[CONTRIBUTION FROM THE MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Solubility, Denaturation and Heterogeneity of Bovine Fibrinogen[1]

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At *p*H values near the isoionic range, native bovine fibrinogen exhibits a solubility behavior which is interpreted as indicating a stable heterogeneity in structural detail. By choice of solvent, fractions have been obtained which retain their solubility differences even after severe treatment, including the action and reversal of the faster denaturation reactions (FR). At ~*p*H 12 fibrinogen denatures as shown by loss of solubility on rapid adjustment to assay *p*H values between *p*H 6.5 and 8.0. Two major classifications of denaturation reactions are distinguished on the basis of their respective rates, the assay conditions under which insolubility appears, and reversibility. The FR require lower assay *p*H values to reveal insolubility and are completely reversible at any *p*H below the minimum denaturing *p*H provided that the molecules so affected remain in solution. Even for partial reversal, the slower denaturation reactions (SR) require a prolonged treatment at a *p*H intermediate between the denaturing *p*H and *p*H 8.0. As reported previously[10] this *p*H is near *p*H 10.8. The result of such treatments is a variety of reversal products, some clottable but differing from native in decreased solubility near the isoelectric range and others soluble in this range but non-clottable. After action of the FR, the fibrinogen is distributed so that all of it is insoluble at assay *p*H 6.5. With increasing assay *p*H the fraction of initial protein which remains in solution (F_s) increases until all is soluble at ~*p*H 8.0. At any assay *p*H in this range, F_s is independent of the saturating body. After the FR, fractions may be prepared by choice of assay *p*H. The supernatant fraction regains native solubility. Reexamination of the solubility effects of the FR on these fractions reveals that each variety retains those structural characteristics which determine its position in the assay *p*H sequence. Analyses of fractions shows that solubility heterogeneity and the heterogeneity revealed by the FR are not directly related. An expansion of the molecule at high *p*H is indicated by alterations in sedimentation coefficient and viscosity; alterations in optical activity being observed at the same time. Since the major changes in all occur during the time required essentially to complete the FR, an expansion mechanism involving the loosening of large submolecular units is preferred. The changes in physical properties observed at the end of the SR are reversed on adjustment to *p*H 10.8, the sedimentation coefficient and specific rotation being equivalent to those of the native protein brought to the same *p*H.

Introduction

Much of the evidence available from studies of physical and chemical properties suggests that, at worst, proteins of a given designation are paucidisperse. We might expect, then, that appropriate fractionation would produce a molecularly homogeneous population. In spite of its obvious utility, there is no real justification for making the basic assumption that all such molecules will be identical in the sense that simple chemical compounds are identical, as has been pointed out by a number of writers.[3-6] Colvin, *et al.*,[6] have reviewed the evidence and conclude that in no case can such perfect homogeneity be proven. That a native protein consists of a wide variety of molecules, possibly identical in their primary structures and differing slightly in their secondary and/or tertiary structures, becomes more probable as the size of the molecules increases. The size of the molecule also influences views of the structural alterations attending denaturation, the classical manifestation of which is a decrease in or loss of solubility under conditions where the native protein is soluble. Since the early work of Hopkins[7] several kinds of physical and chemical alterations have been associated with a decrease in solubility. Often the latter is not made manifest under the conditions producing denaturation due either to electrostatic energy barriers, which prevent close approach, or to chemical agents which decrease interaction energy. Several reviews have considered denaturation.[8,9] While the native protein is generally accepted as having available a limited number of conformations all closely related and interconvertible from the structural standpoint, the denatured protein is considered to have available many conformations, covering a much wider structural range; thus, that denaturation introduces randomness into an initial set of microcrystalline structures. An understanding of the degree of unfolding or expansion which takes place during denaturation, both in the intermediate and final states, is of greatest importance where the alterations in properties are found to be reversible, for it is difficult to reconcile reversibility with gross disorganization of structure. It is not necessary, furthermore, to postulate extreme disorganization even when marked changes in solubility or biological activity occur.

In an earlier publication,[10] it was reported that bovine fibrinogen undergoes several interesting changes at high *p*H. The molecules fragment irreversibly if conditions are strenuous enough, but at *p*H 12.2, low ionic strength, and near 0° there is a rapid (less than 20 minutes) expansion, leading to a reduced sedimentation coefficient without reduction in molecular weight, and a logarithmic decay of solubility on assay in phosphate buffer at *p*H 7.7, $\mu = 0.1$. Solubility and clottability, as well as native sedimentation behavior, are to a large extent recovered by treatment for several hours at an intermediate *p*H, the optimum value of which was found to be 10.8.

The existence of a critical intermediate *p*H and incomplete reversibility suggest that the condi-

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tions used were more drastic than one would expect to be associated with complete reversibility, reversible equilibria, etc. A further investigation of the characteristics and reversibility of alkaline denaturation revealed a heterogeneity on the part of fibrinogen. This observation of heterogeneity suggested that an examination of the solubility of native fibrinogen near the isoionic *p*H range be undertaken. The results of these studies are reported here.

Materials and Methods

Materials.—For most of the present work the fibrinogen was prepared as previously described[10] essentially according to the method of Laki[11] from Armour Bovine Fraction I. For certain comparison experiments, as will be noted, fibrinogen was also prepared by the method of Blombäck and Blombäck[12] from Armour Fraction I, as well as from the fresh blood of a single animal. The final preparations, at concentrations of 10–20 mg./ml., were dialyzed against appropriate buffers for immediate use or for storage at −20°.

Protein concentration was regularly measured as optical density at 280 mμ, this being related to weight concentration by the use of the factor 0.61 mg./ml./OD unit. This factor assumes 6% residual water after drying at 100° for 24 hr. (constant weight). Clottable protein was determined at *p*H 7 using about 1 mg./ml. total protein and 0.1 NIH unit/ml. thrombin. Concentration of the supernatant was determined at 4 hr. clotting time after compaction and removal of the fibrin. Preparations ranged from 92 to 98% clottable.

For several preparations supernatant concentrations were examined at times between 1.5 and 48 hr. Clottability did not vary by more than 1% over this period. In addition, all clots remained unchanged in appearance over a period of two weeks or more.

Most of the experiments required that the starting solutions be fairly concentrated (10–20 mg./ml.) but at low ionic strength ($\mu < 0.01$). To keep the fibrinogen in solution under these conditions, a somewhat elevated *p*H was required. For this purpose a buffer of 0.01 *M* glycine titrated to *p*H 9.2 with KOH was used. This will be referred to as standard glycine buffer.

