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LIPID COMPOSITION AND MICROVISCOSITY OF SUBCELLULAR FRACTIONS FROM RABBIT THYMOCYTES

DIFFERENCES IN THE MICROVISCOSITY OF PLASMA MEMBRANES FROM SUBCLASSES OF THYMOCYTES

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Summary

There are indications from freeze-fracture experiments that subclasses of rabbit thymocytes show different mobilities of plasma membrane components. Consequently, one would expect differences in the fluidity of the plasma membrane. For this reason, rabbit thymocytes were separated on a Ficoll/Metrizoate gradient yielding three subclasses representing various levels of cell differentiation. These thymocyte subclasses did not show any significant differences in the degree of fluorescence polarization using the probe 1,6-diphenyl-1,3,5-hexatriene. The fluorescence polarization of the plasma membrane may be overshadowed by the contribution of all cellular lipids due to penetration of the fluorescent probe into the cell. Therefore, plasma membranes were isolated from rabbit thymocytes using a cell-disrupting pump, differential centrifugation, and sucrose density gradient centrifugation. As shown by biochemical and electron microscopical analyses, plasma membranes with a high degree of purity were obtained. As expected the plasma membrane fractions showed a higher microviscosity than the other subcellular fractions. This was attributed to a higher cholesterol to phospholipid molar ratio and a higher degree of saturation of phospholipid fatty acid chains.

Subsequently, the microviscosity was measured of plasma membrane preparations obtained from two main subclasses of thymocytes representing mature and immature lymphocytes. The immature thymocytes yielded two plasma membrane fractions with higher microviscosity than the mature cells.

Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene.

This finding is in line with earlier observed differences in the glycerol-induced clustering of intramembranous particles.

Furthermore, the results of this study support the view that the fluorescence polarization technique applied to whole cells does not exclusively monitor the plasma membrane.

Introduction

On the basis of their physical characteristics, i.e. size, buoyant density, and electrophoretic mobility, thymic lymphocytes can be separated into various subclasses [1—4]. A continuous linear density gradient of Ficoll and Metrizoate has been developed in our laboratory which yields three subclasses of rabbit thymocytes with peak densities of approx. 1.067, 1.077, and 1.084 g/ml [5,6]. The two cell populations with the lower densities are enriched in cortisone-resistant mature lymphocytes (compare Ref. 7). In freeze-fracture experiments, the plasma membranes of lymphocytes from the lightest fraction and of thymic lymphocytes isolated from cortisone-treated animals showed the greatest glycerol-induced redistribution of intramembranous particles compared with the smaller cortisone-sensitive lymphocytes of the fraction with density 1.084 g/ml. In later experiments it was found that intramembranous particles in plasma membranes of cells from the fraction with density 1.077 g/ml could also be redistributed by glycerol; in all instances significant differences in glycerol-induced redistribution were only seen in the external fracture face and not in the protoplasmic fracture face (De Groot, C., personal communication). This effect might depend on differences in microviscosity of the plasma membranes of these subclasses of thymocytes. We therefore directed our investigation at the determination of this parameter using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH).

In this report we show that the microviscosity measured in whole cells did not reveal any significant difference between the three subclasses of rabbit thymocytes which can be separated on a continuous Ficoll/Metrizoate gradient.

Because changes in plasma membrane microviscosity might be become obscured when the fluorescent probe is taken up by all lipids being present in the cell, we decided to isolate the plasma membranes. The cells were homogenized with the aid of a cell-disrupting pump [8]. Subcellular fractions were obtained by differential and isopycnic centrifugation and analyzed biochemically and electron microscopically. The microviscosity of the various subcellular fractions was also estimated. When it was clear that plasma membranes with a high degree of purity could be obtained, the technique was applied to subclasses of rabbit thymocytes. It was found that two plasma membrane fractions obtained from the most dense cells in a multistep sucrose density gradient exhibited a significantly higher microviscosity than all plasma membrane fractions obtained from lymphocytes of the fraction with density 1.077 g/ml. This result is in accordance with the observed differences in glycerol-induced redistribution of intramembranous particles in the plasma membrane, and might bear relevance to the maturation of thymic lymphocytes.

Materials and Methods

Isolation and separation of thymocytes

Thymi were taken from female Chinchilla rabbits 8—10 weeks old and immediately placed in ice-cold Hanks' balanced salt solution (Grand Island Biological Company, Grand Island, NY). The cell suspensions were prepared by pressing pieces of tissue through six layers of 20 denier nylon gauze and collecting the cells in cold medium. The cells were washed twice by centrifugation at $300 \times g_{av}$ for 10 min. Cell viability ranged from 85—90% as measured by the eosin exclusion test.

For the separation of thymocytes into their subclasses a continuous Ficoll/Metrizoate gradient was used slightly modified after Leene et al. [5]. The gradient was prepared in 40-ml centrifuge tubes by layering different mixtures of solutions A and B. Solution A consists of 12.7% (w/v) Ficoll (mol. wt. $4 \cdot 10^5$, Pharmacia Fine Chemicals, Uppsala), 7.8% (w/v) sodium metrizoate (32.8% (w/v) solution, Nyegaard and Co., Oslo), 18.6 mM Tris-HCl, pH 7.4, and 0.2% (w/v) bovine serum albumin. Solution B contains 9.1% (w/v) sodium metrizoate, 18.6 mM Tris-HCl, pH 7.4, and 0.2% (w/v) albumin. The densities of solutions A and B were 1.102 and 1.055 g/ml, respectively; the osmolarity of both solutions was 320 mosM. The different layers in the gradient were from bottom to top: 7 ml A, 5 ml A/B (3 : 1), 5 ml A/B (1 : 1), 5 ml A/B (1 : 3), and 4 ml B. The gradient was stored at 0—4°C and used the next day. A 2 ml sample of an ice-cold cell suspension in Hanks' balanced salt solution (maximally $2 \cdot 10^8$ cells/ml) was layered on top of the gradient. The gradient was then centrifuged for 40 min in a MSE Mistral 6L centrifuge at $2300 \times g_{max}$ at 4°C. Cells were collected from the gradient and washed twice. Three subclasses were obtained with peak densities of approx. 1.067, 1.077, and 1.084 g/ml, and will be denoted as fractions I, II, and III. The relative amounts of cells in fractions I, II, and III were approx. 5, 15, and 80%, respectively.

Isolation of plasma membranes

The procedure followed for the isolation of plasma membranes from lymphocytes was adopted from a number of methods in the literature [8—12] and is schematically depicted in Fig. 1. An ice-cold cell suspension (maximally 10^8 cells/ml) in Hanks' balanced salt solution was pumped through a cell disruptor (Stansted Fluid Power Ltd., Stansted, Essex; model A0 612, disrupting valve 516) at a disrupting pressure of 750 lb/inch^2. At this pressure no clumps or aggregates of cellular material due to damaged nuclei were seen in the phase contrast microscope while almost all cells were disrupted as judged by the uptake of eosin by the remaining nuclei. After sedimentation of the nuclear, mitochondrial, and microsomal fractions by differential centrifugation, the microsomal pellet was suspended in 3 ml Tris buffer (10 mM Tris-HCl, pH 7.4) to which 80% (w/v) sucrose (in Tris buffer) was added until a concentration of 40% (w/v) was reached as determined by the refractive index. Then this suspension was homogenized by hand in a tight-fitting all-glass homogenizer until no aggregates were visible by phase contrast microscopy. After adjusting the volume with 40% (w/v) sucrose (in Tris buffer) to the value stated in Fig. 1, the resulting suspension was used as the bottom layer in the

