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PHYSICAL CHARACTERISTICS OF MITOCHONDRIAL MEMBRANE PROTEIN DISSOLVED IN CHLOROFORM–METHANOL

P. J. CURTIS*

Beatson Institute for Cancer Research, Royal Beatson Memorial Hospital, Glasgow, C.3. (Great Britain)

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SUMMARY

Polystyrene fractions of known narrow molecular weight ranges have been used to characterise methylated Sephadex G-75 and G-100, which have previously been shown to separate the protein components of mitochondrial membranes dissolved in chloroform–methanol. The membrane proteins behave as compact globular macromolecules on gel filtration in chloroform–methanol.

Optical rotatory dispersion studies show the protein components have 50% α-helicity which agrees well with recent estimates of the α-helix content of whole membranes.

However, viscosity studies indicate that the membrane proteins cannot exist as impermeable spheres which is the form of water-soluble globular proteins.

INTRODUCTION

The use of organic solvents to study membrane proteins has been investigated on the basis of the considerable indirect evidence that the major interaction between protein and phospholipid is hydrophobic[1–5]. More recent results indicate more directly that some proteins of the red cell membrane penetrate the phospholipid bilayer[6,7]. Thus some membrane proteins certainly exist within a hydrophobic environment. For such proteins water is an unsuitable medium in which to study them. Detergents have proved very useful in studies of membrane bound enzymes, particularly as many enzymes retain their activity, but the physical state of the protein is very difficult to determine. Replacing the hydrophobic environment provided by phospholipids with an organic solvent does not involve a large change and so the membrane proteins may retain their native structures.

There are a number of reports of organic solvents being used to dissolve membrane proteins[2,8–15] but little more has been attempted except for the separation of protein from phospholipid on Sephadex LH-20[2,12]. In this paper some preliminary evidence is presented that mitochondrial membrane protein dissolved in chloroform–methanol is mainly disaggregated, but retains secondary and tertiary structure.

* Present address: Institut für Molekularbiologie der Universität Zurich, Hönggerberg, 8049 Zürich, Switzerland.

MATERIALS AND METHODS

Polystyrene fractions of known average molecular weight were kindly supplied by Dr. D. Ballard, I. C. I. Runcorn. Diethyl pyrocarbonate was obtained from Bayer as Baycovin; bovine serum albumin, ovalbumin and lysozyme from Sigma and trypsin from Worthington.

The preparation of rat liver mitochondrial membranes and the solution of membrane protein in chloroform–methanol (2:1, v/v) has been described earlier[15]. Methylated Sephadex G-75 and G-100 were prepared using the method of NYSTRÖM AND SJÖVALL[16]. It was found worthwhile to sieve Sephadex before swelling using the fraction obtained between sieves 120–200 BS. Two sizes of columns were used; 2 cm × 100 cm, used at 4° for membrane protein, normally gave a flow rate of approx. 5 ml/h, and was equilibrated with chloroform–methanol (2:1, v/v) containing 10 mM acetic acid; and a 1 cm × 30 cm column was used at room temperature with polystyrene with a flow rate of 12 ml/h and equilibrated with chloroform–methanol (3:1, v/v). The elution of polystyrene was followed by the absorption at 262 nm. The partition coefficient, σ, was calculated from $\sigma = (V_e - V_o)/V_i$; V_e, elution volume; V_o, void volume; V_i, internal volume of Sephadex permeable to small molecules, calculated from $V_c - V_o$, where V_c is elution volume for a small molecule.

