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The Subcellular Distribution of Adenylate and Guanylate Cyclases in Murine Lymphoid Cells†

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ABSTRACT: Membrane vesicles can be prepared from murine lymphoid cells by nitrogen cavitation and fractionated by sedimentation through nonlinear sucrose density gradients. Two subpopulations of membrane vesicles, PMI and PMII, can be distinguished on the basis of sedimentation rate. The subcellular distribution of adenylate and guanylate cyclases in these membrane subpopulations have been compared with the distribution of a number of marker enzymes. Approximately 20–30% of the total adenylate and guanylate cyclase activity is located at the top of the sucrose gradient (soluble enzyme), the remainder of the activity being distributed in the PMI and PMII fractions (membrane-bound enzyme). More than 90% of the 5′-nucleotidase and NADH oxidase activities detected in lymphoid cell homogenates are located in PMI and PMII fractions, whereas succinate cytochrome *c* reductase activity is detected only in the PMII fractions. In addition, β-galactosidase activity is distributed in the soluble and PMII fractions of the sucrose density gradients. On the basis of the fractionation patterns of these various enzyme activities, it appears that PMI fractions contain vesicles of plasma membrane and endoplasmic reticulum, whereas PMII fractions contain mitochondria, lysosomes, and plasma membrane vesicles. Approximately 30–40% of the adenylate and guanylate cyclase activities in PMII can be converted to a PMI-like form following dialysis and resedimentation through a second nonlinear sucrose gradient. Adenylate and guanylate cyclases can be distinguished on the basis of sensitivity to nonionic detergents.

The experiments described here are preliminary to developing methods to study how lymphocyte mitogens interact with cell surface membranes and initiate the intracellular biochemical changes that lead to DNA synthesis and cell division. A large number of agents have now been shown to exert selective mitogenic activity on murine bone-marrow-derived (B[1]) (Andersson et al., 1972; Coutinho et al., 1974;

† This work was supported by Grants AI 13383-01 and AI-05875 from The National Institutes of Health, National Science Foundation No. BMS72-01899, American Cancer Society No. BC-30, and National Institutes of Health CA-11176. *Received September 11, 1975.*

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[1] Abbreviations used: B and T lymphocytes, bone-marrow-derived and thymus-derived lymphocytes, respectively; LPS, bacterial lipopolysaccharide; cyclic GMP, guanosine 3′:5′-monophosphate; PBS, phosphate buffered saline; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PMI and PMII, the first and second plasma membrane fractions, respectively; NADH, nicotinamide adenine dinucleotide reduced form.

Diamantstein et al., 1974; Strong et al., 1974; Watson and Riblet, 1974) and thymus-derived (T) (Janossy and Greaves, 1971; Greaves and Bauminger, 1972) lymphocytes. Several lymphocyte mitogens have been reported to retain their stimulatory activity when conjugated to Sepharose beads (Greaves and Bauminger, 1972; Möller et al., 1975) indicating the mitogens interact with the surface membranes of lymphocytes to exert their physiological activity. Any agent that interacts with the surface membrane of lymphocytes and stimulates the initiation of DNA synthesis and cell division must activate a coordinated sequence of biochemical changes that allow a cell to progress through a division cycle. It is evident that binding of mitogens to lymphocytes is insufficient for physiologic activity since mitogens appear to bind to most cell types but activate only selective classes of lymphocytes to cell division (Yahara and Edelman, 1972; Gronowicz et al., 1974; Watson and Riblet, 1975). How the binding of the mitogen to cell surface components results in the transmission of signals to the interior of the cell or which intracellular biochemical events are modified by mitogen are not known. The addition of concanavalin A (Hadden et al., 1972) or bacterial lipopolysaccharide (LPS) (Watson, 1975) to lymphocytes has been reported to cause rapid increases in the intracellular levels of guanosine 3′:5′-monophosphate (cyclic GMP). Since cyclic GMP itself is mitogenic for B lymphocytes (Watson, 1975), it has been suggested that the changes in the levels of this cyclic nucleotide may be involved as an intracellular mediator of a mitogenic signal delivered to cells by LPS (Watson, 1975). However, the interaction of antigen-sensitive B lymphocytes with antigen has revealed several aspects of the developmental commitment of these cells: these cells are either induced to mature to antibody-forming cells or rendered noninducible (paralyzed) by antigen (Walters et al., 1972; Chiller and Wiegle, 1973; Katz et al., 1974). In view of the mitogenic properties of cyclic GMP and also the observation that agents that elevate cyclic AMP levels in B lymphocytes inhibit the effects of cyclic GMP, it has been suggested that the intracellular ratio of cyclic AMP to cyclic GMP may regulate the inductive and paralytic pathways of antigen-sensitive cells (Watson et al., 1973; Watson, 1975). In addition to mitogens, the external stimuli that are associated with the physiological activation of lymphocytes probably also result from interactions at the cell surface membranes. If these signals directly affect cyclic nucleotide levels in cells, then the enzymes involved in their synthesis, adenylate and guanylate cyclases, may be located in the plasma membranes linked in some manner to cell surface receptors. It is these enzymes that may be important in the delivery of signals from the exterior to the interior of cells. While there is considerable evidence that adenylate cyclases exist in mammalian cells predominantly in a membrane-bound form, guanylate cyclases have been detected in both soluble and membrane-bound forms (Hardman and Sutherland, 1969; White and Aurbach, 1969; Hardman et al., 1971; Robison et al., 1971).

