The first primary band has been shown to be capable of displacement to the edge of the visible region; this would seem to clear up confusion in the literature where the more displaced bands have been identified solely with the secondary absorption.

An empirical semi-quantitative relationship has been developed involving the interaction of substituent groups in the displacement of the first primary band for *p*-disubstituted compounds where the groups are of complementary types—*i. e., ortho–para vs. meta* directing.

The displacing effect ($\Delta\lambda$) of a substituent group on the primary band of benzene has been associated with a directional displacement of electrons to or from the benzene ring, depending on the electron attracting or contributing character of the group. The effect of simple ionization on band displacement has been tentatively cited to corroborate this principle.

The kind of electronic interaction responsible for band displacement has been associated with mesomerism or resonance rather than with simple coulombic displacement effects.

It was noted that the order of substituent groups obtained from the magnitude of the derived displacement values ($\delta\lambda_0$) correlates well with that of the $\Delta\sigma$ values given by Price.

DETROIT, MICHIGAN RECEIVED MAY 19, 1947

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[CONTRIBUTION FROM DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

# Preparation and Properties of Serum and Plasma Proteins. XV. Some Factors Influencing the Quantitative Determination of Fibrinogen[1,2]

BY PETER R. MORRISON

Fibrinogen is the plasma protein which forms fibrin under the action of thrombin. Among the plasma proteins it readily may be identified not only by this, its physiological activity, but also by its very low coagulation temperature and its solubility characteristics which make it the most easily salted out of all the plasma proteins. Each of these three properties has been made the basis of various quantitative methods for its estimation in plasma, but all are subject to certain errors. These errors may lead to measurements which are too low due to incomplete precipitation of the fibrinogen, or too high due to the occlusion of other plasma components. Their magnitude can, therefore, only be evaluated by a study using purified components. The large-scale fractionation of human plasma[3] has both emphasized the need for a well-defined analytical method and provided quantities of stable concentrated fibrinogen and thrombin for its investigation. Many procedures have been proposed for the determination of fibrinogen.[4]

However, despite this great interest which has attached to fibrinogen and the many analytical techniques for it which have been described, the influence of the concentration of the reactants and of the presence of other plasma components has apparently never been systematically studied.

Since it is clear that the ultimate analysis must be based on the functional characteristic which defines fibrinogen—the ability to form fibrin under the action of thrombin—this study largely concerns itself with the yield of that reaction under various conditions. The effect of variation of fibrinogen and thrombin concentrations, and *p*H, and of the presence of contaminants, largely other proteins, is considered. These effects are of importance in determining fibrinogen both in solu-

(1) This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(2) This paper is Number 61 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

(3) E. J. Cohn, *Chem. Rev.*, **28**, 395 (1941); *Science*, **101**, 51 (1945); *Am. Scientist*, **33**, 61 (1945).

(4) These techniques may be classified as follows:

(A) Separations by heat coagulation. Analysis by dry weight (Frederick, "Recherches sur la Coagulation du Sang," Gand. Paris and Leipzig, 1878. Cited by Gram, *J. Biol. Chem.*, **49**, 279 (1921). G. H. Whipple, *Am. J. Physiol.*, **33**, 50 (1914)).

(B) Separation by salting out. Analysis (a) by dry weight (W. Reyhe, "Nachweis und Bestimmung des Fibrinogens," Inaugural Dissertation, Strassburg (1898). Cited by Lewinski, *Arch. ges. Physiol.*, **100**, 611 (1903)); (b) by nitrogen (Lewinski, *ibid.*, P. N. Howe, *J. Biol. Chem.*, **57**, 235 (1923); M. Florkin, *ibid.*; **87**, 629 (1930); W. L. Campbell and M. I. Hanna, *ibid.*, **119**, 15 (1937)).

(C) Separation by clotting (calcium chloride). Analysis (a) by dry weight (H. C. Gram, ref. 4A); D. P. Foster and G. H. Whipple, *Am. J. Physiol.*, **58**, 407 (1921); (b) by nitrogen (G. E. Cullen and D. D. Van Slyke, *J. Biol. Chem.*, **41**, 387 (1920); P. E. Howe, *ibid*, **49**, 93 (1921); T. B. Jones and H. P. Smith, *Am. J. Physiol.*, **94**, 144 (1930)); (c) by tyrosine (H. Wu, *J. Biol. Chem.*, **51**, 33 (1922); H. Wu and S. M. Ling, *Chinese J. Physiol.*, **1**, 161 (1927)); (d) by turbidity of the clot (K. Klinke and G. Elias, *Z. ges. exptl. Med.*, **77**, 717 (1913); K. K. Nygaard, R. Gathe and Th. Guthe, *Skand. Arch. physiol.*, **83**, 195 (1940); K. K. Nygaard, "Hemorrhagic Diseases. Photo-electric study of Blood Coagulability." C. B. Mosby Co., St. Louis, Mo., 1941).

(D) Separation by clotting (thrombin). Analysis by dry weight or nitrogen. This procedure has been used routinely in this Laboratory since 1941 when it became necessary to determine the fibrinogen content of various plasma fractions; K. Laki, *Studies Inst. Med. Chem. Univ. Szeged*, **2**, 27 (1942); L. Szecsenyi-Nagy, *Biochem. Z.*, **317**, 185 (1944).

(E) Separation by electrophoresis. (E. Stenhagen, *Biochem. J.*, **32**, 714 (1938); L. G. Longsworth, T. Shedlovsky, D. A. MacInnes, *J. Exptl. Med.*, **70**, 399 (1939); J. T. Edsall, R. M. Ferry and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 557 (1944); H. F. Deutsch and M. B. Goodloe, *J. Biol. Chem.*, **161**, 1 (1945)).

tions such as plasma where it constitutes only a few per cent. of the total protein, and in purified fibrinogen, where the impurities may constitute only a few per cent.

## Materials and Methods

The proteins used were products of the fractionation of human plasma by ethanol precipitation at low temperatures.[5] Fraction I, containing about 60% of fibrinogen[6] and preparations purified by reprecipitation containing 90 to 94% were used.[7] The thrombin was purified from Fraction III-2[8] and contained 10 to 15 units[9] per mg. of protein. Although the actual thrombin in these preparations may comprise one per cent. or less of the total protein, they were sufficiently free of the lytic enzyme of plasma[11] often associated with thrombin. Thus samples allowed to clot for forty-eight to seventy-two hours, 10 to 20 times the period usually employed, showed no loss in yield.