Solubility.—Solubility measurements were made over a range of saturating body, temperature, ionic strength and *p*H. Sodium acetate buffers were used. Two general mixing methods were employed, dialysis and direct mixing. In the former, a series of samples of different fibrinogen concentrations were dialyzed together against a chosen solvent. Dialysis sacks were wiped with tissue and weighed in a closed bottle before and after dialysis so that correction could be made for changes in volume. Maximum deviations of 3% were observed when this technique was tested by controls in which no precipitation occurred. The second method involved the direct mixing, in a series of vials, of acetate buffer, fibrinogen solution and the standard glycine dialysate of the fibrinogen. The total amount of the last two was held constant and the saturating body determined by the proportion of fibrinogen solution. This was theoretically less satisfying than the dialysis method but gave identical results and was much less tedious. Protein in solution was determined after equilibration at constant temperature and with gentle rocking for 12–16 hr.

Denaturation Kinetics.—Samples of fibrinogen (10–20 mg./ml.), dialyzed against standard glycine buffer, were taken to the desired denaturing *p*H by the addition of 0.31 *N* KOH. An atmosphere of nitrogen was maintained above the solutions to prevent carbon dioxide absorption. After successive time intervals, measured aliquots were removed and plunged into large (10×) volumes of chosen assay solvents. After equilibration for at least 1 hr., sediments were removed by centrifugation and supernatant concentration measured.

Ultracentrifuge runs were made in the Spinco Model E Ultracentrifuge using schlieren cylindrical lens optics. For high *p*H runs a Kel-F centerpiece was used. The rotor temperature was measured at intervals during the runs by the radiation method of Waugh and Yphantis.[13]

Optical rotation measurements were made in a Rudolph model 200 photoelectric polarimeter.

Viscosity measurements were made in a capillary viscometer having an outflow time of 18.9 sec. for 7 ml. of distilled water at 3.5°.

Results

Solubility studies were taken after much of the information to be presented under denaturation had been obtained. However, they are simpler to interpret, are independent of denaturation studies and contribute to an understanding of denaturation in such a way as to suggest that they be presented first.

Solubility.—The solubility of untreated purified fibrinogen is shown in Fig. 1, which gives the data for several solvent conditions ranging from that of curve 1, a poor solvent, to curve 7, a relatively

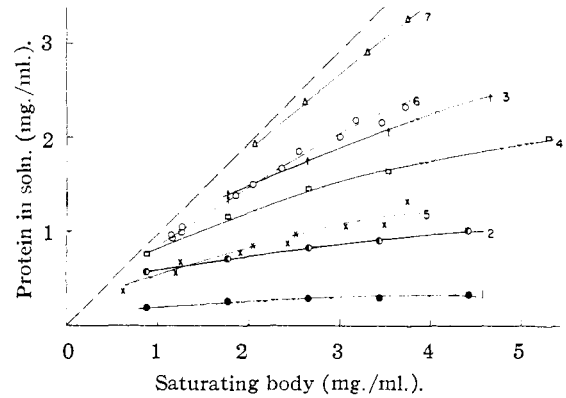

Fig. 1.—Typical solubility curves for fibrinogen: (1) *p*H 5.5, $\mu = 0.75$, $T = 0.0°$; (2) *p*H 5.5, $\mu = 0.37$, $T = 0.0°$; (3) *p*H 5.45, $\mu = 0.21$, $T = 0°$; (4) *p*H 5.1, $\mu = 0.30$, $T = 15°$; (5) *p*H 5.5, $\mu = 0.045$, $T = 21°$; (6) *p*H 5.5, $\mu = 0.09$, $T = 21°$; (7) *p*H 5.5, $\mu = 0.18$, $T = 21°$.

good solvent. It is apparent that a considerable manipulation of solubility may be obtained at these *p*H values, which are near the isoelectric range, by appropriate choice of conditions. The dotted line of Fig. 1 represents the initial portion of the solubility curve for a pure component. For such a system the solubility limit would be indicated by a horizontal line. It is apparent that under none of the conditions used does fibrinogen behave as a single component as defined by the phase rule.

Each curve requires that fibrinogen be at least a two component system and, to a first approximation, each can be pictured as such, in which case extension of the nearly linear solubility curve to the vertical axis would give the solubility of the less soluble component and the slope of the curve would be a direct measure of the fraction of the total protein represented by the more soluble component. By varying the solvent conditions, however, essentially any slope from zero to one can be produced. Since each curve implies a division into less soluble (<1 mg./ml.) and more soluble components, it is evident that the fraction of the whole falling into one or the other of the components is a function of the solvent conditions. This, in turn, means that there are more than two varieties of molecules present (possibly many).

(11) K. Laki, *Arch. Biochem. Biophys.*, **32**, 317 (1951).

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If this interpretation is *correct*, it should be possible to obtain fractions with different solubility characteristics. Such fractionations have been accomplished by dialysis and direct mixing methods, which gave similar results. Ingredients were mixed as for a solubility measurement, but in larger proportions, equilibrated overnight and the precipitates removed. Supernates were concentrated by precipitation with 30% saturated ammonium sulfate. Both fractions were dissolved in potassium phosphate buffer *p*H 7.5, μ = 0.3, dialyzed *versus* standard glycine buffer, and solubility re-examined.

Figure 2 shows a typical result. In Fig. 2a the solid line represents the solubility curve of the initial fibrinogen in the solvent used for fractionation and the points represent the solubility of the supernatant and precipitant fractions for several saturating bodies under the same solvent conditions. Shown in Fig. 2b are solubilities of the same three materials under another set of solvent conditions.

