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FRACTIONATION OF MEMBRANE VESICLES

II. A METHOD FOR SEPARATION OF MEMBRANE VESICLES BEARING DIFFERENT ENZYMES BY FREE-FLOW ELECTROPHORESIS

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Summary

Free-flow electrophoresis was used to subfractionate membrane vesicles from calf thymocyte plasma membranes. The fractionation resulted in a separation of vesicle populations bearing four different enzymes: alkaline nitrophenylphosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1), γ-glutamyltransferase (EC 2.3.2.2), (Mg^{2+} + Na^+ + K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) and acyl-CoA:lysophosphatidylcholine acyltransferase (acyl-CoA:1-acylglycero-3-phosphocholine-*O*-acyltransferase, EC 2.3.1.23). The specific content of cholesterol and total phospholipid coincided with the distribution of membrane-bound protein. However, vesicles migrating towards the cathode had a higher molar ratio of cholesterol to phospholipid (0.75) compared to those migrating to the anode (0.55). Sodium dodecyl sulphate-gel electrophoresis of pooled vesicle fractions also demonstrates distinct differences in their protein pattern. Electron-micrographic thin sections show that the vesicle populations have a similar morphology and size distribution.

These results are discussed in terms of heterogeneity of the original thymocytes, contamination with intracellular membranes and a heterogeneous structure of the plasma membrane.

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Introduction

Evidence is accumulating showing that the components of the outer cell membrane are not distributed homogeneously but that the cell membrane consists of distinct areas with specialized structure and function [1—3]. We reported previously [4] an attempt to isolate such functional entities as concanavalin A-receptor bearing membrane vesicles using affinity chromatography on concanavalin A-Sepharose. Because this type of subfractionation was not due to specific interactions between sugar moieties and the ligand alone and, moreover, it was complicated by the liberation of bound concanavalin A, we decided to try another principle of subfractionation of plasma membranes. Since the sugar moieties of glycoproteins determine the surface charge of the cell membrane we tried subfractionation in an electrical field. The most suitable method for separation of membrane vesicles of heterogeneous size seems to be free-flow electrophoresis according to Hannig and Heidrich [5].

In this paper we report on the fractionation of membranes mainly derived from plasma membranes from calf thymocytes. We show that this fractionation procedure results in a separation of membrane vesicles bearing different enzyme activities. Furthermore, these subfractions show differences in their protein pattern, lipid content and also in their binding properties for concanavalin A.

Materials and Methods

1. Isolation of subcellular components from calf thymus cells

The preparation of subcellular components from thymocyte suspensions was performed after cell disruption using nitrogen cavitation as described previously [6] with the following modification: Instead of resuspending the cells in a sucrose-containing medium a saline buffer was used consisting of 152 mM NaCl, 4.17 mM KCl, 0.5 mM $MgCl_2$ and 10 mM HEPES (4-(hydroxyethyl)-piperazinyl-ethane-2-sulfonic acid), pH 7.2. After removal of nuclei at $220 \times g_{av}$, 15 min, (1500 rev./min, rotor JA-14, Beckman) and of mitochondria and other large granules at $19200 \times g_{av}$, 20 min (14000 rev./min, rotor JA-14, Beckman) microsomal membranes were pelleted at $177000 \times g_{av}$, 60 min (50000 rev./min, rotor 60 Ti, Beckman).

After two hypotonic shocks with 10 mM and 1 mM HEPES buffer, pH 7.2, the microsomal membranes were subfractionated by centrifugation on a discontinuous sucrose gradient [7], which consisted of three layers containing 45, 35 and 0% (w/w) of sucrose in 10 mM HEPES buffer, pH 7.2. After centrifugation for 2 h at $177000 \times g_{av}$ in a fixed angle rotor (Beckman, 60 Ti) two membrane bands and a pellet were obtained. The upper fraction (S_1) was located at the interface between 0 and 35% (w/w) sucrose and the medium band (S_2) at the interface between 35 and 45% (w/w) sucrose. All electrophoretic separations described in the following were carried out using fraction S_1.

2. Electrophoresis of membrane vesicles

The electrophoretic separation of membrane vesicles was performed in a free-flow electrophoresis apparatus according to Hannig and Heidrich [5]

(Elphor VaP-5-, Bender and Hobein, Munich, G.F.R.). Before applying the membranes to electrophoresis they were washed twice with electrophoresis buffer at $177000 \times g_{av}$, 60 min, to remove the sucrose. The electrophoresis buffer consisted of 10 mM triethanolamine, 10 mM acetic acid, pH 7.2. As electrode buffer the same buffer, but 10 times the concentration, was used. The electrophoretic separation was carried out in a chamber of 0.3 mm width at 1300 V (130 V/cm) and 90 mA at +5.5°C. The flow rate was 2.0 ml $\times$ h^{-1}, which corresponds to a total amount of 180 ml $\times$ h^{-1} for 90 fractions. The membrane suspension (6—8 mg membrane protein/ml) was injected at a rate of 1.35 ml $\times$ h^{-1}.

Electrophoresis of intact cells was performed in the same apparatus using a chamber of 0.7 mm width and a different buffer system [8].

3. Biochemical determinations

Protein, total cholesterol, total phospholipid, the activities of the acyl-CoA: lysophosphatidylcholine acyltransferase and of the alkaline nitrophenylphosphatase and the binding parameters of concanavalin A (number of binding sites and affinity) were determined as described previously [9]. The activity of γ-glutamyl transferase was determined according to G. Szasz [10] using l-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine (Boehringer, Mannheim, G.F.R.) as substrates. (Mg^{2+} + Na^+ + K^+)-ATPase activities were measured in a coupled optical assay according to Schoner et al. [11].

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out using commercially available gradient gels (Pharmacia, Frankfurt, G.F.R.) ranging from 4 to 30% polyacrylamide. 50—100 μg of membrane protein were applied on each gel. Buffer composition, electrophoretic conditions, gel calibration and the staining procedure was identical with the methods described by Knüfermann et al. [12].

