have found that at a given concentration of methyl orange, serum albumin adsorbs about three times as much dye as the same weight of β-lactoglobulin, and that ovalbumin and pepsin show no detectable adsorption of methyl orange. We have found that serum albumin is very easily unfolded by urea while pepsin and ovalbumin are unfolded with much more difficulty and the behavior of β-lactoglobulin is intermediate between those of ovalbumin and serum albumin.

This similarity is interesting in the light of the recent suggestion of Karush[13] that serum albumin is able to change the conformation of its adsorption sites to fit the contours of whatever molecules are available for adsorption. He calls this behavior "configurational adaptability." It is very possible that the ease with which urea unfolds serum albumin is a manifestation of a lack of rigidity in the serum albumin molecule which would also make possible configurational adaptability.

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The Kinetics of Protein Denaturation. IV. The Viscosity and Gelation of Urea Solutions of Ovalbumin[1]

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The viscosities of ovalbumin solutions in urea increase very considerably with time. The first stage of this change is caused by the unfolding process which is responsible for the change in optical rotation, but the viscosity continues to increase after the optical rotation has reached a constant value. This difference in behavior is more marked, the higher the protein concentration and is caused by the gradual aggregation of the unfolded protein molecules. The factors affecting this aggregation were studied in two ways: (1) by observing the rate of gelation at high ovalbumin concentrations, and (2) by studying the effect of protein concentration on the viscosity change induced by urea. The effect on aggregation of electrolytes, *p*H, urea concentration and various oxidizing and reducing agents have been investigated. In accordance with the results of Huggins, Tapley and Jenson on serum albumin, it was found that the reaction between sulfhydryl and disulfide groups is an important cause of aggregation. Other factors (electrostatic charges and hydrogen bonds) also seem to play an important role, especially at lower urea concentrations. When the sulfhydryl-induced aggregation is suppressed by *p*-chlormercuribenzoate, the optical rotation and viscosity change at nearly the same rates. Values of the intrinsic viscosity of the unassociated, denatured ovalbumin molecule have been obtained under various conditions. The behavior of the viscosity subsequent to changes in both the protein concentration and the urea concentration has also been studied.

In Part I[3] an account has been given of the kinetics of the change of the optical rotation of ovalbumin in urea solutions. This change is undoubtedly a consequence of the unfolding, or denaturation, of the ovalbumin molecule and it therefore offers a convenient means of studying this interesting process. The structural changes which accompany denaturation are, however, probably very complex and as Neurath, *et al.*,[4] have pointed out, it is therefore desirable to investigate denaturation by studying simultaneously the changes in different properties which depend on the structure of the protein in different ways. The optical rotation depends on the position of the groups in the vicinity of the asymmetric carbon atoms in the protein. A convenient complementary property is the viscosity increment of the protein solution, which is sensitive to the over-all molecular shape and compactness.[5] Whereas a change in the optical rotation of a protein indicates a change in the internal relationships of the atoms, a change in the viscosity of a protein solution indicates a change in the shape of the external envelope of the molecule.

Many workers have noted and studied the very considerable increase in the viscosity of ovalbumin and serum albumin solutions in the presence of urea.[6-11] The increase is often at least partially time-dependent.[11] It furnishes very strong evidence for the generally accepted view that denaturation involves uncoiling of the relatively tightly folded native protein molecule to a more open and extended form.

Using ovalbumin and serum albumin in urea we have found that the viscosity invariably continues to increase with time long after the optical rotation has reached a steady value (compare Fig. 2 of this paper with Fig. 1 of Part I). This paper and the next one are concerned with a detailed examination of this difference in behavior and with its explanation.

There are two possible ways of accounting for such a result: (1) the initial unfolding of the molecule might be followed by a gradual aggregation involving only a few points of contact between molecules; or (2) the initial unfolding might produce a molecule consisting of flexible loops held together by a few intramolecular cross links which then gradually rupture, permitting the molecule to take on more extended shapes (Fig. 1). Neither of these possible secondary changes should affect the optical

(1) This article is based upon the theses submitted by H. K. Frensdorff and M. T. Watson in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Princeton University.

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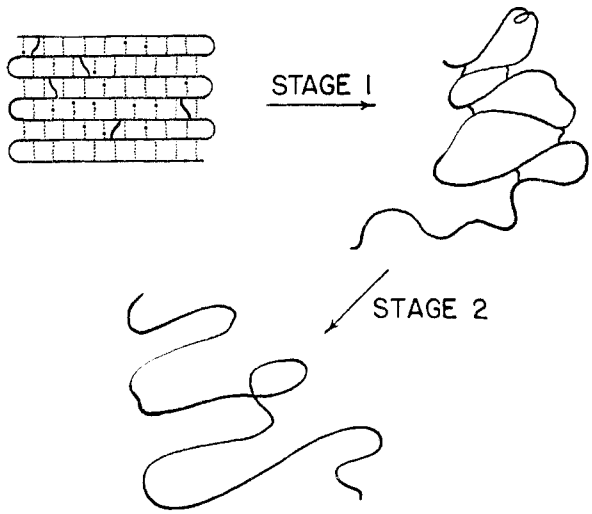

Fig. 1.—Possible stages in the denaturation of a protein. Stage 1 represents the initial unfolding reaction caused by rupture of many weak linkages and Stage 2, the subsequent "loop-opening" caused by rupture of a relatively few strong intramolecular cross links.

rotation very much, since this property is sensitive only to changes in the vicinity of an appreciable fraction of the asymmetric centers in the molecule. On the other hand both aggregation and loop opening can make the viscosity increase.

Some evidence has been advanced that aggregation does not take place in the urea denaturation of ovalbumin and serum albumin. Burk and Greenberg[12,13] concluded from osmotic pressure measurements at comparatively low protein concentrations that the molecular weight of these two proteins remains unchanged when they are treated with 6.66 *M* urea at 0°. Neurath, Cooper and Erickson[9] came to the same conclusion on the basis of viscosity and diffusion measurements on serum albumin in 8 *M* urea at 25°. Rothen[14] obtained similar results for ovalbumin by means of sedimentation–diffusion measurements. All of these results involved, however, extrapolations to zero protein concentration and therefore refer to the state of the molecule at infinite dilution.

In spite of this evidence, it is well known that urea solutions of ovalbumin and serum albumin usually form gels when the protein concentration is high enough.[15] In other words, pronounced aggregation must occur under certain conditions. Huggins, Tapley and Jensen[16,17] have investigated the gelation of serum albumin and some other proteins. They found that addition to serum albumin of as little as one equivalent of any class of reagents which destroys sulfhydryl groups will prevent the formation of gels in concentrated urea, and they ascribe gelling to an exchange reaction: "By means of a chain reaction between protein–SH and protein–SS groups a reticulum is knitted together consisting of intermolecular SS bonds which hold together the extended protein chains." Halwer[18] has recently presented evidence for the formation of intermolecular disulfide bonds in heat coagulated ovalbumin.

In this paper we shall show that a concentration-dependent aggregation occurs during the urea denaturation of ovalbumin and that this accounts for the difference in behavior of the optical rotation and the viscosity. It will be shown that except possibly at the highest urea concentration the aggregation is caused by hydrogen bonding as well as by intermolecular disulfide bonds. In the next paper the somewhat different behavior of serum albumin will be investigated from this point of view.

