Biochimica et Biophysica Acta, 603 (1980) 36—51

BBA 79008

PLASMA MEMBRANE HETEROGENEITY IN ASCITES TUMOR CELLS

ISOLATION OF A LIGHT AND A HEAVY MEMBRANE FRACTION OF THE GLYCOGEN-FREE EHRLICH-LETTRÉ SUBSTRAIN

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(Received June 24th, 1980)

Key words: Plasma membrane isolation; Fluidity; Electron microscopy; Sialic acid; Phospholipid; (Ascites tumor cell)

Summary

In this work we report on the isolation of two plasma membrane fractions of a glycogen-free substrain of Ehrlich-Lettré ascites cells, a light fraction sedimenting in a sucrose gradient at 1.10 g/ml, and a heavy fraction sedimenting at 1.16 g/ml. 95% of the cells were broken without any significant damage to the nuclei by a combination of short-term swelling and mild Dounce homogenization. A 12 000 × *g* postnuclear pellet (PII) containing major portions of the plasma membrane marker enzymes, 5′-nucleotidase, ouabain-sensitive (Na^+ + K^+)-ATPase and the alkaline phosphatase, was prepared by differential centrifugation. The two plasma membrane fractions were obtained by centrifugation on a discontinuous sucrose gradient, from which they were further purified on a linear sucrose gradient applying sedimentation velocity conditions only. Enrichment factors for the three marker enzymes were between 5- and 14-fold for the light fraction and between 3- and 7-fold for the heavy fraction with an overall yield of 1—4% and 0.5—1.7%, respectively, of cellular protein. Contamination of both fractions with nuclear material was minor. Mitochondrial contamination was about 8% for the light material and somewhat higher for the heavy material. In the light fraction, co-sedimentation of lysosomal and Golgi marker enzymes was detected. The presence of membrane structures of these organelles could not be confirmed definitely by electron microscopy. Differences in sialic acid content and phospholipid composition within the two fractions, especially in the relative proportion of lecithin to sphingomyelin, suggests differences in membrane fluidity. The light material showed mostly

unit membrane vesicles in thin-section and freeze-etch electron microscopy, whereas the heavy fraction mainly consisted of sheet-like membrane fragments.

Introduction

Plasma membranes play an important role in many biological processes such as transport and permeability events, adhesiveness and contact inhibition, agglutination, antigeneity and others. For all these reasons it is of great interest to obtain highly purified membranes in, for example, compositional and modification studies. Apart from the efficiency of diverse membrane preparation procedures with respect to yield and purity of the isolated membranes, a major discrepancy still existing between the results of researchers is related to the degree of enrichment of the membranes. The values vary between 10—15-fold [1—3] on the one hand and about 20—25-fold [4,5] on the other, and no good explanation has been given for these variations.

Plasma membranes from ascites tumor cells were isolated for the first time by Hoelzl-Wallach and Ullrey [6] from a microsomal pellet. In recent times, a couple of papers appeared in which the authors reported on the isolation of ascites cell membrane partly from a microsomal [2,7] and partly from a nuclear pellet [8].

In this paper we describe the isolation and characterization of the plasma membranes of a glycogen-free subline of ascites cells [9] using a mixture of the mitochondrial and microsomal fraction in which most of the membrane material co-sedimented. After a mild disruption of the cells [10], a combination of discontinuous and linear gradient centrifugations allowed us to separate two plasma membrane fractions which were characterized by their biochemical and enzymatic composition and their ultrastructure. Preliminary results of this work have been reported elsewhere [11], in a forthcoming paper, comparative studies of the plasma membranes of this glycogen-free subline with a glycogen-containing subline will be published.

Materials and Methods

Isolation of ascites cells. Male MIRC mice, about 5 weeks of age, were used in this study for the in vivo propagation of the glycogen-free [9] Ehrlich-Lettré ascites tumor cells. The cells were harvested 7 days after inoculation. They were centrifuged at 4000 rev./min (1800 × *g*) for 10 min to remove the ascites fluid, and then washed once with Ringer buffer, pH 7.4.

Reagent grade substrates such as 5'-AMP, ATP, *p*-nitrophenyl phosphate, cytochrome *c*, glucose 6-phosphate, β-glycerophosphate and RNAase-free sucrose were used for the enzyme tests and were purchased from Serva, Heidelberg, F.R.G.

Cell disruption. The cells were swollen in 12 vol. (related to the volume of the packed cells) of 2 mM EDTA, pH 7.0, for 5 min at 5° C, and then they were centrifuged at 4000 rev./min (1800 × *g*) for 15 min. The packed swollen cells were taken up in 6 vol. of 18 mM Tris-HCl buffer, pH 8.0, containing 25 mM NaCl and 0.5 mM $CaCl_2$, and then homogenized with 12 up and down strokes

in a Dounce homogenizer with a loose-fitting pestle. The cells were disrupted to about 90—95% as has been estimated by phase-contrast microscopy. To avoid possible breakage of the nuclei in the hypotonic homogenization medium, the homogenate was centrifuged immediately.

Subcellular fractionation. The homogenate was centrifuged at 2000 rev./min ($450 \times g$) for 2 min to sediment the major portion of the nuclei (PI). This procedure was repeated with the supernatant fraction (SI) at highly increased centrifugal speed (2500 rev./min, $700 \times g$) to remove any remaining nuclear material (PI'). The combined nuclear pellets (PI + PI') were washed with an equal volume (same as SI) of 18 mM Tris-HCl buffer pH 8, containing 25 mM NaCl and 0.5 mM $CaCl_2$, and the washing fluid (SI'') was combined with the nuclear-free supernatant (SI'). This material (SI' + SI'') was then subjected to centrifugation at 12 000 rev./min ($12\,000 \times g$) for 15 min in a Beckman J21 centrifuge using a JA-20 rotor. The pellet (PII) obtained was taken as starting material for further purification of the plasma membranes by successive discontinuous and linear sucrose density gradient centrifugations.

The isolation of mitochondria and microsomes as reference fractions was performed in a manner similar to the procedure described by Charalampous et al. [12].

