[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

# The Conversion of Fibrinogen to Fibrin. I. Influence of Hydroxyl Compounds on Clotting Time and Clot Opacity[1]

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The reaction between fibrinogen and thrombin to form a solid structure of fibrin is modified by the presence of glycerol or other hydroxyl compounds so that the clotting time is prolonged and the clot opacity, a measure of coarseness of structure, is markedly decreased.[2] It has been suggested that hydroxyl compounds reduce the attractive forces between fibrinogen molecules and diminish their tendency toward side-by-side association during clotting, resulting in a network of fine rather than coarse strands.

This phenomenon has now been further studied by examining the effects of a large number of hydroxyl compounds on clotting time and clot opacity. Correlations are drawn between molecular structure and effectiveness in modifying the clotting process, and tentative conclusions can be reached concerning the nature of the interactions involved.

## Materials and Methods

Bovine fibrinogen (Fraction I[3]) and thrombin were furnished through the kindness of Dr. J. D. Porsche of Armour and Company, to whom we are deeply indebted for the gift of these materials. In Fraction I preparation C-185A the protein was 79% fibrinogen, as determined by the assay method of Morrison[4]; in preparation C-739, it was 65%. Thrombin preparation C-173B had an activity[5] of 4.7 units per mg.

Glycerol, mannitol, pentaerythritol, trimethylene glycol and pinacol were obtained from the Eastman Kodak Company; 2-methyl-1,3-pentanediol, 2-methyl-2,4-pentanediol, 1,3-butanediol, and bis-(2-hydroxyethyl) sulfide from Carbide and Carbon Chemicals Corporation[6]; pentamethylene glycol, hexamethylene glycol, and polyvinyl alcohol (Elvanol 72-51) from E. I. du Pont de Nemours and Co.; and tetrahydrofurfuryl alcohol from the Quaker Oats Co.[6] A sample of tetramethylene glycol was kindly given us by Professor Homer Adkins. The other compounds were commercial products of reagent grade. Most of the reagents were used without further purification. Trimethylene glycol, tetrahydrofurfuryl alcohol and pentamethylene glycol were redistilled.

Fibrinogen solutions were prepared by dissolving the stock powder (which had been dried from a frozen solution in citrate buffer) in water at a concentration of about 3% protein, dialyzing against a large volume of sodium chloride solution (usually 0.45 *M*) with several changes for twenty-four hours to remove citrate, and clarifying by filtration through filter pads. After filtration, each stock solution was assayed[4] for fibrinogen, and the *p*H was measured. Aliquots were then diluted to give samples with chosen concentrations of fibrinogen, salt, and added reagent. The reagents (hydroxyl compounds) were added in the form of moderately dilute solutions containing 0.45 *M* sodium chloride (the *p*H having been adjusted to 6.3 if necessary), to minimize possible injurious effects of local changes in composition. In every case, the *p*H of the mixture containing the highest concentration of reagent differed by not more than ±0.1 unit from the corresponding control with no reagent. Thrombin solutions were prepared by dissolving the stock powder in 0.45 *M* sodium chloride, usually at a concentration of 20 unit/cc.

Each sample, after addition of thrombin, had a volume of 5 cc. and contained 5 g./l. of fibrinogen, 1 unit/cc. of thrombin, and 0.45 *M* sodium chloride. The clotting time was taken as that required to develop a characteristic rigidity in a test-tube of 1.40 cm. i.d. The opacity,[2] or extinction coefficient, was measured in the same tube at a wave length of 6000 Å. with a Beckman spectrophotometer[7] at intervals, sometimes before as well as after the moment of clotting. The clots were kept at room temperature, which varied from 22 to 25°.

## Results

**Effect of *p*H and Ionic Strength.**—Since previous studies[2] of the dependence of clot opacity and clotting time on reaction conditions had been made with human rather than bovine fibrinogen and thrombin, preliminary experiments were made to show that the effects of *p*H and ionic strength were qualitatively the same for both species. For the bovine, as for the human system, both opacity and clotting time decreased with increasing *p*H; with increasing ionic strength, clotting time increased and opacity passed through a minimum. However, at *p*H 6.3 and ionic strength 0.45, the bovine clotting time was roughly twice as long and the clot opacity twice as great as the human; a higher *p*H, and/or a higher salt concentration, was required for bovine than for human fibrinogen to effect the changes in properties representing the transition from coarse to fine structure.[2] This difference has also been noted by Edsall and Lever.[8]

Preliminary experiments showed that, with bovine as with human fibrinogen, addition of glycerol prolonged the clotting time and diminished clot opacity. These effects were observed at various values of *p*H and ionic strength. At the average unadjusted *p*H of our solutions, 6.3, the sensitivity of opacity to added glycerol was greatest near an ionic strength of 0.45, the value selected for all experiments with other reagents.

