THE PROBLEM OF THE LIPOID THROMBOPLASTINS*

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For a long time, it was accepted that calcium and cephalin activate prothrombin. There were secondary questions about the identity of the phosphatide and whether it was important that the phosphatide be bound to protein. Otherwise the issue was closed. Now the basic premise is in sufficient doubt to warrant a restatement of the problem in the light of newer observations.

" . . . I have come upon a fact which is of the very highest importance not only for the question of the coagulation of the blood but for the very much greater question of the nature of the chemical processes in protoplasm, which constitute life. This fact is that lecithin, a body omnipresent in protoplasm, can bring about coagulation." So wrote Wooldridge, in 1883.[28] The material he thus caused to coagulate was specially treated plasma from dogs injected with peptone. The plasma of peptonized dogs was also used by Howell[10] to assay the coagulant activity of his cephalin preparations. At that time, Howell employed one of Nolf's terms and wrote of "thromboplastic substances" as "the extractible substance or substances in the tissues which facilitates the process of clotting." This is considerably more general than his previous description[9] of "thromboplastic substance or thromboplastin, which neutralizes the antithrombin. . . ." Then, and ever since, "thromboplastin" has been a rather broad and variable term. It is now applied to diverse materials from fixed tissue or blood which accelerate coagulation, especially those which accelerate production of thrombin.

As discussed by Howell, a confusing set of reports had accumulated by 1912. One group of investigators dealt with heat-stable thromboplastins found in alcohol or ether extracts, while others used heat-labile, water-soluble preparations. Among the latter were Morawitz[29] and Mellanby,[19] who designated their aqueous extracts thrombokinase; and it was clear that they considered thrombokinase to be a direct activator of prothrombin.

If these old definitions are accepted, it may be considered that a thrombokinase would activate pure prothrombin, aided only, if at all, by calcium. In this sense, a thrombokinase is a specific kind of thromboplastin. There may be other thromboplastins which would not activate pure prothrombin.

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* From the Laboratory of Pathology, Yale University School of Medicine. Aided by a grant from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.
Received for publication April 11, 1950.

Howell preferred to consider that, basically, there was only one type of thromboplastin. He proposed that the heat-labile material was merely the thromboplastic lipid in combination with protein. However, Rumpf[30] believed that the two had distinct effects. Whereas the aqueous extracts were usually much more active than the lipoid extracts, the latter were reported to be more effective in the clotting of lipid-poor plasma. To this, an incomplete answer was offered by the assumption that the lipid was more effective when combined with protein. Today it is still frequently stated that prothrombin is activated by a lipoprotein.

In the years 1916-17, it was reported by Dale and Walpole,[2] by Douglas and Colebrook,[3] and by Heard[8] that trypsin accelerated blood coagulation. But the time was not ripe, and this remarkable finding was lost in the shadow of lipoid thromboplastin for two decades. The isolation of trypsin as a crystalline protein (not a lipoprotein!) by Kunitz and Northrop[15] offered a finer means for investigation. Promptly, Eagle and Harris[6] found that crystalline trypsin activated their crude prothrombin. Conversion to thrombin had also been accelerated by cephalin, with a similar crude prothrombin.[4]

Thus, Eagle[5] was confronted with a dilemma much like that faced by Howell twenty-five years previously. There were now two sharply distinct classes of thromboplastins: one represented by a crude ether-soluble cephalin derived from brain; one represented by crystalline trypsin. Which, if either, was a true thrombokinase? Eagle favored trypsin and was puzzled by cephalin.

Whereas trypsin is known to activate proenzymes, no lipid is known to do so. Therefore, it is more attractive to consider that trypsin is a direct activator of prothrombin, and that the thrombokinases found in blood and tissue preparations may be somewhat similar enzymes. As logical as this may seem, it has not been widely accepted. The principal arguments against this idea will be analyzed later in this paper.

Recently, Ferguson[7] has discussed the results of his detailed studies and added some experiments performed with Folch's subfractions of crude brain cephalin. These leave open the possibility that true cephalin is a potent thromboplastin, as Howell believed. To date, no clarification of the problem has been achieved by the purification of brain lipids, other than the separation of anticoagulants.