The $12\,000 \times g$ pellet (PII) was taken up in 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, and 2 mM EDTA, and was made up to 20% (w/v) in sucrose concentration. Portions of 20 ml of the resulting mixture with a protein concentration of 1 mg/ml were layered on top of a gradient consisting of 20 ml of 20, 25, 33, 35 and 45% (w/v) sucrose solutions in 150-ml Pyrex centrifuge tubes. The gradients were centrifuged at 4000 rev./min ($1800 \times g$) for 2.0 h in the Christ centrifuge (Heraeus-Christ, Osterode, F.R.G.). The membrane-enriched fractions equilibrated at the 20% sucrose band and at the 35/45% interphase, whereas the major protion of mitochondrial material remained in the 30 and 35% sucrose bands. The fractions were collected by hand with a 50 ml syringe, diluted with Tris-HCl buffer, pH 7.4, to about 10% (w/v) sucrose, and the material was pelleted at 20 000 rev./min ($32\,000 \times g$) for 30 min in a Beckman L5-50 centrifuge using a JA-21 rotor.

The combined 20% band and 35/45% interphase of the plasma membrane-enriched fractions were further fractionated on a linear sucrose gradient ranging from 20 to 50% (w/v) sucrose. The material suspended in 20 mM Tris-HCl buffer, pH 8, containing 0.25 M sucrose and 2 mM EDTA with a protein concentration of 1 mg/ml was brought to 20% (w/v) sucrose and layered over the gradient. Centrifugation was performed in an SW 27 rotor at 8000 rev./min ($8450 \times g$) for 42 min using a Beckman L5-50 centrifuge. The gradients were fractionated into 5-ml fractions, and after dilution the material was pelleted at 20 000 rev./min for 30 min as previously performed.

Enzymatic assays. 5'-Nucleotidase was analyzed according to the method of Lauter et al. [13]. The enzyme assay was optimized with respect to reaction time (linear up to 20 min with a breaking point), protein concentration (300 μg), $MgCl_2$ (20 mM) and 5'-AMP (10 mM) concentrations. The mixture of $(Na^+ + K^+)$- and Mg^{2+}-ATPases was measured by using the procedure of Forte et al. [8], and differentiated from pure $(Na^+ + K^+)$-ATPase by the addition of 1 mM ouabain. The enzyme test was optimized for protein concentration (200

μg) and reaction time (30 min). Saturation kinetics were found for the ATP/$MgCl_2$ concentration (1 : 1 molar ratio) between 1 and 10 mM and for NaCl/KCl (11 : 1 molar ratio) between 30 and 110 mM. Cytochrome *c* oxidase was measured according to the method of Beaufay et al. [14] in a Beckman Acta CIII spectrophotometer using cytochrome *c* reduced with $NaBH_4$. The enzyme activity was calculated according to the method of Cooperstein and Lazarow [15]. The reaction was linear up to 10 μg of protein and between 2 and 20 μM cytochrome *c* (Fe^{2+}). The analysis of glucose-6-phosphatase [16] showed linearity up to 400 μg of protein and 40 min of incubation time with saturation between 15 and 40 mM glucose 6-phosphate.

Analytical methods. Protein was determined by using the method of Lowry et al. [17], using bovine serum albumin (Serva) as standard protein. Corrections were made for deletorious effects of buffer solutions by using the corresponding solutions as blanks. P_i was measured both by using the procedure of Bartlett [18] and that of Eibl and Lands [19]. DNA as marker for nuclear material and RNA as marker for the rough endoplasmic reticulum and ribosomes were measured according to the method of Schmidt and Thannhäuser [20] with slight modifications for the application to smaller quantities. Sialic acid was analyzed by using the method of Warren [21]. The lipids were extracted by using the procedure of Folch et al. [22], and the phospholipids quantified by phosphorus analysis after two-dimensional thin-layer chromatographic separation. Cholesterol was determined by gas-liquid chromatographic analysis using a 1.80 m stainless-steel column packed with 3% OV-17 on Chromosorb Q (80-100 mesh) and N_2 as carrier gas (Haeffner, E.W., unpublished data). For quantitative analysis, a cholestane/cholesterol mixture (1 : 4 molar ratio) was used as internal standard.

Electron microscopy. Membrane suspensions were prepared in Tris-EDTA buffer, pH 7.4, and fixed in buffered 2.5% glutaraldehyde. The material was pelleted at 40 000 × *g* for 1 h at 4°C in a Beckman L75 centrifuge (Beckman Intruments Inc., Spinco Division). The pellets were post-fixed with buffered 1% OsO_4, dehydrated in acetone, and then embedded in Spurr embedding medium. Thin sections were cut on a Reichert OMU-3 ultramicrotome and counterstained with uranyl acetate. Sections from top to bottom were routinely examined in a Siemens 102 electron microscope. For freeze-etching, the membranes were also fixed in suspension with 2.5% veronal acetate-buffered glutaraldehyde. The pellets were treated with 20% glycerol in veronal acetate buffer, frozen in Freon 22 and transferred to liquid N_2. The specimens were fractured at −100°C and replicated after 90 s of etching.

Results

Initial studies using a combination of the methods of Mamaril et al. [10] and Forte et al. [8] for cell disruption and centrifugation showed that the main portion of plasma membrane material identified by marker enzyme measurements was localized in the 12 000 × *g* fraction. Attempts to separate both fractions simply by differential centrifugation were only partly successful. Our aim then was to purify and characterize the plasma membranes from this post-nuclear fraction.

TABLE I

DISTRIBUTION OF PROTEIN AND ENZYME ACTIVITIES IN VARIOUS FRACTIONS AFTER DIFFERENTIAL CENTRIFUGATION OF THE HOMOGENATE

The specific activities are expressed in nmol/min per mg protein. Values given are means ± S.D. Figures in square brackets refer to the number of experiments with duplicate analyses. *F*, enrichment factor. Figures in parentheses refer to the percent distribution.

