Gel Electrophoresis Studies of Bovine Brain White Matter Proteolipid and Myelin Proteins†

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ABSTRACT: Bovine white matter proteolipid and myelin preparations were studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the presence and absence of urea. Each observed band was analyzed by means of the Ferguson relationship (log R_F *vs.* % gel concentration). The major proteolipid band and the DM-20 protein observed in myelin by Agrawal *et al.* (*J. Neurochem. 19*, 2083 (1972)) were present in white matter proteolipid preparations and were found to have molecular weights of 30,000 and 25,000, respectively. In myelin, higher and lower molecular weight bands which were different from the Wolfgram and basic proteins were also observed. Data on the multiple bands in the white matter proteolipid are consistent with the possible existence of an oligomeric series having a theoretical monomeric molecular weight of 5000. The major proteolipid protein band appears to be the hexamer and exists as two isomeric forms in the presence of a reducing agent and 8 M urea. In the absence of reducing agent, there was no change in the amounts of either lower or higher molecular weight bands, suggesting that the disulfide bonds in the proteolipids are intramolecular. Ferguson plots revealed a higher free electrophoretic mobility for the proteolipids than for water-soluble standard proteins. Under these circumstances, the molecular weights of proteolipids should be obtained by a comparison of the retardation coefficients of proteolipids with those of standard proteins rather than by a comparison of their relative mobilities.

Proteolipids are a class of lipoproteins which are soluble in chloroform–methanol and insoluble in water (Folch-Pi and Lees, 1951). They are most abundant in brain white matter where they constitute the major protein component of the myelin sheath. Proteolipids are characterized by a high content of neutral amino acids which may contribute to their hydrophobic nature and consequently to their insolubility and tendency to aggregate in aqueous solvents. The use of conventional fractionation techniques such as ion exchange or gel filtration column chromatography is thus precluded for the purification of proteolipids so that, at the present time, both the number of molecular species and the molecular weights of white matter proteolipid remain uncertain. Ultracentrifugation and moving boundary electrophoresis have revealed a major and a minor component in bovine white matter proteolipid apoprotein which could represent specific states of aggregation of a single protein or the presence of more than one protein (Folch-Pi and Stoffyn, 1972). Several investigators have observed a single proteolipid band upon polyacrylamide gel electrophoresis of isolated myelin or white matter proteolipid preparations from bovine, human, or rodent brains (Thorun and Mehl, 1968; Einstein *et al.*, 1968; Braun and Radin, 1969; Gonzalez-Sastre, 1970; Waehneldt and Mandel, 1970; Eng, 1971). However, multiple bands have also been reported for bovine, rodent, and porcine proteolipids (Greenfield *et al.*, 1971; Morell *et al.*, 1972; Reynolds and Green, 1973; Nicot *et al.*, 1973). Agrawal *et al.* (1972) recently demonstrated on SDS polyacrylamide[1] gels a protein band, designated as DM-20, which has a mobility intermediate between the proteolipid protein and myelin basic protein of several animal species. Since they also observed this band in the chloroform–methanol extract of rat brain, the DM-20 is, by definition, a proteolipid and its presence suggests heterogeneity in the proteolipid protein. To obtain more information on the nature of the multiple bands observed, we have carried out a detailed study of bovine white matter proteolipid and myelin proteins upon SDS polyacrylamide gel electrophoresis in the presence and absence of urea.

Our approach has been to make use of the Ferguson relationship

$$\log R_F = Y_0 - K_R T$$

where R_F is the relative mobility of a protein species, Y_0 the y intercept or free mobility, K_R the slope of the line or the retardation coefficient, and T the per cent total acrylamide concentration. Theoretical treatment of this relationship and its applicability to gel electrophoresis have been thoroughly reviewed (Chrambach and Rodbard, 1971). Briefly, for any protein, a plot of log R_F *vs.* T (Ferguson plot) results in a straight line the slope of which is proportional to molecular size. Separation of two protein species based on size gives nonparallel lines having a constant Y_0. On the other hand, separation based on charge differences results in two parallel lines. In situations where both size and charge effects are operative, the two lines cross at a point where the mobilities of the two species are indistinguishable. In the presence of SDS, proteins can be separated on the basis of size only if their charge to mass ratio is constant and the protein–SDS complexes behave like rod-like particles in which the particle length is a function of molecular size (Reynolds and Tanford, 1970a,b). A number of proteins, which have physical and chemical properties that interfere with SDS binding, show deviations upon SDS gel electrophoresis (Tung and Knight, 1971; Panyim and Chalkley, 1971; Furthmayer and Timpl, 1971). The Ferguson plot can reveal such deviations and

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; Temed, *N,N,N',N'*-tetramethylethylenediamine; HMW, high molecular weight band; LMW, low molecular weight band; PLP, proteolipid protein band; BP, basic protein.

provide additional information on the protein. The present paper reports the application of the Ferguson plot to the analysis of the multiple bands observed in white matter proteolipid and myelin.

Experimental Section

Preparation of White Matter Proteolipid and Myelin. Bovine white matter proteolipid was prepared by the emulsion-centrifugation method of Folch *et al.* (1959) but with omission of the final organic solvent extractions. Briefly, a washed total lipid extract of the tissue was evaporated to dryness *in vacuo*, emulsified in cold distilled water, and centrifuged at 4000*g* for 45 min at 4°. The emulsification and centrifugation were repeated until the supernatant fluid was only slightly opalescent. The pellet was suspended in cold distilled water and centrifuged at 200*g* for 10 min, and the final pellet was lyophilized. The preparations contained about 40% protein. Bovine myelin was prepared from frozen brain white matter by the method of Autilio *et al.* (1964) up to the stage of crude myelin. The final pellet was washed five times with water and lyophilized.

Analytical Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in a water-cooled 18-tube unit (Hoeffer Instrument Co., San Francisco, Calif.). Gel tubes (12.5 × 0.5 cm) were soaked in chromic acid overnight, rinsed thoroughly with distilled water, washed with 0.2% Photo-Flo solution (Eastman Kodak Co.), and oven dried before use. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine (Temed), and ammonium persulfate were obtained from Bio-Rad Laboratories (Richmond, Calif.). Tris(hydroxymethyl)aminomethane was obtained from Sigma Chemical Co. (St. Louis, Mo.). Urea and sodium dodecyl sulfate of ultrapure quality were purchased from Schwarz/Mann (Orangeburg, N. Y.).

