ISOLATION OF PLASMA AND NUCLEAR MEMBRANES OF THYMOCYTES

II. Biochemical Composition

ARIANE MONNERON and JACQUES d'ALAYER

From the Département de Biologie Moléculaire, Institut Pasteur, 25, Rue du Docteur Roux, 75724 Paris Cedex 15, France

ABSTRACT

Thymocyte plasma and nuclear membranes obtained by the procedure described in the accompanying paper were analyzed for their biochemical composition. Plasma membranes were very rich in phospholipid, cholesterol, sialic acid; they did not contain nucleic acids. In comparison, nuclear membranes had a lower phospholipid to protein ratio and contained much less sialic acid and cholesterol. 50% of the cellular cholesterol and of the membrane-bound sialic acid were found in the plasma membranes, 14% in the nuclear membranes. Live cells were labeled with ^{131}I, and the acid-insoluble radioactivity was followed in the subfractions. A good correlation with the distribution and enrichment of plasma membrane marker-enzymes was obtained. Label enrichment was about 50-fold in the two lightest of the three plasma membrane fractions. 60% of the label was contained in the plasma membranes, only 4% in the nuclear membranes. Cross-contamination of these two types of membranes was thus negligible. Sodium dodecyl sulfate-gel electrophoresis revealed three different patterns specific for, respectively, plasma membranes, the microsomal-mitochondrial fraction, and nuclear membranes. Each pattern was characterized by a set of proteins and glycoproteins, among which high molecular weight glycoproteins could be considered as marker-proteins of, respectively, 280,000, 260,000, and 230,000 daltons. ^{131}I-labeling of live cells tagged with a very high specific activity three glycoproteins of mol wt 280,000, 200,000, and 135,000 daltons. Nuclear membranes prepared from labeled isolated nuclei had a set of labeled proteins completely different from plasma membranes.

KEY WORDS lymphocyte · plasma membrane · nuclear membrane · biochemical composition · glycoproteins · radioiodination

In the preceding paper, we described a procedure for thymocyte subfractionation, yielding within a short time and with a high recovery, plasma membranes on the one hand, and nuclear membranes on the other (40). On the basis of ultrastructural and enzymatic studies, these membranes appeared to be well separated from mitochondria, microsomes, and other organelles. The biochemical characterization of the subcellular fractions is reported in this paper. The distribution

 J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/78/0401-232 $1.00

pared with that of marker-enzymes. Radioactive iodine labeling of live cells was performed, and the labeled proteins were followed in the subcellular fractions in order to trace the components of the cell surface. Finally, the electrophoretic patterns of the proteins of the different types of membranes were studied, and it was possible to assign to each membrane fraction one or several marker proteins.

MATERIALS AND METHODS

Cellular and Subcellular Fractionation

The procedures applied for cellular and subcellular fractionation were described in the accompanying paper (40).

Analytical Procedures

Protein determinations were performed according to Lowry et al. (33). Lipids were extracted from the preparations with 20 vol of chloroform-methanol 2:1 (wt/vol) and purified according to Folch et al. (18). The lipids were solubilized in chloroform and analyzed for cholesterol content according to Kates (29), and for phospholipid content according to Ames and Dubin (4). Phospholipid phosphorus was converted to phospholipid (PLP) by using a multiplication factor of 25.

Sialic acid was measured according to Svennerholm (50) and Warren (55) with some modifications. The samples were hydrolyzed in 0.1 N HCl for 1 h at 80°C, neutralized, mixed with 10–15 vol of ice-cold ethanol, and kept at −20°C for at least 2 days. By this procedure, the bulk of nucleic acids and some other contaminants were precipitated, whereas sialic acid was not. The alcoholic supernates were then applied to 3 g wet AG 2-X8 Biorad resin columns (Bio-Rad Laboratories, Richmond, Calif.), and eluted with 0.6 N formic acid. The eluates were lyophilized and subjected to the Warren procedure. The spectrum of the developed color was identical, in all membrane fractions, to that given by synthetic *N*-acetyl-neuraminic acid (Sigma Chemical Co., St. Louis, Mo.). The yield of the method was 75%. In the fractions containing large amounts of nucleic acids (homogenate, nuclei), sialic acid could not be safely estimated, due to some persisting traces of nucleic acids. Therefore, enrichment and yield of sialic acid were given (*a*) as compared to the homogenate (uncorrected values), and (*b*) as compared to the sum of the sialic acid recovered in fractions 1 to 4 and in nuclear membranes (corrected values).

RNA was determined according to Mejbaum (35), as modified by Moulé (41). DNA was measured by the method of Burton and Peterson (8).

Sodium Dodecyl Sulfate Acrylamide Gel Electrophoresis

Slab gels, accommodating 12 samples, were made according to Laemmli (31) and Neville (42). Acrylamide linear gradients were from 6 to 12%. A 3% stacking gel was used. Acetone-delipidated samples (30 min at −20°C) and nondelipidated samples were compared. Dissolving buffers were, in the Laemmli system: Tris-HCl pH 8.9, 300 mM, EDTA 1%, sodium dodecyl sulfate (SDS) 1%, β-mercaptoethanol 1%; in the Neville system: sodium carbonate 50 mM, SDS 1%, β-mercaptoethanol 1%. The samples were boiled for 2–5 min and mixed with bromophenol blue and sucrose (final concentration 20%). A constant current of either 15 or 30 mA was applied. Gels were either fixed with methanol (50%) acetic acid (7.5%) and stained with Coomassie Blue, or processed according to Fairbanks et al. (16) for periodic acid-Schiff (PAS) staining of the glycoproteins. Gels submitted to PAS staining could be stained in a second step by Coomassie Blue, provided they were carefully rinsed (0.2% sodium metabisulfite, 1 or 2 days; 7.5% acetic acid, several hours). Some gels were processed according to Kelly and Cotman (30), in order to visualize mannose-containing glycoproteins by the sequential application of concanavalin A, horseradish peroxidase, and 3,3′-diaminobenzidine. Nonglycoprotein marker proteins were not stained. The gels were scanned in a Vernon gel scanner, model PHI-5 (Paris).

Radioiodination of Cells and of Nuclei

Freshly dissociated cells were washed several times in cold Hanks' medium and suspended at a concentration of 1.5×10^8 cells/ml. 6 ml of this suspension were layered over a cushion of 8 ml of a metrizoate sodium-Ficoll solution (Lymphoprep, Nyegaard, Oslo), and centrifuged at 15°C for 10 min at 400 *g*. The cellular layer obtained at the interface was diluted with Hanks' solution containing 20 mM glucose. Damaged cells (trypan blue positive) accounted for 0.5–1% of the cells at most.