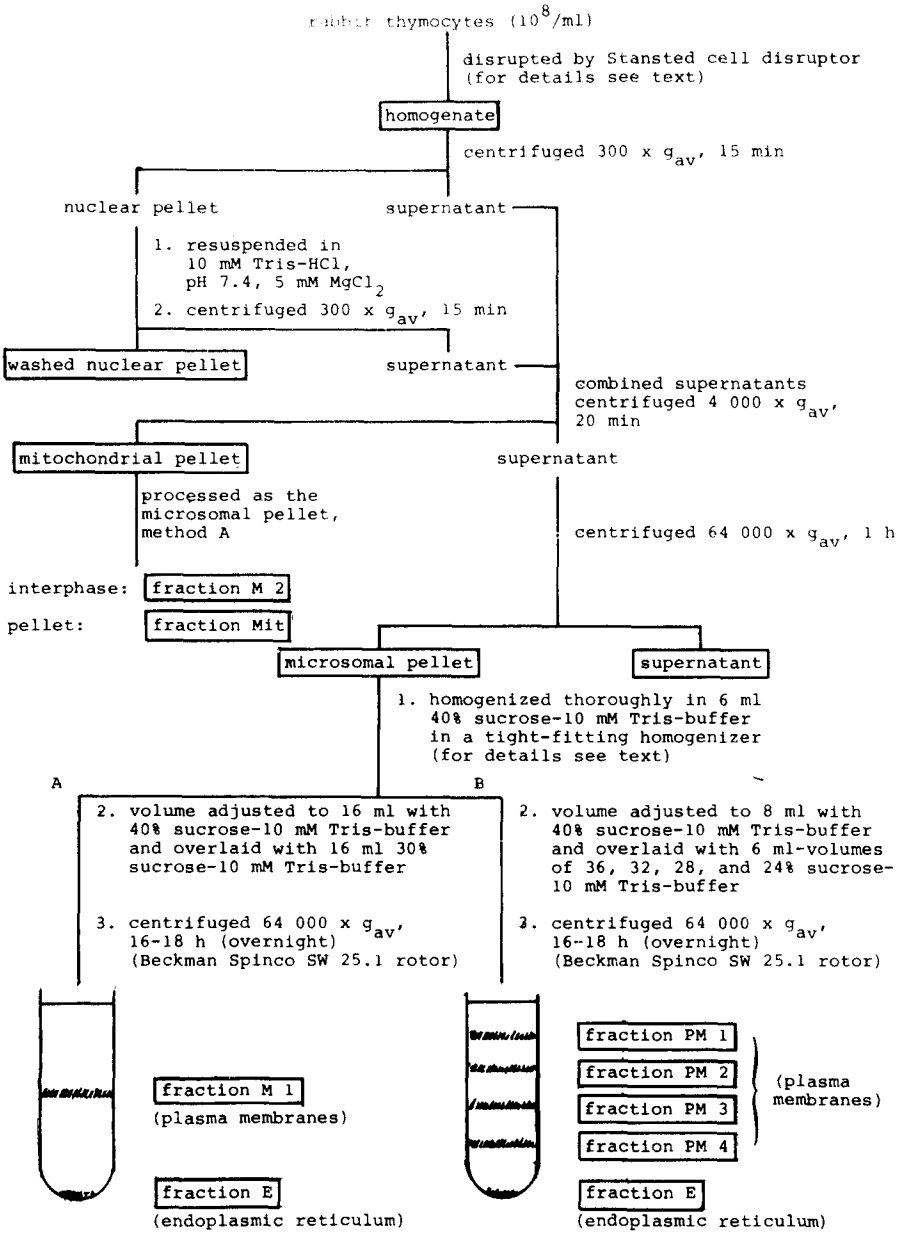

Fig. 1. Scheme for the isolation of plasma membranes from rabbit thymocytes.

gradient. In some experiments the mitochondrial pellet was treated in the same way. All steps were performed at 0—4°C.

Biochemical analyses

Protein was measured according to Lowry et al. [13] using bovine serum albumin (Sigma Chemical Company, St. Louis, MO; fraction V) as a standard,

and sodium dodecyl sulfate (Applied Science Laboratories Inc., State College, PA) as a protein-dissolving agent [14].

DNA and RNA were separated according to Schneider [15]. RNA was estimated by the orcinol reaction and DNA with the diphenylamine reagent according to Giles and Myers [16]. Standards for the DNA and RNA determinations were from Sigma Chemical Co. (calf thymus DNA) and from BDH Biochemicals Ltd. (yeast RNA, sodium salt).

Lipids were extracted from frozen-dried material according to Folch et al. [17] and stored dissolved in chloroform/methanol (2 : 1) under nitrogen at −30°C. Total phospholipid was determined according to Turner and Rouser [18] using the micro-method. Cholesterol was assayed enzymatically. To the dried residue of a sample from the lipid extract 100 μl ethanol were added followed by 1 ml enzyme reagent from the Merckotest cholesterol kit (Merck AG, Darmstadt). This reagent contains cholesterol esterase, cholesterol oxidase, and a colour reagent. After 1 h standing at room temperature the absorbance was read at 365 nm.

One-dimensional thin-layer chromatography on prewashed silica gel plates in petroleum ether 60/80/diethyl ether/acetic acid (40 : 60 : 0.1, v/v) was applied for the separation of the neutral lipids from the phospholipids which remain at the origin. The lipids were visualized under ultraviolet light after spraying the thin-layer plates with aqueous 0.01% Rhodamine 6G. After drying the plates in a nitrogen atmosphere, the phospholipid spots were scraped off and processed for gas chromatography as described before [19,20] using 10% BCl_3 or 14% BF_3 in methanol for the transesterification step (both reagents were from Applied Science Laboratories Inc., State College, PA).

Gas chromatography. The fatty acid methyl esters were analyzed with a Varian Aerograph 1520 gas chromatograph equipped with flame ionization detectors. The liquid phases were 10% SP 2330 and 10% SP 2340 (Supelco, Inc., Bellefonte, PA), both on Chromosorb W-AW, 100—120 mesh, and packed in 5 ft × 1/8 inch stainless steel columns. Conditions of chromatography were: flow rate of carrier gas (nitrogen) 20 ml/min, column temperature programmed from 160 to 200°C within 20 min, injector temperature 200°C, detector temperature 240°C. Peaks were quantified with an Infotronics model CRS-204 electronic integrator and identified by comparison with standard fatty acid methyl esters.

Membrane marker enzymes. The following membrane marker enzymes were used: Mg^{2+}-ATPase, $(Na^+ + K^+)$-ATPase, 5′-nucleotidase, and thymidine 5′-phosphodiesterase for plasma membranes, glucose-6-phosphatase and NADH dehydrogenase for endoplasmic reticulum, succinate dehydrogenase for mitochondria, and β-*N*-acetylglucosaminidase for lysosomes.

Total Mg^{2+}-ATPase activity was measured by following the release of inorganic phosphate from ATP, according to Kimelberg and Papahadjopoulos [21]. For the estimation of $(Na^+ + K^+)$-ATPase additional incubations were carried out in the presence of 1 mM ouabain which is a specific inhibitor of this enzyme. The assay of 5′-nucleotidase (AMPase) was conducted basically according to Michell and Hawthorne [22]. The incubations were carried out in the presence of 0.3% Triton X-100 (Eastman Organic Chemicals, Rochester, NY); this raised the activity by 30% (compare Ref. 23). Thymidine 5′-phospho-

diesterase was estimated according to Erecińska et al. [24] in the presence of 0.1% Triton X-100. The substrate was thymidine 5′-*p*-nitrophenyl phosphate (Sigma Chemical Co.) and the increase of absorbance at 400 nm was registered by a recording spectrophotometer.

