

Paper electrophoretic and enzymatic studies on blood serum, venom and liver of "Bothrops jararaca" *

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Large amounts of riboflavin have been found in blood serum of "*Bothrops jararaca*" (25) (26) and it has been shown that almost all of the flavins are bound to proteins (20). NEUMANN and HABERMANN (11) in paper electrophoretic studies of the venom have found two protein fractions and one protein-free area in the electropherogram which presented a bright yellow fluorescence, probably due to flavins.

In a previous report (24) we have presented some results obtained for the electrophoretic analysis of blood serum of *B. jararaca* in which fluorescent areas due to flavins were also noted. Since xanthine oxidase (XO) and L-amino acid oxidase have a flavin adenine-dinucleotide (FAD) as prosthetic group, the estimation of these enzymes was accomplished in order to see if they could account for the flavins occurring in the blood serum and venom of this snake.

Methods and Results.

1). Enzymes Assay:

XO activity of the liver, blood serum and venom was measured by the manometric method of Axelrod and Elvehjem (1) as previously described (22). The liver of "*Bothrops jararaca*" was homogenized with five volumes of distilled water in a Warren blender. After incubating the homogenate at 37°C for 45 minutes, in order to partly suppress the endogenous respiration, 1.5 ml of the homogenate, corresponding to 250 mg of fresh tissue, was placed in the Warburg flask. The side arm was filled with 0.2 ml of 0.05 M xanthine solution and a folder filter paper was placed in the center well containing 0.2 ml of 20% KOH solution. In all experiments the phosphate buffer pH 7.4 was substituted for pyrophosphate pH 8.6 according to the observation of Dhungat and Sreenivasan (2) that pyrophosphate buffer inhibit the endogenous respiration of the homogenates.

* This work is dedicated to Prof. HENRIQUE B. ARAGÃO.

