Biochemical and Enzymatic Characterization of Thymic and Splenic Lymphocyte Plasma Membranes from Inbred Rats†

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ABSTRACT: Purified splenic and thymic lymphocytes from the ACI and F344 strains of inbred rats were disrupted by controlled hypotonic treatment, and their plasma membranes were prepared by sucrose density gradient centrifugation. The plasma membrane preparations were highly purified as judged by the structural appearance of the smooth membrane vesicles, by the 10- to 15-fold enrichment of 5′-nucleotidase, which cytochemically localized exclusively in the plasma membranes of intact lymphocytes, by the high cholesterol to phospholipid molar ratio (0.7–1.0), and by the very low specific activities of the enzymes associated predominantly with mitochondria, lysosomes, and endoplasmic reticulum. The protein and the lipid contents of the membranes were 48–55 and 37–48%, respectively. The total lipid content of plasma membranes was characteristically higher in thymic than splenic lymphocytes from both ACI and F344 strains. The specific activity of 5′-nucleotidase was similar in splenic lymphocyte membranes of the ACI strain, and in both the thymic and splenic lymphocyte membranes of the F344 strain. In contrast, the thymic lymphocyte membranes in the ACI strain showed half as much 5′-nucleotidase specific activity. Cytochemical results indicated that the 5′-nucleotidase is located on the outside surface of the lymphocyte plasma membranes.

The biological role of lymphoid cells involves antigenic recognition, antibody synthesis, and the mediation of cellular immune reactions. The evidence for the importance of the lymphoid cell membranes in these reactions has been reviewed recently (Warner, 1974). Previous studies on the mechanism of action of antigen and on the genetic control of the antibody response in rats (Gill et al., 1970) led us to undertake a detailed chemical and immunochemical investigation of the membranes of lymphocytes from these animals in order to explore the genetically controlled differences in their structure and reactivity.

This paper describes a new isolation procedure for lymphocyte membranes. The cells were disrupted by controlled hypotonic treatment, and the membranes were isolated by sucrose gradient centrifugation. Comparative chemical and enzymic analyses of the isolated membranes from splenic and thymic lymphocytes of the ACI and F344 strains of genetically inbred rats of both sexes were performed. The results showed some characteristic biochemical and enzymic differences between these membranes. Cytochemical studies showed that the enzyme 5′-nucleotidase is located exclusively in the plasma membranes of the lymphocytes and can serve as a good marker enzyme.

Materials and Methods

Separation of Lymphocytes and Preparation of Plasma Membranes. Male and female inbred ACI and F344 rats, 8 to 10 weeks old, were used. Spleens and thymuses from 15 to 20 animals were removed and immediately put in 15–20 ml of 10 m*M* Tris-0.15 *M* NaCl (TBS),[1] pH 7.4 at 4°. The organs were minced finely, and a cell suspension was obtained by pressing the minced tissue gently with a spatula through a coarse stainless steel mesh with dropwise addition of TBS. The cell suspensions were made up to 40–50-ml with TBS, and 3–4-ml aliquots were put on 4–5 ml of a 1:2 solution of 39% (v/v) Hypaque and 9% (w/v) Ficoll (density 1.16 g cm^{-3}) and centrifuged at $400g_{av}$ for 30 min. The interface was removed, washed twice with TBS, and suspended in 10 ml of TBS. All of the cells were viable as measured by the Trypan Blue dye exclusion test, and they were small and medium-sized lymphocytes without any contamination by erythrocytes or macrophages. It was essential to dilute the original cell suspension to 40–50 ml and to use only 3–4 ml of the diluted suspension on the Hypaque–Ficoll gradient solution in each tube in order to remove all red cells.

The washed lymphocytes were suspended in 10 ml of TBS at 0°, and 2 vol of 10 m*M* Tris-HCl (pH 7.4) was added slowly while stirring the cell suspension. The mixture was allowed to stand at 0° for 5 min, and then 10 m*M* Tris–1.5 *M* NaCl was added to restore the NaCl molarity to 0.15. The cells were homogenized in a loosely fitting glass homogenizer with a Teflon rod using 10 to 15 gentle strokes. The homogenate was centrifuged at $300g_{av}$ for 15 min to remove the cell nuclei. The sediment was resuspended in 30 ml of TBS, homogenized further with 5 strokes, and centrifuged as before. The two supernatants, free of nuclei, were combined and centrifuged at $4000g_{av}$ for 20 min to remove the mitochondria. The mitochondrial pellet was resuspended in 30 ml of TBS and centrifuged again at $4000g_{av}$ for 20 min. The two supernatants, free of nuclei and mitochondria, were combined and centrifuged at

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[1] Abbreviations used are: NADH, reduced nicotinamide adenine dinucleotide; 5′-AMP, adenosine 5′-phosphate; 2′-AMP, adenosine 2′-phosphate; 3′,5′-AMP, adenosine 3′,5′-cyclic phosphate; TBS, 10 m*M* Tris–0.15 *M* NaCl (pH 7.4).

20,000g_{av} for 1 hr. The pellet was dissolved in 20 ml of 10 m*M* Tris-HCl (pH 7.4) mixed with an equal volume of 40% (w/v) sucrose in the same buffer, and homogenized with 5 gentle strokes. The material was layered on a 25–50% (w/v) discontinuous sucrose gradient with 5% steps in three cellulose nitrate tubes and centrifuged in a Beckman SW 25.1 rotor at 4° for 15–18 hr at 88,000g_{av}. The different interfaces were removed in 30–40 ml of 10 m*M* Tris-HCl buffer (pH 7.4) and centrifuged at 20,000g_{av} for 1 hr. This step was repeated twice, and the final pellets were dissolved in the Tris buffer for subsequent studies.

The material at the 30–35 and 35–40% sucrose interfaces consisted entirely of smooth membrane vesicles, as seen by electron microscopy, and it had a high cholesterol content, the highest specific activity of 5′-nucleotidase, and the lowest content of nucleic acids and of the enzymes associated with mitochondria, lysosomes and endoplasmic reticulum. The interfaces were combined and used as the purified plasma membrane fraction. Subsequently, the sucrose gradient step was simplified: the microsomal fraction (20,000g_{av} pellet) was mixed with an equal volume of 60% sucrose, layered on 40% sucrose solution, and centrifuged; the 30–40% interface was taken as the plasma membrane fraction.

Biochemical Analyses. The total protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. RNA and DNA were extracted by the procedure of Munro and Fleck (1966). The RNA concentration was estimated spectrophotometrically by assuming an absorbance of 1.00 at 260 nm for 32 μg/ml of RNA, and the DNA was determined by the diphenylamine method (Burton, 1956) using calf-thymus DNA as the standard. Carbohydrate was estimated by the anthrone reaction (Scott and Melvin, 1953). Lipid was extracted by the method of Folch et al. (1957), and the total lipid was determined gravimetrically as well as colorimetrically (Bragdon, 1951). Cholesterol was determined by the method of Bowman and Wolf (1962). Lipid phosphorus was determined by the method of Ames (1966), and the phospholipid content was estimated assuming 15 μg of phospholipid per μg of phosphorus (Kopaczyk et al., 1966).

Enzymatic Assays. The 5′-nucleotidase (EC 3.1.3.5) was assayed by the method of Michell and Hawthorne (1965). Glucose-6-phosphatase (EC 3.1.3.9) and acid phosphatase (EC 3.1.3.2) activities were estimated by the potassium fluoride–EDTA inhibition method (Hübscher and West, 1965). The inorganic phosphorus released in these reactions was determined by the method of Ames (1966). Succinate dehydrogenase (EC 1.3.99.1) and NADH dehydrogenase (EC 1.6.99.3) were assayed by the method of Earl and Korner (1965) and Wallach and Kamat (1966), respectively. All enzymes were assayed within 24 hr of membrane isolation.

