Studies on the Activation and Purification of Blood Fibrinolysin

By M. ROCHA E SILVA AND C. RIMINGTON

Department of Chemical Pathology, University College Hospital Medical School, London

(Received 26 November 1947)

The presence, in normal plasma or serum, of a fibrinolytic agent that can be activated by chloroform, was first reported by Delezenne & Pozerski (1903 *a, b*). This effect of chloroform was further investigated by Nolf (1905, 1908) who also observed activation of this fibrinolytic capacity of the blood in dogs given peptone intravenously, provided the liver was excluded from circulation either by clamping the thoracic aorta and inferior vena cava ('thoracic animal'), or simply by interrupting the circulation through the liver by manual compression of the vessels going into the organ. More recently, Tagnon (1942) showed that the activity of this fibrinolytic enzyme was connected with the euglobulin fraction of the blood, since it might be fully recovered by isoelectric precipitation (pH 5.5) after dilution of the plasma with 10 vol. of distilled water. A comparison of this fibrinolytic factor with the so-called 'lytic factor' described by Milstone (1941) as the effective fibrinolytic agent of material isolated from certain strains of β -haemolytic streptococci (streptokinase), was undertaken by Kaplan (1944), Christensen (1944-5), and Christensen & MacLeod (1944-5). Confirmatory evidence of identity appears in several papers (e.g. Macfarlane & Pilling, 1946; Loomis & Smith, 1946).

Further reports have stressed the importance of the activation of this fibrinolytic enzyme in several physiological and pathological conditions. Using the 'fibrinolytic protamine test', it has been shown (Rocha e Silva, Andrade & Teixeira, 1946) that activation of fibrinolysin occurs in anaphylactic and peptone shock, and that a definite relationship seems to exist between the appearance of the fibrinolytic effect and other characteristics of anaphylactic and peptone shock. In traumatic and burn shock also there is a spontaneous activation of the fibrinolytic enzyme of the blood, the intensity of which bears some relationship to the gravity of the shock condition (Tagnon, Levenson, Davidson & Taylor, 1946). Using normal and sensitized guinea pigs, Ungar (1947) demonstrated activation of a fibrinolytic enzyme when pieces of certain organs (liver and lung) were placed *in vitro* in contact respectively with peptone or with the homologous antigen. Macfarlane & Biggs (1946), using the technique of Macfarlane & Pilling (1946), reported activation of plasma fibrinolysin in a number of subjects from a hospital popu-

lation including cases awaiting operation, undergoing pretreatment and after actual operation, also some accident and miscellaneous pathological cases. A recent report by Ferguson, Travis & Gerheim (1947) ascribes a weak trypsin-like thromboplastic activity to the fibrinolytic enzyme present in normal blood, suggesting participation of this proteolytic enzyme (plasma tryptase) in blood coagulation. The possibility that activation of this enzyme may play a role in the liberation of histamine was recently suggested by Scroggie, Jaques & Rocha e Silva (1947) on the basis of the fact that a trypsin inhibitor from soya beans partially inhibits the release of histamine from cells to plasma when peptone is added to rabbit blood.

The desirability of securing highly active preparations of this fibrinolytic enzyme, in order to study more thoroughly its biological properties, was the main reason for undertaking this study. When the experiments were nearing completion, a paper appeared by Loomis, George & Ryder (1947) in which they described a method for purification of blood fibrinolysin by combining fractionation by ammonium sulphate with several isoelectric precipitations in high dilutions with distilled water. Under present circumstances, we cannot undertake a comparative study of our results with those of the American workers. We have, however, investigated a sample of fibrinolysin supplied by Loomis, and have shown that we can raise its activity appreciably by using a new method of fractionation, as described in the text. Although we did not attain a final purification of the enzyme, the facts described in the present paper may assist a further attack upon the problem.

