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STUDIES ON PLASMA MEMBRANES

XIX. ISOLATION AND CHARACTERIZATION OF A PLASMA MEMBRANE FRACTION FROM CALF THYMOCYTES

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SUMMARY

A plasma membrane fraction was isolated from calf thymocytes by a modification of the method of Wallach and Kamat (Wallach, D. F. H. and Kamat, V. B. (1966) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 8, pp. 164–172, Academic Press, New York). Fractions were examined electron microscopically and subjected to chemical and enzymic assays.

With respect to the cell homogenate and the final microsomal fraction, respectively, the plasma membrane fraction was enriched by a factor 23 and 5.1 in cholesterol, 11 and 2.4 in phospholipid, 5.1 and 4.2 in sialic acid, 20 and 5.2 in Mg^{2+}-ATPase (EC 3.6.1.3), and 8 and 2.6 in 5′-nucleotidase (EC 3.1.3.5). Succinate:cytochrome *c* oxidoreductase (EC 1.3.99.1) was lacking, and DNA was hardly if at all present in the plasma membrane fraction. The major part of the RNA found in this fraction (30 $\mu g \cdot mg^{-1}$ protein) was concluded to be an authentic component of the plasma membrane.

The concept of membrane "markers" was briefly considered and the conclusion was reached that by current criteria and electron microscopic evidence the plasma membrane fraction obtained from calf thymocytes consisted of reasonably clean plasma membranes.

INTRODUCTION

From the point of view of cell interactions mediated by the cell surface, lymphocytes are very interesting cells to study. One approach is through the isolation of the surface membrane. Isolation of plasma membranes from free cells requires a different methodology from that developed for cells of solid tissues, such as liver, on which much progress has been made in the last decade. A basic method for isolation of plasma membranes from free cells is the one originally devised by Wallach and Kamat[1] for Ehrlich ascites tumor cells. Our experience shows that this method should be adjusted to meet the particular requirements set by the type of cells under study.

The cells studied in the present investigation are calf thymocytes, chosen with

the aim to develop a "model" for plasma membrane isolation from free lymphocytes to be later adapted to the study of peripheral lymphocytes and leukemic cells. Since calf thymocytes contain large nuclei, but a small amount of cytoplasm and fragile membranes, prone to damage that easily leads to clumping of the free cells *in vitro*, it was necessary to vary the suspending medium and the pressure for cell disruption (nitrogen cavitation method) to find the optimal conditions. The separation of the plasma membranes from the microsomes as a function of the density of the Ficoll solution was also studied. Fractions were examined electron microscopically and subjected to chemical and enzymic assays.

Very recently, plasma membranes have been prepared by Allan and Crumpton[2,3] from lymphocytes of pig lymphnodes and pig and human thymus. To achieve cell disruption the tissues were pressed through a perforated plate; this method is not suitable for free lymphocytes at which we aimed. Dods *et al.*[4] isolated plasma membranes from a leukemic cell line following mild glutaraldehyde fixation of the disrupted cells. This method risks a partial or total inactivation of some biological (antigenic?) functions of the membrane. Most recently, when our experimental work was nearing its end, Ferber *et al.*[5] also published a modification of the Wallach–Kamat method for isolating plasma membranes from pig and calf lymphnode lymphocytes.

MATERIALS AND METHODS

Twice glass-distilled water was used in all experiments. Chemicals used were of analytical grade.

Preparation of thymocyte suspensions

Thymuses were removed within a few minutes after the death of the calves in the slaughter house, and were put directly into ice. The temperature was maintained within 0–4 °C throughout all further steps. After removal of pieces of fat and blood vessels, the thymus was cut into small pieces and finally minced with scissors in Hanks solution (Oxoid). The resulting suspension was freed from clumps of cells and connective tissue by filtration through stainless steel gauze, 18 threads (thickness 0.5 mm) per cm^2, followed by two filtrations through one and four layers of surgical gauze (18 threads/cm^2), respectively. The filtered cell suspension was kept overnight in the presence of 10% defatted (by centrifugation) human serum; at this stage cell counts were made in a hemocytometer. Early the next morning the cells were centrifuged at $250 \times g_{max}$ for 10 min and after sucking off the supernatant the pellet was washed twice with 5 vol. of Hanks solution. The cells were suspended in Hanks solution at a concentration of about $7.5 \cdot 10^{10}$ cells (corresponding to 25 ml packed cells) per 100 ml. As judged by trypan blue exclusion, more than 95% of the cells were viable.

Cell disruption

A maximum of 330 ml of the thymocyte suspension was put into a chilled pressure vessel, designed and made by Baskerville and Lindsay Ltd, Manchester, based on the description given by Hunter and Commerford[6], equilibrated with 500 lb/$inch^2$ (36 atm) N_2 for 15 min under constant gentle stirring, and cell disruption was brought about by slowly releasing the suspension from the pressure vessel.

Isolation of microsomes (Fraction P_1)

Immediately after cell disruption the homogenate was distributed over 8 polycarbonate tubes of a SS-34 rotor and centrifuged at $13500 \times g_{max}$ (10500 rev./min) for 15 min in a Sorvall RC2-B centrifuge. The resulting pellets, consisting of nuclei, mitochondria and lysosomes, were discarded. The supernatants were centrifuged at $105000 \times g_{max}$ (30000 rev./min in the 30 rotor of a Beckman L2 ultracentrifuge) for 75 min. The resulting pellets were suspended in 10 mM Tris–HCl buffer, pH 8.6, using a small hand-driven homogenizer of 15 ml content equipped with a Perspex pestle, and centrifuged in 4 tubes of the 30 rotor at 30000 rev./min for 75 min. Each pellet was resuspended in sufficient 1 mM Tris–HCl buffer, pH 8.6, to fill one tube of the 40 rotor; the rotor was driven at 40000 rev./min ($145000 \times g_{max}$) for 90 min to yield the pelleted Fraction P_1. For analysis the Tris washes and the original $105000 \times g_{max}$ supernatant were concentrated by ultrafiltration in an Amicon cell equipped with a Diaflo^R membrane PM-10.

