

Coagulation studies on „Reptilase”, an extract of the venom from *Bothrops jararaca*

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Snake venoms contain a variety of enzymes of biological importance. Earlier investigations in this field were recently reviewed by Porges (1953). In certain snake venoms one or several of these enzymes promote or inhibit the coagulation of the blood. Among the clot-promoting enzymes some have been found to enhance the coagulation by accelerating the conversion of prothrombin to thrombin while on the other hand others promote the conversion of fibrinogen to fibrin. Eagle (1937) found that certain venoms such as from *Bothrops jararaca* act like thrombin and have simultaneously an effect on the conversion of prothrombin to thrombin. He believed the action to be a proteolytic one like that of papain on fibrinogen or trypsin on prothrombin.

Rocha e Silva and Andrade (1945) as well as Breda, Bernardi and Sala (1951) likewise pointed out that the venom from *Bothrops jararaca* possessed both thrombin and thromboplastin activity.

Klobusitzky (1935) and later Klobusitzky and König (1936) succeeded in separating the coagulating principle of the venom of *Bothrops jararaca* from the neurotoxic activity. Klobusitzky thought this coagulating principle to be an enzyme, haemocoagulase, which accelerates the clotting of whole blood and coagulates oxalated plasma.

The preparation „Reptilase“ (Pentapharm, Basel) is said to contain the clot promoting agent of the venom of *Bothrops jararaca* in a purified form. Deutsch (1955) found this preparation to have a thrombin-like effect and probably also a thromboplastin effect. Clinical experience in the treatment of haemorrhages with this drug has been described by Bruck and Salem (1954), Fleischhacker (1954), Heiss (1954) and Glaninger (1955).

In this investigation the influence of Reptilase on prothrombin conversion, its thrombin action on pure fibrinogen, as well as its action on tosylarginine methyl ester and lysine ethyl ester was studied. The N-terminal aminoacids of the fibrin formed by Reptilase were also analysed with the phenylthiohydantoin method of Edman.

Materials

Fibrinogen. — Bovine fibrinogen prepared according to Blombäck and Blombäck (1956) with a coagulability of 99.3 per cent. A 0.4 per cent solution of fibrinogen in imidazole buffer of pH 7.25 and ionic strength 0.15 was used for the clotting tests.

Heparin. — Heparin Vitrum, Stockholm, containing 108 international units of heparin per milligram.

Plasmas. — Human and bovine plasma were used. Citrated plasma was prepared by mixing one volume of 3.8 per cent sodium citrate with nine volumes of blood, and then centrifuging at 1150 g for 20 minutes at 4° C. $\text{Ca}_3(\text{PO}_4)_2$ adsorbed human oxalated plasma and stored oxalated plasma were prepared in the manner described by Nilsson and Wenckert (1954).

Thromboplastin. — Thromboplastin was prepared according to Owren (1947).

Tosylarginine methyl ester (TAME) was prepared according to Bergmann, Fruton and Pollok (1939) and Troll, Sherry and Wachman (1954).

Lysine ethyl ester (LEe) was prepared according to Werbin and Palm (1951).

Experimental

The effect of Reptilase in the second stage of the blood coagulation

The following test-systems were used in the investigation of the thrombin-like activity of Reptilase.

Fibrinogen. — To 0.2 ml of fibrinogen solution was added 0.2 ml of Reptilase solution of varying concentrations. The activity, as determined in a water bath at 37° C, was compared with that of a standard thrombin solution.

The undiluted Reptilase solution was found to have an activity corresponding to 0.75 N. I. H. (National Institute of Health) units of thrombin per ml.

Plasma. — The same tests as above were performed with the exception that the fibrinogen solution was replaced by human citrated plasma. Coagulation was obtained with Reptilase in this system, too, and the thrombin effect of Reptilase was found to be the same as with the fibrinogen solution. This thrombin effect was also obtained when prothrombin-free human plasma (adsorbed with $\text{Ca}_3[\text{PO}_4]_2$) and asbestos-filtered bovine plasma (Owren and Aas [1951]) were used as test-bases.

Reptilase and antithrombin. Since Reptilase behaves like thrombin, we tried to neutralize its action with the antithrombins of normal plasma. Eagle (1937) found that the coagulating activity of native venom was not neutralized by normal plasma or by plasma from pepton shocked animals.

