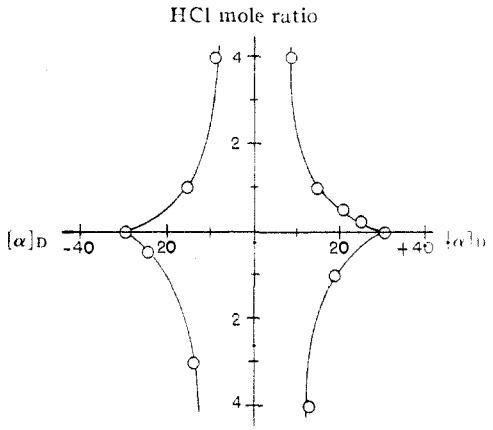

Fig. 1.—The optical rotation of the two isomers of *m*-tyrosine with different ratios of acid or base. The left hand curve corresponds to the L- or natural isomer and the right curve to the D- or unnatural configuration. In each instance the amino acid was present in 0.05 *M* solution.

N-Acetylcyclohexylalanine.—A solution of 11.1 g. (0.05 mole) of acetamido-*m*-hydroxycinnamic acid in 150 ml. of glacial acetic acid was reduced at 40 pounds pressure for two hours in the presence of 0.25 g. of platinum oxide catalyst. The catalyst was removed by filtration and the acetic acid by vacuum distillation. Crystallization of the thick oil from boiling water yielded plate crystals melting at 174–175°. A Millon's test was negative. Herbst and Shemin[13] record a melting point of 178° (cor.).

Anal. Calcd. for $C_{11}H_{19}O_3N$: N, 6.57. Found: N, 6.45, 6.43.

β-DL-Cyclohexylalanine.—Five grams of the above reduction product was refluxed twelve hours in 100 ml. of *N* hydrochloric acid. The amino acid was obtained by isoelectric precipitation. The 4.3 g. obtained melted at 229–230° with decomposition and gave a negative Millon test and a positive ninhydrin reaction.

Anal. Calcd. for $C_9H_{17}NO_2$: N, 8.18. Found: N, 8.09, 8.18.

N-Formyl-DL-cyclohexylalanine.—The formyl derivative was prepared from 4 g. of the amino acid according to the procedure of Clarke.[9] The residue upon recrystallization in 10 ml. of water, gave 3.2 g. (80%) of crystals melting at 135–136°.

Anal. Calcd. for $C_{10}H_{17}O_3N$: N, 7.03. Found: N, 7.13, 7.07.

N-Benzoyl-DL-cyclohexylalanine.—The above amino acid was benzoylated in the usual fashion and the resulting derivative exhibited a melting point of 184–185°. Herbst and Shemin[13] reported a value of 182–183.5°.

AMES, IOWA RECEIVED DECEMBER 22, 1949

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Streaming Orientation Studies on Denatured Proteins. III. Denaturation of Ovalbumin in the Presence of Urea[1]

BY JOSEPH F. FOSTER AND EDWARD G. SAMSA[2]

Studies have been made on the streaming birefringence of ovalbumin denatured in the presence of concentrated aqueous solutions of urea. The data indicate both unfolding and aggregation of the molecules. At *p*H values near 2.5 or above 9.0 aggregation is least serious and the measurements indicate that the preparations are nearly homogeneous with respect to molecular length. Such values of length lie invariably in the range 500–700 Å., a value taken as characteristic of the unfolded molecule in the absence of aggregation. At *p*H 2.5 denaturation for one hour at 37° in 7.5 *M* urea yields lengths which are substantially independent of protein concentration. This provides further suggestive evidence that the reaction under these conditions is essentially an intramolecular unfolding.

In the preceding papers of this series[3] an investigation was made of the heat denaturation of ovalbumin using the technique of streaming birefringence. It was shown that interpretation of the results on a molecular basis is not in general possible because of aggregation, even at *p*H values rather far removed from the isoelectric point. Evidence was given, however, that unfolding to lengths of 300 to 600 Å. does occur, depending on the conditions of denaturation.

