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IDENTIFICATION OF A PHOSPHOLIPASE A_1 IN PLASMA MEMBRANES OF RAT LIVER

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SUMMARY

1. A fraction rich in plasma membranes was isolated from the rat liver cell by zonal centrifugation, and the amounts of mitochondria, microsomes, and lysosomes in the fraction were determined by the use of marker enzymes for these organelles. Recovery of the marker enzymes indicated that 94% of the fraction was plasma membranes.

2. The plasma membranes contain a phospholipase that hydrolyzes the 1-acyl ester of phosphatidylethanolamine. Activity of the enzyme is stimulated by Ca^{2+}, and its pH optimum is near pH 9.

3. The acylation reactions of phospholipids that occur in the plasma membranes, together with this report of a phospholipase, provide evidence for a monoacyl–diacyl phosphoglyceride cycle in the plasma membranes.

INTRODUCTION

The procedure developed by NEVILLE[1], and later modified by other workers, made it possible to determine some of the biochemical properties of the plasma membranes of rat liver. Much of the membrane is phosphatidylcholine and phosphatidylethanolamine[2,3], which are important in keeping the membrane intact. Their enzymatic degradation by exogenous phospholipase A in the plasma membranes of intact rat liver cells causes at least one cytoplasmic enzyme to leak out of the cell[1].

The work of HANAHAN[5] and others showed that the two acyl groups of phospholipids turn over independently in the cell to form both the 1-acyl and 2-acyl glycerylphosphorylethanolamine or -choline. SCHERPHOF AND VAN DEENEN[6] and WAITE AND VAN DEENEN[7] fractionated rat liver homogenates and found two phospholipases that formed the monoacyl glycerylphosphoryl derivatives A phospholipase A_1 (specific for the 1-acyl ester) was located in the microsomes, a phospholipase A_2 (specific for the 2-acyl ester) was found in the mitochondria. Phospholipases A_1 and A_2 have also been detected in the lysosomes of the rat liver cell[8–11].

Phospholipid synthesis has also been studied. LANDS AND MERKL[12] found that the microsomes from rat liver contain an acyl transferase catalyzing the synthesis of phosphatidylcholine from monoacyl glycerylphosphorylcholine. In the outer mem-

brane of the mitochondrion there are acylating enzymes that preferentially incorporate an acyl derivative either into the 1- or the 2-position of monoacylglyceryl phosphorylethanolamine[13,14]. These studies demonstrate that monoacyl–diacyl phosphoglyceride cycles exist in the cell. The acylating enzymes and phospholipases in the organelles of the cell may selectively change the nature of the fatty acids at the 1-acyl and 2-acyl positions of the phosphatidylethanolamine or phosphatidylcholine. The altered physical properties of these phospholipids might then affect the properties of the membrane.

Although the other organelles of the cell have been studied, little is known about phospholipid synthesis and degradation in the plasma membranes. STEIN *et al.*[15] and STAHL AND TRAMS[2] reported that acylation reactions occur with phospholipid precursors if plasma membranes are incubated with fatty acids and the necessary cofactors. The ability of a plasma membrane enzyme to degrade phospholipids was unknown. We therefore decided to determine if there were any phospholipases in the plasma membranes of the rat liver cell. We report here a procedure to isolate the plasma membranes of that tissue, and the identification of a phospholipase A_1 in the plasma membranes.

MATERIALS AND METHODS

Isolation of plasma membranes

Plasma membranes of rat liver were isolated from homogenates prepared as follows. One to three rats (male, Dublin, 200–250 g) were decapitated and their livers were perfused with cold 0.01 M $NaHCO_3$ buffer (pH 7.6). The livers were minced in the same buffer and were homogenized in 2 to 3 portions per liver with 8 strokes of a large Dounce homogenizer with a loose-fitting pestle. The homogenate, diluted to 200 ml with the buffer, was filtered once through four layers of gauze and the filtrate was centrifuged at 2000 × *g* for 15 min. The supernatant fraction was discarded.