4. Electron microscopy

The membrane preparations were fixed for at least 1 h in 0.05 M cacodylate buffer, pH 7.1, containing 1% glutaraldehyde followed by 30 min treatment with 1% OsO_4 in barbital-acetate, pH 7.1. The fixed membranes were washed in this buffer, suspended in agar, dehydrated in ethanol according to the procedure of Kellenberger et al. [13], and embedded in Epon [14]. Sections were mounted on copper grids coated with formvar and stained with uranyl acetate [15] followed by lead acetate [16]. The sections were examined in a Philips EM 400 and a Siemens Elmiskop I A.

Results

1. Preparation of plasma membranes

The membrane fraction used for all experiments was mainly derived from plasma membranes. We use the term plasma membrane for that fraction which possesses the classical characteristics of the outer cell membrane: Highest specific content (per mg protein) of cholesterol and phospholipid, the highest mol of cholesterol to phospholipid and the highest specific activity of the thymocyte plasma-membrane marker enzymes alkaline nitrophenylphosphatase and γ-glutamyl transferase.

TABLE I

DISTRIBUTION OF ENZYME ACTIVITIES IN PELLETS AND SUPERNATANTS OF POOLED FRACTIONS AFTER FREE-FLOW ELECTROPHORESIS

Specific activities are expressed as nmol/mg protein per min and total activities as μmol/min and as percentage.

		Lysophosphatidylcholine acyltransferase			Alkaline phosphatase		
		Specific activity	Total activity	%	Specific activity	Total activity	%
A	Pellet	32.0	26.0	95.4	332	773	96.0
	Supernatant	1.0	1.3	4.6	18	32	4.0
B	Pellet	10.0	25.0	95.8	519	1834	96.7
	Supernatant	0.7	1.1	4.2	33	62	3.3
C	Pellet	4.0	2.5	>98.0	2405	2963	94.4
	Supernatant	Trace	Trace	—	106	176	5.6

The uppermost fraction (S_1) obtained by sucrose gradient centrifugation of the original microsomal membranes shows these typical characteristics of plasma membranes (Tables II and III). The pellet of the sucrose gradient (S_3) consists mainly of ribosomes as shown in the electron-micrographs (Fig. 1c) and contains, according to SDS-polyacrylamide gel electrophoresis, high amounts of low molecular weight proteins (10000—40000, Fig. 2). Sucrose gradient fraction S_2 is apparently a mixture also possessing ribosomes (Fig. 1b). Fraction S_1, exclusively used for all studies on separation by free-flow electrophoresis, contains predominantly proteins of higher molecular weight (Fig. 2). Electron-microscopic thin sections indicate that this fraction consists mainly of vesicles with a diameter of 30—200 nm (Fig. 1a). The original microsomal fraction used for the preparation of plasma membranes contains apparently all protein bands which were found in the separated fractions.

2. Protein profile and re-electrophoresis of membranes

The protein profile of membranes after electrophoresis mainly consists of a uniform but rather broad peak (fractions 6—30, Fig. 3a). Light scattering (500 nm/500 nm, right angle) of the fractions shows a similar distribution which indicates that the protein profile is due to particle-bound protein only (Fig. 3a).

To rule out the possibility that the fractionation is caused or influenced by changes of the surface charge induced by the electrical field, we carried out two different control experiments: (a), Two separated single fractions were re-electrophoresed and (b), two separated single fractions were mixed and then again separated by electrophoresis.

Fig. 3 shows that in both cases no gross changes of electrophoretic mobility of these vesicle populations were observed. This shows that the surface charge of the membrane vesicles is stable under the separation conditions used and that other artefactual events such as disintegration (Fig. 3b) or fusion (Fig. 3c) of membrane vesicles do not occur.

A slight broadening of peaks was observed during re-electrophoresis which is partly caused by diffusion. Diffusion of the injected material without an electrical field is approximately three fractions.

TABLE II

CHEMICAL COMPOSITION OF DIFFERENT FRACTIONS FROM PLASMA MEMBRANE PURIFICATION AND FROM FREE-FLOW ELECTROPHORETIC SEPARATION

After electrophoretic separation, fractions were pooled (A, B, C) as described in Fig. 4. In addition, results of analyses of fractions of different purification steps are given. Total contents of microsomes and S_1 are also expressed as percent of the homogenate and for pooled fractions A—C as percentage of the sum of total contents of fractions A, B, C.

	Protein		Cholesterol			Phospholipid			Molar ratio cholesterol/ phospholipid	Concanavalin A binding	
	Total (mg)	%	(nmol/mg protein)	Total (μmol)	%	(nmol/mg protein)	Total (μmol)	%		Maximum sites (molecules/ mg protein)	Kass. (l/mol)
Homogenate	1298.0	100	25.6	33.2	100.0	77.5	100.6	100	0.33	—	—
Microsomes	28.3	2.2	346.0	9.8	29.4	492.0	13.9	13.8	0.70	—	—
Sucrose gradient fraction S_1	8.2	0.6	788.0	6.5	19.4	1069.0	8.8	8.7	0.74	—	—
A	2.3	32.8	730.0	1.7	26.7	1330.0	3.1	36.7	0.55	$2.0 \cdot 10^{14}$	$82 \cdot 10^6$
B	3.5	49.8	940.0	3.3	52.3	1080.0	3.8	45.3	0.87	$4.2 \cdot 10^{14}$	$37 \cdot 10^6$
C	1.2	17.3	1080.0	1.3	20.9	1230.0	1.5	17.9	0.88	$8.2 \cdot 10^{14}$	$22 \cdot 10^6$

TABLE III

ENZYME ACTIVITIES OF DIFFERENT FRACTIONS FROM PLASMA MEMBRANE PURIFICATION AND FROM FREE-FLOW ELECTROPHORETIC SEPARATION

After electrophoretic separation fractions were pooled (A, B, C) as described in Fig. 4. In addition, results of analysis of fractions of different purification steps are given. Total activities of cell suspensions of different thymi show a biological variation of approximately 80%. Therefore, a statistical evaluation was not performed. However, the relative specific activities (purification factors) revealed the same value. Specific activities are expressed as nmol/mg protein per min. Total activities are given as μmol/min as percentage of the homogenate (microsomes and S_1) and for pooled fractions A—C as percentage of the sum of total activities of fractions A, B, C.

