Sedimentation, Viscosity and Partial Specific Volumes of Membrane Proteins and Lipoproteins

By A. D. BROWN AND A. NETSCHEY

Department of Microbiology, University of New South Wales, Kensington, Sydney, and Department of Physical Chemistry, University of Sydney, Sydney, New South Wales, Australia

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1. Lipoproteins or defatted proteins from the membrane of *Halobacterium halobium* were dissolved in 0.02M-phosphate buffer, pH 7.4, with or without urea (8M). Physicochemical measurements were made on these solutions with and without addition of sodium chloride. 2. In the presence of 8M-urea the lipoproteins could be resolved into two sedimenting groups. The faster group had an average apparent molecular weight of 390 000; sedimentation coefficients of the slower group could not be determined. 3. Proteins that had been defatted by extraction with a chloroform-methanol mixture could also be resolved into two groups distinguished by their sedimentation velocities. Sedimentation coefficients of both groups were readily determined. The average apparent molecular weight of the faster group of defatted proteins in 8M-urea was 340 000 and that of the slower group was 97 000. It is concluded that attachment of lipid does not affect the state of aggregation of the proteins in 8M-urea. 4. Intrinsic viscosities of lipoproteins and defatted proteins were higher than is customary for globular proteins and suggest expanded highly-solvated molecules, consistent with the 'denaturing' action on the proteins of solutions of low ionic strength.

The membrane of the halophilic bacterium *Halobacterium halobium* disaggregates electrostatically at low ionic strength to give a solution of lipoproteins (Brown, 1963). The availability of such a solution facilitates various physicochemical studies of the membrane that would not otherwise be possible; some have already been reported (Brown, 1965). The present paper describes additional measurements on the complete mixture of lipoproteins and on proteins after removal of lipid. An aim of the work was to obtain information about possible states of aggregation of the membrane components and effects of the lipid on aggregation. Accurate determinations of molecular weights could not be made for components of the mixture, but some approximations were possible.

MATERIALS AND METHODS

Membrane lipoproteins. Membranes were isolated by osmotic lysis of the organisms in the presence of 0.02M-MgCl₂ as described by Brown, Shorey & Turner (1965). Membranes were dissolved by exhaustive dialysis against water at 4° and the resulting solutions freeze-dried. Several separate batches were prepared and mixed; the resulting preparation was used throughout. Some measurements made on other preparations gave slightly different quantita-

tive results, particularly in viscosity, but did not affect the qualitative conclusions. Lipid was removed from protein by five extractions at room temperature with chloroform-methanol (2:1, v/v). The solvent was evaporated and the residual protein was dissolved overnight in 0.02N-NaOH at approx. 4°. The solution was dialysed at 4° to remove NaOH. The protein solution was centrifuged and the deposit washed twice with water at 9750g for 10 min. The supernatant solution and washings were combined and freeze-dried. This preparation, designated 'soluble fraction', was used for all measurements involving defatted protein. The gelatinous pellet, designated 'insoluble fraction', was not used in this work.

Measurements. Except for some preliminary viscosity determinations (see below), all measurements were made in phosphate buffer (Na₂HPO₄-KH₂PO₄, pH 7.4 and *I* 0.06) with other solutes added as stated in the Results section. The sedimentation-velocity measurements were made at a nominal speed of 59780 rev./min. in a Spinco model E analytical ultracentrifuge; the actual average speed was calculated from the number of revolutions. Pressure effects were neglected in the calculation of sedimentation coefficients. In calculations involving sedimentation coefficients, protein concentrations were equated to the total concentration of the mixture. This practice was preferred to an estimate of the concentration of a measurable sedimenting component, since it was considered to be more realistically related to levels of molecular interaction and it avoided complications introduced by the Johnston-Ogston effect. Viscosity was

measured at $20 \pm 0.01^\circ$ with a suspended level viscometer, BS/IP/SL(S) no. 3 (flow time for water, 134 sec.). Flow times were not corrected for densities of the solutions; at the protein concentrations used, errors caused by this omission were negligible. Calculations of reduced and intrinsic viscosities used as η_0 flow times for the appropriate solvent in each case. Partial specific volumes were calculated from solution densities at $20 \pm 0.01^\circ$ by using the equation;

$$1 - \bar{v}\rho = \frac{(1 - w) dm}{m dw}$$

where $\bar{v}$ is the partial specific volume of solute, w is the weight fraction of solute, m is the mass of liquid in the pycnometer (capacity 11.5 ml.) and ρ is the density of solution. A.R.-grade chemicals were used throughout without further purification. For the calculation and expression of viscosities, protein concentration is expressed as % (mass of solute/volume of solution). Where it has been necessary to express concentrations for other purposes both % (w/v) and % (w/w of solution) have been given.