Separation of plasma membranes (Fraction M) and microsomes (Fraction P_2)

Fraction P_1 was suspended in 1 mM Tris–HCl buffer of pH 8.6 containing 1 mM $MgSO_4$, at a concentration of 4–6 mg protein/ml, and dialyzed against this buffer for 2 h. The dialyzed suspension was layered on 2 vol. of Ficoll solution (Pharmacia) of density 1.05 at 25 °C (or other densities as indicated under Results), prepared according to Wallach and Kamat[1], and containing 1 mM Tris–HCl, 1 mM $MgSO_4$, pH 8.6, in cellulose nitrate tubes fitting the SW25-1 rotor and centrifuged at 25000 rev./min for 15 h. In some experiments 2 ml of suspended Fraction P_1 was layered on 3 ml Ficoll solution in SW50 rotor tubes and centrifuged at 50000 rev./min for 4 h. After centrifugation two main fractions had been separated: one at the Ficoll-buffer interface, designated Fraction M, and the other as pellet, designated Fraction P_2. In some experiments the intermediate Ficoll layer was also analyzed. Fraction M, collected by using a Pasteur pipette with a bent tip, was diluted 5-fold with 10 mM Tris–HCl, 10 mM $MgSO_4$, pH 8.6, centrifuged in a SW50 rotor at 50000 rev./min for 40 min, and the precipitated material was suspended in 1 mM Tris–HCl buffer, pH 8.6, for analysis. The intermediate fraction and Fraction P_2 were treated likewise, except that for the latter fraction 1 mM Tris–HCl buffer, pH 8.6, was used throughout.

Chemical determinations

Protein was determined by the method of Lowry *et al.*[7], as used by Emmelot and Bos[8], with bovine serum albumin (Armour Pharmaceutical Co. Ltd) as standard. DNA and RNA were extracted according to the Schmidt–Thannhauser procedure as described by Munro and Fleck[9]. For the determination of RNA an absorbance of 1.000 at 260 nm was assumed to be equivalent to 32 μg RNA/ml. DNA was measured according to Ceriotti[10].

For measuring phospholipid and cholesterol, aliquots of fractions were extracted with chloroform–methanol (2:1, v/v) (Merck) as described by Folch *et al.*[11]. Phospholipid phosphorus was determined according to the method of Morrison[12], with subsequent extraction of the blue pigment in an appropriate volume of *n*-amylalcohol and measuring the absorbance at 795 nm instead of 822 nm[13,14]. Total cholesterol was determined by the Liebermann–Burchard reaction as de-

scribed by Stadtmann[15] with cholesterol (British Drug Houses Ltd) as standard. Cholesteryl esters and cholesterol of Fraction M were separated on thin-layer silicagel, eluted as described previously[14], and measured separately, with cholesteryl palmitate (Fluka A.G.) serving as standard for the cholesteryl ester determination. Sialic acid was determined by the thiobarbituric acid method according to Warren[16] after hydrolysis in 0.05 M H_2SO_4 at 80 °C for 60 min.

Enzymes

Mg^{2+}-ATPase (EC 3.6.1.3) and (Na^+-K^+)-ATPase activities were determined at pH 7.4 as described before[17], P_i being measured by the method of Fiske and SubbaRow[18]. 5′-Nucleotidase (EC 3.1.3.5) was measured at pH 7.4 as described previously[17]; the P_i liberated by this enzyme and by glucose-6-phosphatase was measured by the method of Ames[19]. In all cases blank values were subtracted. Glucose-6-phosphatase (EC 3.1.3.9) was determined according to Swanson[20], and succinate:cytochrome *c* oxidoreductase (EC 1.3.99.1) according to De Duve *et al.*[21]. NADH oxidoreductase (EC 1.6.4) was measured according to Wallach and Kamat[1]. Neutral (pH 7.2) and acid (pH 5.0) phosphatase (EC 3.1.3.1 and EC 3.1.3.2) were determined according to Emmelot and Bos[22], and Ostrowski and Tsugita[23], respectively, using disodium *p*-nitrophenyl phosphate (Light) as substrate.

Electron microscopy

Small parts of calf thymus were fixed at the slaughter house as rapidly as possible after the death of the animal. The tissue blocks, as well as free cells prepared from cooled thymuses in the laboratory, were fixed in 2% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.3). Pellets of the fractions were fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, rinsed with the same buffer and postfixed for 1.5 h in 1% OsO_4. During OsO_4 fixation the pellets were carefully dislodged from the tubes and cut into small pieces. After dehydration in a graded series of ethanol the specimens were embedded in a mixture of Epon 812 and Araldite. The pellets were embedded in such a way that each thin section covered the whole depth of the pellet from top to bottom. Thin sections were stained with uranylacetate and lead hydroxide, and examined in a Philips EM 300 microscope at 80 kV.

RESULTS

Medium and nitrogen pressure for cell disruption

The sucrose containing medium used by Wallach and Kamat[1] for suspension of Ehrlich ascites carcinoma cells prior to their disruption by the intracytoplasmic nitrogen cavitation method, was not suitable for calf thymocytes. The latter cells aggregated irreversibly and DNA leaked out. Addition of 0.2 mM $MgSO_4$ or 2 mM $CaCl_2$ (to protect membranes[1,24,25]) to the sucrose medium did not improve the results, nor was phosphate-buffered saline (pH 7.4) with or without 2 mM $CaCl_2$ very suitable, although it was somewhat superior to the sucrose media. The best results were obtained with Hanks solution as suspending medium; the very slight agglutination which was observed after washing and centrifuging the cells in this medium did not affect the subsequent cell disruption and particle isolation. Intro-

duction of the thymocytes suspended in Hanks solution into sucrose medium also led to cell aggregation and DNA leakage, in spite of precautions as advocated by Ferber *et al.*[5] who were also faced with the fragility of their lymphocytes (see also ref. 2). Accordingly, cell disruption by the nitrogen cavitation method was carried out in Hanks medium. To this end, a pressure of 500 lb/inch^2 (36 atm) proved to be optimal in terms of yield of Fraction P_1 (to be used as a source of plasma membranes) while leaving the nuclei intact. The time of equilibration was even more critical: pressure release after 15 min leaved nearly all nuclei intact, but when pressure was released after 20 min DNA started to leak from the nuclei resulting in glue formation and counteracting yields.

