Vipera ammodytes*	< 2	18
Fibrinolysin	*Vipera ammodytes*	< 2	15
Acetylcholinesterase	*Naja haje*	< 2	—
Lecithinase A	*Echis carinatus*	< 2	22

Fig. 11 — Protection by antienzyme-sera.

VENOMDOSE (γ) TOTAL VOLUME 0,2ml	I.E. TUR. HYALURONIDASE	MICE DEAD	MICE ALIVE
—	100	0	20
20 γ	—	0	20
20 γ	5	0	20
30 γ	—	0	20
30 γ	5	0	20
40 γ	—	6	14
40 γ	5	8	12
40 γ	10	5	15
50 γ	—	8	12
50 γ	5	8	12
50 γ	10	9	11
60 γ	—	12	8
60 γ	5	10	10
60 γ	10	12	8
70 γ	—	14	6
70 γ	5	14	6
70 γ	10	13	7
80 γ	—	20	0

Fig. 12 — Snake venom and hyaluronidase.

Serum against	Species	Neutralized by 1 ml antiserum	
		dcl	Enzyme activity of dcl
Toxin	*Crotalus terrificus*	100	—
Toxin	*Echis carinatus*	16	—

Fig. 13 — Protection by antitoxin-sera.

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34. VENOM AND ANTIVENIN SPECIFICITY: MODERN CONCEPT

AFRÂNIO DO AMARAL

(*Brazil*)

An expression of the changes our knowledge of biological phenomena constantly undergoes may be found in the gradual evolution of the notion of specificity of venoms and antivenins since the beginning of the present century.

At the implantation stage of Serumtherapy, when Calmette (1894), based on tests and injections made with the first batches of antivenin he had prepared to counteract the effects of the envenomation caused by "Cobra" (*Naja naja*), stated the anti-neurotoxin was devoid of specificity, a serious generalization of the concept thus expressed by a such a prominent pioneer of Science, commenced rapidly spreading out. Fortunately, Calmette's radical theory did not last long. As a matter of fact, since 1897 and, more precisely, 1901, following Vital Brazil's original demonstration in favour of the opposite view, a succession of experimental studies was published by specialists from all over the world (McFarland, 1901; Rogers and Lamb, 1906; Ishizaka, 1907; Noguchi, 1909; Arthus, 1911; Gomes, 1920; Houssay and Negrete, 1923; Amaral, 1921-27, besides other investigators at a more recent period) showing specificity to be a normal phenomenon between venoms and antivenins. The adoption and generalization of this conception, however, did not occur before successive technical advancements were made in Bio-Chemistry and Physico-Chemistry so as to dissipate many doubts and remove serious obstacles researchers had encountered in their way.

The existence of specificity in this field is no longer a matter of controversy. Any restriction still to be heard in this respect is rather a reaction to prove the notion of specificity to be broader than we were at first led to suppose.

It is true we admit that this property, normally confined to the morphological range of any species of veneniferous animal, is apt eventually, but partly anyhow, to reach closely allied forms through the intervention of factors leading to speciation.

Be as it may, such an extensiveness would in no case cross the boundaries of the genus wherein those forms happen to have been placed. In thesis, such a limitation does exist in view of the fact that the specificity principle implies the solidary and uniform intervention of the entire succession of activities on the part of the numerous constituents of the molecules forming every venom, most of which have a protein nature.

The behaviour of such substances in regard to heat, diffusion, dialysis, chemical affinities and bio-immunologic reactions is so consistent as to have led researchers to include them in the group of the "antitoxinogens" (Zinsser, 1923).

In the course of numerous investigations it was possible to connect venom nocuousness towards the organism of other animals with the presence of certain toxins and especially of a large series of enzymes.

The number of enzymes found in venoms has been steadily increasing as a direct result of many improvements introduced in laboratory technic, which are rendering their identification possible.

The very number of toxins that have been recognized in a few venoms, such as "crotoxin" connected with the South American rattlesnake (Slotta *et al.*, 1938) has increased through further researches (Gonçalves, 1950; Neumann and Habermann, 1955), "crotamin" (1) and "crotactin" deserving special mention as new principles.

As a matter of fact, variation in the composition of that crotalic venom was foreseen some forty years ago (Amaral, 1925-6) (2), when the presence of yellow pigment was consistently noticed in that excretion so as to characterize the rattlesnake population found in North-Eastern Brazil.

Further knowledge of the chemical constitution of that venom was enhanced by the application of improved and more sensitive technical processes such as electrophoresis (Slotta, 1938; Gonçalves, 1950), chromatography and double microdiffusion (Schenberg, 1959-63). Under the stimulus received from such findings, the characterization of other venoms was also attempted with a view to identifying the nature of their chemical constituents, the constancy of which, connected with genetic factors, is used as a means for telling apart specimens representing even sub-racial forms.

As regards the presence of immunologic variants already traced in some venoms (that of *B. neuwiedii*, for instance), their possible connexion with racial differences is a question under investigation (Schenberg, 1963).

In view of the existence of variations in the content of the venom from different specimens of the same morphologic species, even the composition of mixtures or batches (prepared either at different periods or at only one occasion) of several samples of a definite venom even though it be secured from numerous specimens all from the known species range may not always be the same (Schoettler, 1951). This variability is likely to assume a clinal or ecologic character related to the "niche" where those specimens have been captured (Amaral, 1956). In the light of these observations it is easy to admit the existence of "biological races" in such species.

A good example of that occurrence we pointed out (Amaral, 1956, WHO/B5/373) while comparatively examining two populations of *B. jararaca*, one from Cruz Machado (Paraná), the other from Timbó (Santa Catarina), separated from each other by the Iguaçu river. Morphologically, they were undistinguishable. Pharmacologically, although the venom from specimens really from C. Machado showed a toxicity (MLD) comparable to that from specimens really from Timbó following intra-venous inoculation (rapid blood-clotting and toxinic effects), the latter venom was about 50% more deadly than the former when given hypodermically (slow, general enzymic effects). This is one of the reasons for us not to advise the use, either in titration of venoms or in standardization of antivenins, of intravenous injections, since this is apt to provoke the primary intervention of substances involved in the blood clotting mechanism.

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(1) Acting like "apamin", the bee-venom basic polypeptid.

(2) Rev. Mus. Paulista, **15**:91; Bull. Antivenin Inst. of America, 1928, **3**:6.

Moreover, the neurotropic venoms (typically toxiniferous), when kept under ordinary, uncontrolled, conditions, are likely to keep their activity for a long time, whilst the cytotropic ones (typically enzymophorous) gradually lose activity. This inactivation as measured through toxicologic tests may reach 60 to 80% of the original figure.

In the light of these facts the conception of homogeneity and stability of the general composition of venoms (at least those under scrutiny) is no longer tenable.

Specificity reflects a combination at definite proportions of a series of principles in any venom; its derangement will follow any handling or treatment that may be partly or totally destructive to such biochemical principles by enhancing splitting or cleavage of their basic constituents and by altering their balance and normal ratio.

Although most of such principles are known to act as "antitoxinogens" a few of them (including mucin, an impurity mixing with them through the much used and abused process of oversqueezing the snake glands) appear to be non-antigenic. This might explain why, in certain cases, not all of the active principles are neutralized even by an otherwise potent uni-specific antivenin. Moreover, even uni-specific antivenins, when given late in the course of ophiotoxicoses, are likely not to neutralize those new noxious substances resulting from the interaction of preformed, normal constituents of the venom with the victim's tissue and blood proteins.

In their present general connotation, zootoxicoses represent chain-reactions initiated by proteinases and intensified by the intervention of other enzymic substances (besides specific toxins) successively acting on different tissues as well as on the very products issuing from cell lysis (Amaral, 1959) (3).

Due to the variable composition of the venom from specimens representing a definite morphologic species but proceeding from different clines or "niches" the preparation of an uni-specific antivenin for exclusive use in the corresponding area appears to deserve consideration. However, this ideal solution, no matter how justifiable it may be from a scientific standpoint, really is economically unsound. Indeed, it would involve the necessity of multiplying beyond acceptable limits the collecting and preserving in a separate container every individual venom (this without mentioning the influence of possible seasonal variations in venom composition) to be employed in immunizing any group of animals for serum production.

In view of the economic contra-indication to the routine use of such a solution, one might resort to the following expediency:

a) securing, preferably through electric stimulation, the venom from specimens proceeding from the greatest number of localities lying within the recognized range of the corresponding species;

b) preparing antivenins for the greatest number of species (races and subraces) within a certain genus so as to possibly cover all the types and subtypes of toxic and antigenic representative principles of that group (multi-specific but uni-generic antivenins);

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(3) Ciência e Cultura, 11:176.

c) titrating, biometrically, every batch of antivenin against either the venoms employed in immunizing the animals or at least that in the molecule of which the greatest number of active principles common to that group may be found.

Due to the complexity of their composition, venoms seem to stimulate, at variable degrees, the production of neutralizing substances: anti-neurotoxic, anti-cytotoxic or anti-enzymic. In certain venoms, especially in the highly enzymophorous group, the rate of inert, non-antigenic, substances appears to be higher than in the toxiniferous group. This may explain why it happens quite often for the antivenins produced even through an advanced immunization procedure not to be so potent as the usual bacterio-antitoxins. In South America, the anti-cytotoxic potency of antivenins happens to be ponderally higher than the anti-neurotoxic potency. As a matter of fact, among venoms used in immunization the neurotoxic ones have a greater activity, as expressed in MLDs, than the cytotoxic.

For titrating antivenins the best process — although not yet the ideal nor equally efficient for every case — among those thus far devised and developped implies the biometric calculation of the results secured on white mice, all homozygotic young males of the same weight, serially injected subcutaneously with decreasing dilutions of antivenin mixed with a fixed dose of the corresponding venom (× MLDs), this antigen being lyophilized and preserved away from light and humidity (Amaral and Schoettler, 1956, WHO/BS/364).

Brazilian contribution — The Brazilian contribution to the advancement of this branch of Science has a rather long and uneven evolution. At first, it coincided with the very growth of this Institute, which happened to become one of the world centres devoted to the study of zootoxicoses thanks to V. Brazil's pioneering activity. This fact notwithstanding, the present stage of our knowledge has not resulted either from a rapid progress or from unforseen discoveries. On the contrary, it has come about through several, successive and discontinuous attempts at abating the surrounding gloom until a few rays of light would appear that started clarifying this highly tangled field of investigation.

We are just beginning to unhide a few of the curious mechanisms, which, by intervening with the development of the most complex phenomena connected with the physiopathologic activities of venoms, have for so long a time kept among the *Naturae arcana* (the *Physeos mysteria* of Aristotelianism) the real meaning of those toxic secretions in their multiple effects on the human and animal tissues.

Our modest role and meager share in those developments had incipience, firstly while we had the luck to be one of V. Brazil's collaborators at Butantan (as a matter of fact, we represent the only survivor of that group still to be interested in zootoxicoses); secondly, as the result of a mere accident, after we were promoted to head the Butantan Laboratory of Medical Zoology, previous to our being commissioned in this Institute's directorship as one of V. Brazil's first successors. In that double capacity have we been both an instrument and a witness in the amazing, gradual unveiling of the secrets surrounding the phenomena of venoms.

Although quite long, the history of our "prise de contact" with problems related to venom and toxin titration, is not sufficiently known. For this reason we feel that, at the last quarter of our life of scientific researcher, we ought at least to touch upon the series of the most impressive experiences we had while dealing with such questions. And so, it might be pertinent for us to do it at

the very moment so large a group of exponential figures in the field of zoo-toxicoses is attending this International Symposium. This will also give us an opportunity to explain to all of you, particularly those who have shown interest in learning the scientific evolution of Butantan particularly for the last 40 years or so (which were complicated by the world's greatest economic depression and political instability leading to the recent international conflict), the changes that have taken place here, to wit:

By the middle of 1919, when V. Brazil decided to retire from the position he so much elevated in excellence at this Institute, we were engaged in an investigation, on the mosquitoes of São Paulo, at the Parasitological Laboratory under J. Florêncio Gomes. Much to our misfortune, Gomes was then caught by the epidemic influenza called "espanhola" and died from it. We were thus compelled, all of a sudden, to assume the heavy duties of chief of Medical Zoology, this being Butantan's main Section.

1 — As though this responsability were not sufficient to challenge our youth's energy, one more, out of the six laboratories existing at Butantan at that remote period, that devoted to Tetanus Serumtherapy had also be assigned to us, as one more contribution to avoid Butantan's work from collapsing.

There, while using the process of Anderson and Rosenau for testing tetanus toxin on guinea-pigs reared at the Institute Breeding Station, we came across an extraordinary case of individual variation as disclosed by their capacity to react against the established MLD of that antigen.

2 — In the 1919-21 period we found the *B. jararaca* venom, as extracted from several specimens, purified by centrifugation and intravenously injected (according to the technic used here) into adult pigeons (350 g) (of the race *Columba livia domestica*) not tc show consistent toxicity, its MLD varying sometimes between 0.016 and 0.025 mg.

3 — In that period we also verified that the venom of *B. insularis*, a Crotalid we described from Queimada Grande Island, where it lives on trees and feeds on small birds, was much more toxic to the pigeon than the venom of the homologous "jararaca" of the mainland, living on the ground and feeding generally on rodents (4).

4 — In 1921, we showed that the experimental intoxication caused by the Texas rattler (*C. atrox*) venom, although yielding much more markedly to the injection of the specific antivenin (that we had just prepared by using a batch of venom sent by our good collaborator, R. Ditmars, of the Bronx Zoo), also favourably reacted to the injection of the antivenin specific for the S. A. rattler still called *terrificus*. The cross-tests as performed intravenously into pigeons showed the following striking differences in toxicity:

A)	Venoms: *atrox* MLD	0.200 mg (in weight)	
	terrificus MLD .	0.001 mg (in weight)	
B)	Sera: anti-*atrox* titre ...	15 MLD (3,000 mg in weight)	of *atrox* venom
		23 MLD (0.023 mg in weight)	of *terrificus* venom
	anti-*terrificus* titre	1.000 MLD (1,000 mg in weight)	of *terrificus* venom
		6 MLD (1,200 mg in weight)	of *atrox* venom (5)

(4) Col. Trab. Inst. Butantan, 1920, 2:53; Anex. Mem. Inst. Butantan, Ofiologia, 1921, I: 44, 88.

(5) Col. Trab. Inst. Butantan, 1921, 2:171.

These observations, which date from about 45 years ago, were all borne in our mind and, if not published, registered in our note-books for further investigations whenever we could find the means for correctly performing them and subjecting their results to scientific analysis. No matter how faithfully we had accomplished our student's duties at College and the Medical School, we could not satisfactorily explain the amazing facts we have just tried briefly (not to tire this audience) to point out, in the light of the knowledge of biological phenomena prevailing at least in our midst at that really remote period of our activity.

Faced with the impossibility of finding the reason for such differences and variations, and led by the desire to penetrate the secrets underlying those intricacies, we decided to take advantage of the traveling prize we had received from the Medical School in order that we could take post-graduate courses at the outstanding University centres and research laboratories both in North America and Europe, our longer stay abroad being facilitated by a special fellowship we received from the International Health Board. We had thus a chance carefully to study different subjects connected with this Institute's work line and especially to enlighten our mind concerning bio-tests and titrations.

Through the personal contact we succeeded in establishing with a few of the great scientists of that time, not only (and particularly) at Harvard University but at other American and European institutions, we learned the ways and technic of investigation as followed by such prominent men as Th. Barbour (Comp. Zoology) — with whom we enjoyed the privilege of collaborating for several years — and G. Parker (Comp. Physiology) — in Cambridge; W. Cannon, C. Drinker, W. Porter and A. Redfield (Exp. Physiology), W. T. Richards and E. Bovie (Physico-Chemistry), E. J. Cohn (Physiol. Chemistry), H. L. Henderson (Blood Phys.-Chemistry), E. W. Wilson (Bio-Mathematics), R. Pearl (Bio-Statistics), W. Castle (Genetics), H. Zinsser (Immunology), M. Rosenau (Serology), E. E. Tyzzer (Comp. Pathology), R. Strong (Trop. Medicine) — all in Boston; J. Macleod and F. Banting (Bio-Titration:insulin) — in Toronto; E. V. McCollum (Nutritional Chemistry) — in Baltimore; Wm. Park (Serumtherapy) — in New York; J. Kolmer (Lab. Testing) — Philadelphia and E. C. Kendall (Hormonal Biochemistry) — in Rochester, our experience in Europe having covered the Lister Institute (London), Inst. Pasteur (Paris), St. Serum Inst. (Copenhagen), Inst. f. Schiffs-u. Tropenhygiene (Hamburg) and Instituto Sieroterapico (Milano). During our second sojourn abroad and before returning home we talked with some of those teachers and investigators over the main problems — both of technic and personnel — we had to face at Butantan. In Europe, we also secured advise as to the selection of a group of specialists we wanted to invite to come to São Paulo as collaborators in our plan of expanding this Institute so as to transform it into a centre of Experimental Medicine especialized in Human Pathology. This seemed, indeed, to be the logical, the necessary step for us to take, in the development of the ideal that led V. Brazil to found this institution. We thus attempted to catch up with the never so mobile trend scientific investigation was already revealing at that time.

That plan as presented to and approved by our Government following their invitation for us to return and modernize Butantan, called for the organization, to start by 1931, of whole new Departments to deal, respectively, with Experimental Genetics (and Cyto-Embryology), Bio-Chemistry (and Pharmacology), Physico-Chemistry, Immunology (and Serum-Therapy), Virus (and Virus-The-

rapy), Physio-Pathology (with Physiology, Endocrinology and Histo-Pathology), Parasitology (with Entomology) and Medical Botany (with Pharmacognosia), most of which were then a novelty in our "milieu".

Needless to say that the dynamics of that transformation implied the real integration of research through a close cooperation of the numerous scientists involved therein, with the hope that it might set an example in an environment such as ours, so well known for its individualism. A sketch of that plan may be found in Mem. Inst. Butantan, **VI**, 1931 *et* **X**, 1935 in their "Noticiário" section.

Concerning venoms and antivenins: we immediately decided to synchronize the action of the new group of scientific collaborators and to take advantage of the modern laboratory equipment we were installing at Butantan, in order to tackle the fundamental problem of properly analysing the chemical composition of venoms with a view to purifying them in such a way as to make it possible for the exact properties of their active constituents to be pharmacologically determined. Moreover: the dream we started to cherish at Harvard as early as 1924 called for the eventual synthetic production of pure principles to be applied as antitoxinogens instead of whole, crude, venoms, so that the preparation of really specific antivenins could leave the empiric stage in which it lay for so long a time, and follow a really scientific direction, thus also opening the way for the establishment of a rational procedure in venom titration and antivenin standardization.

Besides several promising colleagues such as Lemos Monteiro, J. Travassos and Vallejo-Freire (Virus), Flavio da Fonseca and Paulo Artigas (Parasitology and Entomology), J. R. Valle and R. F. Mello (Physiology and Endocrinology) and others, the following specialists came to work here: G. v. Ubisch (Prof. Heidelberg Univ.), in Genetics; K. H. Slotta (Prof. Breslau Univ.) and his assistants H. F.-Conrat and G. Szyska, besides Kl. Neisser (Berlin Univ., assistant to Nobel Prize Prof. A. Windaus), in Bio-Chemistry; D. v. Klobusitzky (Frankfurt Univ. Prof. W. Pauli's pupil) and P. Koenig (Wien Univ.), in Physico-Chemistry; Prof. Thales Martins (Inst. Oswaldo Cruz, Rio) and M. F. Amorim (São Paulo Med. Faculty), in Physio-Pathology; the great Prof. Pirajá da Silva (Bahia Med. Faculty, retired), in Medical Botany, besides the famous Prof. Ludwig Fraenkel (Breslau Univ.), who joined us as a volunteer at the Endocrinological Laboratory; the dynamic Werner Schoettler (who as a student at Berlin Univ. had already been one of our collaborators through the Antivenin Institute of America); and, at a later date, the cautious Saul Schenberg, who, like, W. Schoettler, became engaged in pharmacological experimentation.

Results — Among the many facts that were brought to light at Butantan at that period, besides many others but unrelated to the object of this Symposium, the following seem to deserve special mention at this time:

a) At the Genetic Dept., v. Ubisch showed the guinea-pigs reared at our Breeding Station not to represent a homozygotic colony but to descend from an extensive and long-standing hybridization between *Cavia porcellus* and *C. rufescens*, hence their variable response to toxin and venom.

b) Our local pigeon is about twice as susceptible to *B. jararaca* stabilized venom as its N. American homologous, in the light of comparative experiments we

made at Butantan Ophiologic Dept., at Harvard Univ. while teaching there and at the Antivenin Institute of America in its organization period.

c) At the Physico-Chemical Dept., "bothropotoxin" as a blood coagulant was prepared from *B. jararaca* venom by v. Klobusitsky and Koenig.

d) At the Bio-Chemical Dept., Slotta and colls., started the isolation of the neurotoxic principle from *terrificus* crotalic venom, the substance still bound to phospholipase A having been called "crotoxin" and "crotactin" (N. *et* H.), when separated from it. This pioneering piece of work has enhanced further investigators in Brazil and abroad (M. Gonçalves, Ribeirão Prêto Med. School, 1950; A. Barrio, Inst. Malbran, 1954; S. Schenberg, 1959, O. V. Brazil *et al.*) to develop the analysis of that substance and recognize "crotamin" also as an active principle in that venom.

e) At the Ophiological Dept., an extensive study of the Brazilian and the Neotropic serpents was made, two general Check-Lists having appeared preparatory to the publication of our "Iconographic Catalog of the Serpents of Brazil" with coloured plates and Portuguese and English texts (in press). In the 1929-1937 period, our official journal "Memórias do Instituto Butantan" was issued quite regularly, numerous studies having been published in its volumes IV to XI (8) as original contributions from some Departments of this Institution. Vol. IV of our "Memórias" covered 2,764 pages, all taken up by the afore-mentioned Check-Lists besides many other articles prepared by the Director of this Institute and Chief of its main Ophiological Department. Previous to that period, a preliminary study as based on the examination we carried out from 1920 to 1924 of the differential characters of over 6,000 specimens (mostly living ones) of the main species of Neotropic pit-vipers of the genus *Bothrops*, which had been misidentified by the great G. A. Boulenger (in Cat. Sn. Brit. Mus. IV, 1896), was published as No. 2 Contribution from the Harvard Institute for Tropical Biology and Medicine, 1925. That revision pointed out many misleading differences particularly connected with the ontogenetic evolution and frequent individual variations in the chromatic characters of those pit-vipers. And as a complement to such a work we published an article (in Amer. J. Trop. Med., **IV**, 5 1924) on the differentiation of *B. atrox*, *B. jararaca* and *B. jararacussu* venoms by their M. L. D., coagulability by heat, proteolytic, hemolytic and hemocoagulant activities, venom-antivenin cross-neutralization and serum-precipitin tests.

f) At the Ophiological and Physio-Pathological Depts., the method of treating human ophiotoxicoses was scrutinized and, following improvement, has of late been introduced into the routine work at the Butantan Infirmary.