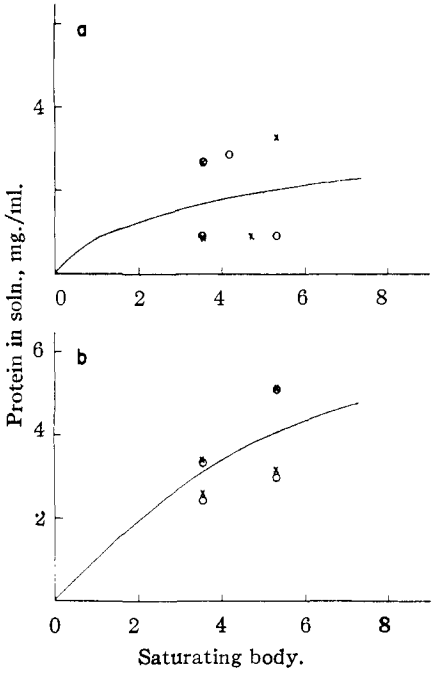

Fig. 2.—Solubility fractionation: (a) solubility of starting material (solid line), supernatant fraction (upper points) and precipitant fraction (lower points) in the solvent used for fractionation, sodium acetate, *p*H 5.10, μ = 0.3, T = 15°; (b) solubility of the same three materials in a second sodium acetate solvent, *p*H 5.5, μ = 0.2, T = 15°. Points marked x represent material that has been through an alcohol precipitation (see text).

In these experiments and in others to be presented, both solubility and saturating body are expressed as total rather than clottable protein. This is justified by the fact that the non-clottable protein segregates in a manner similar to the clottable protein, as shown by the fact that the control and both fractions assayed about 93% clottable. The solubility of the supernatant fraction is characteristic of a mixture, as expected, since it contains the saturation quantities of the relatively insoluble component as well as most of the soluble component. It is enriched with soluble component, however, since it is more soluble than the starting material. The precipitant fraction has low solubility, which in the solvent used for fractionation is nearly independent of the saturating body. That it is nevertheless a mixture can be seen from its behavior in the second solvent.

Before accepting these differences in solubility as indicating a heterogeneity on the part of native fibrinogen, the preparative procedure should be examined for the possibility that a single fibrinogen species present in the animal is modified in various ways to produce heterogeneity. That this is not the case is indicated by an extensive series of studies. Comparison solubility studies were made of fibrinogen prepared from (a) the fresh plasma of a single cow by the method of Blombäck and Blombäck[12] (b), from Armour Fraction I by the method of Blombäck and Blombäck and (c) by the method of Laki.[11] No significant differences were observed. Fibrinogen fractions, such as those illustrated in Fig. 2, were subjected to treatments, including alcohol precipitation corresponding to the original isolation procedure, lyophilization, lyophilization followed by reflux petroleum ether extraction, and treatment with ethylenediaminetetraacetate sodium. These treatments were undertaken to examine not only the possibilities of structural alterations due to preparative procedures, but also the alterations in properties which might be traced back to the influences of bound lipides and divalent cations. No significant alterations in solubility were observed.

It has been frequently observed that different preparations of fibrinogen, though equal in total clottable protein, differ in the finer details of their clotting characteristics. The following results indicate why this might be expected. More soluble and less soluble fibrinogen fractions were prepared as described above and dialyzed, along with unfractionated starting material, against a *p*H 7 buffer. Clotting times were measured by a method used routinely in this Laboratory for several different concentrations of each preparation. The results are shown in Table I. The differences in clotting time are clearly significant, the less soluble fraction forming a clot most rapidly.

TABLE I

CLOTTING TIMES OF SOLUBILITY FRACTIONS[a]

Sample	Concn. (mg./ml.)	Clotting times (sec.)
Starting material	1.01	18.5, 18.7, 18.5, 17.3, 17.9, 18.4, 19.2, 18.7
	0.90	18.1, 17.7, 17.6, 18.4, 18.5, 19.0, 18.5, 18.4
	.80	18.4, 18.6, 18.7
Supernatant fraction	.95	22.1, 20.7, 22.5
	.90	21.3, 21.2, 21.6, 22.6, 22.0, 22.0, 21.9, 22.5
	.80	21.3, 21.3, 21.4
Precipitant fraction	.90	16.3, 16.2, 16.1, 16.4, 16.5, 16.3, 16.6, 16.4
	.80	16.4, 16.3, 16.3

[a] Fractionated at *p*H 5.10, μ = 0.3, T = 15°, saturating body = 6 mg./ml.

Denaturation.—The denaturation reactions described by Fitzgerald, *et al.*,[10] take 60 minutes to go essentially to completion. These reactions appear to be first order except during a brief initial period when the loss in solubility is excessive. It is noted also that a single assay *p*H of 7.7 was used and that this assay *p*H is well above the isoelectric range. These facts suggest that faster denaturation reactions might be occurring which would be revealed by lowering the assay *p*H. Such is the case, for if assay *p*H 6.5 is used, virtually all of the fibrinogen becomes insoluble within 4 minutes for denaturation at *p*H 12. Our present purpose is to describe certain properties of the faster denaturation reactions (FR) and slower denaturation reactions (SR).

The solubility behavior of fibrinogen denatured by fast reactions, slow reactions, heat, urea, etc., is different from that of the native material where, as described in the preceding section, a significant amount of solute is in equilibrium with the solid phase. In the range of assay solvents used (*p*H 6.5–8.0, μ = 0.1–0.3) solubility of a particular denatured variety is either high or negligible. Thus the fraction (F_s) of the total protein which remains in solution in a particular assay solvent is virtually independent of the saturating body. This fraction, then, represents a definite set of molecular varieties.

Figure 3 shows the results obtained from studies of a denaturation carried out at *p*H 12, 3.5°, 0.04 ionic strength and 15 mg. of protein per ml.

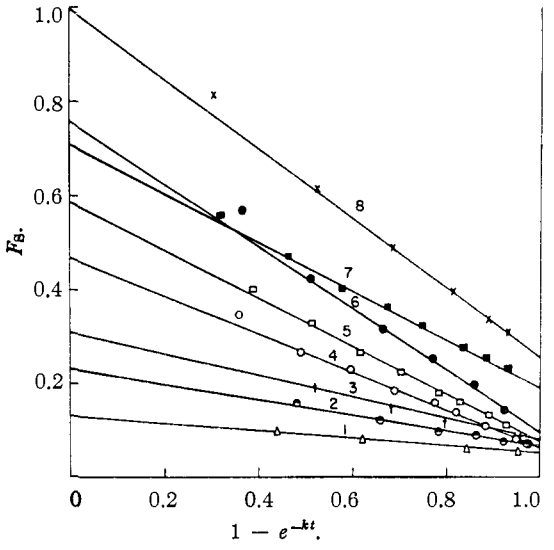

Fig. 3.—Denaturation at *p*H 12.0, μ = 0.04, T = 3.5°, c = 10–20 mg./ml. ($k = 10^{-3}$ sec.$^{-1}$). Assayed in potassium phosphate buffer at: (1) *p*H 6.53, μ = 0.1; (2) *p*H 6.93, μ = 0.1; (3) *p*H 7.15, μ = 0.1; (4) *p*H 7.3, μ = 0.1; (5) *p*H 7.4, μ = 0.1; (6) *p*H 7.6, μ = 0.1; (7) *p*H 7.4, μ = 0.3; (8) *p*H 7.5, μ = 0.3.

