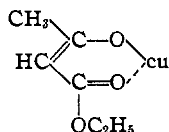
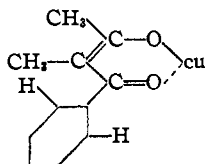
It is of interest now to comment on the two very wide deviations from the line A of Fig. 4, namely, acetoacetic ester and C-methylbenzoylacetone. Part of the deviation is undoubtedly due to the value of K_D which, for these two compounds, having small amounts of enol relative to the other compounds falling near line A, must be considerably smaller than the true value for the enol form. The rest of it, probably about half, must be attributed to other causes.

When we write the structure of the chelate of acetoacetic ester



we see that the enolate (or benzenoid) resonance involving the copper is greatly interfered with by the participation of one of the C=O groups in the very strong ester resonance, thus accounting for the very large decrease in the stability of the chelate.

When the structure of the C-methyl benzoylacetone chelate is examined



using what information we have concerning C to C bond distances and angles, it becomes apparent that, for the completely coplanar structure which would be required for the effective contribution of the enolate (or benzenoid) resonance involving the copper, there should be quite an appreciable interference between the C-methyl group and its neighbors on either side, *i. e.*, the phenyl hydrogen atom and the end methyl group. This interference would tend to distort the coplanar molecule and thus decrease the contribution of the enolate (or benzenoid) resonance and hence decrease the stability of the chelate.

There are other smaller or second order effects which would influence this enolate (or benzenoid) resonance, such as the simple dissymmetry caused by any one-sided substitution, the interaction of the phenyl and carbonyl groups in benzoylacetone, the resonance interaction of a nitro group in salicylaldehyde, etc. It is very likely that these effects contribute to the scattering of the points from the lines in Fig. 4. But the essential nature of the conclusions cannot be changed by these smaller effects.

Summary

1. The stability against dissociation into their component ions, of twenty-one chelate compounds of copper, has been determined.

2. It has been shown that in addition to their character as bases, a resonance effect involving the copper plays a very important role in determining the stability of these compounds.

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[CONTRIBUTION FROM THE DIVISION OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

Studies on Protein Denaturation. I. Electrophoretic Study Kinetics at Neutrality of Heat Denaturation of β-Lactoglobulin[1]

BY DAVID R. BRIGGS AND ROBERT HULL

In the course of some orientation experiments preliminary to a study of the electroviscous properties of heat denatured β-lactoglobulin, a Tiselius electrophoresis diagram was made on a sample of this protein which had been titrated to *p*H 7.0 (no foreign salts present), heated for forty minutes at 80°, then dialyzed at 6°, against successive amounts of phosphate buffer, *p*H 6.9, ionic strength 0.1, and the electrophoresis carried out under the final equilibrated buffer. While the native protein was an electrophoretically homogeneous material, the heat denatured protein was found to consist of two distinct components, each nearly electrophoretically homogeneous, but differing from each other in mobility by a ratio of approximately 2:3. The slow component had a mobility very near to that of the native protein. By repeating this experiment, varying only the time of heating, it was found that the ratios of the two components varied with time of heating, the fast fraction increasing progressively in amount at the expense of the slow fraction as time of heating was increased (see patterns D_{10}, D_{11} and D_{22}, Fig. 1).

During the denaturation process, conducted as it was at a *p*H removed from the isoelectric point of the native protein (I. E. P. = *p*H 5.2), little change in the appearance of the protein solution took place. A slight opalescence developed which seemed roughly to parallel in degree the amount of fast fraction formed. Qualitatively it was observed that the rate of conversion of slow to fast form during the heating was a function of *p*H of the solution, ionic strength of the solution, time of heating and temperature of heating. The mobilities (after dialysis to standard conditions)

(1) Paper no. 2245, Scientific Journal Series, Minnesota Agricultural Experiment Station.

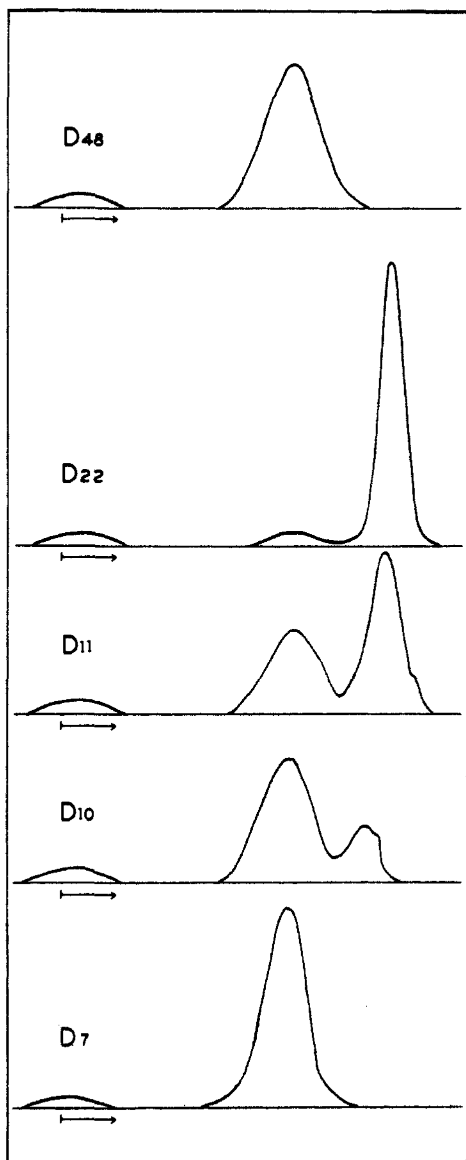

Fig. 1.—Tiselius patterns, descending, of native and heat denatured β-lactoglobulin: D7, native protein; D10, D11 and D22, protein heated at 80° for 10, 20 and 150 minutes, respectively; D48, protein heated at 99° for forty minutes. Electrophoresis time = 6800 seconds, at 5.41 volts per cm.

of the two fractions obtained, however, seemed to be independent of the factors governing the rate of conversion.

