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Studies of the Denaturation and Partial Renaturation of Ovalbumin

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1. The denaturation of ovalbumin by the reagents sodium dodecyl sulphate and guanidinium chloride was investigated, by following the changes in sedimentation velocity, optical rotatory dispersion and viscosity as a function of denaturant concentration. 2. With sodium dodecyl sulphate both the optical-rotatory-dispersion parameters a_0 and b_0 become more negative, the sedimentation coefficient decreases and the viscosity increases; significant differences in the denaturation profiles are observed. The change in each parameter is indicative of only limited denaturation. 3. With guanidinium chloride the transition occurs over the concentration range 1–4M: more extensive changes occur in all the physical parameters than with sodium dodecyl sulphate. The values of a_0 and b_0 are indicative of complete denaturation. Reduction by mercaptoethanol produces only minor further changes. 4. Renaturation was attempted from both denaturants, the removal of reagent being accomplished reversibly by controlled slow dialysis. Partial renaturation was observed, but aggregated or insoluble material was produced in both cases at relatively low concentrations of denaturant. Similar behaviour was observed with fully reduced protein in guanidinium chloride–mercaptoethanol; complete renaturation could not be brought about even at very low protein concentrations.

The concept that the amino acid sequence of a protein determines its conformation (Lumry & Eyring, 1954; Crick, 1958) is now fully accepted, but the subsidiary question of whether the conformation represents an unqualified free-energy minimum is less certain (cf. Tanford, 1968, 1970). On the one hand, the many successful reversible denaturation experiments, notably those of Anfinsen and his associates on ribonuclease (see, e.g., Anfinsen, 1967) lend powerful support to the proposition; on the other, difficulties and very variable yields found in renaturing several enzymes (see, e.g., Ullmann & Monod, 1969; Cook & Koshland, 1969; Teipel & Koshland, 1971*a*) suggest that kinetic or other extrathermodynamic factors may be critically involved, at least in these cases. Although a number of larger proteins have been successfully renatured under conditions that do not indicate any specific pathways of folding (see, e.g., de Crombrugghe & Edelhoch, 1966; Andersson, 1969; Kohn, 1970), there is no doubt that the evidence in favour of the proposition is strongest for proteins of molecular weight under 20000. In these circumstances it appeared that a study of a protein of intermediate molecular weight would be of interest, and accordingly the globular protein ovalbumin, which has a single polypeptide chain of molecular weight 45000, was selected. The denaturants used in this study were sodium dodecyl sulphate and guanidinium chloride. Sodium dodecyl sulphate produces limited unfolding, and in excess induces an extended conformation (Reynolds & Tanford, 1970*a*); guanidinium chloride, at much higher concentrations, destroys all detectable non-covalent structure (Tanford, 1968).

Studies of the denaturation of ovalbumin by urea have been reported by Simpson & Kauzmann (1953), Steven & Tristram (1959) and Gagen & Holme (1964); Simpson & Kauzmann (1953) and Imahori (1960) refer to the irreversibility of the denaturation. The interaction between ovalbumin and sodium dodecyl sulphate has been investigated by several workers; earlier studies are summarized by Ray (1968). Schellman *et al.* (1953) studied the kinetics of denaturation of ovalbumin by guanidinium chloride, and several reports have appeared quoting the values of various physico-chemical parameters for ovalbumin in this medium (see below); however, a systematic study of the system has been lacking.

Materials and Methods

Crystallized ovalbumin (lot nos. LB0751, LK1650 and ME1751; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) was used. It gave single symmetrical boundaries in the ultracentrifuge, but sedimentation-equilibrium experiments and gel chromatography on Bio-Gel P150 (BioRad Laboratories, Richmond, Calif., U.S.A.) indicated the presence of a small amount of high-molecular-weight material. Results given apply to the unfractionated protein unless otherwise specified. Both sodium dodecyl sulphate and guanidinium chloride were obtained as

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specially purified reagents from BDH Chemicals Ltd. (Poole, Dorset, U.K.). The sodium dodecyl sulphate, when dissolved in buffer at I 0.1, was shown to pass freely across a dialysis membrane. The buffer used throughout was composed of (final concentrations) 16.5mM-Na_2HPO_4, 16.5mM-NaH_2PO_4 and 33mM-NaCl to give pH6.8 and I0.10. Addition of guanidinium chloride to a concentration of 6M lowered the pH to 5.3, and such solutions were used in the determination of the denaturation profiles; all renaturation experiments, however, were performed after restoration of the pH to its initial value by the addition of 0.1M-NaOH. This did not change the guanidinium chloride concentration significantly. Dialysis tubing (Visking 18/32in) was pretreated by soaking it in a concentrated solution of sodium dodecyl sulphate for 24h (for use with sodium dodecyl sulphate) or by soaking in hot 0.5M-acetic acid for 30min (for use with guanidinium chloride), followed in both cases by extensive washing with water.

Protein concentrations were determined from extinction measurements by using the coefficients $E_{280} = 0.737$ litre·g^{-1}·cm^{-1} (native and sodium dodecyl sulphate-denatured protein) and $E_{280} = 0.754$ litre·g^{-1}·cm^{-1} (protein in 6M-guanidinium chloride). These were the values found for dialysed protein solutions whose concentrations had been defined by refractometric measurements; the value $R = 1.850 \times 10^{-4}$ litre/g was used for the specific refractive increment, R, at 546nm. Measurements were made with a differential refractometer (Polymer Consultants Ltd., Nottingham, U.K.) modified to give a Rayleigh interference pattern in the image plane.

Detergent concentrations were determined, in the absence of protein, by the colorimetric method of Graham & Whitney (1959). For solutions containing protein the total refractive increment was first measured, relative to buffer. The contribution of protein to this quantity was calculated from the protein concentration determined from an extinction measurement; subtraction then gave the contribution of sodium dodecyl sulphate, which was converted into the concentration by use of the value $R = 1.21 \times 10^{-4}$ litre/g for the specific refractive increment of sodium dodecyl sulphate (Anacker *et al.*, 1964).

