STUDIES ON THE FIBRINOLYSIN-ANTIFIBRINOLYSIN SYSTEM IN SERUM

I. Action of the Anterior Pituitary, Adrenal Cortex, and Spleen*

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The accumulated evidence indicates that liberation of a proteolytic enzyme from its inactive precursor plays a part in anaphylactic and anaphylactoid processes (1–5). It is well known that fibrinolysin (also called plasmin, serum protease, and serum tryptase) is inactivated by an inhibitor present in serum (antifibrinolysin, antiplasmin, antiprotease, antitryptase).

The observations reported in the present paper indicate that the rate of inactivation of fibrinolysin by antifibrinolysin is controlled by the pituitary and the adrenal cortex and they suggest that this action is mediated by the spleen.

Methods and Materials

Activation of profibrinolysin was induced by a procedure described previously (5). To 2 ml. of guinea pig serum 0.4 ml. of a 10 per cent peptone[1] solution was added. After 2 minutes' contact, the mixture was diluted to 48 ml. with distilled water and adjusted to pH 5.2 with 0.5 per cent acetic acid to precipitate the euglobulin fraction which contains fibrinolysin. After centrifugation, the precipitate was redissolved in saline buffered at pH 7.4 (phosphate buffer diluted to 0.1 M with 0.85 per cent NaCl solution). This solution made up to 1.5 ml. was warmed up to 37.5°C. and pipetted into an Ostwald viscosimeter containing 2 ml. of a 3 per cent solution of gelatin placed in a constant temperature bath (37.5°C. ± 0.05). Flow times were measured 4 to 6 times during 20 minutes. Further details of the technique, calculations, definition of the unit and the standardization curve used in this study were reported in a previous publication (5). Results of these experiments are expressed as units of fibrinolysin released by ml. of serum.

Antifibrinolysin activity of serum was estimated by a similar technique. Purified fibrinolysin was prepared according to the method of Loomis, George, and Ryder (6).[2] Fibrinolysin dissolved in buffered saline and kept at −30°C. for several days lost its activity slowly. Before using it for antifibrinolysin estimation the enzyme solution was standardized every day. Amounts corresponding to 8 to 10 units were then mixed with 25 μl. of the serum to be tested.

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[1] Proteose peptone Difco.

[2] Part of the work was done with fibrinolysin kindly supplied by Mr. E. C. Loomis of the Research Laboratories, Parke, Davis and Company.

The volume was made up to 1.5 ml. with saline. After contact for 5 minutes the mixture of enzyme and serum was pipetted into the viscosimeter which contained the gelatin. The effect of this mixture on viscosity was determined as described in the previous paragraph. The number of units inhibited was obtained by subtracting the potency of the fibrinolysin-serum mixture from that of fibrinolysin alone.

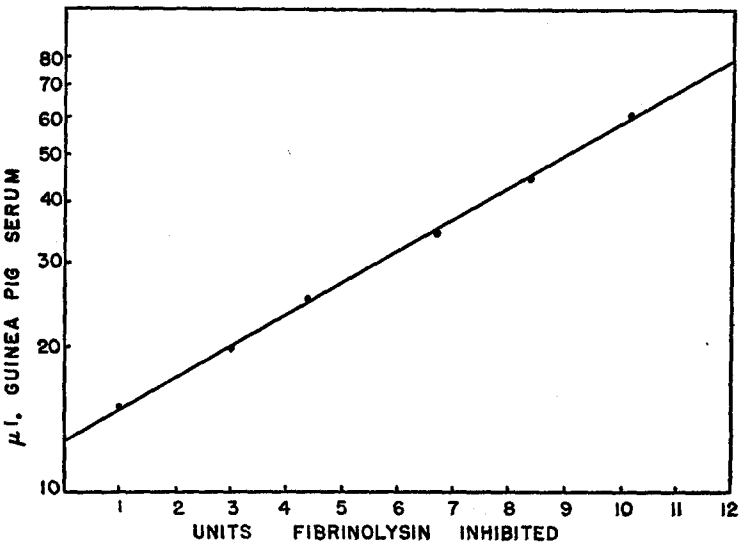

FIG. 1. Inhibition of fibrinolysin by guinea pig serum after 5 minutes' contact. Abscissa: units of fibrinolysin inhibited; Ordinate: μl. of guinea pig serum (log scale).

