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PREPARATION OF TWO PLASMA MEMBRANE FRACTIONS FROM ASCITES TUMOUR CELLS BY GEL CHROMATOGRAPHY ON SEPHACRYL S-1000

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SUMMARY

Two plasma membrane fractions from ascites tumour cells with differences in vesicle size were isolated by gel-exclusion chromatography on Sephacryl S-1000. Fraction 1 appeared in the void volume and had a vesicle diameter in the range 300—400 nm. Fraction 3, with an equilibrium constant (K_d) of 0.58, consisted of vesicles between 100 and 200 nm in diameter as measured by routine size analysis with the electron microscope and by calibration of the column with latex beads. The appearance of two plasma membrane fractions could also be confirmed by iodination of the surface membrane prior to fractionation. This gel chromatographic procedure represents a rapid and convenient method for the isolation of membrane material, which was enriched between five- and fourteen-fold based on the specific activity of the membrane-bound marker enzymes. Fraction 1 contained small amounts of lysosomal and Golgi membranes, and fraction 3 some material of the Golgi apparatus and the endoplasmic reticulum. The major portion of the contaminating membraneous material remained on the column and could be eluted with high salt buffer. The two plasma membrane fractions revealed some differences in 5'-nucleotidase specific activity and in the protein pattern, especially in the higher molecular weight range, as shown by sodium dodecyl sulphate gel electrophoresis.

INTRODUCTION

Recently it has been reported [1] that gel exclusion chromatography on Sephacryl S-1000 can be used for the fractionation of heterogeneous populations of phospholipid vesicles. We have applied this method for the preparation and purification of plasma membranes from tumour cells, which we formerly isolated by sucrose density gradient centrifugation [2]. In the previous work we described the existence of two plasma membrane fractions of ascites tumour cells, which we called "light" and "heavy" membranes. The light

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membranes were shown to represent mainly vesicular and the heavy ones sheet-like structures. Gel chromatography of an enriched plasma membrane preparation of ascites tumour cells on Sephacryl S-1000 yielded two membrane vesicle fractions. These two fractions, which contained vesicles of different sizes, were analysed for marker enzyme activities, for contamination with other membraneous material, for cholesterol and phospholipid composition and the protein pattern. They were also compared with the light and heavy membranes prepared by sucrose density gradient fractionation. Some differences between the two membrane fractions indicate that the gel chromatographic procedure allows the separation of specific domains from a heterogeneous population of plasma membrane material.

EXPERIMENTAL

Materials

Reagent-grade substrates such as 5'-AMP, ATP, *p*-nitrophenyl phosphate, glucose-6-phosphate, thiamine pyrophosphate and β-glycerophosphate were used for the enzyme tests and were purchased from Serva (Heidelberg, F.R.G.). Sephacryl S-1000 superfine was obtained from Pharmacia (Freiburg, F.R.G.), lactoperoxidase from Boehringer (Mannheim, F.R.G.) and $Na^{125}I$ from Amersham (Braunschweig, F.R.G.).

Isolation of ascites cells

Male NMRI mice, ca. five weeks of age, were used in this study for the in vivo propagation of the glycogen-free and the glycogen-containing Ehrlich—Lettré ascites tumour cells [3]. The cells were harvested seven days after inoculation. They were centrifuged at 1800 *g* (4000 rpm) for 10 min to remove the ascites fluid, and then washed once with Ringer buffer (pH 7.4).

Gel chromatography of membraneous material

Cell disrupture and the preparation of a nuclear-free 12 000 *g* pellet was performed as described in detail elsewhere [2]. The plasma membrane-containing 12 000 *g* pellet was placed on a 30 × 2 cm I.D. glass column from Serva, filled with Sephacryl S-1000 superfine. Between 10 and 60 mg of the above material could be fractionated. Fractions of 3 ml were collected, which were eluted at 4°C with Tris-buffered isotonic sucrose (pH 7.4) at a flow-rate of ca. 40—50 ml/h. The material was recorded with a UV detector at 280 nm using a Gilson chromatography unit, and this procedure was routineously applied. In initial experiments the eluent was also analysed by protein measurements, which gave elution profiles similar to those from the UV detection. The yield of total material eluted from the column varied between 15 and 20% on a protein basis. Some additional membrane material could be eluted from the column with 0.5 *M* sodium chloride in 10 μ*M* Tris—HCl (pH 7.4), which consisted mainly of mitochondrial and lysosomal material and of some of the endoplasmic reticulum. After collection of the plasma membrane-containing fractions, the material was pelleted by ultracentrifugation at 104 000 *g* for 3 h, and the pellets were taken up in Tris-buffered isotonic sucrose solution (pH 7.4). The column could be used repeatedly, if the gel was regenerated with ca. three bed volumes of

0.5 *M* sodium chloride, 10 m*M* Tris—HCl (pH 7.4), containing 0.1% sodium dodecyl sulphate (SDS). The regeneration procedure was carried out at room temperature.