	Lysophosphatidylcholine acyltransferase			Alkaline phosphatase			γ-Glutamyl transferase			Succinate dehydrogenase
	Specific activity	Total activity	%	Specific activity	Total activity	%	Specific activity	Total activity	%	Specific activity
Homogenate	7.0	9.09	100.0	39.8	51.66	100.0	0.6	0.78	100.0	3.5
Microsomes	20.0	0.57	6.3	655.0	18.54	35.9	10.1	0.29	37.2	2.0
Sucrose gradient fraction S_1	25.0	0.21	2.3	1233.0	10.11	19.6	29.0	0.24	30.8	0.05
A	51.0	0.12	60.0	350.0	0.81	7.6	6.7	0.02	11.8	<0.01
B	21.0	0.07	35.0	740.0	2.59	24.2	35.0	0.12	70.6	<0.01
C	7.0	0.01	5.0	6100.0	7.32	68.3	22.3	0.03	17.6	<0.01

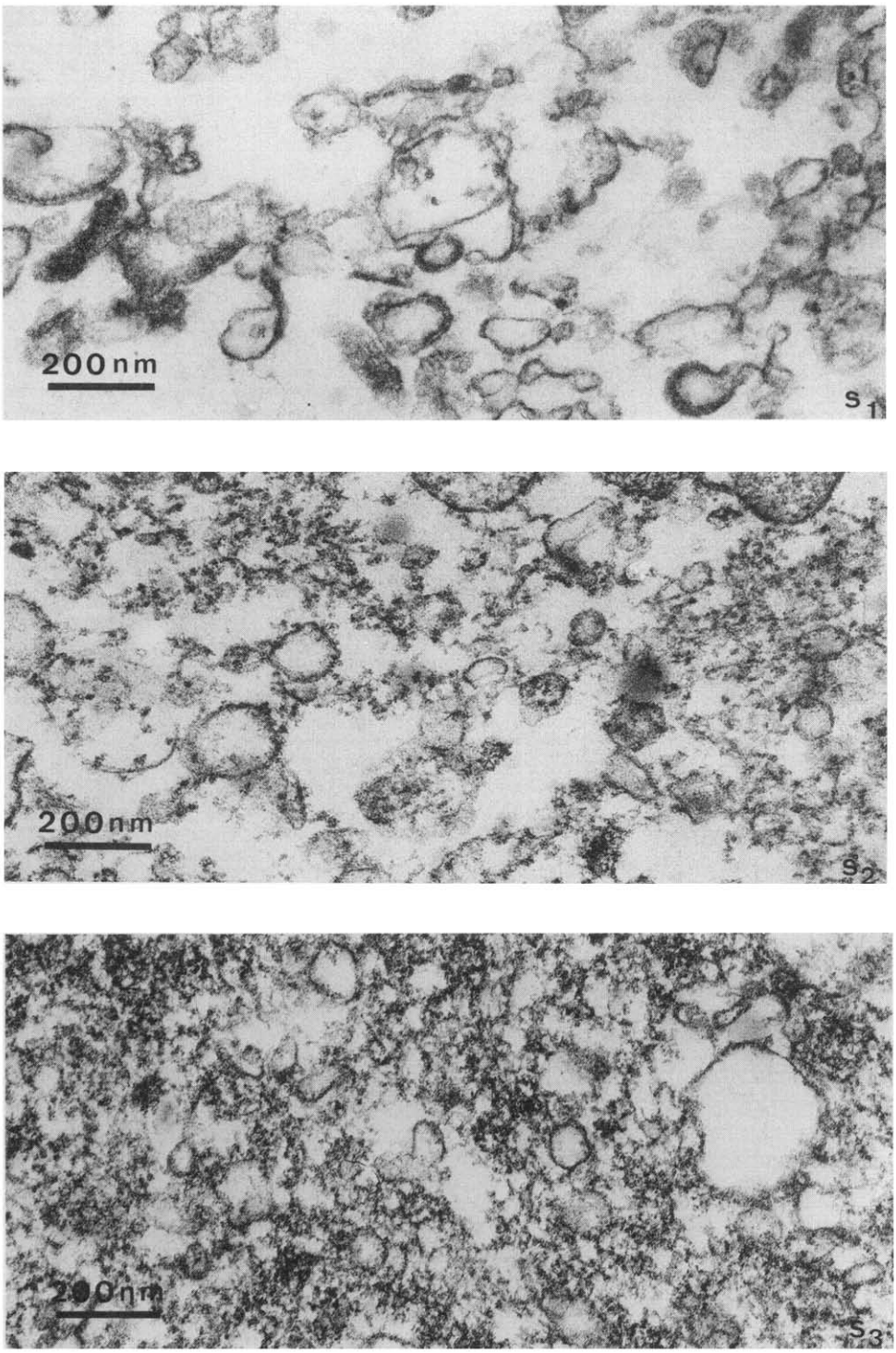

Fig. 1. Electron-micrographs of fractions from sucrose gradient. (a), Fraction S_1 characterized as plasma membranes; (b), fraction S_2, not well characterized; (c), fraction S_3 (pellet), mainly ribosomes.

