ANTI TRYPTIC COMPONENTS OF BLOOD

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It is well known that the blood serum has the property of retarding the digestive activity of trypsin. Landsteiner[1] reported that this effect was associated with the albumin fraction of serum. Hedin[2, 3] found many similarities between the inhibition of trypsin by adsorption on charcoal and inhibition of trypsin by serum albumin and therefore came to the conclusion that the equilibrium between trypsin and serum albumin was governed by adsorption phenomena, *i.e.*, the trypsin is adsorbed by the serum albumin as it is by the charcoal. The correctness of this interpretation is questioned by Hussey and Northrop[4] after a detailed study of the mechanism. They have adduced evidence which suggests that the inhibitive agent in serum combines with trypsin to form an inactive but dissociable compound. The conditions of equilibrium are apparently goverend by the law of Mass-Action.

It has been shown by Schmitz[5] that the enzyme trypsin present in plasma is blocked by an inhibition body, whose action is in no way related to the anti-tryptic action of serum albumins. This inhibitor could be separated from the serum albumins by precipitation of proteins with trichlor-acetic acid whereby the inhibitor remains in solution. This can also be prepared by ultra-filtration of an acid solution of plasma proteins obtained by acetone precipitation. By this process, the inhibitor passes into the albumin-free filtrate. This inhibitor is thus strongly analogous to the trypsin-inhibitor obtained by Kunitz and Northrop[6] from the pancreas. The reaction between Schmitz plasma-inhibitor and crystalline trypsin shows that it runs quantitatively as in the case of Northrop-inhibitor from the pancreas. The one important difference between the two inhibitors is that plasma-inhibitor does not inactivate chymo-trypsin from the pancreas as opposed to the pancreas-inhibitor which reacts with chymo-trypsin bringing about a gradual fall in activity. The inhibitor associated with the serum albumin also inactivates chymo-trypsin and hence is similar only in this property to the inhibitor from pancreas. The inhibitor present in native serum is however differentiated from either the inhibitor from pancreas or the inhibitor isolated from plasma by Schmitz. According to Hedin[7] the inhibition of trypsin appears only when ferment and serum are mixed first and after an interval the substrate is added. On the other hand, the other two inhibitors

act only if they are added to the ferment substrate mixture when the digestion is in progress. The destruction of trypsin by serum (albumin) is therefore sharply differentiated from the inhibition by the substance isolated from blood plasma. In blood plasma, there are therefore two inhibition mechanisms side by side, of which one is probably (according to Schmitz) by unspecific adsorption of the ferment on serum albumin and difficulty of elution and the other by a quantitative combination with the ferment specifically. In order to detect the presence of the second inhibitor, either free inhibitor should be present in plasma or it should be separated from the trypsininhibitor compound.

During the course of a search for a simple substance which might retard the activity of specific proteases, it was discovered by Horwitt[8] that heparin, the physiological anticoagulant is a trypsin-inhibitor. The effects of heparin on the hydrolysis of casein by crystalline trypsin and chymo-trypsin isolated by beef pancreas, have been studied. No inhibition of trypsin is obtained unless the heparin is allowed to remain in contact with the alkaline trypsin solution for about 30 minutes before the two are added to casein. This inhibition of trypsin by heparin has many similarities with the mechanism of inhibition of trypsin by the inhibitor isolated from plasma. The heparin-trypsin addition complex like the trypsin-inhibitor compound may be dissociated by acidifying to pH 3 for about 30 minutes to recover all the tryptic activity. Like the plasma-inhibitor, heparin does not inhibit chymotrypsin. This property of heparin is very interesting since trypsin catalyses the clotting of recalcified plasma while chymotrypsin does not. It has been reported by Horwitt that trypsin and heparin are mutually antagonistic in a sample of plasma and that clotting will not occur unless the amount of trypsin added is more than enough to neutralise the effect of heparin.

It is evident from the foregoing account that there are present in blood, three different substances having the property of retarding the activity of trypsin. The presence of these substances acquires an added significance in the light of my (Iyengar, N. K.)[9] observations on the presence of trypsin in plasma and its possible role as the physiological thrombo-plastic substance.

