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ANALYSIS OF THE PROTEINS IN THYMOCYTE PLASMA MEMBRANE AND SMOOTH ENDOPLASMIC RETICULUM BY SODIUM DODECYLSULFATE–GEL ELECTROPHORESIS

R. SCHMIDT-ULLRICH[b], E. FERBER[a], H. KNÜFERMANN[a], H. FISCHER[a] and D. F. HOELZL WALLACH[b]

[a]*Max-Planck-Institut für Immunbiologie, Freiburg, Stübeweg 51 (Germany) and* [b]*Division of Radiobiology, TUFTS-New England Medical School, 136, Harrison Ave Boston (U.S.A.)*

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SUMMARY

We have purified the plasma membranes and membranes of endoplasmic reticulum from calf and rabbit thymocytes and from calf mediastinal lymph node lymphocytes. We disrupted the cells by the "nitrogen cavitation method" and prepared a microsomal isolate by differential centrifugation. We fractionated this by isopycnic ultracentrifugation in dextran gradients into membrane vesicles, PM_1 and PM_2, most likely derived from plasma membrane and a fraction, ER, most likely originating from endoplasmic reticulum. More than 80% of the microsomal 5′-nucleotidase and acid *p*-nitrophenylphosphatase concentrates in the PM_1 and PM_2 fractions; alkaline *p*-nitrophenylphosphatase, another presumptive PM marker, is concentrated in the PM_1 fraction. These data are confirmed by the lactoperoxidase radioiodination of intact rabbit thymocytes followed by subcellular fractionation. The specific content of phospholipids (822 nmoles/mg protein) and cholesterol (1032 nmoles/mg protein) is highest in PM_1 and PM_2 plasma membrane fractions. NADH-oxidoreductase, our endoplasmic reticulum marker, is clearly enriched in gradient pellet.

The membrane proteins were separated by electrophoretic molecular sieving in sodium dodecylsulfate–polyacrylamide gel electrophoresis, containing dithiothreitol (sodium dodecylsulfate–polyacrylamide gel electrophoresis). We numbered the 10 major protein components of the "microsomal fraction" (apparent molecular weights between 280000 and 15000) from 1–10 according to their decreasing molecular weights. Of these proteins, those with higher molecular weight, predominantly glycoproteins, appear in the PM_1 fraction, while the endoplasmic reticulum fraction contains mainly low molecular weight components.

INTRODUCTION

Immunoglobulins and other macromolecules of biological interest appear to form integral parts of some lymphoid cells' plasma membranes, at least during

Abbreviation: HEPES, 4-(hydroxymethyl)-1-piperazinylethane-2-sulfate.

certain developmental and physiological states. This knowledge has engendered numerous ingenious experiments informing about the mobility, temperature sensitivity, and stability of membrane immunoglobulins, as well as certain membrane antigens [1, 2]. Nevertheless, we still need to learn, in molecular terms, how these substances affect membrane structure, how they behave and influence their neighbours after binding specific antigens, as well as how the lymphocyte membrane changes in response to diverse mitogens, i.e. during the steps which initiate the conversion of an "immunocompetent" into an "immunoactive" one.

Some clues as to the participation of plasma membrane lipid metabolism derive from the studies described in [3, 4] on the metabolic effects of concanavalin A. However, biomembranes comprise subtly interacting lipid–protein complexes, whose specific responses indubitably lie in their complexes, as well as possible associated carbohydrates.

Part of our investigations on the structure and function of lymphocyte plasma membranes involves the characterization of their membrane proteins. We here present some of our initial studies on thymic lymphocytes, a relatively uniform population of seemingly quiescent lymphoid cells. Moreover, these cells are well suited for the gentle, controlled plasma membrane isolation procedure [5, 6] adapted to normal lymphoid cells by Ferber et al. [7].

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, all chemicals, water, and biologicals were of the highest purity grade available; 4-(hydroxymethyl)-1-piperazinylethane-2-sulfate (HEPES), methylene blue, Coomassie Brilliant Blue, sodium dodecylsulfate, acrylamide, *N,N'*-methylene-bisacrylamide, *N,N'*-diallyltartrate diamide, dithiothreitol, and tetranitromethane were obtained from Serva, Heidelberg. Dextran-150 was obtained from Pharmacia, Frankfurt. Lactoperoxidase was obtained from Sigma Chemical Co., St. Louis, Mo.; Ficoll from Pharmacia, Sweden, Isopaque (Sodium Metrizoat, 75%) from Nyegaard and Co., Oslo. Substrates and reagents for marker enzyme assay were used as in [7]. Sodium Iodide (^{125}I), carrier free, 20–140 mCi/ml was obtained from Buchler Amersham, Braunschweig.

Lymphocytes

Lymphocytes from calf or rabbit thymus were collected within 1 h after death as in [7]. In all cell isolations and fractionations the temperature was maintained at 0–4 °C. Free lymphoid cells were harvested after grinding tissue fragments with a Tenbroeck glass homogenizer (Bellco, clearance 0.01–0.015 cm) in phosphate-buffered saline (pH 7.2). In most cases fewer than 10% of the lymphocytes showed damage by dye-exclusion tests. When the proportion was greater, the dead cells were eliminated by a Ficoll–Isopaque gradient, density 1.08. $2 \cdot 10^9$ pelleted cells were first suspended in 0.5 ml phosphate-buffered saline (pH 7.2), and then in 5 ml of a 9% Ficoll, 24% Isopaque (v/v, 24:10) mixture, underlaid by 5 ml of the same mixture and overlaid by 5 ml phosphate-buffered saline (pH 7.2) (Resch, K., Huber, A. and Ferber, E., unpublished). Centrifugation was at 2400 rev./min for 20 min (International PR 6 centrifuge).