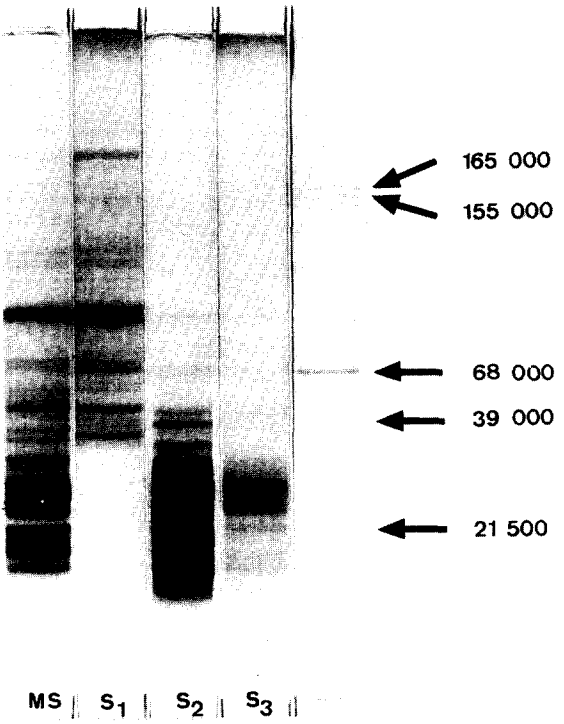

Fig. 2. SDS-polyacrylamide gel electrophoresis of sucrose gradient fractions. Gradient gels containing 4—30% polyacrylamide were used. MS, unfractionated crude membranes. S_1, S_2, S_3, upper, middle and pellet fraction after sucrose gradient centrifugation. As reference proteins were used: Trypsin inhibitor from soybean (M_r 21500), RNA-polymerase from *Escherichia coli* α-subunit (M_r 39000), bovine serum albumin (M_r 68000), RNA-polymerase β-subunit (M_r 155000) and RNA-polymerase β'-subunit (M_r 165000). These reference proteins were purchased from Boehringer (Mannheim, G.F.R.).

3. Enzyme distribution

The specific activities of four enzymes which occur in thymocyte plasma membranes were determined, i.e., alkaline nitrophenylphosphatase [17,18] γ-glutamyl transferase [19], $(Mg^{2+} + Na^+ + K^+)$-ATPase [20], and acyl-CoA: lysophosphatidylcholine acyltransferase [6].

It is not established that the latter enzyme is localized exclusively in the plasma membrane. Total activities (Table III) found in purified plasma membranes could support this notion. However, since the specific activity is increased during purification of plasma membranes one could also suggest that the low total activities are caused by an inactivation during the membrane preparation.

According to these experiments it is possible to separate vesicle populations bearing different enzymes by free-flow electrophoresis (Fig. 4). Two enzymes are completely separated from each other (acyl-CoA:lysophosphatidylcholine acyltransferase and alkaline nitrophenylphosphatase) while the γ-glutamyl transferase and the $(Mg^{2+} + Na^+ + K^+)$-ATPase are only partially separated. The specific activities of the alkaline nitrophenylphosphatase (fractions 35—45) and of the lysophosphatidylcholine acyltransferase (fractions 14—28) did not coincide with the main protein peak but were clearly separated from each other. The γ-glu-

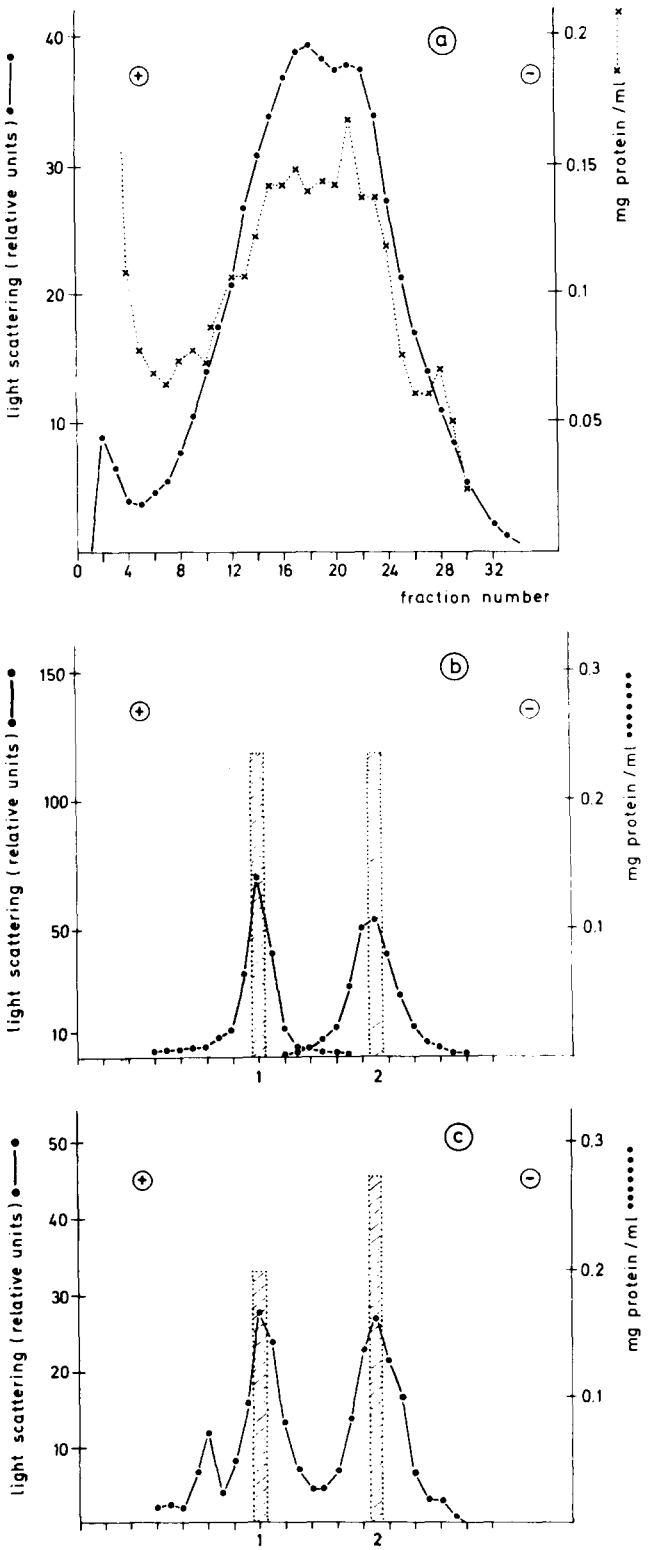

Fig. 3. Free-flow electrophoresis of sucrose gradient fraction S_1. (a), Comparison of the protein distribution (dotted line) with light scattering (solid line) at 500/500 nm, right angle; (b), re-electrophoresis of two single fractions: Columns 1 and 2 indicate two fractions from a previous electrophoretic separation; (c), re-electrophoresis of two single fractions: Columns 1 and 2 indicate two fractions from a previous electrophoretic separation which were mixed and re-electrophorezed (solid line).

tamyl transferase and the $(Mg^{2+} + Na^{+} + K^{+})$-ATPase were located between the main protein peak and the alkaline nitrophenylphosphatase peak partly overlapping with them. It should be mentioned that Fig. 4 shows the specific activities of these enzymes. When total enzyme activities are plotted the acyl-CoA:lysophosphatidylcholine acyltransferase and the alkaline nitrophenylphosphatase are also clearly separated from the main protein peak in contrast to the total activities of the γ-glutamyl transferase and the ATPase which coincided with the protein peak.