EXPERIMENTAL

Preparation of material. Ox or human serum or plasma was obtained by prolonged centrifugation of fresh blood. After dilution with 10 vol. of distilled water and adjustment of pH to 5.3-5.5 with dilute acetic acid, the globulin precipitate, which settled overnight in the ice box, was collected by centrifugation and redissolved in one third of the original volume of 1% (w/v) NaCl (subsequently referred to as 'saline') and carefully neutralized with 0.1 N-NaOH. Chloroform (0.1 vol.) was added and the globulin solution was left for 3 or 4 days at room temperature with occasional shaking. The aqueous layer was then again diluted with distilled water (10 vol.), adjusted to pH 5.3, and the precipitate

allowed to settle in the ice box. It was centrifuged, re-dissolved in 0.2 of the original volume of saline and neutralized. This 'activated globulin solution' was used for further purification.

Fractionation with ammonium sulphate. Appropriate amounts of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ were added to the 'activated globulin solution'. After standing for 1 hr. at room temperature, some 'celite' was added and the precipitate filtered off. The filtrates were dialyzed (cellophan) against several changes of 1% (w/v) solution of NaCl, neutralized and tested for fibrinolysis.

Fig. 1. Time-dilution curve of the activated ox-globulin preparation OxGl_{10} (concentrated 3 times). From data corresponding to dilutions 1:8, 1:16 and 1:32, one can calculate the potency of the preparation as equal to 40 units/ml.; $U=10c/t$, where $c=1/D$, D being the dilution, and t the time required for complete fibrinolysis.

'Low-ionic' isoelectric precipitation. Isoelectric precipitation at low concentration of inorganic ions was performed, as described above, by diluting the activated globulin solution with distilled water (10 vol.) and adjusting to pH 5.0-5.5.

'Saline' isoelectric precipitation. The precipitate of the activated globulin solution was dissolved in 1% (w/v) NaCl solution at pH 7.4, acetic acid added to bring the pH to 5.5-5.0 and the precipitate centrifuged and redissolved in neutral saline.

Delipidization of serum or plasma was performed according to the technique of McFarlane (1942) by adding ether (0.3 vol.), shaking and freezing to -25° or -30° with solid CO_2 (dry ice); after slow thawing at room temperature, the mixture was centrifuged and the clear bottom layer was

carefully collected and diluted with 10 vol. of distilled water (pH 5.3) and the precipitate collected and treated in the usual way with 0.1 vol. of chloroform. As a rule, a duplicate sample of the original serum or plasma (non-delipidized) was treated in exactly the same way and the final activity of both samples compared.

Tyrosine was determined according to Folin & Ciocalteu (1927) in 0.1 or 0.2 ml. of an appropriate dilution of the material, N-NaOH (5 ml.), distilled water (4 ml.) and the phenol reagent (1 ml.) being successively added. After 20 min. standing, the colour was read in a Pulfrich spectrophotometer and the tyrosine concentration determined from a standard curve.

Cholesterol was estimated by the method of Bloor (1928), using the Pulfrich spectrophotometer for the final reading of the colour.

Fibrinolytic activity. The fibrinolytic tests were performed in small Wassermann tubes at 37° . To the solution of the enzyme to be tested (0.5 ml.), a 0.2% (w/v) solution of human fibrinogen (0.3 ml.) and two drops of a thrombin solution (adjusted to produce clot in 1 min.) were added. The time for total lysis was measured and the number of units calculated. We found it convenient to define the unit as the amount of enzyme which produces total lysis in 10 min. under the conditions stipulated above (cf. Fig. 1).

RESULTS

Precipitation by ammonium sulphate

The whole of the active enzyme contained in the activated globulin fraction is precipitated between 25 and 37% saturation (room temperature) with ammonium sulphate (Table 1). In a quantitative examination the precipitates at 0-40% and 0-50% saturation with ammonium sulphate were collected, redissolved in saline and dialyzed for 20 hr. The activity of the original solution was almost fully recovered.

