XXIX. SOME EXPERIMENTS ON FACTORS CONCERNED IN THE FORMATION OF THROMBIN.

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The experiments described in this paper were undertaken as part of an investigation into the causes of the toxicity acquired by serum from normal animals, when treated in certain ways. These toxic properties have been supposed by a number of investigators to be related to the phenomena of the anaphylactic reaction, and the name "anaphylatoxin" or "serotoxin" has been applied to the hypothetical toxic constituent. Some have supposed that the toxic substance is a protein cleavage-product; others that the toxicity is due to some undefined alteration in the physical properties of the colloidal system. We may remark that it is not as yet clear, that the toxic action produced by the various treatments, to which the serum has been exposed by different workers, is in all cases the same. Our attention was first directed to the toxic action, which fresh guinea-pig's serum very regularly acquires when shaken with about twice its volume of chloroform, as described in a series of papers by Jobling [1914] and his co-workers. We were struck by the conspicuous part played, in the toxic action of such a serum, by massive intravascular clotting. The occurrence of clots in the heart is mentioned, indeed, incidentally by these authors, but is evidently regarded by them as a subsidiary feature of the action. It may be so. We decided, however, to begin our proposed analysis of the action by examining this aspect of the effect, and were thus led to make a series of experiments on the phenomena of blood-clotting in vitro, and the effect, on the factors concerned, of treating plasma with chloroform, along similar lines to those which have been found to produce toxicity of sera.

A few preliminary experiments were made with mammalian serum and plasma. The investigation was complicated, however, by the necessity of

using a calcium precipitant to secure a plasma which did not coagulate spontaneously. We turned, therefore, to the plasma of the fowl, which several investigators have used on account of its stability when carefully prepared. The examination of the changes produced in it by treatment with chloroform necessitated the preparation of a series of the individual clotting-factors in as nearly pure a condition, in a physiological sense, as was practicable; and we were thereby at once involved in a consideration of different theories of blood-coagulation. For the mere definition of the factors concerned in normal clotting, and of the criteria of physiological purity to be applied to them, involves at least a provisional acceptance of one of the theories of their nature and function.

Our purpose in this preliminary paper, therefore, is to describe the methods which we have employed in preparing test-solutions of the clotting factors, and the information which we have obtained, by their use, concerning the processes occurring in a plasma treated with chloroform. The results so obtained seem to us incidentally to give very clear evidence against one recently propounded conception of the clotting-process. Later, when external conditions are more favourable to the steady pursuit of such an investigation, we hope to apply these methods and results to the study of induced toxicity of sera, and of the problem of anaphylaxis.

Theories of Blood Coagulation.

We are not immediately concerned with the mode of origin, or the precise nature of the interaction between the clotting-factors. So many reviews of the large literature of the subject have appeared, that we need only indicate briefly the main points of the two conceptions between which we attempt a decision. The principal current conception was present, in its essential features, in the theory finally put forward by Alexander Schmidt [1892, 1895], at the conclusion of his protracted and classical investigation; though in Schmidt's statement it was obscured by other conceptions which later work has failed to uphold. The theory, in its present relatively clear form, was put forward independently by Morawitz [1904] and by Fuld [1903], and numerous other investigators have contributed evidence as to the mode of origin and interaction of the factors concerned. The theory involves the interaction of three original factors, in addition to calcium ions, and the variety of the nomenclature used by different authors is somewhat bewildering. The factors are (1) fibrinogen, the soluble globulin of plasma which is converted into insoluble fibrin by the action of "thrombin" (fibrin ferment);

(2) an inactive precursor of thrombin, present in circulating blood and in stable plasma, which has been variously called plasmozyme, serozyme, thrombogen, and for which (following Mellanby [1908] and other recent writers) we use Schmidt's term "prothrombin," though this involves some restriction of Schmidt's application of the term; and (3) a substance obtainable from cellular tissues, or from blood-platelets, which in the presence of calcium ions brings about the conversion of the inactive "prothrombin" into the active thrombin. This substance was recognised by Schmidt, and by him called "zymoplastic substance." It corresponds to the "*A*-Fibrinogen" of Wooldridge, and has been variously termed thrombokinase (Morawitz), cytozyme [Fuld and Spiro, 1904], and, more recently, "thromboplastin" [Howell, 1910]. We use here, again following Mellanby, Morawitz's term "thrombokinase," or more briefly "kinase," for this factor.

An alternative conception, put forward in recent years by Howell [1911], and elaborated in a series of communications from his laboratory, differs from the foregoing in the importance attributed to "antithrombin." The presence in plasma or serum of factors inhibiting the coagulant effect of thrombin on fibrinogen had been recognised by a number of earlier investigators, and their action collectively attributed to "antithrombin." Howell supposes that prothrombin is converted into thrombin simply by the action of calcium, and that this action is prevented in circulating blood, or in stable avian or reptilian plasma, by the fact that prothrombin is associated with antithrombin. The rôle of the kinase (thromboplastin), according to Howell's view, is to dissociate this complex, by combining with the antithrombin, freeing the prothrombin to the action of the calcium ions.

It is clear that an acceptance of one or other of these theories is implicit in any definition of the clotting-factors, when an attempt is made to prepare them separately. Howell, for example, regards a preparation as pure prothrombin, in respect of its activity, if it fails to clot fibrinogen when added in the absence of calcium ions, but clots it promptly if an ionised calcium salt be added as well. He would regard a prothrombin, which fails to produce clotting under such conditions, and needs the further addition of kinase for its activation, as contaminated with antithrombin. Adherents of what we may, for brevity's sake, term the Morawitz theory would conclude, on the other hand, that the activation of Howell's "prothrombin" by calcium ions alone was clear proof of its contamination with kinase; it might even be kinase only, without any prothrombin, if the fibrinogen used had not been carefully freed from the prothrombin which is readily precipitated with it.

Preparation and Testing of Reagents.

1. *Fibrinogen.* The only difficulty in preparing an adequate fibrinogen solution is to get it absolutely free from prothrombin. Prothrombin is either itself a euglobulin, or is closely associated with one. Mellanby, indeed, appears to regard fibrinogen and prothrombin as so closely associated, that he frankly abandons the possibility of separating them, and uses as "fibrinogen" a preparation from which prothrombin can be separated by clotting the fibrinogen with thrombin.

This practice has the disadvantage that the decision, whether a given solution contains thrombin or kinase, is frequently a matter of indirect inference; and when both are present in the preparation being examined, the presence of adventitious prothrombin in the reagent fibrinogen makes the interpretation of results excessively complicated and difficult.

To obtain a fibrinogen free from this defect we used a device indicated by Bordet and Delage [1912]. These authors showed that an oxalated plasma, exposed to the adsorbing influence of precipitated barium sulphate, becomes indefinitely stable on recalcification, and will no longer clot when kinase is added in any amount, but yields a good clot with thrombin.

A plasma so treated ought, therefore, to yield a fibrinogen free from prothrombin. We find that it does so. A fair amount of the fibrinogen is apparently lost on the adsorbent barium sulphate; but enough remains to make easy the preparation of a strong fibrinogen solution. As a precipitant of fibrinogen we have used an equal volume of saturated sodium chloride, following Hammarsten [1879]. Mellanby suggests that Schmidt's earlier method of precipitating by dilution and acidification gives a less denaturated product. Our experience with oxalated rabbit-plasmata is quite contrary to this. We found that dilution and acidulation, even with the greatest care to attain the optimum reaction, produced in such plasmata a scanty precipitate, in an inconveniently large volume of fluid. This precipitate, collected by the centrifuge, was seldom perfectly soluble in neutral saline, and became less so if it were taken up in dilute sodium carbonate, and reprecipitated by dilution and acidulation. We therefore adopted the following routine procedure, which is similar to that employed by Howell, except that the plasma was freed completely from prothrombin before precipitation.

Rabbits were etherised and bled from the carotid artery, through a paraffined cannula, into large centrifuge tubes. 10 cc. of a watery solution containing 1 % potassium oxalate with 0·9 % sodium chloride were put into

each tube as a preliminary. If the volume of blood obtained exceeded 90 cc., an extra amount of the oxalate solution was added as soon as the 100 cc. mark was attained. The oxalated blood was cooled and thoroughly centrifuged. The plasma was carefully pipetted off, rejecting the layer immediately in contact with the corpuscles and platelets.