In contrast, as prothrombin is purified, it reaches a state where it is readily activated by trypsin, but not by crude cephalin. A purification program which has been in progress many years has offered repeated opportunities to elaborate on this finding.

The thromboplastin studied

The lipoid thromboplastin studied was similar to that used by Eagle[4] as crude cephalin. It represented that fraction of brain which was soluble in ether but not in acetone. As such, it contained, among other things, both cephalin and lecithin. Possibly there are diverse lipids and lipoproteins with distinct thromboplastic effects; and these thromboplastins may be accompanied by inhibitors. It is proposed to neglect these complexities temporarily and to focus on the main issue: Is this thromboplastin a direct activator of prothrombin? If not, then what else could be a direct activator? And how can one account for the undoubted acceleration caused by the lipid?

It should be mentioned, however, that this thromboplastin is different from the brain thromboplastin commonly used in clinical tests. Findings in this laboratory tend to corroborate those of Lewis and Ferguson[18] who reported that the first aqueous extract of acetone-dried brain contains protein factors of significance in coagulation. Leathes and Mellanby[16] had previously shown that an aqueous brain extract contained what they considered to be a kinase, precipitable at pH 5.5 and not soluble in ether. They further indicated[17] that the kinasic action was probably fortified by the presence of brain lipid.

In short, there is strong indication that an aqueous suspension of brain contains a thromboplastic factor in addition to the ether-soluble lipoid thromboplastin as used in this work.

It has occasionally been reported that lipid also augments thrombin activity. This raises the question whether the acceleration of thrombin production is spurious, due merely to a shift in the thrombin assays caused by the lipid. Results in this laboratory have agreed with the general experience that the thrombin is actually produced more rapidly, and that the effect is greater than could be accounted for on the basis of the observed shift in thrombin assays.

Preparation of the thromboplastin. The following is an outline of the most recent preparation: 150 gm. fresh calf brain was washed, minced, and triturated in a mortar for 10 minutes with 150 ml. acetone (analytical reagent). The acetone was decanted and trituration repeated successively with 5 more 150 ml. portions of acetone. The residue was suspended in 750 ml. ether (U.S.P.) and allowed to stand overnight. The ether extract was filtered and evaporated under vacuum at 35°C. The residue was twice extracted with 450 ml. portions of acetone for 15 minutes and finally dissolved in 100 ml. ether. This solution was evaporated under vacuum at room temperature and kept under suction for several hours to approach complete removal of the ether. The yellow residue, weighing 3.75 gm., was triturated with 37.5 ml. veronal-buffered saline, and the mixture was centrifuged, yielding a milky suspension which was stored frozen.

Lipid and prothrombin

It was observed repeatedly that the apparent activation of crude prothrombin by lipoid thromboplastin plus calcium depended upon the presence of contaminating material in the prothrombin. If the contaminating material was destroyed or removed, the prothrombin would lose its responsiveness to the lipid. Responsiveness to lipid could be restored by adding an appropriate protein fraction from plasma.

At the time these investigations were begun, the best available method for purifying prothrombin was that of Mellanby.[20] In this, the prothrombin was precipitated with the euglobulins, then extracted from the precipitate

with very dilute calcium bicarbonate. With modifications,[29] batches of 39 liters could be prepared at one time on a routine basis.

The calcium bicarbonate extracts were studied extensively. Fresh extracts were promptly converted to thrombin following addition of lipoid thromboplastin and calcium chloride. However, aged extracts were not activated by lipid plus calcium, although they were still activated by an aqueous extract of lung, as noted in 1938 (unpublished). Later the same year, Seegers *et al.*[31] reported that their prothrombin was activated promptly by lung extract but very slowly by cephalin.

The significant material contaminating the prothrombin could be destroyed or removed not only by aging, but also by heating or by adsorption of the prothrombin on magnesium hydroxide followed by thorough washing. Figure 1A, a 1939 experiment, shows that although the lipid did not activate heated prothrombin, it accelerated activation in the presence of prothrombin-poor plasma globulin. Figure 1B portrays a 1947 experiment. Here, the reagents had been prepared in a manner quite different from that of earlier years, as described elsewhere.[24] For reasons discussed at that time, the prothrombin-poor plasma globulin has been called prothrombokinase. In Figure 1B the pattern of results is the same; however, the apparent activation by lipid and calcium was not eliminated as completely as before.

