7. Caffeine and chlorocaffeine are the only purines which react as described with haem, CO-protohaem, porphyrins and metalloporphyrins. No other purine, out of about 20 tested, including theobromine, theophylline and 1:3:7-trimethyl uric acid, gives any of these reactions.

8. The fact that chlorocaffeine behaves in this respect like caffeine, shows that the H in position 8 can be replaced by Cl but not by O₂.

9. Of all the iminazole compounds tested, only pilocarpine, and to a lesser degree 1-methyl iminazole, react with the porphyrin in the same way as caffeine; but, unlike caffeine, with haematin, haem and CO-haem, they give parahaematin, haemochromogen and CO-haemochromogen compounds respectively.

10. The fact that globin and certain serum proteins react with porphyrins in the same manner as

caffeine, while none of their amino-acid constituents, including histidine, gives this reaction, suggests that (a) either these proteins contain an additional, not yet isolated, constituent having these properties, or (b) that the iminazole ring of histidine, when the latter is incorporated in a polypeptide chain, may have some of the properties of the iminazole ring of caffeine and of pilocarpine.

11. The bearing of all these properties of caffeine on the mechanism of caffeine diuresis is discussed.

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The Action of Proteolytic Enzymes on Fibrinogen Solutions

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It has been recognized for some years that a proteolytic enzyme system may be concerned with blood coagulation. Wright [1915] noted that blood coagulation could be inhibited by the addition of trypsin, and Douglas & Colebrook [1916] confirmed this observation, but demonstrated that smaller quantities of trypsin had the effect of *shortening* the clotting time.

Further studies of the effect of trypsin on blood coagulation have been reported by Tyson & West [1937], Eagle & Harris [1937], and Mellanby & Pratt [1938]. Though differences are evident between these investigations all the authors agree that trypsin in sufficient amounts prevents coagulation,

and assume that the fibrinogen is destroyed. More recently, Tagnon, Davidson & Taylor [1942] have demonstrated the presence in chloroform-treated serum of a proteolytic enzyme which appears to be derived from the plasma globulins. This enzyme, like trypsin, prevents coagulation in large amounts, while in smaller amounts it hastens coagulation with subsequent digestion of the clot.

The work reported here is concerned mainly with the effect of pepsin on fibrinogen solutions and on plasma at pH values of 5.0–5.6. This limited pH range is necessary because at values below 5.0 fibrinogen tends to precipitate, and at values higher than 5.6 pepsin is inactivated.

The results indicate that under suitable conditions pepsin reacts immediately with fibrinogen, the product differing from fibrinogen in not being precipitable by 20% saturation with $(\text{NH}_4)_2\text{SO}_4$, or by half-saturation with NaCl. The fibrinogen modified by peptic action is referred to in this paper as 'non-precipitable fibrinogen' (i.e. not precipitable by low concentrations of salts), as compared with untreated fibrinogen, which is referred to as 'precipitable fibrinogen'.

METHODS

Plasma and serum. Horse plasma (oxalated and centrifuged) or serum was used, in most cases from horses which had not responded well to diphtheria immunization.

Solutions of fibrinogen. To 1000 ml. of plasma were added 300 ml. of sat. $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifuging, and dissolved in 1000 ml. of saline. The precipitation and solution were both repeated twice [cf. Warner, Brinkhous & Smith, 1935-6]. The final solution was adjusted to pH 5.4 by cautiously adding acetate buffer (pH 5.0) and was centrifuged before use.

'Chloroform serum' was prepared as described by Tagnon [1942].

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

pH values were determined by means of a glass electrode.

Estimation of salt-precipitable fibrinogen. Salt-precipitable fibrinogen in the plasmas and digest mixtures was assayed approximately by precipitation with 20% sat. $(\text{NH}_4)_2\text{SO}_4$, and collection of the precipitate by centrifugation. The precipitate was dried at room temperature by rolling on a No. 50 Whatman filter paper, was treated with ethanol for 5 min. and finally dried at 110° to constant weight. Though the presence of foreign material (e.g. $(\text{NH}_4)_2\text{SO}_4$) was unavoidable in the dried precipitate, the method in our hands proved sufficiently accurate for the purpose for which it is used in this work.

