Mem. Inst. Butantan, 29:143-163. 1959.

COAGULANT AND FIBRINOLYTIC ACTIVITY OF ANIMAL VENOMS; DETERMINATION OF COAGULANT AND FIBRINOLYTIC INDEX OF DIFFERENT SPECIES (*) (**)

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The existence of different activities in snake venom such as blood-coagulating, proteolytic, hemolytic and neurotoxic was recognized for a long time by many researchers. Noc had concluded in 1904 (7), by "in vitro" experiments, that the venom anticoagulant activity was a consequence of its proteolytic activity. By adding venoms to oxalated plasma, he observed that some of them, after having coagulated the plasma, provoked lysis of the clot and this lysis was proportional to the venom concentration. Other venoms inhibited the coagulation of plasma and no posterior clotting could be obtained. In the first case proteolytic enzymes lysed fibrin while, in the latter, these enzymes decomposed fibrinogen before its transformation to fibrin. However in 1909, Vital Brazil and Pestana (1) by experiments on the same subject were able to destroy the proteolytic activity of *Bothrops jararaca* venom on gelatine, by heating 100° C, without damaging the coagulant activity. They inferred that no parallelism existed between the proteolytic and the coagulant activity. This conclusion was confirmed by the observation that some coagulant venoms such as that of *Crotalus durissus terrificus* did not have proteolytic activity on gelatine (1). Besides, these two properties did not keep the same relation in venoms of different species.

The dissociation between the coagulant and the proteolytic action was substantiated by Houssay and Negrete in 1918 (5), who considered these two properties to be distinct from the aglutinant, toxic and hemolytic actions.

(*) This research was done with the aid of National Council of Research (CNPq) and Research Fund of Instituto Butantan.

(**) Presented to the "Symposium on Animal Venoms" of the Brazilian Society of Advancement of Sciences, at the 10th annual meeting of July, 8, 1958.

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These authors (5) also confirmed the lack of parallelism between both activities in different venoms, since they found that the neutralization of these activities by antivenom serum followed different ways. They observed as well non parallel differences in these venom properties in venoms of related species. In 1928 Vital Brazil and Vellard (2) found that this dissociation between coagulant and proteolytic properties was also valid for the venom of *Agkistrodon contortrix* and other species. In fact, heating at 75° C destroyed the proteolytic activity of these venoms without affecting their coagulant activity.

In conclusion, the knowledge of these facts lead to assume that the proteolytic fractions were distinct from the coagulant ones, while the anticoagulant effect was merely a consequence of the proteolytic fractions provoking fibrinogenolysis (before coagulation) or fibrinolysis (after coagulation).

Eagle (3) compared snake venoms having coagulant activity to two proteolytic enzymes having such activity: trypsin (thromboplastic activity-transformation of prothrombin to thrombin) and papain (thrombinic activity-transformation of fibrinogen to fibrin). He observed also that the venom proteolytic activity on gelatine was directly proportional to its ability to coagulate or destroy fibrinogen. Thus the author (3) found reason to believe that the coagulant properties would depend on the venom proteolytic enzymes.. This view has been supported by Rocha e Silva (9), who admitted a close analogy between the coagulant effect of snake venoms and the proteolytic enzymes; in consequence, he (9) attributed all effects of snake venoms on blood coagulation to their proteolytic enzymes.

Thus, the almost established notion about the existence of two different substances changed towards the opposite view, *i.e.*, that the coagulant power is merely a consequence of the proteolytic activity. The purpose of this paper was to investigate the parallelism between coagulant and proteolytic activity of snake venoms and of some other species of poisonous animals and to look for an eventual correlation between the appearance of necrotic lesions observed in patients accidentally bitten by poisonous animals of different species and these enzymatic properties of the venoms.

MATERIAL AND METHODS

Venoms. Tests were carried out using desiccated venoms of the Instituto Butantan stock. The venoms were dissolved in sodium chloride 0,85% just before the use.

Standard Venom. The venom of *Bothrops jararaca* was arbitrarily chosen as standard for the coagulant and fibrinolytic index determination, since it is easily available and has the two activities in very high degree. This venom was extracted from a number of snakes by squeezing their glands; the venom obtained was immediately vacuum-dried at room temperature. Afterwards it was stored dry in the dark. The standard is variable under such conditions but it could be better compared to venoms already existing at the Institute

which are obtained by this method. Each time a determination was carried out with a different venom, the results were compared to the activity of the standard measured on the same day and under the same experimental conditions. Under these conditions, the index indicates really the relative power degree of venoms, but the absolute values should not be considered.

Coagulant activity. Coagulation tests were carried out by Laki's method (6) slightly modified. Fresh oxalated horse plasma (2 mg of potassium oxalate per ml of blood) was used. Determinations were made on an excavated plexiglass plate, maintained in a water-bath at 37°C, using equal volumes of plasma and venom solutions (0,2 or 0,1 ml). All determinations were made in duplicate.

In order to compare all venoms the coagulating index (*CI*) of the sample analysed was calculated. This is defined as the ratio:

$$CI = \frac{C_s}{C_t}$$

cn which C_t and C_s are respectively the concentrations (mg/ml) of the sample analysed and that of the "standard" venom which produce plasma-clotting in 120 seconds. In all ases the values of C_t and C_s were obtained graphically from the respective curve drawn by plotting the logarithm of the concentration of venom and the logarithm of the corresponding clotting time. The data to draw both graphs were obtained by experiments made concomittantly. At the zone of 120 seconds a straight line was obtained for all venoms, (fig. 1) this being the reason why the period of 120 seconds was chosen.

Fibrinolytic Activity. The fibrinolytic activity of different venoms was determined by measuring the remaining fibrin by Quick's method (8) after the action of 1 ml of venom solution during 60 minutes at 37°C on the washed fibrin clot. This clot was obtained from 1 ml of oxalated horse plasma (2 mg of potassium oxalate /ml of blood) diluted with 17 ml of distilled water, recalcified with 2 ml of calcium chloride M/10. The resulting fibrin, was determined spectrophotometrically by its tyrosine content. The fibrinolysis curves were made with absolute quantities of fibrin lysed by different proportions of venom.

The fibrinolytic index (*FI*) was calculated as the relation between the amount of fibrin in mg lysed per mg of problem venom and of standard venom (*B. jararaca*):

$$FI = \frac{\text{mg of fibrin lysed by 1 mg of problem venom}}{\text{mg of fibrin lysed by 1 mg of standard venom (}B.\ jararaca\text{)}}$$

RESULTS

Coagulant Activity. Clotting time data of *B. jararaca* venom in several dilutions were plotted on a log-log scale. A. "diphasic" curve was obtained (curve 1 of fig 1), with unheated venom of *B. jararaca,* in the sense that high concentrations of the venom, such as 10 mg/ml, did not provoke coagulation; as dilution increases the clotting times become shortened up to an optimum, after which the clotting time rises with the increase of the dilution. It is likely that this "diphasic" curve is due to the interaction of the venom proteolytic and coagulant actions. In high concentrations (10 mg/ml) the venom digests fibrinogen before it is transformed to fibrin (later addition of thrombin does not clot the plasma). Clotting appears by

adding less concentrated venom (5mg/ml) but is immediately followed by lysis of the clot. The proteolytic activity decreases with increasing dilution since this property is weaker than the coagulant one, thus the coagulant activity appearing more neatly. This explains the "zone phenomenon" described by Rocha e Silva (10) with *Bothrops atrox* venom.

This hypothesis can explain the regular "non diphasic" curves obtained with venoms having very weak fibrinolytic activity and relatively evident coagulant action as that of *Crotalus d. terrificus* (white venom) (curve 4 of fig. 1). It also explains the reason why *B. jararaca* venom gave a regular curve after having been heated at 63° C during 5 minutes in M/15 acetate buffer solution, pH 5.0, showing almost absence of another factor (curve 6 of fig. 1). Heating destroys the greatest part of proteolytic enzymes. But a remainder of these substances was evidenced by the small inflexion of the curve and the fibrinolytic capacity when high concentration was used.

At the point where dilutions provoked clotting in 120 seconds, the curves of different venoms were all regular, *i.e.*, the venoms were sufficiently diluted to avoid the proteolytic activity. Therefore this was the clotting time used for determination of the coagulant index (table 1, 2 and 5).