The surface membrane and endoplasmic reticulum of mammalian cells can be converted into vesicles by nitrogen cavitation (Kamat and Wallach, 1966; Graham, 1972). In this paper we describe the fractionation of vesicles prepared from murine lymphoid cells by density gradient centrifugation. A number of enzyme activities with known subcellular distributions have been used to analyze the cellular origin of the various membrane fractions detected. Most of the adenylate and guanylate cyclase activity is located in a membrane-bound form. Since the membrane preparations retain enzymatic activities characteristic of intact cells, the plasma membrane vesicles may provide a system for examining the mechanisms involved in the delivery of membrane-mediated signals to lymphocytes.

Materials and Methods

Mice. Balb/c mice were bred at The Salk Institute, San Diego. C3H/HeJ, C3HeB/FeJ, and C57BL/6 mice were purchased from Jackson Laboratories, Bar Harbor, Maine.

Plasma Membranes. Mouse spleen cells were prepared in the following way. Thirty spleens from 6–8 week old mice were teased apart in 20 ml of a balanced salt solution (Mishell and Dutton, 1967). All subsequent steps were performed at 4 °C. The cells were then filtered through a wire mesh to break up cell clumps and then collected by centrifugation (800*g*, 5 min). Erythrocytes were removed from the spleen cells by NH_4Cl treatment (Shortman et al., 1972). The spleen cell pellet ($\sim 3 \times 10^9$ nucleated cells) was resuspended in 60 ml of 0.17 M NH_4Cl. After 10 min at 4 °C, the cell suspension was divided into two portions in glass centrifuge tubes and 10 ml of 50% fetal calf serum in a phosphate buffered saline (PBS) solution was layered under each suspension. The cells were collected by centrifugation (800*g*, 5 min) and washed twice with PBS. The cell pellet was then suspended at a ratio of 1 volume of pellet to 9 volumes of buffer described by Ferber et al. (1972) containing 0.02 M Hepes (pH 7.4), 0.13 M NaCl, and 0.0005 M $MgCl_2$ and was dispersed by drawing the cells up and down through a 5-ml pipet. The cells were homogenized by nitrogen cavitation in an Artisan instrument, using a nitrogen pressure of 800 psi. After the cell suspension had been under pressure for 5 min, the suspension, still under pressure, was delivered to the atmosphere dropwise from the homogenizer, and the solutions were returned to isotonicity by adding an equal volume of 0.5 M sucrose. At this time, EDTANa_2 (pH 7.0) was added to a final concentration of 0.002 M.

After homogenization, the broken cell suspension was centrifuged at 3200*g* for 5 min to remove nuclei and unbroken cells. The post-nuclear supernatant (3–5 ml containing 10–50 mg of protein) was then layered on top of a nonlinear sucrose gradient consisting of a 10-ml linear 60–30% gradient, a 10-ml barrier of 30% sucrose, and a 12-ml linear 30–10% sucrose gradient. The gradient contained 0.005 M Tris-HCl (pH 7.2) and 0.001 M EDTANa_2.

The gradient was centrifuged for 90 min at 82 500*g* (25 000 rpm in a Beckman SW27 rotor). Gradient fractions of 1.2 ml were collected in an Isco fraction collector, Model 640, by upward displacement with a solution of 32% sucrose and 23% KI (w/w) and stored at 4 °C. Two plasma membrane populations were resolved in this sucrose gradient. The first plasma membrane fraction (PMI) peaked at 28–30% sucrose, and the second plasma membrane fraction (PMII) having moved through the 30% barrier is located at 40–42% sucrose. It should be noted that these two plasma membrane populations also contained endoplasmic reticulum, (PMI) lysosomes, and mitochondria (PMII).

Adenylate and Guanylate Cyclase Assays. Each gradient fraction was analyzed for adenylate and guanylate cyclase activities by conversion of a nonradioactive nucleoside triphosphate to the cyclic nucleotide which was then measured by a radioimmune assay (Steiner et al., 1972). Adenylate cyclase assays were performed in 0.1-ml volumes and contained a mixture of 0.05 ml of gradient fraction and 0.05 ml

of reaction mixture. The reaction mixture contained 0.08 M Hepes-Na (pH 7.6), 0.006 M $MgCl_2$, 0.002 M ATP, 0.001 M dithiothreitol, 0.02 M theophylline, 0.01 M caffeine, 0.03 M creatine phosphate, and 4 μg of creatine phosphokinase. Reactions were incubated for 15 min at 37 °C and then terminated by addition of 0.1 ml of ice-cold 0.05 M sodium acetate (pH 6.2) and subsequent boiling for 2 min. The extracts were centrifuged for 10 min at 5000*g*, and duplicate samples (0.075 ml) were removed from the supernatant for the radioimmune assay.

Guanylate cyclase assays were also performed in 0.1-ml volumes, containing a mixture of 0.05 ml of gradient fraction and 0.05 ml of reaction mixture. This reaction mixture contained 0.08 M Hepes-Na (pH 7.6), 0.006 M $MnCl_2$, 0.0004 M GTP, 0.001 M dithiothreitol, 0.02 M theophylline, 0.01 M caffeine, and 0.1% Triton X-100. In some experiments 0.03 M creatine phosphate and 4 μg of creatine phosphokinase were also added. Reactions were incubated for 15 min at 37 °C. To avoid the problem of nonenzymatic conversion of GTP to cyclic GMP during a subsequent boiling step (Kimura and Murad, 1974), reactions were terminated by the addition of 0.01 ml of 10% perchloric acid and 0.1 ml of ice-cold 0.05 M sodium acetate (pH 6.2). The samples were then boiled for 2 min and centrifuged for 10 min at 5000*g*, the supernatants removed and neutralized with 1 M $KHCO_3$, and duplicate samples (0.075 ml) removed for the radioimmune assay.

