FRACTIONATION OF THE VENOM OF *BOTHROPS JARARACA* BY AMMONIUM SULPHATE. PURIFICATION OF SOME OF THE FRACTIONS OBTAINED *

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It is well known that the venom of *Bothrops jararaca* shows many enzyme activities [for a review, see Zeller (23) and Slotta (21)]. Only in recent years, however, partial separation of some of these activities have been reported. Holz and Raudonat (14), who compared the proteolytic and blood-clotting activities of fractions obtained from the venom by precipitation with ammonium sulphate, were able to show that the "proteolytic" activity was precipitated at a lower concentration of ammonium sulphate than the blood-clotting activity; the two enzymes involved were called respectively "Protease" and "Koagulin". Hamberg and Rocha e Silva have shown (7 and 8) that heating a venom solution of *B. jararaca* destroys its caseinase activity while not affecting its ability to hydrolyse benzoyl-L-arginine-methyl ester, a finding which indicates that, in this venom, there should be two different proteolytic activities. Henriques, Lavras and Fichman (10) have also presented evidence for the presence of two proteolytic enzymes in the venom of *B. jararaca*, as they separated by precipitation with ammonium sulphate two proteolytic fractions with different specificities. They found that the fraction precipitated at the level of saturation of 0.40-0.50 showed marked caseinase and low benzoyl-L-arginine amidase (BAAmidase) activity, while the fraction precipitated between 0.70 and 0.80 saturation presented high BAAmidase and low caseinase activity. The fraction having higher BAAmidase activity was subsequently purified 52 times as compared to the crude venom (11). This enzyme, which was called *Bothrops* protease A, has no detectable hydrolysing activity on casein, is very

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active on BAA, and hydrolyses gelatine. After comparing the fibrinolytic and clotting activities of venoms of *B. jararaca* collected and/or kept under different conditions Rosenfeld, Hampe and Kelen (19) also concluded that the proteolytic and blood clotting activities are due to different constituents. The blood-clotting enzyme was studied by Habermann (6) who was able to purify it 10 times and by Henriques, Fichman and Henriques (9) who, by means of electrophoresis on starch column, had concluded that *Bothrops* protease A and the blood clotting enzyme are two separate entities.

This paper reports on the distribution of the toxic activity, the known proteolytic enzymes (caseinase and *Bothrops* protease A), coagulating fraction, 5-nucleotidase and ATPase activities, in the fractions obtained when venom solutions are precipitated with increasing concentration of amnomium sulphate.

MATERIAL AND METHODS

Proteolytic activity. This activity was measured using casein or benzoyl-L-arginine amide as substrate. The caseinase activity was measured by the method of Kunitz (16) as previously described (11). For the BAAmidase activity the method of Schwert, Neurath, Kaufman & Snoke (20) was adapted to the diffusion apparatus of Tompkins & Kirk (22) as described by Henriques *et al* (11). The caseinase and *Bothrops* protease specific activities were calculated as previously described (11).

Coagulating activity. The coagulating activity was measured and the coagulating specific activity calculated as described by Henriques, Fichman & Henriques (9).

Hydrolytic activity on adenosinephosphates. Adenosinetriphosphate (ATP) in the form of the crystalline disodium salt was obtained from Sigma Chemical Co. Adenosine 5-monophosphate (AMP, free acid) was obtained from General Biochemicals Inc. The hydrolytic activity on these substrates was measured by the method of Lowry, Robert, Wu, Hixon & Crawford (17) slightly modified. When testing the hydrolysing activity on ATP, in the mixture buffer-substrate the buffer was substituted by 0.1 M 2-amino-2-methyl-1, 3-propanediol pH 9.2 containing $MgCl_2$ in 2mM concentration. When testing the hydrolysing activity on AMP the buffer used was tris-HCl 0.1 M, pH 8.0, containing $MgCl_2$ in 2mM concentration. The ATP-ase and 5-Nucleotidase specific activities were calculated by dividing the quantity of inorganic phosphate, liberated by the enzyme from the respective substrate, by the amount of protein contained in the sample.

Toxic activity. Male mice weighing 20-25 g were injected intraperitoneally with the solution to be tested. In all cases 0.05 M cacodylate buffer was used as solvent. All solutions were prepared in such a way that, per g of body weight, all experimental animals included in the same assay were injected with equal volumes of solution. For all quantitative assays 20 mice were used per dosis, the distribution of animals into groups being done with the aid of a table of random numbers. The mortality was recorded up to 24 hours after the injections. All assays were submitted to probit analysis, since a preliminary experiment showed straight-line relationship between the logarithm of the dosis and the mortality in probits (table 3).