In parallel, lymphocyte nuclei were prepared according to Blobel and Potter (6), using a 73% sucrose cushion, and resuspended in TKM buffer containing 20 mM glucose, at the same concentration as cells. Cells and nuclei, at a final concentration of 7×10^6/ml, were incubated at 4°C for 20 min with lactoperoxidase (Sigma Chemical Co., type V, 4–40 μg/ml), glucose oxidase (Sigma Chemical Co., 0.5 U/ml) and carrier-free $Na^{131}I$, 40 mCi/ml (The Radiochemical Centre, Amersham, England) according to Hubbard and Cohn (25). At the end of the iodination step, the dead cell count did not exceed 1%. The cells and the nuclei were then centrifuged through fetal calf serum containing 50 mM Na I, to wash them free from the radioactive iodination medium. They were further rinsed four to five times in 50 ml of Hanks' solution (cells) or TKM (nuclei) containing 50 mM NaI. The cells and nuclei were then mixed with twice their number of unlabeled cells or nuclei, and fractionated as previously described (40), the 35% sucrose layer being omitted so as to get fractions 1 and 2 combined in one fraction called P. Throughout the fractionation steps, acid-insoluble radioactivity was mea-

sured by the "disk batch" method (25). Counting was performed in an Intertechnique Gamma spectrophotometer CG 30 (Paris, France). Cells, nuclei, and fractions were delipidated, dissolved in SDS-containing buffer, and subjected to electrophoresis in Neville-type gels. The gels were stained, dried on a drying plate, and autoradiographed (Kodirex film, Kodak, Paris, France). Exposure times were from 12 h to 5 days.

Materials

Chemicals were obtained from the same sources as previously indicated (40). Acrylamide and bis-acrylamide were obtained from Koch-Light Lab. Ltd, England. Molecular weight markers were pig thyroglobulin (mol wt 320,000), calf myosin (mol wt 200,000), β-galactosidase (mol wt 135,000), β-phosphorylase (mol wt 94,000), kindly provided by J. Torresani, R. Whalen, A. Ullmann, and M. Morange. Other molecular weight marker proteins were obtained from Boehringer, Mannheim, Germany. Fetal calf serum was obtained from Flow Laboratories, Scotland, and carrier-free $Na^{131}I$ from the Radiochemical Center, Amersham, England.

RESULTS

As shown in the preceding paper (40), a first gradient yielded directly from the homogenate the bulk of plasma membranes in the floated fractions 1 to 3. Plasma membranes were most enriched in the two lightest fractions 1 and 2 (P), but some Golgi vesicles co-purified with them. Two other fractions, E and 4, composed mainly of microsomes, ribosomes, and mitochondria, were obtained from the same gradient, as well as a pellet of pure nuclei. From these DNase-treated nuclei, nuclear membranes were floated in a second gradient, yielding a light fraction, called LNM, and a denser fraction, HNM.

Composition of the Fractions

Tables I to IV give the composition in proteins, PLP, cholesterol, sialic acid, and nucleic acids of the subcellular fractions, and the distribution of such components among them.

PLP represented more than 50% of the dry weight of fractions 1 and 2, which contained 3.6 and 2 μmol PLP/mg protein (Tables I and II). Cholesterol accounted for 12% of the dry weight of fractions 1 and 2, amounting to 1.2 and 0.9 μmol/mg protein (Tables I and II). PLP was enriched 36-fold, and cholesterol 42-fold, in these two fractions (Table IV). Fractions 1 and 2 contained, respectively, 26 and 18 μg of sialic acid/mg protein, which corresponded to a 56-fold average enrichment (Tables II and IV). Fractions 1 and 2 had an exceedingly low RNA content, and no DNA (Tables I and II).

By comparison, fraction 3 had a lower phospholipid to protein ratio, 0.65 as compared to 1.86 in combined fractions 1 and 2. However, the cholesterol to phospholipid ratio slightly increased in fraction 3 (Table II). As compared to fractions 1 and 2, the sialic acid content was lower, 8 μg/mg protein, whereas the RNA content was higher (106 μg/mg protein) (Table II).

The plasma membrane fraction (1 to 3) thus contained 40% of the total PLP, and close to 50%

TABLE I

Composition of Thymocyte Subcellular Fractions, as a Percentage of Dry Mass

	Protein	PLP	Cholesterol	Sialic acid (A)*	RNA	DNA
Homogenate	75.2	3.9	0.7	0.06	1.5	18.6
Plasma membranes						
1	23.4	64.7	11.2	0.6	0.1	0
2	33.8	53.0	12.2	0.6	0.4	0
3	51.9	33.7	8.3	0.4	5.5	0.06
Fractions E + 4	68.9	19.3	3	0.2	8.1	0.6
Nuclei	76.5	3.5	1.5	Not measurable	0.54	18.2
Nuclear membranes						
LNM	41.2	52	5	0.2	1.5	0
HNM	73.8	21.3	1.5	0.2	3.0	0.04

The values given are means of at least six separate experiments. The SEM is about ± 10% of each value, except for nuclei for which data are more scattered (±25% for PLP and cholesterol).

* See Materials and Methods (A, uncorrected values).

of the total cholesterol and sialic acid (Table III).

The composition of the nuclear fraction appears in Table I. DNA amounted to 18.2% in dry weight of this fraction (84% of the homogenate DNA was recovered in the gradient, 83.7% in the nuclear fraction). The PLP and cholesterol content of nuclei was totally recovered in the nuclear membranes floated from the DNase-treated nuclei. As compared to plasma membranes, nuclear membranes had a lower phospholipid to protein ratio (0.88), a much lower cholesterol to phospholipid molar ratio (0.19 in LNM and 0.14 in HNM, Table II), and a relatively low sialic acid content, 3–5 μg/mg protein (Table II). No free sialic acid could be detected in nuclei. Nuclear membranes contained about 14% of the total cholesterol and membrane-bound sialic acid, as compared to about 50% in plasma membranes (Table III).

Fractions E and 4, studied for comparison and shown by electron microscopy to contain ribosomes, fibrils, tubules, as well as rather dense membranous organelles, had a lipid to protein ratio and a sialic acid to protein ratio much lower than those of the floated fractions. Fraction 4 contained the highest percentage of RNA.

SDS Gel Electrophoresis of the Fractions

To allow for careful comparison of apparent molecular weights, slab gradient gels were prepared in two different systems, according to Laemmli (31) or Neville (42). All molecular weights given in daltons, were determined by following the migration of proteins with a known molecular weight up to 320,000. No correction was made

TABLE II

Composition of Thymocyte Plasma and Nuclear Membranes*

	PLP		Cholesterol		Cholesterol to PLP	Sialic acid	RNA	DNA
	(mg)	*(μmol)*	*(mg)*	*(μmol)*	*(ratio)*	*(mg)*	*(mg)*	*(mg)*
Plasma membranes								
1	2.76	3.6	0.48	1.24	0.34	0.026	0.004	0
2	1.57	2.0	0.36	0.93	0.46	0.018	0.012	0
3	0.65	0.84	0.16	0.41	0.49	0.008	0.106	0.001
Nuclear membranes								
LNM	1.26	1.63	0.12	0.31	0.19	0.005	0.036	0
HNM	0.29	0.37	0.02	0.05	0.14	0.003	0.041	0.001

* mg or μmol/mg protein.

