MECHANISMS INVOLVED IN FIBRIN FORMATION*

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Since the publication (9), some 12 years ago, of a standardized procedure for the study of blood coagulation reactions (in vitro), which has been the basis of a large number of investigations in the senior author's laboratories during the intervening years, there have been significant advances toward the purification of many of the clotting factors. In particular, these advances now permit us to qualify the statement of our 1938 paper that (as compared with the rate of the thrombin-fibrinogen reaction) ... “the consideration of amount of fibrin formed... is governed by a different set of factors, notable among which are questions related to quantity of thrombin and weight of the clot (Eagle (7)), neither of which can as yet be dignified with a biochemical significance...” Thrombin (30) and prothrombin (32) of great potency and reasonably satisfactory purity (12) are now available from bovine plasma. Thrombins of other species, e.g. rabbit (29), human (5), in our experience, have proved somewhat less reliable (see below) for research studies. The Human Plasma Fractionation program, developed at Harvard under the direction of Dr. E. J. Cohn (4) and colleagues (8), has yielded successful fibrinogen (and fibrin) preparations and these technics have been adapted to bovine plasma, especially in the instance of “fibrinogen” (fraction I) now commercially available from the Armour Laboratories (24). J. D. Ferry and Morrison (13) and Morrison (27) utilized the Harvard human plasma fractions to obtain a considerable body of data concerning the thrombin-fibrinogen reaction and many aspects of fibrin formation and fibrin yields. Our independent studies on materials of bovine and other species have been carried on for a number of years and the data accumulated for the present publication should be reviewed in the light of the Harvard investigations. It will be noted that we have carried certain confirmatory experiments somewhat further into technical details and have added new data on the topics of “fibrinoplastic” factors, the (alleged) “profibrin” question, and problems relating to thrombin stability.

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Methods.—Clotting times were measured with a stop-watch from the moment of addition of thrombin to the first appearance of definite fibrin filaments or gel (9). Fibrin yields were determined as follows: After a contact period of 1 hour (or otherwise stated), the fibrin clot was carefully recovered on a dissecting needle, washed in distilled water, digested 10 to 20 hours with 4 ml. of a modified (Mehl's (26)) biuret reagent, diluted to 10 ml., and read with the Klett-Summerson photoelectric colorimeter, (No. 540 filter), after previous calibration against gravimetric (dry weight of clot) and macro Kjeldahl (fibrin N) determinations. All tests, unless otherwise wise stated, were performed at 37°C. (water bath) at pH = 7.7 (borate buffer), and the usual clotting mixture was 1 ml. fibrinogen + 1 ml. thrombin.

Reagents.—Fibrinogen (F): Armour's bovine plasma fraction I (24) (BF), containing about 30 per cent of citrate and 50 to 55 per cent "clottable" fibrinogen, was purified, to 97 per cent clottable protein (average), by triple precipitation respectively with 25, 20, 20 per cent saturation with ammonium sulfate, centrifugal separation of precipitates, and resolution in 0.9 per cent NaCl, with several hours' dialysis of the final solution, at 0-2°C., against saline or borate buffer. Prothrombin-free fibrinogen (F2): was obtained by one or two preliminary adsorptions of BF with freshly prepared BaSO4 (19), followed by the above fractionation procedures, in the cold room.

Controls.—Proving the complete absence of fibrin formation in unmixed F2 solutions, these were always run in all experiments involving studies of the reactivity of various thrombins, etc., and particularly when employing very weak thrombins.

Thrombin Preparations.—Thrombins from various sources and differing in potency and in purity (see text) included: T1, an especially pure bovine thrombin obtained by the "spontaneous" activation, over 18 months in an ordinary refrigerator, of a 1:1000 (original) solution in borate buffer of one of Dr. W. H. Seeger's (32) highly purified prothrombins; T3, bovine thrombin (Upjohn's (6)) and T3, bovine thrombin (Parke, Davis' (31)), supplied through the courtesy of Dr. J. T. Correll and Dr. E. A. Sharp, respectively; T4, rabbit thrombin, lyophilized "hemostatic globulin" (Lederle (29)), courtesy of Dr. I. A. Parfentjev; T5, human thrombin, National Institute of Health Standard, courtesy of Dr. Roderick Murray (28).

Buffer Solutions.—I. Borate buffer solution: pH = 7.7, specific resistance 170 ohm-cm. (at 21°C.), effective ionic strength 0.055, containing, per liter, 11.25 gm. H3BO3, 4 gm. Na2B4O7.12H2O, 2.25 gm. NaCl, is our standard solvent and diluent (12). II. Clark's (3) well known buffers: (a) phthalate-NaOH; (b) KH2PO4-NaOH; (c) boric acid, KCl-NaOH, were used for special pH studies (see text). III. 0.2 M KH2PO4 and Na2HPO4 were used in the experiments of Table X.

Salt Solutions.—These were prepared accurately from reagent quality salts.

Acacia.—A purified (Ca-free) acacia was used to prepare a 16 per cent stock solution (aqueous) from which dilutions were made with borate buffer for testing the "fibrinoplastic" effects of colloids (Table VI).

Enzyme, etc.—XT: Crystalline trypsin (pancreatic), courtesy Dr. M. Kunitz (25) (Rockefeller Institute), 0.25 per cent stock solution in borate buffer preserved in frozen state at −20°C.

NBI: Navy bean inhibitor (antitryptic), courtesy of Dr. D. E. Bowman (2) (Indiana University), 0.1 per cent stock solution, in borate buffer, of crystalline acetone-insoluble factor.
Alcohol: Ethyl alcohol, U.S.P. (95 per cent), in dilution 1:4 (by volume) is referred to as 25 “per cent” alcohol (cf. reference 35).

**Effects of Varying Concentrations of Fibrinogen**

In the experiments summarized in Table I four dilutions of relative fibrinogen strength 4:3:2:1 were clotted with a very weak thrombin (T1: 0.001 per cent) at pH = 7.7 (borate buffer) and temperature 37°C., noting clotting times and fibrin yields after 1 hour contact time. Despite the fact that the fibrinogen dilutions extended into the region of suboptimal clotting times, the final fibrin yields, in each case, were 100 per cent of the expected values, dilution being allowed for.