In about 35 experiments the same separation pattern always was observed. After the separation all enzymes were still found to be membrane-bound and were not solubilized during the separation procedure. This is documented in Table I which shows that about 95% of the total activities could be pelleted by centrifugation at 177000 × g_{av}, 60 min. In order to control whether the separation of membrane vesicles bearing different enzymes reflects a heterogeneity of the original cell population we electrophoresed intact thymocytes by free-flow

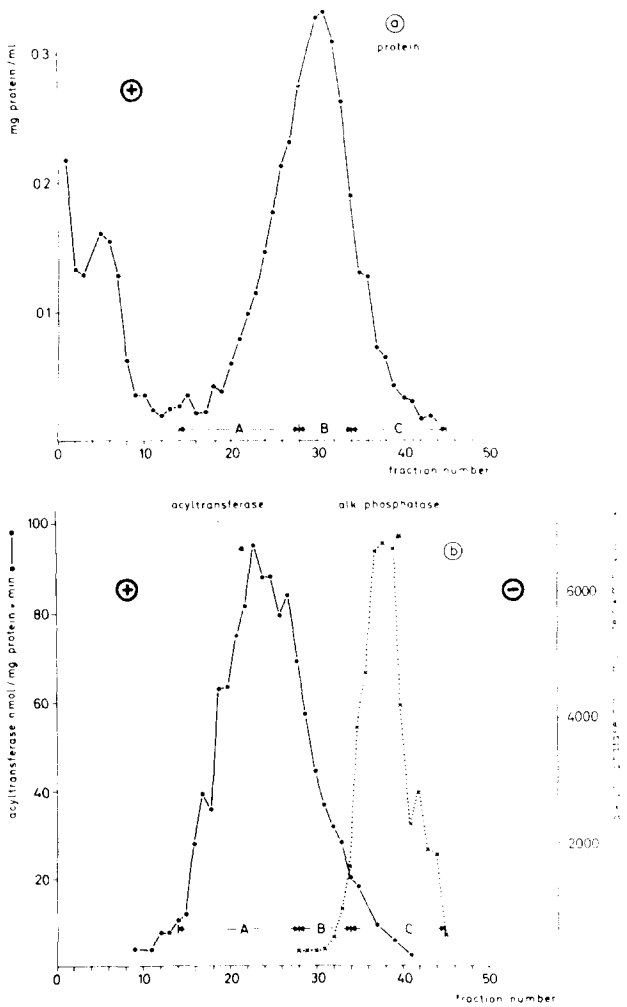

Fig. 4, a and b. See opposite page for legend.

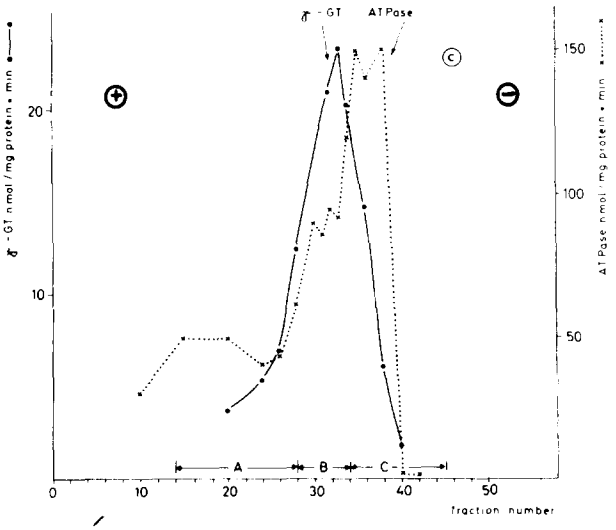

Fig. 4. Free-flow electrophoresis af sucrose gradient fraction S_1. Separation patterns of protein (a) and membrane-bound enzyme (b and c). (b). Solid line, acyl-CoA:lysophosphatidylcholine acyltransferase; dotted line, alkaline nitrophenylphosphatase. (c). Solid line, γ-glutamyl transferase; dotted line, (Mg^{2+} + Na^+ + K^+)-ATPase. Enzyme activities are expressed as nmol/mg protein/min. The peak fractions were pooled for further biochemical analysis (see Tables II and III) as indicated: A, vesicles bearing acyl-CoA: lysophosphatidylcholine acyltransferase (fractions 14—28); B, bulk membrane fraction (fractions 29—34); C, vesicles bearing alkaline nitrophenylphosphatase (fractions 35—45).

electrophoresis. The distribution of the alkaline nitrophenylphosphatase gives no indication that a specific cell population can be separated by free-flow electrophoresis which contains the total activity in this enzyme. As shown in Fig. 5, the specific activity per cell is nearly equal in all fractions. However, this

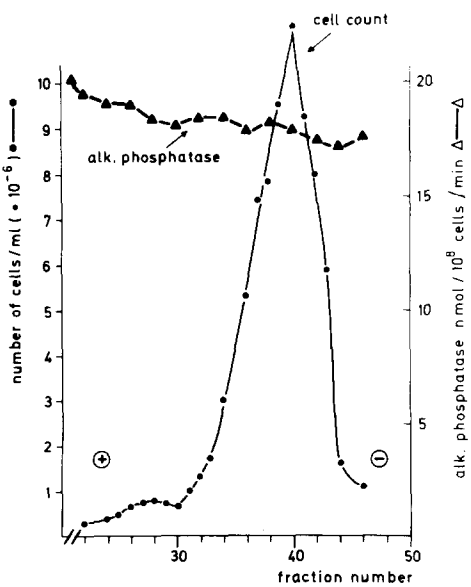

Fig. 5. Free-flow electrophoretic separation of intact thymocytes. Solid line, cell number/ml of fraction; dotted line, specific activity of alkaline nitrophenylphosphatase (nmol/10^8 cells per min).

type of experiment is not completely comparable with the separation method of membranes since different buffer systems were used.