Electron Microscopy. The various samples were fixed at 4° for 1 hr in 3% phosphate-buffered glutaraldehyde. Small blocks were then post-fixed with 1% phosphate-buffered OsO_4 for 2 hr at 4°, dehydrated in cold ethanol, and embedded in an Epon–Araldite mixture. Thin sections were stained with 3% uranyl acetate in water for 30 min and with lead citrate for 10 min. The electron micrographs were taken with a Phillips EM 300 at 80 kV utilizing the double condenser system.

Cytochemical Localization of 5′-Nucleotidase. Washed lymphocytes were fixed for 2 hr at 4° in 3% glutaraldehyde or 3% formaldehyde prepared from paraformaldehyde and buffered to pH 7.5 with 100 m*M* Tris-acetate. The cells were then washed with six changes of the cold buffer over a period of 3 hr and incubated with 2 m*M* 5′-AMP, 2 m*M* $Mg(NO_3)_2$, and 2 m*M* $Pb(NO_3)_2$ at 37° for 30 min to 1 hr. In order to avoid precipitation, lead nitrate was added to the incubation mixture while stirring in a vortex mixer. Controls included cells incubated with 5′-AMP and magnesium nitrate, with 5′-AMP and lead nitrate, or with magnesium nitrate and lead nitrate under identical conditions. Another set of controls utilized cells incubated with 2′-AMP or 3′,5′-AMP, which are analogs of 5′-AMP, in the presence of magnesium nitrate and lead nitrate. After incubation, the cells were washed with Tris-acetate buffer and post-fixed with 1% OsO_4 for 2 hr at 4°. Electron micrographs were taken of the thin sections with and without uranyl acetate and lead citrate staining. Similar procedures were followed for isolated membranes.

Chemicals used included: sodium diatrizoate (Hypaque), Winthrop Laboratories, New York, N.Y.; Ficoll, Pharmacia Fine Chemicals, Uppsala, Sweden; sodium β-glycerophosphate and 2,6-dichloroindophenol (sodium salt), Fisher Scientific Co., Pittsburgh, Pa.; β-nicotinamide adenine dinucleotide (reduced form) and glucose 6-phosphate, Sigma Chemical Co., St. Louis, Mo.; 5′-AMP, 2′-AMP, and 3′,5′-AMP, P-L Biochemicals, Milwaukee, Wis.; glutaraldehyde, Ladd Research Industries, Burlington, Vt.

Results

Membrane Preparation. The purified cells from spleen and thymus consisted of small and medium sized lymphocytes free of any significant contamination by macrophages, granulocytes, or red cells. The splenic and the thymic lymphocytes, as observed by electron microscopy in thin sections, showed a clear difference in their surface characteristics. Most of the thymic lymphocytes showed a smooth contour, but the splenic lymphocytes had quite characteristic surface projections (Figures 1A and B). Thirty micrographs containing splenic or thymic lymphocytes were scanned, and 140 of 300 spleen cells and 31 of 300 thymic cells showed these projections.

The lymphocytes purified on the Hypaque–Ficoll gradient showed only 30–40% disruption by Trypan Blue staining even after extensive homogenization in TBS. The yield of membrane from this material was 0.5–0.6% of the homogenate protein, which was less than the yield obtained by Allan and Crumpton (1970) by the same membrane purification technique but without prior cell purification on Hypaque–Ficoll gradients. In contrast, the controlled hypotonic treatment disrupted virtually all cells and improved the yield and the quality of the membranes, as judged by electron microscopy and by enzymatic analyses. The hypotonic treatment resulted in larger membrane vesicles than those obtained by cell homogenization in isotonic media.

In order to explore the extent of cell disruption by the hypotonic treatment, equal numbers of cells were incubated in different concentrations of NaCl at 0° for 5 min. The cells were examined by the Trypan Blue test after restoration of NaCl molarity to 0.15 *M*. The results are shown in Figure 2: 75–80% of the cells were partially broken after incubation in 50 m*M* NaCl. This method did not show any disruption of the intracellular organelles. An electron micrograph of the nuclear pellet (Figure 1C) showed that the nuclear structure was maintained.

The 4000g_{av} pellet (Figure 1D) showed intact mitochondria and other cytoplasmic organelles in the form of smooth

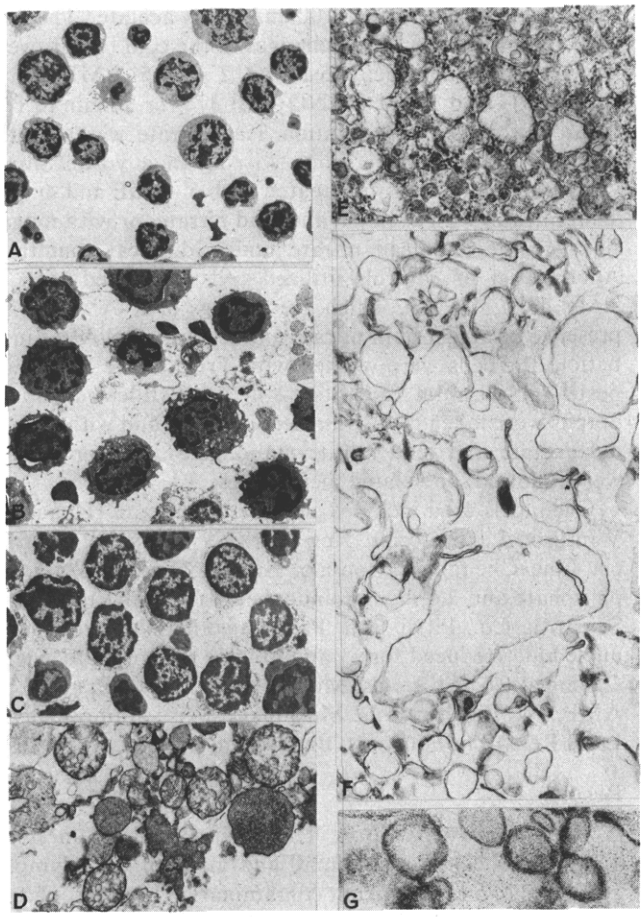

FIGURE 1: (A) Thymic lymphocytes show smooth contour (×1250). (B) Splenic lymphocytes show surface projections (×1700). (C) Nuclear pellet reveals that most of the nuclei are intact (×1850). (D) Mitochondrial pellet shows intact mitochondria with other cytoplasmic organelles (×7400). (E) Microsomal pellet shows smooth and rough membranes without any mitochondrial contamination (×17,800). (F) Plasma membrane fraction shows vesicles of smooth membranes without any rough membranes or free microsomes (×19,650). (G) Membrane fraction at a higher magnification displays this typical membrane triple-layered structure (×66,900).

membranes, lysosomes, and sometimes rough endoplasmic reticulum. The microsomal pellet (Figure 1E) contained smooth membrane vesicles, rough endoplasmic reticulum, and dense amorphous material. The 50% sucrose pellet and the 45–50 and 40–45% sucrose interfaces contained smooth membrane vesicles, rough endoplasmic reticulum, and other amorphous structures in different proportions. The 35–40 and 30–35% sucrose interfaces consisted almost entirely of closed smooth membrane vesicles and a few amorphous structures. These fractions were free from any visible contamination by mitochondria, rough endoplasmic reticulum, or free ribosomes. A typical micrograph of the 30–40% interface is shown in Figure 1F. A high magnification micrograph of this sample (Figure 1G) demonstrates the triple-layered membrane structure. The 25–30 and 20–25% sucrose interfaces, plus the 20% sucrose top, contained a very small amount of material. In the thymic membrane preparations, these three fractions contained 0.2–0.3% of the protein in the homogenate, and the splenic preparations had 0.1% or less. These materials, which showed some smooth vesicles, low 5′-nucleotidase activity, and high lipid content, were not analyzed further.

