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FAILURE OF LIPOID THROMBOPLASTIN TO ACT AS COFACTOR FOR TRYPSIN IN THE ACTIVATION OF PROENZYMES*

Trypsin is known to activate certain proenzymes, including trypsinogen and chymotrypsinogen.[13] It also activates prothrombin.[1, 9] In the case of prothrombin activation, platelets have been shown to complement the effect of trypsin. Small quantities of trypsin and platelets together exert a much greater effect than the sum of their separate effects.[9] Lipoid thromboplastin has been found to complement trypsin in a similar manner.[2, 11]

The mechanism of this complementary effect is unknown. Therefore, it is of interest to inquire whether lipoid thromboplastin functions as a simple cofactor for trypsin in the activation of proenzymes, generally.

MATERIALS AND METHODS

Buffer: 0.9 per cent NaCl; 0.02M veronal buffer, pH 7.4.

Prothrombin preparations: These materials were obtained by modifications of the methods previously described for prothrombin preparation II[10] and prothrombin preparation III.[11] Preparation II was obtained by taking the material left in solution after isoelectric precipitation at pH 4.6 and aging four days at 5° C.; whereas preparation III was derived from the material precipitated at pH 4.6.

Lipoid thromboplastin (lipid): A cephalin-containing fraction from bovine brain, prepared as described previously.[9]

Crystallized trypsin: Supplied by Armour and Co., Chicago. Three hundred mg. of this material, containing about 120 mg. protein (plus 180 mg. magnesium sulfate) was dissolved in 20 ml. 0.0025M HCl and dialyzed against cold 0.0025M HCl.

Crystallized chymotrypsinogen: Prepared by Armour and Co. by a process involving four crystallizations from ammonium sulfate solution followed by two crystallizations from alcohol. It was reported by the manufacturer to contain less than 0.3 per cent ash. One hundred twenty-six mg. was dissolved in 10 ml. 0.0025 M HCl to give the stock solution.

Crystallized trypsinogen: Prepared according to the method of Kunitz and Northrop[6] by Worthington Biochemical Laboratory, Freehold, New Jersey.

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Inhibitor-free trypsinogen: Prepared from crystallized trypsinogen in this laboratory according to the method described by Kunitz[4]; 570 mg. of moist filter-cake were dissolved in 5.7 ml. 0.005M HCl to give the stock solution.

Assay for thrombin: As described.[8]

Rennet activity of chymotrypsin: As described by Northrop, Kunitz, and Herriott.[13]

Proteolytic activity: Casein digestion method of Kunitz.[5]

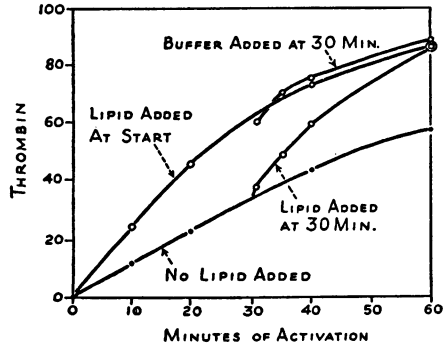

FIG. 1. Activation of prothrombin by trypsin without added lipoid thromboplastin, and with lipoid thromboplastin added at different times. One-tenth ml. prothrombin was mixed with 0.7 ml. buffer, 0.1 ml. of a 1:100 dilution of lipoid thromboplastin, 0.1 ml. of a 1:1,000 dilution of stock trypsin, and 0.1 ml. $CaCl_2$ 0.0275M. In a parallel experiment, buffer was substituted for lipoid thromboplastin. A separate pair of experiments was set up at the same time and in the same way, except that each contained 0.1 ml. less of buffer. This pair was allowed to incubate for 30 minutes without removal of samples. Then, 0.1 ml. lipoid thromboplastin was added to the mixture which had not received the lipid at the start; and 0.1 ml. buffer was added to the one which had started with added lipid. The activation mixtures contained 0.00055 mg. trypsin per ml. All tests were performed at room temperature.

EXPERIMENTAL

Figure 1 deals with the activation of prothrombin by trypsin. There it is apparent that lipoid thromboplastin, added either at the start or during the activation of prothrombin, increased the rate of thrombin production. When the lipid was added at 30 minutes, the curve of thrombin production did not shift suddenly to the upper curve, but approached it gradually. If the lipid effect had been due to a direct influence on the estimation of thrombin, the curve would have shifted immediately.