TABLE III

Subcellular Distribution of Main-Membrane Components and of Acid-Insoluble ^{131}I*

	Proteins		PLP		Cholesterol		Sialic acid‡ A		Sialic acid‡ B		^{131}I	
Plasma membranes												
1	0.2		10.6		10.3		5.4		10.5		38	
2	0.61	1.64	18.8	39.8	23.8	48.4	12.3	25.3	23.8	49		60.1
3	0.83		10.4		14.3		7.6		14.7		22.1	
Fractions E + 4	6.45		34.8		30.3		19.0		36.8		34.5	
Nuclear membranes												
LNM	1		24.3		13		4.7		9.1			
HNM	0.64	1.64	3.5	27.8	1.4	14.4	2.6	7.3	5	14.1		4.1
Recovery§	9.7		103		93		52		100			98.7

* % of the homogenate.

‡ See Materials and Methods (A, uncorrected values; B, corrected values).

§ Recovery in fractions 1 to 4 and in nuclear membranes.

TABLE IV

*Relative Enrichments Over Homogenate of Plasma Thymocyte Membrane Components in Plasma and Nuclear Membrane Fractions**

	PLP	Cholesterol	Sialic acid‡ A	Sialic acid‡ B	Alkaline phosphatase	Ouabain-sensitive ATPase	γ-glutamyl transferase	Acid-insoluble ^{131}I
Plasma membranes								
1 + 2	36	42	29	56	72	45	47.3	47
3	12.5	17.2	10.7	20.5	23.5	28	23.5	27
Nuclear membranes	17	8.8	8.6	16.5	6.6	3.2	0.5	Not measurable

* Homogenate values taken for 1.

‡ See Materials and Methods (A, uncorrected values; B, corrected values).

of lipids, sialic acid, and nucleic acids was comfor the estimation of the molecular weight of glycoproteins.

Three different and reproducible patterns emerged, one characteristic for plasma membranes, (especially fractions 1 and 2), one for the microsomal-mitochondrial fractions E and 4, and one for nuclear membranes. They are shown in Figs. 1-3.

Plasma membranes (1 and 2) displayed at least 35 Coomassie Blue stained bands. Band A of mol wt 280,000, band C of mol wt 68,000-70,000, band D of mol wt 45,000, and several lower molecular weight bands were prominent. Rather diffuse but characteristic bands were located around mol wt 200,000 and 135,000 (Figs. 1 and 2). These two regions, as well as band A, were Schiff positive. Concanavalin A binding allowed the detection of many more glycoproteins than the Schiff procedure, including band C (Fig. 3). Densitometric profiles of glycoproteins stained by both methods were not comparable.

Microsomes and mitochondria were present mainly in fractions E and 4 (40). Highly enriched mitochondrial and microsomal fractions were prepared from the homogenate (24) and analyzed in parallel in gels (not shown). As expected, they proved to be quite different from one another, but fractions E and 4 clearly displayed the two sets of proteins. Their gel electrophoresis pattern was characterized by major bands of mol wt 230,000 (band B), 50,000-52,000, and 45,000 (Figs. 1 and 2). Concanavalin A only weakly stained these major bands, but sharply contrasted bands at mol wt 200,000, 130,000-150,000, and 68,000-70,000 (Fig. 3). The Schiff procedure was not sensitive enough to detect bands in these fractions. Fractions E and 4 completely lacked band A, a

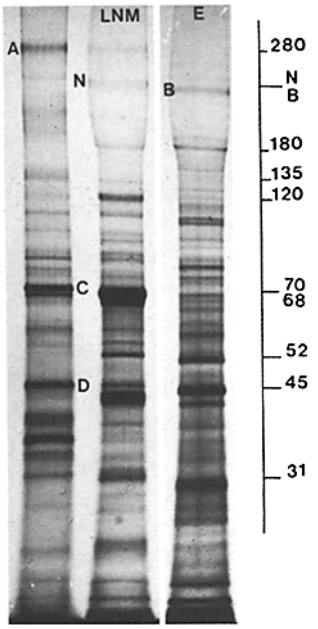

FIGURE 1 SDS gel electrophoresis, Laemmli-system, acrylamide gradient 6-12%, Coomassie Blue staining. From left to right: fraction 1 (lightest plasma membrane fraction), light nuclear membranes, fraction E. Scale, mol wt × 10^{-3}.

major plasma membrane component. Fraction 3 had a pattern intermediate between those of fractions 1-2 and of fractions E-4, displaying, for instance, both bands A and B.

Nuclear membranes had again a very different pattern (Figs. 1 and 2). Major bands were found at mol wt 260,000 (band N), 120,000, 68,000-70,000, 52,000, and 42,000. The region from 68,000 to 70,000 daltons clearly contained many components (Fig. 1). However, the bands of mol wt 260,000 68,000-70,000, and 42,000 de-

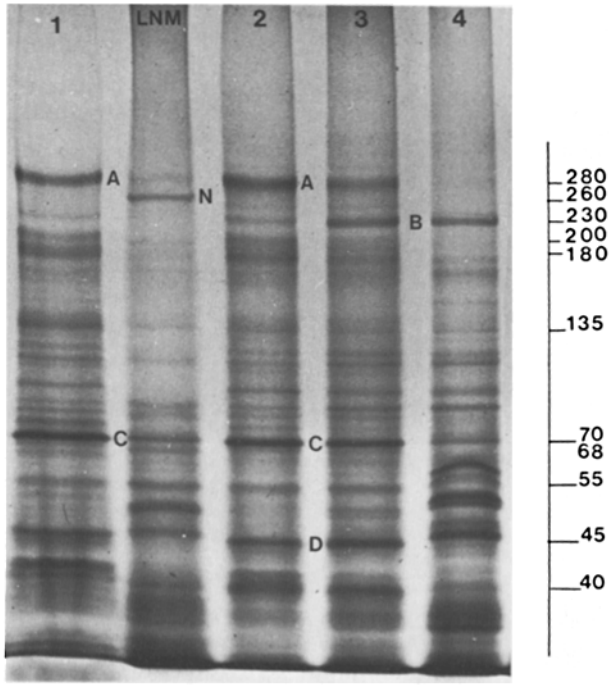

FIGURE 2 SDS gel electrophoresis, Neville system, acrylamide gradients 6–12%, Coomassie Blue staining. From left to right: fraction 1, light nuclear membranes, fractions 2, 3, 4. Scale, mol wt × 10^{-3}.