Each line refers to a particular set of assay conditions; the group to studies mainly of the effects of alterations in the assay *p*H and to an extent (curves 7 and 8) alterations in ionic strength. The ordinate records F_s, the fraction of total protein which is soluble, and the abscissa the quantity ($1 - e^{-kt}$) where t is in seconds and k is a rate constant calculated from the experimental data on the assumption that the SR follow first-order kinetics. Several of the initial points lie above the straight lines, a consequence of the fact that the FR are not instantaneous. According to the assay *p*H and ionic strength each reaction curve extrapolates to a particular value of F_s at t = 0. This value of F_s should depend only on the characteristics of the FR.

That such is the case is shown by the results summarized in the lower curve of Fig. 4. Here, denaturing *p*H values ranging from 11.92 to 12.08 were used and assays carried out in a single solvent

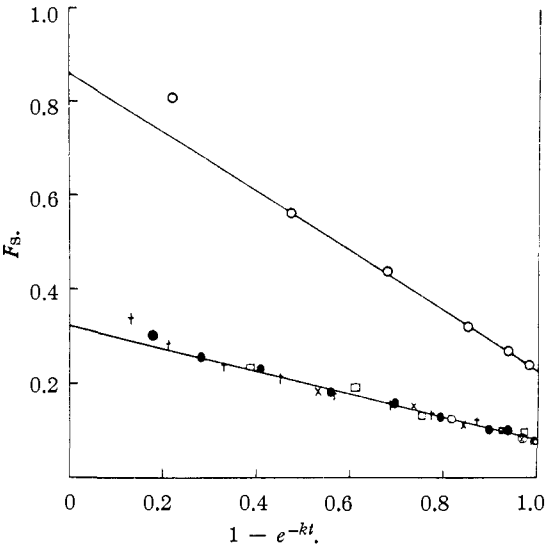

Fig. 4.—Effect of varying the denaturing *p*H (lower line): (†) *p*H 11.92, $k = 5.0 \times 10^{-4}$ sec.$^{-1}$; (●) *p*H 11.98, $k = 7.7 \times 10^{-4}$ sec.$^{-1}$; (□) *p*H 12.00, $k = 12.3 \times 10^{-4}$ sec.$^{-1}$; (x) *p*H 12.06, $k = 17.0 \times 10^{-4}$ sec.$^{-1}$; (○) *p*H 12.08, $k = 28.8 \times 10^{-4}$ sec.$^{-1}$. Assay solvent potassium phosphate buffer *p*H 7.15, μ = 0.1. Denaturation kinetics (upper line) of material recovered as soluble after denaturation for 5 minutes at *p*H 12.0 and using an assay solvent at *p*H 7.15, μ = 0.1. This kinetic experiment was performed at the same denaturing *p*H and with the same assay solvent.

at *p*H 7.15. Although the SR varied widely in rate, as shown by the variation in k from 5 to 28.8 $\times 10^{-4}$ sec.$^{-1}$, all points lie on a single line. From the common intercept it is apparent that the set of structures produced by the FR is independent of the denaturing *p*H over the range used. Thus, after a short time at the denaturing *p*H, the population of molecules is distributed in a sequence of denatured forms, each characterized by an assay *p*H (for constant μ) below which it is insoluble.

A given assay *p*H thus makes a definite selection from the fast denatured varieties existing at *p*H 12. Two possibilities are apparent: the distribution of denatured varieties could be produced from (1) a single variety of native fibrinogen or (2) a distribution of native varieties. That the latter is the case and that the distributions of native and denatured proteins are not a collection of tautomeric configurations is evident from the following investigations. The FR, as will be shown, are completely reversible. This allows a fractionation based on the FR and a chosen assay *p*H. The lower line of

Fig. 4 represents denaturation kinetics of the starting material, the assay conditions being *p*H 7.15 and $\mu = 0.1$. The starting material was treated for 5 minutes ($1 - e^{-kt} = 0.3$) at *p*H 12.0 and the soluble components recovered after using the same assay conditions followed by sufficient time for renaturation of the supernatant protein. This renatured protein was concentrated and denaturation kinetics examined under the conditions initially specified. The upper curve of Fig. 4 was obtained. Alternative 1 above would require that the renatured protein be comparable to the starting protein, and alternative (2) that the renatured protein should yield a line extrapolating to unity at $t = 0$; (*i.e.*, at this assay *p*H no formation of precipitate of protein affected only by the FR). That alternative 2 is the case is apparent. The observed small deviation from unity in ordinate intercept might result from a combination of a slight decrease in assay *p*H, the specific choice of k or the inclusion of some material partially recovered from the SR (see below). The sequence of denatured forms, therefore, represents a one-to-one transformation of a sequence of native forms.

We have presented evidence to the effect that heterogeneity, as observed through studies of solubility in the isoelectric range, is not due to the procedures used in preparing fibrinogen. A similar conclusion is now drawn again, for no significant differences in denaturation kinetics were observed for the same preparations. Furthermore, the denaturation characteristics of a fraction such as that used in the studies of Fig. 4 were unaffected by alcohol precipitation.

Studies so far described have dealt with fast and slow denaturation reactions near *p*H 12. Both sets of reactions are *p*H sensitive: at *p*H 11.8 the rates of the SR are 0.1–0.2 of those found at *p*H 12. The FR are also reduced in rate but in a manner which immediately focuses attention on the complexity of this sequence. For example, in Fig. 5 are shown a series of assay *p*H lines obtained for denaturation at *p*H 11.8. It should be noted at once that these lines result from the FR since the denaturation so produced is completely reversible. The SR would produce downward deviations in the lines of Fig. 5 on the extreme right-hand side (*i.e.*, at longer times). Thus, extrapolation of these lines to infinite time give values of F_s which would occur at the completion of the FR in the absence of the SR. These are, as expected, in agreement with the values of F_s obtained by extrapolating the lines of Fig. 3 (the SR) to zero time. The fact that the zero time extrapolations of the FR shown in Fig. 5 are not unity but occur in a *p*H assay sequence shows that the FR of Fig. 5 are preceded by still faster reactions which require a lower assay *p*H to give a particular F_s.