RESULTS

Solubility of membranes. Some lipoprotein solutions remained faintly turbid after exhaustive dialysis against water. These preparations were centrifuged for 1 hr. at 17000g at about 4° . The deposit, which was always less than 5% of the preparation, was very dark in colour, sometimes almost black with a minor proportion of a viscous dark-purple material associated with it. The deposit almost certainly did not originate in the membranes. Some of it seemed to have been carried over from the growth medium although the viscous material was apparently of intracellular origin. It is therefore unlikely that separation of a residual insoluble material caused the removal of a component essential to the structure of the whole membrane and so to the interpretation of the results. When the freeze-dried lipoproteins were redissolved in water or dilute phosphate buffer the resulting solution was slightly turbid and its sedimentation pattern (see below) was slightly different from that previously obtained with lipoprotein solution that had been concentrated but not dried (Brown, 1963). Solutions of redissolved lipoprotein were used without additional fractionation. When dried lipoproteins were redissolved in buffered 8M-urea, clear solutions were obtained.

About 65% of the defatted protein was obtained in soluble form. After being dried, the soluble fraction dissolved readily in water, dilute phosphate buffer and buffered 8M-urea. The insoluble fraction remained insoluble in all three solvents.

Sedimentation. Patterns of sedimentation of lipoproteins and defatted protein in buffer with or without urea (8M) are shown in Fig. 1. Lipoprotein in phosphate buffer (Fig. 1a) had one major component that sedimented at a measurable rate.

Fig. 1. Representative sedimentation patterns at 20° and various time-intervals after reaching maximum speed (59780 rev./min.). The phase-plate angle was 60° in all cases. (a) Lipoprotein (0.72%) in 0.02M-phosphate buffer; (b) lipoprotein (0.81%) in buffered 8M-urea; (c) defatted protein (1.27%) in buffered 8M-urea; (d) defatted protein (0.93%) in 0.02M-phosphate buffer; (e) defatted protein (1.35%) in buffered 8M-urea + NaCl (0.5M).

During acceleration of the centrifuge, a fast component appeared and accumulated on the bottom of the cell. Lipoprotein in buffered 8M-urea had two components, one forming a sharp peak and the other a slow broader one (Fig. 1b). Sedimentation-velocity measurements were obtained only for the sharp faster peak; no reliable measurements of the slower component could be made. Addition of sodium chloride to these two solvents affected sedimentation rates but not the number of peaks observed. Defatted protein had two distinct and measurable components both in the presence (Fig. 1c) and absence (Fig. 1d) of 8M-urea. Boundary shapes, as well as sedimentation velocities, were affected by urea and also by sodium chloride (Fig. 1e). Sedimentation coefficients (S_{20}^0) are listed in Table 1 for lipoprotein and defatted protein. Table 2 shows an effect of sodium chloride on the sedimentation coefficients of lipoprotein; the partially corrected values show that sodium chloride at concentrations greater than 0.2M produced no significant effect. Table 3 shows effects of urea and of sodium chloride on uncorrected sedimentation coefficients of defatted protein.

Viscosity. The viscosity of lipoprotein dissolved in water and adjusted to pH 7.0 with sodium hydroxide but without any other adjustment to

ionic strength was very high (a reduced viscosity as high as 55 at 25° was encountered at a protein concentration of 0.03%), but decreased sharply on successive determinations or on agitation. When the ionic strength was raised even slightly, as in 0.02M-phosphate buffer, viscosities were much lower and reproducible. Intrinsic viscosities, $[\eta]$, are listed in Table 4: urea increased $[\eta]$ of lipoprotein about twofold and that of defatted protein about 2.5-fold. The effects of sodium chloride on the reduced viscosities are shown in Table 5. In both

Table 1. *Sedimentation coefficients of the measurable components of lipoprotein and defatted protein*

The sedimentation coefficient at zero protein concentration in water or 8M-urea is given as appropriate. The sedimentation coefficient of lipoprotein in phosphate buffer is the mean of determinations at three concentrations that did not extrapolate smoothly to zero concentration. Other values of S_{20}^0 were obtained from extrapolations of curves described by the equation $1/S = (1 + kc)/S^0$ where c is protein concentration and k is a constant. For the fast lipoprotein component in buffered 8M-urea, k is 0.164; for the fast component of defatted protein in buffered 8M-urea, k is 2.23; for the slow component of defatted protein in buffered 8M-urea, k is 2.11.

Material	Solvent	S_{20}^0 (s)
Lipoprotein	0.02M-Phosphate buffer	5.05
Lipoprotein fast component	Buffered 8M-urea	2.50
Defatted-protein fast component	Buffered 8M-urea	2.78
Defatted-protein slow component	Buffered 8M-urea	1.19

Table 2. *Sedimentation coefficients of lipoprotein (fast component) in buffered 8M-urea plus sodium chloride*

For the partially corrected sedimentation coefficients the reference liquid was buffered 8M-urea.

