Myer, F., and Barnard, D. (1971), *Arch. Biochem. Biophys. 143*, 116.
Némethy, G., and Scheraga, H. A. (1962a), *J. Phys. Chem. 66*, 1773.
Némethy, G., and Scheraga, H. A. (1962b), *J. Chem. Phys. 36*, 3401.
Noguchi, H., and Yang, J. T. (1971), *Biopolymers 10*, 2569.
Parker, K. D., and Rudall, K. M. (1957), *Nature (London) 179*, 905.
Pauling, L., and Corey, R. B. (1951), *Proc. Nat. Acad. Sci. U. S. 37*, 729.
Pederson, D., Gabriel, D., and Hermans, J., Jr. (1971), *Biopolymers 10*, 2133.
Quadrifoglio, F., and Urry, D. W. (1968), *J. Amer. Chem. Soc. 90*, 2760.
Rosenheck, K., and Doty, P. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1775.
Rossman, M. G., Adams, M. J., Buehner, M., Ford, G. C., Hackert, M. L., Lantz, P. J., Jr., McPherson, A., Jr., Schevitz, R. W., and Smiley, I. E. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 179.
Sarkar, P. K., and Doty, P. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 981.
Scheraga, H. A., Némethy, G., and Steinberg, I. Z. (1962), *J. Biol. Chem. 237*, 2506.
Senti, F. R., Eddy, C. R., and Nutting, G. C. (1943), *J. Amer. Chem. Soc. 65*, 2473.
Snell, C. R., and Fasman, G. D. (1972), *Biopolymers 11*, 1723.
Spackman, D. H., Stein, H. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Tanford, C. (1970), *Advan. Protein Chem. 24*, 1.
Tooney, N., and Fasman, G. D. (1968), *J. Mol. Biol. 36*, 355.
Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D., and Davidson, B. (1966), *Biochem. Biophys. Res. Commun. 23*, 163.
Walrafen, G. (1968), *in* Equilibria and Reaction in Hydrogen Bonded Solvent Systems, Covington, A., and Jones, P., Ed., London, Taylor and Frances, p 9.
Widmer, U., and Lorenzi, G. P. (1971), *Chimia 25*, 236.
Wooley, S.-Y. C., and Holzwarth, G. (1970), *Biochemistry 9*, 3604.

Bovine β-Lactoglobulins in Urea Solution. Denaturation at pH 5.2 and 3.5†

H. A. McKenzie* and G. B. Ralston‡

ABSTRACT: The denaturation reactions of bovine β-lactoglobulins A, B, and C in urea solution at pH 5.2 and 3.5 are compared. The optical rotation and viscosity, and their rate of change with time, are strongly dependent on urea concentration. In 7 M urea at pH 5.2 the optical rotation change may be broken down by kinetic analysis into a primary and a secondary stage. The primary change does not follow simple first-order kinetics, but may be decribed by the sum of two exponential terms. Its half-time decreases slightly with increasing protein concentration. The primary denaturation is not a two-state process and involves sequential and/or parallel reactions. In this stage the rotation change is largely reversible but on prolonged reaction the extent of reversibility decreases with increasing reaction time, the kinetics of renaturation always being complex. Each variant is more readily unfolded by urea at pH 3.5 than at pH 5.2. The change in optical rotation with time, at pH 3.5 and urea concentrations where the reaction is sufficiently slow for kinetic measurements to be made, consists of a rapid primary and a much slower secondary stage. The primary change at 578 nm is sometimes apparent first order, but that at 260–300 nm is not first order. The presence of stable intermediates at the end of the primary stage is demonstrated by analysis of ORD curves. On prolonged reaction irreversible products, due to -SH/-SS- interchange, are produced at pH 3.5, but to less extent than at pH 5.2. The order of kinetic and thermodynamic stability of the variants is determined, and a general mechanism for their behavior in urea is proposed.

The work described here arose out of two series of investigations in this laboratory. One series is concerned with the chemical evolution of individual milk and blood proteins (McKenzie, 1967, 1971). The other is concerned with comparative studies of the denaturation and aggregation of proteins under a variety of conditions with the aim of gaining a better understanding of the mechanism of these processes and hence of some aspects of protein structure (McKenzie and Ralston, 1971). The rationale of the investigations is that the change with time of several experimental parameters, sensitive to different properties of the protein molecule, is studied under varying conditions. The effects of denaturant and its concentration, pH, temperature, and group specific reagents, and the reversibility of the reactions are determined. If any theory of protein denaturation ("unfolding") is generally valid it should be applicable to proteins containing cysteine and cystine residues as well as to those not containing them. Thus both groups of proteins are being investigated in this laboratory. The unfolding of the former group is

† From the Department of Physical Biochemistry, Institute of Advanced Studies, Australian National University, Canberra City, A.C.T., Australia. *Received May 30, 1972.* This research forms part of a Ph.D. Thesis submitted by G. B. R. to the Australian National University, Oct 1969.

‡ Recipient of an Australian Commonwealth Graduate Scholarship (1966–1969). Present address: Chemistry Department, University of Wisconsin, Madison, Wis. 53706.

usually the more difficult to investigate since such proteins tend to undergo intra- and intermolecular –SH/–SS– interchange reactions. Hence care must be taken to disentangle unfolding reactions from interchange effects. We have included the β-lactoglobulins in our investigations of the former group since we are making an extensive study of their chemical evolution. The β-lactoglobulins occur as a wide range of genetic variants with interesting structural features, and exhibiting subtle transitions and varying association–dissociation as the pH is altered. We are concerned here with the behavior in urea solution of the three best known genetic variants (A, B, and C) of the bovine protein.

Near the isoionic point (pH ~5.2) each of these variants exists in solution primarily as a dimer consisting of two similar polypeptide chain monomer units of 18,000 daltons, and their conformations appear to be broadly similar. The amino acid residue differences between the variants are: A↔B = Asp↔Gly, Val↔Ala; B↔C = Gln↔His. A partial amino acid sequence has been determined by Frank and Braunitzer (1967). More recently a modified amino acid sequence including the location of the single cysteine and two cystine residues in each monomer unit has been presented by McKenzie *et al.* (1972). The behavior of each variant in the absence of urea is asymmetric with respect to the isoionic point (McKenzie, 1971). At low pH the protein is stable and appears to undergo no appreciable conformational change down to pH 2, but there is an increasing tendency for the dimer to dissociate rapidly and reversibly to the monomer below pH 3.5. The behavior in neutral and alkaline solution is quite different from that at low pH, the protein undergoing conformational changes, and, at higher pH, interchange reactions.

As well as investigating the variants in urea at pH 5.2, we have also studied them at pH 3.5, where it was anticipated that interchange reactions would be negligible. In the course of this investigation Pace and Tanford (1968) reported that the urea denaturation of the A variant at pH 2.5–3.5 is a reversible two-state process. We shall show here that the unfolding reactions at pH 5.2 and 3.5 are not generally two state, and that they may be followed by appreciable irreversible changes, including –SH/–SS– interchange even at pH 3.5.

Materials

Reagents and Glassware. All reagents were of analytical grade. However, ammonium sulfate used in protein fractionation and solubility studies was Mann enzyme grade. Special precautions were taken to avoid contamination by trace metals, particularly copper(II) (McKenzie and Murphy, 1970).

Protein Preparations. Each of the bovine β-lactoglobulins studied was isolated from the milk of a cow homozygous in that variant, using method Ia of Armstrong *et al.* (1967). The moist crystals were held at 2°. In order to minimize possible deterioration, protein that was to be held for an appreciable length of time was dissolved, precipitated with ammonium sulfate, and stored as paste. When required for use the paste was dissolved, dialyzed, and recrystallized as described by Armstrong *et al.* (1967). The purity of each preparation was checked by the starch gel ($NaOH–H_3BO_3$) electrophoresis method of Bell (1967) and usually also with the semidiscontinuous buffer system of Ferguson and Wallace (1963).