It is apparent that the thromboplastic activity of the lipid depended upon at least one impurity in the prothrombin, which could be removed and restored at will. Moreover, the prothrombin plus its impurities produced thrombin without the added lipid, showing that the direct activator of prothrombin was present in, or obtainable from, the blood derivatives. This suggested that any effect the lipid had on production of thrombin was either indirect or secondary. For example, it might be considered that the lipid activated prothrombokinase and thus indirectly hastened activation of prothrombin.

Lipid and prothrombokinase

Collingwood and MacMahon[1] wrote of prothrombokinase in 1912, and proposed that it was converted to thrombokinase in the first step of a three-stage clotting mechanism. 1. Prothrombokinase → Thrombokinase; 2. Prothrombin → Thrombin; 3. Fibrinogen → Fibrin. Recently, the stages they postulated have been separated and used for a three-stage analysis.[24] In this, calcium is added to crude prothrombokinase and the mixture is assayed for thrombokinase activity at successive intervals. This technique makes it possible to chart the conversion process and, further, to enquire whether lipoid thromboplastin activates prothrombokinase.

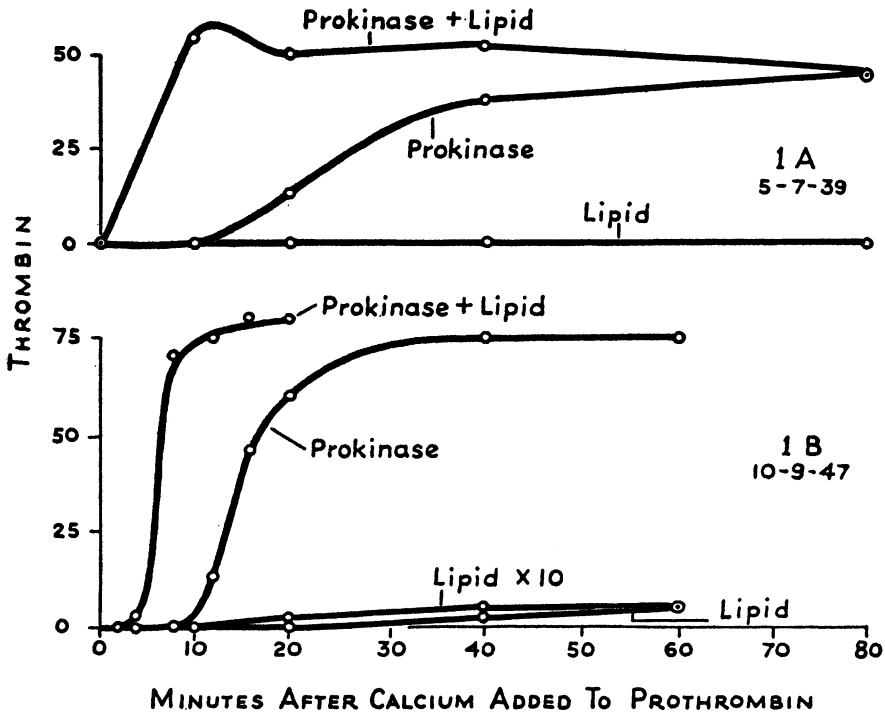

FIG. 1. Activation of prothrombin by prothrombokinase, lipoid thromboplastin, and calcium. All experiments were performed with calcium chloride present. Prokinase = prothrombokinase.