Table 1. *Precipitation of fibrinolysin by different concentrations of ammonium sulphate at room temperature*

Materials added (ml.):						
Activated ox globulin	10	10	10	10	10	10
Ammonium sulphate (saturated solution)	—	5	6	7	8	10
Saline	10	5	4	3	2	—
Conc. ammonium sulphate (% satn.)	—	25	30	35	40	50
Lytic power of filtrates (units/ml.)	40	42	30	11	—	—

Influence of temperature on the activation of fibrinolysin after removal of the chloroform

The activity of the preparation was very seldom fully developed immediately after the chloroform treatment. Usually, the activity increased slowly, if the preparation was left a few days at room temperature. This spontaneous increase in activity was much slower if the material was kept in the ice box,

and very rapid if maintained at 38°. Figs. 2 and 3 illustrate such spontaneous increase in activity of the enzyme at 0°, at room temperature and at 38° respectively. The shape of the curve in Fig. 3 suggests an autocatalytic process, since the increase in activity is slow initially and rapid after 60–90 min.

Fig. 2. Spontaneous activation at room temperature of preparation $O\alpha Gl_{11}$ after removal of chloroform.

After the maximum is attained, the activity of the enzyme slowly decreases again, possibly owing to the action of the products of the proteolysis. As a routine procedure, all preparations, after removal of the chloroform and precipitation at pH 5.3 with 10 vol. of distilled water, were maintained for 2–4 hr. at 38°. We have, however, observed that some of the preparations changed very little after incubation, probably because they were fully activated while in contact with the chloroform.

Christensen (1946) demonstrated that chloroform destroys the inhibitor normally present in plasma or serum. The shape of the curve of Fig. 3 disproves the idea that the further spontaneous activation might

Fig. 3. Spontaneous activation at 0 and 38° of preparation $O\alpha Gl_{12}$ after removal of chloroform.

be due to a further destruction of the inhibitor, since in that case the maximal increase in activity would occur at the beginning of the incubation. That the enzyme is still present in the solution after the chloroform treatment, partially as an inactive precursor (pro-fibrinolysin) ready to undergo autocatalytic activation, seems to be the simplest conclusion to be drawn from our study of the effects of temperature upon the activation of the enzyme. All attempts to produce a spontaneous activation of the globulin solution without the chloroform treatment, even by incubating the original globulin solution for several hours at 38°, have failed. In a few experiments we have tried to activate the globulin solution with carbon tetrachloride and also with ether or ethanol, but in no case was any significant activation detectable by fibrinolysis tests.

Isoelectric fractionation in saline

Repeated low-ionic precipitation of an activated globulin (AGI) failed to raise its potency appreciably. However, by carrying out saline isoelectric pre-

Table 2. Saline isoelectric fractionation of α globulin preparation $O\alpha Gl_{15}$

Conditions of experiments	Fibrinolysin (units/ml.)	Tyrosine (mg./ml.)	Fibrinolysin (units/mg. tyrosine)
I. Solution $O\alpha Gl_{15}$, after chloroform treatment	10	2.5	4
II. Above, after precipitation with 10 vol. distilled water (pH 5.5) and incubation at 38° (4 hr.)	65	2.2	30
III. Above, after isoelectric precipitation with 10 vol. distilled water ('low-ionic isoelectric precipitation')	85	1.7	47
IV. Above, after isoelectric precipitation in 1% (w/v) saline (three times) ('saline isoelectric precipitation')	24	0.07	343

cipitation, we obtained a rapid increase in activity/mg. tyrosine up to a maximum attained after three or four reprecipitations at pH 5.0-5.3.

Table 2 shows the increase in activity of the oxglobulin preparation $OxGl_{15}$, after chloroform treatment, incubation at 38° for 4 hr., reprecipitation at pH 5.5 with 10 vol. of distilled water and three precipitations in saline, at pH 5.1. A similar experiment with the preparation $OxGl_{18}$, in which incubation at 38° preceded low-ionic isolation precipitation, led to the same result, i.e. a marked increase in potency only when saline was substituted for distilled water.