The potency ratio was calculated by means of the equation

$$M = \overline{x_S} - \overline{x_T} - \frac{\overline{y_P} - \overline{y_D}}{b} \quad (1)$$

in which M is the logarithm of the potency ratio; $\overline{x_S}$, the weighed average of logarithm of the dosis of the "standard", which in all cases was the starting material; $\overline{x_T}$ the correspondig value for the "unknown", which usually was a fraction derived therefrom; $\overline{y_P}$ and $\overline{y_D}$ are the weighed average probits recorded for the "standard" and "unknown" respectively and b is the common slope of the two regression lines. Since $\overline{x_S}$ and $\overline{x_T}$ were the logarithm of the dosis in μg/g of respectively the starting material and its fractions, it is easy to see that the anti-logarithm of M is the purification index of the fraction analysed. Therefore

$$I.P. = \text{anti-log } M. \quad (2),$$

in which $I.P.$ is the purification index. Since the value of g was always higher than 0.1, the equation for the exact fidutial limits (2) had to be used in all assays.

Fractionation of the venom with ammonium sulphate. A 2% solution of the venom in 0.05 M sodium cacodylate buffer, pH 6.2 was fractionally precipitated with ammonium sulphate. The venom used was approximately one month old. When collected, it was immediately dried *in vacuo* over $CaCl_2$ at room temperature. The whole procedure of fractionation and subsequent dialysis were performed at 5-10°. Solid ammonium sulphate, in small portions, was stirred with the venom solution to give the desired percentage saturation. The amount of $(NH_4)_2SO_4$ necessary for each case was calculated from the table of Green & Hughes (3). The precipitates were separated by centrifugation, dissolved in a small volume of 0.05 M sodium cacodylate buffer, pH6.2, transferred to a cellophan tubing and dialysed against the same buffer until the diffusates became free of NH_4^+ ions. The dialysed solutions, when turbid, were centrifuged to separate any precipitate of denaturated protein.

Electrophoresis on starch columns. The same technique as described previously by Henriques *et al* (11) was used, varying the buffer and direction of the electric current in each special case.

RESULTS AND DISCUSSION

The fractionation of the proteins of *B. jararaca* venom with ammonium sulphate from a solution in 0.05 M cacodylate buffer, pH 6.2, permits a partial separation of the coagulant, ATP-ase and 5-nucleotidase fractions. The caseinase fraction is partially inactivated by cacodylate. The *Bothrops* protease A is not altered by this buffer; however for this enzyme a better method of separation was already obtained (11). Table 1 shows that, by precipitation with amonium sulphate from a venom solution in cacodylate buffer as described in this paper, the following activities are partially separated: caseinase, coagulating enzyme, ATP-ase, 5-Nucleotidase and *Bothrops* protease A, between respectively 0.40-0.45, 0.60-0.65, 0.65-0.70 and 0.70-0.80 saturation with ammonium sulphate.

TABLE 1. *Comparison of caseinase, coagulant, ATP-ase, 5-Nucleotidase* and Bothrops *protease A (benzoyl-L-arginineamidase) activities of the various fractions precipitated with ammonium sulphate.*

Relative specific activity is the specific activity of each fraction calculated from the analytical data obtained if the corresponding specific activity (see text) of the starting material is equaled to one.

Fraction	Saturation with $(NH_4)_2SO_4$	Relative specific activities				
		Caseinase	Clotting	ATP-ase	5-Nucleo-tidase	Bothrops protease A
1	None	1.0	1.0	1.0	1.0	1.0
2	0.35-0.40	0.9	0.3	—	—	0.3
3	0.40-0.45	1.1	0.7	—	—	0.6
4	0.45-0.50	0.9	0.9	—	—	0.8
5	0.50-0.55	0.4	1.3	0.7	0.4	1.0
6	0.55-0.60	0.2	2.1	1.7	1.0	1.6
7	0.60-0.65	0.2	2.4	3.0	2.7	2.2
8	0.65-0.70	0.2	1.8	0.2	5.0	3.1
9	0.70-0.80	—	—	—	—	4.8
10	0.80-1.00	—	—	—	—	0.7

Proteolytic enzymes. The existance of two proteolytic enzymes has been previously demonstrated in the venom of *B. jararaca* (10). One of them, which we call "caseinase", due to its high hydrolytic power on casein, seems to be the same as the "protease" found in the *Bothrops* venom by Holtz & Raudonat (14). Those authors separated the "protease" at 0.40 saturation with ammonium sulphate while Henriques *et al* (10) obtained the fraction most active on casein at 0.40-0.45 saturation with ammonium sulphate. The other proteolytic enzyme identified, *Bothrops* protease A, when purified, has no detectable activity on casein, is very active on L-benzoyl-arginine-amide and hydrolyses gelatine. This last enzyme has been obtained, prepared with an activity approximately 50 times greater than that of the crude venom.