1A. For the experiment of May 7, 1939, the prothrombin was prepared by adding 0.9% of sodium chloride to a calcium bicarbonate extract, aging 3 days in the refrigerator, and heating in a 60°C. water bath for 5 minutes. The prothrombokinase was prepared from the residue left after extraction with calcium bicarbonate. The fresh residue was almost completely dissolved in a volume of oxalated saline equal to half the original volume of plasma, heated to 56°C. to remove fibrinogen, and treated with 21 gm. ammonium sulfate per 100 ml. The resulting precipitate was dissolved in distilled water equal to one-fourth the volume of original plasma and dialyzed against oxalated saline. Control tests showed no prothrombin in this reagent at the concentration employed. The concentration of lipid in the activation mixtures represented a 1/120 dilution of 1% stock suspension. The activation procedure was similar to that described in 1942.[22]

1B. For the experiment of October 9, 1947, prothrombin and prothrombokinase reagents were prepared and tested as described in 1948.[24] The most rapidly activated mixture contained 0.8 ml. prothrombin, 0.1 ml. prothrombokinase, 0.1 ml. of a 1/100 dilution of a 10% stock suspension of lipid and 0.1 ml. 0.0275 M calcium chloride. For the "prokinase" curve, the mixture was the same, except that 0.1 ml. buffer was substituted for lipid. For the "lipid" curve, buffer was substituted for prothrombokinase. For the "lipid x 10" curve, 0.1 ml. of a 1/10 dilution of stock lipid suspension was used, with no prothrombokinase added.

Results are shown in Figure 2. After calcium was added to prothrombokinase, there was a latent period, followed by a rapid increase in thrombokinase activity. When lipid was included there was a shorter latent period and a higher peak of thrombokinase activity. When ten times as much lipid was used, there was a pronounced elevation of the thrombokinase peak with relatively little effect on the latent period. Despite a rapid decline, the

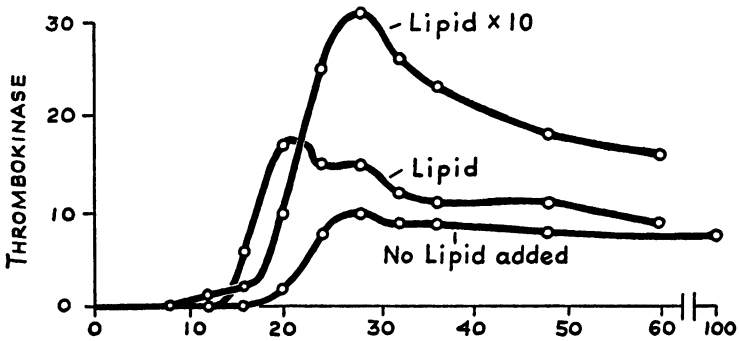

FIG. 2. Effect of lipoid thromboplastin on the prothrombokinase activation curve. The materials and methods were those previously described,[24] except that only one absorption with barium sulfate was done in the preparation of the prothrombokinase. For the "no lipid added" curve: 2.0 ml. prothrombokinase plus 0.2 ml. 0.0275 M calcium chloride. For the "lipid" curve: 1.8 ml. prothrombokinase, 0.2 ml. of a 1/100 dilution of a 10% suspension of lipid, plus 0.2 ml. calcium chloride. For the "lipid x 10" curve: 1.8 ml. prothrombokinase, 0.2 ml. of a 1/10 dilution of lipid, plus 0.2 ml. calcium chloride. As will be noted, the "no lipid added" mixture had 1.1 times as much prothrombokinase per ml. as the others. Separate experiments in which the prothrombokinase was constant gave the same pattern of results. The relative thrombokinase values were not reduced to percentage of an arbitrary standard.

thrombokinase activity remained, for the duration of the experiment, above that of the mixture with "no lipid added."

If the principal effect of the lipid were simply the direct activation of prothrombokinase, the curves should have risen from the origin and been about ten times as steep with the tenfold quantity of lipid. Since an autocatalytic mechanism or a chain reaction is involved,[26] an indirect effect, such as protection or enhancement of an autocatalytic factor, could account for as much shortening of the latent period as was observed. The materials were crude; and the explanation of the results would probably be complex. In

any case, the results do not encourage the view that the principal effect of the lipid is the direct activation of prothrombokinase.

