Molecular-Weight Estimates of Milk-Fat-Globule-Membrane Protein–Sodium Dodecyl Sulphate Complexes by Electrophoresis in Gradient Acrylamide Gels

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The molecular weights of milk-fat-globule-membrane proteins solubilized in sodium dodecyl sulphate were estimated by gradient gel electrophoresis. Standard curves were calibrated from both protein and glycoprotein markers of known molecular weight. Six major proteins were observed with Coomassie Blue staining and six with periodic acid-Schiff staining. The behaviour of the membrane proteins and the marker proteins was compared on several different single strength sodium dodecyl sulphate–polyacrylamide gels between 3 and 12% (w/v). The results were used to calculate the free electrophoretic mobility and retardation coefficient of each protein. Glycoprotein markers had a significantly lower mean free electrophoretic-mobility value than the protein markers. Three of the milk-fat-globule-membrane glycoproteins were shown to be independent of any of the Coomassie Blue-stained bands. On the basis of a comparison of the free electrophoretic-mobility and retardation-coefficient values of markers and unknown proteins the most appropriate standard curve for molecular-weight estimation was chosen.

Since its introduction by Shapiro *et al.* (1967) the technique of sodium dodecyl sulphate–polyacrylamide gel electrophoresis has been widely used as an apparently simple, reliable method for the estimation of protein molecular weights. However, an increasing number of proteins have been shown to behave anomalously on sodium dodecyl sulphate–polyacrylamide gels. Examples of such anomalies have been described by Banker & Cotman (1972) and include the behaviour of membrane glycoproteins.

The molecular weight of a glycoprotein on sodium dodecyl sulphate–polyacrylamide gels, estimated from a non glycoprotein standard curve, depends on the acrylamide concentration in the gel (Bretscher, 1971; Segrest *et al.*, 1971; Kobylka & Carraway, 1972; Evans & Gurd, 1973). Segrest *et al.* (1971) found that as acrylamide concentration increased from 5 to 12.5% (w/v) the difference between true and observed glycoprotein molecular weight became smaller. This approach of improving glycoprotein molecular-weight estimation has limitations because glycoproteins with a molecular weight higher than 100000 cannot be estimated in gels of 10% (w/v) or above (Dunker & Rueckert, 1969). The use of gradient gels appears to be a means of overcoming this problem (Rodbard *et al.*, 1971).

The detection of anomalous protein behaviour on sodium dodecyl sulphate–polyacrylamide gel from measurements of free electrophoretic mobility and retardation coefficient has been described (Neville, 1971; Banker & Cotman, 1972). This approach offers two advantages; first, a means of determining whether a gel band that stains with both a general

protein stain and with a carbohydrate stain represents the same protein and secondly, an aid to selection of the most appropriate markers from which molecular-weight estimates are made.

The milk-fat-globule membrane apparently contains a number of glycoproteins (Kobylka & Carraway, 1972; Anderson *et al.*, 1972). Molecular-weight estimates for milk-fat-globule-membrane proteins have been calculated solely from non-glycoprotein markers and include values in excess of 100000 (Kobylka & Carraway, 1972; Anderson *et al.*, 1972).

The objective of the work was to determine the molecular weights of milk-fat-globule-membrane protein–sodium dodecyl sulphate complexes by using protein and glycoprotein markers that had similar free electrophoretic mobilities and retardation coefficients to the membrane proteins.

Materials and Methods

Materials

Acrylamide, *NN'*-methylene diacrylamide and *NNN'N'*-tetramethylethylenediamine were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; avidin, bovine serum albumin, horse-radish peroxidase (EC 1.11.1.7), human transferrin, lysozyme (EC 3.2.1.17), myoglobin, ovalbumin and pepsin (EC 3.4.23.1) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; glutamic acid dehydrogenase (EC 1.4.1.2), and liver alcohol dehydrogenase (EC 1.1.1.1) were obtained