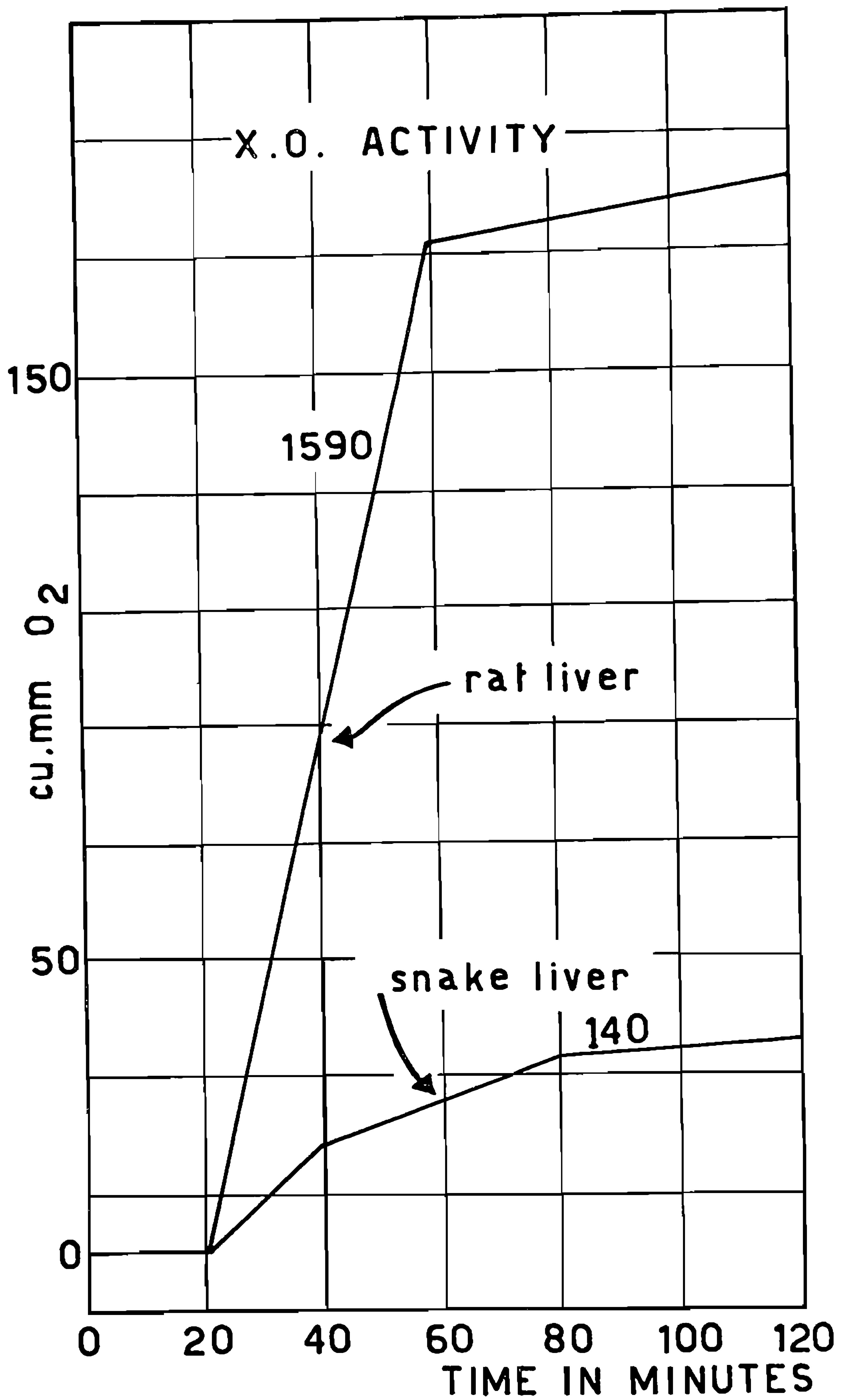


Fig. 1 — Xanthine oxidase activity of snake and rat livers. Numbers on the curves represent cu.mm O₂/g dry wt./hour.