In a few experiments we used human blood, defibrinated or kept citrated. In the latter case, no attempt was made to clot the plasma before the chloroform treatment, but at the end of this treatment the whole fibrinogen was found to be destroyed by the active enzyme. Table 3 shows a protocol of an experiment of isoelectric fractionation of human globulin solution (HGL_2) by three precipitations in saline at pH 5.3.

As seen from the examples given, repeated precipitations at pH 5.0 in saline raise the activity to a maximum which may be reached with two precipitations (cf. Fig. 4) or may require more. A limitation of this method of purification is that, when the initial activity of the enzyme was low, the maximum attained was also rather low. The only plausible explanation for this is that the inactive form of the enzyme (pro-fibrinolysin) is also precipitated in the same range of pH as the active form in such a way that further separation of the two substances by this type of precipitation becomes impossible. Table 4 records all our results with isoelectric precipitation in saline of different preparations of activated ox globulin and one human globulin preparation.

Fig. 4. Activation of preparation $OxGl_{18}$ and progressive purification by isoelectric precipitation in saline. Abscissae: 1, after chloroform treatment; 2, after incubation at 38° for 6 hr.; 3, after precipitation in saline at pH 5.0; 4, after repetition of 3; 5, after further repetition of 3; 6, after further repetition of 3.

It is quite clear from Table 4 that, if one starts with a very active preparation, purification by isoelectric precipitation in saline works very well, but

Table 3. Human globulin solution (HGL_2) treated with chloroform and fractionated at pH 5.0 in saline

Conditions of experiment	Fibrinolysin (units/ml.)	Tyrosine (mg./ml.)	Fibrinolysin (units/mg. tyrosine)
I. Activated human globulin	60	0.58	110
II. Above, after precipitation in saline at pH 5.0	50	0.25	200
III. Above, after reprecipitation in saline at pH 5.0	27	0.13	207

Table 4. Increase in activity by fractionation at pH 5.0-5.5 of different globulin preparations

Material	Activity (units/mg. tyrosine)	
	After chloroform and incubation at 38°	After isoelectric precipitation in saline (no. of reprecipitation in brackets)
$OxGl_{14}$	17	65 (4)
$OxGl_{15}$	65	343 (3)
$OxGl_{16}$ I	50	132 (3)
$OxGl_{16}$ II	24	158 (3)
$OxGl_{18}$ I	1	9 (4)
$OxGl_{18}$ II	15	48 (3)
Human GL_2	110	207 (2)
Parke Davis preparation (Loomis <i>et al.</i> 1947)	114	300 (2)

when the initial activity is low, a less satisfactory result is obtained even after several isoelectric precipitations. That this method of purification is different from that recently described by Loomis *et al.* (1947) can be shown by the fact that fractionation in saline at the isoelectric point of a sample of one of the Loomis preparations, increased the specific activity three times. We feel, therefore, that purification by isoelectric precipitation in saline has a useful place, in combination with other methods, in the preparation of purified fibrinolysin.

in the usual way by isoelectric precipitation with 10 vol. of distilled water at pH 5.5. The precipitate collected by centrifugation was redissolved in saline, neutralized and submitted to the chloroform treatment, since there was no evidence that the simple treatment of delipidization was enough to activate the enzyme. Simultaneously, a sample of the original serum or plasma was submitted to identical treatment (delipidization excluded) and the final activities of both samples compared. The results (Table 5) show that a previous delipidization of the

Table 5. *Effect of a previous delipidization of serum or plasma by ether at -25° upon the activity of the enzyme resulting from subsequent chloroform treatment*

Exp.	Material used	Fibrinolysin		Cholesterol (mg./100 mg tyrosine)
		(units/ml.)	(units/mg. tyrosine)	
I	$DOxGl_{21}$ (delipidized)	15	8.4	14.0
	$OxGl_{21}$	1	0.7	2.0
II	$DHGl_4$ (delipidized)	33	36.4	17.0
	HGl_4	20	25.0	9.0
III	$DHGl_5$ (delipidized)	55	55.0	12.5
	D_2HGl_5 (twice delipidized)	5	5.0	12.5
	HGl_5	9	10.0	3.7
	HGl_5E (ether saturated)	10	11.0	112.0
IV	$DHGl_3$ (delipidized)	40	88.8	8.3
	HGl_3	33	43.0	4.9
V	$DOxGl_{20}$ (delipidized)	12	14.0	28.0
	$OxGl_{20}$	0.5	0.5	15.0