In various successive stock solutions prepared as described above, the clotting times without added reagent varied from six to nine minutes for

(1) This work was supported in part by the Research Committee of the Graduate School of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

(2) J. D. Ferry and P. R. Morrison, THIS JOURNAL, **69**, 388 (1947).

(3) E. J. Cohn and others, *ibid.*, **68**, 459 (1946); J. B. Lesh and J. D. Porsche, reported at the 110th Meeting of the American Chemical Society, Chicago, Sept. 9-13, 1946.

(4) P. R. Morrison, THIS JOURNAL, **69**, 2723 (1947).

(5) Minimum requirements of the National Institute of Health for Dried Thrombin, Division of Biologics Control, National Institute of Health, Bethesda, Md., 3rd ed., 1946.

(6) We are grateful to those manufacturers who furnished complimentary samples of reagents.

(7) We are indebted to Professors V. W. Meloche and J. W. Williams for the use of their spectrophotometers, and to Mr. Meredith Miller for help in some of the measurements.

(8) J. T. Edsall and W. F. Lever, private communication.

preparation C-185A, and from eleven to fourteen minutes for C-739. The corresponding opacities (six hours after addition of thrombin) varied from 0.5 to 1.2 cm.$^{-1}$ for C-185A, and from 1.0 to 1.2 cm.$^{-1}$ for C-739. Most of the data reported here were obtained with the latter preparation, whose opacity was more nearly reproducible. In any case, the relative effects of different added reagents could be expressed independently of the variability of the stock solutions, by the methods of calculation described below.

**Clotting Time.**—Most of the hydroxyl compounds prolonged the clotting time ($t_C$); a plot of log $t_C$ against concentration of reagent ($c$, in g./l.) gave a straight line at low concentrations (Fig. 1). At higher concentrations, the slope usually decreased somewhat. Two reagents—starch and polyvinyl alcohol—decreased the clotting time; here also log $t_C$ was a linear function of $c$ (Fig. 1).

TABLE I

EFFECT OF HYDROXYL COMPOUNDS ON CLOTTING TIME

Fraction I Preparation C-739 except where otherwise noted

| Reagent | Expt. | d log $t_C$/d$c$ (g./l.)$^{-1}$ | d log $t_C$/d$m$ (mole/l.)$^{-1}$ |
|---|---|---|---|
| 2-Methyl-1,3-pentanediol | 49 | 0.083 | 9.8 |
| Hexamethylene glycol | 34 | .078 | 9.2 |
| | 36 | .082 | 9.7 |
| | 47 | .075 | 8.9 |
| Cyclohexanol | 43 | .080 | 8.2 |
| Pinacol | 32 | .085 | 10.0 |
| | 46 | .060 | 7.1 |
| | 51 | .060 | 7.1 |
| Trimethylene glycol | 49 | .070 | 5.3 |
| Tetrahydrofurfuryl alcohol | 45 | .063 | 6.4 |
| Mannitol[b] | 33 | .07 | 13 |
| Pentaerythritol[b] | 51 | .06 | 8 |
| Glucose[b] | 51 | .06 | 11 |
| Sucrose[b] | 51 | .06 | 20 |
| Pentamethylene glycol | 45 | .055 | 5.7 |
| bis-(2-Hydroxyethyl) sulfide | 47 | .044 | 5.3 |
| Propanol | 37 | .039 | 2.3 |
| | 40 | .050 | 3.0 |
| Butanol | 37 | .036 | 2.7 |
| | 40 | .051 | 3.8 |
| 1,3-Butanediol | 43 | .043 | 3.9 |
| Tetramethylene glycol | 46 | .040 | 3.6 |
| 2-Methyl-2,4-pentanediol | 11[a] | .033 | 3.9 |
| Propylene glycol | 41 | .020 | 1.5 |
| Ethylene glycol | 30 | .018 | 1.1 |
| Diethylene glycol | 43 | .017 | 1.8 |
| Glycerol | 7[a] | .009 | 0.9 |
| | 33 | .012 | 1.1 |
| | 34 | .013 | 1.2 |
| | 36 | .010 | 1.0 |
| | 38 | .016 | 1.5 |
| | 41 | .014 | 1.3 |
| Ethanol | 6[a] | .008 | 0.35 |
| | 40 | .010 | 0.46 |
| Starch | 39 | − .050 | −8.2[c] |
| Polyvinyl alcohol | 51 | − .14 | −5.9[c] |

[a] Fraction I Preparation C-185A. [b] No straight line; initial slope estimated. Slope falls off sharply above 4 g./l. [c] (moles monomer residue per liter)$^{-1}$.