The effect on aggregation of varying the experimental conditions was studied in two ways: (1) by observing the time required for gelation to occur, and (2) by measurement of the viscosity changes accompanying denaturation, paying particular attention to the effects of varying the protein concentration.

Experimental

The results of the viscosity experiments are expressed in terms of the *reduced viscosity*

$$\eta_{red} = \eta_{sp}/c = \frac{1}{c}\left(\frac{\eta}{\eta_0} - 1\right)$$

where c is concentration of protein in % (g. per 100 ml. solution); η_{sp} is specific viscosity; η is viscosity of the protein–urea–buffer mixture; η_0 is viscosity of the "solvent," *i.e.*, a solution of the same composition as the protein–urea–buffer mixture, except that distilled water has been added in place of the protein stock solution. The ***intrinsic viscosity*** is the limiting value of the reduced viscosity as c approaches zero. Throughout this paper reduced viscosities and intrinsic viscosities will be expressed in units of (g. per 100 ml.)$^{-1}$.

The viscosimeters were of the Ostwald type. In order to minimize the danger of clogging the capillary with solid particles which might have been produced by surface denaturation, capillaries of relatively large bore (about 1.2 mm.) were used. The outflow end of the capillary projected into the reservoir bulb in such a way as to form a dead space in the lower part of the bulb. This served as a trap for solid particles. Water times of 40 to 55 seconds at 30° were obtained by making the capillaries 80 to 125 cm. long and bending into several loops. The viscosimeters were cleaned with fresh, filtered chromic acid solution and thoroughly rinsed and dried before each run. When not in use they were kept filled with chromic acid solution or distilled water.

They were mounted rigidly in a 30.0° thermostat which held the temperature constant to within ±0.005°. The outflow times obtained with different solvent preparations of a given composition generally checked within ±0.08 sec.

The ratio η/η_0 was assumed to be equal to the ratio of the outflow time of the protein solution to that of the solvent. This neglects the kinetic energy effect (which is small because of the large capillary bores) and the small effect of the protein on the density of the solution; it usually leads to a reduced viscosity too low by about 1 to 2%. In the most unfavorable case (*viz.*, the native protein) the error so introduced may be as great as 5%.

Ovalbumin was prepared from fresh hens' eggs either by the method of Kekwick and Cannan[19] or by that of Sørensen and Høyrup.[20] It was recrystallized four times. The crystals were stored either air-dried or, more commonly, as a paste wet with half-saturated ammonium sulfate solution. The compositions of the solutions were determined after dialysis by drying weighed samples of solution to con-

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stant weight at 100°. No significant difference between batches of ovalbumin prepared by the various methods could be noted.

All protein solutions were stored under saturated toluene vapor in the refrigerator. Fresh urea solutions were prepared for each experiment. Urea concentrations are expressed in moles per liter of solution at room temperature. The urea–buffer mixtures and the protein stock solution were warmed to 30° before mixing, and in some cases were filtered. A 7-ml. sample was then pipetted into the viscosimeter. The filtration step was omitted when it became apparent that the results were not affected by it. The first viscosity reading could usually be taken within 2 or 3 minutes of mixing.

Since salts have a considerable effect on the kinetics of denaturation, it is desirable to maintain as low a buffer concentration as is consistent with reasonably good *p*H control and with suppression of the electroviscous effect. A buffer concentration of 0.05 *M* was felt to be adequate for this purpose, since Bull[21] showed that 0.02 *M* sodium chloride suppresses the electroviscous effect in native ovalbumin.

The *p*H values were measured with a Beckman Model G *p*H meter. The *p*H of the various buffers employed varies with the urea concentration. The buffer capacity was usually sufficient to prevent *p*H changes greater than 0.2 *p*H units within 1500 minutes.

The comparatively large size of the viscosimeter capillaries resulted in velocity gradients of the order of 100 sec.$^{-1}$. In order to be sure that the measured viscosities were not dependent on the velocity gradient, the viscosity of a representative solution was measured under several pressure heads. The results in Table I show that the relative viscosity is not greatly, if at all, affected by the velocity gradient, so the flow is essentially Newtonian.

TABLE I

DEPENDENCE OF VISCOSITY ON PRESSURE HEAD

1.5% ovalbumin denatured for 1800 minutes in 10 *M* urea and borate buffer (*p*H 10.2). Pressure head varied by changing volume of solution in the viscometer.

Vol. of soln., ml.	7	12	17
Outflow time for water, sec.	44.59	58.61	86.52
Outflow time for protein soln., sec.	171.7	225.9	330.0
Relative viscosity	3.85	3.86	3.81

Results and Discussion

A. Gelling Experiments.—Ovalbumin forms gels in 10 *M* urea and 0.05 *M* 1:1 NaH_2PO_4:Na_2HPO_4 buffer if the protein concentration is 3% or more. The results reported in this section were obtained in an effort to investigate the various pertinent factors in a systematic manner.

The gelling times reported here are only approximate, because the transition from a viscous liquid to a gel is a gradual one. Fortunately the rate of viscosity increase becomes very great as the gelling point is approached, so that the interval between extreme viscosity and definite gelation is usually quite short.

All of the results in this section were obtained using the same batch of ovalbumin. Unless otherwise mentioned, the experiments were performed on 3% ovalbumin solutions in 10 *M* urea at 30.0°. Gelling was considered to have taken place when the solution failed to flow on inverting the test-tube (dimensions 10 × 100 mm.).

1. Effect of Electrolytes.—The effects of various electrolytes on the gelling time are summarized in Table II. Two important effects may be noted: (a) increasing the electrolyte concentration accelerates gelling, and (b) electrolytes with more highly charged anions are more effective than those with monovalent anions. This effect is so strong that gelling occurs at a protein concentration as low as 2% in the presence of 0.20 *M* sodium sulfate. The cationic charge seems to be unimportant, since the gelling time is the same in 0.10 *M* NaCl as in 0.05 *M* $CaCl_2$.

(21) H. B. Bull, *Trans. Faraday Soc.*, **36**, 80 (1940).

TABLE II

EFFECTS OF ELECTROLYTES ON THE GELLING TIME

(3% ovalbumin in 10 *M* urea, no buffer, *p*H 7.6–8.0)

Electrolyte	Concn. moles/l.	Gelling time, hr.	Electrolyte	Concn. moles/l.	Gelling time, hr.
Na_2SO_4	0.20	0.5	$CaCl_2$	0.05	24
$K_4Fe(CN)_6$	.10	2	Na_2HPO_4	.025	24
NaCl	.50	3	NaH_2PO_4	.025	
Na_2SO_4	.05	11	NaCl	.050	40
$CaCl_2$	.20	18	None	...	[a]
NaCl	.10	24			

[a] Fails to gel.

The role played by electrolytes may be understood if one assumes that they decrease the repulsions between the electrical charges on the molecules and permit them to approach more closely. It is, however, somewhat puzzling that the anion should be more important than the cation at a *p*H above the isoelectric point, where the protein carries a net negative charge. It may be that the anion exerts its effect by being adsorbed at certain critical sites which carry positive charges (despite the net negative charge of the molecule).