Effect of delipidization of the original serum or plasma

The great variability of the results obtained after activation of the serum or plasma with chloroform induced us to study more thoroughly the mechanism by which chloroform activates the fibrinolysin precursor (pro-fibrinolysin). Christensen (1946) has shown that chloroform destroys the inhibitor, thus allowing spontaneous activation of the enzyme. It is, however, quite probable that the inhibitor, or at least one of the inhibitors, might be combined with the enzyme precursor. We have assumed as a working hypothesis that this linkage might be of a lipoprotein nature, and that chloroform might act by loosening such a linkage. We have, therefore, delipidized serum or plasma in an attempt to activate the enzyme, using first the technique of Hardy & Gardiner (1910) as modified by Hartley (1925). After treatment of the original serum or plasma with a mixture of ether and ethanol at -12° , the material obtained was entirely free from cholesterol or phospholipid, but had no fibrinolytic activity, nor could it be activated by chloroform. We, therefore, decided to try the milder delipidization described by McFarlane (1942), which consists of treating the plasma or serum with ether at -25° or -30° . After this treatment, the globulin fraction was prepared

material definitely increased the fibrinolytic activity after chloroform treatment. In one of the samples of human plasma (D_2HGl_5), after complete thawing, the underlying plasma was submitted again to the cold-ether treatment with a definite decrease in the resulting activity. If one combines this result with that obtained by the Hardy & Gardiner (1910) method, it seems clear that a very mild initial delipidization will improve the final activity, but, if the process of delipidization is repeated, a permanent inactivation of the enzyme occurs.

From the data of Table 5, it might appear that a relationship exists between the final activity of the preparation and its cholesterol content. This seems to be true for most of the experiments in which the untreated material was compared with the delipidized one. In the experiment in which the plasma was saturated with ether (HGl_5E), without freezing, the amount of cholesterol present was, however, far greater than that in the delipidized fraction, whereas the final fibrinolytic activity was rather low. It is known that McFarlane's (1942) method does not remove all of the lipids present in serum or plasma. In our experiments 20–40 % of the cholesterol was not removed by this treatment. When the globulin fractions were prepared from delipidized serum or plasma, this residual cholesterol appeared to be concentrated in the globulin fraction; very frequently

Table 6. *Cholesterol contents of untreated and delipidized plasma or serum and their globulin fractions*

Material	Cholesterol content (mg./100 mg. of tyrosine)			
	Plasma or serum		Globulin fraction	
	Untreated	Delipidized	Untreated material	Delipidized material
Human P_4	31.0	5.0	9.0	17.0
Ox S_{21}	8.7	3.1	2.0	14.0
Ox S_{20}	18.0	6.0	15.0	28.0
Human P_3	—	—	4.9	8.3
Human P_5	34.0	7.0	3.7	12.5
DHP ₅ (twice delipidized)	34.0	2.5	3.7	12.5

there was more cholesterol in the globulin fraction derived from a delipidized serum or plasma than in that from normal untreated plasma or serum (Table 6). This might be due to the fact that the material, from which the globulin fraction was precipitated, was saturated with ether. In favour of this possibility we might mention the result obtained with the ether-saturated material HGI_5E (Exp. III, Table 5) which contained much more cholesterol than any one of the other fractions obtained from the same human plasma. The results were, however, so paradoxical that a further study of this phenomenon is desirable. For the time being, however, we can accept the explanation that the presence of ether modifies the precipitability of a possible lipoglobulin material that might bear some relationship to the fibrinolytic enzyme.