These and previous[11] results eliminate the possibility that the lipid effect depends on a spurious increase in thrombin values. It must be concluded that the net production of thrombin is actually faster in the presence of the lipid.

When crystallized chymotrypsinogen was used as the proenzyme, the results were quite different. Even though calcium chloride was included, the lipid had no effect on the activation of chymotrypsinogen by trypsin, as shown in Figure 2. The comparatively large amounts of protein materials used in this experiment were dictated by the standard methods used. If less chymotrypsinogen had been used, the rennet (milk-clotting) activity would have been hard to measure in the early stages of activation.

Consequently, a separate series of experiments was done in which the activation of chymotrypsinogen was followed by estimating its increasing proteolytic activity. Four pairs of tests covered the range of 57 to 456 micrograms of chymotrypsinogen per ml. activation mixture. In each case, the proenzyme was activated by 0.00055 mg. of trypsin per ml. activation mixture, the same concentration as that used for the prothrombin activation of Figure 1. Lipoid thromboplastin was added to one test of each pair. In no case did the lipid make any significant difference in the activation of chymotrypsinogen.

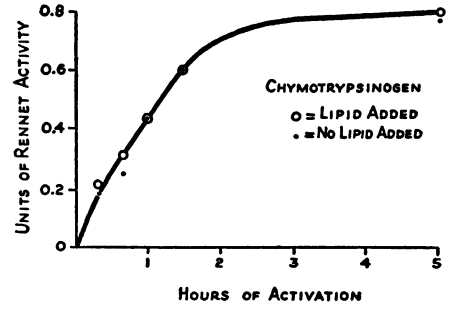

FIG. 2. Activation of crystallized chymotrypsinogen by crystallized trypsin, with and without lipoid thromboplastin. One ml. stock chymotrypsinogen was mixed with 6 ml. buffer, 1 ml. $CaCl_2$ 0.0275M, and 1 ml. of a 1 : 100 dilution of lipoid thromboplastin. Activation was begun by adding 2 ml. of a 1 : 200 dilution of stock trypsin. In the control, 1 ml. buffer was substituted for the thromboplastin. There were 1.1 mg. chymotrypsinogen and 0.0055 mg. trypsin in each ml. activation mixture. The activations were carried out at room temperature; but the serial tests for rennet activity were performed at 35° C.

Certain aspects of trypsinogen activation seemed to offer a more favorable chance of detecting an effect. In the first place, ionic calcium is known to be required for the effect of lipoid thromboplastin[10]; and it is also known to be favorable for the activation of trypsinogen.[7] Secondly, because it is autocatalytic, the activation of trypsinogen is sensitive to a slight, early acceleration. A cofactor, even if it remained effective for only a short time, would shift the sigmoid portion of the curve to an earlier position. It would have an effect similar to that of seeding the reaction with a small amount of trypsin. No such effect is evident in Figure 3.

As these negative findings accumulated, it was necessary repeatedly to verify the activity of the stock thromboplastin. By chance, one recheck was negative—the stock lipid now had little or no effect on the activation of prothrombin by crystallized trypsin. However, further tests soon showed that it was the prothrombin reagent which had changed, rather than the lipid. This was originally encountered with prothrombin preparation I,[10] a material obtained by adsorption with magnesium hydroxide.[8] When fresh, this preparation gave a pair of curves like that of prothrombin preparation III in Figure 4. However, after several days' storage at 5° C. it gave a pair of curves like that of prothrombin preparation II. This difference in

reaction-pattern has been encountered many times since, on several occasions with prothrombin preparations tested simultaneously.

During work on purification of prothrombin, this reaction-pattern has been used as another means of characterizing prothrombin fractions. Various preparations have shown varying degrees of difference in their thrombin production curves, with and without added lipoid thromboplastin.