Occasional observations have been reported of what appear to be similar phenomena with other proteins. Tiselius diagrams made on denatured proteins have sometimes, but not invariably, indicated changes in the electrophoretic velocity of the denatured protein from that of the native protein. In a few cases the denatured protein has been shown to migrate as two distinct fractions which vary in relative amounts as the conditions of denaturation are varied. Longsworth,[2] while observing that "completely heat, or acid, denatured ovalbumin has mobilities at *p*H 6.80 and *p*H 10.28 but slightly less than the native form," found, however, that ovalbumin when allowed to dialyse in a buffer of *p*H 12.81 gave Tiselius diagrams indicating two distinct components. The slower fraction increased in amount at the expense of the faster fraction as the time of dialysis at this *p*H was increased. Cooper and Neurath[3] present Tiselius diagrams which indicate the presence of two electrophoretically distinct fractions after heat denaturation of horse serum albumin in alkaline media. In this case, neither fraction had quite the same mobility as the native protein and the ratios of the two components varied at constant temperature, ionic strength, and time of the denaturation treatment, with the ionic strength of the solution against which electrophoresis was carried out. When blood serum is heated at 65° for increasing periods of time, Van der Scheer, Wyckoff and Clark[4] found a progressive formation of a new component, "C," at the expense of both the albumin and the globulins, and which migrated at a rate intermediate to those of the native albumin and globulin fractions. Ultraviolet irradiation of serum results in similar changes, according to studies made by Davis, Hollaender and Greenstein.[5]

While such observations indicate that the formation of electrophoretically changed components during some kinds of denaturation of proteins may be a commonly occurring phenomenon, no quantitative studies have as yet been made to clarify the nature of the reactions involved in their formation. In the present paper, the kinetics of the process giving rise to a fast from a slow component in heat denatured β-lactoglobulin have been followed under specific conditions of *p*H and ionic strength of the protein solution. Relative amounts of slow and fast fractions in the denatured protein have been estimated from their Tiselius diagrams.

Experimental

β-Lactoglobulin was prepared according to a modification of the method of Palmer[6] and was recrystallized three times. A Tiselius diagram on this protein showed it to be electrophoretically homogeneous (Fig. 1, pattern D_7). Solutions of this native protein, at approximately 0.5% concentration in buffer solution, were placed in cellophane sacs and allowed to equilibrate against a large volume of the buffer (at 6°). A sample of the equilibrated protein solution (25–35 cc.) was then warmed to room temperature, placed in a small flask and subjected to vacuum until the sample boiled vigorously. Dissolved air was thus removed. This procedure was found to be necessary prior to heating to the denaturation temperature in order to prevent a considerable part of the protein from being

(2) L. G. Longsworth, *Ann. N. Y. Acad. Sci.*, **61**, 267 (1941).

(3) G. R. Cooper and H. Neurath, *J. Phys. Chem.*, **47**, 383 (1943).

(4) J. Van der Scheer, R. W. G. Wyckoff and F. L. Clark, *J. Immol.* **40**, 39 (1941).

(5) B. D. Davis, A. Hollaender and J. P. Greenstein, *J. Biol. Chem.*, **146**, 663 (1942).

(6) A. H. Palmer, *ibid.*, **104**, 359 (1934).

adsorbed on air bubbles and collecting as a film of insoluble protein at the surface of the protein solution. This precaution succeeded in keeping all the protein in solution, thus eliminating any question as to whether one or the other of the components in the denatured protein might be preferentially collected in the film of insoluble protein obtained when degassing was not done. The deaerated sample was then placed in a large test-tube, connected with an air condenser and immersed in a water-bath maintained at the selected denaturation temperature. The protein solution was brought to the temperature of the bath quickly by rotating the test-tube for a minute or so after the tube was immersed in the bath. After a selected interval of time, the tube containing the sample was removed and cooled quickly in contact with ice water. The sample was then placed in the Tiselius electrophoresis cell under the buffer against which it was originally equilibrated and the electrophoresis pattern developed at 4.0°.

That very little change in the protein or buffer concentrations of the solutions occurred during the degassing and heating procedures was evidenced by the fact that the areas under the ϵ and δ boundaries deviated little from those obtained in cases where equilibration against the buffer was allowed to be attained subsequent to the denaturation treatments.

From the Tiselius patterns, as obtained by the Longsworth scanning method, total area under the curve for a particular experiment compared with total area under the curve of a pattern obtained on a standard protein solution of known protein concentration gave the total protein concentration of the solution used in the particular experiment. Concentrations of the components present in the particular experiment could then be obtained from measurements of areas under the peaks corresponding to each component, augmented by the fractional amount of each component corresponding to the ϵ or δ boundaries.[7] Thus, in the descending pattern, a near approximation of these concentrations could be obtained by use of the following equations

$$C_t = A_t \frac{C'_t}{A'_t}$$

$$C_s = \left[A_s\left(1 + \frac{A_\epsilon}{A_s + A_f}\right)\right]\frac{C'_t}{A'_t}$$

$$C_f = \left[A_f\left(1 + \frac{A_\epsilon}{A_s + A_f}\right)\right]\frac{C'_t}{A'_t}$$

where C_t, C_s and C_f = concentration of total, slow and fast fractions, respectively, of protein in the particular experiment. C'_t = concentration of total protein in the standard solution in which protein concentration was determined by an independent method. A_t, A_s, A_f and A_ϵ = total area and areas under slow, fast and ϵ peaks, respectively, in the Tiselius patterns for the particular experiment. A'_t = total area under curve in the Tiselius pattern on the standard protein solution.