A standard suspension of barium sulphate was prepared as follows. 31·5 g. of barium chloride were precipitated with a calculated slight excess of sodium sulphate, and washed repeatedly with 1 % sodium chloride solution by decantation, until the washings were free from sulphate. The total volume was then made up with 1 % sodium chloride to 200 cc. To every four volumes of oxalated plasma were added three volumes of the well-shaken suspension of barium sulphate.

The mixture of plasma and barium sulphate was well stirred, and allowed to stand for some hours—usually over night. It was then centrifuged clear, and the plasma poured off from the heavy barium sulphate. To each volume of the now diluted plasma was added an equal volume of a saturated solution of pure sodium chloride. Precipitation occurs rather slowly. As soon as separation was complete—i.e. usually after about an hour—the precipitate was separated with the help of the centrifuge, dissolved in 2 % sodium chloride, and again precipitated with an equal volume of saturated sodium chloride. The precipitate was collected again and dissolved in a convenient volume of 2 % sodium chloride. A trace of sodium hydrate was added if necessary to assist solution, and a measured equivalent quantity of standard hydrochloric acid then added to neutralise. The solution was centrifuged clear from any flakes of denaturated material, if such were present, and finally diluted with water to a content of 1 % sodium chloride. Such a solution is, apparently, indefinitely stable if kept cold. It yields a hard clot in a few minutes if a drop of active thrombin be added to a few drops of the fluid. The following represents the usual test and its result:

Fibrinogen solution 5 drops + 1 % sodium chloride 10 drops + thrombin solution 1 drop. Put into bath at 37°.—Clotted firmly in 2½ minutes.

[In recording subsequent experiments we adopt the convention of using only the name of a clotting factor to indicate the stock solution of it here described. The figure following it indicates the number of drops added. Saline = 1 % pure sodium chloride. Calcium = 1 % calcium chloride. All determinations were done in a water-bath at 37°.]

On the other hand, neither of the other clotting-factors can be made to clot such fibrinogen solutions when added separately. With either prothrombin

and calcium, or kinase and calcium, using these terms as defined below, no trace of clotting could be observed, however long the incubation was continued.

2. *Prothrombin.* It is evident that the definition of "prothrombin" involves adhesion to one or the other of the two theories mentioned above. Since one of our objects was to discover additional facts which would not, as are so many of those hitherto available, be explicable on either theory, we were obliged to make our definition in a sense experimental. Previous workers have obtained prothrombin preparations, which either failed altogether to clot fibrinogen with calcium only, or did so extremely slowly, and had their action enormously accelerated by kinase. On the Morawitz theory, such preparations are prothrombin, either pure, or contaminated by traces of thrombin. On Howell's view, they are prothrombin which cannot become thrombin in the presence of calcium, on account of associated antithrombin. If, therefore, we could prepare a prothrombin which showed no sign of activation with calcium alone, which rapidly yielded thrombin when kinase in addition was added, and which contained no demonstrable antithrombin, we should have good reason for not accepting Howell's view. There would remain, in regard to that view, only the duty of showing how Howell's evidence in its favour could be explained along other lines. We are convinced that we have on numerous occasions obtained such preparations of prothrombin, practically free alike from thrombin and from antithrombin, not activated by calcium alone, but readily by kinase in addition. One method of obtaining such is the treatment of fowl's plasma with chloroform under appropriate conditions, which we discuss in a later section. Such preparations, however, are very unstable, for reasons which we there discuss. Moreover the conditions governing their production are so complex, that there is an element of uncertainty in the method, as we shall also show later.

Mellanby's method of preparing prothrombin from fowl's plasma is admirable as a practical process. From the controversial point of view it has two defects. It is difficult to add just so much thrombin that all the fibrinogen is removed from the solution containing it and prothrombin, and no excess of thrombin left. One easily obtains prothrombin with some fibrinogen, still capable of yielding a weak clot when kinase and calcium are added; and one easily obtains prothrombin with so little thrombin, that a quantity of the preparation, which clots fibrinogen in a few seconds after treatment with kinase and calcium, will clot it only after half an hour or

more, when added with calcium alone. But this presence of a trace of thrombin gives to the action of the kinase the appearance of a mere acceleration. And this is important, as being capable of reconciliation with Howell's ideas; since Mellanby's method unmodified gives no guarantee against the presence of some antithrombin, albeit in traces which would not seem to us significant. By a slight modification, however, prothrombin, free alike from perceptible traces of thrombin or antithrombin, can be obtained.

In a fowl anaesthetised with ether the main artery of the leg is exposed, isolated, tied, and clamped proximally to the ligature with bull-dog forceps. The surface is washed carefully with oxalated saline, a cut with clean scissors made between ligature and bull-dog clamp, and the interior and cut surface washed through a capillary pipette with the oxalated saline. A clean glass cannula is then tied in and the blood allowed to flow straight into a large centrifuge tube. In this the blood is rapidly cooled to nearly 0° C., and is then centrifuged for about 15 minutes at 4000 revolutions per minute with a mean radius of 20 cm. The clear plasma is pipetted off. If stored cold, such a plasma seems to remain indefinitely stable. It does not clot at 37° C. within any reasonably limited time of observation. If diluted with two or three volumes of water, it usually clots after some hours.

A mixture of fibrinogen and prothrombin can be precipitated from such a plasma, as Mellanby describes, by diluting with twenty volumes of water and bringing the reaction to the isoelectric point of the globulins by adding acid. The association of fibrinogen and prothrombin in this precipitate is regarded by Mellanby as indicating their association as a preformed complex in the plasma. The force of this suggestion seems to us to be weakened by certain observations of our own. We have already described how fibrinogen can be prepared free from prothrombin by adsorbing the latter with barium sulphate. Mellanby showed that prothrombin was left in solution when a fibrinogen-prothrombin mixture was clotted with thrombin. We have now to add evidence that the two can be separated by fractional precipitation, brought about by careful reduction of electrolyte content. It seems to us, therefore, that the regularity with which fibrinogen and prothrombin occur together, in precipitates obtained by diluting and neutralising plasma, merely signifies that prothrombin has the solubilities of a euglobulin, like fibrinogen. It appears, however, to be soluble in a weaker solution of pure sodium chloride than is required to dissolve fibrinogen. The details of an actual preparation will explain the point.

50 cc. of clear, fresh fowl's plasma were diluted with 1 litre of distilled

water. One-tenth normal acetic acid was then added till maximum precipitation was obtained, 15·75 cc. being required. The precipitate was collected by the centrifuge, rubbed up with distilled water and again separated. It was then dissolved in 18 cc. of 1 % pure sodium chloride solution. The solution still showed a trace of the yellow colour of the original plasma. 11 cc. of it were poured into a glass vessel and warmed to 37° in a water-bath. A stock solution of thrombin was added, one drop every half-hour, till a weak clot began to form. The solution was then removed and allowed to stand at laboratory temperature over night. Next morning the now solid clot was broken up, and the serum removed. The object was to remove most of the fibrinogen without leaving any excess of thrombin in the serum. This was apparently attained, since five drops of the serum failed to clot five drops of fibrinogen solution in several hours. The serum, however, still contained some fibrinogen, since a sample yielded a soft clot in a few minutes on adding kinase and calcium. It also contained a remnant of "antithrombin," as we shall show, and was, therefore, further treated as follows. It was first dialysed for 4 hours against distilled water in a collodion tube. A fairly copious precipitate formed, and a specimen of the supernatant fluid on testing was found to be relatively weak in prothrombin, and to possess a weak, but distinct antithrombic action. The contents of the dialysing tube were therefore poured out and spun in the centrifuge. The supernatant fluid was then poured off, being retained for a test of its antithrombic action, and the precipitate was dissolved in 10 cc. of 1 % sodium chloride. On dilution with 10 cc. of distilled water no precipitation occurred; but on adding a further 10 cc., reducing the sodium chloride content to 0·33 %, a flocculent precipitate was formed. This contained practically all the fibrinogen; for the supernatant fluid, separated clear by the centrifuge, no longer gave any trace of clot with kinase and calcium, though it contained a powerful prothrombin. This fluid was, indeed, such a powerful prothrombin that it was diluted with twice its volume of 1 % sodium chloride, for routine use. Experimental details may be given of the tests made of its properties, since the results seem to us to constitute a very clear refutation of Howell's theory. On this theory, the solution, having all the properties of prothrombin in a high degree, should (*a*) yield thrombin on treating with calcium ions alone, or (*b*) contain at least a definitely recognisable amount of antithrombin. We find no trace of either action.

