

## 23. THE INFLUENCE OF SNAKE VENOMS OF FIBRINOGEN CONVERSION AND FIBRINOLYSIS

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The studies on the influence of snake venoms seem at the present time to be a special branch of the coagulation theme. Most of the work has been devoted to the procoagulant action of the venoms. Far less numerous are the papers dealing with anticoagulant properties of snake venoms.

In some of our previous works we were able to show, that the *in vitro* anticoagulant action of this second group is at least partially due to the destruction of prothrombin. However the role of the fibrinolytic properties of these venoms still remain to be elucidated. In this respect the most active proved to be those from *Agkistrodon piscivorus*, *Agkistrodon contortrix* and surprisingly also the venom of *Vipera lebetina*. The results obtained indicated that the fibrinolytic compound of these three toxins are mutually very similar as regards the quality of their action and differences are merely of quantitative nature, the venom of *Agkistrodon piscivorus* being the most potent.

The first approach to the estimation of fibrinolytic properties of any substance is the classical method of the native and heated fibrin plates, where plasminogen has been destroyed by the heat. We can see the lytic areas produced by 0.1% solution of the toxins (Fig. 1). *N* = native plates, *H* = heated. In the center trypsin in 0.01% for comparison.

At first one would conclude that the difference indicates the presence of the plasminogen activating system in the toxins. That was also our interpretation of these findings till we had the opportunity to work with purified plasminogen.

Plasminogen has been added both to the fibrinogen solution of which the plates have been made and/or it had been incubated with the venoms before dropping them on the plates. In neither case there was any significant difference in the lytic areas produced by venoms with or without plasminogen, i.e. the latter was not activated by the venoms (Fig. 2).

*FBG + PLG* = plasminogen added to the fibrinogen solution,

*TOX + BLG* = plasminogen incubated with toxins, *N* native plate.

If the bovine fibrinogen solution is incubated with venoms it gradually loses its ability to be converted into fibrin by thrombin, i.e. it is either denatured or split-off. There is no significant difference in the course of this action if plasminogen incubated for 10 min at 37°C either with toxin or toxin alone is added to the fibrinogen solution. From all these results it can be concluded, that the venoms have practically no plasminogen activating properties (Fig. 3).



By addition of 0.01% solution of epsilon amino kaproic acid (EAK) fibrin clot lysis from the test tube wall is prolonged from 5 to 8 min. Without EAK the venom acts synergically with plasmin. This is reflected in the shortening of the lysis time to 4 to 3 min. Toxins alone would cause the clot lysis only after a considerably longer period (over 20 min.), which corresponds to their own fibrinolytic activity. On the other hand toxins added to the test tube simultaneously with EAK are able, to a certain extent, to paralyse the inhibitory effect of a specific fibrinolysis inhibitor which EAK is known to be (Fig. 4).

#### CORRELATION BETWEEN THE VENOMS, PLASMIN AND EPSILON AMINO KAPROIC ACID IN LYSIS TIME OF A FIBRIN CLOT

The dynamism of clot formation and its lysis can be very well observed through the method described by Gruedlinger in which the clot formation and lysis is assessed by plotting changes of the turbidity (measured photometrically at 350  $\mu\text{m}$ ) of the tested system against time. This method is definitely more accurate and therefore we have used it to ascertain the fibrinolytic properties of the venoms — plasmin mixtures and their blocking by various inhibitors of the proteolytic enzymes. We can observe that the venoms are able to paralyse the inhibitory effect of soya-bean inhibitor (SBI), Trasylol (TRA) and EAK (Fig. 5).

#### SYNERGIC ACTION OF VENOM WITH PLASMIN AND RESTRICTION OF THE INHIBITORY EFFECT OF VARIOUS INHIBITORS BY VENOM

To get an idea about the quantitative relations between the lytic activities of toxins, plasmin and trypsin and about the influence of the different inhibitors exerted on these enzymes an arbitrary unit has to be established. For this purpose the turbidimetric method was rather time consuming and therefore we have used the fibrinogenolytic properties of these active substances.

From the curves in Fig. 6 we can see that the fibrinogenolytic activity of 300 U/cc of plasmin can be compared in case of *Agkistrodon piscivorus* venom with 0.1 mg/cc and in case of *A. contortrix* venom with 0.25 mg/cc. Adhering to these quantitative relations, we have added to the tested system various inhibitors in different concentrations. Both plasmin and trypsin are inhibited by 0.01% EAK, 0.001 SBI, 250 U/TRA, whereas even hundred times stronger concentration of these inhibitors had practically no effect upon the fibrinogenolytic activity of venoms (Fig. 6).

#### QUANTITATIVE RELATION BETWEEN PLASMIN & VENOMS AND DIFFERENT INHIBITORS

We were further interested if the split products which result from the action of venoms upon fibrinogen are of a nature similar to those produced by the lytic action of plasmin, at least if there is an antithrombin VI activity which is attributed to the polypeptide D.

As can be seen in Fig. 7 fibrinogen split products of both lytic agents have been added to fibrinogen and thrombin solution and the increasing turbidity indicated the course of fibrin formation, i.e. the activity of thrombin. From the curves we can see that in case of snake venoms a considerable activity of antithrombin VI is present within split products of toxin fibrinogenolytic action (Fig. 7).



## ANTITHROMBIN VI ACTIVITY OF SPLIT PRODUCTS PRODUCED BY ACTION OF SNAKE VENOMS UPON FIBRINOGEN

Beside the fibrinolytic properties the tested venoms are known to possess a fair amount of proteases. It was of interest to compare the proteolytic activity of the venoms with the lytic activities of plasmin and trypsin and to find out how the toxin proteases are affected by inhibitors. Proteolysis has been assessed by a slightly modified method of Anson Mirsky using casein as substrate. We can observe that the caseinolytic proteases of the venoms are not inhibited by inhibitors, unlike trypsin, which is (Fig. 8).

### ACTION OF DIFFERENT INHIBITORS UPON VENOM PROTEASES AND TRYPSIN

To be at least partially sure that both the activities, fibrinolytic and proteolytic could be attributed to the same enzyme we had to isolate the active substances from the venom. After several attempts, using different separative procedures the most convenient method proved to be starch gel electrophoresis. From the curves showed in Fig. 9, we can see that both these activities always go along. The lytic fractions had no other enzymic activities. They were roughly 50 times less toxic than the whole venom and the lytic enzymes are 150 times more concentrated than in the whole venom, as could be computed from the protein content. These results are similar in all the three venoms. We were nevertheless not able to separate completely the haemorrhagins from these lytic fractions. Besides the proteolytic enzymes there is a different enzyme — the esterase splitting TAME — present in the venoms. This activity does not go parallel with the protease. Both these results are in full agreement with the findings obtained recently by Japanese authors for fractions of Habu snake venoms (Fig. 9).

### STARCH GEL ELECTROPHORETIC PATTERNS OF *Agkistrodon piscivorus* VENOM AND THE ENZYMIC ACTIVITIES OF THE FRACTIONS

In the experiments *in vivo* we have injected sublethal doses of *Agkistrodon piscivorus* venom (400  $\mu$ g/100 gr) subcutaneously into white rats. In these animals the routine blood coagulation check-up has been performed in time intervals of 30 min. 2 h. and 24 h., respectively. Except for a slight hypercoagulability of the whole blood in the first 30 min. there were no significant changes in clotting time of plasma in experimental animals compared with the control group.