Chemical and Enzymatic Analyses. The data in Table I represent the average of two typical experiments, each having about 5×10^9 lymphocytes isolated from 20 spleens. The yield of protein in the membrane fraction was more than 1% of the total homogenate protein. About 1 mg of membrane protein was obtained from 10^9 splenic lymphocytes, but only about 0.5–0.6 mg of protein was obtained from the same number of thymic cells. As shown in Table I, 75–80% of the total DNA was recovered in the $300g_{av}$ pellet. This corroborated the electron microscopic finding that very few of the nuclei were disrupted during the hypotonic treatment and subsequent homogenization. The RNA content of the microsomal supernatant was 45–55% of the homogenate RNA. The RNA to protein ratio in the $20{,}000g_{av}$ supernatant and in the 40% sucrose bottom (which contained mostly rough endoplasmic reticulum) was 2 to 3.5 times greater than in the membrane. The microsomal fraction ($20{,}000g_{av}$ pellet), which consisted mainly of the 40% sucrose bottom and the 30–40% sucrose interface (plasma membrane fraction), contained 15–20% of the homogenate cholesterol. The cholesterol to protein ratio was much higher in the plasma membrane than in any other fraction. The phospholipid content in the microsomal fraction was 15–20% of the homogenate, and the phospholipid to protein ratio also was highest in the plasma membrane fraction.

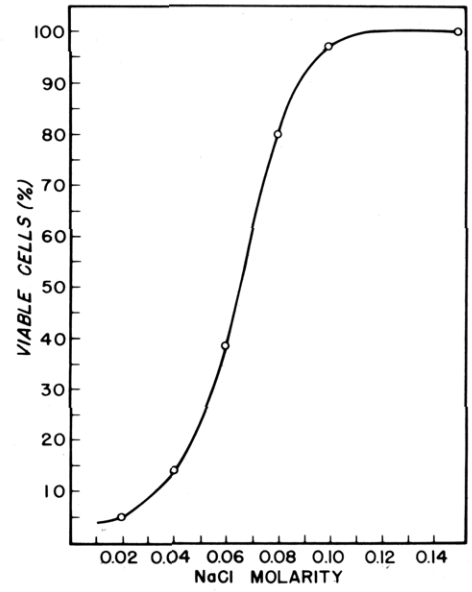

FIGURE 2: The curve shows the viability of splenic lymphocytes at different NaCl concentrations.

The five enzymes 5′-nucleotidase, succinate dehydrogenase, acid phosphatase, glucose-6-phosphatase, and NADH dehydrogenase were assayed in different subcellular fractions to estimate the purity of the membrane fraction and the amount of contamination by mitochondria, lysosomes, and endoplasmic reticulum (Table I). More than 40% of the 5′-nucleotidase in the homogenate was recovered in the microsomal fraction, and 40–50% of this was recovered in the plasma membrane fraction (the 30–40% sucrose interface). The specific activity in this fraction was 12 to 15 times higher than in the homogenate. About 40–50% of the succinate dehydrogenase, which is known to be associated with mitochondria, was recovered in the mitochondrial fraction where its specific activity was highest. Less than 0.05% of the total homogenate activity was recovered in the plasma membrane fraction. The specific activity in the mitochondrial fraction was 50 to 100 times greater than in the membrane fraction. The mitochondrial fraction contained about 60% of the total acid phosphatase activity and showed the

Table I: Chemical and Enzymic Analyses of the Different Subcellular Fractions of Rat Spleen Lymphocytes.[a]

	Homogenate	Nuclear Pellet	Mitochondrial Pellet	$20,000g_{av}$ Supernatant	Sucrose Bottom	Plasma Membrane	Total Recovery (%)
Protein							
Recovery (%)	100	11.1	26.5	39.7	9.2	1.47	87.0
DNA							
Recovery (%)	100	78.9	13.7	4.47	0.93	0.21	98.2
μg/mg of protein	57.9	412	29.8	6.50	5.87	8.05	
RNA							
Recovery (%)	100	7.46	23.1	53.0	11.8	0.87	96.2
μg/mg of protein	41.8	28.2	36.4	45.6	53.6	24.6	
Cholesterol							
Recovery (%)	100	6.99	27.7	18.7	11.7	7.98	73.1
μg/mg of protein	42.8	27.2	45.1	20.1	54.4	232	
Phospholipid							
Recovery (%)	100	8.20	27.8	17.1	15.3	5.35	73.7
μg/mg of protein	147	109	154	63.3	245	534	
Cholesterol/phospholipid (molar ratio)	0.58	0.50	0.59	0.64	0.44	0.87	
5′-Nucleotidase							
Recovery (%)	100	6.43	39.0	15.4	22.9	14.30	98.0
Specific activity	0.67	0.39	0.99	0.26	1.67	9.49	
Succinate dehydrogenase							
Recovery (%)	100	8.36	43.7	16.6	3.03	0.05	71.7
Specific activity	0.216	0.163	0.356	0.090	0.071	0.007	
Glucose-6-phosphatase							
Recovery (%)	100	8.07	17.2	59.2	8.26	0.77	93.5
Specific activity	0.266	0.193	0.173	0.396	0.239	0.139	
Acid phosphatase							
Recovery (%)	100	4.91	57.3	18.7	18.5	0.83	100.2
Specific activity	1.08	0.48	2.34	0.51	2.18	0.60	
NADH dehydrogenase							
Recovery (%)	100	8.05	51.9	18.2	10.3	1.08	89.5
Specific activity	11.57	8.41	22.68	5.30	12.92	8.47	

[a] The experiments were performed with material obtained from 20 spleens yielding about 5×10^9 lymphocytes and the results represent the average of two typical experiments. Absolute yields from homogenate were: 234 mg of protein, 13.5 mg of DNA, 9.8 mg of RNA, 10.0 mg of cholesterol, 34.4 mg of phospholipid. Total activities of the enzymes in homogenate, expressed as micromoles of product liberated per hour, were: 5′-nucleotidase, 157; succinate dehydrogenase, 50.5; glucose-6-phosphatase, 62.2; acid phosphatase, 253; NADH dehydrogenase, 2707. Specific activities were expressed as micromoles of product liberated per hour per milligram of protein.

highest specific activity. The membrane fraction contained less than 1% of the total homogenate content of acid phosphatase. The specific activity of this enzyme was 4 to 5 times greater in the mitochondrial fraction than in the plasma membrane fraction. The largest proportion of the glucose-6-phosphatase activity (50–60%) was in the $20,000g_{av}$ supernatant. The plasma membrane fraction contained less than 1% of the total activity. The specific activity in the different subfractions was relatively evenly distributed; only the specific activity in the membrane fraction was about one-third of that in the $20,000g_{av}$ supernatant. The mitochondrial fraction contained 40–50% of the NADH dehydrogenase activity, and the plasma membrane contained 1% or less. The specific activity in the membrane fraction was about one-third of that in the mitochondrial fraction.

Biochemical and Enzymic Composition of the Isolated Membranes. Tables II and III contain the results of biochemical and enzymatic analyses of the plasma membrane fractions of the splenic and thymic lymphocytes of the two strains. The DNA content in all the samples was very low (5 to 10 μg/mg of protein), and the content of RNA ranged from 20 to 37 μg/mg of protein. The cholesterol to phospholipid molar ratio in most of the samples was close to 0.9. Table IV summarizes the chemical compositions of the different samples and shows that they were all very similar. The DNA contamination in all the membranes was very low (0.2–0.5%), and the RNA content was within 1–2%. The carbohydrate content varied from 2 to 5%. The protein content ranged from 48 to 55%, and the lipid content, from 37 to 48%. In males and females of each strain, the thymic membranes had a slightly, but consistently, higher total lipid content than the splenic membranes. The percentage of protein and of carbohydrate was greater in the splenic cell membranes.

Figure 3 shows the variation in 5′-nucleotidase activity in the plasma membrane with respect to pH. Both splenic and thymic lymphocyte membranes showed a similar broad pH optimum. The enzyme activity is highly dependent upon divalent cations, since the addition of EDTA almost completely abolished the activity. With the substrates 2′-AMP and 3′,5′-AMP, no enzymatic activity was found in the plasma membranes over the same pH range; hence, the membranes do not contain nonspecific phosphatase activity. Some characteristic organ differences in the mean 5′-nucleotidase activities are shown in Tables II and III. In the ACI strain twice as much activity was found in splenic as in thymic lymphocyte membranes. However, in the F344 strain the mean values found for thymic lymphocyte membranes were similar to or slightly higher than those found for splenic membranes. These mean differences for the F344 strain were not statistically significant, although within individual experiments, as shown in Table V and discussed below, the activity in thymic membranes was always significantly higher.