Cell rupture

Here we proceeded as in [7], except for a few specific modifications. The washed cells were suspended at a concentration of $5 \cdot 10^7$–$1 \cdot 10^8$ cells/ml in 0.075 M KCl, 0.065 M NaCl, 0.25 mM $MgCl_2$ and 0.01 M HEPES buffer (pH 7.4). We could not employ sucrose as in [7], since this promotes irreversible aggregation of thymocytes. The cell suspensions were exposed to 30 atm N_2 for 20 min at 4 °C with gentle stirring. After return to 1 atm, EDTA was added to a final concentration of 0.001 M.

Subcellular fractionation

Subcellular fractionation was done at 0–4 °C. To avoid aggregation artifacts, we did not freeze samples prior to dissolution in sodium dodecylsulfate for electrophoresis.

First we sedimented nuclei + mitochondria + lysosomes at $6 \cdot 10^5$ g·min (Spinco–Beckman Centrifuge, J 21). The "microsomal fraction" was pelleted thereafter at 10^7 g·min (Spinco ultracentrifuge, L2 65, Rotor 50.1). We released material trapped within, or loosely bound to, membrane vesicles by exposure to hypoosmotic media in combined use with shearing forces, using first exposure to 0.01 M HEPES buffer (pH 7.5), after resuspension of the microsomes in a small volume of this buffer with a 2-ml syringe and a needle of 0.7-mm inner diameter, pelleting at 10^7 g·min, and then identical washing in 0.001 M HEPES buffer (pH 7.5). The material from $5 \cdot 10^9$ cells was then suspended in 1 ml 0.001 M HEPES buffer (pH 7.5), and dialysed for 90 min against 0.001 M HEPES buffer, 0.001 M $MgCl_2$ (pH 8.2). We then layered 1 ml of this suspension upon a continuous 0.7 ml dextran gradient of density 1.00–1.09, underlaid with a 2.3-ml cushion of 1.09-density dextran. The buffer throughout the system was 0.001 M HEPES buffer, 0.001 $MgCl_2$ (pH 8.2).

Centrifugation was for 10^8 g·min (Spinco ultracentrifuge, L2 65, Rotor SW-56) and yields two plasma membrane bands, PM_1 and PM_2 (0.2 ml and 0.15 ml volume, respectively) (Fig. 1) near the dextran buffer border, and a pellet (0.3 ml volume), comprised primarily of endoplasmic reticulum fragments. Fraction 1 (1.2 ml volume), the supernatant, contains mainly soluble material. Fraction 3 (2.15 ml volume) within the dextran barrier lacks material of definite characteristics. The gradient is fractionated from the upper portion of the tube with 1-ml syringe and a needle of 0.5-ml inner diameter.

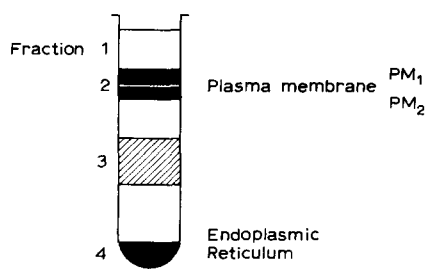


Fig. 1. Distribution of microsomal vesicles in dextran (density 1.09) with superimposed continuous dextran gradient (density 1.09–1.00). Fraction 1, predominantly soluble proteins; Fraction 2, plasma membrane PM_1 and PM_2 near the border of continuous and discontinuous gradient; Fraction 3, intermediate fraction; Fraction 4, endoplasmic reticulum.

Chemical analyses

Protein assays on various subcellular fractions were carred out fluorometrically [8] or by the ninhydrin method [9]. Lipid extractions were as in [10] and lipid phosphorus was determined by the method of Lowry et al. [11]; cholesterol was determined as in [12]. The relative RNA content of diverse gradient fractions was estimated after polyacrylamide gel electrophoresis and fixation according to [13], using 0.04% methylene blue in 0.1 M sodium acetate buffer (pH 4.7).

Enzyme assays

We utilized 5'-nucleotidase (EC 3.1.3.5), determined as in [14] as plasma membrane marker. As additional markers we estimated acid *p*-nitrophenylphosphatase (EC 3.1.3.2) as in [15] and alkaline *p*-nitrophenylphosphatase (EC 3.1.3.1) essentially as in [16] but using 1 M diethanolamine buffer (pH 9.5) [17].

We employed NADH-oxidoreductase (EC 1.6.4.3), estimated as in [6] as marker for endoplasmic reticulum, succinate dehydrogenase (EC 1.3.99.1) as mitochondrial marker [18] and β-glucuronidase (EC 3.2.1.31), assayed as in [19] to monitor lysosomal contamination.

Lactoperoxidase radioiodination of intact rabbit thymocytes

As extrinsic plasma membrane marker we employed the lactoperoxidase radioiodination method using H_2O_2 as substrate principally as in [20, 21], but adapting the method to our preparative scale. Rabbit thymocytes were carefully freed from dead cells as previously described in this paper. To $5 \cdot 10^7$ cells in 0.5 ml phosphate buffered saline (pH 7.2), first 0.05 ml lactoperoxidase (60 mg/ml phosphate-buffered saline), then 0.02 ml H_2O_2, 0.0018%, and finally 0.01 ml $Na^{125}I$ (0.02 mCi ^{125}I) was added. After 10 min reaction time another 0.02 ml H_2O_2 was added. Reaction time was at 30 °C for 20 min. After reaction, the cells were washed 3 times in 10 ml ice-cold phosphate-buffered saline (pH 7.2), containing 10 mM KI, to remove non-covalently bound ^{125}I. Aliquots of the iodinated cells were mixed with unlabelled ones to get enough material for membrane preparation. For analyses of ^{125}I distribution within subcellular fractions during membrane isolation, thrice washed trichloroacetic acid (10%) precipitates were counted in the Packard autogamma spectrometer, Model 3002. To exclude the possibility of penetration of lactoperoxidase through the plasma membrane during the labelling procedure, e.g. by pinocytosis, rabbit thymocytes were treated with auto-radioiodinated lactoperoxidase, dialysed thrice against 2000 ml phosphate-buffered saline (pH 7.2) every time at 4 °C, for at least 36 h. Cells were incubated in the presence of non-radioactive KI using identical conditions as described above.