**TABLE I**

**Effects of Varying Fibrinogen Concentration on Clotting Time and Fibrin Yield**

<table>
<thead>
<tr>
<th>Final Concentration of Fibrinogen (per cent)</th>
<th>Clotting time (37°C.)</th>
<th>Fibrin yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.25</td>
<td>230</td>
</tr>
<tr>
<td>2</td>
<td>0.19</td>
<td>230</td>
</tr>
<tr>
<td>3</td>
<td>0.13</td>
<td>240</td>
</tr>
<tr>
<td>4</td>
<td>0.06</td>
<td>360</td>
</tr>
</tbody>
</table>

*Fibrin yield: percentage of expected value (dilution allowed for), after 1 hour at 37°C.

**TABLE II**

**Effects of Varying Thrombin* Concentration on Clotting Time and Fibrin Yield**

<table>
<thead>
<tr>
<th>Relative Thrombin Concentration</th>
<th>Clotting time (37°C.)</th>
<th>Fibrin yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>18 sec.</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>63 &quot;</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>341 &quot;</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>30 min.</td>
</tr>
</tbody>
</table>

*Thrombin: highly purified preparation T1 (see text).
†Fibrin yield: percentage of expected value, after 10 days at 37°C.

**Effects of Varying Concentrations of Thrombin**

A. The experiments of Table II were obtained with successive tenfold dilutions of the purest thrombin obtainable (T1: see Reagents), clotting an equal (1 ml.) volume of 0.5 per cent fibrinogen at 37°C. and pH = 7.7 (borate buffer). The fibrin yield was assayed after a 10 day contact period and it is highly significant that, despite the 1000-fold variation in relative thrombin concentrations and clotting times ranging from 18 seconds to 30 minutes, the final fibrin yields were identical and complete (100 per cent) in all cases. This
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shows that, with a sufficiently good thrombin and fibrinogen, the ultimate fibrin yield is quite independent of the thrombin concentration.

B. In the experiments of Table III, a less pure thrombin (T₄; see Reagents), known to be contaminated with a trace of fibrinolytic protease, was used. Several parallel tests, with varying contact periods, were run at each thrombin dilution, the latter varying from 1:1 (0.5 per cent T₄) to 1:2048. Clotting times ranged from 10 to 870 seconds. With diminishing thrombin concentrations, the 1 hour fibrin yield was maximal only with the first two tests and was reduced with each successive thrombin dilution. With longer contact periods, however, the fibrin yields were more and more complete. With the two strongest thrombins (1:1 and 1:4), the fibrin yields in 8 and 24 hours

TABLE III

Fibrin Yields after Varying Contact Periods with Impure Thrombin (Fibrinolysin-Containing) at Various Dilutions

37°C.; pH = 7.7 (borate buffer).

<table>
<thead>
<tr>
<th>Relative dilution of thrombin*</th>
<th>Clotting time (sec.)</th>
<th>Fibrin yields after contact periods (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 hr.</td>
</tr>
<tr>
<td>1</td>
<td>1:1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>1:4</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>1:128</td>
<td>111</td>
</tr>
<tr>
<td>4</td>
<td>1:256</td>
<td>190</td>
</tr>
<tr>
<td>5</td>
<td>1:1024</td>
<td>610</td>
</tr>
<tr>
<td>6</td>
<td>1:2048</td>
<td>870</td>
</tr>
</tbody>
</table>

* Thrombin 1:1—0.5 per cent solution of T₄ (see Reagents).

were progressively lessened, evidently due to fibrinolysis, which was later observed in some similar tests. The lysin, however, was diluted out in the case of the weaker thrombins.

C. We have a number of data to show that thrombin instability is a factor occasionally interfering in these tests. This topic will be considered in a later section and the point relevant to the present observations is illustrated, incidentally, in tests 3 and 4 of Table IX. Only under these exceptional circumstances do we find incomplete fibrin formation due to the technical circumstance of a thrombin preparation losing its potency before it has a chance to complete the fibrinogen conversion.

Course and Kinetics of the Thrombin-Fibrinogen Reaction

By setting up a series of similar thrombin-fibrinogen mixtures, employing relatively weak thrombins to slow down the reaction, we obtained fibrin yields after successive contact periods, varying from shortly after the first visible
clotting to the point at which fibrin formation was virtually complete. These data are presented graphically in Fig. 1, with the logarithm of the concentration of remaining fibrinogen (computed as difference) plotted against the contact periods. For a chemical reaction of the first order, the curves should be linear (Laki (22)), and our data show this satisfactorily for four different thrombin concentrations (1:1, 1:2, 1:3, 1:5), the clotting times ranging from 40 to 435 seconds, and the contact periods, over which fibrin recoveries were followed, extending up to 50 minutes. The tendency for curvature to appear with the lower residual substrate concentrations near the end of the curves is of minor significance and indicative of secondary phenomena which are not investigated in this study.

Conditions Modifying the Thrombin-Fibrinogen Reaction

I. Temperature.—In a typical experiment, in which thrombin and fibrinogen mixtures were allowed to react at 5°, 31°, 37°, and 50°C., respectively, with clotting times decreasing from 57 seconds to 18 seconds, the 1 hour fibrin yields were all practically identical and nearly complete (97 to 98 per cent). Another 5°C. mixture was rapidly frozen immediately after clotting and stored for an hour at −20°C. On subsequently thawing and recovering the fibrin, the same assay value was recorded.

II. pH.—Varying the pH from 5.5 to 10.0 in a series of tests with standard Clark's buffers (see Reagents) was without significant effect on the fibrin yields, which were all over 90 per cent after 1 hour contact period (at 37°C.).
Clotting times showed the usual slight evidence of an optimum (pH 7.5–8) with only minor prolongation until below pH 6.0.