Table II: Comparison of the Chemical and Enzymic Composition of the Isolated Plasma Membrane Fractions of Thymocytes and Splenic Lymphocytes from F344 Female and Male Rats.[a]

Chemical Components and Enzymes	F344 Female		F344 Male	
	Spleen	Thymus	Spleen	Thymus
DNA (μg/mg of protein)	9.50 ± 1.46 (3)	3.70 ± 1.49 (3)	8.07 ± 1.91 (3)	5.03 ± 1.27 (3)
RNA (μg/mg of protein)	20.3 ± 1.00 (4)	26.7 ± 1.00 (4)	24.6 ± 1.90 (3)	26.4 ± 1.45 (3)
Carbohydrate (μg/mg of protein)	52.0 ± 4.77 (4)	47.2 ± 4.59 (4)	101.1 ± 6.67 (5)	82.7 ± 2.65 (5)
Lipid (μg/mg of protein)	833 ± 47.4 (3)	992 ± 44.4 (3)	772 ± 36.0 (4)	825 ± 91 (5)
Cholesterol (μg/mg of protein)	178 ± 18.4 (4)	213 ± 13.1 (5)	228 ± 6.04 (3)	232 ± 15.1 (3)
Phospholipid (μg/mg of protein)	529 ± 35.8 (3)	561 ± 50.1 (3)	500 ± 38.7 (3)	534 ± 18.3 (3)
Cholesterol/phospholipid (molar ratio)	0.67 ± 0.03	0.76 ± 0.04	0.91 ± 0.04	0.87 ± 0.03
5′-Nucleotidase	10.39 ± 1.33 (5)	10.92 ± 1.74 (6)	8.06 ± 1.13 (5)	11.4 ± 2.36 (5)
Glucose-6-phosphatase	0.319 ± 0.056 (3)	0.622 ± 0.068 (3)	0.139 ± 0.026 (3)	0.471 ± 0.030 (3)
Acid phosphatase	3.23 ± 0.16 (3)	2.66 ± 0.23 (3)	0.78 ± 0.16 (3)	0.94 ± 0.11 (3)
Succinate dehydrogenase	0.015 ± 0.007 (3)	0.032 ± 0.012 (3)	0.002 ± 0.002 (3)	0.005 ± 0.005 (3)
NADH dehydrogenase	8.10 ± 0.84 (3)	8.24 ± 0.33 (3)	8.47 ± 1.17 (3)	6.03 ± 1.27 (3)

[a] 10^9 splenic lymphocytes yielded 40–50 mg of homogenate protein, and an equal number of thymocytes yielded 30–40 mg of homogenate protein. The specific activities represent micromoles of product liberated per hour/per milligram of protein. The phospholipid molecular weight was assumed to be 775. The results are presented with standard deviations (SD), and the number of experiments is given in parentheses.

Table III: Comparison of the Chemical and Enzymic Composition of the Isolated Plasma Membrane Fractions of Thymocytes and Splenic Lymphocytes from ACI Female and Male Rats.[a]

Chemical Components and Enzymes	ACI Female		ACI Male	
	Spleen	Thymus	Spleen	Thymus
DNA (μg/mg of protein)	6.48 ± 1.68 (8)	7.97 ± 1.62 (8)	5.30 ± 2.69 (3)	8.00 ± 2.01 (3)
RNA (μg/mg of protein)	36.8 ± 4.81 (8)	33.6 ± 7.07 (8)	32.7 ± 0.62 (3)	32.3 ± 0.51 (3)
Carbohydrate (μg/mg of protein)	93.3 ± 9.78 (9)	82.1 ± 13.24 (9)	96.6 ± 10.79 (3)	70.2 ± 7.11 (3)
Lipid (μg/mg of protein)	678 ± 39.7 (8)	807 ± 50.8 (9)	793 ± 39.7 (3)	975 ± 60.1 (3)
Cholesterol (μg/mg of protein)	168 ± 19.4 (9)	235 ± 31.9 (7)	234 ± 2.8 (7)	223 ± 6.1 (7)
Phospholipid (μg/mg of protein)	381 ± 32.2 (7)	481 ± 24.3 (7)	533 ± 5.8 (3)	493 ± 3.5 (3)
Cholesterol/phospholipid (molar ratio)	0.88 ± 0.06	0.98 ± 0.07	0.88 ± 0.01	0.90 ± 0.01
5′-Nucleotidase	10.19 ± 1.46 (12)	5.20 ± 1.92 (14)	10.23 ± 2.35 (7)	5.81 ± 1.88 (7)
Glucose-6-phosphatase	0.131 ± 0.033 (6)	0.237 ± 0.035 (5)	0.505 ± 0.16 (3)	0.634 ± 0.173 (5)
Acid phosphatase	1.18 ± 0.18 (5)	0.79 ± 0.08 (5)	1.88 ± 0.14 (3)	2.61 ± 0.11 (3)
Succinate dehydrogenase	0.024 ± 0.004 (10)	0.029 ± 0.006 (12)	0.007 ± 0.004 (4)	0.020 ± 0.008 (4)
NADH dehydrogenase	13.30 ± 3.14 (10)	11.43 ± 2.20 (10)	3.03 ± 0.35 (4)	3.20 ± 0.14 (4)

[a] Like the F344 rats, 10^9 purified lymphocytes yielded 40–50 mg of homogenate protein in the case of the splenic cells, and 30–40 mg of homogenate protein in the case of thymocytes. The results are presented as described in Table II.

The quantitative significance of the compositional differences shown in Tables II and III is illustrated in Table V: in each experiment, spleens and thymuses were taken from the same animals, membranes prepared, and both enzymic and chemical estimations done simultaneously. The observed differences in the 5′-nucleotidase activity were highly significant. In most of the experiments, the difference in lipid composition between the splenic and thymic membranes was significant. The differences in carbohydrate content were not significant, although the thymic membranes were consistently found to contain slightly less carbohydrate than the splenic membranes. The differences in total lipid content were also reflected by differences in the phospholipid and cholesterol content in most of the cases (Tables II and III).

Analysis of phospholipid composition by thin-layer chromatography was done on ACI female splenic lymphocyte membranes, and the relative amounts of each type were: phosphatidylethanolamine, 22%; phosphatidylserine and phosphatidylinositol combined, 15.7%; lecithin, 50.3%; and sphingomyelin, 12%. The choline-containing phospholipids (lecithin and sphingomyelin) comprised about 62% of the total membrane phospholipid, and the ratio of lecithin to sphingomyelin was 4:1. These results show a remarkable similarity with the lipid composition of rat erythrocyte membranes (Nelson, 1967).

Cytochemical Localization of 5′-Nucleotidase. The cells incubated in the presence of lead, magnesium, and 5′-AMP showed reaction products on their plasma membranes. In the cells fixed with glutaraldehyde, deposits of the reaction products on the membrane were discontinuous (Figures 4A and B) and on some cells (Figure 4A) the grains were finer than on others (Figure 4B). In cells fixed with formaldehyde, reaction products on the membranes were relatively continuous (Figure 4C). When a large number of cell sections were scanned, very fine granules were found in the heterochromatin of some of the cells fixed with glutaraldehyde (Figure 4D), while such granules were absent from others (Figure 4E). No nuclear staining was found in cells fixed with formaldehyde (Figure 4C). The cells incubated

Table IV: Relative Chemical Compositions as Percent of the Plasma Membrane Fraction of Splenic and Thymic Lymphocytes from Rats of the Two Strains and Both Sexes.