One of the chemical agents often used for denaturing globular proteins is concentrated aqueous urea.[4] In this medium the denatured proteins usually remain soluble, a result which is of particular interest since it suggests that aggregation might be less serious in such media. The present paper summarizes some of the principal results of a study of the streaming birefringence of ovalbumin denatured in aqueous urea.

Experimental

Preparation of Solutions.—The weighed samples of ovalbumin[5] were dissolved in a small volume (x) of buffer or water, usually about 2.5 ml. The urea, usually 10.5 g., was dissolved in ($15 - x$) ml. of buffer or dilute HCl and brought to 37°. The solutions were mixed, held for the desired period of time at the desired temperature and cooled rapidly to 20–25°. Glycerol (42.0 g.) was then added, the solution filtered through sintered glass to remove any traces of floating debris, centrifuged at 20,000 × *g* to remove any smaller suspended impurities, and degassed by evacuating under a water aspirator. Such a solution is approximately 7.5 *M* in urea at the denaturation stage, and the final solution is 58.9% in glycerol (viscosity 18.6 centipoise at 25° as determined with an Ostwald pipet).

Streaming Birefringence Measurements.—The instrument used has been described previously.[3,6] In some of the measurements a new outer cylinder providing an annular gap of 0.50 mm. replaced the previously used cylinder (gap 1.0 mm.). This modification was desirable because of the lower birefringence obtained in the urea-containing media

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(2) Postdoctoral Fellow, Dept. of Chemistry, Iowa State College, 1950–1951. Present address: A. E. Staley Mfg. Co., Decatur, Ill.

(3) (a) J. F. Foster, and E. G. Samsa, THIS JOURNAL, **73**, 3187 (1951); (b) E. G. Samsa and J. F. Foster, *ibid.*, **73**, 3190 (1951).

(4) For a review of the literature in this field see H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

(5) The ovalbumin was recrystallized at least three times with $(NH_4)_2SO_4$ by the method of Sørensen and Høyrup (*Compt. rend. trav. lab. Carlsberg Sér. chim.*, **12**, 12 (1917)), dialyzed free of salt and lyophilized.

(6) J. F. Foster and I. H. Lepow, THIS JOURNAL, **70**, 4169 (1948).

at a given gradient (see below). This gap approximately doubles the gradient at a given r.p.m. but still does not impose too difficult an optical problem and appears to be a good compromise. The details of the measuring technique were the same as previously used.

Results and Discussion

Effect of Urea Concentration.—Figure 1 shows the effect of varying urea concentration in the heat denaturation of 0.60 g./100 ml. ovalbumin for 5.5 min. at 100° in veronal buffer (*p*H 8.0, ionic strength 0.1 before addition of urea). The particle lengths are seen to decrease pronouncedly with increasing urea concentration, a result which is attributed to decreased aggregation at the higher urea levels. At 4.0 *M* urea an apparently homogeneous system is obtained under these conditions and the length, 700 Å., is roughly comparable to the lengths which have been found under other conditions of heat denaturation in which aggregation is thought to be unimportant.[3]

A further important point brought out by Fig. 1 is the reduction in the reduced birefringence (Δ/fc)[7] brought about by the urea. The values obtained on samples denatured in 4.0 *M* urea are only about one-third those obtained in the absence of urea. At higher urea levels the reduction in birefringence is even more serious. This renders measurements at high urea concentration rather difficult. This effect is probably due to a reduction in the form-birefringence although the possibility of a reduction in optical anisotropy of the protein arising from complex formation with urea cannot be ignored.

In the studies on heat denaturation it was found that heating in the presence of the glycerol led to less aggregation,[3b] presumably because of the lower concentration at the heating stage. Similar experiments in the case of urea denaturation indicated that the presence of glycerol during denaturation promotes aggregation. Samples containing as high as 6.6 *M* urea in 55% glycerol in general yielded lengths which were very dependent on gradient and well above 600 Å. It appears preferable to denature in aqueous urea at 7.5 *M*, then to dilute with glycerol before making the optical measurements. Some aggregation takes place following dilution with glycerol; however, this is not a serious problem if the measurements are made within an hour or two following addition of the glycerol.