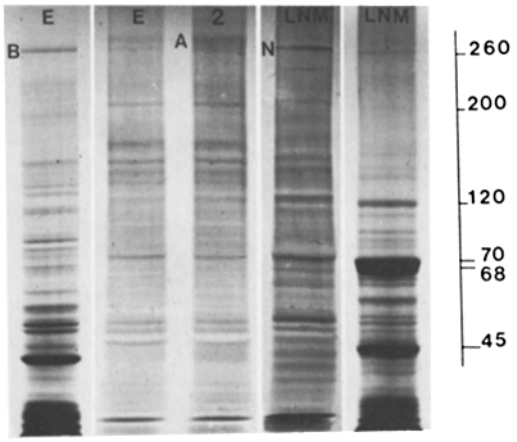

FIGURE 3 SDS gel electrophoresis, Laemmli system, acrylamide gradient 7–12%. Two identical gels were run in parallel, using the same power supply. One was stained for proteins with Coomassie Blue, one was processed according to Kelly and Cotman for glycoproteins (concanavalin A binding) (30). From left to right: fraction E, Coomassie Blue; fraction E, glycoprotein stain; fraction 2, glycoprotein stain; light nuclear membranes, glycoprotein stain; light nuclear membranes, Coomassie Blue stain. Scale, mol wt × 10^{-3}.

creased in intensity when DNase treatment was lengthened or took place at room temperature (Fig. 2). Band N was intensely stained by both the Schiff procedure and concanavalin A binding, this last technique revealing additional glycoproteins at mol wt 120,000, 68,000–70,000, and 52,000. Nuclear membranes lacked band B, a major component of the microsomal-mitochondrial fraction, and inconstantly contained a faint band A.

Each of the gel patterns described was reproducible, from the point of view of both their set of components and the relative proportions of these components. In particular, the high molecular weight glycoproteins A, B, and N, well separated on Neville-type gels, were characteristic for each fraction. Controls were performed to assess the validity of the estimated molecular weight. The addition, throughout the subcellular fractionation, of 0.1 mM phenylmethylsulfonylfluoride, a drug which inhibits proteases (chymotrypsin, trypsin), did not change the electrophoretic pattern. Fractions 1, 2, and 4 were subjected to DNase I and high ionic strength under the same conditions as the nuclear membranes. Although densitometry revealed the fading of some bands, such treat-

ment did not modify the apparent migration of the components. Electrophoresis of fraction I mixed with LNM was performed; the pattern obtained resulted from the superposition of the two sets of bands, but there was no shift in the apparent molecular weight of any constituent. Finally non-delipidated samples provided protein gel electrophoresis patterns similar to those of delipidated samples, indicating that proteins above mol wt 30,000 were not dissolved in the acetone phase.

[^{131}I]*Iodine Labeling of Cells and Nuclei*

Live cells were iodinated by the lactoperoxidase method, in order to identify membrane components located at the cell surface (25). Isolated nuclei were iodinated in parallel, to compare labeled nuclear membranes with labeled plasma membranes. Such an analysis was completed by the estimation of intracellular labeling performed throughout the fractionation steps.

One of the most critical conditions to insure a predominant cell surface labeling was the integrity of the cells. This appears clearly in Table V, which shows the distribution of acid-insoluble iodine in the subcellular fractions of two separate experiments, one performed on a cell population comprising 4–5% dead cells (experiment 1), the other performed on a cell population with only 0.5% dead cells (experiment 2). 60% of the acid-insoluble radioactivity was present in the plasma membrane fractions in experiment 2, 28% in the plasma membrane fractions in experiment 1. In the case of live cells, the specific radioactivity of fraction P was about 50 times higher than that homogenate. Still, about 30–35% of the acid-insoluble label was found in the microsomal-mitochondrial fraction, and 5% in nuclei. When isolated nuclei were labeled, 80% of the acid-insoluble label was recovered in the nuclear membrane fractions.

Autoradiographs of fractions from cells labeled in vivo from two separate experiments are shown in Figs. 4 and 5. As the specific activity of the fractions decreased dramatically from the surface towards the inside of the cell, the protein load had to be modulated to obtain comparable autoradiographs for all fractions on one slab gel. (10 times more proteins for fraction 4 than for plasma membranes, at least 200 times more for nuclear membranes.)

Fig. 4 was derived from an experiment in which labeling was performed at 20°C in the presence of lactoperoxidase, 4 μg/ml. The homogenate electrophoretic pattern showed two major bands at mol wt 45,000 (band D) and 31,000, and additional bands especially in the high molecular weight region, corresponding to bands seen in the P fraction. The plasma membrane fractions (P) displayed three highly radioactive bands in the high molecular weight region, of mol wt 280,000 (band A), 200,000, and 135,000. Aside from these major components, four other bands of mol wt 165,000, 90,000, 45,000, and 31,000 were strongly labeled, and several others were less radioactive. The same bands were seen in fraction E, although the specific radioactivity of each band was much lower, and especially so for the 280,000 mol wt band. In fraction 4, all the high molecular weight bands characteristic of the plasma membranes were no longer seen (except when the gel

TABLE V

Acid-Insoluble [^{131}I]Iodine: Distribution and Specific Activity‡ in Subcellular Fractions*

	Experiment 1			Experiment 2		
	Yield		Specific activity	Yield		Specific activity
	(%)			(%)		
Plasma membranes						
1 + 2	9.4	27.2	11.6	38	60	47.3
3	17.8		21.4	22.1		26.6
Fraction E	22.4	39.6	9.8	13.8	34.5	5.3
Fraction 4	14.2		3.9	20.7		5.4
Nuclei	33		0.54	5.1		0.08

In experiment 1, 4–5% of the iodinated cells were trypan blue positive, in experiment 2, only 0.5%.

* Expressed as a percentage of acid-insoluble [^{131}I]iodine in the homogenate (100%).

‡ Expressed by taking the homogenate specific activity as 1.

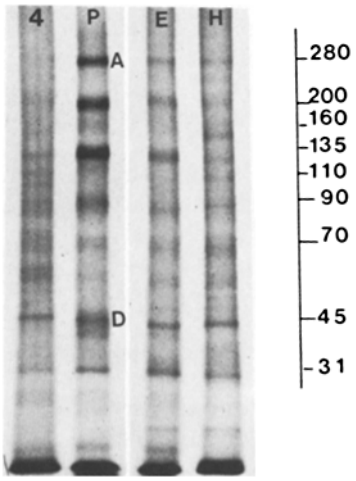

FIGURE 4 Autoradiograph of a gel containing fractions from ^{131}I-labeled cells. (99% live cells). Cells were labeled at 20°C. Neville system, acrylamide gradient 6–12%. From left to right: fraction 4, fraction P (1 + 2), fraction E, homogenate. Scale, mol wt × 10^{-3}.

was loaded with high amounts of proteins); band D was present, as well as several bands of low specific activity located between mol wt 135,000 and 50,000.

Fig. 5 was taken from an experiment in which labeling was performed at 4°C in the presence of lactoperoxidase, 40 μg/ml. An aliquot of cells, washed three times after labeling, was subjected to electrophoresis. The pattern obtained was comparable to that of a homogenate of cells labeled at 20°C, except for an additional, strongly radioactive doublet corresponding to the iodinated lactoperoxidase, seemingly tightly bound to the cells. As to the subcellular fractions, the patterns were almost identical to those obtained from cells labeled at room temperature. The three major labeled protein species in plasma membranes (mol wt 280,000, 200,000, and 135,000) were seen in more inner fractions with sharply decreasing intensities, band A disappearing almost completely in fractions 4 and LNM.