III. Salt Effects.—The tests of Tables IV and V were made (at 37°C.) with the following clotting mixtures: (1) 1.0 ml. F (in 0.9 per cent NaCl), 1.0 ml. salt (dilutions of 2 M NaCl or 1 M CaCl₂ (aqueous) with borate buffer to double

<table>
<thead>
<tr>
<th>Added NaCl (final concentration)</th>
<th>Total ionic strength</th>
<th>Clotting time 1 hr.</th>
<th>Fibrin yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sec.</td>
<td></td>
<td>percentage</td>
</tr>
<tr>
<td>0 (buffered only)</td>
<td>0.128</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>0.016 molar</td>
<td>0.139</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>0.931 &quot;</td>
<td>0.151</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>0.062 &quot;</td>
<td>0.184</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>0.125 &quot;</td>
<td>0.241</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td>0.25 &quot;</td>
<td>0.364</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>0.5 &quot;</td>
<td>0.614</td>
<td>27</td>
<td>100</td>
</tr>
<tr>
<td>1.0 &quot;</td>
<td>1.1</td>
<td>56</td>
<td>98</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Added CaCl₂ (final concentration)</th>
<th>Total ionic strength</th>
<th>Clotting time 1 hr.</th>
<th>Fibrin yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sec.</td>
<td></td>
<td>percentage</td>
</tr>
<tr>
<td>0 (buffered only)</td>
<td>0.128</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>0.004 molar</td>
<td>0.14</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>0.008 &quot;</td>
<td>0.153</td>
<td>3½</td>
<td>100</td>
</tr>
<tr>
<td>0.016 &quot;</td>
<td>0.174</td>
<td>3½</td>
<td>100</td>
</tr>
<tr>
<td>0.031 &quot;</td>
<td>0.229</td>
<td>7½</td>
<td>100</td>
</tr>
<tr>
<td>0.062 &quot;</td>
<td>0.312</td>
<td>7½</td>
<td>98</td>
</tr>
<tr>
<td>0.125 &quot;</td>
<td>0.496</td>
<td>20½</td>
<td>98</td>
</tr>
<tr>
<td>0.25 &quot;</td>
<td>0.864</td>
<td>134</td>
<td>98</td>
</tr>
<tr>
<td>9*</td>
<td>0.5</td>
<td>1.6</td>
<td>32 hrs.</td>
</tr>
</tbody>
</table>

* Contact time: 1 hour, except in 9 (72 hours).

the strengths stated, (3) 0.1 ml. T₂ (10 units). The final concentrations of added salt and ionic strengths of the mixtures are computed.

Non-specific salt effects are seen in the inhibitory action (prolongation of clotting times) with increasing amounts of added NaCl and with the higher concentrations of CaCl₂. The 1 hour fibrin yields are not significantly affected, except in the case of the strongest CaCl₂ (Table V, test 9), with which clotting was all but prevented and the fibrin yield after a contact time of 72 hours was only two-thirds complete.
Specific salt action is seen, first, in the definite acceleration of clotting times in the 0.008-0.031 \text{M} range of \text{CaCl}_2 additions (Table V, tests 3, 4, 5). Secondly, the much greater retardation of clotting by 0.25 \text{M} \text{CaCl}_2 (total ionic strength = 0.864) than by 1.0 \text{M} \text{NaCl} (total ionic strength = 1.1) must indicate also that excess \text{Ca}^{++} has a specific inhibitory effect above that due merely to the increase in ionic strength.

IV. Fibrinoplastic Colloids, e.g. Acacia.—Table VI illustrates the effects of increasing amount of purified (Ca-free) acacia, in the presence of the usual borate buffer. Clotting times are decreased with each addition of colloid, which is a typical example of what we have long called “fibrinoplastic” effect (10). Fibrin yields are not affected, however, except for a minor (<10 per cent) increase which may be attributed to some acacia becoming occluded in the clot. The data suggest a limit to this occlusion when the amount of fibrin is unvarying.

V. The (Alleged) “Profibrin Question.”—The experimental phenomena by which Apitz (1), in particular, sought to establish the existence of a “profibrin” or “soluble fibrin” precursor of the true clot and, incidentally, to claim support for the “denaturase” theory (1, 37) of the thrombin-fibrinogen reaction, were reinvestigated in the present studies. Our methods closely followed the German workers' but added certain important control experiments, together with determinations of fibrin yields.

A. Salt Inhibition Experiments.—Table VII shows clotting time tests on (1) prothrombin-free fibrinogen (F_2; see Reagents) mixed with thrombin; compared with controls; (2) thrombin alone; (3) fibrinogen alone. The materials were incubated (at 24°C.) for the stated periods (½ to 20 minutes) with 4.5 per cent NaCl and then diluted with 10 volumes of distilled water containing, in (2) and (3), the other reagent needed for the clotting process. Thus, all mixtures were equivalent as to final concentrations of salt, borate buffer (pH = 7.7), thrombin, and fibrinogen (original). The thrombin-fibrinogen

<table>
<thead>
<tr>
<th>Acacia concentration</th>
<th>Clotting time</th>
<th>Fibrin yield (1 hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>per cent</td>
<td>sec.</td>
<td>percentage</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>23</td>
</tr>
</tbody>
</table>
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mixture (1), undiluted, clotted in 18\frac{1}{4} minutes at 24°C. The diluted samples of this mixture, after the various incubation periods, clearly show the phenomena of Apitz, namely, marked progressive shortening of clotting times. Controls (2) show that thrombin is not significantly affected by the salt. Controls (3), however, show that the fibrinogen does undergo some alteration in "reactivity" as the result of addition and incubation with strong NaCl. As confirmed by repeated experiments of this type, the reactivity of the fibrinogen was always lessened by salt, as judged from the longer clotting times in (3).