Effect of *p*H and Buffer Ions.—Samples denatured near the isoelectric point, even in 7.5 *M* urea solution, show definite evidence of aggregation. In particular, the samples appear very heterogeneous in length. (The lengths are very concentration dependent.) At *p*H values above 9.0 and particularly in the range 2.0–2.5 aggregation was found to be relatively unimportant.

(7) Here Δ is the observed angular rotation of the Senármont Compensator, f the magnitude of the orientation function corresponding to the observed extinction angle and c the protein concentration in g. per 100 ml. For further details the first paper of this series should be consulted. In previous publications[3] the authors have used the term "intrinsic birefringence" to refer to the quantity Δ/fc. However, this term has been used more commonly to designate that part of the total birefringence due to the optical anisotropy of the oriented molecules or particles in distinction to the "form birefringence." For this reason the term "reduced birefringence" appears preferable. Strictly the term should apply to the quantity $n_e - n_0/fc$, so that the numerical values given in the tables are only relative, not absolute values.

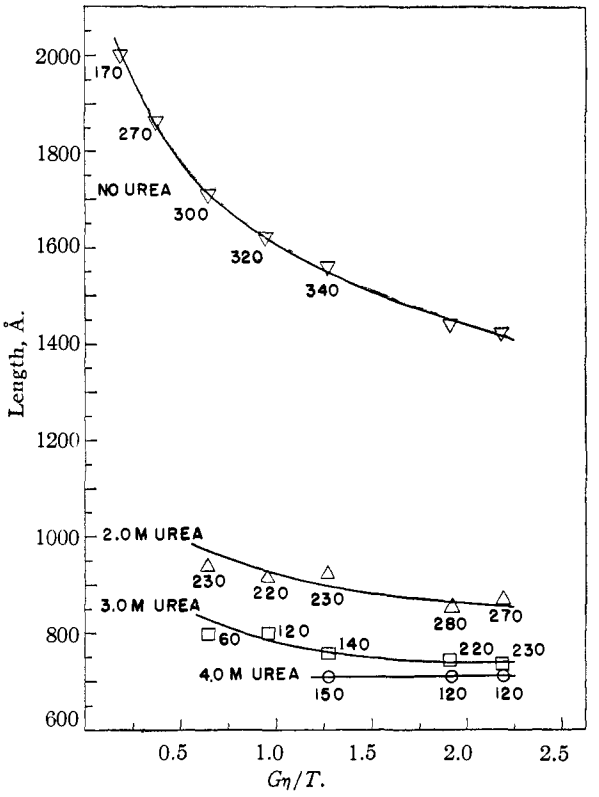


Fig. 1.—Heat and urea denaturation of ovalbumin at 100° for 5.5 min. and *p*H 8.0 (using veronal buffer). Concentration during denaturation: ovalbumin 0.60 g./100 ml.; urea as shown; glycerol 0%. Concentration during optical measurements: ovalbumin 0.19 g./100 ml.; urea 0 *M*, 0.63 *M*; 0.95 *M*; 1.27 *M*; glycerol 70% (by wt.). The Δ/fc values are indicated adjacent to experimental points.

These two *p*H ranges were singled out for further investigation. There are some buffer effects, though these were not studied in any detail. Phosphate is worse from the standpoint of aggregation than veronal at comparable *p*H. Low ionic strength is preferable; however, it is desirable to use a buffer in experiments in the alkaline range to minimize changes in *p*H which would result from slight decomposition of the urea.

Effect of Temperature.—A large number of studies were carried out at 100° and are perhaps best considered as heat denaturation experiments in which aggregation is repressed to a greater or less extent by urea. Later investigations indicated the unfolding to be conveniently fast at 37° and aggregation to be much slower than at 100°. Other than those given in Fig. 1, the results presented were all obtained at the lower temperature.