EXPERIMENT 89 (*b*).

Test of solution (*S*) *for thrombin.*

(*a*) Preformed.

Fibrinogen 5 + saline 7 + *S* 5.—No clot in 1 hour.

(*b*) Formed in presence of calcium ions.

Fibrinogen 5 + saline 7 + calcium 1 + *S* 5.—No clot in 1 hour.

Test for prothrombin.

S 5 + calcium 1 + kinase 2—incubated together 10 minutes; then added: Fibrinogen 5 + saline 7.—Hard clot in 30 seconds.

Test for "antithrombin." In using, for convenience, the term "antithrombin," we have no intention of committing ourselves to the assumption that plasma or serum contains a definite substance, having a specific affinity for thrombin, and a neutralising effect on its action. What is definitely known is, that plasma and serum contain some substance or substances which hinder and delay clotting. Some may, and probably do, act by becoming physically associated with the thrombin; others may hinder the gelation of the fibrin. Howell seems to assume that the substance, which delays the action of formed thrombin, is the same as that which, on his theory, hinders the combination of prothrombin with calcium to form thrombin. The reason for this assumption is not clear to us, but a good deal of Howell's evidence is directed to showing that kinase weakens the effect of the factor delaying the action of formed thrombin. We are, therefore, entitled to suppose that the presence of this factor in a prothrombin, which is not activated by calcium ions alone, is essential to his argument. This factor has been shown by Howell, among others, to be stable at 60°; and since our thrombin contains traces of kinase, all solutions containing prothrombin had to be freed from the latter by heating to this temperature before their antithrombic value could be fairly tested. In comparative tests, like the following, the preparations were heated side by side, in the same water-bath, being immersed and removed together. The thrombin solution was diluted so that one drop, added to the standard fibrinogen dilution, caused clotting in about 10 minutes. In addition to the reagents already specified, we prepared heated fowl plasma, and heated solution of prothrombin (*S*), the heating being carried out by immersion in a water-bath at 60° for 20 minutes. The following are the tests and their results:

EXPERIMENT 89 (*b*) (*continued*).

1. (Control.) Fibrinogen 5 + saline 12 + diluted thrombin 1.—Clotted in 11 minutes.

2. Fibrinogen 5 + heated prothrombin 7 + saline 5 + thrombin 1.—Clotted in 11 minutes.

3. Fibrinogen 5 + heated plasma 7 + saline 5 + diluted thrombin 1.—No clot in 3 hours. Found clotted next morning—16 hours.

It is quite clear, then, that the prothrombin solutions contain no recognisable fraction of the substance inhibiting thrombin action, which can be demonstrated in the original plasma. When the original fibrinogen + prothrombin precipitate, obtained according to Mellanby's directions, was purified by redissolving, and then again precipitating by dialysis, the supernatant fluid from the dialyser was found to contain a trace of the antithrombin, as the following results show. The solutions were heated together to 60° as before, but a thrombin of twice the strength was used in this experiment.

1. (Control.) Fibrinogen 5 + saline 12 + thrombin 1.—Clotted hard in $5\frac{1}{2}$ minutes.

2. Fibrinogen 5 + saline 5 + heated prothrombin 7 + thrombin 1.—Clotted hard in $5\frac{1}{2}$ minutes.

3. Fibrinogen 5 + saline 5 + heated supernatant fluid from dialyser 7 + thrombin 1.—This also showed signs of clotting after $5\frac{1}{2}$ minutes in the thermostat, but the clot was soft, and was not properly hard even in 1 hour.

4. Fibrinogen 5 + saline 5 + heated plasma 7 + thrombin 1.—An extremely soft clot began to form after 11 minutes, and was still soft after 1 hour.

We have found, in experiments on the effect of varying quantities of antithrombin, that a delay of the firm setting of the clot appears with amounts which no longer clearly delay its first appearance. We feel entitled, therefore, to conclude that small quantities of the antithrombin of plasma adhere to the precipitate of fibrinogen and prothrombin, obtained from the plasma by dilution and adjustment of reaction. These, however, can be removed by reprecipitating the mixed euglobulins by dialysis. There is no indication of the formation of thrombin from the prothrombin during the process, nor on the subsequent addition of calcium salts. On the subsequent addition of kinase, thrombin is formed, and, if fibrinogen is present, this is clotted. A further series of tests may be quoted, to illustrate this last point, together with the other properties of a preparation of prothrombin made according

to Mellanby's directions, without further purification. This specimen, when freshly prepared, contained some thrombin, and probably, from previous experience, traces of antithrombin. After keeping in the cold for a few days, no thrombin or antithrombin could be detected in it. Physiologically it was now a pure and powerful prothrombin, though its activity in this respect had also undergone slight decay.

EXPERIMENT 88.

1. *Test for prothrombin.*

Solution 5 + calcium 1 + kinase 2.—Incubated 10 minutes.

Then added fibrinogen 5 + saline 7.—Clotted in 1½ minutes.

2. *Test for preformed thrombin.*

Fibrinogen 5 + saline 10 + solution 5.—No clot in 3 hours.

3. *Test for formation of thrombin with calcium ions, but no kinase.*

Fibrinogen 5 + saline 7 + solution 5 + calcium 1.—No clot in 1 hour.

Then added: kinase 2—clotting began in 12 minutes, and was complete in 17 minutes.

4. *Test for antithrombin*, in solution heated to 60° for 8 minutes.

As a control we used, in this case, a fowl-plasma which had lost some of its antithrombin value by keeping. It was heated to 60° for 20 minutes.

Control 1. Fibrinogen 5 + saline 12 + diluted thrombin 1.—Clotted hard in 18 minutes.

Control 2. Fibrinogen 5 + heated fowl-plasma 7 + saline 5 + diluted thrombin 1.—Weak, soft clot in 1½ hours.

Test. Fibrinogen 5 + heated solution 7 + saline 5 + diluted thrombin 1. —Clotted hard in 18 minutes.

Here, then, we have again a prothrombin, which contains no demonstrable antithrombin. It is activated by kinase in the presence of calcium to a powerful thrombin, but even prolonged incubation with calcium alone fails to produce any thrombin from it; the only effect of such long incubation, with calcium and fibrinogen, is an apparent delay in the subsequent activating effect of kinase.

3. *Thrombin.* We have not prepared a pure thrombin solution. We required thrombin mainly as a test for the continued presence of fibrinogen in a solution, which has failed to clot with other reagents, and for testing the antithrombic effect of various preparations. Provided the latter contained

no prothrombin, which was excluded by previous heating, the existence in our thrombin of a trace of kinase, which it undoubtedly contained, was immaterial. It was prepared by whipping the fibrin from rabbit's blood, as shed from the artery; washing the fibrin till white by kneading with water; and then extracting with 8 % sodium chloride, according to Howell's modification of Gamgee's method. The thrombin so obtained was, as a rule, unnecessarily powerful for general use. It was usually kept in the strong solution, and portions diluted as required. Diluted with an equal volume of water, it gave a solution of which one drop caused firm clotting of our standard mixture of 5 drops of fibrinogen + 10 drops of saline in 2 or 3 minutes. For detecting small amounts of antithrombin it was diluted further, so that one drop clotted such a mixture in 10 to 20 minutes. After heating to 60° the strong solution showed no thrombic action, failing to clot pure fibrinogen solutions in any period. A weak activating (kinasic) effect on prothrombin in the presence of calcium remained after such treatment.