The only pronounced difference was in the activity of euglobulin fraction in animals 30 min. after toxin application. Surprisingly the fibrinogen content was not altered in the sense of a decrease. On the contrary we could observe a significant rise in fibrinogen content in animals 24 h. after application. Both these findings can, in our opinion, be attributed to the stress reaction caused by toxin (Fig. 10 and 11).

By means of a 10% solution of formaldehyde in 60% alcohol applied in rats jugular vein an artificial thrombosis can be produced. 24 h. after operation on the average in 60% of so treated animals a thrombus can be found. The same amount of venom (400  $\mu$ g/100 gr) has been injected in the animals 10 h., before or 10 h. after the operation. The toxin increased the amount of incidence



of thrombosis from 60% to 100%. This is probably caused by the action of the haemorrhagins exerted on the vessel wall rather than by the rise in the fibrinogen content.

It was surprising that venoms, which have *in vitro* a remarkable fibrinolytic activity are in animals unable to produce any change in their fibrinogen content, this being so even in amounts far exceeding those which have been used in experiments *in vitro*. It indicated the presence of a specific inhibitor in the blood which could inactivate the venom fibrinolysins. From the fig. 12 we can see that this seems to be the case. Fibrinogenolytic activity of both the most potent venoms i.e. *Agkistrodon piscivorus* and *A. contortrix* practically ceases in the presence of sera from different animals, human being included (Fig. 12).