Coagulating factor. Experiments published previously (12), as well as those reported in table 1, indicate that the blood clotting enzyme can be distinguished from the two proteolytic activities known to be present in the venom of *B. jararaca.* The blood clotting activity may, however, be due to a third proteolytic enzyme; this hypothesis has been strengthened by the findings of

Blombäck and Westermarck (1) who found that the clotting of fibrinogen by "Reptilase" (pharmaceutical product distributed by Pentapharm), a preparation of the blood-clotting enzyme obtained from *Bothrops* venom, is accompanied by the liberation of one of the peptides known to be liberated during the action of thrombin on fibrinogen.

ATP-ase and 5-Nucleotidase. The curve of pH — activity (Fig. 1) shows that using the buffers employed by Myers & Slater (18) the activity reached

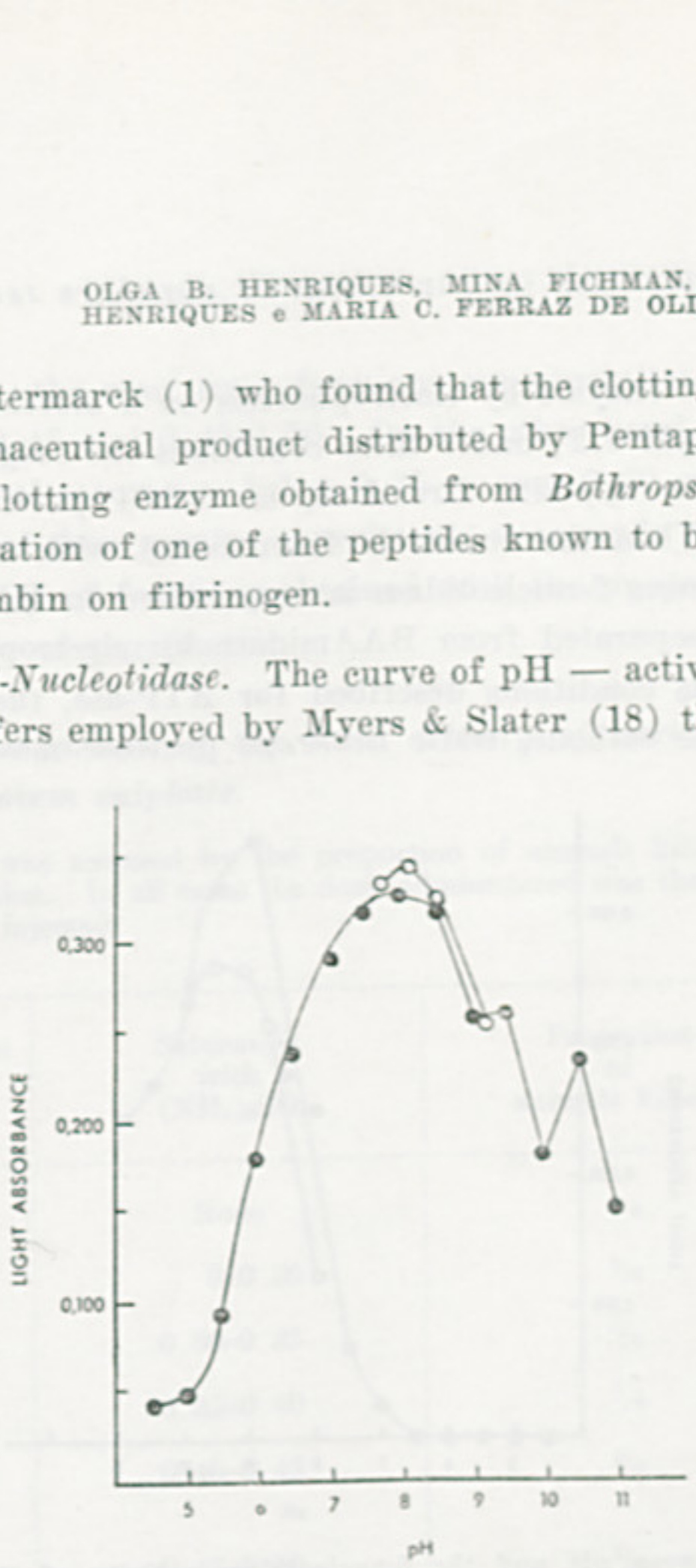

Fig. 1. Relation between pH and the ATP-ase activity of the venom of *Bothrops jararaca*. ●——● with the buffer mixture of Myers and Slater (18). ○——○ with 0.1 M ammediol buffer mixture containing $MgCl_2$ at the final concentration of 0.002 M.