According to Seegers, Johnson and Fell (1954) inactivation of thrombin can be explained as follows:

- 1) adsorption of thrombin on the fibrin clot;
- 2) in the presence of excess heparin and heparin-cofactor thrombin has no effect on fibrinogen;

3) in plasma and serum an antithrombin is found, which can neutralize large quantities of thrombin. This so called „progressive antithrombin“ does not require the presence of heparin for its action.

1) *Adsorption of thrombin on fibrin.* To 1 ml of 0.96% fibrinogen solution was added 3 ml of Reptilase solution and after the clot had formed the thrombin activity of the supernatant was determined at different intervals. The experiment was repeated with corresponding amounts of bovine thrombin (0.75 N. I. H. units per ml). Still after 30 minutes the thrombin effect remained unchanged in the supernatant of the clot in the Reptilase sample. On the other hand, no thrombin activity was demonstrable in the sample clotted with bovine thrombin.

2) *Heparin and heparin-cofactor.* Reptilase was added to human citrated plasma containing heparin in increasing concentration. (Test-system: 0.2 ml citrated plasma + 0.2 ml heparin solution of increasing concentration + 0.2 ml Reptilase solution.) The mixture of heparin and plasma was incubated for 3 minutes in a water bath at 37° C before the Reptilase was added. The thrombin effect of the Reptilase solution was not inhibited even if large amounts of heparin were added (Fig. 1). Bovine plasma, which contains four or five times more of the heparin-cofactor than does human plasma, was also used as test-base, but neither in this system did heparin exert any inhibiting effect on Reptilase. By adding both Reptilase and thrombin to the heparin-plasma mixture it was checked that Reptilase did not have any heparin or heparin-cofactor neutralizing effect, i.e. bovine thrombin is inactivated by heparin + cofactor even in the presence of Reptilase.

3) *„Progressive antithrombin“.* To 1 ml of human serum was added 3 ml of Reptilase or 3 ml of a thrombin solution. The samples were placed in a water bath at 37° C, and the thrombin activity was determined on fibrinogen solution at different intervals after the addition of serum. From Fig. 2 it is apparent that, unlike thrombin, Reptilase was not inactivated by the serum antithrombin. A thrombin solution containing 20 times the Reptilase-thrombin activity per ml was inactivated by this amount of serum in 12 minutes.

Reptilase and fibrinolytic activity. The fibrin clots formed from 0.2% fibrinogen solutions and from plasma after the addition of Reptilase did not dissolve on incubation for 48 hours at 37° C. Tested on fibrin plates (Astrup and Müllertz [1952], and Lassen [1952]) the Reptilase did not show any plasmin, plasminogen or plasminogen-activator activity. The test method used has been described in detail by Blombäck and Blombäck (1956).

The effect of Reptilase in the first stage of blood coagulation

The following experiments were performed to investigate whether or not Reptilase had any action in the first stage of coagulation. The recalcification

time of plasma was determined after addition of Reptilase and thrombin solution of equivalent strength (0.75 N. I. H. units/ml), respectively. In these tests the Reptilase and the thrombin were added to the plasma simultaneously with the calcium. The results are given in Table I. It is known that thrombin shortens the recalcification times of plasma (Rosenfeld and Jánssky [1952]; Ratnoff [1953, 1954]). So did Reptilase, but not more than did an equivalent amount of bovine thrombin solution. Reptilase was also tested in serial dilutions, but no shortening of the coagulation times in comparison with the controls was obtained. The recalcification times of hemophilic plasma, platelet-free plasma and prothrombin-free plasma were also determined in the presence of Reptilase. Clotting was not accelerated more than by addition of an equivalent amount of thrombin. The one-stage prothrombin time of these various plasmas was not shortened by Reptilase.

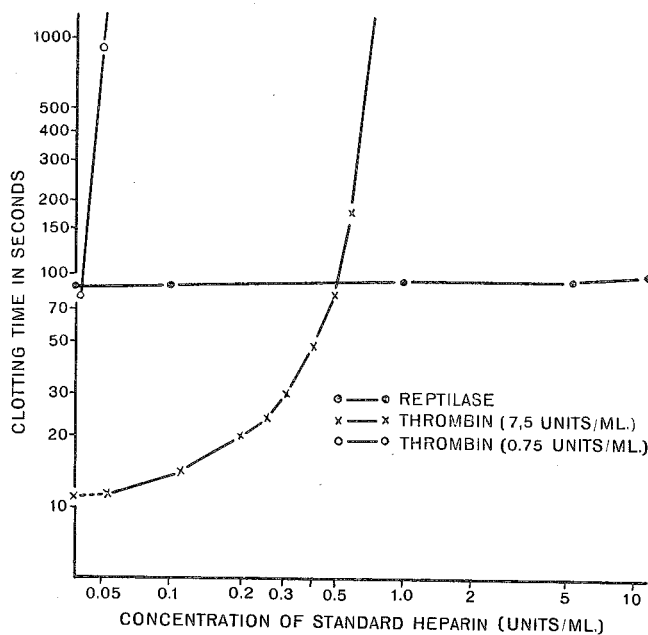


Fig. 1: The effect of heparin on the clotting time of human citrated plasma in the presence of Reptilase and bovine thrombin