All bothropic venoms and that of *Lachesis muta muta* showed the *B. jararaca* venom curve type. Only the activity varied between the different species (fig. 2). While these venoms showed a "diphasic" curve when a concentration of 1 mg per ml was used, the same concentration of *Crotalus* venoms did not show "diphasic" curves, in spite of having almost the same coagulant activity as some bothropic venoms. With the *Crotalus* venoms a "diphasic" curve was obtained only with concentration of the order of 10 mg/ml indicating a very weak proteolytic activity in these venoms.

The coagulation curves permitted to separate the venoms in 3 groups according to the following criteria:

I — Lack of coagulant activity on oxalated plasma even when there was a certain proteolytic action (*Agkistrodon, Micrurus,* table 5).

II — a markedly coagulant activity with small proteolytic action, giving a "non diphasic", practically straight curve (*Crotalus,* fig. 1).

III — marked coagulant and proteolytic activities showing "diphasic" curves (*Bothrops, Lachesis,* fig 2).

Results obtained with venom of young and adult *B. jararaca* showed a marked difference of coagulant activity, this action being higher in the venom of young snakes (curve 4 and 5 of fig. 3), giving a coagulant index as high as 3,92 (table 2).

The influence of ageing on the coagulant property of the venom was observed comparing recent venom used right after having been vacuum dried,

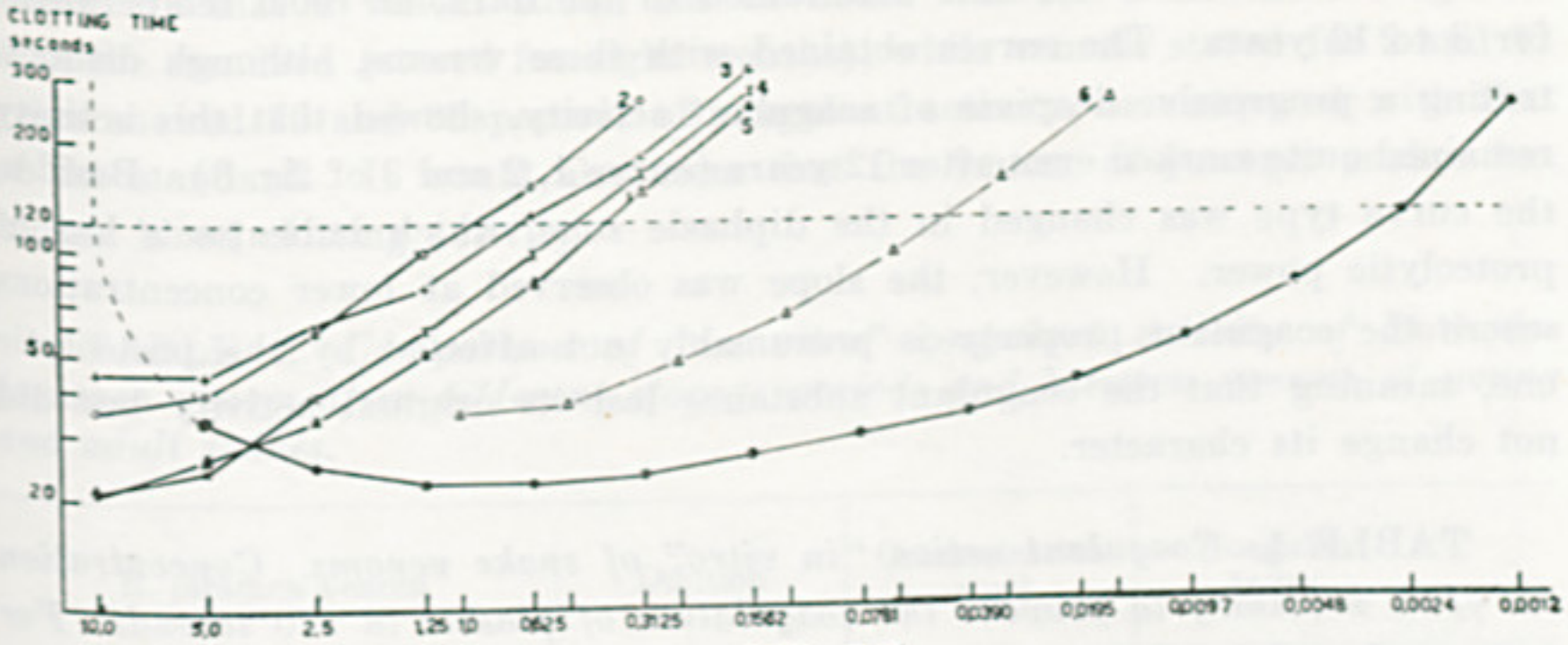

Fig. 1. Relation between venom concentration and plasma clotting time, on log-log scale, obtained with venoms of *B. jararaca* (standard) curve 1; *C. durissus terrificus* (Marajó island) curve 2; *C. durissus durissus* curve 3; *C. durissus terrificus* (white venom) curve 4; *C. durissus terrificus* (yellow venom) curve 5; *B. jararaca* fractionated by heating at 63°C, 5 minutes, pH5.0 curve 6.

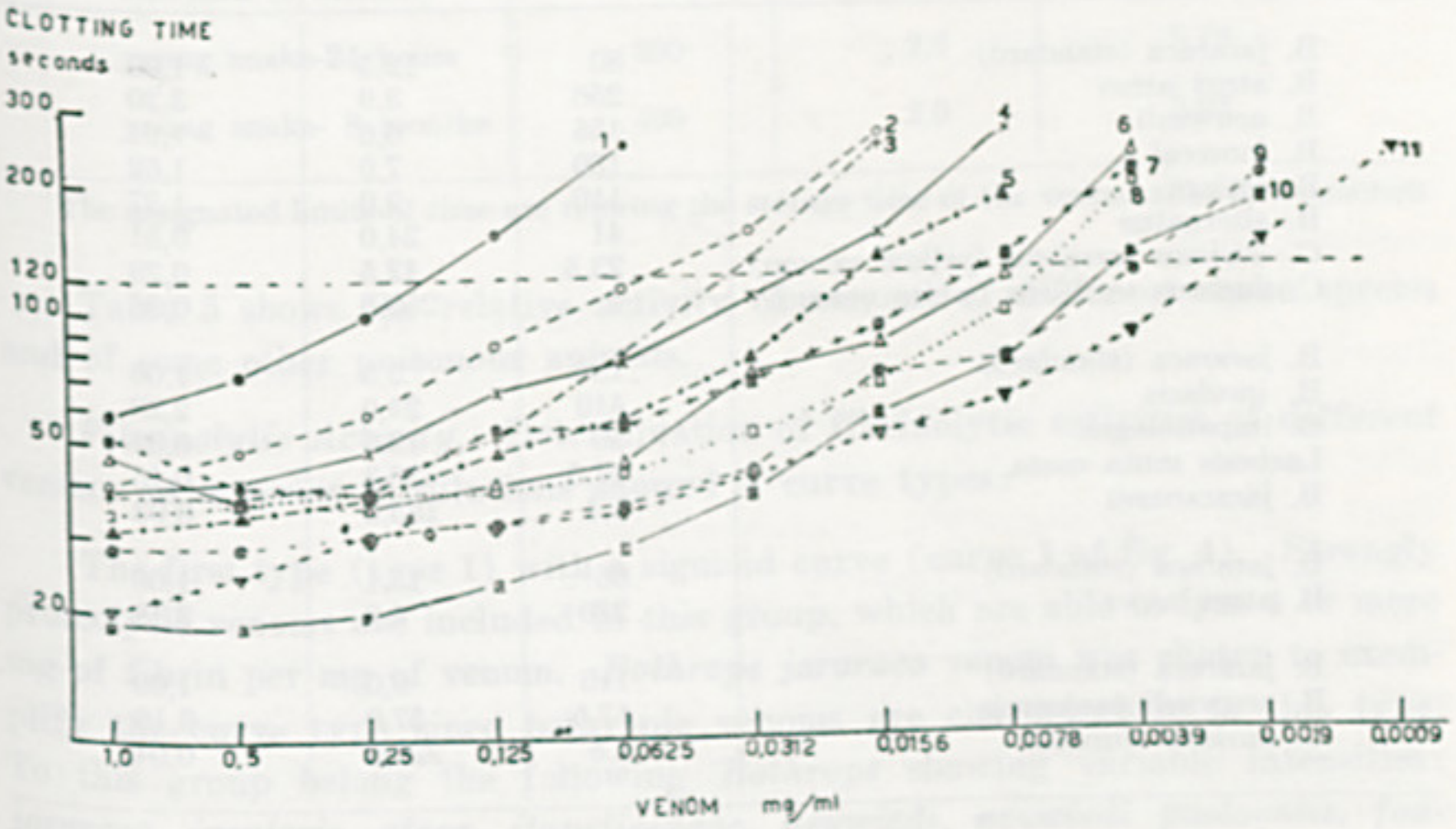

Fig. 2. Relation on log-log scale between venom concentration and plasma clotting time obtained with venoms of many Bothrops species and Lachesis: *B. jararacussú* curve 1; *B. neuwiedi paoloensis* curve 2; *Lachesis muta muta* curve 3; *B. alternatus* curve 4; *B. itapetiningae* curve 5; *B. fonsecai* curve 6; *B. cotiara* curve 7; *B. neuwiedi* curve 8; *B. atrox atrox* curve 9; *B. atrox asper* curve 10; *B. insularis* curve 11.

