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# METHYLATION OF PHOSPHOLIPIDS IN MICROSOMES OF THE RAT AORTA

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**The methylation of phospholipids by *S*-adenosyl-L-methionine was characterized in microsomes prepared from strips of rat aorta. In the presence of 0.5 μM *S*-adenosyl-L-methionine, endogenous phosphatidylethanolamine was methylated to form three products: phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine. In the presence of 150 μM *S*-adenosyl-L-methionine the methylation activity increased more than 50-fold and the principal radioactive product was phosphatidylcholine. Optimal activity was at pH 9 and no magnesium requirement was detected. Exogenous phosphatidylethanolamine, phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine served as substrates for the enzyme. The methylation of exogenous phosphatidyl-*N*,*N*-dimethylethanolamine proceeded at a slower rate. Incubation of trypsin with the aorta microsomes reduced the enzymatic activity and reduced the relative yield of phosphatidyl-*N*-monomethylethanolamine. Phospholipase C degraded the methylated phospholipids, but phosphatidyl-*N*,*N*-dimethylethanolamine appeared to be less accessible to the phospholipase. The phospholipid methylation activity was inhibited by the addition of *S*-adenosyl-L-homocysteine or by L-homocysteinethiolactone. When intact strips of rat aorta were incubated with L-[*methyl*-$^3$H]methionine, [$^3$H]methyl groups were incorporated into phospholipids. This incorporation was inhibited when L-homocysteinethiolactone was added to the incubation. Polarized fluorescence of diphenylhexatriene in aorta microsomes was measured to determine the apparent membrane fluidity. When intact strips of aorta were incubated with methionine or with L-homocysteinethiolactone, methionine enhanced and L-homocysteinethiolactone decreased apparent fluidity of the microsomal membranes. Phospholipid methylation activity was examined in aorta microsomes prepared from genetically spontaneous hypertensive SHR strain rats. Phospholipid methylation activity was substantially greater in the SHR aorta microsomes than in microsomes prepared from Wistar-Kyoto WKY control strain aorta. Membrane fluidity was greater in the SHR aorta microsomes than in the WKY aorta microsomes. The hypothesis that phospholipid methylation activity influences fluidity of membranes and the possible involvement of methylated phospholipids in aorta membrane functions are discussed.**

## Introduction

Recent studies carried out by Axelrod and coworkers [1–3] have demonstrated the synthesis of phosphatidylcholine by the methylation of phos-

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Abbreviations: PME, phosphatidyl-*N*-monomethylethanolamine; PDE, phosphatidyl-*N*,*N*-dimethylethanolamine; PC, phosphatidylcholine.

phatidylethanolamine in biological membranes. In the tissues that were examined, the synthesis of phosphatidylcholine from phosphatidylethanolamine in biological membranes was found to involve at least two distinct enzymes. One enzyme methylates phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine (PME), has a high affinity for the methyl donor, *S*-adenosyl-L-methionine, and requires magnesium. The second enzyme transfers two methyl groups to PME to form phosphatidylcholine (PC). Phosphatidyl-*N*,*N*-dimethylethanolamine (PDE) is an intermediate product. The second enzyme has a lower affinity for *S*-adenosyl-L-methionine and does not require magnesium. The two enzymes and their products are asymmetrically distributed in the membranes. The methylation of phosphatidylethanolamine to PME in the erythrocyte alters membrane fluidity as determined by the measurement of the polarized fluorescence of diphenylhexatriene [4].

Phosphatidylcholine is formed principally via the transfer of a phosphocholine group from CDPcholine to a diacylgycerol molecule [15]. Phospholipid methylation activity in the liver is very high and there it contributes significantly (20–40%) to the net synthesis of phosphatidylcholine [6].

Evidence is accumulating which indicates that changes in phospholipid methyltransferase activity occur in the transduction of a variety of signals acting at the cell surface [7–16]. Membrane phospholipid methylation appears to be widespread, having been identified in reticulocytes [7], erythrocytes [2], leukocytes [8,14], synaptosomes [17], lymphocytes [10] bovine adrenal medulla microsomes [1], rat liver microsomes [43], platelets [18] and in kidney cortex brush-border membranes [40].

Vascular muscle is an excitable tissue and phospholipid methylation may conceivably play a role in transducing signals modulating vascular muscle contraction. In the present study, phospholipid methylation activity was examined in microsomes prepared from rat aorta. The polarized fluorescence of diphenylhexatriene in rat aorta microsomes was also determined. Aortas obtained from genetically hypertensive SHR strain rats were included in the study.