Experimental Methods

Measurements of pH. pH measurements were made with a Leeds and Northrup pH meter, Type 7664, or a Cambridge Instrument Co. bench model pH meter. Calibration of each electrode assembly was made by the procedures recommended by Bates (1964). No attempt was made to set up a separate scale for pH measurements in concentrated urea solution, and no theoretical interpretation is made of the apparent pH values obtained in urea solution. The composition of a given buffer system was varied with urea concentration so that the apparent pH for the system was constant, *e.g.*, when urea was absent a pH of 5.2 was obtained with 0.065 M CH_3COOH–0.05 M CH_3COONa and for 7 M urea the composition was 0.097 M CH_3COOH–0.05 M CH_3COONa.

Preparation of Urea Solutions. Since urea undergoes hydrolysis to cyanate (Werner, 1913) the following precautions were taken to minimize the presence of cyanate in the urea used. Samples of Mallinckrodt urea were selected for low cyanate content and then further purified by recrystallization from 70% (v/v) aqueous ethanol. Stock solutions 10 M in urea were made up freshly each day and at no stage in the solution process was the temperature allowed to rise about 35°.

Protein Stock Solutions. These were made by dissolving *ca.* 0.2 g of moist β-lactoglobulin crystals in 1 ml of 0.25 M NaCl solution. The solutions were made in a 5-ml beaker without stirring to minimize surface denaturation, and then filtered through a 0.45-μm Millipore filter (previously washed and dried).

Protein Concentration. The concentration of native protein solutions was determined from the absorbance at 278 nm, using the absorbance indices given by Bell and McKenzie (1967). The protein concentration in urea solutions was checked from the absorbance at 278 nm, using a value of $A_{1cm}^{1g/dl} = 9.5$ determined for known concentrations of the B variant in urea solution at pH 3.5–8.9. In all cases corrections for light scattering, which was small, were made.

Reaction Mixtures. Sufficient 10 M urea was added to a 5-ml Quickfit conical flask to give the desired final urea concentration. This was followed by the relevant volumes of water, stock buffer solution ($CH_3COOH–CH_3COONa$ in the pH 5.2 experiments, NaCl–HCl for pH 3.5), and sodium chloride solution. The stoppered flask was then immersed in a water bath at the desired temperature. When thermal equilibrium was attained protein stock solution was then added with rapid mixing by swirling, with care to avoid foaming. Except where otherwise stated all experiments were carried out at $I = 0.1$.

Optical Rotation. Most of the reaction kinetic experiments were carried out with a Perkin-Elmer Model 141 photoelectric polarimeter at 578 nm. Water-jacketed cells, thermostated to the desired temperature with water circulated from an external bath, were used. The temperature inside the cell was measured with a nichrome constantan thermocouple, and was constant to ±0.05–0.10°. In the later stages of the work a Cary Model 60 recording spectropolarimeter was acquired and optical rotatory dispersion (ORD) measurements were made over the range 200–600 nm, as well as kinetic measurements at fixed wavelengths in this range. Rotations in the ORD measurements were expressed in terms of the mean residue reduced rotation [m'] (for definitions, see McKenzie, 1970). Refractive index corrections were made using the tables compiled by Fasman (1963) and an interpolation procedure based on an approximation of the Lorenz–Lorentz relationship (Partington, 1953).

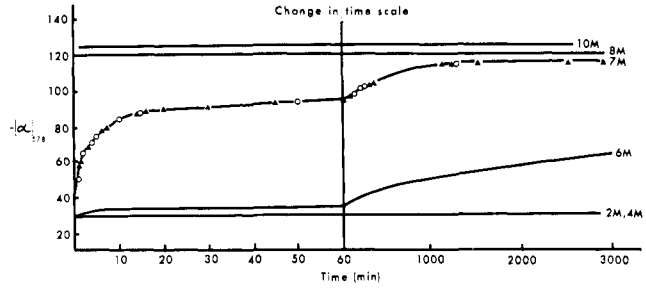


FIGURE 1: Effect of time on specific rotation at 578 nm, $[\alpha]_{578}$ of bovine β-lactoglobulin B in urea solutions of various concentration (shown on each curve) at pH 5.2 and 25°. The effect of previously denatured protein on the rotation change is also shown for 7 M urea solution. ▲ signifies native protein in 7 M urea; O, native protein *plus* previously denatured protein. Note the change of time scale at 60 min.

Viscosity. Two sets of Ostwald type capillary viscometers, with a 2-ml sample volume, were used. One set had flow times of *ca.* 65 sec for water at 25° and the other flow times of *ca.* 65 sec at 3°. The viscometers were located by suitable V-blocks in a thermostat bath controlled to ±0.02°. Results were expressed in terms of the viscosity number (reduced viscosity) in milliliters/gram.

Results

Reaction at pH 5.2

Effect of Urea Concentration on Optical Rotation of B at pH 5.2 and 25°. The specific optical rotation at 578 nm of bovine β-lactoglobulin B (10 g/l.) was determined as a function of time at 25° and pH 5.2 (CH_3COOH–CH_3COONa–NaCl, $I = 0.1$). Urea concentrations of 4 M and less had little effect on the specific rotation, as shown in Figure 1. There was a slow increase in levorotation with time in 6 M urea, but even at the end of 48 hr a final value of the rotation had not been reached. The levorotation increased rapidly in 7 M urea during the first 30 min and then changed slowly for the next 48 hr. In 8 and 10 M urea there was an immediate (less than 1 min) increase in rotation and no further change occurred in the next 48 hr.

The rotation change during the first 50 min in 7 M urea was examined by the method of Guggenheim (1926), giving a nonlinear plot indicating that the kinetics were not simple first order. The rate of change of rotation decreased appreciably with increasing time, curvature becoming clearly evident in this plot after a few minutes reaction. The results also failed to satisfy second-order kinetics tests.

The overall rotation change was broken down into a primary and secondary stage by the method of Simpson and Kauzmann (1953). The primary stage, which had a half-time of *ca.* 1.7 min (at 10 g/l.), did not follow simple first-order kinetics. However, its time course could be described by an equation containing two exponential terms

$$[\alpha] - [\alpha_f] = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t} \quad (1)$$

where $[\alpha]$ is the specific rotation at a time t, $[\alpha_f]$ is the value of the specific rotation at the end of the primary stage, A and B are constants, and λ_1 and λ_2 are two parameters. A typical example of the analysis of results in terms of this equation is shown in Figure 2a. The overall primary and secondary stages could not be described satisfactorily using three exponential terms.

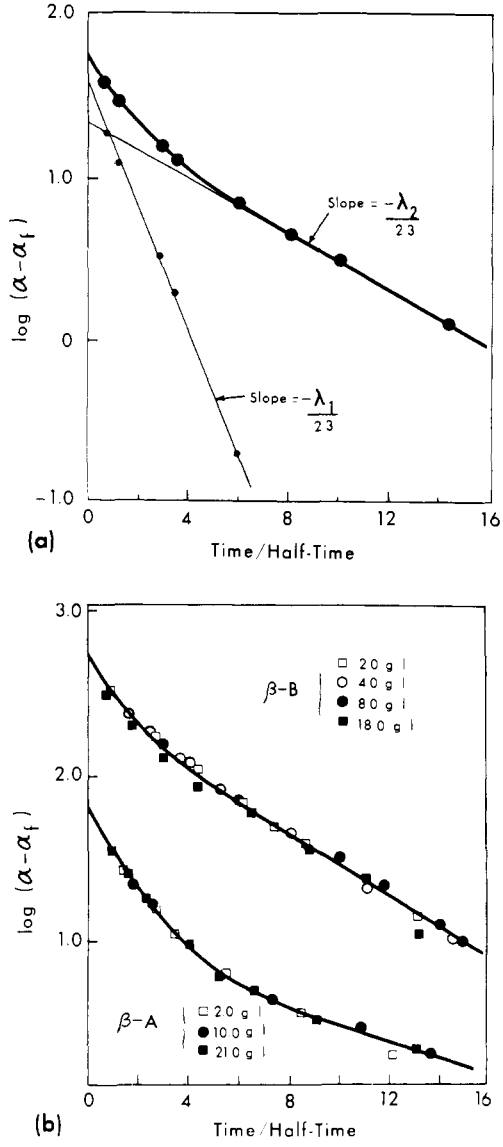


FIGURE 2: (a) Plot of log $(\alpha - \alpha_f)$ *vs.* the normalized time parameter (time/half-time) for the *primary* rotation change of bovine β-lactoglobulin B (8 g/l.) in 7 M urea, pH 5.2, 25°. The half-time is 1.7 min. Extrapolation of the curve for the later period of the primary stage to zero time yields a straight line of slope $-\lambda_2/2.3$. By a subtraction procedure the points lying on the line of slope, $-\lambda_1/2.3$, are obtained. (b) Plots of log $(\alpha - \alpha_f)$ *vs.* time/half-time for bovine β-lactoglobulins A and B at different protein concentrations over the range 2–20 g/l. at pH 5.2, 25°. (The plots for B have been displaced one decade for clarity.) Values of $t_{1/2}$ are given in Table II.

