coagulable in the presence of plasma containing Ca^{++} ions.

2. Alkali destruction of the enzyme restores to the fibrinogen the power of forming clots under suitable conditions, and of being precipitated by low concentrations of salts.

3. Certain other proteolytic enzymes react similarly under suitable conditions.

I am indebted to the Medical Director of this Institute for providing facilities for carrying out these experiments, and to Mr L. N. Owen who has performed much of the experimental work.

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The Separation of Fibrinogen from 'Pepsin-inhibitor'

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Numerous methods for the purification of fibrinogen by precipitation with $(\text{NH}_4)_2\text{SO}_4$ or NaCl have been described [Florkin, 1930; Warner, Brinkhous & Smith, 1935-6; Ferguson, 1938-9; and Ferguson & Erickson, 1939]. Florkin [1930] used solubility in salt solutions as the criterion of purity of his preparations.

In a previous paper [Hind, 1943] it was shown that pepsin, when present in sufficient amounts, would protect fibrinogen against precipitation at low salt concentrations, and that a solution of partially purified fibrinogen required less enzyme per g. of fibrinogen to effect this protection than did fibrinogen present in plasma. In the present work we show that a substance (or substances) present in plasma competes with fibrinogen for the enzyme. The substance (or substances) having this inhibiting effect on pepsin is termed 'pepsin inhibitor' in the following account. Some properties of the pepsin inhibitor and its separation from fibrinogen are described.

METHODS

Horse plasma (centrifuged and oxalated) and serum were used.

Pepsin was a commercial (1/3000 U.S.P.) product.

Estimation of the salt-precipitable fibrinogen and the preliminary and semi-quantitative tests between pepsin and fibrinogen were carried out as previously described [Hind, 1943].

Purified fibrinogen. 1000 ml. of centrifuged plasma were precipitated with 21.6% sat. $(\text{NH}_4)_2\text{SO}_4$. The

precipitate was collected on the centrifuge and dissolved in 1000 ml. of saline. The precipitation and solution were repeated 4 times. On redissolving for the last time, however, only 500 ml. of saline were used.

Pepsin inhibitor and serum globulins. The residues obtained from the purified fibrinogen were precipitated with 50% sat. $(\text{NH}_4)_2\text{SO}_4$ and the globulin precipitate was filtered off with kieselguhr, and pressed. The kieselguhr-protein mixture was then extracted 3 times with 200 ml. quantities of water and the solution dialysed overnight. The dialysed solution was made up to 1000 ml., i.e. the original volume of the plasma used.

Serum. Serum was prepared from 400 ml. of plasma, by adding CaCl_2 and pressing the clot. The serum gave no precipitate with 21.6% sat. $(\text{NH}_4)_2\text{SO}_4$.

Heated serum was prepared by heating 150 ml. of oxalated serum to 58° for 2 min.

Defibrinated plasma. 400 ml. of plasma were heated to 56° for 2 min. and centrifuged until clear.

RESULTS

The effect of repeated precipitation of fibrinogen on its reaction with pepsin

1000 ml. of plasma were clarified by centrifuging at 5000 r.p.m. for 5 min., and 200 ml. were removed for subsequent tests. Solid $(\text{NH}_4)_2\text{SO}_4$ to 21.6% saturation was dissolved in the remaining 800 ml. The fibrinogen precipitate was collected by centri-

fuging and redissolved in normal saline. It was made up to 800 ml. and 200 ml. were removed. The precipitation and solution were repeated twice more. The volume was made up in each case to correspond to the volume which had been precipitated, and at each stage 200 ml. of solution were removed.

Fig. 1. Pepsin titration curves of fibrinogen solutions after successive reprecipitations. Curves 1-4 were obtained with plasma, once precipitated fibrinogen, twice precipitated fibrinogen and thrice precipitated fibrinogen respectively. The data plotted were obtained by subtracting the weight of fibrinogen precipitated in the presence of pepsin from that precipitated in its absence. This difference was plotted against the volume of 1% pepsin which had been added.

Fig. 2. Pepsin titration curves of fibrinogen solutions from different plasmas. ∇ Fibrinogen reprecipitated 3 times; $\circ$ fibrinogen reprecipitated 3 times; $\triangle$ fibrinogen reprecipitated 3 times; ∇ fibrinogen reprecipitated 4 times; $\triangle$ fibrinogen reprecipitated 3 times; $+$ fibrinogen reprecipitated 4 times; $\times$ fibrinogen reprecipitated twice; $\bullet$ plasma (typical curve).

After the determination of the approximate values for the amount of pepsin required to prevent the salt precipitation of the fibrinogen in 2 ml. of each fraction, quantitative determinations were carried out. 50 ml. of solution of each fraction were used, and after treating with the predetermined

amount of 1% pepsin for 10 min., the precipitable fibrinogen was estimated. The curves obtained from the results are given in Fig. 1. The weight of fibrinogen protected against precipitation represents the difference in weight between a control precipitate and the precipitate obtained from a pepsin-fibrinogen mixture. This difference in weight is plotted against the amount of pepsin which produced this change. It will be seen that at each successive stage of purification less enzyme was required to protect the fibrinogen against salt precipitation, and that at least 4 or 5 precipitations would be necessary to effect complete separation of the fibrinogen from the pepsin inhibitor.