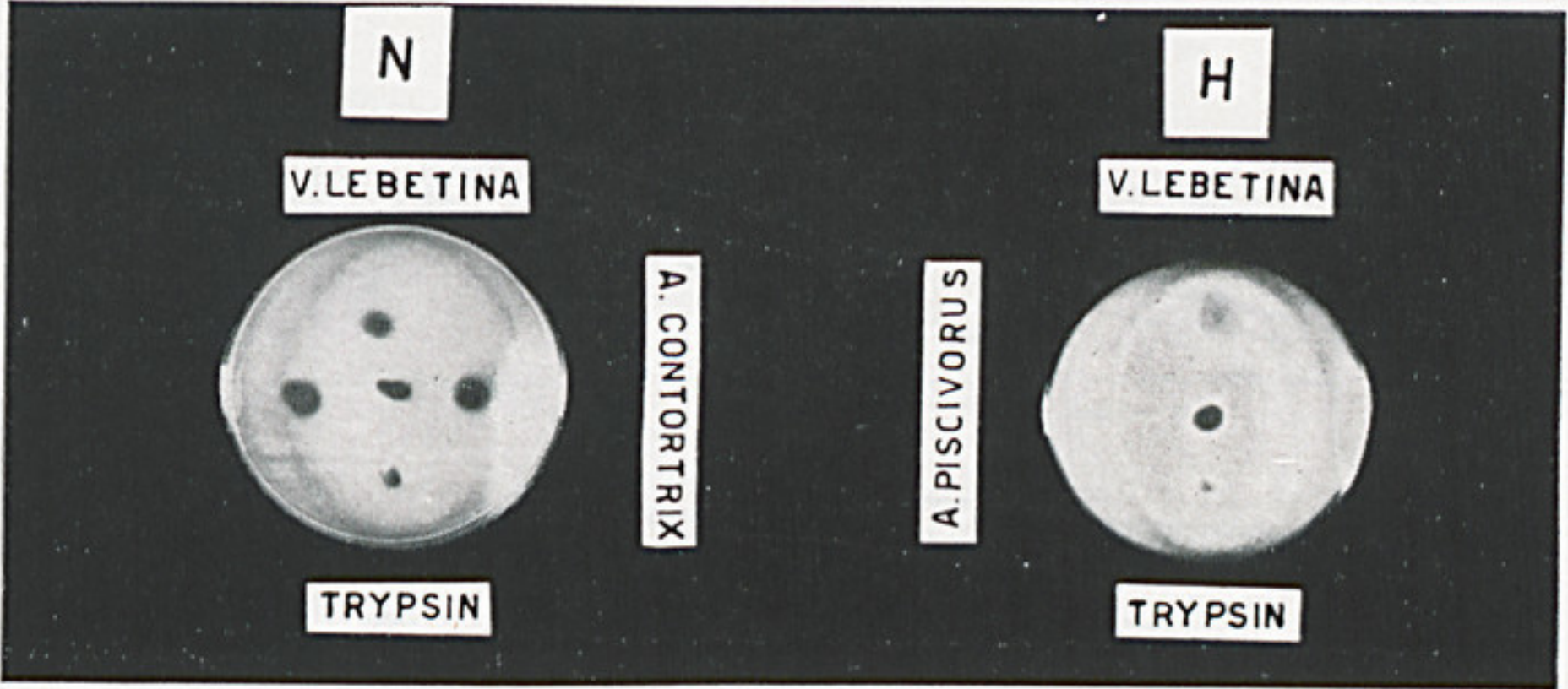


Fig. 1

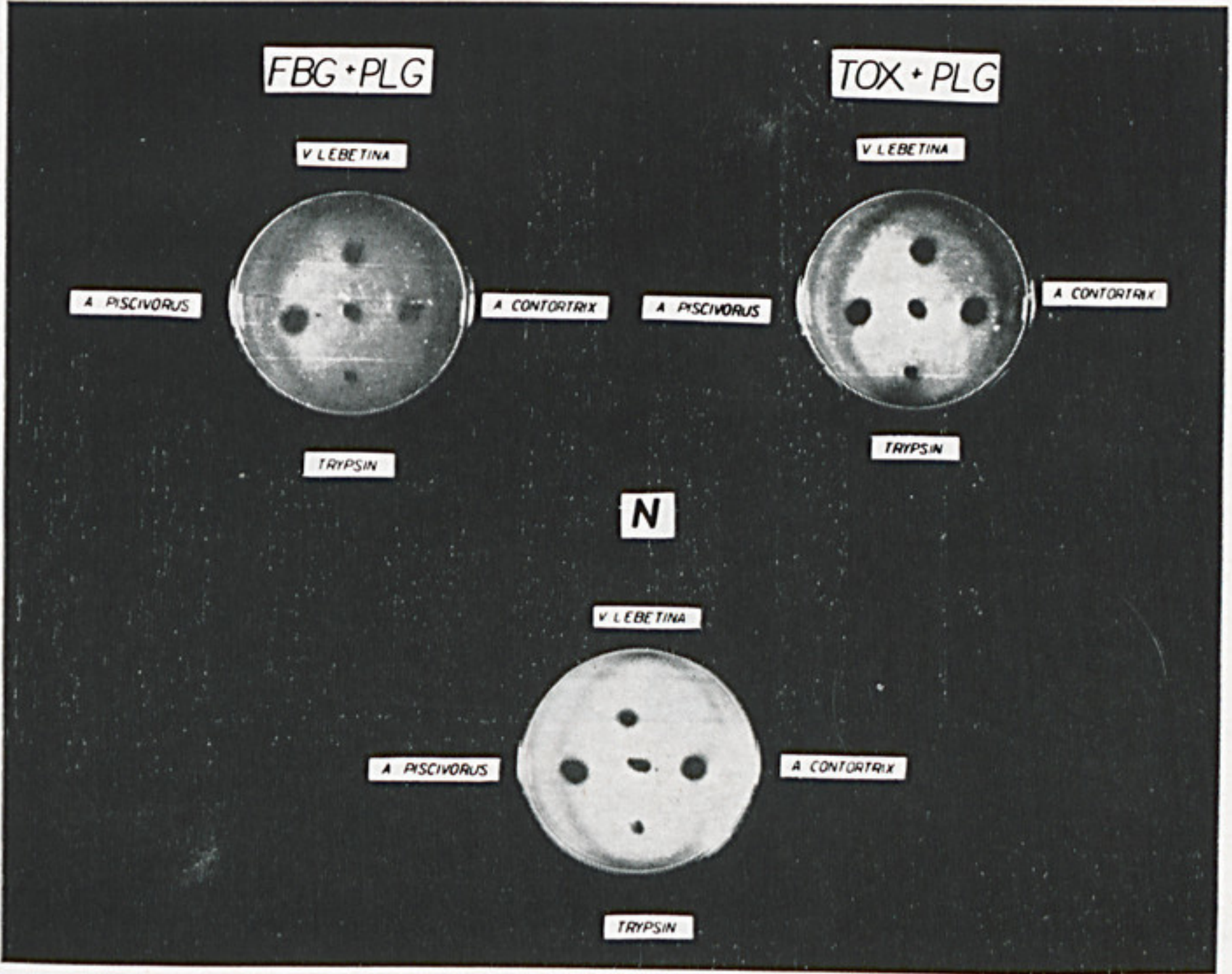


Fig. 2



THE INFLUENCE OF SNAKE VENOMS ON FIBRINOGEN CONVERSION  
AND FIBRINOLYSIS