The part played by trypsin in blood in the inactivation of insulin has been discussed by Iyengar and Scott.[10] In view of this dual role of trypsin in blood, the action of the anti-tryptic components of blood in retarding coagulation or in prolonging the hypoglyceimic effect of insulin is worth investigating. The object of this paper is to study first of all the *in vitro* effect of these trypsin-inhibitors on the action of trypsin in (i) catalysing the coagulation of blood and (ii) in the destruction of insulin. The action of heparin as a general anti-coagulant is well known and therefore need not

be considered. The action of heparin in checking the destruction of insulin by trypsin has not been reported, although the possibility is indicated by Horwitt (*Science*, 1940). Long before Horwitt's work was published, while I was working in the Connaught Laboratories on the destruction of insulin in blood, the way in which heparin would behave in a reaction between trypsin and Insulin, attracted my interest, firstly, because the work on heparin was going on in the laboratory and secondly because Ferguson[11] has shown that different amounts of heparin can inhibit clotting of citrated dog plasma by crystalline trypsin under varying conditions of calcium and cephalin mobilization. A study of the action of the Schmitz-inhibitor on the coagulation of blood plasma by trypsin, would be exceedingly interesting. The effect of adding this inhibitor to a mixture of insulin and trypsin on the course of the destruction of the hormone has also been studied.

Materials and Methods

Preparation of the trypsin-inhibitor from plasma (according to Schmitz).—5 Litres of oxalated blood after keeping overnight in a refrigerator are mixed with 15 litres of 0·25 NH_2SO_4 to dissociate ferment inhibitor compound. The dark coloured solution is kept overnight on ice. 242 gm. of $(NH_4)_2SO_4$ per litre (40% saturation) are then added. The total albumins are precipitated. The precipitate is filtered and thrown away. To this yellow coloured filtrate, 205 gm. of $(NH_4)_2SO_4$ per litre are added. When this is kept for a few days in an ice-chest a fine flocculent precipitate separates. It is filtered under suction on a hardened filter-paper. The precipitate which is very small in quantity is dissolved in a few c.c. water (6 c.c.), the turbid solution is mixed with 4 c.c. saturated $(NH_4)_2SO_4$ solution and filtered. The filtrate is salted out by the addition of 2 gm. of $(NH_4)_2SO_4$ per 10 c.c. The white precipitate is filtered by suction on small hardened filer-paper. The precipitate is dissolved in 5 c.c. water and the solution is mixed with equal volume of 5% trichlor-acetic acid. This is kept for 30 minutes and the precipitate formed during this period is filtered and thrown away. The clear filtrate is brought to pH 3·0 by the addition of a few drops of 5 N NaOH. The inhibition body is then salted out by the addition of 5·6 gm. ammonium sulphate per 10 c.c. The precipitate is filtered through a hardened filter-paper. A fairly good quantity of the inhibitor (about 100 mg.) was obtained by undertaking the preparation in four different batches.

Method of insulin assay employed.—The mouse convulsion method was employed. Since we were concerned only with comparative effects, a large number of animals were not considered necessary for the test. On an average, 50 mice were used, 25 for test solution and 25 for standard.

Method of study of the prolongation of hypoglyceimic effect.—These tests were made by studying the effect of the test solution on the blood sugar of rabbits and comparing with the blood sugar reducing effect of a similar dose of standard insulin at the end of the same period. The mean blood sugar figure for each hour is calculated both for rabbits injected with the standard and for rabbits injected with test solution. Each figure is then converted to a percentage of the mean initial figure. The point at which the blood sugar has returned to normal in the case of rabbits injected with standard insulin is taken. At this point the blood sugar of rabbits receiving the test solution is noted. The percentage of this value to the initial blood sugar gives an idea of the prolongation of the hypoglyceimic effect when compared with standard insulin.

The effect of the addition of the inhibitor isolated from plasma, on the coagulation of citrated plasma by trypsin, has been studied first. In order to be able to study this, it is imperative to determine the optimum concentration of trypsin required to bring about coagulation in the shortest time. The importance of this has been pointed out by Eagle and Harris[12] who have determined such an optimum concentration using Digestive Ferment Company's trypsin 1 : 110 and in the absence of calcium. Ferguson and Erickson[13] have shown that the enzyme trypsin is much more potent in the presence of ionised calcium. As it was intended to study the effect of the trypsin-inhibitor on the coagulation of citrated plasma by tryspin in the presence of calcium, it was considered of interest to find out the optimum concentration of the trypsin employed, (B.D.H.) that is necessary to bring about coagulation of citrated plasma in the presence of calcium. Accordingly the following experiments were undertaken :—

TABLE I

Coagulation time of 1 *c.c. plasma—varying quantities of trypsin solution (B.D.H. trypsin* 10% *in salt solution)*

Quantity citrated plasma	Quantity of $CaCl_2N/10$	Quantity of trypsin solution	Clotting time
c.c.	c.c.	c.c.	secs.
1	0·25	0·75	32
1	0·25	0·60	19
1	0·25	0·50	35
1	0·25	0·40	80
1	0·25	0·30	95

The total volume was made up to 2 c.c. in each case.