Concentrations of guanidinium chloride were determined refractometrically after dilution to approx. 10mg/ml. The value $R = 1.71 \times 10^{-4}$ litre/g was used for the specific refractive increment (Kielley & Harrington, 1960).

Viscosities were measured in extended-type Ostwald viscometers (Creeth & Knight, 1967) having water flow times of 70–150s at 25±0.005°C; all observations were made at this temperature. The flow times were determined with the aid of an autoviscometer (Hewlett-Packard Ltd., Slough, Bucks., U.K.) embodying photoelectric detection and electronic timing systems.

Sedimentation-velocity and sedimentation-equilibrium experiments were carried out in a Beckman-Spinco model E ultracentrifuge, standard procedures (e.g. Creeth, 1964; Creeth & Knight, 1967) being followed. The sedimentation coefficients of native and sodium dodecyl sulphate-denatured ovalbumin were determined in single-sector Dural or Kel-F cells at 25°C and 59780rev./min. For solutions containing guanidinium chloride double-sector cells were used to enable a correction to be made for the influence of the strongly curved base-line on the position of the peak. Sedimentation-equilibrium experiments were carried out on dialysed solutions, generally at 25°C, in 12mm or 30mm double-sector cells with filled-Epon centre-pieces and sapphire windows. Cells were filled with a micrometer syringe to give column heights close to 3mm; in the absence of guanidinium chloride fluorocarbon oil FC43 was used to provide a transparent cell-bottom. A blank run was always carried out. The speed was chosen to yield a meniscus concentration of about 0.5 fringe (moderately high-speed equilibrium) or about 5 fringes (low-speed equilibrium). The time required for the attainment of equilibrium was calculated by the method of Van Holde & Baldwin (1958). In experiments that would otherwise have been inconveniently prolonged the overspeeding procedure of Hexner *et al.* (1961) was employed, with the restriction that the higher speed was not greater than 1.5 times the operating speed. The attainment of equilibrium was always confirmed directly. All calculations referring to ultracentrifuge determinations were programmed for automatic processing by a desk-top computer (Olivetti Programma 101).

The value used for the partial specific volume of native ovalbumin was $\bar{v} = 0.746$ml/g (Charlwood, 1957); values for protein–sodium dodecyl sulphate complexes were calculated on the basis of the weight composition (Schachman, 1957). The value $\bar{v} = 0.865$ml/g was used for the partial specific volume of sodium dodecyl sulphate (Anacker *et al.*, 1964). In the presence of guanidinium chloride as a third component the quantity $(1-\bar{v}\rho)$ necessary for the evaluation of molecular weights from sedimentation-equilibrium experiments was replaced by $(1-\phi'\rho^\circ)$, where ϕ' is the apparent specific volume and ρ° the solvent density (Casassa & Eisenberg, 1964). The value $\phi' = 0.738$ml/g was used, by analogy with the behaviour of other proteins for which accurate comparison of ϕ' and $\bar{v}$ (native) has been made (Kielley & Harrington, 1960; Marler *et al.*, 1964; Reisler & Eisenberg, 1969). In correcting sedimentation coefficients measured in 6M-guanidinium chloride to standard conditions $\bar{v}$ was replaced by ϕ'. When experiments were done at temperatures below 25°C values of $\bar{v}$ and ϕ' were calculated assuming a

decrease of 0.3 μl/g per degree, as found for a number of other proteins (Svedberg & Pedersen, 1940; Taylor & Lowry, 1956; Hunter, 1967).

O.r.d.* measurements were made at six wavelengths in the range 365–578nm with a spectropolarimeter constructed to the design of Malcolm & Elliott (1957). The observed optical-rotation values were converted into mean residue rotations by using a mean residue weight $M_0 = 112$ and dispersion values for water obtained from *International Critical Tables*; for solutions containing guanidinium chloride the latter values were modified as suggested by Tanford *et al.* (1966). The results were plotted according to the Moffitt & Yang (1956) equation, with $\lambda_0 =$ 212nm; values of a_0 and b_0 were obtained as the intercept and slope respectively of the straight lines that were always found.

Denaturation with sodium dodecyl sulphate was carried out by dialysing the protein solution against a large excess of sodium dodecyl sulphate; typical concentrations were 10mg/ml for protein and 5mg/ml for sodium dodecyl sulphate. The disulphide bonds were not reduced. In the absence of protein the concentration of sodium dodecyl sulphate inside the dialysis sac rose rapidly (2h) to 1.1mg/ml, but increased thereafter only very slowly (1.5mg/ml after 40h). The critical micelle concentration of sodium dodecyl sulphate in 0.10M-NaCl is about 0.5mg/ml at 25°C (Anacker *et al.*, 1964; Emerson & Holtzer, 1967); it is recognized that transfer of detergent across a dialysis membrane does occur above the critical micelle concentration (Harrap & O'Donnell, 1954; Mysels *et al.*, 1963), but that this is a slow process in the presence of excess of salt. Thus the value 1.1mg/ml may include a proportion due to micelles, although no micellar component could be detected by sedimentation-velocity analysis. Similarly when protein-containing sacs were dialysed against sodium dodecyl sulphate at 5mg/ml the sodium dodecyl sulphate concentration within the sac rose rapidly to values much greater than 1.1mg/ml, but no micellar component could be detected. The failure to detect micelles probably arises from the relative insensitivity of the optical system.

By comparison of the total refractive increment of a protein–sodium dodecyl sulphate mixture with that of the component that migrates in a sedimentation-velocity experiment, it is possible (by successive approximation) to determine the free uncombined sodium dodecyl sulphate whether this is in monomer or micellar form. The extent of interaction between the protein and sodium dodecyl sulphate may then be expressed in terms of a binding ratio, denoted r, defined as the number of mol of sodium dodecyl sulphate bound/mol of protein. With the one exception noted, this procedure was followed to obtain the values of r quoted in the Results section. At higher values of r it was found that the concentration of free sodium dodecyl sulphate was always in the region of 1mg/ml, in agreement with the behaviour of protein-free dialysis systems.

