Mem. Inst. Butantan
Simp. Internac.
33(1):339-347, 1966

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.

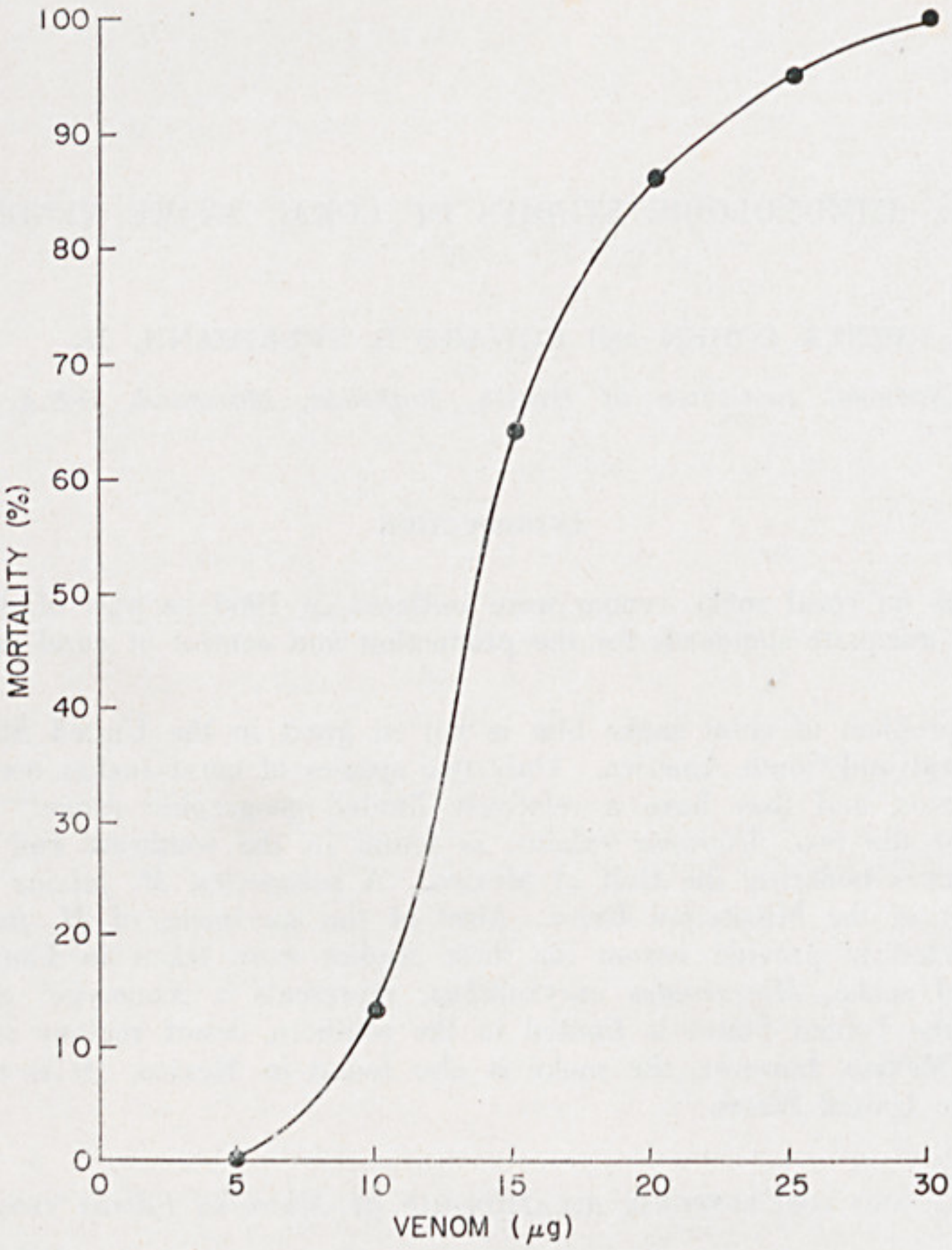

Fig. 1 — Dose-response relationship of *Micrurus fulvius* venom in mice.

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.

