TRYPSIN-KINASE IN BLOOD

By N. K. Iyengar, M.Sc.

(From the Biochemical Standardization Laboratory, Government of India, Calcutta)

Received January 21, 1942

(Communicated by Prof. G. Sankaran)

Schmitz[1] has isolated a trypsin-inhibitor from beef blood and has shown that it is probably a polypeptide of low molecular weight. This inhibitor exists in combination with trypsin in the circulating blood, thus inactivating most of the enzyme. The author makes the statement that this trypsin-inhibitor is present in blood in excess of the amount required for the complete inactivation of trypsin present in blood. If this were the case, the acetone precipitate of the plasma should not possess any proteolytic activity. Iyengar[2] and Scott have reported a definite, though small, tryptic activity in the plasma proteins obtained by precipitation with acetone. This however does not disprove the existence of the inhibitor, the presence of which has been substantially confirmed by us in our experiments on the proteolytic activity of various blood fractions. Trypsin circulating in plasma is only partially neutralised by the inhibitor and a small portion of the trypsin is left free to exert its activity. The significance of this free plasma-trypsin in physiological and pathological conditions, is under investigation.

The proteolytic system in blood has been the subject of extensive investigations by Schmitz, who has experimentally demonstrated a combination of trypsin with inhibitor to form a trypsin-inhibitor compound which as such is inert. The enzyme is liberated in an active form by treatment with trichlor-acetic acid bringing the pH of the plasma to 3 and breaking the compound into its constituents. If the plasma-trypsin is to play a role in the physiological processes, there must be a mechanism in the body to bring about such a breakdown of the trypsin-inhibitor compound. The pH of the blood *in vivo* is at no time as low as 3, and hence the mechanism of activation of plasma-trypsin *in vivo* if present must be other than by acidification. Schmitz envisages the possibility of the presence in blood of a ' kinase ' similar to the trypsin-kinase present in the intestinal secretions bringing about the activation of trypsinogen of the pancreas. No direct evidence has been adduced to support the above hypothesis. According to this author,

the 'kinase' also combines with the trypsin-inhibitor present in the blood, and when present in such a combination, it is not capable of liberating the trypsin from the trypsin-inhibitor compound. Summing up the above observations, it is seen that there is present in plasma a trypsin-inhibitor compound and a kinase-inhibitor compound, both of which are unable to exert their respective actions. It is presumed that the inhibitor is the same chemically in both the compounds.

Pope[3] during the course of his work on the purification and concentration of diptheria anti-toxin, has detected the presence of a proteolytic enzyme in fibrin clots. Since this enzyme lyses the fibrin, Pope named it fibrinolysin. The enzyme fibrinolysin is also reported to be present in the culture medium in which *Streptococcus* is grown. Judging from the properties of this enzyme, I am of opinion that the nomenclature, fibrinolysin, can be employed only to the enzyme which digests specifically the fibrin protein, and does not attack other proteins. The proteolytic enzyme present in the fibrin clot appears to resemble trypsin in its pH optimum and in its action on other proteins. Trypsin, it may be stated, can also digest the fibrin clot. I am of opinion, therefore, that the so-called fibrinolysin reported by Pope is nothing but plasma-trypsin in its free and active state.

Schmitz has put forward a very interesting hypothesis to explain the presence of trypsin in the fibrin clot.

According to Schmitz, during clotting, the trypsin-inhibitor compound is adsorbed on the fibrin clot. The kinase-inhibitor compound which is also present in plasma, is broken up into its constituents with the result that only kinase which is probably of a protein nature gets adsorbed on the clot while the inhibitor which is a polypeptide of low molecular weight remains in the serum. The components of the proteolytic system present in the fibrin clot are: (i) trypsin-inhibitor compound and (ii) trypsin-kinase. Under these circumstances, it is reasonable to visualise an interaction of the two components if the fibrin is suspended in a buffer suitable for kinase action. This reaction can be represented by the following equation:

$$\text{Trypsin-inhibitor compound} + \text{Kinase} \leftrightharpoons \text{trypsin} + \text{Kinase-inhibitor.}$$

It can be seen from this equation that trypsin is liberated in an active form during the process of clotting by the mechanism described above. This fascinating possibility is based on the assumption that the trypsin-kinase is present in the blood, for which no direct evidence has been presented as yet. As I was engaged in a fairly detailed study of the proteolytic system present in the whole blood, it occurred to me that it might be worth while to

investigate the various blood fractions for the presence of this kinase. In pursuance of this idea, the following constituents of blood have been examined :

(i) Red Blood Corpuscles.