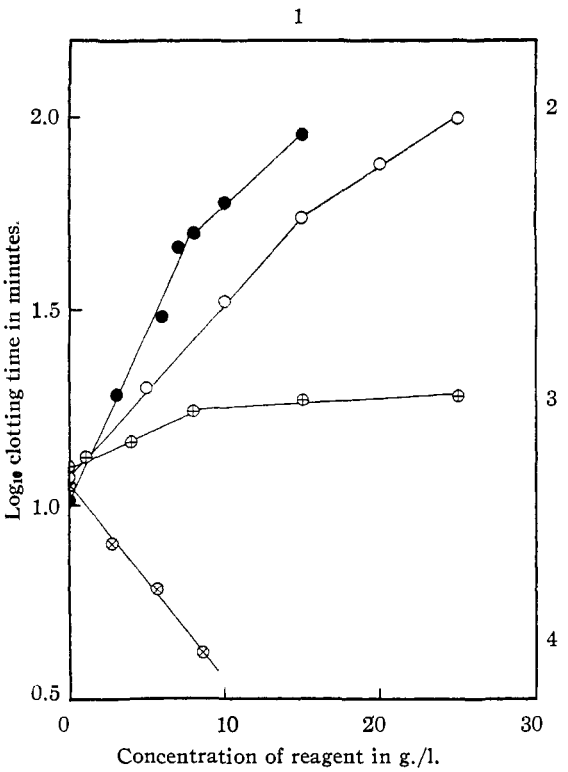



Fig. 1.—Logarithm of clotting time (in minutes) plotted against concentration of reagent in g./l.: 1, hexamethylene glycol; 2, 1,3-butanediol; 3, ethylene glycol; 4, starch.

The initial slopes of plots such as Fig. 1 were taken as measures of the effectiveness of the various reagents in altering the clotting time. They are summarized in Table I in decreasing order. Except where noted, only those experiments in which at least three points fell on a straight line are included; some other experiments, in which erratic results were obtained, have been omitted.

With the same reagent, different stock fibrinogen solutions usually gave lines with similar slopes, as shown by the fair agreement in values of d log $t_C$/d$c$ in successive experiments. The slopes are recorded both as d log $t_C$/d$c$ and d log $t_C$/d$m$, where $m$ is the concentration in moles per liter. Since most of the compounds do not differ greatly in molecular weight, the order of decreasing values is about the same in both cases. The extremes represent rather marked effects; thus, hexamethylene glycol at a concentration of 5 g./l. prolongs the clotting time by a factor of 2.5, while polyvinyl alcohol at the same concentration shortens the clotting time by a factor of 3. At higher concentrations, beyond the linear range, far greater changes are observed; polyvinyl alcohol shortens $t_C$ by as much as a factor of 10, and hexamethylene glycol prolongs it indefinitely. How-

ever, these larger effects are not so convenient for characterizing reagents.

**Change of Clot Opacity with Time.**—In the course of the conversion process, the opacity rises steadily before the moment of clotting and continues to rise for a long time thereafter.[2] Its increase with time is illustrated in Figs. 2 and 3 for systems containing varying amounts of 1,3-butanediol. In the early stages (Fig. 2) the opacity rises linearly with time; the moment of clotting, indicated by a cross, occurs during this linear increase.[9] In the later stages (Fig. 3) the opacity appears to approach a limiting value, but in the presence of higher concentrations of glycol the opacity is still rising after twenty-four hours.

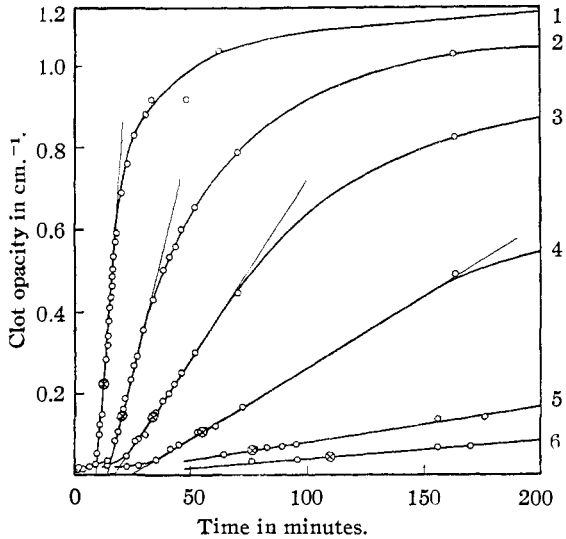



Fig. 2.—Clot opacity plotted against time, for different concentrations of 1,3-butanediol in g./l. as follows: 1, 0; 2, 5; 3, 10; 4, 15; 5, 20; 6, 25. The crosses denote clotting times.