The Dounce homogenizer was used to resuspend the pellet. The resuspended pellet was diluted to 200 ml with the buffer. The diluted precipitate *plus* a 200 ml buffer overlay were successively pumped onto a discontinuous density gradient composed of 200 ml of 0.59 M sucrose, 300 ml of 1.02 M sucrose and 765 ml of 1.33 M sucrose. The gradient, the sample, and the overlay were each loaded into the Spinco Ti-15 rotor which was rotating at 2500 rev./min. After the overlay had been applied, the head was accelerated to 5000 rev./min, maintained at that speed for 12 min, then decelerated to 2500 rev./min. The gradient was removed in ten 100-ml aliquots, designated "Fractions 1–10", by pumping 1000 ml of 1.33 M sucrose to the outer edge of the rotor bowl. Sucrose concentrations were determined refractiometrically and 5′-nucleotidase was assayed to determine which fractions contained the plasma membranes. All aliquots were diluted with an equal volume of the bicarbonate buffer and centrifuged at 50000 × *g* for 30 min. The precipitates were resuspended in the $NaHCO_3$ buffer.

Isolation of microsomes and rough- and smooth-surfaced endoplasmic reticulum

The microsomes and rough-surfaced and smooth-surfaced endoplasmic reticulum were isolated by the procedure of DALLNER *et al.*[20]. The supernatant fraction was

obtained by centrifuging a homogenate at 20000 × *g* for 30 min in 0.3 M sucrose containing 10 mmoles $MgCl_2$ per liter. The supernatant fraction was centrifuged at 250000 × *g* for 60 min. The pellet from this treatment (the microsomes) was resuspended in 0.3 M sucrose and layered on a solution of 1.5 M sucrose that contained 10 mmoles $MgCl_2$ per l. This discontinuous gradient was centrifuged at 250000 × *g* for 60 min to produce a fluffy layer at the 0.3 M–1.5 M sucrose interface, the smooth surfaced endoplasmic reticulum, and a pellet, the rough surfaced endoplasmic reticulum. Each of these was removed from the tubes, resuspended in 0.3 M sucrose and recentrifuged at 250000 × *g* for 60 min. The pellet in each case was resuspended in 0.3 M sucrose.

Marker enzyme assays; protein and phosphorus determinations

Marker enzymes were assayed to determine the content of subcellular organelles in the homogenate, the precipitate obtained at 2000 × *g*, and Fractions 1–10 of the gradient. NADPH cytochrome *c* reductase (EC 1.6.99.1) was used for microsomes, cytochrome oxidase (EC 1.9.3.1) for mitochondria, and acid phosphatase (EC 3.1.3.2) for lysosomes. These assays were performed as noted by WAITE[16]. The marker enzyme for plasma membranes was 5′-nucleotidase (EC 3.1.3.5), assayed at pH 7.5 as described by MICHELL AND HAWTHORNE[17]. Inorganic phosphorus was determined by the procedure of EIBL AND LANDS[18], protein by the method of LOWRY *et al.*[19]

Incubation procedures; chromatographic techniques

The substrate for the phospholipase reactions was 2-[1′-^{14}C]linoleyl phosphatidylethanolamine, prepared and purified as described previously[7]. Hydrolysis of this substrate with the venom of *Crotalus adamanteus* had shown that more than 95% of the [1-^{14}C]linoleic acid was incorporated at the 2-position of the phosphatidylethanolamine. The following conditions were used in phospholipase assays, except as noted in the legends to the accompanying data: a 1.0 ml mixture containing 20–100 μg of protein, 60 nmoles of phosphatidylethanolamine (300 counts/min per nmole), 2 μmoles of $CaCl_2$, 100 μmoles of glycine–NaOH buffer (pH 9), was incubated 20 min at 37°. The phosphatidylethanolamine was added as an ultrasonicated suspension in water[9].

The lipids were extracted from the phospholipase incubation mixtures by the technique of BLIGH AND DYER[21], dried under nitrogen, and chromatographed with standards on silica gel G plates, first in chloroform – light petroleum – glacial acetic acid (70:30:2, by vol.), then in chloroform – methanol – water (70:30:4, by vol.). The respective portions of the silicic acid containing the fatty acid, monoacyl glycerylphosphorylethanolamine, and phosphatidylethanolamine, were scraped into scintillation vials and counted.