Two gel systems were used. The procedure for the SDS gels was essentially that of Weber and Osborne (1969), as modified by Agrawal *et al.* (1972), and for the SDS–urea gels that of Swank and Munkres (1971). The detailed composition and running conditions for each system are outlined below. Total concentrations of acrylamide (% *T*) and cross-linking agent (% *C*) are described by the notation of Hjerten (1962).

SDS Gels. (*T* = 7.0–14.5%, *C* = 2.7%). A stock buffer (pH 7.1) was prepared containing 1.95 g of $NaH_2PO_4 \cdot H_2O$, 5.12 g of Na_2HPO_4, and 2.0 g of SDS per liter. A stock acrylamide solution was prepared by dissolving 11.675 g of acrylamide and 0.325 g of *N,N'*-methylenebisacrylamide in 25 ml of stock buffer and diluting to 40 ml with distilled water. For gels containing 7.0, 8.5, 10.0, 11.5, 13.0, and 14.5% *T* the following volumes of stock gel solution (ml) were pipetted into each of six graduated centrifuge tubes: 1.4, 1.7, 2.0, 2.3, 2.6, 2.9. To each tube was added approximately 9 μl of Temed and sufficient running buffer (stock buffer diluted 1:1 with distilled water) to a volume of 5 ml. Each tube was then degassed for 1 min on a water aspirator. To form gels, 1 ml of catalyst solution (1.2 mg of ammonium persulfate per ml of running buffer) was added to each tube, the contents were mixed gently, and aliquots were pipetted into three electrophoresis tubes to a height of 9.5 cm. The gels were layered with 2-butanol to obtain a smooth surface. Polymerization was complete within 30 min with circulating tap water for cooling. After sample application, upper and lower reservoirs were filled with running buffer and electrophoresis was carried out for 16 hr at a constant current of 12 mA/18 tubes.

SDS–Urea Gels (*T* = 7.0–14.5%, *C* = 3.6%). A stock buffer was prepared with 34.9 g of Tris, 10.2 ml of H_3PO_4, and 1.5 g of SDS adjusted to pH 7.0 with Tris and diluted to 300 ml with distilled water. A urea–buffer solution was prepared by adding 7.2 g of urea to 3 ml of stock buffer and diluting to 15 ml with distilled water. A urea–acrylamide stock solution was prepared containing 12 g of urea, 3.615 g of acrylamide, 0.135 g of *N,N'*-methylenebisacrylamide and 5 ml of stock buffer and diluting to 25 ml with distilled water. All solutions were degassed as described above. To prepare gels of 7.0, 8.5, 10.0, 11.5, 13.0, and 14.5 % *T*, the following volumes (ml) of urea–acrylamide solution were pipetted into each of six graduated centrifuge tubes: 2.8, 3.4, 4.0, 4.6, 5.2, 5.8. To each tube was added approximately 9 μl of Temed and sufficient urea–buffer solution to bring the volume to 5.8 ml. To form gels, 0.2 ml of catalyst solution (6 mg of ammonium persulfate per ml of urea buffer) was added to each tube and three gels polymerized for each % *T* as described above. Stock buffer was diluted 1:4 with distilled water to constitute the running buffer. Current was set at 18 mA/18 tubes and electrophoresis carried out for 16 hr.

Sample Preparation. Myelin or white matter proteolipid containing approximately 3 mg of protein was partially delipidated by extracting three times with 3 ml of anhydrous ether and three times with 3 ml of acetone. The residue was dried with nitrogen and emulsified in a medium containing 100 mg of SDS, 150 mg of dithiothreitol, and 800 mg of sucrose per 10 ml of distilled water (solution A) at a concentration of 1 mg of protein per ml. To obtain complete solubilization the mixture was heated in a boiling water bath for 5 min. Each gel surface was rinsed carefully with distilled water and 20 μl of sample applied followed by 10 μl of Bromophenol Blue (0.01% aqueous solution) as a tracking dye. Unreduced samples were treated similarly with the omission of dithiothreitol from solution A. Protein standards were conalbumin, catalase, glucose-6-phosphate dehydrogenase, myoglobin, beef heart cytochrome *c* (Sigma Chemical Co.), chymotrypsinogen A, and ovalbumin (Worthington Biochemical Corp.). Approximately 1.5 μg of each standard in 20 μl of solution A was applied to the gels. Purified rabbit large basic protein (molecular weight 18,400) was a gift from Dr. M. W. Kies.

Staining and Destaining. After electrophoresis, gels were removed from the tubes and the positions of the tracking dye marked by a small piece of copper wire. Gels were stained individually for 24 hr in 0.25% Coomassie Blue (Sigma Chemical Co.) dissolved in 50% (v/v) methanol containing 10% (v/v) acetic acid. Rapid destaining was carried out in the same solvent system in a diffusion destainer (Hoeffer Instrument Co.) for 8 hr followed by slower destaining in 5% (v/v) methanol containing 7.5% (v/v) acetic acid overnight. The position of the copper wire as well as each stained protein band was measured immediately after destaining.

Preparative Gel Electrophoresis. Preparative fractionation of white matter proteolipid was performed in an apparatus manufactured by Shandon Southern Instruments, Inc. (Sewickley, Pa.). The procedure for the preparation of the 7% SDS–urea gels described above was scaled up to provide sufficient material to form a gel ring of 6.5 cm in height. Sample preparation was identical with that for analytical gels except that 15 mg of protein was applied. The elution buffer was the same as the running buffer and the flow rate was adjusted by gravity to produce 2.4 ml/tube in 13 min. A current of 50 mA was applied for 24 hr. Fractions were read at 280 nm in a Beckman DB spectrophotometer. Aliquots of 100 μl from selected fractions were concentrated *in vacuo*, taken up

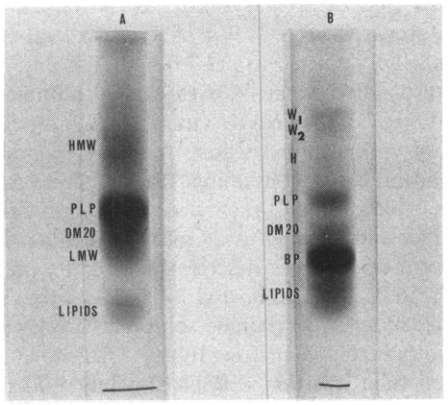

FIGURE 1: SDS polyacrylamide gel patterns of (A) white matter proteolipid, 20 μg of protein; (B) central nervous system myelin, 20 μg of protein. Electrophoresis was carried out as described under Methods. Gels shown contain 10% total acrylamide and 2.7% *N,N'*-methylenebisacrylamide. HMW, high molecular weight band; PLP, major proteolipid band; DM-20, protein band observed by Agrawal *et al.*; LMW, low molecular weight band; W_1, W_2, Wolfgram proteins; H, high molecular weight band in myelin; BP, basic protein. The basic protein binds more dye per mg of protein than does other myelin proteins so that visual observation of gels gives an incorrect impression of the amount of basic protein present.

in 50 μl of solution A, boiled for 5 min in a water bath, and applied to the gels.