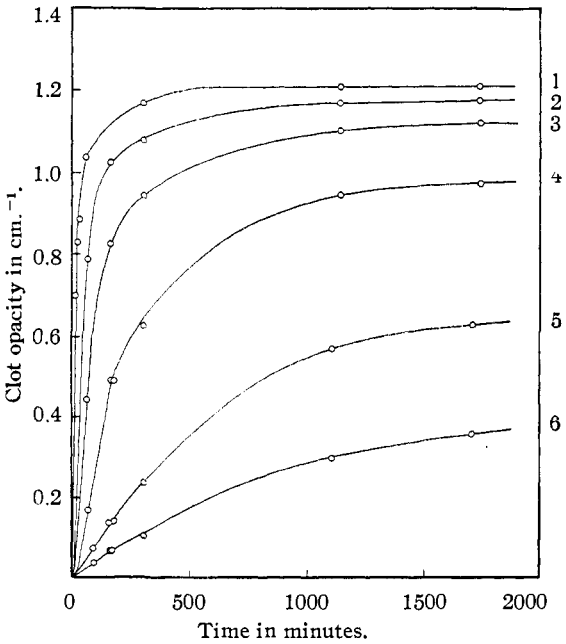



Fig. 3.—Clot opacity plotted against time, for different concentrations of 1,3-butanediol (for longer time periods). Key to curves same as in Fig. 2.

**Change of Clot Opacity with Concentration of Added Reagent.**—The butanediol decreases markedly the slope of the linear portion of the opacity–time curve, and it also decreases the opacity attained after a given time interval. This result may be partly attributed to a decrease in the rate of formation of the fibrin structure (as measured by the prolonged clotting time) and partly to a change in the character of the structure itself. We seek a measure of the latter effect alone. It could be provided by a comparison of the final opacity values after conversion is complete. However, it is difficult to ascertain when the opacity attains its maximum value; in the presence of relatively high concentrations of hydroxyl compounds, the rise may continue for several days, and it is undesirable to prolong experiments for such periods because of possible instability and bacterial action.

Accordingly, to gage the effect of added reagents on the clot structure, we have adopted the procedure of comparing opacities *at the clotting time*

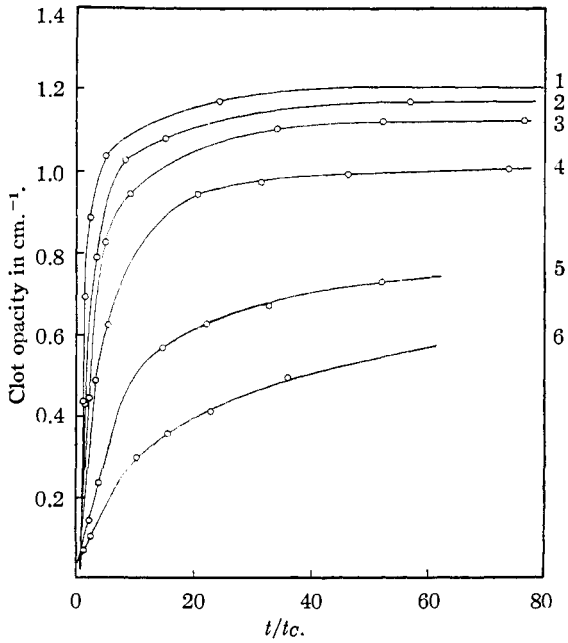



Fig. 4.—Clot opacity plotted against $t/t_c$ for different concentrations of 1,3-butanediol. Key to curves same as in Fig. 2.

(9) Oster (*J. Colloid Sci.*, **2**, 291 (1947)) has shown that the opacity should increase linearly with time when molecules are growing by condensation polymerization, provided the molecular sizes are all small compared with the wave length of light. However, it cannot be inferred that the conversion of fibrinogen to fibrin is a condensation polymerization, because the above provision is not satisfied.

($t = t_C$) or at equal multiples thereof.[10] When the opacity data of Figs. 2 and 3 are plotted against $t/t_C$ instead of $t$, the influence of the glycol appears less pronounced (Fig. 4). Here its effectiveness in decreasing coarseness of structure has been isolated, as a first approximation, from its effectiveness in decreasing the rate of the conversion process.