The active constituents in the Fraction I and thrombin preparations were concentrated roughly 15 to 20-fold relative to plasma. They were always freshly dissolved from the dry state. Other fractions and purified components were utilized in occlusion measurements. Certain of these were obtained as pastes or concentrated solutions which had been stored in the cold. A sodium chloride–phosphate buffer system in which the phosphate constituted one-third of the total ionic strength[12] was used in most cases. The final *p*H was measured on the syneretic fluid using a glass electrode. The temperature was not controlled.

In the most convenient procedure the fibrinogen sample was transformed into a flat fibrin clot by the action of thrombin under specified conditions. After clotting it was transferred to a supporting cloth and allowed to synerize, spontaneously or under pressure, forming a membranous film 10 to 20 $\mu$ in thickness. The thinness of this film facilitates any washing or extraction procedure. After washing or extraction it is removed from the cloth, dried, and weighed. The fibrin may be estimated gravimetrically or from its nitrogen content.[13] Colorimetric reactions such as the biuret reaction[14] or the tyrosine reactions with Folin's phenol reagent[4C,c] may also be used.

The gravimetric yield was 1 to 2% higher than that obtained by Pregl or biuret analysis and this difference corresponds to the residual weight after ignition of 1.5 to 2%, since the nitrogen factor used in computing the Pregl and biuret analyses had been determined on an ash-free basis.[15] In this study, values will refer to uncorrected gravimetric determinations.

The procedure described above may be applied to any fibrinogen-containing solution; it is quick and reproducible, and requires no special training or apparatus. It has been particularly convenient in studies of the kinetics of the clotting reaction. With care, duplicate results on clots weighing 20 mg. agreed to $\pm 0.1$ mg., the accuracy of the balance used.

The detailed procedure as standardized for routine analysis of Fraction I[16] is as follows.

A volume of solution[18] containing about 20 mg. of fibrinogen was delivered accurately into a shallow dish of about 40 sq. cm. in area and containing 0.15 *M* sodium chloride to give a final volume of 20 cc. Two units of thrombin solution was added with thorough stirring to ensure complete mixing. After two to sixteen hours the clot was carefully loosened in the dish and turned out onto a porous cloth of fine texture (*e. g.*, finest muslin, bolting silk). The cloth and clot were transferred to an absorbent pad (*e. g.*, filter pulp) on which the liquid from the clot was passively absorbed, usually in five to fifteen minutes. The cloth and the adhering fibrin membrane were successively immersed in 0.15 *M* sodium chloride and distilled water for fifteen-minute periods to remove other proteins and salt, respectively. The membrane was finally rolled off the cloth, coiled and deposited in an appropriate container. (Spot plates are convenient.) After drying sixteen hours at 110° the coil was weighed to 0.1 mg.

(5) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(6) The other proteins present in Fraction I are albumin, 7%; $\alpha$-globulin, 8%; $\beta$-globulin, 15%; and $\gamma$-globulin, 9%.[5] Although far from pure, the preparations used in this study were stable in regard to both spontaneous clotting and lysis.

(7) Before drying 96 to 98% of the protein in these preparations was clottable, but after drying the value was only 90 to 94%. Electrophoretic analysis showed 2 to 5% of $\beta$- and $\gamma$-globulin.

(8) (a) J. L. Oncley, M. Melin, D. Richert, J. Cameron and P. M. Gross, THIS JOURNAL, in preparation; (b) J. T. Edsall and S. G. Miller, unpublished experiments.

(9) The thrombin unit employed in this Laboratory[10] is, within the limits of experimental error, identical with that used by Seegers.[10] It is now defined in terms of a standard thrombin preparation. Each new preparation is assayed at serial dilution against the standard by clotting tests with fibrinogen.

(10) Minimum requirements of the National Institute of Health for Dried Thrombin, Divison of Biologics Control, National Institute of Health, Bethesda, Maryland, 3d. ed., 1946.

(11) L. R. Christensen and C. M. MacLeod, *J. Gen. Physiol.*, **28**, 559 (1945).

(12) Dry preparations of Fraction I and purified fibrinogen contain salt, largely sodium citrate, which comprised about 10% of the ionic strength at the dilution (1 g./liter of fibrinogen) and ionic strength (0.15) used in most of these experiments.

(13) As by Pregl or Nessler techniques; see for example, J. P. Peters and D. D. Van Slyke, "Quantitative Clinical Chemistry. Vol. II, Methods," Williams and Wilkins Co., Baltimore, Md., 1938,

(14) We have employed a modification using ethylene glycol as described by J. Mehl, *J. Biol. Chem.*, **157**, 173 (1945). Since the clot dissolves directly in the single biuret reagent, this is a particularly convenient technique. A concentrated albumin solution, previously compared to fibrinogen by Pregl nitrogen determination, served as a standard.

(15) E. Brand, B. Kassel and L. J. Saidel, *J. Clin. Invest.*, **23**, 437 (1944), report a factor of 5.92 (15.9% nitrogen) for washed fibrin, while Cohn, *et al.*,[5] report a factor of 6.08 for Fraction 1. In studies on fibrinogen an average factor of 6.00 has been used for both Fraction I and purified fibrinogen.

(16) As usually prepared, Fraction I has a *p*H of about 6.3 which, together with the other conditions, provides a clot with optimal mechanical properties for this method. In choosing these conditions the tensile strength, adherence, and friability of the clot have been considered.[17] Clots prepared under other conditions may be considerably more difficult to handle although the procedure is still applicable.

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

(18) Since any particulate matter such as cells, cell debris, or protein aggregates will be carried down by the clot, it is important that the unknown solution be carefully clarified before use.

## Experimental

When not specifically mentioned the conditions of clotting were those just described, namely:

| | |
|---|---|
| Fibrinogen concentration | = 1 g./liter |
| Thrombin concentration | = 0.1 unit/cc. |
| Ionic strength | = 0.15 |
| *p*H | = 6.3 |
| Temperature | = 23–26° |
| Length of clotting | = 4 to 5 hours |

For the purpose of this study, the yield under these "standard" conditions was taken as a reference point and yields under other conditions were referred to this value. The exact quantity of fibrinogen used in individual samples varied, in different experiments, between 18 and 21 mg.