From the patterns for the ascending boundaries similar calculations (using the area under the δ peak in place of that under the ϵ peak) should yield equivalent values of C_t, C_s and C_f, provided the components migrate independently in the solutions. This proved to be the case within a small degree of variation. Values of protein concentrations as given in the tables are average values of those calculated using the descending and ascending patterns.

Mobility values have been calculated from the distances in the descending leg of the cell from the starting boundary to the point of maximum ordinate of the peak corresponding to the fraction for which the mobility is given.

Order of the Reaction Giving Rise to the Fast Fraction when Native Protein Is Heated at 80°.—Preliminary experiments had indicated that the formation of the electrophoretically fast fraction proceeded at a readily measurable rate when the native β-lactoglobulin was denatured at 80°. A series of experiments using 0.5% protein solutions in a phosphate buffer of pH 6.9 and 0.1 ionic strength was made at 80 ± 0.5° for times of heating varying from ten minutes to three hours. From the Tiselius patterns, obtained on each, the values of C_t, C_s and C_f were computed and are given in Table I.

These data were analyzed graphically to determine the order of the reaction. Best agreement was found when the data were plotted according to the equation for a second order process, *i. e.*, $(C_t - C_s)/C_tC_s = C_f/C_tC_s = kt$, where values of C_f/C_sC_t are plotted against time of heating, t, as in Curve A, Fig. 2. Fair agreement with a straight line passing through the origin is evident. Values of k, the rate constant calculated for a second order reaction, are also given in Table I. With the exception of the shortest time periods, the values of k are in fair agreement but show a tendency to increase somewhat with time of heating.

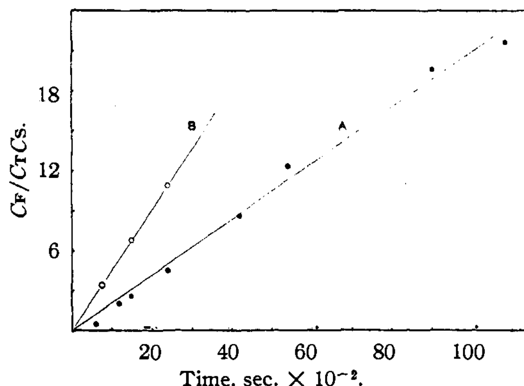

Fig. 2.—C_f/C_tC_s *versus* time curves (second order reaction graphs); (A) for native β-lactoglobulin after heating for various times at 80°, and (B) for primary denatured β-lactoglobulin (pretreated at 99° for ten minutes) after heating for various times at 70°.

Dependence of Reaction Rate upon Temperature.—A series of experiments was made wherein the temperature to which the protein was exposed during the denaturation process was varied stepwise from 60 to 99°, the protein concentration, pH and ionic strength of the solutions being the same as in the rate experiments at 80°. The duration of heating was also maintained constant at forty minutes.

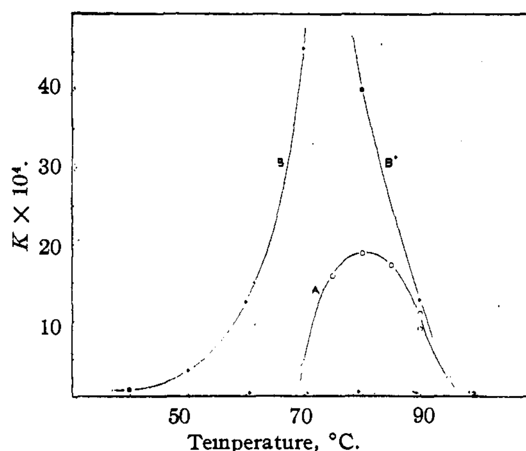

Fig. 3.—Reaction constants for the conversion of slow to fast forms of β-lactoglobulin (calculated for second order reaction) at various temperatures: (A) for native β-lactoglobulin after heating for forty minutes, (B and B') for primary denatured β-lactoglobulin (pretreated at 99° for ten minutes).

(7) L. G. Longsworth and D. A. Mac Innes, THIS JOURNAL, **62**, 705 (1940).

TABLES I–V

ELECTROPHORETIC MOBILITIES OF NATIVE, SLOW AND FAST HEAT DENATURED FORMS OF β-LACTOGLOBULIN AND KINETIC DATA ON THE REACTION INVOLVED IN THE CONVERSION OF THE SLOW TO THE FAST DENATURED FORM

m = mobility at 4° measured at the descending boundaries (cm.^{2}/volt seconds × 10^5), T = temperature of heating (°C.), t = duration of heating (seconds × 10^{-2}), C_t, C_s, C_f = concentration of total protein and of the slow and fast forms, respectively (grams per 100 cc. of solution), k = rate constant calculated for a second order reaction (seconds^{-1} grams %$^{-1}$ × 10^4), A = activation energy (calories), μ = ionic strength.

I. NATIVE PROTEIN HEATED AT 80°, pH 6.9, μ = 0.1, FOR VARYING TIMES

Expt.	T	t	C_t	C_s	C_f	C_f/C_tC_s	m_s	m_f	k
D10	80	6	0.53	0.43	0.11	0.47	6.36	8.51	7.8
D11	80	12	.62	.27	.34	2.01	6.45	9.07	16.8
D13	80	15	.56	.22	.34	2.66	6.42	8.92	17.7
D6	80	24	.62	.16	.45	4.52	6.41	9.22	18.8
D21	80	42	.56	.10	.46	8.55	6.31	9.12	20.3
D14	80	54	.60	$.07^1$	.53	12.36	6.30	9.16	22.9
D22	80	90	.49	$.04^6$	.45	19.6	6.48	9.40	21.7
D23	80	108	.49	$.04^2$	.45	21.7	6.40	9.30	20.1