Denaturation with guanidinium chloride was done either by mixing the protein with a concentrated guanidinium chloride solution or by dialysis. When a concentration of guanidinium chloride between 0 and 6M was obtained by mixing, a stock solution (7.4M) was added slowly, with continuous stirring, to the protein solution. All guanidinium chloride solutions were made up in the phosphate–chloride buffer.

The renaturation procedure was applied to protein initially in dialysis equilibrium with an appropriately high concentration of denaturant. Pure buffer was then added to the continuously stirred outer solution while the total volume was kept constant by means of an overflow. The denaturant concentration in the outer solution was thus lowered in a slow and continuous manner and, since both denaturants passed freely across the membrane, removal of denaturant from the protein proceeded in a similar way. Typical conditions were: protein, 20ml; outer solution, 2 litres; buffer flow rate, 20ml/h. Removal of sodium dodecyl sulphate to a concentration where $r = 20$, or of guanidinium chloride to a concentration of 2M, then required about 8 days. Experiments were done at a protein concentration of 10mg/ml and 21°C, unless otherwise noted.

Results

Denaturation with sodium dodecyl sulphate

After prolonged dialysis (8 days) an equilibrium value, $r = 175$mol of sodium dodecyl sulphate/mol of ovalbumin ($\equiv 1.1$ g/g), was reached. This is appreciably higher than the value of 0.9g/g found for the non-reduced protein by Pitt-Rivers & Impiombato (1968), presumably as a result of the higher concentration of sodium dodecyl sulphate used in the present work. The denaturation profiles, i.e. the changes in the o.r.d. parameters, the sedimentation coefficient and the reduced viscosity that accompanied binding of sodium dodecyl sulphate are shown in Fig. 1(*a*)–(*c*). It is noteworthy that the change in a_0 is virtually complete at $r \sim 20$, at which point s, $\eta_{red.}$ and, less significantly, b_0 are almost unchanged. Similar values were obtained when denaturation was done by mixing instead of dialysis.

In these experiments the proportion of free sodium dodecyl sulphate was not determined, and the binding is expressed accordingly in terms of the ratio of the total sodium dodecyl sulphate inside the sac to the protein, and denoted r_t. A measurable difference between r and r_t occurs only at higher values of r, and

*Abbreviation: o.r.d., optical rotatory dispersion.

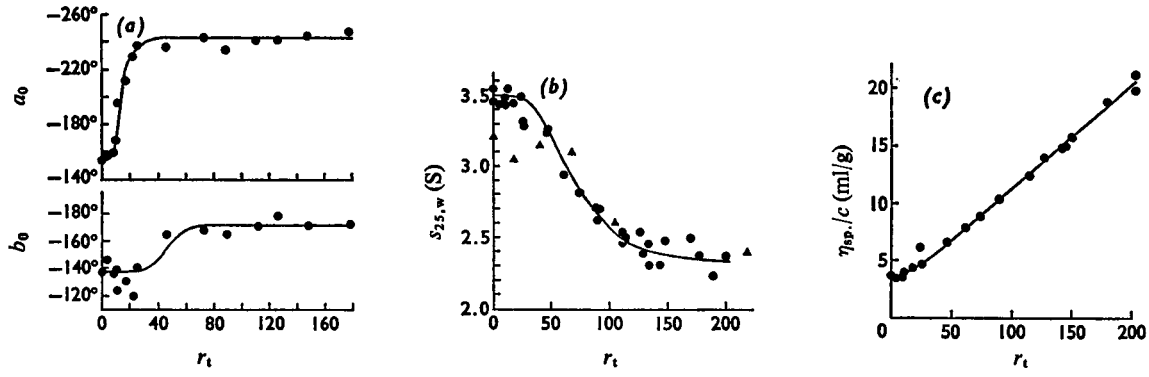

Fig. 1. *Denaturation profiles for the system ovalbumin–sodium dodecyl sulphate*

(*a*) O.r.d. parameters, a_0 and b_0. (*b*) Sedimentation coefficient, $s_{25,w}$, at a protein concentration of 10mg/ml: ●, present work; ▲, results obtained by Aoki & Hori (1959). (*c*) Reduced viscosity, ($\eta_{sp.}/c$), at a protein concentration of 10mg/ml. The abscissa in each case is the molar ratio, r_t, of total sodium dodecyl sulphate (free and bound) to protein.

neither the shape nor the significance of the curves given in Fig. 1 is affected.

At values of r below 50 about 30% of the protein migrated in sedimentation-velocity experiments as a component faster than the main peak. This behaviour is shown in Fig. 2, together with a result for a higher value of r, where the faster-migrating material is no longer evident. After resolution of the peaks the sedimentation coefficients found were 3.3 and 4.4S. These values are consistent with those expected for the end-to-end dimerization of molecules of essentially native conformation (see, e.g., Creeth & Nichol, 1960). Only non-covalent interactions can be involved in the aggregation, because additional detergent dissociated the complex. A similar aggregation, although at pH5.4 rather than 6.8, was found by Ray (1968). It cannot arise from contamination by metal ions from the ultracentrifuge cells (cf. Gordon & Ottesen, 1961), as Kel-F fluorocarbon polymer centre-sections were used.

The concentration-dependence of the sedimentation coefficient was determined for the complex with $r = 130$. The stock solution, of concentration 14mg of protein/ml, was found to contain 1.0mg of free sodium dodecyl sulphate/ml; dilutions were therefore made with buffer containing this concentration of sodium dodecyl sulphate. The results are shown in Fig. 3, together with previously reported values for the native protein (Miller & Golder, 1952; Creeth & Winzor, 1962). The lines are described by the equations:

$s_{25,w} = 3.87(1-0.0062c)$ S (native, results of Miller & Golder, 1952).