An attempt was made to make a similar kinetic analysis of the kinetic results for 6 M urea solution at 25°, but the values for the primary rotation failed to converge.

Comparison of Rotation Change for A, B, and C Variants at pH 5.2. Curves for optical rotation at 578 nm *vs.* time for the A variant, at 25°, were similar to those for the B variant over the urea concentration range 0–7 M. However, the reactions in 6 and 7 M urea were more rapid and extensive for A than for B. In 7 M urea solution, the rate and extent of rotation change for the A, B, and C variants were in the order A > B > C.

An analysis of the curves for the A and C variants in 7

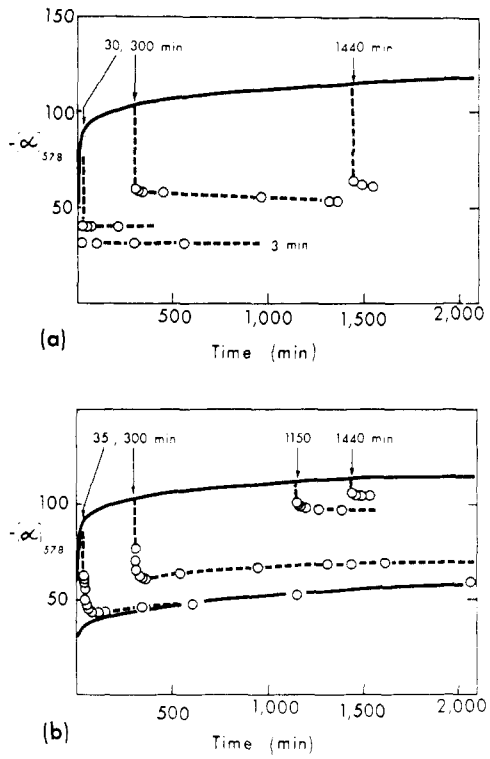


FIGURE 3: Effect of dilution of a solution of bovine β-lactoglobulin B (10 g/l.) in 7 M urea, pH 5.2, 25° to (a) 2 M urea, (b) 6 M urea, at the times indicated by the arrows. The lower solid curve in (b) is for the denaturation reaction in 6 M urea.

M urea by the method of Simpson and Kauzmann (1953) gave similar departure from first-order kinetics to that of the B variant, typical results for A and B being shown in Figure 2b. Values of the half-times and final values of rotation for the primary reaction are shown in Table I.

Effect of Protein Concentration on Rotation Change. The rotation changes for β-lactoglobulin A and B in 7 M urea were examined at different protein concentrations over the range 2–20 g/l. The reaction for each protein concentration was resolved into a primary and secondary stage. The curves of log ($\alpha - \alpha_f$) *vs.* a normalized time parameter for the primary reactions had similar curvature as can be seen from Figure 2b. It can be seen from Table II that the half-times for the primary reaction of each variant increase slightly with the approximately tenfold increase in protein concentration studied.

The rates of the secondary reactions increased slightly with increasing protein concentration. A final specific rotation of *ca.* −117° was attained after 24 hr in all cases.

TABLE I: $t_{1/2}$ and $[\alpha_f]_{578}$ for the Primary Reaction of A, B, and C in 7 M Urea, pH 5.2, 25°.

	Variant		
	A	B	C
Concn (g/l.)	10.0	8.3	6.5
$t_{1/2}$ (min)	1.1	1.7	4.0
$[\alpha_f]_{578}$ (deg)	−97.5	−93.1	−80.0

TABLE II: Dependence of $[\alpha_f]$ and $t_{1/2}$ on Protein Concentration for the Primary Reaction in 7 M Urea, pH 5.2, 25°.

β-Lactoglobulin A			β-Lactoglobulin B		
Concn (g/l.)	$[\alpha_f]$ (deg)	$t_{1/2}$ (min)	Concn (g/l.)	$[\alpha_f]$ (deg)	$t_{1/2}$ (min)
2.0	−96	0.7	2.1	−95	1.2
			4.3	−92.5	1.3
10.0	−97.5	1.1	8.3	−93	1.7
20.8	−101	1.6	18.1	−83	2.3

Effect of Denatured Protein on Rotation Change at pH 5.2. It was shown that the deviation from first-order kinetics for the rotation change in 7 M urea was not due to inhibition of the reaction by products of denaturation. A solution of the B variant in 7 M urea, pH 5.2, was held at 25° for 2 days. An aliquot (0.2 ml) of this solution was then added to a 7 M buffer mixture (2 ml), followed immediately by native protein solution, and the change of rotation with time was determined. When the contribution of the previously denatured protein was subtracted from the total rotation change, the resultant curve of specific rotation *vs.* time was indistinguishable from that of the normal reaction as shown in Figure 1.

Reversibility of Rotation Change at pH 5.2. When the reaction mixtures for higher urea concentrations at pH 5.2 were diluted with water in the early period of reaction, the rotation change was very rapid and was completely reversible ($[\alpha]_{578}$ *ca.* −32°). The degree of reversibility decreased with increasing reaction time prior to dilution (Figure 3a). At the end of the primary denaturation stage (*ca.* 30 min) reversal was still largely complete. After 300 min the rotation decreased rapidly to −59° on dilution and this was followed by a slow decrease to −44°.

Complex kinetics were observed for the reversal reaction on dilution of the denaturation reaction mixture from 7 to 6 M, and the complexity increased with increasing reaction time prior to dilution. When a solution of B variant (9 g/l.) in 7 M urea at pH 5.2 and 25° was diluted to 6 M after 35 min of reaction, the decrease in levorotation could be resolved into two stages: an immediate change from 95 to 68°, followed by a slower change. The latter followed apparent first-order kinetics for the first 25 min, $t_{1/2}$ being 5 min. The levorotation passed through a minimum at 42°, then increasing and merging with the normal forward reaction in 6 M urea (Figure 4b). The presence of a minimum was also observed when the dilution to 6 M was made after 300 min (*i.e.*, well into the secondary stage), but the rotation did not revert to the normal 6 M curve (Figure 3b). When the dilution to 6 M was made after *ca.* 20 hr reaction there was only a slow small decrease in levorotation (to 95°).

When the reaction for the B variant (10 g/l.) in 10 M urea was allowed to proceed for 1 min and then the mixture was diluted to 7 M the levorotation decreased to a minimum value of 92°, with a subsequent slow increase. This value is virtually the same as that (93°) estimated for the primary "forward" reactions in 7 M urea.

Effect of Temperature on Rotation Change at pH 5.2. The change with time of the specific levorotation at 578 nm was followed for the B variant (11.0 g/l.) in 6 M urea at pH 5.2 over the temperature range 3–45°. The rate of change de-

TABLE III: Effect of Temperature on Initial Rates of Change in $[\alpha]_{578}$ of B (11.0 g/l.) in 6 M Urea at pH 5.2.