Polyacrylamide-gel electrophoresis was performed as described by WEBER AND OSBORN[17], using 0.1 M phosphate buffer, pH 7.4, containing 0.1 % sodium dodecyl sulphate. Gel concentration was 7.5 % acrylamide. Protein in chloroform–methanol was prepared for electrophoresis by first precipitating the protein by addition of ethanolamine; the precipitate was dissolved in a minimal volume of acidified methanol, chloroform was removed by a flow of nitrogen and was followed by 8 M urea to a suitable volume. The sample was then made 1 % sodium dodecyl sulphate, 1 % β-mercaptoethanol and 0.01 M phosphate, heated at 37° for 2 h and finally dialysed overnight against 0.01 M phosphate, pH 7.4, containing 0.1 % sodium dodecyl sulphate and 0.1 % β-mercaptoethanol. Bovine serum albumin and ovalbumin were polymerised by the method of WOLF *et al.*[18] to give additional standards for the estimation of molecular weights. The protein was dissolved in water to give 5 mg/ml and mixed with diethyl pyrocarbonate (0.02 ml/ml solution) for 3 min at room temperature, using a Vortex whirlimixer. The solutions were made 1 % sodium dodecyl sulphate and 1 % β-mercaptoethanol and dialysed for 4–6 h against 0.01 M phosphate buffer, pH 7.4, containing 1 % sodium dodecyl sulphate and 1 % β-mercaptoethanol. The solutions were then heated at 37° for 2 h before dialysis overnight against 0.01 M phosphate buffer, pH 7.4, containing 0.1% sodium dodecyl sulphate and 0.1 % β-mercaptoethanol. Dimers and trimers of both proteins were seen in gels. For electrophoresis the sample was prepared by mixing 0.1 ml 0.01 M phosphate buffer, pH 7.4, containing approx. 50 μg membrane protein, with 5 μl 0.05 % bromophenol blue, 10 μl β-mercaptoethanol and 1 drop glycerol; 0.1 ml was applied to each gel. Electrophoresis was carried out at 10 mA per tube for 2.5 h. At the end of a run, the position of bromophenol blue was marked by a cut in the gel, which showed later on the record of the scan of the gel. The gels were fixed and stained by standing overnight in 50 % methanol containing 7 % acetic acid and 0.25 % naphthalene black. The gels were destained electrophoretically in 50 % methanol containing 7 % acetic acid and then allowed to swell overnight in 7 % acetic acid. The stained bands

were recorded using a Joyce:Loebl ultraviolet scanner and a Servoscribe recorder.

Viscosity measurements were made using a U-tube capillary viscometer, capacity approx. 3 ml, at 25° ± 0.1° with flow times of about 160 sec. Optical rotatory dispersion measurements were made using a Bendix polarimeter in a 10-mm cell at protein concentrations 1–2 mg/ml at 22° in the visible region (600–300 nm) and were treated according to the procedure of MOFFIT AND YANG[19]; λ_0 was assumed to be 212 nm and b_0 as −630 for 100 % α-helix.

Protein was measured by the biuret procedure[20], and by dry weight for the viscosity studies.

RESULTS

Using a mixed solvent, chloroform–methanol limits the physical techniques that can be used to study the membrane protein; for example ultracentrifugation and light scattering are not suitable. The separation of molecules by gel filtration has been shown to depend on molecular size[21], which for a homologous series of macromolecules correlates well with molecular weight. Exhaustively methylated Sephadex G-75 and G-100 has been used to separate membrane proteins dissolved in chloroform–methanol (2:1, v/v)[15], but there are no suitable proteins that could be used to standardise such columns. For this purpose polystyrene fractions with narrow molecular weight ranges were used. Chloroform–methanol (3:1, v/v) was used as a solvent in the experiments as this solvent was the closest one could approach to the solvent used for membrane protein and still dissolve all the polystyrene fractions. It was noted that methylated Sephadex G-75 and G-100 gave the same unpacked bed volume in chloroform–methanol (3:1, v/v) as in chloroform–methanol (2:1, v/v). Both modified Sephadexes were used in columns of 1 cm × 30 cm; the sample applied was 0.5 mg in 0.5 ml and the eluted material followed by the absorption at 262 nm. It was noted that for both methylated Sephadex G-75 and G-100, the elution volume for polystyrene, mol.wt. 51000, was very close to that for polystyrene, mol.wt. 97000, so that it was assumed that polystyrene, mol.wt. 97200 marked the void volume. Acetone was added to a number of samples and its elution volume used to determine the internal volume. In addition the intrinsic viscosity was determined for each fraction; and from the intrinsic viscosity the radius, R_E, of an equivalent hydrodynamic sphere was calculated from

$$[\eta] = \nu \cdot \tfrac{4}{3}\pi \cdot R_E^3 \frac{N}{M} \text{ (ref. 22)}$$

where ν is the viscosity increment and equal to 2.5 for a sphere, N is Avogadro's number and M is the molecular weight of the solute (Table I). The plot of partition coefficient against R_E gave a straight line (Fig. 1), showing that the methylated Sephadex retained the basic property of separating macromolecules according to their size.