from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; chymotrypsinogen was obtained from Worthington Biochemical Corp., Freehold 2, N.J., U.S.A.; calf fetuin and orosomucoid were gifts from Dr. P. Andrews; glyco- α -lactalbumin was a gift from Dr. T. E. Barman; α -lactalbumin and β -lactoglobulin were gifts from Dr. R. L. J. Lyster; Navy-bean glycoproteins 1 and 2 were gifts from Dr. A. T. Andrews; lactoperoxidase (EC 1.11.1.7) was prepared from raw milk by the method of Morrison & Hultquist (1963); Coomassie Blue G-250 (Xylene Brilliant Cyanin G, C.1. 42655) and Fuchsin Basic were obtained from Raymond A. Lamb, Alpert, Middx., U.K. All remaining reagents used were of the highest purity available from BDH Chemicals Ltd., Poole, Dorset, U.K.

Standard proteins. The non-glycoprotein (protein) and glycoprotein markers investigated, together with the molecular weights used in subsequent calculations, are shown in Table 1. Markers (5mg/ml) were dissolved in 0.05M-sodium phosphate buffer (pH7.0) containing 5mM-EDTA, 5mM-2-mercaptoethanol and 3% (w/v) sodium dodecyl sulphate and kept at room temperature for 1h before use. About 10 μ g of protein was applied to the gel.

Milk-fat-globule membrane. Washed cream containing 50% (w/w) fat was prepared and treated with sodium dodecyl sulphate by methods reported previously (Anderson *et al.*, 1972) to yield a detergent-soluble extract of milk-fat-globule membrane. In addition, a number of other disruptive agents were examined for their ability to solubilize the membrane

proteins. These reagents included dimethyl sulphoxide, NaCl, butan-1-ol and Triton X-100. The amount of total membrane nitrogen solubilized was determined for each reagent (Anderson *et al.*, 1972).

A soluble fraction of milk-fat-globule membrane was prepared by cooling washed cream (Anderson *et al.*, 1972) to 4°C, rewarming to 40°C and centrifuging at 25000g (r_{av} 5.5cm) for 1.5h at 30°C. Three layers, a pellet, a slightly opaque supernatant and a residual fat layer, were obtained. The supernatant was removed after solidifying the fat by cooling at 4°C and freeze-dried. The soluble fraction was then treated with sodium dodecyl sulphate, as previously described for freeze-dried membrane (Anderson *et al.*, 1972).

Membrane samples were studied on 3, 4, 5, 6, 7, 8, 10 and 12% (w/v) single-strength gels and 4–12% (w/v) gradient gels.

Gel electrophoresis

Polyacrylamide gels were prepared in precision bore, borosilicate glass tubes, internal diameter 4.5mm, length 70mm. At each gel concentration used the amount of bis-acrylamide present was 5% (w/w) of the total acrylamide. Acrylamide solutions for single-strength gels were made up in 0.05M-sodium phosphate buffer (pH8.0) containing 5mM-EDTA, 5mM-2-mercaptoethanol and 0.1% (w/v) sodium dodecyl sulphate (buffer A). Polymerization was initiated by the addition of 0.1% (v/v) tetramethylethylenediamine and ammonium persulphate (20mg/

Table 1. *Molecular weights of the protein and glycoprotein markers*

The protein numbers referred to below correspond to the numbers used to identify markers in the Figures

Protein	Protein number	Molecular weight	Reference
α -Lactalbumin	1	14176	Lyster (1972)
Lysozyme	2	14300	Weber & Osborn (1969)
Myoglobin	3	17200	Weber & Osborn (1969)
β -Lactoglobulin	4	18363	Lyster (1972)
Chymotrypsinogen	5	25700	Weber & Osborn (1969)
Pepsin	6	35000	Weber & Osborn (1969)
Liver alcohol dehydrogenase	7	41000	Weber & Osborn (1969)
Glutamic dehydrogenase	8	53000	Weber & Osborn (1969)
Bovine serum albumin	9	68000	Neville (1971)
Lactoperoxidase	10	93000	Paul (1963)
Avidin	11	15600	Glossman & Neville (1971)
Glyco- α -lactalbumin	12	16800	Barman (1970)
Navy-bean glycoprotein 2	13	30000	A. T. Andrews (personal communication)
Navy-bean glycoprotein 1	14	36000	A. T. Andrews (personal communication)
Horse-radish peroxidase	15	40000	Paul (1963)
Ovalbumin	16	43000	Weber & Osborn (1969)
Orosomucoid	17	44100	Glossman & Neville (1971)
Calf fetuin	18	50500	Glossman & Neville (1971)
Human transferrin	19	82000	Glossman & Neville (1971)

g of acrylamide). Water was layered on to the gel solution and after polymerization was complete it was replaced by buffer A for storage purposes.