In this connexion we may say that our first personal contribution towards rationalizing specific therapeutics of envenomation consisted in the establishment of the following fundamental principle that had thus far been overlooked: the dose of any antivenin to be given to a patient (either human or animal) must be inversely proportional to his body weight, since the lighter the victim proportionately the greater the concentration of the venom in his tissues (Amaral — in *Bull. Antivenin Inst. America*, **1**:77, 80, 1927, et *Mem. Inst. Butantan*, **5**:223, 1930).

g) Through the joint work of the Ophiological and Pharmacological Laboratories, an analysis of the most rational process for testing venoms and titrating antivenins has been under way for many years although not with the desirable continuity.

As explained in a special report we prepared in collaboration with W. Schoettler for publication through the WHO (BS/364/1956) and now brought up to date, in that work a few preliminary precautions must be taken so as to eliminate many causes of error and reduce variants to an acceptable minimum, to wit:

A) *Concerning the snake:*

a) Specimens must proceed from or be secured in definitely identified places;

b) When taken into the laboratory, every specimen must be kept undisturbed and under constant environmental conditions, in a separate cage (with number and full data on tag), to be properly fed and cared for (WHO/BS/373/1956).

B) *Regarding the venom:*

a) Forceful extraction must be avoided to prevent mixing the proper excretion with other gland constituents and mucus;

b) For titration and standardization purposes, it is advisable to secure the venom by means of the electrically induced bite through a special rubber membrane so as to avoid injuring the snakes teeth and exceeding the normal limits of pressure set by the natural contraction of the muscles involved in the bite mechanism;

c) The venom, ejected into a laboratory glass, must be immediately and successfully centrifuged and dehydrated, either from the liquid or from the frozen state, under vacuum at either room or lower temperature, or through Stokes' lyophilizer at 0.1 mm Hg;

d) As to storage, apparently keeping in neutral-glass tubes filled with N at athmospheric pressure and kept in dark at $-10^{o}C$ is a safe way to warrant preserving every active principle in a venom. For obvious reasons, the desirable establishment of any "reference preparation" must be based on a rationally extracted and properly preserved venom.

C) *Respecting venom testing:*

Among the technics thus far devised for this purpose there seems to deserve preference, for reasons both economic and biologic, the probit method through hypodermic injections into standard homozygotic mice, every precaution being taken to warrant a correct statistical computation of results and all tangible causes of error being avoided, inclusive such shortcomings as: age/weight variations; sex differences (pregnant female mice usually being more resistant than the male); greater or lesser local trauma, with loss of

plasma as held up in the oedema area (resulting particularly from injection of enzymophorous venoms), responsible for the dehydration followed by collapse of some of the test animals; environmental physical factors prevailing at the breeding quarters, etc.

D) *As to antivenin titration:*

Preliminary, the following facts must be borne in mind: a) The MLD of a venom type per unit of weight varies from species (perhaps also from race to race) of animal; the relative potencies of antivenins, for reasons until unknown and calling for further investigation, may show variations when assayed on different species of laboratory animal; different animals show different susceptibility to different venom types. b) The absolute and relative resistance of man to venoms is unknown. c) The intimate mechanism of death of man and even laboratory animals in every type of ophiotoxicosis is still a matter of speculation and so is its possible and complete inhibition by antivenin.

REMARKS — In view of so many fundamental and unknown factors being involved in the mechanism of death caused by the numerous types of venom, it seems necessary to extend our comparative scrutiny to larger animals such as dogs and monkeys as an approach to our learning the ways man reacts to venoms and antivenins. We feel that, at the present stage of our knowledge, even the method (which we consider reasonable) based on the determination of the relative potency of an antivenin by assaying various amounts of serum against a fixed dose of venom would be generally accepted only when its efficiency should pass a test performed on larger animals. In this connexion we might mention that, while organizing the Antivenin Institute in the U.S.A. some 40 years ago, we decided to resort to *Rhesus* monkeys on which to test the activity of the first batches of the Nearctic antivenin we had prepared, in order to give satisfaction to the officers of the Hygienic Laboratory, in Washington, as to the therapeutic efficacy of that product. Concerning dogs and monkeys as testing animals, one important aspect that seems to have been overlooked is that, in order for the most tangible variants to be removed, it would be necessary for those animals also to be reared at Breeding Stations so as to warrant the formation and maintenance of homozygotic stocks.

CONCLUDING HINTS — The reorganization of Butantan always as a State institution, having commenced in 1931, was near completion by 1937, having thus passed a most difficult period following the 1930 Revolution and opening the era of successive political crises, social unrest and economic distress Brazil started to experience. Much to our regret and shame, at the very moment we were beginning to get the fruit of our reorganizational plan, there came the well-known "coup d'État" of 1937, responsible for the improvised statization of this country. As happened in Europe all through traditional centers of culture such as Germany, Italy and other countries, in Brazil the new regime made havoc and preferred negative selection as the rule for filling positions of responsability. Instituto Butantan as a State organization was profoundly affected: many of the members of its scientific staff were displaced. Whilst some of them luckly were engaged by local Biological Laboratories and others were made teachers at

Medical Schools, a few had to leave Brazil to continue serving Science. Amongst these we may mention G. v. Ubisch who went to work in Genetics at Leyden University; K. H. Slotta, who, as the head of the Biochemical Dept. of the Miami University Medical School, immediately took up chemical analysis of the ative constituents of blood involved in coagulation; and H. F.-Conrat, who joined, at the University of California, the group under Prof. W. M. Stanley, with whom he soon succeeded in separating and re-synthetizing the active molecule of a virus of vegetable mosaic; not to mention Prof. L. Conrat, who left for Uruguay where he continued cooperating towards the progress of Medical Science.

In more recent years, scientific research was taken up again on Physio-pathology by G. Rosenfeld and his assistants, on Bio-chemistry by S. B. Henriques and his group, as well as on Cyto-genetics, first under the leadership of Prof. G. Schreiber with collaboration from H. Belluomini and others, and lately by W. Beçak and assistants, on Arachnidology by W. Bücherl and on Ophiology by A. R. Hoge.

Fifteen years had not yet elapsed when, in view of the Brazilian reconstitutionalization, we made a new attempt at modernizing Butantan (cf. "Noticiário" in *Mem. Inst. Butantan*, **XXVI**, 1954). In order that it might be freed from strange influences used to causing periodic slowing crises at Butantan, we tried to have it changed into an autarchic organization. Unfortunately, Brazil, still as an underdevelopped country in want of an organized, watchful public opinion apt to uphold any sensible plan of scientific investigation, proved not to be ready yet to take Science seriously or even to comprehend our programme to keep this institution true to the sprit that dictated its foundation, as an homage rendered to its first director, in whose honour this International Symposium is being held. Anyhow, Butantan scientific structure has suffered so deeply, that, following our decision to retire from official duties here, after having honestly and faithfully tried to serve Brazil for 50 years, and confine our attention to the international organizations (International Commission on Zoological Nomenclature, in London, and World Health Organization, in Geneva) wherewith we have long been connected, our successor, Dr. A. Vallejo-Freire, decided to devote his energies to the extreme ("heróico" as it is called in Portuguese) plan of establishing here a foundation whereby research might recover vitality. This is our earnest hope, which we feel is shared by you all, who know the progress of human knowledge to lie on investigation.

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35. VENOM AND ANTIVENOM POTENCY ESTIMATION

P. A. CHRISTENSEN

The South African Institute for Medical Research, Johannesburg, South Africa

It is common knowledge that venoms contain a wealth of enzymes and other biologically active substances, and that their potency in terms of such substances may be ascertained by measuring their effect on suitable substrates. Most, if not all, the active venom components are antigenic and an antivenom's potency could refer to its ability to inhibit the effect of any such substance, but in this discussion of potency measurement of venoms and antivenoms, I intend to confine myself to the measurement of the lethal effect of venoms and the life-saving properties of antivenoms as assayed in animals. Furthermore, having very limited experience with venoms of scorpions and spiders which constitute a minor problem in Southern Africa I will be basing my arguments on snake venoms and their antivenoms and draw my examples from work with African venoms.

For the determination of venom potency we are restricted to observing either the proportion of animals dying from suitably chosen doses, or the time to death of animals, given larger graded doses of venom.

If one used the quantal response method, i.e. observed the percentage death caused by graded doses of venom, the magnitude of the median lethal dose (LD50) for a venom would of course depend on which animal one employed for the test, rabbit, guinea-pig, mouse or pigeon, to mention some of the more commonly used animals, and also the order of toxicity of different venoms could not be expected to be the same irrespective of which animal was used because the susceptibility to different venoms may vary from one kind of animal to another. Furthermore, the order of toxicity for a series of different venoms tested in one kind of animal may depend on the route of injection. As an example one could mention that different *Bothrops* venoms examined by Schöttler here at Institute Butantan could be placed in one order of toxicity when the mice were injected intravenously and in a different order when the test was carried out subcutaneously. This, as he pointed out, would indicate that the main lethal factor differed for the two routes of injection (Schöttler, 1955/6, 1958).

In this respect, African venoms behave in a more regular fashion. Table 1 shows the intravenous and subcutaneous LD50 determined in mice weighing from 16 to 18 g for the venoms of the important African snakes. The values for some *Naja* species from the Asian mainland and the Philippines which show a certain relationship to African *Naja* venom have been included for comparison.

These particular values refer to venom samples set aside as laboratory reference preparations and other samples may give slightly different values but the order of potency for different venoms is quite typical. It is as the table 1 shows closely the same for the two routes of injection which gives no reason to

think that different components are active in the two ways of testing. The very slight difference between the intravenous and subcutaneous LD50 for the elapid venoms is presumably explained by a small molecular size and rapid absorption from the subcutis whereas the larger toxins in viper venoms are surprisingly ineffective subcutaneously.

It is always interesting to have an idea about the order of toxicity of venoms from different snakes for different kinds of animals, but nobody would attempt to compare the potency of two different venoms and expect to gain more than an impression of their relative toxicity. Venoms from different species are totally dissimilar and one cannot obtain a potency estimate which is valid in the accepted sense of this term. Even the comparison of the toxicities of samples of venom from the same kind of snake may not be a simple matter.

If two preparations containing one and the same toxin in different concentrations were compared one would expect to get the same estimate of their relative potency irrespective of the kind of animal used and the route of injection, as long as the animal is susceptible to the toxin and both preparations are tested by the same route, but this cannot be taken for granted in the case of venoms which contain more than a single lethal toxin.

If a venom contains two toxins, it could happen that one is the dominant toxin in one experimental animal, the other in another, and one toxin may be particularly active intravenously, the other subcutaneously. The relative contents of the two toxins may not be the same in two samples of the same venom in which case the apparent relative toxicity will depend on the test animal and the route of injection. It is well known that such variations in venom composition do occur; the most striking and extreme example is the crotamine-secreting and non-secreting South American rattlesnakes demonstrated by Brazilian workers (Gonçalves, 1956; Schenberg, 1959).

In order to increase the likelihood of comparable results it is therefore necessary to define the exact technical details of the tests with regard to test animal, and route of injection. Factors such as the injected volume, the speed of intravenous injection, and the temperature at which the injected animals are kept must be kept in mind as possible sources of variation. Working with mice and African venoms, I have never observed signs of significant differences in the susceptibility of males and females, but Dossena (1950) in Switzerland claimed that female mice, rats and guinea-pigs were more resistant than males to *Naja nivea* venom and according to Schöttler (1952) there is an indication that this applies to *Crotalus* and *Bothrops* venoms also, yet Henriques and her coworkers (1959) noted later a higher LD50 for male mice, but stressed, as did Schöttler, that the results were inconclusive, and it is probably fair to conclude that the sex of the mice used in venom work is of no great importance.

The results of toxicity tests carried out in mice under similar conditions in different laboratories on identical venom samples have occasionally given widely discrepant LD50 values indicating differences in the susceptibility between strains of mice. This, in itself, need not mean that two laboratories cannot arrive at the same relative potency estimate for two venom preparations, but it is just possible that the susceptibility of mice to the different toxins in a venom could vary independently, which could lead to complications, particularly in antivenom potency assays but also in toxicity tests, if in some mouse strains a minor toxin took on the role of the dominant toxin, even if this may be unlikely.

Whichever the test animal and whatever the route of injection it is common practice to compare the toxicities of different venom preparations in parallel line assays by converting the observed mortalities into probits which are linearly related to the logarithm of the venom dose, and I should like to call attention to an interesting suggestion made by Schöttler (1958) to overcome the difficulty that arises when only a single group of animals showing partial survival separates groups showing 0% and 100% mortality. If for instance 10 mice are used per dose and the two lower doses, d_1 and d_2, fail to kill any mice, d_3 kill some, and the higher doses d_4 and d_5 kill all the 10 mice, Schöttler suggests that the response to d_2 could be recorded not as 0% but as 2.5% because an expected mortality of from 0% to 5% would result in no deaths when only 10 mice are tested, and similarly the response to d_4 could be recorded as 97.5%, the mean of the expected mortalities 95 and 100%, which would result in no survivors.

The magnitude of the LD50 depends on the effect of the dominant toxin or on its combined action with other components if they happen to have some form of joint action, but it gives no information about the toxicity of minor toxins which cannot be determined except after their isolation although it sometimes may be possible to get a crude estimate of the order of toxicity in terms of whole venom from the results of neutralization tests with antivenoms and some examples of this will be presented later.

The higher the toxin dose the shorter the time to death, and everybody routinely examining the lethal effect of toxins will have some idea about the amount injected from the time it takes to kill the animal. There is however, no simple relationship between dose of venom and death-time. If the time to death (T) is plotted in a graph against the dose (D) one gets a hyperbolic curve with asymptotes determined by a dose (d) which kills in unlimited time and a death-time (t) for an infinitely large dose. Many workers early in this century sought a general formula to express this relationship and the English immunologist A. T. Glenny suggested in 1914 that the different solutions were modifications of a formula.

$$(D - d)^{\alpha} (T - t) = K,$$

where alpha and K are constants characteristic for each toxin.

In order to make use of the relationship between dose and death-time for the estimation of the relative potency of different preparations of a toxin, it would be desirable to transform the doses or death-times, or both of these, in such a way that they become linearly related.

Working with bacterial toxins, Ipsen (1941) found that the equation

$$(D/d - 1)^{\alpha} (T/t - 1) = K$$

fitted his data better than Glenny's formula. The parameters in this equation, d, t, α, and K, of which the three last are constants, may be determined graphically (Ipsen, 1941; Christensen and Finney, 1953), and a function of the death-time, Ipsen's *logarithmic dose-time equivalent*, $f(T)$, is defined as

$$f(T) = \log D - \log d = \log (Kt/(T - t)^{\frac{1}{\alpha}} + 1).$$

This logarithmic dose-time equivalent is linearly related to the logarithm of venom dose over a fairly wide range of doses and has been used successfully in

potency assays of venoms from Africa and Formosa (Christensen and Finney, 1953; Christensen, 1955; Lin, 1956).

Also a simpler function of the death-time, namely the logarithm of the death-time, is for some venoms linearly related to the logarithm of the dose over a range of doses wide enough to make it equally suitable for use in such assays (Christensen and Finney, 1953; Christensen, 1955).

This graded response method is very satisfactory in that results with fairly narrow limits of error are obtained with very few mice in two or three hours, but also this method has its limitations in work with whole venom. The dose (d) which kills in infinite time is presumably determined by the dominant toxin, but as one moves to larger and larger venom doses, more and more other toxins will come into play and the dose-time relationship must be determined by the combined actions of several toxins, with the result that two preparations of venom from the same species which have not exactly the same toxic pattern could give graded response lines which are not truly parallel, just as one cannot expect that a purified toxic fraction will yield a curve which only differs with regard to position from the curve obtained with the starting material, crude venom. An example of this was recently presented by Boquet and his co-workers (1966) in experiments with *N. nigricollis* venom and a purified lethal component of this venom called alpha-toxin.

Hopes of using this method to determine the amount of unbound toxin in under-neutralized venom-antivenom mixtures, which apart from its theoretical interest might offer a means of obtaining a quick, even if rough, estimate of antivenom potency, are therefore not likely to be fulfilled, nor is the method likely to be of any great value as a screening test during the purification of venom toxins, but it may be of value as an additional means of selecting venoms with similar properties and of checking stored preparations such as those set aside for the assay of antivenom potency.

The preparation of antivenom for the first time just before the turn of the century was a natural sequence to the discovery of bacterial antitoxins, and the preparation, purification, and potency assay of antivenoms have ever since closely followed the lines adopted for bacterial antitoxins, though not with the same measure of success.

As far as potency assay is concerned, this has not been for lack of interest, because a large proportion of venom literature has dealt with the specific and paraspecific potency of antivenoms, but it is regrettable that much of the earlier work must be considered wasted because the particular behaviour of venoms and antivenoms was either ignored or misinterpreted.

Whenever it is possible it is always desirable to replace methods which demand the use of animals with tests carried out in glassware and similarly tests carried out in for instance the skin of animals are preferable to tests which necessitate their death as long as these ways of testing measure the same thing. But the lethal venom toxins have not, as yet, been shown to have any specific enzyme activity or other effect which can be measured in such simpler tests. As far as African antivenoms are concerned, their ability to inhibit various effects of venoms, such as their haemolytic, proteolytic, coagulant or anti-coagulant, and haemorrhagic actions, will not even serve as a guide for their protective titre. Some venoms are however more likely to lead to permanent damage due to tissue destruction than to loss of human life and one cannot exclude that for instance

the anti-haemorrhagic potency of a serum would be a better indicator of its therapeutic value than its ability to save the life of mice, but standardization in this respect is not as simple as it might seem because the venom haemorrhagin is not a single factor; those interested in this aspect should consult the recent study of Kondo and his co-workers (1965b).

The precipitation of the flocculation type occurring in mixtures of venoms and antivenoms has been studied by numerous workers and there is no doubt that the lethal toxins are capable of flocculating with their antitoxins as exemplified by the early studies by Calmette and Massol (1909) on the liberation of toxin from such floccules, and by Bier's work with purified *Crotalus* toxin at Instituto Butantan (Bier, 1947), but the reaction is of little practical importance in work with crude venom because of the multitude of individual antigen-antibody flocculations which take place, making interpretation in terms of titre impossible.

The potency of antivenoms has therefore to be assessed in animals and, as for bacterial antitoxins, the assay usually involves the preparation of a set of mixtures containing either a fixed amount of serum and increasing amounts of venom or a constant amount of venom and varying amounts of serum, which arrangement one adopts is rather immaterial for the result.

The mixtures can be injected at once, but are often left for some time at a certain temperature in order to allow antigen and antibody to combine before the mixtures are tested for toxicity. This is probably of little importance when one is dealing with a venom and its specific antivenom, particularly if this is a refined serum from hyperimmune horses, but it may, as will be shown, sometimes influence the result. The outcome may also depend on the route by which the venom-antivenom mixtures are injected, and some workers favour the subcutaneous route, because the snake injects its venom under the skin rather than into the blood stream. Some go further, and condemn the injection of ready-made mixtures as an artificial procedure which should be abandoned in preference for a method by which the animals are tested for protection by injecting venom and serum separately, thus immitating the sequence in naturally occurring snakebite. There may be something in favour of such arguments, but it is by far the simplest to mix the reagents before injection, a principle adopted in work with others toxins and antitoxins and practised in most venom laboratories, and it is reasonable to assume that a preparation found to be truly better by this method would also be more effective in the treatment of snakebite.

Most laboratories express the potency of a serum as the amount of venom neutralized by a unit volume of serum using a particular venom preparation set aside for this purpose. Whether the amount of venom or the amount of serum is kept constant is, as already stated, largely immaterial for the result, but in order to have a meaning, the estimate of potency should be independent of the conditions of the test, and if a certain amount of venom is found to be neutralized by a given amount of serum, then any multiple of this amount of venom should require the same multiple of serum for its neutralization, as originally stated by Ehrlich. It was soon seen that this rule did not apply to venoms and antivenoms. Among the first to realize this was Vital Brazil who observed that certain antivenoms neutralized relatively more venom in higher dilutions than in stronger concentrations, observations which were confirmed and extended by many workers both in this and other countries.

This "law of combination in multiple proportion" implies that the "neutral points" obtained by testing serum with venom at many concentration levels will

fall on a straight line if recorded graphically in a system of co-ordinates in which the axes express the concentrations of serum and venom in the injected mixtures.

Even without the addition of serum it takes a certain amount of venom to kill, and the neutralization line cannot, therefore, pass through the origin but must cut the dose axis at some distance from this point. If the neutral points were recorded to indicate mixtures just toxic enough to kill half the injected animals, the neutralization line should intersect the dose axis at a point corresponding to the LD50 of the venom. In assays carried out at a single dose level, this would be of no consequence if the curve is linear and the assay carried out at a level high enough to ensure that the injected mixtures contain a large number of lethal doses. This is the case when bacterial antitoxins are assayed in the usual way, but with antivenoms it may not be possible to employ a very large test dose because the serum potency is too low. Under such circumstances account must be taken of the fact that the neutralization curve cuts the dose axis at some distance from the point of origin, otherwise it will obviously lead to results which seem to disobey the law of multiple proportions and give titres which are exaggerated and which seemingly increase with decreasing number of lethal doses in the injected mixtures. Although others may have realized this, it was left for Banić and Ljubetić (1938) to stress that the amount of serum required to protect a mouse from the effect of two lethal doses is larger than twice the amount protecting against one lethal dose, and to suggest a method of assay which made allowance for this. Later the same year their method was modified by Ipsen (1938) who proposed to determine the slope of the neutralization curve which expresses the titre of a serum.