Polyacrylamide gel electrophoresis in sodium dodecylsulfate

We fractionated membrane proteins/peptides in 1% sodium dodecylsulfate containing 40 mM Tris–20 mM acetate–2 mM EDTA buffer (pH 7.4, using 7% polyacrylamide cross-linked with *N,N'*-methylene-bisacrylamide (2.5%) or with *N,N'*-diallyltartardiamide (3.75%) in the form of round gels 6 mm in diameter. To determine the presence of –S–S– bridges, we carried out sodium dodecylsulfate–polyacrylamide gel electrophoresis with and without 0.04 M dithiothreitol. As standard proteins we employed human immunoglobulin G (155000 daltons), phos-

phorylase A (92000 daltons), bovine serum albumin (69000 daltons), ovalbumin (45000 daltons), α-chymotrysinogen (22500 daltons), myoglobin (17500 daltons) and cytochrome *C* (13000 daltons). The molecular weight determinations of the membrane proteins were done in polyacrylamide cross-linked with *N,N'*-diallyltartardiamide, because these gels provide good, simultaneous resolution of both high and low molecular weight proteins/peptides as shown in Table VI.

Coomassie brilliant blue was employed as general protein stain [13] and glycoproteins were localized by periodate–Schiff method [13]. Gels were scanned at 620 nm for Coomassie staining and 560 nm for periodate–Schiff reagent staining using a Gilford scanning attachment (Model 240) and integrating under the curves with a Hewlett Packard integrator (Model 3370 B). Identical amounts of protein (quantified fluorometrically) were applied to each gel.

Radioactive ^{125}I analyses of separated iodinated membrane proteins, prestained with o-phthalaldehyde according to [22] were done by cutting the gels into single protein bands.

Protease assays

According to Bowers [23] thymus contains appreciable levels of cathepsin D. We have tested for this and other proteases, using three different methods. This was particularly crucial for the peptide analysis of the endoplasmic reticulum. First we assayed concentrates of microsomal supernatant as in [24], using 1% bovine serum albumin in 1.35 M sodium acetate, 0.02 M $(NH_4)_2SO_4$ (pH 3.5) as substrate. The microsomal supernatant was concentrated 10-fold using a Diaflo ultrafiltration membrane, Type PM-10, (Amicon Corp., Lexington, Mass.), bringing the protein concentration to 20 mg/ml. 2 ml of the concentrate were then incubated with 1 ml bovine serum albumin (10 mg) at 37 °C for 60 min. After addition of trichloracetic acid to 10% at 0 °C, the precipitate was removed by filtration through Whatman No. 2 filter paper. Soluble peptides and/or amino acids were determined by the ninhydrin method after prior neutralisation with 0.5 M NaOH. In the controls, the trichloroacetic acid was added to the microsomal supernatant before the bovine serum albumin. As a positive control we used trypsin (3 mg/ml) instead of the microsomal supernatant. As second criterion we inhibited cathepsins nonspecifically using tetranitromethane. Here we followed [25, 26], nitrating the substrate, as well as possible contaminating enzymes, with tetranitromethane. To do this, we added the tetranitromethane (in 96% ethanol) during cell rupture to the final concentration of 1.2 mg/ml homogenate. We then proceeded with our usual subcellular fractionation and sodium dodecylsulfate–polyacrylamide gel electrophoretic analyses. As a third test we incubated various fractions at 37 °C for 60 min. Here we examined (1) intact cells; (2) fresh, whole homogenate; (3) the supernatant after low-speed centrifugation having removed the nuclei and large granules. After the incubations, the material was worked up as usual and analysed by sodium dodecylsulfate–polyacrylamide gel electrophoresis.

Analysis of the protein composition of material released by osmotic shock

For sodium dodecylsulfate–polyacrylamide gel electrophoretic analysis of osmotically released material, this was centrifuged at $5 \cdot 10^7$ g·min and then concentrated using Diaflo ultrafiltration membranes Type PM-10.

TABLE I

PREPARATION OF PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM BY FRACTIONATION OF CALF THYMOCYTE MICROSOMES

Values are given from 3 separate preparations. Specific activity is given per mg protein. About 3% of total homogenate were found in washed microsomes.

Fraction	Protein		5′-Nucleotidase *			Acid phosphatase ** (*p*-nitrophenylphosphate)			NADH-oxidoreductase ***		
	Total (mg)	% distribution	Spec. act.	Total act.	% distribution	Spec. act.	Total act.	% distribution	Spec. act.	Total act.	% distribution
Microsomes	9.65	100.0	6.7	64.6	100.0	157.4	1518.9	100.0	214.7	2071.8	100.0
Fraction 1	0.52	5.4	5.0	2.8	4.3	0	0	0	19.9	10.3	5.0
Fraction 2 (plasma membrane)	4.84	50.2	11.0	53.2	82.4	254.6	1232.3	81.0	104.4	505.3	24.4
Fraction 3	2.86	29.6	2.5	7.2	11.1	23.5	238.8	15.7	360.3	1030.5	49.7
Fraction 4 (endoplasmic reticulum)	1.43	14.4	0	0	0	33.4	47.4	3.1	367.6	525.7	25.4

* nmoles P_i liberated/min.
** nmoles *p*-nitrophenylphosphate liberated/min.
*** nmoles NADH liberated/min.

RESULTS

Enzyme markers

Table I provides the distribution of the various marker enzymes, as well as that of total protein, after the dextran gradient fractionation. We obtained a 10–15-fold increase in the specific activity in the microsomal fraction in comparison to that in the cell homogenate. Since values determined in the homogenate were very low and therefore difficult to measure, we did not enter them in Tables I and II. About 80% of the microsomal 5′-nucleotidase and acid *p*-nitrophenylphosphatase locates in the low density bands which were accordingly considered enriched in plasma membrane. The separate determination of the alkaline *p*-nitrophenylphosphatase in the PM_1 and PM_2 fractions showed PM_1 to exhibit a 50% higher specific activity (Table II). In contrast, about 75% of the NADH-oxidoreductase lies in the dextran barrier and the pellet (Table I). The specific enzymatic activities in the case of isolated calf thymocyte membranes lie generally lower than in previously studied lymphocytes [7]. None of the fractions gave evidence of contamination by mitochondrial and/or lysosomal markers. Our gradient procedure thus appears to provide an adequate, if not perfect, separation of plasma membrane and endoplasmic reticulum and satisfactory elimination of other membranous organelles.

