ISOLATION OF PLASMA AND NUCLEAR MEMBRANES OF THYMOCYTES

I. Enzymatic Composition and Ultrastructure

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ABSTRACT

The purpose of this work was to isolate thymocyte plasma membranes at high yield and purity to study specific surface molecules in their structural context. A procedure was developed in which 92–95% of the cells were disrupted by homogenization in a dense viscous medium, while nuclei remained intact. Differential centrifugation of the homogenate was avoided; instead, only a brief (2 h) centrifugation at equilibrium-density of membrane components was used. Five fractions were obtained, three by flotation. Membrane-bound enzymatic activities indicated a 60–80% yield of plasma membranes in the three floated membrane fractions, which comprised 1.6% of the homogenate protein. Enrichment factors for three ectoenzymes, alkaline phosphatase, γ-glutamyltransferase, and ouabain-sensitive adenosine triphosphatase were respectively, 70–74, and 40–50 in the two lightest fractions. Nuclear membranes were then isolated from the remaining whole nuclei and were found to be enriched in esterase and NADH-cytochrome c reductase.

Plasma membranes and light nuclear membranes appeared as pure unit-membrane vesicles in thin sections and freeze-etching electron microscopy. Some aggregation of intramembranous particles occurred in plasma membrane vesicles.

KEY WORDS lymphocyte · plasma membrane · nuclear membrane · marker enzymes · ultrastructure · subcellular fractionation

T cells play a central role in immunological processes. They are responsible for the recognition of many antigens and respond to them by activating surface signals and releasing soluble factors which trigger B cells in various ways (7, 30). They are also the effectors of cellular immunity. Such functions are mediated by the plasma membrane. Work is in progress in several laboratories to characterize the receptors of antigens, now known as the products of the Ir genes, and the surface signals, presumably related to the molecules responsible for histocompatibility (38). To study such molecules, it appears likely that they must be maintained in their structural context. Therefore, it is essential to obtain purified plasma membranes of T cells, and of their precursors, the thymocytes. T and B cells, despite their profound biological differences, are morphologically indistinguishable (1, 5). Thymocytes, the precursors of T cells, resemble very much circulating lymphocytes when isolated in a culture medium. Therefore, methods
for isolating lymphocyte plasma membranes are common to both T and B cells, and isolated thymocytes. A certain number of preparative procedures for lymphocyte plasma membranes have been published (2, 3, 14, 18, 20, 22, 24, 25, 28, 31, 39-41, 45, 48, 52), some of which are analyzed in a general review (13). Some of these methods yield reasonably purified plasma membranes, but too often at the expense of the recovery. Low yields are specially crucial drawbacks, as the amounts of the cells to be studied may be quite low if subclasses of T cells are to be analyzed (11).

We present here a simple and rapid method for lymphocyte fractionation, which allows for high recovery of purified plasma membranes, as well as other fractions such as nuclear membranes. The cellular components are characterized by their ultrastructure, biochemical, and enzymatic compositions, which permit us to define specific markers. In this first paper, we will describe the fractionation procedure, the morphology of the different fractions, and their enzymatic composition. A second companion paper will deal with the biochemical composition and the analysis of the protein content of such fractions (34). A third paper describes the distribution of adenyl-cyclase activity in the fractions.¹

MATERIALS AND METHODS

Isolation of Thymocytes

Calf thymus from freshly slaughtered animals was cut with a razor blade into pieces, 2-5 mm², rinsed several times in cold Hanks' medium, and agitated into a 50-fold volume of this medium containing 10% fetal calf serum and antibiotics (penicillin G, 20,000 U/l; streptomycin 50 mg/l) at 4°C. Lymphocytes progressively moved from the tissue pieces into the culture medium. After 1-3 h, the medium was filtered through eight layers of surgical gauze. Cells were collected by centrifugation (600 g, 10 min) and rinsed in Hanks' medium without serum, one to three times. Generally, 90% of the cells were viable, as judged by trypan blue exclusion. Thymocytes represented at least 92% of the cell population; the preparations were contaminated by a few macrophages, which were eliminated by filtering the cell suspension on glass wool, and a few polymorphonuclear cells.

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.

Cell Fractionation

Fractions were generally prepared in TKM buffer (10) consisting of 50 mM Tris-HCl pH 7.4, 25 mM KCl, 5 mM MgCl₂, or in STKM (TKM buffer containing sucrose).

Disruption of Cells: The cell pellet was suspended in 70% (wt/vol) STKM, and adjusted to 60% sucrose. Homogenization in an ice-cold glass-teflon Potter homogenizer (clearance 130-180 μm) consisted of eight strokes at 4,500 rpm. The efficiency of the method was assessed by counting the number of whole cells relative to the number of nuclei in electron micrographs of the pelleted homogenate. About 4-8% of the cells were not completely disrupted.

Subcellular Fractionation: The homogenate was adjusted to 40% sucrose, which corresponds to the density of the mitochondria obtained in such conditions. 5-10 ml were layered over a cushion of 5 ml of 73% sucrose (2.15 M) (10). Above the homogenate, a discontinuous gradient of respectively, 6 ml each of 35% STKM, 22.5% STKM, and TKM was layered (Fig. 1). The gradient was centrifuged for 2 h at 130,000 g in a Spinco SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 4°C. Conspicuous bands were obtained at all four interfaces. The sucrose layers between the bands were clear. Fractions were collected from the top with a gradient collector or a pipette. Each band was diluted at least fourfold with TKM, centrifuged for 30 min at 360,000 g, 4°C, and the pellet was resuspended in a small volume of buffer by homogenization. The clarified 40% sucrose layer was processed in the same way and yielded a pellet called E. Fractions were either immediately frozen in liquid nitrogen or used at once.