For the mitochondrial membrane proteins (Fig. 2) it was only possible to relate partition coefficient to molecular weight. The molecular weights of the membrane proteins were estimated by a comparison of the mobilities of their bands with those of known proteins after sodium dodecyl sulphate–polyacrylamide-gel electro-

phoresis. It was then necessary to determine the elution volume from which the partition coefficient of each protein could be calculated. It had been noted before that the stain bound by a protein band in a gel was proportional to the amount of protein, though the bound stain was different for equal amounts of different proteins[23]. This relation was found to hold for each of the membrane proteins. Total membrane protein was applied to different gels in increasing amounts. After electrophoresis, staining and recording the stained gels it was found that the peak heights of each band were proportional to the amount of protein applied to the gels (Fig. 3). An equal volume was therefore taken from alternate fractions from the effluent of a column of methylated Sephadex G-100 to which mitochondrial membrane protein had been applied. The protein in each sample was treated in an identical manner for gel electrophoresis, and recording of the stained gels was carried out at the same setting of the recorder so that the peak heights in the scan reflected the quantities of each protein eluted in each sample. A plot of the peak heights for each band indicated the elution profile and the elution volume of the protein corresponding to that band (Fig. 4). The void volume was taken as indicated by the peak of nucleic acid, obtained from readings of 260 nm/280 nm of the first peak; this peak always precedes or corresponds to the peak for Band 1. The internal volume was determined from the elution volume of the third small peak that contains no protein and absorbs maximally at 400 nm. From the elution profiles it was noted that there appeared a small amount of most of

TABLE I

Mol. wt. of polystyrene	*Partition coefficient*		*Intrinsic viscosity* (cm^3/g)	R_E (Å)
	Methylated Sephadex G-75	*Methylated Sephadex G-100*		
97200	0	0	30.0	77.2
51000	0.02	0.02	21.3	55.5
19800	0.20	0.29	13.4	35.5
10300	0.44	0.49	9.2	24.6
4800	0.68	0.68	5.9	16.5

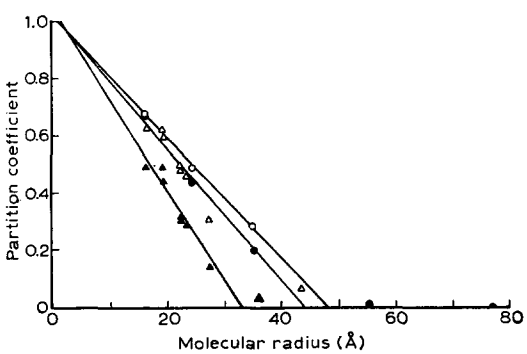

Fig. 1. Comparison of partition coefficient with molecular radii. ▲, water-soluble proteins on Sephadex G-75; △, the same on Sephadex G-100 (data from ANDREWS[24] and LAURENT AND KILLANDER[25]); ●, polystyrene fractions on methylated Sephadex G-75; ○, the same on methylated Sephadex G-100.

the bands in the first peak; this was possibly due to aggregation, to the existence of small amounts of an extended form of each band, or to binding to the small amount of nucleic acid present in the first peak. It was therefore difficult to determine the elution volume for the Bands 3–11 with much accuracy. However, Table II shows the results obtained for methylated Sephadex G-75 and G-100. A comparison of these data with that obtained for water-soluble proteins (Fig. 5) on Sephadex (taken from ANDREWS[24]) indicates that the methylated Sephadex must have larger pore sizes as the protein in chloroform–methanol cannot occupy a smaller volume than protein of similar molecular weight in water. This observation is supported by a comparison of the plot of molecular radii of water-soluble proteins[25] against their partition coefficients with the corresponding plot for the polystyrene fractions (Fig. 1).