Fig. 1 shows a linear relationship between the number of units of fibrinolysin inhibited and the concentration of serum when the latter is plotted on a logarithmic scale. When 25 μl. of serum did not cause inactivation or did inactivate all the fibrinolysin, smaller or larger amounts were tried. Results are expressed in units of fibrinolysin inactivated by 25 μl. of serum. As it is not certain that the linear relationship is valid for amounts of serum higher than 60 μl., extrapolation for 1 ml. did not seem to be justified.

Blood was collected from guinea pigs by heart puncture and allowed to clot. The serum was then used either immediately after separation from the clot or within 48 hours. In the latter case the serum was kept frozen at −30°C. In experiments with rats, the animals were decapitated and bled through the vessels of the neck.

Anterior pituitary, adrenocortical, and splenic substances were injected subcutaneously and blood samples were taken at stated intervals after treatment.

EXPERIMENTAL

Action of Adrenocorticotrophic Hormone (ACTH) and Cortisone on Activation of Profibrinolysin.—When the activation of profibrinolysin by peptone in sera of guinea pigs treated with ACTH[3] and cortisone[4] was compared with that in

[3] ACTH, Lot 57A potency 250 per cent, kindly supplied by Dr. John R. Mote of Armour and Company.

[4] Saline suspension of cortisone acetate 2.5 per cent kindly supplied by Dr. J. M. Carlisle of Merck and Company.

sera of normal guinea pigs no significant differences were found. In a previous study (5), eight guinea pig sera activated with peptone showed a mean release of 4.95 ± 0.94 units of fibrinolysin per ml. as measured by the fibrinogenolytic method. Using the viscosimetric method the sera of five normal guinea pigs showed 5.6 ± 0.35 units of fibrinolysin. 1 hour after injection of 50μg. ACTH in eight guinea pigs, the serum fibrinolysin was 5.15 ± 0.76 units. 3 hours after

TABLE I

Antifibrinolytic Power of Sera from Guinea Pigs Treated with ACTH and Adrenocortical Hormones

Substance	Dose per kg.	Interval	Units fibrinolysin inhibited per 25 μl. serum	±S.E.	No. of animals
		hrs.			
Normal guinea pigs	—	—	4.7	0.40	17
ACTH	1 μg.	1	4.7	0.125	5
	5 μg.	1	6.4	0.39	5
	20 μg.	1	10.1	0.33	5
	20 μg.	2	7.3	0.12	5
	1 mg.	2	8.2	0.57	5
	1 mg.	4	7.2	0.42	5
	1 mg.	6	6.0	0.49	4
Cortisone acetate	1 mg.	3	6.8	0.90	5
	10 mg.	1	7.4	0.43	5
	10 mg.	5	8.9	0.62	5
Desoxycorticosterone acetate	1 mg.	2	4.6	0.625	5
	5 mg.	1	4.4	0.37	5
	5 mg.	5	4.5	0.79	5
Corticosterone	0.6 mg.	3	4.6	0.92	5
Corticosterone acetate	2 mg.	1	5.0	0.87	5
	2 mg.	5	4.8	0.75	5

injection of 10 mg. of cortisone acetate in five guinea pigs the fibrinolysin release was 5.2 ± 0.86 units. These observations indicate that neither of these hormones influences the amount of fibrinolysin liberated from profibrinolysin by the addition of peptone to serum.

Action of ACTH and Adrenocortical Hormones on Fibrinolysin Inactivation.— In seventeen guinea pigs the mean antifibrinolytic action of serum was 4.7 ± 0.40 units per 25 μl. Estimation of antifibrinolytic activity in the serum of animals injected with ACTH and cortisone shows an increase which was statistically highly significant. Twenty-five guinea pigs were injected subcutaneously

with varying amounts of ACTH and it is seen in Table I that 20 μg./kg. of ACTH produces a significant rise in antifibrinolytic activity. The maximum effect appears to be reached within 1 hour after injection and then decreases rapidly. In contrast, the effect of cortisone acetate increases with time. This difference is probably due to the fact that ACTH dissolved in saline reaches the circulation rapidly while cortisone in suspension is absorbed slowly. Table I shows the effect of cortisone acetate in ten guinea pigs and it is also seen that neither desoxycorticosterone acetate,[5] nor corticosterone[6] shows any activity at the dosages employed.