The bulky nuclear pellet was resuspended in buffer, using a motor-driven homogenizer (five strokes, 4,500-5,000 rpm), and the nuclei were counted. They were suspended in cold TKM, made 50 mM in MgCl₂, and stirred in the cold with DNAse I, at a concentration of 1 mg/10⁶ nuclei, for 10-30 min. This is a modification of the method of Berezney et al. (9). The suspension was then made 0.3 M in MgCl₂ and 52% in sucrose, placed in SW 27 rotor tubes, and overlaid by 47% STKM and TKM buffer, each containing 0.3 M MgCl₂. The gradient was centrifuged at 4°C for 2 h at 130,000 g. The light nuclear membranes (LNM) floated at the buffer - 47% sucrose interface. The heavy nuclear membranes (HNM) floated at the 47% sucrose-nuclear suspension interface. The bands were collected, diluted with cold distilled water, and centrifuged for 1 h at 360,000 g. The pellets were resuspended in TKM buffer and frozen.

Enzymatic Assays

Protein determinations were performed according to Lowry et al. (27). The enzymes assayed in the different fractions are listed in Table I. The fractions tested had been stored in liquid nitrogen, and were thawed once.
The activities of phosphatases were determined by the release of inorganic phosphate measured by the procedure of Chen et al. (12). The method of Tashima (43) was used for membrane fractions to avoid TCA precipitation. Adenosine triphosphatase (ATPase) activities were assayed by three different methods, one of which (4) used \[^{32}P\]ATP, 8 Ci/mmol. The liberated \[^{32}P\]i was counted in an Intertechnique SL 31 liquid scintillation counter (Intertechnique, 78 Plaisir, France) by using Cerenkov radiation. Ouabain-sensitive ATPase was assayed with a final concentration of 1 mM ouabain. Galactosyltransferase was assayed with N-acetyl-glucosamine as an acceptor. Tritiated uridine-5'-diphospho-galactose (UDP-galactose) had a specific activity of 1.23 Ci/mmol. High-voltage electrophoresis was carried out at 2,000 V in 1% tetaborate buffer, pH 9.0 (37). Glutamyltransferase was measured in the presence of an exogenous acceptor, glycyI-glycine, 30 mM (44).

Electron Microscopy

Cellular and nuclear pellets were fixed in cold 1.8% glutaraldehyde in cacodylate buffer (pH 7.4, 100 mM) for 20-60 min, followed by 1% osmium tetroxide in the same buffer for 1-2 h in the cold. Membrane suspensions were prepared in phosphate solutions (pH 7.4, 50 mM) instead of Tris-HCl solutions. They were fixed in 1.8% glutaraldehyde in phosphate buffer, centrifuged at 40,000 g for 30 min, at 4°C in a Beckman centrifuge (Beckman Instruments, Inc., Spineo Div.). The thin pellets were cut into small pieces along the axis of

**Table 1**

**Enzymes Used as Membrane Markers**

| EC*    | Name                          | Substrate                          | Substrate concentration | pH of assay | Reference |
|--------|-------------------------------|------------------------------------|-------------------------|-------------|----------|
| 3.1.3.5 | 5'-Nucleotidase               | Sodium 5'-AMP                      | 3 mM                    | 7.5         | (16)     |
| 3.1.3.6 | 3'-Nucleotidase               | Sodium 3'-AMP                      | 3 mM                    | 7.5         | (16)     |
| 3.6.1.3 | Na⁺-K⁺-Mg⁺-ATPase             | Tris-ATP                           | 2 mM                    | 7.4         | (16, 4)  |
|        |                               | p-nitrophenylphosphate (Tris salt) | 3 mM                    | 7.5         | (36)     |
| 3.1.3.2 | Alkaline phosphonooesterase  | Sodium β-glycerophosphate          | 20 mM                   | 9.5         | (21)     |
|        |                               | Sodium p-nitrophenylphosphate     | 2 mM                    | 10.0        | (23)     |
| 2.3.2.1 | γ-Glutamyl-transpeptidase     | γ-glutamyl-p-nitroanilide           | 0.25 mM                 | 8.0         | (44)     |
| 3.1.1.6 | Acetyltransferase             | α-nitrophenylacetate               | 3 mM                    | 7.4         | (6)      |
| 3.1.3.1 | Acid phosphomonoesterase      | Sodium β-glycerophosphate          | 50 mM                   | 5.0         | (16)     |
|        | Thiamine pyrophostase         | Thiamine pyrophosphate chlorid e    | 0.4 mM                  | 7.4         | (29)     |
| 2.4.1.67 | Galactosyltransferase         | UDP-galactose                      | 70 mM                   | 7.2         | (37)     |
| 3.2.1.30 | N-acetyl-β-glucosaminidase   | p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside | 6 mM                  | 4.5         | (6)      |
| 1.9.3.1 | Cytochrome oxidase            | Reduced cytochrome c               | 35 μM                   | 7.4         | (42)     |
| 1.6.2.1 | NADH-cytochrome c reductase   | Oxidized cytochrome c              | 0.1 mM                  | 7.5         | (42)     |
| 1.3.99.1 | Succinate dehydrogenase       | 2,6-dichlorophenol indo phenol     | 0.9 mM                  | 7.2         | (19)     |

EC* Report of the Commission of Enzymes of the International Union of Biochemistry.
were dehydrated in acetone and flat-embedded in Epon. From top to bottom in a Siemens 101 electron microscope. For freeze-etching, cells and membranes were fixed either in suspension or as pellets with 2% glutaraldehyde at room temperature. Pellets were impregnated with cold 25% glycerol in phosphate buffer (pH 7.4, 100 mM) for 1½-3 h, frozen in Freon, and stored in liquid nitrogen. The specimens were fractured at -115°C and replicated with or without etching. The replicas were digested in concentrated bleach, or in 40% nitrous acid or 2 M NaCl, and rinsed several times.