Sucrose gradient fractionation

The material, suspended in 20 m*M* Tris—HCl buffer (pH 8) containing 0.25 *M* sucrose and 2 m*M* EDTA with a maximum protein concentration of 1 mg/ml, was brought to 20% (w/v) sucrose and layered on top of the gradient. Centrifugation was performed in an SW27 rotor at 8450 *g* (8000 rpm) for 42 min using a Beckman L5-50 ultracentrifuge. The gradients were fractionated into 2-ml fractions with a Buchler Auto Densi Flow IIC. After enzyme activity determinations the material was collected according to the activity profile, diluted with Tris buffer (pH 7.4), and then pelleted at 103 000 *g* (36 000 rpm) for 3 h using a Ti45 rotor.

Enzymatic assays

5′-Nucleotidase and alkaline phosphatase were analysed according to the method of Lauter et al. [4]. The $(Na^+ + K^+ + Mg^{2+})$ATPase was measured by using the procedure of Forte et al. [5], and differentiated from pure $(Na^+ + K^+)$ATPase with 1 m*M* ouabain as inhibitor. The incubation mixture (except the substrate) was preincubated in the presence of 0.001% saponin for 5 min at 25°C to solubilize the ATPase activity, and consequently to render it accessible both to substrate and ouabain. The analysis of glucose-6-phosphatase and acid phosphatase was followed by the procedures of De Duve et al. [6], and thiamine pyrophosphatase by the method of Meldolesi et al. [7]. Protein was determined by the method of Lowry et al. [8], and inorganic orthophosphate (P_i) by the procedure of Eibl and Lands [9]. The lipids were extracted according to the procedure of Folch et al. [10], and the phospholipids quantified by phosphorus analysis after two-dimensional thin-layer chromatographic separation using silica gel G-covered plastic sheets from Merck (Darmstadt, F.R.G.) [11]. Cholesterol was quantified according to Haeffner and Hoffmann [12].

Polyacrylamide gel electrophoresis

Electrophoretic analysis was performed in 10% gels at pH 8.8 and in the presence of 0.1% SDS according to Laemmli [13]. Staining was carried out by using standard technique with Coomassie brilliant blue.

Iodination experiments

Iodination of cell surface membranes was performed by the lactoperoxidase-catalysed procedure [14] using $Na^{125}I$ [specific activity 13—17 mCi/μg (100 mCi/ml)]. Amounts of ca. $2 \cdot 10^9$ cells washed with phosphate-buffered saline were incubated in 10 ml of the same buffer in the presence of 1 mCi $Na^{125}I$ and 650 μl (3.25 mg) lactoperoxidase for 1.5 h at 37°C. During the first 25 min, six 20-μl volumes of a 0.03% perhydrol solution were added. The reaction was stopped with 200 μl of mercaptoethanol, the cells were washed at least four times and then ruptured and fractionated to obtain the 12 000 *g* pellet for the gel chromatographic preparation of the plasma membranes. Radioactivity was counted in an LKB automatic gamma counter.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of the post-nuclear fraction (12 000 *g* pellet) on a Sephacryl S-1000 column. Determination of plasma membrane marker enzymes such as 5′-nucleotidase, ouabain-sensitive (Na^+ + K^+)ATPase and alkaline phosphatase yielded two activity peaks, designated fractions 1 and 3, which have consistently been obtained throughout these fractionation studies. For simplicity, only the activity profile of 5′-nucleotidase, and in addition the radioactivity distribution of the iodinated cell surface, is shown in the figure. The intermediate fraction between 1 and 3 did not contain plasma membranes. Additional material could be eluted from the column with high salt buffer, but this material contained mostly mitochondrial and lysosomal membranes.