4. *Thrombokinase.* As a source of kinase, following Mellanby's recommendation, we used the fowl's testis, stripping off the capsule with its blood-vessels. The pulp was allowed to stand at room temperature for a day, and then treated by Howell's method—i.e. it was ground up with sand, dried, and then rubbed up with glycerol. The mixture was centrifuged and the turbid supernatant fluid, unfiltered, was diluted as required with two volumes of distilled water. Such dilutions appeared to keep well for a week or two, and then suddenly to lose their effect. The strong glycerol extract seemed to be indefinitely stable.

The Effect of Chloroform on Plasma, etc.

As stated in the introduction, our ultimate object is a closer analysis of the effect of extraction with chloroform, and other forms of treatment, in imparting toxicity to sera. A more immediate interest was given to the matter, from our present point of view, by the discovery that shaking with chloroform, which has been shown by Jobling and his associates to destroy antitrypsin in serum, very rapidly and completely destroys the antithrombic action of serum or plasma. Since we first observed this effect, a paper has appeared by Minot [1915], in which he shows that shaking oxalated mammalian plasma with a little chloroform will frequently induce clotting. He, rightly in our opinion, attributes this to the effect of some preformed thrombin, which is able to act when the antithrombin has been destroyed.

He further reaches the conclusion that chloroform does not convert prothrombin into thrombin as Howell [1910] had originally supposed. Minot's meaning seems to be that the mere removal of antithrombin, in the absence of calcium, does not activate Howell's preparation of prothrombin, of which we consider the properties in another section.

Our own first experiments with fowl's plasma gave results which, at first sight, seemed to be strongly in favour of Howell's theory. On shaking a small volume of fowl's plasma with twice its volume of chloroform, keeping the mixture warm in a water-bath at 37°, we found that, after about 10 minutes, the emulsion suddenly acquired a semi-solid consistency, as though clotting had occurred. The now permanent emulsion was kept at 37° for about an hour, and then removed to centrifuge tubes, and spun rapidly for about 15 minutes. The clear, supernatant watery layer was pipetted off and tested. It was found to possess a very powerful thrombin action. After dilution to five times its volume, one drop clotted the standard fibrinogen mixture in 2 minutes. After heating to 60° the preparation was found to be devoid of antithrombic action. This observation lends itself readily to interpretation in accordance with Howell's views. Fowl's plasma is known to contain much prothrombin, and a good deal of antithrombin. According to Howell, it is this latter which prevents the prothrombin from becoming thrombin by union with calcium ions. We have removed the antithrombin by chloroform treatment, and thrombin is formed. To that extent, therefore, the expectation is fulfilled. A few further experiments, however, sufficed to dispel this plausible interpretation of the effect.

In the first place, the fluid obtained by chloroform treatment of the fowl-plasma, readily produced clotting of many times its own volume of the plasma from which it was produced, or of any other stable fowl-plasma. For example, the following mixtures were placed in the bath at 37°, with results as indicated:

EXPERIMENTS 48 AND 49.

1. Fowl's plasma—5 drops.

Saline 1 drop.—No clot in 24 hours.

2. Fowl's plasma—5 drops.

Chloroform-treated fowl's plasma (as above)—1 drop.—Clotted firmly in $1\frac{1}{2}$ minutes.

3. Fowl's plasma—10 drops.

Chloroform-treated fowl's plasma—1 drop.—Clotted firmly in $2\frac{1}{2}$ minutes.

If the activation of the plasma were to be explained as the result of removal

of antithrombin, the result in tube No. 3 would oblige us to conclude, that the loss of not more than 1/11 of the total antithrombin present in a sample of the plasma allows the formation of sufficient thrombin to clot the whole in a few minutes. And this conclusion would be wholly at variance with all other experience of the neutralising effect of antithrombin on the action of thrombin. If we are dealing with a sample of antithrombin, of which eleven drops so delay the action of a given dose of thrombin that the clotting-time is lengthened from, say, 10 minutes to 5 hours, then we shall find that ten drops of the same antithrombin prolong the clotting-time to $4\frac{1}{2}$ or $4\frac{3}{4}$ hours, perhaps; but that clotting should occur in $2\frac{1}{2}$ minutes with ten drops, and be altogether inhibited by eleven drops of an antithrombic preparation from plasma or serum, is an impossible result.

It might possibly be urged that this argument only applies to the free antithrombic constituent of serum or plasma, which survives heating for some minutes to 60°; and that the possibility remains of prothrombin being associated in plasma with some other thermolabile inhibitory factor, which is similarly liable to the destructive action of chloroform. Such a suggestion, however, has no direct bearing on either the evidence or the theory which we are discussing. The only factor, of whose destruction by chloroform we have direct evidence, is thermostable; and it is an essential feature of Howell's view, if we rightly understand it, that it postulates an association between prothrombin and this thermostable antithrombin, which is not readily destroyed at 60°; and that it attributes to the effect of this factor the inability of calcium alone to activate a prothrombin preparation. It is easy, moreover, to dispose of the suggestion by experiment. If the activation of fowl-plasma by chloroform were due to the removal of an antithrombic factor of any kind, it should clearly be possible to activate, by similar treatment, a prothrombin solution prepared from that plasma. This we have repeatedly attempted, with no hint of success. If a prothrombin, prepared from fowl's plasma according to the indications of a previous section, be shaken with chloroform, either in the cold or at 37°, the prothrombin is fairly rapidly destroyed, but no trace of thrombin appears. As long as activity of any kind remains, it is that of prothrombin—i.e. of a substance which clots fibrinogen only after treatment with calcium and kinase, and never with calcium alone.

We may conclude, then, that the activation of fowl-plasma by chloroform is due neither to the removal of antithrombin, revealing a previously neutralised thrombin, nor to direct action of the chloroform on the prothrombin. To elucidate the nature of the action we decided to examine the different

layers of the plasma-chloroform emulsion, after different periods of treatment at varying temperatures, for the presence of the several known clotting-factors, using the set of reagents previously described.

It should be stated at once that the results obtained, though all pointing to the same conclusion, showed considerable variation. After shaking a specimen of stable fowl-plasma for a given time and at a given temperature with chloroform, separating the emulsion by the centrifuge, and then examining separately the clear supernatant watery layer, and the emulsion of more or less denaturated material separating with the chloroform-layer, we found it impossible to prophesy, in any given case, the nature and distribution of the clotting factors which we should find. It varied, not only with the plasma from different fowls, but with the same sample of plasma, after different periods of storage in the cold-room. The reason of this variability in the result will be sufficiently clear, when we have described the experiments which demonstrate the complexity of the processes at work. We mention it here in explanation of the fact, that we choose, for extended description, experiments in which the conditions made it possible to follow clearly the stages of the production of thrombin. No results were obtained which were inconsistent with the conception thus obtained; but many in which intermediate stages were missed, or in which, owing to changes which will later become clear, the process was incomplete.

The procedure adopted was, in its general features, identical in all cases. To one volume of the plasma we added two volumes of chloroform. It is quite probable that a much smaller volume would have effected the changes which we describe; but to experiment with different proportions would have made the number of tests required unmanageably great. After the two had been shaken together under the conditions of the particular experiment, the mixture was thoroughly centrifuged. The clear watery layer could then be poured off clean from the stiff emulsion which formed the upper portion of the chloroform-layer. The watery layer was, if necessary, freed from traces of dissolved chloroform by blowing air over it. The chloroform-layer was then stirred, and the free, fluid chloroform poured away. The curdy emulsion left was then rubbed up with a volume of 1 % sodium chloride solution equal to twice the volume of plasma taken and over this a vigorous stream of air was blown until all chloroform was removed. Part of the material passed into solution during this process; part, in most cases, was rendered permanently insoluble by the denaturating effect of the chloroform.

We had, then, for examination the supernatant watery layer, and the

saline extract of the chloroform-layer. These will be referred to as "watery layer" and "chloroform-layer" respectively.

A preliminary, incomplete experiment may first be quoted.

EXPERIMENT 52.

A sample of fowl-plasma was shaken with chloroform till a permanent emulsion was formed, and this was left over night at laboratory temperature. Next morning the mixture was centrifuged and the two layers examined. The watery layer had a rather weak thrombin-action, as the following experiment shows:

Fibrinogen 5 + saline 5 + watery layer 1.—Clotted in 12 minutes.