Materials
Reagent grade chemicals were obtained from the following sources: sucrose, density gradient grade, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.; inorganic salts, and solvents, Prolabo, France; ethanol and acetone, Merck AG, Darmstadt, West Germany; glutaraldehyde, Fisher Scientific Co., Pittsburgh, Pa.; deoxyribonuclease I, cytochrome c and 32P]adenosine triphosphate and tritiated UDP-galactose, a gift from Janssen Pharmaceutica, Beerse, Belgium; levamisole was a gift from Janssen Pharmaceutica, Beerse, Belgium; 2,6-dichlorophenol-indophenol (DCIP), Fluka; L-3,-glutamyl-p-nitranilide, Boehringer, Mannheim, Germany; p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, Koch-Light Lab., England; levamisole was a gift from Janssen Pharmaceutica, Beerse, Belgium.

RESULTS
In our attempt to quantitatively prepare purified plasma membranes, we developed a procedure based on the purification of so-called marker-enzymes, and their recovery in high yield. Preliminary results were obtained by directly floating membranes from the homogenate into a continuous sucrose gradient. This yielded two major bands, one at 10-20% sucrose (density 1.034-1.080) and the other at 32-35% (density 1.126-1.136). On the basis of enzymatic studies, we improved the separation by using the discontinuous gradient described under Materials and Methods. Five fractions were obtained, three of them by flotation (Fig. 1). Band 1 was at the interface TKM-22.5% sucrose; band 2, at the interface 22.5-35%; band 3, at the interface 35-40%. These three floated bands were milky and white. Band 4, at the interface 40-73%, was much thicker, and yellow.

From the DNase-treated nuclei a membranous band was obtained at each of the two interfaces of the second gradient. The lightest one, LNM, was at the interface buffer-40% sucrose, the densest one, HNM, at the interface 40% sucrose-nuclear suspension. When the LNM and HNM samples were centrifuged at equilibrium in STKM linear gradients, the LNM membranes banded at a density of 1.124 and the HNM at a density of 1.19. However, when the HNM fraction was submitted to a further treatment by RNAse A and DNase I (at 37°C, 30 min), and recentrifuged in a high MgCl₂, sucrose gradient, its position in the gradient shifted to that of LNM.

Enzymatic Analysis of the Fractions
Enzymes having a highly specialized function often have a unique cellular localization, whatever the tissue or species studied, and are used as marker enzymes for specific membranes. We investigated a series of such enzymes (Table I), and related our data to a previous cytochemical study at the ultrastructural level (32). Tables II to VI give the specific activity, enrichment over homogenate, and yield of the tested enzymes in the different fractions.

(a) VALIDITY OF THE DATA

PHOSPHATASES: In the case of enzymes displaying zero-order kinetics, initial velocity was measured. When phosphate release was the parameter chosen to follow phosphatase activity, a difficulty occurred. As a result of the addition of substrates, be it mono-, di-, or triphosphate nucleotides, purified membrane fractions, especially fractions 1 to 3, released a large amount of inorganic phosphate within a few seconds (Fig. 2). Thereafter, the slope of the curve abruptly changed to a lower value which remained constant for 30-60 min. This phenomenon could be explained partly by the high specific activity of alkaline phosphatase (Table II), still very active at neutral pH. The addition of a potent inhibitor of alkaline phosphatase, levamisole (0.1 mM), indeed suppressed the fast early release of phosphate, the following slow release being unaffected (Fig. 2). We also found that the amount of products released during the first few seconds was not inhibitory by itself (be it phosphate or nucleoside). When whole homogenates were incubated, an uptake of phosphate, not a release, occurred during the first few minutes after addition of the substrate (Fig. 3). Later, the release of phosphate
proceeded linearly with time and was proportional to the amount of homogenate. Clearly, the addition of phosphorylated compounds triggered more than one enzyme. We therefore calculated phosphatase activities from the second, linear part of the curves. The validity of this observation was further confirmed by the study of ATPase activity by three different methods. The release of phosphate from ATP (4, 16) was biphasic. The release of paranitrophenol (36), which was linear, was identical with that of phosphate in the second part of the curve. When calculated in the same way, the $K_m$ value for 5'-AMP of 5'-nucleotidase measured in fractions 2 and 4 was found to be 25–30 mM, comparable to that of the rat-liver purified enzyme (17).

**DIFFERENCES IN MEMBRANE PREPARATIVE PROCEDURES:** As DNAse and high ionic strength were used to prepare nuclear membranes, aliquots of all other membrane fractions treated in the same way were assayed. The enzyme activities and contents were not modified by the procedure.

(b) **IDENTIFICATION OF PLASMA MEMBRANE MARKERS**

Table II illustrates the distribution of four classical plasma membrane markers, alkaline phosphatase, $\gamma$-glutamyltransferase, ouabain-sensitive ATPase, and 5'-nucleotidase, and of the enzyme 3'-nucleotidase shown to be present on the thymocyte surface by cytochemistry (32). The three floated fractions were considerably enriched in all five enzyme activities, especially fractions 1 and 2. Levamisole-sensitive alkaline phosphatase was purified 70- to 74-fold in the two lightest fractions, $\gamma$-glutamyltransferase and ouabain-sensitive ATPase, 40- to 50-fold. Enrichment factors were lower for 5'- and 3'-nucleotidases. The floated fractions, comprising 1.6% of the cellular proteins, contained 60–80% of the first three plasma membrane markers. They were thus considered as the plasma membrane fractions. Their degree of purity was then assessed by measuring the amount of enzymes considered as markers for mitochondrial inner membrane (cytochrome oxidase, succinate dehydrogenase, Table III), endoplasmic reticulum (esterase, NADH-cytochrome c reductase, Table IV), Golgi apparatus (galactosyltransferase, thiamine pyrophosphatase, Table V) and lysosomes (N-acetyl-$\beta$-glucosaminidase, Table VI). Fractions 1 and 2 were the least contaminated by intracellular membranes, except that they contained substantial amounts of Golgi markers (30% of the total thiamine pyrophosphatase activity and 22% of the total galactosyltransferase activity).
### Table II