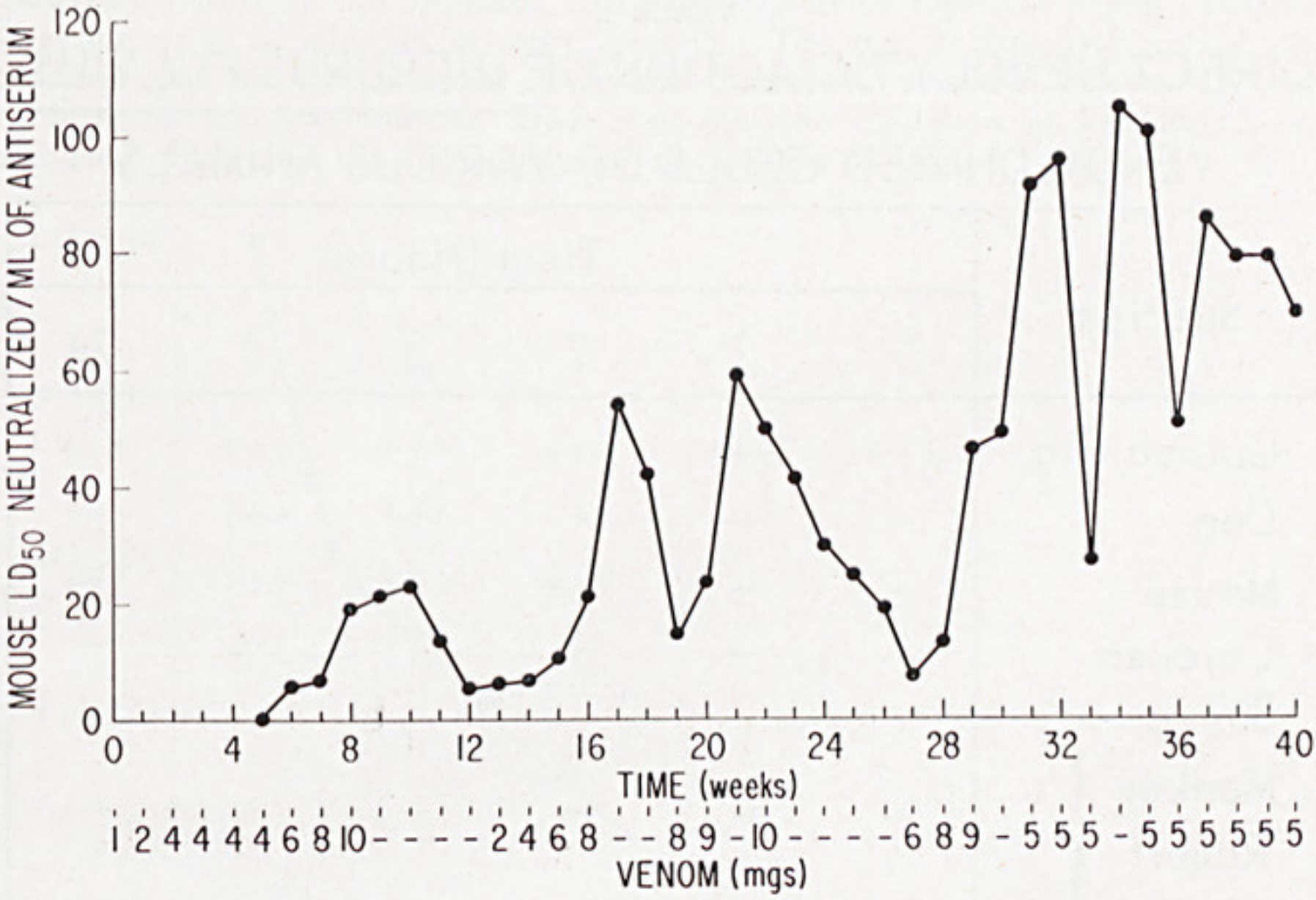

Fig. 2 — Production of neutralizing antibody against *Micrurus fulvius* venom in goats.

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.