Results

SDS Gels. Typical gel patterns of white matter proteolipid and myelin are shown in Figure 1. Four protein bands are observed in white matter proteolipid: a diffuse high molecular weight band (HMW), the major proteolipid protein band (PLP), the DM-20, and a low molecular weight band (LMW) immediately below the DM-20. The zone indicated as lipids is white and is sometimes lightly stained. In myelin, six bands are observed: a slow moving doublet (W_1, W_2) designated as the Wolfgram proteins, a somewhat diffuse band H, the PLP, the DM-20 and the heavily stained basic protein (BP). The position of the basic protein was verified by comparison with an authentic basic protein standard. A lipid zone is also present.

The Ferguson plots of standard proteins and the bands observed in white matter proteolipid and myelin are shown in Figure 2. Each point is derived from the average relative mobility of three runs which are reproducible to within $\pm 3\%$. Mobilities for the high molecular weight bands are less reproducible especially at high % *T* (13.0 and 14.5%) where they deviate considerably from linearity. Such points are therefore rejected in obtaining the least-squares regression line for each protein band. Figure 2B shows that W_1 and W_2 give lines that are separate from the other lines suggesting that the Wolfgram proteins of myelin are absent from the proteolipid preparation. The line for HMW in white matter proteolipid and that for H in myelin appear to differ from one another but the diffuse nature of both bands limits the accuracy of these lines. The lines for PLP and DM-20 from white matter proteolipid and myelin are coincident indicating that the corresponding proteins are identical in the two preparations. The line for band LMW is close to but not coincident with that of the myelin basic protein. Further proof for their nonidentity was obtained by coelectrophoresis of pure rabbit basic protein with white matter proteolipid: the LMW clearly migrated more slowly than the basic protein and therefore differs from it. The lines for H, PLP, DM-20, LMW, and basic protein have approximately the same Y_0 (1.18 $\pm$ 0.01 S.D.) as those of chymotrypsinogen A, myoglobin, and cytochrome *c*. The Y_0 of higher molecular weight bands HMW, W_1, W_2, as well as glucose-6-phosphate dehydrogenase, ovalbumin, catalase, and conalbumin increases with increasing molecular size.

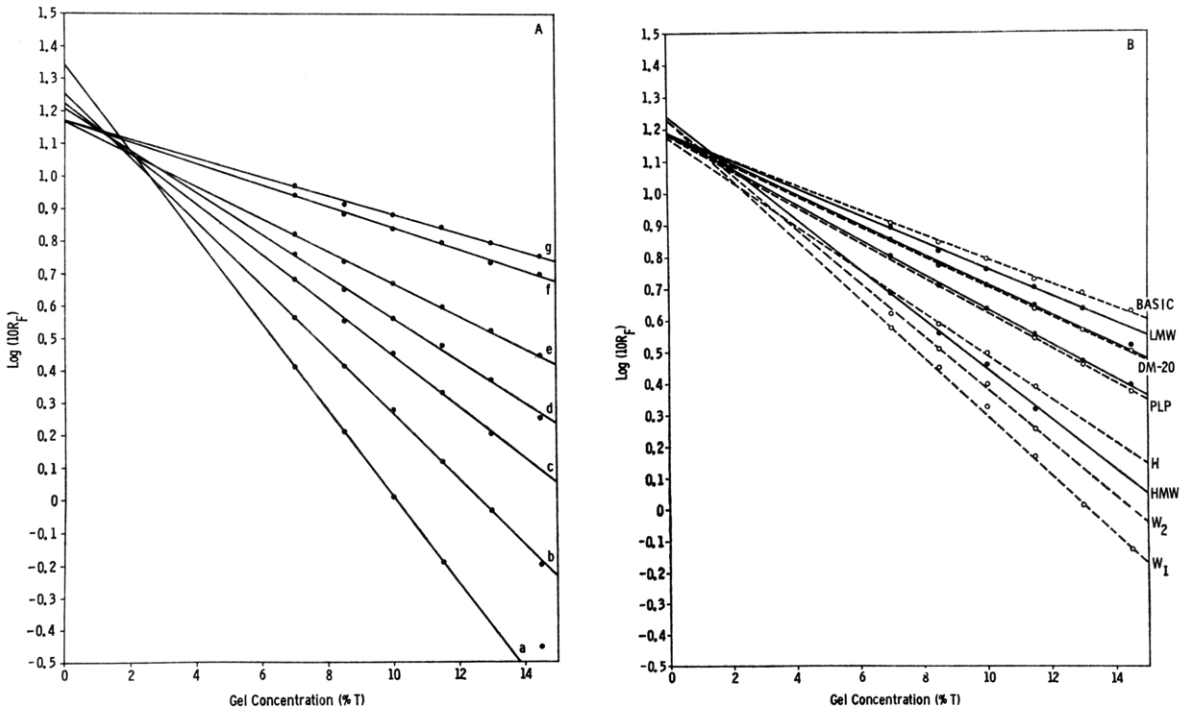

FIGURE 2: Ferguson plots of bands observed upon electrophoresis in SDS polyacrylamide gels. (A) Plot of standard proteins: (a) conalbumin, (b) catalase, (c) ovalbumin, (d) glucose-6-phosphate dehydrogenase, (e) chymotrypsinogen A, (f) myoglybin, (g) cytochrome *c*. (B) Plot of white matter proteolipid (-●-●-●-) and myelin proteins (-O-O-O-).