#### Plasma Membrane Markers

| Protein of homogenate (30) | Alkaline phosphatase (levamisole sensitive) (6) | γ-glutamyltransferase (5) | Ouabain-sensitive ATPase (15) | 5'-nucleotidase (12) | 3'-nucleotidase (10) |
|----------------------------|-----------------------------------------------|--------------------------|--------------------------------|---------------------|---------------------|
| %                          |                                               |                          |                                |                     |                     |
| Homogenate cells           | 175 ± 24 mg/10 m² | 0.382 ± 0.113 | 0.045 ± 0.005 | 0.110 ± 0.03 | 0.08 ± 0.02 | 0.024 ± 0.006 |
| 1 ×                         | 1 ×                                           | 1 ×                      | 1 ×                            | 1 ×                 | 1 ×                 |
| 100%                       | 100%                                          | 100%                     | 100%                           | 100%               | 100%               |
| Fraction 1                 | 0.20 ± 0.03                                   | 28.3 ± 3.5 | 2.05 ± 0.18 | 4.27 ± 1.45 | 2.72 ± 1.05 | 0.68 ± 0.26 |
| 74 ×                       | 41.5 ×                                        | 39 ×                    | 34 ×                           | 28 ×               |                     |
| 15%                        | 9%                                            | 8%                      | 7%                             | 6%                 |                     |
| Fraction 2                 | 0.61 ± 0.10                                   | 27.6 ± 3.6 | 2.22 ± 0.22 | 5.21 ± 2.00 | 2.85 ± 1.11 | 0.71 ± 0.28 |
| 72 ×                       | 49.3 ×                                        | 47 ×                    | 36 ×                           | 30 ×               |                     |
| 44%                        | 30%                                           | 27%                     | 22%                            | 18%                |                     |
| Fraction 3                 | 0.83 ± 0.14                                   | 9.14 ± 1.6 | 1.05 ± 0.21 | 3.08 ± 0.91 | 2.46 ± 0.73 | 0.62 ± 0.18 |
| 24 ×                       | 23.5 ×                                        | 28 ×                    | 31 ×                           | 26 ×               |                     |
| 20%                        | 20%                                           | 23%                     | 26%                            | 21%                |                     |
| Fraction 4                 | 2.60 ± 0.84                                   | 1.5 ± 0.3  | 0.37 ± 0.03 | 0.74 ± 0.31 | 0.38 ± 0.08 | 0.08 ± 0.02 |
| 4 ×                        | 8.3 ×                                         | 7 ×                    | 4.7 ×                           | 3 ×                |                     |
| 7%                         | 21%                                           | 18%                     | 12%                            | 9%                 |                     |
| Fraction E                 | 3.85 ± 0.56                                   | 1.18 ± 0.3  | 0.175 ± 0.02 | 0.52 ± 0.13 | 0.37 ± 0.09 | 0.04 ± 0.01 |
| 3 ×                        | 3.9 ×                                         | 5 ×                    | 4.7 ×                           | 2 ×                |                     |
| 12%                        | 15%                                           | 19%                     | 18%                            | 6.5%               |                     |
| Nuclei                     | 61.20 ± 2.50                                  | 0.064 ± 0.050 | Not detectable | Not detectable | 0.006 ± 0.005 | Not detectable |
| 0.17 ×                     |                                               | 0.08 ×                |                                |                     |                     |
| 10%                        | 0%                                            | 0%                     | 5%                             | 0%                 |                     |
| LNM                        | 1.00 ± 0.17                                   | 3.20 ± 1.0  | 0.03 ± 0.08 | 0.44 ± 0.10 | 0.35 ± 0.01 | 0.11 ± 0.01 |
| 8.3 ×                      | 0.7 ×                                         | 4 ×                    | 4.4 ×                           | 5 ×                |                     |
| 8%                         | 0.7%                                          | 4%                     | 4.4%                            | 4%                 |                     |
| HNM                        | 0.64 ± 0.11                                   | 1.5 ± 0.6  | 0.01 ± 0.01 | 0.22 ± 0.05 | 0.26 ± 0.01 | 0.08 ± 0.01 |
| 4 ×                        | 0.2 ×                                         | 2 ×                    | 3.3 ×                           | 3 ×                |                     |
| 2.5%                       | 0.15%                                         | 1.3%                   | 2.1%                            | 2%                 |                     |
| Soluble gradient           | 28 ± 4                                        | 0%                     | 0%                             | 0%                 | 30%                 |
| Yield in gradient          | 99                                            | 108%                   | 100%                            | 91.5%               | 96.5%               |

Specific activities are given in micromoles of product released per milligram of protein per hour for phosphatases, in micromoles of product (p-nitroaniline) formed per milligram per hour for γ-glutamyltransferase.

**Legend for Tables II to VI.** Numbers in parentheses refer to the number of experiments. In each column corresponding to one enzyme, the specific activity and the SEM is given in absolute values, on the first line. The enrichment of different fractions, calculated by taking the homogenate specific activity for one (IX), is given on the second line. The yield relative to the homogenate is given in percent on the third line. The sum of the activities recovered in the gradient is also given as a percent of the activity present in the homogenate. All phosphatase and nucleotidases were 100% inhibited by 100 mM NaF, except for alkaline phosphatase which was inhibited by 0.1 mM levamisole.

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**Figure 3** Kinetics of phosphate release by the homogenate after addition of 3'-AMP. During the first few minutes, an uptake of inorganic phosphate occurred.

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Fraction 3 contained lower amounts of Golgi markers but was contaminated to a slightly larger extent by endoplasmic reticulum vesicles and lysosomes. In summary, a high yield of plasma membranes was obtained in the three floated fractions, but Golgi vesicles co-purified with them to some extent.