SUMMARY

1. Experiments on activation and purification of the fibrinolytic enzyme of ox and human plasma or serum showed that the enzyme is associated with the globulin fraction precipitated between 25 and 37 % saturation with ammonium sulphate at room temperature.

2. After treatment with chloroform, the enzyme preparation can be further activated by incubation

at 38° for a few hours. The activation process follows the course of an autocatalytic reaction.

3. 'Low-ionic isoelectric precipitation' (10 vol. of distilled water at pH 5.5) was compared with 'isoelectric fractionation in saline'. The former effected slight purification, whereas the latter rapidly enhanced enzymic potency, indicating that the enzyme is probably connected with a less soluble fraction of the total euglobulin fraction of plasma or serum.

4. A previous mild delipidization of plasma or serum led to a definitely higher activity of the enzyme after the chloroform treatment. A more complete delipidization appeared to destroy the pro-ferment, since no activation could be obtained by subsequent treatment with chloroform.

5. Some relationship has been found between fibrinolytic activity and cholesterol content, especially in those fractions derived from mildly delipidized plasma or serum.

One of us (M. R. e S.) wishes gratefully to acknowledge the tenure of a British Council Research Fellowship during the course of this work. Our thanks are due to Dr R. A. Kekwick for generous supplies of purified fibrinogen and of thrombin, to our colleague Dr M. Maizels, for arranging the supply of human blood and to Dr C. E. Work for helpful discussions on many problems. Mr J. Evans kindly performed the cholesterol analyses.

REFERENCES

- Bloor, W. R. (1928). *J. biol. Chem.* **77**, 53.
 Christensen, L. R. (1944-5). *J. gen. Physiol.* **28**, 363.
 Christensen, L. R. (1946). *J. gen. Physiol.* **30**, 149.
 Christensen, L. R. & MacLeod, C. M. (1944-5). *J. gen. Physiol.* **28**, 559.
 Delezenne, C. & Pozerski, E. (1903a). *C.R. Soc. Biol., Paris*, **55**, 690.
 Delezenne, C. & Pozerski, E. (1903b). *C.R. Soc. Biol., Paris*, **55**, 693.
 Ferguson, J. H., Travis, B. L. & Gerheim, E. B. (1947). *Proc. Soc. exp. Biol., N.Y.*, **64**, 302.
 Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
 Hardy, W. B. & Gardiner, S. (1910). *J. Physiol.* **40**, lxxviii.
 Hartley, P. (1925). *Brit. J. exp. Path.* **6**, 180.
 Kaplan, M. H. (1944). *Proc. Soc. exp. Biol., N.Y.*, **57**, 40.
 Loomis, E. C. & Smith, R. M. (1946). *J. biol. Chem.* **163**, 767.
 Loomis, E. C., George, Jr., C. & Ryder, A. (1947). *Arch. Biochem.* **12**, 1.
 McFarlane, A. S. (1942). *Nature, Lond.*, **149**, 439.
 Macfarlane, R. G. & Pilling, J. (1946). *Lancet*, **2**, 562.
 Macfarlane, R. G. & Biggs, R. (1946). *Lancet*, **2**, 862.
 Milstone, H. (1941). *J. Immunol.* **42**, 109.
 Nolf, P. (1905). *Arch. int. Physiol.* **3**, 1.
 Nolf, P. (1908). *Arch. int. Physiol.* **6**, 306.
 Rocha e Silva, M., Andrade, S. O. & Teixeira, R. M. (1946). *Nature, Lond.*, **157**, 801.
 Scroggie, E., Jaques, L. B. & Rocha e Silva, M. (1947). *Proc. Soc. exp. Biol., N.Y.*, **66**, 326.
 Tagnon, H. J. (1942). *J. Lab. clin. Med.* **27**, 1119.
 Tagnon, H. J., Levenson, S. M., Davidson, C. S. & Taylor, F. H. L. (1946). *Amer. J. med. Sci.* **211**, 88.
 Ungar, G. (1947). *Lancet*, **1**, 708.