**Reaction Time.**—Our concern with the time course of the clotting reaction is (1) in providing a sufficient period for completion of the reaction within our limits of error, and (2) if this is not feasible, in estimating its degree of completion. In Fig. 1 curves are presented showing the yield of fibrin with time under several conditions of *p*H and fibrinogen and thrombin concentration. Clotting may be prolonged under conditions of low *p*H, low temperature, high ionic strength and low fibrinogen or thrombin concentration. The significance of the rate of fibrin formation under various conditions and its relation to the properties of the resultant clot are considered elsewhere.[17]

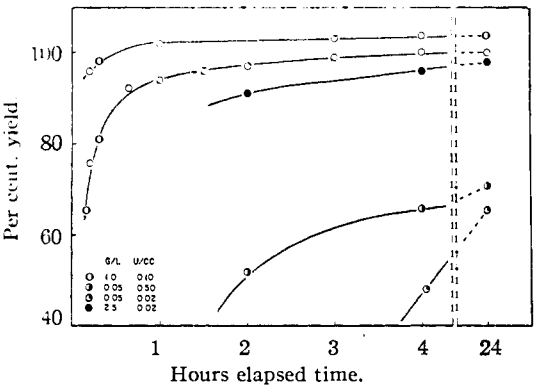


Fig. 1.—The effect of variations in *p*H, fibrinogen concentration and thrombin concentration on the clotting of fibrinogen. Ordinate, per cent. yield referred to a four hour value under "standard" conditions; abscissa, clotting time in hours. Fibrinogen and thrombin concentrations as noted; fibrinogen in grams per liter times 1.07; thrombin, units per cc. Upper curve, *p*H 7.2; all others, 6.3.

**The Concentrations of the Reactants.**—The yields obtained with various concentrations of thrombin and purified fibrinogen at a *p*H of 6.3 are shown in Fig. 2. In the ranges studied the fibrinogen concentration was a more critical variable than the thrombin concentration. This series was clotted for five hours, which appears to be a sufficient time to allow conversion to approach completion in all samples, except at the lowest fibrinogen concentrations (0.05, 0.10 g./liter) and

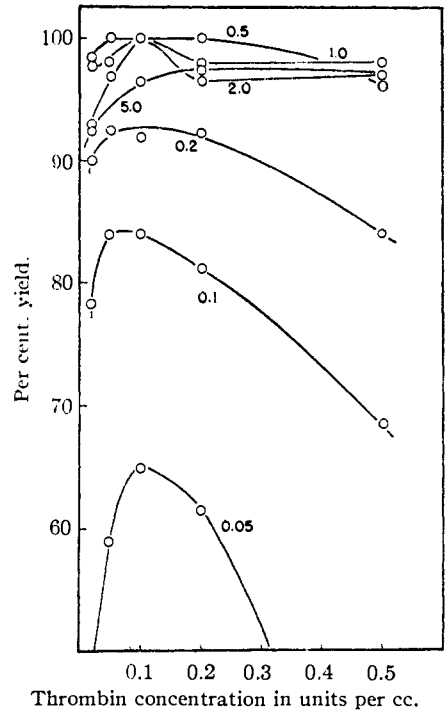


Fig. 2.—The yield of fibrin as a function of the fibrinogen and thrombin concentrations. Clotting time, five hours; salt, 0.15 *M* sodium chloride; *p*H of stock, 10 g./liter, purified fibrinogen solution, 6.34. Ordinate, per cent. yield referred to five hour value under "standard" conditions; abscissa, thrombin concentration in units per cc.; contours, fibrinogen concentration in grams per liter times 1.01.

the lowest thrombin concentration (0.02 unit/cc.). In the most unfavorable case (note Fig. 1), the sample containing 0.05/liter of fibrinogen and 0.02 unit/cc. of thrombin, a yield of 75 to 80% (instead of 65%) was obtained after clotting for fifty hours.

Figure 3 presents a yield-contour diagram whose points have been interpolated from Fig. 2 and a similar plot of per cent. yield against the fibrinogen concentration with thrombin contours. The insensitivity to changes in thrombin concentration is further emphasized. The area representing conditions under which the most satisfactory yields may be expected is bounded by fibrinogen concentrations of 0.5 and 2.0 g./liter and thrombin concentrations of 0.05 and 0.20 unit/cc. Reduced yields may be expected at both higher and lower concentrations. The standard conditions lie in the center of this area. A yield-contour diagram based on three series of measurements with Fraction I at this *p*H was very similar to that for purified fibrinogen except that at the higher fibrinogen and thrombin concentrations values slightly larger than 100% (100 to 103%) were obtained. This apparent increase in yield correlates with the much larger amount of non-clottable protein present in Fraction I as compared to purified fibrinogen.

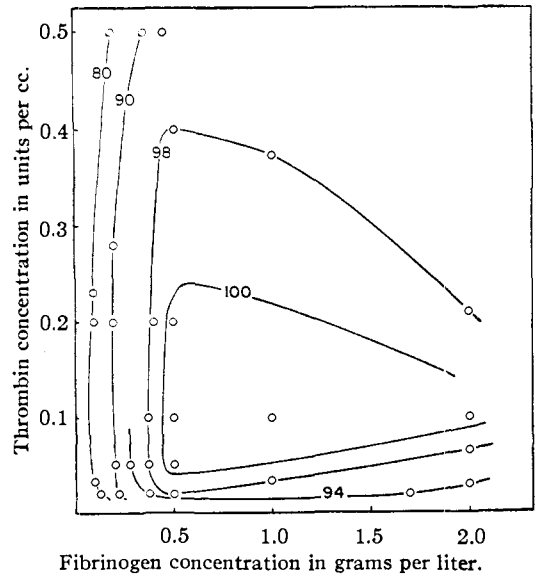


Fig. 3.—Contour diagram of the yield of fibrin as a function of fibrinogen and thrombin concentration. Individual points interpolated from fibrinogen contours in Fig. 2 and from thrombin contours plotted in a similar manner against per cent. yield and fibrinogen concentration.