If salt linkages between positively charged groups on one molecule and negatively charged groups on another were important in causing aggregation, one would expect that electrolytes would inhibit aggregation, since they should stabilize the charged groups either by direct combination with them or by surrounding them with an atmosphere of gegenions (Debye–Hückel effect). The fact that electrolytes promote aggregation therefore indicates that salt linkages play an insignificant role in causing aggregation.

2. Effect of *p*H.—The data in Table III illustrate the effect of changes in *p*H on the time required for gelling. 0.10 *M* sodium chloride was present in all samples so that the addition of acid or base caused but small variations in the ionic strength.

TABLE III

EFFECT OF *p*H ON THE GELLING TIME

(3% ovalbumin in 10 *M* urea, 0.1 *M* NaCl, no buffer)

Added acid, HCl, equiv./mole (45,000 g.) protein	*p*H	Gelling time, days	Added base, NaOH, equiv./mole (45,000 g.) protein	*p*H	Gelling time, days
150	3.0	[a]	7.5	7.1	2
75	3.6	[a]	11	8.0	1
30	4.9	6	15	8.9	0.5
15	5.5	5.5	21	9.6	0.5
7.5	5.8	5	30	10.5	1
0	6.3	4	38	10.8	[a]
			60	12.0	[a]

[a] Fails to gel.

The absence of gelation at the lowest and highest *p*H's is undoubtedly caused by the mutual repul-

sions between the highly charged molecules. At the highest *p*H values some hydrolysis might also occur. The isoelectric point for urea-denatured ovalbumin is at *p*H 5.7,[7] while the optimum for gelation is considerably higher, at *p*H 9. This shift of the optimum from the *p*H of lowest net charge indicates that some phase of the gelling reaction must be favored by high *p*H. We shall see that this behavior is probably associated with the ionization of the sulfhydryl groups in the denatured protein.

3. **Influence of Urea Concentration.**—The results presented in Table IV show that the optimum urea concentration for gelation is about 8 *M*. In fact, even 2% ovalbumin will gel in 7.5 *M* urea.

TABLE IV

EFFECT OF UREA CONCENTRATION ON THE GELLING TIME

(3% ovalbumin in 0.05 *M* 1:1 sodium phosphate buffer)

Urea concn., moles/l.	*p*H	Gelling time	Half time for unfolding[b]
1	7.0	[a]	.....
3	7.1	[a]	.....
5	7.2	[a]	10 days
6	7.3	10 days	10 hr.
7	7.4	6 hr.	1 hr.
8	7.4	4 hr.	9 min.
9	7.5	7 hr.	1.5 min.
10	7.5	24 hr.	0.5 min.

[a] Fails to set in 14 days. [b] From the change in the optical rotation in 0.03 *M* 9:1 Na_2HPO_4:NaH_2PO_4 buffer.[3]

Optical rotation measurements[3] reveal that the rate of unfolding of ovalbumin in urea solution decreases very rapidly with decreasing urea concentration (see last column of Table IV). Gelling appears to be promoted by decreasing urea concentration between 10 and 8 *M;* apparently the gelling reaction, which is much slower than the unfolding reaction, is rate determining. Below 8 *M* urea, however, unfolding may be sufficiently slow to delay gelation. These observations indicate that, as one would expect, unfolding must occur before the gelling reaction can take place. It is, however, significant that the ratio of the gelling time to the half time for unfolding does not remain constant below 8 *M* urea.

Concentrated urea solutions are good solvents for denatured proteins,[22] presumably because they decrease hydrogen bonding between the protein molecules. The fact that gelling is more rapid in 8 *M* urea than in 10 *M* urea indicates that hydrogen bonds or similar interactions contribute to the forces holding the gel together, at least for urea concentrations below 8 *M*.

4. **Oxidizing and Reducing Reagents.**—During the study of the influence of electrolytes it was noted that, in spite of the high charge on its anion, 0.10 *M* potassium ferricyanide failed to cause gelling. This led to an investigation of other oxidizing and reducing agents, all of which acted similarly: 0.05 *M* sodium sulfite, 0.02 *M* cysteine hydrochloride and 0.02 *M* glutathione, all in the presence of 0.05 *M* phosphate buffer, as well as 0.01 *N* iodine with 0.025 *M* potassium iodide and 0.05 *M* sodium sulfate failed to produce gelling in two weeks, while the corresponding solutions without the reducing or oxidizing agents gelled in 24 hours or less. This is even more remarkable because most of the reagents used were electrolytes.

On the other hand, if the protein is first denatured and then an oxidizing agent is added, immediate gelling occurs. This was observed when 0.01 *N* iodine was added after the protein had been treated with 10 *M* urea and 0.05 *M* phosphate buffer for one hour.

Consideration of the kind of aggregation reaction which could be so completely inhibited by both mild oxidizing and reducing agents leads one to suspect that sulfhydryl groups are involved. Hence it appeared desirable to test this hypothesis using reagents specific for sulfhydryl and disulfide groups.

5. **Sulfhydryl and Disulfide Reagents.**—The following reagents were also found to prevent gelling in the presence of 0.05 *M* phosphate buffer (*p*H 7.5) and 10 *M* urea: 0.05 *M* sodium cyanide, 0.02 *M* sodium iodoacetate and 0.01 *M* sodium *p*-chloromercuribenzoate (hereafter referred to as PCMB). Of these reagents, cyanide splits SS bonds, iodoacetate forms a thioether with SH groups, and PCMB forms a mercaptide with SH groups.[23]

Ovalbumin contains 5 cysteine residues per molecule.[24] Hence 0.01 *M* PCMB in 3% ovalbumin solution corresponds to a threefold excess over the number of available SH groups. Since it is very strongly bound by the protein,[25] several lower concentrations were tried, and it was found that as little as 1 mole PCMB per mole of albumin was sufficient to prevent gelling, while 4 moles were necessary to prevent a considerable viscosity increase. At high electrolyte concentrations (0.20 *M* sodium sulfate), however, even 0.01 *M* PCMB did not prevent gelling, though it delayed it from $^1/_2$ hour to 20 hours.

Every reagent discussed in this as well as in the previous section removes either SH or SS groups. Although only PCMB is specific for SH,[23] it is hard to avoid the conclusion that SH and/or SS groups are necessary for the gelling reaction in 10 *M* urea. The same conclusion was reached by Huggins, Tapley and Jensen[16] for plasma albumin. The nature of the reaction leading to gelation will be discussed after further experimental evidence has been presented.

6. **Experiments in the Absence of Air.**—A number of experiments were performed in the virtual absence of air in order to ascertain the contribution of air oxidation of sulfhydryl groups. The protein stock solution was placed in the side bulb of a Thunberg tube and all other reagents were placed in the main tube. The tube was evacuated, intermittently over a period of several hours. The solutions were then mixed under vacuum, and the tubes were kept at 30.0°. As shown in Table V, this treatment delayed gelling somewhat though it did not prevent it in any instance.

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(23) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Revs.*, **41**, 151 (1947).

(24) H. L. Fevold, *Advances in Protein Chem.*, **6**, 188 (1951).