TABLE II

CONCENTRATION OF REAGENT REQUIRED TO REDUCE OPACITY BY ONE-HALF

Fraction I Preparation C-739 except where otherwise noted

| Reagent | Expt. | $c$ (g./l.) $t/t_C = 1$ | $c$ (g./l.) $t/t_C = 10$ | $m$ (mole/l.) $t/t_C = 1$ | $m$ (mole/l.) $t/t_C = 10$ |
|---|---|---|---|---|---|
| bis-(2-Hydroxy-ethyl) sulfide | 32 | .. | 3 | .. | 0.02 |
| | 47 | | 2 | | .02 |
| Pinacol | 32 | .. | 3 | .. | .03 |
| | 51 | | 4 | | .03 |
| Pentaerythritol | 9[a] | .. | 6 | .. | .05 |
| | 33 | | 4 | | .03 |
| | 51 | | 4 | | .03 |
| Pentamethylene glycol | 45 | .. | 5 | .. | .05 |
| Cyclohexanol | 42 | 4.0 | 6 | 0.04 | .06 |
| Hexamethylene glycol | 10[a] | .. | 6 | .. | .05 |
| | 34 | .. | 7 | .. | .06 |
| | 36 | 4.6 | 7 | .04 | .06 |
| Trimethylene glycol | 48 | .. | 6 | .. | .08 |
| | 49 | | 7 | | .09 |
| Glucose | 51 | .. | 8 | .. | .04 |
| Sucrose | 51 | .. | 8 | .. | .02 |
| Tetrahydrofurfuryl alcohol | 45 | .. | 8 | .. | .08 |
| 2-Methyl-2,4-pentanediol | 33 | .. | 9 | .. | .08 |
| Mannitol | 33 | .. | 10 | .. | .05 |
| 2-Methyl-1,3-pentanediol | 49 | .. | 11 | .. | .09 |
| Butanol | 40 | 13.5 | 12 | .18 | .16 |
| Propanol | 37 | 10.0 | .. | .17 | .. |
| | 40 | 10.5 | .. | .17 | .. |
| 1,3-Butanediol | 43 | 14.5 | 19 | .16 | .21 |
| Glycerol | 7[a] | .. | 14 | .. | .15 |
| | 38 | 12.0 | 23 | .13 | .25 |
| | 41 | .. | 21 | .. | .23 |
| | 42 | 11.7 | 18 | .13 | .20 |
| Tetramethylene glycol | 46 | .. | 24 | .. | .27 |
| Triethylene glycol | 34 | .. | 64 | .. | .43 |
| Diethylene glycol | 34 | .. | 67 | .. | .63 |
| | 43 | .. | 73 | .. | .69 |
| Propylene glycol[b] | | | | | |
| Ethylene glycol[c] | | | | | |
| Dipropylene glycol[d] | | | | | |

[a] Fraction I Preparation C-185A. [b] Depresses the opacity by 13% at $c = 25$ g./l. [c] Depresses the opacity by 11% at $c = 40$ g./l. [d] Depresses the opacity by 14% at $c = 40$ g./l.

(10) It would be preferable to compare opacities at equal extents of reaction, corresponding to times when a given proportion of fibrinogen had been converted to fibrin. However, this is impractical, if not impossible, to determine at present.

The effects of different concentrations at time intervals which are equal multiples of the clotting time are represented by intersections of the curves of Fig. 4 with vertical lines. Opacities obtained in this way are plotted against the concentration in Fig. 5 for $t/t_C = 1$, 10, 30 and 50. The sigmoid shape of the upper curves was characteristic of most of the other reagents studied.

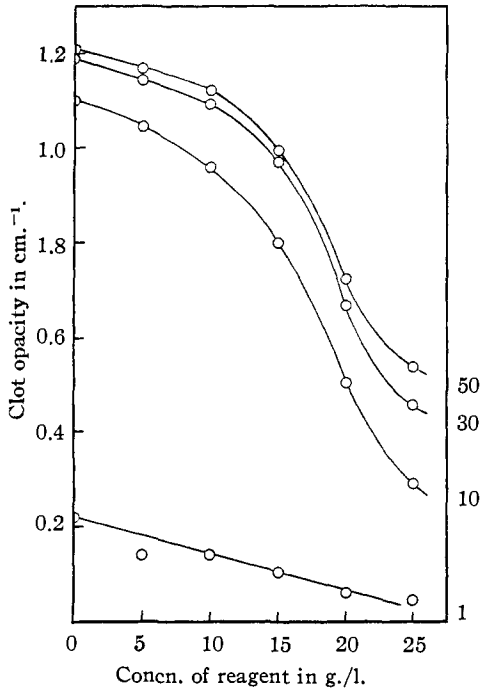



Fig. 5.—Opacity plotted against concentration of 1,3-butanediol, at various values of $t/t_C$ as indicated.

**Comparison of Reagents.**—To provide a simple comparison, the concentrations of reagents in g./l. required to reduce the capacity to half the control value (with no added reagent) at $t/t_C = 1$ (*i.e.*, at the moment of clotting) and at $t/t_C = 10$ were interpolated from graphs of the type of Fig. 5. These results are given in Table II, together with the corresponding concentrations expressed as moles per liter. Fewer values are available at $t/t_C = 1$ because of the difficulty of making numerous opacity measurements in a short time interval in the vicinity of the clotting time. At $t/t_C = 10$, they range from 2 to 3 g./l. (0.02 *M*) for bis-(2-hydroxyethyl) sulfide to 70 g./l. (0.6 *M*) for diethylene glycol. This particular comparison is striking because the two molecules differ only in the central atom, which is oxygen and sulfur, respectively. Several compounds at the end of the table are so weakly effective that the opacity is not diminished to one-half the control value within the concentration range studied. In some cases the range was limited by solubility.

The two compounds of high molecular weight, starch and polyvinyl alcohol, which shorten the

clotting time, increase the opacity when compared at constant $t$ but are without effect when compared at constant $t/t_C$.