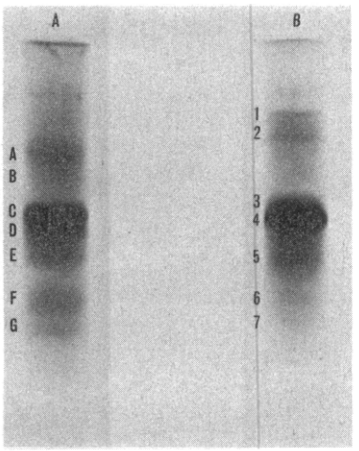

FIGURE 3: SDS–urea polyacrylamide gel patterns of (A) white matter proteolipid, 20 μg of protein; (B) central nervous system myelin, 20 μg of protein. Electrophoresis was carried out as described under Methods. Gels shown contain 10% total acrylamide, 3.5% *N,N'*-methylenebisacrylamide, and 8 M urea. See text for description of bands A–G and 1–7.

SDS–Urea Gels. In the presence of 8 M urea, seven bands are observed for both white matter proteolipid (A–G) and myelin (1–7) (Figure 3). The bands are generally sharper and more reproducible than those in the SDS gels. Figure 4 shows the Ferguson plots of the standard proteins and the bands of white matter proteolipid and myelin. The standard proteins have an average Y_0 of 1.13 ● 0.03 S.D. (Figure 4A). Of the seven lines of white matter proteolipid, only C and D are parallel and all except line C have approximately the same Y_0, 1.26 ± 0.01 S.D. (Figure 4B). The parallel Ferguson plots for bands C and D, which visually appear on the gels as a doublet, indicate that these two bands are separating on the basis of charge differences.

Preparative electrophoresis of white matter proteolipid provided information for the identification of the bands seen on urea gels and their relation to those seen on SDS gels. Figure 5 shows the elution profile of a preparative separation together with the patterns on analytical gels, both with and without urea, of selected fractions from the elution. Peak I contains tracking dye and lipid material. Peak II shows mainly bands F and G in the SDS–urea gels and gives a diffuse band corresponding to the position of LMW in the SDS gels. The initial portion of peak III is enriched in band E which corresponds to the DM-20, but also contains some of bands C and D which together are equivalent to the PLP band seen in the SDS gels. The latter portion of peak III contains bands C and D with little or no trace of band E (DM-20). In all of the fractions from peak III, the analytical gels showed higher molecular weight bands which correspond to bands A and B or to HMW in the respective gel systems. These bands probably result from aggregation of lower molecular weight forms since their amounts appeared to increase when the fractions were tested several weeks after elution. However, we have not been able to obtain a fraction containing only high molecular weight material. It is possible that at the sample concentration used for preparative electrophoresis (3 mg of protein/ml) the high molecular weight material is so highly aggregated that it is not eluted.

In myelin, the seven lines of the Ferguson plot fall into three categories according to their values of Y_0 (Figure 4C). Lines 1 and 2 have the same Y_0, 1.02; lines 4 and 5 have a Y_0 of 1.13. Lines 3, 6, and 7 are coincident with lines C, F, and G of white matter proteolipid, respectively, and have a Y_0 of 1.26.

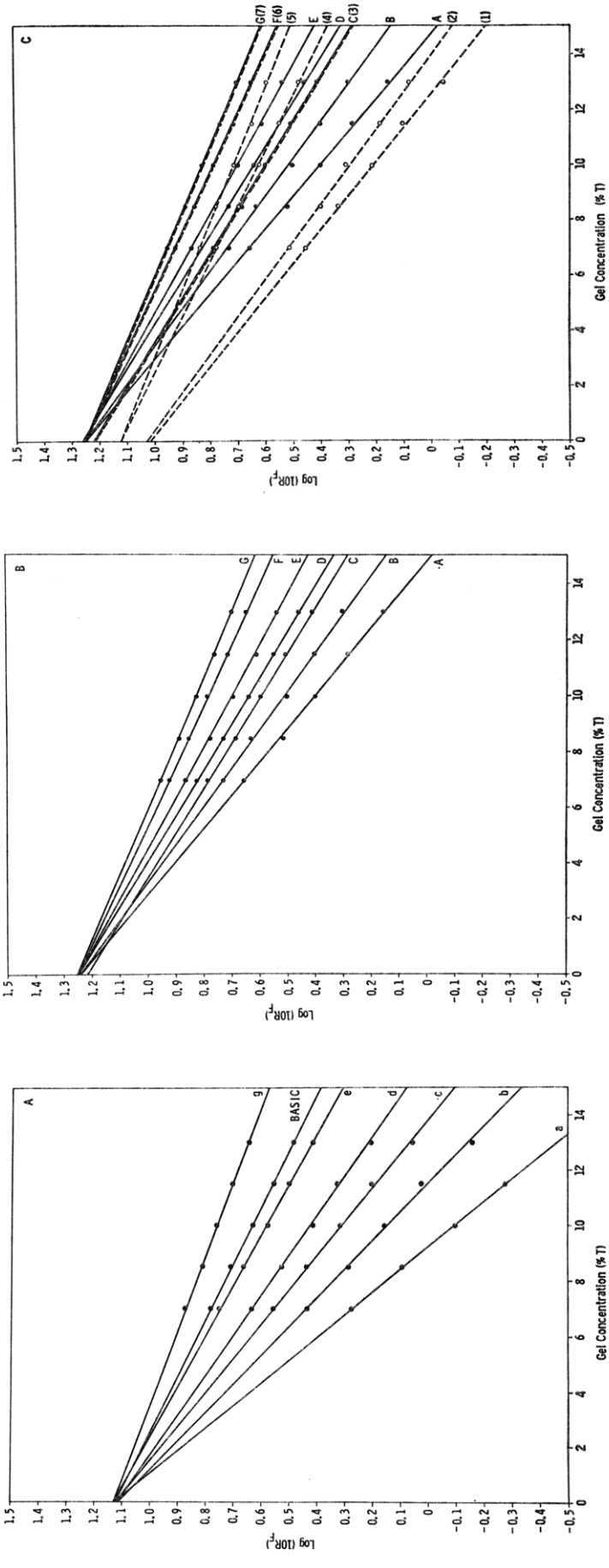

FIGURE 4: Ferguson plots of bands observed upon electrophoresis in the SDS–urea system: (A) standard proteins, notations as in Figure 2A (basic = rabbit large basic protein); (B) white matter proteolipid; (C) white matter proteolipid (-●-●-●-) and myelin proteins (-O-O-O-).