II. NATIVE PROTEIN HEATED AT pH 6.9, μ = 0.1, AT VARIOUS TEMPERATURES

Expt.	T	t	C_t	C_s	C_f	C_f/C_tC_s	m_s	m_f	k
D7	65	42	0.59	0.59	0	...	6.33	...	0
D9	70	24	.46	.31	0.14	1.02	6.43	9.20	4.25
D17	75	24	.53	.18	.36	3.82	6.36	8.85	15.9
D18	85	24	.53	.16	.36	4.16	6.30	8.77	17.3
D19	90	24	.52	.24	.28	2.19	6.46	9.05	9.1
D8	90	36	.54	.17	.38	4.09	6.54	9.12	11.4
D20	95	24	.50	.39	.12	0.60	6.53	8.74	2.5
D29	99	6	.42	.42	0	...	6.45	...	0
D48	99	24	.38	.38	0	...	6.40	...	0
D38	Native	..	.42	.42	0	...	6.30	...	0

III. NATIVE PROTEIN HEATED TO 99° FOR TEN MINUTES, COOLED AND HEATED AT THE VARIOUS TEMPERATURES, AT pH 6.9, μ = 0.1

Expt.	T	t	C_t	C_s	C_f	C_f/C_tC_s	m_s	m_f	k	k_2/k_1	A
D37	40	144	0.48	0.30	0.18	1.27	...	...	0.88	3.94	27,500
D28	50	36	.56	.33	.23	1.24	6.48	8.70	3.46	3.61	27,400
D26	60	36	.55	.16	.39	4.50	6.35	8.63	12.5	3.63	29,300
D39	70	24	.57	$.07^9$	.49	10.90	6.44	9.25	45.4	Ave. =	28,000
D43	70	7.8	.51	.18	.33	3.51	6.51	9.12	45.0		
D44	70	15	.47	.11	.36	6.84	6.38	8.90	45.6		
D42	80	24	.49	$.08^6$	.40	9.60	6.36	9.16	40.0		
D45	90	24	.53	.20	.33	3.10	6.46	8.95	12.9		

IV. NATIVE PROTEIN HEATED AT 80°, μ = 0.1, TIME = FORTY MINUTES AT VARIOUS pH VALUES. ELECTROPHORESIS CARRIED OUT AT SAME pH

Expt.	T	t	C_t	C_s	C_f	C_f/C_tC_s	m_s	m_f	k	pH
D41	80	24	0.49	0.06^3	0.42	13.7^6	5.85	8.55	57.4	6.6
D24	80	24	0.51	0.45	0.05^6	0.25	7.70	10.10	1.04	8.0

V. NATIVE PROTEIN HEATED AT 80°, pH 6.9, TIME = 40 MINUTES AT VARIOUS IONIC STRENGTHS

Expt.	T	t	C_t	C_s	C_f	C_f/C_tC_s	m_s	m_f	k	μ
D46	80	24	0.41	0.01^8	0.39	60.0	6.40	9.30	250.0	0.2
D47	80	24	0.46	0.27	0.19	1.55	6.30	8.62	6.5	0.05

Values of C_t, C_s and C_f for these experiments are given in Table II. Values of $(C_f/C_tC_s) \times 1/t = k$, the rate constant, calculated upon the assumption that the conversion of slow to fast fraction at all these temperatures was proceeding in accordance with a second order process, are plotted in Fig. 3 (Curve A) against the temperature at which the protein was heated. Two facts are to be noted from these experiments. First, the reaction giving rise to the fast component is absent if the native protein is not heated above 65° but appears in small degree at 70° and increases in rate between 70 and 80°. Secondly, the rate of the reaction reaches a maximum at 80–85° and diminishes rapidly at higher temperatures to zero at 99° (see pattern D_{48}, Fig. 1).

Irreversibility of the Reaction giving Rise to the Fast Protein.—From the form of the rate constant *versus* temperature curve shown in Fig. 3, Curve A, it might be thought that the process was a reversible one, being completely reversed at the boiling temperature of water. Although the rate curve obtained from the data of Table I showed no indication that an equilibrium was being approached, an experiment was made to find out whether or not, once the fast fraction had been formed at lower temperatures, heating at 99° would convert the fast component back to the slow component. A sample of the protein was denatured under the same conditions as in experiment D_6, Table I, and was then heated for two hours at 99° No decrease in the proportion of fast fraction was found.

This indicated that failure to obtain the fast fraction at 99° was not due to a shift in equilibrium of a reversible reaction, but rather to some other condition which increased sharply in intensity at higher temperatures, and prevented the reaction that gives rise to the fast fraction from occurring.

Denaturation a Two-step Process.—Since the rate of formation of the fast fraction seemed to proceed, if at all, with extreme slowness at 60 or 65° and then increased rapidly from 70–80°, it was suspected that the fast fraction was not formed directly from native protein but from some intermediate denatured state. It is commonly considered that denaturation of proteins proceeds with a high temperature coefficient in the region of 65–70°. If an initial denaturation had to occur before the fast fraction could be formed, this would explain why the rate of fast fraction formation was zero below 65° and very much accelerated above this temperature.

A series of experiments was made to find out if, once the protein had been allowed to undergo primary denaturation, formation of the fast fraction might proceed at temperatures below 65°. In these experiments the protein solutions were heated initially to 99° for ten minutes. This was considered as time enough for primary denaturation to be completed, but it had already been shown (Experiment D_{48}, Table II) that even after forty minutes at this temperature, no detectable amount of fast fraction was formed. The hot protein solution was cooled very rapidly in an ice-bath and was then brought back to temperatures of 40, 50, 60 and 70° (in separate experiments) and the rates of formation of the fast fraction determined. These data are given in Table III and shown in Fig. 2, Curve B. Once the protein had been primarily denatured, the conversion into the fast fraction proceeded at temperatures lower than 65°. The temperature coefficients for the temperature intervals 40–50, 50–60 and 60–70° were of the same order of magnitude, k_2/k_1 having values of 3.9 to 3.6 (see Table III). The energy of activation for this reaction, as calculated from the Arrhenius equation, $A = (RT_2T_1/T_2 - T_1) \log_e k_2/k_1$, is of the order of 28,000 calories. This places the reaction in the category of ordinary chemical reactions.