a plateau at pH 9.0-9.5, decreasing at higher pH values. These values are similar to those of Zeller (22) who reported an optimal pH range of 8.3-9.5. Using 0.05 M ammediol, the pH optimum was found to be also around 9.0. The pH optimum found for 5-nucleotidase, using either the buffers of Myers and Slater (18) or 0.05 M tris-HCl buffer, was 8.0 (Fig. 2). This value is similar to the pH optimum found by Gulland and Jackson (4) for the 5-nucleotidase of Russel's viper venom, using diaethyl-barbiturate and borate buffers. ATP-ase and 5-nucleotidase proved to be thermolabile as they are completely destroyed when the venom solution is heated to 87° in 0.05 M cacodylate buffer, pH 6.2. As mentioned before ATP-ase is partially separated from 5-nucleotidase by fractional precipitation with ammonium sulphate, being precipitated together with the coagulating enzyme. The ATP-ase can be sepa-

rated from the last enzyme by electrophoresis on a starch column in 0.05 M cacodylate buffer, pH 6.2; under these conditions the coagulating enzyme and ATP-ase migrate in opposite directions, an 8-fold purification of ATP-ase being obtained. When the venom is fractionated with ammonium sulphate, the fraction containing 5-nucleotidase is very active on BAA. However 5-nucleotidase can be separated from BAAmidase by electrophoresis in a starch column in the same conditions described for ATP-ase, the 5-nucleotidase migrating towards the cathode, while *Bothrops* protease moves anodically.

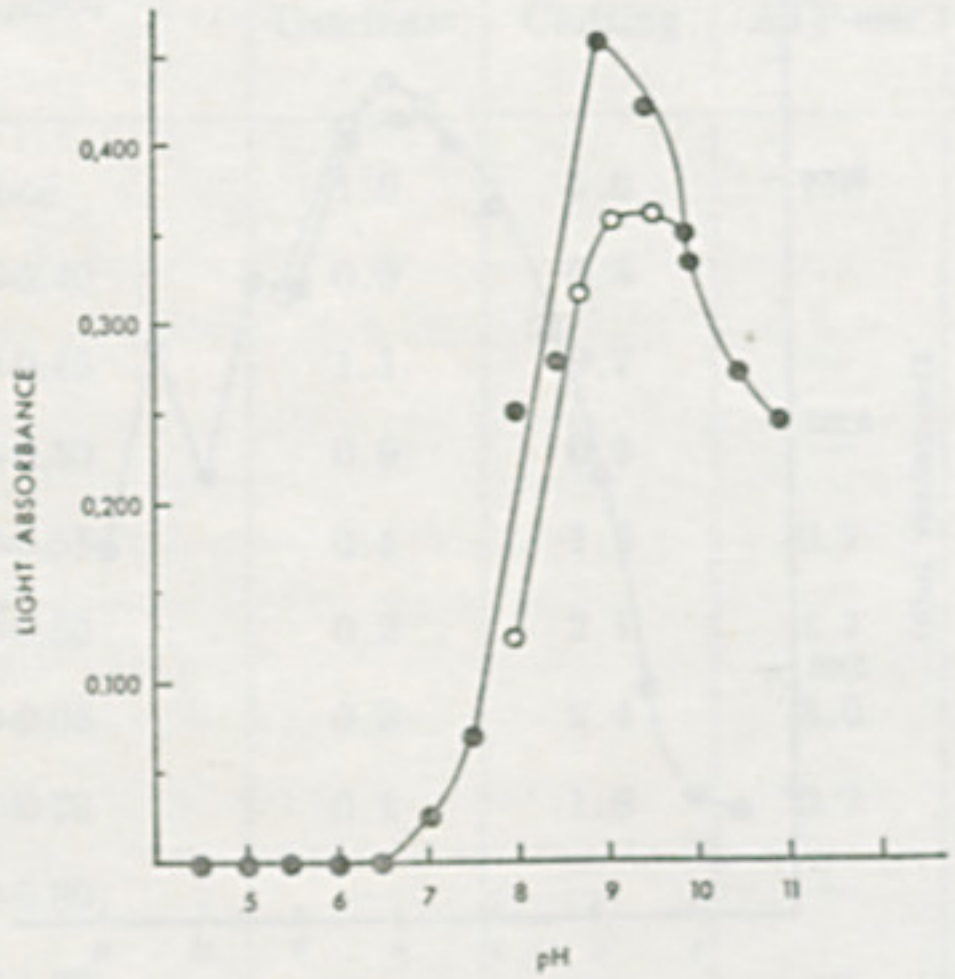

Fig. 2. Relation between pH and the 5-nucleotidase activity of the venom of *Bothrops jararaca*. ●——● with buffer of Myers and Slater (18). O——O with 0.1 M Tris buffer mixtures containing $MgCl_2$ at the final concentration of 0.002 M.

It must be noted that it seems easier to obtain 5-nucleotidase free from ATP-ase than vice-versa, since the fraction with highest ATP-ase specific activity (3 times as active as the starting material) had a 5-nucleotidase specific activity 2.5 as high as the original venom; on the other hand, the fraction with highest 5-nucleotidase specific activity (5 times as high as the starting material) had an ATP-ase specific activity 1/5 of that of original venom. Therefore, all our ATP-ase fractions contained 5-nucleotidase, a circunstance which does not allow us to exclude the possibility of ATP-ase of *Bothrops* venom being a β-γ- adenosine-triphosphatase, as found to be the case for the ATP-ase of cobra venom (15).