Estimation of heat-precipitable fibrinogen. Normal fibrinogen is precipitated by heating in solution to 56° for 2 min. For the estimation of heat-precipitable fibrinogen the precipitate was collected by centrifuging and dried as for salt-precipitable fibrinogen.

Temperature of enzyme-fibrinogen mixtures. Throughout this work the enzyme-fibrinogen mixtures were made up at room temperature (18°).

RESULTS

The effect of pepsin on plasma

In order to determine the amount of pepsin which would render non-precipitable the fibrinogen in a given quantity of plasma, both pepsin and plasma were adjusted to pH 5.4. To 10 ml. quantities of

plasma in test tubes was added a 1% pepsin solution, beginning with 1 ml. in the first tube and finishing with 10 ml. in the last. All volumes were adjusted to 20 ml. with water. 2 ml. quantities were removed from the tubes at intervals and these were tested for fibrinogen by the addition of 0.5 ml. of sat. $(\text{NH}_4)_2\text{SO}_4$ solution. This range usually included the end-point, giving a precipitate in the tubes with the smaller, but none in those with larger, amounts of pepsin. The end-point changes little after the first 5 min. during periods up to 24 hr. The results of a typical experiment are given in Table 1.

Table 1. *The reaction between plasma fibrinogen and 1% pepsin at pH 5.4 and 18°*

Time from beginning of exp. min.	ml. pepsin/10 ml. plasma				
	1	2	3	4	5
8	+++	+++	++	++	++
30	+++	+++	++	++	++
120	+++	+++	+	+	+
840	+++	++	+	+	+

	ml. pepsin/10 ml. plasma				
	6	7	8	9	10
8	+ -	+ -	-	-	-
30	-	-	-	-	-
120	-	-	-	-	-
840	-	-	-	-	-

The data indicate the precipitates obtained on the addition of $(\text{NH}_4)_2\text{SO}_4$ to 20% saturation.

+++ = heavy precipitate, ++ = moderate precipitate, + = light precipitate, + - = opalescence, - = no precipitate.

In order to render the tests quantitative, larger digest mixtures were prepared corresponding to selected tubes which, in the preliminary test, had precipitated with 20% sat. $(\text{NH}_4)_2\text{SO}_4$. Usually 10 or 20 times as much of each reagent was employed in the larger digest mixtures. 100 ml. of the digest were removed at 5 and 15 min. after mixing, and added to 25 ml. of sat. $(\text{NH}_4)_2\text{SO}_4$ solution. A control experiment was done with plasma and water only. The results obtained in a typical experiment are given in Table 2.

Table 2. *The effect of increasing amounts of pepsin on the amount of fibrinogen precipitable from plasma by 20% saturation with $(\text{NH}_4)_2\text{SO}_4$ at pH 5.4 and 18°*

ml. of 2% pepsin/200 ml. plasma	Fibrinogen precipitable from 100 ml. of digest mixture (g.)	
	After 5 min.	After 15 min.
0	0.2458	—
20	0.1498	0.1500
40	0.064	0.044
60	Nil	Nil

Vol. of each digest mixture was made up to 400 ml. with H_2O .

The effect of pepsin on solutions of fibrinogen

Solutions of fibrinogen were treated in a similar manner to the plasma. In a typical experiment tests showed that between 1 and 1.2 ml. of 0.2% pepsin prevented the precipitation, by $(\text{NH}_4)_2\text{SO}_4$, of the fibrinogen in 5 ml. of solution. Having determined these approximate values larger digest mixtures were prepared. After standing at room temperature for 10 min., the precipitable fibrinogen was estimated in 100 ml. of each mixture. The results are given in Table 3.

Table 3. *The effect of increasing amounts of pepsin on the amount of fibrinogen precipitable from fibrinogen solutions by 20% saturation with $(\text{NH}_4)_2\text{SO}_4$ at pH 5.4 and 18°*

ml. of 0.2% pepsin/150 ml. of fibrinogen solution	Fibrinogen precipitable from 100 ml. of digest mixture after 10 min. (g.)
0	0.2855
3	0.2210
9	0.1315
21	0.0285

Vol. of each digest mixture was made up to 210 ml. with H_2O .