to dry venoms stored in flasks maintained in the dark, at room temperature, for 3 to 12 years. The curves obtained with these venoms, although demonstrating a progressive decrease of coagulant activity, showed that this activity remained quite marked even after 12 years (curve 1, 2 and 3 of fig. 3). Besides, the curve type was changed in the diphasic zone, which indicates a loss of proteolytic power. However, the slope was observed at lower concentrations where the coagulant property is presumably not affected by the proteolytic one, meaning that the coagulant substance lost its original activity but did not change its character.

TABLE 1. *Coagulant action "in vitro" of snake venoms. Concentration in. γ/ml necessary to provoke the coagulation of plasma in 120 seconds. For tests perfomed in different days the value of standard venom* (B. jararaca) *in the same day was used as reference.*

Clotting time — 120 seconds

Venom	Dilution	Concentration γ/ml	Coagulant Index
B. jararaca (standard)	.80	12,5	1,00
B. atrox atrox	.256	3,9	3,20
B. neuwiedi	.155	6,6	1,94
B. fonsecai	.130	7,6	1,62
B. cotiara	.110	9,0	1,37
B. alternatus	.41	24,0	0,51
C. durissus terrificus (yellow venom)	.23,5	42,5	0,29
C. durissus terrificus (white venom)	.4	250,0	0,05
B. jararaca (standard)	.180	5,5	1,00
B. insularis	.410	24,0	2,28
B. itapetiningae	.55	18,1	0,31
Lachesis muta muta	.27,5	36,3	0,15
B. jararacussu	.5,4	185,0	0,03
B. jararaca (standard)	.66	15,1	1,00
B. atrox asper	.210	4,8	3,18
B. jararaca (standard)	.110	9,0	1,00
B. neuwiedi paoloensis	.17,5	57,0	0,16
C. durissus durissus	.4,5	220,0	0,04

Tables 1 and 2 summarize the data presented in figure, 1, 2 and 3 in terms of the blood-coagulating index, already described. It can be seen that only snakes of the genus *Bothrops*, such as *B. neuwiedi, B. fonsecai, B. cotiara, B. insularis, B. atrox atrox, B. atrox asper,* may have stronger blood-coagulating activity than that of the venom of *B. jararaca.* It may also be seen

(table 2) that a venom stored for 24 hours was 2,6 times more coagulant than the standard (stored for eight months), while venoms stored for 2 and 12 years showed 70 and 30 percent of coagulant activity, as compared with that of the standard. It would also seem that the venom of younger snakes has higher blood clotting activity.

TABLE 2. *Comparison of "in vitro" coagulant activity of* Bothrops jararaca *venom after different storage periods and between venoms of young and adult snakes.*

B. jararaca venom	Dilution	Concentration γ/ml	Coagulant Index
24 hours	.240	4,2	2,6
8 months (standard)	.90	11,1	1,0
2 years	.64	15,6	0,7
12 years	.23	43,5	0,3
standard (8 months)	.125	8,0	1,0
young snake-24 hours	.390	2,6	3,12
young snake- 8 months	.490	2,0	3,92

The assignated limits of time are refering the storage time of the venom after its extraction.

Table 5 shows the relative activity of venoms of different snake species and of some other poisonous animals.

Fibrinolytic Activity. Determination of fibrinolytic activities of different venoms in a series of dilutions showed 4 curve types:

The first type (type I) with a sigmoid curve (curve 1 of fig. 4). Strongly proteolytic venoms are included in this group, which are able to lyse 4 or more mg of fibrin per mg of venom. *Bothrops jararaca* venom was chosen to exemplify the curve type since bothropic venoms are characteristic to this type. To this group belong the following *Bothrops* showing variable intensities: *jararaca, insularis, atrox, itapetiningae, neuwiedi, neuwiedi paoloensis, fonsecai, jararacussu,* and also *Trimeresurus flavoviridis, Vipera lebetina, Crotalus durissus terrificus* (yellow venom) and *C. durissus durissus* (fig. 5).

The second type (type II) with an exponential curve (curve 2 of fig. 4), were presented by proteolytic venoms, which were able to lyse 2 mg of fibrin per mg of venom. Characteristic to this type was the venom of *Bothrops*

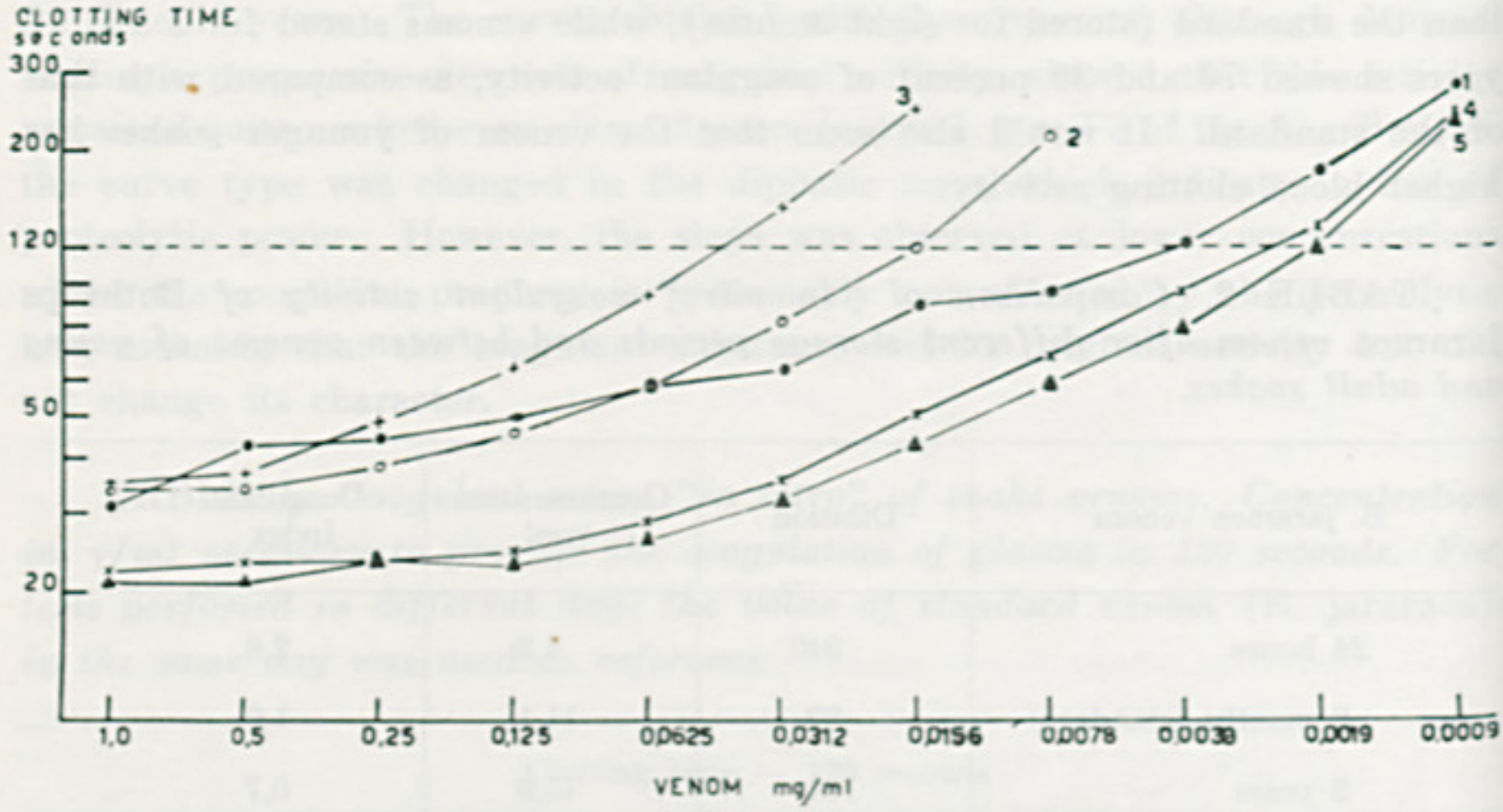

Fig. 3. Influence of the snake age and of ageing of venom on the relation between plasma clotting-time and concentration of *Bothrops jararaca* venom — curves 1,2 and 3 correspond to venoms collected from adult snakes kept respectively for 1 day, 2 and 12 years after extraction. Curves 4 and 5 correspond to venoms collected from young snakes and kept respectively for 1 day and 8 months after extraction.

cotiara belonging also to the group *B. alternatus* and *Lachesis muta muta* venoms (fig. 6).