Cyclic AMP and cyclic GMP measurements were made using the radioimmune assay of Steiner et al. (1972), purchased in assay kits from Collaborative Research, Boston, Mass. Negligible cross-reaction was observed with ATP using the antiserum against cyclic AMP (10^5 pmol of ATP were recorded as 0.2–0.5 pmol of cyclic AMP). It should be noted that occasional antisera against cyclic AMP from Collaborative Research have shown higher cross-reactivity with ATP, but these were not used. However, GTP cross-reacted with the antiserum against cyclic GMP such that 10^5 pmol of GTP were recorded in the range of 2–4 pmol of cyclic GMP. To avoid high backgrounds in the radioimmune assay, it was not possible to use more than 0.0002 M GTP in the final reaction volumes. In experiments where the effect of various cations were examined, these cations were added directly to the final assay mixture. The background values determined in assays containing nucleotide triphosphates but no enzyme source have been subtracted from the data presented. Results have been recorded as picomoles of cyclic nucleotide synthesized per 15 min per milliliter of gradient fraction. The synthesis of cyclic nucleotides increased linearly in these assay conditions up to 15 min of incubation using 10–200 μg of protein. The products synthesized and measured as cyclic AMP and cyclic GMP were authentic as shown by digestion with cyclic 3′,5′-phosphodiesterase after separation from caffeine by chromatography over Dowex resin (Bio-Rad AG 1-X8, 200–400 mesh, formate form) as detailed elsewhere (Watson, 1975). In these experiments theophylline was left out of the assay mixture. The determination of the amount of protein in sucrose gradient fractions was made using the method of Lowry et al. (1951).

Adenylate and guanylate cyclases were also assayed by conversion of [α-^{32}P]ATP or [α-^{32}P]GTP to the corresponding ^{32}P-labeled cyclic nucleotide. Reaction mixtures were identical with those above except that 3×10^7 counts/min of 0.01 mM [α-^{32}P]ATP or [α-^{32}P]GTP replaced the nonradioactive nucleoside triphosphates. At the end of the reaction period, each incubation mixture was boiled and centrifuged, and the supernatant was layered on an aluminum oxide column (0.4 × 4 cm) equilibrated with 0.06 M Tris-HCl (pH 7.5) buffer. The cyclic nucleotides were eluted with 6 ml of 0.6 M Tris-HCl (pH 7.5) buffer directly onto a Dowex Ag 1-X8 column (0.4 × 4 cm) (formate form) equilibrated with H_2O. Each column was washed with 10 ml of H_2O and the cyclic AMP eluted with 10 ml of 2 N formic acid while cyclic GMP was eluted with 10 ml of 4 N formic acid. Samples were lyophilized and radioactive measurements made using a toluene–terphenyl mixture in a scintillation counter (Watson, 1975). Starting with 3×10^7 counts/min of ^{32}P-labeled nucleoside triphosphate, less than 100 counts/min of nucleoside triphosphate was found in the final 2 or 4 N formic acid eluant.

To determine if the radioactivity eluted in the 2 or 4 N formic acid fractions was contained in cyclic AMP and cyclic GMP, respectively, the lyophilized samples were redissolved in 0.05 ml of H_2O and chromatographed on poly(ethylenimine) thin-layer plates (Brinkman Instrument Co., Polygram Cell 300 PEI) in a solvent of 0.2 M LiCl by ascending chromatography. The positions of the cyclic nucleotides were located by ultraviolet adsorption of marker samples, and the corresponding areas for the ^{32}P-labeled samples were cut out, eluted with 1 ml of 0.06 M Tris-HCl (pH 7.4), and counted directly with a scintillation fluid.

Other Enzyme Assays. 5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) was assayed by the method of Avruch and Wallach (1971), with the modification that the reaction mixture was pH 7.5 and contained 0.001 M Tris-HCl buffer, and the concentration of AMP was 0.02 mM. Succinate cytochrome *c* reductase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) was also assayed using the methods of Avruch and Wallach (1971). NADH oxidase (reduced NAD:(acceptor) oxidoreductase, EC 1.6.99.3) was assayed in a 1-ml volume containing 0.01 M Tris-HCl buffer, pH 7.4, and 0.007 M $KFeCN_6$ and the appropriate amount of enzyme. The reaction was started by adding 0.0001 mol of NADH (Sigma Chemicals) and the initial rate of the reaction determined at 340 nm. β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), a lysosomal enzyme, was determined using methyl umbelliferyl-β-D-galactoside as substrate according to the method of Ho et al. (1972) using 0.022 ml of sucrose gradient fraction per determination and with the inclusion of 0.1% Triton X-100 for full enzyme expression.

Results

Subcellular Distribution of Adenylate and Guanylate Cyclases. When guanylate cyclase activity was originally detected in a variety of tissues, it was located in membrane-free extracts prepared from cells (Hardman and Sutherland, 1969; White and Aurbach, 1969). A large increase in guanylate cyclase activity was obtained after treatment of membrane-containing cell fractions with nonionic detergents (Hardman et al., 1971). This is revealed in our data presented in Figure 1A. When sucrose-gradient fractions, prepared as described above, were assayed for guanylate cyclase activity in the absence of Triton X-100, most of the activity detected was located at the top of the sucrose gradient. However, when 0.2% Triton X-100 was included in the assay mixtures, two additional regions of guanylate cyclase activity were detected, in the positions of PMI and PMII (see later), and these activities were maximal at 28 and 40% sucrose (Figure 1A).