The results in Tables 2 and 3 indicate that fibrinogen in plasma requires more enzyme per g. to render it non-precipitable than that in solutions of fibrinogen prepared by our method.

A comparison of the amount of fibrinogen precipitable from pepsin-treated plasma by $(\text{NH}_4)_2\text{SO}_4$ and by heat in the presence of active and alkali-inactivated enzyme

In order to furnish data as to the fate of the fibrinogen on pepsin treatment, the effect of heat precipitation of the fibrinogen in pepsin-treated plasma was compared with that of salt precipitation. Experiments were carried out at pH 5.4, at which pepsin is comparatively stable, and at pH 8.2, at which pepsin is inactivated [Northrop, 1930].

Three digest mixtures were prepared by adding to three 200 ml. quantities of plasma at pH 5.4 80, 120 and 160 ml. of 1% pepsin. The volumes of all the mixtures were adjusted to 400 ml. with water.

Estimation of the salt-precipitable fibrinogen.

(a) At pH 5.4. 100 ml. quantities of the above mixtures were removed and the salt-precipitable fibrinogen estimated.

(b) At pH 8.2. Only the mixture containing the greatest amount of pepsin, and therefore the least amount of salt-precipitable fibrinogen, was studied. 100 ml. of this were removed, its pH was adjusted to 8.2, and it was allowed to stand at room temperature for $3\frac{1}{2}$ hr., before estimation of the precipitable fibrinogen. This time interval was necessary since the non-precipitable fibrinogen was not immediately rendered precipitable on raising the pH to 8.2.

Estimation of heat-precipitable fibrinogen. (a) At pH 5.4. A 100 ml. quantity of the mixture containing the largest amount of pepsin was heated at 56° for 2 min.

(b) At pH 8.2. 100 ml. quantities of all three mixtures were adjusted to pH 8.2, and heated at 56° for 2 min.

The weights of the fibrinogen precipitates obtained under the conditions given above are shown in Table 4.

Table 4. *A comparison of the amounts of fibrinogen precipitable from digest mixtures by heat and by salt at pH 5.4, and at pH 8.2*

Volume of 1% pepsin solution/ 200 ml. plasma ml.	Precipitate with $(\text{NH}_4)_2\text{SO}_4$ from 100 ml. of mixture g.		Precipitate by heating at 56° for 2 min. from 100 ml. of mixture g.	
	At pH 5.4	At pH 8.2	At pH 5.4	At pH 8.2
160	0.0472	0.2292	0.2498	0.2968
120	0.1608	—	—	0.2928
80	0.2648	—	—	0.2838
0	0.2808	—	—	0.2872

The effect of pepsin treatment on the clotting of plasma

Reference has already been made to the work of other investigators on the effect of active trypsin on blood coagulation. The effect of a proteolytic enzyme on plasma coagulation in the presence of active enzyme, and also following inactivation of the enzyme by mild alkali treatment, has now been studied. To 35 ml. of plasma were added 45 ml. of 1% pepsin. The solution after 2 min. gave no precipitate in 20% sat. $(\text{NH}_4)_2\text{SO}_4$. The pH of the mixture was then raised to 8.2. 0.1 ml. of CaCl_2 solution (sp. gr. 1.2) was added to 10 ml. of the mixture, which gave a clot after incubation at 37° for 25 min. The addition of 5 ml. of normal horse serum to 10 ml. of the mixture to which CaCl_2 had been added, gave a clot in less than 5 min. at 37°.

Efforts to clot plasma at pH values of 5.0–5.6 in the presence of active pepsin have failed consistently, whereas after inactivation of the pepsin by alkali, clots have been obtained at pH values of 5.5, 6.2 and 7.2.