Preparation of microsomes (Fraction P_1)

The time interval between cell disruption and the $13500 \times g_{max}$ centrifugation was kept to a minimum because of the risk of particle aggregation. Addition of EDTA (6 mM final, in view of the composition of Hanks medium) immediately after cell disruption[1,5,26] was omitted since it caused some clumping in the homogenate. This omission did not affect the properties of the subsequently isolated plasma membranes. Since addition of EDTA to the supernatant resulting from the $13500 \times g_{max}$ centrifugation did not affect the results either, it was not applied. The gelatineous character of the $13500 \times g_{max}$ pellet did not allow its resuspension in fresh Hanks medium, so that no more microsomal material could be salvaged from the pellet. Fraction P_1 was prepared by centrifugation of the $13500 \times g_{max}$ supernatant at $105000 \times g_{max}$ followed by two Tris washings as described under Materials and Methods. Each washing reduced the degree of packing of the subsequent pellet.

The main chemical composition and the specific activities of a number of enzymes of Fraction P_1, the $13500 \times g_{max}$ supernatant and the cell homogenate, are listed in Tables I and II. With respect to the cell homogenate, Fraction P_1 was enriched 9.8-fold in cholesterol, 6.2-fold in phospholipids, 8.4-fold in Mg^{2+}-ATPase, 11.3-fold in NADH oxidoreductase, and 4.8-fold in 5'-nucleotidase. Recoveries of chemical and enzymic determinants are included in Table VI. Fraction P_1 contained

TABLE I

CHEMICAL COMPOSITION OF HOMOGENATE, $13500 \times g_{max}$ SUPERNATANT AND FRACTION P_1 PREPARED FROM CALF THYMOCYTES

Data are the mean ± S.D., with numbers of different experiments in parentheses.

Compound	*Homogenate*	*13500 × g_{max} supernatant*	*Fraction P_1*
Sialic acid*	12.9 ± 1.5 (4)	15.6 ± 5.0 (6)	28.9 ± 3.8 (6)
Cholesterol**	17.0 ± 1.7 (4)	31 ± 2 (2)	167 ± 11 (5)
Phospholipid*	153 ± 10 (5)	163 ± 17 (4)	948 ± 88 (6)
RNA**	45.7 ± 13.4 (3)	64.0 ± 2.4 (4)	114 ± 39 (7)
DNA**	99.4 ± 9.7 (3)	22.8 ± 2.1 (3)	3.8 ± 0.7 (3)

* Expressed as nmoles·mg^{-1} protein.
** Expressed as μg·mg^{-1} protein.

TABLE II

ENZYMIC ACTIVITIES OF HOMOGENATE, 13500 × g_{max} SUPERNATANT AND FRACTION P_1 PREPARED FROM CALF THYMOCYTES

Enzymic activities are expressed as μmoles of product liberated·mg^{-1} protein·h^{-1} (at 37 °C) except for entries marked* which represent nmoles substrate utilized·mg^{-1} protein·min^{-1} (at 20 °C). n.d., not determined.

Enzyme	*Homogenate*	*13500 × g_{max} supernatant*	*Fraction P_1*
5′-Nucleotidase	0.32 ± 0.13 (6)	1.17 ± 0.12 (5)	1.53 ± 0.11 (4)
Mg^{2+}-ATPase	1.3 ± 0.5 (8)	3.2 ± 0.9 (5)	10.9 ± 2.9 (6)
p-Nitrophenylphosphatase, pH 7.2	1.23 ± 0.10 (4)	2.4 ± 0.6 (4)	7.3 ± 0.4 (4)
p-Nitrophenylphosphatase, pH 5.0	2.45 ± 0.30 (4)	6.3 ± 0.9 (4)	14.2 ± 0.6 (4)
Glucose-6-phosphatase	0.31 ± 0.05 (4)	0.83 ± 0.22 (5)	0.66 ± 0.09 (4)
NADH oxidoreductase*	54.1 ± 18.9 (5)	164 ± 35 (5)	613 ± 88 (5)
Succinate:cytochrome *c* oxidoreductase*	7.4 ± 1.3 (4)	n.d.	1.6 ± 0.6 (4)

2.8% of the total protein, 27.7% of the cholesterol, 17.5% of the phospholipid, 7% of the RNA, but only 0.11% of the total cellular DNA. Comparison of enzyme recoveries in the 13500 × g_{max} supernatant and the P_1 fraction derived therefrom (washed 105000 × g_{max} pellet) shows that an appreciable amount of supposedly membrane-bound enzyme activity, most pronouncedly 5′-nucleotidase and glucose-6-phosphatase activities, had not been sedimented. Analysis of the 105000 × g_{max} supernatant (soluble fraction) and the Tris washes of the 105000 × g_{max} pellet for Mg^{2+}-ATPase, 5′-nucleotidase, glucose-6-phosphatase and sialic acid, showed these fractions to contain the remainder of enzymic activities and sialic acid not accounted for by Fraction P_1. The 5′-nucleotidase and glucose-6-phosphatase activities and sialic acid were about equally divided between soluble fraction and Tris washes, whereas the ATPase activity was contained in washes only. The use of *N*-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid (HEPES) buffer of pH 7.5, as advocated by Ferber *et al.*[5], for washing the 105000 × g_{max} pellet yielded similar properties of and recoveries in Fraction P_1 with respect to these enzymes and sialic acid.

Separation of plasma membranes (Fraction M) and microsomes (Fraction P_2)

Four different Ficoll densities (*d* at 25 °C = 1.09, 1.07, 1.05 and 1.035) were tested for their effectiveness in further fractionation of the P_1 fraction, suspended in buffer, into a plasma-membrane (Fraction M) and microsomal (Fraction P_2) fraction by isopycnic centrifugation. The specific activities and concentrations of Mg^{2+}-ATPase, NADH oxidoreductase, sialic acid and cholesterol in Fraction M, accumulating at the Ficoll–buffer interface, and the microsomal Fraction P_2 (pellet) obtained in each case, were measured (Table III). The use of the two lowest Ficoll densities yielded the lowest specific activity of NADH oxidoreductase (*cf.* ref. 26) and the highest specific activity and concentrations of Mg^{2+}-ATPase, sialic acid and cholesterol in Fraction M, but at the expense of a lower recovery of all deter-

minants. Since in other tissues, such as liver, Mg^{2+}-ATPase, sialic acid and cholesterol are relatively more concentrated in plasma membranes than in microsomes, it may be concluded on the basis of these "relative markers" that Fraction M contains the thymic plasma membranes and that these are relatively best separated from microsomes (Fraction P_2) at the lower Ficoll densities.