4. Cholesterol and phospholipid composition

The profiles of the total phospholipid and cholesterol content in general coincide with the protein distribution curve (Fig. 6). The only difference is found in the more negatively charged region where some protein material occurs which contains no cholesterol and phospholipid. The nature of this material is not yet clear. One possibility would be that peripheral membrane proteins are dissolved during the separation procedure.

Interestingly, the specific content (per mg of protein) of both phospholipid and cholesterol increases with more positively charged vesicle populations and shows a peak in the region of the protein peak (Fig. 6). On the other hand, the molar ratio of cholesterol to phospholipid shows a small but significant increase with a maximal value coinciding with the most positively charged membrane vesicles.

5. Characterization and comparison of pooled fractions

The fractions containing membrane vesicles bearing different enzymes or the main protein peak respectively, were pooled as indicated in Fig. 4 (pool A: fractions 14—28; pool B: fractions 29—34; pool C: fractions 35—45). Electronmicrographic thin sections show membrane vesicles of similar size distribution (Fig. 7) and a similar morphology as unfractionated membranes (S_1 in Fig. 1a). Futhermore, the similar particle size in all three fractions (30—200 nm) confirms the theoretical and experimental results from Hannig et al. [21] that the separation principle of particles by free-flow electrophoresis is not dependent on the particle or vesicle size.

SDS-polyacrylamide gel electrophoresis of the pooled fractions also results in distinct differences (Fig. 8). Compared with pooled fraction B (main protein-containing fraction) fraction A shows an increased content of membrane protein-containing fraction) fraction A shows an increased content of membrane proteins of molecular weights of approx. 200000 and aprox. 60000 (see Fig. 8). Fraction C differs from fraction B in a higher content of membrane proteins in the region of 20—40000.

In unfractionated membranes (S_1) (Fig. 2) all these proteins could be detected by SDS-polyacrylamide gel electrophoresis.

The increased content of distinct membrane proteins in fraction A and B correlates well with the different enzyme activities of these fractions. Depending on how the individual fractions were pooled, fraction A exhibits nearly all of the acyl-CoA:lysophosphatidylcholine acyltransferase activity and fraction C the total alkaline nitrophenylphosphatase activity (Table III). This means that free-flow electrophoresis results in a further enrichment of membrane-bound enzymes as can be seen from the increase of their specific activities. The factor of enrichment for the alkaline nitrophenylphosphatase was approximately 4 compared with unfractionated purified membranes (S_1). The overall enrichment of this enzyme during the membrane preparation procedure (homogenate to fraction C) was approximately 100—150, even though the enzyme was still membrane-bound.

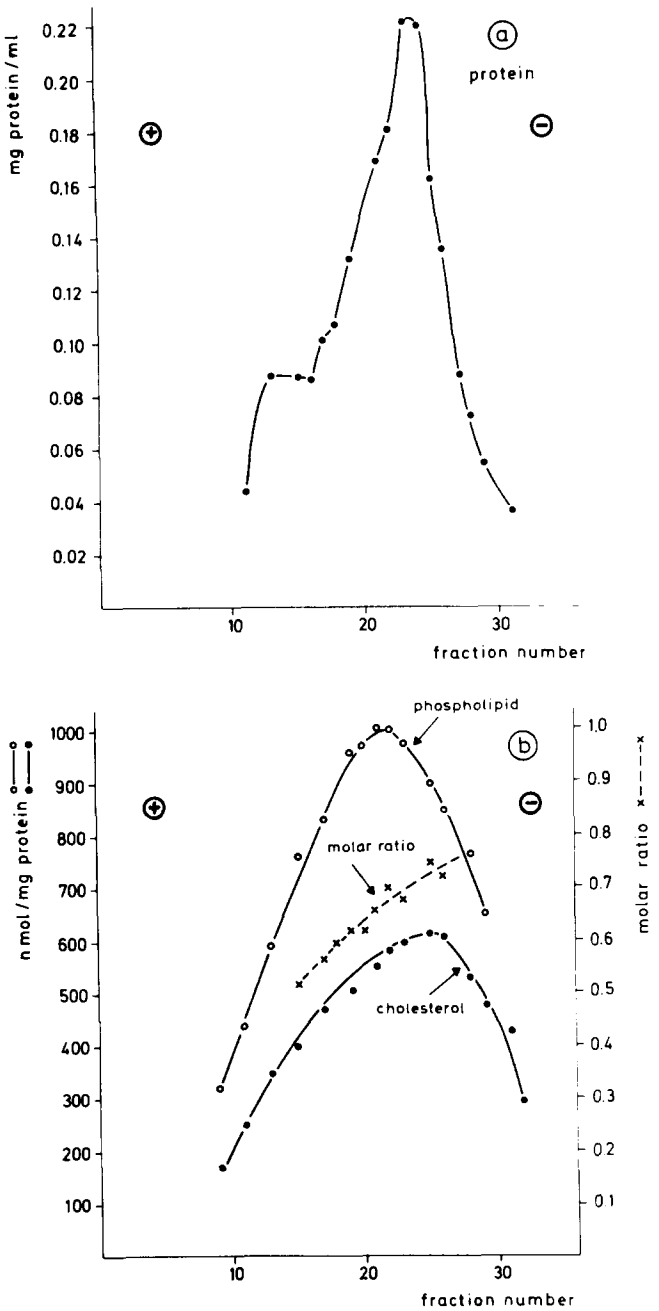

Fig. 6. Free-flow electrophoresis of sucrose gradient fraction S_1. Separation patterns of protein (a) and lipids (b). (b). ○———○, Total phospholipid (nmol/mg protein). ●———●, total cholesterol (nmol/mg protein); X———X, molar ratio of cholesterol to phospholipid.