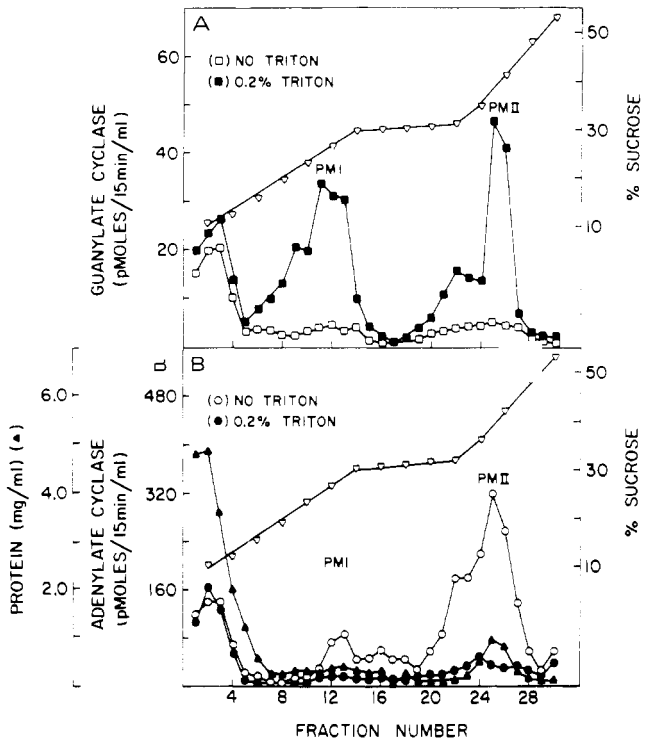

FIGURE 1: The distribution of adenylate and guanylate cyclase activity in sucrose density gradient fractions. The post-nuclear supernatant from spleen cell homogenates was fractionated by sucrose gradient centrifugation and assayed for adenylate and guanylate cyclases as detailed in Methods. Each gradient fraction was assayed in the presence and absence of 0.2% Triton X-100. Enzyme activities are expressed as picomoles of cyclic nucleotide per 15 min per milliliter of gradient fraction. (A) Guanylate cyclase activity; (B) adenylate cyclase activity.

In contrast, adenylate cyclase activity has been located mainly in plasma membrane fractions in other cell types (Robison et al., 1971). When homogenates prepared from murine lymphoid cells are fractionated by sucrose-gradient sedimentation, three regions of adenylate cyclase activity are observed (Figure 1B). The first is the upper (less dense) region of the gradient and is designated the "soluble" fraction; the second activity (PMI) is maximal at 28–30% sucrose and the third (PMII) at 40% sucrose. The presence of 0.2% Triton X-100 in the adenylate cyclase assay inhibits the enzymatic activity detected in PMI and PMII regions of the gradient. The positions of adenylate and guanylate cyclase activities in these sucrose gradients are virtually identical.

The experiment described in Figure 2 shows the effect of different concentrations of Triton X-100 on adenylate and guanylate cyclase activity in the PMI region of the sucrose gradient. Adenylate cyclase is extremely sensitive to Triton X-100; concentrations of more than 0.01% markedly decrease enzyme activity. However, guanylate cyclase activity is generally increased 5- to 20-fold in the presence of 0.05–0.5% Triton X-100 (Figure 2). Similar results were obtained using the PMII region of the sucrose gradient (data not shown). However, adenylate and guanylate enzyme activities in the soluble region (Figure 1) were not markedly affected by Triton X-100 concentrations in the range of 0.01 to 0.5% (data not shown).

The data expressed in Figure 1 show the total enzyme activities detected in 1 ml of each sucrose gradient fraction. When the specific activities of adenylate and guanylate cyclases are determined in each gradient fraction (picomoles

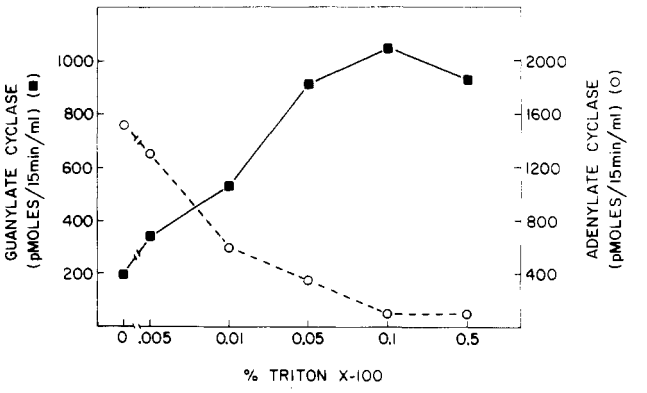

FIGURE 2: Effect of Triton X-100 on adenylate and guanylate cyclase activities in PMI sucrose gradient fractions.

of cyclic nucleotide synthesized per milligram of protein per 15 min), it can be seen that these are highest in the PMI and PMII regions of the sucrose gradients (Figure 3). The specific activity of both of these enzymes is generally at least tenfold greater than that of the same enzyme activity in the soluble fraction. The apparent division of adenylate and guanylate cyclase specific activities into two peaks at 40% sucrose (PMII) is due to the decline in enzyme specific activity (fraction 24, Figure 3). This decline is caused by a large increase in protein concentration (Figure 1B), probably a result of the large mitochondrial contribution to this fraction (see Figure 6).