TABLE VII

"Profibrin" Formation in NaCl, Fibrinogen, Thrombin Mixture

<table>
<thead>
<tr>
<th>Mixture (with salt)</th>
<th>Added (with water)</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 min.</td>
</tr>
<tr>
<td>1* Thrombin + fibrinogen</td>
<td>(Water only)</td>
<td>150</td>
</tr>
<tr>
<td>2 Thrombin</td>
<td>Fibrinogen</td>
<td>190</td>
</tr>
<tr>
<td>3 Fibrinogen</td>
<td>Thrombin</td>
<td>240</td>
</tr>
</tbody>
</table>

* Mixture (1) clotted in 18\frac{1}{4} minutes (24°C.).

TABLE VIII

Effects of Heating with NaCl and Subsequent Dilution on Clotting Times (at 37°C.)

<table>
<thead>
<tr>
<th>Dilution</th>
<th>&quot;Heated&quot; fibrinogen</th>
<th>Control fibrinogen</th>
<th>1 hr. (37°C.) fibrin yields</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>4:4</td>
<td>38</td>
<td>226</td>
</tr>
<tr>
<td>2</td>
<td>3:4</td>
<td>43</td>
<td>235</td>
</tr>
<tr>
<td>3</td>
<td>2:4</td>
<td>48</td>
<td>240</td>
</tr>
<tr>
<td>4</td>
<td>1:4</td>
<td>52</td>
<td>360</td>
</tr>
</tbody>
</table>

* The fibrinogen concentration in 4:4 control was 0.25 per cent.

as compared with (2). Quite the reverse is the case when fibrinogen is partly denatured by mild heating (see below).

B. Salt-Heating Experiments.—In the second type of approach, we followed Apitz's (1) technic of heating fibrinogen (F₂) in 5 per cent NaCl for 12 minutes at 48°C. Clotting times (at 37°C.) and fibrin yields (after 1 hour contact time) were tested, in parallel experiments, at four dilutions (1:2:3:4) with 5 per cent NaCl of (a) the "profibrin" (i.e. heated F₂) and (b) a control, consisting of fibrinogen plus salt but unheated. The data are given in Table VIII. In the heated ("profibrin"-containing) series (1) the clotting times are much shorter than in the controls (2), thus showing the marked effect of partial thermal
denaturation upon the "reactivity" of the clotting system. There is no effect, however, upon the 1 hour fibrin yields, which were 100 per cent complete in all tests. The similarity of these results to the experiments with added "fibrinoplastic" colloid (acacia) in section IV may be pointed out. In addition to the opposite type of effects on fibrinogen reactivity of NaCl (A) and heating (B), it may be noted that whereas the former, with thrombin (Table VII, test 1) clotted after a number of minutes, despite the high salt content, the latter ("profibrin") solutions were stable for several days and formed true fibrin only after the addition of thrombin, which is evidence against thermal denaturation being able to cause the same kind of change in fibrinogen as that induced by thrombin.

TABLE IX
Clotting Times and Fibrin Yields, with Varying Thrombin Concentrations, on Salt-Heated (and Control) Fibrinogen

<table>
<thead>
<tr>
<th>Relative thrombin dilution</th>
<th>Clotting times, 37°C.</th>
<th>Fibrin yields (5 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salt-heated fibrinogen</td>
<td>Control fibrinogen</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1:1*</td>
<td>78 sec.</td>
</tr>
<tr>
<td>2</td>
<td>1:10</td>
<td>430 &quot;</td>
</tr>
<tr>
<td>3</td>
<td>1:100</td>
<td>60 min.</td>
</tr>
<tr>
<td>4</td>
<td>1:1000</td>
<td>300 &quot;</td>
</tr>
</tbody>
</table>

*Thrombin 1:1—0.0125 per cent T₄.

C. Fibrin Yields from Salt-Heated Fibrinogen.—In the tests of Table IX, clotting times and 5 day fibrin yields were determined on a single batch of "profibrin"-containing fibrinogen (obtained by heating for 12 minutes at 48°C. in 5 per cent NaCl, as above), as compared with unheated, but similarly salted, controls. Four successive decimal dilutions of a weak thrombin (1:1 = 0.0125 cent T₄) were employed. The marked shortening of clotting times in the salt-heated fibrinogen, as compared with the controls, again indicates the above-mentioned "fibrinoplastic" effect. Fibrin yields were 100 per cent with the less dilute thrombins, but in the case of the two weakest thrombins the 120 hour (presumably maximal) fibrin yields were greater in the heated fibrinogen. It is perhaps significant that a 9 per cent yield was obtained for unheated fibrinogen with 1:100 T and heated fibrinogen with 1:1000 T, the two clotting times (325; 300 minutes) being very similar. It is probable that we are here dealing with a very weak and unstable thrombin which loses its activity before completing the fibrin formation and that clotting times and fibrin yields may be correlated to some extent under these exceptional circumstances.

D. Acidification Experiments.—Laki and Mommaerts (23) performed an
### Effects of Acidification and Rere-neutralization with Phosphate Buffers on Thrombin-Fibrinogen Mixtures

**A. Control of Dilution Effects**

<table>
<thead>
<tr>
<th></th>
<th>Water (distilled)</th>
<th>Fibrinogen (F)</th>
<th>Thrombin (T: 20 units/ml.)</th>
<th>Clotting time (at 24°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml.</td>
<td>ml.</td>
<td>ml.</td>
<td>sec.</td>
</tr>
<tr>
<td>1</td>
<td>0.8</td>
<td>0.2</td>
<td>0.2</td>
<td>11.2</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
<td>0.2</td>
<td>0.2</td>
<td>15.4</td>
</tr>
<tr>
<td>3</td>
<td>1.6</td>
<td>0.2</td>
<td>0.2</td>
<td>20.0</td>
</tr>
</tbody>
</table>

**B. Dilution after Neutralization**

<table>
<thead>
<tr>
<th></th>
<th>Time after neutralization with 0.4 ml of 5% Na₂HPO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 sec.</td>
</tr>
<tr>
<td>1</td>
<td>147</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
</tr>
</tbody>
</table>

* Neutralized before addition of fibrinogen and thrombin.

**C. Varying Periods of Incubation with Acid Phosphate**

Temperature 24°C; pH during incubation: (indicated); pH after neutralization with Na₂HPO₄: 6.8; water added with neutralizing agent: as indicated; clotting times (seconds) at 24°C.