Samples were prepared by heating initially for ten minutes at 99°, cooling to 80 and 90°, respectively, and then heating for forty minutes more at these temperatures. Here, as in the case of the native protein (Table II), the rate of formation of fast fraction was repressed. These data are given in Table III and plotted in Fig. 3, Curve B′. When the influence of the process giving rise to the primary denatured protein is eliminated by the initial heating for ten minutes at 99°, it is apparent that the maximum in the rate of formation of fast fraction occurs in the neighborhood of 75°. The repression influence which so greatly decreases this rate at 80° and above apparently is non-functional at 70° as evidenced by the fact that the value of k_2/k_1 between 60 and 70° is so near that expected from the ratios obtained at lower temperature ranges.

Order of the Reaction giving Rise to Fast Fraction from Primary Denatured Protein at 70°.—In the experiments (Table I) in which the rate of formation of the fast fraction from native protein at 80° was studied, the observed rate was actually the resultant of three factors which could now be recognized as influencing the rate under these conditions, *i. e.*, (1) the rate of formation of primary denatured protein from native protein, (2) the rate of formation of fast denatured protein from the slow primary denaturation product, and (3) a repression influence upon this second process which appears at the higher temperatures (above 70–75°).

The first and third of these factors may be eliminated if the protein is subjected to 99° for ten minutes, cooled and then heated at 70°. A study of the rate of formation of fast fraction under these conditions should give data from which the order of the single process, *i. e.*, slow denatured protein to fast denatured protein, could be obtained. In Table III are given results of three such experiments in which the times of heating at 70° were thirteen, twenty-five and forty minutes. Values of C_t, C_s and C_f for these cases were analyzed graphically, as before, to determine the order of this reaction. As indicated in Curve B, Fig. 2, where values of C_f/C_sC_t *versus* t are plotted (see also k values given in Table III), this reaction is described accurately as a second order process.

Significance of the Decreased Rate of Conversion of Slow into Fast Forms at Higher Temperatures (85–100°).—From the high rate of increase in the specific reaction rate (k) at which conversion of slow to fast denatured forms occurs at lower temperatures (40–70°), it would be predicted that at 95–100°, the value of k for this reaction should be very high indeed. Deviation from the expected high values of k can already be detected at 80° and it is found that at 99° the rate of conversion has reached a value near, if not actually equal to, zero. A maximum value of k occurs at 80–85° when the native protein is heat denatured and at about 75° when the primary denatured protein is heated. Such a reversal of sign in the value of dk/dt could result if the reaction were reversible and the equilibrium, slow $\rightleftarrows$ fast, were displaced radically to the left at higher temperatures. However, the reaction has been demonstrated to be non-reversible. Since a second order process appears to be involved, a possible explanation for the observed dependence of k on temperature could rest upon the postulate that at higher temperatures some force comes into action which serves to block the reaction by decreasing the probability of mutual contact of the reactants. The activation energy functional at lower temperatures becomes no longer sufficient at higher temperatures because of this new repulsion force which must be increasing in magnitude very rapidly at temperatures above 70–80°. What the nature of this force may be cannot be said with any degree of certainty. That there *may* exist some relationship between k and the charge (and electrokinetic potential) of the protein micelle is indicated by the two following sets of experiments.

Results of three experiments wherein the native protein was heated for forty minutes at 80° in phosphate buffers of ionic strength = 0.1 but at pH values of 6.6, 6.9 and 8.0, respectively, are given in Table IV and Experiment D6, Table I. The rate of formation of fast form (starting with the native protein and calculating k as a second order reaction constant) is greatly influenced by the pH of the solution. (In these experiments, the electrophoretic analysis was carried out at the same pH as the heating process and under the buffers against which the protein solutions had been equilibrated during preparation. The mobilities therefore are for the slow and fast fractions at the pH values designated.)

Similarly, three experiments were made in which the protein was heated at 80° for forty minutes in phosphate buffers of pH 6.9 but at ionic strengths of 0.05, 0.1 and 0.2, respectively. These samples were all equilibrated, following the heat treatment, against a phosphate buffer of pH 6.9, ionic strength = 0.1, and the electrophoresis carried out under this buffer. Results are given in Table V and Experiment D6, Table I. The rate of formation of fast fraction from native protein (k again calculated as rate constant of a second order process) is markedly influenced by the ionic strength of the solution in which denaturation is brought about.

The apparent marked dependence of the rate of formation of fast fraction from native protein upon pH and salt concentration, as observed here, is strongly reminiscent of similar dependence of the rates of primary denaturation of many proteins upon these factors (see review by Neurath[8] pp. 189 *et seq.*). The data reported in Tables IV and V are exploratory and definite conclusions as to their significance and interpretation must await further experimentation. In both these sets of experiments, however, the rate of formation of fast fraction is greatest under conditions where, at ordinary temperatures, the electrokinetic potential at the micelle–solution interface is lowest (*i. e.*, at low pH and at high ionic strength) and is suppressed where this potential is highest. The suppression of the rate of this reaction at high temperatures might be ex-

(8) H. Neurath, *Chem. Rev.*, **34**, 157 (1944).

