PROTHROMBIN AND PLASMA TRYPSIN

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It is now generally recognized that the coagulation of blood fibrinogen is brought about by thrombin produced from plasma prothrombin by the action of thrombo-plastin and calcium. Prothrombin is present in the blood stream in a soluble and inactive form. The exact nature of prothrombin has not been elucidated, but its preparations contain globulin protein which may be precipitated by dilution and acidification, or removed from plasma by colloidal adsorption. Like other plasma proteins, prothrombin is formed in the liver but with the help of vitamin K.

Mellanby[1] has prepared highly active prothrombin from beef blood and described its properties. It has been found to give well marked protein reactions. Being a protein in nature, it would be naturally of great interest to study the action of proteolytic enzymes on prothrombin. Mellanby however does not appear to have conducted any experiments to test the action of proteolytic enzymes, since he found that prothrombin is rapidly destroyed at 38° C. in concentrations of acid and alkali in which pepsin and trypsin act.

Pope[2] has demonstrated the action of pepsin and trypsin on plasma proteins at a pH, away from the optima of the action of these enzymes. The enzymic degradation of the proteins may not take place but there is a possibility of the disaggregation which can be demonstrated by a change in the properties such as critical denaturation temperatures, etc. In the case of prothrombin, since it is shown that its biological activity is very labile, any slight action on the prothrombin protein may be reflected in its biological activity. The biological activity referred to is its capacity to form thrombin, in the presence of calcium and thrombo-kinase.

Douglas and Colebrook[3] reported that blood coagulation was accelerated by the addition of trypsin. Wall-Schmidt-Leitz[4, 5] and his co-workers also reported that trypsin accelerated blood coagulation. They came to the conclusion that thrombin was a proteolytic enzyme either identical with, or related to, trypsin and that coagulation was due to the enzymic hydrolysis of fibrinogen to an insoluble modification. Tryspin is considered to have

accelerated coagulation in so far as it hastened this hydrolysis. Other proteolytic enzymes like papain were found to be active. Eagle and Harris[6] have made a detailed study of the action of trypsin on blood coagulation, in order to throw some light on the mechanism of physiological coagulation. Trypsin does not coagulate fibrinogen but apparently reacts with plasma prothrombin to form the physiological coagulant thrombin. Papain directly acts on fibrinogen to form an insoluble modification resembling fibrin. It appears therefore that trypsin acts as a thrombo-plastic enzyme in assisting blood-clotting. The possibility of trypsin being a thrombin can be discounted in view of the complete inability of the former to clot fibrinogen meticulously free from prothrombin (Ferguson[7]) even when calcium and cephalin are also added. The possible rôle of trypsin in bringing about the activation of prothrombin solutions has been brilliantly discussed by Ferguson.[8] Prothrombin solutions used in his experiments were found to contain 8 to 30 mg. per cent. of phospholipid. This amount of phospholipid if supplied in the form of added cephalin solution would be powerfully thrombo-plastic, while prothrombin solutions containing phospholipid in firm combination with proteins require the mediation of trypsin for the formation of thrombin. It is therefore concluded by Ferguson that trypsin splits off cephalin from its inert protein combinations and make it 'available' for the activation of prothrombin. In the words of Ferguson, the action of trypsin consists in the "mobilization of cephalin and calcium at the colloidal surface of the protein (prothrombin) substrate where the close juxta-position of the three components permit of the formation of thrombin *via* an intermediary prothrombin-calcium-cephalin complex or compound". According to this concept, the tryptic action is brought into line with the classical processes of thrombin formation. Schmitz[16] and Iyengar and Scott[11] have demonstrated the presence of trypsin in blood plasma. One can therefore consider plasma-trypsin as part of the normal physiological clotting mechanisms.

A study of the action of plasma-trypsin on prothrombin at pH 7·2, the physiological pH of blood, will therefore throw light on the formation of thrombin on the one hand and also on the inactivation of prothrombin on the other. The present work was undertaken to elucidate the rôle, if any, of plasma-trypsin, in the physiological clotting mechanism. Incidentally, it may also be possible to discuss in the light of the results obtained the mechanism of the synthesis of prothrombin.

The experiments reported in this paper can be broadly divided into two groups. In view of the thromboplastic activity of trypsin, the possibility of the trypsin in plasma acting as the physiological thrombo-plastin circulating

in blood can be visualised, and experiments have been designed to test this hypothesis. In order to test this hypothesis, the clotting times of oxalated plasma on mere recalcification in a large number of samples of blood, from different human subjects as well as from other species, have been determined. Simultaneously the trypsin content of each sample of plasma was also estimated. Secondly, the action of plasma-trypsin on the prothrombin present in plasma has been studied, to see whether any enzymic degradation of the prothrombin protein is a necessary adjunct to the physiological inactivation of prothrombin activity. Since prothrombin is rapidly destroyed at a temperature of 36° in a solution of pH 8·4, the optimum for trypsin, specific action of plasma-trypsin on the prothrombin protein could be evaluated, only if the enzyme action was allowed to take place at pH 7·2, a reaction at which prothrombin solutions are definitely known to be stable. The action of high-grade commercial trypsin on purified prothrombin solutions has also been studied.