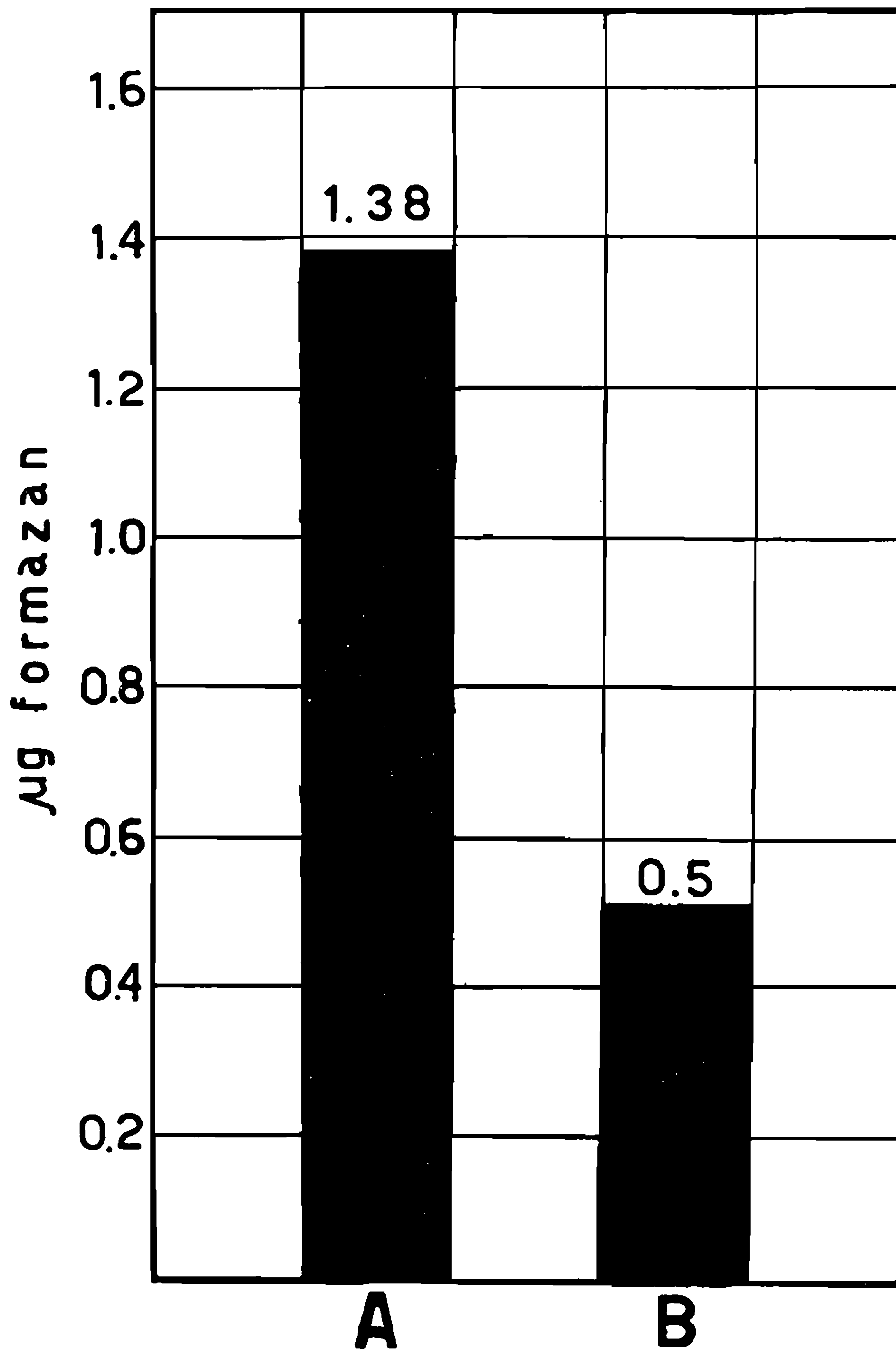


Fig. 2 — Xanthine dehydrogenase activity. A: Rat liver; B: Snake liver.