Effect of Time of Heating.—In Figs. 2 and 3 are shown results for samples heated for varying lengths of time at the two *p*H values found to give minimal aggregation, 2.5 and 9.0. Veronal buffer (0.1 ionic strength before addition of urea) was used at the high *p*H. The low *p*H was obtained by adding the calculated amount of dilute HCl. At the higher *p*H the sample at 30 minutes is homogeneous within experimental error. At one hour there is seen to be a hint of polydispersity and by 2.3 hours it is serious. At *p*H 2.5 the 30-

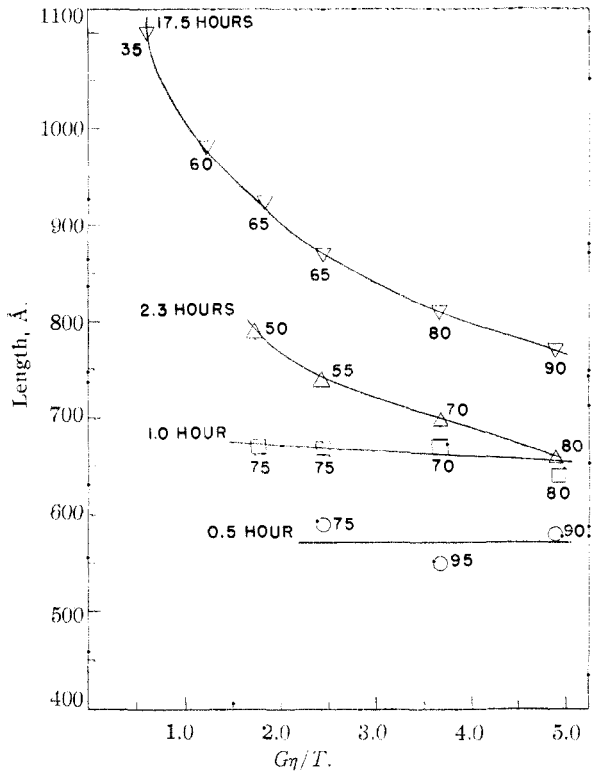


Fig. 2.—Urea denaturation of ovalbumin at 37° and *p*H 9.1 (using veronal buffer). Concentration during denaturation: ovalbumin 1.18 g./100 ml.; urea 7.5 *M*; glycerol 0%. Concentration during optical measurements: ovalbumin 0.48 g./100 ml.; urea 3.1 *M*; glycerol 58.9% (by wt.). The Δ/fc values are indicated adjacent to experimental points.

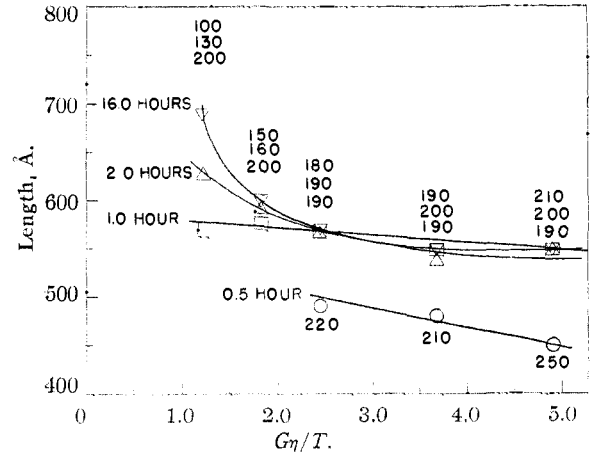


Fig. 3.—Urea denaturation of ovalbumin at 37° and *p*H 2.5 (using HCl). Concentration during denaturation: ovalbumin 1.18 g./100 ml.; urea 7.5 *M*; glycerol 0%. Concentration during optical measurements: ovalbumin 0.48 g./100 ml.; urea 3.1 *M*; glycerol 58.9% (by wt.). The Δ/fc values are indicated adjacent to experimental points.

minute sample is polydisperse, becoming substantially homogeneous at one hour. Even at 16 hours the heterogeneity under such conditions is not very great and shows up only at the lower gradients. Those systems which are substantially homogeneous again are seen to be in the range 500–600 A. The heterogeneous but short lengths observed in the half-hour sample may be an indication of the presence of partially unfolded units. (The undenatured ovalbumin is too short to contribute.) It seems probable that the changes taking place up to one hour of heating at either of these *p*H values can be interpreted as an unfolding and this process appears to be substantially complete by that time.