The method assumes linearity of the neutralization curves and however important they were, these studies did not solve the difficulty; they may in fact have retarded progress by goading experimentors to assume that linearity is the rule, which it obviously is not.

Neutralization curves may sometimes be perfectly linear but if examined over a sufficiently wide dose range they will commonly show a change of slope, sometimes abrupt, but often gradual, giving the impression of a gentle curvature convex towards the venom axis. Some workers realized that curvature rather than linearity is the rule and attempted to describe the relationship between serum dilution and amount of venom neutralized in mathematical terms (Houssay and Negrete, 1921), and Eichbaum (1947) observed a linear relationship between the logarithm of the amount of neutralized venom (y) and the logarithm of the serum dilution (x) effecting this neutralization according to the equation, $\log y = \log a + b \cdot \log x$, where a and b are constants, a being the amount of venom neutralized by 1 ml of undiluted serum.

This tendency to accept nonconformity with the law of combination in multiple proportions as a common phenomenon in antigen-antibody reactions may have been due to a confusion of certain concepts. This law states, as already mentioned, that if a certain amount of toxin is neutralized by a given amount of serum then any multiple of this amount of toxin will require the same multiple of serum for its neutralization. But, as Jerne wrote in his monograph dealing with avidity, "this law does not imply, as it might be thought and as it is sometimes understood, that the amount of toxin neutralized by antitoxin is proportional to the quantity of antitoxin added" (Jerne, 1951). This, which Jerne called "the principle of proportional neutralization", would mean that the amount of serum required exactly to neutralize a fixed amount of venom would be exactly

twice the amount which could neutralize half the venom in the mixture. Jerne showed that although neither the "law" nor the "principle" are followed as a general rule, the conformity with both law and principle is so good in the particular case of sera from hyperimmune animals that deviations would not be disclosed by ordinary measurements.

All antivenoms routinely examined fall into the category of hyperimmune sera and there is no reason to expect gross deviations from combination in multiple proportions especially not as the concentration range which can be tested is very restricted even with the best antivenoms available. In any case, the type of curvature seen with venoms and antivenoms is convex towards the venom axis, not as for non-avid sera convex towards the serum axis. Furthermore, why should one get perfectly linear curves with some antivenoms but not with others, and why should the anti-coagulant property of a serum tested with a coagulant venom follow the law of multiple proportions but the protective activity fail to do so, as recorded by Eichbaum in experiments with *Bothrops* venom (Eichbaum, 1947).

The explanation for the particular shape of antivenom titration curves should have been clear if more attention had been paid to the observation made by Schlossberger, Bieling and Demnitz (1936). They noted that the amount of venom neutralized by some sera would increase with increasing serum dose up to a point beyond which further serum addition was without effect because such sera lacked antibody to some venom component. Schöttler observed the same happening with *Bothrops jararaca* venom and came to the same conclusion, but appeared to accept lack of combination in multiple proportions as not surprising but "a rather common phenomenon of antigen-antibody reactions" (Schöttler, 1952).

The curve drawn in figure 1A shows the same situation for *Bitis lachesis* antivenom tested intravenously in mice. The amount of venom neutralized increased as the serum dose was increased to 0.2 ml, but twice this amount of serum had no further effect because the serum, a raw serum from a single horse, contained no antibody to a venom component which began to assert itself when the venom dose was increased beyond about 0.3 mg. Two toxins were involved, a dominant toxin which determined the LD50 of the venom and which was neutralized according to the law of multiple proportions, as indicated by the lower left part of the curve, and another toxin of which an LD50 was contained in about 0.5 mg of whole venom and which was not at all neutralized by this serum. The curve will not show a sharp angle as indicated in figure 1B because the minor toxin acts before a dose of 0.5 mg is reached. A hypothetical dose-mortality curve for this minor toxin has been drawn in the lower right corner of figure 1B in order to ease the explanation. If the minor toxin had been removed from the venom without affecting the dominant toxin, one would expect that 0.45 mg of venom would require about 0.67 ml of serum to be neutralized to such an extent that 50% of the tested mice would survive the effect of the mixture. With the minor toxin present — and assuming that the dose-mortality curve for this toxin is correct — this dose, 0.45 mg of venom, would contain enough minor toxin to kill 25% of the mice which survived the effect of the free dominant toxin. This would mean that not 50% but only about 37% of the mice would survive and that more serum would have to be added to give an "over-all" survival rate of 50%. This extra addition of serum would be a little smaller for a dose slightly less than 0.45 mg, and a little larger for a dose bet-

ween 0.45 mg and 0.5 mg, and thus the curve would show the gentle change of direction shown in Figure 1A and not a sharp angle as in Figure 1B.

The part of the curve determined by the minor toxin would not be vertical if the serum contained measurable amounts of antibody to this component but would show some slope. The steepness of the slope would depend on the concentration of specific antibody to this component in the serum.

This interpretation deals adequately with all situations encountered when antivenoms are tested with crude venoms, and it was substantiated some years ago by results obtained in experiments with *Naja nivea* venom which contains three antigenically distinct toxins, called alpha, beta and gamma toxin in decreasing order of toxic importance. Tested with specific serum this venom gave the usual type of neutralization curve showing a change of slope, quite abrupt as often is the case with elapid venoms. The curve consisted of two linear parts, one due to the neutralization of alpha toxin, the other due to the neutralization of gamma toxin; the concentration of antitoxin to the beta toxin was too high in this serum to interfere in the assay (Christensen, 1953). Further and very neat evidence for the correctness of this explanation of the shape of venom-antivenom neutralization curves was supplied by Kochwa and his co-workers (1959), who showed that a rabbit after immunization with *Vipera xanthina palestinae* venom yielded a serum which due to the absence of antibody to the venom's neurotoxin gave a neutralization curve of the type shown in Figure 1A but produced a serum which conformed with the law of multiple proportions after antigenic stimulation with purified neurotoxin.

The position with regard to the neutralization of venoms by antivenoms may be summarized as follows:

All venoms contain more than one and often several antigenically different toxins, each of which combines with its antitoxin according to the law of multiple proportions.

The neutralization curve will have its origin at a point on the venom dose axis corresponding to the LD50 of the most dominant toxin, which will be in agreement with the LD50 of whole venom unless several antigenically distinct toxins have joint action and all contribute to the effect produced by the LD50 of whole venom.

The neutralization curve is only truly linear if the serum under test contains relatively least antibody to the dominant toxin, and a valid estimate of the relative potency of different sera will be obtained if they all give this type of curve.

In cases where a serum contains relatively less antibody to a component of minor toxic importance, the curve will show a change of direction if the potency of the tested serum allows the use of sufficiently high venom doses. The potency estimate will depend on which of the components is active in the test but a valid estimate of the relative potency of different sera can be obtained if corresponding parts of the neutralization curves are compared.

The point at which the curve begins to change direction and the steepness of the changed slope will depend on the magnitude of the LD50 of the minor component in terms of whole venom and on the concentration of antitoxin to this component. Extrapolation to the venom axis will give an estimate of the amount of venom which contains an LD50 of this component.

Further changes in direction may occur if the serum's concentration of antitoxin to even less toxic components is still lower.

The abruptness with which the curve changes direction depends on the steepness of the dose-response curves for the toxins. This interaction of different toxin-antitoxin systems explains why the endpoint in potency assays may be perfectly sharp at lower concentration levels, become "woolly" in a certain range, and regain sharpness at higher levels, or in other words, why the toxin-antitoxin response curve may appear flatter and the results show signs of heterogeneity at some concentration levels.

An estimate of the relative potency of two sera will be invalid if the chosen test level involves different venom components for the two sera.

Having determined the neutralization curve for a serum intravenously in mice one would expect to get a curve of similar shape if the test was repeated subcutaneously or even carried out in another animal if the relative toxic importance of the different components was the same by either route and for different animals, which could be true for some venoms but not for others.

I have not had an opportunity to test different animals in this way, but I have examined one serum with different cobra venoms and another with two viper venoms intravenously and subcutaneously in mice.

Explanation of the tables

Table 1 — See text for details.

Table 2 — Shows the intravenous and subcutaneous potency of four sera relative to that of the *N a j a* antivenom standard, calculated from the ED50 of the sera tested with a dose of venom fixed at five times the LD50. The venoms listed in Table 2 have been separated into four groups. The two venoms in the first group, *Naja naja* venoms from Thailand and the Philippines, are grouped together because they both gave simple linear neutralization lines in the tests by both routes with standard serum. This in itself does not mean that they only possess one lethal toxin but it means either that their main toxin by far supersedes any other toxin or that the standard contains relatively least antitoxin to the dominant toxin in these venoms and the table shows that the ratio between relative titres for a serum determined by the two routes is for all sera as close to the value 1 as one would expect in toxin-antitoxin titrations. The observation that the order of increasing potency of the four sera against the Thai *N a j a* venom is 1, 2, 3, 4 but for *N. naja philippinenses* venom 1, 4, 3, 2 indicates that the two venoms dominant toxins are antigenically distinct.

The two venoms in the second group, those of Indian *N. naja* and *N. melanoleuca*, are placed together because two venom components were demonstrable by both routes in the tests with standard serum, but for both venoms the subcutaneous and intravenous neutralization curves were almost parallel. For the Indian *N a j a* venom the order of increasing potency of the sera is 1, 2, 3, 4, as in the case of *N a j a* venom from Thailand and the numerical value for a serum is in fair agreement for the two venoms which were about equally toxic, indicating that the dominant toxin in these two venoms is in all probability identical. With a test dose of 30 or 40 micrograms it is likely that only the dominant toxin was active in the tests.

Reference to Figure 8 justifies the assumption that the second *N. melanoleuca* component was active in the tests which explains the different order of potency of the sera, here 4, 1, 3, 2, but for each serum the two values showed good agreement as could be expected if the neutralization curves for the two routes were parallel for all the sera.

The venoms in the third group, those of *N. nivea* and *H. haemachatus*, are akin because only the dominant toxin was active subcutaneously. Higher doses of *N. nivea* venom than those used would have caused discrepant results because the second toxin would have been active intravenously, as it is, agreement between paired results is good for this venom yet the order of potency for the four sera, if significant, would indicate that the dominant toxins of *N. naja* and *N. nivea* venoms are antigenically different, which in any case I think they are.

The values for *H. haemachatus* venom shows signs of disagreement for results between routes and between sera which were to be expected knowing that only the dominant toxin is involved subcutaneously whereas either of the two toxins could be active intravenously, depending on the properties of the sera.

Some irregularities of the data entered in Table 2 for the paraspecific venoms in the last group were to be expected because the ratio of subcutaneous to intravenous titre of sera tested with such venoms not only differs from unity but varies from one serum to another, as discussed in relation to Figures 9 and 10.

Why is it that some venoms behave intravenously and subcutaneously in the expected manner with such sera and others do not? The answer seems to lie in the problem of specificity.

The standard was prepared from the sera of horses already immune to the venoms of *N. nivea* and *H. haemachatus* and subsequently immunized with *N. naja* venom, and tested with these venoms it behaved as expected. The satisfactory result obtained with *N. naja* venom from the Philippines and *N. melanoleuca* venom could be explained by a close antigenic relationship between these venoms and one or more of the venoms used as antigens in the preparation of the standard, whereas the poor result obtained with *N. nigricollis* and *N. haje* venoms could be due to qualitative differences between these two venoms and those used as antigens, resulting in a lack of firmness of antibody binding.

If this is the case, one would expect sera prepared with the venoms of *N. haje* and *N. nigricollis* to behave in a "specific" way in tests with the homologous venom, unless the lack of firmness of antibody binding is due to peculiarities in the structure of the toxin molecules, and not only the fault of the serum.

In order to test this, the preparation of monovalent sera against these two venoms was begun. The results with serum prepared with *N. nigricollis* venom are unfortunately not yet available, but the results obtained intravenously and subcutaneously with *N. haje* antivenom, a pepsin-treated solution of globulin from one horse after two short courses of immunization, are shown in fig. 11.

Table 3 — In each of these experiments, a set of mixtures was prepared with a fixed amount of venom and graded volumes of serum and these mixtures were injected intravenously in mice in a volume of 0.5 ml under varying conditions as outlined in Table 3. Nine mice were used per mixture for each set of conditions, a fast injection lasted less than 2 seconds, a slow injection between 15 and 20 seconds.

The result of the first experiment confirmed that these factors are unimportant with *N. naja* venom and specific anti-venom. The second experiment indicated that the mice receiving warm mixtures of *N. haje* venom and standard serum fared better than those receiving cold mixtures and, even if it is not statistically significant, the difference between the ED50 values for the first two groups in the second experiment suggested that the speed of injection might influence the result and this was confirmed by the third experiment. In the last of the experiments recorded in Table 3, *N. haje* venom was tested with the specific monovalent serum at the extremes of the test conditions using two venom doses, 105 and 150 micrograms. For both doses the ED50 was smaller when the mixtures were warm and injected slowly after having stood for an hour, but not significantly smaller than for the cold mixtures injected rapidly immediately after preparation.

The conclusion to be drawn from these experiments in that technical details such as the time and temperature at which the mixtures are held before injection, and the speed with which they are injected are at most of little importance if a venom is tested with specific serum, and that the titre assigned to such sera will be about the same whether the mixtures are injected under the skin or into a vein. But sera with paraspecific action will bind the venom loosely and the result will depend on all these factors.

The paraspecific venom-antivenom complex is presumably dissociating when injected intravenously whereas the mixture injected under the skin will benefit from incubation at a high temperature and become concentrated because water is more rapidly absorbed than the venom-antivenom complex which therefore will have less tendency to dissociate.

The discussion has so far only been concerned with variations encountered in tests with a particular set of venoms set aside as laboratory test preparations but variations in the composition of venom from the same species will probably prove the greatest difficulty to uniform antivenom potency control. Figure 12A and B and Figure 13 are included here to exemplify this.

TABLE 1 — THE INTRAVENOUS AND SUBCUTANEOUS LD_{50} IN MOCROGRAM OF DIFFERENT SNAKE VENOMS FOR MICE WEIGHING FROM 16 g TO 18 g

VENOM	INTRAVENOUS		SUBCUTANEOUS	
	LD_{50}	5% fiducial limits	LD_{50}	5% fiducial limits
Naja naja philippinenses	3.3	3.7 — 2.9	3.6	4.1 — 3.6
Naja naja (Thailand)	5.9	6.6 — 5.4	6.6	7.4 — 5.7
Naja naja (India)	6.2	6.8 — 5.9	7.7	9.0 — 6.6
Naja nivea	9.7	10.8 — 9.1	12.3	13.0 — 11.6
Naja melanoleuca	16	18.5 — 14.9	25	28.5 — 22.0
Naja haje	21	23.5 — 19.2	29	33.8 — 25.6
Naja nigricollis	23	28.9 — 19.9	48	51.4 — 43.5
Hemachatus haemachatus	29	35.1 — 26.1	30	34.4 — 26.0
Dendroaspis polylepis	8.3	8.9 — 7.7	9.3	10.7 — 8.3
Dendroaspis viridis	12.0	13.3 — 10.9	13.5	15.2 — 12.2
Dendroaspis jamesoni	16	18.6 — 14.4	17	19.3 — 15.6
Dendroaspis angusticeps	44	57.5 — 37.8	57	64.9 — 49.1
Bitis lachesis	9.7	10.6 — 8.8	74	107.5 — 59.4
Bitis gabonica	11.5	12.9 — 10.4	100	117.0 — 85.5
Bitis nasicornis	25	28.3 — 22.8	184	209.5 — 163.7
Echis carinatus	22	30.9 — 16.3	177	177.4 — 134.3

TABLE 2 — THE POTENCY OF FOUR SERA RELATIVE TO THAT OF STANDARD *Naja* ANTIVENOM DETERMINED INTRAVENOUSLY AND SUBCUTANEOUSLY IN MICE WEIGHING FROM 16 g TO 18 g USING A TEST DOSE OF FIVE TIMES THE LD_{50} OF DIFFERENT VENOMS

VENOM	Route	Test dose μg	SERUM 1	2	3	4	Stand.
N. naja (Thailand)	intraven.	30	0.64	0.73	0.79	0.96	1
	subcut.	35	0.67	0.78	0.87	0.90	1
N. n. philippinenses	intraven.	16.5	0.70	1.65	1.15	0.85	1
	subcut.	17.5	0.78	1.57	1.13	0.91	1
N. naja (India)	intraven.	30	0.53	0.73	0.86	0.94	1
	subcut.	40	0.72	0.84	1.01	1.00	1
N. melanoleuca	intraven.	80	0.86	1.56	0.97	0.62	1
	subcut.	125	0.90	1.47	1.03	0.65	1
N. nivea	intraven.	50	0.65	0.90	0.71	0.96	1
	subcut.	60	0.72	0.94	0.72	0.90	1
H. haemachatus	intraven.	150	1.20	2.00	1.36	0.89	1
	subcut.	150	0.91	1.59	1.09	0.96	1
N. haje	intraven.	105	1.08	1.79	2.03	0.91	1
	subcut.	145	0.89	1.53	1.06	0.72	1
N. nigricollis	intraven.	115	1.17	1.59	1.04	0.70	1
	subcut.	240	0.92	1.15	0.93	1.15	1

TABLE 3 — THE EFFECT ON THE ASSAY RESULT OF THE CONDITIONS OF THE TEST WITH REGARD TO SPEED OF INJECTION AND THE TIME THE VENOM-SERUM MIXTURES ARE HELD AT DIFFERENT TEMPERATURES BEFORE INTRAVENOUS INJECTION IN MICE

VENOM	Test dose in µg	Serum	Conditions of test		ED_{50} in µl	5% fiducial limits
Naja naja	30	standard	fast, immediate,	16°	13.7	
			slow, 60 mins.	37°	13.9	14.6 — 13,1
Naja haje	105	standard	fast, 20-40 mins	20°	64.0	233 — 58.6
			fast, 20-40 mins	20°	57.4	64.6 — 56.7
			slow, 90 mins.	37°	50.5	55.0 — 41.5
Naja haje	105	standard	fast, 60 mins.	37°	58.5	67.6 — 54.5
			slow, 60 mins.	37°	48.4	51.4 — 43.4
Naja haje	105	monovalent	fast, immediate	16°	101.6	106.3 — 96.3
			slow, 60 mins.	37°	92.4	98.2 — 86.7
Naja haje	150	monovalent	fast, immediate	16°	166	177 — 158
			slow, 60 mins.	37°	161	170 — 152

Explanation of the figures

Figure 1A & B — See text for details.

Figure 2 — Shows the neutralization curve obtained with *N. naja* venom from India and a particular serum which recently was established as the international standard for *Naja* anti-venom potency. The upper curve was determined intravenously, the lower curve subcutaneously, and the slopes of corresponding parts of the two curves agree quite well, which means that the titre of the serum expressed in mg venom neutralized per ml would be closely the same whichever way the mixtures were injected, but, being different for the two venom components, the value would depend on the level of the test.

Figure 3 — The curves for a sample of *N. naja* venom obtained from Thailand are shown in Figure 3. Only one venom component is active throughout the tested range and the titre in mg per ml would not only agree quite closely irrespective of the route of injection but would also be independent of the concentration level.

Figure 4 — Shows the same to apply to results obtained with a sample of *N. naja* venom obtained from the Philippines. That the subcutaneously determined curve happens to be placed above the other is probably due to experimental variation as the two tests were not carried out at the same time, and not because this venom is more toxic subcutaneously than intravenously.

Figure 5 — The curves for *N. nivea* venom are shown in Figure 5. It appears that two venom components operate intravenously but only the dominant toxin is active subcutaneously. Maybe the effect of the second component would have shown up if even more concentrated solutions had been injected, but this component is obviously of little or no importance subcutaneously in mice. Expressed as mg neutralized per ml, the titre of the serum would be about the same for the two routes at low dose levels, but disagreement would be found at higher levels where two different properties of the serum would be compared.

Exactly the same position applied to the results obtained with *Hemachatus haemachatus* venom (Figure 6) and with two viper venoms, those of *Bitis gabonica* and *B. lachesis*, tested against a polyvalent *Bitis-Echis* antivenom (Figure 7).

Figures 6 & 7 — It could reasonably be suggested that the potency of sera against venoms such as those giving the results shown in Figures 5, 6 and 7, ought to be tested subcutaneously in mice if the second venom component is equally non-toxic injected under the skin of human beings, as happens in snakebite.

Figure 8 — The results for *N. melanoleuca* venom, shown in Figure 8 are very similar to those obtained with *N. naja* venom (Figure 1) but the parts of the curves due to the second component show signs of divergence resulting in a slightly higher estimated titre in the subcutaneous test.

Figure 9 — Even more marked differences in slope for the parts of the curves determined by the second component was seen when *N. haje* venom was tested by the two routes (Figure 9), and the route of injection would clearly have to be specified if the titre in terms of weight of reference venom neutralized per ml of serum was to have any meaning. Even if the titre were lower, it could be argued in cases like this that the sera should be tested intravenously because the neutralization in persons treated for snakebite probably takes place in the blood-stream rather than at the site of the lesion.

If the ratio of subcutaneous to intravenous titre remained constant from one serum to another, this situation could also be quoted in support of the use of a reference preparation of serum rather than venom; but this is not necessarily so.

Figure 10 — As an example, Figure 10 shows the subcutaneous and intravenous *N. nigricolis* antivenom potency of two sera of which the one was the *Naja* antivenom standard, the other a similar preparation of unmodified serum from one horse. The coinciding intravenous curves for the two sera, shown farthest to the left in Figure 10, indicate that the two sera were equally potent against both the toxic venom components. As far as the dominant toxin is concerned, this was confirmed by the subcutaneous test, but tested by this route the potency of the raw serum appeared to be far superior against the venom's second toxin. If two different laboratories were to test these two sera with the same preparation of venom, one laboratory intravenously the other subcutaneously, the expected result would be in disagreement about the absolute amount of dominant toxin neutralized by the two sera but agreement with regard to their relative potency against this component, a situation which would favour the expression of potency in terms relative to the potency of a standard serum. As far as the second venom component is concerned, the two laboratories would report conflicting results whether the titre was expressed in relative terms or as the amount of venom neutralized per ml, and nothing would be gained by the use of a serum standard.