Further enrichment and purification of the two plasma membrane fractions

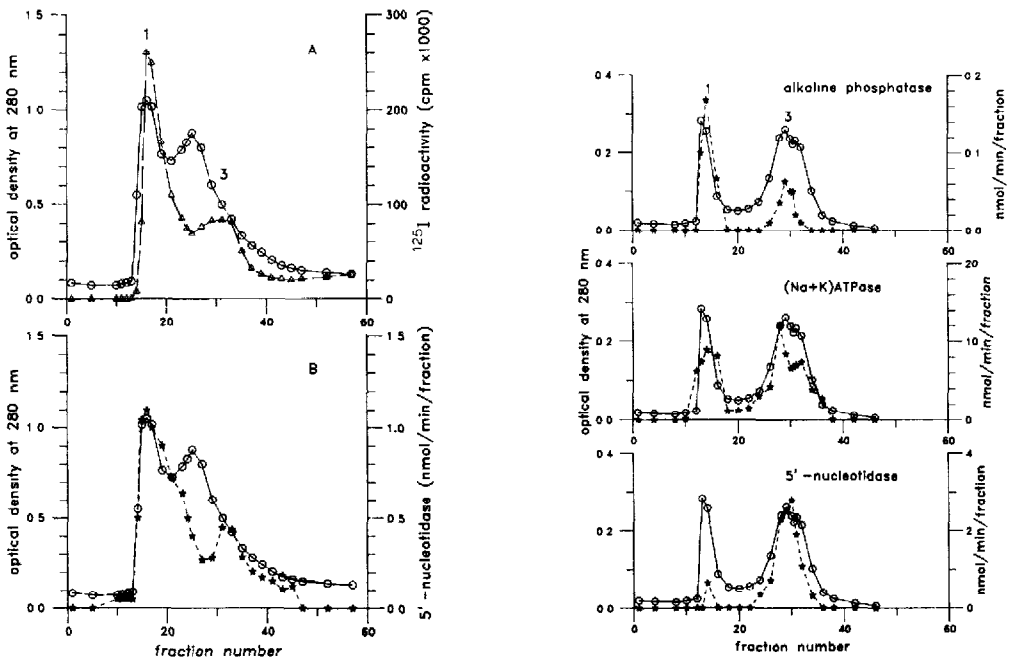

Fig. 1. Gel chromatography of enriched plasma membranes (12 000 *g* pellet) from the glycogen-free subline on Sephacryl S-1000. A 30 × 2 cm I.D. glass column from Serva filled with Sephacryl S-1000 was used for the fractionation of ca. 35 mg of membrane material. Fractions of 3 ml were collected, which were eluted with Tris-buffered isotonic sucrose (pH 7.4) at a flow-rate of 50 ml/h. The material was recorded with a UV detector at 280 nm. The yield of plasma membranes eluted from the column varied between 15 and 20% on a protein basis. (A) Distribution of protein (○) and ^{125}I-radioactivity (△); (B) 5′-nucleotidase activity (★) and distribution of protein (○).

Fig 2. Rechromatography of a combination of fractions 12—22 (first activity peak) and fractions 30—42 (second activity peak) of the first gel chromatography on Sephacryl S-1000. The amount of protein varied between 10 and 20 mg. The conditions were essentially the same as described in Fig. 1. The yield of both membrane fractions was ca. 15%. The marker enzymes tested were alkaline phosphatase, (Na+K)ATPase and 5′-nucleotidase (stars). The protein elution profile is marked with circles.

was obtained after a second gel chromatography of the combined two fractions from the first fractionation experiment (see Fig. 1). Fig. 2 shows the result of this study, in which the activity distribution of the three plasma membrane marker enzymes is given, revealing a complete separation of the two membrane fractions into activity and mass peaks. In case of the (Na^+ + K^+)ATPase there is some indication even for a third activity peak. Table I summarizes the specific activities of the marker enzymes in comparison to the homogenate and the 12 000 *g* pellet; in the case of 5'-nucleotidase the data are given for both ascites cell sublines, which have previously been shown to contain markedly different activities [15]. The degree of enrichment of both plasma membrane fractions is from five- to fourteen-fold based on the activities of the membrane-bound enzymes. Data about contamination of this finally purified material with other membranes are given in Table II. Some activity of thiamine pyrophosphatase (Golgi vesicles) and glucose-6-phosphatase (endoplasmic reticulum) was found in membrane fraction 3. A small amount of acid phosphatase was observed, primarily in fraction 1, indicating minor contamination of fraction 1 with lysosomal material.