(25) L. R. MacDonnell, R. B. Silva and R. E. Feeney, *Arch. Biochem.*, **32**, 288 (1951).

TABLE V

EFFECT OF AIR ON THE GELLING TIME

(3% ovalbumin in 10 *M* urea, no buffer)

Electrolyte	Gelling time, hr. Without air	With air
0.50 *M* NaCl	8	3
0.20 *M* Na_2SO_4	3/4	1/2

The solution when saturated with air contains oxygen equivalent to about twice the concentration of protein SH. This treatment reduced the concentration of dissolved oxygen to a value which was far below the SH concentration. Consequently, these experiments show that air oxidation is not necessary for gelation, though it augments it under ordinary conditions.

7. **The Properties of the Gel.**—The gels were usually quite clear; only those with high electrolyte or low urea concentration showed some opalescence. Most of them became rather stiff on standing. The stiff gels invariably failed to show any sign of melting when heated to 100°. Some of the weaker gels, however, melted at temperatures between 60 and 100°; it was not possible to ascertain whether this melting was reversible.

Pieces of a typical gel dissolved when immersed in dilute alkali or sodium sulfite; when added to dilute acid they contracted into opaque clots; when placed in 10 *M* urea they swelled somewhat but did not dissolve even on heating. A small sample, dialyzed against several changes of distilled water, swelled a little, became slightly opalescent, but maintained its gel-like consistency; the dialyzed gel dried to a hard, horny, clear mass which reverted to a gel when immersed in water.

Gels are believed to consist of three-dimensional networks which entrap considerable amounts of solvent. In the case of ovalbumin gels the network must be formed by cross-linkages between polypeptide chains. These cross-linkages may consist of either chemical bonds, secondary forces localized at a few points in the molecules or non-localized secondary forces.[26] The absence of a reversible melting point in the stronger gels shows that chemical bonds can by themselves provide adequate cross-linkages. The great influence of sulfhydryl reagents shows that these chemical cross-linkages are disulfide bonds. But the melting of the weaker gels on warming shows that secondary forces must also be involved in the gelation (although it is possible that disulfide bonds will break on warming).

These observations suggest that while disulfide bonds bind the polypeptide chains into a firm three-dimensional network, secondary forces also contribute appreciably to the mechanical strength of the gel.

8. **The Nature of the Aggregation Reaction.**—Our results with ovalbumin are entirely analogous to those obtained with plasma albumin by Huggins, Tapley and Jensen.[17] We agree with their conclusion that the intermolecular SS bonds which play an important role in holding the gel together are formed by the exchange reaction

SS(Intramolecular) + SH = SH + SS(Intermolecular)

The effect of varying the urea concentration, however, indicates that hydrogen bonding, or similar forces, also contribute materially to the gelling process. Indeed, at the lower urea concentrations it may become the controlling factor.

The exchange reaction between cysteine and dithiodiglycolic acid, as well as its reverse, has been studied in detail.[27] Since the rate is much enhanced by increasing the *p*H, it is probable that the exchange goes *via* the mercaptide ion

$$RS^- + R'SSR' = RSSR' + R'S^-$$

At 30° and *p*H 6 complete equilibrium is reached in less than four hours. Direct exchange between two disulfides apparently does not take place, as no reaction could be observed at *p*H 6 between cystine and dithiodiglycolic acid even in the presence of traces of cysteine, thioglycolic acid and cupric ion.[27]

A number of other reactions have been reported which seem to involve a similar exchange between a disulfide and a mercaptide ion. These include the catalyzed interchange between polysulfide polymers and mercaptans which occurs with great ease at room temperature[28]; the interchange between propyl disulfide and decyl mercaptan, which is very slow even at 140°,[29] but is markedly catalyzed by mercaptides[30]; and the mercaptide-catalyzed polymerization of cyclic ether disulfide and cyclic formal disulfide, which occurs very rapidly at room temperature.[30]

The existence of an exchange reaction in denatured ovalbumin is in agreement with all the observed facts. Unfolding has to take place before exchange can occur, in accordance with the well-known observation that the sulfhydryl groups in native ovalbumin are masked to almost all reagents but become available for reaction after denaturation by urea or guanidine hydrochloride.[15] Sulfhydryl reagents prevent the reaction by removing the necessary SH groups, and SS-splitting reagents remove the required disulfide groups. Higher *p*H favors the exchange reaction by increasing the ionization of the sulfhydryl groups; this probably accounts for the location of the optimum for gelling at a *p*H well above the isoelectric point.

As discussed above, air oxidation can provide a small portion of the necessary SS links. If it were rapid enough, it could undoubtedly contribute many more, as is illustrated by the immediate gelling of 3% protein solution, denatured in 10 *M* urea for an hour, when an excess of iodine was added to oxidize the unmasked SH groups.

Huggins, Tapley and Jensen[31] found that gelling of serum albumin is *accelerated* under anaerobic conditions. This points to one of the important differences between ovalbumin and serum albumin. While the former possesses 5 SH groups per molecule,[24] the latter contains only one such group.[32]

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(31) E. V. Jensen, V. D. Hospeklorn, D. F. Tapley and C. Huggins, *J. Biol. Chem.*, **185**, 411 (1950).

(32) W. L. Hughes, Jr., H. A. Saroff and A. L. Carney, THIS JOURNAL, **71**, 2479 (1949) (reference to unpublished work by Hughes).

Consequently, air oxidation removes the necessary SH group of serum albumin and prevents the exchange reaction for this protein. In the case of ovalbumin, however, removal of one or several SH groups does not prevent the exchange reaction; if oxidation creates intermolecular SS bonds, it even augments gelation.

B. Viscosity Measurements. 1. Effect of Urea Concentration.—The changes with time of the reduced viscosity of ovalbumin in 7, 8.5 and 10 *M* urea were first studied using 0.05 *M* 1:1 phosphate buffer (*p*H 6.9 to 7.5 depending on the urea concentration) and a protein concentration of 1%. The results are shown in Fig. 2.

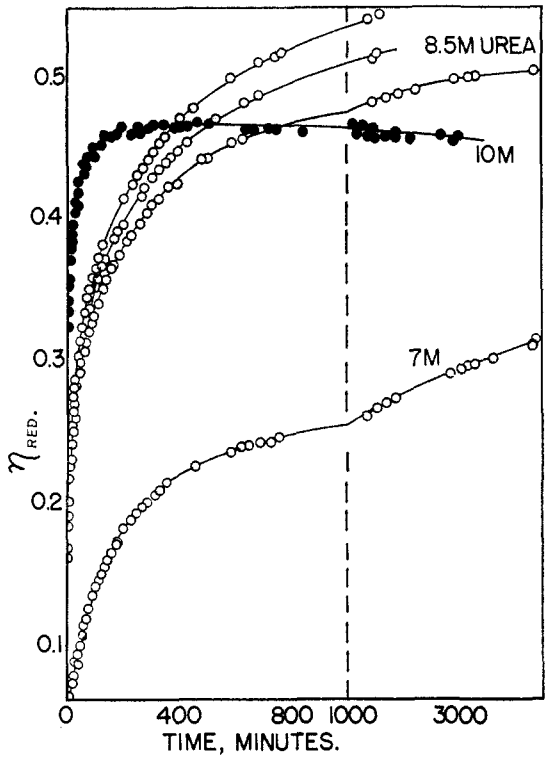

Fig. 2.—Effect of 7, 8.5 and 10 *M* urea on reduced viscosity (in g.$^{-1}$ 100 cc.) of 1% ovalbumin at 30°, *p*H 7.0–7.6 (0.05 *M* sodium phosphate buffer containing equal parts of mono- and dibasic salts). (Runs shown in this figure were done in triplicate at each urea concentration. No reason can be given for the discordant results in 8.5 *M* urea, but it is not believed to be caused by any gross error in experimental technique.) Time scale changes at 1000 min.