Temp (°C)	Rate (deg min^{-1})
10	8.7
20	4.7
25	0.8
35	0.03
45	0.07

creased with increasing temperature until 35° when it increased again over the range 35–45°. At 3° the rate of change was very rapid and the extent of change was greater than at the higher temperatures, the final rotation being 128° (*cf.* 114° at 10°). A gradual increase in levorotation was observed over 48 hr at temperatures between 20 and 45°, and no estimate of the final rotation could be made. Determination of the initial rates of change was possible for temperatures from 10 to 45°, and these are shown in Table III.

The changes in rotation for the A and B variants in urea at 3.8° were more rapid and extensive than those for the corresponding urea concentrations at 25°. In 7 M urea the rotation for B reached −130° in less than 2 min, this rotation being independent of concentration over the range 1.0–19.2 g/l. The levorotation of C increased more slowly, its final value of 133° being only obtained after 12 min.

The rotation changes of A and B in 6 M urea followed apparent first-order kinetics (linear log plot) and no secondary change was observed. On the other hand in 5 M urea primary and secondary changes occurred, although the latter was not extensive (4°). The primary stage could be described by two exponential terms. The first-order log plots for B in 5 M urea are shown in Figure 4. Both the rate of change of specific levorotation and the final value attained in 5 M urea decreased slightly with increasing protein concentration. A fivefold increase from 2.0 to 10.0 g/l. resulted in a change in half-time from 15 to 25 min.

Optical Rotatory Dispersion at pH 5.2. The levorotation of β-lactoglobulin increased over the wavelength range 313–578 nm in urea solutions above 4 M. The rate and extent of this increase were paralleled by decreases in the parameter, a_0, of the phenomenological equation of Moffitt and Yang (1956). However, the parameter, b_0, remained approximately constant. In Figure 5 values of a_0 and b_0 are shown for the B variant in urea concentrations from 0 to 10 M at 25°, at times when the final rotation at 578 nm of the primary stage for each urea concentration had been reached. These values correspond to equilibrium values for reversible denaturation prior to appreciable irreversible changes occurring.

The ORD of the A, B, and C variants in 0.1 M NaCl at pH 5.2 are very similar over the wavelength range 215–320 nm, although there are small differences in magnitude of rotation between the variants (C > B > A). The dispersion for the native B protein is shown as the upper curve in Figure 6. It is characterized by a deep trough near 230 nm with two minima (230 and 237 nm) and by weak Cotton effects between 280 and 300 nm. The change with time in the dispersion of the B variant in 7 M urea at pH 5.2 is also shown. Within 3 min of mixing, the bimodal trough near 230 nm was replaced by a single trough near 228 nm. With increasing time of reaction the levorotation at all wavelengths increased and the trough shifted further toward 225 nm, and at the end of 20 hr was

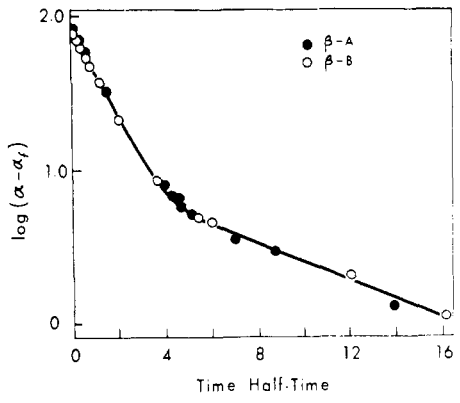


FIGURE 4: Plot of log ($\alpha - \alpha_f$) *vs.* time/half-time for the primary rotation change of β-lactoglobulins A and B (10 g/l.) in 5 M urea at pH 5.2, 3.8°. For the A variant $t_{1/2}$ = 17 min and $t_{1/2}$ = 27 min for B.

no longer detectable. Similar changes occurred in the region 260–320 nm, the weak Cotton effects being considerably diminished within the first 3 min of reaction. Subsequently, they changed at a slower rate, being still detectable at 60 min, but they had disappeared after 20 hr.

Effect of N-Ethylmaleimide on Rotation at pH 5.2. In the presence of *N*-ethylmaleimide (2 mol/mol of β-lactoglobulin sulfhydryl group) there were more rapid and extensive optical rotation changes for bovine β-lactoglobulin in 6 and 7 M urea than in the absence of sulfhydryl reagent (Figure 7). There were no secondary changes and the primary change followed apparent first-order kinetics in 6 M urea.

The effect of variation of the urea concentration after various reaction times was studied for the B variant (10 g/l.) in 7 M urea containing *N*-ethylmaleimide (1.12×10^{-3} M) at pH 5.2 and 25°. Lowering the urea concentration from 7 to 2 M after 150 min of reaction resulted in a rapid reversal of specific levorotation to 33°, a value which is close to that of the native protein at pH 5.2 (Figure 7). When urea concentration was lowered from 7 to 6 M after 30 and 300 min of reaction, the levorotation rapidly fell at both times to 108° without any further change with time (Figure 7). This value is close to the final value of levorotation (107 ± 1°) for the denaturation of the protein in 6 M urea containing *N*-ethylmaleimide. These results may be contrasted with those for the

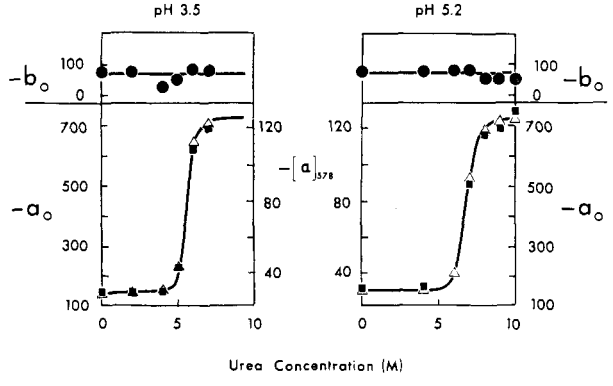


FIGURE 5: Effect of urea concentration on the specific rotation at 578 nm, $[\alpha_f]_{578}$ (Δ), and the parameters a_0 (■) and b_0 (●) of the Moffitt–Yang equation (1956) at the end of the primary reaction for bovine β-lactoglobulin B at pH 3.5 and 5.2.

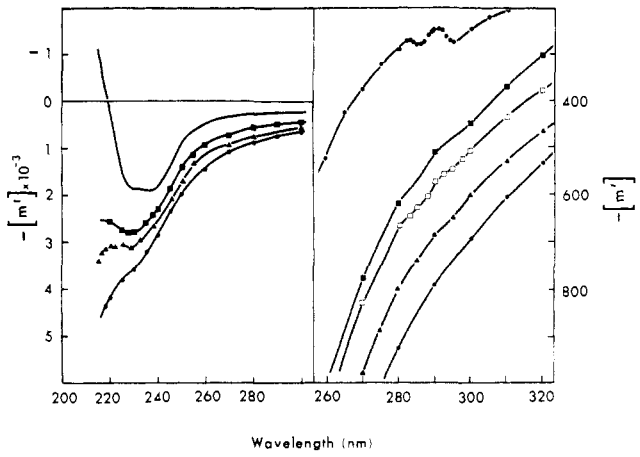


FIGURE 6: Changes in the ORD of bovine β-lactoglobulin B (1.3 g/l.) in 7 M urea, at pH 5.2 and 25°, with time of reaction. Plot of reduced mean residue rotation ([m'] in cm² dmol⁻¹) *vs.* wavelength at the following times: 3 min (■); 15 min (□); 60 min (▲); 20 hr (●). The upper curve shows the ORD of the native protein at pH 5.2.

reaction in the absence of *N*-ethylmaleimide where increasing irreversibility is shown with increasing reaction time (Figure 3).