Extrinsic labelling of rabbit thymocytes by radioiodination

The data of extrinsic labelling are consistent with those obtained from intrinsic marker enzymes. The total recovery of covalently bound ^{125}I within the subcellular fractions was about 70% the remainder of the radioactive material apparently adsorbing to plastic and glass tubes. Of this recovered activity we found 18% in the nuclei + large granules, 30% in the soluble cytoplasmic proteins and 52% in the microsomal fraction, the last showed a 13-fold increase in specific activity in comparison

TABLE II

SUBFRACTIONATION OF CALF PLASMA MEMBRANES. ACTIVITY OF ALKALINE *p*-NITROPHENYLPHOSPHATASE

Data are given from 3 separate preparations. Specific activity is given per mg protein.

Fraction	Protein		Alkaline phosphatase* (*p*-nitrophenylphosphate)		
	Total (mg)	% distribution	Spec. act.	Total act.	% distribution
Microsomes	9.65	100.0	87.5	827.6	100.0
Fraction 1	0.52	5.4	24.2	12.6	1.5
Fraction 2					
PM_1	2.97	30.8	176.9	525.4	63.5
PM_2	1.87	19.4	107.3	200.7	24.2
Fraction 3	2.86	29.6	24.7	70.5	8.5
Fraction 4 (endoplasmic reticulum)	1.43	14.4	16.4	23.4	2.8

* nmoles *p*-nitrophenylphosphate liberated/min.

to that in the cell homogenate (Table III). After subfractionation of the microsomes in the dextran gradient, the PM_1 fraction exhibited the highest absolute and specific activity $12.5 \cdot 10^4$ cpm/mg protein; this represents a 140% increase in comparison to the specific activity of the PM_2 fraction and a 7-fold increase in comparison to that of endoplasmic reticulum (Table IV). A penetration of lactoperoxidase to a considerable amount during the labelling procedure, can be excluded since less than 0.3% of the radioactivity added as ^{125}I-labelled enzyme was found intracellularly.

RNA

The RNA remaining in the final microsomal fraction distributes almost fully into the pellet of the dextran fractionation, with only 7–9% in the plasma membrane isolates. Fig. 2 gives the distribution of RNA after sodium dodecylsulfate–polyacrylamide gel electrophoresis.

TABLE III

LACTOPEROXIDASE ^{125}I RADIOIODINATION OF INTACT RABBIT THYMOCYTES. DISTRIBUTION OF ^{125}I WITHIN THE SUBCELLULAR FRACTIONS

Data are obtained from 4 preparations. Microsomes are counted after having been freed from cytoplasmic proteins. Activity liberated by hypotonic shocks is added to the values obtained for the supernatant.

Subcellular fraction	Protein		cpm		Spec. act. (cpm/mg protein)
	Total (mg)	%	Total	%	
Homogenate	234.5	100.0	$9.2 \cdot 10^5$	100.0	$3.9 \cdot 10^3$
Nuclei+Large granules	91.3	38.9	$1.2 \cdot 10^5$	13.5	$1.4 \cdot 10^3$
Microsomes	6.4	2.7	$3.4 \cdot 10^5$	36.2	$5.2 \cdot 10^4$
Supernatant	121.5	51.8	$2.1 \cdot 10^5$	23.1	$1.7 \cdot 10^3$
Recovery	219.3	93.5	$6.7 \cdot 10^5$	72.9	

TABLE IV

LACTOPEROXIDASE ^{125}I RADIOIODINATION OF INTACT RABBIT THYMOCYTES. SUBFRACTIONATION OF MICROSOMES INTO PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM

Values are given from 4 preparations.

Fraction	Spec. act. (cpm/mg protein)
Microsomes	$5.2 \cdot 10^4$
Fraction 1	$7.4 \cdot 10^3$
Fraction 2	
PM_1	$12.5 \cdot 10^4$
PM_2	$5.4 \cdot 10^4$
Fraction 3	$2.3 \cdot 10^4$
Fraction 4 (endoplasmic reticulum)	$1.7 \cdot 10^4$

Phospholipids and cholesterol

Table V presents the phosphatide and cholesterol content of the various membrane fractions. Both lipids are markedly enriched in the plasma membrane fraction (822 nmoles phosphatide and 1032 nmoles cholesterol per mg protein, respectively). The cholesterol/phosphatide ratio near one appears typical for many plasma membrane isolates.

Summarizing, the two low-density fractions (PM_1 and PM_2) contain about 50% of the "microsomal" protein. Fraction 1, above the gradient, comprises soluble proteins only and Fraction 3, the dextran barrier, approaches the characteristics of the pellet, i.e. those of endoplasmic reticulum.