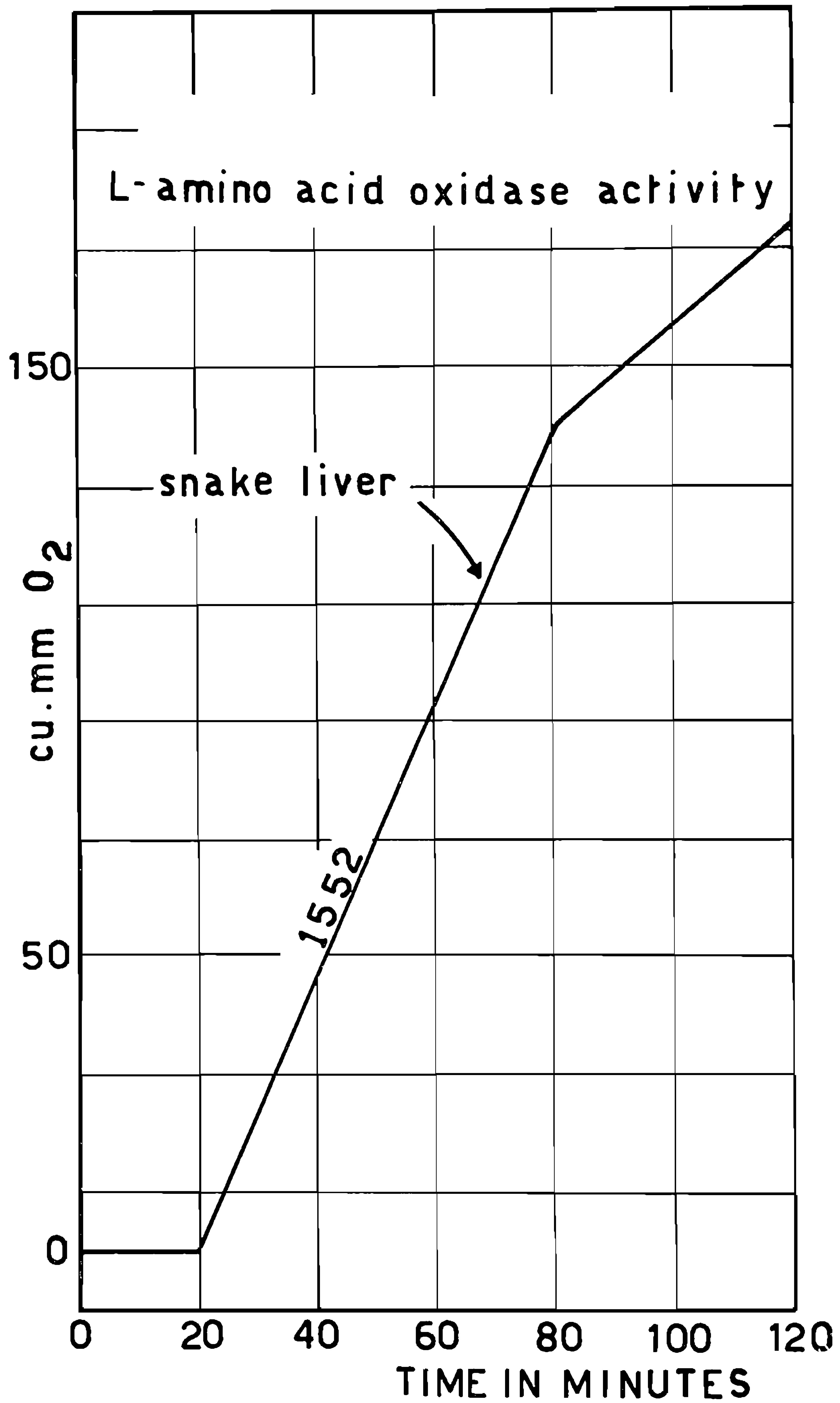


Fig. 3 — L-amino acid oxidase activity of snake liver. The number on the curve represents cu.mm O₂/g dry wt/hour

All flasks were filled with enough pyrophosphate to give a final volume of 3.0 ml. After equilibrating the flasks for 10 minutes at 37°C the first reading was taken and the side arm substrate tipped in. The subsequent measurements were made every 20 minutes for 120 minutes. Liver XO activity was then calculated as oxygen consumption and the results expressed in cu.mm $\frac{1}{2}$ g. dry weight hour at 37°C. The determination of XO activity in blood serum was made in the same way, using 2ml of serum and a final volume of 3ml.

Xanthine dehydrogenase (XD) activity was assayed using the colorimetric method of Villela described elsewhere (21). The Thunberg tubes were filled with 1ml of liver homogenate and enough pyrophosphate buffer pH 8.6 to give a final volume of 2.0ml. The side arm contained 0.1ml of the xanthine solution (0.05M) and 0.3ml of 0.1% triphenyltetrazolium chloride (TTC) solution. After the air was evacuated for 5 minutes the tube was placed in a water bath at 37°C for 10 minutes and the substrate and TTC tipped in.

The formazan produced after 30 minutes of incubation was extracted with petroleum ether after acidification with acetic acid. The extracted color was read in a spectrophotometer at 480 m μ and the results as μ g formazan 30 minutes at 37°C mg dry wt.

XO and XD activities were detected in the liver of *Bothrops jararaca*, the venom and blood serum being practically devoided of the XO and XD activities. The liver of "*Bothrops jararaca*" contains both activities as mentioned above. We have found values of 140 cu.mm $\frac{1}{2}$ g dry wt. hour for XO and 0.4 to 0.6 μ g formazan g dry wt. 30 minutes at 37°C for XD. The values found here are lower than those reported previously for rat liver (21) (22) (23). These results are plotted in Fig. 1 and Fig. 2 in comparison with those of the normal rat liver.