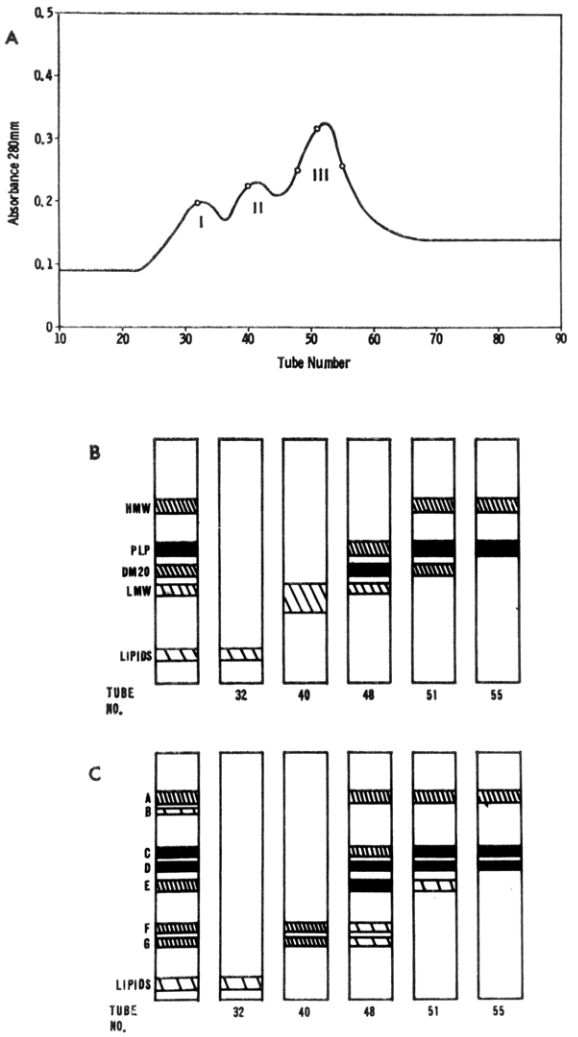

FIGURE 5: Elution profile of preparative electrophoresis of white matter proteolipid and comparison of gel patterns of selected fractions using the two gel systems described under Methods: (A) elution profile of preparative separation of white matter proteolipid on a 7% SDS–urea gel column, protein load was 15 mg; (B) analytical SDS–gel patterns of selected fractions from the elution; (C) analytical SDS–urea gel patterns of the same samples as in (B). Degree of shading corresponds to intensity of bands.

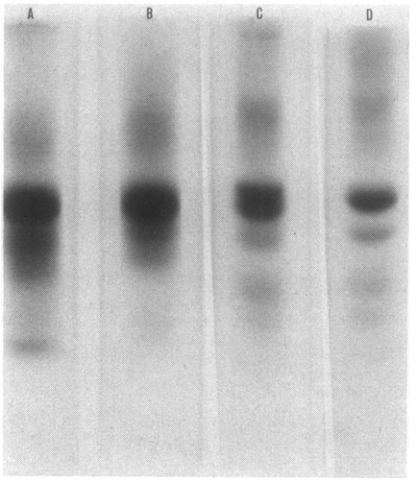

FIGURE 6: Polyacrylamide gel patterns of white matter proteolipid in the presence and absence of reducing agent; 20 μg of protein was applied on each gel: (A) and (B), SDS gels, reduced and unreduced, respectively; (C) and (D), SDS–urea gels, reduced and unreduced, respectively. "Reduced" samples were dissolved in solution A as described under Methods. "Unreduced" samples were treated in the same manner except that dithiothreitol was omitted from solution A.

Band 4 was identified as the basic protein since its line of the Ferguson plot is coincident with that of the authentic basic protein used as a standard. Depending on the gel concentration the basic protein can obscure one or the other of the two PLP bands. In gels of 10–13% T, the basic protein overlaps with band D whereas in gels of 7 and 8.5% T it overlaps with band C. Coelectrophoresis of pure basic protein with white matter proteolipid in a 10% T SDS–urea gel further confirms the position of the basic protein band. The basic protein overlaps band D and the latter is consequently not visible as a separate band. Figure 4C also shows that the line for band 5 is not coincident with that of band E and therefore band 5 apparently does not correspond to DM-20. It could be related, however, to the basic protein since their respective lines have the same Y_0. Bands 1 and 2 give lines that are separate from any others and, as in the case of the SDS gels, these are designated as the Wolfgram proteins.

Band Patterns of Reduced and Unreduced White Matter Proteolipid. The band patterns of reduced and unreduced white matter proteolipid were studied in the two gel systems (Figure 6). In SDS gels the band patterns of the reduced and unreduced samples do not differ from one another. In SDS–urea gels, bands C and D, which are seen as a doublet when reducing agent is present in the sample, appear as a single band when the reducing agent is omitted. Other bands do not differ.

Molecular Weight Determination. Two different linear relationships can be used to plot calibration curves for the determination of molecular weights: (1) log molecular weight *vs.* R_F which gives valid results only when the free electrophoretic mobilities (Y_0) of the standard and unknown proteins are the same and (2) K_R *vs.* molecular weight, in which K_R is the slope of the Ferguson plot and is independent of Y_0. Calibration curves based on these two relationships are shown in Figure 7 and the molecular weights calculated therefrom for the bands observed in white matter proteolipid and myelin are listed in Table I. The molecular weights can be interpreted only in relation to the Ferguson plots of the respective bands (Figures 2 and 4). In SDS gels, the two methods of plotting the data give approximately the same results except for some discrepancies in the values for bands in the higher molecular weight region where the Y_0 values of the standards and the unknowns differ. The average molecular weight determined for the PLP at various gel concentrations is 30,000 ± 540 S.D. (n = 12) and for DM-20 is 24,300 ± 640 S.D. (n = 9).

In the SDS–urea gels, the molecular weights determined for the bands in white matter proteolipid differ according to the method of calculation. Specifically, lower molecular weights are obtained from the log molecular weight *vs.* R_F plot (method 1). Since the average Y_0 of the standard proteins is lower than that of the white matter proteolipid bands in this gel system (Figure 4A and B), comparison of their respective relative mobilities is not valid. In addition, the two bands which show parallel Ferguson plots (bands C and D) are separating by charge and not by size and correct molecular weights for these bands can be expected only from the K_R *vs.* molecular weight relationship (method 2). Values determined by this method for bands A, C and D, and E are comparable to those obtained on the SDS gels for HMW, PLP, and DM-20, respectively. Band F has a slightly lower molecular weight than does the LMW band.