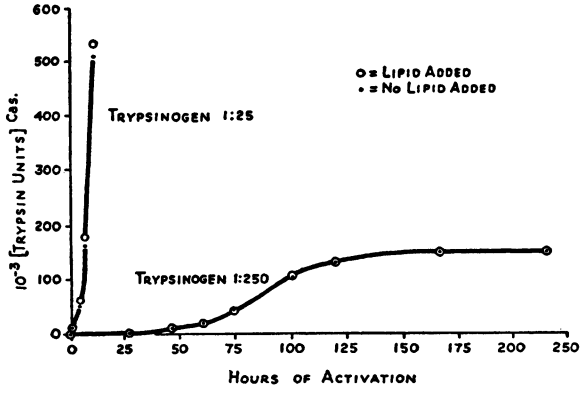

FIG. 3. Autocatalytic activation of crystallized trypsinogen, with and without lipoid thromboplastin. Stock trypsinogen was diluted 1:2.5 and 1:25 with 0.005M HCl; 0.5 ml. diluted trypsinogen was mixed with 3.5 ml. buffer, 0.5 ml. of a 1:100 dilution of lipoid thromboplasti nand 0.5 ml. $CaCl_2$ 0.2M. In the controls, buffer was substituted for thromboplastin. The curves are labelled according to the final dilutions of stock trypsinogen in the activation mixtures. They correspond respectively to trypsinogen concentrations of the order of 0.025 and 0.25 mg. per ml. activation mixture. Activations were carried out at 4-5° C. Serial tests for proteolytic activity were done at 35° C.

DISCUSSION

In the experiments reported, lipoid thromboplastin failed to behave as simple cofactor for trypsin in the activation of chymotrypsinogen, trypsinogen, and prothrombin preparation II. But the lipid *seemed* to behave as cofactor for trypsin in the activation of prothrombin preparation III.

Conceivably, the prothrombin molecules in preparation II may have been different from those in preparation III. Or the difference in behavior may have depended on the impurities in the prothrombin preparations. In the latter case, several mechanisms other than simple cofactor action would be conceivable.[9] The possibility that the lipid takes part in a more complex cofactor effect is not eliminated. And the possibility that the lipid combats an anticoagulant is still open.

Further investigation of these possibilities would be more profitable as the purification of clotting factors progresses. Meanwhile, the difference in

rate of thrombin production, observed with and without lipid, serves as an additional means of characterizing prothrombin fractions. This applies specifically to those cases in which crystallized trypsin is used as the activator and the prothrombin contains little, if any, thrombokinase as a contaminant.

It is of particular interest to note the trend of the evidence concerning the function of lipoid thromboplastin. Howell, himself, did not believe that his

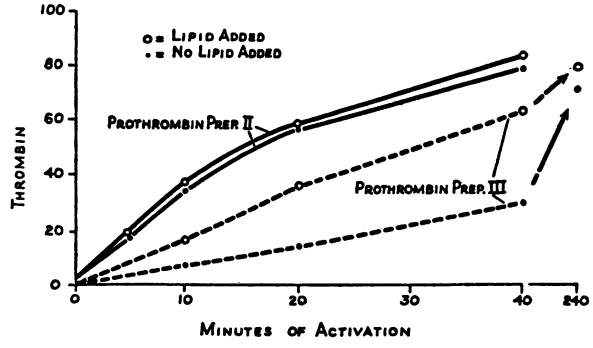

FIG. 4. Activation of two different prothrombin preparations by crystallized trypsin, with and without added lipoid thromboplastin. One-tenth ml. prothrombin preparation III was mixed with 0.7 ml. buffer, 0.1 ml. of a 1:100 dilution of lipoid thromboplastin, 0.1 ml. of a 1:1,000 dilution of stock trypsin and 0.1 ml. $CaCl_2$ 0.0275M. In the control, buffer was substituted for thromboplastin. On a different day, 0.2 ml. prothrombin preparation II was mixed with 0.6 ml. buffer, 0.1 ml. lipoid thromboplastin, 1:100, 0.1 ml. trypsin, 1:1,000 and 0.1 ml. $CaCl_2$ 0.0275M. The control had buffer instead of thromboplastin. All tests were performed at room temperature.

"cephalin" was a direct activator of prothrombin.[8] It has long been known[14] that partially purified prothrombin is activated only very slowly in the presence of calcium and "cephalin." A negative result after an incubation period of 80 minutes has been reported.[9] More recently, it has been possible in this laboratory to obtain a prothrombin preparation which was less than one per cent activated after 28 hours with calcium and lipoid thromboplastin at room temperature.[12] Now an example has been presented (Fig. 4) in which the lipid had very little effect, even as an accessory factor for the production of thrombin.

SUMMARY

In the experiments reported, lipoid thromboplastin failed to function as simple cofactor for trypsin in the activation of crystallized chymotrypsinogen, crystallized trypsinogen, and certain preparations of prothrombin.

With other preparations of prothrombin, the lipid seemed to act as cofactor for trypsin; but this may well have been due to some effect other than simple cofactor action. Since different prothrombin preparations behaved differently in the test system, this system has been used as an additional means of characterizing prothrombin fractions.

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