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TABLE III
Mitochondrial Inner Membrane Markers

| Plasma membranes | Nuclear membranes | Total recovery |
|------------------|-------------------|---------------|
|                  | Fractions         | Nuclei        | LNM    | HNM    |               |
| Homogenate       | 1                 | 2             | 3      | Fraction E | Fraction 4    | Nuclei | LNM | HNM |
| Cytochrome       |                  |               |        | 126 ± 52 | 145 ± 41     |        |     |     |
|oxidase (3)       | 1x                |               |        | 1.6±      | 1.8±         |        |     |     |
|nmol/mg/min       | 100%              | able          |        | 1.6%      | 1.15%        | 77%    |     |     |
| Succinate de-    |                  |               |        | 12±3      | 5±            |        |     |     |
|hydrogenase (3)   | 1x                | measurable    |        | 1.6±      | 4±            | 118%   |     |     |
|                  | 100%              | 0%            |        | 7%        | 2.56%        |        |     |     |

*Fractions were preincubated for 10 min with DCIP and KCN 0.2 mM.
†Cytochrome oxidase and succinate dehydrogenase could not be reliably measured in nuclear fractions, the amount of nuclei needed being high enough to interfere with the spectrophotometer recordings.

(c) Analysis of the Nuclear Membrane Fractions

The main evidence for the nuclear origin of such membranes rested on the purity of the nuclear preparation as assessed by electron microscopy (see below). As shown in the accompanying paper (34), the membranes contained in the nuclear suspension were totally recovered in bands LNM and HNM. Nuclear membrane marker-enzymes are unfortunately not yet known. Tables II to V show that a discrepancy existed between the activities measured in nuclei and in the derived membrane fractions where some were higher. This phenomenon could partially be due to the clumping of nuclei in the incubation media. Esterase activity was more enriched in nuclear-membrane fractions than in any other fraction (by a factor of 16.5 in LNM), and NADH-cytochrome c reductase activity was also high (enrichment factor of 12.5 for LNM). Plasma membrane marker enzymes were enriched four- to eight-fold in nuclear membrane fractions, except for γ-glutamyltransferase. The lysosomal contamination was negligible; a few Golgi vesicles were present. Assessment of the mitochondrial contamination was based on the measurements of two enzymes linked to the inner membrane of these organelles, cytochrome oxidase and succinate dehydrogenase. Both enzymes were present and slightly enriched in the nuclear membranes. As expected, the contamination was somewhat variable from one experiment to the other, probably depending very much on the homogenization step. Berezney and co-workers have shown that bovine nuclear membranes contain cytochrome oxidase as a true component (8, 9). Our lymphocyte nuclei were too contaminated by mitochondria to be analyzed in this respect (Fig. 14).

(d) Other Fractions

The other fractions were analyzed for the sake of completeness, irrespective of their heterogeneity. Fractions E and 4 comprising, respectively, 2.6 and 4% of the total proteins, each contained about 15% of the plasma membrane markers. Taken together, they contained 75–90% of the mitochondria, half of the endoplasmic reticulum markers and of the lysosomal marker, as well as about 30% of the total Golgi vesicles.

We selected N-acetyl-γ-glucosaminidase as lysosomal marker rather than the classical acid phosphatase for the following reason. A β-glycerophosphatase active at pH 5.0 was present in all membrane fractions (Table VI). This distribution did not parallel that of N-acetyl-β-glucosaminidase, which was found mainly in fractions E and 4. We suspected that among the several phosphatases found in high levels in the floated membrane fractions 1 to 3, some were active within a broad pH spectrum, thus obscuring the presence of a specific acid phosphatase associated with lysosomes.

Ultrastructural Study of the Fractions

**Thymocytes:** Due to the huge volume occupied by the nucleus in the cell, about 60% (35), only two types of membranes are well represented in lymphocytes: the plasma membrane and the nuclear envelope (Fig. 4). The rough endoplasmic reticulum is represented mainly by the outer leaf-
### Table IV

**Endoplasmic Reticulum Markers**

| Fractions | Plasma membranes | Nuclear membranes | Total recovery |
|-----------|------------------|-------------------|---------------|
| Homogenate | Fractions | Esterase (μmol/mg/h at 37°C) | NADH-cytochrome c reductase (μmol/mg/h at 20°C) |
| 1 | 2 | 3 | E | 4 | Nuclei | LNM | HNM | Soluble |  |
| 6.7 ± 0.5 | 15.4 ± 1 | 26.6 ± 2.4 | 27.8 ± 4.9 | 12.1 ± 2.6 | 13.9 ± 3.7 | 6.7 ± 1.2 | 10.3 ± 12.5 | 42.6 ± 11.8 |  |
| 1 x | 2.2 x | 3.8 x | 4.1 x | 1.9 x | 2.1 x | 1 x | 16.5 x | 6.4 x |  |
| 100% | 0.6% | 2.7% | 4.1% | 9% | 9% | 64% | 16.5% | 4.1% | 10% 89% |
| 0.1 ± 0.03 | 0.6 ± 0.1 | 0.7 ± 0.1 | 1.1 ± 0.15 | Not measured | 1.1 ± 0.1 | 0.04 ± 0.03 | 1.3 ± 0.2 | 0.5 ± 0.1 | Not measured |
| 1 x | 6 x | 7 x | 11 x | 11 x | 0.4 x | 12.5 x | 5.1 x | 81% |
| 100% | 1.2% | 4% | 9% | 42% | 25% | 12.5% | 3.3% |  |