To aliquots of 0.2 ml of human citrated plasma were added 0.2 ml of heparin solution in increasing concentration. These mixtures were then allowed to stand 3 minutes in a water bath at 37° C after which 0.2 ml of Reptilase solution or 0.2 ml of a thrombin solution containing 7.5 N. I. H. units of thrombin per ml or 0.2 ml of a thrombin solution containing 0.75 N. I. H. units of thrombin per ml was added.

Furthermore, Reptilase did not correct the prolonged one-stage prothrombin time of stored plasma as might have been expected if it had had any factor V activity.

If Reptilase has any thromboplastin effect it might be expected to be capable of correcting the poor prothrombin consumption of hemophilic blood for example. Therefore the following experiment was performed: 1 ml of Reptilase was added to 1 ml of normal and to 1 ml of hemophilic plasma. These mixtures were then recalcified by addition of 1 ml of 30 mM CaCl_2 solution and kept at 37°C for 2 hours. The prothrombin consumption test was then performed according to Biggs and Macfarlane (1953). Control experiments were performed in which Reptilase was replaced by thrombin solution (0.75 N. I. H. units/ml) or by saline.

100 per cent of the prothrombin remained in the hemophilic serum even on addition of Reptilase. Neither did Reptilase have any influence on the prothrombin consumption of normal plasma.

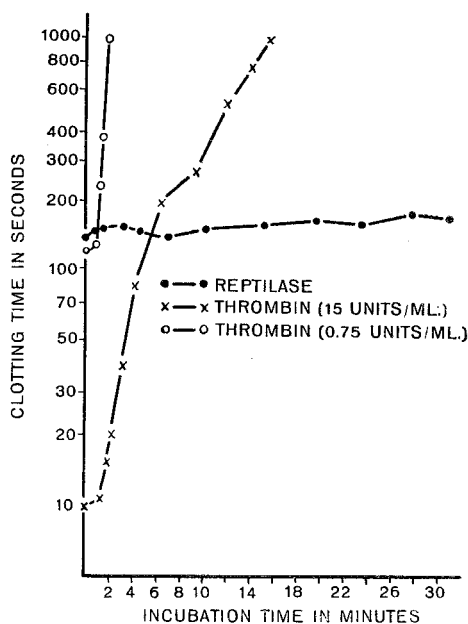


Fig. 2: The inactivation of Reptilase and bovine thrombin by human serum

To aliquots of 3 ml of human serum were added 1 ml of Reptilase solution, 1 ml of thrombin solution containing 15 N. I. H. units of thrombin per ml and 1 ml of thrombin solution containing 0.75 N. I. H. units of thrombin per ml. At various intervals after addition of the Reptilase and the thrombin aliquots of 0.2 ml were removed from the serum samples / kept at 37°C / and added to clotting tubes containing 0.2 ml of fibrinogen solution, and the clotting times were recorded.

The effect of Reptilase on the synthetic substrates tosylarginine methyl ester (TAME) and on lysine ethyl ester (LEe)

Like many other proteolytic enzymes, e. g. trypsin, thrombin can split tosylarginine methyl ester into tosylarginine and methanol (Sherry and Troll

Table I:
The effect of Reptilase and thrombin on the clotting time of plasma in the presence of calcium

Sample of plasma	Clotting time in seconds of aliquots of 0.2 ml of plasma on addition of										
	0.2 ml of thrombin (0.75 units/ml)	0.2 ml of Repti- lase	0.2 ml of saline + 0.2 ml of 30 mM CaCl ₂	0.2 ml of saline + 0.2 ml of thrombin		0.2 ml of saline + 0.2 ml of Reptilase		0.2 ml of 30 mM CaCl ₂ *) + 0.2 ml of thrombin		0.2 ml of 30 mM CaCl ₂ *) + 0.2 ml of Reptilase	
				0.75 units/ml	0.075 units/ml	dil. 1 : 1	dil. 1 : 10	0.75 units/ml	0.075 units/ml	dil. 1 : 1	dil. 1 : 10
Citrated human normal plasma	55	57	190	107	>1000	94	>1000	46	129	47	134
Haemophilic A plasma	52	55	1170	105	—	92	—	58	—	72	—
Ca ₃ (PO ₄) ₂ adsorbed human plasma	—	—	No clot	127	—	105	—	42	—	40	—
Bovine asbestos filtered plasma	—	—	No clot	334	—	317	—	62	—	135	—

*) CaCl₂ and thrombin (or Reptilase) were added simultaneously to the plasma sample.