It is evident from the above table that 0·6 c.c. of a 10% solution of the trypsin used, coagulates 1 c.c. citrated plasma in the shortest interval of 19 seconds in the presence of $CaCl_2$. Having thus determined the optimum amount of trypsin, we proceeded to study the effect of the Schmitz-inhibitor, isolated from plasma on the above process.

TABLE II

B.D.H. Trypsin—10% solution in 0·85% NaCl. 5 mg. of the trypsin-inhibitor dissolved in 10 c.c. of normal saline

Citrate plasma	$CaCl_2$N/10	Trypsin	Inhibitor solution	Clotting time
c.c.	c.c.	c.c.	c.c.	secs.
1	0·25	0·6	..	22
1	0·25	0·6	0·3	35
1	0·25	0·6	0·5	45
1	0·25	0·6	0·65	48
1	0·25	..	..	90

The volume was made up in each case to 2·5 c.c.

In the above experiments, the solutions were mixed in the following order: Plasma—Trypsin—Inhibitor—Calcium.

There was thus no interval allowed for the combination of trypsin and the inhibitor. The inhibition brought about in the above experiments does not appear to be complete. The maximum inhibition that has taken place (column 4) is still far from complete as can be seen from the blank experiment carried out with neither trypsin nor inhibitor. As it is known that the inhibition of the trypsin brought about by this inhibitor is not instantaneous, and a certain amount of time is required to effectively block the enzyme, it was decided to incubate the enzyme solution and the inhibitor solution for a period of 1 hour at 30° C. This incubated mixture was used in the following experiments.

TABLE III

Plasma	$CaCl_2$N/10	Trypsin+ Inhibitor solution (*a*) (5 c.c. Trypsin + 5 c.c. Inhibitor sol. containing 2·5 mg. inhibitor)	Trypsin (10% Sol.)	Clotting time
c.c.	c.c.	c.c.	c.c.	secs.
1	0·25	..	..	95
1	0·25	..	0·6	22
1	0·25	1·2	..	70
		Trypsin+Inhibitor solution (*b*)	(5 c.c. Trypsin+5 c.c. Inhibitor sol. containing 5 mg.)	
1	0·25	1·2	..	82
		Trypsin+Inhibitor solution (*c*)	(5 c.c. Trypsin+5 c.c. Inhibitor sol. containing 10 mg.)	
1	0·25	1·2	..	85

When the inhibitor is incubated with trypsin before use, the blocking of the effect of trypsin on coagulation is more effective. The amount of the inhibitor required to check the trypsin completely can be calculated from solution (*b*). 0·6 mg. will prevent practically completely, 0·6 c.c. of 10% B.D.H. trypsin from exerting its catalysing effect on the coagulation of citrated plasma. It is therefore clear that the plasma-inhibitor has got the property of inhibiting the coagulation action of trypsin. Ferguson[11] has reported that heparin can inhibit clotting of citrated dog plasma by crystalline trypsin. The plasma-inhibitor can therefore be considered analogous to heparin in this respect.

The close analogy of thrombo-plastin and trypsin has been observed by Ferguson and fresh evidence has been adduced in support of this by Iyengar in his work on plasma-trypsin and prothrombin. In view of this similarity of the behaviour of thromo-plastin and trypsin in the process of coagulation, and in the light of the above results, the question whether the plasma-inhibitor can inhibit the thrombo-plastic action of the Russel viper venom, suggested itself to the author.

The following experiments were carried out to test this possibility: -

TABLE IV

Oxalated plasma	Russel Viper Venom	$CaCl_2$ N/40	Clotting time
c.c.		c.c.	secs.
0·2	1 in 20,000 Russel Viper Venom 1 in 20,000 *plus* inhibitor (5 c.c. Venom sol. *plus* 2 mg. inhibitor) kept for one hour at 25° C.	0·2	14
0·2	0·2 c.c.	0·2	15
0·2	nil	0·2	80

It is evident from the above table that the plasma-inhibitor cannot prevent the thrombo-plastic activity of Russel viper venom. In this respect, the similarity between trypsin and thrombo-plastin breaks down.