Material and Methods

Preparation of purified prothrombin.—This was prepared according to Mellanby. 2 Litres of freshly bled ox blood is well shaken in a bottle containing 20 c.c. of 20% neutral potassium oxalate, and the plasma separated by high-speed centrifugalisation. The plasma is diluted with 10 vols. of distilled water and brought to pH 5·3 (the iso-electric point of prothrombin) by the addition of 1% acetic acid. The supernatant fluid is then poured off and the dilute suspension of globulin is then centrifuged. The precipitate is then resuspended in distilled water, equal to half the volume of the original plasma. Dilute calcium bicarbonate solution is now added to an equal volume of the globulin suspension, mixed by gentle shaking and allowed to stand for about 10 minutes. The suspension is rapidly filtered through a series of coarse filter-papers. The prothrombin is precipitated from the rest of the solution by the addition of 1% acetic acid until the pH is approximately 5·3. The precipitate is then centrifuged, and the mass is quickly dried by treatment with acetone.

Preparation of fibrinogen.—This was prepared according to Eagle and Harris. Repeated precipitations with 1·5 vols. of saturated NaCl yielded a satisfactory product. This failed to coagulate on the addition of Ca and tissue extracts but was promptly coagulated on the addition of thrombin. The final product was brought to a concentration of 0·9% with respect to NaCl by proper dilution.

Trypsin.—Digestive Ferments Company's trypsin 'Trypsin 1: 110' was used in the experiments in which the proteolytic action of purified prothrombin solutions was studied.

Estimation of prothrombin.—After many trials with Quick[9] and Fullerton[10] techniques Iyengar *et al.*[17] have evolved an improved and convenient method for the determination of prothrombin time. This consists in adding to 0·2 c.c. plasma maintained at 38° C., 0·2 c.c. of a solution of 1 in 20,000 Russel viper venom in 0·025*M* $CaCl_2$ solution. The interval between the addition of the calcium thrombo-plastin solution and the first appearance of the fibrin web is taken as the prothrombin time. The shorter this interval, the greater the prothrombin content.

Estimation of plasma trypsin.—2 c.c. plasma was precipitated with 8 c.c. acetone, and centrifuged. The precipitate was washed with acetone and allowed to dry for a few minutes. The whole precipitate was then mixed up, with 10 c.c. of phosphate buffer of pH 8·4, the optimum for plasma-trypsin (Iyengar[11] and Scott) in a glass pestle and mortar to get a uniform suspension. 3 c.c. of this suspension was pipetted to a test-tube containing 3 c.c. of 10% trichlor-acetic acid. The contents are well shaken and filtered. The nitrogen in 3 c.c. filtrate is estimated by the micro-Kjheldal method. Few drops of toluene are added to the remainder of the suspension and incubated at 37° C. for 48 hours. At the end of this period, 3 c.c. of this suspension is mixed with 3 c.c. of 10% trichlor-acetic acid, filtered and the nitrogen in 3 c.c. of the filtrate estimated. The increase in non-protein nitrogen is taken as a measure of tryptic activity of plasma. It is seen that by the above method, the free trypsin, in plasma, is precipitated by acetone along with plasma protein. If the solids are suspended in the buffer of pH 8·4, and incubated, the autodigestion of the protein takes place giving rise to an increase in non-protein nitrogen. Tryptic activity is expressed in terms of increase in N.P.N. for 100 c.c. of blood plasma.

Although the measurement of tryptic activity is carried out at pH 8·4, its action on prothrombin has been studied at pH 7·2 for the reasons enumerated in the early part of the paper. While we were engaged in a routine determination of prothrombin levels in plasma of a large number of cases, the clotting time of plasma on mere recalcification without the addition of thrombo-kinase, was noted in every case. It was found that this varied over a wide range while the prothrombin time itself with the addition of an optimum amount of thrombo-kinase, was remarkably constant with very slight variations. Can this variation be due to the varying amounts of trypsin present in blood plasma? This has been put to test and the results are given below:

Experimental

TABLE I

Relationship between 'Clotting time on mere recalcification of plasma' and the free trypsin content of plasma