Toxic activity

Results published previously had shown (13) that, when the venom of *B. jararaca* is fractionated with ammonium sulphate under the conditions des-

cribed in this paper, the most toxic fractions are precipitated at the levels of saturation of 0.40-0.45 and 0.45-0.50. On the other hand, the fractions precipitatated at levels of saturation below 0.35 or over 0.60 are much less toxic (table 2). In view of these results, experiments were made in order to measure accurately the toxicity of fractions obtained with the purpose of studying the yield and the degree of purification.

TABLE 2. *Comparison of the toxic activity of various fractions precipitated with ammonium sulphate.*

The toxic activity was assessed by the proportion of animals killed when 5 mice were injected with each fraction. In all cases the dosis administered was the same, as judged from the amount of protein injected.

Fraction n.°	Saturation with $(NH_4)_2SO_4$	Proportion of animals killed*
1	None	4/5
2	0–0.30	0/5
3	0.30–0.35	0/5
4	0.35–0.40	1/5
5	0.40–0.45	3/5
6	0.45–0.50	5/5
7	0.50–0.55	1/5
8	0.55–0.60	0/5
9	0.60–0.65	1/5
10	0.65–0.70	0/5
11	0.70–0.80	0/5

* The denominators represent the number of mice used in the assay.

Relationship between the dosis of Bothrops *venom and mortality rate.* LD_{50} of the venom was measured in mice. For this purpose two assays were made, one on female and the other on male mice; the results of these assays are presented in table 3. From the non significant values of χ^2 obtained, it was concluded that a straight-line relationship may be assumed to apply between the logarithm of the dosis and the mortality in probits. The female

mice seemed to be slightly more "heterogenous" than the male ones since the values of χ^2 obtained were 2.27 and 0.68 respectively. The values of LD_{50} obtained were 4.81 μg/g for females and 8.15 μg for males. While this seems to indicate that the male mice are more resistant to the venom than the males, it must be mentioned that the two assays were made in different days and no steps were taken to fix the progeny of the animals used in these experiments: therefore no definite conclusion can be drawn from the data from table 3 in this respect.

TABLE 3. *Determination of the L. D_{50} of* Bothrops *venom in mice.*

For each assay 80 animals were distributed into 4 groups of 20, with the aid of a table of randon numbers. The venom solutions were administered intraperitoneally, each animal receiving the same volume per g. of body weight. The dosis are expressed in terms of protein administered. The mortality rate registered is the mortality observed up to 24 hours after the injections.

Assay no.	Group no.	Dosis μg/g	Mortality rate		Results of probit analysis
			%	Empirical probit	
I	1	6.03	5	3.36	$\chi^2[2]=0.680(0.95>P>0.50)$
	2	6.87	10	3.72	$LD_{50}=8.15$ μg/g
(Male mice)	3	7.83	45	4.87	Fiducial limits 7.67-8.65
	4	8.93	75	5.67	
II	1	5.70	45	4.87	$\chi^2[2]=2.27(0.50>P>g0.10)$
	2	6.50	70	5.52	
(Female mice)	3	7.40	65	5.39	$LD_{50}=4.81$ μg/g
	4	8.40	90	6.28	Fiducial limits 3.54-6.63

Fractional precipitation of the toxic activity. Table 4 gives the results of an assay of the toxicity of fractions separated from *Bothrops* venom by precipitation with ammonium sulphate. It can be seen that the crude venom (sample 1) gave mortality rates of 50% and 70% for the dosis of 4.67 μg/g and 5.60 μg/g respectively. At the dosis 2.98 μg/g the fractions precipitated at the levels of saturation of 0.40-45 (sample 2) and 0.45-0.50 (sample 3) gave mortality rates of 35 and 40% respectively, while at the higher dosis of 3.58 μg/g both fractions produced mortality rates of 70%. Finally, the

observed mortality rates with the fraction precipitated at 0.50-0.55 (sample 4) saturation were 45 and 75% for the dosis of 3.58 μg/g and 4.30 μg/g respectively.

TABLE 4. *Assay of toxicity of fractions obtained from* Bothrops *venom by fractional precipitation with ammonium sulphate.*

A total of 160 mice were used in this assay. The animals were divided into eight groups of 20 mice with the aid of a table of random numbers. For each sample 40 mice were used. 20 of which receiving a lower, while a second group of 20 received a higher dosis of the material. The ratio higher/lower dosis was the same for all samples. The animals of any group received the same volume of solution administered intraperitoneally and were observed for 24 hours after the injections. The dosis are expressed in terms of protein.