[1954]). Under standard conditions 1 N. I. H. unit of thrombin will split about 1 μ M of the same substrate in 10 minutes (Sherry and Troll [1954]). It was found that Reptilase could split TAME and that 1 ml of the undiluted extract released 3 micromoles of acid from TAME in 10 minutes (Table II).

Table II:

Digestion time	μ M of acid released by 1 ml of Reptilase	μ M of acid released by 1 ml of Reptilase in 10 minutes
30 min	10.00	3.33
60 min	16.80	2.79
120 min	25.00	3.00

Thrombin has no measurable activity on lysine ethyl ester (Sherry and Troll [1954]). Nor did Reptilase split this ester. The digestion was performed in imidazole buffer (pH 6.5), and the free carboxylic groups were titrated (Troll, Sherry and Wachman [1954]).

N-terminal aminoacids in fibrin formed by the action of Reptilase

Using Sanger's technique (1945) Bettelheim and Bailey (1951, 1952), Lorand and Middlebrook (1951, 1952) found the N-terminal aminoacids of bovine fibrinogen to be glutamic acid and tyrosin. After the coagulation with thrombin the glutamic acid had disappeared and glycine appeared. The amount of tyrosin was unchanged.

Using Edman's (1950) phenylisothiocyanate-method Blombäck and Yamashina (1956) found the N-terminal aminoacids in bovine fibrinogen to be glutamine, glutamic acid and tyrosin. In fibrin glycine replaced the glutamyl residues. The ratio between the tyrosin and glycine in fibrin was found to be 1 : 2.

We considered it of interest to investigate the N-terminal aminoacids formed by the action of Reptilase on bovine fibrinogen. For this purpose we used Edman's method. The phenylthiohydantoins of the N-terminal aminoacids were identified by means of paper chromatography in two different solvent systems (Edman-Sjöqvist). The technique used is described by Blombäck and Yamashina. Fibrin formed with Reptilase had qualitatively the same N-terminal amino-acids as the product obtained with thrombin, namely tyrosin and glycine. However, the ratio between tyrosin and glycine was 1 : 1, thus differing from that of fibrin clotted with thrombin.

Discussion

It was apparent from the experiments that Reptilase had a thrombin-like effect in the second stage of the coagulation even when tested on a highly purified fibrinogen solution free from other clotting components. Earlier investigators also found this venom-extract to have a thrombin effect but suggested that in addition it should promote the prothrombin conversion. However, our experiments did not indicate any such effect. Thus the effect of Reptilase was the same in the presence and in the absence of prothrombin in the plasma (Table I). Contrary to what might have been expected if it had any factor V activity, Reptilase did not correct the prolonged one-stage prothrombin time of stored plasma.

The shortening of the recalcification time of plasma on addition of Reptilase could be explained entirely by the thrombin activity of the preparation (Table I). Thromboplastin is able to correct the poor consumption of prothrombin in hemophilic plasma. Reptilase had no influence at all on the prothrombin consumption of hemophilic plasma or of normal plasma.

As already mentioned, Reptilase seems to act as an enzyme in the same manner as thrombin in the conversion of fibrinogen to fibrin. Several proteolytic enzymes including thrombin are known to split synthetical substrates, e. g. tosylarginine methyl ester. We found Reptilase also to split this substrate. Since no fibrinolytic activity could be demonstrated in the Reptilase preparation, this splitting could not be ascribed to fibrinolytic enzymes. Like thrombin, Reptilase did not split lysine ethyl ester.

The study of the N-terminal aminoacids of fibrinogen and fibrin formed by thrombin and Reptilase, respectively, showed at least qualitatively a similarity in the mode of action, thus the glutamyl residues in fibrinogen disappeared and were replaced by glycine in the fibrin. However, quantitatively, there was a difference since the ratio between tyrosin and glycine in the thrombin-fibrin was 1 : 2, but in the Reptilase-fibrin 1 : 1.