In experiments with Fraction I, clotted at *p*H 6.7, 7.1 and 7.3, the yield was also very insensitive to changes in thrombin concentration between 0.05 and 0.20 unit/cc. Although the effect of fibrinogen concentration was less regular and differences of 2 to 4% were observed, the standard concentration of 1.0 g./liter was the most uniformly satisfactory throughout this range of *p*H.

In several highly purified fibrinogen preparations, 96 to 98% of the total protein was found in the fibrin after clotting under standard conditions. Since electrophoretic analysis had shown 2 to 5% of $\beta$- and $\gamma$-globulin, this demonstrates that upon clotting under these conditions the fibrinogen was quantitatively precipitated as fibrin. Lower yields obtained under other conditions (Fig. 2) suggested 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. Such a product would be particularly expected at low fibrinogen concentrations and high thrombin concentrations and would account for the falling off of the curves in Fig. 2 under these conditions. At fibrinogen concentrations of 0.02 and 0.03 g./liter, gel fractions of as low as 10 to 20% have been found,[19] *i. e.*, only 10 to 20% of the fibrinogen present was converted into insoluble clot structure. Under these conditions allowing additional time for clotting or adding more thrombin gives no further yield.

While the formation of a clot on the addition of thrombin is clear proof of the presence of fibrinogen, the converse is not true. Thus, at fibrinogen concentrations below 0.05 g./liter the result may be a transient clot, a precipitate, or no change. This fact has not been sufficiently recognized, and several investigators have cited the absence of a clot after the addition of a second supply of the clotting agent as proof of completeness of yield.

**The Effect of *p*H.**—Although the *p*H very effectively changes the properties of the clot,[17] it has much less influence on the yield. Figure 4 compares yields of both purified fibrinogen and Fraction I at various *p*H values. The increase in yield at higher *p*H values which is much more evident in Fraction I probably depends on the occlusion of non-clottable protein rather than on some property of the fibrinogen–thrombin reaction. Figure 4 emphasizes the importance of allowing a sufficient clotting time at low pH values. After twenty-four hours, values of 99 to 100% were obtained at *p*H 6.0.

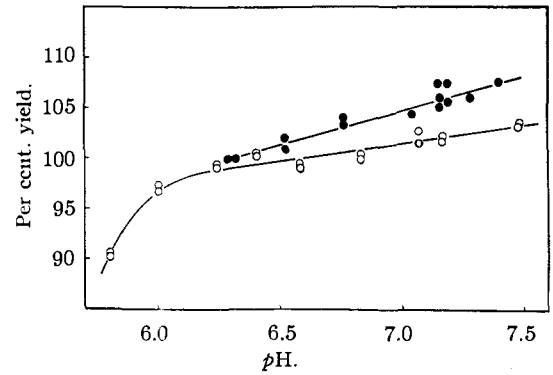


Fig. 4.—The yield of fibrin as a function of *p*H. Thrombin concentration, 0.1 unit per cc.; clotting time four hours; ionic strength, 0.10 sodium chloride, 0.05 phosphate. Open circles, purified fibrinogen, concentration, 0.97 g./liter; closed circles, Fraction I, fibrinogen concentration, 0.95 to 1.22 g./liter; value at *p*H 6.3 = 100% yield.

**The Occlusion of Other Proteins.**[20]—Other proteins are carried down by the fibrin to varying extents. Thus, although fibrinogen is colorless, fibrin from plasma may be grossly colored by occluded substances. It has long been known that thrombin itself is partly carried down by the fibrin and this fact led some observers to question the enzymatic nature of the clotting reaction. Plasmin, the proteolytic enzyme of plasma,[11] is largely occluded by the fibrin and this phenomenon has been utilized in its preparation.[21] Likewise

(19) Observations by Mr V. Kimel.

(20) The retention of other proteins by fibrin will be referred to as occlusion. The use of this term does not presume any specific mechanism and this retention cannot be clearly designated as adsorption, occlusion or chemical binding as usually defined but appears to combine several elements. There is insufficient evidence at hand to warrant a discussion of these alternatives.

(21) D. Richert, S. G. Miller and J. T. Edsall, unpublished experiments.

the antihemophilic factor[22,23] which occurs largely in fraction I appears to be almost completely carried down by the fibrin.

Occlusion has been studied quantitatively by clotting fibrinogen in the presence of known amounts of other proteins. The increase in the gravimetric yield is shown in Fig. 5. The occlusion varied widely with the nature of the protein added but was proportional to the amount added in almost all cases. Thus, under a given set of conditions, each protein or protein fraction was characterized by having a specific percentage carried down by the fibrin. Since this percentage, termed the occlusion factor, $\alpha$, is usually independent of the amount of protein added, it may be determined by a single occlusion measurement. In describing these experiments the amount of added protein is expressed as its *relative concentration, i. e.*, the ratio of its concentration to that of the fibrinogen. The occlusion factor will then be defined by the relation.

$$\alpha = (Y - 100)/R = \Delta Y/R$$

where $Y$ is the *apparent yield of fibrin in per cent.* and $R$ is the *relative concentration of added protein.* $\alpha$ is a pure number and is represented by the slopes of the curves in Fig. 5. Physically, it defines in per cent. the fraction of the added protein which is occluded. When occlusion was measured under other than "standard" conditions, the value obtained under these conditions in the absence of added protein was always taken as the base; *i. e.*, it was subtracted from the value obtained in the presence of the protein to determine the amount of occlusion.