**Inhibition.**—Several of the most effective reagents, at moderate concentrations, inhibit clotting altogether. These compounds can be characterized by the minimum inhibiting concentration required to prevent clotting for at least twenty-four hours (Table III); this is practically equivalent to inhibition indefinitely, since mixtures unclotted after one day have been observed to remain unclotted for weeks. It is surprising that these simple alcohols interfere so effectively with the reaction between fibrinogen and thrombin, while others at higher concentrations permit clotting within an hour or less: mannitol and glucose at 80 g./l., and ethylene glycol and glycerol at 200 g./l.

TABLE III

MINIMUM INHIBITING CONCENTRATIONS[a]

| Reagent | $c$, g./l. | $m$, moles/l. |
|---|---|---|
| Hexamethylene glycol | 32–43 | 0.27–0.36 |
| Pentamethylene glycol | 48–60 | .46– .58 |
| bis-(2-Hydroxyethyl) sulfide | 61–81 | .50– .66 |

[a] The first concentration is the highest observed that permits clotting, and the second is the lowest that prevents clotting for at 24 hours, at $p$H 6.2–6.4, ionic strength 0.45.

**Reversibility of Effects of Hydroxyl Compounds.**—The reagents studied do not appear to cause any irreversible changes in either fibrinogen or thrombin, as shown by the following experiments with several of the more powerful compounds. (*a*) Solutions of fibrinogen with the standard concentration, $p$H, and ionic strength, containing hexamethylene glycol, pentamethylene glycol, and bis-(2-hydroxyethyl) sulfide at concentrations somewhat above their minimum inhibiting levels, were allowed to stand six hours and then dialyzed against large volumes of sodium chloride–phosphate buffer of ionic strength 0.45. The dialyzed solutions showed no evidence of precipitation, and, upon addition of thrombin, clots of normal opacity and clotting time were obtained. (*b*) Similar solutions of fibrinogen were dialyzed against large volumes of 0.45 $M$ sodium chloride without buffer. In this case, the $p$H fell to 5.5, (as did that of fibrinogen solution with no reagent present), and portions tested with thrombin did not clot; but, after the $p$H was readjusted to 6.2, clots of normal opacity and clotting time were obtained. (*c*) Solutions of thrombin (20 units/cc.) containing the above inhibitors at concentrations somewhat above their minimum inhibiting levels were allowed to stand twenty hours and then used to clot fresh fibrinogen solutions with the standard procedure. Since the concentration of reagent was reduced twenty-fold in mixing with the fibrinogen, its influence should have been slight unless the thrombin had been inactivated; clots of nearly normal opacity and clotting time were in fact obtained, showing that the thrombin had not been affected. (*d*) Mixtures of fibrinogen and thrombin containing the inhibitors which had been kept without clotting for as long as a month were dialyzed against 0.45 $M$ sodium chloride. Clotting soon took place, although the opacity was of course lower than normal because the inhibitor had not been completely removed.

When opaque control clots, formed with the standard procedure in cellophane tubing, were dialyzed against 0.45 $M$ sodium chloride containing pentamethylene and hexamethylene glycols at their minimum inhibiting concentrations, no changes in opacity were apparent, showing that once the coarse structure is established these reagents are powerless to alter it.

When transparent clots, formed in cellophane tubing from fibrinogen solutions containing slightly less than the minimum inhibiting concentrations of the above reagents, were dialyzed against 0.45 $M$ sodium chloride, a gradual increase in opacity occurred after one or two days. It is not yet certain whether this represents a coarsening of the fine structure already established or a continued polymerization of smaller units which had remained unattached to the network structure.

## Discussion

Interpretation of these results will be based on the concepts previously developed,[2] that the conversion of fibrinogen to fibrin is essentially a polymerization; that the rod-like fibrinogen molecules (about 35 × 700 Å.) enter the fibrin structure without any profound changes in their own shape; and that their end-to-end junction is accompanied by varying degrees of side-by-side association. The more lateral association, the coarser are the strands of the final network.

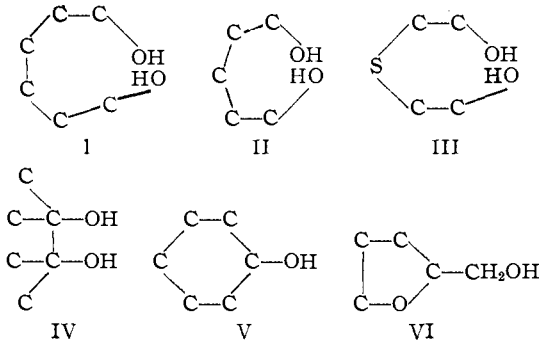
Since the hydroxyl compounds studied do not produce any irreversible change in fibrinogen or thrombin, it may be concluded that their effects on the clotting process are due to interaction involving van der Waals forces. This interaction might interfere with the primary reaction between fibrinogen and thrombin, or it might modify the normal interactions between fibrinogen molecules which determine the geometrical details of polymerization. In either case, the simplest assumption is that the volume of the reagent molecule prevents the two protein molecules from approaching closely enough for their normal combination to occur.