Other findings, e. g. their behaviour towards antithrombin, also revealed differences between the properties of thrombin and Reptilase. Thus Reptilase was not inactivated by heparin + cofactor, or by the plasma antithrombin. Furthermore Reptilase did not seem to be adsorbed on the fibrin clot.

In view of the fact that Reptilase has only a thrombin-like effect on the coagulation and that the thrombin activity of the commercial preparation corresponds to only 0.75 N. I. H. units of thrombin per ml, i.e. the activity of 0.03 ml of activated plasma, its use as a haemostaticum is unwarranted.

Summary

Reptilase — the clot-promoting extract of the venom of *Bothrops jararaca* — was found to have a thrombin-like activity. No effect could be demonstrated in the first phase of coagulation. Unlike bovine thrombin it was not inactivated by heparin + heparin-cofactor or by the antithrombin of normal plasma. Analyses of N-terminal aminoacids in the fibrin formed by Reptilase revealed the same aminoacids as in the fibrin formed by bovine thrombin. Quantitative differences found may reflect different specificity of the two enzymes.

It is concluded that the use of Reptilase as a haemostaticum is unwarranted.

We wish to thank Professor Erik Jorpes for valuable advice and support in this investigation.

Résumé

La "Reptilase" est une substance coagulante extraite du venin du *Bothrops jararaca* dont l'action s'avère être similaire à celle de la thrombine. La Reptilase n'intervient pas dans la première phase de la coagulation. A l'opposé de la thrombine bovine, elle n'est pas inactivée par l'héparine et son cofacteur ni par l'antithrombine naturelle du plasma. L'analyse des acides aminés N-terminales de la fibrine formée par la Reptilase, démontre qu'il s'agit d'acides aminés identiques à ceux formés par la thrombine. Les différences quantitatives peuvent être causées par une différence de spécificité des deux enzymes. L'emploi de la Reptilase comme hémostatique n'est pas recommandable.

Zusammenfassung

Reptilase, der gerinnungsfördernde Extrakt aus dem Gift von *Bothrops jararaca* hat eine thrombinähnliche Wirkung, es konnte jedoch keine Wirkung auf die 1. Phase der Gerinnung gezeigt werden. Im Gegensatz zu Rinderthrombin wird Reptilase nicht durch Heparin + Heparin-Cofaktor oder durch Antithrombin aus normalem Plasma inaktiviert. Die Bestimmung der endständigen Aminogruppen im Fibrin, das durch Einwirkung von Reptilase gebildet wurde, ergab dieselben Aminosäuren wie im nach Einwirken von Rinderthrombin entstandenen Fibrin. Es bestehen aber quantitative Unterschiede, die eine unterschiedliche Spezifität der beiden Fermente widerspiegeln.

Die Verwendung von Reptilase als Hämostyptikum ist somit nicht gerechtfertigt.

References

- Astrup, T. and Müllertz, S.: The fibrin plate method for estimating fibrinolytic activity. *Arch. Biochem. Biophys.* 40: 346 (1952).
 Bailey, K., Bettelheim, F. R., Lorand, L. and Middlebrook, W. R.: Action of thrombin in the clotting of fibrinogen. *Nature (Lond.)* 167: 233 (1951).