TABLE I

THE OCCLUSION OF PLASMA FRACTIONS BY FIBRIN

| Fraction[a] | Principal component[a] | Prepn. no. | Occlusion,[b] % *p*H 6.3 | *p*H 7.1 |
|---|---|---|---|---|
| Serum | | 2/44 | | 2.4 |
| | | 3/44 | 1.6 | 2.5 |
| II | γ-Globulin | S53-2 | 0.1 | (−0.3) |
| V | Albumin[c] | ACB-26 | (− .1) | .2 |
| | | Com. 1 | 0 | .1 |
| III-0 | β-Euglobulin[d] lipoprotein | S362 | ..[e] | 26 |
| IV-1 | α-Lipoprotein[d] | H168 | 4.7 | 14 |
| | | | 5.0 | 11.5 |
| IV-4 | (α-Globulin) | H161 | 1.7 | |
| | (β-Globulin) | H167 | (−0.2) | 1.7 |

[a] The albumin and γ-globulin were purified proteins containing less than 5% of other electrophoretic components; Fraction III-0 euglobulin contained 65–70% of $\beta_1$-globulin with 75% lipid, the remainder being largely lipid-free $\beta_1$-globulin; Fraction IV-1 contained about 45% of an $\alpha_1$-globulin with 35% lipid together with an equal amount of lipid-free $\alpha_1$ protein. Fraction IV-4 is very heterogeneous, containing a small amount of albumin together with roughly equal amounts of $\alpha_1$, $\alpha_2$ and $\beta_1$ globulins. Further information regarding these fractions will be found in references (5) and (8a) and in subsequent papers in this series. [b] The occlusion factor, $\alpha$, as measured in a clot formed from Fraction I at 1.0 g./liter fibrinogen, 1.0 unit/cc. of thrombin and 0.15 ionic strength. Figures represent the average of four measurements each made at a different relative concentration of the fraction in question. Concentrations 1, 2, 5 and 10 were employed in most series; for albumin the series was 5, 10, 20 and 40. [c] Crystalline bovine serum albumin; all other fractions were of human origin. [d] The concentrations of lipoprotein were based on total weight of protein and lipid, as was the occlusion measurement. If, for a homogeneous lipoprotein, these quantities were expressed in terms of protein alone, or of nitrogen, the same occlusion factor would be obtained. [e] At *p*H 6.3 the clot was dissolved by the plasmin in Fraction III-0. Although the value measured at *p*H 7.2 is the highest recorded for any fraction, the actual value may be even higher because of this lytic action.

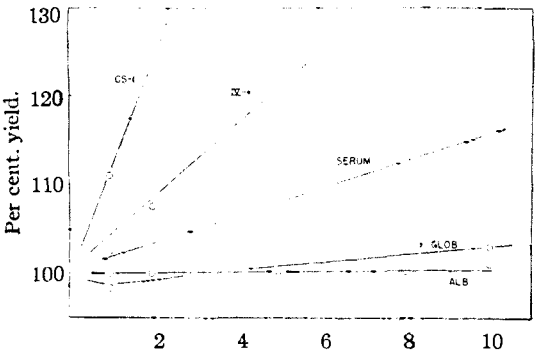


Fig. 5.—The apparent yield of fibrin as a function of the amount of added protein for several fractions and proteins at *p*H 6.3. Ordinate, per cent. yield referred to the "standard yield"; abscissa, concentration of added protein relative to the concentration of fibrinogen (or grams per liter). CS-1 refers to the insoluble subfraction from the non-clottable protein in Fraction I (note Table II). The other proteins and protein fractions are described in Table I.

The occlusion factors for the proteins shown in Fig. 5 and for other proteins, both under standard conditions and at *p*H 7.2, are summarized in Table I. Values range from 0 for serum albumin to 26% for Fraction III-0 euglobulin. In several proteins at *p*H 7.2, the occlusion falls off at higher concentrations of occluded protein, and in these cases only the linear portion of the curve has been used in determining the slope. With Fraction IV-1 at *p*H 7.2 the curve is linear up to an apparent fibrin yield of 246% (at a relative concentration of 10), the largest absolute increase thus far observed. Serum gives linear curves up to relative concentrations of 20. In this case and with serum albumin, with which relative concentrations as high as 40 have been used, no impairment of clotting has been noted. As shown in Fig. 6 the addition of a considerable amount of the non-clottable portion of Fraction I has no measurable effect on the rate of clotting as determined gravimetrically. The occlusion of this non-clottable protein and certain subfractions thereof are summarized in Table II.

(22) G. R. Minot, C. S. Davidson, J. H. Lewis, H. J. Tagnon and F. H. L. Taylor, *J. Clin. Invest.*, **24**, 704 (1945).

(23) J. T. Edsall, R. M. Ferry and S. H. Armstrong, Jr., *ibid.*, **23**, 557 (1944).

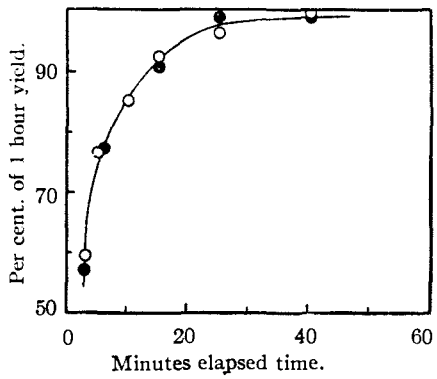


Fig. 6.—The effect of added protein on the rate of fibrin formation. Purified fibrinogen 132B, 7.0 g./liter; *p*H 6.22. Open circles, 1.75 g./liter of non-clottable protein: closed circles, 13.0 g./liter.

TABLE II

THE OCCLUSION OF NON-CLOTTABLE PROTEIN[a] FROM FRACTION I BY FIBRIN

| Preparation | Subfraction[b] | % Occlusion[c] *p*H 6.3 | *p*H 7.2 |
|---|---|---|---|
| A | Whole | 7.9 | |
| | | 7.6 | |
| B | Whole | 5.4 | 9.5 |
| | Insoluble | 11.8 | 18 |
| C | Whole | 2.8 | 5.2 |
| | Insoluble | 10 | 12 |
| | Soluble | (−0.1) | 0.1 |

[a] The composition of Fraction I is given in footnote 6. To prepare this material, Fraction I (3–20 g./liter) was clotted with 0.1 to 0.3 unit of thrombin per cc. The clot was beaten and the protein in the syneretic liquid was precipitated with 20% ethanol at −5°. [b] The non-clottable protein was divided into two fractions respectively insoluble and soluble in 4% ethanol at 0°. The former contains protein which forms a reversible gel when solutions of Fraction I are cooled to 0°. [c] As in Table I.