Viscosity at pH 5.2. The effects of urea concentration, protein concentration, and time on the viscosity number of β-lactoglobulin in urea were examined. It was found that urea concentrations up to 4 M had little effect on the viscosity number of the B variant (3 ml g^{-1} at concentration of 6 g/l.) at pH 5.2 and 25°. Time-dependent increases in viscosity occurred in 6 and 7 M urea. However, in contrast with the optical rotation the viscosity number did not reach a limiting value even after 48 hr in 7 M urea when it had a value of 17 ml g^{-1} at 6 g/l. The viscosity number and its time rate of change in 7 M urea increased with increasing protein concentration (from 6 to 20 g/l.) from times corresponding to the onset of the secondary optical rotation change. Nevertheless, the concentration dependence of the viscosity number was not large even after 2000 min of reaction, *i.e.*, the degree of aggregation was small. No appreciable concentration dependence was observed in 6 M urea.

Reaction at pH 3.5

Optical Rotation at pH 3.5. There was no change in levorotation for β-lactoglobulins A, B, and C at pH 3.5 for urea concentrations in the range 0–4 M except that the rotation of C underwent a small immediate change in 4 M urea and then remained constant. At higher urea concentrations the changes in rotation were greater and much more rapid than those for the corresponding urea concentration at pH 5.2. A primary change, which was completed within a few minutes (<1 min for 8 M urea), was followed by a slow secondary change over the next 3–5 hr until final values of the rotation were obtained, no further change occurring over the next 2 days. The extent of rotation change for the variants studied was in the order C > B > A. However, the rate of change at a given urea concentration was in the order B > C > A.

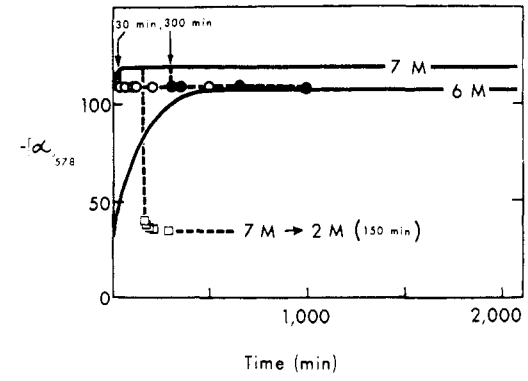


FIGURE 7: Effect of *N*-ethylmaleimide (1.12×10^{-3} M) on the specific rotation change at 578 nm (solid lines) for bovine β-lactoglobulin B (*ca.* 10 g/l.) in urea at pH 5.2, 25°, and the effect of dilution to 6 *M* urea (○) and 2 M urea (□) after various reaction times.

TABLE IV: Values of $[\alpha_f]_{578}$ and $t_{1/2}$ for the Primary Optical Rotation Change at pH 3.5, 25°.

	A		C	
[Urea] (M)	$[\alpha_f]$ (deg)	$t_{1/2}$ (min)	$[\alpha_f]$ (deg)	$t_{1/2}$ (min)
5	−40	15	−69	1.7
6	−98	0.8		

The primary rotation change for the B variant was so rapid at 25° that a kinetic analysis could not be made of it. However, it was possible to determine the values of $[\alpha_f]$ and $t_{1/2}$ for the primary reaction at 25° of the A variant in 5 and 6 M urea and of the C variant in 5 M urea (Table IV). A kinetic analysis was possible for A in 6 M urea and for C in 5 M urea, both of which followed first-order kinetics in the rapid primany reaction at 25°. There was a dramatic change in rate between the rapid primary reaction and the secondary reaction as we have shown previously in plots of log $[\alpha - \alpha_f]$ *vs.* time (Figure 3a of McKenzie and Ralston, 1971). The break in such plots occurred at a point corresponding to a $[\alpha]_{578} = -95°$, a value close to that of −97° for $[\alpha_f]$ determined by the method of Simpson and Kauzmann (1953). The integral first-order plot (over a period of 48 hr) for the secondary reaction was curved.

Reversibility of Rotation Change at pH 3.5. A solution of the B variant (9.2 g/l.) in 7 M urea, pH 3.5, was held at 25° and samples were diluted to 2 M urea with NaCl–HCl buffer (I = 0.1) after reaction times of 10 min, 100 min, and 24 hr (Figure 8). After 10-min exposure to 7 M urea the specific rotation (*ca.* 125°) was completely reversible to that of the native protein at pH 3.5 (*ca.* 28°). Longer exposures to 7 M urea resulted in a reduction in the degree of reversibility, but to a smaller extent than at pH 5.2. After 24 hr a value of −50° was obtained on dilution (*cf.* −62° after dilution from 7 M urea, pH 5.2, 24 hr).

After 30 min in 7 M urea at pH 3.5 a sample of solution was diluted to give a urea concentration of 6 M, causing an immediate change in rotation to −115°, a value similar to that attained finally in 6 M urea.

Effect of Temperature on Rotation Change at pH 3.5. The effect of temperature on the half-time of the primary reaction was determined for the B variant in 5 M urea over the range 3–60° (Table V). A kinetic analysis of the primary rotation change was possible at 3.5, 10, and 60°, first-order kinetics being followed in each case. The extent of the rotation change was a minimum near 35° (*cf.* pH 5.2), $[\alpha]_{578}$ only changing from −28 to −35°. This may be contrasted with the change to −130° at 3.5°, a value attained at 25° only in urea concentrations greater than 7 M.

TABLE V: Effect of Temperature on $[\alpha_f]_{578}$ and $t_{1/2}$ for the Rotation Change of the B Variant in 5 M Urea at pH 3.5.

Temp (°C)	$[\alpha_f]$ (deg)	$t_{1/2}$ (min)
3.5	−130	1.0
10	−115	2.6
25	−44	<1
35	−35	<1
45	−40	<5
60	−81	0.6

The temperature dependence of the rate of rotation change did not parallel the temperature dependence of the extent of the change. At 3.5° the rate was greater than that at 10°, but the rate increased again at temperatures above 10°. The primary reaction was completed within 10 min at 25° and 2 min at 35°. The rate decreased above 35°, the reaction half-time being less than 2 min.

Optical Rotatory Dispersion at pH 3.5. In the wavelength range 313–578 nm the levorotation of β-lactoglobulin increased when the urea concentration was greater than 4 M at pH 3.5. The values of $[\alpha]_{578}$, a_0, and b_0 at the end of the primary reaction for various urea concentrations are shown in Figure 5, for convenience in comparison with values at pH 5.2.

The ORD curves in the wavelength region 215–320 nm for the native A, B, and C variants are generally similar to one another and to the ORD curves at pH 5.2. In 7 and 8 M urea at 27° there was an immediate increase in levorotation for the A, B, and C variants: the final rotations were attained within 1 min of mixing, and there was no further change in rotation with time. The final ORD curves for the three variants were qualitatively similar to one another and to the corresponding curves at pH 5.2 (*cf.* Figure 6). In 5 and 6 M urea at 27° a rapid time-dependent change in rotation was observed, followed by a much slower change for the next 48 hr (typical curves in Figure 9). Although the weak Cotton effects in the 260–300-nm region and the trough near 230 nm disappeared in 7 and 8 M urea, the Cotton effects were still discernible in 5 M urea, even after 48 hr, and the trough became deeper but was no longer bimodal (Figure 9).

It was not practicable to make a kinetic analysis of the initial change with time of the 280–300-nm Cotton effects for the protein in 5 M urea at 25°, but it was possible to do so for the A variant at pH 3.6 and 3.8°. Plots of log $[\alpha - \alpha_f]$ *vs.* time at 260, 285, 290, and 300 nm were nonlinear, indicating that simple first-order kinetics were not being followed. However, the curves for the changes at the several wavelengths were parallel to one another.