Fibrinogenolysis by toxins and plasminogen

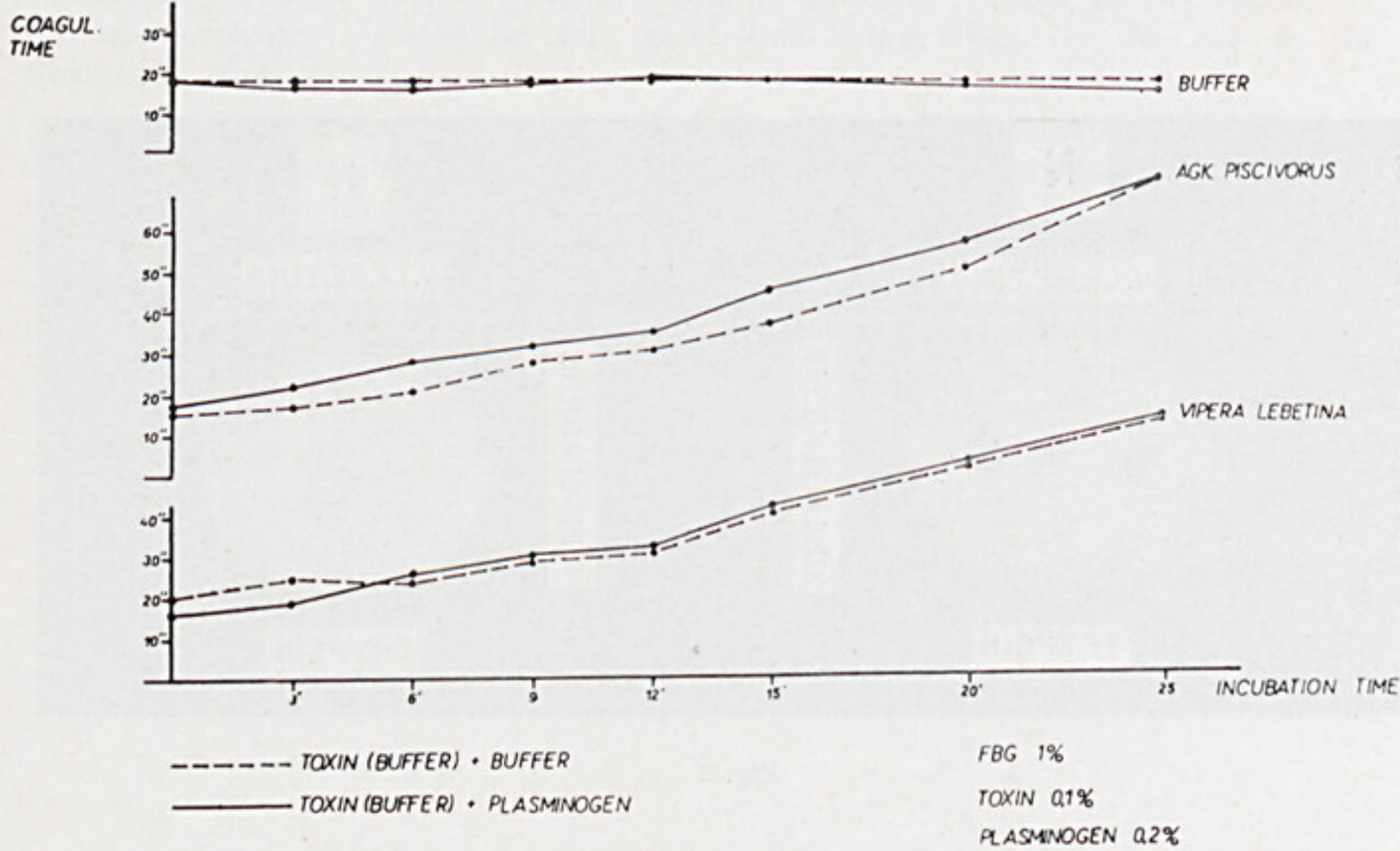


Fig. 3

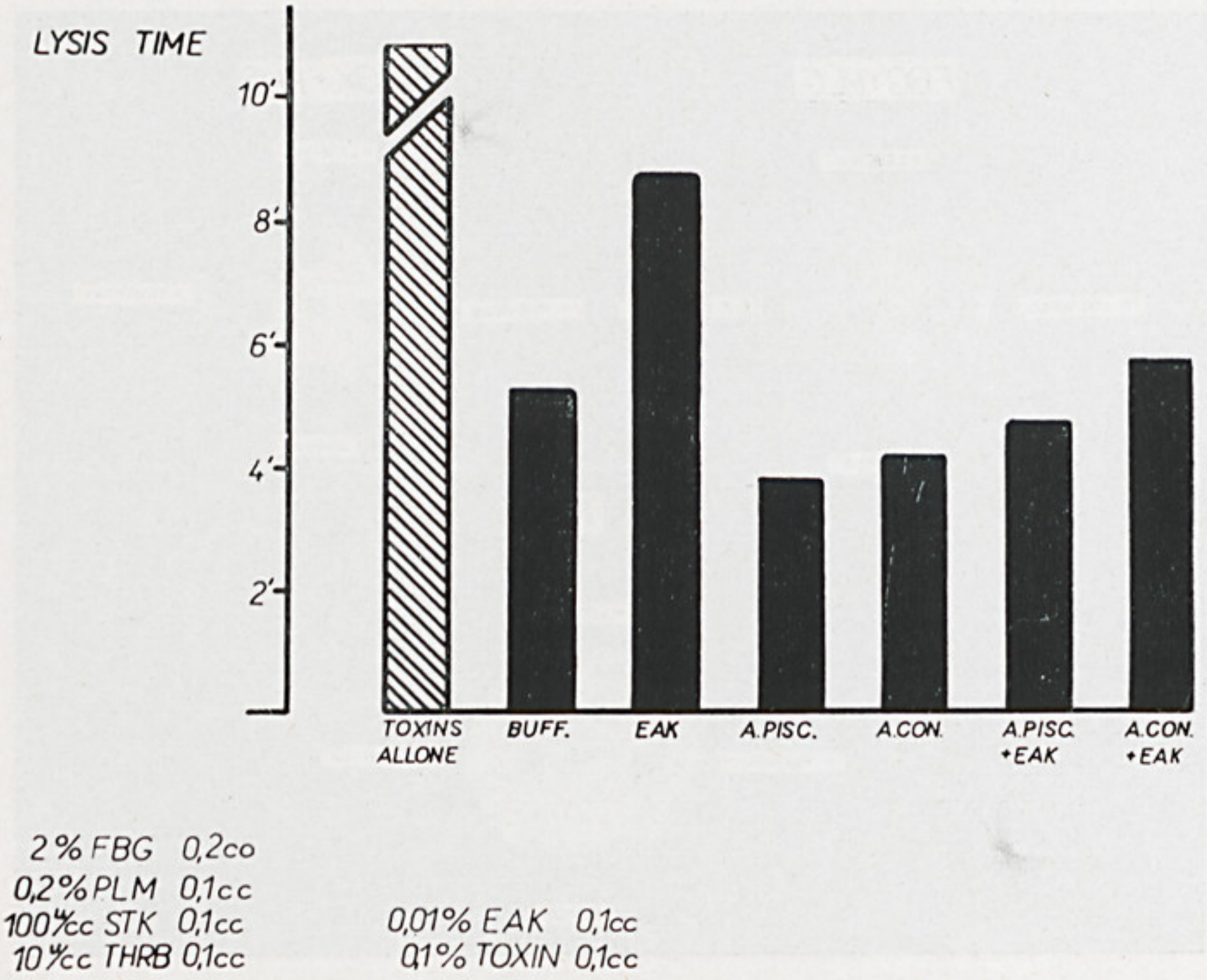


Fig. 4



FBG • PLM • TOX (BUFF) • INHIB (BUFF)