<table>
<thead>
<tr>
<th></th>
<th>Acid mixture (pH)</th>
<th>Water</th>
<th>Incubation period with acid phosphate</th>
<th>Control†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ml.</td>
<td>1 min.</td>
<td>1 min.</td>
</tr>
<tr>
<td>1</td>
<td>F + T (5.1)</td>
<td>0.8</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>F + T (5.1)</td>
<td>0.4</td>
<td>165</td>
<td>170</td>
</tr>
<tr>
<td>3</td>
<td>F + T (5.1)</td>
<td>0.4</td>
<td>78</td>
<td>83</td>
</tr>
<tr>
<td>4</td>
<td>F + T (5.1)</td>
<td>0.4</td>
<td>48</td>
<td>36</td>
</tr>
<tr>
<td>5§</td>
<td>F (control) (5.1)</td>
<td>0.4</td>
<td>48</td>
<td>54</td>
</tr>
<tr>
<td>6§</td>
<td>T (control) (4.5)</td>
<td>0.4</td>
<td>48</td>
<td>42</td>
</tr>
</tbody>
</table>

† See 0 time column in section B of table.

§ In controls 5 and 6 the fibrinogen and thrombin were separately incubated with the acid phosphate and the appropriate reagent (thrombin or fibrinogen, respectively) added with the diluted Na₂HPO₄.

The experiment with phosphate buffers in which a thrombin-fibrinogen mixture was incubated with KH₂PO₄ at pH = 5.1 and samples removed after successive intervals of time, rere-neutralized (pH = 6.8) with Na₂HPO₄, and the clotting times observed to get shorter and shorter, in a manner resembling the...
results of Apitz's salt inhibition experiments (see section A, above). Recognizing that these "acidification" experiments did not take into account factors of dilution, of salt content, and possible effects on the thrombin and fibrinogen individually, we have repeated similar experiments on a number of occasions but with care to include the necessary controls. Some typical data are reproduced in Table X, the experiments of sections A, B, and C 1, 2 being performed on one occasion and those of C 3-6 on another, but with very similar reagents.

The essential phenomenon of Laki and Mommaerts is shown in tests C 1 and C 4 but is absent in C 2 and C 3. There are some clotting time fluctuations in C 2, C 3, B 1, and C 5 which seem to be of comparable magnitude and may perhaps be explained as a minor variability arising as the result of the action of the acid salt on the fibrinogen, possibly correlated with the fact that a slight turbidity appeared in the mixtures. After several hours a trace of fine flocculent deposit was observed but no clotting occurred in the acid mixtures even after several days.

The most significant effects are those of dilution. In the absence of salt effects due to the phosphate buffers, the effect (section A) is simply a lengthening of clotting time caused principally by the thrombin dilution, confirming data of a previous paper (9). The data of section B show a marked inhibitory effect of the buffer salts, undoubtedly due to the usual non-specific "salt effects" noted earlier. It is very striking that the reaction, which has begun and advanced in the "latent phase" during the period following neutralization (even without dilution), is rapidly accelerated to the point of visible clotting by subsequent dilution with water. The longer the neutralized mixture has been in the latent period and the greater the subsequent dilution, the more dramatic is the shortening of the clotting time (section B). In other experiments, where the pH was reduced to a few tenths pH unit above 5.1 or where weaker phosphate solutions were tried, the acidified mixtures clotted, albeit rather poorly and often after many minutes, without neutralization. In such experiments, the clotting acceleration phenomenon of Laki and Mommaerts was much more dramatic than in the tests cited in Table X.

Some Factors Affecting Thrombin Stability

As the result of many years of experience with a wide variety of thrombin preparations and clot-testing conditions, it has become apparent that most of the difficulties encountered in assigning a quantitatively significant potency or "reactivity" to any particular thrombin preparation (granted adequate standardization of the fibrinogen and test conditions) can be explained by circumstances which may be considered under four heads:

1. Antithrombin.—While the best modern thrombins (e.g. (30)) can be prepared antithrombin-free, inhibitor(s) of this type accompany the cruder
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preparations and are, of course, very important in plasma or serum, being best controlled by dilution (36). The markedly progressive loss of thrombin added to serum is classically explained as due to "antithrombin" but the underlying mechanisms are inadequately understood. That this loss can be prevented to a remarkable degree by 25 "per cent" ethyl alcohol, as recently pointed out by Sternberger (35), is clearly shown in the data of Table XI.

### TABLE XI

**Inhibition of Serum Antithrombin by 25 Per Cent Ethyl Alcohol**

Tests on purified fibrinogen (F2) at 26°C., pH = 7.7 (borate buffer) 0.5 ml. Mixture (at stated incubation periods) + 0.5 ml. F2; clotting times, in seconds.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min.</td>
</tr>
<tr>
<td>Thrombin (0.2 unit/ml.) + serum + 25 per cent ethyl alcohol</td>
<td>16 sec.</td>
</tr>
<tr>
<td>Thrombin + serum (control)</td>
<td>16 sec.</td>
</tr>
</tbody>
</table>

### TABLE XII

**Thrombin Deterioration and Effects of Wettable Surface (Glass vs. Silicone)**

Clotting times (seconds) for 0.25 cc. fibrinogen (0.5 per cent) + 0.25 cc. thrombin (A and B), after incubation of thrombins under the stated conditions, at 37°C., for the periods noted.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min.</td>
</tr>
<tr>
<td>A: 1 Glass (pyrex) tubes</td>
<td>14.8 sec.</td>
</tr>
<tr>
<td>2 Silicened (pyrex) tubes</td>
<td>14.9 sec.</td>
</tr>
<tr>
<td>3 Added powdered glass</td>
<td>17.7 sec.</td>
</tr>
<tr>
<td>B: 1 Glass (pyrex) tube</td>
<td>30.0 sec.</td>
</tr>
<tr>
<td>2 Silicened (pyrex) tube</td>
<td>30.0 sec.</td>
</tr>
<tr>
<td>3 Added powdered glass</td>
<td>39.7 sec.</td>
</tr>
</tbody>
</table>

The final serum dilution was 1:5 and in the control, an equivalent amount of alcohol (12.5 "per cent") was added to the fibrinogen.