Membrane Composition	ACI Female		ACI Male		F344 Female		F344 Male	
	Spleen	Thymus	Spleen	Thymus	Spleen	Thymus	Spleen	Thymus
Protein	55.1	51.8	51.9	48.0	52.2	48.3	52.5	51.6
Lipid	37.4	41.8	41.1	46.8	43.5	47.9	40.5	42.5
Carbohydrate	5.14	4.25	5.01	3.37	2.70	2.28	5.30	4.26
RNA	2.03	1.74	1.70	1.55	1.06	1.29	1.29	1.27
DNA	0.36	0.41	0.27	0.38	0.50	0.18	0.42	0.26

Table V: Comparison of Chemical Compositions of the Splenic and Thymic Cell Membranes.[a]

Strain and Sex	Expt. No.	5′-Nucleotidase (μmole of P_i/hr per mg of Protein)			Lipid (μg/mg of Protein)			Carbohydrate (μg/mg of Protein)		
		Spleen	Thymus	Significance[b]	Spleen	Thymus	Significance[b]	Spleen	Thymus	Significance[b]
ACI	I	7.6 (1)	4.4 (1)					103.0 ± 12.5 (2)	88.5 ± 1.1 (2)	–
female	II	9.8 ± 0.24 (3)	2.9 (1)		607 ± 11 (2)	924 ± 45 (2)	+	113.2 ± 14.8 (2)	105.7 ± 2.8 (2)	–
	III	9.4 ± 0.50 (2)	6.3 ± 0.22 (2)	+	681 ± 22 (2)	757 ± 35 (2)	+	96.6 ± 5.5 (3)	72.2 ± 12.6 (3)	±
	IV	10.2 ± 0.21 (3)	4.8 ± 0.10 (3)	+	677 (1)	738 ± 55 (3)		75.1 ± 3.3 (2)	57.1 ± 3.0 (2)	+
ACI	I	12.1 ± 0.21 (3)	6.5 ± 0.29 (3)	+	793 ± 39 (3)	974 ± 61 (3)	+	82.6 ± 16.0 (3)	70.6 ± 14.3 (3)	–
male	II	9.1 ± 0.46 (4)	7.1 ± 0.27 (4)	+	728 ± 59 (3)	816 ± 55 (3)	+	96.7 ± 10.8 (3)	70.2 ± 7.2 (3)	+
F344	I	13.0 ± 0.17 (3)	13.8 ± 0.07 (3)	+	811 ± 41 (2)	967 ± 6 (2)	+	53.8 ± 2.2 (2)	48.9 ± 7.1 (2)	–
female	II	11.6 ± 0.54 (6)	12.9 ± 0.16 (5)	+	829 ± 47 (2)	1003 ± 51 (2)	+	50.2 ± 7.1 (2)	45.5 ± 1.0 (2)	–
F344	I	6.6 ± 0.14 (5)	8.1 ± 0.27 (5)	+	842 ± 14 (3)	900 ± 4 (3)	+	80.4 ± 8.9 (4)	67.7 ± 3.5 (4)	±
male	II	5.1 ± 0.22 (3)	9.1 ± 0.30 (3)	+	736 ± 45 (2)	773 ± 52 (2)	–	101.1 ± 6.7 (3)	82.4 ± 2.9 (3)	±

[a] In each experiment as shown in column 2, spleens and thymuses were obtained from the same set of animals, the membranes prepared, and their chemical composition studied at the same time. The results are expressed with standard deviations, and the numbers in parentheses indicate the number of measurements on each sample. *P* value (significance) represents probability of difference between the two membranes. [b] +, $P \leq 0.01$; ±, $0.01 < P \leq 0.05$; –, $P > 0.05$.

with either lead and magnesium, 5′-AMP and magnesium, or 5′-AMP and lead did not show any precipitate (Figure 4G). Similarly, the cells incubated with the analogs 2′-AMP or 3′,5′-AMP in the presence of lead and magnesium did not show any reaction. The unstained sections and the sections stained with uranyl acetate and lead citrate gave similar results. Heavy reaction products similar to those found on the intact cells were found also on the free membrane vesicles which occurred in these preparations (Figure 4F).

Figure 5 presents the cytochemical results with the isolated plasma membranes. The membranes incubated with magnesium, lead, and 5′-AMP showed heavy deposition of reaction products on the vesicles (Figure 5A). The lead grains were localized mostly on the outer surface of the membrane vesicles (Figures 4F and 5B), although in some vesicles the reaction products were found on the inner surface as well. The control sample with magnesium and lead did not show any reaction product (Figure 5C). No difference could be detected between the thymic and splenic cells of the two strains.

When lymphocytes were mildly homogenized, fixed in glutaraldehyde, and incubated in the presence of lead, magnesium, and 5′-AMP, reaction products were found on the plasma membranes of the broken cells, but not on the cytomembranes, mitochondria, or nuclear membranes (Figure 6).

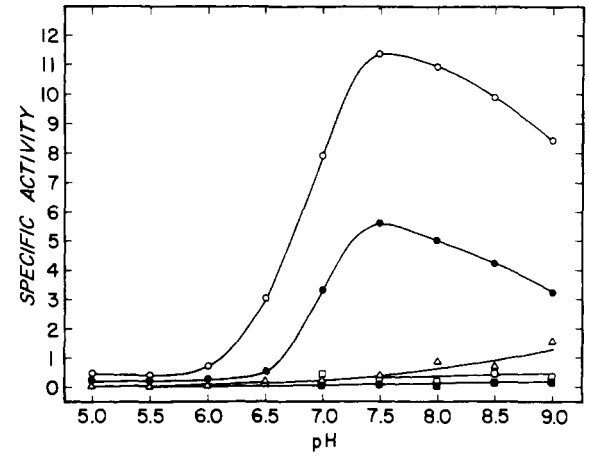

FIGURE 3: Specific activities of 5′-nucleotidase in the isolated plasma membranes at different pH values of the incubation medium. The incubation medium contained 100 m*M* Tris-HCl buffer, 100 m*M* KCl, 10 m*M* sodium potassium tartrate, 10 m*M* $MgCl_2$, and 5 m*M* of the respective substrates. The curves (○) and (●) represent the activity for ACI female splenic and thymic cell plasma membranes, respectively, with 5′-AMP. The curves show almost identical activity variation with respect to pH and have a broad pH optimum. The curve (△) shows the enzyme activity in the presence of 5 m*M* EDTA. The curves (□) and (■) represent the activities when 3′,5′-AMP and 2′-AMP were used, respectively, as substrates in place of 5′-AMP.

Discussion

Lymphocytes and Their Plasma Membrane Isolation. Goldschneider and McGregor (1973) and Balch and Feldman (1974) found that in the rat almost all (>95%) thymocytes and 35–50% of splenic lymphocytes have the immunologic properties of T-cells, and the other splenic lymphocytes are B-cells. We obtained similar results for the ACI