The kinetics of synthesis of cyclic AMP and cyclic GMP by the plasma membrane fractions were assayed by the conversion of [α-^{32}P]nucleoside triphosphate to the corresponding cyclic nucleotide. The synthesis of ^{32}P-labeled cyclic GMP or ^{32}P-labeled cyclic AMP is linear for 15 to 20 min in reaction mixtures containing either the soluble fraction, PMI or PMII (data not shown).

Characteristics of PMI and PMII. When PMI is pooled, dialyzed against 0.04 M Hepes (pH 7.6) buffer, and resedimented in a second, identical nonlinear sucrose gradient, 65–80% of the adenylate and guanylate cyclase activities are located in their original positions (Figure 4B), the remainder being located in the soluble region of the gradients. When PMII is pooled, dialyzed against the same buffer, and then resedimented in a second gradient, some 30–40% of the PMII is converted to a fraction that sediments in the PMI region of the sucrose gradient (Figure 4C), the remainder sedimenting in the original PMII position. 5′-Nucleotidase is distributed in a pattern similar to those of the cyclases after dialysis (data not shown).

When the total adenylate and guanylate activity is determined in the post-nuclear supernatant from a lymphoid cell homogenate before layering on a sucrose density gradient and compared with the total activity recovered from gradient fractions following sedimentation, there are approximately a twofold increase in total guanylate cyclase activity and a threefold increase in total adenylate cyclase activity (Table I). This may be due to a variety of reasons, for example, the removal of inhibitors from the PMI and PMII enzymes, or a decrease in phosphodiesterase (PDE) activity, although PDE inhibitors were included in the assays. The specific activity of guanylate cyclase (picomoles per 15 min per milligram of protein) for the PMI enzyme represents an increase of approximately 20-fold over that in the original post-nuclear supernatant (Table I). There is a similar large increase in the specific activity of adenylate cy-

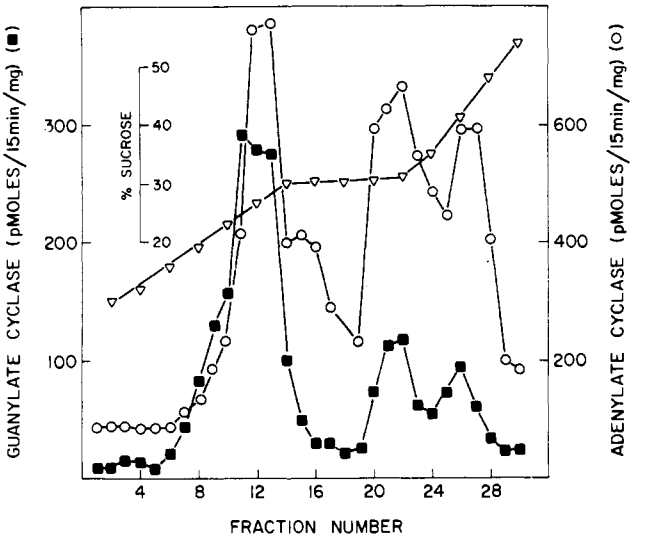

FIGURE 3: The specific activities of adenylate and guanylate cyclase in sucrose density gradient fractions. The data are expressed in picomoles of cyclic nucleotide per 15 min per milligram of protein for each gradient fraction.

clase in PMI. In contrast, there is only a seven- to ninefold increase in adenylate and guanylate cyclase specific activities in PMII. The data presented in Table II summarize the distribution of adenylate and guanylate cyclase activities in the soluble, PMI and PMII regions of sucrose gradients. These data are assessed from three separate experiments, using either BALB/c, C3H/HeJ, or C57BL/6 spleen cells as a source of lymphocytes. Finally, the data presented in Table II summarize the variation in specific activity of adenylate and guanylate cyclases in soluble, PMI and PMII fractions from three separate experiments. Both the distribution of adenylate and guanylate cyclase activities in these sucrose density gradients, as well as their specific activities, are reasonably constant in different experiments.

Subcellular Distribution of Other Enzyme Activities. The sucrose gradient fractions prepared from lymphocyte homogenates were assayed for other enzyme activities with known subcellular distributions. 5′-Nucleotidase has been located on the plasma membrane of many mammalian cells (Essner et al., 1958; Avruch and Wallach, 1971; DePierre and Karnovsky, 1974; Misra et al., 1974; Trams and Lauter, 1974) and has been shown to exclusively reside on the plasma membrane of lymphocytes (Misra et al., 1974, 1975). NADH oxidase is located on the endoplasmic reticulum and the outer mitochondrial membrane (Wallach and Winzler, 1974); succinate cytochrome *c* reductase is located on the inner mitochondrial membrane (de Duve et al., 1955).

5′-Nucleotidase activity was found in two peaks, the activities being maximal at 28–30% and 40–42% sucrose (Figure 5), coincident with the adenylate and guanylate cyclase activities in the fractions designated as PMI and PMII (Figure 1). No 5′-nucleotidase activity is observed in the upper portion of the gradient. These data suggest that 5′-nucleotidase is in a membrane-bound rather than a soluble form. NADH oxidase activity was also located only in the PMI and PMII regions of the sucrose gradient (Figure 5). Maximal NADH oxidase activity in PMI was not coincident with 5′-nucleotidase activity in the same region of the gradient.

