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FIBRINOLYSIS INDUCED BY BRAIN EXTRACTS.

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IN a previous paper from this Institute (Fantl and Simon, 1948) it was shown that under the influence of electro-convulsions a transient fibrinolytic activity appeared in human plasma whilst fibrinogen was not attacked. R. H. Mole (1948) reported recently that the fluidity of blood post mortem in cases of sudden death was also due to the dissolution of fibrin by an enzyme which did not split fibrinogen. Because of the significance which a specific fibrinolytic enzyme may have in the prevention of thrombo-embolic conditions, further studies of the mechanism of fibrinolysis were carried out.

It appears most likely that the appearance of the fibrinolytic enzyme occurs through a mechanism similar to that suggested by Christensen (1945), and Biggs, McFarlane and Pilling (1947), who assume that the enzyme precursor is a normal blood constituent, whilst a kinase is introduced following certain stimuli or pathology. A search for the source of the fibrinokinase was undertaken. The most consistent results were obtained by the use of extracts of human brain. A description of the experimental procedure follows.

EXPERIMENTAL.

Portions of human brain after removal of blood vessels and pia mater were rinsed with isotonic saline and ground with several lots of neutral acetone. The powder was introduced into ampoules which were sealed under vacuum and stored in a refrigerator. Prior to use a 6 per cent suspension was prepared by extraction with 0·85 per cent NaCl at 37° C. for 15 min. with occasional stirring. The suspension was centrifuged at 2700 G. for 10 min. and the supernatant fluid used. Little activity remained in the sediment. Plasma specimens were obtained from normal human donors by addition of 1 vol. 0·1 M sodium oxalate to 9 volumes of venous blood, and the cellular elements were removed by centrifugation. In one series of experiments 0·1 ml. oxalated plasma was added to

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1·3 ml. 0·85 per cent NaCl, and after addition of 0·1 ml. 0·25 M calcium chloride, containing 1 g. merthiolate in 400 ml., a small glass rod was inserted, the contents were mixed and incubated at 37° C. until the contents gelled. The fibrin clot was wound around the glass rod and 1 ml. of the brain extract added.

In a second series of experiments the fibrin was isolated on the glass rod in a similar manner to the first series, but before brain extract was added, the serum was removed and the clot rinsed with isotonic saline until soluble serum proteins were removed. This washed clot was incubated with brain extract.

A third series of experiments were carried out. These were similar to the first except that no calcium chloride was added and incubation was carried out for periods in which complete fibrinolysis occurred in the first series. Usually after 18 hours calcium chloride was added to these specimens.

TABLE I.—*Influence of Brain Extract on Fibrinolysis.*

Number of plasma specimens tested.	*Fibrinolysis occurring.* (1) In presence of serum.	(2) In absence of serum.	*Fibrinogenolysis occurring* (3).
54	54	15	1*

* Tested in 10 cases only.

The results given in Table I (Col. 1) indicate that an extract of human brain contains a principle which, combined with a serum component, is capable of dissolving fibrin. This occurred in 4 to 16 hours at 37° C. That the process was dependent upon the presence of serum can be judged from Column 2. When the serum was removed fibrinolysis occurred in only approximately one-fourth of all cases tested. This result would indicate that the extracts contained little, if any, enzymes capable of attacking fibrin. They suggest that serum contains the precursor and brain the kinase. The last column, Table I, indicates that in contrast to the breakdown of fibrin, fibrinogen was not attacked in the majority of cases. These results could be verified with four different brain specimens. A fifth brain preparation, however, obtained from a child who died from leukaemia, was inactive. The following properties of the brain component were noticed. Heating to 70° C. for 15 min. destroyed the activity, as can be seen in Table II.

TABLE II.—*Heat Inactivation of Brain Fibrinokinase.*

Incubation temperature of brain extract.	Fibrinolysis time in hours.	Prothrombin time in seconds.
18° C.	18	17
70° C.	∞	20

In this instance the brain suspension was centrifuged at 540 G. In addition to the fibrinolysis tests the brain extracts were used as thromboplastin for the estimation of prothrombin. The results in the third column of Table II indicate that the thromboplastic property was not greatly influenced by heating.