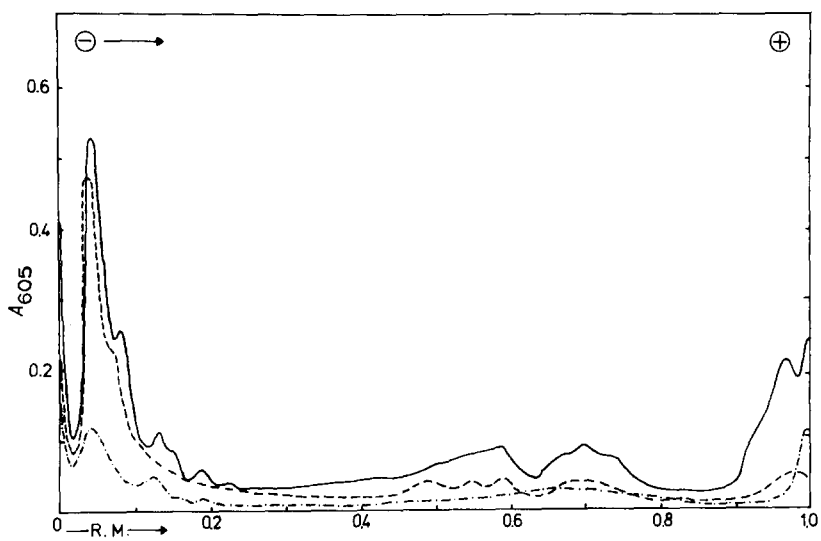
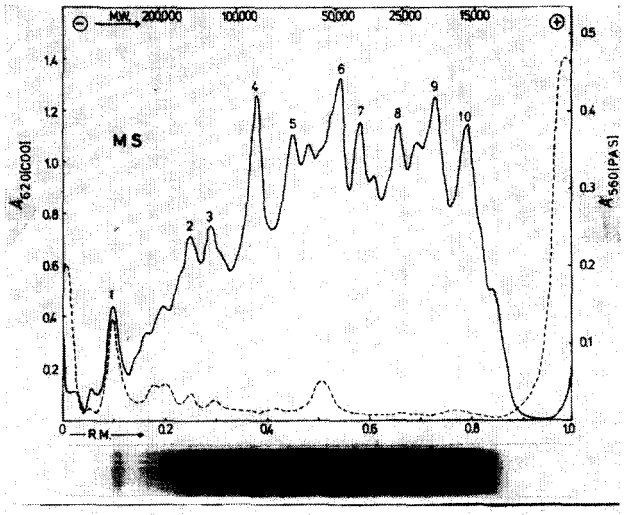


Fig. 2. Relative distribution of RNA in sodium dodecylsulfate–polyacrylamide gel electrophoresis, according to methylene blue staining. 40 μg protein was applied to each gel. The abscissa gives relative mobility (R.M.), the ordinate absorbance scanned at 605 nm. ———, Microsomes; –·–·–, plasma membrane; – – – –, endoplasmic reticulum, 7% polyacrylamide gel cross-linked with *N,N'*-methylene-bisacrylamide.

TABLE V

PHOSPHOLIPID AND CHOLESTEROL CONTENT OF THE FRACTIONS OBTAINED FROM THE DEXTRAN GRADIENT

Fraction	Protein		Phospholipid			Cholesterol		
	Total (mg)	%	nmoles/mg protein	Total (nmoles)	%	nmoles/mg protein	Total (nmoles)	%
1	0.98	10.1	393	385.1	7.3	580	568.4	7.1
2	5.15	53.0	822	4233.3	80.2	1032	5314.8	66.6
3	2.57	26.4	150	386.1	7.3	573	1478.9	18.5
4	1.02	10.4	272	276.1	5.2	615	624.2	7.8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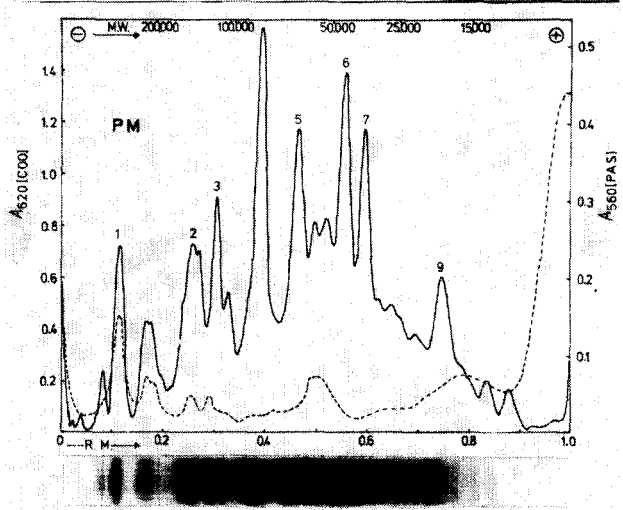
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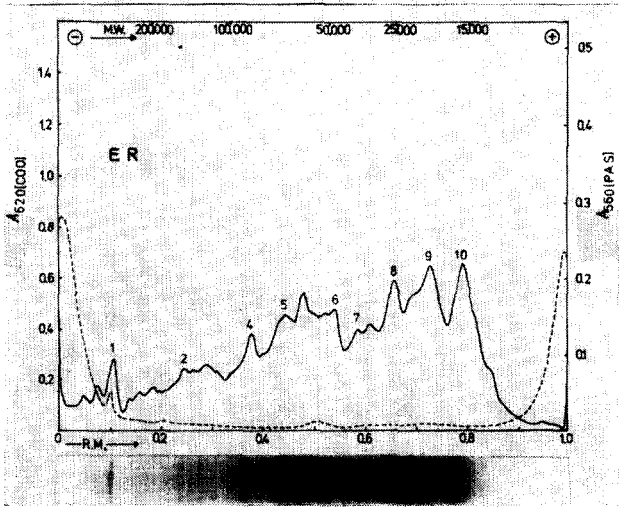
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Sodium dodecylsulfate–polyacrylamide gel electrophoresis of the gradient fractions

Fig. 3 depicts the electropherograms of microsomes, plasma membranes and endoplasmic reticulum derived from calf thymocytes; densitometric scans of the stained gels are presented concurrently.

TABLE VI

SODIUM DODECYLSULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS OF DIVERSE CALF THYMOCYTE MEMBRANE FRACTIONS

The apparent molecular weights were determined in acrylamide–*N,N'*-diallyltartardiamide gels.