RESULTS

Relative specific activities of the 4 marker enzymes in each of the 10 fractions of the zonal gradient obtained from one rat liver are depicted in Fig. 1. The lysosomes (acid phosphatase used as the marker enzyme) and microsomes (NADPH–cytochrome *c* reductase used as the marker enzyme) were confined mainly to the loading zone, the volume of the sample and the overlay. NADPH–cytochrome *c* reductase activity

was slightly increased in Fractions 9 and 10 relative to Fractions 4–8. The plasma membranes with the highest specific activity of 5′-nucleotidase (the plasma membrane marker enzyme) were found in Fractions 9 and 10. The specific activity of 5′-nucleotidase in Fraction 10 was twice as great as that of Fraction 9. The mitochondria (cytochrome oxidase used as the marker enzyme) entered the gradient and were found primarily in Fractions 5–8. Under these conditions the mitochondria sedimented slower than the plasma membranes but faster than the lysosomes and microsomes. Longer centrifugations caused much of the mitochondria to sediment with the plasma membranes.

45 % of the protein loaded into the rotor was recovered from the gradient in Fractions 1–10. Cell nuclei and intact cells sediment much faster than the plasma membranes and organelles[22]. They went through the gradient, collected on the rotor wall, and so were not recovered when the gradient was collected from the rotor.

Table I shows the total activities and percent recovery of the 4 marker enzymes

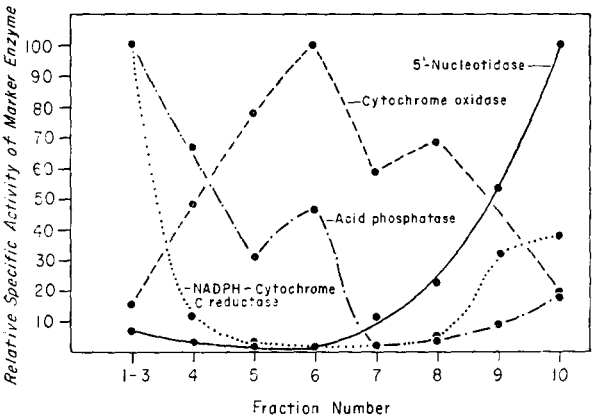

Fig. 1. Relative specific activities of the marker enzymes in each fraction obtained from a zonal preparation performed with one rat liver. The relative specific activities were calculated by setting the highest value found for each enzyme at 100% and determining the relative specific activities as a ratio to that value. The highest value for each marker enzyme, expressed as nmoles of product formed per min per mg of protein, was as follows: NADPH-cytochrome *c* reductase, 81.3; cytochrome oxidase, 1295; 5′-nucleotidase, 1365; acid phosphatase, 48.2.

TABLE I

TOTAL MARKER ENZYME ACTIVITIES AND PERCENT RECOVERY IN THE HOMOGENATE, PRECIPITATE OBTAINED AT 2000 × *g*, AND FRACTION 10 OBTAINED FROM THE ZONAL CENTRIFUGE PREPARATION

Total activity units are nmoles product formed per min per fraction. Percent recovery values (%) were calculated as the total activity in each fraction divided by the total activity in the homogenate, times 100.

Sample	*Protein (mg)*	*5′-Nucleotidase*		*Cytochrome oxidase*		*NADPH–cytochrome c reductase*		*Acid phosphatase*	
		Total Activity	%	*Total activity*	%	*Total activity*	%	*Total activity*	%
Homogenate	1620	$6.38 \cdot 10^4$	100	$4.15 \cdot 10^5$	100	$5.07 \cdot 10^4$	100	$4.38 \cdot 10^4$	100
Precipitate, 2000 × *g*	540	$4.27 \cdot 10^4$	67	$2.57 \cdot 10^5$	62	$2.34 \cdot 10^4$	46	$3.20 \cdot 10^3$	9.2
Fraction 10	3.74	$5.11 \cdot 10^3$	8.0	$9.59 \cdot 10^2$	0.23	$1.14 \cdot 10^2$	0.22	$3.40 \cdot 10^1$	0.078