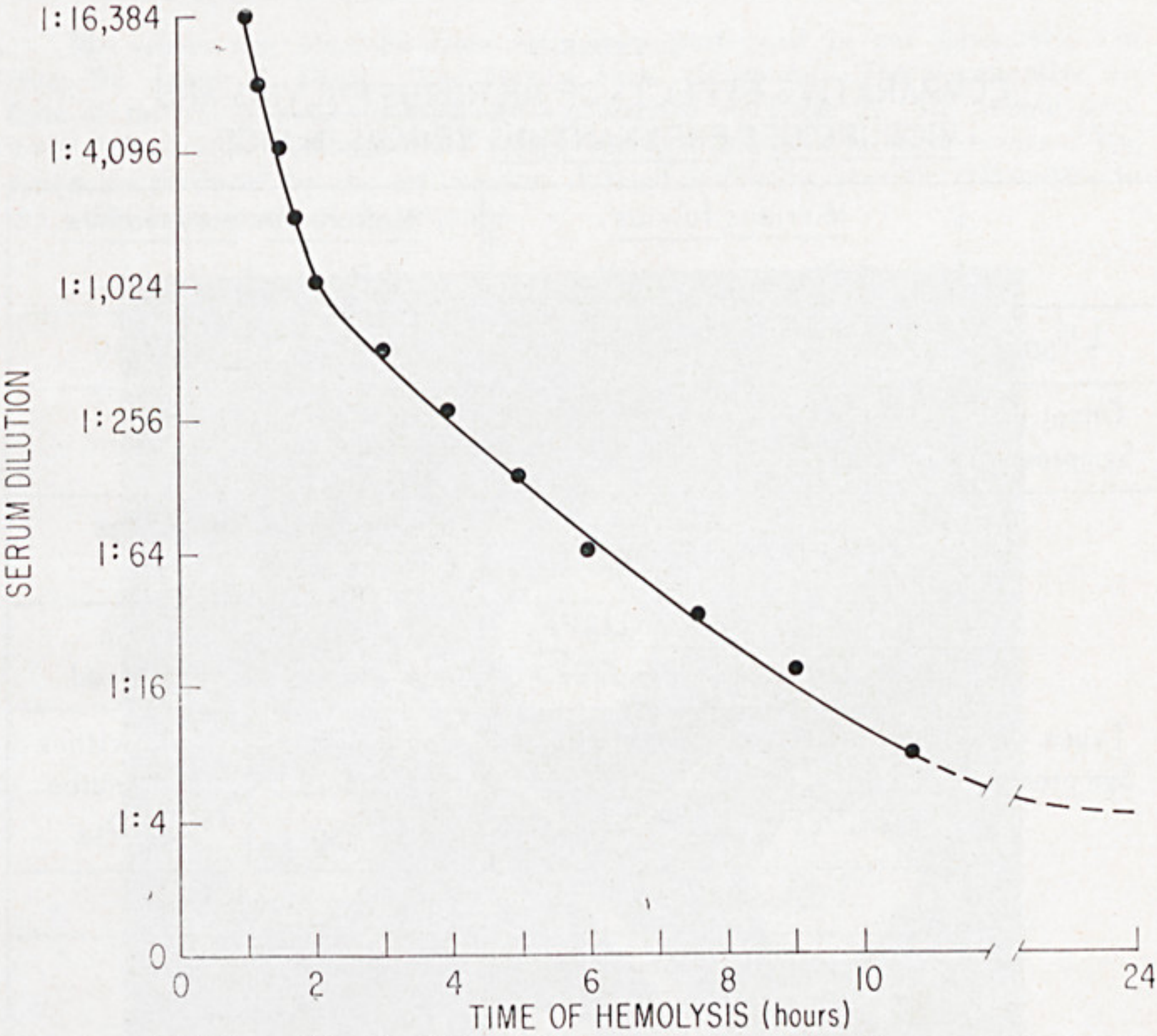

Fig. 3 — Inhibition of the direct hemolytic factor in *Micrurus fulvius* venom by normal rabbit serum.

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.