TABLE VI

DIFFUSION CONSTANTS, SEDIMENTATION CONSTANTS, MOLECULAR WEIGHTS, FRICTIONAL RATIOS AND INTRINSIC VISCOSITIES OF NATIVE, SLOW DENATURED AND FAST DENATURED FORMS OF β-LACTOGLOBULIN

Protein form	$D^{4.7}_{Buffer} \times 10^7$	$D^{20}_{H_2O} \times 10^7$	$s^{20}_{H_2O} \times 10^{13}$	V_{20}	M_s	f/f_0	$\eta sp/c$
Native (accepted values)[a]	...	7.3	3.12	.751	41,500	1.26	0.037
Native (found)	4.00	6.5	3.12[b]	.751[b]	46,500	1.36	...
Slow (10 min. at 99°)	1.78	2.9	5.58	.751[b]	187,000	1.95	.086
Slow (40 min. at 99°)	1.77	2.9	...	...	.....	...	.081
Fast (60° for 4 hr.)	1.11	1.8	15.2	.751[b]	820,000	1.89	.079
Fast (70° for 2 hr.)	0.92	1.5	...	...	.....	...	.082

[a] Taken from T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, London, 1940. [b] Assumed values.

plained if it were assumed (without actual evidence) that the electrokinetic potential increases dis-proportionately in this higher temperature range.

Diffusion Constants and Sedimentation Velocity Constants on the Slow and Fast Denatured Forms of the Protein.—Two samples of the slow or primary denatured forms of the β-lactoglobulin were prepared by heating 0.5% solutions of the protein for ten and forty minutes respectively at 99° in phosphate buffer of pH 6.9 and ionic strength = 0.1 Under these conditions none of the fast type of the denatured protein was formed.

Two samples of the fast form of the denatured protein were prepared by heating similar solutions of protein in the buffer for ten minutes at 99° following by heating for four hours at 60° and two hours at 70°, respectively. Under these conditions no electrophoretically detectable amount of the slow form remained in the preparations. Almost no opalescence developed during the conversion at 60° while a considerable degree of opalescence developed in the sample converted at 70°.

On each of these samples, together with a sample of the native protein in the same buffer, the diffusion constant was determined at 4.7°, against the equilibrium buffer, using the Neurath diffusion cell[9] and the procedure described by Longsworth[2] in which scanned pictures of the diffusion boundary are taken at intervals, the values of the maximum ordinates (H_m) of the dn/dx patterns plotted against the reciprocal of the square root of the time, and the diffusion constant calculated from the slope (a) of the straight line obtained and the area (A) under the dn/dx curve, according to the equation, $D = 1(A)^2/4\pi a$.

When values of H_m were plotted against $1/\sqrt{t}$ for these experiments, the linear relationship required by theory was excellent in each case. The dn/dx curves for the native protein approximated closely the theoretical curves for a homogeneous substance and the values of D calculated by several of the various methods[7] using the pattern obtained after the longest time of diffusion were very nearly identical. The dn/dx curves for the slow denatured form were such as to indicate a low degree of heterogeneity, while those for the fast denatured form indicated a greater degree of heterogeneity. Values of D as calculated by the maximum ordinate method and corrected to 20° against water are given in Table VI.

Sedimentation velocity constants were determined on the preparation of slow form which had been heated for ten minutes at 99° and on the preparation of fast form which had been converted at 60°. The Beams-Pickels ultracentrifuge was employed in these determinations. Each sedimented with a single boundary, the slow form showing a small degree of heterogeneity, the fast form showing a greater degree of heterogeneity (as indicated by the broadening of the boundary with distance sedimented). Sedimentation velocity constants, s, obtained in these experiments are given in Table VI corrected to 20° and water.

Particle weights and frictional ratios, as calculated from $D^{20}_{H_2O}$ and $s^{20}_{H_2O}$, for each of these protein forms are included in Table VI; also the specific viscosities/concentration values for the solutions.

In view of the fact that the value of $D^{20}_{H_2O}$ obtained (6.5×10^{-7}) for the native β-lactoglobulin is not in very good agreement with the value (7.3×10^{-7}) given by Polson,[10] and the fact that both of the denatured forms showed heterogeneity, slight in the case of the slow form and quite definite in the case of the fast form, the values of M_s and f/f_0 given in Table VI should not be taken to indicate more than a qualitative relationship. Even on a qualitative basis, however, it is clearly indicated that the primary denaturation process is accompanied by an approximately fourfold increase in particle weight without any appreciable change in mobility. The observation that no change occurred in the diffusion constant of the slow form, whether it was prepared by heating at 99° for ten minutes or for forty minutes, can be taken to indicate that in the initial denaturation process the protein cannot agglomerate beyond a limit of four molecular units of the native protein, and that it does agglomerate to this extent very quickly at 99°. The process giving rise to the fast form is accompanied by a further increase in particle weight. In this case, there is evidence, from the diffusion constant decrease which accompanies the increase in opalescence of the preparation (at 70 *versus* at 60°), that once conversion of slow to fast form has occurred, further agglomeration can proceed indefinitely, but with no further change in electrophoretic mobility.

That the increase in hydration and/or asymmetry which results in an increase in the frictional ratio is confined to the primary denaturation process, and is not much changed during the process in which the slow denatured form is converted to fast denatured form, is indicated both by the values of f/f_0 calculated from $s^{20}_{H_2O}$ and $D^{20}_{H_2O}$ and by the intrinsic viscosity data obtained upon these samples.

The Primary Denaturation Process.—Because it migrates with a mobility so nearly identical with that of the native protein from which it is formed through one or more steps, not much can be learned by electrophoretic analysis about the reaction rates involved in the formation of the final primary denaturation product which acts as the precursor of the fast form.

If we assume, in experiments D9 and D17 where the native protein was heated at 70 and 75°, respectively, for forty minutes, that the conversion of native to primary denaturation product is the limiting process determining the amount of fast fraction formed at these temperatures, we can get a rough idea of the rate increase in the over-all primary denaturation process in this temperature range. [The assumption that the process (or series of processes), native → primary, may be the limiting process at these temperatures is indicated by the fact (see Fig. 3, Curve A *vs.* Curve B) that the process, primary → secondary is so much greater in cases (Curve B) where the native → primary process has already been completed.] Calculations show that in D17 (at 75°) C_f/C_t is more than twice that in D9 (at 70°).