L-amino acid oxidase activity was determined manometrically by the method of Warburg and Christian (27). In all determinations the pyrophosphate buffer pH 8.3 was substituted for Tris (hydroxymethyl) amino methane buffer pH 7.2 according to the observations of Singer and Kearney (14) (16) that pyrophosphate inhibits the L-amino acid oxidase activity of snake venom. The side arm of the Warburg flask was filled with 0.2 ml of 0.1 M L-leucine and the other experimental conditions were the same used for the XO activity determinations.

L-amino acid oxidase activity of the liver gave values of 1552 cu.mm $\frac{1}{2}$ g dry wt. hour. These results are shown in Fig. 3.

The assay of L-amino acid oxidase activity of blood serum of the snakes gave values averaging 15.6 cu.mm $\frac{1}{2}$ ml 60 minutes. Recovery experiments were undertaken by adding known amounts of the venom (L-amino acid oxidase activity of which has been previously determined) to the serum. The results were quite satisfactory as shown in Table 1.

TABLE I

L-Amino acid oxidase activity of blood serum of *B. jararaca*

Snake venom	Blood serum	2ml blood serum + 1 mg snake venom	Recovery
182.1	15.6	196.3	99.2%
cu.mm O ₂ /mg/hour	cu.mm O ₂ /2 ml	cu.mm O ₂ /hour	

2) Paper Electrophoresis:

Paper electrophoresis was carried out in an apparatus of the sandwich-type described previously (13), with the modifications outlined before (12). The electrode vessels were filled with equal amounts of buffer and the liquid levels balanced. We have used about 750 ml of 0.06 M veronal, pH 8.6 in each vessel where the two ends of the filter paper dipped into buffer solution. The glass plates were coated with a very thin layer of silicone grease with a thick layer on the edge of each plate in order to produce a seal. In all experiments Whatman 3MM filter paper sheets 10cm X 57cm were used. With this arrangement four samples of serum could conveniently be run at one time.

The filter paper was moistened with the buffer and blotted to remove any excess of liquid. It was laid across one of the glass plates allowing 11cm of paper on each end. The apparatus was then allowed to come into hydrostatic equilibrium before application of the serum. With the aid of a micro-pipet, 0.01ml of blood serum was applied on previously marked dots across the center of the paper. The other plate was then placed on top and these were held together by means of six pinch clamps equally distributed along the plates. The power pack was turned on and a potential difference of 220-250V was used, which should give 2-2.5mA in our experimental conditions. The electrophoresis proceeds for 12-16 hours.

After the electrophoretic separation the paper was carefully removed and placed into an oven at 100°-120°C in order to fix the proteins to be stained. The dye-elution technique of Kunkel and Tiselius (5) was employed with some modifications. The filter paper was placed in a tray of 1% bromophenol blue in alcohol saturated with mercuric chloride for 15 minutes. It was then washed with 0.5% acetic acid till the background became white. The strip was again dried and after this it was cut transversely into several narrow sections of 0.5cm. The small sections were eluted with 5ml of 2% sodium carbonate in 50% ethyl alcohol. The eluates were read at 600 m μ , in a spectrophotometer and the readings plotted against distance along the strip as usual. The detection of fluorescent bands in the paper electropherogram was accomplished by exposing the unstained and dried strip to ultraviolet light.

Paper electrophoresis of blood serum gave a spectrum of 7 components and two of these fluoresced when exposed to ultraviolet light. Comparison with human blood serum showed that the fluorescent fractions have a migration rate corresponding to human serum albumin and beta globulin fractions. Fig. 4 shows a typical pattern of the electrophoretic separation. In our experimental conditions it was found that sharply separated zones are difficult to achieve.