In 7 *M* urea the reduced viscosity immediately after adding the urea is about 0.04, a value close to that of the native protein.[33] At the higher urea concentrations the viscosity changes too rapidly to permit an accurate estimate of its initial value, but there is no reason to believe that it is far from that of the native protein. Evidently the ovalbumin molecule can exist for an appreciable time with essentially its native shape even at relatively large urea concentrations. The optical rotation of ovalbumin is also unchanged immediately after the addition of urea.[8]

Figure 2 clearly shows that the rate of viscosity change during the early stages of the reaction increases rapidly with the urea concentration. This behavior is entirely analogous to that encountered with the optical rotation, and is consistent with the supposition that the initial increase in viscosity is caused primarily by unfolding of the protein molecule.

In 10 *M* urea the viscosity change ceases after about 200 minutes when the protein concentration is 1%. The change in the optical rotation under the same conditions is, however, complete in only about 20 minutes (*cf.* Fig. 9 of Part I). A similar sluggishness of the viscosity change is found at the other urea concentrations when the protein concentration is above 1%. As we shall see, it is a consequence of a time-dependent aggregation which is pronounced in 1% ovalbumin and which has no effect on the optical rotation.

When the protein concentration is 1%, a higher viscosity is attained in 8.5 *M* urea than in either 10, 7.5 or 7 *M* urea. This is also undoubtedly a result of aggregation, and is consistent with the results of the gelling experiments, which showed (Table IV) that aggregation is most pronounced in 8 *M* urea.

2. Effect of Protein Concentration.—Figure 3 illustrates the viscosity changes observed when ovalbumin at various concentrations is denatured in 10 *M* urea. The very great concentration dependence of the reduced viscosity is apparent in this figure. The 3% solution gelled in about 1500 minutes; the inset in Fig. 3 shows that the rate of viscosity change first decreases and then increases without limit.

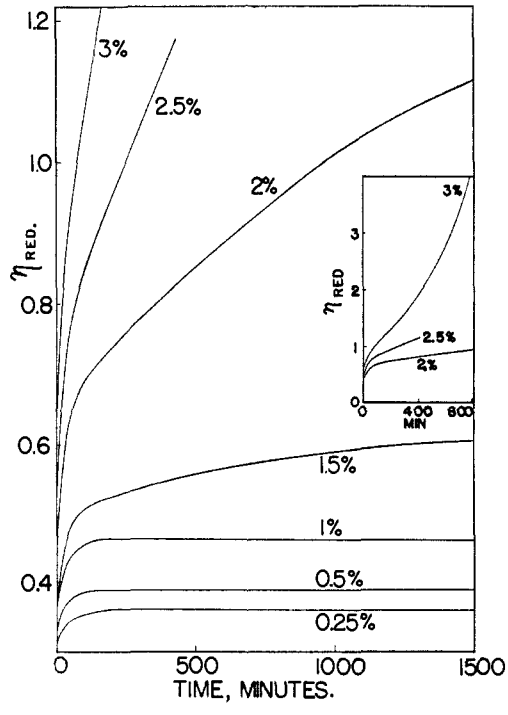

Fig. 3.—Effect of protein concentration on the change in reduced viscosity of ovalbumin in 10 *M* urea at 30°, buffer as in Fig. 2. The reduced viscosity of 2% ovalbumin levelled off at 1.18 g.$^{-1}$ 100 cc. after 3000 min.

Several relations have been proposed to describe the variation of the viscosity of high polymer solu-

(33) A. Polson, *Kolloid Z.*, **88**, 51 (1939).

tions with concentration. Huggins[34] proposed an equation

$$\eta_{red} = [\eta] + k'[\eta]^2 c$$

where η_{red} is the reduced viscosity, $[\eta]$ is the intrinsic viscosity, c is the polymer concentration and k' is a dimensionless constant characteristic of a given solute–solvent system and ordinarily independent of the molecular weight. This equation can be expected to hold only at low concentrations, since second order and higher terms in concentration have been neglected. In agreement with this equation, linear polymers in good solvents are found to give a linear relation between the reduced viscosity and the concentration for concentrations up to about 1.5% in polymer.[35] Values of 0.3 to 0.8 for k' are obtained. At higher concentrations an empirical equation, first suggested by Martin

$$\eta_{red} = [\eta] \exp(k'[\eta]c)$$

has been reported to hold very well for a number of polymer systems up to concentrations of 5% and beyond.[36]

Curves A, B and E of Fig. 4 show that the final viscosities of ovalbumin in urea deviate considerably from the Huggins equation at protein concentrations below 1.5%. It is found that these data cannot even be made to fit the Martin equation (curve D of Fig. 4). Evidently some factor operates here which is different from the purely hydrodynamic interactions existing in normal polymer solutions. This factor is undoubtedly aggregation of the same kind as is responsible for gelling at higher protein concentration. The aggregation varies strongly with the protein concentration, and the concentration dependence of the reduced viscosity thus provides a convenient means of investigating aggregation. Furthermore, since the osmotic pressure measurements of Burk and Greenberg[13] and the sedimentation–diffusion measurements of Rothen[14] show that urea-denatured ovalbumin is unaggregated at infinite dilution of protein, the extrapolated reduced viscosity at infinite dilution must correspond to the intrinsic viscosity of the unaggregated denatured ovalbumin molecule.

The intrinsic viscosity of denatured ovalbumin at *p*H 7.6 is 0.33 (g./100 cc.)$^{-1}$ in 10 *M* urea and 0.24 (g./100 cc.)$^{-1}$ in 7.5 *M* urea. This shows that the unaggregated molecule is more tightly coiled in 7.5 *M* urea than in 10 *M* urea, probably because there is more intramolecular hydrogen bonding at the lower urea concentration.

3. Effect of *p*H.—On increasing the *p*H from 7.6 to 10.2 the intrinsic viscosity of denatured ovalbumin in 10 *M* urea increases from 0.33 to 0.38 (Fig. 4). This increase might arise from a "swelling" of the randomly coiled denatured molecule caused by the electrostatic repulsions associated with the increased net charge of the molecule. Such a "swelling" effect is well known from studies of polyelectrolytes.[37]

(34) M. L. Huggins, THIS JOURNAL, **64**, 2716 (1942).

(35) C. E. H. Bawn, "The Chemistry of High Polymers," Interscience Publishers, Inc., New York, N. Y., 1948, p. 167; I. Harris, *J. Polymer Sci.*, **8**, 353 (1952).