Though the yield of the two plasma-membrane marker enzymes alkaline nitrophenylphosphatase and γ-glutamyl transferase was similar in purified membranes (gradient fraction S_1), both enzymes were clearly separated from each other after free-flow electrophoresis. Usually the γ-glutamyl transferase

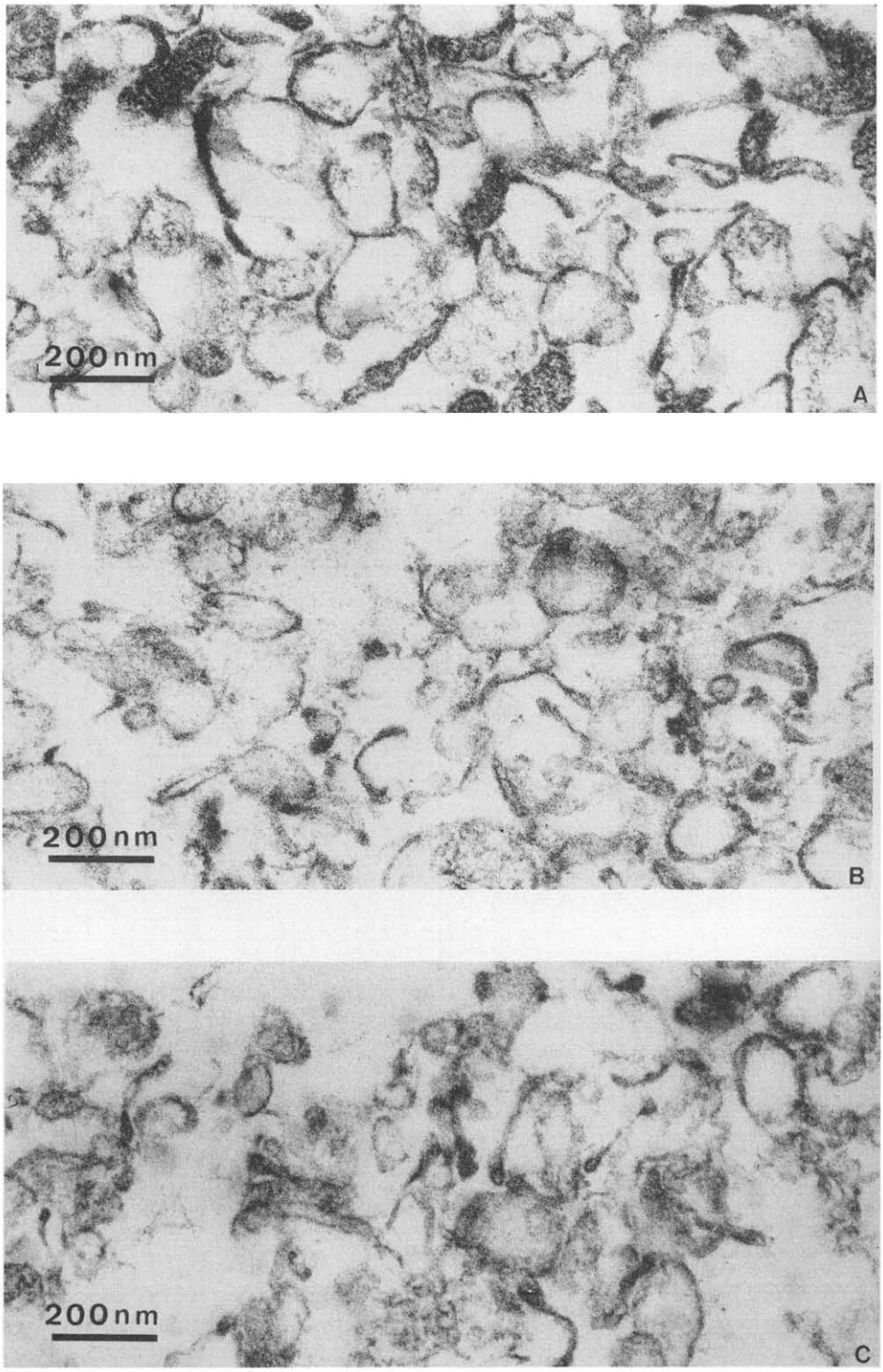

Fig. 7. Electron-micrographs of pooled fractions A—C according to Fig. 4.

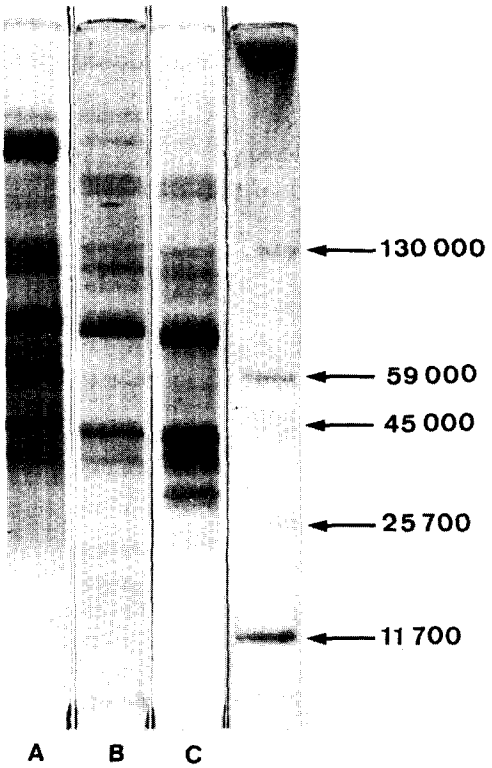

Fig. 8. SDS-polyacrylamide gel electrophoresis of pooled fractions A—C. Gradient gels containing 3—30% polyacrylamide were used. Pools A—C, see Fig. 4. As reference proteins were used: Cytochrome *c* (M_r 11700), chymotrypsinogen A (M_r 25700), ovalbumin (M_r 45000), catalase (M_r 59000) and β-galactosidase (M_r 130000) all purchased from Boehringer (Mannheim, G.F.R.).

was preferentially found in pool B (70.6%), whereas the alkaline nitrophenylphosphatase was recovered mainly in pool C (68.3%).