The third type (type III) with a straight-line relationship between the amount of fibrin hydrolysed and the logarithm of the concentration (curve 3 of fig. 4). The characteristic venom was the one of *Agkistrodon piscivorus* being also included in this group the venom of *Bothrops atrox asper* (Costa Rica) and *Vipera ammodytis montandoni* (fig. 7).

The fourth type (type IV) is that of weakly proteolytic venoms giving the third curve type being however less active (curve 3B of fig. 4). To this group belong the venoms of *Micrurus frontalis, Naja naja, Vipera russellii, Crotalus durissus terrificus* (white venom) and *Crotalus durissus terrificus* of Marajó island (fig 9).

Analysis of fibrinolytic activity curves of *Bothrops jararaca* venom stored for variable periods of time (table 4) showed fundamental differences. The fresh venom, *i.e.*, whose activity was determined right after having been vacuum dried, showed a sigmoid curve of type I (curve 1 of fig. 4, curve 3 of fig 5 and curve 1 of fig. 8). After a little more than 2 months storage it gave

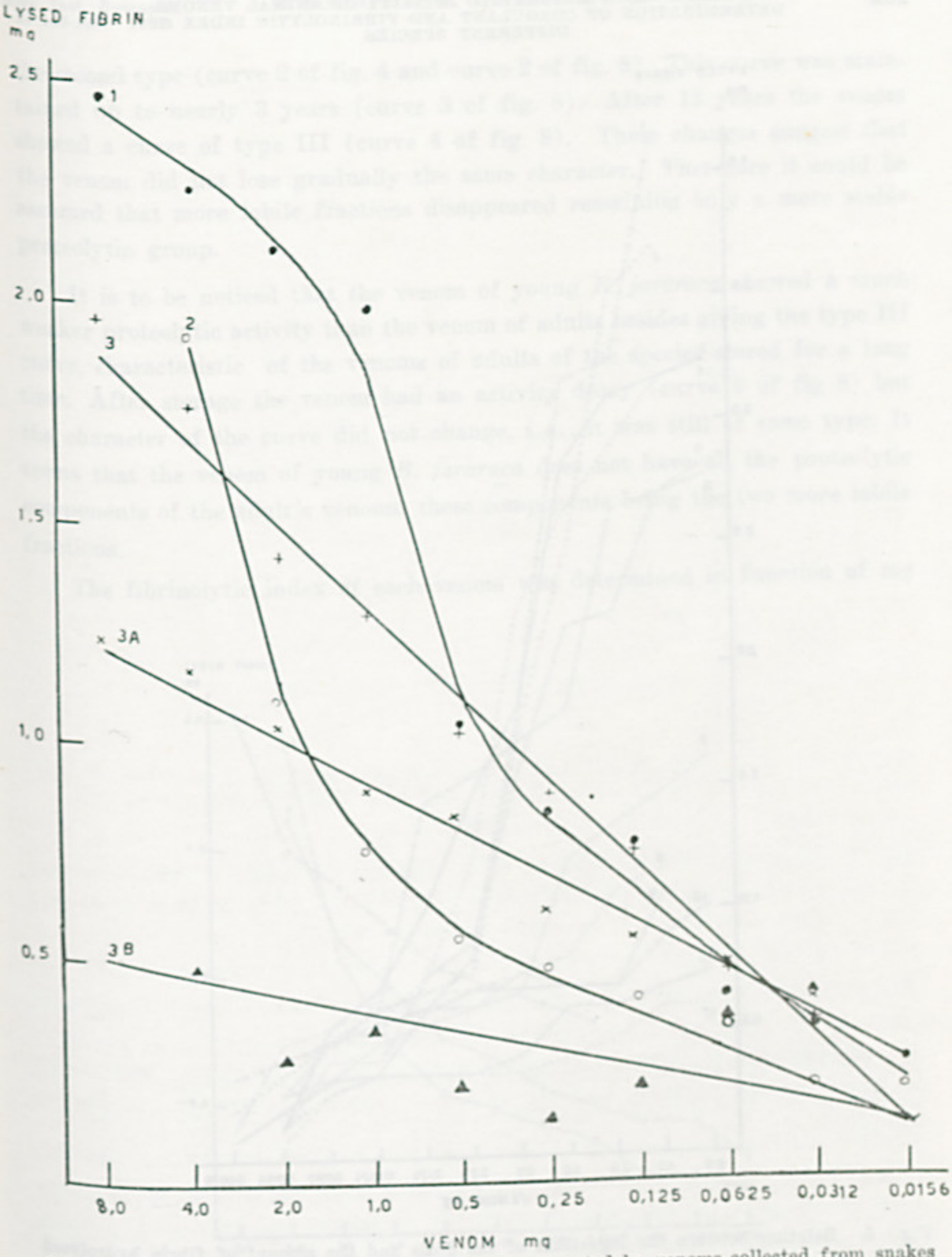

Fig. 4. Types of curves of fibrinolytic activity presented by venoms collected from snakes of different species. Relation between the logarithm of the dosis and the amount of fibrin lysed by the venoms of *B. jararaca* (standard) type I curve 1; *B. cotiara* type II curve 2; *A. piscivorus* type III curve 3; *B. jararaca* stored for 12 years type III curve 3A; *C. durissus terrificus* (white venom) type IV curve 3B.