Experiments with trypsin, 'chloroform serum' and papain

The treatment of plasma with trypsin (B.D.H.) at pH 8.0 showed that at room temperature this enzyme behaved similarly to pepsin, but since this enzyme, unlike pepsin, is not easily inactivated, no effort has been made in this case to determine whether the change produced is similar to that produced by pepsin. Other workers [Eagle & Harris, 1937], however, have declared that the fibrinogen is

destroyed by an excess of this enzyme, though it may be that in the efforts to prove its presence by heating, true digestion was brought about. Experiments have shown that at room temperature the amount of fibrinogen not precipitable by 20% saturation with $(\text{NH}_4)_2\text{SO}_4$ is approximately proportional to the amount of trypsin added. The reaction proceeds rapidly, and little difference is noted in the weight of fibrinogen precipitable at 1, 15 and 30 min.

An experiment with 'chloroform serum' at pH 6.0 showed that between 2 and 3 ml. of this would prevent the precipitation of fibrinogen in 1 ml. of plasma, whereas a control experiment with untreated serum gave a heavy precipitate of fibrinogen at all levels.

The results with papain at pH 5.6 were similar to those with trypsin.

DISCUSSION

The results above show that pepsin and fibrinogen react at pH values of 5.0–5.6 in such a way as to render the fibrinogen non-precipitable at 20% saturation with $(\text{NH}_4)_2\text{SO}_4$. The amount of fibrinogen rendered non-precipitable is roughly proportional to the amount of enzyme added, and after the initial reaction the amount of fibrinogen modified by the enzyme is not appreciably increased during a subsequent period of 30 min. Inactivation of the pepsin by alkali restores to the fibrinogen the property of being precipitated by 20% sat. $(\text{NH}_4)_2\text{SO}_4$.

The alteration in the fibrinogen may be reversible, since, after alkali destruction of the enzyme, clots have been obtained with fibrinogen solutions which, in the presence of the active enzyme, had given no precipitate with 20% sat. $(\text{NH}_4)_2\text{SO}_4$, or with 50% sat. NaCl. No clot was obtained in pepsin-treated fibrinogen solutions unless the enzyme was first inactivated by alkali.

The conclusion drawn from these observations is that the enzyme combines with (or is adsorbed by) the fibrinogen, protecting it against precipitation at low salt concentrations, and against coagulation in plasma on the addition of Ca^{++} .

Solutions of partially purified fibrinogen require less pepsin per g. fibrinogen to produce this change than does plasma. Tagnon [1942] showed that more 'chloroform serum' was necessary to prevent the 'lysis' of fibrinogen in plasma than in purer fibrinogen solutions, and hence assumed some antilytic substance in plasma. It has been found in the present investigation that with many plasmas a certain amount of pepsin must be added before any fibrinogen is protected against precipitation, indicating that more than one substance must be competing for the enzyme. A comparison of graphs

obtained for three separate experiments with different plasmas, by plotting the amount of fibrinogen rendered non-precipitable by low concentrations of salts, under the influence of pepsin, against the amount of 1/3000 pepsin present, illustrates this point (Fig. 1).

Fig. 1. The influence of the presence of pepsin on the precipitability of fibrinogen in solution by low concentrations of salts. Curves 1–3 represent different experiments with three different preparations of horse plasma.

The extension of the above work to other proteolytic enzymes is of interest in view of the discovery of Gorter, Maaskant & Campagne [1936] that trypsin has an explosive-like action on the spreading of fibrinogen in monomolecular films. Other authors have observed that trypsin and 'chloroform serum' can inhibit the coagulation of plasma, and the present work shows that trypsin and an enzyme present in 'chloroform serum' can protect fibrinogen against precipitation by low concentrations of salts. This raises the question as to whether the reaction between these enzymes and fibrinogen is similar to that between pepsin and fibrinogen, and whether inactivation of these enzymes would restore the power of clotting to solutions of fibrinogen which had been rendered non-coagulable in the presence of the active enzyme.

SUMMARY

1. Pepsin reacts with fibrinogen at pH 5.0–5.6 in such a way that the fibrinogen is rendered non-precipitable with 20% sat. $(\text{NH}_4)_2\text{SO}_4$ and non-

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-