In contrast, succinate cytochrome *c* reductase activity

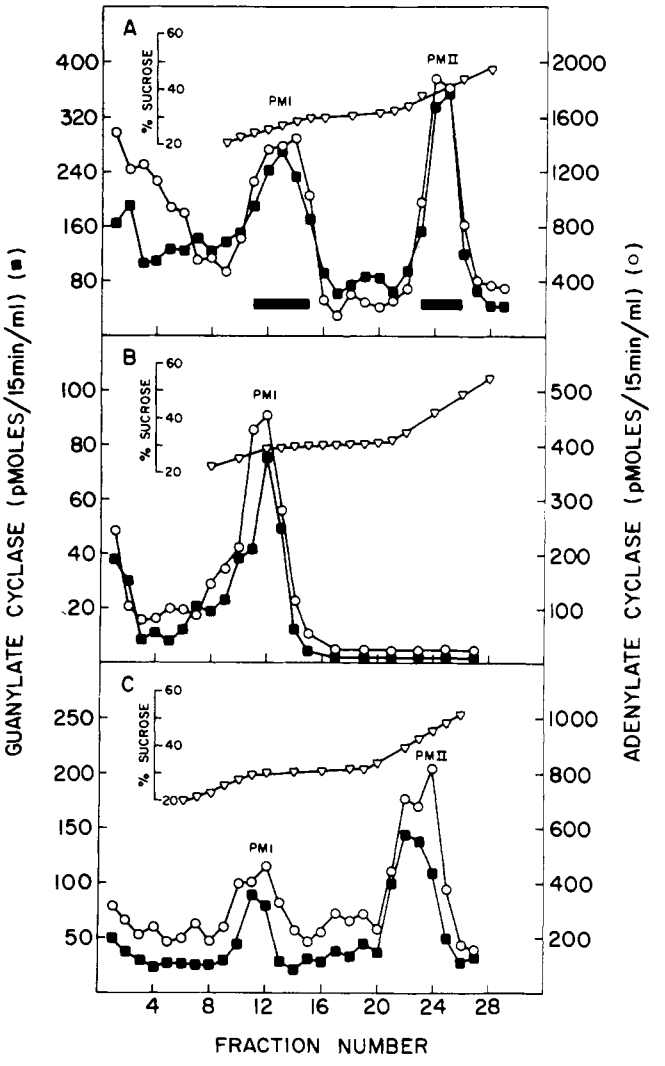

FIGURE 4: The sucrose gradient fractionation of membrane vesicles and the resedimentation of PMI and PMII gradient fractions. (A) Fractionation of the post-nuclear supernatant. The gradient fractions were assayed for adenylate and guanylate cyclase activities and then the gradient fractions under PMI and PMII marked with the solid bars were separately pooled and dialyzed against 0.04 M Hepes (pH 7.4). (B) The pooled and dialyzed PMI fractions were resedimented in a second sucrose density gradient. Fractions were then assayed for adenylate and guanylate cyclase activity. (C) The pooled and dialyzed PMII fractions were resedimented in a second sucrose density gradient and the gradient fractions assayed for adenylate and guanylate cyclase activity. All guanylate cyclase assays were performed using 0.2% Triton X-100.

was detected only in the PMII region, maximal activity between 40 and 42% sucrose (Figure 6). β-Galactosidase, the lysosomal marker (de Duve et al., 1955), was found both in the soluble and PMII regions of the sucrose gradient (Figure 6).

Discussion

Nitrogen cavitation of cells results in the conversion of the surface membrane and endoplasmic reticulum into vesicles (Kamat and Wallach, 1966; Graham, 1972). Using the conditions described in this paper, murine splenic lymphocytes were lysed, leaving nuclei and mitochondria relatively intact, and, following removal of the nuclei, the homogenates containing the membrane vesicles and mitochondria were fractionated by sucrose gradient sedimentation. Two membrane-containing regions (PMI and PMII) were

Table I: Subcellular Distribution of Adenylate and Guanylate Cyclase Activities Following Sucrose Density Gradient Sedimentation.

Fractions Assayed	Protein (mg)	Total Cyclase Activity (pmol/15 min)		Percent Total Cyclase Activity		Specific Activity of Cyclases (pmol/15 min/mg)		Enhancement Cyclase Activity[a]	
		Guanylate	Adenylate	Guanylate	Adenylate	Guanylate	Adenylate	Guanylate	Adenylate
Post-nuclear supernatant	67.2	1120	4160	100	100	16.6	62.0	1.0	1.0
Total recovered gradient	67.2	2740	14184	100	100	40.7	211.0	2.4	3.4
Soluble	60.2	1013	5520	36.9	38.9	16.8	91.6	1.01	1.47
PMI	2.2	856	4536	31.2	31.9	389	2061	23.4	33.2
PMII	6.8	871	4128	31.7	29.1	128	607	7.7	9.7

[a] The enhancement of cyclase activity refers to the increase observed above the activity of the postnuclear supernatant, which has been given a relative index value of 1.0.

Table II: Distribution of Adenylate and Guanylate Cyclase Activities in Sucrose Density Gradients.