The destruction of insulin by blood has been investigated by a large number of workers (Schmidtz,[14] Karelitz,[15] Fraudenberg[16] and Black[17]). The destructive principle has been shown to be a proteolytic enzyme of a tryptic nature (Iyengar and Scott[10]). The presence of the anti-tryptic agents in blood assumes an added significance in the light of this property of blood. Advantage of the anti-tryptic effect of blood serum has been taken by Murlin and Hawley[18] to protect the insulin from destruction by trypsin and they claim that the hormone can be absorbed from alimentary tract of depancretized dogs. Harned and Nash[19] have reported that when

insulin is mixed with anti-trypsin prepared from the round worm of swine (*Ascaris lumbricoides*), and given by stomach tube, it causes a marked decrease in sugar output. These authors have also been able to demonstrate that the physiological activity of insulin can be protected from destruction by trypsin, if the enzyme has been previously incubated with this anti-trypsin.

The possible prolongation of insulin action by inhibitors of proteolytic activity has not been studied by any of the above workers, but is merely mentioned by Horwitt in his work on heparin as an anti-tryptic agent. Since the inhibitor we are working with occurs in blood, it would be exceedngly interesting to study the (1) *in vitro* effect of a mixture of trypsin and inhibitor on the course of the destruction of insulin, and (2) the *in vivo* effect of injecting into rabbits a mixture of insulin and the inhibitor and observe the prolongation if any, of the hypoglycæmic effect as compared with standard insulin of an identical dosage. The following experiments have therefore been conducted.

TABLE V

Inhibition of the tryptic digestion of Insulin by plasma-inhibitor
The solutions were incubated for 2 hrs. at 38° C.
The pH of the solutions were maintained at pH 8 by the addition of Phosphate buffer

	Increase in N.P.N mg.	Destruction of Insulin Protein %	Destruction of Insulin Activity %
Trypsin + Insulin (5 mg. or 110 units)	0·32	40	90
Trypsin + Inhibitor (3 mg.) incubated for 1 hr. and then Insulin (5 mg.) added ..	0·08	10	40

The influence of the inhibitor on the course of the destruction of physiological activity of insulin and also on the digestion of the insulin protein has been studied in the above table. The digestion of the insulin protein is only 10% in the presence of the inhibitor whereas 40% digestion takes place if the trypsin is not previously incubated with the inhibitor. While there is a definite inhibition of the tryptic destruction of the physiological activity of insulin, the inhibitor has not been able to prevent it completely. It is generally recognised that a large part of the activity of insulin is destroyed during the early stages of digestion of insulin protein. The fact that 40% of the activity is destroyed even in the presence of the inhibitor shows that the plasma-inhibitor is not capable of blocking the earliest stages of tryptic action. Nevertheless the results obtained clearly show that the inhibitor checks the tryptic digestion of insulin protein.

Having thus established that the plasma-inhibitor can partially block the tryptic destruction of insulin, the possibility of the practical applications of this finding was next investigated. If the hypoglycæmic effect of insulin could be prolonged by the simultaneous injection of the inhibitor, it would be of great practical utility in the treatment of diabetic subjects. The amount of the inhibitor circulating in the blood is not enough to block all the trypsin present in blood. In addition the trypsin present in platelets (Iyengar and Scott) is released into the blood. If therefore an additional amount of the inhibitor is administered with insulin, the hormone may not all be destroyed so quickly as it normally happens. The method of study of the prolongation of the hypoglycæmic effect has already been described.

TABLE VI

Effect of Plasma-inhibitor in the prolongation of the Hypoglycæmic effect of Insulin

Expt. No.		Blood sugar 6 hrs. after injection expressed as percentage of the original blood sugar level
1	1 Unit Insulin injected	100
2	1 „ „ „	90
3	1 „ „ „	94
4	1 „ „ + 2 mgs. Inhibitor ..	95
5	1 „ „ + 4 „ „ ..	100
6	1 „ „ + 5 „ „ ..	90

There is practically no prolongation of the hypolgycæmic effect since in all cases with varying amounts of the inhibitor, the blood sugar has been restored to the original level within a period of 6 hours after the injection of the mixture.

Similar experiments were conducted using heparin in place of the inhibitor, in view of similarity of its behaviour towards trypsin.