(36) R. S. Spencer and J. L. Williams, *J. Colloid Sci.*, **2**, 117 (1947).

(37) J. J. Hermans and J. T. Overbeek, *Bull. soc. chim. Belg.*, **57**, 154 (1948); W. Kuhn, O. Künzle and A. Katchalsky, *Helv. Chim. Acta*, **31**, 1994 (1948).

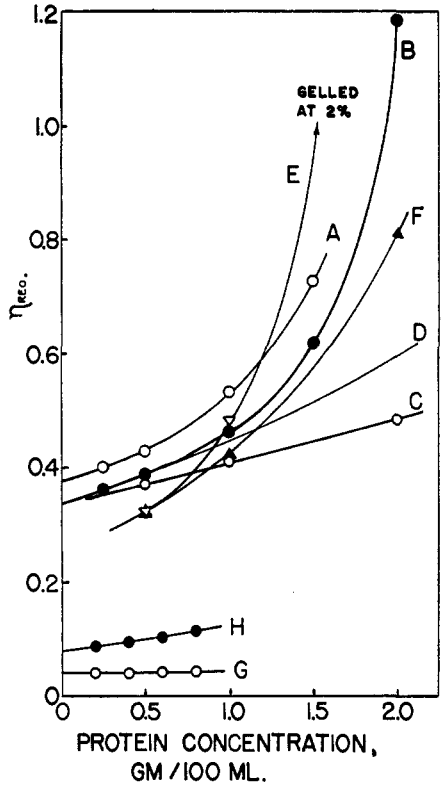

Fig. 4.—Effect of ovalbumin concentration on final reduced viscosity attained under various conditions at 30°: A, 10 *M* urea, 0.05 *M* borate buffer, *p*H 10.2; B, 10 *M* urea, 0.05 *M* phosphate buffer, *p*H 7.6; C, same as B but with 0.005 *M* PCMB; D, calculated curve using Martin equation with $k' = 0.873$, $[\eta] = 0.335$—corresponding to slope at lowest concentrations on curve B; E, 7.5 *M* urea, phosphate buffer, *p*H 7.3, reduced viscosities at 500 min.; F, same as D but with 0.005 *M* PCMB; G, native ovalbumin in 0.2 *M* phosphate buffer, *p*H 8, from Bull.[10]; H, ovalbumin denatured in 9.5 *M* urea for one hour, then diluted to low urea concentration in 0.2 *M* phosphate buffer, *p*H 8, from Bull.[10].

At *p*H values above 11 the viscosity first increases with time, passes through a maximum after about 20 minutes, and then gradually decreases, the rate of the decrease being greater, the higher the *p*H. For 1% ovalbumin in 10 *M* urea the maximum reduced viscosity was 0.55 at *p*H 11.5 (0.05 *M* Na_3PO_4–Na_2HPO_4 buffer) and 0.70 at *p*H 12.3 (0.05 *M* NaOH). These values lie above the final reduced viscosities for 1% ovalbumin given in Fig. 4, showing that the electrostatic "swelling" is probably increasingly important at these *p*H values. (The possibility that increased aggregation is responsible for these high viscosities is unlikely since the gelling experiments show (Table III) that aggregation is decreased above *p*H 10.)

That the decrease in viscosity with time at high *p*H is irreversible is shown by the following experiment: ovalbumin was exposed to 10 *M* urea at *p*H 12.3 for various times and then boric acid was added to bring the *p*H down to about 10.8. If the boric acid is added after 25 minutes (at which time the reduced viscosity at *p*H 12.3 has reached its maximum value of 0.70) a reduced viscosity of 0.60 is obtained. If it is added after 1300 minutes (at which time the

reduced viscosity at pH 12.3 has fallen to 0.35) a reduced viscosity of only 0.27 is found. If the ovalbumin had been exposed to only borate buffer at pH 10.8 its reduced viscosity would have levelled off at about 0.66. This behavior indicates that rupture of some alkali-labile linkages in the protein (possibly peptide bonds) takes place above pH 11.[38]

4. **Effect of Sulfhydryl Reagents.**—Figures 4 and 5 show the effect of 0.005 M PCMB on the viscosity of ovalbumin in 10 M urea at pH 7.5. (This amount of PCMB is approximately twice the amount needed to react with all of the sulfhydryl groups at the highest protein concentration used here.) The initial change in the viscosity is accelerated, but apparently the PCMB has no effect on the intrinsic viscosity of the denatured protein. It does eliminate the abnormal increase in the reduced viscosity at high protein concentrations, however, and the concentration dependence of the reduced viscosity is now consistent with the Huggins equation, giving a normal value, 0.69, for the Huggins constant, k'. This behavior is consistent with the hypothesis that PCMB reacts with sulfhydryl groups and thereby prevents aggregation by inhibiting the exchange reaction of SH with SS. A similar effect is obtained, presumably for obvious related reasons, with iodine and sodium cyanide (Fig. 5), although these reagents do not seem to be as effective as PCMB in reducing the viscosity in 2% ovalbumin. The effect of cyanide in reducing the viscosity of ovalbumin in urea has been noted by Fredericq and Desreux.[39]

Apparently PCMB eliminates practically all of the aggregation in 10 M urea for ovalbumin concentrations at least as great as 2%. It is interesting to consider next the effect of PCMB at a somewhat lower urea concentration, where the unfolding occurs more slowly and where aggregation due to hydrogen bonding should be more noticeable. Figure 6 compares the behavior of ovalbumin in 7.5 M urea in the absence and presence of PCMB.

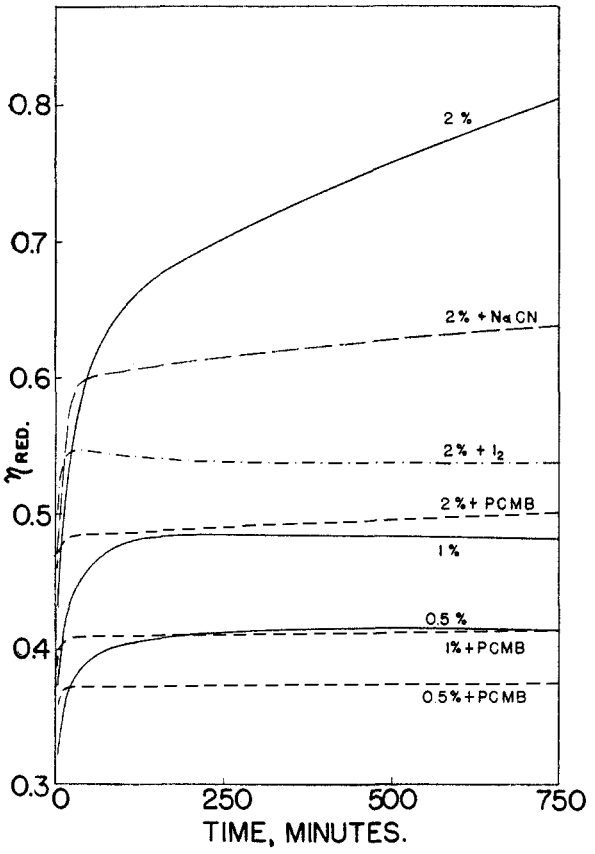

Fig. 5.—Effect of various sulfhydryl reagents on the reduced viscosity of ovalbumin in 10 M urea at 30°; buffer as in Fig. 2. Protein concentration as indicated. Dashed curves correspond to sulfhydryl reagent concentrations as follows: 0.005 M PCMB, 0.005 N I_2 + 0.012 M KI, 0.02 M NaCN.