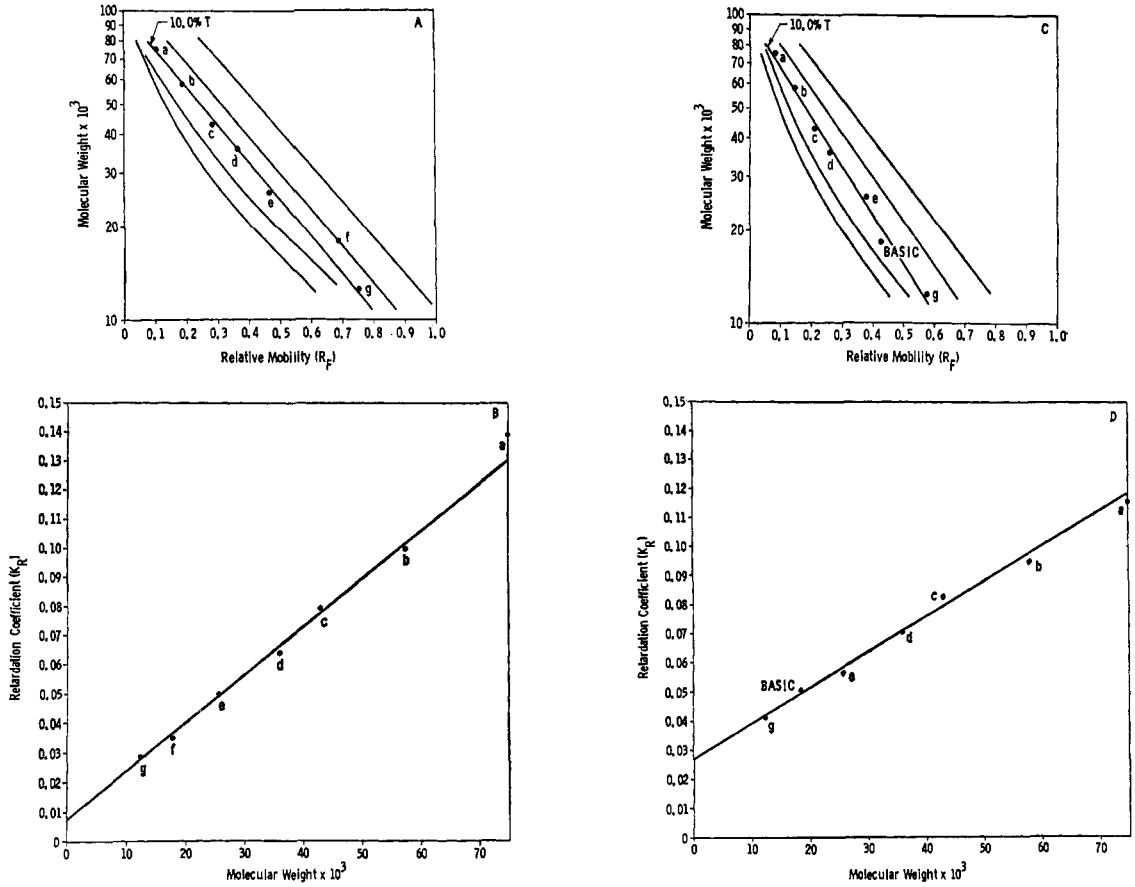

FIGURE 7: Molecular weight calibration curves of standard proteins, notations as in Figure 2A: (A) and (B) SDS gels; (A) log molecular weight *vs.* R_F (method 1), (B) K_R *vs.* molecular weight (method 2); (C) and (D) SDS–urea gels, (C) log molecular weight *vs.* R_F (method 1), (D) K_R *vs.* molecular weight (method 2). For clarity, only points for 10% gels are identified in method 1.

In myelin the situation is complicated both by the proximity of the heavily staining basic protein band (band 4) to the proteolipid doublet and by our inability to locate the DM-20. The Wolfgram type proteins (bands 1 and 2) have a lower Y_0 than the standards and therefore higher molecular weights are obtained from method 1 than from method 2. On the assumption that only the latter method is theoretically valid, it is apparent that the molecular weights calculated for the Wolfgram proteins differ depending on the presence or absence of urea in the gel system, an observation which cannot be explained by the present data. The proteolipid bands 3, 6, and 7 all give lower molecular weights by method 1 for the reasons noted above for the white matter proteolipids. On the other hand, the molecular weights obtained for these bands upon plotting the data according to method 2 are the same as those for comparable bands in white matter proteolipid. Furthermore, the major proteolipid band (band 3) has a molecular weight identical with that obtained on SDS gels, *i.e.*, 30,000. The basic protein of myelin shows a Ferguson plot which is coincident with that of pure rabbit basic protein. The molecular weight of the myelin basic protein as determined from the log molecular weight *vs.* R_F plot of the SDS–urea gels is higher than the accepted value. In the molecular weight region below 20,000, the standard curve exhibits a change of slope with a consequent molecular weight deviation which can amount to as much as 18% (Swank and Munkres, 1971). Since we did not use a sufficient number of standards to specifically establish a valid standard curve for this region, an error in the molecular weight of the basic protein is to be expected. Using method 2, a value of 19,800 is obtained which is close to that of the basic protein standard (18,400). Band 5 has the same Y_0 as the basic protein but has a small K_R which explains in part the different molecular weights obtained by the two methods of calculation. Although we cannot identify this band, it apparently does not correspond to DM-20.

Evidence for an Oligomeric Series. A mathematical relationship between molecular weight and band number has been developed for linear aggregates based on the relationship between K_R and molecular weight. Theoretical and experimental justification of this relationship has been provided by Ugel *et al.* (1971). When a plot of K_R *vs.* band number is extrapolated to zero band number, the value obtained for K_R is identical with that obtained from extrapolating the K_R *vs.* molecular weight plot of the standard proteins to zero molecular weight. Accordingly, we have plotted the K_R for each of the proteolipid bands against arbitrary band numbers and positioned the line thus obtained so that it gives the same intercept as the plot of K_R *vs.* molecular weight of the standard proteins (Figure 8). In both SDS and SDS–urea gels, the major proteolipid band PLP appears as a hexamer, the DM-20 as a pentamer, and LMW as a tetramer. The K_R of the theoretical monomeric form (band number 1) would correspond to a molecular weight of 5000 in both gel systems.