Solutions which are homogeneous with respect to length also yield reduced birefringence values which are substantially independent of gradient. In the heterogeneous systems this quantity increases with gradient in a manner which is in at least qualitative agreement with the theory. A noteworthy point is that the reduced birefringence in the homogeneous solutions at *p*H 2.5 is more than twice that at the higher *p*H. A similar observation was made in the heat denaturation studies.[3] This effect is most probably a consequence of a change in the anisotropy of the protein molecule with the state of ionization. That there should be such a dependence is certainly not surprising and a detailed study of this point should prove interesting.

Effect of Protein Concentration.—Figure 4 summarizes the results of a concentration study at *p*H 2.5 which is in the range giving minimal aggregation. The concentration effects, both with respect to length and reduced birefringence, are relatively small except at the lowest gradient. It seems clear that under this set of conditions aggregation effects are of secondary importance and the predominant process is an intramolecular one.

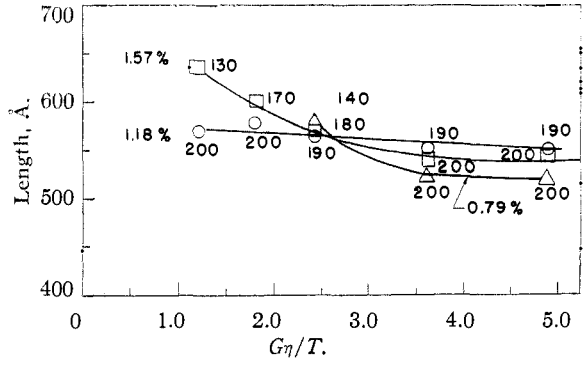


Fig. 4.—Urea denaturation of ovalbumin at 37°, 60 min., and *p*H 2.5 (using HCl). Concentration during denaturation: ovalbumin as shown (% denotes g./100 ml.); urea 7.5 *M*; glycerol 0%. Concentration during optical measurements: ovalbumin 0.64, 0.48, 0.32 g./100 ml.; urea 3.1 *M*; glycerol 58.9% (by wt.). The Δ/fc values are indicated adjacent to experimental points.

Concentration effects were found to be appreciable on the alkaline side of the isoelectric point and were particularly severe in phosphate buffers at *p*H 7.5.

Comparison with Results on Heat Denaturation.—It is seen that the length of the orienting units obtained by urea denaturation of ovalbumin is quite dependent on the conditions. Again, as in the case of heat denaturation,[3] aggregation is a

complicating factor. Those solutions in which aggregation can be assumed to be negligible on the basis of the homogeneity criterion almost invariably yield lengths in the range 500–700 A. This is far less than the value of about 1400 Å. which the protein could yield if it opened up fully to the extended (β-keratin) configuration. On the other hand the 500–700 range is in excellent agreement with the lengths observed in the case of heat denaturation under conditions yielding minimal aggregation, except in the case of denaturation in glycerol-rich media where somewhat shorter lengths were found.[3b]

Acknowledgment.—The authors wish to express their appreciation to the Rockefeller Foundation for the financial support of a Fellowship for one of us (E.G.S.).

AMES, IOWA RECEIVED MAY 10, 1951

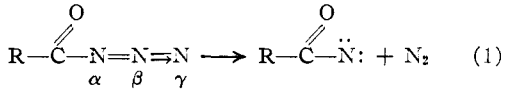
[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, BROOKHAVEN NATIONAL LABORATORY]

An N^{15} Tracer Study of Organic Azide Reactions. I. The Curtius Rearrangement of 3,5-Dinitrobenzazide-N^{15} [1]

BY AKSEL A. BOTHNER-BY AND LEWIS FRIEDMAN

Nitric acid containing 31.4 atom % excess N^{15} has been converted to potassium nitrite which was used in the preparation of 3,5-dinitrobenzazide from 3,5-dinitrobenzhydrazide. Thermal decomposition of the azide gave nitrogen containing all the excess heavy nitrogen isotope, and dinitroaniline of normal isotopic composition. This confirms the currently accepted mechanism of rupture of the α-β N–N bond followed by the elimination of a molecule of nitrogen.