## Methods

*Materials.* Authentic phospholipids were obtained for the identification of the methylated phospholipids. Phosphatidylethanolamine, phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine were obtained from Grand Island Biological Co., Grand Island, NY. These phospholipids were derivatives of egg phosphatidylcholine prepared by exchange of bases in the presence of phospholipase D. Bovine and porcine brain phosphatidylcholine were purchased from Sigma Chemical Co., St. Louis, MO. Phospholipase C (*Clostridium welchii*) was also obtained from Sigma. Trypsin and trypsin inhibitor were obtained from Boeringer-Mannheim Inc., Indianapolis, IN.

*S*-Adenosyl-L-[*methyl*-$^3$H]methionine (82.6 Ci/mmol) was purchased from New England Nuclear Corp., Boston, MA. This preparation of *S*-adenosyl-L-methionine was diluted with 100 mM Tris-glycylglycine buffer (pH 8), to obtain a concentration lower than 2 $\mu$M. Unlabeled *S*-adenosyl-L-methionine purchased from Sigma Chemical Corp., St. Louis, MO was added to labeled *S*-adenosyl-L-methionine to obtain concentrations higher than 2 $\mu$M. L-[*methyl*-$^3$H]Methionine was obtained from New England Nuclear Corp., Boston, MA. Aquasol was obtained from Amersham Corp., Arlington Heights, IL. All other reagents employed were of analytical grade.

SHR genetically hypertensive rats and WKY Wistar-Kyoto normotensive control strain rats were obtained from Taconic Farms, Germantown, NY. Male Sprague-Dawley rats were obtained from Harlan Co., Indianapolis, IN, and Sasco Co., St. Louis, MO.

*Preparation of aortic microsomes.* Rats were killed by guillotine and the aminals were allowed to bleed. A section of thoracic aorta between the heart and the diaphragm was removed. Extraneous connective tissue was removed and aortas were opened longitudinally with scissors. Strips were then washed with ice-cold 0.25 M sucrose. Four or five aortas were pooled for each microsomal preparation. The strips were minced very finely with scissors and then were homogenized in 10 ml of ice-cold sucrose (0.25 M) at 2000 rpm (two series of ten strokes up and down) with a Potter-Elvej-

hem homogenizer and a Teflon pestle. The homogenate was filtered through nitex. The homogenate was centrifuged for 10 min at 3500 rpm (1475 × *g*) in a Sorvall SS 34 rotor and the pellet was discarded. The supernatant was centrifuged in the same rotor at 15 000 rpm (27 000 × *g*) for 10 min. The new supernatant was centrifuged at 105 000 × *g* (40 000 rpm) in a Beckman Spinco type 50 Ti fixed angle rotor in a Beckman L2-65 ultracentrifuge for 60 min. The final pellet was resuspended in 0.1–0.2 ml Tris-glycylglycine buffer pH 8.2, 50 mM, except as otherwise stated. Fractionation of rat aorta microsomes on a sucrose density gradient is described by Moore et al. [36].

*Assay for phospholipid-N-methyltransferases.* These enzymes were assayed by measuring [$^3$H]methyl groups transferred from $^3$H-labeled *S*-adenosyl-L-methionine to the endogenous phosphatidylethanolamine in the membrane. The total volume of reaction mixture (50 μl) was composed of membrane preparation (200–300 μg protein) in 50 mM Tris-glycylglycine buffer (pH 8.2) containing 5 mM $MgCl_2$ and 0.1 mM EDTA and *S*-adenoyl-L-[*methyl*-$^3$H]methionine. The reaction was started by adding *S*-adenosyl-L-[*methyl*-$^3$H]methionine to the membrane suspension in a 6 ml Falcon tube. The final reaction mixture was incubated at 37°C for 60 min. The reaction was stopped by the addition of 0.5 ml 10% trichloroacetic acid and the tubes were kept on ice for 15 min. The tube was centrifuged at 12 100 × *g* (10 000 rpm) in a Sorvall RC-2 centrifuge for 10 min and the supernatant was decanted and discarded. The lipids were extracted from the precipitated protein by 3 ml of chloroform/methanol/hydrochloric acid (2 : 1 : 0.02, v/v) and the tubes were shaken for 10 min on a mechanical shaker. The chloroform/methanol/HCl extract was washed twice by shaking with 2 ml of 0.1 M KCl in 50% methanol. The aqueous phase was aspirated each time and discarded. The 1-ml aliquot of the chloroform phase was transferred to a counting vial and the solvent was evaporated to dryness at 80°C. The residue of phospholipids was dissolved in 10 ml Aquasol and the total radioactivity was measured. The remaining chloroform phase was dried over anhydrous $Na_2SO_4$ and was used for characterization and quantitation of phospholipid products.