Gradient gels [4–12% (w/v) acrylamide] were prepared from 8 and 24% (w/v) acrylamide stock solutions made up in double-strength buffer A containing 0.1% (v/v) tetramethylethylenediamine. The gradient was formed by the method of Margolis & Kenrick (1968), and stabilized by a 6.4–15.8% (w/v) sucrose gradient. Table 2 gives the compositions of the 4 and 12% (w/v) acrylamide solutions used in the gradient mixer. Sucrose and riboflavin solutions were made in deionized water. The gradient was mixed in subdued light and the gels were photopolymerized by placing the gel tubes 5 cm below a 20W fluorescent light-tube.

Buffer A was used as the electrode-compartment buffer. The gels were pre-run for 1 h at 2.5 mA/tube. Running time was varied according to the acrylamide concentration but the current was fixed at 2.5 mA/tube in all cases.

Staining

The gels were fixed in 40% (w/v) trichloroacetic acid for 5 min, washed briefly in water, stained for 15 min with a 0.25% (w/v) solution of Coomassie Blue G-250 (Diezel *et al.*, 1972) in acetic acid-methanol-H₂O (10:50:40, by vol.) (Weber &

Osborn, 1969), and destained in 5% (v/v) acetic acid. This method allowed the major membrane proteins and all markers to be detected 40 min after the end of the electrophoresis run.

Gels were stained for carbohydrate by the periodic acid-Schiff method. Sodium dodecyl sulphate was removed from the gel as suggested by Glossman & Neville (1971). Subsequent steps were performed in the dark at 4°C. The gels were treated with 1% (v/v) periodic acid in 7% (v/v) acetic acid for 1 h, 0.2% (w/v) sodium arsenite in 7% (v/v) acetic acid for 1 h, 0.1% (w/v) sodium arsenite in 7% (v/v) acetic acid for 1 h, stained in Schiff reagent (Zacharius *et al.*, 1969) for 2 h and destained in 0.1% (w/v) sodium metabisulphite in 0.01 M-HCl.

Mobility measurements and calculations

The mobility of all bands was measured relative to α -lactalbumin. This protein migrated to the same position in a given gel concentration irrespective of whether it was loaded singularly or in combination with other markers. When a commonly used dye, Pyronin Y, was used as a reference, it appeared to change the mobility of some markers.

Straight-line relationships were fitted by using the method of least squares outlined by Steel & Torrie (1960). The equation of Ferguson (1962) was used to calculate the free electrophoretic mobility and retardation coefficients of individual proteins.

Results

Milk-fat-globule-membrane solubility

A comparison of the ability of various reagents, which themselves do not contain nitrogen, to solubilize membrane protein is given in Table 3. The most satisfactory reagent in this respect was sodium dodecyl sulphate. Gel patterns obtained from material extracted by the various solvents were similar thus indicating that no selective solubilization occurred.

Table 2. Gradient gel solutions

The components were mixed by volume in the proportions indicated.

Solution	4% Acrylamide	12% Acrylamide
8% (w/v) Acrylamide	7	—
24% (w/v) Acrylamide	—	7
30% (w/v) Sucrose	3	7
0.004% (w/v) Riboflavin	1.15	0.15
Distilled water	3	—

Table 3. Effectiveness of various reagents in solubilizing milk-fat-globule-membrane proteins

The treated material was washed cream containing 50% (w/w) lipid. Conditions at the temperature indicated were those which gave optimum solubilization for each reagent.