Fraction	Protein (mg/ml packed cells)	5′-Nucleotidase		($Na^+ + K^+$)-ATPase		Alkaline phosphatase	
		Specific activity	*F*	Specific activity	*F*	Specific activity	*F*
Homogenate	55.3 ± 10.7 [5] (100)	1.01 ± 0.22 [5] (100)	1	12.6 ± 3.7 [9] (100)	1	0.17 ± 0.04 [4] (100)	
Nuclear (450 × *g* pellet)	15.3 ± 2.4 [5] (30.1 ± 5.4)	0.24 ± 0.14 [5] (14.3 ± 4.4)	0.24	6.5 ± 3.3 [6] (14.6 ± 7.6)	0.52	0.08 ± 0.03 [4] (12.4 ± 6.5)	0.50
P_{II} (12 000 × *g* pellet)	11.9 ± 4.5 [5] (23.7 ± 4.0)	3.48 ± 0.84 [6] (56.3 ± 8.9)	3.45	39.9 ± 11.0 [12] (62.1 ± 8.0)	3.17	0.61 ± 0.09 [5] (40.9 ± 0.9)	3.63
P_{III} (105 000 × *g* pellet)	5.6 ± 1.9 [5] (10.0 ± 2.0)	1.85 ± 0.35 [5] (5.0 ± 1.6)	1.83	10.7 ± 1.8 [3] (3.5 ± 0.7)	0.85	0.08 ± 0.01 [7] (1.0 ± 0.10)	0.50
S_{III} (105 000 × *g* supernatant)	21.7 ± 6.5 [4] (36.2 ± 6.3)	0.41 ± 0.19 [5] (1.4 ± 0.9)	0.41	4.5 ± 1.9 [3] (12.5 ± 4.3)	0.36	0.20 ± 0.06 [7] (44.8 ± 3.0)	1.17
Yield (%)	93.6 ± 8.0 [5]	85.8 ± 5.5 [3]		73.1 ± 7.3 [5]		85.3 ± 12.8 [4]	

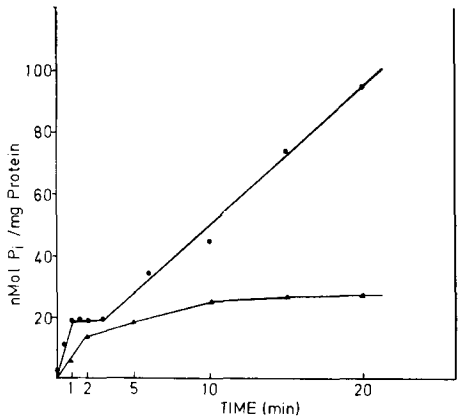

Fig. 1. Time dependence of 5'-nucleotidase activity at pH 7.4 (●———●) and at pH 9.0 (▲———▲). Between 115 and 154 μg protein of an enriched membrane fraction were incubated with 25 mM adenosine 5'-monophosphate for 10 min at 37°C. Aliquots of the incubation mixture were used for phosphorus analysis by using the method of Eibl and Lands [19].

In Table I, data are listed for the percent distribution of protein between the various subcellular fractions after differential centrifugation and of the three measured membrane-bound enzymes, 5'-nucleotidase, ouabain-sensitive (Na^+ + K^+)-ATPase and alkaline phosphatase, including their specific activities. The 12 000 × *g* pellet (PII fraction), comprising about 1/4 of the total protein, proved to contain most of the plasma membrane material, i.e., between 41 and 62% of initial total enzyme activity with very small amounts in the 105 000 × *g* pellet. During this first fractionation step, the plasma membranes were enriched to between 3- and 4-fold. Measurements of 5'-nucleotidase revealed a peculiarity with respect to its time-dependent kinetics at pH 7.4. A fast reaction was observed during the first minute followed by a plateau phase and then again a linear reaction with a decreased velocity (Fig. 1). The plateau phase was at the height observed for the plateau of the enzyme reaction measured at pH 9. It is known that at pH 9 no distinction can be made between 5'-nucleotidase and alkaline phosphatase [24]. The discontinuity in the velocity might be an expression of a mixture of two different enzyme reactions or of cleavage of another substrate present in trace amounts. Table II shows the distribution of DNA as a marker for nuclei and of RNA as a marker for free ribo- and polysomes as well as membrane-attached ribosomes. There was still up to about 6% nuclear material in the PII fraction even though the nuclei had been removed from the cell homogenate by several carefully executed centrifugation steps. The RNA content in this fraction was about 20%. The high RNA value in the reference PIII fraction is striking, however, it is not surprising since it is known that ascites cells have almost no rough endoplasmic reticulum but larger portions of ribo- and polysomes. But this fraction also contains membranous material as has been investigated by electron microscopy (not shown) and by lipid analysis, which gave a phospholipid-to-protein ratio of 0.085. This value is slightly below that which has recently been found by Forte et al. [8]. In Fig. 2 the relative specific activity distribution is given for the three plasma

TABLE II

DISTRIBUTION OF DNA AND RNA IN VARIOUS FRACTIONS AFTER DIFFERENTIAL CENTRIFUGATION OF THE HOMOGENATE

Values are expressed as means ± S.D. Figures in parentheses refer to the number of experiments with duplicate analyses.

Fraction	DNA		RNA	
	μg/mg protein	%	μg/mg protein	%
Homogenate	58.6 ± 21.4 (6)	100.0	101.4 ± 29.3 (4)	100.0
Nuclear (450 × *g* pellet)	298.8 ± 33.5 (6)	82.3 ± 3.4	95.5 ± 19.0 (5)	10.4 ± 3.8
P_{II} (12 000 × *g* pellet)	40.3 ± 7.6 (7)	6.4 ± 0.8	102.3 ± 17.6 (6)	20.0 ± 6.0
P_{III} (105 000 × *g* pellet)	10.4 ± 2.3 (3)	1.7 ± 0.1	715.4 ± 63.2 (4)	51.9 ± 4.9
S_{III} (105 000 × *g* supernatant)	9.1 ± 1.9 (4)	7.7 ± 2.3	71.4 ± 10.9 (3)	21.9 ± 4.1
Yield (%)	66.9 ± 10.1 (3)		85.7 ± 16.4 (4)	