Discussion

The results of this study confirm the existence of multiple bands upon SDS gel electrophoresis of bovine white matter proteolipid. In addition to the major proteolipid protein band PLP which has been observed by all investigators, the DM-20

TABLE I: Molecular Weight Determinations of Proteolipid and Myelin Proteins Based on Different Methods of Calculation.

	SDS Gels			SDS–Urea Gels		
	Bands	log Mol wt *vs.* R_F (Method 1)[a]	K_R *vs.* mol wt (Method 2)[a]	Bands	log Mol wt *vs.* R_F (Method 1)[a]	K_R *vs.* mol wt (Method 2)[a]
White matter proteolipid	HMW	44,000	45,800	A	38,000	48,800
				B	32,000	36,000
	PLP	29,500	29,500	C	24,000	30,800
				D	20,400	29,500
	DM-20	23,800	24,600	E	16,700	25,200
	LMW	19,600	20,000	F	11,600	17,500
				G	9,950	15,000
Myelin	W_1	54,800	51,200	1	52,000	44,300
	W_2	49,000	45,600	2	45,200	35,800
	H	41,300	37,100	3	24,500	29,900
	PLP	30,000	30,600	4	21,800	19,800
	DM-20	23,600	24,300	5	16,200	12,100
	Basic	17,700	18,900	6	11,700	16,800
				7	9,970	14,600

[a] Method 1, molecular weights were obtained from the standard curves of R_F plotted against log of molecular weight shown in Figure 7A for the SDS gels and Figure 7C for the SDS–urea gels. Data are from gels of 10% total acrylamide; method 2, molecular weights were obtained from the standard curves of K_R plotted against molecular weight shown in Figure 7B for the SDS gels and Figure 7D for the SDS–urea gels.

described by Agrawal *et al.* (1972) is also present. The coincidence of the Ferguson plots of the DM-20 from white matter proteolipid and myelin attests to their identity (Figure 2). In white matter proteolipid a third band LMW is observed which is not the same as basic protein and, if it is present in myelin, would be obscured in the SDS gels by the heavily staining basic protein. However, in the SDS–urea gels, LMW appears as bands F and G which correspond to bands 6 and 7 of myelin. Thus, it seems clear that both the DM-20 and LMW observed in white matter proteolipid also occur in myelin. Under a different set of electrophoretic conditions Greenfield *et al.* (1971) and Morell *et al.* (1972) have observed two intermediate bands between the PLP and the basic protein of mouse myelin which could correspond to our DM-20 and LMW bands. The bands observed in the low molecular weight regions do not originate from lipids or lipid–protein interactions since similar bands are observed with proteolipid apoprotein preparations (D. Sakura and J. Folch-Pi, in preparation).

The presence of multiple bands does not necessarily indicate the existence of several proteins. The data relating K_R and band number (Figure 8) support the concept that all the bands observed in white matter proteolipid represent specific states of aggregation of a single protein. Final proof for this suggestion will depend on the development of procedures for aggregating and disaggregating the proteolipids under controlled conditions and on further analysis of the individual bands. In the two gel systems studied, the theroetical monomeric form appears to have a molecular weight of 5000; the major PLP band would then be the hexamer, the DM-20 the pentamer, and LMW the tetramer. The trimer is the lowest band which is consistently observed in SDS–urea gels. However, when these gels are overloaded, two additional fast moving bands are observed below bands F and G, and these would presumably be the dimeric and monomeric forms. The higher aggregates (bands A and B) apparently correspond to the heptameric and nonameric forms. Folch-Pi (1963) has calculated from the amino acid composition of white matter proteolipid a possible monomeric molecular size of 12,500 based on two methionine residues per molecule. If only a single methionine residue occurred, the molecular weight of the monomeric form would be close to the 5000 that we have determined. Further evidence for the existence of the monomer is provided by the fact that a portion of the proteolipid in both the white matter total lipid extract and in myelin is dialyzable through cellophane membranes which are impermeable to molecules above a molecular size of 12,000 (Thompson *et al.*, 1963; Lees *et al.*, 1964; Folch-Pi, 1971). It is of interest that the Neurospora structural protein which is also a membrane protein and has certain features in common with proteolipids has been shown on the basis of gel electrophoresis to consist of an oligomeric series with a monomeric molecular weight of 3,700 (Munkres *et al.*, 1971).

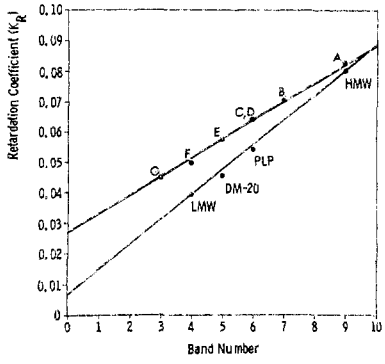

FIGURE 8: Plot of K_R *vs.* band number for bands of white matter proteolipid observed in SDS gels and SDS–urea gels. See text (Results) for explanation of how band numbers were obtained.

In the absence of reducing agent, the band pattern of white matter proteolipid in SDS gels does not differ from that of the reduced sample. In the SDS–urea gels the only difference between the reduced and the unreduced sample occurs in the PLP doublet which appears as a single band in the unreduced

sample. The lower molecular weight bands are present irrespective of reduction and are not produced as a result of reduction of the higher molecular weight material. The different species therefore apparently contain only intramolecular and not intermolecular disulfide linkages. The PLP doublet (bands C and D) observed for the reduced sample in SDS–urea gels gives parallel Ferguson plots indicating that these proteins are isomeric and are separating on the basis of charge. In the unreduced sample, a single band with an intermediate mobility appears. Thus the hexameric PLP is capable of existing in two isomeric forms upon reduction and denaturation in the presence of urea. This would be consistent with the formation of isomers as a result of random recombination of reduced disulfide bonds. A similar situation has been reported for ribonuclease in denaturing concentrations of urea (Haber and Anfinsen, 1962). For the proteolipids, apparently only the hexamer possesses the correct steric arrangement for this recombination to occur. These isomers would have different sites for the binding of SDS or small ligands and therefore would appear as charge isomers. Alternatively, the charge isomers may be a consequence of two conformational states which migrate differently. In phenol–acetic acid–urea gels, the mobility of a myelin protein which appears similar to the proteolipid protein has been shown to be conformation dependent (Anthony and Moscarello, 1971).