$s_{25,w} = 3.50(1-0.022c)$ S (denatured).

For both equations $2<c<13$mg/ml. The roughly threefold increase in the concentration-dependence coefficient is similar to that observed with other denatured proteins (Creeth & Knight, 1965).

Denaturation with guanidinium chloride

Non-reduced protein. The extent of denaturation with increasing concentration of guanidinium chloride was followed primarily by observation of the o.r.d. parameters a_0 and b_0. The results are shown in Fig. 4; in the plateau region at guanidinium chloride concentrations above 4.5M the values of a_0 and b_0 are −544° and +19° respectively. Bemis *et al.* (1966) have reported $a_0 = -585°$ and $b_0 = +55°$ for ovalbumin in 8.6M-urea. Slight turbidity was noted in protein solutions containing 1–2M-guanidinium chloride after they had been left overnight, but solutions at higher guanidinium chloride concentrations were apparently stable. It was found nevertheless, from molecular-weight measurements, that aggregation tended to occur to variable extents, and was not eliminated by iodoacetamide in 25 molar excess relative to the protein thiol concentration.

The viscosity measurements were made on samples containing the least proportion of aggregate: the results are shown in Fig. 5 (curve *A*). The weight-average molecular-weight values for these samples were in the range 49000–51000, compared with 45000 for the native protein, implying a probable maximum of 10% of dimer. The value of the intrinsic viscosity found, 31.6ml/g, must accordingly be regarded as a slight overestimate.

Sedimentation-velocity experiments are necessarily very prolonged in the presence of guanidinium

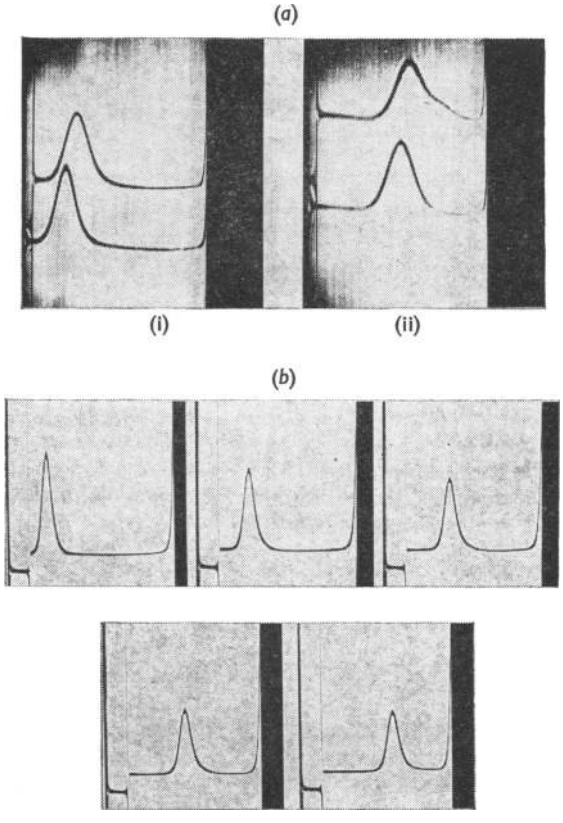

Fig. 2. *Sedimentation-velocity patterns for ovalbumin and sodium dodecyl sulphate-denatured ovalbumin*

(*a*) Native protein (lower trace) and sodium dodecyl sulphate-denatured protein (upper trace), $r = 25$: frame (i) after 57min, frame (ii) after 137min at 59780rev./min; 12mm cells. (*b*) Sodium dodecyl sulphate-denatured protein, $r = 140$: frames at 32min intervals beginning 42min after operating speed (59780rev./min) was reached; 6mm cells. The protein concentration was 10mg/ml in all cases.

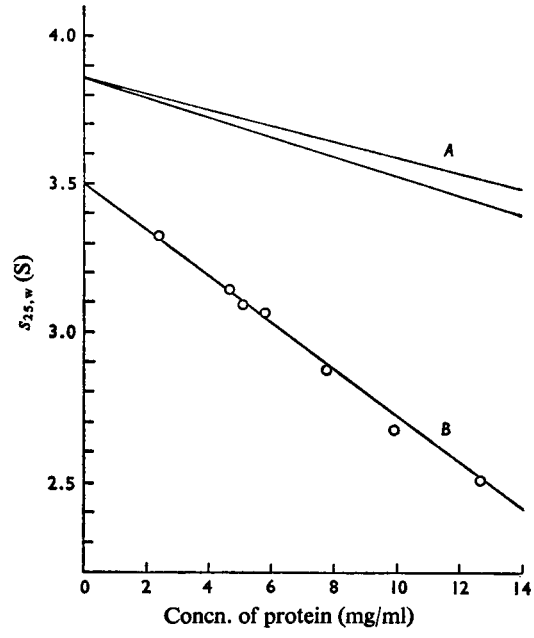

Fig. 3. *Concentration-dependence of sedimentation coefficients of native and sodium dodecyl sulphate-denatured ovalbumin*

Curve *A*, native ovalbumin: lines drawn from results of Creeth & Winzor (1962) (upper line) and Miller & Golder (1952) (lower line). Curve *B*, sodium dodecyl sulphate-denatured ovalbumin (see the text).

chloride (the uncorrected s values are in the range 0.5–1.0S), and only two values were obtained on solutions containing less than 10% of dimer. These values are included in Fig. 6, which refers otherwise to reduced protein (see below).

Reduced protein. Ovalbumin contains one disulphide bond/molecule (Fothergill & Fothergill, 1970); this value is to be preferred over the value of two found polarographically by Winzor & Creeth (1962). In the presence of 0.2M-mercaptoethanol and 6M-guanidinium chloride protein disulphide bonds are broken (Tanford *et al.*, 1967), and many of the problems encountered with non-reduced proteins do not arise. Accordingly, measurements of the three physicochemical quantities were made in this medium: the values are more reliable than those referring to non-reduced protein.