Reagent	Conditions		Time (min)	Percentage of total membrane N ₂ solubilized
	Concentration (%)	Temperature (°C)		
Sodium dodecyl sulphate	3.0 (w/v)	100	3	84
Sodium chloride	4.7 (w/v)	4	1440	54
Sodium deoxycholate*	0.5 (w/v)	40	60	50
Butan-1-ol	80.0 (v/v)	4	60	39
Dimethyl sulphoxide	10.0 (v/v)	20	1440	24
Triton X-100	3.0 (v/v)	100	3	23

* From Anderson *et al.* (1972).

Fig. 1. Diagram of the electrophoretic patterns obtained from a sodium dodecyl sulphate extract and a soluble extract of milk-fat-globule membrane on 4–12% (w/v) gradient sodium dodecyl sulphate–polyacrylamide gels

Major proteins are indicated by the shaded bands. Gels 1 and 2 represent the Coomassie-Blue pattern from the sodium dodecyl sulphate and soluble extracts respectively. The pattern obtained after Schiff staining, represented by gel 3, was the same for both extracts. Approximate molecular weights are indicated by the adjacent scales calculated from Fig. 2 and Table 4 for protein and glycoprotein markers.

Milk-fat-globule-membrane gel patterns

The 4–12% (w/v) gradient gel gave the best resolution of the membrane proteins and this technique, in conjunction with the appropriate data, was used for estimating molecular weights of the major bands. Gel patterns obtained from sodium dodecyl sulphate extracts and a soluble fraction of membrane in gradient gels are shown in Fig. 1. This Figure is a

composite diagram representing pooled results from six experiments in which different milks were used as source material. Some of the minor bands shown in Fig. 1 were not observed in all experiments. There were four major bands (I, IV, V, VI) in the sodium dodecyl sulphate extract, all of which appeared in the soluble fraction, although not apparently as the same proportion of the total protein. Two further

bands (II, III) were major proteins in the soluble fraction pattern but less prominent in the sodium dodecyl sulphate extract. Minor bands in the soluble fraction pattern between proteins V and VI were more clearly defined than those in the sodium dodecyl sulphate extract.

In the gel patterns for some animals band V appeared as a doublet. This was not observed in single-strength gel patterns.

Six Schiff-positive bands occurred in both the sodium dodecyl sulphate extract and the soluble fraction. These were designated glycoproteins A-F.

Fig. 2. Relationship between molecular weight and relative mobility for protein (●) and glycoprotein (○) markers in 4–12% (w/v) gradient sodium dodecyl sulphate-polyacrylamide gels

Proteins II, III, IV and V appeared to have similar relative mobility values to glycoproteins C, D, E and F.

Molecular-weight estimation

The relative mobilities of protein and glycoprotein markers was plotted against the logarithm of their respective molecular weights. These results are shown in Fig. 2. Regression equations were then calculated for both sets of markers. Ovalbumin and transferrin were omitted from the glycoprotein marker regression. To assess the suitability of these regressions for estimating milk-fat-globule-membrane protein and glycoprotein-sodium dodecyl sulphate complexes the mobility of membrane proteins I–VI, A–F and the markers was measured in 3, 4, 5, 6, 7, 8, 10 and 12% (w/v) sodium dodecyl sulphate-polyacrylamide gels. From the results, values of free electrophoretic mobility and retardation coefficient were calculated from the Ferguson (1962) equation $\log R_F = -K_r T + \log M_0$ where R_F = relative mobility, T = percentage acrylamide as described by Hjerten (1962), K_r = retardation coefficient and M_0 = free electrophoretic

Table 4. Free electrophoretic mobilities of the protein markers and milk-fat-globule-membrane proteins

Protein	Free electrophoretic mobility
Protein markers	202.9 ± 0.96
Glycoprotein markers	194.8 ± 1.65
Membrane proteins I–VI	201.3 ± 1.28
Membrane proteins A–C	175.6 ± 0.54
Membrane proteins D–F	201.6 ± 0.50

Fig. 3. Relationship between relative mobility and retardation coefficient at different concentrations of acrylamide for the marker proteins

Plots shown are for 5% (●) and 7% (○) (w/v) gels. (a) Protein markers; (b) glycoprotein markers. Further details are given in the text.