Since the three-stage procedure is new and not fully developed, it is desirable to emphasize that it is nevertheless capable of detecting the kind of effect sought, i.e., a drastic shortening of the latent period with relatively little increase in peak activity. It was originally shown[24] that crude thrombokinase has such an effect on the activation of prothrombokinase. More recently,[27] a highly active protein fraction, which may represent a thrombokinase, has been described under the temporary name of "converter." At *high dilution,* the converter briskly accelerates activation of crude prothrombokinase without greatly elevating the activity peak. Such results make the converter a candidate for the role of prothrombokinase activator. Such results have not been obtained with lipoid thromboplastin.

Lipid and trypsin

It is now quite clear that thrombokinase can be obtained from slaughterhouse blood, although it may be necessary to convert it from an inactive state. The behavior of active thrombokinase is compatible with the possibility that it is an enzyme;[24] and it has usually been possible to substitute crystallized trypsin for blood thrombokinase.

The recent prothrombin preparations, as noted elsewhere, are suspected of containing a trace of thrombokinase or its precursor. Such prothrombins give a slight, limited response to lipid plus calcium. Beyond this low limit, the addition of more lipid does not lead to faster activation (Fig. 1B). In contrast, the prothrombin is activated with increasing rapidity when increasing amounts of trypsin are used (Fig. 3).

This last relationship must be borne in mind in the interpretation of experiments which purport to demonstrate that trypsin does not activate prothrombin. In Figure 3 it is seen that 0.1 microgram of crystallized trypsin did not activate the prothrombin reagent, neither did 0.1 ml. of twice-washed platelets. However, the two added to prothrombin together produced measurable thrombin. Experiments like this have sometimes been taken to imply that trypsin cannot activate prothrombin directly, but must act through the mediation of a blood factor, in this case platelets. That this conclusion is not necessarily valid is evident from Figure 3, where a tenfold quantity of trypsin caused activation without added platelets. In these experiments the range of trypsin concentration corresponded to a very slight degree of proteolytic activity as measured by the method of Kunitz.[14] As further illustrated in Figure 3, larger amounts of trypsin are rapidly destructive.

The necessity for testing several concentrations of enzyme was well recognized by Mellanby and Pratt;[21] therefore their negative results probably deterred subsequent investigators from pursuing the lead offered by trypsin. Using two-fold dilutions of pancreatic juice, they showed that dilutions just beyond the destructive one failed to activate prothrombin. No attempt was

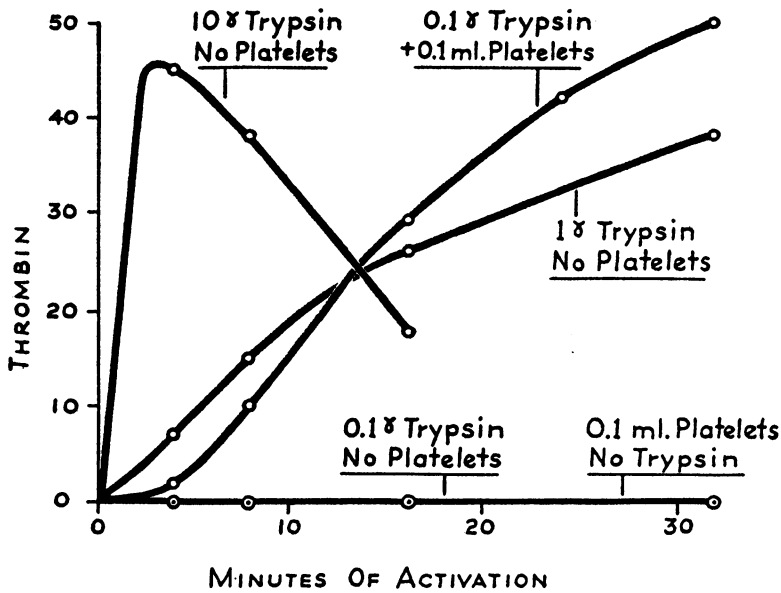

FIG. 3. Activation of prothrombin by trypsin, with and without platelets. 0.4 ml. buffer, 0.4 ml. prothrombin, 0.1 ml. platelets, 0.1 ml. Ca 0.0275 M., and 0.1 ml. trypsin (1/6,000, 1/600 or 1/60). Ca added 15 seconds before trypsin. Twice-washed rabbit platelets prepared as described.[25] One hundred and fifty mg. of crystallized trypsin, containing about 60 mg. protein (plus 90 mg. magnesium sulfate) were dissolved, dialyzed against 0.001 M HCl, and diluted to 10 ml. The figures on the chart represent the approximate amount of trypsin protein added to the activation mixtures.