Concn. of NaCl (M)	Concn. of lipoprotein		Uncorrected sedi-mentation coefficient S (s)	Partially corrected sedi-mentation coefficient S_{20} (s)
	(%, w/v)	(%, w/w)		
0.00	0.916	0.816	1.06	1.06
0.19	0.905	0.801	1.75	1.84
0.53	0.934	0.820	1.73	2.02
0.76	0.974	0.847	1.61	2.09

Table 4. *Intrinsic viscosities of lipoprotein and defatted protein in the presence and absence of 8M-urea*

Solution	Intrinsic viscosity $[\eta]$ (dl.g. ⁻¹)
Lipoprotein in 0.02M-phosphate buffer	0.600
Lipoprotein in buffered 8M-urea	1.16
Defatted protein in 0.02M-phosphate buffer	0.336
Defatted protein in buffered 8M-urea	0.912

Table 5. *Effect of sodium chloride on viscosity of protein solutions in the presence and absence of 8M-urea*

Solution	Concn. of NaCl (M)	Concn. of protein C (%, w/v)	Reduced viscosity
			$\frac{\eta - \eta_0}{\eta_0 C}$ (dl.g. ⁻¹)
Lipoprotein in 0.02M-phosphate buffer	0.75	0.255	0.461
	0.50	0.259	0.424
	0.25	0.259	0.352
	0.00	0.259	0.740
Lipoprotein in buffered 8M-urea	0.75	0.246	0.780
	0.50	0.244	0.760
	0.25	0.240	0.740
	0.00	0.240	1.31
Defatted protein in buffered 8M-urea	0.50	0.252	0.769
	0.00	0.253	1.44

Table 3. *Effects of 8M-urea and sodium chloride on sedimentation coefficients of defatted protein*

Concn. of urea (M)	Concn. of NaCl (M)	Concn. of protein		Uncorrected sedimentation coefficient S (s)	
		(%, w/v)	(%, w/w)	Fast component	Slow component
8	0	1.42	1.27	0.77	0.32
8	0	1.04	0.93	0.87	0.40
8	0.5	1.54	1.35	1.25	0.43
0	0	0.93	0.93	2.89	1.21
0	0.5	0.93	0.91	4.57	1.50

Table 6. *Partial specific volumes of lipoproteins and defatted protein in various solvents*

Solute	Solvent	Partial specific volume $\bar{v}$ (ml.g. ⁻¹)
Lipoprotein	0.02M-Phosphate buffer	0.790
Lipoprotein	Buffered 8M-urea	0.710*
Lipoprotein	Buffered 8M-urea + NaCl (0.5M)	0.754*
Defatted protein	0.02M-Phosphate buffer	0.684
Defatted protein	Buffered 8M-urea	0.688*

* Variable (see the text).

the presence and absence of urea, sodium chloride lowered the viscosity of lipoprotein solutions but there was a minimum in 0.25M-sodium chloride; at higher salt concentrations the viscosity increased slightly. Sodium chloride also decreased the viscosity of defatted protein solution, as shown for one concentration in Table 5.

Partial specific volume. Partial specific volumes are given for five sets of conditions in Table 6. In calculating these values it was found that plots of mass of solution versus protein concentration were linear for the two systems without urea; in these two cases calculation of $\bar{v}$ was straightforward. For the three systems with urea the slope dm/dw decreased progressively with increasing protein concentration (in the ranges 0–0.27% lipoprotein, 0–0.29% defatted protein and 0–0.43% lipoprotein + 0.5M-sodium chloride). The change in slope in urea was least for lipoprotein and greatest for lipoprotein + sodium chloride. The values of $\bar{v}$ given in Table 6 for these three systems were calculated from the slope dm/dw for values of w between 0 and the maximum that was used. The implication of a decrease in dm/dw with increasing w is that $\bar{v}$ increased with increasing protein concentration. This, in turn, is indicative of interaction between solute molecules at higher concentrations and is not unexpected in a complex system.

DISCUSSION

The present methods do not allow accurate molecular-weight determinations to be made of components of a mixture of proteins. It is possible, however, to make some approximate calculations of average molecular weight that can give a general indication of size and provide a basis for comparing the states of proteins examined. Molecular weights were calculated for the materials in 8M-urea by using the Scheraga & Mandelkern (1953) treatment and the equation:

$$M = \frac{8405 S_{20}^{0.3} [\eta]^{\frac{1}{2}}}{(1 - \bar{v}\rho)^{\frac{1}{2}}}$$

where M is molecular weight, S_{20}^0 is the sedimentation coefficient corrected to zero concentration in 8M-urea, $[\eta]$ is the intrinsic viscosity and $\bar{v}$ is the partial specific volume of the protein, both in urea; ρ is the density of the solvent (urea). All values are at 20°. The numeral 8405 is obtained for solution in 8M-urea by using Scheraga's (1961) treatment for flexible-chain molecules in which the factor $\Phi^{\frac{1}{2}}P^{-1}$ is used (instead of the customary β) with a numerical value of 2.5×10^6 . The assumption that the molecules are flexible chains is based on the known denaturing action of urea, a probable absence of cysteine and cystine from the proteins (Brown & Shorey, 1963) and the high intrinsic viscosities listed in Table 4. An additional assumption that has been made is that the intrinsic viscosity of an individual sedimenting component was the same as the average value of the mixture.