Glucose-6-phosphatase and NADH dehydrogenase were assayed according to Hübscher and West [25] and Wallach and Kamat [26], respectively.

Succinate dehydrogenase activity was measured by the method of Earl and Korner [27].

The lysosomal enzyme β-*N*-acetylglucosaminidase was estimated according to Jett et al. [28] with *p*-nitrophenyl-β-*N*-acetylglucosaminide (Sigma Chemical Co.) as the substrate.

Determination of inorganic phosphate. Inorganic phosphate enzymatically released from AMP or glucose 6-phosphate was assayed according to a modification of the method of Lowry et al. [29]. To 0.5 ml aliquots of enzyme incubation mixture containing 5% trichloroacetic acid were added successively with vigorous stirring: 0.1 ml 6 N H_2SO_4, 0.1 ml 0.02 M ammonium heptamolybdate, 0.1 ml 10% ascorbic acid, and 0.2 ml distilled water. The reaction mixture was heated at 100°C for 5 min. After cooling to room temperature the absorbance was read at 820 nm. Standard mixtures with inorganic phosphate and AMP or glucose 6-phosphate blanks were treated in the same way. By this method a mean absorbance (± S.D.) of 0.908 ± 0.012 (n = 8) for 1 μg P_i was obtained. This is higher than in the original method for the determination of inorganic phosphate by Fiske and Subbarow [30], and is due to heating of the reaction mixture [29]. This heating step cannot be carried out in the presence of labile organic phosphates like ATP which is the substrate for ATPases. In this case we employed a modification of the malachite green method by Muszbek et al. [31]. To 0.2 ml aliquots of enzyme incubation mixture containing 5% trichloroacetic acid 0.1 ml of acid sodium molybdate reagent was added under stirring. The acid sodium molybdate reagent consists of three parts of 0.1 M sodium molybdate and one part of 25% (w/v) H_2SO_4. After exactly 15 s 0.1 ml malachite green reagent was added with vigorous stirring, and after exactly 2 min 0.7 ml 7.8% (w/v) H_2SO_4. The malachite green reagent is a 0.05% solution of malachite green (Gurr Searle Diagnostic, High Wycombe Bucks) in 1% polyvinyl alcohol prepared according to Anner and Moosmayer [32]. All steps were carried out at 0°C. The absorbance was read at 625 nm after at least 1 h standing of the complete reaction mixture at room temperature. Standard mixtures of inorganic phosphate and ATP blanks were treated in the same way. This method gave a mean absorbance (± S.D.) of 2.98 ± 0.09 (n = 11) for 1 μg P_i which is about three times higher than in the molybdate-ascorbic acid method described above. The malachite green method could also be applied to the assay of 5′-nucleotidase and glucose-6-phosphatase, but in these cases the reactions should be carried out at room temperature, because at 0°C no colour at all was produced. At room temperature AMP and glucose 6-phosphate still exerted a slight effect on the colour development, but this could be accounted for by including the appropriate amounts of these substrates in the standard mixtures.

Fluorescence polarization measurements

The microviscosity of whole cells and cellular membranes was measured by the fluorescence polarization of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (Koch-Light Laboratories Ltd., Colnbrook Bucks) [33—36]. 100 μl of 2 mM DPH in tetrahydrofuran were added to 50 ml Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 0.9% NaCl) or Hanks' balanced salt solution without pH indicator and sonicated for 30 s in a Mullard sonifier. The cells to be measured were suspended in Hanks' balanced salt solution (without pH indicator); the membranes were present in Tris buffer. Samples (approx. 10^6 cells or 50—200 μg membrane protein in maximally 0.7 ml) were added to 3 ml of the DPH dispersion and incubated for 20 min at 37°C. The degree of fluorescence polarization was measured at 25°C in an Elscint MV-1a microviscosimeter (Elscint Ltd., Haifa). Excitation was from polarized light (366 nm) from a mercury arc. Correction for light scatter was carried out by successive dilutions with Hanks' balanced salt solution or Tris-buffered saline until a plateau value of the degree of fluorescence polarization P was obtained [37]. The value of P was transposed into the fluorescence anisotropy [$r = 2P/(3 - P)$] [33] which is related to the microviscosity $\bar{\eta}$ according to the Perrin equation for rotational depolarization of a non-spherical fluorophor:

$$\frac{r_0}{r} = 1 + C(r)\frac{T \cdot \tau}{\bar{\eta}}, \quad \text{or} \quad \left(\frac{r_0}{r} - 1\right)^{-1} = \frac{\bar{\eta}}{C(r) \cdot T \cdot \tau}$$

where r and r_0 are the measured and the limiting fluorescence anisotropy, T is the absolute temperature, τ is the excited state lifetime of DPH, $\bar{\eta}$ is the microviscosity of the medium where the DPH molecules are embedded, and $C(r)$ is a parameter which relates to the molecular shape of the fluorophor, and has a specific value for each r value. $C(r)$ was estimated from a calibration curve derived from published values for P and $\bar{\eta}$ of biological and artificial membranes. Further is r_0 equal to 0.364 at 25°C, and for τ (25°C) an average value of 10 ns was taken [36]. The microviscosity $\bar{\eta}$ (expressed in poise) can now be calculated.

Electron microscopy

Pellets from plasma membranes and nuclear, mitochondrial, and endoplasmic reticulum fractions were fixed for 1 h in cold 2% glutaraldehyde in 1/15 M Sörensen buffer, pH 7.4, containing 2.55 mM $MgCl_2$. After fixation the pellets were washed with phosphate-buffered sucrose with the same osmolarity (500 mosM) as the fixative, and also containing 2.55 mM $MgCl_2$. Postfixation was carried out in 1% osmium tetroxide. The pellets were dehydrated in ethanol and embedded in Epon. Ultrathin sections were cut on a LKB Ultrotome III and stained with uranyl acetate and lead nitrate. They were examined in a Philips EM 300 electron microscope.

Results

Microviscosity of whole cells from subclasses of rabbit thymocytes

As a rule, the viability of cells isolated from rabbit thymus was lower than 90%. Centrifugation in the continuous Ficoll/Metrizoate gradient caused nearly

all dead cells to be sedimented on the bottom of the centrifugation tube yielding subfractions of thymocytes with a high viability. The degree of fluorescence polarization P was measured and the microviscosity was calculated from the P values. No significant difference (by Student's t-test) in microviscosity could be detected between cells belonging to the subclasses I—III with peak densities of approx. 1.067, 1.077, and 1.084 g/ml (Table I). Cells from the same fractions were left overnight at 4°C in Hanks' balanced salt solution which resulted in a lower viability of all cell fractions. The cell suspensions thus contained more dead cells than before, but as shown in Table I, this had no consequences for the microviscosity. Although fraction III contained less dead cells than the other subclasses, the total cell number was only a fraction of the initial number (data not shown). Obviously, the smaller lymphocytes die more quickly than the larger cells, but their remnants are cleared away very fast. This observation is in agreement with other reports [38].