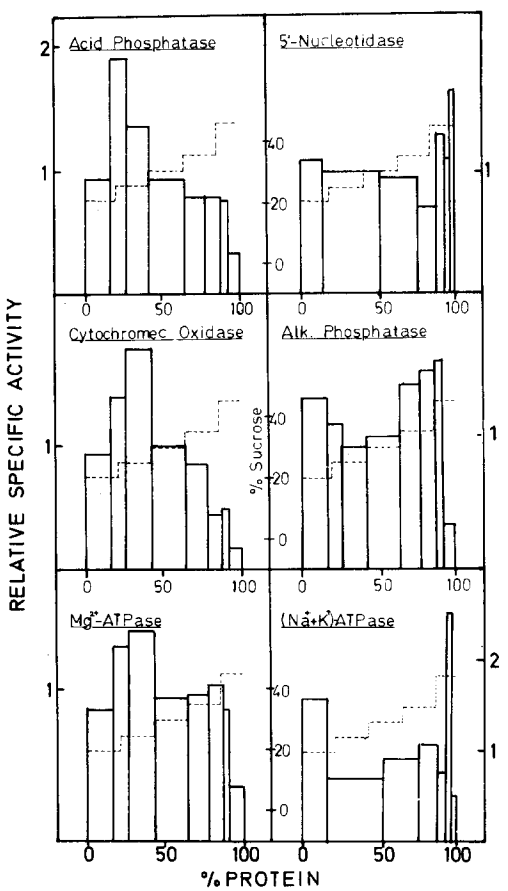

Fig. 2. Distribution of the relative specific activities of the various marker enzymes vs. the protein content (in %) in each fraction of the discontinuous gradient. (- - - - -) Sucrose concentration ranging between 20 and 50%.

membrane markers on the right-hand side of the figure. As can be seen, the enzymes exhibit a biomodal distribution between the first band (20% sucrose) and the interphase 35/45%. The light material had a light-yellow color whereas the heavy material was almost white. The acid phosphatase as a lysosomal marker is localized mainly in the second band (25% sucrose) and the cytochrome *c* oxidase as a mitochondrial marker is localized in the third band (30% sucrose) of the gradient. The Mg^{2+}-stimulated ATPase shows a somewhat mixed distribution between the third band and the lower part of the gradient. A Mg^{2+}-dependent ATPase located at the outer surface of ascites cells and stimulated both by Na^+ and K^+ has been demonstrated a few years ago [24].

In our early fractionation studies, we made the step gradient by starting with 30% sucrose at the top ranging to 60% sucrose at the bottom. Under these conditions we were only able to separate the heavy fraction whereas the light frac-

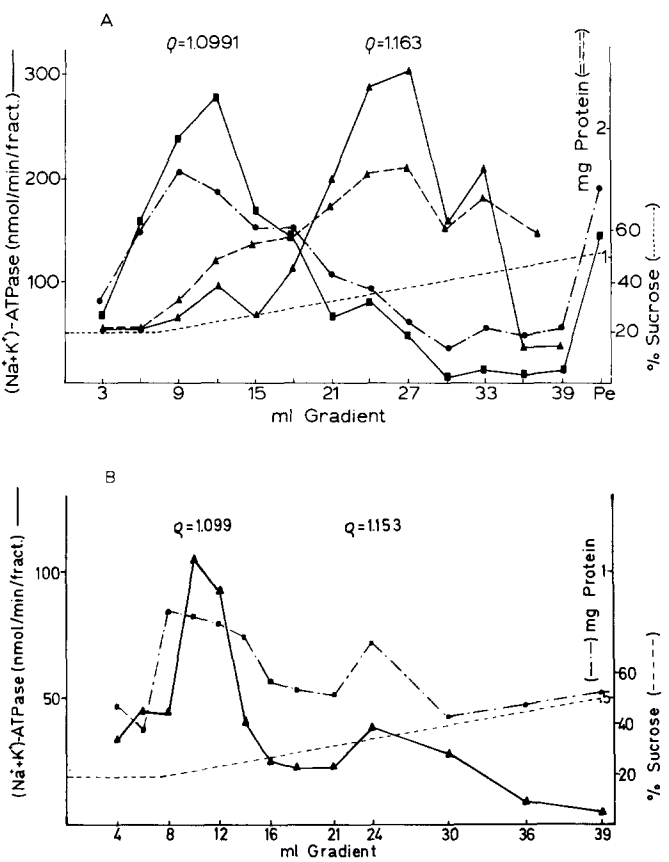

Fig. 3. Distribution of protein and the ouabain-sensitive ($Na^+ + K^+$)-ATPase after centrifugation in a linear sucrose gradient from 20 to 50% (- - - - - -). The upper part (A) shows the light (ρ = 1.099 g/cm^3) and the heavy (ρ = 1.163 g/cm^3) fractions obtained in two separate experiments using different starting material. The lower part (B) shows both membrane fractions obtained in a single experiment. Usually, 7 mg protein at a concentration of 1 mg/ml were applied on top of the gradient.

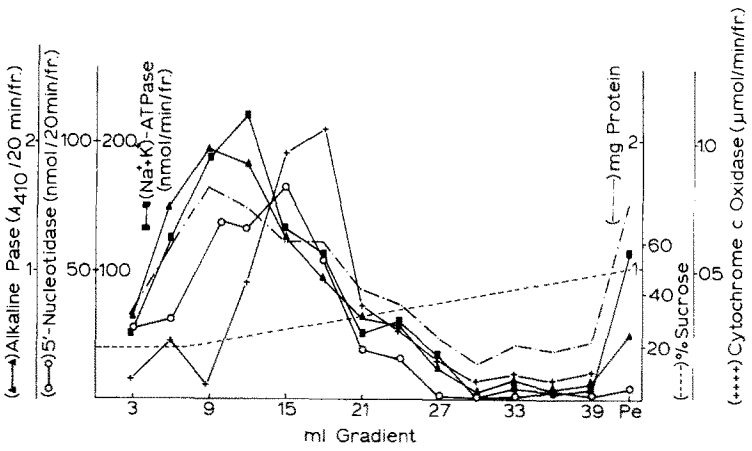

Fig. 4. Gradient centrifugation of mainly light membranes including protein and activity distribution of the three membrane markers, $(Na^+ + K^+)$-ATPase (■———■), 5'-nucleotidase (o———o), and alkaline phosphatase (Alkaline Pase) (▲———▲). Mitochondrial membranes as the major contaminating material measured by cytochrome *c* oxidase activity exhibit sedimentation properties between light and heavy material with $\rho \simeq 1.124$.

tion could not be detected because of overlap with the mitochondrial fraction at the top of the gradient. However, we used the sedimentation properties of the heavy fraction under these conditions to calculate the sedimentation coefficient using the equation of McEwen [25]. We obtained a value (at 5°C) of $9.27 \cdot 10^4$ S which gave us $\omega^2 t = 1.9 \cdot 10^9$. This meant that at a centrifugal rate of $8450 \times g$ (8000 rev./min) we had to centrifuge for about 42 min to separate the heavy fraction from other material, which means mainly the mitochondrial fraction, whereas the light membranes remained close to the top of the gradient. In Fig. 3 the distribution of the light and heavy material, applying the above conditions, is shown on a linear sucrose gradient when the two fractions are centrifuged separately (Fig. 3A) and when the combined fractions are put

TABLE III

SPECIFIC ACTIVITIES AND CONTENT OF MEMBRANE MARKERS IN THE LIGHT AND THE HEAVY FRACTION

F, enrichment factor. Activity expressed in nmol/min per mg protein. Number of experiments with duplicate analyses in parentheses.