made to determine how much of the destruction was due to chymotrypsin and how much to trypsin. So, it is not known whether an activating effect of trypsin should have been expected to dilute out further than the combined destructive effects of chymotrypsin and trypsin. It is known that their activated pancreatic juice contained considerable chymotrypsin, since the average specimen was said to clot milk quite rapidly. Trypsin does not clot milk, chymotrypsin does. It is conceivable that in their experiments there was sufficient trypsin to have an effect on prothrombin in conjunction with

other components of the clotting system. Their conclusion that trypsin releases an active form of thrombokinase may well be correct. But their results with crude pancreatic juice do not exclude the possibility that trypsin can also activate prothrombin directly. No one has yet claimed that crystallized trypsin cannot activate prothrombin.

If it is contemplated that prothrombin is ordinarily activated by an enzyme, it might be considered that the lipid could act as a cofactor. Such a relation of lecithin to the thrombokinase of daboia venom was considered by Leathes and Mellanby.[17] So far, attempts in this laboratory to show that the lipid can act as cofactor for trypsin have not succeeded. Experiments to be reported[28] have failed to show that the lipid acts as cofactor for crystallized trypsin in the activation of either crystallized trypsinogen or crystallized chymotrypsinogen. Little can be said about the lipid and the thrombokinase of blood.

Thus, the mode of action of lipoid thromboplastins remains an unsolved problem. However, the possibilities have not been exhausted. The heightening of the thrombokinase curve by the lipid offers one point of departure. It will be of interest to inquire whether lipid modifies production of thrombokinase, or certain side-effects, or the activity or persistence of the kinase. An entire group of possibilities is embraced by Howell's proposals[9-12] that the lipid would have no effect unless an anticoagulant were also present. Another such mechanism has recently been suggested by Jacox.[13]

Discussion

If the basic clotting mechanism is represented as three primary reactions:

1. Prothrombokinase ⟶ Thrombokinase
2. Prothrombin ⟶ Thrombin
3. Fibrinogen ⟶ Fibrin

there are many things left unexplained. Among them is the mode of action of the lipoid thromboplastins. The foregoing studies on a given type of lipoid thromboplastin were designed to inquire how it impinged on the above basic mechanism. A concurrent review of the literature attempted to locate the origins of certain ideas and the evidence in their favor.

There is no good evidence that lipoid thromboplastin, in the presence of calcium, will activate either prothrombin or prothrombokinase. Nor is there proof that the lipid will act as simple cofactor for thrombokinase or for trypsin.

These views are derived from an extended study involving separation of the factors and reactions of blood coagulation. As these separations

progress, the problem may assume a different aspect. As one example, removal of impurities from prothrombin preparations has almost eliminated their susceptibility to activation by lipid, while responsiveness to trypsin was preserved. It cannot be said that, as further impurities are removed, the prothrombin preparations will not regain their susceptibility to lipid and/or lose their responsiveness to trypsin. The facts at hand do not suggest that this will happen. Nevertheless, it is obligatory to emphasize that the prothrombins studied here have not been pure. Furthermore, a thoroughly documented pure prothrombin has not been described anywhere. Additional uncertainties accumulate as consideration is given to the crudity of lipoid thromboplastins and to the question of lipoid anticoagulants.

As the phosphatide theory has become weaker, there has risen in its place the belief that lipoproteins may have thromboplastic effects which are qualitatively different from those of phosphatides. This is uncertain. The opinion that lipoproteins activate prothrombin is based on the fact that crude thromboplastic activity and lipoprotein are often found together and seem to be altered together by various procedures. However, as here shown the finding that a material accelerates activation of crude prothrombin does not constitute proof that it is the direct activator.