The Ficoll solution of *d* at 25 °C = 1.05 was chosen for further experiments as the best compromise between plasma membrane purity and yield. Specific con-

TABLE III

SPECIFIC ACTIVITIES/CONCENTRATIONS AND RECOVERIES OF Mg^{2+}-ATPase, NADH OXIDOREDUCTASE, SIALIC ACID AND CHOLESTEROL IN FRACTIONS M AND P_2 AS A FUNCTION OF THE FICOLL DENSITY

Concerning specific activity/concentration data are expressed as in Tables I and II. Recovery is expressed as percentage with respect to cell homogenate.

	Fraction M				*Fraction P_2*			
Ficoll density at 25 °C:	*1.035*	*1.05*	*1.07*	*1.09*	*1.035*	*1.05*	*1.07*	*1.09*
	Specific activity/concentration							
Mg^{2+}-ATPase	26.4	25.8	16.7	15.2	3.0	4.4	4.6	7.5
NADH oxidoreductase	509	500	562	678	1130	1050	997	735
Sialic acid	63.2	65.0	42.5	37.4	19.8	15.7	14.5	13.4
Cholesterol	402	390	330	321	64	65	52	53
	Recovery							
Mg^{2+}-ATPase	12.0	13.3	16.7	15.8	3.1	3.3	3.3	5.6
NADH oxidoreductase	5.5	6.2	13.5	16.9	28.4	19.2	17.0	13.2
Sialic acid	2.9	3.4	4.3	3.9	2.1	1.2	1.0	1.0
Cholesterol	14.0	15.4	25.2	25.5	5.1	3.8	2.8	3.0
Protein	0.59	0.67	1.30	1.35	1.36	0.99	0.92	0.97

TABLE IV

CHEMICAL COMPOSITION OF FRACTIONS M, P_2 AND THE INTERMEDIATE FRACTION FROM CALF THYMOCYTES

Data are expressed as in Table I.

Compound	*Fraction M*	*Fraction P_2*	*Intermediate fraction*
Sialic acid	65.8 ± 4.7 (6)	15.7 ± 1.0 (6)	21.4 ± 7.6 (2)
Cholesterol	390 ± 22 (7)	76 ± 15 (7)	109.9 ± 5.1 (2)
Phospholipid	1660 ± 260 (5)	700 ± 160 (6)	900 ± 210 (2)
RNA	30 ± 7 (6)	79 ± 26 (4)	313
DNA	0–0.2 (5)	1.8 ± 1.7 (5)	0

TABLE V

ENZYMIC ACTIVITIES OF FRACTIONS M, P_2 AND THE INTERMEDIATE FRACTION FROM CALF THYMOCYTES

Data are expressed as in Table II. n.d., not determined.

Enzyme	*Fraction M*	*Fraction P_2*	*Intermediate fraction*
5′-Nucleotidase	2.57 ± 0.31 (4)	1.00 ± 0.08 (5)	n.d.
Mg^{2+}-ATPase	25.8 ± 3.5 (8)	5.0 ± 0.9 (5)	5.0 ± 1.3 (2)
p-Nitrophenylphosphatase, pH 7.2	4.6 ± 1.2 (5)	2.2 ± 0.3 (5)	1.6
p-Nitrophenylphosphatase, pH 5.0	10.4 ± 1.9 (3)	3.2 ± 0.1 (4)	2.7
Glucose-6-phosphatase	0.56 ± 0.06 (3)	0.74 ± 0.22 (3)	0.82
NADH oxidoreductase	497 ± 24 (6)	917 ± 97 (6)	682 ± 2 (2)
Succinate:cytochrome *c* oxidoreductase	0.0 ± 0.0 (4)	3.8 ± 2.5 (4)	n.d.

TABLE VI

RECOVERIES OF CHEMICAL AND ENZYMIC COMPONENTS IN THE VARIOUS FRACTIONS OBTAINED FROM CALF THYMOCYTES

Data are given as percent of the total activities/concentrations in the cell homogenate calculated from mean values. In the case of protein also standard deviations and number of experiments (between parentheses) are listed, to illustrate the consistency of recoveries. The protein recovery in the $105000 \times g_{max}$ supernatant was 12.1 ± 1.7% (5). $1.0 \cdot 10^{11}$ cells (corresponding to 32 ± 3 ml packed cells in 9 experiments) contained 1690 ± 240 mg protein (9), 21.8 ± 2.5 μmoles sialic acid (4), 27.3 ± 1.8 mg cholesterol (4), 230 ± 20 μmoles phospholipid (4), 78 ± 16 mg RNA (3) and 187 ± 9 mg DNA (3). n.d., not determined.

Component	*$13500 \times g_{max}$ supernatant*	*Fraction P_1*	*Fraction P_2*	*Intermediate fraction*	*Fraction M*
Protein	16.6 ± 1.6 (8)	2.82 ± 0.20 (10)	0.95 ± 0.12 (7)	0.57 ± 0.06 (2)	0.67 ± 0.06 (6)
Sialic acid	20.0	6.3	1.2	1.0	3.4
Cholesterol	30.3	27.7	4.3	3.4	15.4
Phospholipid	17.7	17.5	4.3	3.4	7.3
RNA	23.3	7.0	1.6	3.9	0.4
DNA	3.8	0.11	0.017	< 0.001	≤ 0.001
5′-Nucleotidase	60.7	13.5	3.0	n.d.	5.4
Mg^{2+}-ATPase	40.9	23.6	3.7	2.2	13.3
p-Nitrophenylphosphatase, pH 7.2	33.0	16.8	1.7	0.7	2.5
p-Nitrophenylphosphatase, pH 5.0	43.8	16.3	1.2	0.6	2.8
Glucose-6-phosphatase	44.5	6.0	2.2	1.5	1.2
NADH oxidoreductase	50.3	32.0	16.1	7.2	6.2
Succinate:cytochrome *c* oxidoreductase	n.d.	0.6	0.5	n.d.	0.0

centrations and activities of constituents of Fractions M and P_2, and occasionally also of the intermediate fraction (Ficoll layer between interface and pellet), are listed in Tables IV and V, and recoveries in Table VI.