Plasma membrane markers	Light membranes	*F*	Heavy membranes	*F*
$(Na^+ + K^+)$-ATPase	130.8 ± 11.1 (7)	10.4	47.34 ± 8.0 (6)	3.8
5'-Nucleotidase	13.46 ± 2.23 (6)	13.5	5.67 ± 1.44 (4)	7.3
Alkaline phosphatase	1.20 ± 0.22 (9)	4.7	0.57 ± 0.12 (6)	2.4
Mitochondrial marker: cytochrome *c* oxidase	115.4 ± 38.9 (5)	0.40	203.5 ± 20.5 (4)	0.71
Lysosomal marker: acid phosphatase	34.64 ± 5.3 (4)	6.9	10.94 ± 2.1 (3)	2.24
Endoplasmic reticulum marker: glucose-6-phosphatase	1.69 ± 0.5 (7)	0.70	0.75 ± 0.04 (2)	0.40
Golgi marker: thiamin pyrophosphatase	12.64 ± 2.6 (3)	6.5	7.40 ± 2.4 (3)	3.3

TABLE IV

CHEMICAL COMPOSITION OF ASCITES CELL HOMOGENATE, 12000 × *g* PELLET AND THE PLASMA MEMBRANE FRACTIONS

Results are given as means ± S.D. Number of experiments with duplicate analyses given in parentheses.

	Homogenate	12000 × *g* pellet	Light membranes	Heavy membranes
Protein (mg/ml packed cells)	55.3 ± 10.7 (5)	11.9 ± 4.5 (5)	0.17 ± 0.06 (6)	0.11 ± 0.04 (6)
Phospholipid/protein (in mg)	0.10 ± 0.04 (6)	0.30 ± 0.07 (4)	0.71 ± 0.08 (5)	0.30 ± 0.07 (4)
Sphingomyelin (% of total phospholipid)	15.6 ± 3.5 (6)	17.3 ± 1.5 (8)	30.3 ± 3.5 (6)	20.65 ± 3.70 (4)
Cardiolipin (% of total phospholipid)	5.2 ± 2.2 (5)	7.3 ± 2.3 (6)	0.66 ± 0.22 (6)	1.99 ± 0.52 (2)
Cholesterol/phospholipid (molar)	0.25 ± 0.01 (2)	0.26 ± 0.02 (2)	0.45 ± 0.11 (2)	0.30 ± 0.06 (3)
Cholesterol/protein (in mg)	0.015 ± 0.006 (3)	0.036 ± 0.003 (2)	0.09 ± 0.03 (4)	0.031 ± 0.01 (4)
DNA/protein (μg/mg)	58.5 ± 21.4 (6)	31.3 ± 16.6 (5)	7.3 ± 3.0 (3)	5.21 ± 0.97 (3)
RNA/protein (μg/mg)	101.4 ± 29.3 (4)	102.3 ± 17.6 (6)	65.9 ± 7.3 (4)	32.62 ± 5.85 (3)
Sialic acid (nmol/mg protein)	8.7 ± 2.7 (6)	13.7 ± 5.9 (7)	20.6 ± 7.1 (3)	7.68 ± 1.41 (3)

on the same gradient (Fig. 3B). It has to be noted that the quantities of membrane material put on the two gradients were not the same in Fig. 3A and B. As a marker for the membranes, the ouabain-sensitive ($Na^+ + K^+$)-ATPase activity is shown. The densities correspond rather nicely to what has been described in the literature [26] for closed vesicles (light fraction) and for open membrane fragments (present in the heavy fraction). In Fig. 4 the activity distribution of all three plasma membrane markers is shown for the light membranes only for the purpose of clarity, and of cytochrome *c* oxidase as the mitochondrial marker. Table III summarizes the membrane enzyme activities and enrichment factors for both fractions including the enzyme activities of other contaminating membranous material. The data indicate that mainly Golgi and lysosomal membranes seem to co-sediment with the plasma membranes. Similar results have been obtained for thymocyte plasma membranes [27]. Table IV summarizes the chemical composition analyses of the two membrane fractions as a balance sheet comparing each of the purification steps. The yield in plasma membrane material on a protein basis was about 1—4% for the light fraction and about 0.5—1.7% for the heavy fraction. The phospholipid-to-protein ratio increased from 1 to 7 (1 to 4 for the heavy membranes) and the sphingomyelin content almost doubled in the light membranes. Taking the values for DNA content, contamination was calculated to be between 1.7 (heavy) and 2.4% (light material). Mitochondrial contamination, as judged by the cardiolipin content, amounted to about 8% for the light material which correlates rather well with the cytochrome aa_3 content measured by differential spectroscopy

TABLE V

PERCENT PHOSPHOLIPID COMPOSITION OF THE LIGHT AND HEAVY PLASMA MEMBRANE FRACTIONS

SpM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Cl, cardiolipin.

	Light membranes	Heavy membranes
SpM	30.33 ± 3.52	20.65 ± 3.70
PC	27.20 ± 4.10	34.81 ± 2.62
PE	27.90 ± 1.80	34.63 ± 3.31
PI	9.04 ± 2.60	6.11 ± 1.80
PS	4.65 ± 1.25	5.67 ± 1.70
Cl	0.66 ± 0.22	1.99 ± 0.52
PC/SpM (molar)	0.90	1.69

(not shown) and also with the cytochrome *c* oxidase activity. The corresponding values for the heavy material were generally somewhat higher. Contamination of both membrane fractions with microsomal membranes, as calculated from the glucose-6-phosphatase activity, seems to be in the same range. Table V compares the phospholipid composition of the two fractions which differ to some extent. For the molar ratio of phosphatidylcholine to sphingomyelin, a value of 0.9 is obtained for the light fraction and 1.69 for the heavy fraction suggesting a greater membrane fluidity and osmotic fragility [28] in the latter.