### Table V

**Golgi Markers**

| Fractions | Plasma membranes | Nuclear membranes | Total recovery |
|-----------|------------------|-------------------|---------------|
| Homogenate | Fractions | Galactosyl-transferase (μmol/mg/h at 37°C) | Thiamine pyrophosphatase (μmol/mg/h at 37°C) |
| 1 | 2 | 3 | E | 4 | Nuclei | LNM | HNM | Soluble |  |
| 1.45 ± 0.7 | 11.6 ± 2.8 | 46.4 ± 14.4 | 21.8 ± 7.2 | Not measured | 8.8 ± 3 | 0.5 ± 0.05 | Not measured | Not measured | 78% |
| 1 x | 8 x | 32 x | 15 x | 6 x | 0.3 x | 19% |  |
| 100% | 1.6% | 20% | 14% | 23% |  |
| 0.055 ± 0.02 | 1.00 ± 0.41 | 2.7 ± 1.10 | 1.15 ± 0.46 | 0.36 ± 0.11 | 0.20 ± 0.10 | Not measured | 0.33 ± 0.05 | 0.38 ± 0.15 |  |
| 1 x | 18 x | 40 x | 31 x | 6.5 x | 3.6 x | detectable | 6 x | 7 x |  |
| 100% | 4% | 26% | 17% | 17% | 14% | 0% | 6% | 4.5% | 0% 78% |
|                     | Homogenate | 1          | 2          | 3          | Fraction E | Fraction 4 | Nuclei     | LNM | HNM | Soluble | Total recovery |
|---------------------|------------|------------|------------|------------|------------|------------|------------|-----|-----|--------|----------------|
| α-N-acetyl-β-glucosaminidase | 0.47 ± 0.10 | 0.55 ± 0.05 | 1.20 ± 0.20 | 2.16 ± 0.74 | 2.73 ± 0.34 | 4.28 ± 0.50 | 0.07 ± 0.07 | 0.3 ± 0.01 | 0.52 ± 0.12 | 30%            |
| (μmol/mg/h at 37°C) | 12%        | 12%        | 48%        | 3%         | 15%        | 35%        | 10%        | 6%  | 6%  | 96%    |                |
| Acid phosphatase   | 0.20 ± 0.02 | 0.50 ± 0.05 | 1.42 ± 0.12 | 1.04 ± 0.07 | 0.94 ± 0.19 | 0.77 ± 0.09 | 0.04 ± 0.02 | 0.42 ± 0.05 | 0.38 ± 0.06 | 0.10 ± 0.01 |
| (μmol/mg/h at 37°C) | 1%         | 25%        | 7%         | 5.2%       | 4.7%       | 3.8%       | 0.2%       | 2.1% | 1.9%| 0.5%   | 62%            |
A thymocyte incubated in a Wachstein and Meisel cytochemical medium (47) for the demonstration of 5'-AMPase. Lead precipitates are strewn on the plasma membrane. G, Golgi apparatus. C, centriole. M, mitochondria. ER, rough endoplasmic reticulum. NE, nuclear envelope. × 23,000.

Figure 4 A thymocyte incubated in a Wachstein and Meisel cytochemical medium (47) for the demonstration of 5'-AMPase. Lead precipitates are strewn on the plasma membrane. G, Golgi apparatus. C, centriole. M, mitochondria. ER, rough endoplasmic reticulum. NE, nuclear envelope. × 23,000.

let of the nuclear envelope (outer nuclear membrane [ONM] as opposed to inner nuclear membrane [INM]). Besides the Golgi apparatus, there is very little smooth endoplasmic reticulum. There are few mitochondria per cell, 3-7 (35), and few lysosomes. Some areas of plasma and nuclear membranes are sometimes almost apposed one to the other. This was well seen on freeze-fracture replicas (Fig. 5). The P and E fracture faces of the plasma membrane were studded in a rather homogeneous way with intramembranous particles of small, 3-5 nm, and large, 7-10 nm, size (Fig. 6). Elongated mounds, 1-50 nm in diameter and 20-50 nm in length, were strewn on the surface of either fracture face. In a small percentage of cells, one to several clusters of up to 40 large particles were seen. The nuclear envelope had a low number of nuclear pores: 3.5-4.2/μm² (Fig. 5). It presented four fracture faces, as already described (33). The E faces were poor in particles; the P faces had many more particles.

Fraction 1

Membrane vesicles were the only structures seen throughout the pellet (Fig. 7). They consisted of an 8-nm thick unit-membrane (Fig. 8) and had
FIGURE 5 Freeze-fractured thymocyte, fixed with glutaraldehyde. P, P face of the plasma membrane. ONM, outer nuclear membrane (E face). INM, inner membrane (P face). Nuclear pores are indicated by arrows. × 45,000.

FIGURE 6 Freeze-fractured adjacent thymocytes, fixed in glutaraldehyde. The P face of the plasma membrane, P, has many more intramembranous particles than the E face, E. × 114,000.
Figure 7 Thin section of band 1 fixed in suspension. Membrane vesicles measure up to 1 μm in diameter. Bar, 1 μm. × 19,000.

Figure 8 Same preparation. The unit membrane is well seen on normally sectioned membranes. Bar, 0.5 μm. × 76,000.
Figure 9  Freeze-fracture. Band 1 fixed as a pellet, consisting of a homogeneous population of vesicles. Bar, 1 µm. × 70,000.

Figure 10  Freeze-fracture. Band 1 fixed as a pellet. A vesicle is shown which bears large aggregates of particles. Bar, 0.5 µm. × 60,000.

Figure 11  Freeze-etched preparation. Band 2 fixed in suspension. A bundle of fibrils may be seen among the vesicles, in the ice, not in the membrane fracture plane. (Arrow). Bar, 0.1 µm. × 138,000.
an average diameter of 0.1-0.5 μm, a few vesicles reaching 1 μm and more in diameter. They were free of adherent material and devoid of content. Occasionally, fine fibrils were seen in connection with the inner face of larger vesicles. Freeze-etching confirmed the homogeneity of the pellets (Fig. 9). Whatever the type of sample preparation (fixed or unfixed membranes), clusters of 20-120 particles surrounded by barren areas were seen on some vesicles (Fig. 10); others showed a rather homogeneous distribution of particles. Here and there, bundles of fibrils 5-11 μm in diameter, up to 0.3 μm in length, 4-6 fibrils per bundle in the plane of the replica, were seen in etched preparations, seemingly converging towards membrane vesicles (Fig. 11).