From the results discussed so far, it appears that whether or not subcutaneous and intravenous tests yield the same result depends on the venoms and sera under test and the next step was to attempt to find the reasons why.

Figure 11 — The two curves are in very good agreement with each other and with the contention that concordance between the intravenous and subcutaneous titres is the rule for specific sera, but not for paraspecific sera.

In all the tests so far recorded no particular attention was paid to the time for which the venom-serum mixtures were left before they were injected, or to the temperature at which they were held, because past experience had shown this to be of little importance, but during these tests the impression was gained that such factors might influence the result with some venoms, *N. haje* venom in particular, and Table 3 summarises some experiments carried out to clarify this point.

Figures 12 A & B — Figure 12 A shows the neutralization of the Johannesburg *B. gabonica* reference venom by three specific sera and Figure 12 B, the neutralization of a sample of *B. gabonica* venom obtained from Institut Pasteur in Garches. One of the three sera is obviously the better in tests with both venoms but the order of potency though almost the same, is reversed for the other two sera indicating basic differences and not just quantitative variations in the composition of the two venom samples.

Figure 13 — Two of these sera were specific against *Echis carinatus* venom also, and tests with *Echis* venom from the same two sources gave the result shown in Figure 13. The relative potency of the two sera may happen to be roughly the same against the second component, but these two venoms must be fundamentally different.

These examples have all been drawn from experiments with African snake venoms but the results are probably applicable to venoms in general, and confirm that true

standardization is impossible as long as we have to test the sera with crude venom preparation. Some form of potency control is, however, urgently needed in order to protect the public against worthless sera, and minimum potency requirements will have to be based on reference preparations of either venoms or sera.

That bacterial antitoxins are standardized on the basis of serum standards is in itself no reason why the use of a venom reference preparation should be rejected. The toxicity and antibody-binding power of dried venoms seem to be extremely stable and the main objections to the use of a reference preparation of venom appear to be firstly, that it may not ensure the correct evaluation of sera in areas where snakes with a different venom composition predominate, and, secondly, that once nearing exhaustion, it would be impossible to replace it by another preparation with identical qualities, as pointed out by Schöttler (1958). To these objections one could add the financial difficulty. An international reference preparation would have to be freely available, and considering the amount of venom required of different species to serve the needs of many laboratories for a number of years, the capital outlay would be substantial. A US dollar would buy about 1,000 intravenous lethal mouse doses of an average priced African venom, just about enough to compare the potency of two sera at two concentration levels. A reference preparation of antivenom is a great deal cheaper to produce, it might be capable of dealing with 'abnormal' venoms, and its eventual replacement, though difficult indeed, would not be impossible. The present *Naja* antivenom standard, the only antivenom standard established so far, is a polyvalent preparation of which 2.69 mg represents the unit of potency against toxic component of *Naja* venom in general.

Its replacement would undoubtedly require the redefinition of the unit not only for each *Naja* species but also for each of the toxic components contained in such venoms, and this would be an extremely difficult undertaking. While on the subject of this particular preparation, and in view of the results just presented, I think that it will prove unsuitable as a standard in tests with paraspecific venoms such as those of *N. haje* and *N. nigricollis*.

The use of antivenom reference preparations will not for the time being do away with the need for reference venoms if independent laboratories are to get concordant results in potency assays and minimum potency requirements are to be defined but, even so, one should welcome the establishment of international reference preparations of antivenoms, and the definition of potency in terms of units because it lends itself to concise information between laboratories, and the time will come when the different venom components have been isolated in quantity and valid potency estimates can be obtained in assays of antivenoms with such purified toxins as already done by Kondo and his co-workers for Habu venom (Kondo *et al.* 1965a).

But potency control is not an international problem; it is a matter for local licensing authorities to decide in consultation with antivenom producers.

It would of course be impossible to suggest a single method of potency control which would be acceptable to everybody and fair to all sera but, in conclusion, I should like to add some remarks with regard to a reasonably practical approach in places where some form of potency control is not already established, and where anti-snakebite sera are accepted on the wording of the label they carry.

There are usually very few producers of antivenom in any country and it should be possible for them to agree to contribute — maybe according to their own requirements — to a common pool of reference venoms to be controlled by the licensing authority. At the same time it would be advisable to put aside suitable freeze-dried sera, which would serve as a means of checking the constancy of the venoms and of settling disputes between producers and authority, and such sera would be of value when new venom preparations had to replace the old; but whether the potency requirements are expressed in terms of absolute or relative potency would be immaterial.

Antivenom potency tests are simple to carry out, require little equipment and space, and the licensing authorities in countries relying entirely on imported antivenoms should acquire a small stock of appropriate reference preparations, lay down their own minimum potency requirements, and demand samples for test before importation is permitted.

The optimal conditions of the potency test, for example with regard to test animal, route of injection, and other technicalities, need not be the same for all venoms but should be defined in detail. The aim would be potency control, not true standardization, and the eventual replacement of the reference venoms by other preparations with closely similar toxicities, graded response curves, and antibody-binding power, would be good enough to guarantee that about the same level of quality was maintained.

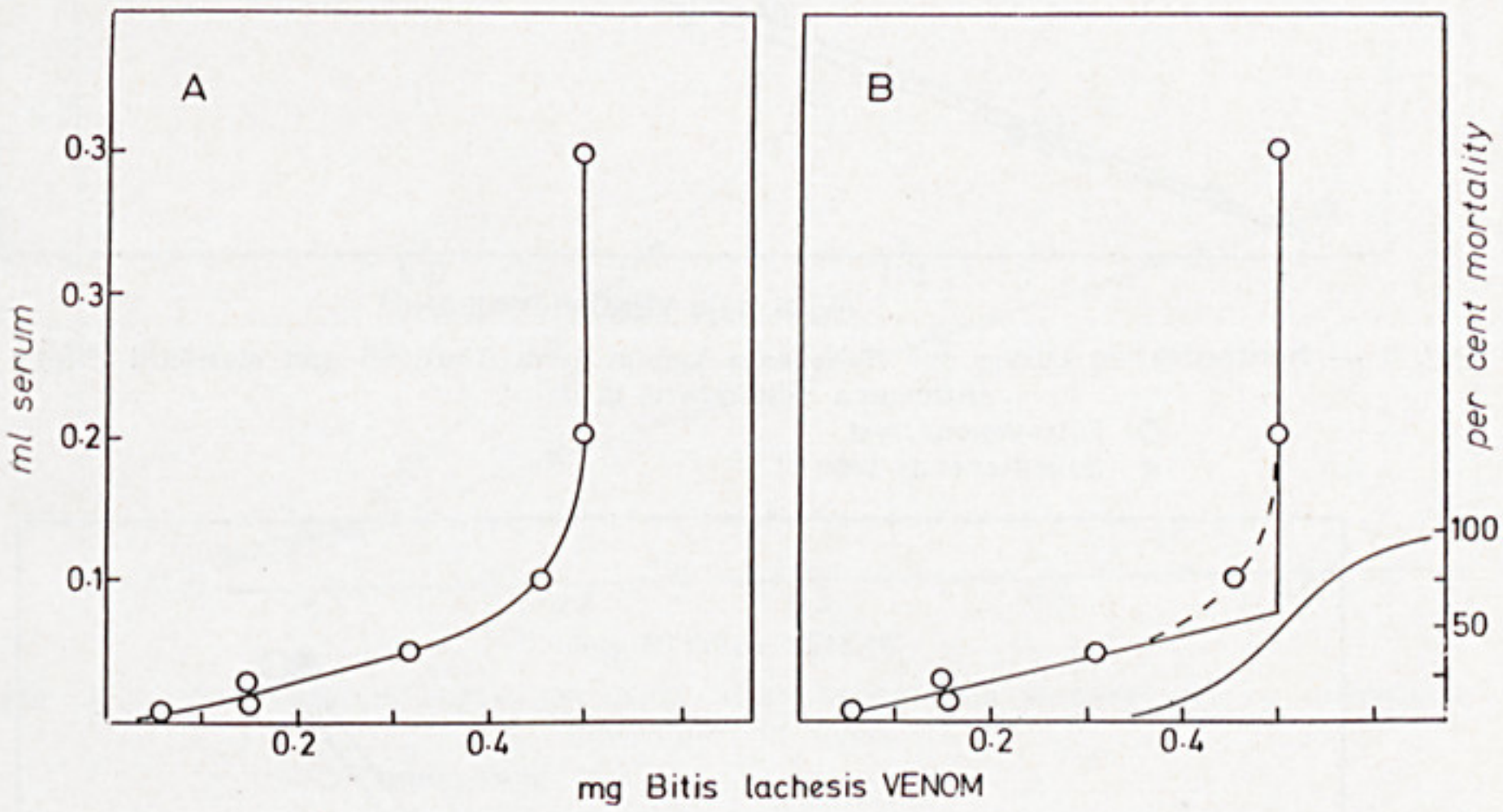


Fig. 1 — A: Neutralization curve obtained in mice tested intravenously with *Bitis lachesis* venom and unmodified serum from a horse. B: See text for details.

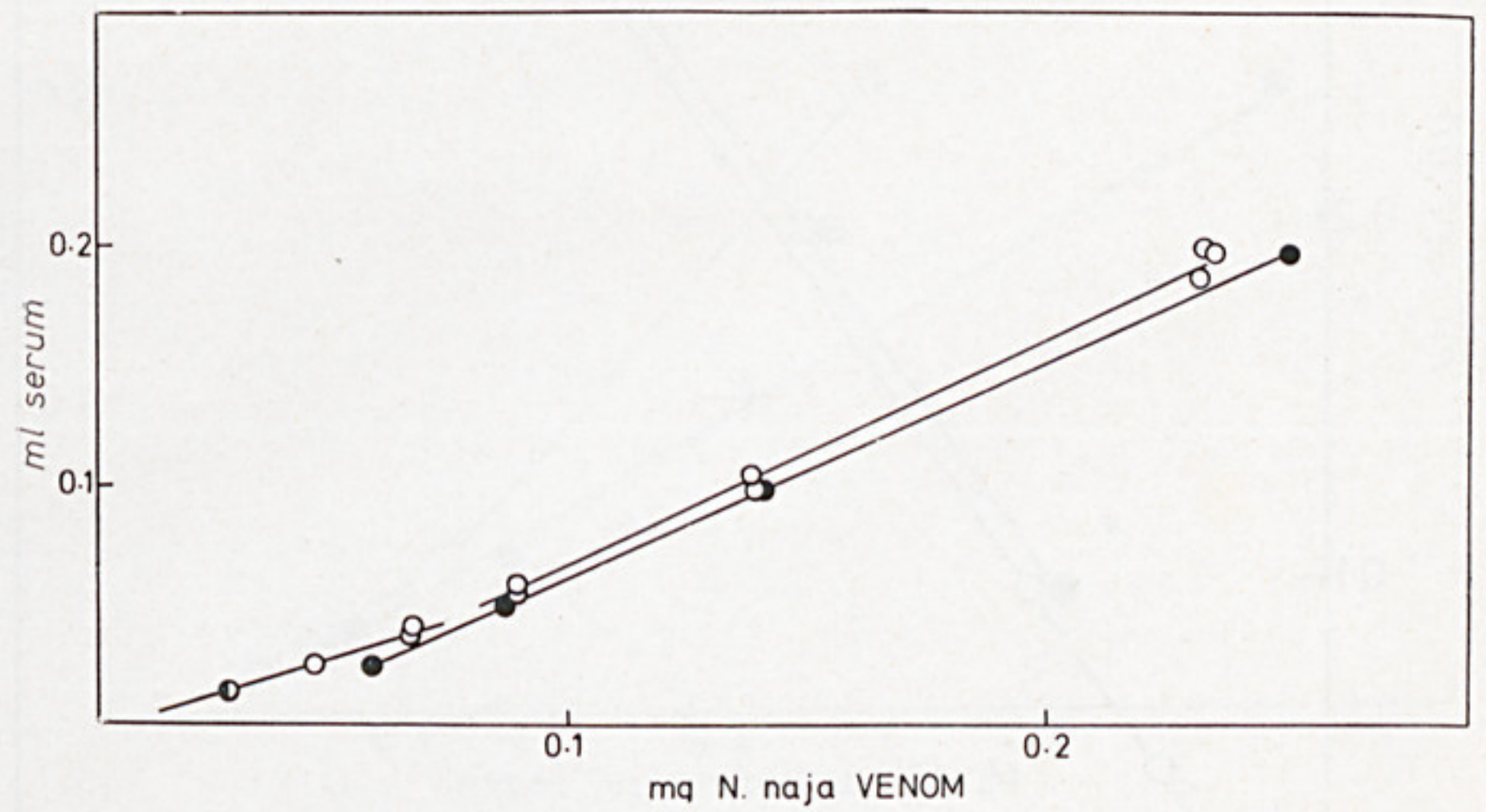


Fig. 2 — Neutralization curves for Indian *Naja naja* venom and standard *N a j a* antivenom determined in mice.

○ Intravenous test

● Subcutaneous test

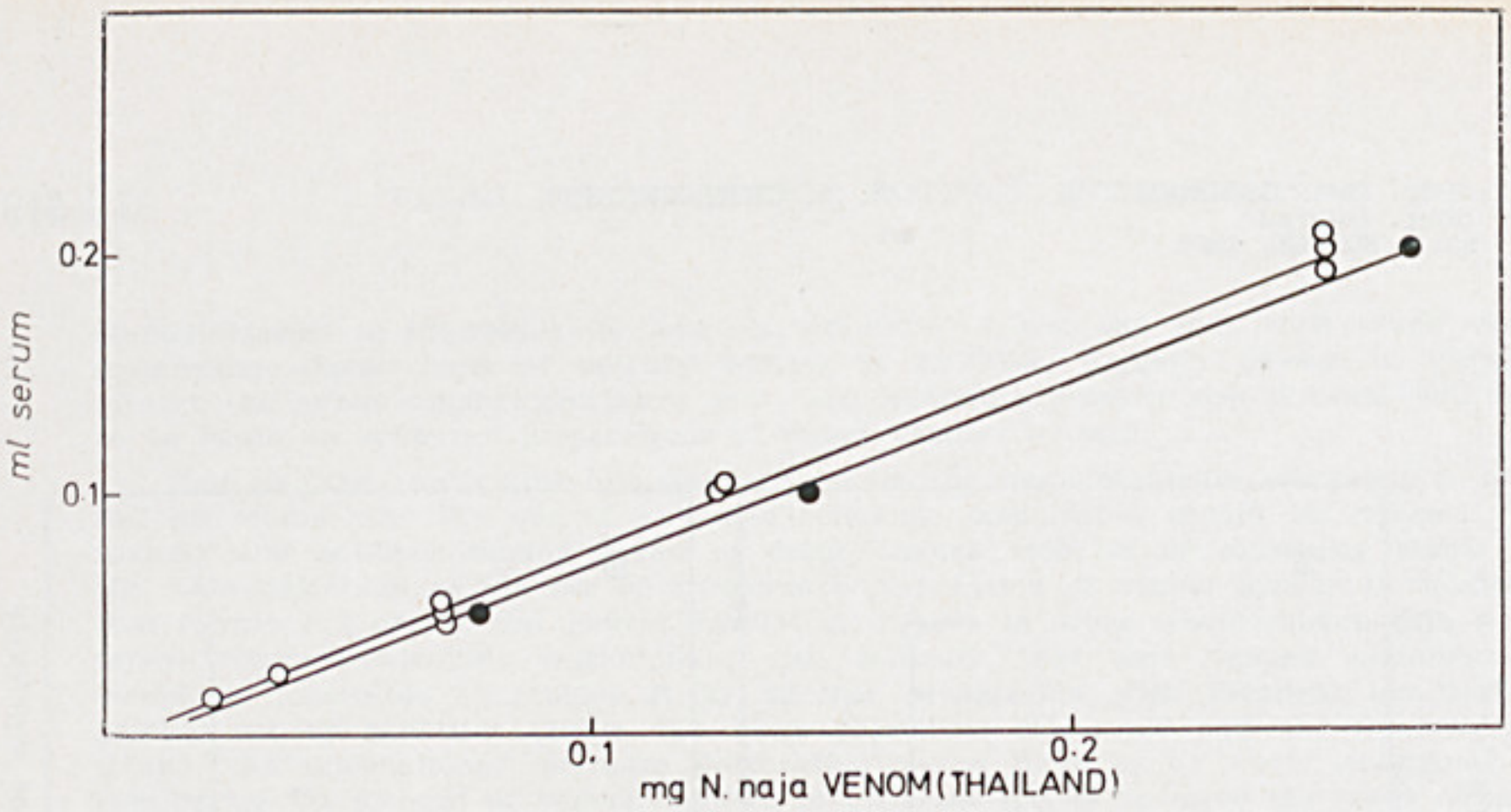


Fig. 3 — Neutralization curves for *Naja naja* venom from Thailand and standard *N a j a* antivenom determined in mice.

○ Intravenous test
● Subcutaneous test

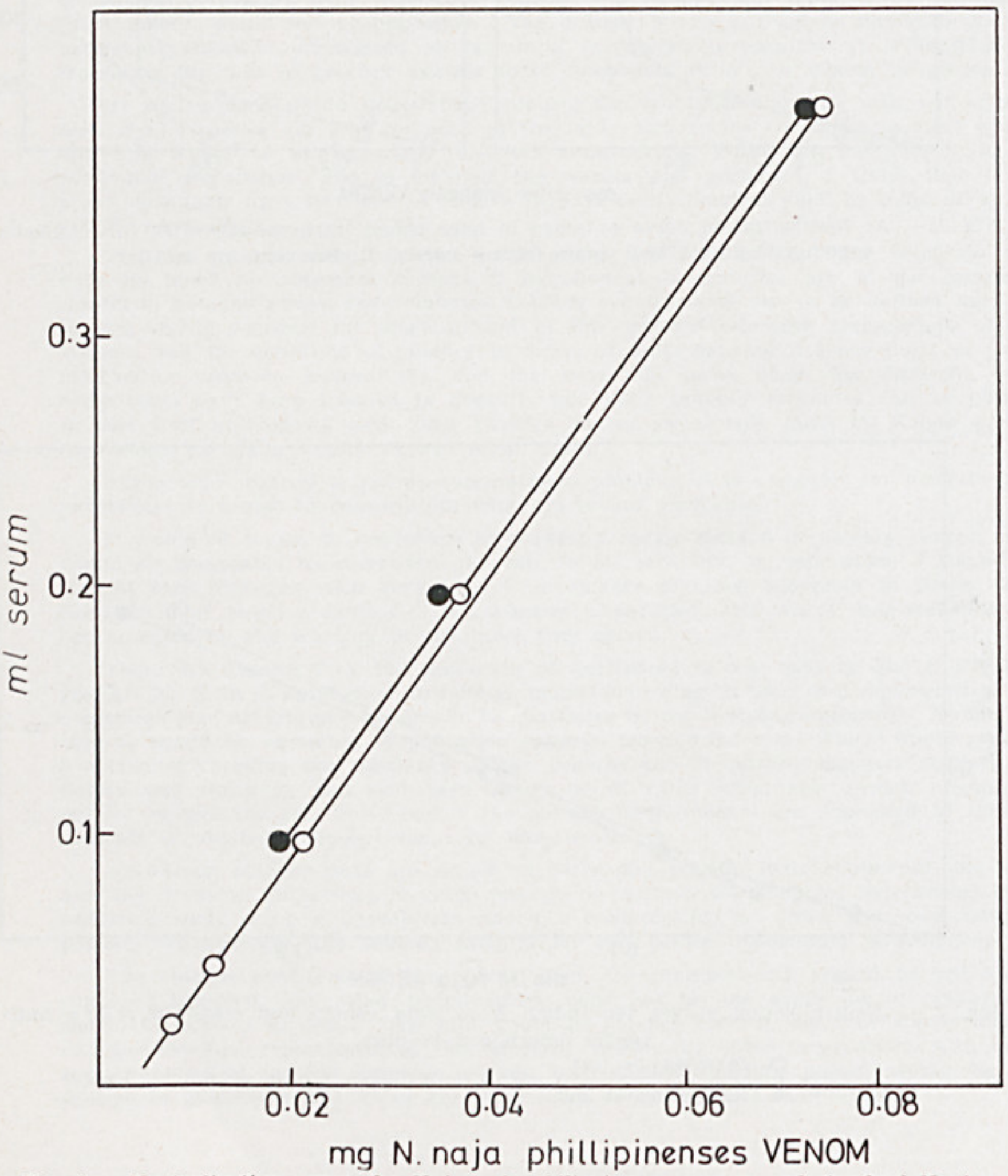


Fig. 4 — Neutralization curves for *Naja naja philippinenses* venom and standard *N a j a* antivenom determined in mice.