The chloroform-layer, on the other hand, was much weaker in this respect:

Fibrinogen 5 + saline 5 + chloroform-layer 1.—Clotted in 50 minutes.

But the chloroform-layer, though having but weak thrombin action, revealed itself, on incubation, with prothrombin and calcium, as an effective kinase.

Prothrombin 5 + calcium 1 + chloroform-layer 2, incubated for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted in 4 minutes.

Here, then, was an indication that the chloroform treatment produces from the stable plasma something which functions as a kinase. In this experiment no attempt was made to study its distribution between the layers; it was merely shown that it was present in the chloroform-layer, which contained little thrombin. We shall show, by quoting other experiments, that this distribution between the layers is very variable. Several of the earliest of our complete experiments gave the impression that the kinase, after the centrifuging of the emulsion, was practically limited to the chloroform-layer. Later, however, we met with cases in which, after apparently identical treatment of a plasma, the watery layer had a very high kinasic value, while that of the chloroform-layer was hardly perceptible. We shall discuss the probable meaning of these discrepancies after illustrating them. For the present, the point of importance is that the chloroform-treatment caused the appearance of kinase in plasma, which certainly did not contain it before, since the untreated plasma was indefinitely stable even at 37°. We may add at once, also, that after treatment with chloroform at 37° for several days, kinase is the sole clotting-factor which can be discovered in either the watery layer, or in what remnant of water-soluble constituents the chloroform-layer then contains. In one such case the incubation was

carried on from a Saturday afternoon to the following Monday morning. After centrifuging, the watery layer was tested as follows:

1. *Test for thrombin.*

Fibrinogen 5 + saline 10 + watery layer 4.—No trace of clotting in 1 hour.

One drop of a known thrombin was then added to the tube and produced a firm clot in a few minutes. We may conclude, then, that the fluid under examination contained no thrombin.

2. *Tests for kinase.*

Prothrombin 5 + calcium 1 + watery layer 2, incubated together for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted firmly in less than 30 seconds.

The clotting-time being so short, the test was repeated, exactly as above, using, however, the watery layer diluted, first four times, and then twenty times. The clotting times were 2 minutes and 16 minutes respectively. Clearly, then, we were left with a very powerfully kinasic solution, while all thrombin, and necessarily all prothrombin, had vanished.

This discovery, of the formation of kinase in plasma treated with chloroform, at once suggested an explanation of the conversion of prothrombin into thrombin in a plasma so treated.

It will be clear that the combination of effects, thus demonstrated as resulting from the action of chloroform, gives us another opportunity of putting to the test the rival theories with which we are here concerned. On the Morawitz-theory the formation of thrombin is obviously explicable by the action of the freshly-liberated kinase on the prothrombin, in the presence of calcium. On Howell's theory the disappearance of the antithrombin would be the decisive feature of the action, the appearance of kinase only having significance, in so far as it assisted this. It occurred to us that a clear indication, in support of one or the other of these views, might be obtained by carrying out the chloroform treatment in the absence of calcium. It seemed probable that antithrombin would still be destroyed, and kinase still formed under such conditions, though activation of prothrombin would not take place till calcium was added. If, then, we could effect a separation of the prothrombin and the newly-formed kinase by the centrifuge, the effect of adding calcium would give us a definite answer; for on Howell's theory the layer containing antithrombin-free prothrombin should form thrombin with the calcium only, while on the Morawitz theory, admixture with the kinase-containing layer should be necessary before activation

occurred. All the conditions indicated have been realised in a series of experiments, and the result seems to us quite decisively against the theory of Howell, and in favour of that of Morawitz.

We may quote first an experiment on the action of chloroform on a further sample of the plasma used in Experiment 52.

EXPERIMENT 53.

To 15 cc. of plasma was added 1·5 cc. of 1 % potassium oxalate. 30 cc. of chloroform were then added, and the whole shaken mechanically at laboratory temperature for 1 hour. It was then thoroughly centrifuged and the two layers examined separately as described above.

Tests of the watery layer.

(1) *For thrombin.*

(*a*) Fibrinogen 5 + saline 10 + watery layer 5.—No clot after 2½ hours.

(*b*) Fibrinogen 5 + saline 10 + calcium 1 + watery layer 5.—No clot after 2½ hours.

Therefore no thrombin was present, and none was formed on addition of sufficient calcium chloride to remove excess of oxalate, and secure the presence of free calcium ions.

On adding two drops of kinase to each of the above tubes, at the end of 2½ hours, (*a*) showed no sign of clotting in a further hour, while (*b*) clotted in about 12 minutes—thus showing that it contained an effective amount of free calcium, as well as prothrombin.

(2) *For prothrombin.*

Watery layer 5 + calcium 1 + kinase 2—incubated together for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted in 4 minutes.

Therefore a moderate amount of prothrombin was present; and since, as shown under (1), no thrombin was formed with calcium alone, no intrinsic kinase can have been present in the watery layer.

(3) *For antithrombin.*

A weak thrombin was used, of which four drops clotted the standard fibrinogen dilution only after 10 minutes.

(*a*) Fibrinogen 5 + saline 11 + thrombin 4 + calcium 1.—Clotted in 10 minutes.

(*b*) Fibrinogen 5 + heated watery layer 8 + saline 3 + calcium 1 + thrombin 4.—Clotted hard in 14 minutes.

(*c*) Fibrinogen 5 + heated fowl-plasma 8 + saline 3 + calcium 1 + thrombin 4.—*Not* clotted in 100 minutes.

The trivial delay of clotting, from 10 to 14 minutes, by the heated watery layer cannot be regarded as indicating the presence of antithrombin. It is an effect such as might be produced by a slight difference in saline concentration, or slight increase of the alkalinity of the mixture. It will be noted that, under the same conditions, the heated original plasma lengthened the clotting-time to over 100 minutes—beyond which period the observation was not continued.

Tests of the chloroform layer.

1. *Tests for thrombin.*

(*a*) Fibrinogen 5 + saline 8 + chloroform-layer 4.—No clot in 30 minutes.

(*b*) Fibrinogen 5 + saline 8 + chloroform-layer 4 + calcium 1.—No clot in 30 minutes.

Therefore no thrombin was present, and none was formed in the presence of calcium.

2. *Test for kinase.*

Prothrombin 5 + calcium 1 + chloroform-layer 4, incubated together for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted in 2 minutes.

Since, therefore, this layer contained plenty of kinase, it is clear that it contained no prothrombin, since the addition of calcium alone (under 1) did not produce any thrombin from it.

We have, therefore, in this experiment, by treating oxalated fowl-plasma with chloroform, produced kinase, which, however, in the absence of calcium, could not activate the prothrombin. Fortunately for the purpose of our enquiry, the kinase, in this instance, is found, after the emulsion has been centrifuged, to be limited to the chloroform-layer. The watery layer contains an adequate prothrombin, free from clearly recognisable antithrombin. Yet this prothrombin is not activated by treatment with calcium only; kinase in addition activates it promptly. We are entitled to conclude, therefore, that the formation of thrombin, when fowl-plasma, containing its normal calcium, is shaken with chloroform, is due to the production of kinase, which secondarily activates the prothrombin. The disappearance of antithrombin is an accidental accompaniment, not an essential feature of the process.

The clean separation of the prothrombin and kinase by the centrifuge, as might be expected, was not seen in every experiment of the kind. We may summarise a few of the varieties of result obtained in a long series of experiments, by indicating once for all that the tests for the different clotting-factors were made according to the following scheme:

Test for thrombin.

Fibrinogen 5 + saline 10 + specimen 4.

Test for prothrombin.

Specimen 5 + calcium 1 + kinase 2. After 10 minutes at 37°, add fibrinogen 5 + saline 5.

Test for kinase.

Prothrombin 5 + calcium 1 + specimen 2, and then as for prothrombin.

Test for antithrombin (on solution heated to 60°).

Fibrinogen 5 + specimen 7 + saline 5 + thrombin 1. (If the specimen was oxalated, calcium was added as well.)

EXPERIMENT 57.