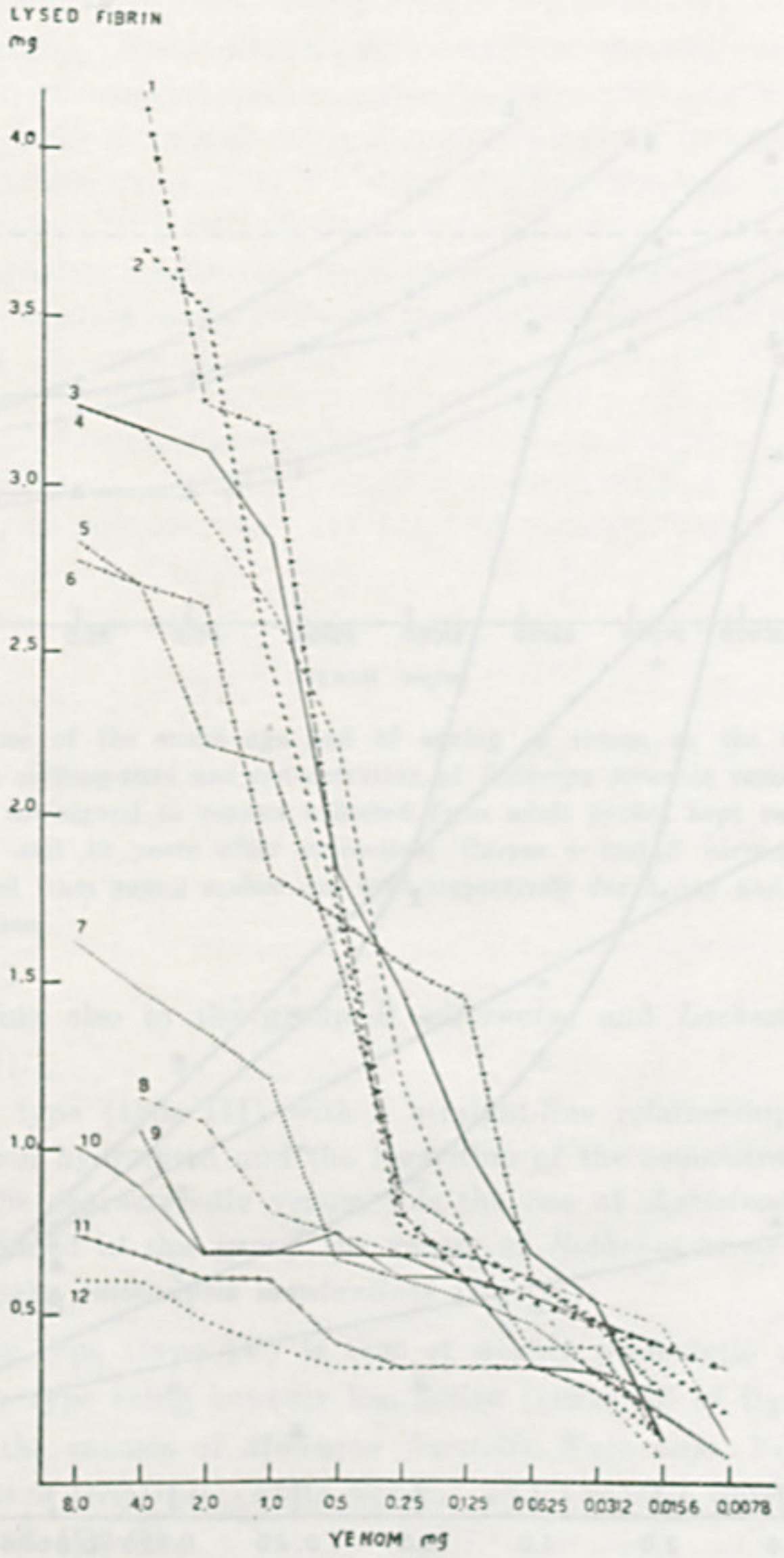

Fig. 5. Relation between the logarithm of the dosis and the amount of fibrin hydrolysed by venoms showing a fibrinolytic curve of type I: *B. atrox atrox* curve 1; *B. neuwiedi* curve 2; *B. jararaca* (standard) curve 3; *B. insularis* curve 4; *B. neuwiedi paoloensis* curve 5; *B. itapetiningae* curve 6; *C. durissus durissus* curve 7; *B. fonsecai* curve 8; *C. durissus terrificus* (yellow venom) curve 9; *V. lebetina* curve 10; *B. jararacussu* curve 11; *T. flavoviridis* curve 12.

the second type (curve 2 of fig. 4 and curve 2 of fig. 8). This curve was maintained up to nearly 3 years (curve 3 of fig. 8). After 12 years the venom showed a curve of type III (curve 4 of fig. 8). These changes suggest that the venom did not lose gradually the same character. Therefore it could be assumed that more labile fractions disappeared remaining only a more stable proteolytic group.

It is to be noticed that the venom of young *B. jararaca* showed a much weaker proteolytic activity than the venom of adults besides giving the type III curve, characteristic of the venoms of adults of the species stored for a long time. After storage the venom had an activity decay (curve 6 of fig 8) but the character of the curve did not change, *i.e.*, it was still of same type. It seems that the venom of young *B. jararaca* does not have all the proteolytic components of the adult's venoms, these components being the two more labile fractions.

The fibrinolytic index of each venom was determined in function of mg

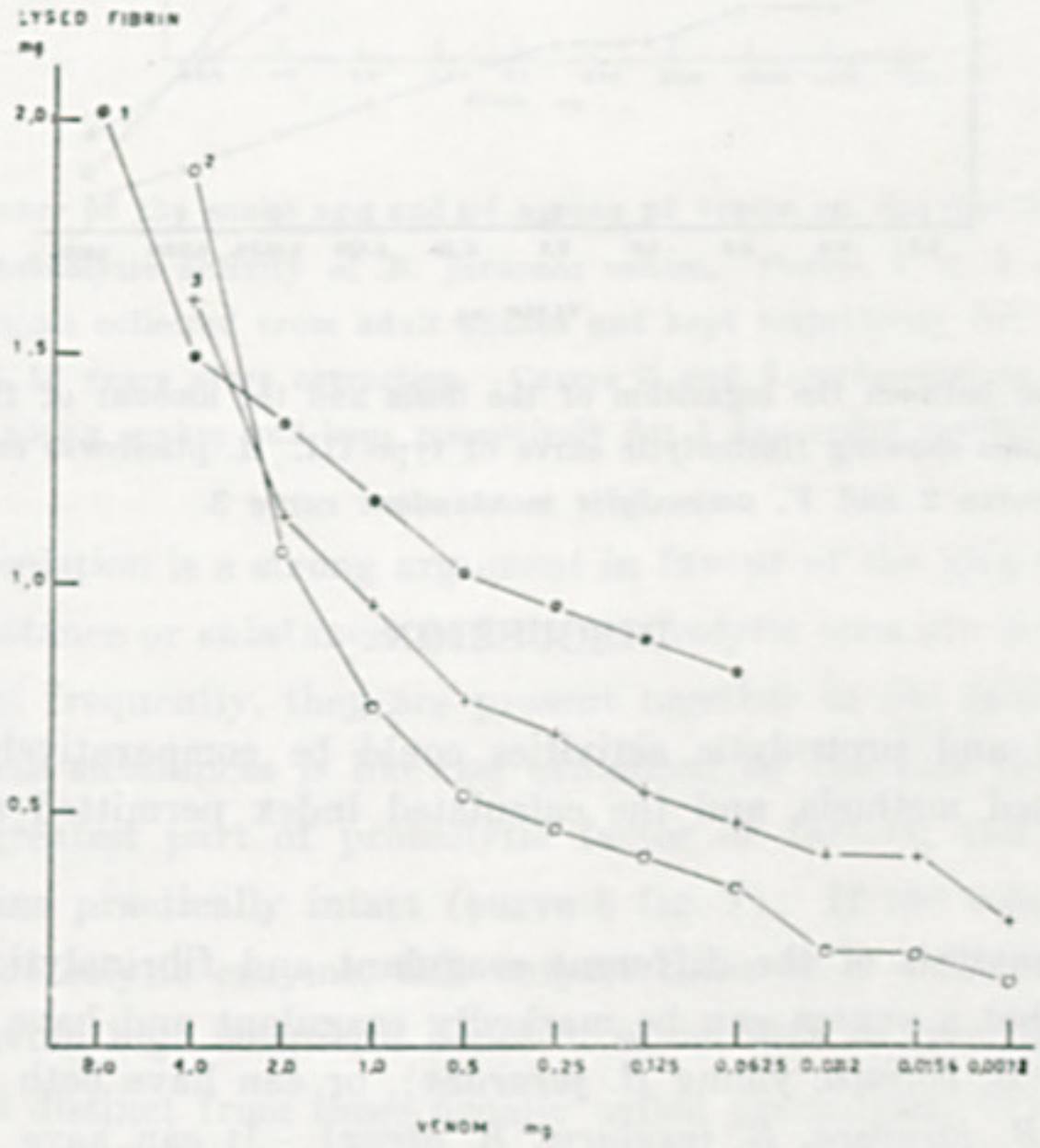

Fig. 6. Relation between the logarithm of the dosis and the amount of fibrin hydrolysed by venoms showing fibrinolytic curve of type II. *L. muta muta* curve 1; *B. cotiara* curve 2 and *B. alternatus* curve 3.

of fibrin lysed by 1 mg of different venoms compared to that lysed by a pooling of *B. jararaca* venom used as standard (table 3, 4, and 5).