**Occlusion Under Different Conditions.**—Fraction IV-1 at relative concentration of 2.0 (2 g./liter under standard conditions) was chosen for comparative measurements of occlusion under various conditions. Under standard conditions the rate of clotting (note Fig. 1) was negligibly influenced by this additional protein. However, under conditions less favorable for clotting it may significantly lower the yield (note below). The effect of *p*H on occlusion has already been indicated in Table I, where values of occlusion for the various proteins at *p*H 7.2 are seen to average about twice those at *p*H 6.3. Figure 7 shows the occlusion of Fraction IV-1 over a wide range of *p*H values. At a *p*H of 7.4 its occlusion was roughly six times that at *p*H 6.2.

The occlusion of Fraction IV-1 at different fibrinogen and thrombin concentrations and at *p*H values of 6.04, 6.25, 6.60 and 7.02 is summarized in Table III. The effect of thrombin concentration was very uniform throughout, with a change from 0.2 to 0.05 unit/cc. reducing the occlusion by one-third to one-half. The effect of the fibrinogen concentration on occlusion,

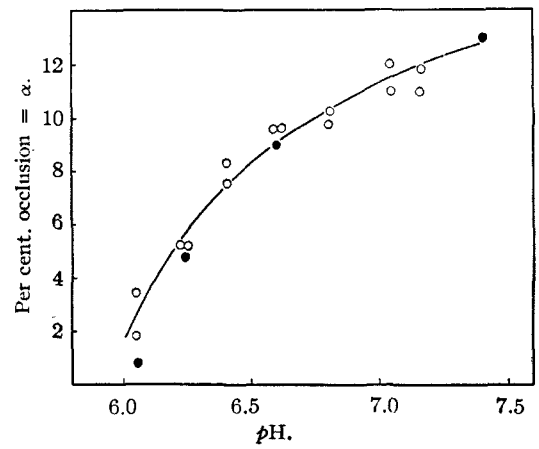


Fig. 7.—The occlusion of Fraction IV-1 as a function of *p*H. Fibrinogen, 1 g./liter, thrombin, 0.1 unit/cc.; relative concentration of Fraction IV-1, 2.0; ordinate, occlusion as per cent. of added protein.

TABLE III

THE PERCENTAGE OF OCCLUSION OF FRACTION IV-1 AT VARIOUS FIBRINOGEN AND THROMBIN CONCENTRATIONS[a]

| *p*H | Thrombin, unit/cc. | Fibrinogen in g./liter 0.2 | 0.5 | 1.0 | 2.0 |
|---|---|---|---|---|---|
| 6.04 | 0.02 | (−3.5) | | | |
| | .05 | (−0.8) | 0 | 0.8 | |
| | .10 | (−1.2) | .2 | −0.5 | |
| 6.25 | .02 | 2.5 | 3.5 | | |
| | .05 | 3.0 | 3.8 | 3.3 | 7.0 |
| | .10 | 3.5 | 5.9 | 5.0 | 7.0 |
| 6.60 | .05 | | | 3.5 | 5.0 |
| | .10 | | 3.8 | 6.2 | 6.4 |
| | .20 | | 6.5 | 7.8 | 8.3 |
| 7.02 | .05 | | 8.2 | 11.0 | 8.8 |
| | .10 | | 10.8 | 14.0 | 13.3 |
| | .20 | | 11.5 | 15.5 | 15.5 |

[a] At a total ionic strength of 0.15, 0.10 sodium chloride, 0.05 phosphate.

as on the yield without added protein, was not so consistent. With fibrinogen concentrations changing from 2.0 to 0.05 g./liter the occlusion was usually reduced by one-third. Values at *p*H 6.25 are presented graphically in Fig. 8. At *p*H 6.04 occlusion has largely disappeared and negative values (*i. e.*, a lower yield than in the sample without added protein) were obtained, particularly at low concentrations of fibrinogen and thrombin.

**The Occlusion of Thrombin.**—The occlusion of fibrin[24] is shown in Fig. 9. Thrombin is much more strongly occluded than the other proteins studied. If this were due to the actual combination of thrombin with the fibrinogen to form fibrin, as has sometimes been suggested, a larger percentage of occlusion would be expected at low

(24) W. H. Seegers, M. Nieft and E. C. Loomis (*Science*, **101**, 520 (1945)) reported the retention by fibrin of up to 20% of thrombin present in very high concentrations (more than 1000 times the concentrations reported here). Because of this great difference in concentration and because the conditions of their experiments were not given in detail, it is difficult to compare the two sets of results.

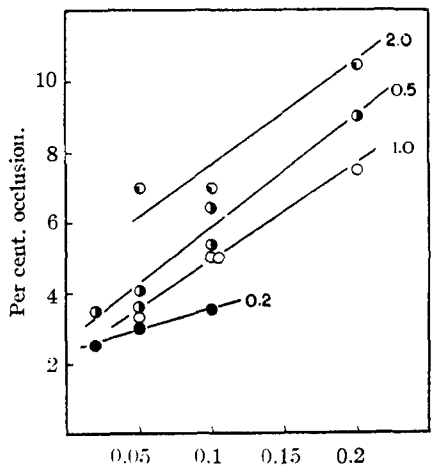


Fig. 8.—The occlusion of Fraction IV-1 as a function of thrombin and fibrinogen concentration; *p*H, 6.25; relative concentration of Fraction IV-1, 2.0; ionic strength, 0.10 sodium chloride, 0.05 phosphate; clotting time four hours; ordinate, occlusion in per cent. of added protein; abscissa, thrombin concentration in units/cc.; contours, fibrinogen concentration in grams per liter times 1.05.

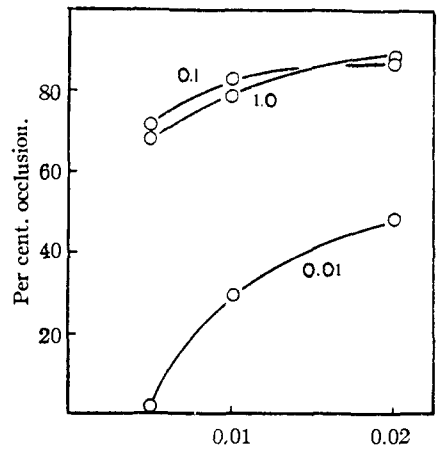


Fig. 9.—The occlusion of thrombin as influenced by its own concentration and that of fibrinogen. Abscissa, occlusion in per cent.; ordinate, initial thrombin concentration in units/cc.; contours, fibrinogen concentration in per cent.; *p*H, 6.3. These dilute thrombin concentrations were measured by clotting tests in which the onset of opacity was taken as the end point. Values in the expressed clot fluid were referred to control solutions of equal initial concentration, and to known dilutions of these solutions.

thrombin concentrations. This is not the case, however, for the effect of lowering either the thrombin or the fibrinogen concentration is to decrease occlusion.