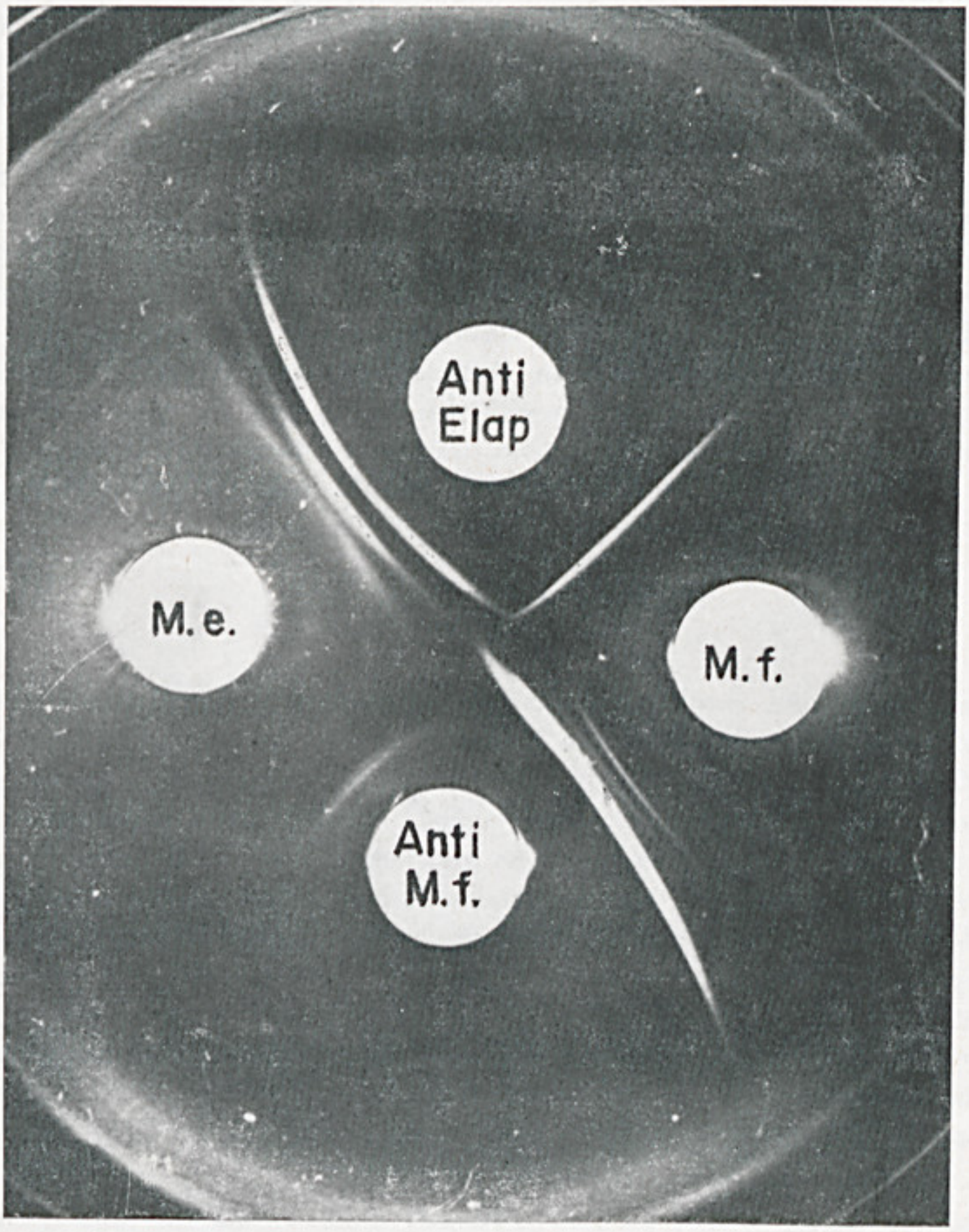

Fig. 4 — Gel-diffusion reaction of *Micrurus fulvius* and *Micruroides euryxanthus* venom. Anti Elap = Sôro Antielapidico. Anti M. f. = Anti *M. fulvius* serum. M. e. = *Micruroides euryxanthus* venom. M. f. = *Micrurus fulvius* venom.

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.

Acknowledgments — The authors gratefully acknowledge the able technical assistance of Mr. William H. Berkeley throughout the course of these studies. Appreciation is expressed to Dr. Raymond D. Zinn, Head, N.I.H. Farm Animal Unit, and Mr. Leonard D. Stuart for maintenance and care of the goats used in immunizations and for periodic collection of blood samples.

References

1. *L. G. Reed & N. Muench — Am. J. Hyg.*, **27**:493, 1938.

2. *P. Cohen, E. B. Heligmann, Jr. & W. H. Berkeley — Nature*, in press.

3. *V. B. Philpot, Jr. — Am. J. Physiol.*, **158**:77, 1949.

4. *E. Condrea, Z. Mammon, S. Aloof & A. DeVries — Israel J. Med. Sci.*, **1**:862, 1965.

5. *E. Condrea & A. DeVries — Biochim. Biophys. Acta*, **84**:60, 1964.

ÊSTE TRABALHO FOI EXECUTADO
NAS OFICINAS DA
TIPOGRAFIA EDANEE S. A.
SÃO PAULO