Table II shows that the action of ACTH is mediated through the adrenals. Whereas 50 μg. of ACTH increased significantly the antifibrinolytic power of

TABLE II

Antifibrinolytic Power of the Serum of Normal and Adrenalectomized Rats Treated with ACTH

Substance	Dose per kg.	Interval	Units fibrinolysin inhibited per 25 μl. serum	±S.E.	No. of animals
		hrs.			
Normal rats	—	—	2.3	0.3	4
ACTH	10 μg.	1	5.1	0.66	4
	50 μg	1	5.25	0.92	6
Adrenalectomized rats	—	—	3.05	0.23	4
ACTH	50 μg	1	2.9	0.38	4

serum in six intact rats, 50 μg. of the hormone failed to produce any effect in four adrenalectomized rats which were maintained on NaCl.

Action of the Spleen.—Earlier studies have shown that the spleen produces two substances, splenin A and splenin B. Splenin A decreases capillary permeability, increases capillary resistance, and shortens bleeding time; whereas splenin B exerts the opposite effect in these tests. Splenin A is probably a derivative of ascorbic acid (7). Splenin B, as recent work at this Institute by Dr. S. A. Karjala has shown, is an ester of two or three fatty acids with a complex alcohol. Normal spleen produces much less splenin B than splenin A; the ratio being about 1:1,000. This ratio is, however, reversed in certain experimental conditions (thyroidectomy, scurvy) in which an excess of splenin B has been found in the spleen and in blood.

Splenin A is released into the circulation under the influence of the pituitary and adrenal cortex (8). Experimental data suggested that certain actions of the adrenal cortical hormones, especially those concerned with capillary function,

[5] DOCA, Roche-Organon, 0.5 per cent solution in peanut oil.

[6] Kindly supplied by Dr. T. Reichstein of Basle.

might be mediated by splenin A (7). Experiments were performed therefore on splenectomized guinea pigs to determine whether the presence of the spleen was required for the action of ACTH and cortisone on fibrinolysin inactivation. Table III shows that splenectomy does not alter significantly the ability of serum to inactivate fibrinolysin. However, when ACTH and cortisone were injected in splenectomized guinea pigs, their serum failed to show any increase in antifibrinolysin.

TABLE III

Antifibrinolytic Power of Sera from Splenectomized Guinea Pigs Treated with ACTH and Cortisone

	Dose per kg.	Interval	Units fibrinolysin inhibited per 25 μl. serum	±S.E.	No. of animals
		hrs.			
Normal guinea pigs	—	—	4.7	0.40	17
Splenectomized guinea pigs	—	—	4.3	0.19	7
ACTH	10 μg.	1	4.5	0.18	7
Cortisone acetate	10 mg.	5	4.3	0.20	6

TABLE IV

Antifibrinolytic Power of Sera from Guinea Pigs Treated with Splenin A

	Dose per kg.	Units fibrinolysin inhibited per 25 μl. serum	±S.E.	No. of animals
Normal controls	—	4.7	0.40	17
Splenin A	10 μg.	9.3	0.16	5
	1 μg.	6.8	0.46	5
Splenectomized guinea pigs	—	4.3	0.19	7
Splenin A	1 μg.	6.8	0.26	4

Blood samples were taken 1 hr. after subcutaneous injection of splenin A.

Splenin A, prepared according to the method described previously (7) was injected subcutaneously into ten normal and four splenectomized guinea pigs. As shown in Table IV this substance produced a significant increase in antifibrinolytic activity. An increase was observed with 1 μg./kg. and an even greater increase with 10 μg./kg. It is also seen that the action of splenin A is not abolished by splenectomy.