## Discussion

The data presented have shown that in the determination of fibrinogen considerable errors can occur both through incomplete precipitation of fibrin and through the occlusion of other proteins. In choosing the optimal conditions for obtaining the true fibrinogen content, the *p*H is clearly the most important variable and the lowest value which will not seriously interfere with clotting is indicated. A *p*H of 6.1 to 6.2 appears optimal. Similar considerations govern the choice of fibrinogen and thrombin concentrations, and values of 1.0 g./liter and 0.05 unit/cc. appear suitable. Under these conditions a generous clotting time of ten to twenty hours should be allowed. Since complete yields are obtained under these conditions, the advantage of decreased occlusion need not be weighed against the disadvantage of decreased yield. These conditions fortunately differ only slightly from those which were largely based on favorable mechanical properties of the clot. In other words, the best conditions for eliminating occlusion also yield a clot with favorable mechanical properties.

**The Mechanism of Occlusion.**—In addition to the analytical importance of occlusion it is of interest in revealing properties both of fibrin clots and of the occluded proteins. Each of the influences which increase occlusion—higher fibrinogen concentration, higher thrombin concentration, and higher *p*H—also changes the properties of the clot toward the "fine" type which has smaller interstices.[25] This correlation between the size of the interstices and the extent of occlusion suggests that physical entrapping is important. We may picture the effective size of the clot interstices becoming smaller and smaller during syneresis until the occluded protein begins to be sieved out of the syneretic fluid by the meshes of the clot. The point at which sieving out begins would, then, depend on the initial size of the clot interstices and the size and shape of the occluded protein. This would occur earlier in a fine clot and would lead to the retention of a larger amount of the protein.

In accord with this interpretation serum albumin and γ-globulin are not occluded at *p*H 6.3, and both will pass through the completely synerized clot which forms a fibrin film.[26] Fibrinogen itself, which is larger and much more asymmetrical, will not pass through such a film. However, its presence in the syneretic fluid from prematurely expressed clots shows that the interstices were *initially* large enough to pass fibrinogen molecules. The fact that certain proteins can be occluded although they have already once been expressed from a clot during preparation is also consistent with the interpretation presented above.

(25) Fibrin clots have been classified[11] as ranging between two extremes, fine and coarse. The former is gelatinous, clear, friable, and non-syneretic. The latter is doughy and opaque, and does not break under pressure but deforms with loss of fluid. These and other properties indicate that the size of the fibrin units and of the meshes in the clot structure are much smaller in fine clots than in coarse clots.

(26) J. D. Ferry and P. R. Morrison, THIS JOURNAL, **69**, 400 (1947).

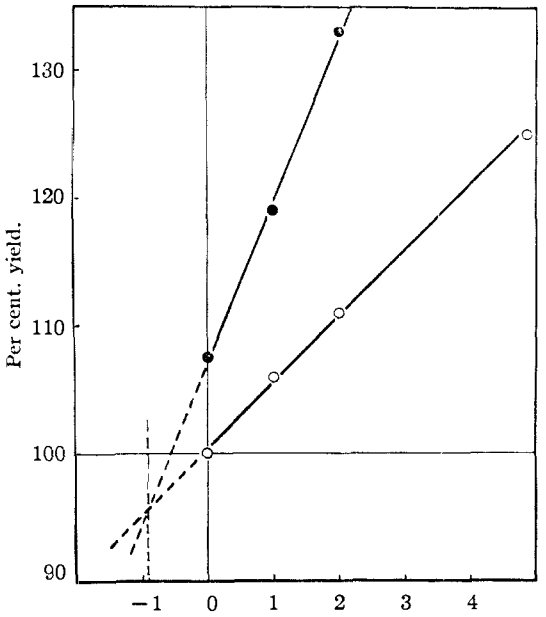


Fig. 10.—Graphic estimation of the occlusion error in Fraction I. Abscissa, relative concentration of *added* non-clottable protein, from Fraction I (equals g./liter at this fibrinogen concentration); closed circles; *p*H 7.1; open circles; *p*H 6.3. Vertical broken line shows the amount of non-fibrinogen protein already in Fraction I, *i. e.*, if 55% of the Fraction I was clottable, then the relative concentration of non-clottable protein was a little less than 1 before any protein was added. Note the excellent agreement between the extrapolated vales at *p*H 6.3 and 7.1.

This non-clottable protein can be separated into two subfractions of which one, comprising the least soluble fifth, has an occlusion factor 3 to 5 fold greater than that of the original material, while the more soluble four-fifths is not occluded at all (Table II). These data and a summation from the amounts and occlusion factors of the various fractions which make up whole plasma, both suggest that the occlusion of a heterogeneous material represents the sum of the occlusions of its component parts.

The fraction which is occluded to the greatest extent is Fraction III-0 euglobulin whose principal component is a $\beta$-lipoprotein. Osmotic pressure, ultracentrifugal and viscosity studies[27] place its molecular weight at more than 1,000,000 which might well account for its large occlusion. The $\alpha$-lipoprotein in Fraction IV-1, which is somewhat less strongly occluded, has an estimated molecular weight[27] of 300,000 or about twice that of $\gamma$-globulin. While these strongly occluded proteins are both large molecules they also contain large amounts of lipid and this, together with their size and shape, may be important in determining occlusion. A further consideration may be the net charge on the molecule since electrostatic repulsion might make the effective size of the fibrin meshes much smaller than their actual physical dimensions.

Thrombin, plasmin and the anti-hemophilic factor, all substances which are part of the clotting complex, are so strongly occluded by fibrin as to point to a specific binding or affinity of some sort. The prosthetic group or structural configuration which fits each of these substances to its special function may well provide this.