With these assumptions the following molecular weights were calculated for the membrane components dissolved in 8M-urea: lipoprotein (fast component only), 390 000; defatted-protein fast component, 340 000; defatted-protein slow component, 97 000. There is therefore no evidence that lipid affects the state of aggregation of the proteins dissolved in 8M-urea or (Table 4) affects greatly the conformation of the proteins in this solvent.

Results obtained without urea were less satisfactory because of aggregation of the lipoproteins. Aggregated material that was 'removed' during acceleration of the centrifuge caused an indeterminate error in the concentration of the solution, and it was presumably this factor that prevented the smooth extrapolation of sedimentation coefficients to zero concentration of lipoprotein (Table 1). For this reason, and because a primary aim of the work was to compare lipoprotein with defatted protein, no attempt was made to determine the molecular weight of defatted protein in simple aqueous buffer. A value for the molecular weight of the lipoprotein, however, was calculated from the average sedimentation coefficient given in Table 1 with a treatment of the foregoing equation appropriate to an aqueous system. Two values were used for the factor $\Phi^{\frac{1}{2}}P^{-1}$ (or β). Assuming that the molecules were flexible chains and that $\Phi^{\frac{1}{2}}P^{-1}$ equals 2.5×10^6 , M is approx. 350 000; assuming that the molecules were relatively symmetrical ellipsoids (which, from Table 4, is unlikely) and that β equals 2.16×10^6 , M is approx. 440 000. It is emphasized that these values, in common with those quoted for the materials in urea, have only qualitative significance because of the many assumptions necessarily made.

The intrinsic viscosities quoted in Table 4 are higher than usually obtained with globular proteins (e.g. Yang, 1961). The lipoprotein and, to a smaller extent, the defatted protein clearly had large

hydrodynamically effective volumes and therefore extended or expanded conformations. The relatively small increases in intrinsic viscosity caused by urea (Table 4) are consistent with an action of this solvent on molecules already considerably 'denatured'. Further, both the absolute values of the intrinsic viscosities as well as the proportional increases caused by urea (Table 4) imply that, in dilute aqueous buffer, the lipoprotein molecules were more fully expanded than the defatted proteins. Such a difference could be caused by the charges on acidic phospholipids of the type detected in *H. cutirubrum* (Kates, Sastry & Yengoyan, 1963) and also in the membrane lipid of *H. halobium* (C. Geczy, unpublished work).

If, as supposed, the proteins are not appreciably cross-linked, a considerable expansion of the molecules at low ionic strength is to be expected. These proteins are in their native state in about 4M-sodium chloride and are released into solution at low ionic strength because of an overall expansion of the membrane. The negative charges responsible for this expansion are located on the proteins (Brown, 1963, 1965) and the proteins themselves must be subject to intramolecular forces caused by these charges. Further, if this interpretation is valid, addition of sodium chloride to solutions of the

protein should cause at least a partial reversal of the process to give more compact conformations of the protein molecules. The described effects of sodium chloride, though generally consistent with such a process, are characteristic of a primary salt effect (Pedersen, 1958) and do not permit an assessment of the extent of any possible conformational changes.

The proteins of the membrane are therefore of two types, one type being about three times larger than the other. There is also reason for believing that at least some of the proteins are very flexible and assume random expanded conformations in aqueous solutions at low ionic strength.

REFERENCES

- Brown, A. D. (1963). *Biochim. biophys. Acta*, **75**, 425.
Brown, A. D. (1965). *J. molec. Biol.* **12**, 491.
Brown, A. D. & Shorey, C. D. (1963). *J. Cell Biol.* **18**, 681.
Brown, A. D., Shorey, C. D. & Turner, H. P. (1965). *J. gen. Microbiol.* **41**, 225.
Kates, M., Sastry, P. S. & Yengoyan, L. S. (1963). *Biochim. biophys. Acta*, **70**, 705.
Pedersen, K. O. (1958). *J. phys. Chem.* **62**, 1282.
Scheraga, H. A. (1961). *Protein Structure*, pp. 23-29. New York: Academic Press Inc.
Scheraga, H. A. & Mandelkern, L. (1953). *J. Amer. chem. Soc.* **75**, 179.
Yang, J. T. (1961). *Advanc. Protein Chem.* **16**, 323.