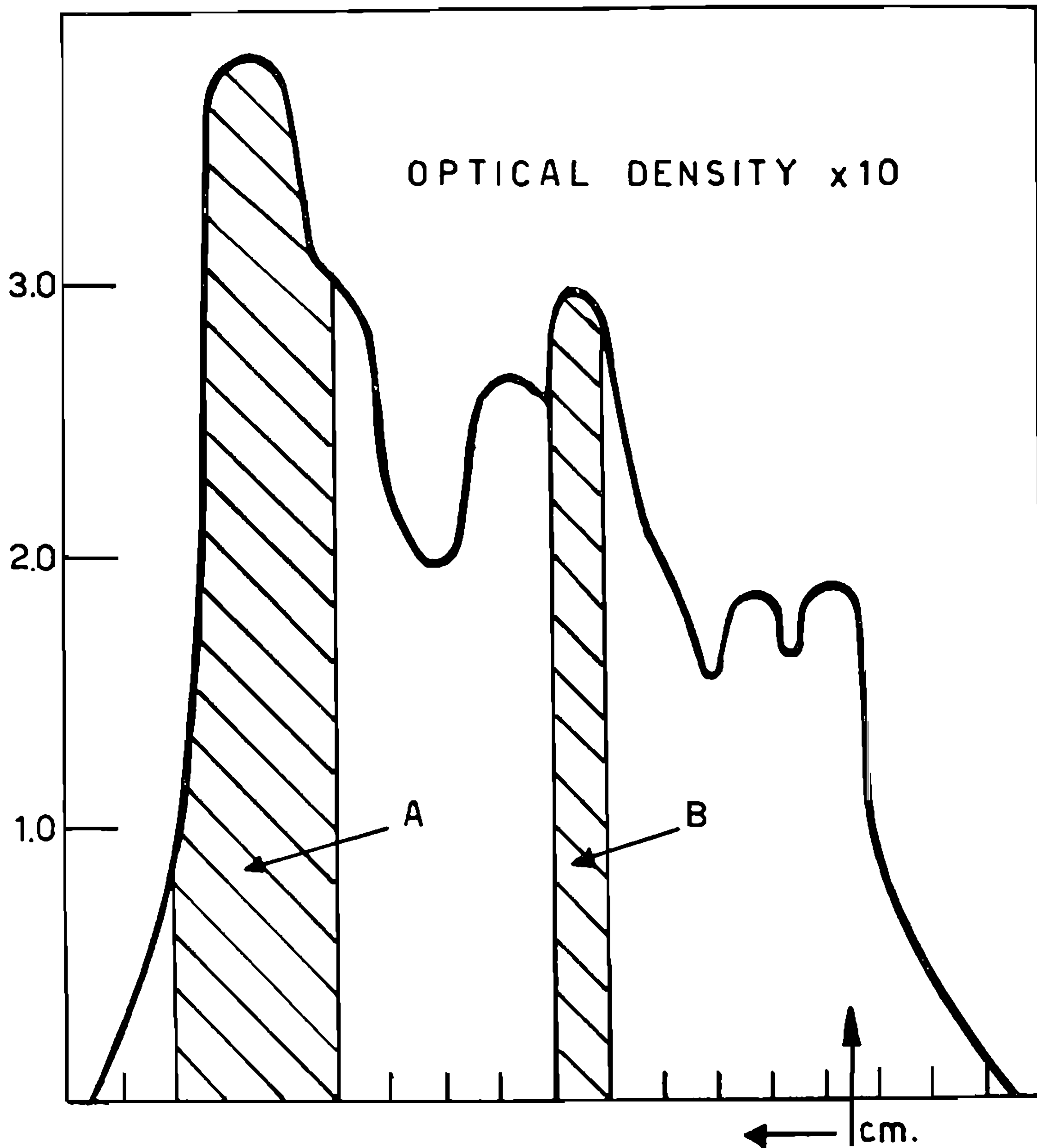


Fig. 4 — Paper electropherogram of blood serum of *B. jararaca*. A and B are the fluorescent fractions corresponding to human serum albumin and β -globulin respectively. The arrows indicate point of application and direction of run

3) Spectrographic Analysis:

The spectrographic analysis was performed in a large Hilger quartz spectrograph. Samples of venom and blood serum were analyzed by arc technique and the results confirmed the negative data obtained for XO and XD activities, since no molybdenum could be detected, even when 50mg of dry venom and 2ml of fresh serum were used.

Table 2 shows the results obtained for the venom and serum.