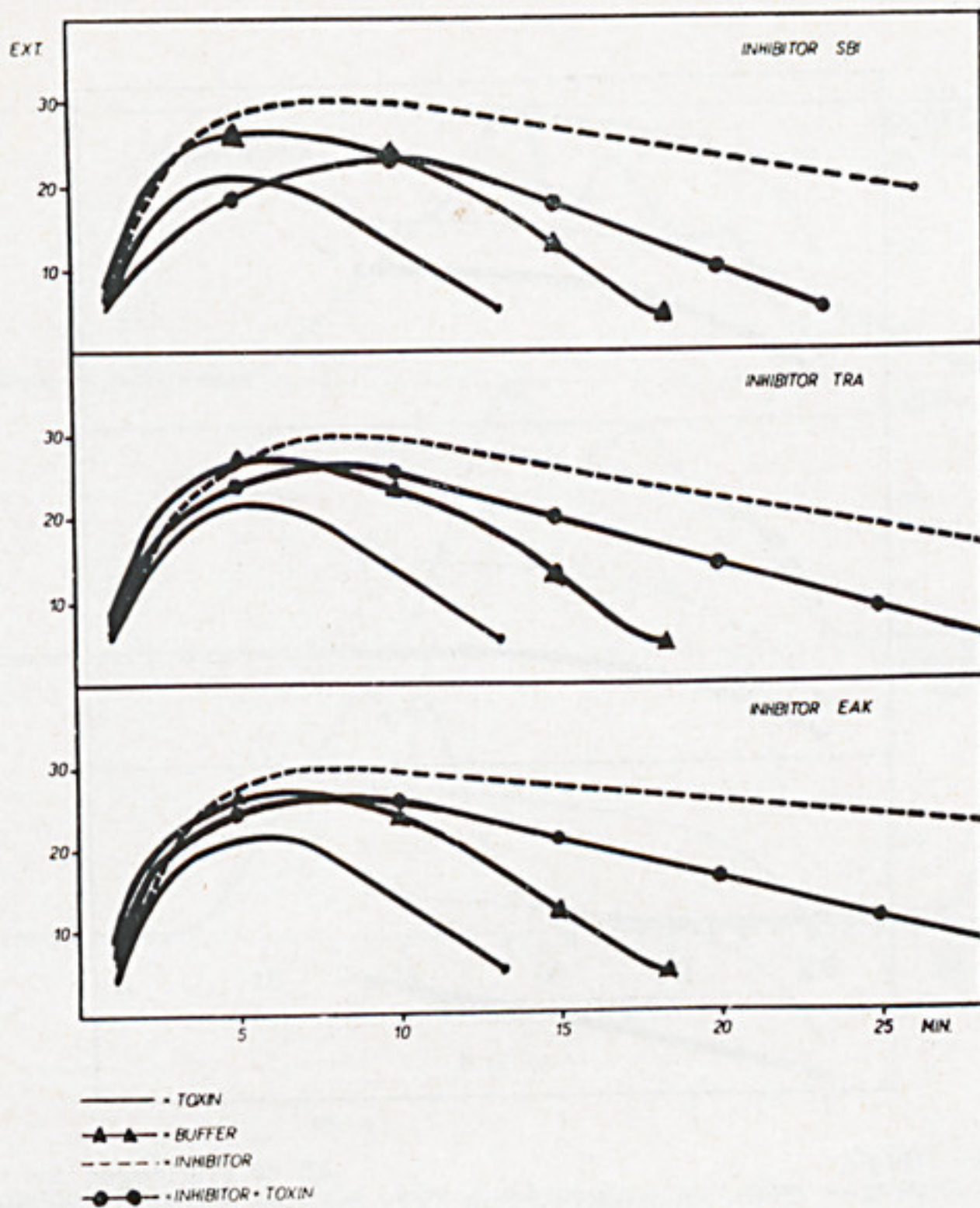
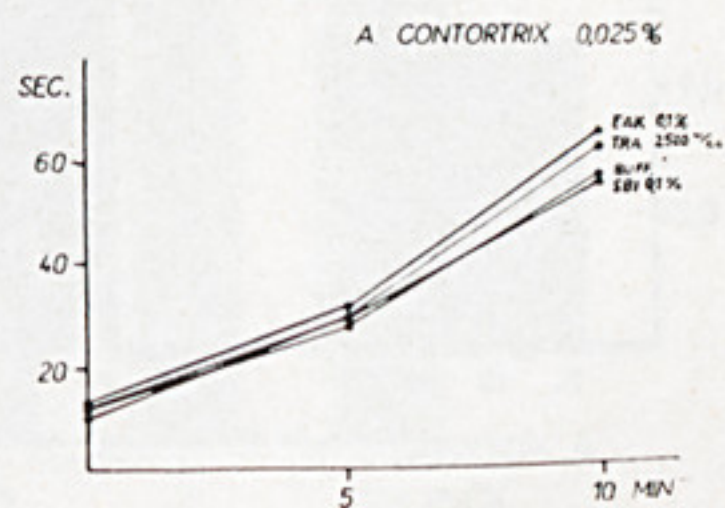
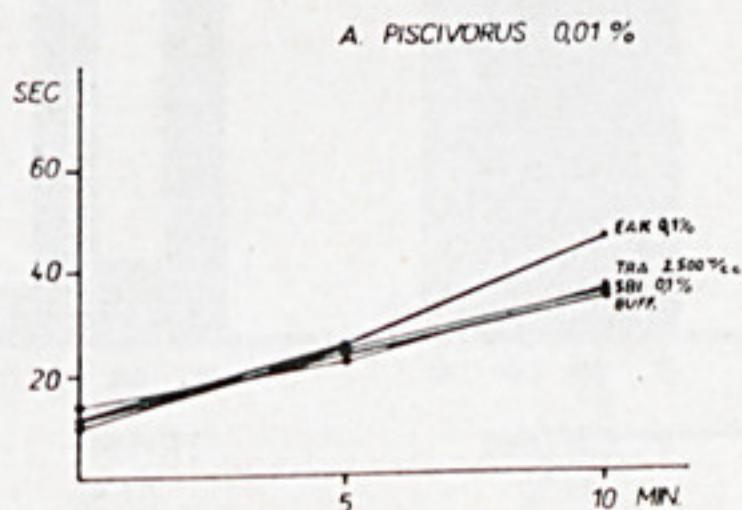
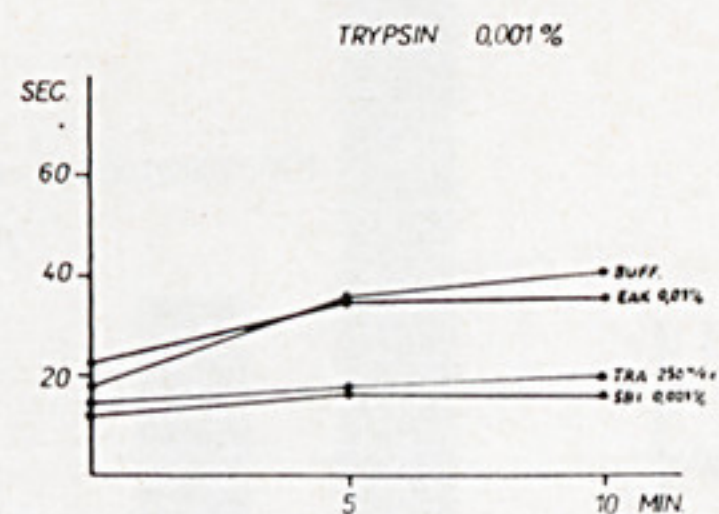
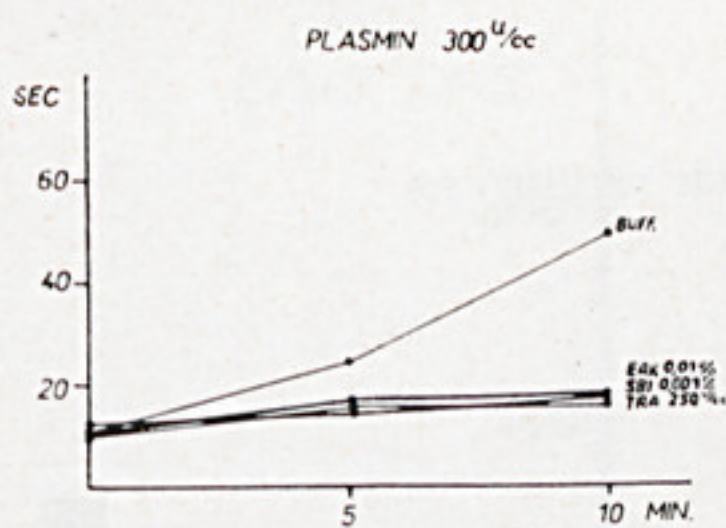