Fraction 2
Fraction 2 was indistinguishable from fraction 1.

Fraction 3
Fraction 3 consisted largely of membrane vesicles similar to those seen in lighter fractions, but a few vesicles with an amorphous content, a few rough endoplasmic reticulum vesicles, and disrupted mitochondria were seen (Fig. 12).

Fraction E
Fraction E resembled fraction 3, but the amount of mitochondria was higher.

Fraction 4
The composition of the pellet was heterogeneous along the axis of sedimentation. A number of shrunken mitochondria were found at the surface. Beneath were rough endoplasmic reticulum vesicles, membrane-bounded droplets of cytoplasm, lysosomes, centrioles, bundles of filaments, and a thick layer of ribosomes (Fig. 13). The few cells that had been incompletely disrupted were at the bottom of the fraction.

Nuclear Pellet
This pellet consisted of nuclei that had retained their shape, envelope, and content (Fig. 14). Almost all the inner nuclear membrane (INM) appeared to be preserved, as well as the pores, whereas part of the outer nuclear membrane (ONM) seemed to be lacking. ONM profiles represented about 30% of the nuclear membrane profiles. A few mitochondria, seemingly trapped by the nuclear envelope, were observed. Mitochondrial profiles represented about 2% of the nuclear membrane profiles. No whole cells were seen in nuclear pellets. However, probable remnants of the plasma membrane could be seen attached to a few nuclei, and represented 1.5-2% of the membrane profiles.

Nuclear Membranes
Thin sections of LNM pellets showed variously sized unit-membrane vesicles, without adherent material or content, 0.1-1 μm in diameter, resembling those seen in bands 1 and 2 (Fig. 15). Pores were not recognized on these small vesicles. Freeze-etching revealed burgeoning vesicles with randomly dispersed particles (Fig. 16). Particle counts revealed several classes of vesicles, from almost barren membranes to vesicles rich in particles. HNM consisted of membrane vesicles, some with adherent fluffy material (Fig. 17). Freeze-etching revealed membrane vesicles with a great variety of shapes, sizes, and particle frequencies, among which the particle-rich surfaces (Fig. 18, arrow) strongly resembled the P face of INM.

DISCUSSION
The method presented here aims at separating, in high yield, purified plasma and nuclear membranes from isolated lymphocytes. It has been worked out with calf thymocytes, but is suitable for fractionation of circulating T or B cells. The assessment of plasma membrane purity and yield did not rely on specific immunological
Figure 15  Thin section of light nuclear membranes fixed in suspension. Some of the vesicles are very contorted or burgeoning. Bar, 1 #m. x 37,000.

Figure 16  Freeze-fracture of light nuclear membranes fixed in suspension. Particles are rather sparse. Bar, 0.2 #m. x 72,000.

Figure 17  Thin section of heavy nuclear membranes fixed in suspension. Fluffy dense material is attached to some of the vesicles. Bar, 1 #m. x 15,000.

Figure 18  Freeze-fracture of heavy nuclear membranes. The vesicles are either poor, or very rich in particles. The arrow points to a surface reminiscent of the P face of the INM. Bar, 0.2 #m. x 72,000.

In freeze-fracture micrographs, the direction of shadowing is indicated by an arrow within a closed circle.
markers which would depend on the developmental or functional state of the cells. It was based on the enrichment and yield of enzymes having a highly specific, basic function, and therefore, a unique cellular localization. The advantages of an analytical, balance sheet approach have been extensively discussed (15). In this respect, because the nuclear envelope is the other major membrane system of thymocytes, it was essential to isolate and study it by comparison with plasma membranes.

The three best plasma membrane marker enzymes in thymocytes appeared to be alkaline phosphatase, γ-glutamyltransferase, and ouabain-sensitive-ATPase, all known ectoenzymes. If the biological significance of the last two enzymes is understood, this is not yet the case for alkaline phosphatase. The finding that both the yield and enrichment of alkaline phosphatase in fractions 1 and 2 were higher than those of the other plasma membrane markers is as yet unexplained. It can possibly reflect a heterogeneous enzyme distribution among the plasma membrane vesicles, alkaline phosphatase purifying preferentially with the lightest fractions. However, an activation of the enzyme in such fractions cannot be excluded. 5’- and 3’-nucleotidases were recovered mainly, but not exclusively, in the plasma membrane fractions. 5’-nucleotidase has been shown to occur in the endoplasmic reticulum of liver cells (49). 3’-nucleotidase has been detected in rat liver plasma membranes, where it is thought to be part of a ribonucleic acid-degrading system of enzymes (51). As thymocytes reutilize the nucleic acids of the many dead cells normally found in the thymus, this enzyme could well play a similar role in thymocytes. However, some soluble activity was detected suggesting the existence of two different 3’-nucleotidases in the cell.

Enzymes specific for nuclear membranes are not known. Because the outer membrane of the lymphocyte nuclear envelope represents the major part of the endoplasmic reticulum of this type of cells, it was expected that the nuclear membrane fraction would contain substantial amounts of esterase and NADH-cytochrome c reductase. This was the case, but the enrichment and yield of esterase were comparatively higher, suggesting for this enzyme a specific function localized in the nuclear envelope.