The o.r.d. parameters were essentially unchanged: $a_0 = -540°$, $b_0 = +33°$. Viscosity measurements were made over a range of concentrations, giving the results shown in Fig. 5 (curve *B*). It is evident that the intrinsic viscosity (34.5ml/g) is slightly, but significantly, higher than the value for the non-reduced protein.

Sedimentation coefficients were also determined over a range of concentrations, giving the results shown in Fig. 6. They may be represented equally well by linear plots of s versus c or of $1/s$ versus c (cf. Tanford *et al.*, 1967), the two lines shown being defined by the equations:

$$s_{25,w} = 2.01\ (1-0.034c)\ \text{S}$$
$$s_{25,w} = 2.35/(1+0.071c)\ \text{S}$$

For both equations $4 < c < 12$mg/ml. The points for non-reduced protein shown on this graph indicate that the sedimentation coefficient is appreciably lower

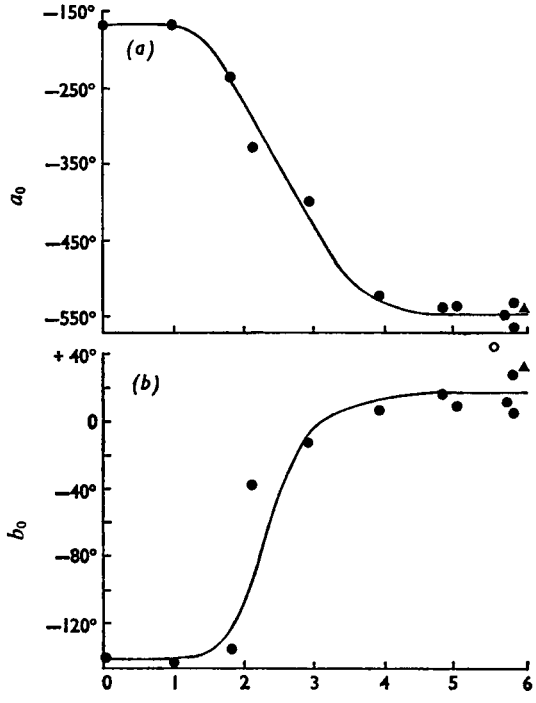

Fig. 4. *Denaturation profiles for the system ovalbumin–guanidinium chloride in terms of the o.r.d. parameters a_0 and b_0*

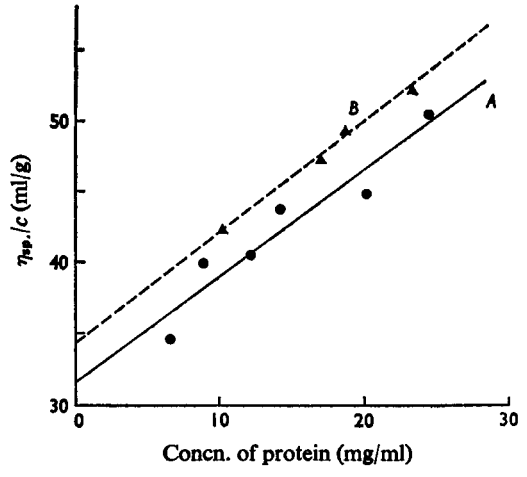

Fig. 5. *Reduced-viscosity plots for ovalbumin in* 6M-*guanidinium chloride*

Curve *A*, non-reduced protein (containing also 50mM-iodoacetamide) (●). Curve *B*, reduced protein (0.1M-mercaptoethanol) (▲). The intercepts on the ordinate axis represent the intrinsic viscosities, the values being 31.6ml/g (curve *A*) and 34.5ml/g (curve *B*).

in the fully reduced state, as would be expected from the correspondingly higher intrinsic viscosity.

Attempted renaturation from sodium dodecyl sulphate

Control experiments showed that sodium dodecyl sulphate was not removed from within a dialysis sac unless the concentration in the outer solution was below a critical value of about 1mg/ml. Therefore, after denaturation, the outer solution was immediately made 1.1mg/ml with respect to sodium dodecyl sulphate, and buffer flow was begun in the manner described above. Typical values of the sedimentation coefficient and the reduced viscosity, which were recorded as the molar ratio of sodium dodecyl sulphate to protein was decreased from $r\sim130$ (fully denatured) to $r\sim25$, are shown in Fig. 7; of the o.r.d. parameters a_0 remained essentially unchanged whereas b_0 moved slightly in the direction indicative of renaturation. The properties of the species existing at $r\sim25$ (referred to as 'partially renatured') are given in Table 1. It should be noted that the value of M: expected for a complex containing 25 detergent ions is 51200; the difference from the value observed probably indicates the presence of approx. 10% of dimer as before.

There is therefore no doubt that a considerable restoration of the native properties has occurred, particularly evident in the viscosity: the species characterized by $r\sim25$ is stable, and must be relatively compact and globular. For values of r less than 25 sedimentation-velocity measurements indicated that aggregation had occurred: the protein was characterized by values of $a_0 = -260°$, $b_0 = -100°$ and $s_{25,w}$ between 4 and 8S. Polydispersity was also indicated by the rapidly spreading boundary in the sedimentation-velocity experiments (Fig. 8).