Fig. 5



1cc FBG  
1cc ENZYME  
0.5cc INHIBITOR } 0.2cc • 0.2cc TRB

Fig. 6



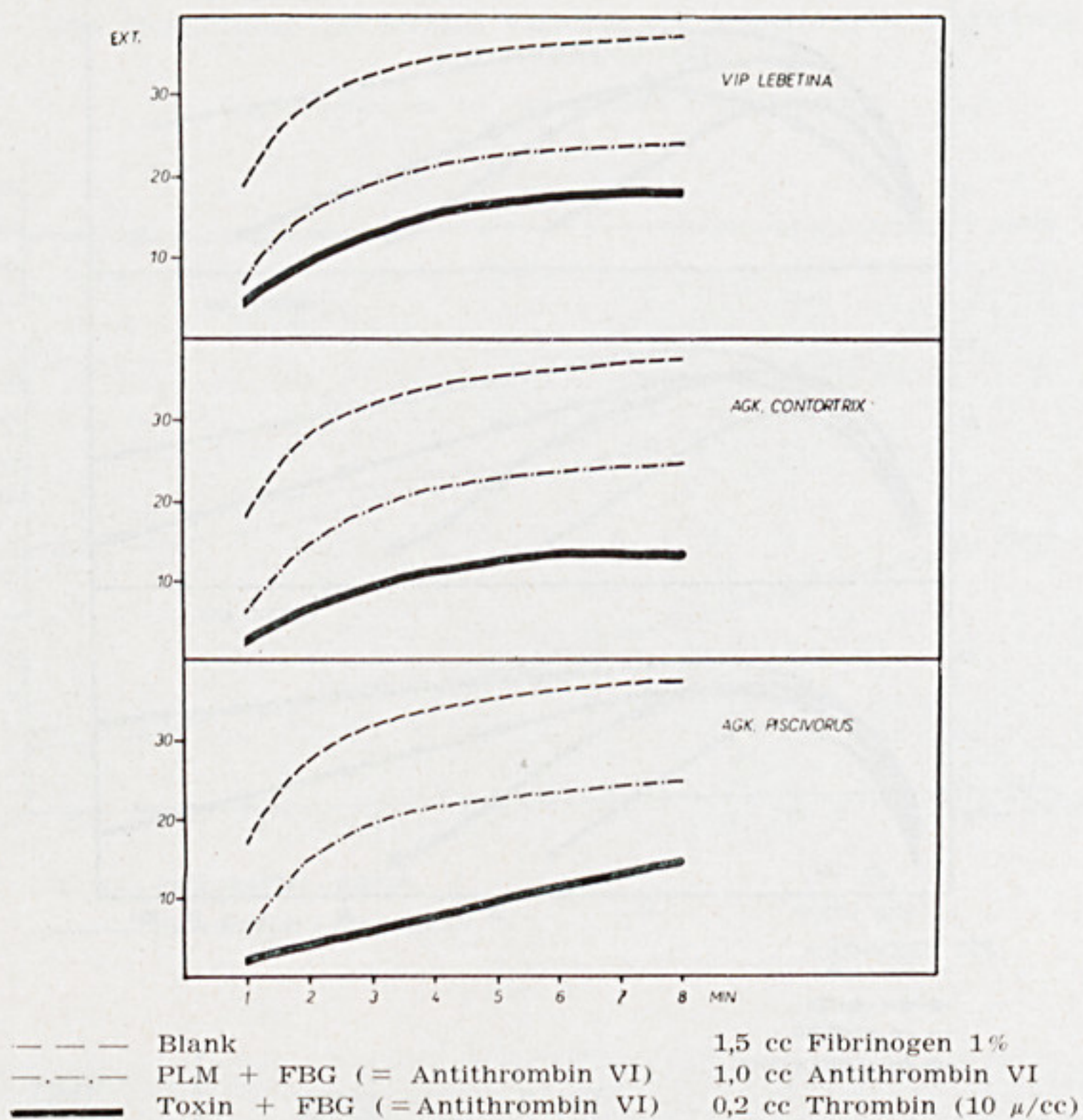


Fig. 7

# PROTEOLYTIC ACTIVITY AFTER INHIBITORS Substrate caseine

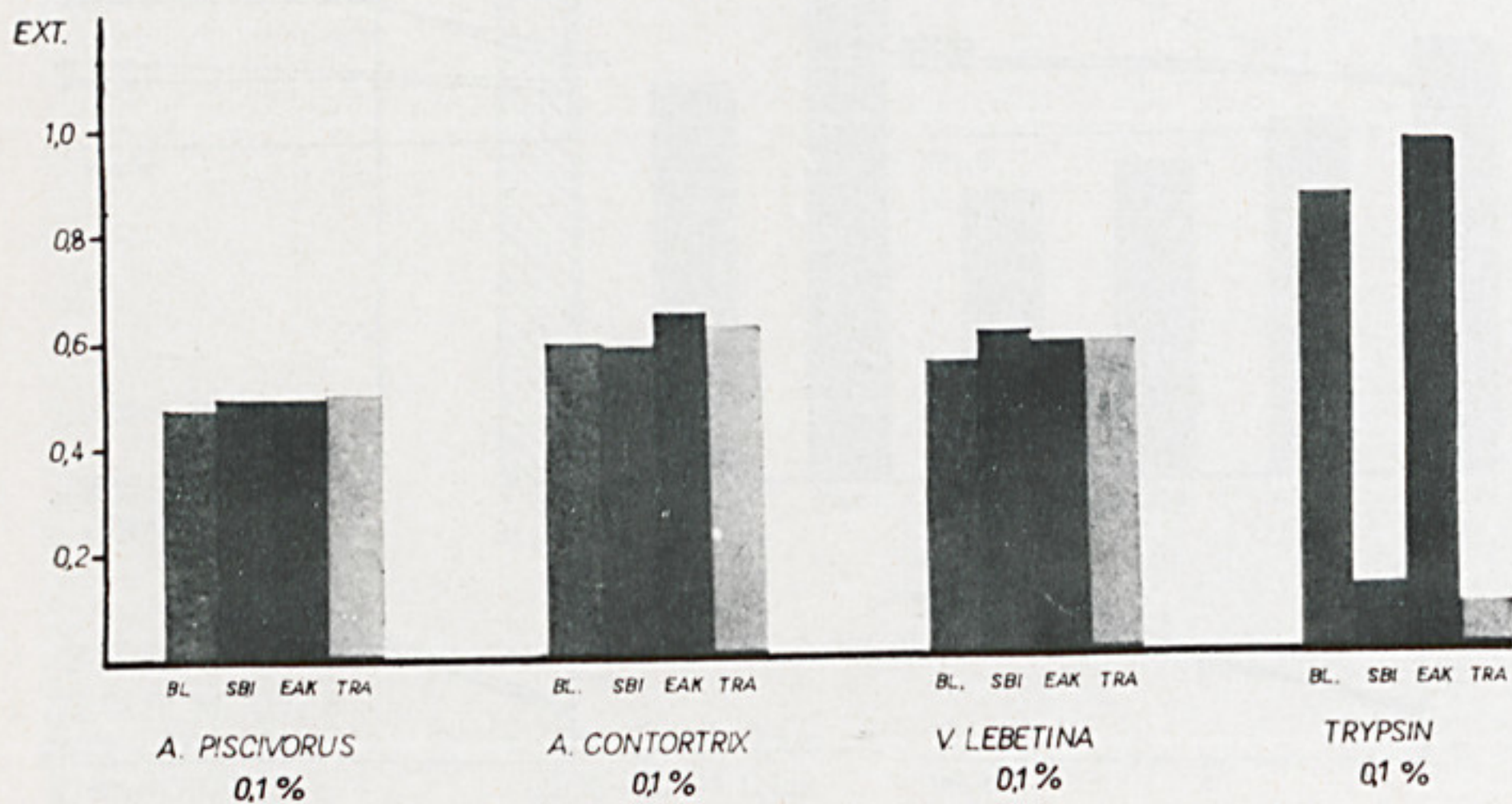