These experiments suggest that the brain component required for fibrinolytic activity is different from the thromboplastin. This could be further substantiated by the use of heterologous brain.

TABLE III.—*Influence of Rabbit and Human Brain Extract on Human Fibrin and Fibrinogen.*

Brain extract used.	Fibrinolysis (1) In presence of serum.	Fibrinolysis (2) In absence of serum.	Fibrinogenolysis (3).
Human	16	2	*Nil*
Rabbit	3	2	,,

Columns (1) and (2)—16 plasma specimens tested.
Column (3) — 7 ,, ,, ,,

In Table III are shown the results obtained from plasma specimens which were tested with both human and rabbit brain extract. It is quite evident that in the majority of cases there was a marked species specificity with regard to brain fibrinokinase. Although in some cases both brain extracts were capable of dissolving isolated fibrin (Column 2), in no case was fibrinogenolysis observed. Prothrombin estimations carried out with human and rabbit brain, however, indicated equal thromboplastic activity.

From the results obtained in a few experiments it would appear that the specificity of the fibrinolytic enzyme produced by combination of the serum precursor with brain fibrinokinase is confined to the undenatured fibrin.

TABLE IV.—*Difference in Reactivity of Native and Heated Fibrin.*

Number of samples.	Fibrinolysis of Native fibrin. (1) In presence of serum.	Fibrinolysis of Native fibrin. (2) In absence of serum.	Fibrinolysis of heated fibrin. (3)
6	6	*Nil*	*Nil*

For experiments in Column 3, Table IV, the fibrin deposited on glass rods was prepared as in previous experiments, but it was removed from its serum and immersed in boiling saline for 5 min. then allowed to cool and returned to the serum. The experimental results indicate quite clearly that the fibrinolysin was able to attack native fibrin only.

In further experiments attempts were made to concentrate the active brain factor. It could be shown that extraction of brain powder with distilled water and, in some cases, 0·4 per cent NaCl, did not produce active extracts, indicating that the principle is not of albumin nature.

Fractionation of saline extracts with ammonium sulphate was sometimes difficult to carry out because sediments would not settle even when centrifuged. In some instances, however, it was possible to obtain filterable precipitates at a concentration of between 35 per cent and 50 per cent ammonium sulphate saturation. Precipitates were dialysed against distilled water and the resulting suspension showed high fibrinolytic activity. The protein concentration of one such suspension was 25 mg. per cent protein N.

The active principle of the saline extract of brain could be adsorbed by alumina gel, but elution with phosphate buffer was unsuccessful.

Barium sulphate suspension did not adsorb the active principle from the brain preparation. Further, the active principle could be precipitated by acetone from the saline extract.

Experimental evidence brought forward is in favour of the assumption that brain fibrinokinase is saline soluble and present in the globulin fraction. Since several workers believe that there is no difference between the susceptibility of fibrinogen and fibrin to fibrinolysins, Christensen (1945), Tagnon, Davidson and Taylor (1942), it seemed of interest to investigate the reactions of a fibrinolysin produced by activation of the plasma precursor by streptokinase.

The potency of the preparation was adjusted to a fibrinolysis time between approximately 1 and 19 hours. Preliminary qualitative experiments indicated that fibrinolysin produced by streptokinase activation attacked fibrin preferentially to fibrinogen. Results for two different streptokinase concentrations are given in the following table.

TABLE V.—*Influence of Streptokinase on Fibrinolysis.*

Streptokinase concentration.	Fibrinolysis time in hours (1).	Fibrinogenolysis (2).
1	1	*Nil*
0·5	1–17	,,

The concentration of streptokinase is expressed in arbitrary units. Four plasma specimens were tested.