**Estimation of Errors Due to Occlusion.**—It should be emphasized that even under the optimal conditions some occlusion will still occur. Further, it may be necessary to form clots under other than these optimal conditions, and there are many values in the literature which were obtained under conditions which were far from optimal. In these cases an estimate of the occlusion error will be of value. Its magnitude in per cent. may be obtained from the relation

$$E = \alpha(P/Y)$$

where $\alpha$ is the *occlusion factor* (*cf.* above); and $P$, the *protein subject to occlusion*, and $Y$, the *apparent yield of fibrin*, are expressed in the same units. The approximation $P = T - Y$ (where $T$ is the *total protein*) is used. The occlusion error for Fraction I at *p*H 6.3 and 7.1 is estimated graphically in Fig. 10 by extrapolation from the apparent yields in the presence of a preparation of its non-clottable portion. The corrected results obtained at the two *p*H values are similar and indicate that a correction of 3 to 5% should be applied to fibrinogen values obtained for Fraction I under the conditions usually employed.

In practice, the occlusion error in an unknown fibrinogen containing solution will be determined by first clotting the solution and removing the clot. A known amount of fibrinogen is then added to the syneretic liquid and the measured occlusion in the second clot defines the original error. In using the occlusion factors of heterogeneous protein mixtures such as the non-clottable portions of Fraction I and plasma, it is assumed that the fraction of any one component occluded is small. If this is not so, the measured occlusion factor will be low because an appreciable portion of the occluded material has already been removed from the supernatant fluid. The calculated correction for occlusion will be correspondingly low. The effect should show up in the apparent attenuation of occlusion as fibrinogen is successively added and clotted out.

Estimation of the error in analyses of plasma is more difficult. Because of the presence of antithrombic activity, the thrombin concentration during clotting cannot be precisely defined. Under the conditions usually employed[13] (*i. e.*, plasma diluted with 30 volumes of 0.15 $N$ or $M$ sodium chloride, with conversion of plasma prothrombin to thrombin) which correspond to a

(27) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Coll. Chem.*, **51**, 184 (1947).

fibrinogen concentration of about 0.1 g./liter, a *p*H of about 7.3, and a relatively high thrombin concentration, both incomplete precipitation of fibrinogen and occlusion of other proteins probably occur. On the basis of this study, values for the fibrinogen content of normal human plasma in the literature may well be too high.

In view of the very high occlusion of the lipoproteins, Fractions IV-1 and III-0, reports of elevated fibrinogen levels in pathological conditions involving concomitant increases in lipoprotein should be viewed with some circumspection. Recent reports[28] of a protein of high molecular weight and considerable asymmetry, which may occur in concentrations up to 50–70% in pathological plasma are pertinent in this connection. At such concentrations a protein with an occlusion factor of only 10 would cause apparent yields 2 to 3 times greater than the actual fibrinogen content.

**Acknowledgments.**—We are indebted to Miss Susan G. Miller for her assistance in many of these analyses and to Drs. E. J. Cohn, J. T. Edsall, and J. D. Ferry for their generous advice.

## Summary

1. Analytical procedures for fibrinogen are briefly summarized and several convenient modifications of technique are described.

2. The yield of fibrin obtained by the action of human thrombin on human fibrinogen, and its modification under various conditions of *p*H and concentration, and by the presence of other proteins, has been studied.

3. At a *p*H of 6.3 complete yields were obtained at fibrinogen concentrations between 0.5 and 2.0 g./liter and at thrombin concentrations between 0.05 and 0.20 unit/cc. Reduced yields were obtained at both higher and lower concentrations of both reactants.

4. Other proteins were carried down by fibrin in amounts which varied widely with their nature. These ranged from a negligible occlusion of serum albumin to a 10–25% occlusion of certain lipoproteins and an almost complete occlusion of several enzymes, including thrombin. In general, the occlusion of each added protein was proportional to its concentration. Certain plasma components were shown to be occluded in amounts sufficient to introduce a considerable error into analyses of plasma fibrinogen.

5. A study of the effect of the fibrinogen and thrombin concentration and of the *p*H on occlusion revealed that in each case the condition which favored occlusion also favored the formation of a "fine" clot. This, and the fact that proteins of high molecular weight and asymmetry are strongly occluded, while smaller, more asymmetrical ones are not, indicates that physical entrapping is important, although the polar and nonpolar interactions of the molecules may also play a part.

6. Optimal conditions for complete yields and minimal occlusion are defined, and a procedure for estimating the extent of occlusion is described.

BOSTON, MASS. RECEIVED MARCH 8, 1947

(28) (a) K. O. Pedersen, "Ultracentrifugal Studies on Serum and Serum Fraction," Uppsala, 1945, p. 39, 121. (b) J. Waldenström, in "The Svedberg, 1884 30/8 1944," Uppsala, 1944, p. 558–573.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

# Studies on Double Refraction of Flow. III. Human Fibrinogen and Fraction I of Human Plasma[1]

BY JOHN T. EDSALL, JOSEPH F. FOSTER[2] AND HERBERT SCHEINBERG

Fibrinogen, as the major constituent involved in the clotting of blood, is a protein of particular chemical and biological interest. In the past, however, its instability has rendered difficult the study of its physico-chemical properties. The large-scale fractionation of blood plasma, employing ethanol at low temperature and low ionic strength,[3] has made possible the separation of fractions rich in fibrinogen, in active and relatively stable form.[4] Such preparations can be dried from the frozen state and thus preserved for months or years. Further subfractionation has yielded preparations in which as much as 96 to 98% of the protein is clottable with thrombin.[5] The high intrinsic viscosity of fibrinogen in solution,[6] and the readiness with which it is converted by thrombin into the complex network of the fibrin clot, indicate that the molecule is elongated

(1) This paper is Number 61 in the series "Studies on the Plasma Proteins" from Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross, and Number XVI in the series "Preparation and Properties of Serum and Plasma Proteins" from the same laboratory. The preparations of Fraction I, purified fibrinogen, and other fractions studied in this work were carried out under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(2) Present address: Department of Chemistry, Iowa State College, Ames, Iowa.

(3) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(4) J. T. Edsall, P. R. Morrison and J. D. Ferry, in preparation.

(5) P. R. Morrison, J. T. Edsall and S. G. Miller, in preparation.

(6) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. & Colloid Chem.*, **51**, 184 (1947).