A sample of the same fowl-plasma as used for Expts. 52 and 53 was oxalated, and then dialysed under pressure against distilled water, till all excess of oxalate was removed. It was brought back to the original volume with 1 % NaCl, and then centrifuged clear. It showed no tendency to spontaneous coagulation, even on adding calcium. It was treated with chloroform for half-an-hour, being kept ice-cold during the treatment. The watery layer contained no preformed thrombin, but, when calcium was added, it clotted fibrinogen after half-an-hour. If kinase were added as well (test for prothrombin) the combination clotted in 30 seconds. This watery layer, however, contained no trace of demonstrable antithrombin. The tube containing it clotted simultaneously, in 4 minutes, with the control tube containing an equal addition of saline; while the tube to which the oxalated and dialysed plasma, heated in parallel, was added, began to show a weak clot only after 53 minutes. The fact that the watery layer showed a trace of activation with calcium only could be fairly attributed to its containing a trace of kinase with its prothrombin. This view was supported by the discovery of kinase in the chloroform-layer. When four drops of the extract from the chloroform-layer were added to five of the watery layer, and the mixture incubated with calcium, it clotted fibrinogen in 9 minutes.

EXPERIMENT 62.

A sample of plasma from another fowl was oxalated, and was treated with chloroform in the cold for 15 minutes. A non-oxalated sample was treated in exactly the same way, and the two watery and chloroform-layers obtained were subject to a parallel series of tests. The watery layers of both contained abundant prothrombin, clotting fibrinogen in 15 seconds after preliminary treatment with kinase and calcium. The oxalated sample contained no thrombin, but a weak thrombin was present in that treated without oxalate (clot in 22 minutes). The chloroform-layers showed a stronger thrombin (6 minutes) in the case of the non-oxalated, none in that of the oxalated sample. Both contained kinase and prothrombin, so that the chloroform-layers, in this experiment presented a rather complex problem.

No antithrombin could be detected in either layer, with either sample.

EXPERIMENT 65.

The same plasma as above was treated, with and without oxalate, with chloroform for $1\frac{1}{4}$ hours at 37°. The watery layers were tested for thrombin, with the result that the sample without oxalate caused clotting in 1 minute, while the oxalated sample had produced no clot in an hour. Neither sample contained any antithrombin, clotting occurring simultaneously in the control, and in the tube containing the two samples. The time in each case was 3 minutes, and the three clots were indistinguishable in firmness. In a fourth tube, with the heated original plasma, a weak clot began to form after 30 minutes, and was still soft after several hours. The oxalated sample contained a fairly active prothrombin, causing clotting in $2\frac{1}{2}$ minutes after treatment with kinase and calcium. Yet, though it contained no antithrombin, it was not activated by calcium alone; the tube was watched for 38 minutes and then abandoned, as no sign of clotting had appeared.

EXPERIMENT 70.

This is quoted as an extreme instance of the variation in distribution of clotting-factors. The first experiment of the detailed series (No. 53) showed, in the case of an oxalated plasma, pure prothrombin in the watery, pure kinase in the chloroform-layer. In this instance, that of a plasma kept frozen for three days after bleeding, and then treated with chloroform for 1 hour at 37°, we find the exactly converse distribution. The finding of prothrombin and kinase, but no thrombin, although the plasma was not oxalated, is especially anomalous. We can only suggest that the precipitation of the

prothrombin with the curdy emulsion, which occurred in this instance owing to some unknown condition, protected it from activation by the liberated kinase, which, owing to some equally unknown condition, appeared in the watery-layer when separation was effected. Whatever be the cause, the watery-layer by itself had no effect on fibrinogen, and was equally inert after treatment with kinase and calcium; when, however, it was digested with prothrombin and calcium, the mixture produced clotting in $1\frac{1}{2}$ minutes, showing an effective kinase. The chloroform-layer was inactive by itself, and did not activate prothrombin, but caused clotting in 3 minutes after it had been treated with kinase and calcium, showing that it contained prothrombin.

Hitherto we have quoted instances in which at least one of the layers, water or chloroform, obtained by the treatment described, contained one of the recognisable clotting factors, practically uncontaminated by others. We met with other cases in which the conditions were less simple. We have records of experiments, for example, in which both layers had some immediate thrombic action, but showed evidence of the presence, in addition, of both prothrombin and kinase, in that their thrombic action was increased by incubation with excess either of kinase or prothrombin, in presence of calcium. A puzzling feature in the behaviour of such samples was that simple incubation, with calcium only, did not greatly increase their content of thrombin; indeed, if incubation were protracted, their activity rather declined. This seems difficult to reconcile with the presence of prothrombin and kinase in them. We believe that the clue to this anomaly is furnished by the fact, which early came to our notice, that the preparations either of prothrombin or of thrombin, but more especially of the former, obtained by the chloroform method, are remarkably unstable; the kinase, by contrast, being remarkably stable under the same conditions. While the prothrombin or thrombin may show a very high potential or developed activity when first obtained, it shows a very rapid decline even at laboratory temperature, which decline is greatly accelerated by incubation at 37°. One experiment, which we have already quoted, showed, as the final stage of this degeneration, a preparation after two days' incubation, in which only kinase could be recognised. We must suppose then, that the solution in which thrombin, prothrombin and kinase can be detected, contains also some factor by which prothrombin and thrombin are continually being destroyed, the former with great rapidity; and that this action is greatly accelerated by incubation. The thrombic action detectable at any period can probably be increased, therefore, either

by accelerating the formation of thrombin, by adding extra kinase before the incubation, or by supplying excess of prothrombin, so that the formation of thrombin, by the kinase already present, does not fail through lack of the rapidly vanishing prothrombin. What, then, is the cause of this peculiar instability of prothrombin and thrombin thus prepared? A hint was afforded by the observation that many of the clots obtained by the use of these preparations, while hardening rapidly on formation, subsequently softened again, and might be completely dissolved in the course of a few hours' incubation. The solution was not a reversal of the clotting process; the mixture now yielded no further clot on the addition of thrombin, and gave no coagulum on heating to 60°. Clearly the initially formed fibrin had been digested. This might, perhaps, have been anticipated from Jobling's demonstration of the liberation of a tryptic ferment in serum shaken with chloroform—an action which we have repeatedly confirmed. And this tryptic action, ensuing on the destruction of the antitrypsin by the action of chloroform, explained not only the instability of the thrombin or prothrombin obtained by this method; it furnished also a possible clue to the appearance of kinase, the resulting activation of the prothrombin, and the whole series of phenomena under discussion. We proceeded to put this possibility to the test, by treating fowl's plasma with trypsin.

Effect of Trypsin on Fowl-plasma.

The trypsin used was the dried preparation of Fairchild, and this was made up as a 1 % solution in water as required. The sample of fowl-plasma used was that employed in Experiment No. 62. This plasma, at the time of the experiment with trypsin, had been kept in the cold-room for a week.

A few preliminary experiments showed that the trypsin itself had no coagulant effect on fibrinogen, and no activating effect on prothrombin in the presence of calcium ions. It contained, therefore, neither thrombin nor kinase.

Like all specimens of natural plasma or serum, the fowl-plasma possessed a marked antitryptic action, and it was necessary, in the first instance, to discover the amount of trypsin required to overcome this effect. It was found that five drops of the 1 % trypsin solution, added to 10 cc. of the plasma, had no apparent effect on the clotting properties during incubation for 1 hour. At the end of that period, a sample of the mixture was as free from activating effect, when added to prothrombin with calcium, as the

original plasma was. When the proportion of trypsin was increased ten times, however, the effect was very soon apparent.

EXPERIMENT 80 (*b*).

To 1 cc. of fowl-plasma were added 5 drops of 1 % trypsin, and the mixture placed in the water-bath at 37°. Samples were removed at intervals, and tested according to the usual scheme for kinase, as follows:

Prothrombin 5 + sample 2 + calcium 1, incubated 10 minutes; then added:

Fibrinogen 5 + saline 5.

The following results were obtained:

With original fowl-plasma.—No clot after several hours.

With sample after 3 minutes' tryptic digestion.—Hard clot in 5 minutes.

With sample after 6 minutes' tryptic digestion.—Hard clot in 2 minutes.