Sample no.	Description	Dosis μg/g	Mortality rate %
1	Starting material*	4.67 5.60	50 70
2	0.40–0.45 fraction	2.98 3.58	35 75
3	0.45–0.50 fraction	2.98 3.58	40 75
4	0.50–0.55 fraction	3.58 4.30	45 75

* Crude venom

These data were submitted to a probit analysis in which the potency of samples 2, 3 and 4 was compared with that of the crude venom (sample 1). It was found (table 5) that one mg of protein of samples 2 and 3 were as toxic as about 1.5 mg of protein of the starting material, while 1 mg of sample 4 was as active as 1.3 mg of protein of the latter. It would therefore appear that the material precipitated between 0.50 and 0.55 saturation with ammonium sulphate is less active than the proteins precipitated between 0.40-0.45 or 0.45-0.50 of saturation. A definite conclusion, however cannot be drawn from the data obtained since there was some overlapping on the confidence limits ($P = 0.80$). Under these circunstances the difference in potency of samples 2 and 3 cannot be regarded as statistically significant, since the probability of the found difference being due to chance is about 0.10. It must also be noted that the two most toxic fractions (samples 2 and 3) contained 47 per cent, and the three fractions combined had 61 per cent, of the total original toxicity of the venom.

TABLE 5. *Results of probit analysis of data included in table* 4.

The toxicity of samples 2, 3 and 4 was compared with that of the material fractionated (sample 1, table 4). Accordingly the potency ratio, obtained by probit analysis, was taken as the index of purification (see text).

Fraction precipitated at	χ^2	Probability of χ^2	Value of g	Index of purification	Fiducial limits of the potency rate		Yield %
					P=0.95	P=0.80	
0.40-0.45	0.80	0.50>P>0.10	0.507	1.517	1.21-1.79	1.36-1.67	23
0.45-0.50	0.03	0.95>P>0.50	0.642	1.544	1.19-1.87	1.38-1.70	24
0.50-0.55	0.23	0.95>P>0.50	0.740	1.301	0.92-1.74	1.17-1.46	13
						Total	60

Reprecipitation of the toxic activity. A reprecipitation of the most toxic fractions with ammonium sulphate was tried in order to obtain further purification. For this purpose, a fraction prepared under the conditions specified for sample 2 (table 4) was refractionated with ammonium sulphate in the usual way in order to obtain separately the material precipitated at the level of saturation of 0.40, between 0.40 and 0.45 and between 0.45 and full saturation. The resulting fractions were compared with the starting material (similar to sample 2, table 4) by means of three biological assays, the results of which are condensed in table 6. The same dosis of 2.80 μg/g and 3.64 μg/g of the starting material was used in all three assays, while the dosis (μg/g) for samples 2 (fraction precipitated at 0.40 of saturation), 3 (fraction soluble at 0.40 of saturation and insoluble at 0.45 of saturation) and 4 (fraction soluble at 0.45 of saturation but insoluble in saturated solution of ammonium sulphate) were respectively 2.92 and 3.64; 2.17 and 2.82; 2.92 and 3.80. It can be seen (table 6) that the observed mortality rates in percent were: a) for the starting material, 40 and 75 in assay I, designed to measure the potency of the fraction precipitated at 0.40 saturation, which produced a mortality rate of 45 and 90; b) for the material precipitated between 0.40 and 0.45 saturation, 40 and 85, while the mortality rates observed for the starting material were 15 and 65 (assay II); c) mortality rates of 15 and 60 for the fraction precipitated between 0.45 and full saturation, while the mortality rates recorded for the starting material were 30 and 70%.

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TABLE 6. *Assays of toxicity of fractions obtained from a fraction similar to sample* 2 (table 3) *by fractional reprecipitation with ammonium sulphate.*

Sample 1, similar to sample 2 of table 4, is the material present in *Bothrops* venom which is precipitated with ammonium sulphate at the level of saturation of 0.45 while being soluble at the level of 0.40 of saturation. Samples 2, 3 and 4 are obtained from this starting material by fractional reprecipitation with ammonium sulphate.

The assays were made under the conditons specified on table 4.

Assay no.	Sample no.	Description	Dosis µg/g	Mortality rate %
I	1	Starting material*	2.80 3.64	40 75
	2	0.40 fraction	2.92 3.80	45 90
II	1	Starting material*	2.80 3.64	15 65
	3	0.40-0.45 fraction	2.17 2.82	40 85
III	1	Starting material*	2.80 3.64	30 70
	4	0.45–1.00 fraction	2.92 3.80	15 60

* Similar to sample 2 (table 4).

When these results were submitted to probit analysis, it was found (table 7) that the fraction precipitated between 0.40 and 0.45 saturation, the most active, had a toxic potency 1.5 higher than the starting material, and that its potency was found to be significantly higher than either that of the fraction precipitated at 0.40 saturation or that of the material precipitated between 0.45 and full saturation. It can also be seen that the total yield of the fraction was 75 per cent, 18 per cent of which is included in the most active fraction.