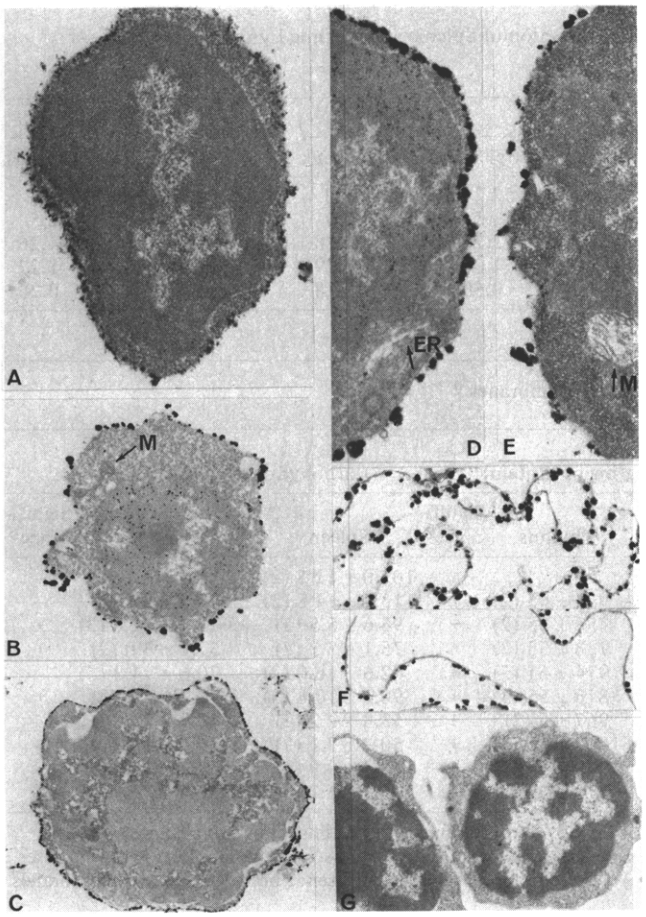

FIGURE 4: (A–F) Lymphocytes incubated in the presence of 5′-AMP, lead and magnesium showing a heavy precipitate of lead phosphate in the plasma membrane. Some cells fixed in glutaraldehyde show fine lead grains only in the heterochromatic regions of the nucleus (B,D), while the others do not show any nuclear staining. The cells fixed in formaldehyde do not show any nuclear staining (C). Plasma membrane vesicles show heavy deposits of reaction products mostly on the outside layer of the membrane (F); in some places, however, lead grains are seen on both sides. (G) Lymphocytes incubated in the presence only of lead and magnesium do not show any reaction products. The symbols in the micrographs are M, mitochondrion, and ER, endoplasmic reticulum. Magnifications: (A) ×16,550; (B) ×7250; (C) ×8950; (D) ×16,450; (E) ×17,350; (F) ×13,050; (G) ×6500.

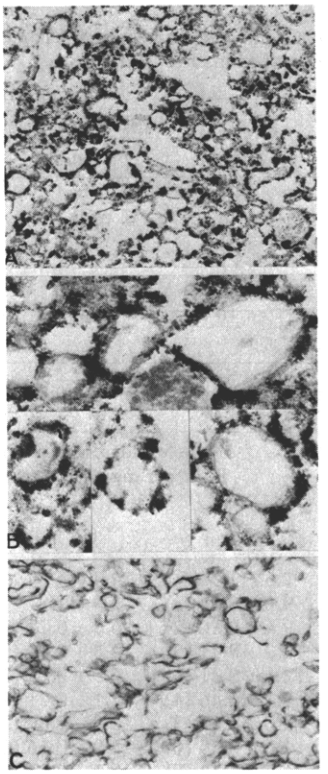

FIGURE 5: (A) Isolated plasma membranes from lymphocytes incubated in the presence of 5′-AMP; lead and magnesium show heavy deposition of the reaction products in all the vesicles (×15,950). (B) Some of the vesicles are shown at a higher magnification to illustrate the finding that the deposition takes place mainly on the outer layer of the membrane (×31,500). (C) Plasma membranes incubated in the presence of lead and magnesium do not show any deposition of reaction products (×13,700).

and F344 strains of rats (Smith et al., 1975). Our electron microscopy results showed that over 90% of thymocytes have smooth contours, whereas there are surface microvilli on about 50% of the splenic lymphocytes. Recent studies by scanning electron microscopy have suggested that human thymus derived lymphocytes (T-cells) have a smooth surface but bone marrow derived lymphocytes (B-cells) have villous projections (Polliack et al., 1974; Lin et al., 1973). The ultrastructural difference that we observed between the thymic and splenic rat lymphocyte populations correlates with their relative T- and B-cell content; thus, these surface features may reflect functional differences between these two cell types.

The method used for cell disruption involved a compromise between shear disruption and the use of a highly hypotonic medium containing sucrose. Similar methods used by Boone et al. (1969) and Johnsen et al. (1974) for HeLa cells and by DePierre and Karnovsky (1973) for guinea pig leukocytes showed good preservation of the cellular organelles. Our method also showed good preservation of nuclei, mitochondria, and other cellular organelles; both the yield and the ultrastructural appearance of the membranes were much better than those obtained by homogenization in isotonic media. For separation of the cellular organelles, we used a differential centrifugation scheme similar to that developed by Allan and Crumpton (1970, 1972) for pig and human lymphocytes. We found that centrifugation at $20{,}000g_{av}$ for 1 hr was sufficient to sediment the microsomal fraction; sedimentation at $88{,}000g_{av}$ for 1–2 hr did not increase the total yield or specific activity of 5′-nucleotidase either in the microsomal fraction or in the final plasma membrane fraction.

The Enzyme 5′-Nucleotidase as a Marker for Lymphocyte Plasma Membranes. Studies on lymphocytes by various investigators (reviewed in Ladoulis et al., 1975) have shown that the plasma membrane fraction is considerably enriched in 5′-nucleotidase activity. The reported recovery of this enzyme in the plasma membrane fractions ranged from 0.3 to 45% even though 40–80% of the total activity was recovered in the microsomal pellet and supernatant combined (see Misra et al., 1974). The sources of variation in the recovery of the enzyme in plasma membranes may be the isolation procedure, the solubility of 5′-nucleotidase during the membrane isolation, or the contamination of the various other fractions by plasma membranes. For example, using the shear homogenization method for pig lymph node lymphocytes, Allan and Crumpton (1970) recovered 10–15% of the total 5′-nucleotidase activity in the plasma membrane fraction, whereas Ferber et al. (1972) using the nitrogen cavitation method for disruption of lymphocytes from the same source recovered about 45% of the enzyme in the

plasma membrane fraction. The latter isolation method provided similar results for calf or rabbit thymocyte membranes (Schmidt-Ullrich et al., 1974). These results indicate that the proper isolation method may improve substantially the recovery of this enzyme in the plasma membrane fraction.

Previous reports of studies on isolated lymphocyte membranes have shown that from 13 to 47% of the total 5′-nucleotidase activity was recovered in the microsomal supernatant (see Misra et al., 1974). Since 5′-nucleotidase is released from membranes by osmotic shock, or even by washing (Cercignani et al., 1974), much of the 5′-nucleotidase in the microsomal supernatant is probably derived from the plasma membrane during cell disruption and fractionation. Furthermore, since nuclear, mitochondrial, and other cellular fractions are always contaminated with plasma membranes, the actual concentration of the enzyme in the plasma membrane fraction of lymphocytes may be underestimated.

While the enrichment of 5′-nucleotidase in plasma membrane is indicative of its preferential localization there, it does not prove that the enzyme is restricted to plasma membranes in lymphoid cells. Cytochemical studies in liver cells have demonstrated that this enzyme is distributed in endoplasmic reticulum, outer mitochondrial membranes, and golgi membranes (Widnell, 1972). Our cytochemical results indicate that in lymphocytes the enzyme is restricted to the plasma membrane. The specificity of the cytochemical assay was established by showing that when 2′-AMP and 3′,5′-AMP, which are analogs of 5′-AMP, were used as substrates, there was no reaction product precipitated on the cell membrane. Nuclear staining occurred in glutaraldehyde-fixed cells, but not in cells fixed with formaldehyde or in control samples. Similar nuclear staining was observed in studies using the lead phosphate method for localization of 5′-nucleotidase in leukocytes, Kupffer cells, endothelial cells, liver cells, and Ehrlich ascites carcinoma cells (see Misra et al., 1974) and rat intestinal epithelium (Millington and Brown, 1967). Such staining is probably artifact due to diffusion of the reaction products into the nucleus and their selective affinity for certain reactive groups within the nucleus (Gomori, 1951). Our results show that formaldehyde fixation eliminates this nuclear staining. Thus, we conclude that the nuclear staining is an artifact and that 5′-nucleotidase is restricted to, and thus a marker for, lymphocyte plasma membranes.