2. **Thrombin Instability.**—It has long been noted (reference 12) that purified thrombin solutions which are amazingly stable (weeks at room temperature) in relatively potent solutions lose activity progressively, over hours or minutes, as they are more and more diluted. In Table XII, we have studied this type of thrombin instability in relation to "wettable" surfaces. In (1) ordinary pyrex tubes were used, in (2) silicened tubes (reference 21), in (3)
powdered glass was added. The data clearly show lessening of the instability in the siliconed tubes and marked acceleration of loss of potency in the presence of powdered glass. Two thrombins were tested.

### TABLE XIII

**Thrombolytic Activity of Various Enzymes**

Volume of all incubated mixtures: 8 ml., adding borate buffer as necessary, and containing 2 ml. T2 (40 units/ml.) together with 2 ml. of added agents specified, namely (XT): crystalline pancreatic trypsin (0.1%); (NBI) navy bean inhibitor (0.1%); 95 per cent (v/v) ethyl alcohol. 0.5 ml. samples added to 0.5 ml. F2 at periods stated. *Clotting times* (seconds) at pH = 7.7 and room temperature 25 ± 2°C. *Clot lysis times* (minutes), in parentheses. *∞* = no lysis in 24 hours.

<table>
<thead>
<tr>
<th>Agent added</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min.</td>
</tr>
<tr>
<td>Buffer only</td>
<td>sec.</td>
</tr>
<tr>
<td>NBI only</td>
<td>104</td>
</tr>
<tr>
<td>XT</td>
<td>104</td>
</tr>
<tr>
<td>XT + NBI</td>
<td>104</td>
</tr>
<tr>
<td>Alcohol + XT</td>
<td>7½</td>
</tr>
</tbody>
</table>

**Thrombin Dilution Series**

Relative dilutions (percentage) of original (10 units/ml.) T2 corresponding to mixture 1 (above).

<table>
<thead>
<tr>
<th>Percentage</th>
<th>100</th>
<th>50</th>
<th>25</th>
<th>10</th>
<th>5</th>
<th>2.5</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotting time, sec</td>
<td>104</td>
<td>18½</td>
<td>33</td>
<td>58½</td>
<td>110</td>
<td>255</td>
<td>454</td>
</tr>
</tbody>
</table>

3. *Thrombolytic Activity*.—While it is recognized (34) that thrombin is remarkably resistant to destruction by the fibrinolytic protase of serum, the observation (12) that thrombolytic activity occurs with sufficiently powerful enzyme acting over a long enough time (several days being usually required for com-
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pletion, so that fibrinolysin deterioration must be allowed for), has been repeatedly confirmed in our laboratories. Pancreatic trypsin, experimentally, is a good thrombinolytic (17) agent and the tests of Table XIII show the effects of this enzyme (XT) on a stable thrombin solution (T₃: 10 units/ml.) and modification by navy bean inhibitor (NBI) and by 25 per cent ethyl alcohol. The progressive lengthening of clotting times in (3) is due to thrombinolysis, which is produced by crystalline trypsin in a concentration (in thrombic mixture) of 0.002 per cent. In (4), navy bean inhibitor (0.02 per cent) completely inhibits both thrombinolysis and lysis of the fibrin clot. The NBI alone has no significant action on the thrombin (2). 25 “per cent” ethyl alcohol (one-quarter volume 95 per cent ethanol in the thrombic mixture) appears to retard thrombinolysis and fibrinolysis to some extent but is not markedly inhibitory (5). The presence of 12.5 “per cent” ethyl alcohol (in T + F mixture) is somewhat “fibrinoplastic” and if this is allowed for, the effects of alcohol on the proteolytic actions of trypsin in these experiments may be regarded as insignificant.

4. Thrombin Destruction by Microorganisms.—The bacteriostatic action of our borate buffer solution is such that we seldom encounter any evidence of the growth of microorganisms in the protein solutions. In records of several thousands of tests, however, we have a few cases in which obvious contamination with dust-borne mold produced a slowly growing white filamentous spherical colony (cf. (12)). Table XIV records the accelerated loss of potency of a not too stable thrombin solution between the 4th and 7th days (at room temperature), compared with a control test in which 25 per cent alcohol, while not preventing the (minor) thrombin deterioration, did avoid any visible microbic contamination and the sharp loss of potency on the 7th day seen in the other test in which a mold colony was first observed on the 4th day.

TABLE XIV

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min.</td>
</tr>
<tr>
<td>Thrombin + borate buffer</td>
<td>50</td>
</tr>
<tr>
<td>Thrombin + buffer + 25 per cent alcohol</td>
<td>50</td>
</tr>
</tbody>
</table>

* Mold colony observed.
It is recognized (37, 27, 13) that (1) fibrin formation is merely incipient (Flory's (15) "gel point") at the onset of visible clotting with a "latent period," occupied by earlier phases of the thrombin-fibrinogen reaction, preceding this, and that (2) much additional time is needed for maximal fibrin yields, particularly under less favorable clotting conditions.

An enzymatic role of thrombin is convincingly demonstrated in the present data in which, with good reagents and test conditions (including adequate contact periods), complete (100 per cent) fibrin yields are obtained over an extremely wide range of thrombin concentrations (over a thousandfold) and of clotting times (3 seconds to 30 minutes). Fibrinolysis, which is not due to thrombin but to a protease contaminant of some less pure thrombin preparations, may be a cause of lessened fibrin yields. Incomplete conversion of fibrinogen because of disappearance of thrombin (discussed later) need be considered only in exceptional cases involving the use of very weak and unstable thrombin solutions.