It had earlier been observed that if a column of methylated Sephadex was equilibrated with chloroform–methanol (2:1, v/v) containing 10 mM HCl, mitochondrial membrane protein eluted only at the void volume[15]. This observation had been

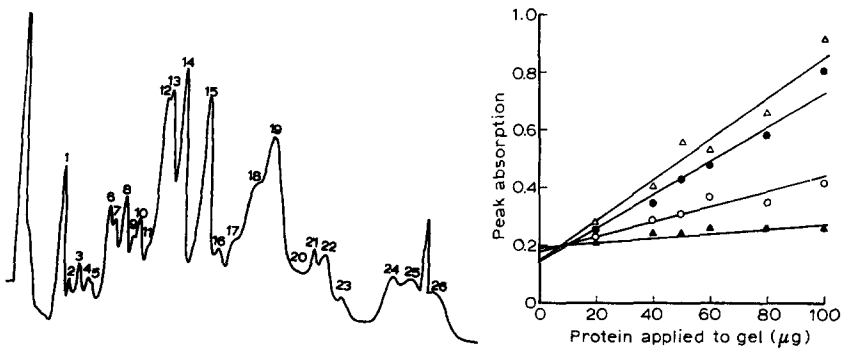

Fig. 2. Scan of 7.5% acrylamide gel with total mitochondrial membrane protein.

Fig. 3. Variation of the peak absorption of individual bands with increasing amounts of total mitochondrial membrane protein added to the gels. ▲, Band 3; ○, Band 8; △, Band 14; ●, Band 19.

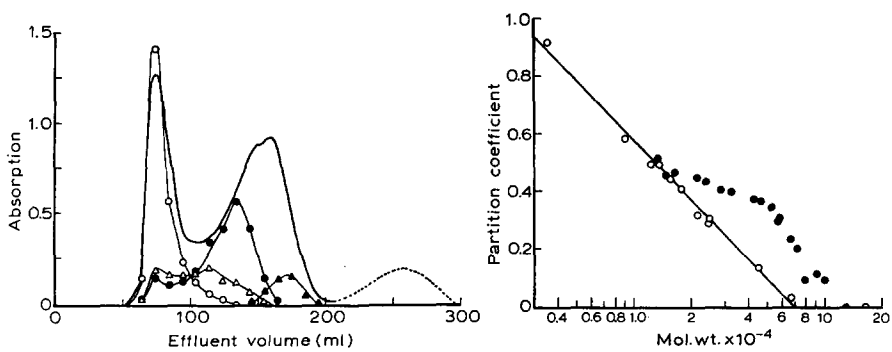

Fig. 4. Elution profile of membrane proteins on a methylated Sephadex G-75 column. ———, $A_{280\ nm}$; — — —, $A_{400\ nm}$; ○, peak absorption of Band 1; △, Band 8; ●, Band 12; ▲, Band 25; from the scans of the gels.

Fig. 5. Comparison of partition coefficient with molecular weight. ○, water-soluble proteins on Sephadex G-75 (ANDREWS[24]); ●, mitochondrial membrane proteins on methylated Sephadex G-75.

extended. It was found that the elution profile of the protein showed 2 peaks only when the HCl concentration was 0.05 mM or less. Similar results were obtained using diethylammonium hydrochloride and $NaNO_3$ (prepared by dissolving crystals in 0.5 ml water and mixing with 240 ml chloroform–methanol (2:1, v/v)); a single peak was obtained when the salt concentration was 1 mM (Fig. 6), while the normal profile appeared when the concentration was 0.05 mM. This change does not appear