in the homogenate, the precipitate obtained at 2000 × *g*, and Fraction 10. 8 % of the 5'-nucleotidase of the homogenate was recovered in Fraction 10 (at the 1.02–1.33M sucrose interface). In this fraction many large membrane fragments and a few mitochondria could be seen by phase contrast microscopy. Less than 0.25 % of the homogenate cytochrome oxidase, NADPH– cytochrome *c* reductase, and acid phosphatase was recovered in Fraction 10. In some preparations more of the plasma membrane-containing fractions were pooled. This yielded a preparation that contained a higher total activity but a decreased specific activity of 5'-nucleotidase (*cf.* Fig. 2 and Table III).

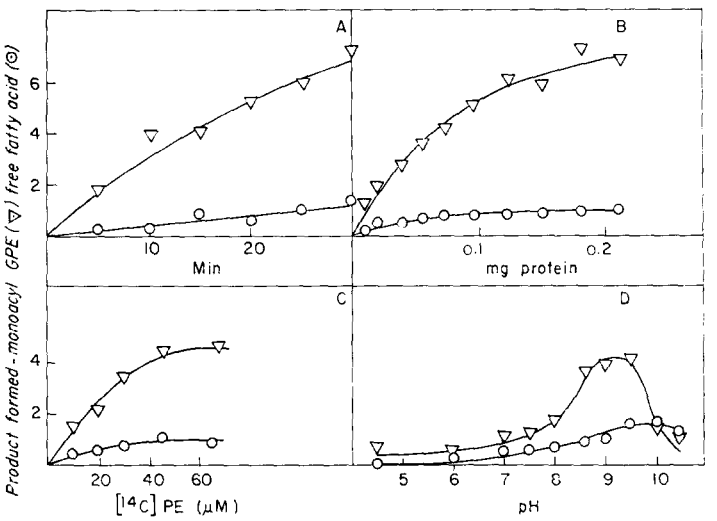

Fig. 2. Determination of optimal conditions for phospholipase A_1 activity. A. The tubes were incubated at 37° for up to 30 min. The 1.0 ml reaction mixture contained 60 nmoles of 2-[1'-^{14}C]-linoleyl phosphatidylethanolamine, 2 μmoles of $CaCl_2$, 100 μmoles of glycylglycine (pH 8.6), and 0.10 mg plasma membrane protein. The formation of 2-[1'-^{14}C]linoleyl glycerylphosphoryl-ethanolamine (monoacyl GPE) and [1-^{14}C]linoleate (free fatty acid) is expressed as nmoles of product recovered. B. Reaction mixture here was the same as in A, incubation was for 20 min at 37°. Formation of 2-[1'-^{14}C]linoleyl glycerylphosphorylethanolamine and [1-^{14}C]linoleate is expressed as nmoles formed per 20 min. C. Tubes were incubated as in A except that the amounts of substrate were varied. The substrate was added as an ultrasonicated suspension in water; 0.180 mg of plasma membrane protein was used. PE = phosphatidylethanolamine D. Reaction conditions were as in A with a 20 min incubation at 37°. 100 μmoles of each buffer was used (sodium acetate to pH 6, glycylglycine to pH 8.6, glycine–NaOH from pH 8.6 to 10.5).

The presence of mitochondria, microsomes, and lysosomes as contaminants of Fraction 10 is quantitated in Table II. This table compares the ratios of total activity of the marker enzyme for the plasma membrane to the total activity of the marker enzymes for the other organelles in the homogenate (Table II-A) and in the membrane-rich Fraction 10 (Table II-B). The increase of the 5'-nucleotidase activity in Fraction 10 relative to that of the other three marker enzymes (Table II-C) is taken as the concentration of the three organelles in Fraction 10 relative to the plasma membranes. From these data we conclude that there was 6 % contamination: 2.5 % of Fraction 10 is mitochondria, 2.7 % is microsomes, 0.9 % is lysosomes (Table II-D); the remaining 94 % is plasma membranes.