Most renaturation experiments were conducted at a protein concentration of 10mg/ml, but identical behaviour was repeatedly observed at 2mg/ml, the lower concentration giving no less extensive aggregation. It was not possible to work at concentrations of less than 2mg/ml with these methods of observation. The aggregate could be completely dispersed by treatment with sodium dodecyl sulphate, when the sedimentation coefficient returned to the value expected for a monomeric fully sodium dodecyl sulphate-denatured species; it follows that the aggregation must be entirely non-covalent in origin. Thus, although the precautions taken to prevent irreversible aggregation through covalent-bond formation were adequate, and the renaturation process was conducted as nearly reversibly as possible, non-

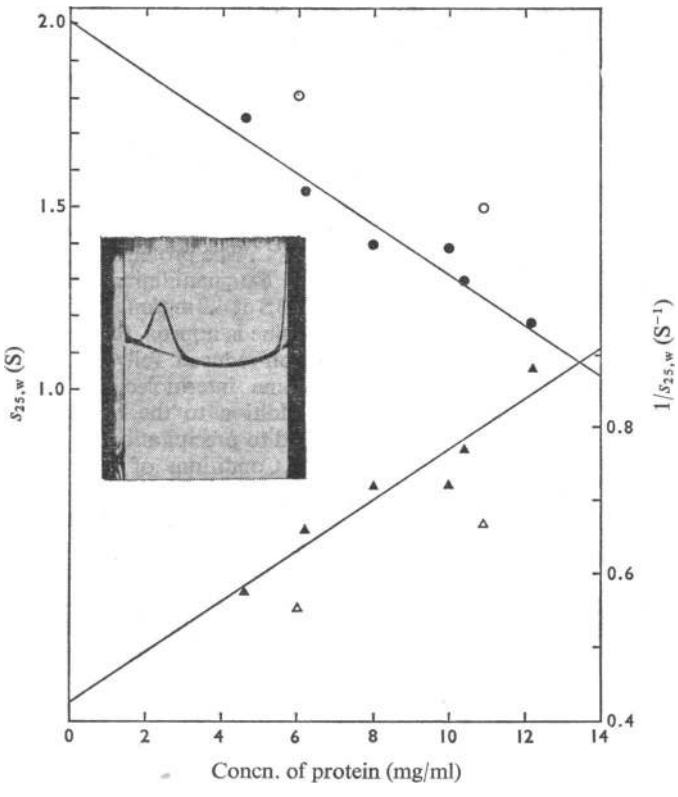

Fig. 6. *Concentration-dependence of sedimentation coefficients of reduced ovalbumin in* 6M-*guanidinium chloride containing* 0.2M-*mercaptoethanol*

●—● and ▲—▲, Reduced ovalbumin; ○ and △, non-reduced ovalbumin (see the text); ● and ○, $s_{25,w}$; ▲ and △, $1/s_{25,w}$. Inset: typical sedimentation velocity pattern, obtained after 5.5h at 59780rev./min; the protein concentration was 6.2mg/ml.

covalent aggregation was not prevented. The temperature only remains as a variable: hydrophobic bonds are less stable at low temperatures (Nemethy & Scheraga, 1962; Bethune, 1965), so that the possibility of their breakage and reformation should be enhanced by lowering the temperature. Accordingly a renaturation experiment was conducted in the same manner as before, but the temperature was lowered in parallel with the sodium dodecyl sulphate concentration (for reasons of solubility) from 21° to 4°C. Aggregation was observed in this experiment, however, to the same extent as before. It was therefore concluded that full renaturation from the sodium dodecyl sulphate-denatured state was not possible in the concentration range 2–10mg/ml; further decrease of the protein concentration to the value found essential in the renaturation of enzymes from urea solution was not considered likely to succeed, in view of the large association constant for the binding of sodium dodecyl sulphate to proteins. It therefore appeared that the guanidinium chloride medium (where the association constant is low) offered greater promise.

Attempted renaturation from guanidinium chloride

Guanidinium chloride exchanges very readily across a dialysis membrane, and accordingly the renaturation process was commenced by the slow dilution of the outer solution in the dialysis system from its original value of 6M. Samples of the protein solution were taken and characterized, as before, as the guanidinium chloride concentration decreased. The results varied only trivially between successive experiments: a typical series of results is given in Table 2. The sedimentation coefficient and the o.r.d.

parameters show some trend towards recovery of the values characteristic of the native state as the guanidinium chloride concentration decreases below 4M; however, further decrease in the guanidinium chloride concentration is accompanied by unmistakable evidence of aggregation and the formation of insoluble products. The sample in equilibrium with 2.4M-guanidinium chloride, in the example quoted, was insoluble at 21°C, the temperature of the renaturation experiment, but dissolved at 4°C, suggesting that the lower temperature might be advantageous. An experiment conducted at 4°C throughout gave only the minor improvement of reaching 2.0M-guanidinium chloride before precipitation of the protein occurred, rather than the value of 2.4M at 21°C. The protein thus precipitated was fully soluble in 6M-guanidinium chloride, in which it had $s_{25,w}$ 2.3S at 4.9mg/ml. Reference to Fig. 6 shows that this value is approx. 0.5S greater than that expected for non-reduced fully unfolded ovalbumin, indicating some intermolecular covalent-bond formation in addition to the hydrophobic-bond formation that led to precipitation.

Conditions of lower protein concentration were therefore investigated; additionally, the disulphide bonds were reduced, as this was the only way their status could be defined unambiguously. The renaturation was attempted with chromatographically purified protein at a concentration of 0.2mg/ml, in the presence of 10mM-EDTA and 15mM-2-mercaptoethanol with continuous deoxygenation, at 4°C. In this

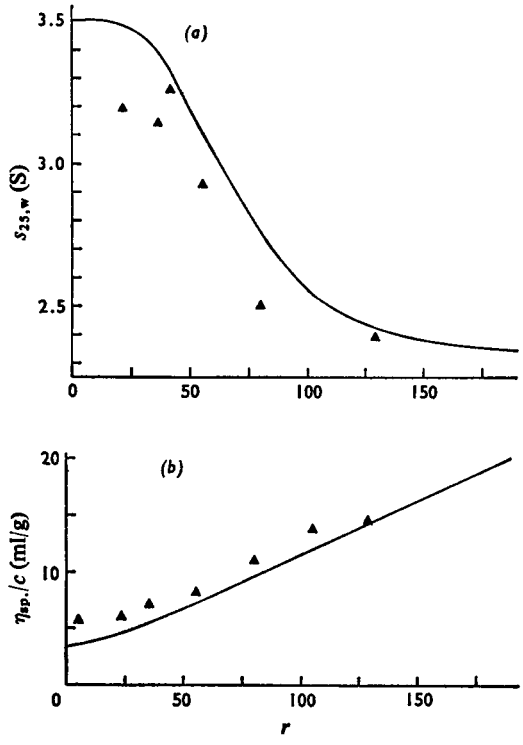

Fig. 7. *Renaturation profiles of sodium dodecyl sulphate-denatured ovalbumin*

(*a*) Sedimentation coefficient. (*b*) Reduced viscosity. The smooth curves represent the denaturation profiles (i.e., obtained with r increasing from initial value of zero), whereas the points were obtained during renaturation (r decreasing from initial value of 130).