Most of the reaction products seen on isolated plasma membrane appear to be located on the outer side of the vesicles. In a few areas, however, the reaction products were found on both sides. This finding is consistent with those of Benedetti and Delbauffe (1971) who found histochemically that 5′-nucleotidase in liver cells was located on the outer surface of the plasma membrane lining the bile spaces and on the inner surface of liver tight junctions. Similarly, Widnell (1972) reported that the reaction products on liver cell microsomal vesicles were localized on the outside surface, and Gurd and Evans (1974) arrived at the same conclusion by immuno-inhibition experiments on liver cell plasma membrane and pig lymphocyte plasma membrane. Since glycoproteins are oriented toward the external surface of the plasma membrane, the cytochemical finding that the 5′-nucleotidase is on the outer side of the membrane vesicles suggests that it is an external glycoprotein. Controversy, however, surrounds the issue of the biochemical nature of this enzyme, since most investigators isolated it as a lipoprotein (Widnell and Unkeless, 1968) while a few found it to be a glycoprotein (Gurd and Evans, 1974).

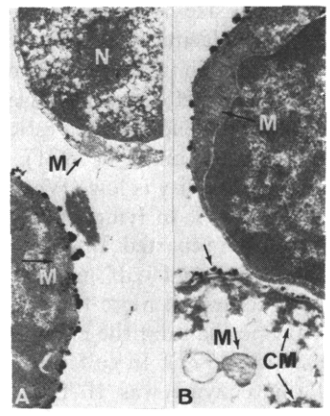

FIGURE 6: (A and B) Partially homogenized lymphocytes incubated in the presence of 5′-AMP, lead and magnesium. The plasma membranes of intact cells and broken cells (arrow) both show reaction products whereas there are none on mitochondria (M), cytomembranes (CM), or on the nuclear (N) membranes; magnifications: (A) ×8100; (B) ×11,950.

We have made two observations during our experiments which do not support the supposition that the cells are impermeable to substrate. First, we have observed after incubation of partially homogenized preparations (Figure 6) that reaction products are not found on cytoplasmic membranes within broken cells. Secondly, we have observed that the nuclei of some cells stain with lead, as discussed above, but that this phenomenon occurs only in the presence of substrate. We interpret these findings as evidence for the penetration of substrate into our fed lymphocyte preparations. In support of this contention studies have shown that osmium-fixed liver tissue (Essner et al., 1958) as well as unfixed isolated liver cells (Trams and Lauter, 1974) are permeable to 5′-AMP and furthermore positive cytochemical reactions have been shown in rat liver lysosomes of formaldehyde-fixed cells (El-Aaser and Reid, 1969) as well as in mitochondria of rat intestinal epithelium fixed in glutaraldehyde (Millington and Brown, 1967). Cytochemical studies of isolated plasma membranes of liver cells clearly localized 5′-nucleotidase simultaneously on the external surfaces of the membrane and on the cytoplasmic side at the tight junctions (Benedetti and Delbauffe, 1971). These reports clearly indicate that fixed cells and membrane vesicles are permeable to both substrate and lead, and support our conclusion that 5′-nucleotidase is localized to the plasma membranes of lymphocytes.

Membrane Purity. The distribution of enzymes in various subcellular fractions has been a major criterion in assessing the purity of the isolated membranes. We used the following enzymes as markers for different subcellular components: 5′-nucleotidase for plasma membranes, NADH dehydrogenase and glucose-6-phosphatase for endoplasmic reticulum, succinate dehydrogenase for mitochondrial membranes, and acid phosphatase for lysosomes. As a second criterion of plasma membrane purity, we utilized the cholesterol to phospholipid molar ratio.

The 5′-nucleotidase in our purified plasma membrane preparations was enriched 12- to 15-fold. Similar results were obtained for lymphocyte plasma membranes from pig mesenteric lymph node (Allan and Crumpton, 1970), human tonsil (Demus, 1973; Lopes et al., 1973), and human thymus (Allan and Crumpton, 1970).

Small and medium size lymphocytes contain a small amount of smooth endoplasmic reticulum, which is a potential source of contamination in any plasma membrane preparation. The enzyme NADH dehydrogenase is considered a relatively good marker for endoplasmic reticulum (Wallach and Kamat, 1966; Brunette and Till, 1971), especially when glucose-6-phosphatase activity is low (Wallach and Kamat, 1966; Ferber et al., 1972). In lymphocytes, however, conflicting results have been reported. In lymphocytes from pig mesenteric lymph nodes and calf mediastinal nodes, the plasma membranes contained more than twice the specific activity of this enzyme than did the endoplasmic reticulum fraction (Ferber et al., 1972). In calf thymocytes, the specific activity of this enzyme was 10 times greater in the plasma membrane than in the homogenate and only half of that in the microsomal fraction (van Blitterswijk et al., 1973). Warley and Cook (1973), who studied normal lymphoid and acute lymphoblastic leukemia cells of mice, found that the specific activity of this enzyme was increased in the plasma membrane compared to that in the cell lysate, and it was the same as that in the endoplasmic reticulum fraction. In a more recent study on calf and rabbit thymocytes, however, Schmidt-Ullrich et al. (1974) reported that the specific activity of this enzyme was 3–4 times greater in the endoplasmic reticulum fraction than in the plasma membrane. Considering these results, our plasma membrane preparations showed a high order of separation from smooth endoplasmic reticulum.

The total recovery of NADH dehydrogenase in the plasma membrane fraction is another criterion of purity. The specific activity in the homogenate and in the sucrose bottom, which contained the rough endoplasmic reticulum and some smooth membranes, was one and a half times greater than in the plasma membrane. Wallach and Kamat (1966) and Brunette and Till (1971) found 3.7 and 3.6% of this enzyme in the plasma membranes from Ehrlich ascites tumor cells and L-cells, respectively, and considered the plasma membranes reasonably free from endoplasmic reticulum. In our preparations, we obtained 1% or less of this enzyme. In calf thymocytes, van Blitterswijk et al. (1973) found more than 6% of the total activity in the plasma membrane and suggested that it may be more valid to compare the specific activity of this enzyme on the basis of phospholipid content. We calculated on this basis that the sucrose bottom fraction contained 3–4 times greater specific activity of NADH dehydrogenase than the plasma membrane fraction.

Glucose-6-phosphatase is another marker for endoplasmic reticulum. More than 50% of the total enzyme activity was recovered in the $20{,}000g_{av}$ supernatant, whereas the plasma membrane fraction contained less than 1%. The specific activity in the plasma membrane was half of that in the homogenate or in the sucrose bottom fraction and one-third of that in the $20{,}000g_{av}$ supernatant. These results are in agreement with those for pig lymphocyte (Allan and Crumpton, 1970), calf thymocyte (van Blitterswijk et al., 1973), and human tonsil lymphocyte (Demus, 1973).

Succinate dehydrogenase is a marker for mitochondrial membranes. Our plasma membrane preparations contained only a trace amount (ca. 0.05%) of the total activity, and the specific activity was very low compared to that in the mitochondrial fraction. The microsomal fraction contained 1–3% of the total activity. In pig lymphocyte membranes, the microsomal pellet was reported to contain more than 1% of the total activity, but none was detected in the plasma membrane fraction (Allan and Crumpton, 1970). Similarly, in the calf thymocyte membrane, 0.5% of the total activity was recovered in the microsomal fraction, but the plasma membrane fraction was free of succinate dehydrogenase (van Blitterswijk et al., 1973). On the other hand, a significantly higher specific activity of succinate dehydrogenase was found in the human tonsil lymphocyte membrane (Demus, 1973) and in the membranes of human normal and leukemic lymphocytes (Marique and Hildebrand, 1973).

Acid phosphatase is a useful lysosomal marker enzyme. In the pig lymphocyte, Allan and Crumpton (1970) found that the mitochondrial pellet is the richest in this enzyme. In human lymphocytes, Marique and Hildebrand (1973) found 9% of the lysosomal material associated with the purified membrane. On the other hand, Ferber et al. (1972) did not find any acid phosphatase activity in the microsomal fraction of pig lymphocytes. In our preparation, more than half of the enzyme was recovered in the mitochondrial fraction, and electron micrographs of this fraction showed a large number of lysosomes. The microsomal fraction contained 15–20% of this enzyme, but the membrane fraction contained less than 1%. The specific activity of the plasma membrane fraction was about one-fourth of that in the mitochondrial fraction or in the sucrose bottom. Thus, our preparations were essentially free from lysosomal contamination.