The currently accepted mechanism of the Curtius rearrangement of acyl azides is briefly outlined by equations (1) and (2).



(1)

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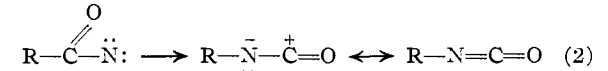
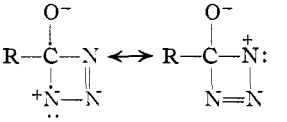


(2)

III

A similar mechanism serves to account for the gross behavior of molecules undergoing the closely parallel Hofmann, Lossen and Wolff rearrangements. These mechanisms have evolved from studies on: (a) the products obtained from the rearrangement of optically active compounds[2]; (b) kinetics of the rearrangements[3]; (c) tracer studies using isotopic carbon[4]; and (d) rearrangements in the presence of free radicals.[5] Such studies yield information concerning the general mechanism of the azide rearrangement, but leave the specific details of the role of the nitrogen atoms in the reaction essentially undetermined.

It has been assumed that the initial decomposition of the acyl azide (I) proceeds by rupture of the α-β N–N bond, yielding an unstable intermediate (II) and a molecule of nitrogen. It is possible that the activated azide undergoing decomposition might have a cyclic structure such as IV.



IV

The existence of such an intermediate would lead to an exchange of nitrogen atoms between the α- and γ-positions of the nitrogen chain. An alternative path for such an exchange is *via* the solvolysis of the acyl azide followed by an intermolecular exchange of azide ions.

An investigation of the products of thermal decomposition of a 3,5-dinitrobenzazide-N^{15} has been carried out. The azide was prepared by the treatment of 3,5-dinitrobenzhydrazide with nitrous acid enriched with N^{15}. It is probably correctly formulated as 3,5-dinitrobenzazide-γ-N^{15}; however, there is at present no evidence to exclude the possibility of nitrogen-isotope exchange between the β- and γ-positions. The nitrogen produced in the decomposition was analyzed with a Consolidated Nier Isotope Ratio mass spectrometer, scanning the individual peaks magnetically. The nitrogen contained approximately 0.1% $N^{15}N^{15}$, 31.1% $N^{15}N^{14}$, and the rest $N^{14}N^{14}$. The 3,5-dinitrophenyl isocyanate was hydrolyzed to 3,5-dinitroaniline, which was found to contain no excess N^{15} above the natural abundance of 0.4%.

The fact that all of the excess N^{15} in the azide appeared in the nitrogen in the reaction products indicates that the initial dissociation does take place by rupture of the α-β N–N bond. It shows that no exchange takes place between the α- and γ-positions, and consequently eliminates from consideration both the cyclic structure IV, and the possibility of intermolecular exchange of azide ion. Furthermore, this coupled with the small amount of $N^{15}N^{15}$ implies that the β-γ N–N bond remains intact throughout the reaction. Nitrogen atoms produced by this bond rupture would in recombination lead to an equilibrium distribution of

(1) Work performed under the auspices of the Atomic Energy Commission.

(2) E. S. Wallis and S. C. Nagel, THIS JOURNAL, **53**, 2787 (1931); L. W. Jones and E. S. Wallis, *ibid.*, **48**, 169 (1926); E. S. Wallis and W. W. Moyer, *ibid.*, **55**, 2598 (1933); R. P. Bell, *J. Chem. Soc.*, 835 (1934).

(3) E. W. Barrett and C. W. Porter, THIS JOURNAL, **63**, 3434 (1941); L. W. Jones and E. S. Wallis, *ibid.*, **48**, 169 (1926); C. W. Porter and L. Young, *ibid.*, **60**, 1497 (1938).

(4) C. Huggett, R. T. Arnold and T. I. Taylor, *ibid.*, **64**, 3043 (1942).

(5) G. Powell, *ibid.*, **51**, 2436 (1929); E. S. Wallis, *ibid.*, **51**, 2982 (1929).