The Presence of Stable Intermediates at pH 3.5. An attempt was made to determine if stable intermediates were present immediately after the end of the rapid primary stage of denaturation, prior to the onset of any appreciable interchange reactions. The ORD of the native protein, the fully denatured protein, and the protein partly denatured at an intermediate urea concentration were determined. The intermediate urea concentration was chosen to give a predicted percentage denaturation on the basis of the reduced mean residue rotation at wavelengths above 260 nm as an index of denaturation and assuming no intermediates were present. An ORD curve calculated from these conditions of denaturation was compared with the experimental ORD curve. The A and B variants were studied at 50% denaturation. The curves for the B variant have been presented elsewhere (McKenzie and Ralston, 1971). The curve calculated for 50% denaturation on the basis of rotation at 578 nm was in close agreement with the experimental curve at wavelengths above 260 nm, but below 250 nm the curves deviate markedly from one another. The results for the A variant were similar. In the case of the C variant a urea concentration was chosen to give an estimated 75% denaturation. Again there is a marked discrepancy between the calculated and experimental curves below 250 nm, the deviation being greatest near 230 nm. There is a crossover point at 220 nm (Figure 10).

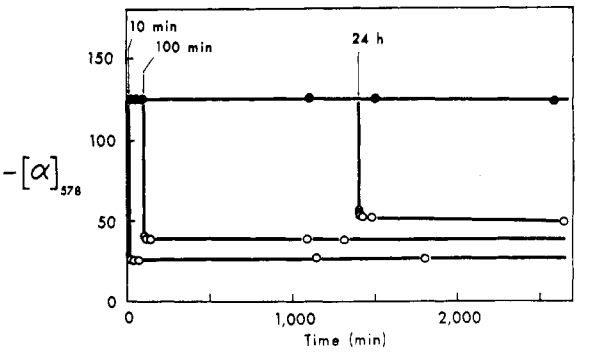


FIGURE 8: The change in specific rotation at 578 nm, $[\alpha]_{578}$, with time following dilution to 2 M in urea of a solution of bovine β-lactoglobulin B (9.2 g/l.) in 7 M urea at pH 3.5 and 25° after reaction times of 10 min, 100 min, and 24 hr.

Viscosity at pH 3.5. The viscosity number of solutions of B variant (8.0–8.9 g/l.) showed an immediate increase in urea solutions above 4 M at pH 3.5. This increase was followed by further slow increases, that were protein concentration dependent and continued long after the optical rotation change had ceased. The rate of change in 7 M urea at 25° for 20.6 g/l. protein concentration was appreciably greater than for one of 8 g/l., the viscosity number increasing from 20 to 27.5 ml/g in 48 hr for the former as compared with 17 to 21 ml/g.

Discussion

Comparison of Reactions at pH 5.2 and 3.5. Near the isoionic point (*ca.* pH 5.2) the optical rotation and viscosity of

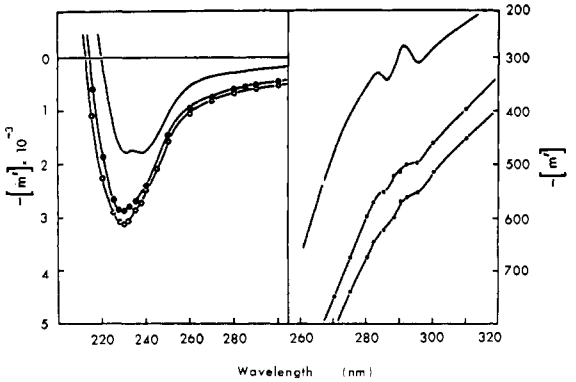


FIGURE 9: The ORD of bovine β-lactoglobulin A (1.1 g/l.) in 5 M urea at pH 3.5, 27°, at the following reaction times: (●) 10 min; (○) 48 hr. The upper curve, without experimental points, is the ORD of the native protein at pH 3.3.

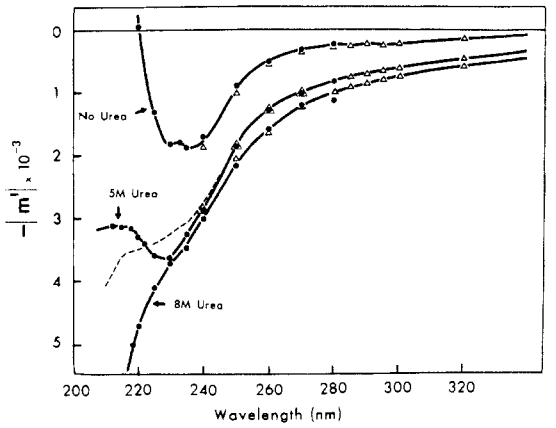


FIGURE 10: The ORD of bovine β-lactoglobulin C (1.2 g/l.) at pH 3.5 and 27° in 0, 5, and 8 M urea. The dashed line represents the curve calculated for 75% denatured protein based on rotation changes above 260 nm for the protein in 0 and 8 M urea: (△) 1-cm cell; (●) 0.1-cm cell.

bovine β-lactoglobulins A, B, and C undergo time-dependent changes in urea (>4 M), and the changes are strongly dependent on urea concentration. In general the optical rotation change does not follow simple first-order kinetics, and this is true even for reaction times of several minutes. It has been possible, under some conditions of urea concentration and temperature, to resolve mathematically the optical rotation change for extended reaction times into a primary change and a secondary change. The primary stage is rapid (*e.g.*, $t_{1/2}$ *ca.* 1–2 min, 7 M urea at 25°) and can be represented by two exponential terms. Thus it consists of (at least) two sequential or parallel reactions (for the significance of the λ parameter of eq 1 in each case, see Frost and Pearson, 1953). The slow secondary stage cannot be represented by a single exponential form. Only under one set of conditions examined at pH 5.2, namely the reaction with 6 M urea at 3.8°, is the rotation change apparent first order, with no secondary change.

The reaction at pH 3.5 has similarities to, and differences from, the reaction at pH 5.2. Again the changes are strongly dependent on urea concentration. However, they occur more readily and the primary changes are more rapid. There is a sharper break between the rates of the primary and secondary change. When the rotation changes at 578 nm are of intermediate magnitude the primary stage can be described by two exponential terms. In urea concentrations causing nearly maximum rotation change at 578 nm, and where the rate is not too fast for conventional kinetic measurements, the primary stage consists of a single first-order step. The primary rotation change at wavelengths between 260 and 320 nm for the A variant in 5 M urea at pH 3.5 and 3.8° does not follow simple first-order kinetics. Similar results to those obtained in urea at pH 3.5 (HCl–NaCl) have been obtained also at pH 3.8 (formate buffer) (Ralston, 1969).

The strong dependence of the denaturation on urea concentration and the occurrence of a temperature of maximum stability to urea have also been observed for other proteins and several explanations of them advanced (see, *e.g.*, Hopkins, 1930; Lauffer, 1943; Simpson and Kauzmann, 1953; Kauzmann, 1954, 1956; Tanford, 1968).

On the basis of the present and other work (pH 3–9) we conclude that the order of thermodynamic stability of variants in urea at pH 3.5 is A > B > C, the kinetic order of stability being A > C > B. At pH >4.5 the thermodynamic and kinetic stabilities are in the order C > B > A. An explanation for this order has been advanced by Ralston (1969).

The Primary Change. In the introductory statement we drew attention to the possible complications in the interpretation of the denaturation (unfolding) reactions that can arise from the occurrence of –SH/–SS– interchange reactions in proteins containing these groups. During the primary stage the optical rotation change for β-lactoglobulin is reversible, but with the onset of the secondary stage the change becomes increasingly irreversible. On the basis of other studies (see below), we conclude that the interchange reactions are not significant during the primary stage. However, they do make an important contribution to the observed behavior of the protein during the slow secondary stage. We shall consider this further below, but first examine the observations during the primary stage in terms of current theories of protein denaturation.