Modification of the geometry of polymerization would cause differences in structure, which are measured roughly by changes in opacity at constant $t/t_C$ (Table II). Interference with the fibrinogen–thrombin reaction would affect the clotting time (Table I), but the latter could also be changed by differences in association geometry, so that both influences must be considered in interpreting the effects of reagents on the time of

clotting.[11] Actually, the orders of effectiveness in the two tables are rather similar.

**Relation of Effectiveness to Structure.**—As Pauling has emphasized,[13] in van der Waals interactions steric factors are overwhelmingly important; and it should be possible to correlate the observed effects with molecular shape and arrangement of hydroxyls. The most profound effect is complete inhibition by hexamethylene glycol (I), pentamethylene glycol (II), and bis-(2-hydroxyethyl) sulfide (III). Their similarity in structure is evident, with two terminal hydroxyls separated by a chain about 8 to 9 Å. long when fully extended.

The above three compounds appear near the top in Table I and Table II, together with pinacol (IV), cyclohexanol (V), and tetrahydrofurfuryl alcohol (VI). These six molecules present rather similar contours if the glycols are in the ring configuration with the hydroxyls aligned in a hydrogen bond




It seems probable that this is the configuration which interacts strongly with one of the proteins to impede clotting; it represents a bulky mass of paraffin material with either one hydroxyl or two associated hydroxyls at one side.

Near the bottom of each table appear ethylene and propylene glycol and glycerol, with other compounds containing a relatively high proportion of hydroxyl to carbon. From the relative inactivity of these reagents, it appears that the non-polar areas of the more powerful substances are essential to their interaction; it may be supposed that the site on the protein which is blocked involves both a non-polar side chain and a hydrogen bond-forming group.

The compounds mannitol, pentaerythritol, glucose, and sucrose, which are bulky but carry many hydroxyls, are anomalous in that they strongly decrease opacity but have relatively little effect on the clotting time except at quite low concentrations.

**Interpretation of Opacity Changes.**—Since most of the effective reagents not only prolong clotting but also decrease the opacity at constant $t/t_C$, it seems probable that they interact with fibrinogen rather than with thrombin, and moreover that they interfere with side-by-side association of fibrinogen molecules even more than with end-to-end junction.[14] It is easy to see how the existence of several sites for interaction with reagent could produce this result (Fig. 6); when a moderate proportion of sites is covered, the probability of finding an unimpeded side is less than that of finding an unimpeded end. The picture of Fig. 6 also explains qualitatively the shape of the curves of opacity against concentration (Fig. 5). If the interaction between sites and reagent can be expressed by an association constant $k$, then the probability of a free side (and hence the opportunity for association to give coarse network strands) is proportional to $1/(1 + kc)^n$, where $c$ is the concentration and $n$ the number of sites on a side. With increasing $c$, this fraction at first decreases slowly, and then falls rather rapidly toward zero.

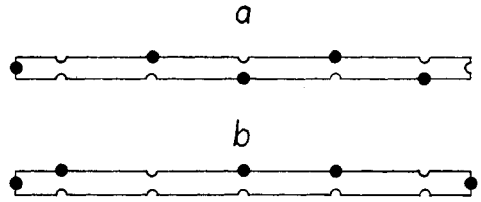



Fig. 6.—Illustration of greater interference, by reagents, with side-by-side than with end-to-end combination. If there are $n$ interaction sites on each side of a fibrinogen molecule and 1 on each end, and a proportion $p$ is blocked by reagents, the probability of a free end (a) is $1 - p$ but the probability of a free side (b) is $(1 - p)^n$.

**Effect of Starch and Polyvinyl Alcohol.**—The two reagents of high molecular weight are clearly in a class by themselves. Their effect in decreasing the clotting time is shared by a number of other macromolecules, termed "fibrinoplastic" by Ferguson.[15] No more detailed interpretation is offered than the possibility suggested previously[2] that the polymer molecules serve as additional cross-links which permit the network to be established at an earlier stage of the reaction.

## Summary

1. The effects of twenty-five hydroxyl compounds on the conversion by thrombin of bovine fibrinogen to fibrin have been investigated by measurements of clotting time and clot opacity.

2. All the reagents except two prolong the

(11) This discussion implies that the extent of reaction at the moment of clotting or gel point[12]—*i. e.*, the proportion of fibrinogen which has been converted to fibrin, or the proportion reacted of whatever specific groups on the fibrinogen molecule are involved—does not depend greatly on the presence of modifying reagents. It is impossible to test the validity of this assumption at present; it probably does not invalidate any of the qualitative conclusions drawn here.