Optimal assay conditions were determined for the phospholipase; the data in Fig. 2 were obtained with a pooled plasma membrane fraction that had a 5′-nucleotidase specific activity of 528 nmoles/min per mg, and a phospholipase A_1 specific activity of 4.0 nmoles/min per mg. With preparations of plasma membranes with higher 5′-nucleotidase specific activity than those used in the data of Fig. 2, (*cf.* Table III) the specific activity of the plasma membrane phospholipase A_1 was correspondingly greater. Throughout this work we observed that incubation of plasma

TABLE II

RATIOS OF TOTAL 5′-NUCLEOTIDASE ACTIVITY TO TOTAL MARKER ENZYME ACTIVITY IN THE HOMOGENATE AND IN FRACTION 10

The ratios of the total 5′-nucleotidase activity to the total activities of each of the marker enzymes (*cf.* Table I) in Fraction 10 (B) are divided by the same ratios in the homogenate (A). These three quotients (C) show the amount of purification of the plasma membranes as compared to the other organelles. The reciprocal of the quotients is an index of the contamination of Fraction 10 by the organelles, which were normalized to show the overall purity of the plasma membranes (D). The data were normalized by taking the sum of the reciprocals of the quotients of the 4 fractions (C, plasma membranes equal to 1). Each reciprocal was divided by the sum of the reciprocals, times 100.

	5′-Nucleotidase / NADPH–cytochrome c reductase	*5′-Nucleotidase / Cytochrome oxidase*	*5′-Nucleotidase / Acid phosphatase*
A. Homogenate	1.26	0.154	1.45
B. Fraction 10	44.8	5.33	150.0
C. Fraction 10 (B) / Homogenate (A)	35.5	34.6	103.5
	% *Contamination*		
	Microsomes	*Mitochondria*	*Lysosomes*
D. Fraction 10	2.5	2.7	0.9

TABLE III

SPECIFIC ACTIVITIES OF 5′-NUCLEOTIDASE AND PHOSPHOLIPASE A IN THE FRACTIONS OBTAINED IN THE PLASMA MEMBRANE ISOLATION

Specific activities in nmoles of product formed per min per mg of protein. Phospholipase A activity was measured by the formation of 2-[^{14}C]linoleyl glycerylphosphorylethanolamine and [^{14}C]linoleate. In the incubations, done as described in MATERIALS AND METHODS, 20 μg plasma membrane protein or 100 μg of the precipitate obtained at 2000 × *g* was used.

Sample	*Specific activity*		
	5′-Nucleotidase	*Phospholipase A*	
		[^{14}C]Linoleate	*2-[^{14}C]Linoleoyl glyceryl-phosphorylethanolamine*
Homogenate	39.3	n.d.*	n.d.*
Precipitate, 2000 × *g*	79.0	0.78	1.56
Fraction 10	1365	2.30	16.8

* Not determined.

membranes with radiolabeled phosphatidylethanolamine produced more 2-[1′-^{14}C]-linoleyl glycerylphosphorylethanolamine than [1-^{14}C]linoleic acid (Fig. 2). This indicates that the plasma membranes contain a phospholipase A that acts at the 1-acyl position of the substrate. Because of its specificity this plasma membrane phospholipase is classified as a phospholipase A_1[23]. There was also a small amount of activity specific for the 2-acyl position, caused by either a phospholipase A_2, or a lysophospholipase, or both. Fig. 2A shows that phospholipase A_1 activity was proportional to incubation time up to 30 min. Phosphatidylethanolamine hydrolysis was proportional to the quantity of plasma membrane protein present to as much as 0.1 mg (Fig. 2B). Substrate concentration was rate-limiting below 40 μM (Fig. 2C). The optimum pH for the formation of 2-[1′-^{14}C]linoleyl glycerylphosphorylethanolamine is between pH 8.6 and 9.5, that for the formation of [1-^{14}C]linoleic acid is between pH 9.5 and 10.0 (Fig. 2D). There is little phospholipase A activity at lower pH's. The phospholipase A_1 was also found to be stimulated by Ca^{2+}. If plasma membranes were incubated in the presence of 2 mM EDTA rather than 2 mM $CaCl_2$ only 20% as much substrate was hydrolyzed.