The cholesterol:phospholipid molar ratio is another major criterion for membrane purity (Coleman and Finean, 1966). Our rat lymphocyte plasma membrane fraction contained 5 to 8 times more cholesterol than other cellular fractions, and the molar ratio of cholesterol to phospholipid was highest in the plasma membrane fraction.

These results indicate that our membrane preparations are quite pure on the basis of both enzymatic and compositional criteria. The preparations contained a very small amount of DNA, and contamination by RNA was within the range found in lymphocyte membranes from other sources (Allan and Crumpton, 1970; Ferber et al., 1972; van Blitterswijk et al., 1973; Demus, 1973).

Biochemical Differences. Our results showed a consistent difference in lipid and carbohydrate content between thymic and splenic lymphocyte membranes. Consonant with these observations, we have also found characteristic electroporetic differences in the glycoprotein components of the splenic and thymic lymphocyte membranes following analysis by polyacrylamide gel electrophoresis (Ladoulis et al., 1974). Since the rat thymus cells are homogeneous by immunologic criteria (>99% positive for thymic antigen) and the splenic lymphocytes contain about 50% thymus-derived lymphocytes, the biochemical differences between thymic and splenic lymphocyte membranes may reflect, among other things, maturational or functional differences between thymus- and marrow-derived lymphocytes. Other evidence that the membranes of these two lymphocyte populations differ biochemically includes the findings that thymic lymphocytes have much less surface immunoglobulin (Warner, 1974), are less susceptible to detergent lysis (Cone and Marchalonis, 1974; Smith et al., 1975), have greater electrophoretic mobility (Wioland et al., 1972), and have a significantly thicker glycoprotein coat (Santer et al., 1973) than splenic lymphocytes.

In the ACI strain, 5′-nucleotidase activity in the splenic cell membranes was almost double that of the thymic cell membranes, whereas it was similar to or slightly lower than the activity in the thymic cell membranes of the F344 strain. The thymocyte membranes in the ACI strain showed

lower activity than the splenic cell membranes. Similar differences were reported by Allan and Crumpton (1970) for lymphocyte membranes from pig thymus and lymph node and by Ferber et al. (1972) and Schmidt-Ullrich et al. (1974) for membranes from calf thymocytes and mediastinal node lymphocytes. The observed differences in 5′-nucleotidase activity with respect to organ and strain seem to be real because: (a) in many experiments (Table V) we analyzed and compared the results for the splenic and the thymic membranes from the same set of animals; (b) the membrane preparations were similar as observed by electron microscopy; (c) the 5′-nucleotidase activity in the homogenates showed differences similar to those in membrane fractions; and (d) the level of the subcellular contamination as indicated by other enzymatic activities did not vary between preparations. The reason for the observed differences, however, is not clear. The difference between thymic and splenic lymphocyte membranes in the ACI strain might be attributed to different proportions of T- and B-lymphocytes but in the F344 strain there is no significant difference. In fact, Quagliata et al. (1974) did not find any correlation between the relative proportion of T- and B-lymphocytes and the specific activity of 5′-nucleotidase in their recent work on normal and leukemic lymphocytes from human. There were no significant differences in activity with respect to sex. Emmelot and Bos (1971) did not find any difference in 5′-nucleotidase activity in the liver plasma membranes from male and female rats, but Solyom and Lauter (1973) reported that in rat liver plasma membranes the specific activity of this enzyme was significantly higher in males than in females.

The possible functions of 5′-nucleotidase have been reviewed recently by Goldberg (1973). Since this enzyme in *Escherichia coli* hydrolyzes uridine diphosphate glucose (UDPG), a major cofactor in the biosynthesis of the membrane carbohydrates, it may have a role in the metabolism of membrane glycoproteins and glycolipids in eukaryotic cells also. As described in the previous section, 5′-nucleotidase is a lipoprotein and/or glycoprotein, and there is some evidence that its activity is lipid dependent (Widnell and Unkeless, 1968). A dependence of enzyme activity on phospholipid has been found in many cases (Tanaka et al., 1971). Lipid may stabilize a particular conformation of an enzyme which is necessary for its catalytic activity, or it may prevent random aggregation of the enzyme protein and thus any decrease in its activity. The difference in lipid and carbohydrate content, and possibly the micellar distribution of the lipid in the membrane, could be the source for the differences in 5′-nucleotidase activity between splenic and thymic lymphocyte membranes.

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Induction of Antibody against Actin from Myxomycete Plasmodium and Its Properties†

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ABSTRACT: Plasmodium actin was highly purified by gel filtration of crude G-actin on Sephadex G-100 followed by ultracentrifugation after polymerization in the presence of 1 *M* urea and 1 m*M* ATP. Purified actin showed a single band in the sodium dodecyl sulfate gel electrophoretic pattern. Antibody against this purified actin was induced in rabbits. The antibody obtained was immunologically monospecific for plasmodium actin, judging from the following results. (1) The addition of the antibody to a plasmodium F-actin solution increased the turbidity of the mixed solution, showing the formation of the antibody–actin complex. (2) In immunodiffusion and immunoelectrophoresis, the antibody formed single precipitin lines with the purified actin preparation and with the crude actin extract from the acetone-dried powder of plasmodium. (3) The antibody inhibited polymerization of plasmodium G-actin. (4) Plasmodium F-actin filaments were decorated with antibody in electron micrographs. The antibody reacted not only with plasmodium F- and G-actin, but also reacted with sea urchin egg actin, but it did not react with actin from rabbit striated muscle.

Actin has been isolated from plasmodium of the myxomycete using its specific binding to muscle myosin (Hatano and Oosawa, 1966) and by column chromatography (Adelman and Taylor, 1969). This F-actin filament is decorated with heavy meromyosin (HMM)[1] from muscle to form the arrowhead like structures (Nachmias et al., 1970).

On the other hand, microfilaments, around 60 Å in diameter, have been observed in electron micrographs of living and glycerinated plasmodium of the same myxomycete (Wohlfarth-Bottermann, 1962; Rhea, 1966; Nagai and Kamiya, 1966). They existed as bundles of several hundreds of filaments in the gel layer of plasmodium. Nagai and Kamiya (1968) showed that when glycerinated plasmodium was treated with ATP in the presence of Mg^{2+}, the bundle was broken into small blocks in which filaments made dense aggregates similar to superprecipitated actomyosin of muscle. Using the method developed by Ishikawa et al. (1969), Alléra et al. (1971) ascertained that microfilaments in question were F-actin filaments. Namely, these filaments were decorated with HMM from muscle to form the arrowhead like structures. Similar actin filaments have been identified in many eukaryotic cells by this method (Pollard et al., 1970; Pollard and Korn, 1973a,b; Pollard and Weihing, 1974; Comly, 1973; Schroeder, 1973; Tilney et al., 1973; Williamson, 1974; Palevitz et al., 1974).

However, there are some technical problems in this method. For example, when actin exists in the soluble part of protoplasm, it or a part of it dissolves out during glycerination before the application of HMM. Some authors have pointed out the possibility that actin exists in the state of monomer or oligomer (Jockusch et al., 1971; Tilney et al., 1973) or in the different states of polymer from F-actin (Hatano et al., 1967; Hatano and Totsuka, 1972; Tilney, 1975). If actin exists in such states in vivo, it will be very difficult to show the existence of actin, even if they were decorated with HMM.

We have tried to produce the antibody to actin from plasmodium in order to examine the precise localization of actin in plasmodium immunohistochemically. We report in this paper the induction of antibody against highly purified actin from plasmodium and describe some properties of the antibody.

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[1] Abbreviations used are: HMM, heavy meromyosin; PBS, phosphate buffered saline.