Based on enzymatic data, the calculated enrichment and yield of plasma membranes provided by our method were high; within 3 h from the cell collection, 60–80% of the plasma membranes were obtained in a fraction containing 1.6% of the cellular proteins. Such enrichment factors and yields are higher than those obtained by other published methods. Table VII summarizes the results found in the literature. In relation to these comparative data, a few methodological problems that occur in lymphocyte subcellular fractionation will be discussed.

Firstly, because of the complexity of organs in the immune system, purification of the relevant cells is a prerequisite. This precludes the use of disrupting techniques in which whole tissues are subjected to disruption (2, 3, 24).

Secondly, due to the small size of thymocytes and the comparatively huge volume occupied by the nucleus in the cell, the difficulty of breaking these cells open without damaging the nuclei is well known. Yet a satisfactory yield of plasma membrane requires that all cells be disrupted, while nuclei stay undamaged. Homogenization of hypotonically shocked cells has been used (2, 3, 14, 25, 28, 31, 52), but the efficiency of the procedure has been evaluated in only one case where it was 25% (52). Nitrogen cavitation gave apparently better results (18, 39, 45), but the efficiency of breakage was not documented and the gelatinous character of the nuclear pellet indicated the lysis of nuclei (45). Specially devised lymphocyte cell disruptors have been described (41). Here we have shown that a simple homogenization in a motor-driven Potter homogenizer disrupted more than 90% of the cells and yielded structurally well-preserved nuclei, provided a viscous medium was used. This medium which was 1.8 M sucrose and 5 mM MgCl₂ probably accounted for the high cell breakage and the absence of DNA leakage.

Thirdly, a survey of the literature suggests that the first step generally used in plasma membrane isolation, a low-speed differential centrifugation designed to separate nuclei and mitochondria from other membranes, was responsible for important losses in plasma membranes; some authors used the pellet of this first centrifugation as their starting material (14, 52), whereas the majority of authors used the supernate. The very fact that both procedures yielded plasma membranes shows that the rationale for this step is not clear. In the method described in this paper, the absence of any differential centrifugation was probably one
TABLE VII

Main Features of Some Published Lymphocyte Plasma Membrane Purification Methods

| Source          | Preparation time (h) | Protein (%) | 5'-Nucleotidase Enrichment | Yield (%) | E.M. |
|-----------------|----------------------|-------------|----------------------------|-----------|------|
| Pig lymph node; human tonsils | 30 | 10 | 3/2 | 22-24 | 14-15 | 30-32 | 24 | 24 | 26 | 3-4 | 1½ | 3 |
| Human tonsils   | 1.6                  | 1.22        | 2.1                        | 0.75      | 0.5   | 0.7   | 1.5 | 0.6 | 0.67 | - | - | 1.6 |
| Pig lymph node  | 11.6, 3.9            | 13.8        | 25                         | 44        | 22    | 12.3  | 29  | 8-17 | 51  | 8  | 4  | 6-8 | 31-36 |
| Rat thymus      | 15-30                | 12          | 44.5                       | 35        | 9.5   | 7     | 18  | 14  | 31  | 4.6 | 0.7 | - | 55 |
| Calf thymus     | +                    | -           | -                          | +         | +     | +     | -   | +   | +   | +  | +  | +  | +  |

The "percent of protein" and the yields represent the recovery of proteins or of 5'-nucleotidase from the homogenate.
of the main factors responsible for the high yield of plasma membrane obtained. Still, 20–40% of the plasma membrane vesicles were not recovered in the floated fractions, but stayed in fractions E and 4. Could the floated plasma membranes be still considered as representative of the whole cellular surface? The higher density of the un-floated vesicles could be explained either by the presence of cytoplasmic content or by a special composition of the membrane vesicles. In the latter case, a selection of some plasma membrane areas in floated vs. dense fractions would have occurred. Thymocytes, however, do not have structural plasma membrane specializations. Freeze-fracture did not reveal a higher density of intramembranous particles in the denser vesicles as compared to the lighter. The first explanation, cytoplasmic trapping, therefore seemed most likely.

Another problem concerning quantitative recoveries of marker enzymes is related to the orientation, or sidedness, of the membranes in the isolated vesicles. Nonpermeant substrates should indeed be available to ectoenzymes only in “right-side out” vesicles. In our case, repeated freezing and thawing did not enhance significantly the enzymatic recoveries, suggesting that plasma membrane vesicles were mostly “right-side out”. Freeze-etching has been used to differentiate P from E faces of isolated vesicles (26). In our case, the clumping of some intramembranous particles made the use of this method hazardous. Lectin columns have been used by Walsh and co-workers, but they do not allow a separation of plasma membrane “inside-out” vesicles from Golgi vesicles, the presence of which was not searched for (46). In our case, we have demonstrated that plasma membranes and Golgi vesicles co-purify to some extent.

In the method presented here, as in most other subcellular fractionation techniques, homogenization and processing of cells and fractions were performed at 4°C. It is possible that a temperature-induced phase separation of membranes into particle-rich and particle-poor areas occurs, especially in the case of the rather fluid plasma membrane of isolated cells. Chilling below 22°C has been shown to produce by itself smooth areas on both faces of the two leaflets of the lymphoid cell nuclear envelope (50). Clumps of intramembranous particles were seen in our freeze-fractured preparations of plasma and nuclear membranes. Such structural modifications should be kept in mind as the relative topology of some elements, such as receptors and adenyl-cyclase molecules, might be changed.

However, a high degree of purity of lymphocyte plasma membranes, as well as a high recovery, can be attained by a simple and rapid procedure. Such membrane fractions should be useful to raise antilymphocyte sera containing antibodies directed mainly against cell surface components. They also represent the first purification step in the isolation of specific surface molecules.

In the companion paper, we show by sodium dodecyl sulfate-gel electrophoresis that some proteins are characteristic of plasma membrane fractions, whereas others are found only in nuclear membranes or in microsomes (34).

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This paper is dedicated to the memory of Dr. Jean-Pierre Tranzer.

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