Table III shows the specific activities of 5′-nucleotidase and the phospholipases obtained in the isolation procedure. The specific activity of the 5′-nucleotidase of the membranes in Fraction 10 is 1365 nmoles/min per mg, and is 35 times the specific activity of the homogenate, and 17 times the 5′-nucleotidase specific activity of the precipitate obtained at 2000 × *g*. The range of the specific activity of 5′-nucleotidase in several preparations was from 1000 to 1365 nmoles/min per mg. The phospholipase A_1 is 11 times as active as the precipitate; the phospholipase A_2 or lysophospholipase is 3 times as active as the precipitate. The discontinuity between the purification of the plasma membranes and the phospholipase A_1 and the phospholipase A_2 or lysophospholipase might be expected, since the precipitate obtained at 2000 × *g* contains many mitochondria and microsomes (*cf.* Table I).

As correlative evidence that the phospholipase A_1 was located in the plasma membrane, phospholipase A_1 activity was compared to the 5′-nucleotidase activity in each of the aliquots obtained in a zonal preparation (Fig. 3). The specific activity

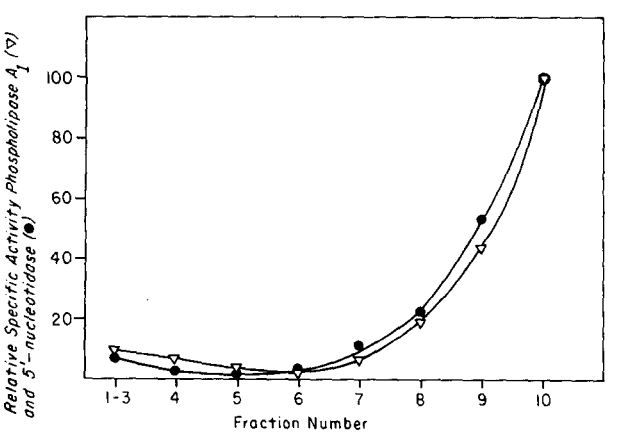

Fig. 3. Comparison of phospholipase A_1 and 5′-nucleotidase acitvity in fractions from the gradient. 0.05 to 0.20 mg protein was used in the incubations. The relative specific activities were determined as indicated in the legend to Fig. 1.

of the phospholipase A_1 in each fraction obtained from the gradient parallels the 5'-nucleotidase specific activity in that fraction, although the phospholipase A_1 activity is somewhat higher than the 5'-nucleotidase activity in Fractions 1–4, the microsome-rich fractions.

The plasma membrane phospholipase A_1 is similar to the microsomal phospholipase[7]. Each hydrolyzes the 1-acyl ester of phosphatidylethanolamine, and each has a pH optimum on the alkaline side of neutrality. Table IV shows the specific activities of the phospholipases A_1 and 5'-nucleotidase from plasma membranes, microsomes, and rough- and smooth-surfaced endoplasmic reticulum as isolated by the procedure of DALLNER *et al.*[20]. The small amount of plasma membranes in the microsomes and endoplasmic reticula indicates that the phospholipase A_1 activity found there does not come from contaminating plasma membranes. The small amount of microsomes in the plasma membranes of Fraction 10 (Table II) indicates that not more than 2 to 3 % of the phospholipase A_1 activity of the plasma membranes could have been caused by contaminating microsomes.

TABLE IV

SPECIFIC ACTIVITIES OF PHOSPHOLIPASE A_1 AND 5'-NUCLEOTIDASE IN THE PLASMA MEMBRANES, MICROSOMES, AND ROUGH- AND SMOOTH-SURFACED ENDOPLASMIC RETICULUM OF RAT LIVER

Incubations were performed as described in MATERIALS AND METHODS with 20 μg of plasma membrane protein or 30 μg protein of the rough- and smooth-surfaced endoplasmic reticulum. Phospholipase A_1 activity is expressed as nmoles of 2-[^{14}C]linoleyl glycerylphosphorylethanolamine produced per min per mg of protein.