Controversies on the theory of protein denaturation have centered on the two-stage mechanism and reversibility (see, *e.g.*, Neurath *et al.*, 1944). In one theory the two states (native and denatured) are those relevant to the particular type of denaturation under consideration, and its essential features were qualitatively stated with admirable clarity by Anson (1945, p 382, lines 1–11). More sophisticated treatments of the theory have been given by more recent workers (*e.g.*, Lumry *et al.*, 1966; Brandts, 1967, 1969, Tanford, 1968, 1970). In considering two-state theory there have been two main approaches. In the first one the two states are considered to depend not only on the type of denaturation (*e.g.*, thermal denaturation), but also on its particular conditions (*e.g.*, the particular temperature). In applying this criterion to equilibrium properties it usually involves some extrapolation of the properties of the native and denatured stages (*e.g.*, Jackson and Brandts, 1970). In the second one, the two states are considered to depend only on the kind of denaturant (*e.g.*, Tanford, 1968; Tsong *et al.*, 1970).

The kinetic and thermodynamic criteria that can be applied to test the two-state hypothesis have been reviewed elsewhere. We have also pointed out that, while the two-state hypothesis appears to be valid for some proteins under certain conditions, there is a compelling body of evidence that the denaturation of a number of proteins does not meet the two-state criteria (McKenzie and Ralston, 1971).

A necessary, but not sufficient, condition for the two-state theory is that the kinetics of the forward and back reaction be apparent first order. For each of the bovine β-lactoglobulin variants studied in urea, we have shown that the kinetics for primary optical rotation change, both for the forward and back reactions, do not generally meet this criterion. We have not tabulated values of the parameters A and B and λ_1 and λ_2 (eq 1) for the forward reaction because to utilize these further in the elucidation of the reaction mechanism we would require values for the reversal reaction. Unfortunately renaturation reaction is more complex and has a very rapid initial phase. Its elucidation would need the application of rapid reaction methods (see Tsong *et al.*, 1971, for an application that has been made to demonstrate a non-two-state rapid unfolding for ribonuclease A since the present work was carried out).

Another criterion for the two-state process is that the rate of change of different properties of the protein should be parallel to one another. We have shown elsewhere that changes in properties such as optical rotation and ultraviolet absorp-

tion at several wavelengths do not parallel one another for β-lactoglobulin in urea at pH 5.2 even during the primary change (Ralston, 1969; McKenzie and Ralston, 1971).

The presence of stable intermediates in urea at pH 3.5 has been established by studying the ORD of products of denaturation at the end of the primary stage, before there is appreciable formation of irreversible products. The experimentally determined ORD curves for conditions in which it was expected there would be 45 and 75% denaturation on the basis of rotation at 578 nm do not agree with the calculated ORD curves *below* 250 nm. In calculating these curves corrections have been made for solvent refractive index effects, but it has been assumed that the extrapolated hypothetical conformation of the native protein in the intermediate urea concentration (5–5.5 M) is the same as that in the absence of urea. This can be justified for the A and B variants where their reduced mean residue rotations do not change significantly in 0–4 M urea. However, there is some change for the C variant in 4 M urea at pH 3.5; nevertheless the calculated and experimental curves agree above 250 nm, but not below. Several workers (Urnes and Doty, 1961; Bell and McKenzie, 1964; Timasheff *et al.*, 1966; McKenzie, 1967) have concluded from ORD studies that native bovine β-lactoglobulin contains segments of α-helical, β, and disordered chain. The difference between the observed and calculated ORD for the protein at intermediate urea concentrations is compatible with the presence of excess ordered structure in the partially unfolded protein. Under these conditions a particular region of the molecule may unfold before other regions in a sequential type mechanism, although parallel reactions may occur. They may also arise from heterogeneity in the protein. When work described here was carried out it was believed that each genetic variant was homogeneous. Subsequent to its completion it was found that the -SH group and one of the two -SS- bridges in each variant occur at alternate positions in a 1:1 ratio (McKenzie *et al.*, 1972). Part of the complexity in kinetics could arise from this source. However, on the basis of the analysis of ORD curves and work in progress, we are inclined to the view that the main mechanism of the primary stage involves sequential, rather than parallel, reactions.

When *N*-ethylmaleimide is present during the urea denaturation apparent first-order kinetics are obeyed. This arises from readjustment of primary reaction rates in the modified protein (which may no longer be regarded as native protein). There are no secondary changes in rotation for the forward reaction, and reversal is complete. It is important to realize that, while the -SH group is involved in interchange reactions (especially at pH >7), it plays an important role in maintaining the stability of the molecule. The marked loss of stability of the bovine protein when it is blocked by a variety of reagents has been recently demonstrated (Pantaloni, 1962; Roels *et al.*, 1968; Ralston, 1972). Immediate unfolding in concentrated urea also occurs for the A and B variants of porcine β-lactoglobulin which has no -SH group (Bell *et al.*, 1970).

The Secondary Stage. In the early stage of the reaction in 7 M urea the optical rotation change is rapidly and almost completely reversed to that of the native protein on dilution of the urea to 2 M. On dilution of the urea from 7 to 6 M at pH 5.2 the changes are complex: the levorotation decreases very rapidly at first and then more slowly, passing through a minimum, and then gradually merges with the normal 6 M denaturation curve. It is evident that, despite the high levorotation attained early in 7 M urea, the protein is not yet in the same state as is attained in 6 M urea during the secondary stage of the reaction.

The slow secondary rotation change reflects the driving to the right of unfolding reactions by the removal of products in interchange reactions (see Scheme I). During this period the concomitant viscosity increase becomes increasingly concentration dependent. The slow viscosity increase observed in 6 and 7 M urea at pH 3.5 continues long after the optical rotation has reached a final value. We have found that increasing loss of solubility of the protein in 2 M ammonium sulfate with increasing reaction time is also accompanied by a decrease in reversal of the solubility when the urea is diluted with water prior to adding the stopping solution. The latter effect reflects an increase in interchange reactions. It is prevented by addition of *N*-methylmaleimide to the reaction mixture.

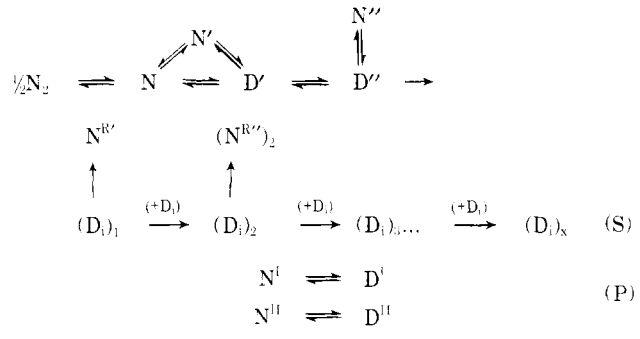
Further evidence for interchange reactions during the secondary stage has come from zone electrophoresis studies. The slow moving bands that are produced increase in number and amount with increasing reaction time and pH over the range 3–9. They are virtually eliminated when *N*-ethylmaleimide is present or when the products are reduced with 2-mercaptoethanol. Following the work of Huggins *et al.* (1951) the -SH/-SS- interchange reaction in proteins is well known in neutral and alkaline solution. It is generally believed that interchange does not take place at low pH, except when strong acid (*e.g.*, 10 M HCl) is present. However, it does take place at pH 3–3.5 for bovine β-lactoglobulins in urea, and it may be a more important factor in other proteins at low pH than is generally realized. The degree of aggregation in urea at pH 3.5 and 5.2 is usually not great as can also be concluded from the present viscosity measurements. We have also shown from chromatographic separation of irreversible products and peptide mapping studies that intra- and intermolecular -SH/-SS- interchange reactions occur, and found evidence that the unfolding proceeds *via* the monomer (Ralston, 1969; McKenzie and Ralston, 1973).