Isolation and characterization of plasma membranes and other subcellular fractions from rabbit thymocytes

In the initial experiments plasma membranes were isolated in a one step sucrose gradient consisting of 30 and 40% (w/v) sucrose [9,10] yielding one plasma membrane fraction (M1) at the interphase of the two layers. As shown in Table II, the yield of plasma membrane protein was approx. 1%. This rather low yield is in agreement with other investigations [8,9,39,40] where the amount of protein recovered in the plasma membranes ranged from 0.6 to 1.7%. The plasma membrane fraction was enriched in the marker enzymes 5'-nucleotidase and Mg^{2+}-ATPase (Table III). They contained practically no ouabain-sensitive ($Na^+ + K^+$)-ATPase since the enzymic activities were practically the same in the absence and presence of 1 mM ouabain (data not shown). Thymidine 5'-phosphodiesterase was too low in all fractions for accurate measurements. The plasma membranes contained no glucose-6-phosphatase, β-*N*-acetylglucosaminidase, and succinate dehydrogenase. Further, they were not contaminated by DNA and contained only a slight amount of RNA (Table II). The presence of RNA is in accord with other reports [9,10,

TABLE I

DEGREE OF FLUORESCENCE POLARIZATION (P) OF DPH AND MICROVISCOSITY ($\bar{\eta}$) IN SUBCLASSES OF RABBIT THYMOCYTES

Thymocytes were fractionated on twelve continuous Ficoll/Metrizoate gradients as described in the text. The fractions from three tubes were combined, thus giving four samples for each subclass. Data are expressed as mean ± S.D. of the quadruplicate samples. A. Measurements immediately after isolation of the cells. B. Measurements after standing at 4°C during the night.

		P (25°C)	$\bar{\eta}$ (25°C) (poise)	Cell viability (%)
A. Fraction I	(1.067 g/ml)	0.284 ± 0.002	3.73 ± 0.04	93
Fraction II	(1.077 g/ml)	0.280 ± 0.003	3.60 ± 0.08	97
Fraction III	(1.084 g/ml)	0.285 ± 0.005	3.79 ± 0.16	96
B. Fraction I		0.283 ± 0.002	3.70 ± 0.05	39
Fraction II		0.278 ± 0.003	3.56 ± 0.08	75
Fraction III		0.282 ± 0.004	3.68 ± 0.12	84

TABLE II

CHEMICAL ANALYSES OF THE DIFFERENT SUBCELLULAR FRACTIONS OF RABBIT THYMOCYTES

Data are expressed as mean ± S.D. with number of preparations in parentheses. The experiments were performed with material obtained from one thymus at a time with an average weight of 2.46 g yielding $42.5 \cdot 10^8$ cells (range $35—60 \cdot 10^8$). The homogenate contained 102.0 ± 11.7 (n = 10) mg protein/$5 \cdot 10^9$ cells. n.d., not determined.

	Protein of homogenate (%) (10)	DNA (μg/mg protein) (4)	%	RNA (μg/mg protein) (4)	%	μg/mg protein	
						Phospholipids (6)	Cholesterol (6)
Homogenate	100	253 ± 14	100	73 ± 19	100	n.d.	n.d.
Nuclear pellet	38.8 ± 3.1	634 ± 46	90.6 ± 6.0	27 ± 5	11.8 ± 3.1	51 ± 7	2.6 ± 0.5
Mitochondrial pellet	6.8 ± 1.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Microsomal pellet	5.5 ± 1.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
64 000 × g_{av} supernatant	38.9 ± 4.0	5.0 ± 1.3	0.7 ± 0.2	148 ± 13	79.6 ± 6.0	n.d.	n.d.
Recovery (%)	91.2 ± 4.3		—		—		
Plasma membrane fraction M1	1.14 ± 0.17	0	0	50 ± 23	0.5 ± 0.1	491 ± 109	144.8 ± 12.3
Endoplasmic reticulum fraction E	2.90 ± 0.89	36 ± 22	0.4 ± 0.2	102 ± 10	3.8 ± 0.7	265 ± 12	51.7 ± 6.7
Plasma membrane fraction M2	0.17 ± 0.04	n.d.	n.d.	n.d.	n.d.	411 ± 127	78.1 *
Mitochondrial fraction Mit	5.92 ± 0.95	110 ± 31	4.4 ± 1.1	20 ± 3	2.0 ± 0.5	114 ± 8	11.4 ± 1.4
Recovery (%)	—		96.3 ± 5.4		98.9 ± 9.5		

* Mean of two determinations.

TABLE III

ENZYMIC ANALYSES OF DIFFERENT SUBCELLULAR FRACTIONS OF RABBIT THYMOCYTES

Data are expressed as the means of duplicate or triplicate experiments. Specific activities are expressed as μmol product liberated · mg^{-1} protein · h^{-1}.

	Homogenate	Nuclear pellet	Plasma membranes fraction		Endoplasmic reticulum fraction E	Mitochondrial fraction Mit
			M1	M2		
5'-Nucleotidase						
Specific activity	0.052	0.036	1.164	0.740	0.312	0.085
Enrichment (-fold)	1	0.7	22.4	14.2	6.0	1.6
Recovery (%)	100	26.9	31.3	2.7	24.8	10.9
Mg^{2+}-ATPase						
Specific activity	0.55	0.26	12.96	7.90	3.38	1.06
Enrichment (-fold)	1	0.5	23.1	14.4	6.2	1.9
Recovery (%)	100	18.3	35.5	2.7	24.3	13.1
Glucose-6-phosphatase						
Specific activity	0.065	0.070	0.003	0.130	0.481	0.045
Enrichment (-fold)	1	1.1	0.05	2.0	7.4	0.7
Recovery (%)	100	41.8	0.0	0.3	28.5	4.6
NADH dehydrogenase						
Specific activity	4.17	3.92	2.67	6.65	12.36	38.98
Enrichment (-fold)	1	0.9	0.6	1.6	3.0	9.3
Recovery (%)	100	36.5	0.7	0.3	8.6	55.3
Succinate dehydrogenase						
Specific activity	0.144	0.054	0	0.038	0	1.86 *
Enrichment (-fold)	1	0.4	0	0.3	0	13.3
Recovery (%)	100	14.6	0	0.05	0	87.8
β-*N*-Acetylglucosaminidase						
Specific activity	0.128	0.068	0.050	0.043	0.415	0.333
Enrichment (-fold)	1	0.5	0.4	0.3	3.2	2.6
Recovery (%)	100	20.6	0.4	0.05	9.4	15.4

* Succinate dehydrogenase was assayed in the mitochondrial fraction before preparation for sucrose gradient centrifugation, because the activity was virtually abolished by the homogenization procedure.

28,39—41] and can be explained by the presence in the plasma membrane vesicles of entrapped cytoplasm containing soluble and ribosomal RNA. This is confirmed by electron microscopy of the plasma membrane fraction (Fig. 2e). Membrane sheets and vesicles of varying sizes were observed. The smaller vesicles which are more resistent to the disrupting forces during the homogenization of the microsomal suspension contained electron-dense amorphous material. Electron microscopy of the nuclear fraction revealed well-preserved nuclei (Fig. 2a). That some of the nuclei were broken by the disrupting force appied to the cell suspension is shown by electron micrographs of the mitochondrial pellet (Fig. 2b). This pellet contained swollen and disrupted mitochondria (due to homogenization in hypotonic medium) and also electron-dense material which was considered by us as heterochromatin derived from damaged nuclei.