Peptide	Apparent mol. wt	Percent of Coomassie blue staining in membrane fractions			
		MS	PM_1	PM_2	ER
1	280 000	2.9	4.5	3.5	2.9
1.1–1.3	210 000	4.6	5.5	4.5	3.2
2.1–2.3	155 000	5.7	8.0	7.5	5.0
3	120 000	3.9	5.0	3.7	3.1
3.1	108 000	2.2	3.2	2.0	1.6
4	79 000	10.6	15.0	11.0	6.9
4.1	66 000	—	—	1.9	1.2
5	60 000	8.3	10.5	7.0	7.2
5.1	55 000	4.2	4.5	4.5	5.8
6	44 500	9.4	10.4	6.5	4.1
7	36 000	6.4	8.0	7.0	5.0
7.1	32 000	2.8	1.9	2.3	4.0
8	26 500	8.3	3.5	7.5	8.9
8.1	23 500	4.6	1.6	3.4	5.5
9	21 000	9.1	7.0	7.0	8.9
10	17 000	10.4	1.4	8.0	12.1
10.1	15 000	3.3	—	—	3.4

The microsomal fraction exhibits numerous protein/peptide components, of which 10 predominate. Their apparent molecular weights range from 280000 to 15000 and we label them 1–10 according to decreasing molecular weights (Table VI). Many of these components include several less intensely stained bands, designated by subnumerals, e.g. 3.1. Component 2 (apparent molecular weight 155000) invariably comprises three closely spaced bands, designated 2.1, 2.2 and 2.3. After fractionation on the dextran gradient, we recover all sodium dodecylsulfate–polyacrylamide gel electrophoretic components in the various fractions, but observe

Fig. 3. Sodium dodecylsulfate–polyacrylamide gel electrophoresis of calf thymocyte membranes. Microsomes, upper panel; plasma membrane (PM_1), middle panel; endoplasmic reticulum, lower panel. Each panel presents a photograph of the electropherogram and the corresponding densitometric scans. The abscissa gives relative mobility, the ordinate absorbance at 620 nm for Coomassie blue (COO) (———) and 560 nm for periodate–Schiff reagent (PAS) (– – – –). Peptides are numbered 1–10 corresponding to decreasing molecular weight. 7% polyacrylamide gel cross-linked with *N,N'*-methylene-bisacrylamide.

major differences in the protein/peptide distributions of the various separated membrane classes.

The protein/peptide composition of PM_1 comprises almost exclusively the high molecular weight components 1–7; indeed, these make up 90% of the Coomassie staining of the PM_1 gels. In contrast, the endoplasmic reticulum fraction contains mainly protein/peptides of low molecular weight. PM_2 and Fraction 3 occupy intermediate positions.

After separation of ^{125}I-labelled microsomal membrane proteins by sodium dodecylsulfate–polyacrylamide gel electrophoresis, we found ^{125}I labelling selectively in the regions of peptide 1–5.1 of these components. The proteins 2.1–2.3, 4 and 5.1 exhibited the highest specific activities, suggesting that these glycoproteins are exposed to external membrane face in the case of intact thymocytes (Fig. 4).

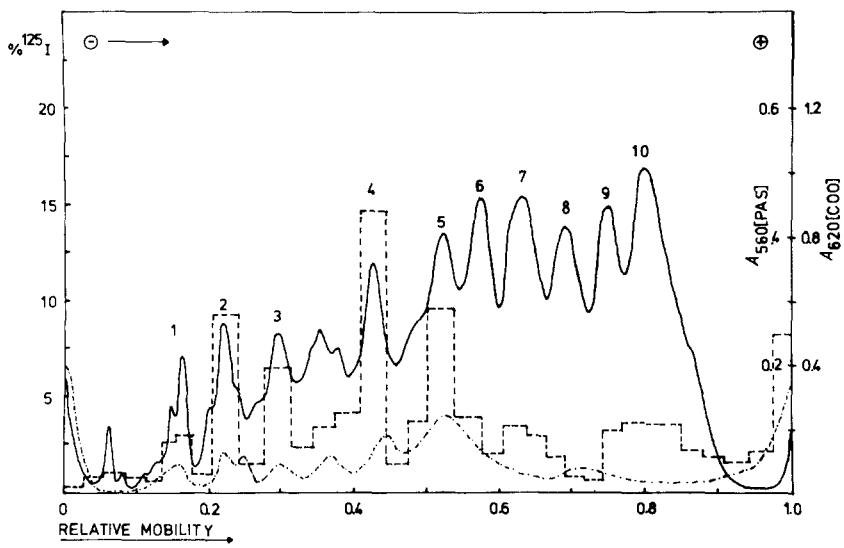

Fig. 4. Sodium dodecylsulfate–polyacrylamide gel electrophoresis of microsomes prepared from ^{125}I-labeled rabbit thymus lymphocytes. Coomassie blue (COO) (———) and periodate–Schiff (PAS) (–.–.–.–) protein pattern are shown in comparison to pattern of ^{125}I radioactivity (– – – –). The abscissa gives the relative mobility, the ordinate the absorption for Coomassie blue (620 nm) and periodate–Schiff reagent (560 nm), and the relative distribution of radioactivity in % ^{125}I, respectively. Proteins are numbered 1–10. 7% polyacrylamide gel cross-linked with *N,N'*-diallyltartardiamide.

Periodate–Schiff reagent staining occurs in the zone of Components 1, 2.1–2.3, 3 and 5.1 and in the case of rabbit thymocytes (Fig. 4) additionally in the zone of Component 4 and suggests a concentration of glycoproteins in the PM_1 membranes. The periodate–Schiff reagent staining at the electrophoresis front derives from the presence of glycolipids, plasmalogens and oxidation products of unsaturated fatty acids.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis of osmotically released membrane proteins

As shown in Table VII, treatment of microsomes with 0.01 M HEPES buffer (pH 7.5), preferentially releases Components 8–10 into a nonsedimentable state.