The conclusions that the urea denaturation of β-lactoglobulin is not a two-state process and that there may also be irreversible changes are in agreement with early studies on pooled β-lactoglobulin (Jacobsen and Christensen, 1948; Linderstrøm-Lang, 1949, 1952; Christensen, 1952; Johansen, 1953; and Kauzmann and Simpson, 1953), but are at variance with the findings of Pace and Tanford (1968) who concluded that the urea denaturation of bovine β-lactoglobulin A is two state at pH 2.5–3.5, and that irreversible reactions do not play a role. Similar views have recently been expressed by Alexander and Pace (1971) for urea denaturation of bovine β-lactoglobulins A and B and caprid β-lactoglobulin. Nevertheless they state (p 2741) that it may not be a two-state process under all conditions. Tanford (1970) appears to have modified his views on two-state denaturation of β-lactoglobulin, suggesting that its guanidine hydrochloride denaturation is not a two-state process. Since our work was completed Ikai and Tanford (1971) have proposed a sequential mechanism for cytochrome *c* involving incorrectly folded intermediate states (see also Tsong *et al.*, 1972). Such forms may possibly exist for β-lactoglobulin (McKenzie and Ralston, 1973).

Summary of Overall Mechanism. The behavior of β-lactoglobulin in urea is complex; nevertheless we consider it is possible to summarize broadly the reactions involved as shown in Scheme I.

Schematic S is a sequential reaction mechanism in which the native β-lactoglobulin dimer, N_2, rapidly and reversibly dissociates to monomer, N. When the urea concentration is

SCHEME I



$\geqslant 5$ M dissociation should be virtually complete (*cf.* Pace and Tanford, 1968) especially at pH $\leqslant 3.5$ and $\geqslant 7$. In any case it is not a rate determining step. During the primary stage of the urea denaturation at pH 5.2 and under some conditions at pH 3.5, the protein unfolds sequentially to denatured forms, D′ and D″. At pH >6.5 it may also involve the transition to the conformational isomer, N′ (McKenzie and Ralston[1]). Form D″ may be in equilibrium with a partially, but incorrectly, unfolded form N″. In the later stages of reaction –SH/–SS– interchange becomes appreciable, especially above pH 7: it may be intramolecular giving rise to monomer, $(D_i)_1$, or intermolecular giving dimer, $(D_i)_2$, trimer, $(D_i)_3$, and x-mer, $(D_i)_x$. When urea is removed $(D_i)_1$ may be partially refolded, giving $N^{R'}$, and $(D_i)_2$ may give $N^{R''}$ (see McKenzie and Ralston, 1973).

In the event that there are two forms of the native molecule, or two portions of a single native molecule, unfolding at different reaction rates, parallel unfolding reactions of the type shown in schematic P would have to be included in the reaction mechanism.

References

Alexander, S. S., and Pace, C. N. (1971), *Biochemistry 10*, 2738.

Anson, M. L. (1945), *Advan. Protein Chem. 2*, 361.

Armstrong, J. McD., McKenzie, H. A., and Sawyer, W. H. (1967), *Biochim. Biophys. Acta 147*, 60.

Bates, R. G. (1964), "Determination of pH in Theory and Practice," New York, N. Y., Wiley.

Bell, K. (1967), *Biochim. Biophys. Acta 147*, 100.

Bell, K., and McKenzie, H. A. (1964), *Nature (London) 204*, 1275.

Bell, K., and McKenzie, H. A. (1967), *Biochim. Biophys. Acta 147*, 109.

Bell, K., McKenzie, H. A., and Ralston, G. B. (1970), *Proc. Aust. Biochem. Soc. 3*, 82.

Brandts, J. F. (1967), *in* "Thermobiology," Rose, A. H., Ed., London, Academic Press, p 25.

Brandts, J. F. (1969), *in* "Structure and Stability of Biological Macromolecules," Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 213.

Christensen, L. K. (1952), *C. R. Trav. Lab. Carlsberg 28*, 37.

Fasman, G. (1963), *Methods Enzymol. 6*, 928.

Ferguson, K. A., and Wallace, A. C. C. (1963), *Rec. Prog. Hormone Res. 19*, 1.

Frank, G., and Braunitzer, G. (1967), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1456.

Frost, A. A., and Pearson, R. G. (1953), "Kinetics and Mechanisms," New York, N. Y., Wiley.

Guggenheim, E. A. (1926), *Phil. Mag. 2*, 538.

Hopkins, F. G. (1930), *Nature (London) 26*, 328, 383.

Huggins, C., Tapley, D. G., and Jensen, E. V. (1951), *Nature (London) 167*, 592.

Ikai, A., and Tanford, C. (1971), *Nature (London) 230*, 100.

Jackson, W. M., and Brandts, J. F. (1970), *Biochemistry 9*, 2294.

Jacobsen, C. F., and Christensen, K. L. (1948), *Nature (London) 161*, 30.

Johansen, G. (1953), *C. R. Trav. Lab. Carlsberg 28*, 335.

Kauzmann, W. (1954), *in* "The Mechanism of Enzyme Action," McElroy, W. D., and Glass, B., Ed., Baltimore, Md., Johns Hopkins, p 70.

Kauzmann, W. (1956), *J. Cell. Comp. Physiol. Suppl. 47*, 113.

Kauzmann, W., and Simpson, R. B. (1953), *J. Amer. Chem. Soc. 75*, 5154.

Lauffer, M. (1943), *J. Amer. Chem. Soc. 65*, 1793.

Linderstrom-Lang, K. (1949), *Cold Spring Harbor Symp. Quant. Biol. 14*, 117.

Linderstrøm-Lang, K. (1952), *Stanford Univ. Publ., Univ. Ser., Med. Sci. 6*, 1.

Lumry, R., Biltonen, R., and Brandts, J. F. (1966), *Biopolymers 4*, 917.

McKenzie, H. A. (1967), *Advan. Protein Chem. 22*, 55.

McKenzie, H. A. (1970), *in* "Milk Proteins," McKenzie, H. A., Ed., Vol. I, New York, N. Y., Academic Press, p 418.

McKenzie, H. A. (1971), *in* "Milk Proteins," McKenzie, H. A., Ed., Vol. II, New York, N. Y., Academic Press, p 257.

McKenzie, H. A., and Murphy, W. H. (1970), *in* "Milk Proteins," McKenzie, H. A., Ed., Vol. I, New York, N. Y., Academic Press, p 127.

McKenzie, H. A., and Ralston, G. B. (1971), *Experientia 27*, 617.

McKenzie, H. A., and Ralston, G. B. (1973), *Aust. J. Biol. Sci. 26* (in press).

McKenzie, H. A., Ralston, G. B., and Shaw, D. C. (1972), *Biochemistry 11*, 4539.

Moffitt, W., and Yang, J. T. (1956), *Proc. Nat. Acad. Sci. U. S. 42*, 596.

Neurath, H., Greenstein, J. P., Putnam, F. W., and Erickson, J. O. (1944), *Chem. Rev. 34*, 157.

Pace, N. C., and Tanford, C. (1968), *Biochemistry 7*, 198.

Pantaloni, D. (1962), *C. R. Acad. Sci. 254*, 1884.

Partington, J. R. (1953), *in* "An Advanced Treatise on Physical Chemistry," Vol. IV, London, Longmans Green, p 72.

Ralston, G. B. (1969), Ph.D. Thesis, Australian National University, Canberra, A.C.T.

Ralston, G. B. (1972), *C. R. Trav. Lab. Carlsberg 38*, 499.

Roels, H., Préaux, G., and Lontie, R. (1968), *Arch. Int. Physiol. Biochim. 76*, 198, 200.

Simpson, R. B., and Kauzmann, W. (1953), *J. Amer. Chem. Soc. 75*, 5139.

Tanford, C. (1968), *Advan. Protein Chem. 23*, 121.

Tanford, C. (1970), *Advan. Protein Chem. 24*, 1.

Timasheff, S. N., Townend, R., and Mescanti, L. (1966), *J. Biol. Chem. 241*, 1863.

Tsong, T. Y., Baldwin, R. L., and Elson, E. L. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2712.

Tsong, T. Y., Baldwin, R. L., and McPhie, P. (1972), *J. Mol. Biol. 63*, 453.

Tsong, T. Y., Hearn, R. P., Wrathall, D. P., and Sturtevant, J. M. (1970), *Biochemistry 9*, 2666.

Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.

Werner, E. A. (1913), *J. Chem. Soc. 103*, 1010.