Fig. 4. Relationship between relative mobility and retardation coefficient at different concentrations of acrylamide for the major milk-fat-globule-membrane proteins

Plots of proteins I-VI (a) are shown for 5 and 7% (w/v) gels and of glycoproteins A-F (b) for 4 and 6% (w/v) gels.

Table 5. Estimated molecular weights of the major milk-fat-globule-membrane proteins

The molecular-weight values calculated in the present experiment, together with the 95% confidence limits of each estimate, are compared with published values. These values are for bands that, on the basis of a comparison of gel photographs, were apparently coincidental with those in the present experiment.

Band number	$10^{-3} \times$ Molecular weight		
	Present experiment	Kobylka & Carraway (1972)	Anderson <i>et al.</i> (1972)
I	169.0 ± 26.2	155	220
II	145.0 ± 21.1		185
III*	94.2 ± 10.6	92	115
IV*	74.6 ± 7.2	80	84
V*	54.6 ± 4.2	65	55
VI	15.0 ± 0.9		16
A*	148.0 ± 10.3		360
B*	112.0 ± 6.6		280
C*	90.0 ± 4.5		220

*Glycoproteins.

mobility. Log relative mobility was used in the form $100 \log 100 R_F$, and was the mean of four determinations for each marker. Calculations were made only from observations in the linear range of the plot for each marker. Deviation from linearity occurred with the larger molecular-weight markers as percentage acrylamide increased. Milk-fat-globule-membrane proteins displayed an apparently linear plot between 3 and 7% (w/v) acrylamide.

The mean free electrophoretic mobility values for the markers and milk-fat-globule proteins are shown in Table 4. A *t*-test comparison showed that the free electrophoretic mobility of the glycoprotein markers was significantly lower ($P < 0.001$) than that of the protein markers and membrane proteins I-VI. The free electrophoretic mobility of membrane

glycoproteins A-C was considerably lower than that of the glycoprotein markers. The individual free electrophoretic mobilities and retardation coefficients of membrane proteins III, IV and V, and glycoproteins D, E and F, were so similar as to suggest that three proteins only are represented by these results. However, the differences between the free electrophoretic mobilities and retardation coefficients of membrane protein II (204.9 and 13.66 respectively) and of membrane glycoprotein C (175.0 and 7.46 respectively) indicated that these bands do not represent the same protein.

Plots of retardation coefficient against relative mobility for the markers and the membrane proteins at two polyacrylamide-gel strengths are illustrated in Figs. 3 and 4. Other gel strengths gave similar

intercept values. These results show that the behaviour of membrane glycoproteins D, E and F, on sodium dodecyl sulphate-polyacrylamide gels was the same as membrane proteins I-VI and the protein markers. Membrane glycoproteins A-C behaved differently from both the protein and glycoprotein markers with perhaps the exception of orosomucoid.

The molecular weights of membrane proteins I-VI were therefore calculated from the protein-marker standard curve in Fig. 1 and membrane glycoproteins A-C from the glycoprotein standard curve. For the purposes of molecular-weight calculations glycoproteins D, E and F were considered to be the same as proteins III, IV and V. The molecular weights and 95% confidence limits of the major milk-fat-globule-membrane proteins calculated from the appropriate gradient polyacrylamide-gel standard curve are shown in Table 5.

Discussion

Previous estimates of molecular weights of milk-fat-globule-membrane protein have been made on single-strength sodium dodecyl sulphate-polyacrylamide gels without reference to the behaviour of markers or unknowns in different concentrations of acrylamide (Anderson *et al.*, 1972; Kobylka & Carraway, 1972; Keenan & Huang, 1972). The disadvantages of such an approach have been discussed by Banker & Cotman (1972) and Neville (1971).