Analysis of the membrane fractions by transmission electron microscopy (no pictures shown) revealed the presence of osmiophilic particles with a diameter of 60—90 nm, which can be classified morphologically as lysosomes of the type of multivesicular bodies, confirming the data on marker enzyme measurements. The ultrastructural studies further showed that both membrane fractions contained mostly vesicles. The vesicles of fraction 1 appearing in the void volume had an average diameter of 300—400 nm, with a few being as large as ca. 600 nm. These data are in agreement with the exclusion limit of Sephacryl S-1000, and similar results have also been reported for phospholipid vesicle separation [1]. The vesicles of fraction 3, with an equilibrium constant (K_d) of 0.58, had an average diameter between 100 and 200 nm.

The yield of both membrane fractions together, starting with the 12 000 *g* pellet, was up to 20% for the first, and ca. 15% for the second gel chromatography based on protein mass. The phospholipid and cholesterol content per unit membrane protein, and the molar C/P ratio for the homogenate and fraction 1 and 3 is given in Table III. These data are generally somewhat lower compared with the values obtained for the light and heavy membrane preparations [2].

The question arose how fractions 1 and 3 from gel chromatography on Sephacryl S-1000 can be compared with the so-called light and heavy membranes isolated by sucrose density gradient fractionation. Taking the vesicle dimensions as the criterion, fraction 1 should resemble the light membranes. This was tested and confirmed by gel chromatography of light membrane material from the density gradient (Fig. 3A). As shown, most of this material represents a very homogeneous peak eluting exactly with the void volume of the column, and only a small fraction was barely included into the gel pores. When, on the other hand, fraction 1 from gel chromatography was analysed on the density gradient, most of the material equilibrated in the upper part of the gradient, indicating again a rather homogeneous fraction (Fig. 4A). Similar experiments were also conducted with fraction 3 from gel chromatography, and the heavy membranes from the sucrose density gradient.

TABLE I

SPECIFIC ACTIVITY DISTRIBUTION OF PLASMA MEMBRANE MARKER ENZYMES BETWEEN FRACTIONS 1 AND 3 AFTER TWO GEL CHROMATOGRAPHIC SEPARATIONS

In case of 5′-nucleotidase the activities are given for both ascites cell sublines. Activity expressed in nmol/min per mg protein. The data represent the mean ±S.D. Number of experiments with duplicate analyses in parentheses. *F* = factor of enrichment.

Enzyme	Homogenate	12 000 *g* pellet	Fraction 1	*F*	Fraction 3	*F*
$(Na^+ + K^+)$ATPase	12.6 ± 3.7 (9) (100%)	39.9 ± 11 (12) (68.1 ± 8.0)*	175.5 ± 51.6 (6) (66.5 ± 19.6)**	13.9	158.5 ± 39.7 (4) (25.4 ± 6.4)	12.6
5′-Nucleotidase, glycogen-free subline	1.01 ± 0.22 (5) (100%)	3.5 ± 0.84 (6) (75.3 ± 8.9)	8.3 ± 2.9 (9) (35.8 ± 12.5)	8.3	7.8 ± 3.0 (8) (14.2 ± 5.5)	7.8
5′-Nucleotidase, glycogen-containing subline	4.89 ± 1.22 (6) (100%)	14.7 ± 3.3 (6) (64.7 ± 14.5)	32.4 ± 16.8 (7) (33.3 ± 17.3)	6.6	9.4 ± 4.4 (4) (4.1 ± 1.9)	1.9
Alkaline phosphatase	0.30 ± 0.08 (4) (100%)	1.08 ± 0.14 (5) (77.4 ± 10)	1.38 ± 0.42 (4) (19.3 ± 5.9)	4.6	1.43 ± 0.08 (3) (8.4 ± 0.5)	4.8
Protein yield (mg)		11.9 ± 4.5 (5)	1.80 ± 0.70 (8)		0.76 ± 0.25 (7)	
Protein yield (%)		(23.7 ± 4.0)	11.30 ± 2.63 (8)		4.10 ± 1.22 (7)	

*Percent activity of homogenate.