Since the first precipitation lead to a 1.5 times purification of the toxic fraction (table 5), and the second caused a purification of similar order (table 7), it should be expected that two successive precipitations with ammonium sulphate should permit the preparation of a toxic fraction 2.3 times more potent than the starting material with an overall recuperation of about 8 per cent of the toxic activity in the most potent fraction.

Comment on the use of mice for the quantitative determination of toxicity of fractions obtained from Bothrops *venom.* In the course of the experiments

TABLE 7. *Results of probit analysis of data included in table* 6.

Te toxicity of samples 2, 3 and 4 was compared with that of the material fractionated (sample 1, table 6). The index of purification is the potency ratio obtained by probit analysis (see explanation of table 5).

Fraction reprecipitated at	χ^2	Probability of χ^2	Value of g	Index of purification	Fiducial limits of the potency ratio	Yield %
0–0.40	0.05	0.95>P>0.50	0.200	0.97	0.86–1.10	38
0.40–0.45	0.10	0.95>P>0.50	0.208	1.48	1.32–1.75	18
0.45–1.00	0.17	0.95>P>0.50	0.269	0.88	0.82–1.15	19
					Total	75

described here it was found that the sensitivity of the mice, raised in this Institute, to *Bothrops* venom, presented considerable variation from batch to batch. This can be seen from the results of the assays included in table 6 and which were already described; in these assay the same starting material (sample 1) was administered to mice on three different occasions at the dosis levels ($\mu g/g$) of 2.80 and 3.64. The mortality rates in per cent were: a) for the lower dosis 40 (assay I), 15 (assay II) and 30 (assay III); and b) for the higher dosis 75 (assay I), 65 (assay II) and 70 (assay III).

For this reason, the quantitative estimation of the toxic potency of fractions obtained from *Bothrops* venom may be time-consuming, since, quite often, several preliminary trials are required in order to chose the best dosis for the final assay. Since this involves also expenditure of material which may be scarce, it seems quite clear that mice should not be used for the fractionation of small amounts of venom.

Sometimes heterogeneity in the same batch is also found. This can be seen in table 8, which summarizes the results obtained in the course of three assays designed for the determination of the potency of sample 2 (tables 6 and 7). While considerable variability between assays is immediately seen, the χ^2 validity test has shown heterogeneity in the batch used in assay III.

Finally the high value for g, obtained in all assays (tables 5, 7 and 8), constitutes another disadvantange of the utilization of mice for the assay of the toxicity of *Bothrops* venom and its fractions. All these considerations cast some doubt on the data based on experiments in mice done with the purpose of obtaining information concerning small scale preparation of the

toxic fraction of this venom. This conclusion is specially true for the separation by paper electrophoresis which seriously limits the amount of material available for the assay.

TABLE 8. *Variability in the sensitivity of mice to the toxic fraction of the venom of* Bothrops jararaca.

The experimental conditons are the same as described in preceding tables. The "standard" is sample 1 of table 6. S_1 and S_2 represent the lower and higher dosis of the standard (2.80 and 3.64 µg/l g). T_1 and T_2 stand for the lower and higher doses of sample 2 of table 6. (2.92 and 3.80 µg/g.)

Assay no.	Mortality rates %				Results of probit analysis				Fiducial Limits
	"Standard"		Sample 2		χ^2	Probability	g	Potency ratio	
	S_1	S_2	T_1	T_2					
I	45	75	65	75	1.16	0.5>P>0.1	0.839	1.50	0.33 – 2.01
II	15	65	40	85	0.10	0.95>P>0.50	0.208	1.50	1.32 – 1.75
III	40	90	55	65	4.52	0.05>P>0.01	0.520	1.60	1.11 – 1.97

SUMMARY

1. — The fractional precipitation of venom solutions of *Bothrops jararaca* with ammonium sulphate permits a partial purification of the toxic activity and of the following enzymic activities of the venom: "caseinase", blood-clotting, ATP-ase, 5-nucleotidase and benzoyl-L-arginine-amidase.

2. — As regards ATP-ase and 5-nucleotidase, the active fractions are obtained between the level of saturation of 0.50 and 0.70 with ammonium sulphate but the optimal concentrations for precipitation are 0.60-0.65 for ATP-ase and 0.65-0.70 of saturation for 5-nucleotidase.

3. — The optimal concentrations for precipitation of caseinase, blood-clotting and benzoyl-L-arginine amidase are respectively 0.40-0.45, 0.60-0.65 and 0.70-0.80.

4. — The toxic activity is optimally precipitated at the level of 0.45-0.50 of saturation with ammonium sulphate.

5. — Different batches of mice proved to vary considerably as regards the sensitivity to the toxic activity of the venom of *B. jararaca* or of its fractions. In consequence, the use of mice for the quantitative estimation of toxicity of this venom or of fractions separated from it, always requires substantial expenditure of material and may be time-consuming.