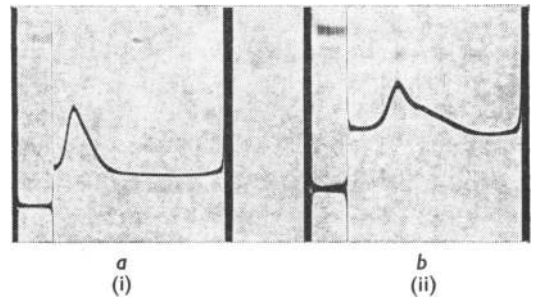

Fig. 8. *Sedimentation-velocity patterns for ovalbumin partially renatured from sodium dodecyl sulphate, r approx. 5*

Frame (i) after 6min, frame (ii) after 30min at 59780rev./min. For the slower component $s_{25,w}$ = 5.1S.

Table 1. *Properties of native, sodium dodecyl sulphate-denatured and partially renatured ovalbumin*

Sample	r	M_w	$s_{25,w}$* (S)	K_s† (ml/g)	a_0	b_0	$(\eta_{sp.}/c)$* (ml/g)
Native	0	44600	3.45	6.2	−170°	−142°	3.72
Denatured	100	—	2.72	22	−244°	−182°	11.5
Partially renatured	25	57000	2.90	17	−226°	−160°	5.0

*At a protein concentration of 9mg/ml.
†$K_s = -(1/s_0)(ds/dc)$.

Table 2. *Properties of guanidinium chloride-denatured and partially renatured ovalbumin*

Concn. of guanidinium chloride (M)	$s_{25,w}$* (S)	a_0	b_0
5.8	1.6	−546°	+12°
3.9	2.21	−401°	−11°
3.3	2.81, 6.8	−407°	−26°
2.4	7.8, 20	(Precipitating)	

*At a protein concentration of 9mg/ml.

system, as before, aggregation and finally precipitation were observed. Sedimentation-equilibrium experiments were performed on the starting material and on two partially renatured samples having guanidinium chloride concentrations of 2.8 and 1.5M respectively. For the fully reduced protein in 6M-guanidinium chloride the molecular weight found was 50000. For the partially renatured samples the values were 52000 (2.8M-guanidinium chloride) and 110000 (1.5M-guanidinium chloride), but the proportion of the protein initially present, to which the values refer, was decreased to 80% and 10% respectively: i.e., most of the protein was lost as high aggregate by the time the guanidinium chloride concentration reached 1.5M.

Discussion

Where comparison is possible, the values of the physical parameters of native and denatured ovalbumin obtained in the present work are in substantial agreement with those found previously. The o.r.d. values for native ovalbumin (Table 1) differ only trivially from those given by Tomimatsu & Gaffield (1965) and Gordon (1968). Meyer & Kauzmann (1962) found higher values for both native and sodium dodecyl sulphate-denatured ovalbumin; if their values for sodium dodecyl sulphate-denatured protein are corrected by the factor necessary to bring the values for native protein into coincidence, one finds $a_0 = -225°$ and $b_0 = -192°$.

Aoki (1956) reported values for the intrinsic viscosity of ovalbumin–sodium dodecyl sulphate mixtures ranging from 4.2ml/g at $r = 40$ to 5.6ml/g at $r = 230$; allowing for the expected concentration-dependence, the agreement with the reduced-viscosity values in Fig. 1(*c*) is satisfactory. Aoki & Hori (1959) measured the variation of the sedimentation coefficient with extent of binding of sodium dodecyl sulphate: their values agree with those in Fig. 1(*b*) except at the lowest values of r, where they found slightly lower sedimentation coefficients.

It is clear, from the changes in the physical parameters, that sodium dodecyl sulphate induces a substantial conformational change. The nature of the change cannot be determined unambiguously from these results, but some insight is obtained by applying the procedure of Scheraga & Mandelkern (1953). For example, the reduced viscosity found for the complex existing at $r = 85$, at a concentration of 10mg/ml, may be combined with the s_0 value quoted and the value of $\bar{v}$ (based on additivity) of 0.79ml/g to give an approximate value for the conformation parameter, β, of 2.37×10^6. Since the intrinsic viscosity, the required quantity in this relationship, must be less than the reduced viscosity, the true value of β must be less than 2.37×10^6; it therefore follows that the indicated conformation is that of a compact particle, rather than that of a random coil or generally expanded particle, for which $\beta = 2.5 \times 10^6$. The hydrodynamically equivalent prolate ellipsoid is characterized by an axial ratio of 9 and a specific volume of 0.94ml/g. Although not conclusive, this analysis shows that the hydrodynamic values are at least consistent with the supposition that the denatured molecule is a compact rod, with minimal solvation.

