KINASE-HEPARIN ANTAGONISM IN VITRO.

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With 1 Figure in the text.

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It is known that putting heparin into a weak solution of toluidine blue dye changes it to violet (Jorpes, 1936). We also know that this reaction is specific to the sulphuric acid esters of great molecular weight and that the phosphoric acid esters do not give it (Lison, 1935).

This reaction can also be utilized histologically, inasmuch as the heparinocytes (Ehrlich's mast cells) in tissue preparations also dye metachromatically with toluidine blue to violet (Holmgren and Wilander, 1937). The reaction itself can be imagined as being that a chemical bond is created between the heparin and the toluidine blue, in consequence of which the toluidine blue suspends the effect of the heparin in the clotting system (Gerendás, Udvardy, Pálos and Csefkó, 1948).

In a previous paper we showed that in vivo the effect of histamine is to diminish the thrombin inactivating capacity of the blood, and this can also be proved in vitro (Csefkó, Gerendás and Udvardy, 1948). From our further investigations it also appeared that in the organism histamine is antagonistic to heparin (Gerendás, Csefkó and Udvardy, 1948a). If in in-vitro experiments the metachromatic colour is produced on the addition of heparin to a toluidine blue solution, this system can be made blue again by a histamine solution. Hence the bond between the histamine and the heparin is stronger than the toluidine blue - heparin bond, for the already developed violet colour turns blue again and if we first mix histamine with heparin it does not develop. According to our calculations about 2 molecules of histamine bind with 1 of heparin. It was therefore supposed that histamine in vivo (similarly to toluidine blue, only stronger) binds the

heparin in the blood, thereby diminishing thrombin inactivation and thus the chemical state of the blood shifts towards clotting.

As it is known from the literature as result of many investigations that the tissue-extract ("Gerinnungsstoff" in the works of FISCHER and ASTRUP, 1935, "kephalin-histon" in those of CHARGAFF, ZIFF and COHEN, 1940, "Kinase" of JORPES, 1947) binds the heparin and suspends, or diminishes its ability to clot, the inference can be drawn that the kinase effect is none other than a histamine-like effect. In this case this effect should be evident in the toluidine blue-heparin system, and further it should also decrease thrombin inactivating velocity.

During our experiments we also noticed that the heparin-toluidine blue bond was fairly labile, among other things at a temperature of 65° C the characteristic violet colour changes to blue, while on cooling it changes back again. The change in colour begins at about 38° C. We were able to use this phenomenon when small amounts of heparin had to be demonstrated. Namely, then the solution with toluidine blue changes only slightly to violet. If, on heating, the solution becomes quite blue and on cooling recovers its first colour, we considered that the change was due to the presence of heparin.

DETAILS OF THE EXPERIMENT.

We investigated on the one hand how brain kinase behaves in the toluidine blue-heparin system, on the other hand how the kinase affects the thrombin-inactivating capacity of the blood, where, according to our findings, an inactivating ferment and heparin take part.

The heparin (Heparinum Vitrum subst.) used for the experiments we received from Professor E. JORPES (Stockholm), the human brain kinase dried with acetone, freshly prepared, from DR. Z. HORN (Budapest), or we prepared kinase ourselves from the brains of rats.

If brain kinase is well shaken up with Ringer solution (100 g dried kinase in 5 ml calcium-free Ringer solution), in a short time two layers form. In the upper layer is a turgid, milk-like fluid, in the lower layer we get the undissolved kinase granules. We investigated what change was to be observed if we poured off the milk-like fluid above the kinase granules and added the violet-coloured toluidine blue-heparin solution to it.

kinase solution — — — — — — — — 0.5 ml
+
0.01 °/oo toluidine blue solution — — — 1.0 ml
2.0 °/oo heparin solution — — — — — 0.2 ml

When the solutions are mixed together the violet colour does not disappear, but its strength diminishes decidedly. It becomes more pronounced after centrifuging the solution. This shows that part of the heparin disappears from the solution but this stops when a balanced concentration is reached.