With sample after 15 minutes' tryptic digestion.—Hard clot in 1½ minutes.

There is clear evidence, therefore, of the production of kinase by the action of trypsin on the plasma. The question at once arises, however, why the formation of the kinase does not result in the clotting of the plasma itself, since the latter contains fibrinogen, prothrombin and calcium. As a matter of fact, the plasma does clot eventually. In the experiment above recorded, the residue of the plasma-trypsin mixture, left in the water-bath, set to a soft clot 5 minutes after removal of the last sample, and therefore 20 minutes after the commencement of the incubation. This clot did not harden, but, on the contrary, soon showed signs of dissolution, and had nearly disappeared at the end of 3 hours. Evidently we were dealing with a complex action of the trypsin, which throws considerable light on the anomalies encountered in dealing with the action of chloroform. The trypsin not only sets free kinase, but simultaneously digests prothrombin, thrombin, fibrinogen, and, later, the fibrin, if any should be formed.

Since several authors have shown that the temperature-coefficient of the action of thrombin above 17° is very small, while that of trypsin is much larger, it occurred to us that the excess of thrombin formed over that destroyed, at any period of the digestion, might be demonstrated by removing samples from the warm bath, and allowing them to cool to the room temperature. This expectation was verified as the following record shows.

EXPERIMENT 80 (*a*).

5 cc. of fowl-plasma + 25 drops of 1 % trypsin, were placed in the bath at 37°, and samples of 0·5 cc. removed, at intervals, to separate tubes, and

allowed to cool. The times indicated are the intervals after removal from the bath at which clotting occurred.

Sample removed immediately.—Did not clot in 3 hours.

Sample removed after 3 minutes' incubation.—Did not clot in 3 hours.

Sample removed after 6 minutes' incubation.—Clotted hard in 22 minutes.

Sample removed after 15 minutes' incubation.—Clotted hard in 5 minutes.

The remainder clotted softly after 20 minutes, without removal from the warm bath, as described above.

In another experiment, using only one-half the proportion of trypsin giving the above results, we detected no production of kinase or other change in the plasma after 30 minutes' incubation. The proportion of trypsin was then increased to that used above (5 drops of 1 % solution to each cc. of plasma) and the formation of kinase, with clotting of the plasma on cooling to room-temperature, occurred as before.

In another experiment the plasma was oxalated, and 1 % trypsin added in the proportion of 5 drops to 2 cc. of plasma. Again nothing occurred after 30 minutes' incubation, and again, after adding a further equal proportion of trypsin, so as to bring the total up to that which proved active with unoxalated plasma, kinase was formed after a further 10 minutes' incubation, as the following test shows (Exp. 84):

Prothrombin 5 + sample 2 + calcium 1, incubated 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted hard in 4 minutes.

Naturally, in the absence of calcium ions, no clotting of the plasma occurred on cooling. Nor could any thrombin be detected by adding four drops to the usual fibrinogen-dilution.

It is quite evident that the results of treating fowl-plasma with trypsin are very similar to those obtained with chloroform, as described in the foregoing section. In both instances we have the liberation of kinase, which, if calcium is present, acts on the prothrombin of the plasma to form thrombin. With the knowledge that one effect of chloroform on plasma is to liberate trypsin, it seems justifiable to regard this as the primary action, to which the appearance of kinase and thrombin are secondary. It then becomes easy to understand why the effect of chloroform, on one batch of plasma, should vary with the time which elapses between the preparation of the plasma and the performance of the experiment. The fact, for example, that a plasma which, when fresh, readily gives a powerful thrombin on treatment with chloroform, may, after cold storage for a week or two, give only prothrombin, readily finds its explanation. In both instances the chloroform treatment

removes the antithrombic and antitryptic actions of the plasma. In the fresh plasma, active trypsin is thereby unmasked, with a resultant production of kinase and, unless calcium has been removed, of thrombin; in the older plasma, we must suppose that the trypsin has undergone decay, so that the only perceptible action of the chloroform is to remove antithrombin, and precipitate fibrinogen, leaving a solution in which prothrombin is the only clotting-factor which can be detected.

The supposed Antagonism between Kinase and Antithrombin.

Howell's [1911] conception of the function of kinase (thromboplastin) starts from the assumption that the substance, or substances, in plasma, which hinder the clotting action of formed thrombin, also hinder the activation of prothrombin by calcium. As we have already indicated, we can find no ground for this assumption, though it is peculiarly difficult to disprove. For the only test of the activation of prothrombin, namely, the appearance of thrombic activity, cannot be applied in the presence of substances by which that activity is inhibited. Howell, however, who considers the function of kinase (thromboplastin) to be the removal of antithrombin, permitting the activation of prothrombin by calcium, supports his conception by evidence designed to show that kinase neutralises the inhibitory action of normal or peptone-plasma, and of hirudin, on the action of formed thrombin.

It will be clear that, if evidence of this effect is to be unequivocal, it must be obtained under conditions which allow the balance between thrombin and antithrombin to be upset in only one way, namely, by the neutralisation of the antithrombin. If there is any possibility of an increase in the amount of thrombin present, the experiment appears to us of no value for the purpose. This criticism would exclude a considerable part of Howell's evidence on this point, in that the experiments were made with oxalated plasma, with the addition of calcium salts. According to Howell, "the acceleration in the time of clotting caused by the thromboplastin cannot be explained by assuming that it served to activate the prothrombin in the oxalated plasma, since the control experiment with calcium chloride showed that this substance alone was sufficient for the purpose." The control experiment in question showed, indeed, that, when calcium chloride was added to the oxalated plasma, sufficient thrombin was formed to clot the plasma in nine minutes. But it surely furnished no proof that the whole of the prothrombin was activated in that time, and that the addition of tissue extract would cause no increase

in the amount of thrombin formed. There is at least a possibility here of the balance between thrombin and antithrombin being upset by addition of thrombin, rather than removal of antithrombin.

The same criticism applies with even greater force to the experiments illustrating the activation of "a preparation containing both prothrombin and antithrombin" by tissue extract. To assume that the action, in this case, is a removal of antithrombin, would be to beg the whole question at issue.

If these experiments are excluded, as giving results which are explicable, with at least equal ease, on the Morawitz theory, there is left, of Howell's evidence, only the series in which fibrinogen solution, or oxalated and dialysed plasma were used, without addition of calcium. In this series the "antithrombin" used was not heated plasma, but hirudin. And the nett result of the experiments is to show that, while a proportion of hirudin, which neutralises the action of thrombin indefinitely, generally does so still, if tissue extract be added as well, smaller proportions, which greatly delay the action of thrombin, generally delay it to a smaller extent in the presence of tissue extract. The degree, in which the tissue extract weakens the hirudin action, may be gathered from the clotting times of parallel experiments.

Hirudin	With tissue extract	Without
1 drop	Less than an hour	2 hours 15 minutes
2 drops	3 hours	6½ hours (weak)
3 „	No clot	No clot
2 „	30 minutes	2–3 hours (imperfect)
4 „	2 hours 15 minutes	No clot in 24 hours

There are other experiments in which the difference produced by "thromboplastin" is much less than this. But, in the best case, what does it amount to? The effect of hirudin, sufficient to prolong clotting beyond 24 hours—say, for practical purposes, indefinitely—is so weakened, that clotting occurs after little more than two hours. But the effect, which the action thus demonstrated is supposed to explain, is something of a different order altogether. A fowl-plasma, for example, which remains stable for weeks or months, clots in a few minutes if some kinase be added. When, further, the notorious instability of hirudin is remembered, together with the presence of proteolytic ferments in tissue extracts, it becomes clear that there are several possibilities needing exclusion, before evidence like the above can be accepted as showing any genuine antagonism between thrombokinase and the antithrombin of plasma.

In our own experiments, made to test the existence of such an antagonism, we attached great importance to the exclusion of prothrombin from all the reagents used. We are testing the resultant action of a mixture of thrombin and antithrombin. If any prothrombin is present, and calcium not excluded, the addition of kinase will increase the balance in favour of thrombin. We may describe in detail an experiment in which these precautions were observed.