TABLE VII

PERCENTUAL DISTRIBUTION OF COOMASSIE BLUE STAINING IN SODIUM DODECYL SULPHATE–POLYACRYLAMIDE GEL ELECTROPHORESIS OF MATERIAL RELEASED BY HYPOOSMOTIC TREATMENT OF CALF MICROSOMES

Peptide	0.01 M *		0.001 M *	
	(150000) ** (%)	(400000) ** (%)	(150000) ** (%)	(400000) ** (%)
1	2.2	0.5	1.9	0.9
1.1–1.3	1.5	0.8	2.7	1.4
2.1–2.3	4.0	2.5	5.4	2.3
3	4.3	1.3	4.5	3.1
3.1	4.1	1.5	2.3	1.9
4	3.9	2.1	4.7	4.4
4.1	3.0	1.9	2.1	2.0
5	2.4	2.1	4.8	4.1
5.1	4.9	5.6	5.6	5.6
5.2		3.3	4.7	3.4
6	12.5	3.4	4.3	5.2
7	5.3	9.6	5.0	8.1
7.1	4.0	—	3.7	2.6
8	13.2	13.0	8.0	8.5
9	10.5	16.1	10.8	16.4
10, 10.1	16.5	27.8	20.5	22.8
10.2	2.0	7.3	1.5	4.8

* Extracted with 0.01 M or 0.001 M HEPES buffer (pH 7.5).
** After centrifugation at 150000 or 400000 $\times g$.

These correspond to 65% of the total protein liberated by the washing step. Components 1–7.1, which appear in traces in the wash supernatant, can be pelleted by a second centrifugation and thus presumably represent particulate material. Treatment with 0.001 M HEPES buffer (pH 7.5), also releases primarily low molecular weight material (Component 8–10), although under these conditions a small amount of high molecular weight material becomes nonsedimentable, even at 10^8 g·min. This could represent solubilisation of some membrane proteins.

Plasma membrane sodium dodecylsulfate–polyacrylamide gel electrophoresis of lymphoid cells from various organs and species

Fig. 5 depicts electropherograms of PM_1 and PM_2 fractions derived from calf and rabbit thymus, and calf mediastinal lymph nodes. Calf thymus and peripheral lymphocytes yield a very similar sodium dodecylsulfate–polyacrylamide gel electrophoretic pattern, but we observe clearly defined, qualitative and quantitative differences in the region of 2.1–2.3. Moreover, Component 6 (apparent mol. wt 42000) is quantitatively more prominent in lymph node cells. Rabbit and calf thymocytes yield generally similar patterns, but Component 1 appears at a lower apparent molecular weight (about 260000) in rabbit cells. Moreover, Component 5.1 appears unduly prominent here and, in opposition to calf cells, Component 6 predominates over Component 7.

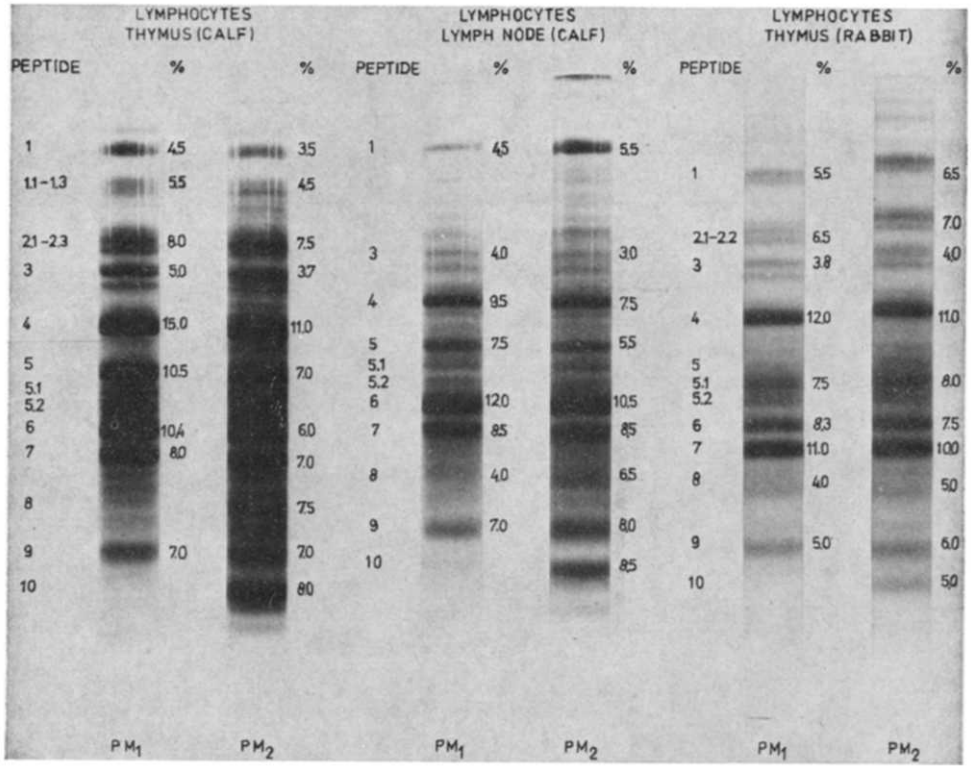

Fig. 5. Sodium dodecylsulfate–polyacrylamide gel electrophoresis of PM_1, PM_2 of calf thymocytes (left), calf mediastinal lymph node cells (middle) and rabbit thymocytes (right). On the left of each gel the peptides are identified numerically and on the right we present their percentual distribution (according to Coomassie blue staining). 7% polyacrylamide gel was cross-linked with methylene-bisacrylamide.

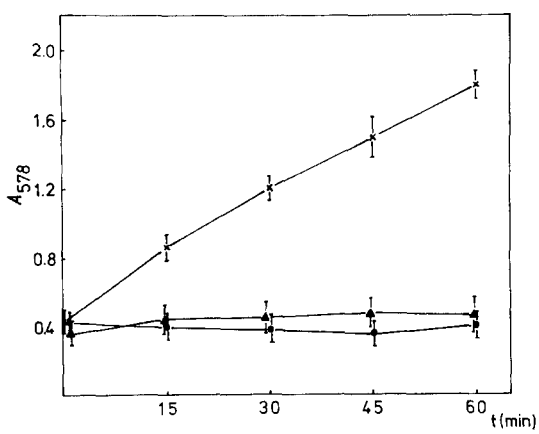

Fig. 6. Proteolytic activity of trypsin compared with a concentrate thymocyte homogenate freed of nuclei, mitochondria and lysosomes. Bovine serum albumin serves as substrate. Proteolysis is presented as a function of time. The low molecular weight material released by proteolysis and not precipitated by 10% trichloroacetic acid is measured by the ninhydrin method. The ordinate gives ninhydrin color (measured at 578 nm); the abscissa gives time in min. The high value in the case of zero proteolysis (absorption 0.4) is due to the hydrolysis of the proteins by the trichloroacetic acid precipitation. × – ×, trypsin (3 mg/ml); ●—●, control (trypsin, precipitated with trichloroacetic acid, before substrate addition); ▲—▲, concentrated cell extract (20 mg/ml).