The endoplasmic reticulum fraction was characterized by the greatest amount of sedimentable RNA and by a high glucose-6-phosphatase activity (Tables II and III). It also displayed a high NADH dehydrogenase activity, as

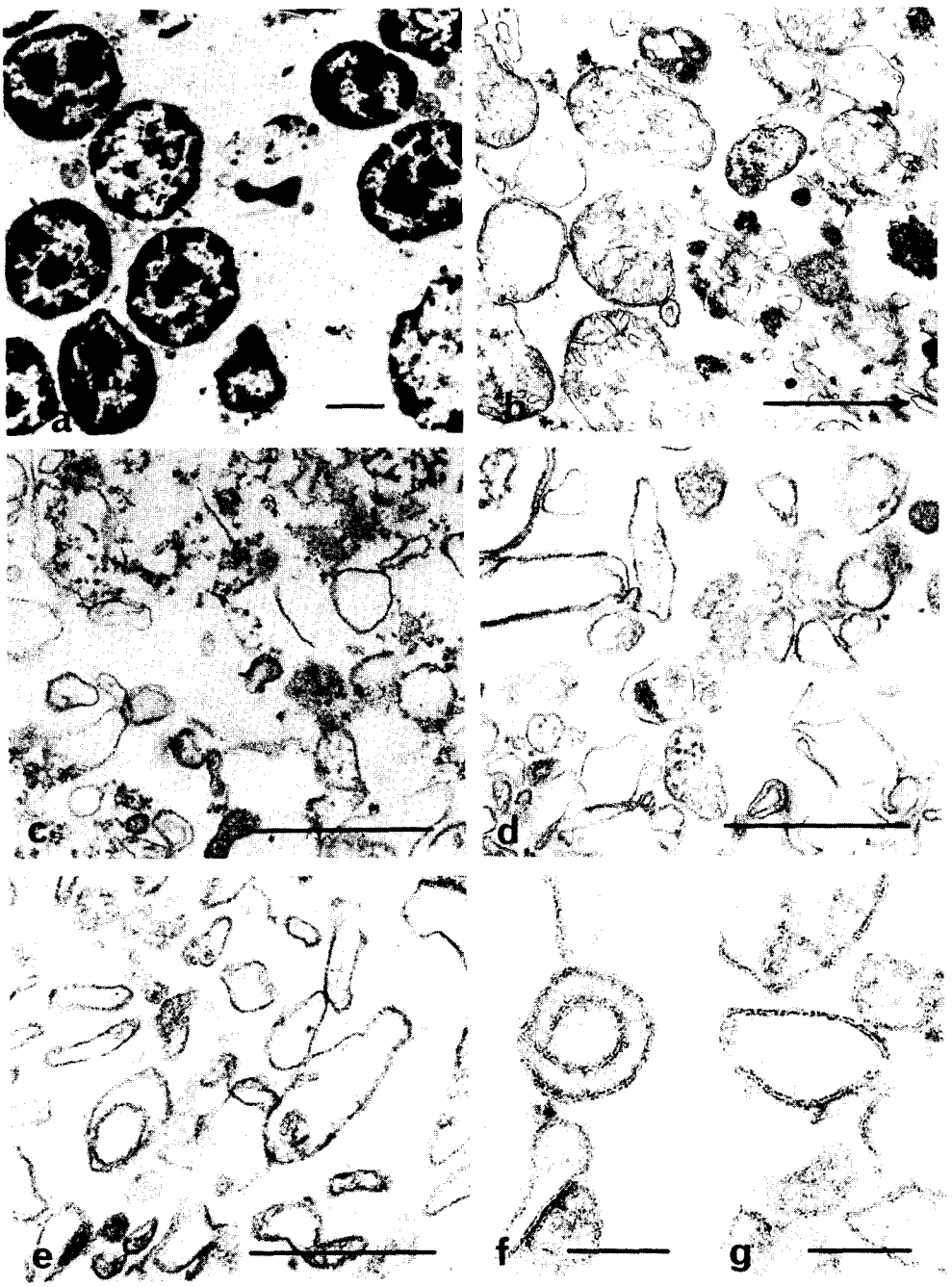

Fig. 2. Electron micrographs of (a) nuclei (bar 2 μm, ×4000); (b) mitochondrial fraction (bar 1 μm, ×19 500); (c) endoplasmic reticulum fraction (bar 0.5 μm, ×51 200); (d) membrane fraction M2 (bar 0.5 μM, ×51 200); (e) plasma membrane fraction M1 (bar 0.5 μm, ×51 200); (f, g) same preparation (bar 0.1 μm, ×139 000; the unit membrane is well seen on normally sectioned membranes).

expected, but it was remarkable that the mitochondrial fraction contained considerably more of this enzyme, beside the greatest amount of succinate dehydrogenase. In the electron microscope, free and membrane-bound ribosomes could abundantly be detected in preparations from the endoplasmic reticulum which also contained many membrane vesicles (Fig. 2c).

The lysosomal enzyme activity assayed with the marker enzyme β-*N*-acetylglucosaminidase was very low in the homogenate and the mitochondrial fraction; it was virtually absent in the plasma membrane and endoplasmic reticulum fractions (Table III).

The mitochondrial pellet contained a small amount of membranous material which floated at the interphase of the 30—40% sucrose gradient. The plasma membrane marker enzymes were less enriched in this fraction (M2) than in the plasma membrane fraction M1 (Table III). Furthermore, fraction M2 was more contaminated with other enzymes than fraction M1 and differed also morphologically from fraction M1 (Fig. 2d).

In the course of preparing plasma membranes via the 30—40% sucrose step gradient we observed that except for the bulk of material at the interphase, some material was also present in the 30% sucrose layer. For this reason we isolated plasma membranes from a 24 to 40% sucrose multistep gradient which became linear and continuous after 16—18 h high speed centrifugation. Plasma membrane fractions were obtained from this gradient at peak densities of 1.096 (PM1), 1.110 (PM2), 1.124 (PM3), and 1.137 g/ml (PM4). As shown in the electron micrographs of Fig. 3, the morphology of fraction PM1 which contained a substantial amount of amorphous material is clearly distinct from that of the other fractions which were composed of membrane sheets and vesicles without adherent material. Fraction PM4 contained a few ribosomes; it further contained more vesicles filled with amorphous material than fractions PM2 and PM3 which were rather alike.

It may be deduced from the densities of the fractions PM1—PM4 that the material recovered at the interphase of a one step gradient composed of 30% sucrose (density 1.113 g/ml) and 40% sucrose (density 1.150 g/ml) will be derived practically only from the fractions PM3 and PM4 in the multistep gradient. Nevertheless, the total yield of protein in fractions PM1—PM4, after the washing procedure, was never higher than in fraction M1 of the 30—40% sucrose one step gradient (Table IV). This confirms earlier observations by us that, for still unknown reasons, sedimentation centrifugation of decreasing quantities of material gives decreasing recoveries.

Lipid composition and microviscosity of plasma membranes and other subcellular fractions from rabbit thymocytes

The highest cholesterol to phospholipid molar ratio was found in the plasma membranes immediately followed by the endoplasmic reticulum (Table V). Mitochondria and nuclei showed, in this order, further decreasing relative cholesterol levels. The cholesterol content is one of the determining factors for the fluidity of biological and artificial membranes [36,42]; increasing the cholesterol content results in a higher microviscosity. This effect is also evident from Table V. Nuclear, mitochondrial, endoplasmic reticulum, and plasma membrane fractions showed increasing microviscosities, in this order.