Fig. 6 shows autoradiographs from labeled plasma membranes and from nuclear membranes derived from isolated, labeled nuclei (I-NM on Fig. 6). The patterns were totally different. The I-NM fraction was characterized by one very radioactive band of mol wt 68,000–70,000. Additional labeled bands were of mol wt 260,000 (band N), 120,000, 110,000, 85,000, 55,000, and 45,000.

It is thus clear that when live thymocytes were labeled, the SDS gel electrophoresis migration patterns obtained from all intracellular fractions resembled the plasma membrane fraction (P) pattern, indicating a low but detectable contamination of inner fractions by plasma membranes.

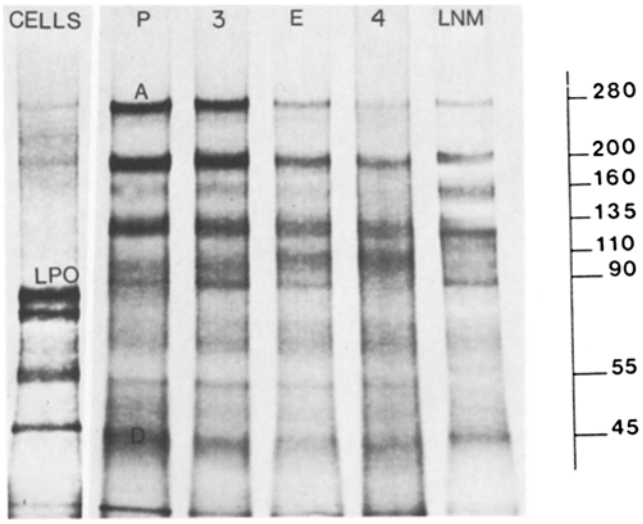

FIGURE 5 Autoradiograph of a gel containing fractions from ^{131}I-labeled cells (99% live cells). Cells were labeled at 4°C, in the presence of 40 μg/ml lactoperoxidase. Neville system, acrylamide gradient 6–12%. From left to right: cells (LPO, lactoperoxidase), fractions P and 3 (plasma membranes), fraction E, fraction 4, light nuclear membranes. Scale, mol wt × 10^{-3}.

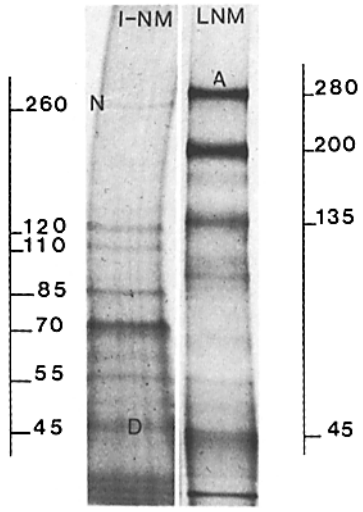

FIGURE 6 Autoradiograph of a gel containing, on the left, light nuclear membrane isolated from ^{131}I-labeled nuclei, on the right, plasma membranes isolated from ^{131}I-labeled cells (labeling at 4°C). Scales, mol wt × 10^{-3}.

However, some intracellular labeling had also occurred, as evidenced by a diffuse labeling seen in autoradiographs from cells, homogenate, and fraction 4. The specific activity of each of the composing bands was generally very low, except for bands of mol wt 160,000 in nuclear membranes, of mol wt 110,000 in fractions E and 4. This indicates that, aside from these two exceptions, the intracellular label was diluted in a very large pool of proteins and represented a rather low background.

On the contrary, electrophoretic patterns of fractions obtained from samples containing 4% (or more) dead cells were very different from those described in the case of live cells. In particular, the high molecular weight species characteristic for plasma membranes were much less labeled than other proteins of generally lower molecular weights, which were not seen in autoradiographs from samples containing less than 1% dead cells. The importance of the intracellular labeling in such samples precluded any conclusion concerning the subcellular localization of the labeled proteins.

DISCUSSION

In the preceding paper, a simple method was described for preparing large quantities of highly purified thymocyte plasma and nuclear membranes (40). Confidence in the assessment of purity and yield was obtained by the convergence of several independent marker-enzyme measurements. The chemical analysis of such membranes was thus warranted.

Among the various membrane types found in eukaryotic cells (14, 23), and in lymphocytes in particular (2, 3, 11, 13, 17, 34, 37, 53), plasma membranes have generally the highest PLP to protein ratio, as well as the highest cholesterol to PLP ratio. Golgi membranes, considered to be plasma membrane precursors to a certain degree, are also rich in PLP and cholesterol. Of all other fractions, thymocyte fractions 1 to 3, containing the bulk of plasma membranes as well as some Golgi vesicles (40), had indeed a higher content of PLP and cholesterol than all other fractions (Tables I to IV). If cholesterol imparts rigidity to membranes, as it is usually assumed, then our finding of a relatively low cholesterol to PLP molar ratio would reflect the great plasticity characteristic of T-cell plasma membranes. The plasma membrane fractions were also, by far, the richest in sialic acid. They contained 50% of the membrane-bound sialic acid (Table III). Values of 5% (44) and 3.4% (53) were reported with other methods. Our plasma membrane fractions were totally devoid of DNA, a result comparable to that of Melera et al. (36). Three fractions enriched in plasma membranes were obtained. The PLP to protein ratio decreased from fraction 1 to fraction 3, as could be expected from their buoyant densities. This was probably explained by an increasing contamination of plasma membrane vesicles by intracellular proteins in fractions of increasing buoyant density (40). However, the cholesterol to PLP ratio slighty increased from fraction 1 to fraction 3, indicating that, besides the varying degrees of plasma membrane purity in the three fractions, some heterogeneity occurred within the plasma membrane vesicles themselves. It may be that disruption of thymocytes leads to segregation of plasma membrane domains which are not strictly equivalent on the basis of their composition. A similar phenomenon has been described in the case of parenchymal cells possessing a defined polarity (57).

When compared to the plasma membranes, the nuclear membranes of thymocytes had a much lower PLP to protein ratio, a common finding in nuclear membranes from various other

cell types (28, 58). However, at least for LNM, the PLP to protein ratio was much higher than previously reported for lymphocyte nuclear membranes (26, 46). Sialic acid was definitely present in the nuclear membranes, but only a small quantity (Tables I to IV). Sialic acid had previously been found in liver nuclei (7, 28), and we have presented ultrastructural evidence for the presence of glycoproteins on the cisternal faces of both nuclear membranes of thymocytes (38). Our method yielded nuclear membranes totally stripped of DNA. Tightly bound RNA was still present, however, although the membranes had been subjected to high ionic strength.