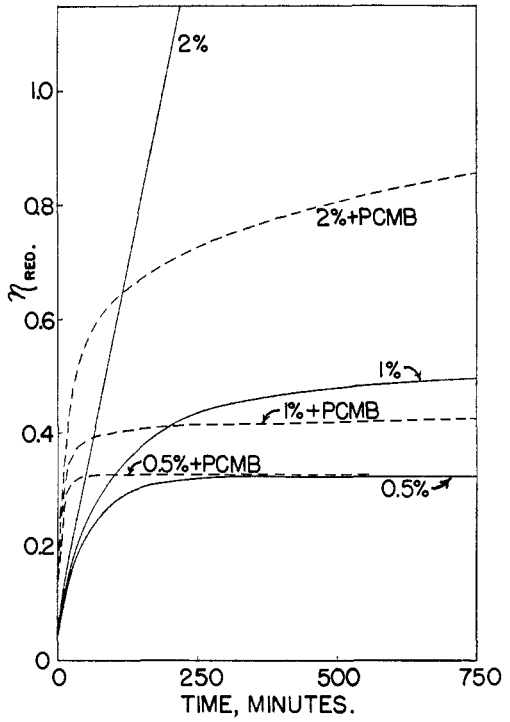

Fig. 6.—Effect of PCMB on the reduced viscosity of ovalbumin in 7.5 M urea at 30°, buffer as in Fig. 2. Protein concentrations as indicated. 0.005 M PCMB present for dashed curves.

The behavior is similar to that found in 10 M urea except that the viscosity fails to level off in 2% ovalbumin, showing that some aggregation probably occurs here. This is shown more strikingly in Fig. 7, where the concentration dependence of the reduced viscosity is shown at various times. In the absence of PCMB (Fig. 7A) the unfolding reaction continues for about 100 minutes, as indicated by the increase in the extrapolated value of the reduced viscosity at infinite dilution (this extrapolated value being unaffected by aggregation and depending only on the shape of the molecule). After 100 minutes the extrapolated value remains con-

(38) In the presence of borate buffers (pH 9 to 11) the change with time of the reduced viscosity of ovalbumin in 10 M urea is complex. It first increases with time, passes through a maximum, then passes through a minimum and finally levels off or very gradually increases. The cause of this behavior is not known. It was not observed with phosphate buffers at pH 11.5, even when sodium borate was added, and it was observed when boric acid was added after exposure of the ovalbumin to pH 12.3 for various lengths of time, as described above.

(39) E. Fredericq and V. Desreux, *Bull. soc. chim. Belges*, **58**, 389 (1949).

stant but aggregation continues to become more and more pronounced, as shown by the increasing upward curvature of the plots. The 2% solution gels after about 1800 minutes.

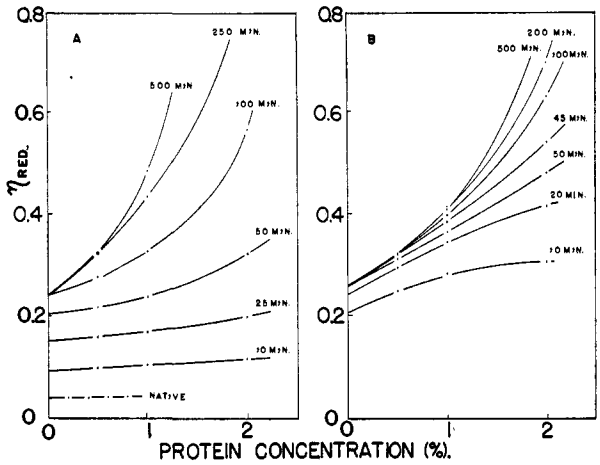

Fig. 7.—Effect of PCMB on the reduced viscosity of ovalbumin in 7.5 *M* urea at 30°, buffer as in Fig. 2: A, control; B, 0.005 *M* PCMB added. Data for native protein from Bull.[10]

In 7.5 *M* urea containing 0.005 *M* PCMB (Fig. 7B) unfolding is complete in about 30 minutes, but in contrast to the behavior in 10 *M* urea, aggregation occurs at higher protein concentrations. In view of the comparative inertness of the sulfhydryl groups in the presence of PCMB, this aggregation is undoubtedly caused by hydrogen bonding, which is favored by lower urea concentrations. Hydrogen bonding also presumably accounts for some of the aggregation which occurs in 7.5 *M* urea in the absence of PCMB. (Gelling occurs in 2% ovalbumin with 7.5 *M* urea, but requires 3% ovalbumin with 10 *M* urea.)

Fredericq and Desreux[39] have reported that chloropicrin prevents some of the increase in viscosity when ovalbumin is treated with 8 *M* urea at *p*H 7. They suggested that chloropicrin oxidizes SH groups to give intramolecular SS links which prevent complete unfolding of the protein. In the light of our results it seems more likely that this behavior is caused by the reaction of the chloropicrin with SH, which inhibits the SH–SS exchange reaction and hence reduces aggregation.

5. Comparison of Kinetics of Optical Rotation and Viscosity Changes.—It is interesting to compare the change with time of the viscosity and the optical rotation under conditions in which aggregation is unimportant, *viz.*, at low protein concentrations and in the presence of PCMB. This is done in Fig. 8. Evidently the two properties change in nearly the same way under these conditions and we can assume that unfolding is the only process which is taking place. Moreover, the acceleration of the rate of unfolding by PCMB shows up to the same degree with both properties. The reason for this acceleration is not known, but may be related to similar effects with other organic substances (see Part I).[3]

6. Reversibility of the Viscosity Changes.—Figure 9 shows the behavior of the viscosity when

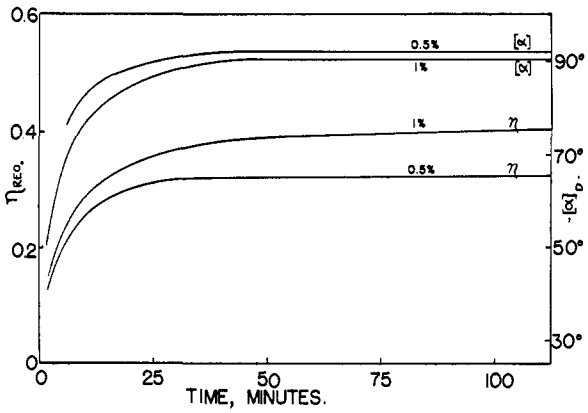

Fig. 8.—Comparison of rate of change of optical rotation and reduced viscosity of ovalbumin 7.5 *M* urea in presence of 0.005 *M* PCMB, buffer as in Fig. 2. Protein concentration as indicated.