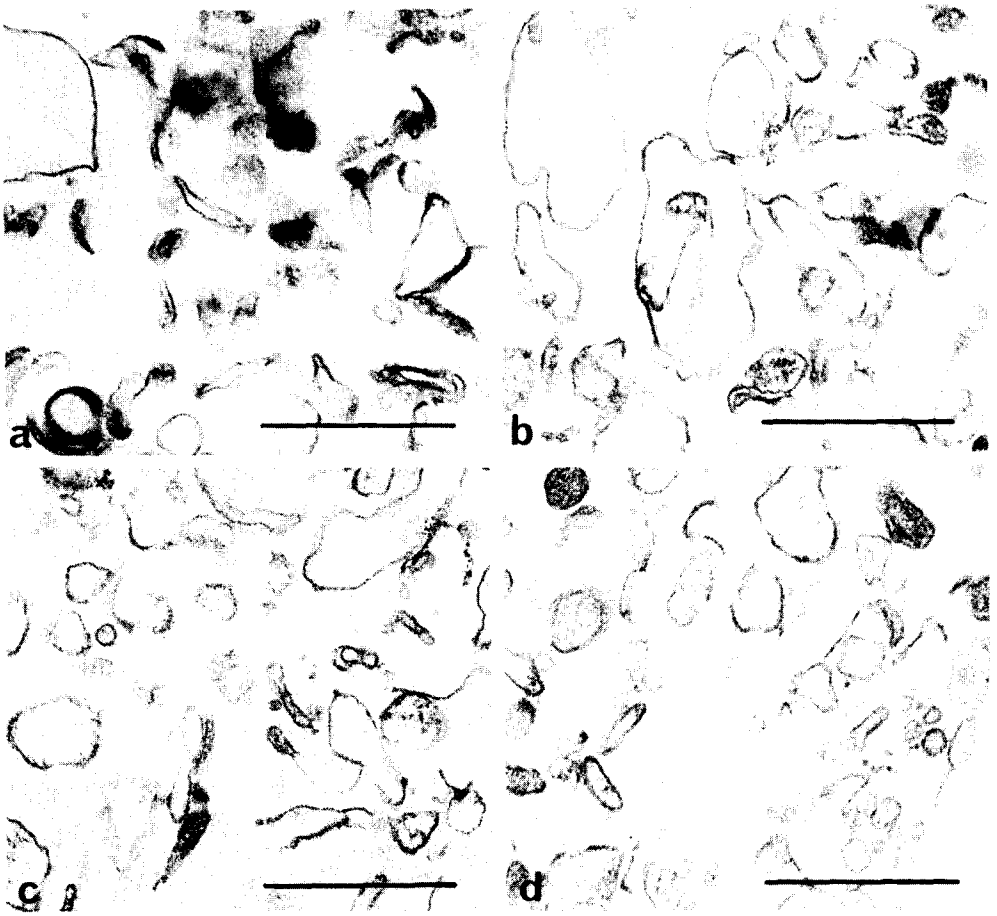

Fig. 3. Electron micrographs of plasma membrane fractions from a 24—40% sucrose multistep gradient. (a) Fraction PM1; (b) fraction PM2; (c) fraction PM3; (d) fraction PM4. Bar, 0.5 μm. ×51 200.

Another important factor which determines the membrane fluidity is the degree of saturation of phospholipid fatty acid chains [42,43]. Enhancement of the degree of saturation is accompanied by an increase of microviscosity. Table VI shows the results of the gas chromatographic analyses of the phos-

TABLE IV

RECOVERY, DEGREE OF FLUORESCENCE POLARIZATION (P) AND MICROVISCOSITY ($\overline{\eta}$) OF PLASMA MEMBRANE FRACTIONS PM1—PM4

Data are expressed as mean ± S.D. from four experiments.

Fraction	Protein (% of homogenate)	P (25°C)	$\overline{\eta}$ (25°C) (poise)
PM1	0.14 ± 0.01	0.309 ± 0.023	4.92 ± 0.97
PM2	0.19 ± 0.02	0.347 ± 0.005	7.74 ± 0.57
PM3	0.29 ± 0.02	0.352 ± 0.003	8.19 ± 0.31
PM4	0.31 ± 0.03	0.354 ± 0.002	8.40 ± 0.29

TABLE V

LIPID COMPOSITION AND MICROVISCOSITY OF DIFFERENT SUBCELLULAR FRACTIONS OF RABBIT THYMOCYTES

Data are expressed as mean ± S.D. with number of experiments in parentheses.

	Fraction			
	Nuclear	Mitochondrial	Endoplasmic reticulum	Plasma membrane M1
Cholesterol to phospholipid molar ratio	0.10 ± 0.02 (6)	0.20 ± 0.02 (8)	0.39 ± 0.04 (7)	0.59 ± 0.04 (7)
Fatty acid distribution of total phospholipids (%):				
Saturated acids	48.0 ± 1.6 (6)	58.5 ± 1.9 (4)	67.4 ± 0.8 (3)	80.3 ± 1.3 (3)
Monounsaturated acids	19.4 ± 1.2 (6)	21.5 ± 3.0 (4)	18.5 ± 1.4 (3)	13.9 ± 1.6 (3)
Polyunsaturated acids	32.8 ± 1.3 (6)	20.0 ± 1.6 (4)	14.1 ± 2.1 (3)	5.8 ± 0.5 (3)
Degree of fluorescence polarization P	0.237 ± 0.011 (5)	0.312 ± 0.004 (5)	0.326 ± 0.009 (7)	0.352 ± 0.002 (7)
Microviscosity $\overline{\eta}$ (poise)	2.32 ± 0.25 (5)	5.05 ± 0.25 (5)	5.98 ± 0.57 (7)	8.17 ± 0.25 (7)

TABLE VI

FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS FROM THE DIFFERENT SUBCELLULAR FRACTIONS OF RABBIT THYMOCYTES

Data are expressed as mean ± S.D. with number of preparations in parentheses. Analyses were performed on two liquid phases (SP 2330 and SP 2340) for each preparation. For fatty acids the nomenclature is according to (1970) Biochem. J. 116, 1; 20 : 4 denotes the acid containing twenty carbon atoms and four double bonds. On SP 2330 18 : 3 and 20 : 4 cannot be separated from 20 : 0 and 22 : 0, respectively. This is possible on SP 2340 which phase, on the other hand, does not discriminate between 18 : 3 and 20 : 1, and between 20 : 4 and 22 : 1. Using both columns the individual fatty acid contributions could be calculated.