It has been stated that the FR are completely reversible. The first evidence for this came from examinations of the supernatants remaining after assays between *p*H 6.5 and 8.0. It was observed that after 0.5 hr., an adjustment of *p*H to *p*H 6.5 did not lead to precipitate formation. Indeed, the renatured fibrinogen in solution in the assay samples is indistinguishable from undenatured controls even in detailed solubility behavior in the vicinity of the isoelectric point. Nor do they differ significantly from the starting materials in clotting time measurements. It should be noted that this is true even though these samples represent definite fractions of the starting material and differ from it in denaturation studies. Further evidence on the reversibility of the FR comes from studies of fractions obtained on the basis of solubility near the isoelectric range. For example, a sample of native fibrinogen was fractionated on the basis of solubility at *p*H 5.5, $\mu = 0.2$, $T = 15°$, and saturating body = 5.3 mg./ml. Half of each fraction was subjected to the FR. A short stay (5 minutes) at *p*H 12 was used to minimize loss due to the SR. A *p*H of 8.5 was used in the "assay solvent" in order to allow reversal of the FR. With respect to the SR, part of the small amount of protein so affected will precipitate at *p*H 8.5 and the remainder with the next downward adjustment to *p*H 6.0. The renatured supernatant protein was then compared with the untreated fractions and with the unfractionated starting protein as to isoelectric solubility. Solubilities under the conditions used for the initial fractionation were determined, with the results shown in Table II. It is striking that the solubilities of the products obtained after reversal of the FR are so closely similar to their undenatured counterparts. The experiments described in this paragraph deal not only with reversibility of the FR but indicate the stability of the observed heterogeneity.

TABLE II

Sample	Solubility[a] (mg./ml.)
Initial unfractional protein	1.48
Supernate, native	2.39
Supernate, denatured and recovered	2.08
Precipitate, native	1.07
Precipitate, denatured and recovered	1.04

[a] Measured under conditions of fractionation—sodium acetate *p*H 5.5, $\mu = 0.2$, $T = 15°$, saturating body = 5.3 mg./ml.

An important result of another set of experiments was that fractions based on solubility near the isoelectric point did not differ significantly from each other or from the starting material in their denaturation characteristics.

Reversibility of the Slower Denaturation Reaction (SR).—The SR appear to correspond to the denaturation reactions studied by Fitzgerald, *et al.*[19] They found that after slow denaturation, solubility in the physiological *p*H range could be in large part recovered by incubation at a narrowly defined intermediate *p*H near *p*H 10.8. This recovery has now been examined more closely. A number of fibrinogen samples were taken to *p*H 12.0 in the usual way. After 1 hr. the *p*H was lowered by the addition of 0.5 *M* glycine buffer, *p*H 9.5, to several *p*H's in the 10–11.5 range. The samples remained under nitrogen at 3.5° for about 16 hr. after which aliquots were assayed in various low *p*H buffers ($\mu = 0.1$, phosphate). The results are assembled in Fig. 6. F_s, the fraction soluble (unity for the starting material), is plotted *versus* assay *p*H for various values of intermediate *p*H.

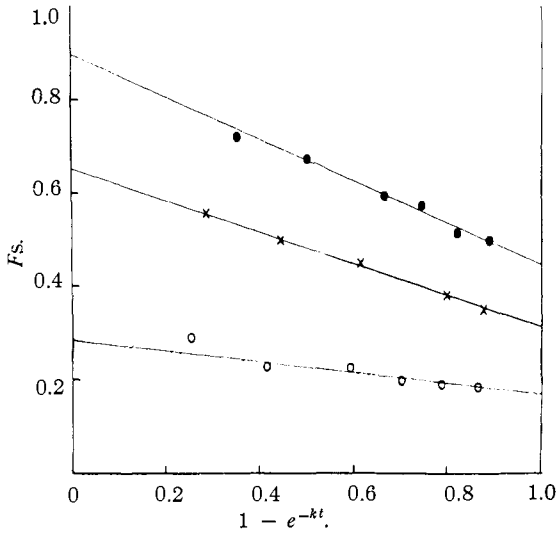

Fig. 5.—Denaturation at *p*H 11.8, μ = 0.04, T = 3.5°, c = 18 mg./ml. (k = 8 × 10^{-4} sec.$^{-1}$). Assayed at: (1) *p*H 6.6, μ = 0.1; (2) *p*H 7.2, μ = 0.1; (3) *p*H 7.4, μ = 0.1.

As in the kinetic experiments, F_s is independent of the saturating body. The uppermost curve is the result of incubation at *p*H 10.8 only, skipping the *p*H 12.0 denaturation. It is important to note that some denaturation is seen to occur here. This denaturation is completely reversible, for which reason we classify it as due to the FR. The optimum intermediate *p*H for recovery, *p*H 10.6–10.9, agrees well with the *p*H of 10.8 previously reported.[10] Figure 6 shows that all treatments at intermediate *p*H values lead to heterogeneity sequences, different varieties precipitating out successively as the assay *p*H is lowered. A complete recovery has clearly not occurred. Some improvement in recovery may be obtained by introducing, after the *p*H 10.8 step, a sojourn at *p*H 9. This improvement, comparable to the amount of protein found to be rendered insoluble by the FR at *p*H 10.8 using native fibrinogen, is probably due to the reversal of reactions similar to the FR.

The bottom curve of Fig. 6, where F_s = 0.18, represents the fraction of initial protein remaining in solution after thrombin is added to assay supernatants after recovery at *p*H 10.8 and adjustment to the assay *p*H. In view of the fact that the original protein is 92% clottable (F_s = 0.08 after thrombin treatment), it is clear that the over-all treatment produces non-clottable protein. This could originate for example as a result of the denaturation–recovery cycle or from enzymatic actions such as are characteristic of plasmin. It has been noted, with respect to the latter, that the soluble non-clottable fraction does not increase with time.

Over the assay *p*H range used the soluble non-clottable protein has a constant value of F_s = 0.18. The differences between this value and values of F_s, for example, points on the curve for *p*H 10.87, give the fractions of the total protein which are soluble and clottable. Evidently protein fractions which are denatured according to strict solubility criteria are nevertheless clottable by thrombin if they are kept in solution.