Electron microscopic studies

Fig. 5 shows a distribution of mostly membrane vesicles in the light plasma membrane fraction with an average diameter of about 0.2—0.5 μm. Only a few adherent structures were found. Some of the vesicles contained fine fibrils or particles of different electron density in their interior, especially in tangential or peripheral cross-sections. Other vesicles tended to round up concentrically and looked like sections of spirals or myelin structures. The thickness of the isolated membranes is less than 10 nm. This corresponds to the data of plasma membranes of ascites cells. Membranes of dictiosomes, known to be extremely thin, could not be identified. Fibrillar material adhering to the membranes (Figs. 5 and 6, fam) possibly characterizes remnants of the surface coat. This material is to be found on the inner face of most vesicles. Assuming a partial preservation of the surface coat during isolation of the plasma membrane fraction, the fibrillar layer provides evidence in favor of a rolled-up plasma membrane which turns the surface layer to the inner face of the vesicles. The granular membrane-adhering material resembles a possible ribosomal contamination which means a co-sedimentation of small vesicles of the rough endoplasmic reticulum or an accidental binding of ribosomes to the membranes during isolation occurs.

The uniformity in size of the light plasma membrane vesicles was confirmed by freeze-etch preparations (Fig. 5b). Membrane particles were randomly distributed. There is no hint of the characteristic content in factured vesicles which could be used for the detection of non-plasma membrane components in the isolates. Deep etching did not reveal ribosomal patterns.

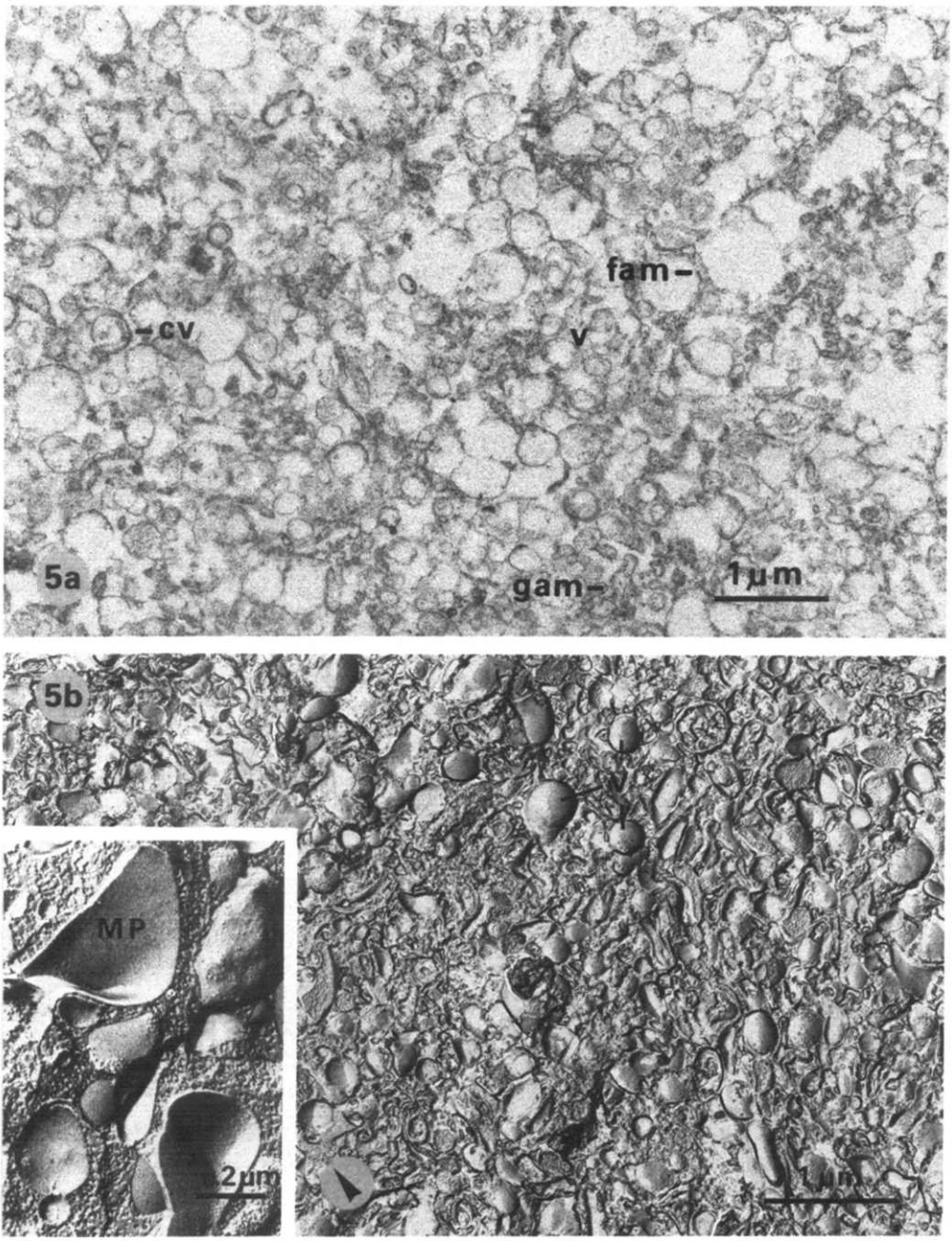

Fig. 5. (a) Thin sectioned pellet of the light material. Membrane vesicles have comparable diameters. v, vesicle; cv, concentric packed vesicles; fam, surface-adhering material, fibrillar; gam, surface-adhering material, globular. Magnification, X16 275. (b) Freeze-etch preparation of a light membrane pellet that confirms the vesicular structure of these membranes. Magnification, X18 600, insert, X46500. The insert demonstrates the regularity of plasma membrane particles (MP). The arrow indicates the direction of shadowing.