Of more practical interest is the demonstration that each type of membrane was characterized by a special SDS gel electrophoresis pattern, and had specific markers, essentially in the high molecular weight domains. In trying to compare our SDS electrophoresis analysis with that of others (9, 10, 13, 32, 47, 49, 52, 54), we sometimes encountered difficulties due to discrepancies in apparent molecular weight estimations, especially in the high range. However, certain assumptions being made, the pattern that we obtained had some features in common with some of the published analyses (9, 10, 32, 47, 49, 54). Our plasma membranes were characterized by a number of high molecular weight glycoproteins. The largest one, band A, of an estimated mol wt of 280,000, was certainly very rich in carbohydrates, as it was strongly Schiff positive and could bind concanavalin A. It was also a major protein species according to Coomassie Blue staining. High molecular weight glycoproteins have been detected on the surface of a number of lymphocyte cells (9, 10, 19–21, 45, 47, 49, 51, 52). The molecular weights of these components were considered to be in the range of 120,000–200,000. Because in such reports gels were not calibrated in the high molecular weight region, the apparent molecular weights of some of the glycoproteins were possibly underestimated. In the 200,000-dalton region, we found several bands, some of them glycoproteins. One co-migrated with calf myosin. Quantitatively, abundant proteins were found in the mol wt 70,000 region. Another major component, band D (45,000 daltons), co-migrated with calf actin. The proteins of mol wt 200,000 and 45,000 could be candidates for membrane-bound contractile proteins, particularly because we detected microfibrils connected with some of the plasma membrane vesicles (40). Barber and Crumpton characterized actin in lymphocyte plasma membrane fractions (5). Myosin has been shown to exist on the surface of cultured fibroblasts (43, 56), and to redistribute in lymphocytes during capping of surface Ig (49). However, the presence of myosin or actin in plasma membrane fractions is not established beyond question, as it could possibly be explained by contamination or adsorption. Data concerning the linkage of contractile proteins to membranes are still missing. In another connection, Zimmerman described a major antigenic material in the mol wt 45,000 region, from lymphoid cell surfaces (59).

To our knowledge, no lymphocyte nuclear membranes have yet been analyzed by gel electrophoresis. The pattern that we obtained for LNM differed in many respects from those of plasma membranes and of band 4. The major glycoprotein N of 260,000 apparent mol wt, best revealed in the Neville system, was unique to nuclear membranes, as was another protein of 120,000 daltons. The quantitatively dominant set of proteins was found to be around 67,000 daltons. In that region, Blobel and co-workers described three proteins of 67,000, 68,000, and 69,000 daltons which they showed to be the pore complex-lamina of nuclei (1, 15). These authors reported a partial solubilization of such proteins by a high concentration of divalent cations. This did not seem to be the case for thymocyte nuclear membranes.

We, thus, have demonstrated that specific "marker" proteins can be assigned to, respectively, plasma membranes and nuclear membranes of thymocytes. This can be taken as proof of a very low degree of cross-contamination of the two types of membrane fractions.

Another, independent proof was obtained from the ^{131}I-labeling of live thymocytes. As already discussed, the integrity of the cells was essential to obtain a very high specific activity in plasma membranes, as opposed to the lowest possible intracellular labeling. Indeed, the substantial trapping of iodine by damaged (trypan blue positive) cells has been well established (22, 25, 27). The distribution and enrichment of acid-insoluble radioactive labels exactly matched that of the marker-enzymes. Table V shows that 60% of the acid-insoluble radioactive label was found in the plasma membrane fractions. The two lightest fractions (1 and 2) had the highest specific activity, close to 50 times that of the homog-

enate (Table V). Such an enrichment factor was very similar to those obtained for plasma membrane marker enzymes (Table IV).

The major radioactive proteins could be safely identified as surface-located components for several reasons. Firstly, their presence and prominence were obvious only when the iodinated samples contained more than 99% live cells. When the proportion of dead cells increased, the corresponding bands in gels were obscured by a high and mostly diffuse labeling in all molecular weight regions. Secondly, proteins of very high specific radioactivity were found only in the light membrane fractions 1 to 3. Thirdly, isolated, radiolabeled nuclei and their derived nuclear membranes displayed entirely different autoradiograph patterns. Such findings confirmed the plasmalemmal origin of light fractions 1 to 3.

However, radioactive components were also found in "inner" fractions, although their specific radioactivity was much lower. The autoradiograph patterns were on the whole similar to that of plasma membranes. This strongly suggests some contamination of inner fractions by plasmalemmal fragments. The presence of a few radioactive proteins, of mol wt 110,000 in fractions E and 4, and mol wt 160,000 in nuclear membranes, which were absent in plasma membrane fractions, remain to be explained. This probably reflects some degree of true intracellular labeling. Radiolabeled proteins found in inner fractions, therefore, derived from contamination by plasma membrane fragments and from internal labeling of a few leaky cells. At present, it is difficult to quantitate these two contributions. However, we are led to conclude that the acid-insoluble radioactivity detected in fractions E and 4, corresponding to 34% of the homogenate label (Table V), was only partially assignable to plasma membranes. This emphasizes the high recovery of plasma membranes attained by our fractionation procedure.

Another intriguing problem concerns the discrepancies found between the autoradiographs of whole cells and plasma membrane fractions. Some major radioactive bands detected in autoradiographs of whole cells were due to adsorption on the cells of lactoperoxidase, and probably glucose oxidase. Others did not correspond to the molecular weight of these two enzymes, and yet were not found in autoradiographs of homogenates or subcellular fractions. They may correspond to calf serum proteins adsorbed on the surface of thymocytes. But they may also correspond to authentic plasma membrane components, which would be lost in the supernates during the repeated washings. We have indications that shedded pieces of membrane which were collected during these steps contained such components. This important problem reflects the difficulties encountered in the radiolabeling of cells in suspension, as opposed to cells fixed on a substrate. Further work is needed to clarify the problem.

While keeping in mind such limitations, it remains that the highly radioactive protein species were characteristic of the plasma membrane fractions. The three major radioactive species corresponded to the three glycoproteins of mol wt 280,000, 200,000, and 135,000 detected in plasma membrane fractions by the Schiff or the concanavalin A staining procedures. Several investigators have reported the presence of labeled, high molecular weight glycoproteins on lymphocyte plasma membranes. Despite discrepancies in molecular weight estimations, some could be compared with our major labeled components. Gahmberg et al. (20), using tritiated borohydride labeling, detected two high molecular weight glycoproteins of 170,000 and 180,000 daltons on the surface of thymocytes and circulating T cells, respectively. Trowbridge et al. (51) revealed proteins of 170,000–190,000 daltons in thymocytes and T cells by iodination, but not in B cells. These proteins were very antigenic. Santana et al. (45) demonstrated the presence of one or two proteins of approximately 200,000 daltons, constituting the major T lymphocyte antigen recognized by a serum raised against thymocyte caps shed into the medium. Gates et al. (21) also found high molecular weight glycoproteins (mol wt 138,000) on the surface of normal human lymphocytes. Crumpton et al. (12) isolated from ^{125}I-labeled murine thymoma cells a plasma membrane fraction which showed peaks of radioactivity stacked in an unresolved region corresponding to a molecular weight larger than 135,000. Other strongly labeled proteins in our thymocyte plasma membrane fractions had molecular weights of 90,000, 45,000, and 31,000 daltons. Tsai et al. (52), studying ^{125}I-labeled mouse leukemia L-1210 cells, found that the major radioactive peptide had a mol wt of 85,000, whereas Fujita et al. (19) claimed that all B-lymphocyte cells, but not T cells, contained large amounts of a surface

glycoprotein of approximately 30,000 daltons. We detected a strongly labeled band of mol wt 31,000 in thymocyte plasma membranes. Crumpton et al. (12) were able to resolve a series of ^{125}I-labeled bands in murine T lymphoma cell plasma membranes, located between 30,000 and 100,000 daltons.