Our fibrinogen was a sample which had been rigidly tested in this direction, and found to be indefinitely stable with calcium and kinase. The antithrombin, obtained by heating fowl-plasma to 60° for 20 minutes, and filtering from coagulated fibrinogen, was subjected to a thorough test. Four tubes were put up, each containing 5 drops of fibrinogen solution, 2 drops of kinase, and 1 drop of calcium chloride (1 %). To these we added 1, 2, 3 and 4 drops respectively of the heated fowl-plasma. There was no sign of clotting in any tube after 24 hours. Therefore, so far as traces of prothrombin can be detected in the presence of antithrombin, we may conclude that the sample was prothrombin free.

The thrombin was obtained by shaking fresh fowl-plasma with chloroform and digesting for 1 hour at 37°. It was an extremely active specimen, and needed diluting many times to render its clotting-time susceptible of accurate measurement. After diluting 10 times, 1 drop clotted the usual fibrinogen dilution in 20 minutes. Two tubes were put up with this amount of thrombin and fibrinogen; to each was added one drop of calcium chloride, and to one of them 2 drops of kinase. Both clotted simultaneously. We may conclude, therefore, that the thrombin contained no remnant of prothrombin. That it contained traces of kinase is probable; but that would not affect the validity of the experiment.

After a few preliminary tests, to get a suitable mixture of thrombin and antithrombin, we put the following mixture into a tube and placed in the bath at 37°:

Fibrinogen 6 + antithrombin 15 + 4 times diluted thrombin 1 + calcium 1. After 10 minutes' incubation we divided the solution into two equal portions, added 2 drops of kinase to one, and returned both to the water-bath. It is peculiarly difficult to time accurately the beginning of the clotting-process in the presence of antithrombin, which not only delays the onset of the process, but also makes the hardening of the clot much slower. No difference, however, could be detected between the two tubes in this experiment. In both, the first signs of increasing viscosity occurred about 20 minutes from the first mixing, and the clots in both tubes were equally firm at 25 minutes.

A similar experiment was made, increasing the antithrombin to 20 drops, with 1 drop of diluted thrombin as before. After 5 minutes' incubation the mixture was divided again, 2 drops of kinase being added to one-half, and 2 drops of 30 % glycerol to the other. In each tube the first thickening began after about 40 minutes, and the clot became firm in about 50 minutes. Several similar experiments were performed, using fibrin-extract as thrombin, and heated rabbit or guinea-pig serum as antithrombin. In no case could we detect any definite acceleration of the clotting process by kinase. Yet the proportions of kinase were such as, when added to indefinitely stable fowl-plasma, produced clotting in a few minutes.

We are compelled, therefore, on this point also, to range ourselves with the earlier observers (Morawitz, Fuld and Spiro), and in opposition to Howell, in our inability to detect any real antagonism between kinase (thromboplastin) and antithrombin.

Howell's Prothrombin.

One of the greatest difficulties of any discussion on blood-clotting is the use of the current nomenclatures without prejudice to the point in dispute. In accordance with the original employment of the term by Schmidt, we have used "prothrombin" to denote the inactive precursor of thrombin, which is activated by kinase in the presence of calcium. Howell regards a preparation showing these properties as a complex of the thrombin-precursor with antithrombin. It is necessary to remember that, having supported this view of the constitution of prothrombin, by evidence which we have already discussed, Howell thereafter applies the name "prothrombin" to preparations which he regards as free from antithrombin, since they need only calcium for their activation. Such a preparation he obtained from cat-plasma by precipitating with acetone, washing the precipitate with ether, and drying in a current of warm air. Though we have followed Howell's directions as closely as possible, we have hitherto failed to obtain from rabbit-plasma a preparation yielding thrombin on treatment with calcium alone, though our preparation was readily activated when treated with kinase in addition. We found no evidence of antithrombic action after the preparation had been heated to 60° for 4 minutes. We hope to be able to obtain a preparation corresponding in properties to Howell's, and propose, in that event, to examine it for the presence of kinase by use of the fibrinogen and prothrombin preparations which we have employed in our other experiments. It is clear that the

value of the evidence, as to the properties of such a preparation, is dependent on the proved freedom from prothrombin of the fibrinogen used. If the test-fibrinogen were contaminated with prothrombin (in the original sense), it is evident that a solution containing only kinase would exhibit the properties of Howell's "prothrombin." And on this particular point, of the freedom of his fibrinogen from prothrombin, Howell's published evidence seems to us to be incomplete. His fibrinogen is described as being prepared by precipitation and reprecipitation from oxalated cat-plasma, with an equal volume of saturated sodium chloride. Rettger's precaution of clarifying the solution, by forming a filmy precipitate of barium phosphate—which possibly would produce the same effect as the barium sulphate used by us—was found unnecessary, since, without it, solutions were obtained which did not clot spontaneously on adding calcium. There is no evidence that they were tested with kinase in addition. This omission seems to us to weaken all the evidence obtained with such preparations; for to assume that prothrombin, if present, would be activated by calcium only, is to assume the point which the solution is used to demonstrate at a later stage of the investigation.

Summary and Discussion.

The following are the main experimental results of this preliminary investigation.

1. Indications have been obtained for the preparation of a set of test-solutions of the different factors concerned in the coagulation of blood-plasma. In particular it has been found possible to prepare

(*a*) Fibrinogen, free from perceptible traces of either prothrombin or kinase, from oxalated mammalian plasma, by adsorbing prothrombin with barium sulphate, and then precipitating the fibrinogen by half-saturation with sodium chloride.

(*b*) Prothrombin, free from perceptible traces of thrombin or antithrombin, by a modification of Mellanby's method.

2. Fresh fowl's plasma, when shaken with chloroform, readily yields a powerful thrombin. If the plasma be previously freed from calcium, prothrombin and kinase are formed by the same treatment, and can often be obtained apart by centrifuging the chloroform from the watery residue. If the treatment with chloroform be continued for some days at 37°, kinase is the only clotting-factor remaining.

3. A similar formation of kinase and (in the presence of calcium) thrombin occurs, when fowl's plasma is treated with an appropriate proportion of trypsin. The effect of chloroform is probably due, therefore, to the liberation of the normal tryptic ferment of the plasma, by the destruction of antitrypsin.

4. We have failed to confirm the antagonistic action of kinase on antithrombin, described by Howell.

So far as these results have an incidental bearing on theories of blood-coagulation, they seem to us to be definitely in favour of the necessity of three factors for the formation of thrombin—prothrombin, kinase and calcium. They are not compatible with the more recent conception of Howell, according to which the function of the kinase is to inactivate the antithrombin, which prevents the activation of prothrombin by calcium.

We may turn from the results of experiment, and conclusions directly deducible therefrom, to some considerations of a more speculative nature.

For the more direct purposes of our investigation, the point of most significance, and of most novel interest, is the demonstration of the appearance of kinase, as the result of slight tryptic digestion, in a body-fluid which previously contained none. It seems possible that this phenomenon will prove to be of considerable physiological interest. Hints of such fermentative production of kinase can be found in earlier observations—for example, in Howell's statement that a tissue yields a more powerfully kinasic extract, if it be allowed to undergo some degree of autolysis at room temperature before extraction. It may be that some degree of protein cleavage is the necessary preliminary to the liberation of kinase from any tissue; and that the ease with which it appears on cell-injury, and the relatively drastic treatment needed for its production in a stable avian plasma, represent a differing delicacy in the poise of the balance between proteolytic ferment and those antagonistic influences which, in the case of the plasma, we summarily describe as "antitrypsin."

Much further experiment is needed to define the relation between this liberation of thrombokinase, and the toxicity acquired by serum and other native proteins, when subjected to a similar partial hydrolysis, and speculation on the basis of existing results would be premature. We have, as yet, no evidence as to which constituent of the plasma is involved in the hydrolytic production of kinase, though evidence, on the one hand, as to association of kinasic action with a lecithin-like substance (according to Howell with the

kephalin-fraction of tissue-lipoid), and, on the other hand, as to the presence of such a constituent in the euglobulins, must be regarded as suggestive. To this suggestion, and to the relation between kinase-formation and the toxicity of variously treated sera and proteins, including the specific toxicity of foreign protein to the anaphylactic animal, we hope to return in future communications.

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