○ Intravenous test
● Subcutaneous test

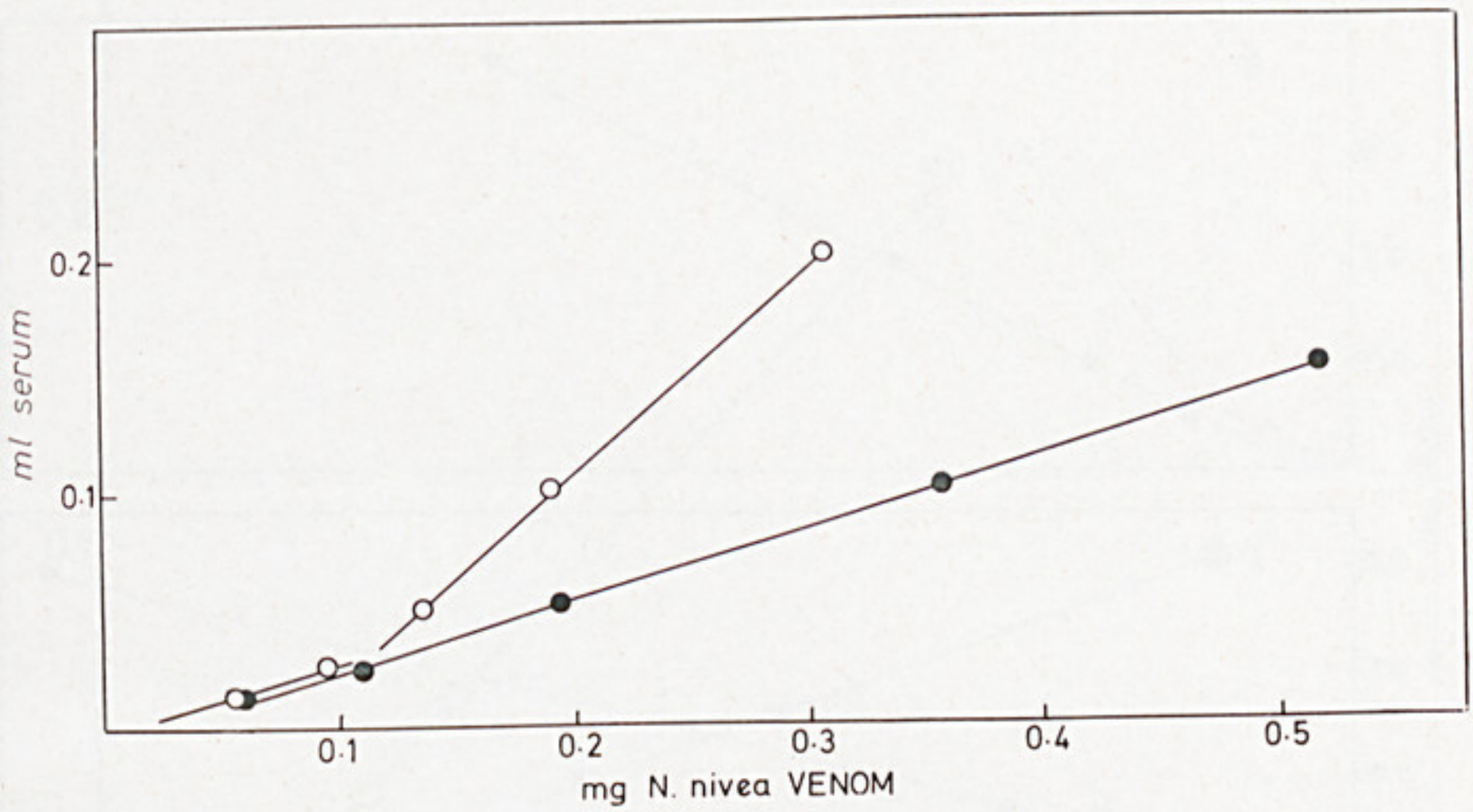


Fig. 5 — Neutralization curves for *Naja nivea* venom and standard *Naja* antivenom determined in mice.

○ Intravenous test
● Subcutaneous test

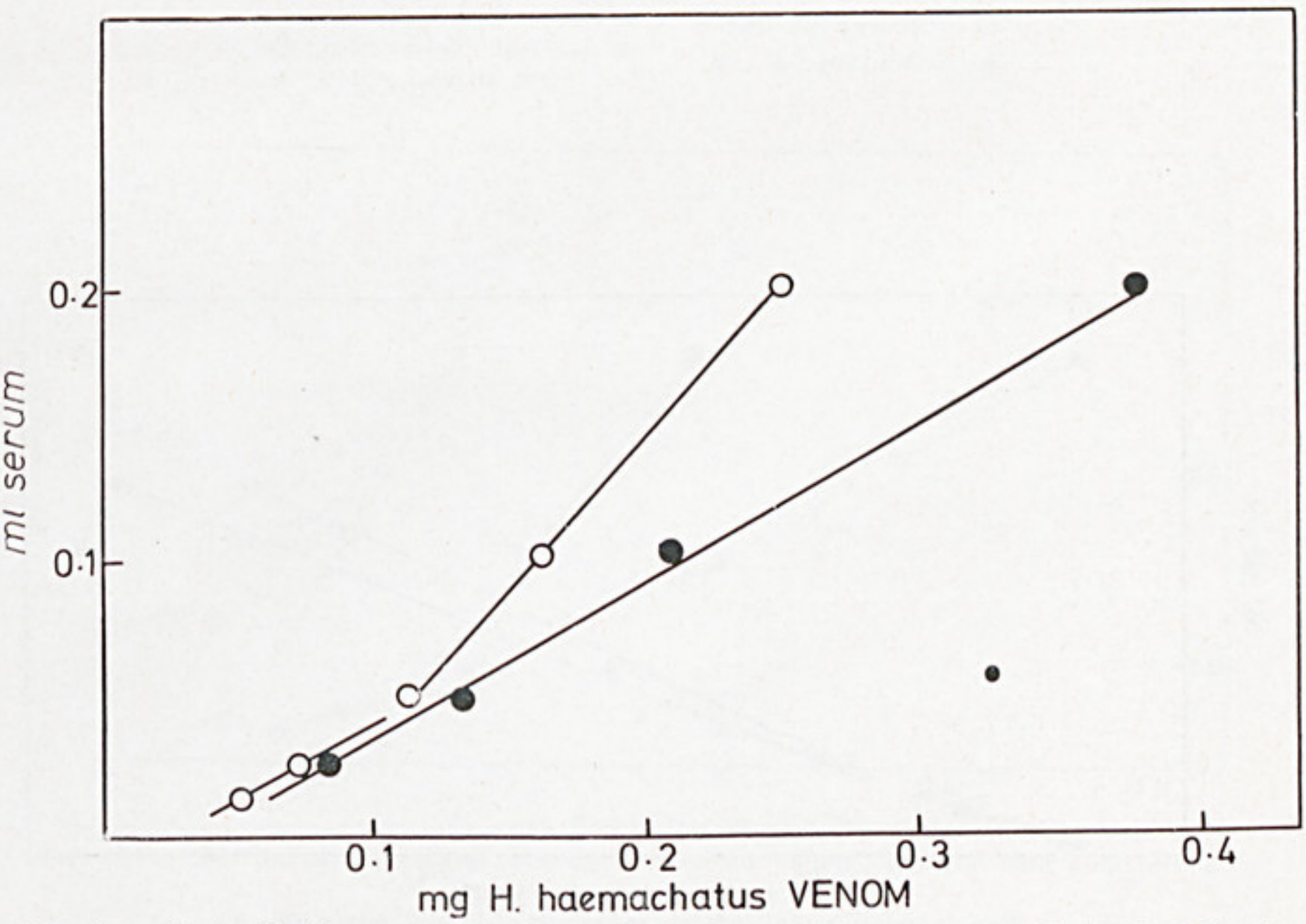


Fig. 6 — Neutralization curves for *Hemachatus haemachatus* venom and standard *Naja* antivenom determined in mice.

○ Intravenous test
● Subcutaneous test

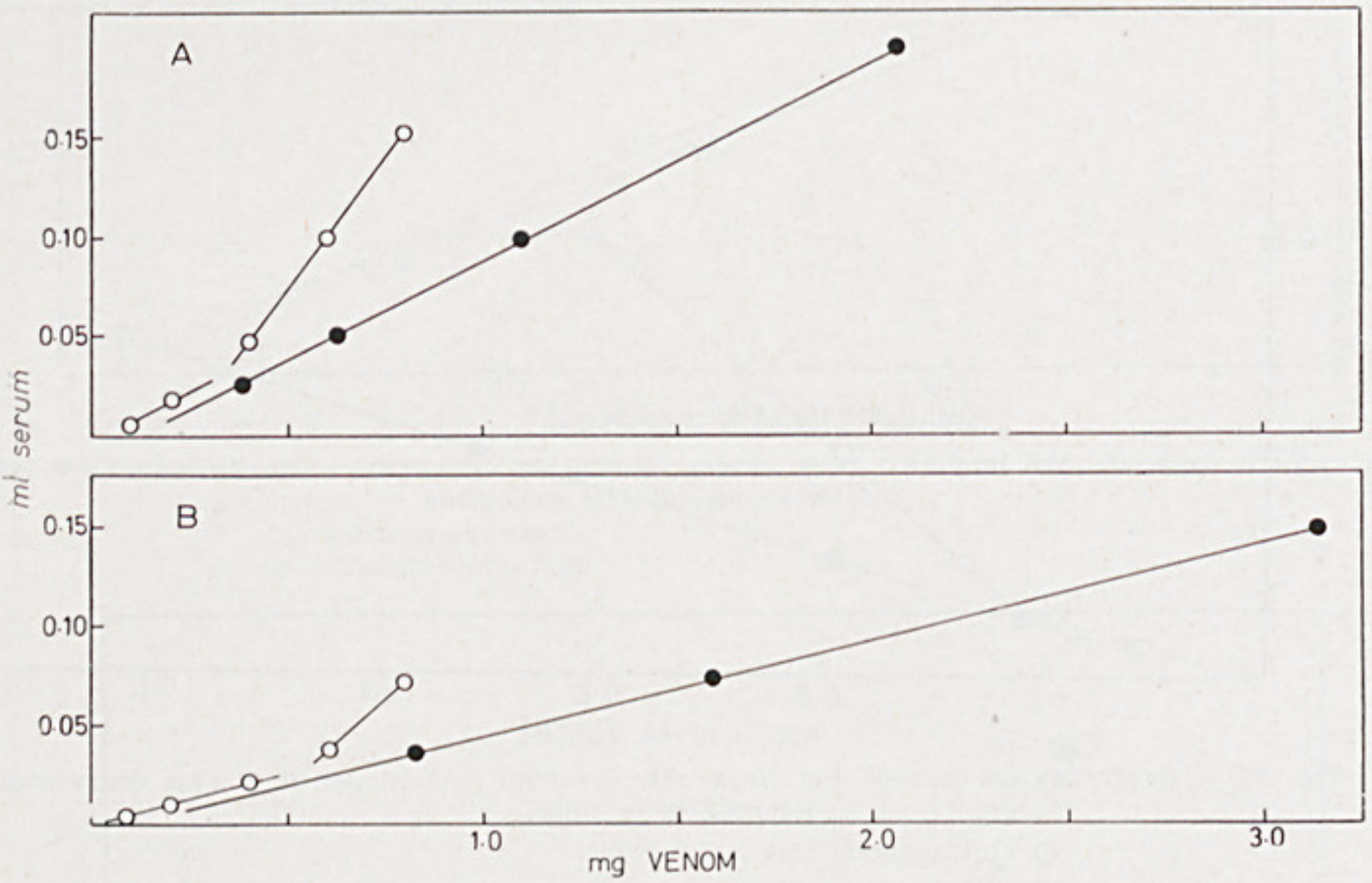


Fig. 7 — Neutralization curves for a polyvalent refined *B i t i s - E c h i s* antivenom tested with *Bitis lachesis* venom (A) and *Bitis gabonica* venom (B) in mice.

O Intravenous test

● Subcutaneous test

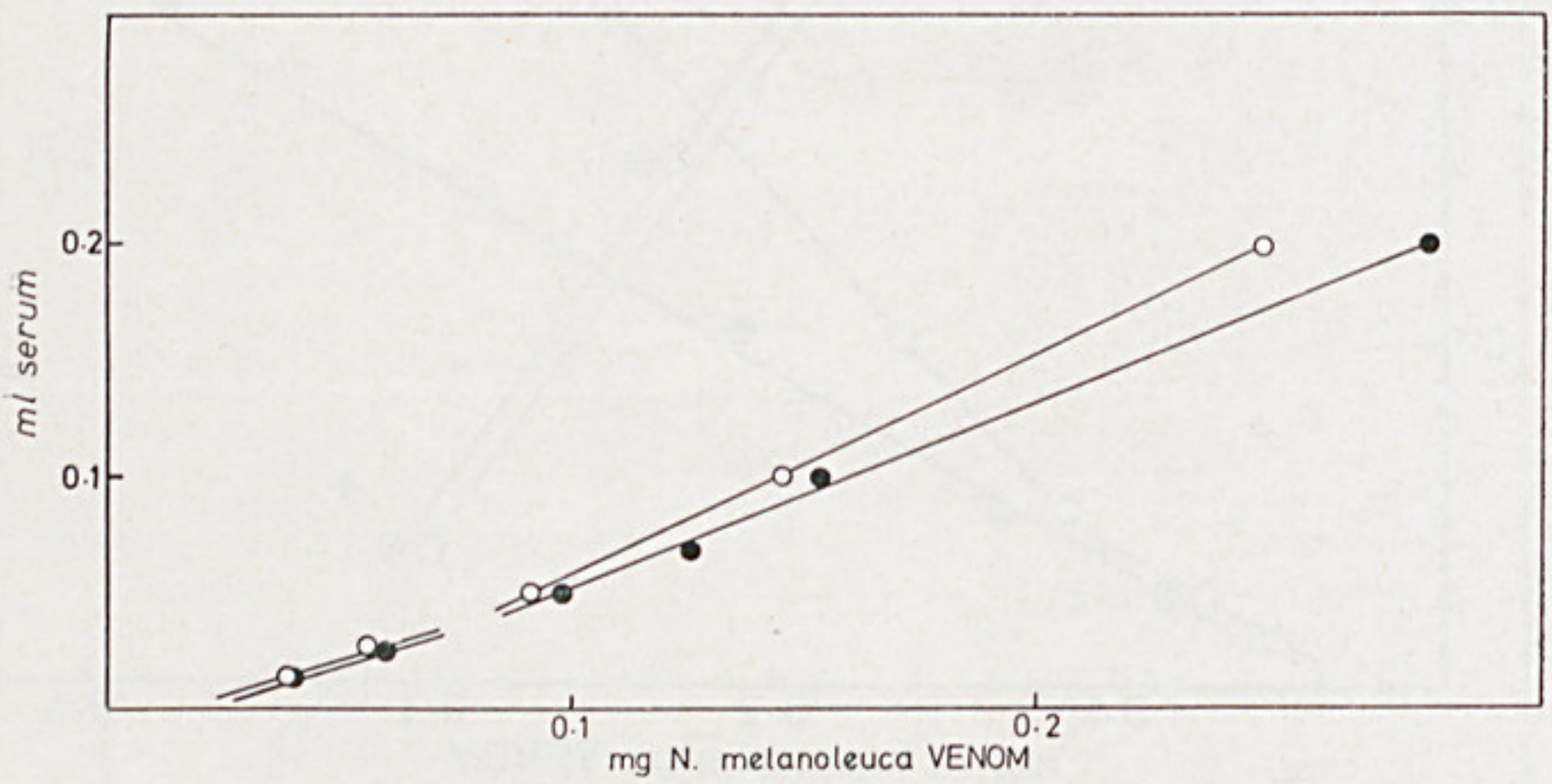


Fig. 8 — Neutralization curves for *Naja melanoleuca* venom and standard *N a j a* antivenom determined in mice.

O Intravenous test

● Subcutaneous test

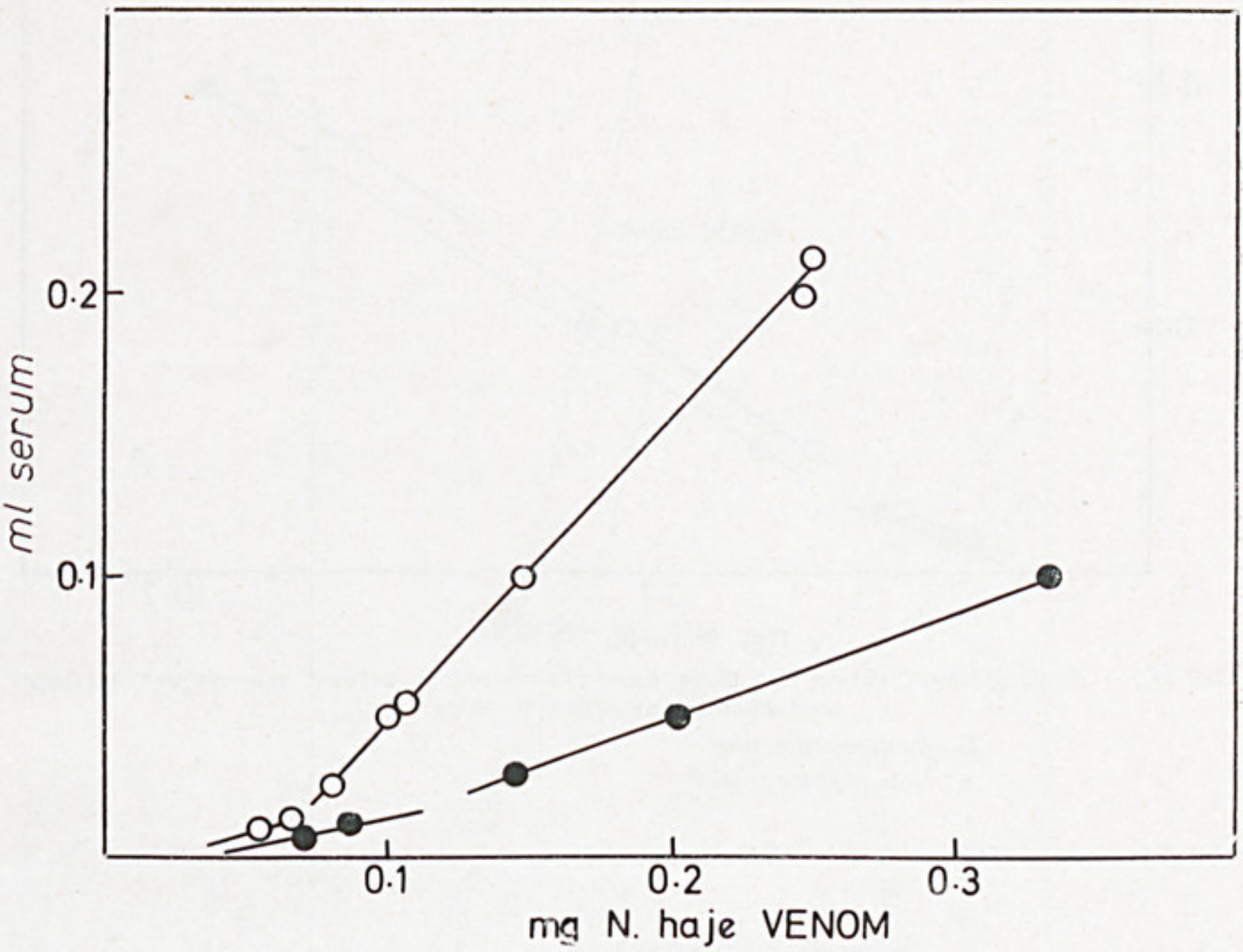


Fig. 9 — Neutralization curves for *Naja haje* venom and standard *N a j a* antivenom determined in mice.

○ Intravenous test
● Subcutaneous test

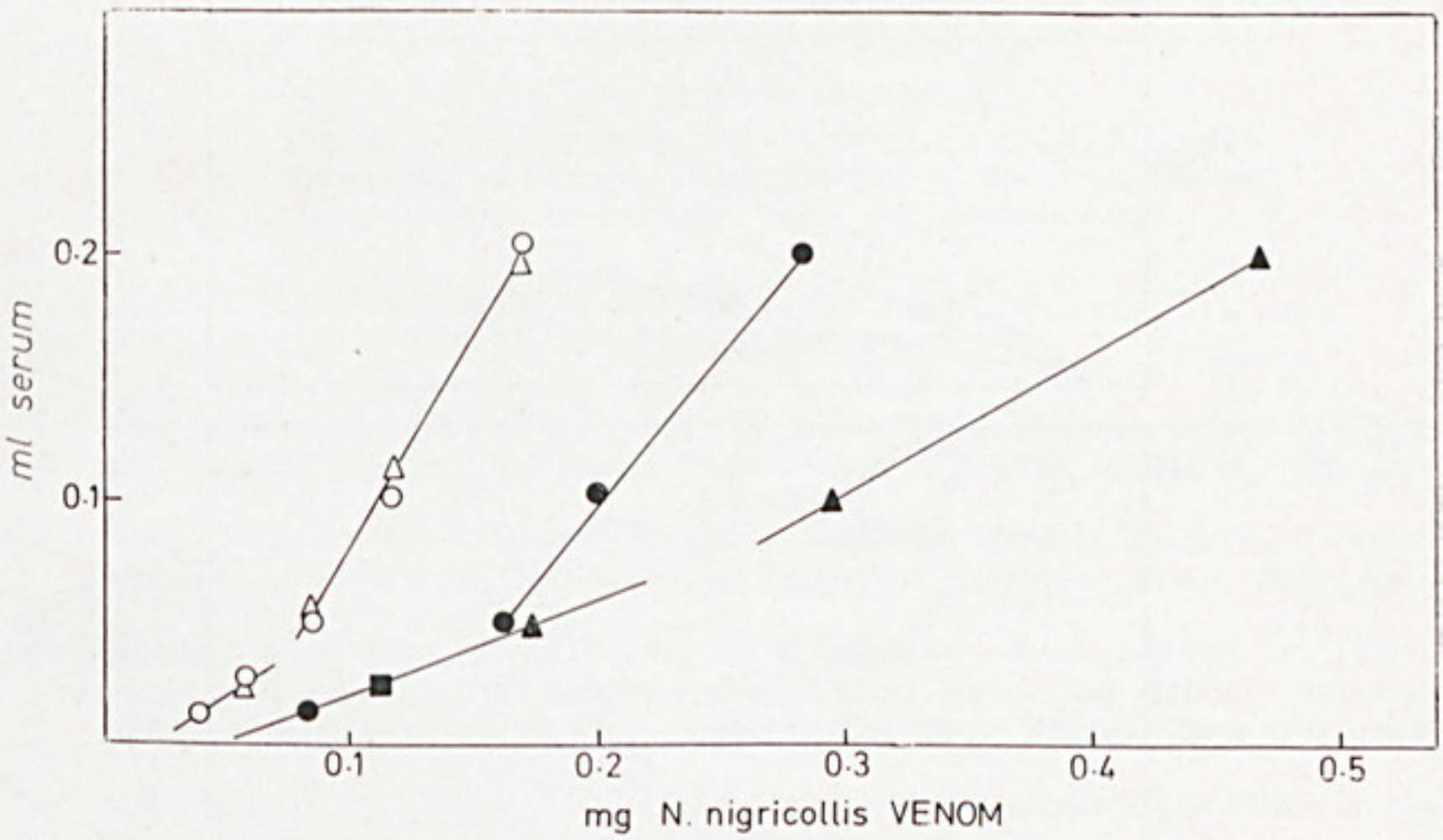


Fig. 10 — Neutralization curves for *Naja nigricollis* venom and two sera determined in mice.