Results in Column 1, Table V, refer to experiments which were carried out in the presence of calcium ions, whilst those in Column 2 were carried out in the absence of calcium ions. They were added to this series when fibrin disappearance was noticed in the Column 1 series. The figures in the table refer to the time in hours until complete fibrin disappearance. The results indicate that fibrinogen was not destroyed in specimens incubated with streptokinase, although fibrin was attacked. Further, the streptokinase was not inactivated during the incubation period because after conversion of fibrinogen to fibrin, fibrinolysis occurred in 1 to 3 hours.

In order to investigate quantitatively the extent of fibrinolysis or fibrinogenolysis the following experiments were carried out.

Into three series of 50 ml. centrifuge tubes was introduced 1 ml. oxalated or citrated plasma (in the latter case specimens were obtained by adding 1 vol. 3·2 per cent sodium citrate to 9 vol. blood). The plasma was diluted with 22 ml. veronal buffer, pH 7·2, and in Series 1, 1 ml. 0·25 M calcium chloride was added. This was a control series.

Series 2 contained, in addition to the components of Series 1, 1 ml. of a suitably-diluted streptokinase preparation.

Series 3 contained diluted plasma and streptokinase but no calcium chloride. All the tubes were incubated at 37° C. When the fibrin gels in Series 2 disappeared completely, thrombin and calcium chloride was added to the tubes of Series 3, and the contents chilled in an ice bath. The tubes were spun as quickly as possible, and the deposited fibrin washed with isotonic saline until protein free. Protein estimations of the isolated fibrin were carried out by the biuret reaction. The results of these estimations are shown in Table VI.

The concentration of streptokinase used for the experiments shown in Table VI produced fibrinolysis in two specimens in 1·5 hours at 37° C. Two more specimens were treated simultaneously and they lysed in 45 min. Following addition of thrombin, clot formation occurred, but the quantitative collection

TABLE VI.—*Recovery of Fibrinogen Following Incubation of Human Plasma with Streptokinase.*

Series 1. Fibrin N, mg. per 100 ml.	Series 3. Fibrin N, mg. per 100 ml.
72	59
40	35

of the fibrin clots was not successful because losses of fibrin due to fibrinolysis occurred during the isolation procedure.

These results indicate that the fibrinolytic enzyme produced by streptokinase activation does attack fibrin in preference to fibrinogen.

DISCUSSION.

Normal body fluids containing the coagulation factors will clot under favourable conditions, but the dissolution of the clot is neither a common nor a physiological property. The exceptions are the dissolution of fibrin in the endometrium and the lytic action of prostatic fluid (Huggins and Neal, 1942). Human blood, after coagulation, ordinarily does not show fibrinolysis. But this process can be induced by a variety of factors *in vivo* as well as *in vitro*.

With regard to the derivation of the fibrinolysin, two possibilities have been suggested. According to one, the enzyme is derived from sources outside the blood, and Mole (1948) believes that endothelial lining of the vascular channels and body cavities are the source of the enzyme which under certain stimuli enters the circulation. Another theory assumes that normal human blood contains an inactive precursor of the enzyme which can be activated by a kinase to the active principle. This assumption has been supported by several independent observations. It originates from the findings of Tillet and Garner (1933) that fibrin clots prepared from human plasma undergo lysis when culture filtrates of certain strains of streptococci are added. It was shown by Milstone (1941) that human plasma contains a factor present in the euglobulin fraction which by combining with the streptococcal component produces fibrinolysis. Christensen (1945) and Christensen and McLeod (1945), from a study of the purified components, concluded that the lytic factor exists in plasma as zymogen and that it is activated by the streptococcal factor called streptokinase in a manner similar to the activation of trypsinogen by enterokinase. This concept has been adopted by Biggs, McFarlane and Pilling (1947) for fibrinolytic activity of human blood produced by physiological stimuli. With regard to specificity, it was observed by Tagnon *et al.* (1942) and by Christensen and McLeod (1945) that the enzyme is active against proteins other than fibrin and the latter authors therefore thought it advisable to abandon the term fibrinolysin and suggested plasmin instead. The experimental evidence brought forward in this paper is in agreement with the assumption that human plasma or serum contains the precursor and the kinase can be supplied from sources outside the blood; in this case, brain. The results show quite clearly that the enzyme appearing in the circulation following electrically-induced convulsions, or activation by brain fibrinokinase attacks fibrin without affecting fibrinogen. Mole (1948) has found that the enzyme present in post-mortem blood has similar properties. Further, fibrinolysin produced by activation of the plasma precursor by streptokinase under our experimental conditions showed also preferential attack of fibrin.