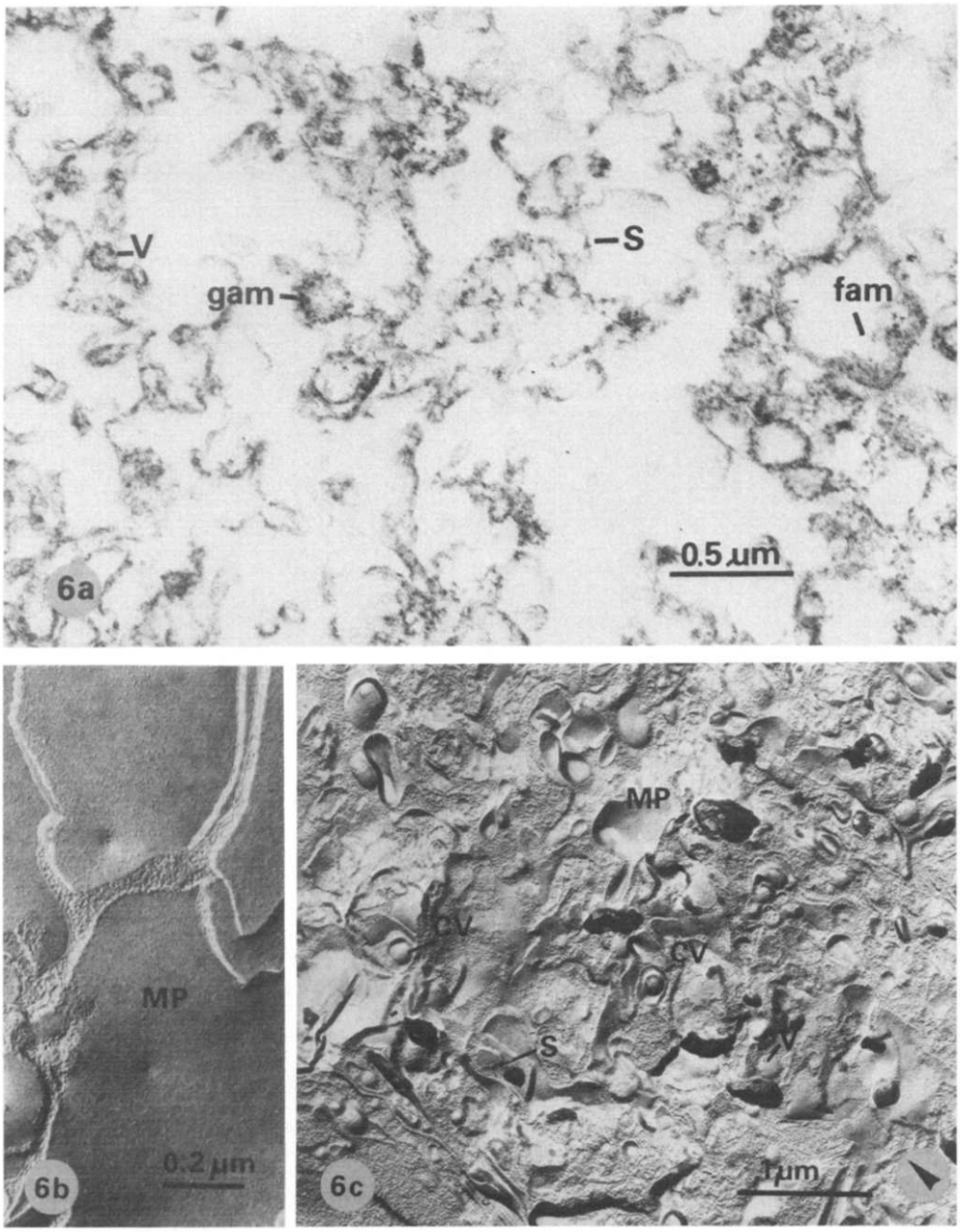

Fig. 6. (a) Thin sectioned pellet of the heavy material. Open structures cause the whole-like areas or piled sheets of membranes. Magnification, X34 130. (b and c) Freeze-etch preparation of a heavy membrane pellet which demonstrates a reduced vesicular appearance of the membranes. b shows the piling of membrane sheets, covered by membrane particles. S, sheet. Other symbols and magnification, see Fig. 5.

The application of the same techniques to the heavy plasma membrane fraction revealed a significant contrast (Fig. 6). In thin sections (Fig. 6a) the structures were found to be more heterogeneous with respect to vesicle diameter and the appearance of sheets. The latter could be demonstrated by

numerous open membrane structures in cross-sections and a lot of more or less tangentially displayed membrane areas which in some cases demonstrated ribosomal (globular) or fibrillar surface components. The ribosomal contamination is higher than in the light membrane fraction, however, typical endoplasmic reticulum systems cannot be identified.

Freeze-etched fractions of the heavy fraction (Fig. 6b and c) support the demonstration of large flat sheets of membranes which were fractured at diverse angles. The number of small, rounded vesicles was drastically reduced. The distribution pattern of membrane particles coincided with that of the light membranes. Greater vesicles or sheets were packed in piles, surely reflecting a similar type of order which was represented by the concentrically packed small vesicles in the light fractions.

Discussion

The plasma membrane isolation procedure described in this paper differs from a great number of other investigations insofar as the membrane material has been purified successfully from a relatively low-speed pellet. Since the sedimentation properties of membranes in general can be influenced by the conditions of cell disruption, we assume that our very mild homogenization step combined with a swelling treatment of the cells is in favor of the observed sedimentation behavior of our plasma membranes. With respect to the size of mostly vesicles in the light material, there was no apparent difference from other membrane preparations. Freeze-etching of the heavy fraction, on the other hand, revealed that this material consisted of more than 50% multilamellar sheets. There are, in addition, indications from affinity chromatography for the existence of two fractions of ascites cell membranes with different binding properties (Tiggemann, R., personal communication) which would support our observations. Plasma membrane heterogeneity has also been documented for lymphocytes using affinity chromatography on concanavalin A-Sepharose [29]. However, in many other membrane preparations including ascites cells this has not been observed, probably because the heavy membranes were removed with the low-speed material [27]. The question as to whether or not the two membrane subfractions originate from different cells or just from different areas of an individual cell cannot be answered definitely from this study. There is evidence which would support either possibility. First, since the differentiated domains within the plasma membrane are well known, a selective breakage of certain membrane fragments during cell disruptions seems conceivable. Compositional differences of our isolated membrane fractions have been found for the polar lipids, cholesterol and sialic acid. The RNA content taken as a contaminant was also different in both fractions. Second, concerning the presence of subclasses, we have some indication showing changes of the cellular surface with the cell cycle (Zimmermann, H.P., personal communication).