(ii) Platelets.

Experimental

(1) Red blood cells from horse were laked with distilled water and precipitated with 4 volumes of acetone at a temperature of 10° C., centrifuged and washed with acetone.

(2) Citrated horse blood was kept in the refrigerator and red blood cells were allowed to settle. The plasma was then taken off and centrifuged at the rate of 3,000 revolutions per minute. The sediment was taken in a jar and allowed to settle in the refrigerator. The supernatant was siphoned off and the sediment resuspended in citrates; this suspension was precipitated with 4 volumes of acetone in the cold, centrifuged and the precipitate again washed with acetone. The precipitate was finally dried at room temperature. This is the platelet preparation.

(3) *Trypsin-inhibitor Compound.*—This is present in plasma as a protein and hence is precipitated by acetone. 100 c.c. of citrated plasma were precipitated by the addition of 400 c.c. of acetone. The precipitate was first washed with aqueous 90% acetone, then with pure acetone and finally dried at room temperature. This preparation has been shown by Iyengar and Scott, to possess slight proteolytic activity.

(4) *Trypsin from Plasma freed from the Inhibitor.*—100 c.c. of citrated plasma were precipitated with 11 volumes of 2·5% trichlor-acetic acid. The precipitate was centrifuged thoroughly freed from the residual nitrogen by washing with 2·5% trichlor-acetic acid. The still moist precipitate is dissolved in 500 c.c. of water and this solution was mixed with two litres of acetone. Upon the addition of a small amount of sodium acetate solution, the plasma was again precipitated and now washed with acetone. This precipitate was dried at room temperature. This preparation has a greater proteolytic activity than preparation 3, since the trypsin-inhibitor compound has been broken up by treatment with trichlor-acetic acid.

1 gm. of each of the above preparations were suspended in 20 c.c. of M/15 phosphate buffer of pH 8·4 and incubated for 18 hours at 37°·5 C. The increase in non-protein nitrogen in each case was as follows:—

TABLE I

Red Blood Cells	Platelets	Trypsin-inhibitor Compound	Plasma-Trypsin freed from the Inhibitor
mg.	mg.	mg.	mg.
2·2	9·48	1·57	3·51

If the kinase is present in red blood cells, it should be able to liberate the trypsin from the trypsin-inhibitor compound when incubated with the latter in M/15 phosphate buffer of pH 8·4. The following experiments were carried out to ascertain the presence of kinase in red blood cells.

1 gm. of the red blood cells was mixed with 1gm. of the trypsin-inhibitor compound preparation, the mixture suspended in 20 c.c. of M/15 phosphate buffer of pH 8·4 and incubated for 18 hours at 37°·5 C. In another experiment 1 gm. of red blood cells was mixed with 1 gm. of the plasma-trypsin preparation freed from the inhibitor, the mixture also suspended in 20 c.c. of M/15 phosphate buffer of pH 8·4 and incubated for 18 hours at 37°·5 C. The increase in non-protein nitrogen in each case was as follows:—

TABLE II

	Increase in N.P.N. mg.
1. Red Blood Cells	2·2
2. Trypsin-inhibitor Compound	1·57
3. Red Blood Cells and Trypsin-inhibitor Compound preparation	3·9
4. Plasma-Trypsin preparation freed from the inhibitor	3·51
5. Red Blood Cells *plus* Plasma-Trypsin freed from the inhibitor	5·95

If kinase was present in red blood cells, the non-protein nitrogen in the case of (3) should be approximately the same as in (5), since the latter represents the combined proteolytic activity of red blood cells and the plasma-trypsin freed from the inhibitor by treatment with trichlor-acetic acid. The increase in non-protein nitrogen in (3) is 3·9 mg. which is approximately equal to the increase in N.P.N. of (1) and (2) added together. The above results definitely show that trypsin-kinase is not present in red blood cells.