○ Standard *N a j a* antivenom tested intravenously, and
● subcutaneously
◆ Unmodified antivenom tested intravenously, and subcutaneously
■ Coincident point

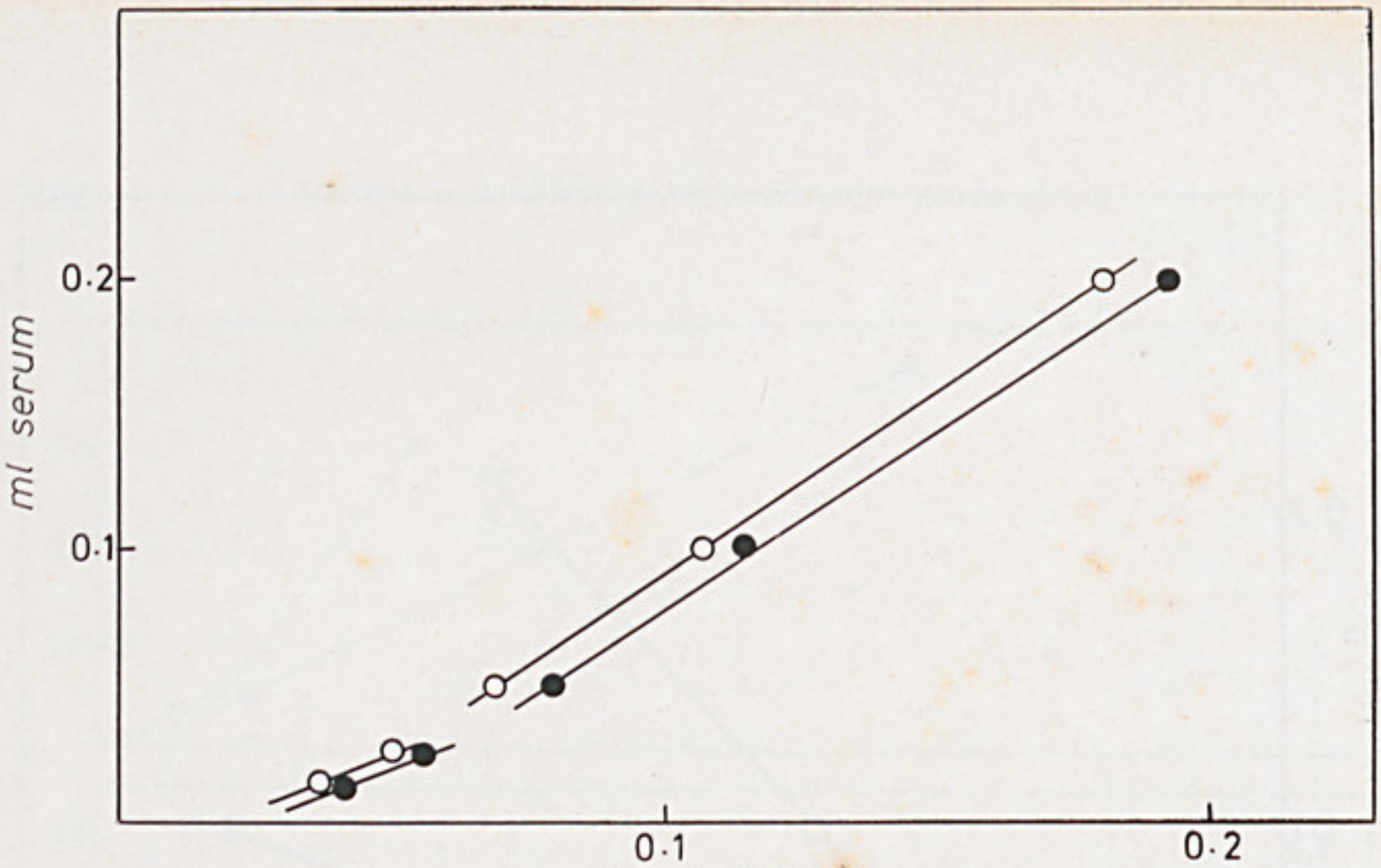


Fig. 11 — Neutralization curves for *Naja haje* venom and a refined monovalent *N. haje* antivenom determined in mice.

○ Intravenous test

● Subcutaneous test

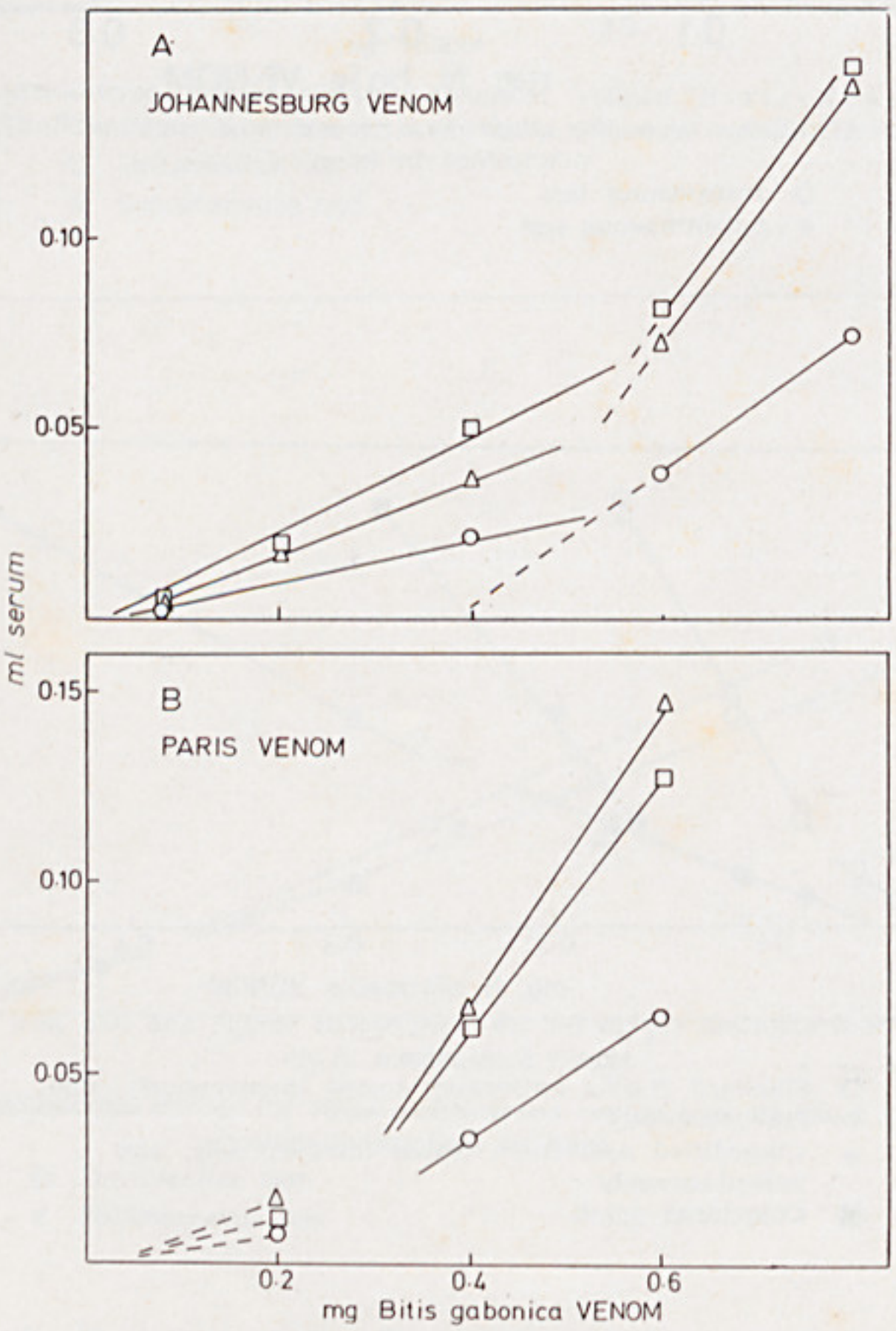


Fig. 12 — Neutralization curves determined intravenously in mice for three polyvalent refined antivenoms tested with *Bitis gabonica* venom from Johannesburg (A) and Paris (B).

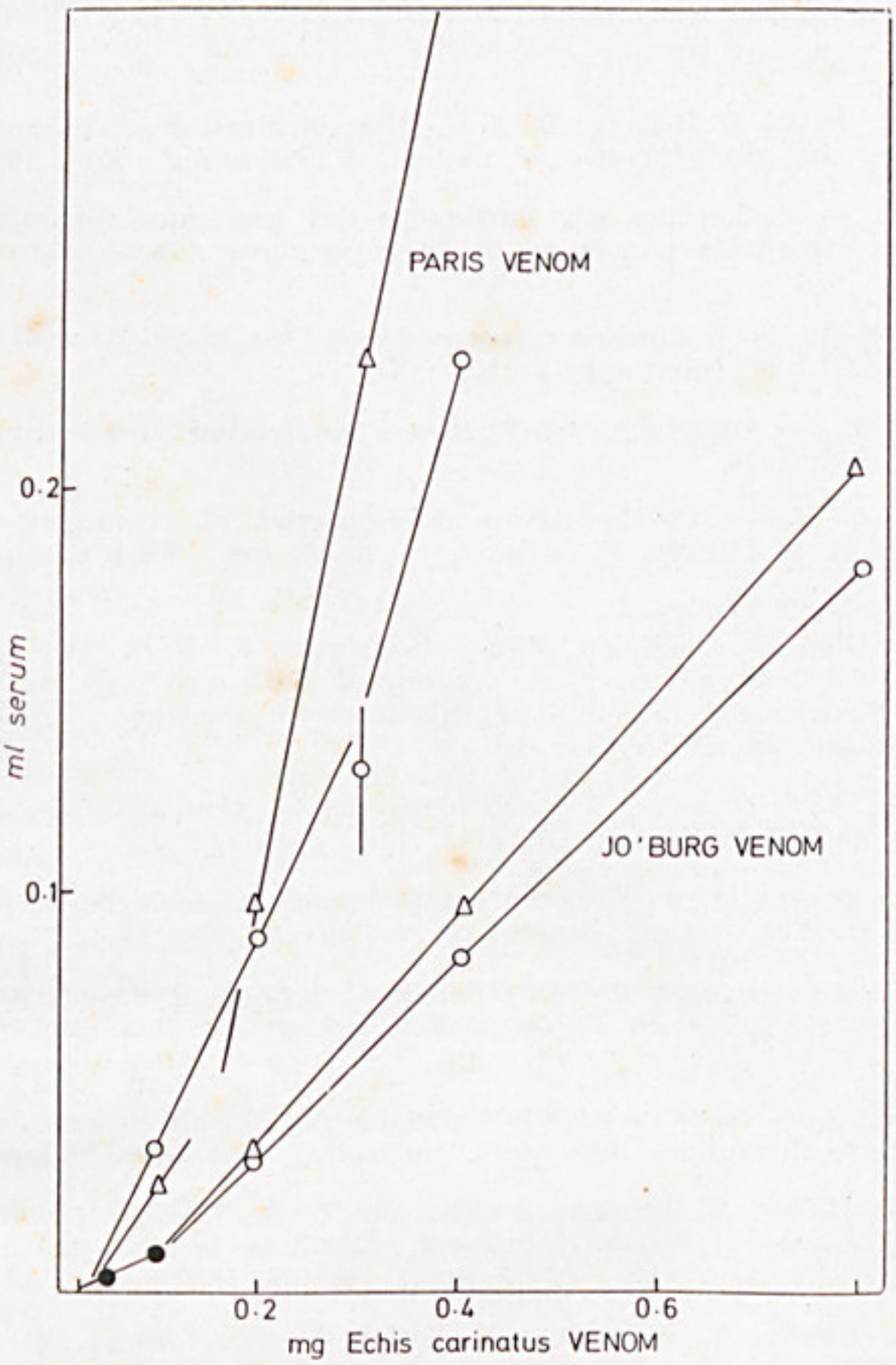


Fig. 13 — Neutralization curves for two polyvalent refined antivenoms determined intravenously in mice with *Echis carinatus* venom from Paris and Johannesburg.

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36. CROSS IMMUNOLOGICAL REACTIONS IN SNAKE-VENOMS

CHALOEM PURANANANDA, PRASIT LAUHATIRANANDA,
SOMSRI GANTHAVORN

Queen Saovabha Memorial Institute, Bangkok, Thailand

The production of antivenine serum has started since 1943 at the Queen Saovabha Memorial Institute, started with antivenine sera against Cobra (*Naja naja*) and Russell's viper (*Viper russellii*). The sera are prepared in monovalent specific form and divalent by mixing the two sera together. Later on sera against Malayan pit viper (*Agkistrodon rhodostoma*), Banded Krait (*Bungarus fasciatus*) and King cobra (*Naja hannah*) were prepared. The polyvalent serum was considered to be unpractical because it is known that the regional distribution of deadly poisonous snakes in Thailand is definite. It is therefore advisable to prepare monospecific serum. By doing this, the patient will get a correct treatment without any surplus of undesirable serum which in turn will expose the patient to serum sickness.

It is therefore important to prove that one serum is efficient for only one kind of venom. This can be proved by cross neutralization in experimental animal and supported by immunoelectrophoresis (Scheidegger, 1955).

Materials and methods

The experiments were carried out both *in vitro* and *in vivo*.

In vitro tests — The venoms used as antigen were of Thailand origin. The neurotoxic group consisted of *Naja naja siamensis* (Thai cobra), *N. hannah* (King cobra) and *Bungarus fasciatus* (Banded Krait). The hemotoxic group consisted of *Vipera russellii* (Russell's viper), *Agkistrodon rhodostoma* (Malayan pit viper) and *Trimeresurus popeorum* (Green pit viper). These venoms were dried in the dessicator under reduced pressure immediately after milking, except *N. n. siamensis* which was lyophilized. Horse antivenine sera specific for each of five venoms were used as antibodies (antivenine against *T. popeorum* has not been produced). These sera were lyophilized and reconstituted in distilled water before use in the experiments.

The microimmunoelectrophoresis method described by Scheidegger (1955) was used in the experiments by putting venoms in both wells (0.2 cm in diameter). Two different venoms were electrophoresed at constant current of 0.6 MA/cm. for 5 hours. Then, the antivenine serum specific for the venom in the upper well (for control) was put in the middle through (0.2×6.7 cm). The electrophoresed venoms were allowed to react with the antivenine serum for about 48 hours and the excess proteins were washed out by physiological saline and

distilled water successively. The slides then were dried and stained by buffalo blue black dye.

In *vivo tests* — Cross neutralization was determined by animal protection tests in white mice. The toxicity of each venom was conducted by injecting varying amounts of venom dissolved in physiological saline into groups of white mice, 3 mice in the preliminary tests and 10 mice in the final test, with a constant volume of 0.5 ml of venom solution for each mouse weighing 16-18 grams. The results were judged by the number of deaths in each animal group 24 hours after injection and the LD_{50} was calculated by the method of Kärbar (The Stated Serum Institute, Copenhagen).

In the neutralization tests, each of three mice was injected intravenously with varying amounts of venom dissolved in 0.25 ml physiological saline mixed with 0.25 ml antivenin solution. The venom-ativenin mixtures were allowed to stand at 37°C for 30 minutes before injecting into mice. Survival of test animals 24 hours after injection was the criterion for judgement of neutralization, and the potency of serum for protection was expressed as mg of venom neutralized by 10 ml of serum.

Results *in vitro* tests

In the reaction between anti-Cobra serum and Cobra and King cobra venom, and of Cobra and Banded Krait venom, there are at least ten precipitation lines, formed between the homologous reactants and a line is formed between the sera and King cobra venom, whereas at least two lines occur between that and Banded Krait venom.

In the reaction between anti-King cobra serum and King cobra and Cobra venom, and that against King cobra and Banded Krait venom respectively, there are at least five lines between the homologous reactants an three lines between sera and Cobra venom, while two lines are formed between that and Banded Krait venom.

In the reaction between anti-Banded Krait serum and Banded Krait and Cobra venom, and that of Banded Krait and King cobra venom respectively, two intense precipitin bands and at least two fainted bands occur between the homologous reactants. There are about six lines between the sera and Cobra venom, while two fainted lines are formed between that and King cobra venom.

In the reaction between anti-Russell's viper serum against Russell's viper and Malayan pit viper, and that of the same homologous venom and Green pit viper venom respectively, at least eight precipitin bands occur between the homologous reactants. A fainted line is formed between the sera and Malayan pit viper, whereas about five fainted lines occur between sera and Green pit viper venom.

In the reaction between anti-Malayan pit viper serum, and Malayan pit viper and Russell's viper venom, and of the same homologous venom and Green pit viper venom respectively, there are at least eight precipitin bands formed between the homologous antigen. Two fainted lines occur between the sera and Russell's viper venom, while about four lines occur between that and Green pit viper venom.

The cross immunological reactions between anti-Cobra sera and Russell's viper venom and vice versa exists, with no precipitation line between the anti-Cobra serum and Russell's viper venom. There are about six fainted lines between anti-Russell's viper sera and Cobra venom. This may be due to the fact that the

In vivo test:

TABLE I — CROSS NEUTRALIZATION OF NEUROTOXIC VENOMS AND THEIR ANTIVENINS

ANTIVENINE	V E N O M S		
	Cobra	King cobra	Banded Krait
Cobra	1 2 4.0 (20)	4.4 (> 4)	2.0 (2)
King cobra	1.0 (5)	4.0 (4)	1.6 (< 2)
Banded Krait	< 0.4 (< 2)	1.6 (< 2)	7.0 (7)

TABLE II — CROSS NEUTRALIZATION OF HEMOTOXIC VENOMS AND THEIR ANTIVENINS

ANTIVENINS	V E N O M S		
	Russell's viper	*A. rhodostoma*	Green pit viper
Russell's viper	5.0 (48)	0	0
A. rhodostoma	0	14.0 (6)	0

TABLE III — CROSS NEUTRALIZATION BETWEEN NEUROTOXIC AND HEMOTOXIC VENOMS AND THEIR ANTIVENINS

ANTIVENINS	V E N O M S	
	Cobra	Russell's viper
Cobra	4.0	1.0 (8)
Russell's viper	0.6 (3)	5.0

1 — Quantities of venoms in mg neutralized by 10 ml of serum. (Judged by survival at 24 hours of all 16-18 grams white mice given intravenous injection of varying amount of venoms in saline plus 0.25 ml antivenim. Venom solutions were mixed and allowed to stand for 30 minutes at 37°C before injecting.)

2 — Amount in LD_{50} of venom.

antiserum possesses antibodies to minor components of antigen. Thus the bands are formed by the intermingling of these components of antigen and antibodies. But in the first case, the antibodies to identical components that constitute in the Cobra venom may not be high enough to form precipitation line with the minor fractions of Russell's viper venom.

Discussion

S. Minton: "1. We find very poor correlation between immunodiffusion lines and neutralization of venom *in vivo.* For example, we find fairly good neutralization of several Naja venoms and Sea Snake Antivenin (Commonwealth Serum Labs) although this serum gives very little in the way of precipitin lines."

"2. The finding of common precipitin antigens between serum of snake and venom of same snake is contrary to our experience."

"3. I am surprised at the number of common antigens between Cobra and Russell's Viper venoms. This is contrary to our experience."

S. Minton: "1. What do you consider a satisfactory neutralization titer in terms of mouse LD/50's neutralized by 1 c.c.?"

"2. How frequently are Bungarus bites seen in Thailand?"

C. Purananda: "1. It was answered by Dr. Krag. Method used in Bangkok is Ipsen's."

"2. Very seldom, because *Bungarus fasciatus* is a docile snake."

A. Shulov: "1. What is the reason of using ponies instead of horses. Did you try donkeys? According to the advice of Dr. Bouquet, Paris we tried with satisfactory results."

"2. Is the percentage of good producers higher among ponies than among horses?"

C. Purananda: "1. Sim, estou de acôrdo. Em meu trabalho mencionei poneys, burros e mulas em quantidade".

"2. Sim. Sòmente não no caso de venenos hematóxicos, onde há melhores resultados com cavalos da China e mulas."

F. Kornalik: "I would like to corroborate Dr. Purananandas' findings about the presence of toxin-like antigens in the serum of King-cobra, which would react with a specific anti-toxin serum. This has been described up till now three times for the serum of *Vipera ammodytes.*"

N. McCollough: "We have not been able to correlate the gravity of serum sickness and the amount of antivenin given. 70-80% of those who receive antivenin become victims of serum sickness and its degree seems to be an individual response. Would you comment."

C. Purananda: "The cases of snakebites after treated with antivenine serum resulted in serum sickness in our country, become important to our notice and we have to be careful about cases of repeated bite. In some cases of bite amongst the snake charmers, who came in with second and third treatment, some of them came with serious symptoms and intravenous injection is indicated. It is therefore very important to reduce the amount of serum used possible."

A. do Amaral: "1. How do you determine the venom toxicity?"

"2. What process of antivenin titration do you use at Bangkok?"

C. Purananda: "1. Replied by Dr. Krag. 2. Ipsen's method."

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37. PROBLEMS IN DETERMINATION OF ANTIHEMORRHAGIC POTENCY OF HABU (*TRIMERESURUS FLAVOVIRIDIS*) ANTIVENINE IN THE PRESENCE OF MULTIPLE HEMORRHAGIC PRINCIPLES AND THEIR ANTIBODIES

AKIRA OHSAKA, HISASHI KONDO, SATORU KONDO, MASAMI KUROKAWA and RYOSUKE MURATA

National Institute of Health, Shinagawa-ku, Tokyo, Japan

Introduction

We demonstrated the presence of two hemorrhagic principles, HR1 and HR2 (1), in the venom of *Trimeresurus flavoviridis*, which is called "Habu" in Japanese. The two principles are not distinguishable by hemorrhagic action on the rabbit skin (2-4) but are distinguishable immunologically from each other (5). Consequently, preparations of Habu antivenine contain the corresponding two antibodies, anti-HR1 and anti-HR2, in varying proportions (5).