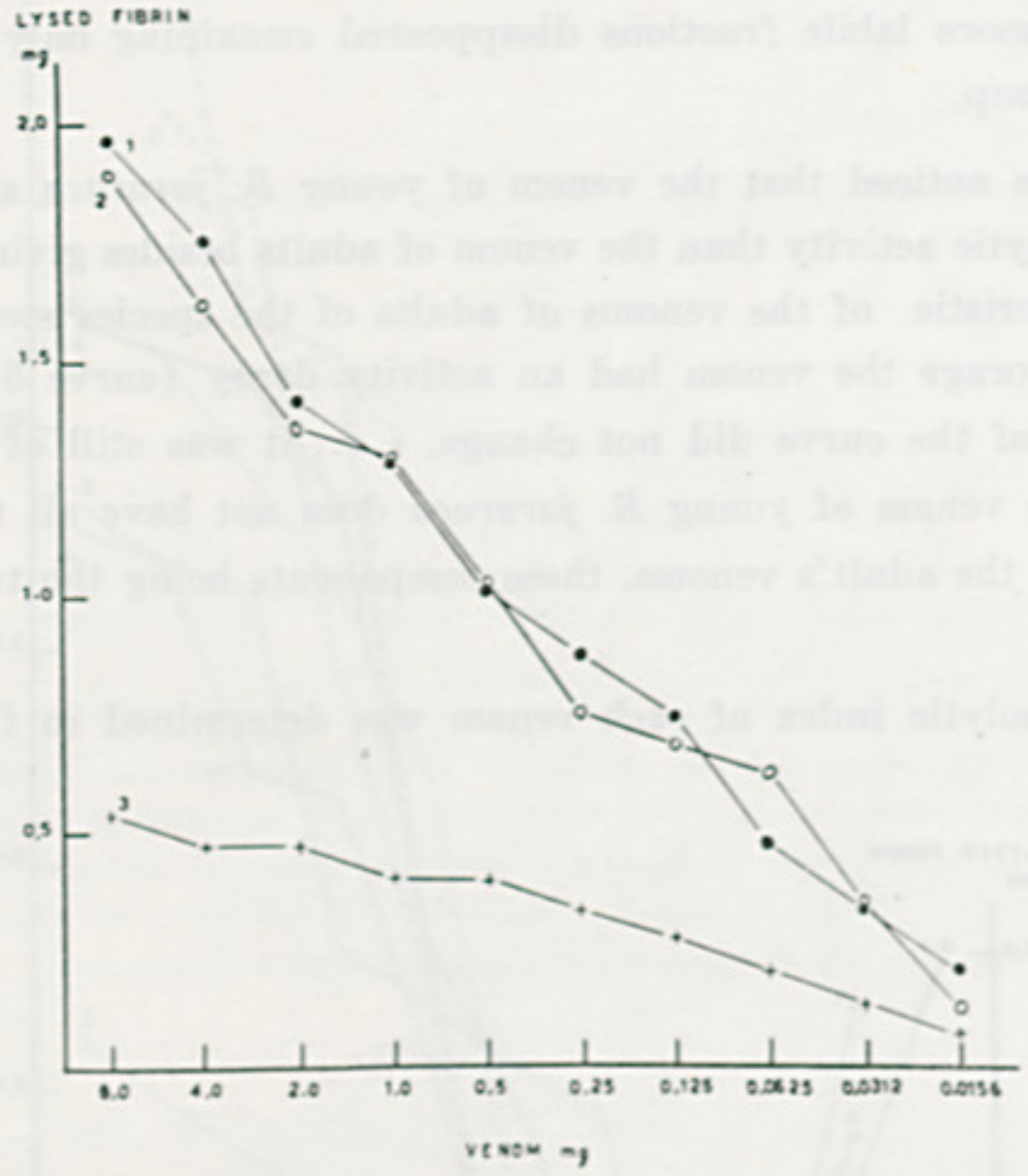

Fig. 7. Relation between the logarithm of the dosis and the amount of fibrin hydrolysed by venoms showing fibrinolytic curve of type III. *A. piscivorus* curve 1; *B. atrox asper* curve 2 and *V. ammodytis montandoni* curve 3.

DISCUSSION

Coagulant and proteolytic activities could be comparatively represented by the described methods, and the calculated index permitted a quantitative appraisal.

A brief analysis of the different coagulant and fibrinolytic index shows very clearly that a venom can be markedly coagulant and have little fibrinolytic activity (*B. cotiara,* young *B. jararaca*), or can have both activities in a high degree (*B. jararaca, B. insularis, B. atrox*). It can have low coagulant activity being however relatively highly proteolytic(*Agkistrodon piscivorus C. d. durissus, B. neuwiedi paoloensis*) or it can be very weak regarding both activities (*C. d. terrificus,* white venom).

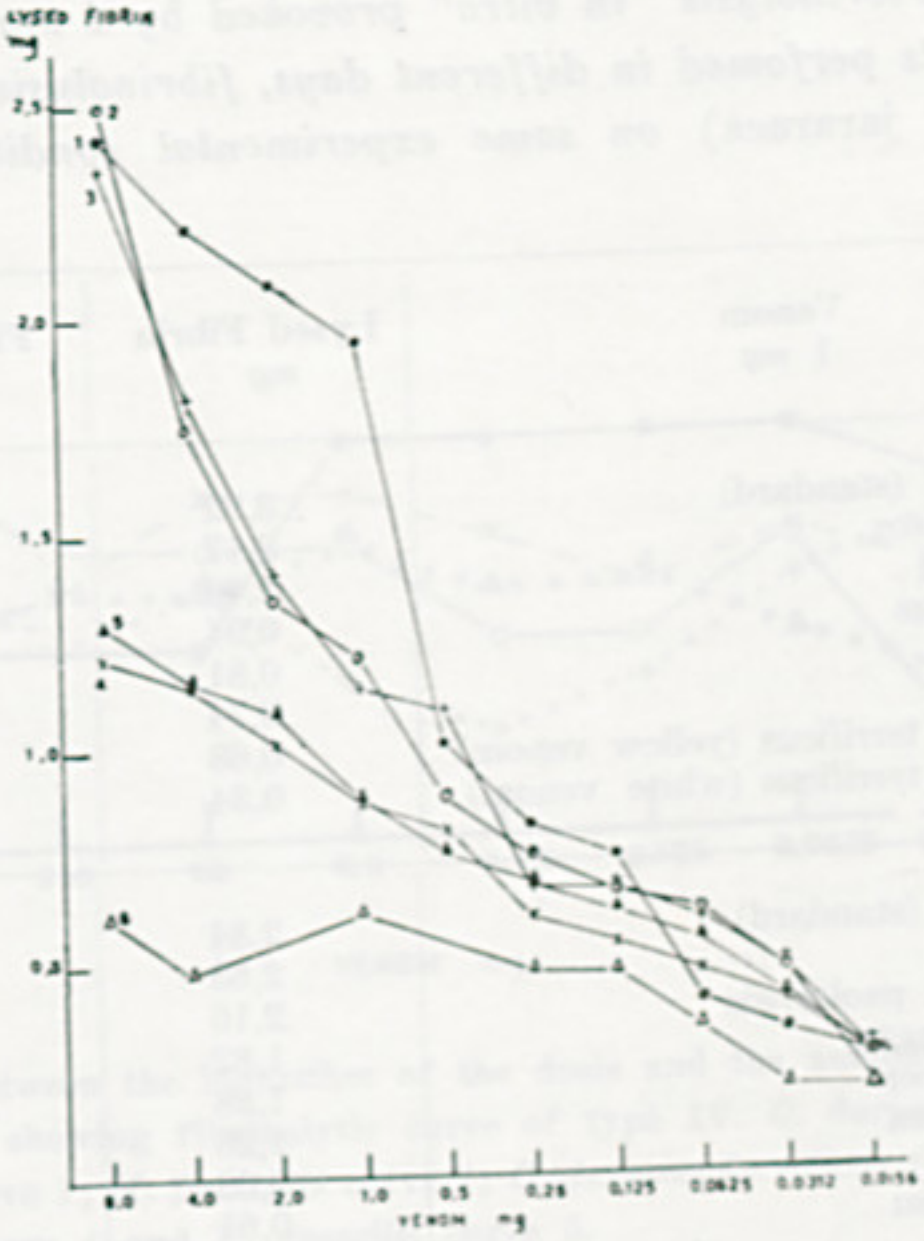

Fig. 8. Influence of the snake age and of ageing of venom on the resulting type of curve of fibrinolytic activity of *B. jararaca* venom. Curves 1, 2, 3 and 4 correspond to venoms collected from adult snakes and kept respectively for 1 day, 8 months, 2 and 12 years after extraction. Curves 5 and 6 correspond to venoms collected from young snakes and kept respectively for 1 day and 8 months after extraction.