From the relation between the intrinsic viscosities and the molecular weights of a range of sodium dodecyl sulphate-denatured proteins, Reynolds & Tanford (1970*a*) showed that a rod-shaped conformation was adopted in the fully unfolded state in the presence of mercaptoethanol. Their values for ovalbumin at 0.4g of sodium dodecyl sulphate/g of protein (equivalent to $r = 70$), although based on a higher value for the viscosity, also gave an axial ratio of 9; it therefore appears that the effect of breaking the one disulphide bond is slight, and the compact rod conformation suggested for the non-reduced species is confirmed. The o.r.d. results are compatible with this conclusion: if b_0 is regarded as an approximate measure of helix content (see, e.g., Jirgensons, 1969), the action of sodium dodecyl sulphate is to increase the α-helix content, in agreement with the results found for other proteins (Visser & Blout, 1971).

The differences in the sodium dodecyl sulphate denaturation profiles revealed by the three physical methods are of some interest. It appears that a_0 must

be sensitive to the environmental changes accompanying the binding of approximately the first 20 sodium dodecyl sulphate residues/molecule, binding that produces relatively little conformational change. The increase in viscosity for values of r greater than 100, where all other parameters have reached plateau values, presumably corresponds to the volume change accompanying the continued binding of detergent to an already extended rod. The absence of any comparable change in s in this region is not unexpected, in view of the relative insensitivity of this parameter, and particularly because the molecular weight and buoyancy factor are affected in opposite ways by binding. No step in the $\eta_{red.}$–r relationship was observed, or any other indication of discontinuity in the binding, although the range of r observed covered that in which Reynolds & Tanford (1970*b*) reported two distinct extents of binding.

Denaturation with guanidinium chloride–mercaptoethanol is recognized as a more complete process than that with sodium dodecyl sulphate: Tanford and co-workers have demonstrated the elimination of all non-covalent structure, with the consequent adoption of a random-coil configuration (Tanford *et al.*, 1967; Kawahara *et al.*, 1967). The results for s and $[\eta]$ found in the present study are in good agreement with those predicted on the basis of this model: by using the equations of Tanford *et al.* (1967) for a polypeptide chain of 378 residues, the predicted value of $[\eta]$ is 35.9, compared with the observed value of 34.3 ml/g. For $s_{25,w}$ the predicted value is 2.07S, compared with 2.18S observed. Thus reduced ovalbumin in 6M-guanidinium chloride is fully denatured: although the physical parameters for non-reduced ovalbumin were not defined so precisely, it is clear that the differences from the reduced protein are small. Non-reduced ovalbumin in 6M-guanidinium chloride must also be almost structureless, a conclusion that suggests that the polypeptide loop maintained by the single disulphide bond forms a small proportion of the total number of residues; Fothergill & Fothergill (1970) showed that one end of the disulphide bond was in the *C*-terminal tetrapeptide, but were unable to locate the other end.

The failure to achieve complete renaturation is the most significant experimental finding. For sodium dodecyl sulphate the result is not surprising, as the interaction of this reagent with proteins is usually irreversible (Friedland & Hastings, 1967; Tarutani & Ui, 1969), markedly different conformations from the native being assumed on its removal (Visser & Blout, 1971).

Although Weber & Kuter (1971) have shown that sodium dodecyl sulphate can be removed from proteins in the presence of 6M-urea (the proteins subsequently being renaturable in the usual way), this does not constitute a renaturation from sodium dodecyl sulphate solution. The guanidinium chloride reagent, however, has been successfully used for many proteins (see, e.g., Anfinsen, 1967), and indeed the experimental conditions with this reagent were identical with those that had led (in this laboratory) to high yields of renatured serum albumin (W. H. Sawyer & J. M. Creeth, unpublished work).

In experiments where the denaturant is removed rapidly, by dilution or dialysis against buffer, the effect of protein concentration is very marked (see, e.g., Anfinsen, 1962). This is to be expected; a partially re-ordered structure, with exposed hydrophobic groups, must have a high tendency to unite with similarly unshielded groups in neighbouring molecules. If denaturant is removed rapidly, the kinetic barriers to subsequent disaggregation and correct folding are prohibitively high. In principle, the maintenance of thermodynamically reversible conditions during removal of the denaturant should overcome this difficulty; if the native state is one of minimum free energy, the dynamic equilibrium existing among the conformations characterizing a particular concentration of denaturant should provide a pathway for the elimination of energetically unfavourable interactions. It is possible that oscillatory conditions analogous to those used for tempering steels may be necessary, as was found by Kuhn *et al.* (1964) in the thermal renaturation of collagen. On the other hand, Ullmann & Monod (1969) have suggested that the conventional definition of a free-energy minimum should be modified, in the context of protein conformation, to refer only to single molecules. However this may be, it is clear that the irreversibility found in the present work is not due to the effects of concentration alone, as the lowest concentration studied, (0.2 mg/ml) where equally irreversible aggregation was found, is well within the range where many enzymes have been successfully renatured. Thus, although experimental inadequacies cannot be ruled out, it seems likely that the failure to renature may arise from causes intrinsic to the protein. In this connexion, several suggestions have been advanced to account for incomplete renaturation of other proteins (see, e.g., Ullmann & Monod, 1969; Teipel & Koshland, 1971*a*), a common feature of which is the postulate that correct folding may involve a specific pathway. For example, such a pathway might arise through primitive folding of the growing polypeptide chain (Levinthal, 1968; Teipel & Koshland, 1971*b*) or through interaction with ligands (Andersson, 1969; Price *et al.*, 1969). When renaturation is possible, primitive folding either does not occur (Taniuchi & Anfinsen, 1969) or does not introduce kinetic barriers to render the thermodynamically stable state inaccessible (see, e.g., Brown & Klee, 1969).

The effects of primitive folding are likely to be more significant with proteins consisting of a single long polypeptide chain, particularly when they are unusually rich in hydrophobic residues, as is true of

ovalbumin (Van Holde, 1964). The implication of the present study is therefore to suggest that ovalbumin belongs to the group of proteins in which extrathermodynamic factors affect the folding of the native molecule; one must recognize, however, that the carbohydrate moiety or the phospho groups on the serine residues may constitute special factors deterring the refolding of this protein.

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