Fig. 8



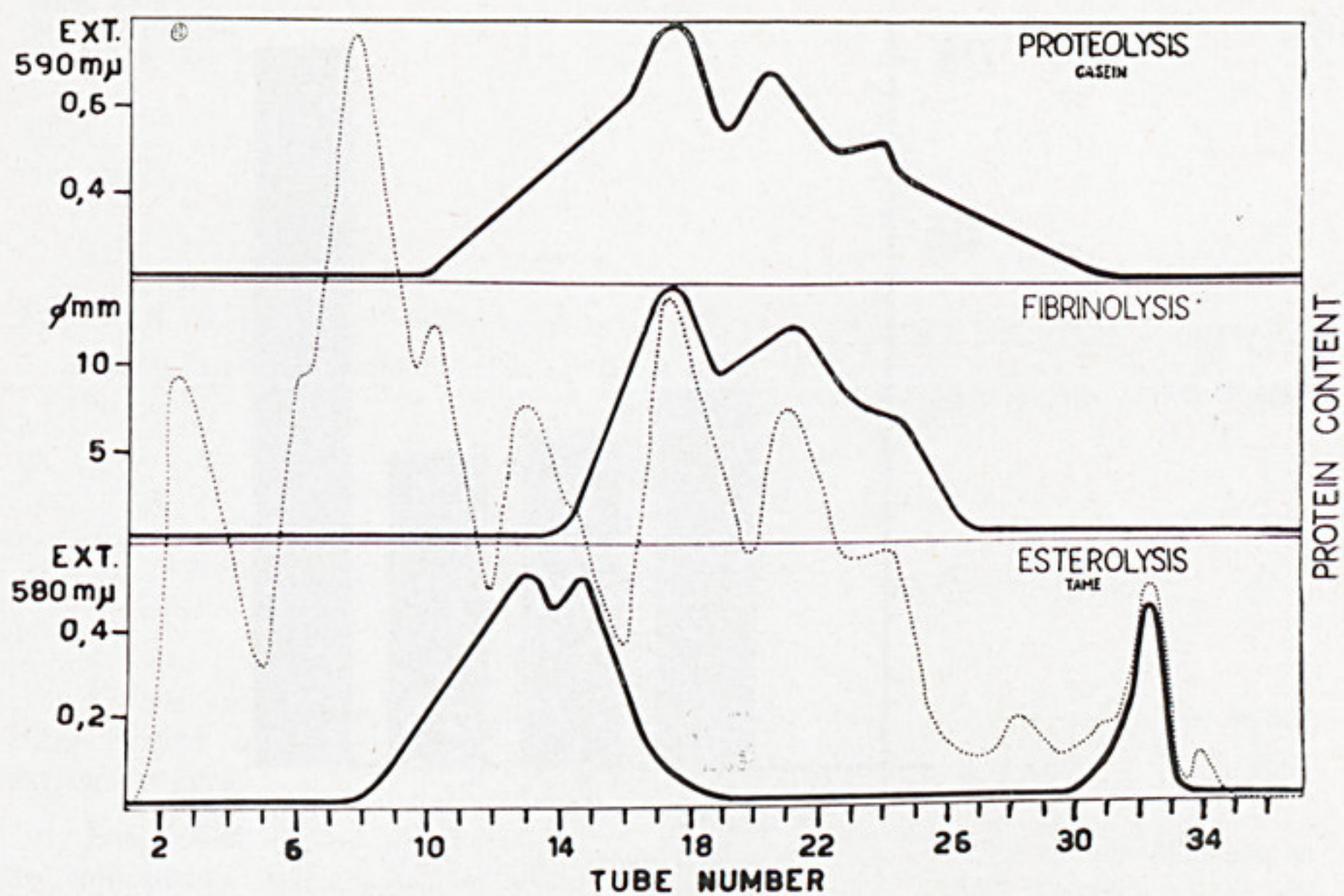


Fig. 9

Euglobulin fraction in rats after 4 mg/g of *Akgistrodon contortrix* venom

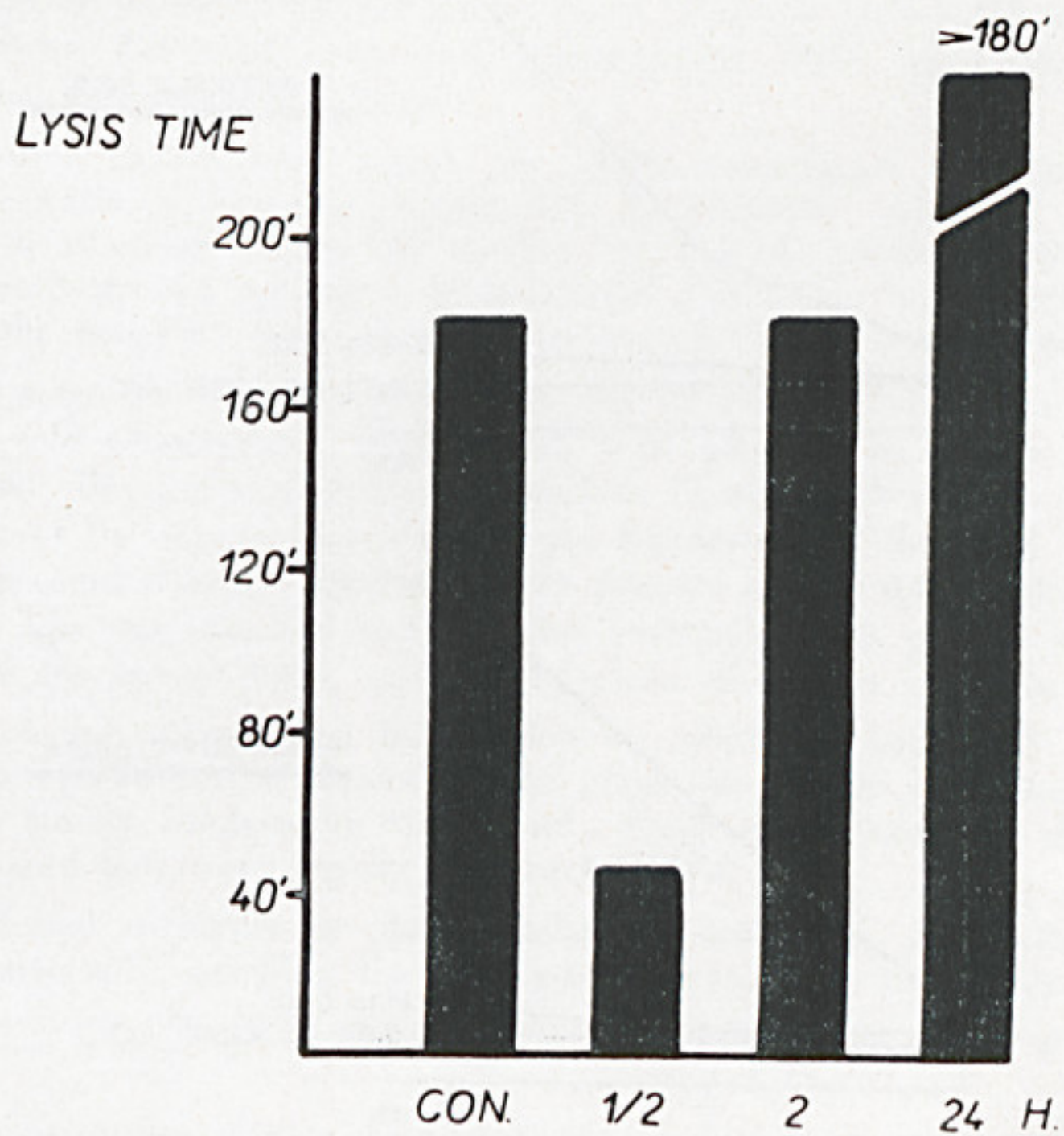


Fig. 10