Fatty acid	Nuclear pellet (6)	Mitochondrial fraction Mit (4)	Endoplasmic reticulum fraction E (3)	Plasma membranes	
				Fraction M1 (3)	Fraction M2 (2)
14 : 0	1.0 ± 0.2	1.5 ± 1.0	1.8 ± 0.7	2.8 ± 1.7	1.8
15 : 0	0.6 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	1.2 ± 0.2	0.9
16 : 0	29.2 ± 2.4	35.1 ± 2.3	44.4 ± 1.8	55.9 ± 1.9	47.5
16 : 1	2.5 ± 0.3	3.1 ± 0.3	2.2 ± 0.6	1.8 ± 0.1	2.1
17 : 0	0.7 ± 0.2	0.9 ± 0.3	1.2 ± 0.5	1.0 ± 0.2	1.0
18 : 0	14.2 ± 1.6	18.9 ± 3.1	17.9 ± 0.5	18.4 ± 2.2	20.3
18 : 1	16.5 ± 0.9	18.1 ± 2.7	16.0 ± 0.9	11.7 ± 1.6	14.5
18 : 2	12.7 ± 1.2	10.2 ± 0.7	7.4 ± 0.7	3.1 ± 0.7	5.5
18 : 3	0.3 ± 0.3	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.3	0.3
20 : 0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.2
20 : 1	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.2	0.2
21 : 0	0.4 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.5
20 : 4	16.6 ± 1.0	7.8 ± 1.1	5.1 ± 1.3	1.7 ± 0.5	3.2
22 : 0	1.4 ± 0.3	0.6 ± 0.2	0.7 ± 0.3	0.6 ± 0.3	1.3
22 : 1	—	—	0.2 ± 0.2	0.1 ± 0.1	—
20 : 5	0.4 ± 0.4	0.2 ± 0.1	—	0.2 ± 0.2	—
22 : 4	1.2 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	0.4 ± 0.2	0.7
22 : 5	1.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	0.1 ± 0.1	0.1

pholipid fatty acid patterns in nuclei, mitochondria, endoplasmic reticulum, and plasma membranes. The most prominent fatty acids in the total phospholipid fractions were 16 : 0, 18 : 0, 18 : 1, 18 : 2, and 20 : 4. In the sequence nuclei — mitochondria — endoplasmic reticulum — plasma membranes the contribution of the saturated fatty acids rose at the expense of the unsaturated acids, particularly the polyunsaturated acids 18 : 2 and 20 : 4. This sequence coincides with that of increasing microviscosity (Table V).

The microviscosity of plasma membrane fractions PM1—PM4 is shown in Table IV. Fraction PM1 displayed a considerably lower microviscosity than the other three fractions. On this ground and because of the ultrastructural appearance (Fig. 3a) we consider this fraction as an impure plasma membrane fraction.

Microviscosity of plasma membranes from subclasses of rabbit thymocytes

Plasma membranes were isolated from thymocyte subclasses and separated in a 24—40% sucrose multistep gradient. The cells of fraction I were too low in number for the isolation of plasma membranes in reasonable amounts for microviscosity determination. For this reason we only used cells from fractions II and III. These fractions very well represented mature and immature lymphocytes. As shown in Table VII, cells from subclass III yielded a plasma membrane fraction PM4 with a significantly higher microviscosity than cells from subclass II ($P < 0.001$ by Student's t-test). A less significant increase in microviscosity ($0.01 < P < 0.05$) is observed for membrane fraction PM3 from cells of subclass III with respect to subclass II. The P and $\overline{\eta}$ values of membrane fractions PM1 and PM2 from cells of subclass III were not significantly different from those of subclass II.

Table VII also shows a stepwise decline in the microviscosity of plasma membranes from higher to lower density. This was not found for cells which were not separated on a Ficoll/Metrizoate gradient (Table IV). The reason for this discrepancy is still unknown.

TABLE VII

DEGREE OF FLUORESCENCE POLARIZATION (P) AND MICROVISCOSITY ($\overline{\eta}$) OF PLASMA MEMBRANES FROM RABBIT THYMOCYTE SUBCLASSES

Plasma membranes were obtained from subclass II (peak density 1.076 g/ml) and subclass III (peak density 1.084 g/ml). The membranes were separated on a 24—40% sucrose multistep gradient and denoted PM1—PM4 as in Fig. 1. Data are expressed as mean ± S.D. from four experiments.

Plasma	P (25°C)		$\overline{\eta}$ (25°C; poise)	
	Subclass II	Subclass III	Subclass II	Subclass III
PM1	0.284 ± 0.008	0.277 ± 0.006	3.74 ± 0.50	3.47 ± 0.40
PM2	0.300 ± 0.007	0.297 ± 0.003	4.44 ± 0.38	4.30 ± 0.28
PM3	0.312 ± 0.006	0.324 ± 0.002	5.07 ± 0.37	5.81 ± 0.19
PM4	0.320 ± 0.003	0.350 ± 0.004	5.55 ± 0.23	8.00 ± 0.52

Discussion

Thymic lymphocytes can roughly be divided into two main subpopulations. The smaller lymphocytes which are localized mainly in the inner cortex are characterized by immunoincompetence, radiation and steroid sensitivity, low glucose transport, high buoyant density, low electrophoretic mobility, and (in mice) high amounts of θ and TL antigens [1—5]. From the larger lymphocytes a considerable proportion is found in the medulla displaying the opposite characteristics. Freeze-fracture experiments conducted in our laboratory on whole cells have further demonstrated that the plasma membranes of the larger cells (present in subclasses I and II with peak densities of 1.067 and 1.077 g/ml, respectively) show the greater glycerol-induced redistribution of intramembranous particles compared with the smaller lymphocytes (present in subclass III with peak density of 1.084 g/ml) (Ref. 5; De Groot, C., personal communication). It has been suggested that the mobility of intramembranous particles in the plane of the membrane is dependent on membrane fluidity [44—47] and the presence of a cytoskeleton [48,49]. Treatment of unfixed lymphocytes with cytoprotectants like glycerol and dimethylsulfoxide has shown to cause perturbation of plasma membranes in a still unknown way resulting in redistribution of intramembranous particles [50,51]. Assuming that the mobility of these particles is influenced by the dynamic character of the membrane it is feasible that membrane fluidity is one of the factors governing the glycerol-induced redistribution of intramembranous particles. It has been inferred from a large number of investigations with different cell types that the fluidity of the plasma membrane is also a prerequisite for many cell reactions like proliferation and differentiation [35,43,52], fusion [53], activity of membrane-bound enzymes [54], transport processes [55], and hormone-activated adenylate cyclase [56]. The role of plasma membrane fluidity in transformation is still questionable [34,35,45,54,57,58] although most authors claim an increased fluidity after transformation. Changing the fluidity of the plasma membrane by manipulating its lipid composition has shown to affect greatly the properties of the cell [59].

On these grounds we hypothesized that the behaviour of cells from subpopulations of thymocytes representing different degrees of maturation might also be influenced by the fluidity of their plasma membranes. The fluidity of biological and artificial membranes may be estimated from the apparent microviscosity which can be calculated from the degree of fluorescence polarization of an appropriate probe like DPH [33,34,60]. Fluorescence decay measurements with this probe [61,62] have shown that the apparent microviscosity which is derived from steady-state measurements using continuous illumination for excitation gives an approximation of the true situation. This is due to restriction on the rotational motion of the fluorescent probe in a certain region such as a cone. In a recent review of the various theoretical and practical aspects of fluorescence polarization Shinitzky and Barenholz [60] conclude: ... 'The steady-state fluorescence polarization method yields weight-averaged fluidity parameters of microscopic regions which by fluorescence decay methods can be resolved, to some extent, to their major components'. And further: 'The degree of fluorescence polarization is a very sensitive and reproducible

parameter which in lipid systems of micelles or membranes can be readily interpreted in fluidity terms'. We were thus reassured that measurement of the degree of fluorescence polarization will yield valuable information concerning the fluidity of biological membranes.