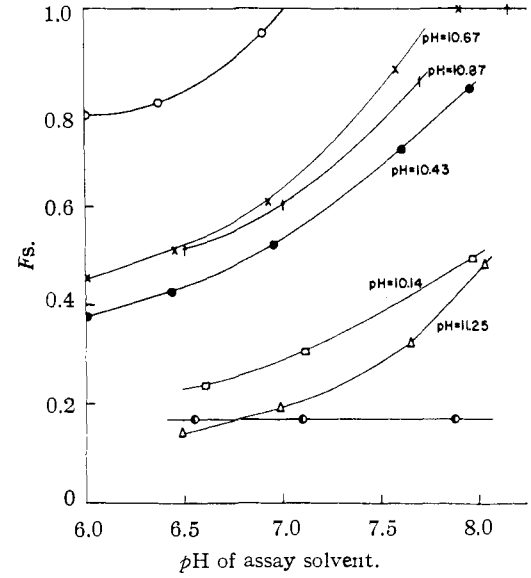

Fig. 6.—Effect of intermediate *p*H on recovery of the SR. Uppermost curve represents assays of starting material incubated at *p*H 10.8 only. Lowest curve represents fraction non-clottable after recovery at *p*H 10.8.

The curves of Fig. 6 show the maximum recoveries obtained at single intermediate *p*H values. Failure to observe complete recovery might be the result of a requirement that the structures existing under denaturing conditions proceed toward the native states by going through a definite series of structural alterations, each involving a small energy barrier. A sudden decrease in *p*H might freeze in non-native structures while a gradual reduction would permit the proper sequence and thus increase recovery of solubility at the lower assay *p*H values. Two experiments were carried out in which, after denaturing for 1 hr. at *p*H 12.0 and 3.5°, an immediate adjustment was made to *p*H 11.6 and thereafter at 30 minutes intervals downward increments of about 0.05 *p*H units were made. Recoveries were not as high as those obtained by using a single treatment at *p*H 10.8.

Recovery of Solubility of Denatured Protein after Precipitation.—The precipitates of denatured protein formed in experiments such as these are uniformly insoluble between *p*H 4 and *p*H 11. At *p*H 12.0, however, they can be brought into solution within 1 hr., after which an incubation for 16 hr. at *p*H 10.8 gives a product which remains in solution at *p*H 8.0 and is to a large extent clottable; qualitatively similar to fibrinogen treated at *p*H 12 and allowed to recover without precipitation.

Physical Changes during Denaturation.—The reduction in sedimentation coefficient at *p*H 12.0 and μ = 0.04 has been attributed to a loosening of the bonds joining relatively large submolecular units, without actual fragmentation.[10] It was suggested there that some looseness of structure might even obtain at *p*H 10.8 and that such looseness might be required for recovery of solubility at low *p*H. Present sedimentation studies have shown that there is indeed a reduction in sedimentation coefficient at *p*H 10.8 (see Fig. 7) from 7.95 S at *p*H 8 to 7.6 S at *p*H 10.8. As the *p*H is increased from *p*H 8

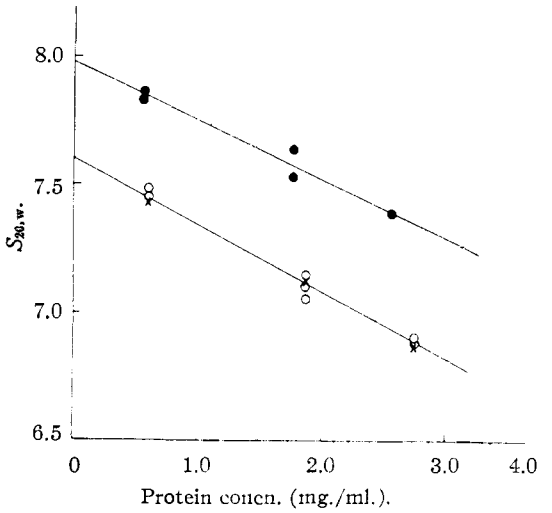

Fig. 7.—Comparison of sedimentation coefficients for fibrinogen at *p*H 7.7 (upper line) and at *p*H 10.8. Points marked O represent material treated at *p*H 12.0.

there is a small but significant increase in negative optical rotation as shown in Fig. 8. These increases take place within minutes after *p*H adjustment. In contrast, above *p*H 10.8 the changes in rotation are larger and strongly time dependent. In view of the small change in sedimentation coefficient, no viscosity measurements were undertaken at *p*H 10.8.

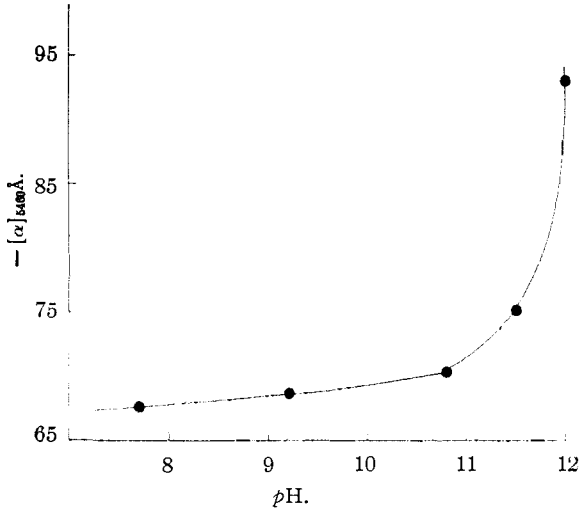

Fig. 8.—Specific rotation at $\lambda = 5460$ as a function of *p*H.

The situation is more complex at *p*H 12. The negative optical rotation increases with time as shown in Fig. 9. The value of $-68°$ at zero time corresponds to the rotation at *p*H 9.2. Within 8 minutes at *p*H 12 the specific rotation is $-84°$, a $\Delta[\alpha]$ of $\sim -16°$ taking place within the time required to complete the FR. During the next 60 minutes, the time required essentially to complete the SR, the rotation changes by the relatively small $\Delta[\alpha]$ of $\sim -4°$. During the next 6 hr. there is a levelling off and an additional increase of about 4°.

The viscosity changes which take place during the first 360 minutes of incubation at *p*H 12 are also shown in Fig. 9. The results are similar to those described for optical rotation, *i.e.*, a large change during the first 8 minutes, a smaller change during the next 60 minutes followed by a gradual levelling off thereafter.