The anomalous behaviour of some membrane glycoproteins in sodium dodecyl sulphate-polyacrylamide gels is also well documented (Segrest *et al.*, 1971; Banker & Cotman, 1972; Evans & Gurd, 1973), but commercially available glycoproteins have rarely been used as molecular-weight markers (Glossman & Neville, 1971). Although the present results show that glycoprotein markers are more satisfactory than protein markers for glycoprotein molecular-weight estimation, their usefulness is limited by anomalous behaviour on sodium dodecyl sulphate-polyacrylamide gels. This is illustrated, for example, by ovalbumin and orosomucoid (see Fig. 3).

Ovalbumin, a commonly used marker for molecular-weight standard curves (Dunker & Rueckert, 1969; Weber & Osborn, 1969; Neville, 1971), contains 3% carbohydrate (Cunningham *et al.*, 1963). However, its behaviour on sodium dodecyl sulphate-polyacrylamide gels was the same as the protein markers used in this study (see Figs. 2 and 3). Membrane glycoproteins D, E and F appeared to be similar in this respect to ovalbumin. Orosomucoid had the highest carbohydrate content (41%, Glossman & Neville, 1971) and the lowest free electrophoretic mobility (186.5) of the glycoprotein markers. Membrane glycoproteins A-C appear to be more

closely related in their behaviour on sodium dodecyl sulphate-polyacrylamide gels to orosomucoid than to other markers.

A decrease in free electrophoretic mobility is thought to be associated with a decrease in sodium dodecyl sulphate binding, thereby decreasing the net negative charge in the protein (Banker & Cotman, 1972). Segrest *et al.* (1971) observed that ovalbumin bound approximately twice as much sodium dodecyl sulphate/g of protein than orosomucoid. The difference in free electrophoretic-mobility values between membrane glycoprotein D, E and F, glycoproteins A-C and orosomucoid might therefore be accounted for by the amount of sodium dodecyl sulphate binding.

It appears that membrane proteins I, III, IV, V are similar on a molecular-weight basis to some of the membrane proteins described by Kobylka & Carraway (1972) (see Table 5). While the gel patterns from the two experiments were similar, we did not observe major protein bands equivalent to those named as band I (mol. wt. 240000) and band VI (mol. wt. 53000) by Kobylka & Carraway (1972). These inconsistencies might be due to differences between either the method of preparation or source material. The data of Keenan & Huang (1972) on milk-fat-globule-membrane-protein molecular weights estimated by the sodium dodecyl sulphate method was not included in Table 5 for two reasons. The protein pattern presented was in the form of a densitometer trace and the range of molecular weight reported (12×10^3 - 100×10^3) was narrow compared with present and published values (Kobylka & Carraway, 1972; Anderson *et al.*, 1972). These factors combined to make comparisons difficult.

The presence of six glycoproteins in the milk-fat-globule membrane agrees with results of Kobylka & Carraway (1972). Previous studies from this laboratory (Anderson *et al.*, 1972) had shown eight glycoproteins to be present but the Schiff staining had been done without the prior removal of sodium dodecyl sulphate. Kobylka & Carraway (1972) reported that one of the smallest glycoproteins, probably equivalent to glycoprotein E, showed a decrease in estimated molecular weight with an increase in gel concentration. Evidence from Table 4 and Fig. 4 suggests, however, that of the six major membrane proteins that stain with Coomassie Blue three (III, IV and V) are the same proteins as glycoproteins D, E and F. The remaining glycoproteins (A-C) did not stain with Coomassie Blue and behaved anomalously on sodium dodecyl sulphate-polyacrylamide gels when compared with protein markers and proteins I-VI. The mean free electrophoretic-mobility value for glycoproteins A-C (175.6) was similar to that calculated from the data of Bretscher (1971) for an erythrocyte-membrane glycoprotein (179.3).

The present results indicate that the molecular-weight estimates for proteins III–VI were valid. However, those for glycoproteins A–C and proteins I and II were less accurate but the best obtainable under the given conditions. Proteins I and II were larger than the heaviest marker used for the standard curve and their estimated molecular weights represent extrapolated values. Glycoproteins A–C behaved anomalously on sodium dodecyl sulphate–polyacrylamide gels relative to the markers available.

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