(12) P. J. Flory, *J. Phys. Chem.*, **46**, 132 (1942); *cf.* J. D. Ferry, "Advances in Protein Chemistry," Vol. IV, p. 60 ff.

(13) L. Pauling, in K. Landsteiner, "The Specificity of Serological Reactions," Cambridge, Mass., 1947, Chapter VIII

(14) This conclusion is supported by preliminary electron micrographs of fibrinogen which has been allowed to react with thrombin in the presence of hexamethylene glycol; very long thin isolated fibers are observed.

(15) J. H. Ferguson, *Ann. N. Y. Acad. Sciences*, **49**, 486 (1948)

clotting time ($t_C$), and diminish the opacity both compared at constant time ($t$) after addition of thrombin and at constant $t/t_C$. The exceptions, starch and polyvinyl alcohol, shorten the clotting time.

3. The effectiveness in prolonging clotting time (expressed by d log $t_C$/d$c$, where $c$ is concentration) and in diminishing opacity (expressed by the value of $c$ at which opacity at constant $t/t_C$ is one-half the control value) is generally greatest for compounds with one or two hydroxyls and several methylene or methyl groups, although specific characteristics are apparent.

4. Three reagents—hexamethylene glycol, pentamethylene glycol, and bis-(2-hydroxyethyl)-sulfide—prevent clotting entirely at moderate concentrations but cause no apparent irreversible changes in either fibrinogen or thrombin.

5. The results are interpreted as due to van der Waals association of the reagents with fibrinogen, and consequent steric interference with both end-to-end and side-by-side union of fibrinogen molecules.

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[CONTRIBUTION FROM THE JOHN HARRISON LABORATORY OF THE UNIVERSITY OF PENNSYLVANIA]

# Cyclization of α-Phenylglutaric Anhydride

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From an extensive study by Attwood, Stevenson and Thorpe[2] on the cyclization of dicarboxylic acids derived from γ-phenylbutyric acid, the generalization was drawn that substitution in the β-position (with respect to the ring) was necessary for cyclization. For example, both β-benzylglutaric acid and α-benzylsuccinic acid gave tetralones on treatment with sulfuric acid; it was postulated that anhydride formation occurred first, and that cyclization then followed under the influence of concentrated acid. The effect of a β-substituent was not clearly defined, but since every instance in which cyclization occurred included this structural feature, the generalization seemed valid.

In the course of work on the synthesis of certain substituted tetralones, we have investigated the cyclization of α-phenylglutaric anhydride. This compound was prepared from phenylacetonitrile by the following steps. Carbethoxylation of phenylacetonitrile with diethyl carbonate by a modification of the usual method[3] gave ethyl phenylcyanoacetate (I). Addition of acrylonitrile to the latter compound provided α-phenyl-α-carbethoxyglutaronitrile (II); this was hydrolyzed and decarboxylated to the corresponding glutaric acid, which was then converted to the anhydride with acetic anhydride. Treatment of α-phenylglutaric anhydride (III) with sulfuric acid gave 4-keto-1,2,3,4-tetrahydro-1-naphthoic acid (IV) in 57% yield.

It was recognized by the English workers that α-phenylglutaric acid or its anhydride should be investigated as a simple test case for their generalization, but unfortunately the method of Fichter and Merckens[4] for the preparation of the acid could not be repeated. Since the original work, instances in which this generalization does not hold have been found; for example, Robinson[5] has described the cyclization of 5-carboxy-4-carboxymethyl-7-phenylheptanoic acid with sulfuric acid to the corresponding tetralone. The present method provides a satisfactory way of obtaining α-phenylglutaric anhydride, and its successful cyclization confirms the view that substitution in the β-position with respect to the ring is not a prerequisite for cyclization. At the same time, the conditions of cyclization may determine the nature of the product. When the anhydride was treated with aluminum bromide in benzene, an intermolecular reaction occurred with formation of α-phenyl-γ-benzoylbutyric acid. It has recently been demonstrated[6] that benzene is a suitable solvent for intramolecular Friedel–Crafts reactions, and this result indicates that sulfuric acid may give results different from those obtained under Friedel–Crafts conditions.

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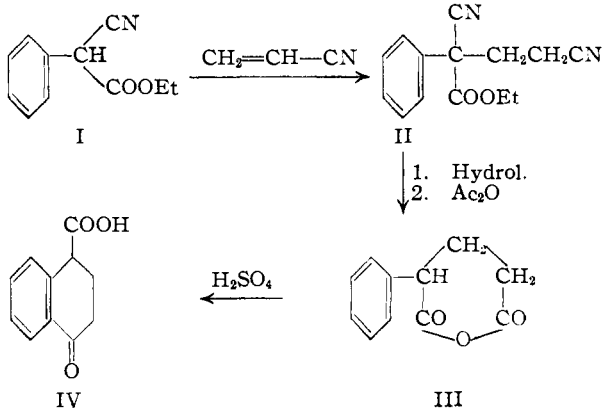


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