TABLE II

Band	*Mol. wt.*	*Partition coefficient*		*Partition coefficient (methylated Sephadex G-100)*	*Viscosity number* η_{sp}/c^*
		Methylated Sephadex G-75	*Methylated Sephadex G-100*		
1	130 000	0	0.02	0.06	367
3	100 000	0.10	0.08	0.11	345
4	91 000	0.12	0.13		
6	79 000	0.09	0.13		
7	76 000	—	0.14	0.17	293
8	72 000	0.21	—	0.22	222
10	66 500	0.24	0.18	0.33	128
12	58 000	0.31	0.39	0.44	76.1
13	57 000	0.30	0.46		
14	53 000	0.35	0.49	0.50	89.5
15	46 500	0.36	0.55	0.56	73.7
16	42 700	0.38	—		
18	36 000	—	0.59	0.61	60.9
19	32 500	0.40	0.63		
20	28 500	0.41	—		
21	25 500		0.65	0.67	51.0
22	24 000	0.44	0.68		
23	21 500	0.45	0.64		
24	16 300	0.47	0.66		
25	14 800	0.46	0.67		
26	13 300	0.52	0.70	0.72	48.7

* The viscosity of succeeding fractions from a methylated Sephadex G-100 column to which membrane protein had been applied, was measured at approx. 1 mg/protein per ml; the partition coefficient, which was calculated from the elution volume of the fraction, was recorded to facilitate the comparison with the partition coefficient of the bands.

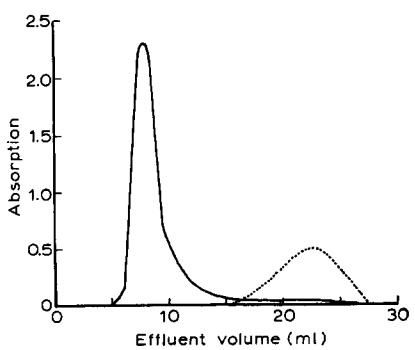

Fig. 6. Elution profile of mitochondrial membrane protein on methylated Sephadex G-100 (1 cm × 30 cm column), equilibrated with chloroform–methanol containing 1 mM $NaNO_3$. ———, $A_{280\ nm}$; – – –, $A_{400\ nm}$.

to be due to nonspecific aggregation because sodium dodecyl sulphate gel electrophoresis of samples taken from the single peak showed that the bands still eluted in the order of their molecular weights (Fig. 7). A single peak at the void volume, but with a little more protein trailing, was also obtained when 2-chloroethanol and dimethylformamide were used as solvents. 2-Chloroethanol normally contains HCl and this may have been responsible for the result in that solvent, but dimethylformamide was deionised by storing over molecular sieve type 4A (BDH) for a month and then used immediately after removal. However, a normal elution profile was obtained when chloroform–methanol (2:1, v/v) containing 1 M water was used.

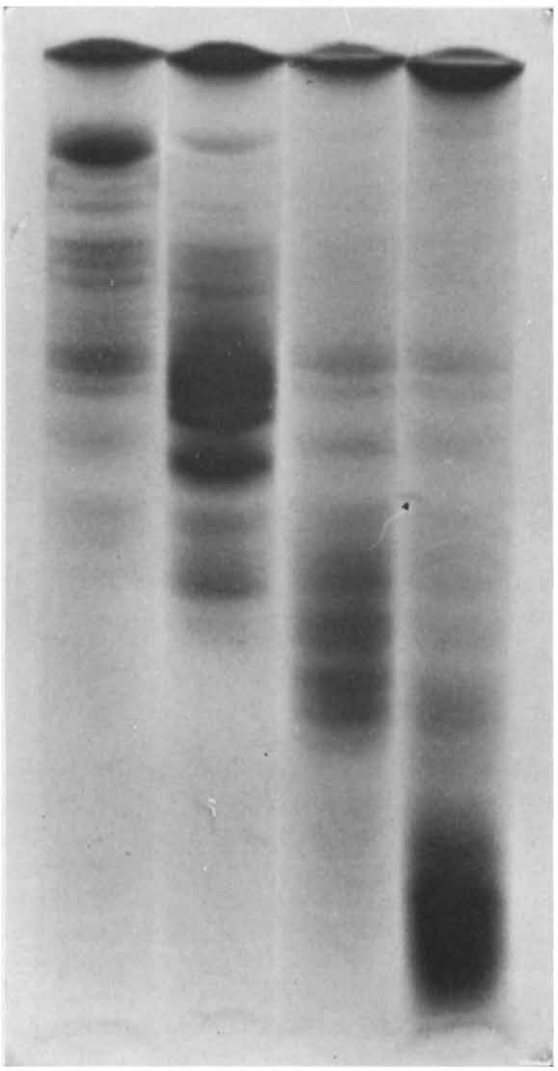

Fig. 7. Polyacrylamide-gel electrophoresis of fractions taken from Fig. 6. From left to right fractions applied to gels were 6 ml, 8 ml, 10 ml and 12 ml.