This dissociation is a strong argument in favour of the idea that the venom coagulant substance or substances and the proteolytic ones are separate entities, although, most frequently, they are present together in the same venom. The duality of these substances is likewise evidenced by the fact that heating can destroy the greatest part of proteolytic factor or factors, and the coagulant activity remains practically intact (curve 6 fig. 1). If the coagulant activity is due to a proteolytic enzyme, this enzyme must be a different one because even when having high coagulant action it is not able to lyse fibrin. Besides, this enzyme is distinct from those usually called proteolytic. This dissociation already mentioned by Vital Brazil and Pestana (1), Houssay and Negrete (5), Vital Brazil and Vellard (2) Rosenfeld, Hampe and Kelen (13) and others was also confirmed by Henriques, Mandelbaum and Henri-

TABLE 3. *Fibrinolysis "in vitro" provoked by 1 mg of different snake venoms. For tests perfomed in different days, fibrinolysis provoked by standard venom* (B. jararaca) *on same experimental conditions was used as reference.*

Venom 1 *mg*	Lysed Fibrin *mg*	Fibrinolytic Index
B. jararaca (standard)	3,92	1,00
B. atrox atrox	3,17	0,81
B. neuwiedi	2,43	0,62
B. alternatus	0,94	0,24
B. fonsecai	0,81	0,21
B. cotiara	0,74	0,19
C. durissus terrificus (yellow venom)	0,68	0,17
C. durissus terrificus (white venom)	0,34	0,09
B. jararaca (standard)	2,84	1,00
B. insularis	2,63	0,93
B. neuwiedi paoloensis	2,16	0,76
B. itapetiningae	1,82	0,64
B. atrox asper	1,28	0,45
L. muta muta	1,28	0,45
V. lebetina	0,68	0,24
B. jararacussu	0,61	0,21
N. naja	0,47	0,17
T. flavoviridis	0,41	0,14
B. jararaca (standard)	2,50	1,00
C. durissus durissus	1,22	0,49
V. ammodytis montandoni	0,40	0,16
V. russellii	0,34	0,14
B. jararaca (standard)	1,89	1,00
A. piscivorus	1,28	0,68
M. frontalis	0,41	0,22
B. jararaca (standard)	1,22	1,00
C. durissus terrificus (Marajó island)	0,27	0,22

ques (4) who were able to isolate a coagulant fraction from the proteolytic fraction of *Bothrops jararaca* venom.

On the other hand, the coagulant and fibrinolysis curves lead to a qualitative demonstration of new points of view about these two activities in venoms. As it was already stressed, the coagulation curves well demonstrated the interaction of proteolytic and coagulant power, and how dilution permitted to evaluate both activities by means of a simple technique. This method can be useful for choosing the more adequate venom for chemical fractionation of

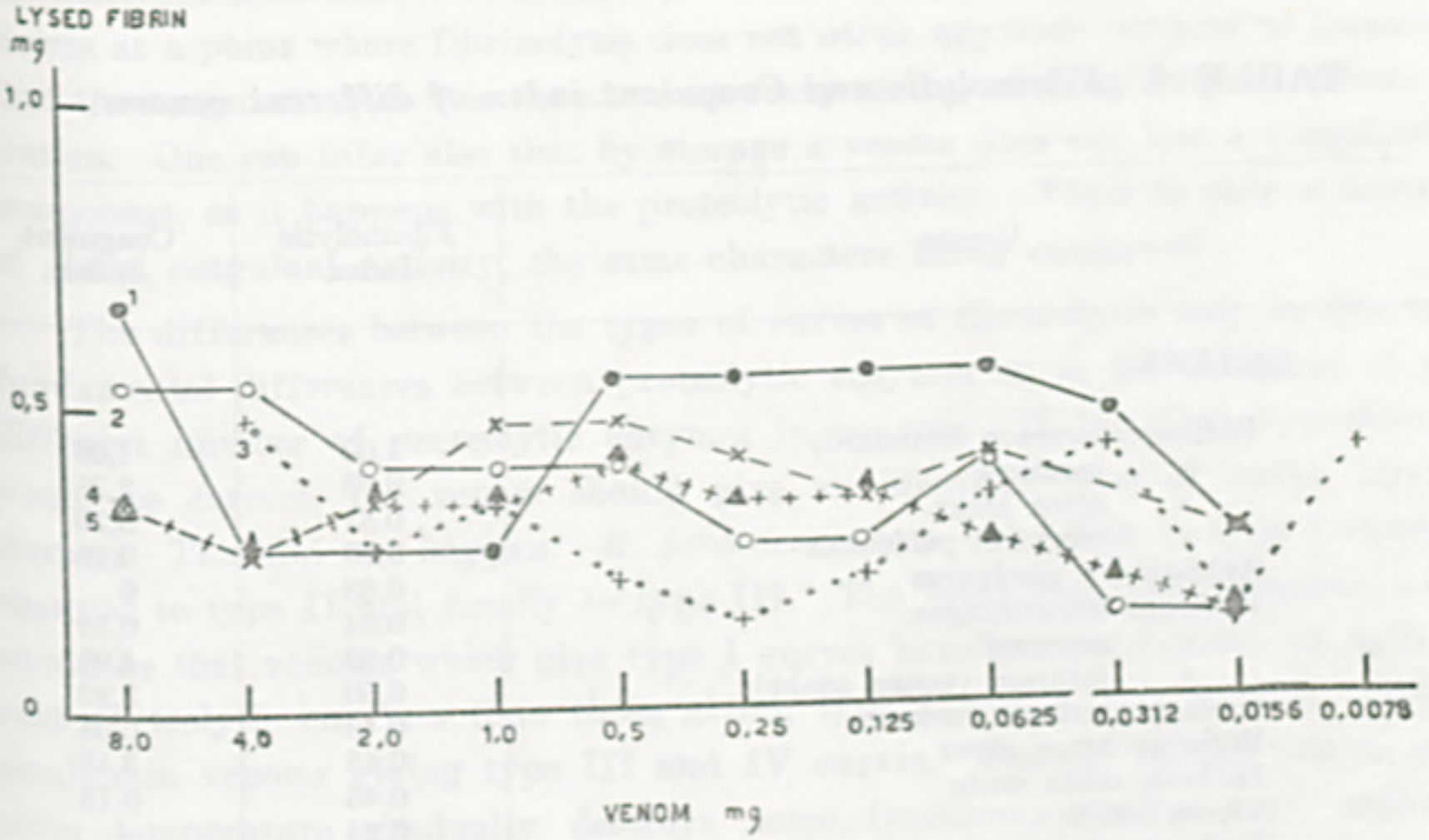

Fig. 9. Relation between the logarithm of the dosis and the amount of fibrin hydrolysed by venoms showing fibrinolytic curve of type IV. *C. durissus terrificus* (Marajó island) curve 1; *M. frontalis* curve 2; *C. durissus terrificus* (white venom) curve 3; *N. naja* curve 4 and *V. russellii* curve 5.

TABLE 4. *Comparison of "in vitro" fibrinolytic activity of* Bothrops jararaca *venom after different storage periods and between venoms of young and adult snakes.*

B. jararaca Venom 1 *mg*	Stored For	Lysed Fibrin *mg*	Fibrinolytic Index
adult snake	24 hours	1,96	1,61
» »	8 months (standard)	1,22	1,00
» »	2 years	1,15	0,94
» »	12 years	0,88	0,72
young snakes	24 hours	0,88	0,72
young snakes	8 months	0,61	0,50

TABLE 5. *Fibrinolytic and Coagulant index of different venoms.*

Venom	Fibrinolytic Index	Coagulant Index
SNAKES		
Bothrops jararaca (standard)	1,00	1,00
» insularis	0,93	2,28
» atrox atrox	0,81	3,20
» neuwiedi paoloensis	0,76	0,16
Agkistrodon piscivorus	0,68	0
Bothrops itapetiningae	0,64	0,31
» neuwiedi	0,62	1,94
» jararaca (young snake)	0,50	3,92
Crotalus durissus durissus	0,49	0,04
Bothrops atrox asper	0,45	3,18
Lachesis muta muta	0,45	0,15
Vipera lebetina	0,24	+
Bothrops alternatus	0,24	0,51
Micrurus frontalis	0,22	+
Crotalus durissus terrificus (Marajó island)	0,22	0,03
Bothrops jararacussu	0,21	0,03
» fonsecai	0,20	1,62
» cotiara	0,19	1,37
Crotalus durissus terrificus (yellow venom)	0,17	0,29
Naja naja	0,17	0
Vipera ammodytis montandoni	0,16	0
Trimeresurus flavoviridis	0,15	0
Vipera russellii	0,14	0
Crotalus durissus terrificus (white venom)	0,09	0,05
SCORPIONS		
Tityus bahiensis	0,17	+
Tityus serrulatus	0,11	0
SPIDERS		
Phoneutria fera	0,25	0
Lycosa erythrognata	0,16	+
FROGS		
Bufo marinus	0,27	+
BEE		
Apis mellifica	0,17	+

+ Clotting only after 12 hours contact

0 Absence of clotting

the different elements. The similarity in the slope of the different coagulation curves at a point where fibrinolysis does not occur anymore permits to assume that the coagulant factor is the same in every snake, varying only its concentration. One can infer also that by storage a venom does not lose a coagulant component, as it happens with the proteolytic activity. There is only a decay of global coagulant activity, the same characters being conserved.