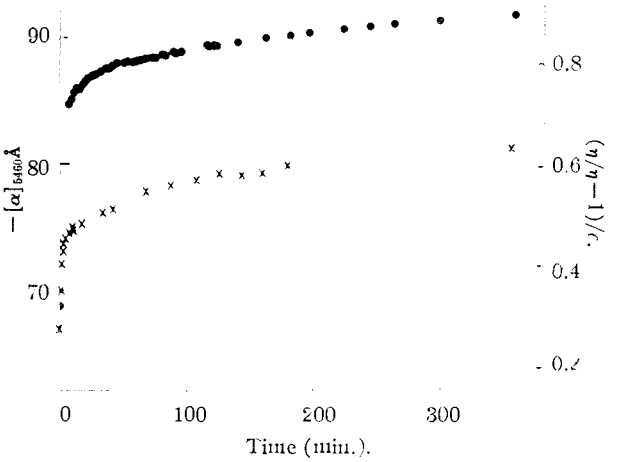

Fig. 9—Viscosity (x) expressed as $(\eta/\eta_0 - 1)/c$, and specific rotation (●) as a function of time. For both, $c = 1.0$ g./100 ml at the denaturing *p*H.

Studies have been made also of the reversal of optical rotation and sedimentation coefficient changes when the *p*H, after 1 hr. incubation at *p*H 12.0, is lowered to *p*H 10.8. The open circles of the lower line of Fig. 7 show that $S_{20,w}$ has returned to its characteristic properties: *i.e.*, the slope and intercept are the same as for a solution brought from *p*H 8 to *p*H 10.8. The recovery in optical rotation is shown in Fig. 10 for intermediate *p*H's 10.8 and

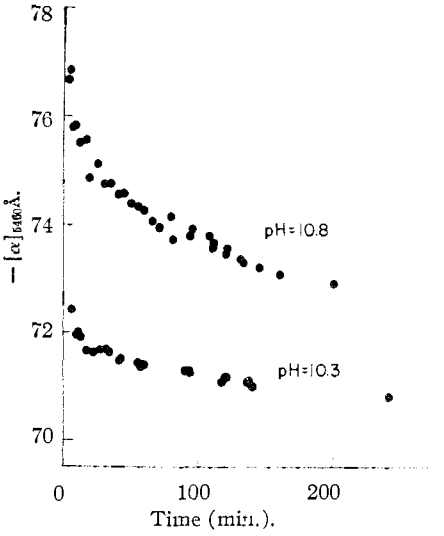

Fig. 10.—Recovery of specific rotation at two intermediate *p*H values after one hour at *p*H 12.0.

10.3. A time dependent decrease in negative rotation takes place, the final values after 16 hr. being the same as for equivalent solutions titrated from *p*H 8.0 to the respective intermediate *p*H values. Only at *p*H 10.8, however, is the time course of recovery of solubility at *p*H 7.7 comparable to the recovery of $-[\alpha]$.

Discussion

Two major classifications of denaturation reactions, designated faster denaturation reactions (FR) and slower denaturation reactions (SR), differ in that: (a) the FR are completely reversible at any *p*H where the molecules so affected are soluble provided the *p*H is below the lowest denaturing *p*H. In contrast, the SR require a special treatment to accomplish recovery. Thus, a critical recovery *p*H of 10.8 is observed. (b) For a particular group of molecules the SR produce structural alterations responsible for insolubility at higher assay *p*H values than do the FR. Thus most of the molecules altered by the SR may be removed at *p*H 8.0, where molecules altered only by the FR remain in solution. (c) The FR invariably have preceded the SR in time. They are essentially complete before the SR have produced extensive effects.

Heterogeneity and the Faster Denaturation Reactions.—Solubility near the isoelectric point may be used to obtain fractions which retain their differing solubility characteristics when re-examined and even when re-examined after recovery from the FR.[14] Similarly, fractions may be obtained after the FR by proper choice of assay *p*H. That these are true fractions is demonstrated by their assay response after resubmission to the action of the FR. Clearly the native fibrinogen exists in a variety of structures and is therefore heterogeneous.

It should be noted however that fractions obtained with the use of the FR and recovery do not differ in solubility characteristics from the starting material. Likewise, solubility fractions examined by *p*H assay after the FR do not differ significantly from the starting material. The two observations of heterogeneity therefore do not depend in the same way on the structural differences present in the initial population.

That the FR produce small modification in structure is indicated by their rapid and complete reversibility and by the results of studies at denaturing *p*H values between 10.8 and 12.0 where, as described, sets of FR are revealed: the faster the set of FR the lower the assay *p*H required to give a chosen value of F_s and presumably the smaller the structural modification involved. In addition, at the lowest denaturing *p*H used we have observed only minor changes in sedimentation coefficient and optical rotation. Clearly, large changes in solubility accompany these small structural modifications. This suggests that the native structure of each variety is established to provide adequate, possibly maximum, solubility. The solubility of the native protein, then, must depend on a surface pattern in which groups contributing to intermolecular attraction are shielded, probably by groups responsible for electrostatic repulsion.

The differences between the surface patterns of varieties might depend either upon small alterations in a common pattern or upon different basic patterns. The former alternative is more attractive. In any case, the structure of the protein is such as to permit modifications of surface pattern but prevent interconversion of patterns even during the physical alterations accompanying the FR. Remarkable are the facts that a variety of patterns can exist, that the molecules having these patterns are soluble and yet that slight modifications in any pattern produce insolubility. It is also clear that at near physiological *p*H the native patterns are the preferred ones so long as modifications are limited to those produced by the FR.

We have noted above that after the FR and use of a particular assay *p*H, those molecules remaining in solution return rapidly to their native states while those which have precipitated remains insoluble up to $\sim$*p*H 11. This is true for all assay *p*H values. It is surprising that the F_s is independent of the saturating body and that a narrow *p*H range exists for each denatured variety below which it precipitates and above which it remains in solution. Two interesting possibilities, to be tested in future experiments, are evident: (a) at a particular assay *p*H certain of the modifications in structure may be locked in by energy barriers between these structures and the native structures, while in other similarly modified varieties energy barriers may not prevent a rapid return to the native structure or (b) the energy barrier to close approach may be highly *p*H sensitive, the *p*H at which the barrier is sufficiently low depending on the modified variety.