This would indicate an over-all activation energy for the

(9) H. Neurath, *Chem. Rev.*, **30**, 357 (1942).

(10) A. Polson, *Kolloid-Z.*, **87**, 149 (1939).

process, native → primary (slow) denatured protein, of about 48,000 calories ($Q_{10} \geq 7.5$) if it is assumed to follow a first order reaction, or of about 63,000 calories ($Q_{10} \geq 14.0$) if a second order process is assumed. This explains why the denaturation process carried out at 80° (Table I) gives the indicated fair agreement with a second order process. At 80° the limiting process at all except the shortest times is that of the transformation of the primary (slow) denatured form into the secondary (fast) form which is a second order reaction.

Notes on the Degree of Electrophoretic Homogeneity in the Slow and Fast Denatured Fractions.—While we have spoken of the slow and fast fractions as being electrophoretically homogeneous, it should be pointed out that, while they are in all cases distinctly different from each other in their mobilities, they, nevertheless, show evidences of some small degree of electrophoretic heterogeneity within themselves.

The slow (primary) form obtained by heating the native protein for forty minutes at 99° gives only one smooth peak in the Tiselius diagram, but the peak is not as sharp as is that obtained under similar circumstances with the native protein. (Compare pattern D7 with pattern D48 in Fig. 1.) This may be taken to indicate a greater degree of electrophoretic heterogeneity in this primary denatured form than is characteristic of native protein.

In cases where conversion of slow to fast form is incomplete, the patterns obtained for the fast fraction show a small degree of secondary peaking, these secondary peaks appearing as bumps or shanks along the sides of the main peak (see patterns D10 and D11, Fig. 1). This undoubtedly indicates some degree of electrophoretic heterogeneity in the fast form which is insignificant compared to the difference between slow and fast but must be recognized as of some significance in itself. When conversion is complete the secondary peaking in the fast fraction is no longer detectable.

When conversion of slow to fast form takes place at lower temperatures (40 to 50°), the electrophoresis patterns indicate a distinctly greater degree of electrophoretic heterogeneity in the fast fraction (increasingly more so the lower the conversion temperature) than is the case when conversion takes place at 60° or higher temperatures.

Electrophoretic Mobilities of the Native and Denatured Forms of the Protein.—Mobilities reported in Tables I, II, III and V were obtained upon the protein forms in solution in phosphate buffer of *p*H 6.9 and ionic strength of 0.1 at 4° and were calculated from the distances through which the boundaries were displaced in the descending leg of the cell from the starting point. From experiments D7 and D38 (Table II) the mobility of the native protein under these conditions was found to be 6.3×10^{-5} cm.^{2}/volt seconds. That of the primary denatured form of the protein, in absence of any of the fast fraction, was found in experiments D29 and D48 (Table II) to be 6.4×10^{-5} cm.^{2}/volt seconds. The mobility of the fast denatured form, when free of any primary denatured fraction (a condition approached in experiments D22, D23 (Table I), D39 (Table III) and D46 (Tabel V), was of the order of 9.3×10^{-5} cm.^{2}/volt seconds. Thus, the limiting ratio of m_s/m_f has the value of 0.69.

When both the slow and fast forms of the denatured protein are present in a sample, there is detected a decrease in the absolute difference in mobilities of the two forms from that obtained when each is present alone. When this difference in absolute mobilities, $m_f - m_s = \Delta m$, is plotted against the fraction of total protein present as the fast form, C_f/C_t, the spot diagram shown in Fig. 4 is obtained. The distribution of points in this diagram is such as to indicate that Δm varies linearly with C_f/C_t. A line, constructed by statistical methods, has been drawn to show this relationship. The equation $\Delta m = K_1 + K_2C_f/C_t$ describes this relationship, where K_1 and K_2 have the values 2.016 and 0.904, respectively. It is probably of significance that the ratio of Δm, where $C_f/C_t = 0$ (*i. e.*, $\Delta m = K_1 = 2.016$), to Δm when $C_f/C_t = 1$ (*i. e.*, $\Delta m = K_1 + K_2 = 2.92$), is identical with the ratio $m_s/m_f = 0.69$, when each is present alone. Qualitatively, the mobility of the slow component is the same whether it is alone or in a mixture. The above effect seems to be that of a hindrance to the movement of the fast form when present in a mixture with the slow, and to be dependent only upon the relative amounts of the two forms present. There is no evidence, from these data, that the effect is in any way related to the absolute concentration of total protein present. Since the intrinsic viscosity increments due to these two forms in solution are almost identical (Table VI) it seems unlikely that this impedance to the migration of the fast form bears any relationship to the viscosity increment due to total protein, though it may well bear some relationship to the viscosity increment due to slow form alone.

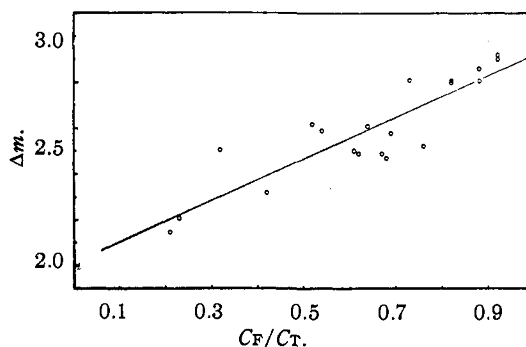

Fig. 4.—Mobility difference between the slow and fast forms of heat denatured β-lactoglobulin as a function of the ratio of fast form to total protein.

In experiments D37, D28 and D26, in which conversion of slow to fast was conducted at 40, 50 and 60°, respectively the two forms separated as electrophoretically distinct fractions, but the Δm values were much lower than in those cases where conversion occurred at 70° or above (and from which the data in Fig. 4 were taken). No explanation can be offered for this observation.