It has been stated that the degree of fluorescence polarization measured with whole cells is directly related to the microviscosity of the plasma membrane, because the fluorescent probe would not penetrate into the cell [52,63,64]. We therefore measured the degree of fluorescence polarization using whole rabbit thymocytes from subclasses separated on a linear continuous Ficoll/Metrizoate gradient. As shown in Table I, no differences could be found between the three subclasses in this respect. Furthermore, the initial viability of the cells had no effect on the degree of fluorescence polarization. For this reason we supposed that the fluorescent probe DPH is not only confined to the plasma membrane, but readily taken up by all cellular elements. Van Hoeven et al. [37] have shown with various cell types that the degree of fluorescence polarization using intact and disrupted cells was practically the same and in all instances lower than of plasma membranes isolated from these cells. They concluded that the fluorescence polarization technique with a lipophilic probe applied to whole cells represents a measure of the average fluidity of all lipids being present in a cell. This conclusion is in accordance with other reports [65—67]. For these reasons we decided to isolate the plasma membranes from the thymocytes.

Because of its ease of handling combined with the production of highly purified plasma membranes from lymphocytes we applied the Stansted cell disruptor [8,68]. The last phase in the isolation procedure consisted of a sucrose density gradient centrifugation. In the initial experiments we applied a 30—40% sucrose one step gradient yielding a plasma membrane fraction enriched in the typical marker enzymes 5′-nucleotidase and Mg^{2+}-ATPase. Ouabain-sensitive ($Na^+ + K^+$)-ATPase activity could hardly be detected. In this respect plasma membranes isolated from rabbit thymocytes resemble plasma membranes isolated from thymocytes of calf [39] and mouse [12]. The plasma membranes were virtually devoid of glucose-6-phosphatase and contained no succinate dehydrogenase and β-*N*-acetylglucosaminidase. They displayed some NADH dehydrogenase activity, in line with other investigations [39,40]. This enzyme was, for that matter, more enriched in the mitochondrial than in the endoplasmic reticulum fraction. From this finding we conclude that NADH dehydrogenase cannot be regarded exclusively as a marker for endoplasmic reticulum. The plasma membrane preparations contained no DNA and only a little RNA, and electron micrographs showed the typical appearance of sheets and vesicles of stripped membranes. In comparison with the other subcellular fractions they were further characterized by the highest cholesterol to phospholipid molar ratio, the greatest amount of saturated fatty acids derived from total phospholipids and, consequently, the greatest microviscosity.

The microviscosity of the plasma membranes amounted to a rather high value ($P = 0.352$; $\bar{\eta} = 8.17$ poise) compared with plasma membranes isolated from other cells. Van Blitterswijk et al. [36] and Van Hoeven et al. [37] found much lower values for plasma membranes from mouse thymocytes ($P = 0.306$) and rat hepatocytes ($P = 0.311$). Only human erythrocytes ($P = 0.332$), rat hepatoma 484A cells ($P = 0.337$), and Novikoff hepatoma cells ($P = 0.351$)

yielded plasma membranes with relatively high values for the degree of fluorescence polarization [37]. Recently, Schroeder [69] found that the inner monolayer of isolated LM cell plasma membranes showed a higher fluorescence polarization ($P = 0.355$) than the outer layer ($P = 0.290$) reflecting differences in the lipid composition of both layers. This may be brought in accord with the finding by De Groot (see Introduction) that for lymphocytes significant differences in glycerol-induced redistribution of intramembranous particles were only seen in the external fracture face and not in the protoplasmic fracture face (de Groot, C., personal communication). As far as we know, no microviscosity values are reported in the literature for plasma membranes isolated from rabbit lymphocytes (or other cells). We think that the relatively high values reported in the present study are related to species differences.

We also isolated a membrane fraction M2 from the mitochondrial pellet. It showed some characteristics of plasma membranes. The specific activities of the typical plasma membrane marker enzymes were, however, lower than for the plasma membrane fraction M1; the cholesterol to phospholipid ratio was also lower. The fatty acid composition of the phospholipid fraction was also different for both membrane preparations. Because of these results and the low yield (0.17% of homogenate protein) we did not involve the membrane fraction M2 in our further studies.

A critical step in the isolation procedure here reported is the preparation of the microsomal pellet for sucrose gradient centrifugation. In this treatment (homogenization by hand in a tight-fitting all-glass homogenizer) aggregates of plasma membranes might be left that were pelleted together with the endoplasmic reticulum material. This might provide an explanation for the high yield of the endoplasmic reticulum fraction E relative to the plasma membrane fraction M1, the distinct activity of plasma membrane marker enzymes in fraction E, the appearance of membrane sheets and vesicles in electron micrographs of this fraction, and its relatively high microviscosity. Recently, Monneron and d'Alayer [70,71] developed a new method for the isolation of plasma membranes from calf thymocytes. The differential centrifugation steps usually employed for the separation of nuclei and mitochondria were omitted. Instead, the homogenate (in 40% sucrose) was directly included in a discontinuous sucrose gradient and a short centrifugation time (2 h) was chosen. By this method plasma membranes were obtained in high yield. We are now trying this method, particularly because the yield of plasma membranes from subclasses of thymocytes was too low for a detailed lipid analysis (see below). In preliminary experiments we found a higher yield of plasma membranes from rabbit thymocytes: 2.6% of the homogenate protein compared with 1.14% in the present investigation. Less endoplasmic reticulum was obtained, but it still showed a high microviscosity. Electron microscopy revealed less pure plasma membrane preparations than in the present study (far more ribosomes were seen), but work is still in progress.

On the basis of the biochemical and morphological data obtained for plasma membranes and the other subcellular fractions we judged the procedure depicted schematically in Fig. 1 adequate for the preparation of plasma membranes from subclasses of rabbit thymocytes. The quantity of cells in fraction I with peak density of 1.067 g/ml (approx. 5% of total cells) was too low for the

preparation of a sufficient amount of plasma membranes for further studies. We therefore only used the cells from fraction II (peak density 1.077 g/ml; approx. 15% of total cells) and fraction III (peak density 1.084 g/ml; approx. 80% of total cells) representing very well mature and immature lymphocytes, respectively (see above). We applied the 24—40% sucrose multistep gradient for the separation of plasma membranes because electron microscopy had revealed distinct characteristics for the fractions denoted as PM1—PM4. The material in the plasma membrane fractions obtained from the cells of fraction II was just enough for reliable microviscosity measurements. It was not sufficient for further detailed lipid analyses. As mentioned before, we are now elaborating the method of Monneron and d'Alayer [70,71] for this purpose.

It may be hypothesized that fractions PM2—PM4 are derived from different domains in the plasma membranes of intact cells. Fraction PM1 is not included because its ultrastructural appearance and apparent microviscosity were markedly different from fractions PM2—PM4. It may be concluded from Table VII that lymphocytes of subclass III possess plasma membrane domains with a significantly higher microviscosity than cells from subclass II. Although the influence of the differences in microviscosity seen for PM3 and PM4 may be lessened by fraction PM2, this effect might be marginal because PM2 comprises only 24% of the total of fractions PM2—PM4 (Table IV). An overall restriction of the mobility of intramembranous particles may thus be possible assuming a greater mobility of these particles in more fluid membrane systems [44—47]. In this case our results can be brought in accord with the previously mentioned finding that the intramembranous particles on the larger cells from subclass II are more readily redistributed by glycerol than on the smaller cells from subclass III. Differentiation of lymphocytes in the thymus might thus be related to changes in plasma membrane microviscosity. It has been stated for other cell types that differentiation is accompanied by changes in microviscosity [35,52] and can even be directed by such changes [53,72].

The here reported difference in microviscosity might also be related to other characteristics of thymocytes. For this reason we direct our further studies to the effect of cholesterol and fatty acids on cellular activities like mitogen-induced stimulation and cell kill by anti-thymocyte-immunoglobulin and complement. We hope that these investigations might clear our insight into the events during the differentiation of lymphocytes in the thymus.

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