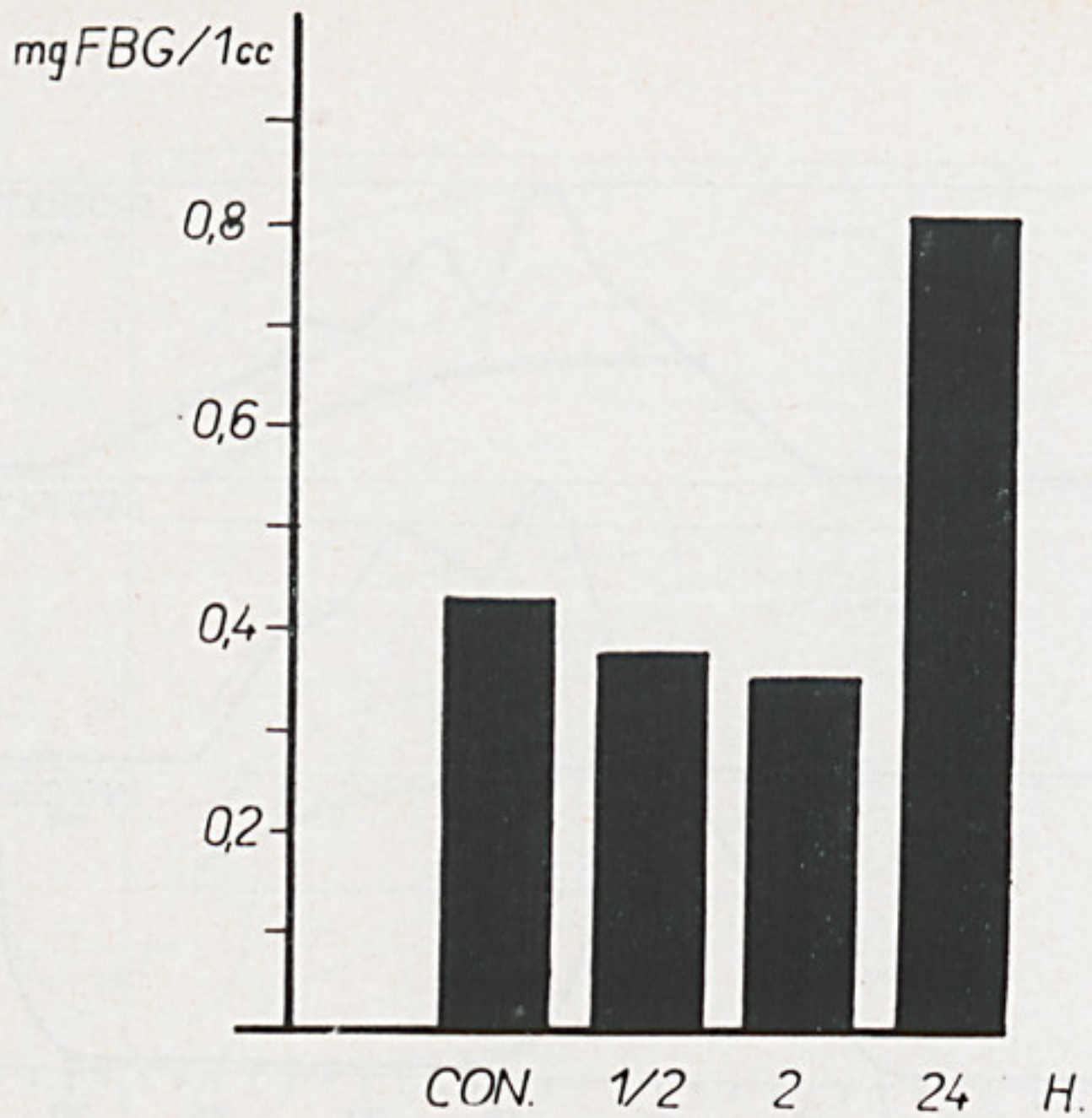
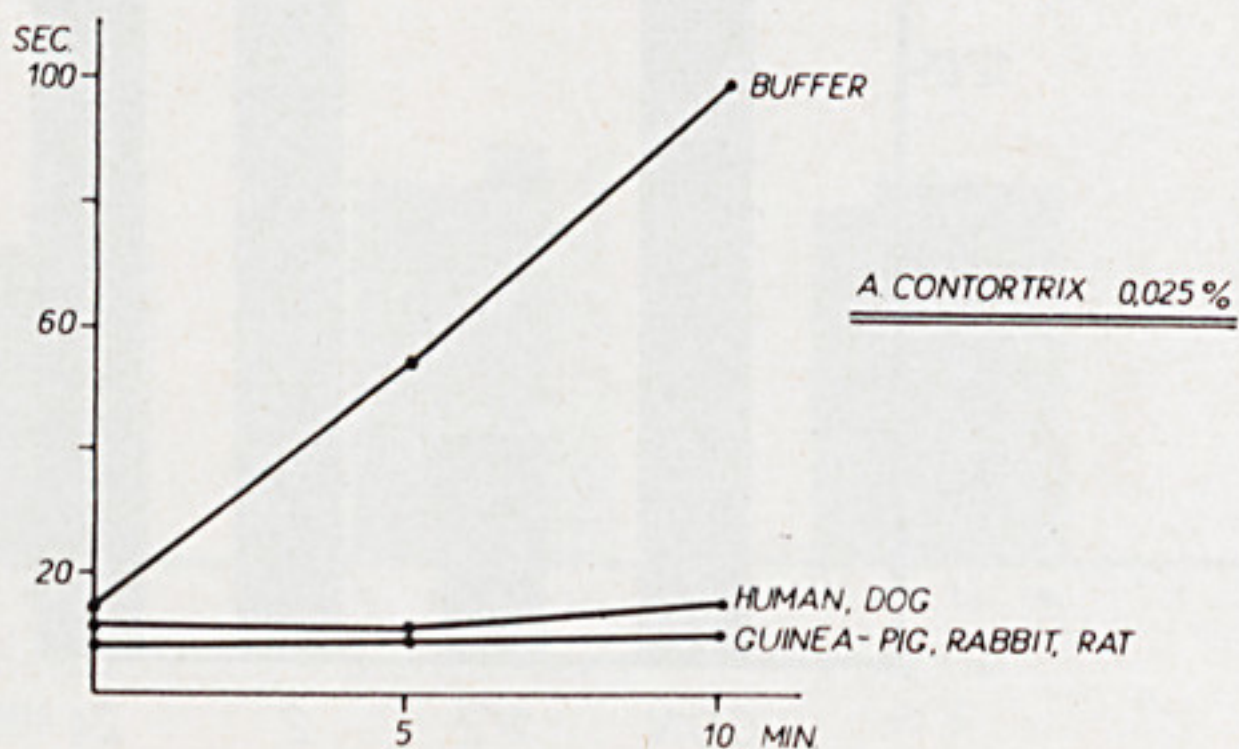
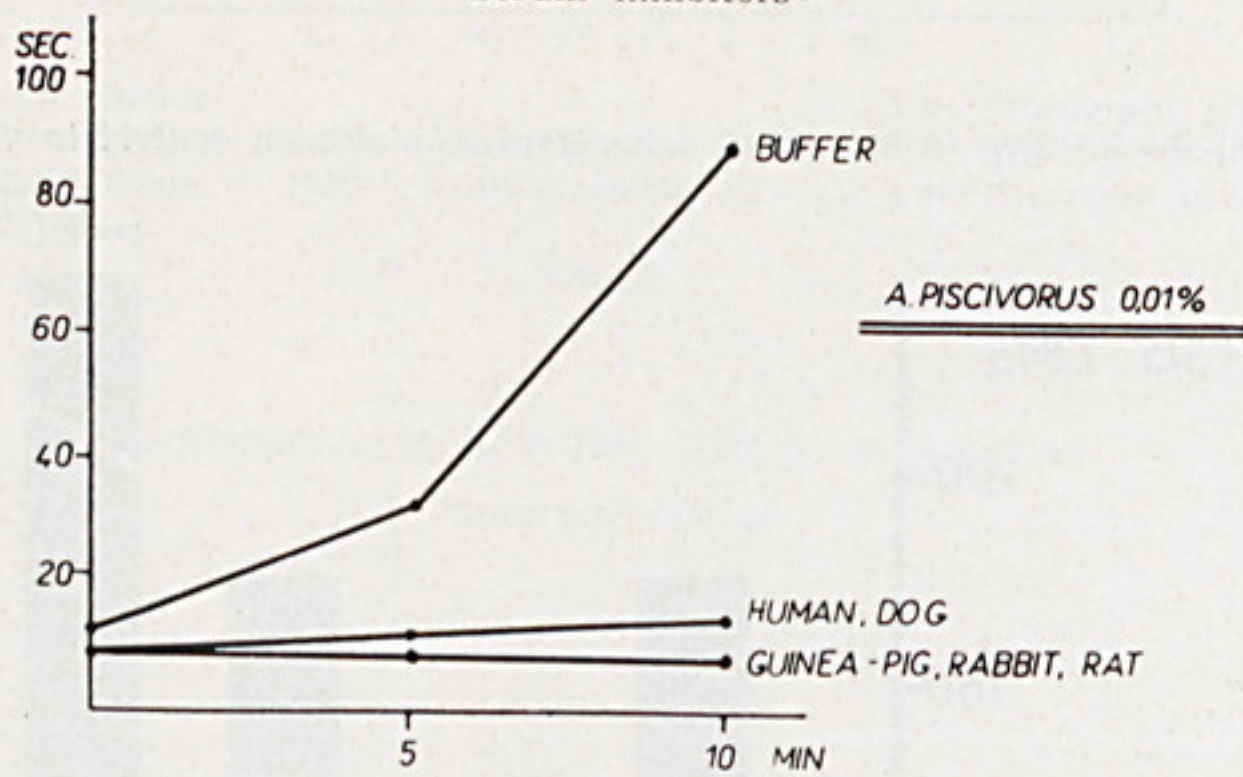


Fig. 11

Serum inhibitors.



1cc TOXIN + 0.5cc PLASMA (BUFFER) + 1cc FBG

0.1cc + 0.1cc THRB

Fig. 12