TABLE VII

Inhibition of tryptic d'gestion of Insulin by Heparin

Solutions were incubated for 2 hrs. at 37° C.	Increase in N.P.N.	Percentage of destruction of Insulin Protein	Approximate percentage of the destruction of physiological activity
	mg.		
Trypsin + Insulin (5 mg.)	0·30	36	90
Trypsin + Heparin (3 mg.) incubated for 1 hr. at 37° C. and then Insulin (5 mg.) added	0·18	22	60

TABLE VIII

Study of the effect of Heparin in the prolongation of the hypoglycæmic effect of Insulin

Expt. No.		Blood sugar 6 hrs. after injection expressed as percentage of the original blood sugar level
1	1 Unit Insulin	100
2	1 ,, ,, ...	90
3	1 ,, ,, ...	94
4	1 ,, ,, +2 mg. Heparin ..	90
5	1 ,, ,, +4 ,, ,, ..	95
6	1 ,, ,, +6 ,, ,, ..	92

Heparin is also not able to prolong the hypoglycæmic effect of insulin. Although the trypsin-inhibitors are able to check the *in vitro* destruction of insulin by trypsin, they are practically useless in slowing down the process of physiological destruction of insulin in the body. This is probably due to the fact that the trypsin present in blood or generated in the blood comes into contact with the insulin first and the destructive process starts before the enzyme can combine with the added inhibitor to form the inactive trypsin-inhibitor compound. If the substrate and the inhibitor are allowed to come into contact with the enzyme simultaneously, the competition between the two for affinity with the enzyme comes into play. In this process of competition, the substrate gets the upper hand. If on the other hand the inhibitor alone is added to the tryspin and incubated for some time an inactive trypsin-inhibitor compound is formed.

Crafford and Jorpes[20] observed that a larger doze of heparin is rendered inactive in the blood shortly after a surgical operation than is the case with the same patient before the operation. This is regarded by them as a clear expression of the tendency to the formation of clots which cause thrombo-embolic complications post-operatively.

The observation that trypsin and heparin are mutually antagonistic and that heparin inactivates trypsin both in its digesting and coagulating action, can be linked up with the finding of Crafford and Jorpes. So far the only known atiheparin agents present in the blood are prothrombin and trypsin. During the early stages of the post-operative period prothrombin is known to decrease if at all any change takes place in the prothrombin content of blood. The changes in the trypsin content of plasma have not been studied. The following table gives the tryspin content of plasma both immediately before and after the operation.

TABLE IX

Nature of operation	Plasma-trypsin before operation expressed as increase in N.P.N. for 100 c.c. plasma	After operation
Obstructive Jaundice	mg. 22·5 18·4 24·8	mg 35·8 31·9 41·2

There is a significant increase in plasma-trypsin immediately after an operation. The increased inactivation of heparin under this condition may reasonably be ascribed to the trypsin content of plasma.

Summary and Conclusions

A review of the anti-tryptic components of blood has been made. There are present in blood three different substances capable of inactivating trypsin. There are (1) A factor in serum associated with the albumins. (2) An inhibitor isolated from plasma which according to Schmitz is a polypeptoid of low molecular weight. (3) Heparin, the physiological anti-coagulant.

The *in vitro* effect of the plasma-inhibitor on the action of trypsin in catalysing the coagulation of blood has been studied in detail. This substance has been found to inhibit this property of trypsin also.

The course of tryptic digestion of insulin in the presence of this inhibitor and heparin has been studied both by following the increase in N.P.N. and also the physiological activity of insulin. The digestion of the insu in protein is not completely checked although a definite inhibition is observed. Since the digestion of insulin takes place even in presence of the inhibitor, or heparin, quite a considerable amount of physiological activity of insulin is destroyed by trypsin even in the presence of the inhibitor, or heparin. This destruction is however very much less than the inactivation of insulin by trypsin under identical conditions but without inhibitor or heparin. The plausible reasons for this observation have been enumerated.

In view of the close analogy between trypsin and thrombo-plastin in their behaviour towards the process of coagulation, the action of the plasma-inhibitor on the thrombo-plastic activity of Russel Viper Venom was studied. The thrombo-plastin of the venom remains quite active even after incubation with the inhibitor. In this respect therefore the analogy between trypsin and thrombo-plastin breaks down.

Attempts have been made to ascertain whether prolongation of the hypoglycæmic effect of insulin can be obtained by injecting a mixture of insulin with the plasma-inhibitor or heparin. A large number of experiments have been carried out to test this important practical application. The results obtained are not very encouraging and there is practically no prolongation of the effect either with the inhibitor or with heparin.

The trypsin content of plasma have been estimated both before and after operation in a number of cases. A significant increase in the enzyme content of the plasma has been observed immediately after operation. It is suggested that this high trypsin content might be responsible for the increased inactivation of administered heparin, observed by Crafford and Jorpes.

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