The presence of easily labeled glycoproteins on the surface of such diverse populations of lymphocytes is certainly of great biological importance, and warrants attempts to purify them. Their isolation should lead to the obtaining of antibodies of potential use in unraveling T cell functions.

When we labeled isolated nuclei, several membrane components were radioactive. The region of mol wt 67,000-70,000 had the highest specific activity. The triplet of proteins studied in that region by Dwyer and Blobel was shown by those authors to be apposed to the inner nuclear envelope membrane, but not to be part of the nuclear membrane itself (15). The pore complex-lamina would thus be a rather exposed structure, neither intramembranous nor buried in chromatin.

The ^{131}I-labeling of thymocytes confirmed the almost complete absence of plasma membrane contamination in nuclear membrane fractions, and the low degree of contamination of the mitochondrial and microsomal fraction by plasma membranes. The extensive characterization of thymocyte membrane types described in this and the accompanying paper (40) provide safe grounds for the study of the distribution of adenylate-cyclase in lymphocytes, a controversial problem. This subject will be discussed in a later paper (39).[1]

We thank J. C. Benichou, S. Guesdon, and G. Rousseau for technical assistance.

The research was supported by grants from the Centre National de la Recherche Scientifique, Laboratoire Associé n° 269, and Délégation Générale à la Recherche Scientifique et Technique (contrat n° 75.7.0040).

Part of this work was presented at the 9th International Congress of Biochemistry, Stockholm, 1973, and at the 15th Annual Meeting of the American Society for Cell Biology, San Juan, Puerto Rico, 1975. (*J. Cell Biol.* 1975. **67**:291*a*).

Received for publication 6 October 1976, and in revised form 22 July 1977.

[1] Monneron, A. and J. d'Alayer. Isolation of plasma and nuclear membranes of thymocytes. III. Subcellular localization of adenylate cyclase in thymocytes. Manuscript in preparation.

REFERENCES

1. Aaronson, R. P., and G. Blobel. 1975. Isolation of nuclear pore complexes in association with a lamina. *J. Cell Biol.* **72**:1007-1011.
2. Allan, D., and M. J. Crumpton. 1970. Preparation and characterization of the plasma membrane of pig lymphocytes. *Biochem. J.* **210**:133-143.
3. Allan, D., and M. J. Crumpton. 1972. Isolation and composition of human thymocyte plasma membranes. *Biochim. Biophys. Acta.* **274**:22-27.
4. Ames, B., and D. Dubin. 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acids. *J. Biol. Chem.* **235**:769-775.
5. Barber, B. H., and M. J. Crumpton. 1976. Actin associated with purified lymphocyte plasma membrane. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **66**:215-220.
6. Blobel, G., and V. R. Potter. 1966. Nuclei from rat liver: isolation method that combines purity with high yield. *Science (Wash. D. C.)* **154**:1662-1665.
7. Bosmann, H. B. 1973. Molecules at the external nuclear surface. Sialic acid of nuclear membranes and electrophoretic mobility of isolated nuclei and nucleoli. *J. Cell Biol.* **59**:601-614.
8. Burton, K., and G. B. Peterson. 1957. The quantitative distribution of pyrimidine nucleotides in calf thymus deoxyribonucleic acid. *Biochim. Biophys. Acta.* **26**:667-668.
9. Chavin, S. I., S. M. Johnson, and A. Holliman. 1975. Plasma membrane-associated immunoglobulins and other polypeptides of pig mesenteric node lymphocytes. *Biochem. J.* **148**:417-423.
10. Chavin, S. I., and A. Holliman. 1975. Reconstitution of pig lymphocyte plasma membranes from solubilized components, with particular reference to membrane-associated immunoglobulins. *Biochem. J.* **152**:267-270.
11. Crumpton, M. J., and D. Snary. 1974. Preparation and properties of lymphocyte plasma membrane. *In* Contemporary Topics in Molecular Immunology. G. L. A. Ada, editor. Plenum Publishing Corporation. **3**:27-56.
12. Crumpton, M. J., J. J. Marchalonis, D. Haustein, J. L. Atwell, and A. W. Harris. 1976. Plasma membrane of a murine T cell lymphoma: surface labelling, membrane isolation, separation of membrane proteins and distribution of surface label amongst these proteins. *Aust. J. Exp. Biol. Med. Sci.* **54**:303-316.
13. Demus, H. 1973. Subcellular fractionation of human lymphocytes. Isolation of two plasma membrane fractions and comparison of the protein components of the various lymphocytic organelles. *Biochim. Biophys. Acta.* **291**:93-106.
14. De Pierre, J. W., and M. L. Karnovsky. 1973. Plasma membrane of mammalian cells. *J. Cell Biol.* **56**:275-303.