In the search for a true thrombokinase, the evidence in favor of lipoproteins is as weak as that for phosphatides. One must hesitate to accept a suspension of tissue particulates as a pure substance. Such material undoubtedly hastens production of thrombin in crude systems. But there has been no successful attempt to isolate its lipoprotein and to show that its lipoprotein activates prothrombin. Caution is further indicated with regard to the notion that the lipoprotein is an enzyme; for there seems to be no established example of a lipoprotein which is an enzyme.

Some of these common assumptions may eventually prove correct; but for the present, they should be recognized as opinions, with some suggestive support from experiments. Then, they will no longer obscure the more plausible view that thrombokinase, the activator of prothrombin, is an enzyme, possibly proteolytic, and quite possibly not a lipoprotein. For this, there is at least as much suggestive evidence, and the further advantage that the idea is in harmony with the general findings of biochemistry. Among the less attractive alternatives, the facts do not exclude a mechanism which requires the simultaneous presence of two or more biologic activators.

With a few exceptions, most recent investigators have turned aside from the lead offered by trypsin. This neglect has had its reasons. The work of Mellanby and Pratt seemed to be devastating and probably discouraged

many. But their experiments were uncontrolled for complications due to the chymotrypsin now known to have been present. Consequently their data do not exclude the possibility that trypsin can activate prothrombin. A further apparent setback occurred when the weight of evidence[28, 29] turned against the view that a fibrinolytic enzyme from blood can function as thrombokinase. This issue is not entirely settled. More important, there is no reason to believe that only one proteolytic enzyme is obtainable from blood. Finally, it has been difficult to demonstrate proteolytic activity where thrombokinase seemed to be present. But the present experiments show that only a minute amount of added enzyme, in terms of micrograms or of proteolytic activity, suffices to produce a large effect on the activation of prothrombin.

The evidence is not adequate to close the problem; rather it should be opened wider. Along with the hypotheses proposed in the past, more attention should be given to the possibility that prothrombin can be activated by one or more enzymes, and that this basic process can be modified in an indirect or secondary manner by phosphatides and by lipoproteins. The relative importance of lipoid thromboplastins in the physiologic control of the clotting mechanism is another question.

Summary

1. In the presence of calcium, either trypsin or crude cephalin seems to activate crude prothrombin. Which, if either, represents a direct activator of prothrombin?

2. Prothrombin is a proenzyme. Trypsin activates some proenzymes. No lipid is known to do so.

3. As prothrombin is purified, it reaches a state where it is readily activated by crystallized trypsin, but not by crude cephalin.

4. In the presence of platelets, one-tenth microgram of trypsin produced a large effect on conversion of prothrombin. Without added platelets, one microgram of trypsin readily activated the prothrombin. Thus, enhancement of trypsin effect by other factors does not prove that trypsin would be ineffective alone, provided the proper quantity was used.

5. The behavior of thrombokinase derived from blood is consistent with the possibility that it is an enzyme.

6. Lipoid thromboplastin accelerated production of thrombin from a mixture of prothrombin, prothrombokinase, and calcium.

7. In three-stage tests the lipid raised the peak of the thrombokinase curve, but caused only minor shortening of the latent period. This does not encourage the view that the lipid activates the prokinase. In contrast, three-

stage tests on a recently described protein fraction from blood have encouraged the view that it may activate the prokinase.

8. Experiments to be published have failed to show that the lipid acts as cofactor for crystallized trypsin in the activation of crystallized trypsinogen or crystallized chymotrypsinogen.

9. The problem has not been clarified by consideration of crude lipoproteins. There is no good evidence that lipoproteins activate prothrombin. There seems to be no established case of a lipoprotein which is an enzyme.

10. The actions of lipoid thromboplastins remain an unsolved problem. Several possibilities are yet to be explored.

Conclusion

Along with the hypotheses proposed in the past, more attention should be given to the possibility that prothrombin can be activated by one or more enzymes, and that this basic process can be modified in an indirect or secondary manner by lipids and by lipoproteins.

It is a pleasure for the writer to acknowledge his indebtedness to Dr. Milton C. Winternitz. The aid and complete freedom offered by Dr. Winternitz made it possible to resume this work at the end of World War II. His friendly interest and encouragement continuously fostered an enthusiastic attack on the problem.

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