The differences between the types of curves of fibrinolysis may be due to fundamental differences between proteolytic enzymes or to the existence of a different number of proteolytic enzymes in venoms. If the first hypothesis would be correct, the venom should give an identical type of curve after storage. This did not happen. *B. jararaca* venom belonging to type I curve changed to type II and finally to type III. The more reasonable explanation would be that venoms which give type I curves have greater number of different proteolytic enzymes than those giving type II curve, this number being smaller in venoms giving type III and IV curves. Storage of dry venom at room temperature gradually destroys some fractions, leaving more stable fractions that do not decay or disappear. The activity decay would therefore depend on the inactivation of some fractions and not on the gradual activity loss of all of them. Thus the old venom, although having high fibrinolytic activity, would not contain certain fractions existing in the fresh venom. This conclusion should be considered when selecting venoms for the preparation of antivenom serum and in experiments done for the purpose of studying immunological aspects of snake venoms. If antivenom sera are prepared with venoms stored like those used in these experiments, it is possible that they do not have antibodies against these fractions. This might explain the observations of Rosenfeld and Leão (11) that antivenom sera do not prevent necrosis produced by venom of *B. jararaca*, possibly because their sera was prepared with old venoms which might not have the more labile component. However, the same reasoning cannot be applied to the coagulant fraction, because, as it was already discussed, no fundamental differences occur due to time of storage, only diminishing of activity.

It must be noticed that coagulant venoms having little proteolytic activity such as *Crotalus durissus terrificus* venom, do not provoke necrotic lesions, while the highly proteolytic but less coagulant venoms, like the *Lachesis muta muta* one, are only able to provoke necrosis when in high concentration, as has been experimentally observed. Venoms having both activities in high degree are those with high necrotic capacity such as the *B. jararaca* venom. It might be concluded that a venom necrotic capacity depends mainly on the simultaneous presence of the blood clotting and proteolytic enzymes in the venom.

The coagulant factor would produce a barrier by the clot, keeping the venom at site where it is injected. This retention keeps the proteolytic factors highly concentrated at the same site, permitting digestion of the surrounding tissues.

In accidents by poisonous animals, the knowledge of a venom coagulant and proteolytic index might permit to foresee the changes of blood coagulability and the occurrence of necrosis, even if there is no clinical record on such animal accident. It permits also an explanation for the absence of necrosis observed in cases of young *Bothrops jararaca* bite. Rosenfeld, Nahas, Fleury and Cillo (12) after clinical observations at the Vital Brazil Hospital of the Instituto Butantan, have seen that in these cases the blood became rapidly incoagulable. Although this clinical finding corresponds more frequently to a very serious case in accidents with adult snakes, resulting from a high volume venom inoculation and having as a consequence great local reaction and risk of necrosis, in cases of bite by young snake these symptoms failed to appear. Indexes of the young *B. jararaca* venom show that though having very strong coagulant activity, C.I. = 3,92 in relation to that of adults of the same species, C.I. = 1,0 its proteolytic activity is the half in relation to that of adults. This indicates that venoms that provoke more frequently blood incoagulability "in vitro" are the more coagulant ones, acting by defibrination, while the more proteolytic venoms produce the same effect by fibrinogenolysis.

SUMMARY

The parallelism between the coagulant and the proteolytic activity of snake venoms and that of some other species of poisonous animals was investigated.

The results presented in form of coagulant and fibrinolytic index permitted to compare both activities and demonstrated the dissociation existing between them. This lack of correlation between the coagulant and fibrinolytic index of each venom permits to affirm that these activities are due to different substances.

The coagulation graphs showed that the fibrinogenolytic substances interfere with coagulation of plasma produced by the venom. The proteolytic substances are less active, because their inhibiting or delaying activity on clotting time by fibrinogen digestion disappears with dilution, remaining more markedly the coagulant activity.

Venoms with practically no fibrinolytic activity, such as the white venom of *Crotalus durissus terrificus* gave the same straight line relationship between the logarithm of venom concentration and the logarithm of the clotting time, as could be theoretically expected from the action of only one substance or a group of substances. The coagulation graph of *B. jararaca* venom became regular after fractionated at about 60° C, pH 5.0 showing an almost total absence of proteolytic factors. The identity of the coagulation curves of the

different venoms, at a dilution where the proteolytic activity does not interfere with the coagulation, suggests that this factor could be the same substance existing in the different animal venoms with such activity. After storage the *B. jararaca* venom showed a decay of activity without changing the character of its curve, which indicates that no disappearance of components occured.

The fibrinolysis curves obtained were of different types. Some venoms gave a sigmoidal curve, some an exponential one others a straight line relationship. These curves do not depend on the fibrinolytic potency of venom, since the type of these curves depends on the type of venom and not on its fibrinolytic potency, thus suggesting that the proteolytic factor, unlike the coagulant factor, varies in the different species, in some perhaps being only one substance and in others a mixture of proteolytic enzymes in variable number and proportion. After storage the *B. jararaca* venom did not show the same type of curve as would be expected if resulting of only one substance, but gave gradually the simpler types of curves, thus demonstrating a gradual disappearance of proteolytic fractions.

The correlation between the coagulant and the proteolytic properties with the necrotic capacity was discussed with reference to its dependence on the coexistence of both activities.

RESUMO

Foi investigado o paralelismo entre as propriedades coagulantes e proteolíticas de venenos de serpentes e de algumas espécies de animais peçonhentos.

Os resultados apresentados sob forma de índices coagulante e fibrinolítico permitiram comparar as duas atividades e demonstraram a dissociação existente entre as mesmas. Essa falta de correlação entre os índices coagulante e fibrinolítico de cada veneno permite afirmar que se tratam de atividades devidas a substâncias diferentes.

As curvas de coagulação demonstraram que as substâncias fibrinolíticas interferem na coagulação do plasma provocado pelo veneno. A medida que se dilui o veneno, sendo as proteolíticas menos ativas, desaparece a ação impediente ou retardadora por digestão do fibrinogênio e aparece mais nìtidamente a atividade coagulante. Venenos pràticamente não fibrinolíticos como o veneno branco de *Crotalus durissus terrificus* apresentaram a mesma relação linear entre o logaritmo da concentração do veneno e o logaritmo do tempo de coagulação e aparentemente igual a uma teórica que seria de esperar na ação de uma só ou um único grupo de substâncias. A curva de coagulação do veneno de *B. jararaca* depois de fracionado a cêrca de 60°C em pH 5,0, passou a apresentar-se regular demonstrando ausência quase total dos fatores proteolíticos. A identidade das curvas de coagulação para os venenos os mais diferentes, nas diluições a partir das quais a atividade proteolítica não interfere, sugere que

êsse fator possa ser uma substância existente nos diversos venenos animais que possuem essa atividade. Após envelhecimento o veneno de *B. jararaca* mostrou apenas uma diminuição de atividade sem modificar a característica da curva. indicando que não há desaparecimento de componente algum.

As curvas de fibrinólise apresentaram alguns tipos diferentes. Certos venenos mostraram uma curva sigmóide, outros curva exponencial e outros ainda retilínea. Essas curvas independem da potência fibrinolítica do veneno, uma vez que o tipo das curvas depende do tipo do veneno e não de sua potência fibrinolítica, o que faz supor que, ao contrário do fator coagulante, o proteolítico varia com as espécies, em algumas talvez sendo uma só substância e em outras sendo uma mistura de enzimas proteolíticos em número e proporções variáveis. Após envelhecimento, o veneno de *B. jararaca* não mostrou o mesmo tipo de curva, como seria de esperar si fosse resultante de uma só substância, e sim passou a dar gradativamente os outros tipos mais simples demonstrando com isso desaparecimento gradativo de fracções proteolíticas, e não simples diminuição dessas substâncias.

A correlação entre as propriedades coagulantes e proteolíticas com a capacidade necrosante foi discutida no sentido de que esta depende principalmente da coexistência das duas atividades.

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