**Percent activity of 12 000 *g* pellet.

TABLE II

ACTIVITY DISTRIBUTION OF MITOCHONDRIAL, LYSOSOMAL, GOLGI AND ENDOPLASMIC RETICULUM MARKER ENZYMES BETWEEN THE TWO MEMBRANE FRACTIONS AFTER TWO FRACTIONATIONS ON SEPHACRYL S-1000

Activity expressed in nmol/min per mg of protein. The data represent the mean ± S.D. Number of experiments with duplicate analyses in parentheses. *F* = factor of enrichment.

Enzyme	Homogenate	12 000 *g* pellet	Fraction 1	*F*	Fraction 3	*F*
Acid phosphatase (lysosomes)	6.36 ± 0.72 (5) (100%)	25.0 ± 10.4 (84.6 ± 35.2)*	13.1 ± 2.6 (2) (7.9 ± 1.6)**	2.1	2.54 ± 0.3 (2) (0.6 ± 0.08)	0.4
Thiamine pyrophosphatase (Golgi vesicles)	2.63 ± 1.71 (6) (100%)	6.82 ± 3.2 (3) (55.8 ± 26.2)	7.7 ± 2.7 (4) (17.1 ± 6.0)	2.9	12.7 ± 3.3 (4) (11.8 ± 3.1)	4.8
Glucose-6-phosphatase (endoplasmatic reticulum)	0.30 ± 0.14 (10) (100%)	0.48 ± 0.26 (8) (34.4 ± 18.6)	0.34 ± 0.13 (6) (10.7 ± 4.1)	1.1	0.49 ± 0.30 (4) (6.5 ± 4.0)	1.6
Cytochrome C-oxidase (mitochondria)	0.38 ± 0.06 (4) (100%)	1.06 ± 0.2 (4) (60.1 ± 11.3)	0.372 ± 0.11 (2) (5.3 ± 1.6)	1.0	0.10 ± 0.03 (2) (0.6 ± 0.2)	0.3

*Percent activity of homogenate.

**Percent activity of 12 000 *g* pellet.

TABLE III

PHOSPHOLIPID AND CHOLESTEROL CONTENT AND THE MOLAR CHOLESTEROL-TO-PHOSPHOLIPID (C/P) RATIO OF THE TWO PLASMA MEMBRANE FRACTIONS 1 AND 3 AFTER FINAL PURIFICATION

Number of experiments with duplicate analyses in parentheses.

	Content per mg membrane protein		C/P ratio
	Phospholipid (mg)	Cholesterol (μg)	
Homogenate	0.10 ± 0.04 (6)	15 ± 6 (3)	0.25
Fraction 1	0.24 ± 0.09 (5)	32 ± 19 (6)	0.28
Fraction 3	0.12 ± 0.05 (3)	54 ± 30 (3)	0.31