The amount of [$^3$H]methyl groups incorporated into phosphatidyl-*N*-methylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine was determined after separation by thin-layer chromatography (TLC). The total radioactivity incorporated into phospholipids was accounted for by the three methylated phospholipids.

*TLC of phospholipids.* The chloroform extract of phospholipids was concentrated under a stream of nitrogen, and chromatographed on silica gel-G plates (Uniplate, Analtech Inc. Newark, DE). The chromatograms were developed in a solvent system comprised of chloroform/propionic acid/*n*-propyl alcohol/water (2 : 2 : 3 : 1, v/v). The solvent was allowed to ascend 6.5 inches. The spots were visualized by exposure to iodine vapor and 0.25-inch sections were transferred to counting vials. 10 ml Aquasol was added to each vial and the radioactivity was counted in a Beckman LS 100 C liquid scintillation system. Authentic samples of phospholipids were chromatographed simultaneously. Radioactivities in the TLC peaks corresponding to phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine were determined and expressed as percentage of total radioactive phospholipids on the TLC plate. To confirm the identity of the radioactive phospholipids separated by TLC, the procedure described by Schneider and Vance was employed [41]. The phospholipid products were hydrolyzed in 6 M HCl at 100°C for 3 h and the free bases were chromatographed on thin-layer plates with *n*-butanol/methanol/HCl/$H_2O$ (50 : 50 : 10 : 10, v/v). In addition several of the samples were analyzed for phospholipid by two-dimensional chromatography employing chloroform/methanol/ammonia (60 : 35 : 5, v/v) followed by chloroform/methanol/acetone/acetic acid/water (50 : 10 : 20 : 10 : 5, v/v) [10].

*Treatment of microsomes with phospolipase C.* Aortic microsomes were incubated at 37°C with *S*-adenosyl-L-[*methyl*-$^3$H]methionine, (1 μM) and other components described for the assay of phospholipid methyltransferase activity. The volume of the incubation was 50 μl. At the end of the 60 min incubation 50 μl 4 mM *S*-adenosyl-L-homocysteine in 0.1 M Tris-HCl buffer (pH 7.4) was added to stop the reaction. Then 100 μl phos-

pholipase C (10 units in 100 $\mu$l of 0.1 M Tris-HCl buffer (pH 7.4)) was added to each tube and incubated at 37°C for 5 min. After 5 min, the phospholipids were extracted from the microsomes and quantitated as described above. Hydrolysis of phospholipids was determined by comparison of the phospholipase treated microsomes with those incubated without phospholipase C.

*Treatment of microsomes with trypsin.* Microsomes suspended in 0.25 M sucrose/0.05 M Tris-HCl (pH 7.4) were incubated with trypsin (1 mg/50 mg microsomal protein) for 30 min at 37°C. At the ends of incubation period, trypsin inhibitor (1 mg/mg trypsin) was added. In control experiments, the inhibitor was added with trypsin at the beginning of the incubation. After trypsin treatment, the phospholipid methyltransferase activity was assayed as described above.

*Preparation of opened microsomal vesicles.* Microsomal vesicles were opened by treatment with 0.4% taurocholate in 0.25 M sucrose buffered with 0.05 M Tris-HCl (pH 7.4). The taurocholate treatment was for 30 min with the incubation tubes on ice. This procedure causes loss of mannose-6-phosphatase latency [19].

*Incubation of rat aorta strips with L-[methyl-$^3$H]methionine.* A section of thoracic aorta was removed, freed of extraneous connective tissue and opened longitudinally with scissors. Two or four arterial strips were employed in each incubation. The strips were incubated in Krebs-Ringer bicarbonate saturated with 95% $O_2$/5% $CO_2$ at 37°C for 30 min. The L-[*methyl*-$^3$H]methionine was then added and the incubation continued for another 60 min. The strips were then washed with 10 mM nonradioactive L-methionine in 0.25 M sucrose and finally washed with 0.25 M sucrose. The aorta strips were homogenized and the homogenate was filtered through nitex. An aliquot of homogenate was employed for extraction of phospholipids and separation of the methylated phospholipids by TLC.