Strain	Fraction of Sucrose Gradient	Percent of Total Cyclase Activity		Specific Activity of Cyclases pmol/15 min/mg	
		Guanylate	Adenylate	Guanylate	Adenylate
BALB/c	Soluble	24	21	50	90
	PMI	37	38	850	1500
	PMII	39	41	325	640
C3H/HeJ	Soluble	28	25	11	105
	PMI	38	35	200	1200
	PMII	34	40	100	560
C57BL/6	Soluble	30	25	43	108
	PMI	35	39	550	1300
	PMII	35	36	171	800

separated on the basis of sedimentation rate. PMI was found between 28 and 30% sucrose, and PMII was found between 40 and 42% sucrose. The physical characteristics of the vesicles in these membrane-containing gradient fractions isolated from murine cells will be described elsewhere (Nilsen-Hamilton and Hamilton, 1976). Two aspects of these membrane preparations are described here: first, the distribution of adenylate and guanylate cyclase activities following sucrose gradient fractionation; second, the distributions of various other enzymatic activities have been analyzed to determine the origins of the membrane vesicles located in the sucrose density gradient fractions designated as PMI or PMII.

The enzymatic activities associated with PMI and PMII reveal several features concerning their membrane content. 5′-Nucleotidase activity is detected only in PMI and PMII, and not in the less dense (soluble) fractions from the sucrose gradients (Figure 5). The enzyme has been shown to exist as an ectoenzyme (with its catalytic site external to the cell) on the surface of guinea pig polymorphonuclear leukocytes (DePierre and Karnovsky, 1974), L cells, mouse neuroblastoma, guinea pig hepatocytes, and HeLa cells (Trams and Lauter, 1974). In addition, cytochemical techniques have been used to show 5′-nucleotidase is restricted to the cell surface of rat splenic and thymic lymphocytes (Misra et al., 1974). Thus, PMI and PMII from mouse lymphocytes may contain most, if not all, of the plasma membrane from the cell homogenates. Succinate cytochrome *c* reductase activity, known to be located on the inner mitochondrial membrane in animal cells (de Duve et al., 1955), is detected only

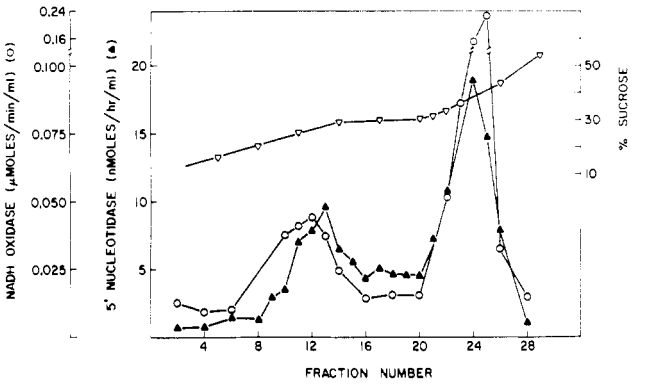

FIGURE 5: 5′-Nucleotidase (▲) and NADH oxidase (○) activities in sucrose density gradient fractions.

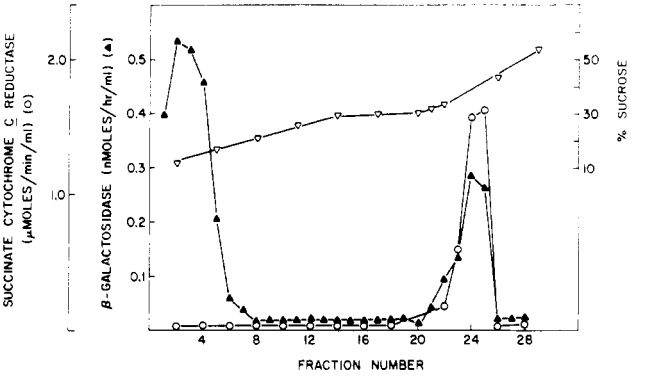

FIGURE 6: Succinate cytochrome *c* reductase (○) and β-galactosidase (▲) activities in sucrose density gradient fractions.

in PMII. Thus, the mitochondria appear to sediment between 40 and 42% sucrose in these gradient conditions. NADH oxidase activity, known to be associated with the endoplasmic reticulum and the outer mitochondrial membrane in mammalian cells (Wallach and Winzler, 1974), was detected in both PMI and PMII. The finding of NADH oxidase in PMII was expected. However, what was interesting was the apparent separation of NADH oxidase and 5′-nucleotidase activity in PMI. This suggests that the two enzyme activities are on separate vesicle populations, most likely endoplasmic reticulum and plasma membrane. Finally, β-galactosidase, the lysosomal marker (Ho et al., 1972), was found both in the upper "soluble" region of the gradient, and in PMII. We interpret this to mean that intact lysosomes sediment with the mitochondria in PMII,

and that β-galactosidase activity in the soluble fraction results from the breakage of the lysosomes during the preparation of the cell homogenate due to the very high nitrogen pressure required to open these cells. In murine fibroblasts there appears to be a positive correlation between pressure during cell breakage and the percentage of β-galactosidase in the soluble fraction (unpublished results).

Adenylate (Robison et al., 1971) and guanylate (Hardman and Sutherland, 1969; Ishikawa et al., 1969; White and Aurbach, 1969; Hardman et al., 1971; Chrisman et al., 1974; Garbers et al., 1974; Nakazawa and Sano, 1974; Pannbacker, 1974) cyclase activities have been detected in a wide variety of tissues. Whereas adenylate cyclase activity has been generally reported to be located in a membrane-bound form, the subcellular distribution of guanylate cyclase activity remains largely unknown. Both "soluble" and "membrane-bound" forms of guanylate cyclase have been detected. Membrane-associated guanylate cyclase has until recently remained undetected because of the chemical treatments that are required in order to reveal enzyme activity. When assayed in the absence of a nonionic detergent, most guanylate cyclase activity is located in the membrane-free cytoplasm of animal tissues (Hardman and Sutherland, 1969; Aurbach and White, 1969). However, in the presence of low concentrations of nonionic detergents, a membrane-bound guanylate cyclase is activated (Hardman et al., 1971).