The enrichment of sialic acid in Fraction M (65.8 nmoles·mg^{-1} protein) was 5.1-, 2.3-, and 4.2-fold in respect of the cell homogenate and Fractions P_1 and P_2, respectively. The corresponding enrichments for cholesterol in Fraction M (390 μg·mg^{-1} protein) amounted to 23-, 2.3- and 5.1-fold, and for phospholipid (1.66 μmoles·mg^{-1} protein) 11-, 1.8- and 2.4-fold. Of the total cellular cholesterol and phospholipid, 15.4 and 7.3%, respectively, were recovered in Fraction M. The cholesterol/phospholipid molar ratio of this fraction amounted to 0.61; the corresponding ratio for Fraction P_2 was 0.27. 5.5% of the cholesterol in Fraction M was present as cholesteryl esters. Of the total cellular RNA 0.4% was found in Fraction M, with a specific concentration of 30 μg RNA·mg^{-1} protein. No RNA or protein was released after suspending Fraction M for 1 h at room temperature in 0.15 M NaCl (*cf.* ref. 8). DNA could hardly, if at all be detected in the M fraction.

The (Na^+–K^+)-ATPase activity of Fraction M varied from 0–4 μmoles P_i·mg^{-1} protein·h^{-1}. The specific Mg^{2+}-ATPase activity amounted to 25.8 ± 3.5 μmoles P_i·mg^{-1} protein·h^{-1}, showing an enrichment of this enzyme in Fraction M of 20-, 2.4-, and 5.2-fold with respect to the homogenate and Fractions P_1 and P_2, respectively. Recovery of Mg^{2+}-ATPase in Fraction M was 13.3%. Also markedly enriched in Fraction M, but showing much smaller recoveries than the former enzyme, were 5′-nucleotidase and nitrophenyl phosphatase activity at pH 7.2 and 5.0. By contrast, Fraction P_2 was enriched in glucose-6-phosphatase and NADH oxidoreductase activities. The mitochondrial marker succinate:cytochrome *c* oxidoreductase was absent from Fraction M.

Storage of Fraction P_1 suspended in 1 mM Tris buffer of pH 8.6 containing 0.25 M sucrose (*cf.* ref. 1) for several days at −25 °C, markedly impaired subsequent yields of Fractions M and P_2.

The data obtained on the intermediate fraction suggest it to contain a mixture of (some) plasma membranes, more microsomal membranes and the highest amount of ribosomes (as indicated by its RNA content).

In an attempt to further purify Fraction M, an aliquot was subjected to a second centrifugation over Ficoll in the SW-50 rotor. The extra run did not materially affect the results: 85% of the proteins was recovered; 5′-nucleotidase, sialic acid and phospholipid per mg protein increased about 10%; the specific activity of Mg^{2+}-ATPase did not change whereas that of NADH oxidoreductase showed a 10% decrease.

Electron microscopy

Calf thymus lymphocytes *in situ* contain a relatively large nucleus surrounded by a thin rim of cytoplasm in which little rough endoplasmic reticulum, many free ribosomes, a small Golgi apparatus and few mitochondria are present. After liberating the cells from the tissue, the cells in suspension were essentially intact and well preserved, being surrounded by an intact plasma membrane (Fig. 1). The 13 500 × g_{max} pellet consisted of nuclei, mitochondria and lysosomes.

The P_1 fraction (Fig. 2) consisted of a mixture of profiles and vesicles of smooth membranes, and ribosome-dotted (rough) membranes, next to free ribosomes. Some

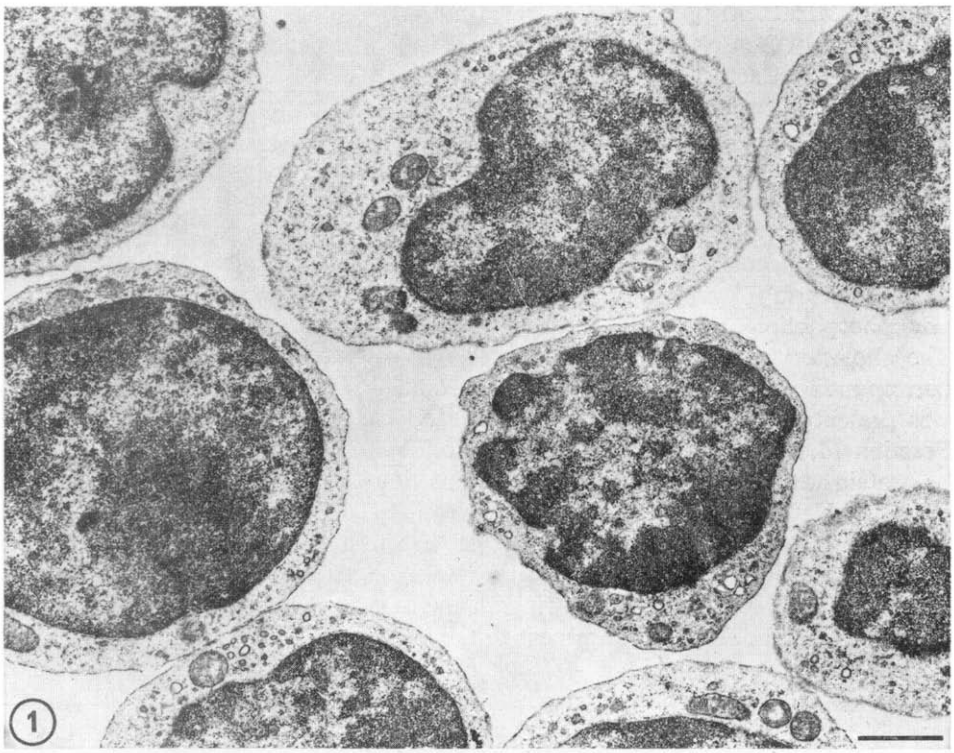

Fig. 1. Free calf thymocytes suspended in Hanks solution. The cells are well preserved and surrounded by an intact plasma membrane. × 11 200.