Fibrinogen concentration does not affect the fibrin yield in the range 0.05 to 0.25 (or 0.5) per cent "clottable" protein, although clotting times with weak thrombins are somewhat prolonged at the lower fibrinogen concentrations. We have not worked with the extremely low fibrinogen concentrations (0.002 to 0.003 per cent) with which Morrison (27) claims incomplete fibrin yields because of "... transformation of a portion of the fibrinogen into a soluble fibrin—i.e., a product in which the degree of polymerization is not sufficient to cause either precipitation or cross-linking into a single continuous structure."

With our (97 per cent) purified fibrinogen there seems no good reason to raise the question of possible "occlusion," except perhaps in our experiments with acacia which do indicate a minor (<10 per cent) error believed due to occlusion (adsorption) of the colloid into the fibrin clot.

Other conditions being constant, thrombin concentration is the most important variable in determining "clotting time."

Fibrin yields afford a method of following the course of the thrombin-fibrinogen reaction only in its later phases, following the first appearance of visible fibrin. Our data (Fig. 1) confirm the suggestions of earlier workers (22, 13, 37) that the kinetics are essentially those of a first order reaction. Many factors operate to modify clotting conditions and alter the clotting time. It is significant that very few of them affect the final fibrin yield. The present data illustrate these points for (1) temperature, (2) pH, (3) salt effects, particularly increasing ionic strengths of NaCl, CaCl₂, etc., (4) "fibrinoplastic" colloids, e.g. acacia, and (5) procedures (e.g. salt-heating) which several European workers (37) claim to involve partial denaturation and the "profibrin" question (see below).

Temperature effects are those of the Arrhenius factor, modifying rate of
reaction without significant effects on the ultimate fibrin yield. \( \text{pH} \) affects (1) clotting time, but with a remarkably broad optimum, unlike most enzyme reactions, (2) the physical properties of the clot (e.g. opacity, rigidity, tensile strength, etc.) associated with the coarseness or fineness of the protofibril aggregations (13, 20), but (3) does not alter the total fibrin yield over a very wide range (6.0–10.0). Correlation of (3) and (1) suggests that it is amount, not quality, of fibrin which determines the “gel point,” i.e. onset of visible clotting, but direct experimental verification of this is lacking. Salt effects are believed to be related to the electrostatic forces along the filamentous fibrinogen molecules (14) and their effects in altering clotting time and physical characters of the clot are evidence of the importance of electrical forces in the “polymerization” processes of fibrin formation. In this phenomenon it is a question of the total Coulomb forces as determined by ionic strength (related to concentration of salt, dissociation, and valency), being otherwise “non-specific.” In addition to this, however, there are “specific” ion effects on the clotting mechanism, exemplified by the acceleration of clotting time by a certain range of \( \text{Ca}^{++} \) and by the inhibitory effects of polyvalent anions, like ferrocyanide, which Ferry (14) agreeing with Glazko and Greenberg (18), admits must be more than the non-specific salt effects. The reasonable conclusion is that “specific” effects probably indicate the operation of electrical charge in bringing thrombin and fibrinogen together to initiate a series of reactions, the chemical details of which are not yet understood.

The evidence for the enzymatic nature of thrombin action, taken in conjunction with the modern physicochemical facts, permits a new insight into some fundamental aspects of fibrin formation, which topic has been excellently presented by J. D. Ferry and coworkers (13, 14). In brief, the essential process is conceived to be a series of increasing degrees of aggregation or “polymerization,” ultimately forming the protofibrils revealed by the electron microscope (20) and the still larger aggregates which appear as filaments or “needles” (quasi-crystalline) visible with the ordinary microscope, especially in darkfield, and with the phase microscope. The “latent phase,” i.e. period before appearance of visible clot and the incomplete fibrin yields at very low concentrations of fibrinogen (Morrison (27)) are best explained by “soluble” fibrin, in the sense that the earliest phases of polymerization are too finely dispersed to precipitate or gel.

The “profibrin” question, of the European theories (1, 37), is a very different idea, based on the supposition that a soluble intermediate or profibrin, with properties of a partly denatured protein, occurs in an initial phase of the thrombin-fibrinogen reaction and is precipitated or gelled by another process. By repeating the types of experiments used by the European workers, but adding control tests and studies of modifying conditions both as to clotting times and fibrin yields, we have come to a reinterpretation of these data.
Firstly, from the above discussion of Coulomb forces in relation to the clotting mechanism, it must be apparent that excess of neutral salts, aided by higher valencies and certain “specific” effects of ionization (electrolytic dissociation) including those due to pH, will change the degree and rate of action of the forces involved in fibrin formation, although not altering its essential character as a polymerization process. Moreover, the magnitude of clot-interfering forces of these kinds must certainly be greatest in the earliest phases of the thrombin-fibrinogen reaction, namely, the “latent period.” It is the length of the latent period which determines the “clotting time,” but mere clotting delay, per se, has very little to do with the ultimate fibrin yield, as repeatedly shown in our data. Thus, the salt inhibition experiments (Apitz (1)) merely denote prolongation of latent period and clotting times by unfavorable “ionic atmosphere” for the thrombin-fibrinogen reaction. Only under the most extreme conditions does this indefinitely inhibit the reaction. In most of the experiments cited, the reaction, though delayed, is still proceeding in the “latent phase.” Subsequent restoration of more favorable clotting conditions, e.g. by dilution, enables the gel point to be reached with apparent acceleration. This is only “apparent,” because it is due to the progress already made in the artificially lengthened latent phase. The acid inhibition experiments (Laki and Mommaerts (23)), according to our experimental analysis, are largely salt effects, quite similar to the above, with only a minor addition to the unfavorable circumstances imposed by the weak acidity. It is dilution as well as “neutralization” of pH which restores the favorable clotting conditions. The clot acceleration phenomenon depends upon the degree to which some reaction between the thrombin and fibrinogen has been permitted during the “inhibitory” period. Our concept does not require any peculiar subdivision into two distinct phases of the clotting process and does not subscribe to any “denaturase” theory. In the sense of the European workers, “profibrin” is to be regarded as a misnomer.