Sample	*Specific activity*	
	Phospholipase A_1	*5'-Nucleotidase*
Plasma membranes	16.8	1365
Microsomes	10.6	66
Rough-surfaced endoplasmic reticulum	13.0	43
Smooth-surfaced endoplasmic reticulum	10.0	189

DISCUSSION

There has been a growing interest in plasma membranes in the last decade, and procedures have been devised to isolate them[1,3,24–27]. One of the latest, that of RAY[25], produces plasma membranes with a high specific activity of 5'-nucleotidase, in good yield. Our zonal isolation procedure yields plasma membranes with a 5'-nucleotidase specific activity that is equally high. The zonal procedure may be more convenient than RAY's[25] since the homogenate is easily prepared and the precipitate obtained at 2000 $\times$ *g* is used on the zonal gradient with no further treatment.

The zonal procedure may also be applicable to the large-scale production of plasma membranes. The plasma membranes from as many as 3 rat livers have been isolated simultaneously on this gradient. One can increase the recovery of the plasma membrane fraction by taking a larger sample from the gradient, although this fraction will have a lower specific activity of 5'-nucleotidase. We estimate that 94 % of the

protein in Fraction 10 is from the plasma membranes, which quantitates the purity of the membranes isolated by this procedure, as well as those isolated by the procedure of RAY[25]. The specific activity of 5'-nucleotidase in an uncontaminated preparation of plasma membranes should therefore be about 1500 nmoles/min per mg protein.

A phospholipase A_1 was present in plasma membranes as shown by formation of 2-[1'-^{14}C]linoleyl glycerylphosphorylethanolamine when plasma membranes were incubated with Ca^{2+} and radiolabeled phosphatidylethanolamine. This activity could not be attributed to a lipase: under the same assay conditions there was no hydrolysis of radiolabeled tripalmitoyl-glycerol. [1-^{14}C]Linoleic acid was also formed, the result of the action of a phospholipase A_2 or a lysophospholipase. These products are formed optimally at different pH's. The plasma membrane phospholipase A_1 has a higher pH optimum than any of the lysosomal phospholipases and requires Ca^{2+} for activity, whereas the lysosomal phospholipase A_1 is inhibited by Ca^{2+} (see refs. 8–10). The mitochondrial phospholipase requires Ca^{2+} and has an optimum pH near 9.0, but it is a phospholipase A_2[9,28]. The plasma membrane phospholipase A_1 is similar to the microsomal phospholipase: both require Ca^{2+}, have an alkaline pH optimum, and are specific for the 1-acyl position of phosphatidylethanolamine[6,7,29]. The small microsomal contamination of the plasma membrane preparations, the low 5'-nucleotidase activity in the microsomes, and the high phospholipase activity in both microsomes and plasma membranes (Table IV) lead us to believe that the same enzyme is present in both organelles.

STEIN *et al.*[15] observed that radiolabeled 1-acyl glycerylphosphorylcholine was acylated when incubated with plasma membranes. The phosphatidylcholine synthesized by the membrane did not exchange with the phospholipid in the medium, and thus appeared to have become a structural component of the membrane. STAHL AND TRAMS[2] similarly found that acylation reactions occur when plasma membranes of rat liver are incubated with acyl-CoA derivatives. The fatty acids were incorporated into phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine. The presence of these acylation reactions, and the phospholipase reported here, constitute evidence for a monoacyl–diacyl phsophoglyceride cycle in the plasma membranes. Such a cycle was first suggested by ROBERTSON AND LANDS[30] for the endoplasmic reticulum. The existence of phospholipases and acylating enzymes in an organelle or cell membrane could provide for the synthesis of specific phospholipids with acyl derivatives of different chain length and saturation. Synthesis and degradation of these phospholipids may be important in the maintenance of the structure and functions of the membrane.

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