Various concentrations of splenin B[7] were injected into twenty-nine guinea

[7] Crystalline splenin B was prepared by Dr. S. A. Karjala from a crude extract kindly supplied by Dr. E. Kaiser and Dr. R. T. Rapala of Armour and Company.

pigs. Table V shows that amounts as low as 10^{-12} mg. reduced the antifibrinolytic activity by about 25 per cent. By using larger amounts of splenin B it was possible to produce statistically significant effects, confirming the trend observed when small amounts were given. The extremely high activity of splenin B was also observed in other tests (7).

TABLE V

Antifibrinolytic Power of Sera from Guinea Pigs Treated with Splenin B

	Dose per kg.	Units fibrinolysin inhibited per 25 µl. serum	±S.E.	No. of animals
Normal controls	—	4.7	0.40	17
Splenin B	10^{-12} mg.	3.4	0.64	4
	10^{-9} mg.	2.5	0.49	5
	2.5×10^{-8} mg.	2.0	0.30	5
	2×10^{-7} mg.	2.3	0.23	10
	2×10^{-4} mg.	2.7	0.40	5

Blood samples were taken 1 hr. after subcutaneous injection of splenin B.

DISCUSSION

These observations deal with the antifibrinolytic power of serum under specified conditions (namely after 5 minutes' incubation with fibrinolysin) and not with the actual antifibrinolysin content of serum. Recent work (9, 10) indicates that complete combination between fibrinolysin and antifibrinolysin *in vitro* requires prolonged contact. With guinea pig serum an incubation of at least 20 minutes is necessary to reach equilibrium between the two components and the complex they form. It is certain, therefore, that estimation after a contact of 5 minutes does not indicate the total antifibrinolysin present. It supplies, however, an estimate of the rapidly available antifibrinolysin. It seems probable that the observed action of these hormones consists in accelerating the combination between the enzyme and its inhibitor. This is illustrated by Fig. 2, which shows the amount of fibrinolysin inactivated after varying intervals of incubation by sera pooled from five normal guinea pigs and from five guinea pigs treated with 10 mg. of cortisone. It is seen that although after 20 minutes' contact both sera have inactivated all the available fibrinolysin, the rate of the reaction between the enzyme and the inhibitor is entirely different. In the serum of control animals the antifibrinolytic action rises slowly; whereas in the serum of animals treated with cortisone 66 per cent of the final value is reached in 1 minute.

These observations indicate that the substances concerned act by catalyzing the combination between fibrinolysin and its inhibitor although they may not

increase the actual amount of antifibrinolysin in serum. The rate of inactivation cannot be determined by the methods usually employed for the estimation of antifibrinolysin because these include either an unspecified time of contact or a maximal one. The use of trypsin instead of fibrinolysin further complicates the interpretation because the kinetics of the serum inactivation of trypsin and fibrinolysin are different (11).

Previous studies have shown that presence of the spleen is required for certain functions of the adrenal cortex: in splenectomized animals cortical hormones do not act on capillary permeability (7) and do not alter lymphocyte and neu-