the protein concentration is reduced after various times, the urea concentration being held constant at 10 *M*. The instantaneous drop in the reduced viscosity is undoubtedly largely caused by a decrease in the hydrodynamic interactions. When 2% ovalbumin is diluted to 0.5% after 60 minutes in 10 *M* urea (without changing the urea or buffer concentrations), the initial instantaneous decrease is followed by a further slow decrease and ultimately a

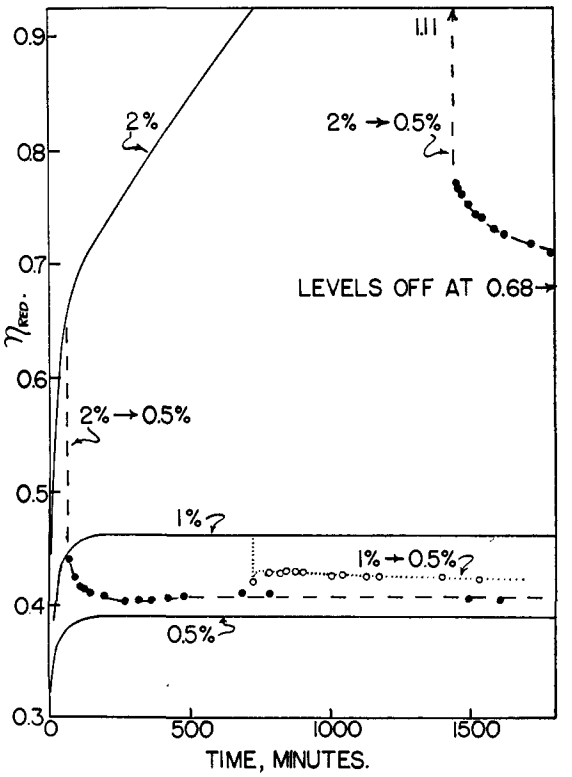

Fig. 9.—Changes in reduced viscosity of ovalbumin in 10 *M* urea at 30° following changes in protein concentration, buffer as in Fig. 2. Solid curves show normal behavior of 0.5, 1.0 and 2.0% ovalbumin in 10 *M* urea. Dashed curves (solid circles) show behavior of 0.5% ovalbumin diluted from 2% after one hour and after one day. Dotted curve (open circles) shows behavior of 0.5% ovalbumin after 720 min. at 1%.

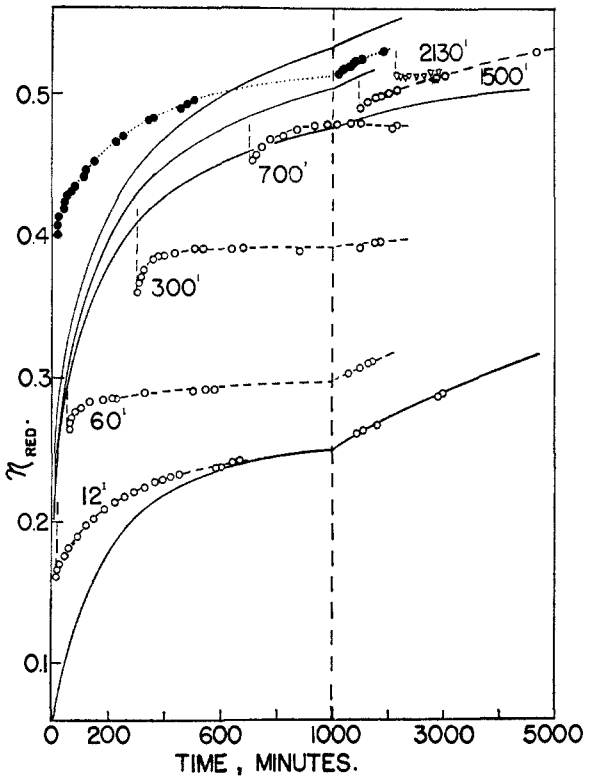

Fig. 10.—Effect of changes in urea concentration on the reduced viscosity of 1% ovalbumin at 30°, buffer as in Fig. 2. Solid curves, normal behavior in 7.0 *M* urea (lower curve) and 8.5 *M* urea (upper curves); dashed curves, behavior on dilution of urea from 8.5 to 7.0 *M* at indicated times; dotted curve, behavior on increasing the urea concentration to 8.5 *M* after one day in 7.0 *M* urea. Time scale changes after 1000 minutes.

viscosity is reached which is not much higher than that obtained in the normal fashion for 0.5% ovalbumin in 10 *M* urea. If, however, the dilution is made after 1440 minutes, the viscosity which is eventually reached is considerably higher. These results show that the gradual aggregation of the protein in 2% ovalbumin is not completely reversible. Fredericq and Desreux[39] have noticed a similar behavior of ovalbumin in 6 *M* urea.

This irreversibility is puzzling if aggregation occurs by means of an exchange reaction between SS and SH, since this reaction should be reversible. The irreversibility may be a result of the gradual disappearance of SH through oxidation by air, since we have observed that the nitroprusside test for free SH is greatly weakened when ovalbumin has been standing in 10 *M* urea at *p*H 7 to 8 for one day.

Some investigations were also made of the reversibility of the viscosity changes with respect to variations in the urea concentration. The significance of these results is obscure, but they are described here because they are interesting and unexpected. Figure 10 shows the behavior in a series of experiments in which 1.21% ovalbumin was exposed to 8.5 *M* urea for various times, after which the urea was diluted with buffer to 7 *M* (giving a protein concentration of 1%). The initial rapid drop in the reduced viscosity probably reflects a contraction of the molecule into a tighter coil because of the increased intramolecular hydrogen bonding, which is also responsible for the fact, already mentioned, that the intrinsic viscosity is smaller in 7.5 *M* urea than in 10 *M* urea. This is in line with the very much lower reduced viscosities (values of the order of 0.1—see curve H of Fig. 4) observed by Bull[10] for ovalbumin which had first been denatured in 9.5 *M* urea for one hour and then strongly diluted with water; in this case the ovalbumin molecule undergoes a strong "contraction." The viscosity changes which occur subsequent to the instantaneous decrease cannot be explained: there is a gradual increase to values not far below those obtaining in 8.5 *M* urea at the moment of dilution. If the dilution is made at 300 min. or later, these values are well above those attained in 7 *M* urea after many days. This might be taken to mean that in 8.5 *M* urea a state is reached after a few hundred minutes which in 7 *M* urea would only be attained in several weeks. That this is not the case is shown by the behavior when the urea concentration is increased to 8.5 *M* after one day in 7 *M* urea (Fig. 10). A reduced viscosity is immediately reached which is well above the value reached in 7 *M* urea after three days, and this is followed by a further increase which is slightly more rapid than that normally observed when ovalbumin has reached the same viscosity with the urea concentration at 8.5 *M* from the beginning. Apparently ovalbumin in 7 *M* urea has attained a state after one day which is similar to, or readily transformed into a form which in 8.5 *M* urea is reached in 200 or 300 minutes. But the state which is reached in 8.5 *M* urea after 300 minutes is not transformed by dilution to 7 *M* urea into the same state as is obtained after one day is 7 *M* urea.

Since the ovalbumin concentrations in these experiments were 1% or more, it is not clear to what extent the results are affected by aggregation and to what extent by the shape of the denatured ovalbumin molecule itself.

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