Should the potency of such an antivenine be titrated against the test toxin containing the two hemorrhagic principles, we must ask what the result of the titration indicates, the potency of exclusively anti-HR1 or anti-HR2, or some other implication (3, 5).

Recently we proposed a model for the mechanism involved in titration of the antihemorrhagic potency of an antivenine containing two antibodies with a test toxin containing two corresponding hemorrhagic principles (5).

The purpose of this presentation is to discuss the general implications of our model in standardizing antivenines and also bacterial antitoxins.

Definition of the terms

The minimum hemorrhagic dose (MHD) of venom is defined as the least quantity of venom in μg causing a hemorrhagic spot of 10 mm in diameter 24 hr after intracutaneous injection into the rabbits (2).

Effective dose (ED) is defined as a quantity of an antivenine in ml which, when mixed with a given dose of a test toxin, makes a mixture producing a hemorrhagic spot of 10 mm in diameter when 0.2 ml is injected intracutaneously into the rabbit (5).

One unit of antihemorrhagic activity is defined as the amount of antivenine containing one ED at the level of 100 MHD (5). The antihemorrhagic potency of an antivenine is expressed as units per ml.

The design for the determination of effective dose (ED) of an antivenine by the rabbit skin test

Table 1 shows an example of the design for determination of effective doses (EDs) of an antivenine by the rabbit skin test (5).

TABLE 1 — AN EXAMPLE OF THE DESIGN FOR DETERMINATION ON EFFECTIVE DOSES (EDs) OF AN ANTIVENINE BY THE RABBIT SKIN TEST

Test toxin : Crude venom (batch No. 48)
Antivenine preparation : Antivenine No. 23

Hemorrhagic activity of test toxin in MHD*	Rabbit No.	Cross-diameters of hemorrhagic spot in mm against varying amount of antivenine					
		0.00063	0.00050	0.00040	0.00032	0.00025	0.00020 ml*
30	1556	±	12 x 12	12 x 13	14 x 13	14 x 15	16 x 15
	1557	±	12 x 13	13 x 13	13 x 15	15 x 16	18 x 16
	1559	–	±	15 x 13	16 x 16	15 x 17	17 x 16
		0.00200	0.00160	0.00125	0.00100	0.00080	0.00063 ml*
100	1556	±	13 x 15	15 x 15	16 x 16	16 x 18	18 x 18
	1557	±	±	11 x 11	13 x 14	16 x 16	18 x 19
	1559	±	11 x 11	15 x 14	17 x 18	19 x 20	20 x 21
		0.00630	0.00500	0.00400	0.00320	0.00250	0.00200 ml*
300	1556	±	13 x 15	15 x 15	15 x 16	17 x 19	18 x 19
	1557	±	±	11 x 11	14 x 15	17 x 18	19 x 21
	1559	±	11 x 11	15 x 15	17 x 17	19 x 19	20 x 23

* Per injected dose

Test toxin solutions containing 30, 100 or 300 MHD per 0.1 ml were prepared. An aliquot from each of these solutions was mixed with the equal volume of each of serial dilutions of the antivenine graded with 1.25-fold intervals. The mixtures were kept standing for 1 hr at room temperature. Each was injected into the depilated back skin of three rabbits at a dose of 0.2 ml and hemorrhage developed was measured after 24 hr from the visceral side of the removed skin as described in the literature.

The ED of the antivenine against each level of test toxin was expressed in the volume of the antivenine reducing the size of the hemorrhagic spot to 10 mm in diameter, when 0.2 ml of the venom-antivenine mixture was injected into the rabbit. In practice, the amount of antivenine in mixture causing a hemorrhagic spot of 10 mm in diameter was determined by interpolation.

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Neutralization curves of antivenines with the crude venom or the partially purified hemorrhagic principles

Effective doses (EDs) of several antivenines were determined with a crude venom (batch No. 48), HR1 and HR2 as test toxins (5). The results were plotted to give the neutralization curves shown in Fig. 1 where the ordinate is ED of the antivenines and the abscissa hemorrhagic activity of the test toxins in MHD, both in logarithmic scale.

Statistical analyses proved that all the neutralization curves obtained with the crude venom as well as the venom fractions (HR1 and HR2) are linear and parallel to each other. This was shown also with a number of other antivenines. The common slope (b) of the neutralization curves was 1.12. (5)

Fig. 1 shows that: 1) the amount of each antivenine required to neutralize a given MHD dose of HR2 was larger than that needed to neutralize an equal dose of HR1; 2) the ED of the three antivenines varied depending on the test toxin used. The ED of antivenine No. 23 was the largest against HR2 and the smallest against HR1 among the three antivenines tested. This situation was reversed with antivenine No. 7. The ED of antivenine No. 9 was between those

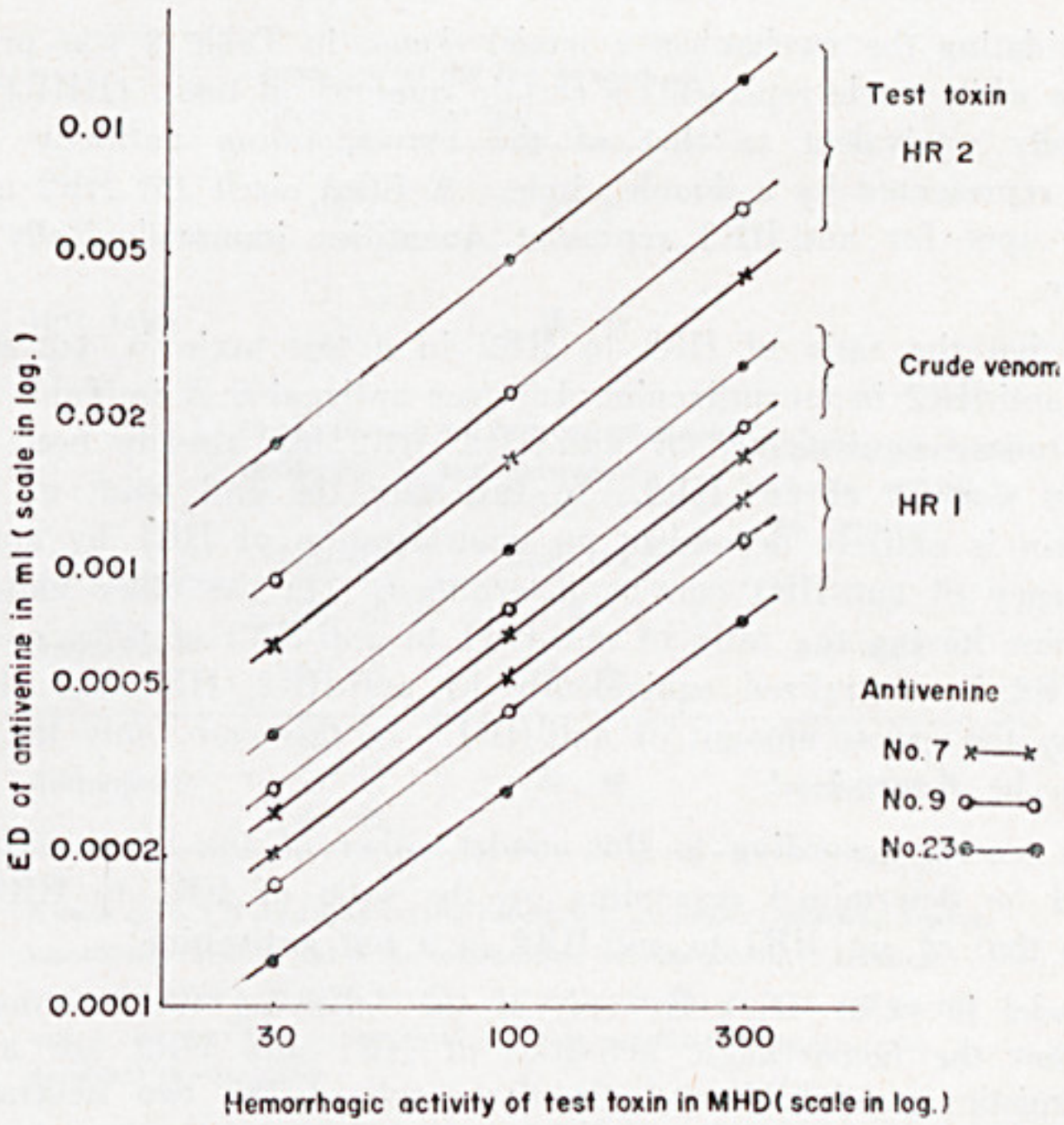


Fig. 1 — Neutralization of antivenine against three test toxins as determined by skin test. Test toxin: Crude venom (batch No. 48) and venom fractions (HR1 and HR2).

of the other two antivenines against either HR1 or HR2; 3) the EDs of the three antivenines against HR2 and against the crude venom were in the same order.

From these results we (5) postulate: 1) that HR1 and HR2 are not distinguishable in hemorrhagic action on the rabbit skin but are distinct immunologically from each other, since the amounts of an antivenine required to neutralize the two principles of an equal MHD dose were different; 2) that each antivenine contains two distinct antibodies corresponding to each of the two hemorrhagic principles; 3) that the ratio of the quantity of the two antibodies differed from one antivenine to another.

Should the potency of such an antivenine be titrated against the test toxin containing the two hemorrhagic principles, the question arises as to what the results of such titration indicate, the potency of only anti-HR1 or anti-HR2, or some other implication.

This question can not be answered unless the mechanism involved in titration of the antivenine potency in the presence of two hemorrhagic principles and the corresponding antibodies is elucidated.

Mechanism involved in titration of antivenine potency in the presence of two hemorrhagic principles and their antibodies

For elucidating the mechanism a model shown in Table 2 was proposed (5). In the table a white circle represents a certain quantity of toxin (HHR1) molecules immunologically equivalent to that of the corresponding antibody (anti-HR1) moleculos as represented by a double circle. A filled circle for HR2 and a circle with a dark spot for anti-HR2 represent quantities immunologically equivalent to each other.

Assume that the ratio of HR1 to HR2 in a test toxin is 4:2 and that of anti-HR1 to anti-HR2 in an antivenine 4:3 (see antivenine A in Table 2). When HR1 is neutralized equivalently by anti-HR1, HR2 has already been neutralized by the excess amount of anti-HR2. In this case the end point of titration in the rabbit skin is entirely dependent on neutralization of HR1 by anti-HR1 and only the potency of anti-HR1 can be determined. On the other hand, with another antivenine having the ratio of anti-HR1 to anti-HR2 of 6:2 (see antivenine B), when HR2 is neutralized equivalently by anti-HR2, HR1 has already been neutralized by the excess amount of anti-HR1. In this case, only the potency of anti-HR2 can be determined.

In other words, according to this model either of the two anti-hemorrhagic activities will be determined depending on the ratio of HR1 to HR2 in a test toxin and on that of anti-HR1 to anti-HR2 in a test antivenine.

This model is valid, however, only if the following four assumptions hold good: 1) that the hemorrhagic activities of HR1 and HR2 are additive but neither synergistic nor inhibitory; 2) that each of the two neutralization reaction systems, HR1 to anti-HR1 and HR2 to anti-HR2, has its own immunological specificity; 3) that formation of toxin-antitoxin complexes of the two systems occurs at the constant molecular ratio of toxin to antitoxin irrespective of the

concentration of the toxin; and 4) that the dissociation constants of the two neutralization reactions are relatively small and of approximately the same magnitude.

The first assumption has been verified by the results of our experiments (5). The results shown in Fig. 1 seem to support the second assumption. The third and fourth assumptions also seem to be valid since the two neutralization reactions followed essentially the "Law of Multiple Proportion" * as shown in Fig. 1, where the slope of each neutralization curve is approximately a unity.

For further verification of the model, an experiment was conducted. In Table 3, antivenines No. 7, 9 and 23 were titrated separately against HR1 and HR2. The ratios of anti-HR1 to anti-HR2 in these antivenines were calculated to be 2.73:1, 4.55:1 and 14.5:1, respectively. When the anti-hemorrhagic potencies of the three antivenines are titrated against the test toxins containing HR1 and HR2 at varying ratios, antihemorrhagic activity against the individual hemorrhagic principles should be determined as predicted in Table 3, if our model (Table 2) holds true.

TABLE 2 — A MODEL FOR THE MECHANISM INVOLVED IN TITRATION OF ANTIVENINE POTENCY IN THE RABBIT SKIN IN THE PRESENCE OF TWO HEMORRHAGIC PRINCIPLES AND THE CORRESPONDING ANTIBODIES

	Proportion of the two hemorrhagic principles in test toxin		
	HR 1	HR 2	
Test toxin	○ ○ ○ ○	● ●	
	Proportion of the two corresponding antibodies in test antivenine		Specific antihemorrhagic activity to be titrated
	Anti-HR 1	Anti-HR 2	
Antivenine A	◎ ◎ ◎ ◎	◉ ◉ ◉	Anti-HR 1
Antivenine B	◎ ◎ ◎ ◎ ◎ ◎	◉ ◉	Anti-HR 2

A white circle (○) represents a certain quantity of toxin (HR 1) molecules immunologically equivalent to that of the corresponding antibody (anti-HR 1) molecules as represented by a double circle (◎). A filled circle (●) for HR 2 and a circle with a dark spot (◉) for anti-HR 2 represent quantities immunologically equivalent to each other.

* The "Law of Multiple Proportion" implies that "if a certain amount of antitoxin neutralizes a certain quantity of toxin, then to neutralize a multiplum of the quantity of toxin the same multiplum of the amount of antitoxin is required" (Jerne, 1951 (6)).

The prediction shown in Table 3 was in exact accordance with the actual results of the experiment, except for antivenine No. 23 in Section 7. The experiment has been published elsewhere (5).

TABLE 3 — THE NEUTRALIZATION REACTIONS DETERMINING THE END POINTS OF TITRATIONS IN THE RABBIT SKIN PREDICTED FROM THE PROPOSED MODEL (TABLE 1)

Section	Test toxin	Antivenine (and the ratio of anti-HR1 to anti-HR2 in the antivenine)		
	The ratios of HR1 to HR2	No 7 (2.73:1)	No.9 (4.55:1)	No. 23 (14.5:1)
4	60:40(1.5:1)	HR 2*	HR2	HR2
5	75:25(3:1)	HR 1*	HR2	HR2
6	83:17(4.9:1)	HR 1	HR1	HR2
7	95: 5(19:1)	HR 1	HR1	HR1

* HR1 is the abbreviation for the neutralization reaction of HR1 to anti-HR1; HR2 is for that of HR2 to anti-HR2.

Conclusion

We conclude that the antihemorrhagic potency of Habu antivenine relative to a standard antivenine can only be determined when the two hemorrhagic principles (HR1 and HR2) are used separately as test toxins instead of a crude venom.

To generalize, all the toxic components in a venom indistinguishable in respect to a biological response should be separated and each component should be used as a test toxin (3, 5). This general conclusion has been fortified by our experiments on determination of antilethal potency of Habu antivenine. (7)

The reasoning thus confirmed is applicable to the assay of antitoxic potency of a polyvalent antivenine. Unless each toxic component in venoms is separated and available as a test toxin, monovalent antivenines should not be combined before determining the potencies of the individual antivenines, or immunization with mixed venoms should be avoided.

It is worth mentioning the suggestion made by Iguchi (8) in 1940 about the possible presence of multiple lethal toxic components in culture filtrate of *Cl. welchii* (*perfringens*) type A and the corresponding antibodies in different proportions in antitoxin preparations; he observed varying antilethal potencies for an antitoxin preparation depending on the test toxin used.

As pointed out by Llewellyn Smith (9) in 1938, the assay of the potency of tetanus antitoxin is also influenced by the test toxin used. Based on similar observations, Petrie (10) in 1943 suggested the multiplicity of composition of tetanus toxin and the consequent multiplicity of antibodies in the antitoxin preparations.

The reasoning described in this presentation should naturally be applicable to the assay of antitoxic activities of various bacterial antitoxins including *Cl. welchii* type A antitoxin and *Cl. tetani* antitoxin.

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38. IMMUNOLOGIC STUDIES OF CORAL SNAKE VENOM

PINYA COHEN and EDWARD B. SELIGMANN, JR.

National Institutes of Health, Bethesda, Maryland, U.S.A.

Introduction

Studies on coral snake venom were initiated in 1964 as part of a program to develop adequate standards for the production and control of coral snake antivenin.

The problem of coral snake bite is not so great in the United States as it is in Central and South America. Only two species of coral snakes occur in the United States, and they have a relatively limited geographic range. The most common of the two, *Micrurus fulvius*, is found in the southeast and westward into the states bodering the Gulf of Mexico. A subspecies, *M. fulvius tenere*, is found west of the Mississippi River. Most of the specimens of *M. fulvius* that were collected to provide venom for these studies were taken in Florida. The other coral snake, *Micruroides euryxanthus*, represents a monotypic genus. Its range in the United States is limited to the southern desert regions of Arizona and New Mexico, however, the snake is also found in Mexico. It is considered rare in the United States.

LD_{50} and dose-response relationship of *Micrurus fulvius* venom

M. fulvius venom was obtained from the Miami Serpentarium, Miami, Florida. It was prepared by freeze-drying pooled, fresh venom from numerous milkings of a large number of snakes.

The LD_{50} for *M. fulvius* venom was determined using 16-18 gm, albino, mice of a randomly bred strain. Six mice were injected intraperitoneally with physiologic saline solutions of the appropriate venom concentration. The results were recorded after 48 hours and the LD_{50} calculated according to the method of Reed and Muench (1). The dose-response relationship is shown in Figure 1. The slope of the curve is fairly steep and the average LD_{50} is 13.0 μg (0.77 μg/gram of body weight).

Antibody response to *M. fulvius* venom in goats

Two Togenberg goats were immunized with subcutaneous injections of *M. fulvius* venom sterilized by filtration and mixed with an equal volume of Amphojel * (aluminum hydroxide gel). The development of neutralizing antibody

* Wyeth Laboratories, Philadelphia, Pennsylvania, U.S.A.

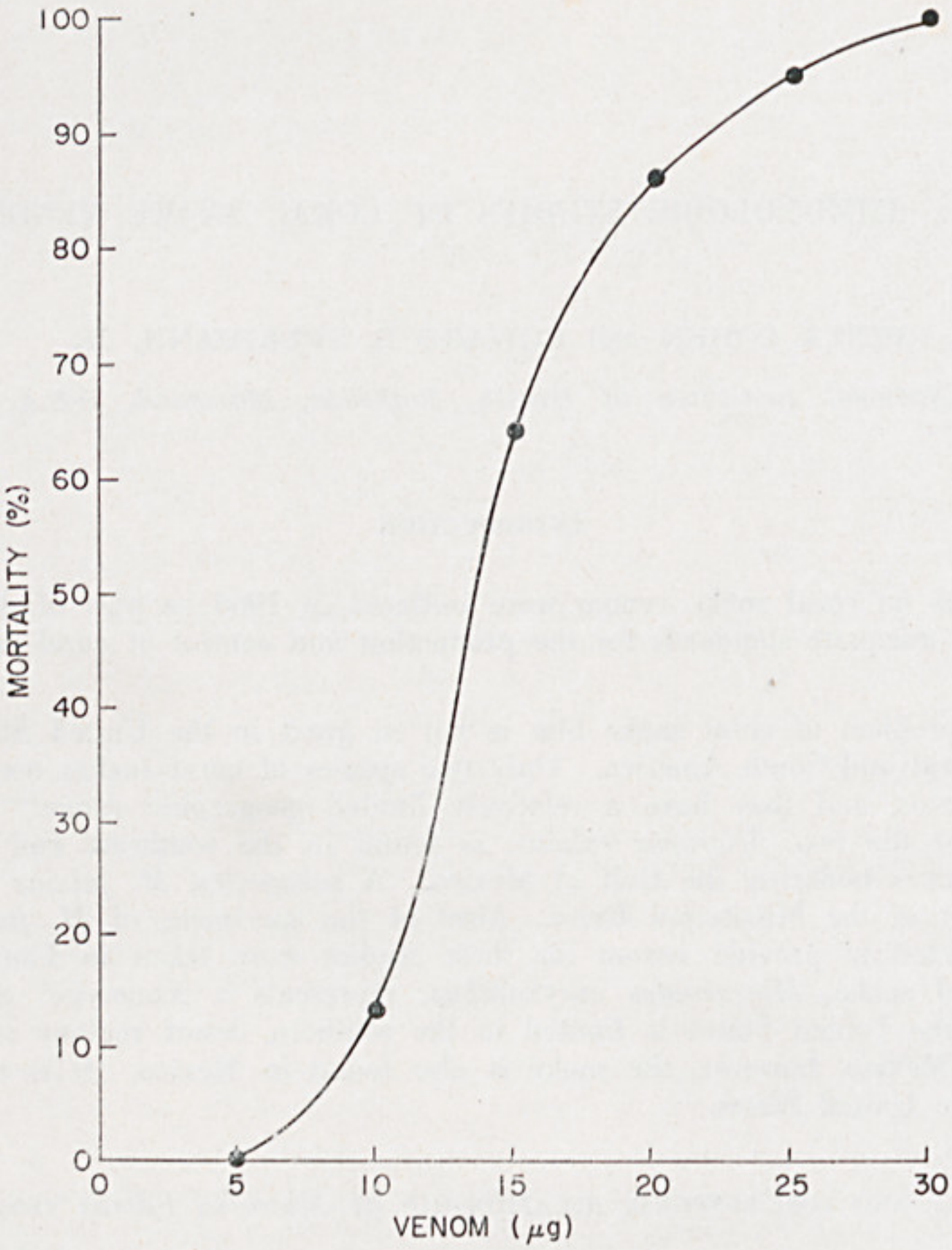


Fig. 1 — Dose-response relationship of *Micrurus fulvius* venom in mice.

was followed weekly. The procedure previously described (2) to determine neutralizing antibody in rabbit serum was also used for the goat serum. Figure 2 shows the immunization schedule and the results of antibody titrations conducted on pools of serum from the two goats. Neutralizing antibody was initially detected during the sixth week of immunization. The highest titer obtained was 105 mouse LD_{50} (1.4 mg of venom) neutralized/ml of antiserum. Earlier work in rabbits yielded an antiserum with a maximum neutralizing potency, *in vitro*, of 38 mouse LD_{50} or approximately 0.5 mg of *M. fulvius* venom per ml (2). The results in goats suggest that considerable fluctuation of antibody levels occurs. However, once the goats have been well stimulated and develop high antibody levels, these high levels can be regained rapidly if regular venom doses are administered. The goats did not exhibit any obvious adverse systemic or local reactions during the course of immunization. They gained weight and the hematocrit value remained normal during periodic tests.