The partially purified major protein component obtained by two successive runs on methylated Sephadex G-100[15], and corresponding largely to Band 12, has been used for viscosity and optical rotatory dispersion measurements. The viscosity measurements were made at 25°. It was routinely noted that the flow time obtained for the solvent alone was longer by 1–2 %, when recorded after a protein solution had been used, compared with the time before any protein solution had entered the viscometer. This difference may be due to adsorption of protein to the capillary wall thus reducing its diameter. For the values shown (Fig. 8) the flow time of the solvent, taken after a protein solution had been used was used in the calculations. The intrinsic viscosity of the major protein component in chloroform–methanol (2:1, v/v) containing 1 mM $NaNO_3$ showed no change in its value. In addition the viscosity of fractions eluted from a methylated Sephadex G-100 column to which mitochondrial membrane protein had been applied was measured (Table II) and showed a marked dependence on the molecular weight of the fraction.

Previously the optical rotatory dispersion of total membrane protein in chloroform–methanol (2:1, v/v) containing HCl had been measured and given a value of 53 % α-helicity[14]. A similar value was obtained for the major protein component in chloroform–methanol (2:1, v/v) containing 10 mM acetic acid, $b_0 = -340$, $a_0 = +100$.

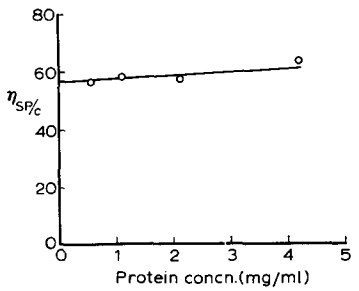

Fig. 8. Variation of viscosity number with protein concentration.

DISCUSSION

Sephadex, which has been exhaustively methylated, still retains its ability to separate molecules according to their size in an organic solvent. A good correlation was obtained between the partition coefficient and molecular radius for polystyrene fractions with methylated Sephadex G-75 and G-100. The methylated Sephadex would appear to have a larger pore size than untreated Sephadex in water; there is a number of possible explanations: (1) Chloroform more completely solvates methylated Sephadex than water with untreated Sephadex; the unpacked bed volume of methylated Sephadex G-100 in chloroform–methanol (2:1, v/v) is 35 ml, in methanol 20 ml, in water 31 ml, and untreated Sephadex G-100 in water 30 ml. (2) However, water is bound more strongly to untreated Sephadex by hydrogen bonding than chloroform to methylated Sephadex by Van der Waals and London forces; so the layer of bound water would be less permeable to macromolecules than the layer of bound chloroform, thus effectively reducing the pore size. Similarly water would bind to protein more strongly than chloroform, again effectively increasing a protein's

size. (3) The value of molecular radius for the proteins will be smaller than the radius of an equivalent hydrodynamic sphere for each protein, and it is the latter value that was measured for the polystyrene fractions.

The pore sizes for methylated Sephadex G-75 and G-100 indicated by the values of hydrodynamic radius obtained for the polystyrene fractions would appear to be reasonable but approximate estimates. Using these values the major protein component, which has a partition coefficient of 0.31 and 0.39 for methylated Sephadex G-75 and G-100 would have an effective hydrodynamic radius of 30–32 Å compared with bovine serum albumin, 36 Å and for ovalbumin, 27 Å. As the major protein component has a molecular weight of 57000[26] which is intermediate between bovine serum albumin and ovalbumin, the protein must be compact and roughly globular. This compact shape is suggested also by the results of gel filtration in the presence of salt, where most of the protein elutes earlier than in the absence of salt. The sodium dodecyl sulphate gel electrophoresis shows that this change is not due to aggregation, so that the change is more likely due to an increase in the size of the protein molecules, from a compact to a more extended shape.