Attention was then directed to the platelet preparation. Similar experiments as in Table II were conducted with the only difference that in place of

red blood cells, the platelet preparation was employed. The results of these experiments are:—

TABLE III

	Increase in N.P.N mg.
1. Platelet preparation	9·48
2. Trypsin-inhibitor compound preparation ..	1·57
3. Platelet preparation *plus* Trypsin-inhibitor compound preparation	13·05
4. Plasma-Trypsin preparation freed from the inhibitor	3·51
5. Platelet preparation *plus* Plasma-Trypsin preparation freed from the inhibitor ..	13·65

The results reported above lend direct experimental evidence for the presence of trypsin-kinase in platelets. The proteolytic activity of (3) is the same as (5) within the limits of experimental error. If kinase was not present in the platelets, the proteolytic activity of (3) should have been represented by an N.P.N. increase of (1)+(2) (*i.e.*, 9·48+1·57=11·05 mg.) whereas the actual increase is 13·05 mg. The increased tryptic activity is due to the extra amount of trypsin released from the associate inhibitor by the kinase that might be present in platelets. The fact that the proteolytic activity of (3) is approximately the same as in (5), further shows that the trypsin-inhibitor compound is practically completely broken up by the platelets.

It may be argued that the increased tryptic activity of (3) may be due to the addition of an increased amount of protein, since the trypsin-inhibitor compound added to the platelet preparation, is merely plasma proteins directly precipitated by acetone. Such a possibility does not however exist, because the platelet preparation itself contains a very large proportion of substrate compared to the quantity of associated trypsin, and the percentage protein broken up by auto-digestion is only 6%. Besides, the enzyme being simultaneoulsy precipitated with the platelet proteins by acetone, the trypsin is already adsorbed on the platelet proteins and will therefore have a preference to digest the associated protein which is present in plenty beyond the capacity of the enzyme present to digest. Iyengar (*I.J.M.R.*, July 1941) has shown that, if to an enzyme preparation containing a large quantity of a susceptible protein substrate another protein which is less susceptible is added, the latter remains practically unattacked. When insulin was added to a platelet preparation (obtained by acetone precipitation) and incubated for a period of 24 hours, the hormone remained practically intact which was demonstrated by the complete retention of its physiological activity. The

plasma proteins obtained by acetone precipitation if incubated in M/15 phosphate buffer undergoes auto-digestion to the extent of only 1%. So, in this case also, the associated trypsin has a plentiful supply of substrate already and the addition of the platelet preparation should not make any difference so far as the tryptic activity of the plasma proteins are concerned. Therefore if the tryptic activity of the mixture of platelets and plasma proteins (obtained by acetone precipitation) is significantly more than the added value, the only possibility is that some interaction between the two has taken place giving effect to this increased activity. This interaction is between kinase probably present in platelets and the trypsin-inhibitor compound known to be present in acetone precipitated plasma proteins as hypothetically visualised by Schmitz. It may be stated that the above results may not be direct convincing evidence for the presence of kinase in platelets, since the kinase has not been isolated from the platelet-proteins and the associated trypsin, but the results reported in this paper lend strong experimental support for the presence of kinase in blood.

Summary

A clear picture of the proteolytic system existing in blood has been presented.

The possibility of the presence of a trypsin-kinase in blood has been discussed.

The red blood cells and the platelets have been examined for the presence of trypsin-kinase.

Red blood cells do not contain the kinase.

The experiments reported in this paper strongly suggest the presence of trypsin-kinase in platelets, which is capable of liberating the trypsin from the inhibitor compound present in acetone precipitated plasma proteins.

BIBLIOGRAPHY

1. Schmitz .. *Zeit. f. Physiol. Chem.*, 1937, **250**, 37.
2. Iyengar, N. K., and Scott, D. A. *Trans. Roy. Soc. Canada*, 1940, Sec. V, **34**, 45.
3. Pope, B. J. .. *Expt. Pathol.*, 1939, **20**, 2, 132.
4. Iyengar, N. K. .. *Indian Journal of Medical Research*, July 1941.