Christensen (1945) obtained equal breakdown rates with fibrin as well as fibrinogen. A possible explanation for the difference in results is that Christensen used fibrinogen isolated by salting out procedures whilst we used diluted plasma as source of fibrinogen. Because of the pronounced specificity the term profibrinolysin, as suggested by Loomis, George and Ryder (1947) for the precursor of the blood enzyme is to be preferred. Turning now to the source of the kinase the evidence submitted allows the conclusion that human adult brain contains this factor. It will be recalled that attempts by previous workers to extract the active tissue component have been unsuccessful (Fleisher and Loeb, 1915; Permin, 1947). Tagnon and Petermann (1949) found the activator in the microsome fraction of rat lungs. The ease with which brain yields the fibrinokinase in contrast to other organs is possibly due to the phospholipids which may have a solubilizing effect on fibrinokinase. In this connection it is interesting to record that Halse (1946) and Kaulla (1947) found the fibrinolysis due to parenteral administration of novocain, or even saline is correlated with an increase of plasmaphosphatides. That we are dealing with a kinase and not a proteolytic enzyme is evident from the fact that fibrin in the absence of serum was not altered by the brain extract. It is true that in some of the experiments the extracts were able to attack isolated fibrin, but this was always smaller than in the presence of serum and therefore cannot be explained solely by the presence of a proteolytic enzyme in brain. It has to be considered also that precipitated fibrin includes other material than fibrin and it is possible that it includes some profibrinolysin. The activation by brain fibrinokinase is species specific. The heat lability suggests that this principle is not identical with the thromboplastin of brain. The physicochemical properties would indicate that it is present in the globulin fraction.

SUMMARY.

Human adult brain contains a factor capable of converting profibrinolysin of human plasma into fibrinolysin. The brain fibrinokinase can be extracted by salt solutions and is present in the globulin fraction. The factor is species specific and not identical with thromboplastin. The enzyme produced by the combination of the plasma with the brain factor breaks down fibrin made by clotting procedures, but shows no action on plasma fibrinogen or heat-denatured fibrin.

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REFERENCES.

BIGGS, R., MACFARLANE, R. G., AND PILLING, J.—(1947) *Lancet*, i, 402.
CHRISTENSEN, L. R.—(1945) *J. gen. Physiol.*, **28**, 363.
Idem AND MACLEOD, C. M.—(1945) *Ibid.*, **28**, 559.
FANTL, P., AND SIMON, S. E.—(1948) *Aust. J. exp. Biol. med. Sci.*, **26**, 521.
FLEISHER, M. S., AND LOEB, L.—(1915) *J. biol. Chem.*, **21**, 477.
HALSE, T.—(1946) *Klin. Wschr.*, 24/25, 121.

HUGGINS, C., AND NEAL, W.—(1942) *J. exp. Med.*, **76**, 527.
KAULLA, K. N. VON.—(1947) *Schweiz. med. Wschr.*, **77**, 313.
LOOMIS, E. C., GEORGE, C., AND RYDER, A.—(1947) *Arch. Biochem.*, **12**, 1.
MILSTONE, H.—(1941) *J. Immunol.*, **42**, 109.
MOLE, R. H.—(1948) *J. Path. Bact.*, **60**, 413.
PERMIN, P. M.—(1947) *Nature*, **160**, 571.
TAGNON, H. J., DAVIDSON, C. S., AND TAYLOR, F. H. L.—(1942) *J. clin. Invest.*, **21**, 525.
Idem AND PETERMANN, M. L.—(1949) *Proc. Soc. exp. Biol., N.Y.*, **70**, 359.
TILLET, W. S., AND GARNER, R. L.—(1933) *J. exp. Med.*, **58**, 485.