Proteases

While trypsin clearly cleaves bovine serum albumin under our conditions, we could demonstrate no peptidase activity in our microsomes or soluble subcellular fractions (Fig. 6). Moreover, nitration effects no change in the sodium dodecylsulfate–polyacrylamide gel electrophoresis of the various membrane fractions. Finally, incubation of diverse fractions at 37 °C for 60 min did not yield sodium dodecylsulfate–polyacrylamide gel electrophoretic patterns significantly different from those obtained when all preparative procedures proceeded at 0–4 °C. Accordingly, we conclude that artifactual proteolysis does not interfere with our analyses.

DISCUSSION

Our data confirm the applicability of the lymphocyte membrane fractionation to thymocytes, developed by Ferber et al. [7]. Moreover, by applying sodium dodecylsulfate–polyacrylamide gel electrophoresis to the diverse membrane isolates and related fractions, we gain some insight into their relative protein composition. Thus, it appears that the low molecular weight peptides 8–10 represent membrane-bound cytoplasmic proteins. This assumption is supported by the peptide composition of the PM_1 fraction. This is the purest plasma membrane fraction according to its specific activity of intrinsic and extrinsic plasma membrane markers and lacks Peptides 8 and 10 almost completely. In contrast, Peptides 1–7 and a small amount of Component 9, of which 1, 1.1–1.3, 2, 3 and 5.1 in the case of calf and 1–4 and 5.1 in the case of rabbit, appear to comprise glycoproteins, all seem closely associated with the membrane "core".

In contrast to the conclusions of Marchalonis et al. [27] based on electron microscopic autoradiography, we do not find the ^{125}I activity exclusively connected with the plasma membrane of thymocytes, but also in other subcellular fractions (Table III). The considerable amount of ^{125}I covalently bound to cytoplasmic proteins and diverse subcellular particles cannot be explained by lactoperoxidase penetrating the plasma membrane, since prolongation of the reaction time does not essentially augment the amount of intracellular ^{125}I-labeled lactoperoxidase. We therefore suspect that some ^{125}I penetrates the plasma membrane and reacts with cytoplasmic proteins. This view is not incompatible with the data given in [27], since the grains in the radioautographs are thicker than the plasma membrane and some grains lie intracellularly. The contamination of cytoplasmic proteins with iodinated solubilized membrane proteins/peptides or with non-pelleting membrane microvesicles after high speed centrifugation ($400\,000 \times g$ for 120 min) can be excluded by electrophoretic separation of cytoplasmic proteins (Table VII) followed by radioactive analyses.

Our data fit those of Allan and Crumpton [28] to the extent that the plasma membranes of diverse species' thymic and peripheral lymphoid cells seem to contain very similar peptide/protein components. This fact is reminiscent of erythrocytes, whose membranes exhibit similar, if not identical patterns [29]. Apparently cells with diverse functions exhibit these differences in their plasma membrane protein composition. However, we cannot confirm the details presented by Allan and Crumpton [30]. We observe a different protein/peptide distribution and a greater

number of well-defined high molecular weight sodium dodecylsulfate–polyacrylamide gel electrophoretic components, whereas we find fewer low molecular weight proteins in our plasma membrane preparation. These differences are apparently due to the different membrane isolation methods used. The 5′-nucleotidase activity is found in the same order of magnitude in our preparations as in [30]. Moreover, we found a considerable amount of alkaline and acid *p*-nitrophenylphosphatase in our plasma membrane preparation (Tables I and II), which is consistent with the data given by van Blitterswijk et al. [31], who also used the "nitrogen cavitation method" for membrane isolation. Since Allan and Crumpton [30] found much lower specific activity for acid phosphatase in their plasma membranes, there may be functional differences in thymocyte plasma membranes of different species.

Our comparisons of sodium dodecylsulfate–polyacrylamide gel electrophoresis with and without 0.04 M dithiothreitol indicate no significant differences, i.e. disulfide bridges do not play a significant role in the protein organisation of the membranes.

Up to the present we have not been able to clearly identify membrane-associated immunoglobulins, since none of the membrane peptides we found between 200000 and 150000 were able to be split into heavy and light chains with the reduction of the disulfide bridges. Conceivably, the glycoprotein, 5.1 (apparent mol. wt. 55000), and the component, 8.1 or 9 (apparent mol. wt 23500 and 21000, respectively), could represent H- and L-chains, but our present methods do not allow us to arrive at a definite conclusion concerning this possibility. With 10^5 IgG molecules per cell [32] it should not be difficult to detect this protein, or its components by sodium dodecylsulfate–polyacrylamide gel electrophoresis [13]. It is conceivable, of course, that IgG is very loosely bound and lost prior to sedimentation of the "microsomal" fraction. Since we also did not find such molecules in the microsomal supernatant containing soluble cytoplasmic proteins or the supernatants after hypotonic shocks, another explanation might be that we mainly investigated a population of immature thymocytes, as suggested by Raff and Cantor [33].

Having developed the technique necessary for adequate analysis of lymphoid cell plasma membranes by sodium dodecylsulfate–polyacrylamide gel electrophoresis, we are now in a position to isolate the major components on a preparative scale and to analyze the consequences of lymphoid cell stimulation by diverse mitogens including specific antigens. Such studies are in progress and are partly described in [34].

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