After that we investigated the remaining kinase granules in the following combination:

kinase residue	1.5 ml
Ca-free Ringer solution	5.0 ml
0.01 °/₀₀ toluidine blue solution	1.0 ml

If we shake this solution very vigorously for some minutes the original blue of the toluidine blue gets a violet tinge. Here too the change in colour is remarkable if by centrifuging the solution we expel the kinase granules which trouble the colours. If now we transfer this violet solution into a test-tube and heat it, it turns blue, and on cooling the original violet colour returns. From this it can therefore be concluded that in the brain tissue itself there is also heparin, or a heparin-like substance, soluble on being shaken up with water. If after this we repeatedly change the liquid above the kinase and again shake the mixture, the toluidine blue freshly put into it will gradually change less into violet. This means that we have exracted the heparin-like material.

If, continuing, we again added to the kinase granules remaining from the previous experiment calcium-free Ringer solution and toluidine blue solution, then heparin solution in the following combination:

Ca-free Ringer solution	5.0 ml
kinase residue	1.0 ml
0.01 °/₀₀ toluidine blue solution	0.5 ml
1.0 °/₀₀ heparin solution	1.0 ml

the fluid became violet-coloured. After vigorous shaking, however, the violet colour of the liquid gradually fades and finally stops at a transitional blue-violet tinge. If now to this same system we add further doses of heparin and again shake well, the violet colour caused by the additional heparin again fades in the course of the shaking. So we can progressively bind 4—5 mg heparin in the same system from which we had dissolved the original heparin, or heparin-like substance. (We did not carry on the experiments to extreme doses.) From this we could conclude that the kinase is capable of binding a considerable amount of heparin.

From comparison of the two experiments therefore it can be said that while on the one hand heparin can be dissolved from the kinase, the kinase can bind heparin from the more concentrated heparin solution. According to this there is a state of equilibrium between the heparin concentration of the kinase and of the solution in the layer above it. The phenomenon occurs equally in preparations made from human and rat brain.

In the other part of our investigations we studied the effect exercised on thrombin inactivation by kinase.

For studying the thrombin inactivation we used a process whereby we followed for 10 minutes, at room temperature, the decrease in activity of a thrombin solution of known activity put to serum, and on the basis of the data so obtained calculated the inactivation reaction velocity factor $k = \frac{1}{t} \log. nat. \frac{Co}{C}$. We refer the reader to our earlier publications on the details of our thrombin inactivation investigations (GERENDÁS, 1948 a, 1948 b). The following experiments were made with fresh serum from hens with fresh kinase taken from the brain of rats. The standard thrombin solution clotted the fibrinogen test in 12 seconds.

In the first experiment we established the normal inactivation of the serum, then investigated the inactivation in the presence of kinase.

Serum	0.3 ml	Serum	0.3 ml
Distilled water	0.2 ml	Kinase solution	0.1 ml
Standard thrombin solution	0.3 ml	Distilled water	0.1 ml
		Standard thrombin solution	0.3 ml

Figure 1 shows the data on clotting time obtained in the course of inactivation in the above experiment in 1, 2, 3, 5, 7 and 10 minutes in logarithmic scale. Calculated from these data the reaction velocity constant of the control curve $k = 0.502$, in the experiment made with kinase $k = 0.332$. From the Figure, as well as from the numerical value of the reaction velocity constant, it appears that the kinase considerably diminishes the thrombin inactivating velocity, as we had supposed.

In what follows we report on experiments made to determine to what extent kinase can decrease thrombin inactivation velocity augmented by heparin.

Serum	0.3 ml	Serum	0.3 ml
Distilled water	0.1 ml	Kinase solution	0.1 ml
Heparin 0.04 mg/ml	0.1 ml	Heparin 0.04 mg/ml	0.1 ml
Standard thrombin solution	0.3 ml	Standard thrombin solution	0.3 ml

The inactivation data obtained are likewise shown in Figure 1. The reaction velocity constant of the heparin curve k = 0.990; the value obtained on the heparin + kinase k = 0.498. Therefore the kinase also decreases the thrombin inactivation reaction velocity augmented by heparin; that is, suspends equally the effect of heparin originally present in the blood and that added to the blood.