The molecular weights which we report for PLP and DM-20 are 30,000 and 25,000, respectively; the same values have been independently obtained for bovine proteolipid apoprotein by D. Sakura and J. Folch-Pi (in preparation). Previously, Eng (1971) reported 24,000 for proteolipid in human myelin and Agrawal *et al.* (1972) obtained 24,000 and 20,000 for proteolipid protein and DM-20 from rat myelin. Recently, Nicot *et al.* (1973) observed band patterns for bovine proteolipid apoprotein similar to ours and reported molecular weights of 25,000 and 20,000 for the two major bands. All of these investigators have determined molecular weights using the plot of log molecular weight *vs.* R_F which is convenient because only a single gel concentration is needed. However, the tendency of proteolipids to aggregate may produce SDS binding properties which are not the same as those of the globular, standard proteins. The present study shows that in SDS–urea gels, the Ferguson plots of the proteolipids have a higher Y_0, and consequently a higher free electrophoretic mobility, than that of the standard proteins. This suggests that the proteolipids, which are hydrophobic membrane proteins, may be binding more SDS per unit weight than the water soluble standards. Under these circumstances, the mobilities of the two types of proteins cannot be directly compared by using the log molecular weight *vs.* R_F plot, and correct molecular weights can only be obtained from the K_R *vs.* molecular weight plot which is independent of Y_0. In SDS gels, the proteolipids have approximately the same Y_0 as the standard proteins in the pertinent molecular weight region. However, even among standard proteins the Y_0 increases with molecular weight. This factor causes a degree of uncertainty which can result in significant differences in molecular weights determined in different laboratories when SDS gels at only a single gel concentration are used. Comparable variations in the free electrophoretic mobilities of a series of standard proteins have been observed by Banker and Cotman (1972) who also pointed out the hazards of depending on a single gel concentration for molecular weight determination without prior validation of the procedures by methods based on the Ferguson relationship. In their gel electrophoretic system, synaptic membrane proteins had the same free electrophoretic mobility as the standard proteins. The present study shows that the behavior of proteolipids on gels differs from that of standard proteins and therefore generalizations for all membrane proteins are unwarranted. In the case of the proteolipids, the plot of K_R *vs.* molecular weight is the method of choice for the determination of molecular weight since it is independent of Y_0.

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Study of *Escherichia coli* Ribosomes by Intensity Fluctuation Spectroscopy of Scattered Laser Light†

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ABSTRACT: The ribosomes of *Escherichia coli* have been studied by the technique of light-scattering intensity fluctuation spectroscopy. A digital photocount correlator was used to analyze the time evolution of the scattered field and thus characterize the hydrodynamic properties of the ribosomes in solution with accurate determinations of D, the translational diffusion coefficient. To selectively study the molecules of interest, the light-scattering technique was combined with the technique of band or zonal sedimentation in a sucrose gradient. The light-scattering experiments were carried out immediately after centrifugation directly on the different macromolecular bands in the sucrose gradient in the centrifuge tube. The values of $D^{\circ}_{20,w}$ determined in this way are $(1.71 \pm 0.03) \times 10^{-7}$ cm²/sec, $(1.90 \pm 0.03) \times 10^{-7}$ cm²/sec, and $(2.18 \pm 0.03) \times 10^{-7}$ cm²/sec for the 70S ribosomes and the 50S and 30S ribosome subunits, respectively. Combining these measured values of D with literature values of the sedimentation coefficients and partial specific volumes, values of the ribosome molecular weights were calculated. These are, in millions, 2.49 ± 0.06, 1.55 ± 0.04, and 0.87 ± 0.02, in the same order as above. Finally, the results of this work were compared to those of other techniques, and discussed in terms of the size, shape, and degree of hydration of the ribosome molecules.

In the work reported here, the ribosomes of *Escherichia coli* are studied by the technique of laser light scattering IFS[1] (see, for example, Clark *et al.*, 1970; Cummins and Swinney, 1970; Chu, 1970; Foord *et al.*, 1970; Pusey *et al.*, 1974). IFS is a recently developed technique in which the coherent radiation of a laser light source is used to probe the dynamics of systems of scatters, in this case, biological macromolecules in solution. The field from each scatterer acts as a reference signal with which we can detect the phase fluctuations in the field from each other scatterer. Molecular motions over distances of the order of a wavelength of light change the relative phases at the detector of the scattered electric field from different scattering centers, thus changing the detected light intensity. The coherence time of the scattered light, the characteristic time scale on which light intensity fluctuates, is thus directly related to the hydrodynamic properties of the scattering molecules. In this study, we used a digital photocount correlator to analyze the scattered field.

For scatterers of linear dimensions small compared to a wavelength of light, such as those studied here, the coherence time of the scattered field is inversely proportional to D, the macromolecular translational diffusion coefficient (Pecora, 1964). For nonspherical molecules of linear dimensions comparable to a wavelength of light, one can investigate rotational diffusion (Pecora, 1968; Cummins *et al.*, 1969; Schaefer *et al.*, 1971), and internal molecular flexing modes (Pecora, 1965; Fujime, 1970).

As a general analytical technique, however, IFS has two inherent limitations.

i. Lack of Specificity. All solute particles, desired and undesired, scatter light. The greatest damage can be done by large aggregates and dust, which, even in minute concentrations, can swamp the signal from the molecules of interest.

ii. Poor Resolution of Different Components. Light-scattering data are relatively insensitive to solution polydispersity. The best one can do in general (Koppel, 1972; Pusey *et al.*, 1974) is to calculate the first two or three moments of the distribution of molecular size (actually the distribution of D); but additional information is needed for a detailed characterization.

In an attempt to overcome the above limitations, the work reported here combines IFS with the technique of band or zonal sedimentation in the ultracentrifuge. For other techniques as well as for light-scattering measurements, band sedimentation on a sucrose gradient has frequently been used as one of the final sample preparative procedures. Traditionally, fractions are collected, dialyzed against buffer to remove the sucrose, and analyzed separately. The experimental work reported here takes the next logical step: The light-scattering analysis is carried out immediately after centrifugation directly on the different macromolecular bands in the sucrose gradient in the centrifuge tube. One can thus selectively study the molecules of interest, separated from a host of other potential scatterers, with an ease and reliability not previously possible.

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[1] Abbreviation used is: IFS, intensity fluctuation spectroscopy.