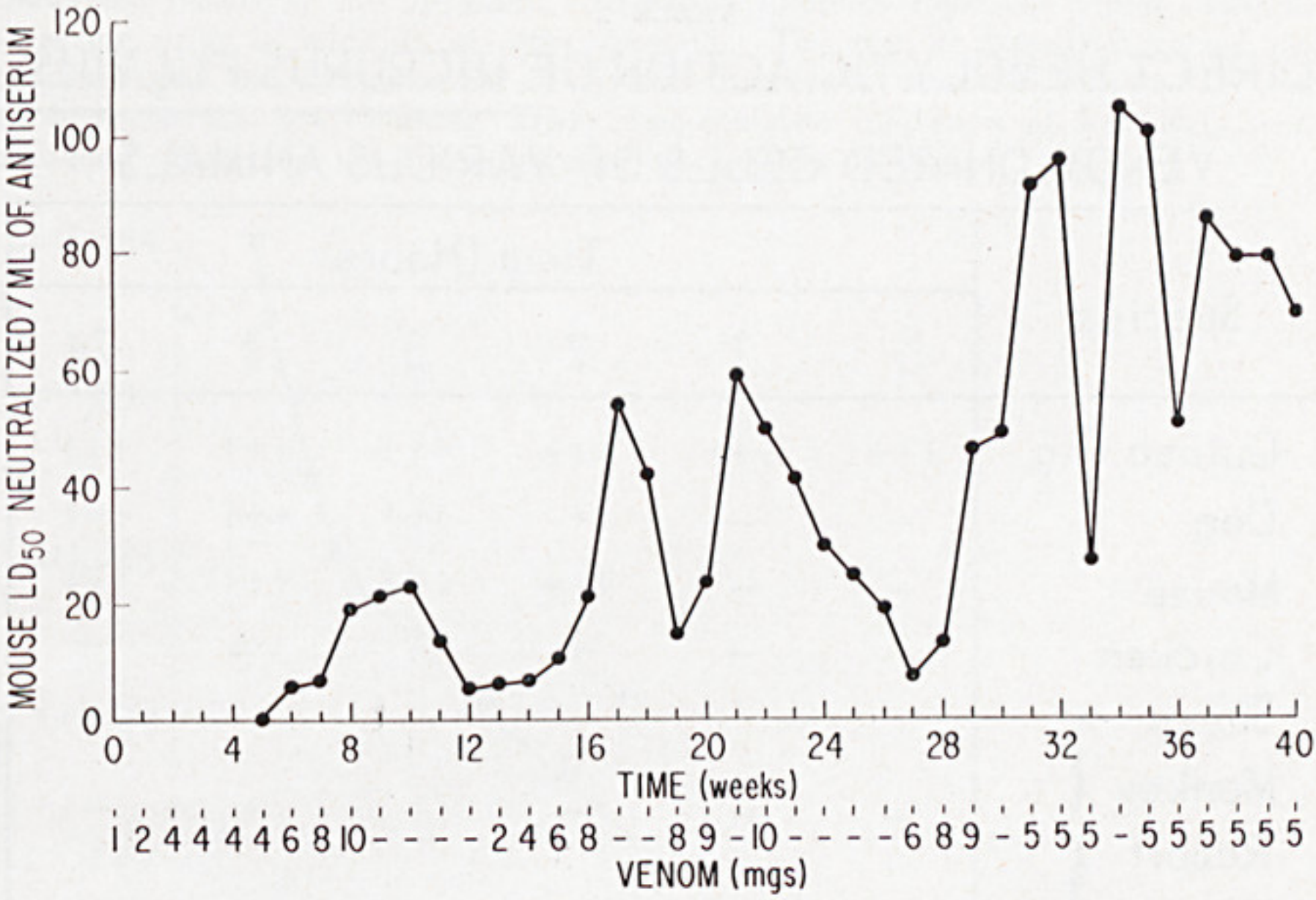


Fig. 2 — Production of neutralizing antibody against *Micrurus fulvius* venom in goats.

Effects of *M. fulvius* venom on washed red cells

Coral snake venom, like most of the venoms of the elapid group, has neurotoxic activity. Limited information is available on its other properties. During experiments with mice we observed that when lethal doses of *M. fulvius* venom were given either intraperitoneally, intravenously or intramuscularly there was evidence of either intravascular hemolysis, or damage to the vascular bed usually seen in the form of bloody urine although hemorrhaging through the nostrils has occurred occasionally. These observations led to testing the effects of *M. fulvius* venom on washed red cells of various animal species.

Three to five day old red cells were washed three times in physiologic saline and resuspended to two percent. Venom was dissolved in physiologic saline in a concentration of 200 μg/ml and 0.5 ml of venom added to 2.5 ml of the red cell suspension. The tubes were incubated at 37°C and periodically observed for hemolysis of the red cells. Table 1 shows that red cells of the guinea-pig, dog, mouse, and chicken were lysed but sheep, rabbit, monkey and human cells were unaffected. Guinea-pig red cells were the most sensitive to the effects of the venom. This direct hemolytic activity, found in various snake venoms (3-5), is distinct from the lytic action of phospholipase A which is also widely found in snake venoms, including coral snake venom. Phospholipase A does not lyse washed red cells but acts indirectly by catalyzing the reaction in which phospholipids, such as lecithin, are converted to lytic substances, like lysolecithin, which cause hemolysis. Both the direct hemolytic factor and phospholipase A were found in *M. fulvius* venom. Although washed rabbit and human red cells were unaffected by this venom, in the presence of egg yolk lecithin these red cells were hemolyzed.

TABLE 1

DIRECT HEMOLYTIC ACTION OF MICRURUS FULVIUS VENOM ON RED CELLS OF VARIOUS ANIMALS

Species	Time (Hours)					
	½	1	2	3	4	24
Guinea Pig	+++	+++	+++	+++	+++	+++
Dog	–	–	+	+++	+++	+++
Mouse	–	–	+++	+++	+++	+++
Chicken	–	–	+	+	++	++
Sheep, Monkey, Rabbit, Man	–	–	–	–	–	–

DEGREE OF HEMOLYSIS

– = None
+ = Slight
++ = Moderate
+++ = Complete

Inhibition of direct hemolytic factor by serum

If antibodies specific for the hemolytic factor are produced, then antiserum should inhibit hemolytic activity. Both rabbit coral snake antiserum and normal rabbit serum were inactivated at 56°C for 30 minutes. Equal volumes of venom in a concentration of 320 μg/ml and serum were mixed and incubated with 0.5 ml of 2% washed guinea-pig red cells at 37°C. Both normal serum and antiserum inhibited hemolysis for 24 hours. However, in the control mixture of only venom and red cells hemolysis occurred in 30 minutes. The results indicate inhibition is nonspecific; it is probably not due to antibody since normal serum produced the same result. Normal human serum produced the same inhibitory activity.

Titration of the serum inhibitor

To determine the titer of the factor in normal serum responsible for inhibition of hemolysis by the venom, two-fold dilutions of inactivated, normal rabbit serum were prepared. Equal volumes of venom in a concentration of 200 μg/ml were mixed with the serum dilutions. The venom-serum mixtures were then added to equal volumes of 2% guinea-pig red cells and incubated at 37°C. Figure 3

shows the results of this titration. All serum dilutions represent initial dilutions of serum prior to addition of other reagents. The rate of hemolysis varied depending upon the concentration of serum. Hemolysis occurred between 10 and 24 hours at the 1:4 dilution. There was complete inhibition of hemolysis with undiluted serum and the 1:2 dilution of serum after 24 hours.

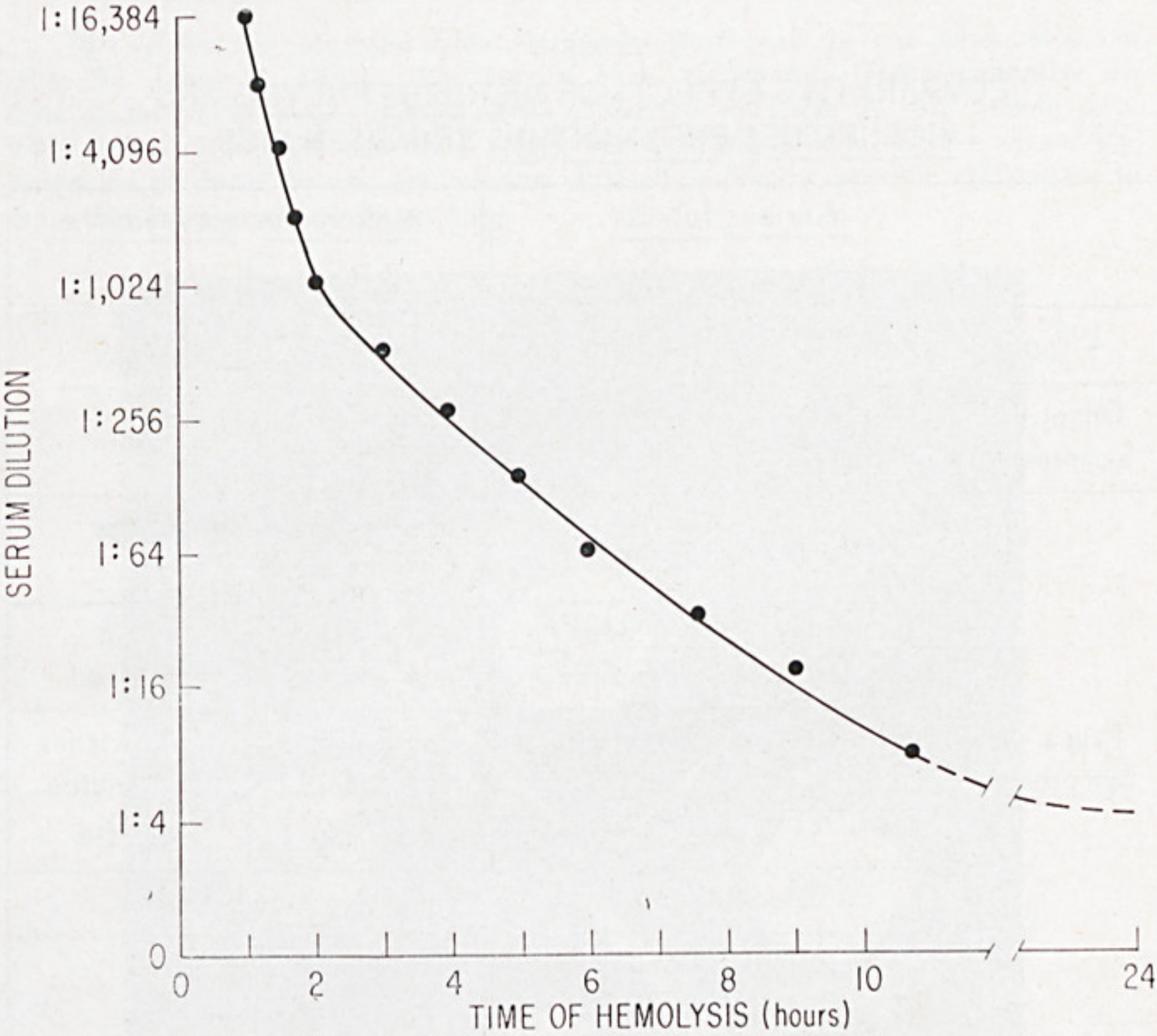


Fig. 3 — Inhibition of the direct hemolytic factor in *Micrurus fulvius* venom by normal rabbit serum.

Inhibitory activity of specific serum fractions

Since crude serum inhibited the direct hemolytic factor in the venom, experiments were conducted to determine which serum fractions were associated with inhibitory activity. Gamma-globulins (Human Fraction II), beta-lipoproteins (Human Fraction III-0), alpha and beta-globulins (Human Fraction IV-4) and bovine albumin were obtained from a commercial source. Saline solutions of these fractions were prepared in concentrations of 10 mg/ml. Two-fold dilutions of the fractions were mixed with equal volumes of venom in a concentration of 200 μg/ml and this mixture was incubated with guineas-pig red cells and observed for 24 hours. Normal human and rabbit sera were tested with these two fractions. The results indicate that the alpha and beta-globulins did not prevent hemolysis,

but at dilutions of 1:2 and 1:4 hemolysis was incomplete. Albumin completely inhibited hemolysis at dilutions of 1:2 through 1:8. Both of these fractions produced hemolysis when undiluted. The reason for this is nuclear. However, it may be due to an osmotic effect or the interaction of venom with the fractions resulting in the release of a lytic agent. The controls of serum fractions plus cells without venom produced slightly darkened cells but no hemolysis.

TABLE 2

COMPARATIVE EFFECTS OF MICRURUS FULVIUS AND MICRURUROIDES EURYXANTHUS VENOMS IN MICE

	Micrurus fulvius		Micruroides euryxanthus	
	I.P.	I.V.	I.P.	I.V.
LD_{50}	13 μg	7 μg	26 μg	18 μg
Onset of Symptoms	5 min.	3 min.	15 min.	3 min.
Types of Symptoms	Slight, labored movement		Immobile – Rear legs appear paralyzed	
	Skin color near normal	Skin deep red	Skin color near normal	Skin deep red
	Death within 4-12 hours at LD_{50}	Death within 3-6 hours at LD_{50}	Death within 1-2 hours at LD_{50}	Death within 45 minutes at LD_{50}
	Bloody urine		No bloody urine	
	Prolonged, labored respiration		Rapid respiration; less labored	

General properties of *M. fulvius* venom

The heat stability of the venom was determined by placing tubes containing *M. fulvius* venom at a concentration of 100 μg/ml of saline in a boiling water bath. Tubes were removed at various times and 0.5 ml (50 μg) injected intraperitoneally into 16-18 g mice. All mice which received venom boiled for 20 minutes died. Mice which received venom boiled as long as 1 hours developed slight signs of distress but recovered. There results indicate that the lethal component in *M. fulvius* venom is highly resistant to heat.

On the assumption that the lethal component is primarily a protein the venom was treated with trypsin. Venom was used in a concentration of 100 μg/ml and crude trypsin was added to the venom to give a final concentration of 1% trypsin. The mixture was incubated 1 hour at 37°C and mice received 0.5 ml (50 μg)

intraperitoneally. Six of seven mice which received the trypsin-treated venom survived. Control mice which received only venom died and those receiving trypsin survived.

Comparative effects in mice and serologic relationship of *Micrurus fulvius* (MF) and *Micruroides euryxanthus* (ME) venoms

Me venom was obtained from extractions performed in our laboratory and from Dr. James R. Dixon, New Mexico State University. The comparative effects of the two venoms in mice are summarized in Table 2. MF venom gave lower LD_{50} values although mice which received ME venom died sooner. The symptoms produced by the two venoms differed indicating possible differences in the lethal components of the venoms.

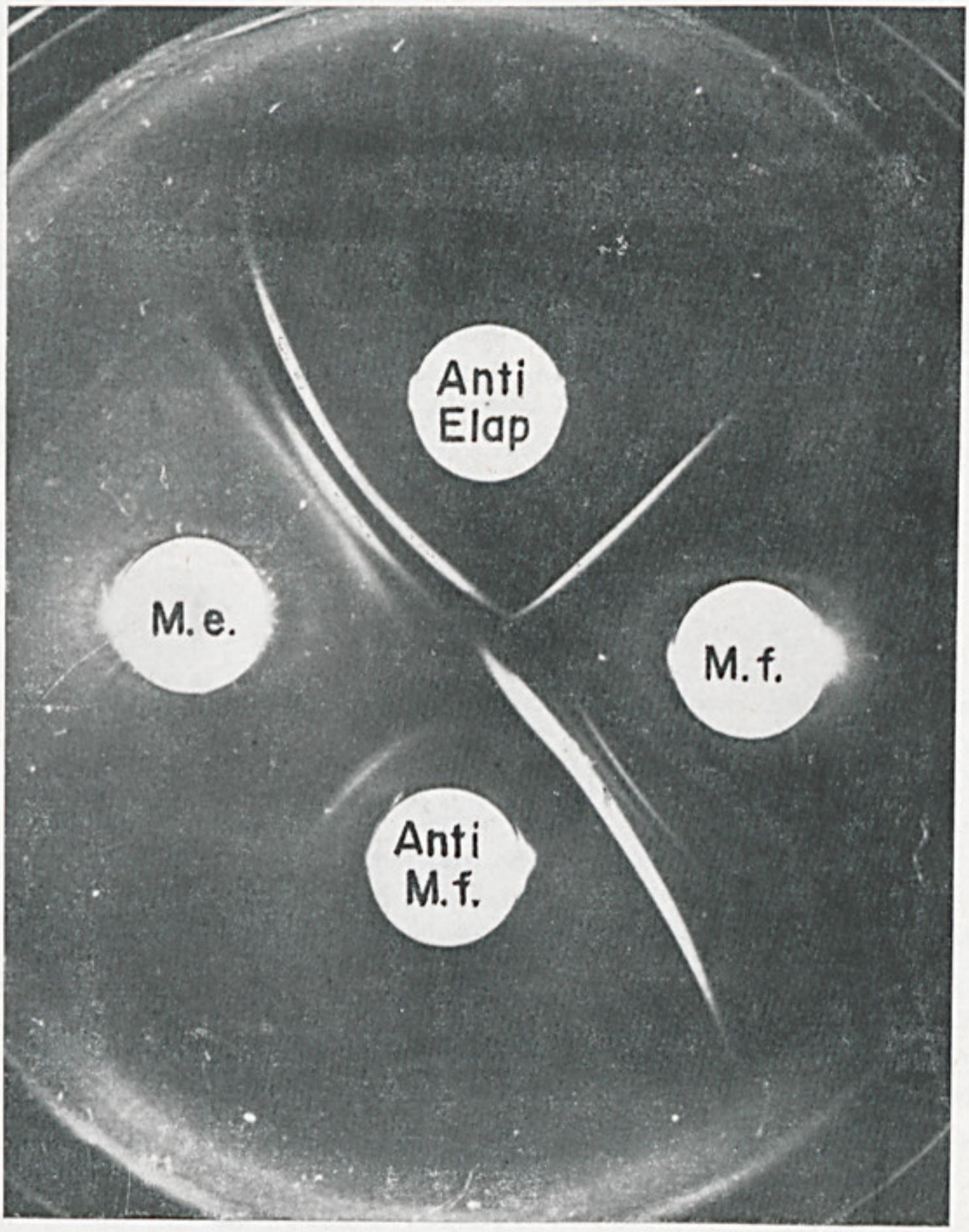


Fig. 4 — Gel-diffusion reaction of *Micrurus fulvius* and *Micruroides euryxanthus* venom. Anti Elap = Sôro Antielapidico. Anti M. f. = Anti *M. fulvius* serum. M. e. = *Micruroides euryxanthus* venom. M. f. = *Micrurus fulvius* venom.

A cross-neutralization test was performed to determine if rabbit anti-MF serum would neutralize ME venom. The test procedures used were previously described (2). Both venoms were used in concentrations of 7.7 LD_{50}/ml. Neither neutralization nor precipitation occurred in the heterologous reaction although both occurred in the homologous MF control reaction. Insufficient ME venom has been collected to produce antiserum and perform the reciprocal cross-neutralization test.

The relationship of the two venoms was also examined by gel-diffusion tests. The venoms were reacted against pooled, rabbit anti-MF serum and against Sôro Antielapídico (Instituto Butantan) produced with South American coral snake venoms. Figure 4 is a photograph of the results of the reactions after 3 days. The MF venom was used in a concentration of 100 μg/ml and the ME venom was freshly collected on a filter paper disc and eluted immediately with saline; the concentration was unknown. The venoms have at least two common antigens. This is based on the strong reactions of identity formed by both venoms with Sôro Antielapídico and the fact that ME venom produced at least two faint bands with MF antiserum. One of these faint bands appears to have formed a reaction of partial identity with one of the bands produced in the homologous MF reaction. This reaction of partial identity suggests the two venoms have some common determinants although there are other antigenic differences. The strong reactions of both venoms with Sôro Antielapídico indicate a serologic relationship between the coral snake venoms of North and South America. The formation of several bands between ME venom and Sôro Antielapídico serum and the presence of only one band in the reaction of MF with this serum may be due to concentration since the actual amount of ME venom used was unknown. However, it may actually indicate that ME venom is more closely related, serologically, to the venoms of South American coral snakes than MF venom.

Summary

Immunologic and serologic studies were conducted on venoms of the two species of coral snakes found in the United States. Antiserum produced in goats gainst *M. fulvius* venom was capable of neutralizing 105 mouse LD_{50}/ml: *M. fulvius* venom contains a direct hemolytic agent. Of the serum fractions examined, inhibitory activity was found in the alpha and beta-globulins and albumin fractions. The lethal component in *M. fulvius* venom is heat stable and susceptible to the action of trypsin. *M. fulvius* and *Micruroides euryxanthus* venoms have at least two antigenic components in common and are serologically related to South American coral snake venoms. However, *Micruroides euryxanthus* venom was not neutralized by antiserum specific for *M. fulvius* venom. *M. fulvius* venom was more toxic and caused *in vivo* hemolysis in mice. *Micruroides euryxanthus* venom did not produce hemolysis but killed mice more rapidly at the LD_{50} dose.

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