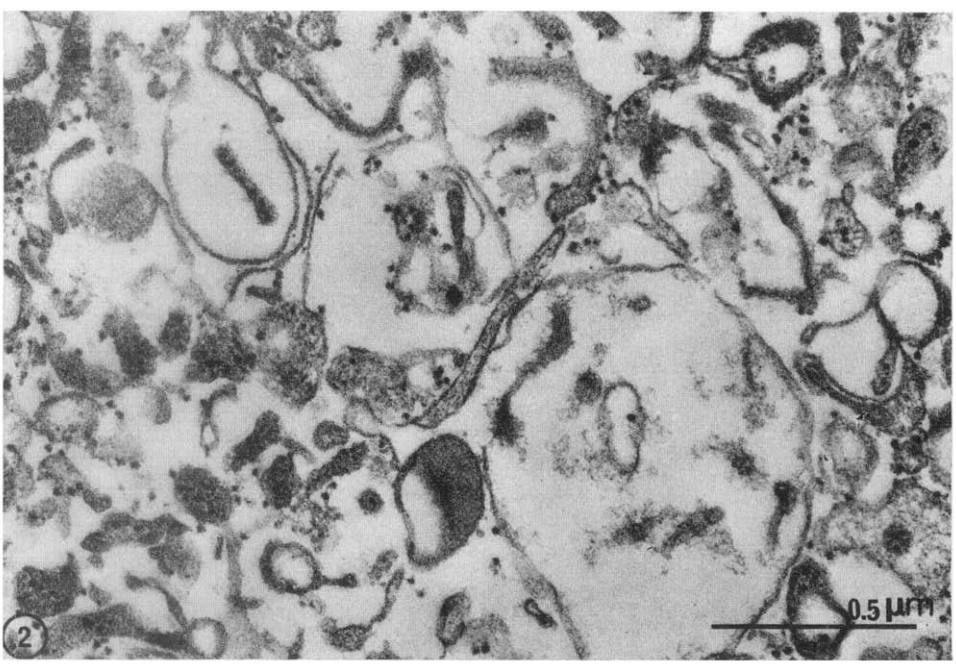

Fig. 2. Thin section through a pellet of Fraction P_1 shows the presence of profiles and vesicles of smooth membranes and ribosome-dotted (rough) membranes. Some vesicles contain amorphous material, presumably trapped cytoplasmic constituents. × 54000.

vesicles contained an electron-opaque amorphous material, presumably cytoplasmic constituents.

The M fraction (Fig. 3) consisted almost exclusively of smooth membranes arranged in short profiles and vesicles, the triple-layered structure of the membrane element was well visible. Few vesicles contained amorphous material and/or some ribosomes. Rough membranes could hardly if at all be detected. Lysosomes and mitochondria were lacking.

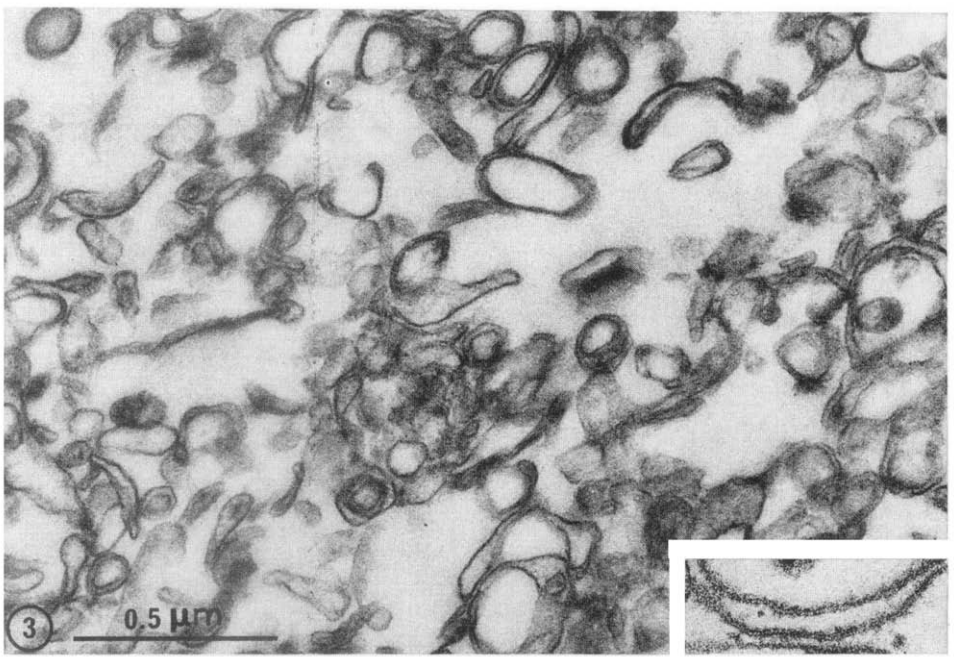

Fig. 3. Thin section through a pellet of Fraction M shows almost exclusively short profiles and vesicles of smooth membranes. × 54000. Inset: the triple-layered structure of the membrane element is well visible. × 108000.

The pelleted P_2 fraction was not homogeneous. The upper part of the pellet contained many ribosomes (polysomes) mixed with few rough microsomal vesicles (Fig. 4a), whereas the lower part contained mainly many rough microsomal vesicles, next to few free polysomes and smooth profiles and vesicles (Fig. 4b).