Secondly, many forms of treatment (e.g. mild heating, strong salts, weak acids, alcohol, etc.) alter the “reactivity” of fibrinogen. Without entering into the vexed question of “denaturation,” it may be suggested that the marked acceleration of clotting time under most of the above conditions (the altered fibrinogen not being rendered non-reactive to thrombin) can be explained by a “fibrinoplastic” effect. By this we mean altered colloidal conditions increasing the action of thrombin and the polymerization of fibrin simply through surface effects (e.g. adsorption) like those demonstrable on the addition of acacia, etc. With the many non-specific colloids available when blood clots under natural conditions, there are numerous possible applications of our idea of “fibrinoplastic” effects on the coagulation mechanism.

In quantitative studies on fibrin formation it is important to know what external factors may interfere with the “reactivity” of thrombin. The complete
effectiveness (e.g. Table II) of extremely minute amounts of thrombin acting over very long periods would hardly be possible if adsorption of thrombin on to the fibrin clot (33) were able to check its action.

Being a protein, it is not unlikely that thrombin in solution may undergo denaturation of some kind, with resulting loss of potency. Not only is this hypothetical, but the amazing constancy of potency of stronger solutions of highly purified thrombins (for example, those giving clotting times, with purified fibrinogen, at room temperature, of 15 seconds or less), strongly indicates that some additional factor or factors must be invoked to account for thrombin instability. Four such factors are studied in the present experiments. The data suggest, first, that the instability of diluted thrombins, as compared with the greater stability of the same preparations in more concentrated solutions, is largely due to adsorption on to the walls of the glass vessels. Potency loss is greatly accelerated by increasing the “wettable” surface by adding powdered glass (etc.). Conversely, there is a significant increase in stability in silicone-treated vessels.

Serum antithrombin progressively weakens thrombic activity and may be a factor in insufficiently purified thrombins. The stabilizing influence of 25 per cent ethyl alcohol may be used to identify this type of antithrombic factor. Proteolysis (thrombinolysis) is another theoretical possibility. Experimentally, this may easily be demonstrated with crystalline trypsin in high dilution, the enzyme action being inhibited by the newer trypsin inhibitors from soybean, navy bean, egg white, etc., but only to a slight extent if at all, by 25 per cent alcohol. In blood serum, there is a proteolytic system (fibrinolysin or “activated” profibrinolysin; antifibrinolysin) and many thrombins and other plasma products show evidence of contamination with this enzyme. Fibrinolysin differs from trypsin not only in origin but in other significant ways so that, despite many similarities, we have now given up our earlier “tryptase” nomenclature (11).

Relevant to the present discussion are the facts, established in our own and other laboratories, that (1) thrombin is extremely resistant to this enzyme. This is perhaps most strikingly illustrated by the fact that traces of thrombin persist in many fibrinolysin preparations, notably the Harvard plasma fraction III-3 and some of our own (and other) lysin preparations. (2) Nevertheless, very potent and stable fibrinolysin, in our experience, does gradually destroy thrombin. It is difficult, in the light of these facts, to implicate contaminant fibrinolysin as a cause of thrombin deterioration, although the possibility persists. More significant, as shown by some of the present experiments, is the chance the enzyme may get to attack the fibrinogen and fibrin and, thereby, reduce the fibrin yield.

Finally, microbic growths, especially air-borne molds, have been shown to cause destruction of thrombin. This obviously is a technical matter, readily
avoided, as a rule, by careful technic and the use of bacteriostatic agents, including our borate buffer, alcohol, etc. In some unpublished experiments, we have failed to find that oxyquinoline sulfate (chinosol) or flavine dyes have any significant effect upon serum antithrombin or other thrombin deterioration in which microbic factors are excluded (cf. Gerendás (16)).

CONCLUSIONS

That the role of thrombin in the conversion of fibrinogen to fibrin is essentially enzymatic, is established not only by the minute amounts of thrombin which are effective but also by the complete independence of fibrin yields and thrombin concentrations over a very wide range of thrombin dilutions and clotting times. The thrombin-fibrinogen reaction, in the phase beyond the "latent period" at least, seems fundamentally "first order." Technical requirements of the experiments leading to these conclusions include: (1) a highly purified (e.g. 97 per cent "clottable") fibrinogen, (2) absence of traces of thrombic impurities in the fibrinogen, (3) absence of fibrinolytic protease contaminant of the thrombin and the fibrinogen, and (4) sufficient stability of the thrombin even at very high dilutions. Four conditions affecting thrombin stability have been investigated.

Fibrin yields are not significantly modified by numerous experimental circumstances that influence the clotting time, such as (1) temperature, (2) pH, (3) non-specific salt action due to electrical (ionic) charges, which alter the Coulomb forces involved in the fibrillar aggregation, (4) specific ion effects, whether clot-accelerating (e.g. Ca++) or clot-inhibitory (e.g. Fe(CN)6--'), (5) occluding (adsorptive) colloids, which have a "fibrinoplastic" action, e.g. (a) acacia and probably (b) fibrinogen which has been mildly "denatured" by salt-heating, acidification, etc.

The data with which several European workers have attempted to substantiate the idea of a two-stage thrombin-fibrinogen reaction with an intermediary "profibrin" (allegedly partly "denatured") have been reanalyzed with controls which lead us to very different conclusions, viz. (1) denaturation and fibrin formation are independent; (2) partial denaturation is "fibrinoplastic" (see above); and (3) conditions of strong salinity and acid pH (5.1) usually do not completely prevent the thrombin-fibrinogen reaction but merely prolong the "latent" phase and lessen the time required for completion of essentially the same reaction (fibrin polymerization) when more favorable clotting conditions are restored.

Thus, our experiments advance the modern concepts concerning the coagulation mechanisms along lines that, for the most part, agree with those of the Harvard physical chemists, and we oppose the European views concerning a two-stage reaction, "profibrin," and "the denaturase theory" of clotting.
REFERENCES

10. Ferguson, J. H., The action of heparin, serum albumin (crystalline), and salmine on blood-clotting mechanisms (in vitro), Am. J. Physiol., 1940, 130, 759.