Human subjects	Case No.	Prothrombin time	Clotting time on recalcification of plasma	Trypsin content as measured by increase in N.P.N. calculated for 100 c.c. plasma
Human subjects		secs.	secs.	mg.
,,	1	10	45	23·5
,,	2	10	42	22·8
,,	3	9	35	24·2
,,	4	8	42	25·8
,,	5	11	65	18·5
,,	6	10	60	19·2
,,	7	13	90	13·4
,,	8	9	65	18·9
,,	9	10	120	10·8
,,	10	8	45	23·5
Rabbit	1	7	35	28·5
,,	2	7	45	25·2
,,	3	8	65	21·4
Monkey	1	9	42	22·8
,,	2	8	45	21·4
Dog	1	9	60	18·3

It will be seen from the table that there is an inverse relationship between the clotting time on mere recalcification and the trypsin content of plasma. This can be noticed only when the clotting time is radically changed as for instance from 45 secs. to 60 secs. or to 90 secs. or to 120 secs. In these instances there is a significant drop in the trypsin content of plasma. Minor changes in clotting time as for instance from 45 to 42 or 35, are not reflected in the trypsin content. In fact, even the tendency may sometimes be in the opposite direction. Such minor changes can only be ascribed to experimental error in trypsin determination, as we are dealing with minute quantities of trypsin from only 2 c.c. plasma. The figures in the table are however magnified since they are calculated for 100 c.c. plasma.

The action of plasma-trypsin on the prothrombin also present in plasma was next studied. The institution of blood banks in many of the larger hospitals has made the clinician largely dependent on stored blood for transfusion. It is now becoming increasingly apparent that such blood is not equivalent in all respects to freshly drawn blood. The normal blood contains a great excess of prothrombin beyond the amount necessary for clotting. The work of Quick[12, 13] has proved a rational basis for the belief that transfusion may diminish certain hæmorrhagic tendencies, associated

with prothrombin deficiency. Prothrombin content of blood stored in the blood bank has been estimated by Rhoad and Panzer[14] at various intervals. Their results have clearly shown that, although the blood is stored at 4° C. in a sterile condition, the prothrombin content is gradually decreased. In a week or more, the blood would be practically useless in the treatment of the acute prothrombin deficiency. The cause of this spontaneous deterioration of prothrombin in blood is not known. Dilute solutions of purified prothrombin appeared to maintain their biological activity quantitatively when stored under identical conditions as in a blood bank. This has been indicated by Mellanby in his experiments on dialysis of purified prothrombin solutions, although the conditions of dialysis are not given. The complete stability of such purified prothrombin solutions kept at a temperature of 4° under sterile conditions, was confirmed by us. On account of the trypsin associated with prothrombin in plasma, the possibility of tryptic action on the prothrombin protein will have to be considered in this connection. It may be pointed out that trypsin in such low concentrations as is present in plasma and at such a low temperature as 7° C. and at a pH of 7·2, slightly removed from the optimum pH of trypsin, cannot be expected to have any action on the prothrombin protein. Pope has shown that even under conditions approaching to the above, a disaggregation of the plasma proteins takes place. Minor degrees of protein cleavage or perhaps mere intramolecular rearrangement may take place even under these conditions. Mellanby has shown that protein is an acid meta-protein or is associated with an acid meta-protein, upon which the preservation of its properties depends. Prothrombic activity is such a delicate property of the protein that any slight change brought about in the protein, may result in the deterioration of its potency. That the trypsin in plasma may be responsible for the reduction of prothrombin in stored blood, can therefore not be rejected summarily.

Prothrombin time of plasma was first determined immediately after the blood was taken. The plasma was then kept in the frigidaire at a temperature of 7° C. and the prothrombin time determined after different intervals. Simultaneously with the prothrombin determination non-protein nitrogen in the plasma was estimated in order to see whether destruction of prothrombin is associated with an increase in N.P.N. as a result of tryptic action. In some cases, the plasma was incubated at 37° C. and the prothrombin destruction and changes in N.P.N. were followed at shorter intervals. In order to ascertain if the rate of prothrombin destruction has any relationship with the trypsin content of plasma, the trypsin was estimated in plasma by the method given above, after autodigestion for 48 hours. The results are given below.

TABLE II (*a*)

Effect of incubation of plasma at 7° C. and 30° C. for different periods, on 'prothrombin time' and corelation between changes in 'prothrombin time', increase in N.P.N. and the free trypsin content of plasma

Case No.	Prothrombin time immediately	Prothrombin time after different intervals. Plasma kept at 7° C.			Increase in N.P.N			Trypsin in 100 c.c. plasma
		1	2	3	1	2	3	
	secs.	secs.	days	secs.				mg.
1	9	10	15	18	nil	nil	nil	23·2
2	8	11	17	25	nil	nil	nil	26·2
3	12	12	15	18	nil	nil	nil	18·5
4	14	14	19	22	nil	nil	nil	17·2
5	13	18	25	42	nil	nil	nil	38·4