DISCUSSION

A plasma membrane fraction (Fraction M) and a microsomal fraction (Fraction P_2) were isolated from a washed $105000 \times g_{max}$ pellet (Fraction P_1) obtained from calf thymocytes. The procedure was examined electron microscopically and the morphology of the various fractions suggested that an effective separation of plasma membranes had been brought about. The marked enrichment of Mg^{2+}-ATPase, 5′-nucleotidase, sialic acid and cholesterol per mg protein of the M fraction (> Fraction P_1 > Fraction P_2 > $13500 \times g_{max}$ supernatant > cell homogenate) allowed the

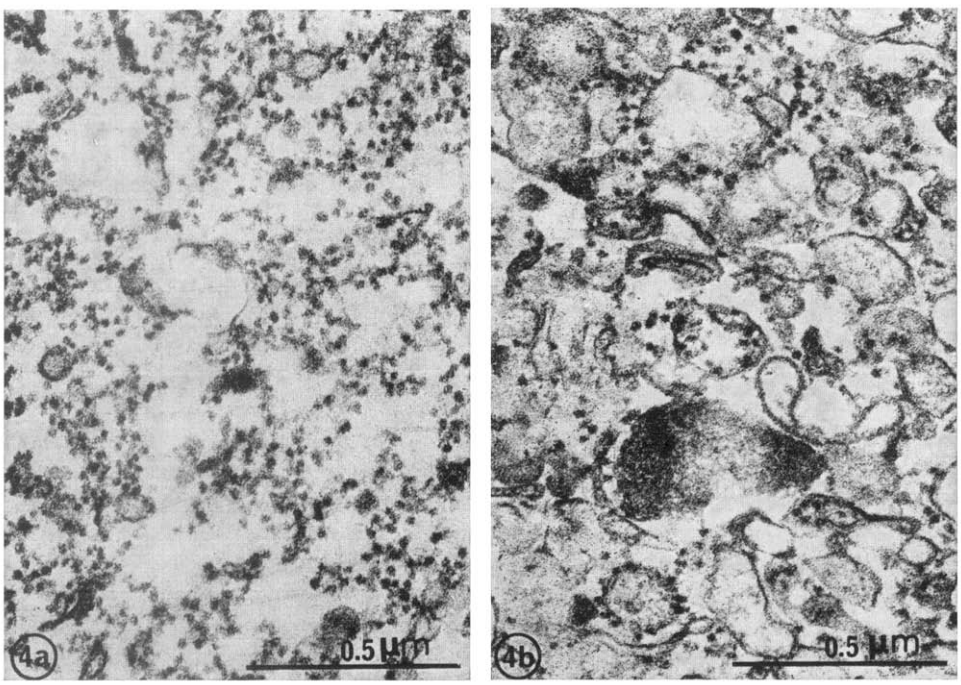

Fig. 4. a. The upper part of a pellet of Fraction P_2 consists of many free polysomes mixed with a few rough microsomal vesicles. × 56000. b. The lower part of a pellet of Fraction P_2 contains mainly rough microsomal vesicles next to free polysomes and some smooth profiles and vesicles. × 56000.

same conclusion. However, the following observations which relate to yield, purity and characterization of the fractions, deserve attention.

Fractions M and P_2 were routinely separated by centrifugation of Fraction P_1 layered on a Ficoll cushion using the SW25 rotor driven at 25000 rev./min for 15 h (overnight). When instead in some experiments (not illustrated) the SW50 rotor was used at 50000 rev./min for 4 h, the recovery of protein in Fraction M was some 30% higher, but its sialic acid and cholesterol specific contents were essentially unchanged. This result may suggest that during our routine isolation a fair amount of plasma membranes interacted with rough microsomes, if not with free (poly)ribosomes during their longer contact (15 h *versus* 4 h) in the presence of Mg^{2+}, and thus were spun down through the Ficoll barrier. The final distribution of the various organelles (based on electron microscopic, chemical and enzymic determinants) is not in disaccord herewith. This, together with the results illustrated in Table III, suggests that one deals with a spectrum of, perhaps partly interacting particles, showing greater or lesser differences in buoyant densities, the separation of which is operationally defined.

For judging the purity of isolated organelles "markers" of enzymic, or occasionally chemical nature are generally employed. "Absolute markers", *i.e.* components being exclusively present in one type of tissue organelle such as cardiolipin and some respiratory enzymes in mitochondria, certain lysosomal enzymes, mixed-function oxydases in liver microsomes, and adenylate cyclase in plasma membranes,

may be relatively scarce. However, "relative markers", *i.e.* components being enriched in a certain organelle, may also be relevant since they characterize that organelle. Thus plasma membranes in general are characterized by a high cholesterol/phospholipid molar ratio[27]. This also applies to our M fraction, this ratio being 0.61, as against 0.28 for the P_2 fraction: reported plasma membrane ratios amount to 0.75 for human thymocytes[3], 1.03 and 1.01 for pig lymphocytes[2,5], and to 0.93 for leukemic cells[4]. It should be noted that the cholesterol/phospholipid ratio of 1.58 calculated from data reported by Ferber *et al.*[5] for pig lymphocyte "microsomes" is uncommonly high, even higher than that found for their plasma membrane fraction. On the basis of cholesterol recoveries our yield of calf thymus plasma membranes is at least 15%.

A conspicuous feature of our plasma membrane preparation is its very high specific content of phospholipid, *i.e.* 1.66 μmoles·mg^{-1} protein, this value being 2.3–4-fold that reported for the lymphocyte plasma membrane preparations obtained by the authors referred to above[2-5]. If not genuine, our data might suggest a loss of protein from membranes during preparation in soluble form or in non-sedimentable membrane particles deficient in phospholipid. Considering conditions, this effect could have been brought about during cell disruption, or during the washing steps of the $105000 \times g_{max}$ pellet. It is therefore of interest that whereas essentially all the cholesterol and phospholipid of the $13500 \times g_{max}$ supernatant was pelleted in Fraction P_1, an appreciable activity of the alleged membrane-bound enzymes, 5′-nucleotidase and glucose-6-phosphatase remained in the $105000 \times g_{max}$ supernatant (soluble fraction) or was recovered in the Tris washes of the $105000 \times g_{max}$ pellet.