Similar curves for fibrinogen preparations from different plasmas are given in Fig. 2.

Preliminary tests with the pepsin inhibitor

The effect of heat on the pepsin inhibitor in serum and in plasma was studied, and the pepsin inhibitor content of plasma was compared with that of serum prepared from the plasma.

Solutions used. (1) 200 ml. plasma + 100 ml. saline. (2) 150 ml. of solution of pepsin inhibitor and serum globulins + 75 ml. of fibrinogen solution. (3) 150 ml. of oxalated serum + 75 ml. of fibrinogen solution. (4) 150 ml. of heated serum + 75 ml. of fibrinogen solution. (5) 150 ml. of heat-defibrinated plasma + 75 ml. of fibrinogen solution. (6) Undiluted fibrinogen solution.

These mixtures were all adjusted to pH 5.3. The fibrinogen content of the solutions was comparable except that (6) contained 3 times as much fibrinogen as the other solutions.

Preliminary tests with 2 ml. quantities of the solutions were carried out against 1.0 and 0.01% pepsin. The lower strength was found necessary, as with some of the mixtures very little pepsin was required to render the fibrinogen non-precipitable. For quantitative determinations 50 ml. of solution were used and, after treatment with appropriate amounts of pepsin for 10 min. at 18°, the precipitable fibrinogen was estimated. The amount of pepsin (g.) required to protect 1 g. of fibrinogen in each of the solutions was calculated and is given in Table 1.

Further tests have shown that serum heated to 55° does not lose its ability to inhibit the action of pepsin in rendering fibrinogen non-precipitable.

Table 1. *The reaction at pH 5.3 between pepsin and different solutions containing fibrinogen*

Fibrinogen-containing solution used	Pepsin required to protect 1 g. of fibrinogen against $(\text{NH}_4)_2\text{SO}_4$ precipitation at 21.6% saturation g.
Plasma	0.9
Fibrinogen	0.02
Solution of fibrinogen and pepsin inhibitor	1.05
Fibrinogen and serum	1.8
Fibrinogen and heated serum	0.06
Fibrinogen and heated defibrinated plasma	0.08

DISCUSSION

Because of the close association between the pepsin inhibitor and fibrinogen, and because fibrinogen solutions could be approximately titrated against pepsin, it was decided to use the pepsin-fibrinogen reaction as a measure of the purity of fibrinogen solutions. The results show that four or five reprecipitations with 21.6% sat. $(\text{NH}_4)_2\text{SO}_4$ are necessary to separate the pepsin-inhibitor from fibrinogen. The concentration of $(\text{NH}_4)_2\text{SO}_4$ (21.6% sat.) used in this work was determined by preliminary experiments to be a safe minimum quantity for the precipitation of the fibrinogen from the plasma used. Most other workers have used 25% saturation. 1 g. of pepsin (1/3000 U.S.P.) will protect approximately 50 g. of our purest preparations of fibrinogen against precipitation with $(\text{NH}_4)_2\text{SO}_4$. Similar values have been obtained by using fibrinogen solutions prepared from different plasmas.

Little or no pepsin inhibitor could be demonstrated in plasma defibrinated by heating, or in serum which had been heated to 58°. However, the pepsin inhibitor is apparently stable at 55°, since serum heated to this temperature showed no loss of potency. It was present, together with the serum globulins, in the filtrates obtained during the preparation of fibrinogen, and was precipitated therefrom by precipitating with 50% sat. $(\text{NH}_4)_2\text{SO}_4$. On adding this precipitate to a purified fibrinogen solution, the reaction between the mixture and pepsin was similar to that between plasma and pepsin. This pepsin-inhibitory action of serum appeared to be greater than that in the plasma from which the serum had been prepared.

SUMMARY

1. A substance ('pepsin-inhibitor') is present in fresh serum and plasma, which inhibits the ability of pepsin to protect fibrinogen against precipitation at pH 5.0-5.6 by low concentrations of salt.
2. This pepsin inhibitor is present in preparations of fibrinogen precipitated with 20-25% sat. $(\text{NH}_4)_2\text{SO}_4$, but separation of it from fibrinogen can be effected by repeated precipitation of the latter with 21.6% sat. $(\text{NH}_4)_2\text{SO}_4$.
3. Heating at 58° for 2 min. destroys almost all the activity of preparations of this 'pepsin-inhibitor'.

I am indebted to the Medical Director of this Institute for providing facilities for carrying out these experiments and to Mr L. N. Owen who has performed much of the experimental work.

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Microdetermination of Vitamin A and Carotenes

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The Carr-Price reaction has been widely used for the estimation of vitamin A and carotenes. The limitations and difficulties of the method have been described by so many that no attempt will be made to review the extensive literature [see Brode & Magill, 1931]. The conventional method, as used by

May, Blackfan, McCreary & Allen [1940] and by Dann & Evelyn [1938] for the determination of β -carotene by measuring the light absorption with a 440m μ filter, is not specific. For example, in blood serum both bilirubin and β -carotene absorb light of wave-length 440m μ .