TABLE II (*b*)

Case No.	Prothrombin time immediately	Prothrombin time after different intervals. Plasma incubated at 30° C.			Increase in N.P.N			Trypsin in 100 c.c. plasma
		1	2	3	1	2	3	
	secs.	hrs. 6	hrs. 24	hrs. 48	hrs. 6	hrs. 24	hrs. 48	mgm.
		secs.	secs.	secs.				
1	10	13	25	40	nil	·37	·51	18·9
2	11	14	28	35	nil	·42	·62	22·5
3	8	10	19	did not clot for 4 minutes	nil	·58	·75	31·2

The results in Table II clearly show that plasma-trypsin slowly inactivates the associated prothrombin even when kept at 7° C. There is no increase in non-protein nitrogen even after a period of 3 days, which indicates that the cleavage of the prothrombin protein has not taken place. Since prothrombin activity has been affected, it should be assumed, that some change of the prothrombin protein has been brought about by the trypsin, which cannot be detected by the ordinary chemical methods employed in studying enzymic degradations of proteins. The biological activity of prothrombin serves in this case as an excellent method of detecting even the most superficial change involving perhaps intramolecular rearrangement of the protein. When the plasma is incubated at 30° C. the prothrombin destruction is considerably accelerated. This is as should be expected since the tryptic activity is enhanced by the higher temperature. At the end of 24 hours, and 48 hours, there is

a definite increase in N.P.N. also and the quantity of prothrombin destroyed is also greater. There is also a very rough relationship between the rate of destruction of prothrombin and the trypsin content of plasma.

In case (3) Table II (*b*) if purified prothrombin was added to the plasma after incubation for 48 hours, the prothrombin time was immediately restored. This clearly shows that in these experiments, it is the prothrombin that has been affected and not any other clotting factor like fibrinogen.

Finally the action of commercial trypsin on purified prothrombin protein has been studied.

200 mg. was dissolved in 250 c.c. water and brought to pH 7·2 by the gradual addition of a dilute solution of sodium carbonate and the volume was made up to 50 c.c. To 25 c.c. of this solution was added 5 c.c. of 10% solution of trypsin and incubated at 37° C. The N.P.N. was determined immediately and at definite intervals. The prothrombin time of this solution was also determined both immediately and after stated intervals by diluting 1 c.c. of the digest to 100 c.c. with 0·9% saline, and activating 0·2 c.c. of this with 0·2 c.c. of a solution of 1 in 20,000 Russel viper venom in 0·025 M $CaCl_2$ and then adding 0·2 c.c. of a solution of fibrinogen.

TABLE III

Action of commercial trypsin on purified prothrombin

Time allowed	Prothrombin time	Increase in N.P.N. in the digest
	secs.	mg.
0	10	..
15 minutes	16	..
30 ,,	25	1·8
45 ,,	40	2·4
1 hr.	62	3·5
2 hrs.	Did not clot in 5 minutes	5·2

The results reported in Table III confirm the conclusion that the prothrobmin property of the protein is so labile that even slight intramolecular rearrangement can disturb the activity.

Summary and Conclusion

The work of Northrop, and Kunitz,[15] Eagle and Harris, and Ferguson, has elucidated the rôle of trypsin in blood coagulation. Experimental evidence has been obtained to show that the trypsin in plasma may be the physiological thrombo-plastin. The thrombo-plastic action of trypsin depends on the amount present in plasma. Since the trypsin content of

plasma is very low, it is definitely below the optimum amount required to have the maximum effect. Being present in sub-optimal amounts, the clotting time on mere recalcification is found to vary with the trypsin content. The prothrombin time is constant irrespective of the plasma trypsin content, since during the determination of this factor, the optimum amount of thrombo-plastin is added in the shape of Russel viper venom. Trypsin is reported to have (Eagle and Harris) no direct coagulative action on purified fibrinogen. The coagulating action of trypsin was found to rest on the fact that it reacts with prothrombin to form thrombin. This thromboplastic action of trypsin is observed only within a comparatively narrow optimum zone of trypsin concentration. The trypsin in plasma is so small in quantity that it is highly probable that it is sub-optimal so far as its thrombo-plastic action is concerned. Hence with varying concentrations of trypsin in plasma in the sub-optimal range, the clotting time of recalcified plasma is found to vary.

The experiments on the incubation of plasma under sterile conditions at a temperature of 0° C. and 30° C. show that the gradual destruction of prothrombin activity takes place. In the early stages, the destruction is not accompanied by any increase in N.P.N. which is the earliest index of protein cleavage. These results are suggestive of the possibility that mere intramolecular changes of the prothrombin protein or mere disaggregation of the protein is enough to bring about a loss in prothrombin activity. This is confirmed by experiments on the tryptic action of purified prothrombin solutions.

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