Allan and Crumpton[2] also found a major portion (43%) of the glucose-6-phosphatase activity of lymphocytes to remain in the $105000 \times g_{max}$ supernatant. If these activities stem from specific membrane enzymes, it follows that their loss from the membranes had occurred preferentially, since the recovery of Mg^{2+}-ATPase and other enzymes in Fraction P_1 was much higher than that of the aforementioned activities (Table VI). However, it is quite well possible that an aspecific phosphatase is present in the cytoplasm of calf thymocytes, which on processing the cells may partly remain soluble and partly be adsorbed onto the membranes or enclosed by membraneous vesicles (to be released by the Tris washes). In this connection it may be significant that about similar proportions of the two aforementioned phosphohydrolase activities (with 5′-AMP and glucose 6-phosphate as substrates) were non-sedimentable, whereas their specific activities in the Tris washes (3.0–3.3 μmoles $P_i \cdot mg^{-1}$ protein$\cdot h^{-1}$) were higher than the corresponding activities of both the M and P_2 fractions. Since no protein was released from the M fraction by 0.15 M NaCl (*cf.* ref. 8), the enzymic activities displayed by this fraction are considered to be intrinsically membranous. However, in view of the possible presence of aspecific phosphatase(s), the "marker" concept should not be applied indiscriminantly for characterizing isolated membranes. For final evaluation specific enzyme properties should be checked in order to rule out (or detect) the presence of aspecific enzymes or isozymes. With this reservation in mind, it appears that 5′-nucleotidase, in spite of its low activity in the present material, satisfies the criterion of a relative plasma membrane marker, as defined above, at least when expressed on a protein basis (specific activity in Fraction M > Fraction P_1 > Fraction P_2 > homogenate). Relatively low specific activities of 5′-nucleotidase have been encountered in the

plasma membrane preparations from many types of cells[2,3,5,26,28], other than hepatocytes[17], leukemic[4] and HeLa[29] cells.

Glucose-6-phosphatase has been considered as a microsomal marker, at least for hepatic tissue (but compare ref. 30). However, in calf thymocytes the activity of this enzyme is low and its distribution among plasma membrane and microsomal fractions is not pronounced; similar findings have been reported for pig lymphocytes[2], and leukemic[4], Ehrlich ascites carcinoma[28], and BHK21[31] cells. The enzyme may be of little significance as marker in these types of cells.

The specific NADH oxidoreductase activity of Fraction M was half that of Fraction P_2 ("microsomes"). Several authors[1,32] have considered this enzyme as a marker for microsomal (endoplasmic reticulum) membranes. However, Molnar *et al.*[28] and Ferber *et al.*[5] found a higher specific NADH oxidoreductase activity in the plasma membrane fractions obtained from Ehrlich ascites carcinoma cells and pig lymphocytes than in the corresponding microsomal (endoplasmic reticulum) fractions. This would rather indicate that the enzyme is intrinsic to these plasma membranes, in contrast to the situation described by Wallach and Kamat[1] for Ehrlich ascites carinoma cells. However, it should be noted that this conclusion is based, in common usage, on enzyme activities expressed per mg protein of a fraction. Actually one deals with membrane-bound enzymes, and it seems more correct when comparing plasma membrane and microsomal (containing many ribosomes) fractions to express data on a phospholipid basis. In doing so (Table VII), it appeared that the specific NADH oxidoreductase of our M fraction amounted to only 23% that of the P_2 fraction. If this would be due to contamination —a conclusion that cannot at this moment be settled definitely for these types of cells— it should be emphasized that contamination of the M fraction cannot be one by rough microsomal membranes but only by smooth ones, or originally rough membranes from which the ribosomes had been stripped during preparation. When expressed on a phospholipid basis, the M and P_2 fractions contained the same specific 5′-nucleotidase activity, and only the Mg^{2+}-ATPase, acid nitrophenyl phosphatase, sialic acid and cholesterol contents were higher in the M than in the P_2 fraction (Table VII); however, for a more proper calculation the phospholipid/protein ratio of the microsomal membranes *per se* should also be known.

TABLE VII

SPECIFIC ACTIVITIES/CONCENTRATIONS IN FRACTIONS M AND P_2 EXPRESSED PER μmole PHOSPHOLIPID

Component	*Fraction M*	*Fraction P_2*
5′-Nucleotidase	1.55	1.43
Mg^{2+}-ATPase	15.5	7.1
p-Nitrophenylphosphatase, pH 7.2	2.8	3.2
p-Nitrophenylphosphatase, pH 5.0	6.3	4.6
Glucose-6-phosphatase	0.34	1.06
NADH oxidoreductase	300	1310
Sialic acid	39.6	22.4
Cholesterol	235	109
RNA	18.1	113
Protein	0.6	1.4

Comparison of the RNA contents of the M and P_2 fractions with the electron microscopic presence of ribosomes in these two fractions, leads to the conclusion that at least most of the RNA in Fraction M (30 μg RNA·mg^{-1} protein) cannot stem from contaminating ribosomes. This conclusion is also based on our unpublished experience with other material (from lactating mammary gland) which showed that if ribosomes, corresponding to the above RNA content, are present in a plasma membrane preparation, such ribosomes should be abundantly demonstrable in the electron micrographs. The possibility that cytoplasmic RNA was electrostatically linked to the membranes of the M fraction by way of Ca^{2+} and Mg^{2+} (initially present in the Hanks solution), could be dismissed on account of the lack of this fraction to release RNA in the presence of 0.15 M NaCl (*cf.* ref. 8). Amounts of RNA similar to that found in our M fraction have been reported for plasma membrane preparations from pig lymphocytes[2,5] and human thymocytes[3], as against much lower amounts in the case of plasma membranes from leukemic cells[4] and mouse and rat liver[8]. It is concluded that the RNA present in Fraction M from calf thymocytes is, at least for the greater part, an authentic constituent of the plasma membrane.

DNA could hardly if at all be detected in Fraction M; we consider the DNA in the calf thymocyte plasma membrane fraction as a contaminant, in contrast to a recent report of Lerner *et al.*[33] on the DNA of the human lymphocyte plasma membrane.

In conclusion, by the current criteria applied in many reports, our M fraction contains reasonably clean plasma membranes, but the question may be raised whether correct criteria are available, or are commonly employed when a new type of cell, other than the much studied rat liver, is investigated.

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