The elution profiles of the bands on methylated Sephadex in chloroform–methanol (2:1, v/v) demonstrate that the protein solution is largely disaggregated. The viscosity measurements which show little or no dependence on concentration support this statement. However, the value obtained for the intrinsic viscosity of the major protein component of approx. 60 cm^3/g, is very different from the value expected for a compact globular protein, the value for which is approx. 2.5 cm^3/g. The gel filtration studies rule out the possibility that the high value for the intrinsic viscosity is due to a highly asymmetrical molecule. One possible explanation is that the protein in chloroform–methanol is globular but freely permeable to the solvent, *i.e.* a free draining coil. Such a structure would account for the high intrinsic viscosity. There are two observations that support this model. For a free draining coil the intrinsic viscosity should be proportional to the molecular weight or to a power of it larger than the first[27]; while for an impermeable sphere the intrinsic viscosity is relatively independent of the mole weight. From Table I, the viscosity number shows a marked dependence on the molecular weight of the fraction measured. The other supporting observation comes from a comparison of the values for the intrinsic viscosity of bovine serum albumin under different conditions. In water the intrinsic viscosity is 3.7 cm^3/g, in urea it is 22 and reduced bovine serum albumin in urea 53[28], while dissolved in acidic chloroform–methanol the value is 40 (unpublished result by P. J. CURTIS). The value in urea would not be due to a marked asymmetry but to a loosening of the interior of the molecule by a partial solvation of hydrophobic groups by urea. This solvation of hydrophobic groups would occur to greater extent in chloroform–methanol. But the –S–S– bridges would still be intact and the molecule would retain a fairly compact shape. Once the –S–S– bridges were reduced the molecule would become a random coil.

How is the structure that has been suggested for the major protein component (and for many of the other components as they have similar properties on gel filtration and viscosity) of a globular, but permeable molecule related to the native structure? Initial measurements of the optical rotatory dispersion of whole membranes showed a dispersion corresponding to an average content of 20% α-helicity[1,2], but more recently corrections have been applied for the effect that the particulate nature of a

membrane has on the dispersion and corrected estimates are 50[29] and 40 %[30]. The dispersion of total membrane protein in 2-chloroethanol[2] and chloroform–methanol[14] indicated 50 % α-helicity. It should be noted that the acid conditions for the last two values would mean the protein was probably in an extended form, so that the α-helix content was determined by the primary sequence of amino acids. Caution should be exercised in comparing these values for they are average values of total membrane protein and the values might be the sums of different components under the various conditions. However, the dispersion of the major protein component corresponded to 50 % α-helix content.

Freeze etching of membranes shows clearly globular units that are probably protein[31,32]. The permeable nature of the membrane protein in chloroform–methanol may reflect the existence of lipid–protein complexes where the lipid permeates the protein. Such a possible structure may exist with β-hydroxybutyrate dehydrogenase, which has a specific requirement for phosphatidyl choline and for incubation at 30° for 30 min in the presence of phosphatidyl choline, 0.1 M β-mercaptoethanol and 4 mM NAD for optimal activation before assaying[33,34]. Such requirements suggest that the interaction between phosphatidyl choline and the enzyme is not a matter of simple binding. It is, however, more likely that the permeable nature is the result of the treatment used to dissolve the membrane protein, in particular the effect of the acid. It is probable that all the charges on the membrane protein extend into the aqueous phase. On addition of the acid in chloroform–methanol, the protein will only have positive charges, presumably on its surface; in the absence of salt, when the protein is relatively globular, the counter ions must remain very close to the bound ions, thus effectively shielding them and counteracting like charge repulsion; in the presence of salt, either because of an overall increase in the dielectric constant or because of a local effect by direct interaction between the free and bound ions, the counter ions are able to move further from the surface resulting in a larger residual positive charge that causes the disruption of the globular protein. A similar explanation may account for the results of gel filtration using 2-chloroethanol and dimethylformamide. The permeable nature of the protein then would be due to the balance between the shielding effect of the counter ions and the disrupting effect of the bound positive ions. However, it is possible that the structure found in the absence of salt is an approach to the native state. Reconstitution of the protein with phospholipid to give enzymatic activity would be the only effective way to examine this problem and so far all attempts to achieve recovery of enzymatic activity have failed (unpublished results by P. J. CURTIS).