Apart from our major effort to isolate plasma membranes representing the entire cellular surface, we were also very interested in setting up balance sheets which would allow us to follow the enrichment of membrane material step by step giving us greater confidence in our procedure. This approach is illustrated very nicely for the marker enzyme activities (Table I) and the chemical com-

position analyses (Table IV) which give almost equal enrichment factors, especially for the phospholipid and cholesterol content on a protein basis. The data obtained in our study generally agree with those reported by others [4,5]. There are indications that the alkaline phosphatase differs in its properties from other plasma membrane marker enzymes. Monneron and d'Alayer [27] found both a higher yield and enrichment of this enzyme in their preparation, suggesting to them a possible heterogeneous distribution among plasma membrane vesicles. Our data, on the other hand, show a lower yield and enrichment of alkaline phosphatase both in the light and the heavy fractions leading to the conclusion that a difference in enzyme distribution seems to be less likely than a deactivation/activation of the enzyme. It should be mentioned that a larger percentage of alkaline phosphatase activity was also detected in the soluble fraction; whether it is solubilized during the isolation procedure or exists per se as a second enzyme cannot be distinguished at present.

Ascites tumor cells are known to have rather large nuclei. Therefore, it was of great importance to keep the nuclei intact as far as possible during the disruption of the cells which under these conditions would minimize a possible contamination of nuclear membranes with our plasma membrane fractions. By using the technique described by Mamaril et al. [10] which was specifically aimed at the preparation of intact nuclei, we were able to remove almost 90% of undamaged nuclear material at the first centrifugation step. The remaining DNA in the final preparation may in part originate from mitochondrial DNA. A possible damaging effect of EDTA on the cell surface architecture during the hypotonic shock of the cells can be excluded, since EDTA has only been known to disrupt membranes after the cells have been homogenized at higher EDTA concentrations and kept under these conditions for longer periods of time [30]. In addition, there are even indications that EDTA was found to be necessary to achieve reproducible separation of plasma membrane vesicles from other components [31].

Acknowledgements

Our thanks are due to Mrs. C. Wöhlert, Mrs. H. Jowanovic and Mr. B. Heck for technical assistance, and to Miss Finze for the electron microscopy. The differential spectroscopy measurements were kindly performed by Drs. E. Jarasch and G. Bruder from the Department of Membrane Biology and Biochemistry of the Krebsforschungszentrum.

References

1 Shin, B.C. and Carraway, K.L. (1973) Biochim. Biophys. Acta 330, 254—268
2 Solomonson, L.P., Liepkalns, V.A. and Spector, A.A. (1976) Biochemistry 15, 892—897
3 Friedberg, S.J. and Halpert, M. (1978) J. Lipid Res. 19, 57—64
4 Rittenhouse, H.G., Rittenhouse, J.W. and Takemoto, L. (1978) Biochemistry 17, 829—837
5 Colombini, M. and Johnstone, R.M. (1973) Biochim. Biophys. Acta 323, 69—86
6 Hoelz-Wallach, D.F. and Ullrey, D. (1964) Biochim. Biophys. Acta 88, 620—629
7 Molnar, J., Marcovic, G. and Chao, H. (1969) Arch. Biochem. Biophys. 134, 524—532
8 Forte, J.G., Forte, T.M. and Heinz, E. (1973) Biochim. Biophys. Acta 198, 827—841
9 Paweletz, N. (1972) Cytobiology 4, 103—115
10 Mamaril, F.P., Dobrjansky, A. and Green, S. (1970) Cancer Res. 30, 352—356

11 Kolbe, K. and Haeffner, E.W. (1976) 10th Int. Congr. Biochem., Hamburg, p. 282
12 Charalampous, F.C., Gonatas, N.K. and Melbourne, A.G. (1973) J. Cell Biol. 59, 421—435
13 Lauter, C.J., Colyom, A. and Trams, E.G. (1972) Biochim. Biophys. Acta 266, 511—523
14 Beaufay, H., Bendall, D.S., Baudhuin, P., Wattiaux, R. and DeDuve, C. (1959) Biochem. J. 73, 628—637
15 Cooperstein, S.J. and Lazarow, A. (1951) J. Biol. Chem. 189, 665—670
16 DeDuve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604—617
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Bartlett, G.R. (1958) J. Biol. Chem. 234, 466—468
19 Eibl, H.J. and Lands, W.E.M. (1969) Anal. Biochem. 30, 51—57
20 Schmidt, G. and Thannhäuser, S.J. (1945) J. Biol. Chem. 161, 83—89
21 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975
22 Folch, J., Lees, M. and Stanley, Y.G. (1957) J. Biol. Chem. 226, 497—509
23 Lee, A., Chance, K., Weeks, C. and Weeks, G. (1975) Arch. Biochem. Biophys. 171, 407—417
24 Ronquist, G. and Ägren, K. (1975) Cancer Res. 35, 1402—1406
25 McEwen, C.R. (1967) Anal. Biochem. 20, 114—119
26 Hoelzl-Wallach, D.F. and Knüfermann, H.G. (1973) The Plasma Membrane: Dynamic Perspectives, Genetics and Pathology, Springer-Verlag, New York, p. 37
27 Monneron, A. and d'Alayer, J. (1978) J. Cell Biol. 77, 211—231
28 Borochov, H., Zahler, P., Wilbrandt, W. and Shinitzky, M. (1977) Biochim. Biophys. Acta 470, 382—388
29 Resch, K., Loracher, A., Mähler, B., Stoeck, M. and Rode, H.N. (1978) Biochim. Biophys. Acta 511, 176—193
30 Matsushita, K., Adachi, O., Shinagawa, E. and Ameyana, A. (1978) J. Biochem. 83, 171—181
31 McKeel, D.W. and Jarett, L. (1970) J. Cell Biol. 44, 417—432