RESUMO

1. — A precipitação fracionada de soluções de veneno de *Bothrops jararaca* com sulfato de amôneo permite uma purificação parcial da atividade tóxica e das seguintes atividades enzimáticas contidas no mesmo veneno: "caseinase", hemo-coagulante, ATP-ase, 5-nucleotidase e Benzoil-L-argininamidase.

2. — Obtêm-se frações com atividade ATP-ásica e 5-nucleotidásica desde o nível de saturação de sulfato de amôneo de 0,50 até 0,70; a julgar entretanto, pelas respectivas atividades à mesma base de concentração protêica, as concentrações ótimas de precipitação de ATP-ase e 5-nucleotidase são respectivamente de 0,60-0,65 e 0,65-0,70 de saturação.

3. — As concentrações ótimas de precipitação das atividades caseinásica, hemocoagulante e benzoil-L-argininamidásica são respectivamente 0,40-0,45; 0,60-0,65; e 0,70-0,80.

4. — A região ótima de precipitação da atividade tóxica fica compreendida entre os limites de saturação de 0,45 a 0,50.

5. — Nas repetições de experiências executadas para a determinação de toxidez de veneno Botrópico, ou frações dêle derivadas, verificou-se a existência de grande variabilidade entre os diversos lotes de camundongos utilizados. Em conseqüência, o uso dêstes animais para a determinação quantitativa da toxidez de veneno Botrópico ou suas frações requer sempre o dispêndio de quantidades substanciais de material e a obtenção do resultado final pode requerer grande gasto de tempo.

REFERENCES

1 — Blombäck, F. R.; and Vestermark, A. — *Arkiv. f. Kemi* 12: 173, 1958.

2 — Finney, D. J. — in Burn, J. H.; Finney, D. J.; and Goodwin, L. G. — *Biological Standardization*, London, Oxford University Press, 1950.

3 — Green, A. A.; and Hughes, W. L. — In *Methods in Enzymology*, vol. 1. p. 67 Ed. by Colowick, S. P. and Kaplan, N. O. New York: Academic Press, Inc., New York, 1957.

4 — Gulland, J. M.; and Jackson, E. M. — *Biochem.* J. 32: 597, 1938.

5 — Habermann, E. — *Arch. exp. Pathol. Pharmakol.* 236: 492, 1959.

6 — Habermann, E. — *Arch. exp. Pathol. Pharmakol.* 234: 291, 1958.

7 — Hamberg, U.; and Rocha e Silva, M. — *Ciência e Cultura* 8: 176, 1956.

8 — Hamberg, U.; and Rocha e Silva, M. — *Arch. in Pharmacodyn.* 110: 222, 1957.

9 — Henriques, O. B.; Fichman, M.; and Henriques, S. B. — *Biochem. J.*, in course of publication.

10 — Henriques, O. B.; Lavras, A. A. C.; and Fichman, M. — *Ciência e Cultura* 8: 240, 1956.

11 — Henriques, O. B.; Lavras, A. A. C.; Fichman, M.; Mandelbaum, F. R.; and Henriques, S. B. *Biochem. J.*, 68: 597, 1958.

12 — Henriques, O. B.; Mandelbaum, F. R.; and Henriques, S. B. — *Nature* (Lond) 183: 114, 1959.

13 — Henriques, S. B.; Fichman, M.; and Henriques, O. B. — *Ciência e Cultura* 10: 162, 1958.

14 — Holtz, P.; and Raudonat, H. W. — *Arch. Pathol. Pharmakol.* 229: 113, 1956.

15 — Johnson, M.; Kaye, M. A.; Hems, R.; and Krebs, H. A. — *Biochem. J.* 54: 625, 1953.

16 — Kunitz, M. — *J. Gen. Physiol.*, 30: 291, 1946.

17 — Lowry, P.; Roberts, N. R.; Wu, M. L.; Hixon, W. S.; and Crawford, E. J. — *J. Biol. Chem.* 207: 19, 1954.

18 — Myers, D. K.; and Slater, E. C. *Biochem. J.*, 67: 558, 1957.

19 — Rosenfeld, G.; Hampe, O. G. and Kelen, E. M. A. — *Memórias do Inst. Butantan* (in the press).

20 — Schwert, G. W.; Neurath, H.; Kaufman, S.; and Snoke, J. E. — *J. Biol. Chem.* 172: 221, 1948.

21 — Slotta, K. — *Experientia*, 9: 81, 1953.

22 — Tompkins, E. R.; and Kirk, P. L. — *J. Biol. Chem.* 142: 477, 1942.

23 — Zeller, E. A. — *The Enzymes*, vol. I, p. 987, Ed. by Sumner, J. B. and Myrbäck, K., Academic Press Inc., New York, 1951.