During a period of 1 hr. at a denaturing *p*H of 12.0 the FR are complete, the SR are essentially complete, the sedimentation coefficient has decreased, the specific viscosity has increased and the specific rotation has decreased. The larger parts of the changes in the latter three take place within the first few minutes and on a time scale comparable to the progress of the FR. In view of the fact that all of the FR appear to be reversible in detail, an expansion mechanism described previously[10] is preferred to a mechanism akin to isotropic swelling in accounting for the initial changes in sedimentation coefficient and viscosity. Thus, expansion is attributed to the loosening of a flexibly connected group of molecular subunits.

Brief examinations of heat coagulated fibrinogen suggest that this protein, dissolved at *p*H 12 in 10 minutes, corresponds closely to native fibrinogen treated at *p*H 12 in the same way. This implies that the structural alterations attending heat coagulation are no more drastic than those corresponding to the FR. Indeed, it might be suggested that the two alterations are of the same nature, precipitation occurring during heat denaturation since the *p*H is low. We have observed that heat denaturation will occur in the absence of coagulation at *p*H 9.2 and $\mu = 0.01$.

Pertinent to the question of heterogeneity are the investigations of Deutsch,[15] who has examined the heat and urea denaturation of another fairly large protein, horse erythrocyte catalase ($M = 270{,}000$). Using a single assay solvent and varying the sever-

(14) Several studies of solubility, such as those conducted by Morrison, *et al.* (P. R. Morrison, S. Shulman and W. F. Blatt, *Proc. Soc. Exptl. Biol. Med.*, **78**, 653 (1951)), have had as their main objective the isolation of fibrinogen from plasma and have not dealt with the homogeneity of the resulting fibrinogen. In an early study, Florkin (M. Florkin, *J. Biol. Chem.*, **87**, 629 (1930)) reported that fibrinogen behaves as a single component. However, these studies were performed under salting-out conditions where the solubility is low. Since large amounts of saturating body were used, no deviations indicative of heterogeneity would be expected.

(15) H. F. Deutsch, *Acta Chem. Scand.*, **5**, 1074 (1951).

ity of the denaturing conditions, Deutsch concluded that his crystalline material was heterogeneous, more stable varieties requiring more extreme conditions for denaturation. Considering our results in peeling off the slowest of the fast reactions by denaturing at *p*H 11.8, it seems likely that the assay *p*H sequence of fibrinogen could be expressed in similar terms. In the absence of evidence to the contrary. Deutsch assumed that the heterogeneity had been introduced during processing and isolation of the catalase.

The Slower Denaturation Reactions.—At the start of the discussion several criteria for distinguishing the FR and SR were given. On the basis of these criteria we conclude that the structural changes involved in the SR are more drastic than those produced by the FR. That they represent a different type of reaction has been made clear.

During the course of the SR the increments in η and $-[\alpha]$ are small compared to the increment concurrent with the FR. Our attention now is on the reversibility of the SR. Of the conditions examined, a sojourn at a critical intermediate *p*H of 10.8 has been most effective in permitting recovery of solubility. In no instance, however, have the solubility properties of the initial population been duplicated and thus many if not all of the molecules present differ in structure from their native progenitors. Little can be said concerning the relationship between the sequences of native and denatured forms or about the potential reversibility of the SR. So far, as attempts are made to reverse the alterations of the SR, apparently non-native modifications are produced at lower *p*H values whose structures are locked in by the development of significant energy barriers between these and native structures. Just as apparent as the incompleteness of reversal is the fact that a significant increase in solubility is generally obtained. This is not surprising, since, as the denaturing *p*H is lowered, one would expect that those groups responsible for attractive energy should satisfy their exposed secondary valences internally so long as precipitation does not occur. The solubility differences between native and recovered protein, just discussed, are not accompanied by observable differences in sedimentation coefficient or optical activity at *p*H 10.8. We recall also that the "recovered" fibrinogen is 90% clottable at *p*H 8, where it is entirely soluble. Solubility, then, is the most sensitive criterion of those used for comparing molecular structures.

Some time ago Caspary and Kekwick[16] reported the dissociation, under near physiological conditions, of freshly prepared human fibrinogen. In many of the experiments described previously and reported here conditions have been used which would be expected to promote dissociation. All of the evidence so far obtained is consistent with the view that bovine fibrinogen is structurally stable up to *p*H 10.8, and well beyond this *p*H if the FR are permitted to reverse. In addition, we have prepared human fibrinogen according to the technique of Caspary and Kekwick and have been unable to observe dissociation under the conditions used (*p*H 8.0, $\mu = 0.35$) or at *p*H values up to *p*H 12.0. Technically, the Archibald procedure[17] was used to determine molecular weights at protein concentration down to 0.04%.

Denaturation Kinetics.—The SR and FR for which data are available are first order in time, suggesting that a single critical molecular event must be sufficient to produce an observed change. Either a particular reaction is available to all varieties or a set of alternative reactions is available, the sum of whose rate constants is the same for all varieties.

It is interesting at this point to examine the kinetics of denaturation of fibrinogen in concentrated urea solutions as reported by Mihályi.[18] The molecules undergo a first-order primary denaturation reation initially observable at low assay *p*H. With time there is a progressive loss of solubility, the same amount of precipitate occurring at progressively higher assay *p*H values. As in our experiments Mihályi found that the fraction of initial fibrinogen assaying as soluble is independent of the saturating body in the assay solvent. It is clear that this result would be obtained if, after a primary denaturing event, a large number of denaturation reactions were available, increasing fractions of which produced insolubility at increasing assay *p*H values. If the progressive reactions observed by Mihályi are comparable to the FR, the *p*H assay effects observed in the two types of denaturation might be comparable. Since reversibility and heterogeneity have not been studied for urea denaturation, no further comparison of the two sets of results is now made.

Origins and Heterogeneity.—In the foregoing, evidence has been presented to show that the observed heterogeneities are not due to the preparative procedures. There remain a variety of interesting possibilities involving, either the synthetic mechanism(s), primary, secondary and tertiary structure, or a process of aging, all molecules being initially identical.

CAMBRIDGE, MASS.

(16) E. A. Caspary and R. A. Kekwick, *Biochem. J.*, **67**, 41 (1957).

(17) H. K. Schachman, "Methods in Enzymology," Vol. 4, Academic Press, Inc., New York, N. Y., 1957, p. 32.

(18) E. Mihályi, *Acta Chem. Scand.*, **4**, 317 (1950).