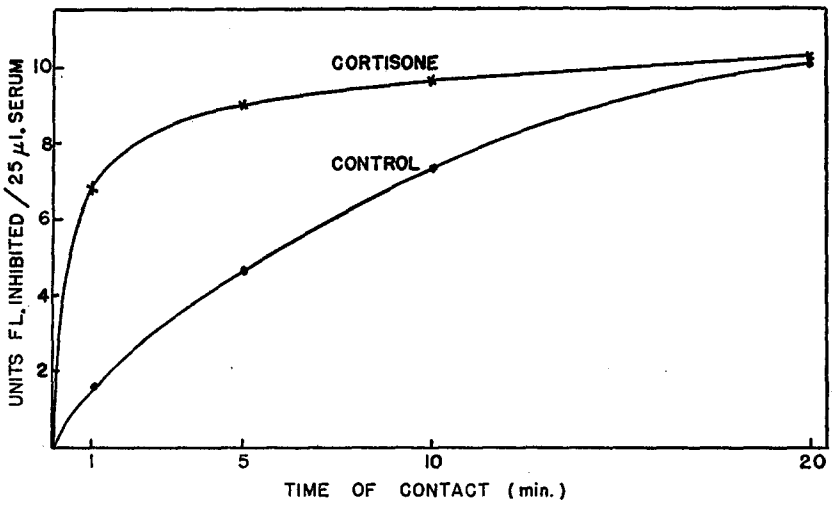

FIG. 2. Inhibition of fibrinolysin by serum from normal guinea pigs and from guinea pigs treated with 10 mg. cortisone acetate 3 hours previously in function of the time of contact between enzyme and serum. Abscissa: time of contact at 37.5°C.; Ordinate: units of fibrinolysin inhibited per 25 μl. of serum.

trophile counts (12, 13). There are also indications that the spleen may take part in fibrinolysin inactivation; for example, Macfarlane has observed that the spleen, of all organs, has by far the highest antifibrinolytic power (14).

The available evidence indicates that the spleen reacts to an adrenocortical stimulus by releasing only splenin A with the exclusion of splenin B (8, 7). The mechanism by which splenin B is liberated is at present unknown, but there is some evidence that the thyroid gland may be involved in it. Normally the spleen synthesizes very little splenin B; however this can be increased by creating a vitamin C deficiency (7). Current studies show that the production of splenin B can also be enhanced by applying stimuli which increase the secretion of thyrotrophic hormone from the pituitary.

It seems improbable that the splenins A and B affect directly the kinetics of

the fibrinolysin-antifibrinolysin system. In certain *in vitro* experiments it was possible to obtain an acceleration of the fibrinolysin-antifibrinolysin reaction by splenin A and its retardation by splenin B (7). However, when purified fibrinolysin and antifibrinolysin were used, the rate of their combination was not changed by either substance. This suggests that the presence of some factor in serum is necessary to obtain this effect from the spleen substances. The nature of this intermediate reaction is being investigated.

In view of the probable part played by fibrinolysin in shock (15–18), and in anaphylactic and anaphylactoid reactions (1–5), the rate of inactivation of the enzyme liberated under these conditions is highly important. The damage which fibrinolysin can produce, either directly by attacking tissues, especially the capillary wall, or indirectly by releasing histamine or other toxic protein breakdown products, is a function of the interval during which it circulates in an active form. It was found in 1944 that ACTH and adrenocortical extracts inhibited the release of histamine-like substances from blood cells (19). This inhibition can be explained by the fact that in animals treated with these hormones fibrinolysin is inactivated more rapidly than in normal animals.

In the light of certain observations histamine seems to be released as a result of a proteolytic process (20–22). The amount of histamine liberated is therefore probably related to the interval during which fibrinolysin is free. These influences are probably most effective in slow reactions like the Arthus or Shwartzman phenomenon or other local sensitization reactions. It seems unlikely that they play any part in acute anaphylactic shock in which profibrinolysin activation is produced rapidly and fibrinolysin exerts its effect before inactivation can even begin.

That the adrenal cortex controls resistance to stress and plays the principal part in the "general adaptation syndrome" of Selye (23), is recognized. Furthermore, it has already been suggested (16) that a release of fibrinolysin is part of the "alarm reaction" which precedes the adaptation phase. It now appears that the rate of the reaction between fibrinolysin and its inhibitor may play a role in the resistance to various noxious stimuli which is usually associated with augmented adrenocortical function.

SUMMARY

Injection of ACTH or cortisone has no effect on the amount of fibrinolysin resulting from the activation of profibrinolysin by peptone.

Antifibrinolytic activity of serum is significantly increased by ACTH or cortisone but not by desoxycorticosterone or corticosterone. No increase in antifibrinolytic power of serum is produced, however, by the injection of ACTH or cortisone in splenectomized guinea pigs.

Splenin A has the same action on fibrinolysin inactivation as ACTH or cortisone. Splenin B has the opposite effect.

This action on the antifibrinolytic power of serum appears to be due to an acceleration of the rate of combination between the enzyme and its inhibitor and not to an actual increase in antifibrinolysin.

The possible significance of these observations is discussed.

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