- Bergmann, M., Fruton, J. S. and Pollok, H.: The specificity of trypsin. *J. biol. Chem.* 127: 643 (1939).
- Bettelheim, F. R. and Bailey, K.: The products of the action of thrombin on fibrinogen. *Biochim. biophys. Acta* 9: 578 (1952).
- Biggs, R. and Macfarlane, R. G.: Human blood coagulation and its disorders. Blackwell Scientific Publ., Oxford, 1953.
- Blombäck, B. and Blombäck, M.: Purification of human and bovine fibrinogen. *Arkiv för Kemi* 10: 415 (1956).
- Blombäck, B. and Yamashina, I.: The determination of N-terminal amino acids during the conversion of fibrinogen to fibrin. *Acta Chem. Scand.*, 1957, in print.
- Breda, R., Bernardi, R. and Sala, F.: Ricerche clinico-sperimentale sull'azione coagulante del veleno di *Bothrops jararaca*. *Giorn. Clin. med.* 32: 882 (1951).
- Bruck, H. and Salem, G.: Reptilase, a haemostatic for prophylactic and therapeutic use in connection with surgical operations. *Wien. klin. Wschr.* 66: 395 (1954).
- Deutsch, E.: Blutgerinnungsfaktoren. Franz Deuticke, Wien, p. 208, 1955.
- Eagle, H.: The coagulation of blood by snake venoms and its physiological significance. *J. exp. Med.* 65: 613 (1937).
- Edman, P.: Preparation of phenyl thiohydantoins from some natural amino acids. *Acta chem. scand.* 4: 277 (1950).
- Edman, P.: Preparation of phenyl thiohydantoins from some natural amino acids. *Acta scand.* 4: 283 (1950).
- Edman, P. and Sjöquist, J.: Identification and semiquantitative determination of phenyl thiohydantoins. *Acta chem. scand.* 10: 1507 (1956).
- Fleischhacker, H.: Concerning the therapy of coagulation disturbances. *Wien. med. Wschr.* 104: 171 (1954).
- Glaninger, J.: About the application of the hemostatic Reptilase in oto-rhino-laryngology. *Msschr. Ohrenheilk.* 89: 63 (1955).
- Heiss, H.: A contribution to the hemostatic therapy in cervical carcinoma. *Wien. med. Wschr.* 104: 833 (1954).
- v. Klobusitzky, D.: Biochemische Studien über die Gifte der Schlangengattung *Bothrops*. *Arch. exp. Path. Pharmacol.* 179: 204 (1935).
- v. Klobusitzky, D. und König, P.: Biochemische Studien über die Gifte der Schlangengattung *Bothrops*. *Arch. exp. Path. Pharmacol.* 181: 387 (1936).
- v. Klobusitzky, D. und König, P.: Biochemische Studien über die Gifte der Schlangengattung *Bothrops*. *Arch. exp. Path. Pharmacol.* 182: 577 (1936).
- Lassen, M.: Heat denaturation of plasminogen in the fibrin plate method. *Acta physiol. scand.* 27: 371 (1952).
- Lorand, L. and Middlebrook, W. R.: Studies on fibrino-peptide. *Biochim. Biophys. Acta* 9: 581 (1952).
- Lorand, L. and Middlebrook, W. R.: Species specificity of fibrinogen as revealed by end-group studies. *Science* 118: 515 (1953).
- Nilsson, I. M. and Wenckert, A.: Demonstration of a heparin-like anticoagulant in normal blood. *Acta med. scand.* 150 (1954). Suppl.
- Owren, P. A.: The coagulation of blood. Investigations on a new clotting factor. *Acta med. scand.* 194 (1947). Suppl.
- Owren, P. A. and Aas, K.: The control of dicumarol therapy and the quantitative determination of prothrombin and proconvertin. *Scand. J. clin. Lab. Invest.* 3: 201 (1951).
- Porges, N.: Snake venoms, their biochemistry and mode of action. *Science* 117: 47 (1953).
- Ratnoff, O. D.: Thrombin accelerator: a clotting factor of plasma deficient in cirrhosis of the liver, multiple myeloma, and eclampsia. *J. Lab. clin. Med.* 42: 933 (1953).
- Ratnoff, O. D. and Potts, A. M.: The accelerating effect of calcium and other cations on the conversion of fibrinogen to fibrin. *J. clin. Invest.* 33: 206 (1954).
- Rocha e Silva, M. and Andrade, S. O.: Estudos sobre a dicoumarina 3,3-metilenobis-(4-hidroxycoumarina). II. Efeito coagulante de venenos dos gêneros *Bothrops* e *Crotalus*

- sôbre o plasma oxalatado de animais normales e tratados pela dicoumarina. Arch. Inst. Biol. **16**: 115 (1945).
- Rosenfeld, G. and J á n s z k y, B.: The acclerating effect of calcium on the fibrinogen-fibrin transformation. Science **116**: 36 (1952).
- Sanger, F.: The free amino groups of insulin. Biochem. J. **39**: 507 (1945).
- Seegers, W. H., Johnson, J. F. and Fell, C.: An antithrombin reaction related to prothrombin activation. Amer. J. Physiol. **176**: 97 (1954).
- Sherry, S. and Troll, W.: The action of thrombin on synthetic substrates. J. biol. Chem. **208**: 95 (1954).
- Troll, W., Sherry, S. and Wachman, J.: The action of plasmin on synthetic substrates. J. biol. Chem. **208**: 85 (1954).
- Werbin, H. and Palm, A.: Trypsin hydrolysis of lysine ethyl ester. J. Amer. Chem. Soc. **73**: 1382 (1951).