When sucrose gradient fractions were assayed for guanylate cyclase activity in the absence of a nonionic detergent, more than 90% of the activity detected was found at the top of the gradient (Figure 1A). This has been termed the "soluble" enzyme fraction. However, when assays were performed in the presence of 0.2% Triton X-100, guanylate cyclase activity was observed in the PMI and PMII regions of the sucrose gradient, coincident with 5′-nucleotidase activity (Figure 1A). The percentage of the total guanylate cyclase activity detected in the soluble, PMI and PMII fractions was reasonably consistent in different experiments and appeared similar when using cell homogenates prepared from different strains of mice (Tables I–II). In general, 25% of the total guanylate cyclase activity detected is found in a "soluble" enzyme form, and the remaining 75% is generally distributed equally between PMI and PMII (Table II), as revealed by the presence of Triton X-100.

No detergent is required to reveal adenylate cyclase activity. This enzyme was also located in "soluble", PMI and PMII fractions, coincident with guanylate cyclase activity (Figure 1B). There are minor differences in the distribution of adenylate and guanylate cyclases. A slightly higher percentage of the total adenylate cyclase activity is generally found in PMI plus PMII than that observed for guanylate cyclase (Table II).

The effects of Triton X-100 on adenylate and guanylate cyclase activities raise several interesting questions. First, why does guanylate cyclase require the detergent for activity? Second, are the activities observed for both adenylate and guanylate cyclases in the soluble, PMI and PMII fractions due to one or several types of each of these enzymes? Concentrations of more than 0.01% Triton X-100 inhibit adenylate cyclase, but stimulate guanylate cyclase activity in PMI (Figure 2) and PMII fractions (data not shown). In contrast the "soluble" forms of these two enzymes are not markedly affected by similar concentrations of Triton X-100 (Figure 1). These data imply that the "soluble" and "membrane-bound" forms of each of these enzymes have different requirements for the expression of activity; whether these data are due to different types of enzymes is not clear. The "soluble" form of guanylate cyclase does not require Triton X-100 for activity. The relevance of this is not clear with respect to the in vivo functioning of guanylate cyclase. The enzyme may, for example, exist in vivo in an inactive state when it is membrane bound, and may require dissociation from the membrane to convert it to an active enzyme. One of the obvious reasons why "soluble" forms of adenylate and guanylate cyclases are found may be that cell homogenization procedures disrupt membrane-bound enzymes yielding an apparently "soluble" form. No 5′-nucleotidase or succinate cytochrome *c* reductase activity is found in a soluble form when the post-nuclear supernatant is resolved on the sucrose gradients (Figure 5). After dialysis of PMI similar proportions (22–33%) of adenylate and guanylate cyclase are released to the soluble fraction (Figure 5). A similar proportion of 5′-nucleotidase is also released into the soluble fraction (data not shown). This may indicate that these three enzymes are intercalated in the membrane to approximately the same extent. If this is the case then the fact that 5′-nucleotidase does not appear in the soluble fraction of the post-nuclear supernatant suggests that the soluble forms of guanylate and adenylate cyclases found in this fraction represent in vivo forms.

It should also be noted that PMI contains both plasma membrane and endoplasmic reticulum (Figure 6). Therefore, until vesicles arising from these subcellular structures are further separated, it is not possible to determine with certainty whether the guanylate cyclase is bound to be the plasma membrane, endoplasmic reticulum, or both. Guanylate cyclase activity in rat liver tissue has also been found associated with plasma membranes and possibly the endoplasmic reticulum (Kimura and Murad, 1975). By contrast, it appears likely from other reports that adenylate cyclase exists predominantly bound to the plasma membrane (Robison et al., 1971), although it is not possible to exclude the endoplasmic reticulum as a source of adenylate cyclase.

Resedimentation of PMI did not result in a change in the sedimentation rate of this membrane-containing fraction (Figure 4). However, when PMII was resedimented in a second gradient, some 30–40% of the adenylate and guanylate cyclase activity in PMII is converted to a fraction in the PMI region of the sucrose gradient. This suggests that the PMII fractions may contain vesicles convertible to PMI. This is most likely due to loss of peripheral or internalized proteins by these vesicles during dialysis.

The adenylate and guanylate cyclase activity in PMI and PMII isolated from murine lymphocytes remains stable when stored at 4 °C over a period of 3 weeks (unpublished observations). The finding that guanylate cyclase activity is found in a membrane-bound form is of major interest in view of the findings that the interaction of bacterial lipopolysaccharides with murine spleen cells (Watson, 1975) and concanavalin A with human peripheral lymphocytes (Hadden et al., 1972) both stimulate cyclic GMP synthesis. If guanylate cyclase is retained in a membrane-bound form in plasma membrane vesicles, it may be possible to examine how the interaction of mitogens, in general, initiates transmembrane signals, by examining whether mitogens lead to specific enzyme activation in plasma membrane vesicles.

Acknowledgments

We thank Dr. R. W. Holley and Dr. M. Cohn for their interest and support during the course of this work.

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