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REFERENCES

1 J. LENARD AND S. J. SINGER, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1825.
2 D. F. H. WALLACH AND P. H. ZAHLER, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1552.
3 D. CHAPMAN, V. B. KAMAT, J. DE GIER AND S. A. PENKETT, *J. Mol. Biol.*, 31 (1968) 101.

4 G. LENAZ, A. M. SECCHI, G. PARENTI-CASTELLI AND L. MASOTTI, *Arch. Biochem. Biophys.*, 141 (1970) 79.
5 D. BRANTON, *Ann. Rev. Plant Physiol.*, 29 (1969) 209.
6 T. L. STECK, G. FAIRBANKS AND D. F. H. WALLACH, *Biochemistry*, 10 (1971) 2617.
7 M. S. BRETSCHER, *J. Mol. Biol.*, 59 (1971) 351.
8 O. O. BLUMENFIELD, *Biochem. Biophys. Res. Commun.*, 30 (1968) 200.
9 D. E. GREEN, N. F. HAARD, G. LENAZ AND H. I. SILMAN, *Proc. Natl. Acad. Sci. U.S.*, 60 (1968) 277.
10 J. A. LOWDEN, M. A. MOSCARELLO AND R. MORECKI, *Can. J. Biochem.*, 44 (1966) 567.
11 A. F. REGA, R. I. WEED, C. F. REED, G. G. BERG AND A. ROTHSTEIN, *Biochim. Biophys. Acta*, 147 (1967) 297.
12 C. SCHNAITMAN, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 412.
13 R. F. A. ZWAAL AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 150 (1968) 323.
14 P. J. CURTIS, *Biochim. Biophys. Acta*, 183 (1969) 239.
15 P. J. CURTIS, *Biochim. Biophys. Acta*, 194 (1969) 513.
16 E. NYSTRÖM AND J. SJÖVALL, *Anal. Biochem.*, 12 (1965) 235.
17 K. WEBER AND M. OSBORN, *J. Biol. Chem.*, 244 (1969) 4406.
18 B. WOLF, P. M. LAUSAROT, J. A. LESNAW AND M. E. REICHMANN, *Biochim. Biophys. Acta*, 200 (1970) 180.
19 W. MOFFIT AND J. T. YANG, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 1043.
20 A. GORNALL, C. S. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
21 G. K. ACKERS, *Biochemistry*, 3 (1964) 723.
22 H. TOMPA, *Polymer Solutions*, Butterworths, London, 1956, p. 275.
23 D. M. FAMBROUGH, F. FUJIMURA AND J. BONNER, *Biochemistry*, 7 (1968) 575.
24 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
25 T. C. LAURENT AND J. KILLANDER, *J. Chromatogr.*, 14 (1964) 317.
26 P. J. CURTIS, *Biochim. Biophys. Acta*, 211 (1970) 575.
27 H. TOMPA, *Polymer Solutions*, Butterworths, London, 1956, p. 273.
28 H. K. SCHACHMAN, *Cold Spring Harbor Symp. quant. Biol.*, 28 (1963) 409.
29 D. W. URRY, L. MASOTTI AND J. KRIVACIC, *Biochem. Biophys. Res. Commun.*, 41 (1970) 521.
30 M. GLASER AND S. J. SINGER, *Biochemistry*, 10 (1971) 1780.
31 D. BRANTON, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 1048.
32 D. W. DEAMER AND D. BRANTON, *Science*, 158 (1967) 655.
33 G. S. GOTTERER, *Biochemistry*, 6 (1967) 2139.
34 G. S. GOTTERER, *Biochemistry*, 6 (1967) 2147.