In no case has there been observed any indication that the denatured protein might be convertible into a form showing a mobility, under the standard conditions, greater than that found for the "fast" fraction described. This is true in spite of the fact that, once the primary denatured form has been converted into the "fast" form, the latter, as judged from the observed increase in turbidity with time of heating, continues to grow indefinitely in particle size.

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Summary

The heat denaturation of β-lactoglobulin, when carried out in the region of *p*H 7 and in a buffer solution of the order of 0.1 ionic strength, involves at least two distinct processes, the products of which can be recognized and their ratios followed by their different electrophoretic mobilities.

The first process (or group of processes) which is initiated only at temperatures above 65°, is not accompanied by any appreciable change in electrophoretic mobility of the protein, but is accompanied by an approximately four-fold increase in particle weight and an increase in frictional ratio. Calculations, based on very incomplete

evidence, indicate the over-all activation energy for this process to be of the order of 48,000 calories, if it is assumed to be a first order reaction, for the temperature change from 70 to 75°. This process does not appear to be repressed at higher temperatures and proceeds readily at 99°.

The second process, which can take place only after the first process has occurred, will proceed at temperatures below that at which the first is initiated. The second process is accompanied by a marked increase in electrophoretic mobility over that of the native protein or that of the product of the first process. It is also accompanied by a further increase (probably unlimited) in particle weight but no marked further increase in frictional ratio. This second process, at temperatures of 70° and below and in phosphate buffer of *p*H 6.9, ionic strength 0.1, proceeds with a constant activation energy of about 28,000 calories and a temperature coefficient between 60 and 70° of about 3.6 (Q_{10} = 3.6). It accurately follows the concentration–time characteristics of a second order reaction. Above a temperature of 75° this process is markedly repressed as the temperature is increased until at 99° it does not take place at all. Increase in *p*H or decrease in ionic strength of the solution, in which the heat denaturation is carried out, depress the rate of this reaction. The postulate is offered that this process is influenced to a marked extent by the electrokinetic potential of the particles of the primary denatured protein. As yet, there is no evidence that the first process of denaturation is so affected.

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On Arsenomethane

BY JURG WASER AND VERNER SCHOMAKER

The question of the structure of arseno compounds, the compounds of arsenic of composition analogous to the azo compounds, has been investigated in the past mainly by molecular weight determinations in solutions[1] and to some extent by studies of their chemical reactions.[2] The chemical considerations appear to have been dominated by the notion that arseno compounds have the structure R—As═As—R, analogous to that of azo compounds R—N═N—R, and it seems that the majority of organic chemical opinion accepts this idea. It is unlikely that arseno compounds actually have the doubly bonded structure, however, for the molecular weight determinations have generally indicated a degree of polymerization, *n* in the formula $(RAs)_n$, of about four or five, and always greater than two. Moreover, the double bond structure violates the general rule, which probably has no important exceptions, that only first row elements (of the periodic table) form what have to be regarded as multiple covalent bonds. For arsenic the remarkable structure of skutterudite,[3] $CoAs_3$ or $Co_4(As_4)_3$, with its four-membered, singly-bonded arsenic rings, provides an example of the working of this rule insofar as the structure might have been expected to involve —As═As— groups. The arsine oxides $(RAsO)_n$ also are subject to confusion in this respect, for they appear to be generally regarded as monomeric although the same arguments apply to them as to the arseno compounds, and some of them have been shown by molecular weight determinations[1] to be certainly polymeric.

It was hoped that on the basis of an electron diffraction study a definite structure could be assigned to arsenomethane[4] $(CH_3As)_n$ that would establish the correct formula for this particular arseno compound. It proved impossible to determine the structure unambiguously on the basis of the diffraction data alone, although the double bond structure could be definitely eliminated. Vapor density measurements were therefore undertaken for the information they might provide regarding the degree of polymerization of arsenomethane in the vapor state. The present study also includes an investigation of some other properties of arsenomethane.

Preparation and Properties

Arsenomethane was prepared by the action of hypophosphorous acid on sodium methylarsenate[5] which had been prepared by refluxing an aqueous solution of sodium arsenite with methyl iodide. The arsenomethane was distilled in a high vacuum and was stored in vacuum-sealed ampoules. An analysis indicated C, 13.22, and H, 3.34 (calculated for $AsCH_3$: C, 13.15; H, 3.36).

Arsenomethane exists in two modifications, a light yellow oil and a brick red to dark brown solid modification, the latter being the more stable at room temperature. If the yellow oil was left standing in contact with air for a period of weeks a red solid slowly formed, which contained less

(1) *E. g.*, F. F. Blicke and F. D. Smith, THIS JOURNAL, **52**, 2946 (1930).

(2) For references see *e. g.*, G. W. Raiziss and J. L. Gavron, "Organic Arsenical Compounds," Reinhold Publ. Corp., New York, N. Y., 1923.

(3) I. Oftedal, *Z. Krist.*, **66**, 517 (1928); *cf.* also *Z. Krist.*, "Strukturbericht," 1931, p. 232.

(4) V. Auger, *Compt. rend.*, **138**, 1705 (1904); W. M. Dehn, *Am. Chem. J.*, **33**, 120 (1905); **35**, 8 (1906), **40**, 109 (1908); E. V. Zappi, *Bull. soc. chim.*, (4) **23**, 322 (1918); F. A. Paneth, *Trans. Faraday Soc.*, **30**, 179 (1934); *J. Chem. Soc.*, 366 (1935); W. Steinkopf and H. Dudek, *Ber.*, **61**, 1906 (1928); A. Valeur and P. Gailliot, *Compt. rend.*, **185**, 956 (1927).

(5) C. S. Palmer and A. B. Scott, THIS JOURNAL, **50**, 536 (1928).