TABLE II

Spectrographic analysis of venom and blood serum of *Bothrops jararaca*.

Venom	Blood serum
Mg	Ca
Na	Si
Ca	Mg
Al	Na (traces)
Mn (traces)	
Si (traces)	

Discussion and Conclusions.

Paper electrophoretic and enzymatic studies have been made by several investigators specially using the venom of different species of snakes. D. von Klobusitzky and P. König seem to be the first investigators to use paper electrophoresis to fractionate snake venom (4). Neumann and Habermann (10) (11) also have used this method for separating "jararaca" and other snake venoms. As previously mentioned, these authors have found fluorescent bands in the electropherogram probably due to flavins. Some enzymes were studied, specially phospholipase. Grassmann and Hanning (3) have also examined a great number of snake toxins by paper electrophoresis and the enzyme activities of the fractions were determined. Special attention was given to lecithinase A and proteolytic activity.

Mochl (6) (7) undertook an extensively electrophoretic and enzymatic study of jararaca toxins. This author studied the enzymatic activities of the different fractions and he was able to detect L-amino acid oxidase, ATP ase, deoxyribonuclease, lecithinase A and proteolytic activities. No reference was made to xanthine oxidase and the serum and liver were not examined. Using densitometric measurements, with ultra-violet light (280 m μ), Michl found a marked increase in optical density in the area corresponding to the starting point of the electropherogram. This fact was explained as due to the prosthetic group (FAD) of L-amino acid oxidase.

It is known that with one exception (18) native flavoproteins, such as L-amino acid oxidase and xanthine oxidase, are not fluorescent (8) (9) (15). The fluorescent spots found in our electropherogram of the snake serum could then be due only to some denaturation of L-amino acid oxidase or to some other flavoprotein not investigated, since no XO could be detected. As a matter of fact, the electrophoretic pattern seems to show that spot A could be due to some denaturation of L-amino acid oxidase if one accepts the same localization of this enzyme activity in

both serum and venom of *B. jararaca*. This is acceptable since Michl showed that the L-amino acid oxidase activity of the venom is localized in the faster migrating protein fraction. Although we did not have conclusive evidence for this statement it seems to be possible since denaturated flavoproteins are fluorescent (17).

The venom of *B. jararaca* is also very rich in riboflavin (19) and appears to be one of the richest known sources of L-amino acid oxidase (28) (17). As in our assays xanthine oxidase could not be detected it seems possible that the flavins are part of the FAD bound only to L-amino acid oxidase, as demonstrated previously by Singer and Kearney (15).

In conclusion we may say that the flavins of blood serum and of the venom of *Bothrops jararaca* are partly linked to the protein fractions as was shown by paper electrophoresis and dialysis experiments. These flavins also seemed to be in the form of FAD bound only to L-amino acid oxidase, since no XO could be detected. Further experiments are now in course to establish the nature of the flavin compounds present in blood serum and venom of some snakes and will be the subject of a future publication.

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Resumo.

Fígado, veneno e sôro sanguíneo de "*Bothrops jararaca*" foram estudados por meio da eletroforese em papel e determinação de atividades enzimáticas. Xantina oxidase e deshidrogenase foram encontradas somente no fígado das cobras. A análise espectrográfica do veneno e do sôro confirmaram os resultados negativos obtidos para xantina oxidase uma vez que não foi encontrado molibdênio. L-amino ácido oxidase foi determinada no fígado, sôro e veneno.

A eletroforese em papel do sôro sanguíneo mostrou que existem 7 frações proteicas, sendo que duas apresentam fluorescência característica de flavinas, quando expostas à luz ultra-violeta.

Em vista dos resultados obtidos é concluído que as flavinas do sôro e do veneno de *Bothrops jararaca* estão na maior parte ligadas às proteínas. Estas flavinas combinadas parecem estar sob a forma de FAD (flavina adenina dinucleotídeo) fazendo parte do grupo prostético da L-amino ácido oxidase, uma vez que não foi encontrada nenhuma atividade de xantina oxidase.

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