The parameters of binding of concanavalin A differ also in the three pooled fractions. The density of concanavalin A binding sites (per mg protein) and the total number of binding sites is highest in pool C, while the affinity for concanavalin A (association constant) is highest in pool A (Table II).

Discussion

Membrane vesicles purified by gradient centrifugation and showing all characteristics of plasma membranes, can be subfractionated by free-flow electrophoresis demonstrating that they consist of a heterogeneous population of membrane vesicles. Since Hannig et al. [21] showed that particles of different size can not be separated by free-flow electrophoresis (which is confirmed by our results) the fractionation principle is based only on differences of the surface charge.

From a more general point of view charge differences may result from the following reasons:

1. The vesicle fractions are of the same composition but consist of right-side-out and inside-out vesicles. In order to obtain an electrophoretic separation the charge difference of both sides has to be high enough.

2. The fractions consist of vesicles of different chemical composition which could result from (a) a heterogeneity of the cell population from which the membranes were obtained, or (b) from a contamination of plasma membranes with intracellular membranes, or (c) from the fact that the plasma membrane itself is heterogeneous, i.e., it consists of areas of different structure.

The fact that the subfractions consist of membranes of different chemical and enzymic composition (protein patterns, phospholipid, cholesterol content, specific activities of enzymes) rules out the possibility that the fractionation is based only on a separation of inside-out from right-side-out vesicles or of vesicles of different size. Furthermore, according to experiments with inside-out membranes from erythrocytes [22] one would expect a protein profile of two separated or partially separated peaks. The protein and lipid distribution of thymocyte plasma membranes, however, showed only one homogeneous, but rather broad, peak.

It is well known that thymocytes consist of different populations which possess different surface antigens [23]. These differences are related to the different stages of cell differentiation. In order to examine the possibility that the membrane separation descibred in this paper is based on a plasma membrane preparation obtained from the different thymocyte populations, the alkaline nitrophenylphosphatase in intact cells was determined. Free-flow electrophoresis of intact thymocytes showed the same specific content of this enzyme in all the electrophoresis fractions. This finding supports our interpretation that the fractionation of membrane vesicles is not caused by the heterogeneity of the thymocytes. However, the electrophoresis of membranes is not completely comparable with that of intact cells because the separation conditions were different (e.g. buffer composition, strength of the electrical field). Therefore, the problem of heterogeneous cell populations has to be studied using a cloned cell line.

It cannot totally be excluded that the plasma membrane is not contaminated with membranes of different origin. However, small thymocytes do not contain appreciable amounts of endoplasmic reticulum [24] and the crude membrane fraction (microsomes, $177000 \times g_{av}$, fraction after hypotonic shock) contains only small amounts of mitochondrial material. Further purification of these crude plasma membranes by sucrose gradient centrifugation results in a complete removal of ribosomes and of mitochondrial material as shown by electronmicrographs (Fig. 1) and by the absence of succinate dehydrogenase activity (Table III). Thus, the fraction with the lowest density exhibits the classical criteria used for identification and characterization of plasma membranes: The highest specific activities of marker enzymes (alkaline nitrophenylphosphatase, γ-glutamyl transferase), the highest specific content of cholesterol and phospholipid and the highest molar ratio of cholesterol to phospholipid.

Following these parameters one could suggest that only pooled fraction C after free-flow electrophoresis represents the pure plasma membrane. But since this fraction contains only 10—20% of the membranes recovered from free-flow electrophoresis it is very unlikely that this fraction exclusively represents pure

plasma membrane. This notion is supported by the fact that the acyl-CoA:lysophosphatidylcholine acyltransferase is found exclusively in pool A. Since this enzyme preferentially is located in the plasma membrane of thymocytes and lymphocytes [6], the pool A definitely has to contain plasma membrane vesicles.

The same holds for the separation of the enzymes alkaline nitrophenylphosphatase and γ-glutamyl transferase which are also known to be localized in the plasma membrane [17—19]. Furthermore, recent experiments using plasma membranes prepared by a different method, according to Wright et al. [25] and Crumpton and Snary [26], also show the same electrophoretic separation profile.

From the above discussion we favour the notion that the subfractionation of thymocyte plasma membranes reflects a heterogeneous structure of the membrane, though it may yet be too early to make final conclusions. It is noteworthy that in concordance with our previously published method of plasma-membrane subfractionation using affinity chromatography on concanavalin A Sepharose [4], free-flow electrophoretic separation also results in a vesicle population exhibiting high affinity binding sites for concanavalin A and high acyltransferase activity [1]. Thus, there seems to be a topological correlation in thymocyte plasma membranes between this special type of concanavalin A-receptor and of the acyltransferase.

The method described here also yields vesicles containing high alkaline nitrophenylphosphatase activity, a high molar ratio of cholesterol to phospholipid, and a high specifc density (per mg protein) of concanavalin A binding sites showing a low affinity for concanavalin A. The functional significance of this topological correlation is as yet unclear.

Similar to the affinity chromatography this separation procedure according to surface charge seems to be a rather specific one. Further advantages of this method, compared with gradient centrifugation techniques, are that it avoids the use of media of high ionic strength (CsCl), high osmotic activity (sucrose) or high viscosity (dextran). The continuous flow allows the separation of large quantities of material in a short time (approximately 15 mg membrane-bound protein/h). In about 35 experiments we found a high reproducibility in the degree of separation of the two marker enzymes (the distance between the peaks of the specific activities ranging from 9 to 14 fractions).

Although we have shown that the electrical field does not cause great artefacts such as membrane desintegration or fusion, we cannot exclude the possibility that peripheral proteins may be removed from the membrane, or that some enzyme activities may be impaired. The method only allows good separations with membranes purified from DNA and RNA; otherwise the vesicles aggregate.

In summary, this method seems promising for solving different problems of membrane structure such as the heterogeneity of membranes, contribution of proteins and lipids to the surface charge [27], problems about the topological correlation of membrane enzymes and lipids, and fusion of membrane vesicles.

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