15. DWYER, N., and G. BLOBEL. 1976. A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. *J. Cell Biol.* **70**:581-591.
16. FAIRBANKS, G., T. L. STECK, and D. F. H. WALLACH. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. **10**:2606-2617.
17. FERBER, E., K. RESCH, D. F. H. WALLACH, and W. IMM. 1972. Isolation and characterization of lymphocyte plasma membranes. *Biochim. Biophys. Acta*. **266**:494-504.
18. FOLCH, J., M. LEES, and Y. G. STANLEY. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**:497-509.
19. FUJITA, S., S. D. LITWIN, and N. HARTMAN. 1975. A glycoprotein associated with the membrane fraction of human B but not T lymphocytes. *J. Exp. Med.* **142**:1416-1424.
20. GAHMBERG, C. G., P. HÄYRY, and L. C. ANDERSSON. 1976. Characterization of surface glycoproteins of mouse lymphoid cells. *J. Cell Biol.* **68**:642-653.
21. GATES, R. E., D. R. PHILLIPS, and M. MORRISON. 1975. The distinguishing characteristics of the plasma membrane are its exposed proteins. *Biochem. J.* **147**:373-376.
22. GONATAS, N. K., J. O. GONATAS, A. STIEBER, J. C. ANTOINE, and S. AVRAMEAS. 1976. Quantitative ultrastructural autoradiographic studies of iodinated plasma membranes of lymphocytes during segregation and internalization of surface immunoglobulins. *J. Cell Biol.* **70**:477-493.
23. HOELZL-WALLACH, D. F., and PECK SUN LIN. 1973. A critical evaluation of plasma membrane fractionation. *Biochim. Biophys. Acta*. **300**:211-254.
24. HOGEBOOM, G. H., W. C. SCHNEIDER, and G. E. PALADE. 1948. Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver. *J. Biol. Chem.* **172**:619-635.
25. HUBBARD, A. L., and Z. A. COHN. 1975. Externally disposed plasma membrane proteins. I. Enzymatic iodination of mouse L cells. *J. Cell Biol.* **64**:438-460.
26. JARASCH, E. D., C. E. REILLY, P. COMES, J. KARTENBECK, and W. W. FRANKE. 1973. Isolation and characterization of nuclear membranes from calf and rat thymus. *Hoppe-Seyler's Z. Physiol. Chem.* **354**:974-986.
27. JULIANO, R. L., and M. BEHAR-BANNELIER. 1975. An evaluation of techniques for labelling the surface proteins of cultured mammalian cells. *Biochim. Biophys. Acta*. **375**:249-267.
28. KASHNIG, D. M., and C. B. KASPER. 1969. Isolation, morphology and composition of the nuclear membrane from rat liver. *J. Biol. Chem.* **244**:3786-3792.
29. KATES, M. 1972. Techniques of Lipidology. T. S. WORK and E. WORK, editors. Elsevier North-Holland, Inc., New York. 360-361.
30. KELLY, P. T., and C. W. COTMAN. 1977. Identification of glycoproteins and proteins at synapses in the central nervous system. *J. Biol. Chem.* **252**:786-793.
31. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. **227**:680-685.
32. LOPES, J., M. NACHBAR, D. ZUCKER-FRANKLIN, and R. SILBER. 1973. Lymphocyte plasma membrane-analysis of proteins and glycoproteins by SDS-gel electrophoresis. *Blood*. **41**:131-140.
33. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
34. MARIQUE, D., and J. HILDEBRAND. 1973. Isolation and characterization of plasma membranes from human leukemic lymphocytes. *Cancer Res.* **33**:2761-2767.
35. MEJBAUM, W. 1939. Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsaüre. *Hoppe-Seyler's. Z. Physiol. Chem.* **258**:117-121.
36. MELERA, P. W., and A. P. CRONIN-SHERIDAN. 1976. Investigation of human lymphocyte plasma membrane associated nucleic acid. *Biochim. Biophys. Acta*. **432**:300-311.
37. MISRA, D. N., C. T. LADOULIS, L. W. ESTES, and T. J. GILL, III. 1975. Biochemical and enzymatic characterization of thymic and splenic lymphocyte plasma membranes from inbred rats. *Biochemistry*. **14**:3014-3024.
38. MONNERON, A., and D. SEGRETAIN. 1974. Extensive binding of Concanavalin A to the nuclear membranes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **42**:209-213.
39. MONNERON A. 1975. Adenyl-cyclase on both plasma and nuclear membranes of thymocytes. *J. Cell Biol.* **67**:291*a* (abstr.).
40. MONNERON, A. and J. d'ALAYER. 1978. Isolation of plasma and nuclear membranes of thymocytes. I. Enzymatic composition and ultrastructure. *J. Cell Biol.* **77**:211-231.
41. MOULÉ, Y. 1953. Dosage spectrophotométrique des acides nucléiques: emploi des acides trichloracétique et perchlorique comme agents d'extraction. *Arch. Sci. Physiol.* **7**:161-188.
42. NEVILLE JR., D. M. 1971. Molecular weight determination of protein-sodium dodecyl-sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.* **246**:6328-6334.
43. OLDEN, K., M. WILLINGHAM, and I. PASTAN. 1976. Cell surface myosin in cultured fibroblasts. *Cell*. **8**:383-390.
44. PRESANT, C. A., and S. PARKER. 1976. Surface alterations in calf thymocytes oxidized by sodium

periodate. *J. Biol. Chem.* **251**:1864–1870.

45. SANTANA, V., N. WEDDERBURN, E. R. ABNEY, and R. M. E. PARKHOUSE. 1976. Identification of a high molecular weight protein on the surface of murine thymus and thymus-dependent cells. *Eur. J. Immunol.* **6**:217–222.
46. SATO, T., T. FUJII, T. MATSUURA, and K. UEDA. 1972. Lipid composition of nuclear membranes isolated from calf thymus. *J. Biochem. (Tokyo).* **72**:1049–1051.
47. SCHMIDT-ULLRICH, R., E. FERBER, H. KNÜFERMANN, H. FISCHER, and D. F. HOELZL-WALLACH. 1974. Analysis of the proteins in thymocyte plasma membrane and smooth endoplasmic reticulum by sodium dodecyl sulfate-gel electrophoresis. *Biochim. Biophys. Acta.* **332**:175–191.
48. SCHREINER, G. F., K. FUJIWARA, T. D. POLLARD, and E. R. UNANUE. 1977. Redistribution of myosin accompanying capping of surface Ig. *J. Exp. Med.* **145**:1393–1398.
49. SMITH, W. I., C. T. LADOULIS, D. N. MISRA, T. J. GILL, III and H. BAZIN. 1975. Lymphocyte plasma membranes from normal and immunized rats. *Biochim. Biophys. Acta.* **382**:506–525.
50. SVENNERHOLM, L. 1958. Quantitative estimation of sialic acids. III. An anion exchange resin method. *Acta Chem. Scand.* **12**:547–554.
51. TROWBRIDGE, I. S., P. RALPH, and M. J. BEVAN. 1975. Differences in the surface proteins of mouse B and T cells. *Proc. Natl. Acad. Sci. U. S. A.* **72**:157–161.
52. TSAI, C. M., K. Y. CHEN, and E. S. CANELLAKIS. 1975. Isolation and characterization of the plasma membrane of L-1210 cells. Iodination as a marker for the plasma membrane. *Biochim. Biophys. Acta.* **401**:196–212.
53. VAN BLITTERSWIJK, W. J., P. EMMELOT, and C. A. FELTKAMP. 1973. Studies on plasma membranes. XLX. Isolation and characterization of a plasma membrane fraction from calf thymocytes. *Biochim. Biophys. Acta.* **298**:577–592.
54. WALSH, F. S., B. H. BARBER, and M. J. CRUMPTON. 1976. Preparation of inside-out vesicles of pig lymphocyte plasma membrane. *Biochemistry.* **15**:3557–3563.
55. WARREN, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234**:1971–1975.
56. WILLINGHAM, M. C., R. E. OSTLUND, and I. PASTAN. 1974. Myosin is a component of the cell surface of cultured cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4144–4148.
57. WISHER, M. H., and W. H. EVANS. 1975. Functional polarity of the rat hepatocyte surface membrane. *Biochem. J.* **146**:375–388.
58. WUNDERLICH, F., R. BEREZNEY, and H. KLEINIG. 1976. The nuclear envelope: an interdisciplinary analysis of its morphology, composition and functions. *In* Biological Membranes, vol. 3, Chapman, D. and Wallach, D. F. H. Edt., Academic Press, Inc., New York. 241–333.
59. ZIMMERMAN, B. 1974. Lymphocyte plasma membranes. I. Characterization of an antigenic plasma membrane constituent on rat thymus cells. *J. Immunol.* **112**:249–259.