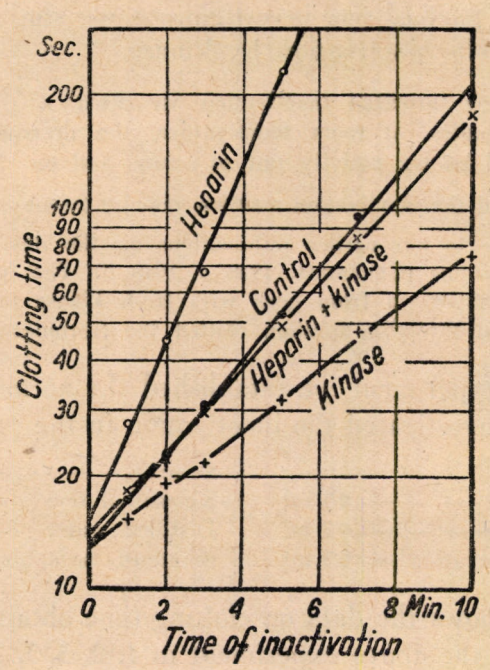

Figure 1. Change in thrombin inactivating capacity of hen serum in the presence of heparin, kinase, and heparin + kinase respectively.

DISCUSSION.

In general so far the conception of the rôle of kinase in blood clotting has been that the kinase transforms the prothrombin into thrombin. But the rôle of kinase in this process is not entirely clear. According to one definition prothrombin becomes active thrombin by binding with kinase and Ca-ions (FISCHER, 1933, 1934); according to others kinase activates the thrombin by eliminating the effect of clot-inhibiting substances (FUCHS, 1930, DEL BAERE, 1932, HOWELL, 1935, DYCKERHOFF and KURTEN, 1936, FISCHER and ASTRUP, 1935). Considering that the

kinase increases the clotting effect exercised on oxalate plasma by added thrombin (HORN and BORSODY, 1948) we must in this case conclude that the kinase effect means the neutralization of the clot-inhibiting substance.

In our opinion the proportions of thrombin formation and disappearance determine the clotting capacity of the blood. If the thrombin disappears rapidly the thrombin level is low and the blood is in a state where it clots with difficulty, or just does not clot. On the other hand, by reducing thrombin inactivation the thrombin level rises and the blood becomes more coagulable.

On the basis of the above experiments it can be concluded that heparin increases thrombin inactivation velocity, while kinase reduces the speed of the process by binding the heparin. According to this, therefore, the kinase not only causes the formation of thrombin, but inhibits its inactivation, thereby creating the thrombin level necessary to bring about clotting.

In this connection we must again point out that in our earlier experiments we also found histamine with kinase to have a similar heparin-binding and inactivation-inhibiting rôle (CSEFKÓ, GERENDÁS and UDVARDY, 1948 l. c., GERENDÁS, CSEFKÓ and UDVARDY, 1948a l. c.). We showed in in vivo and in vitro experiments that histamine increases both the thrombin level and the clotting capacity of the blood, hence acts in just the same way as the kinase has acted in our present experiments. It is beyond doubt after this that histamine — as a physiological substance in the organism -- takes part in regulating blood clotting (GERENDÁS, CSEFKÓ and UDVARDY. 1948b). We consider it possible that in the effect of histamine and of kinase on heparin we have observed two similar systems regulating the process of blood clotting side by side, indeed it is also possible that the two phenomena are based on an effect with an identical chemical radical.

SUMMARY.

By heparin toluidine-blue colour-reaction we established that heparin, or a heparin-like substance dissolves from kinase in Ringer solution. In a similar way we also showed that the kinase thus freed of heparin could bind heparin from a more concentrated heparin solution.

We found that the kinase decreased equally the normal thrombin activating capacity of the blood, and that increased by heparin. It is suggested that the rôle of kinase in the clotting of blood is an inhibition

of thrombin inactivation, whereby the thrombin level is raised to the level necessary to bring about clotting.

We established an analogy between the histamine-heparin antagonism found in our earlier experiments and the present investigation.

Our observations stand close to the conceptions of FUCHS, DEL BAERE, and HOWELL (l. c.) principally, as opposed to those usually found in the literature, namely, that the function of kinase consists in the activation of prothrombin.

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