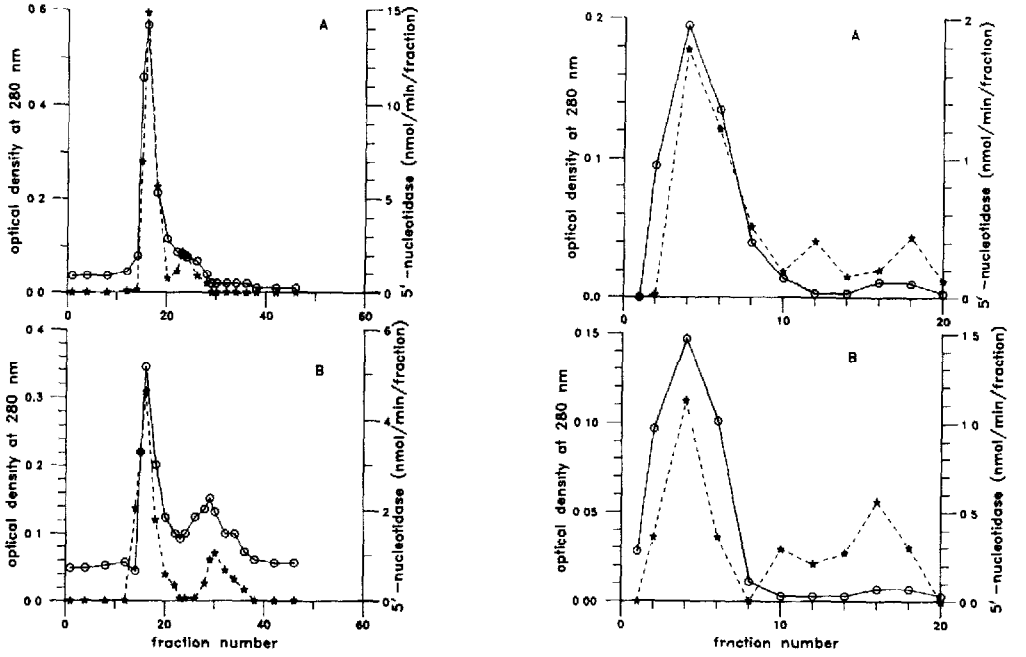

Fig. 3. Gel chromatography of the light (A) and heavy (B) plasma membranes purified by sucrose density gradient centrifugation. About 12 mg of membrane protein were applied to the column and run under the same conditions as described in the legend to Fig. 1. 5'-Nucleotidase activity distribution was used as membrane marker enzyme (stars).

Fig. 4. Sucrose density gradient centrifugation of fraction 1 (A) and fraction 3 (B) of the plasma membrane material isolated by gel chromatography on Sephacryl S-1000. Fractions of 2 ml were collected with a Buchler Auto Densi Flow IIC, and then used for the determination of 5'-nucleotidase activity (stars)

Analysis of heavy membrane material on the column revealed the appearance of two peaks, a major one corresponding to fraction 1 and a minor one at the position of fraction 3 (Fig. 3B). Electron microscopy (pictures not shown) of both fractions showed the typical vesicular structures, suggesting that the formerly open sheets must have formed vesicles in a random fashion during

the gel chromatographic procedure or before. Conversely, the major amount of fraction 3 obtained from gel chromatography equilibrated at the position of the light membranes in the sucrose gradient, as is to be expected (Fig. 4B). It can be summarized that upon gel chromatography on Sephacryl S-1000 two membrane vesicle populations of different sizes are obtained. On the other hand, sucrose density gradient fractionation yields both vesicular material (= light membranes) representing a mixture of fraction 1 and 3 from gel chromatography and open structures (= heavy membranes), which cannot be isolated by gel chromatography.

As the previous data have shown, some differences in enzyme activity, purity and lipid composition are observed between fractions 1 and 3. SDS gel chromatography revealed further differences in the protein pattern of these two fractions, mainly in the higher molecular weight range (Fig. 5, arrows).

It is assumed that this procedure is suitable for the separation of any mixture of plasma membrane vesicle populations from a given cell type.

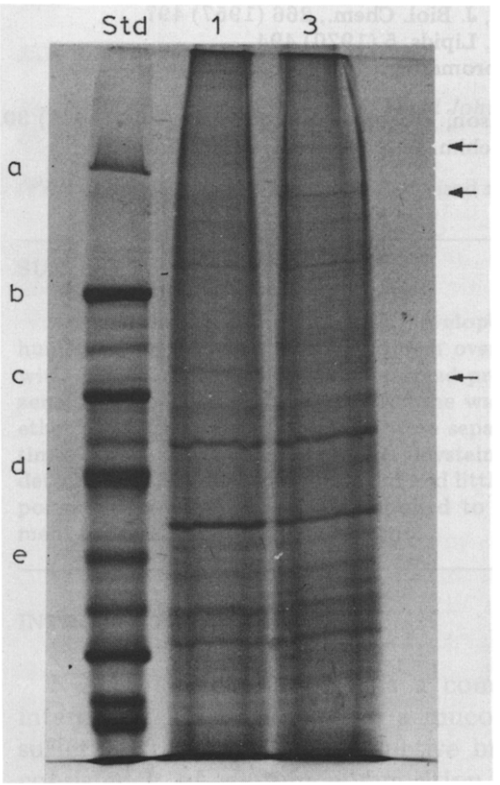

Fig. 5. Polyacrylamide gel electrophoresis of the membrane fractions 1 and 3. About 100 μg of protein of each preparation were placed on the gel, separated and stained according to standard procedures. Protein standard mixture on the left: (a) aldolase, 158 000 daltons; (b) β-galactosidase, 139 000 daltons; (c) bovine serum albumin, 68 000 daltons; (d) ovalbumin, 45 000 daltons; (e) chymotrypsinogen, 25 000 daltons.

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