*Steady-state fluorescence polarization.* Trans-1,6-Diphenyl-1,3,5-hexatriene (Aldrich Chemical Co., Milwaukee, WI) was employed as a probe for determining the steady-state fluorescence polarization, fluorescence anisotropy and for estimation of the structural order parameter [20] of the hydrocarbon region of the lipid layer of the microsomal membrane. 2 mM diphenylhexatriene in tetrahydrofuran was diluted 1000-fold with 50 mM Tris-HCl buffer (pH 7.4). The mixture was stirred vigorously for about 30 mins. This provides a clear, stable, aqueous dispersion of 2 $\mu$M diphenylhexatriene which is essentially void of fluorescence. A microsomal suspension (100 $\mu$g protein/ml) in 50 mM Tris-HCl buffer (pH 7.4) is diluted with an equal volume of 2 $\mu$M diphenylhexatriene and is incubated for 30 min in the dark at 25°C. Steady-state fluorescence polarization measurement is made with an Aminco SPF 500 spectrofluorometer equipped with a polarizing attachment. The excitation and emission wavelengths are 360 and 430 nm, with band-pass 5 and 8, respectively. Fluorescence intensities are measured with polarizers parallel and perpendicular to the vertically polarized exciting beam. The temperature in the cuvettes is controlled with a thermostatically controlled circulating water pump and measured with a thermistor probe. Temperature measurement is accurate to 0.5°C. Steady-state fluorescence polarization ($P$) is calculated as:

$$P = (I_{vv} - I_{vh})/(I_{vv} + I_{vh})$$

where $I_{vv}$ and $I_{vh}$ are observed intensities measured with polarizers parallel and perpendicular to the vertically polarized exciting beam, respectively. The steady-state fluorescence anisotropy ($\gamma_s$) is calculated for the relation

$$\gamma_s = 2P/(3 - P)$$

where $P$ is the fluorescence polarization. Limiting fluorescence anisotropy and lipid structural order parameter are calculated according to the procedure of Van Blitterswijk et al. [20]. Apparent microviscosity and its reciprocal, which is a measure of membrane fluidity, were calculated from polarized fluorescence according to the procedure of Shinitzky and Barentholtz [21].

## Results

*Phospholipid methylation in rat aorta microsomes.*

When rat aorta microsomes were incubated with *S*-adenosyl-L-[*methyl*-$^3$H]methionine and the phospholipids were extracted and separated by

thin-layer chromatography, three radioactive peaks corresponding to the methylated compounds PME, PDE and PC were obtained (Fig. 1). The identity of the methylated products was further established by two-dimensional chromatography and by hydrolysis of the phospholipids and identification of the free bases as described in methods. The rat aorta microsomes were incubated with *S*-adenosyl-L-[*methyl*-$^3$H]methionine at levels which ranged from 0.5 to 150 $\mu$M. The net accumulation of (*methyl*-$^3$H)-labeled PC increased progressively as the level of *S*-adenosyl-L-methionine was increased in the incubation. When the aorta microsomes were incubated with 0.5 $\mu$M *S*-adenosyl-L-[*methyl*-$^3$H]methionine for 60 mins, radioactive methyl groups were distributed in the three phospholipid compounds (40% PC, 25% PDE and 35% PME) (Fig. 1A). When the aorta microsomes were incubated with 150 $\mu$M *S*-adenosyl-L-methionine for 60 mins, there was a 54-fold increase in total phospholipid methylation from that seen with 0.5 $\mu$M *S*-adenosyl-L-methionine. The principal radioactive product was phosphatidylcholine (59% of the total radioactivity) (Fig. 1B).

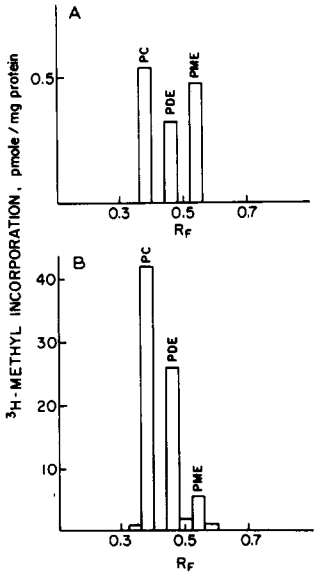


Fig. 1. Chromatographic profile of (*methyl*-$^3$H)-labeled phospholipids separated by thin-layer chromatography after the incubation of Sprague-Dawley rat aorta microsomes with *S*-adenosyl-L-[*methyl*-$^3$H]methionine. The ordinate is pmol [$^3$H]methyl incorporated into phospholipids per mg microsomal protein. (A) Incubation with 0.5 $\mu$M *S*-adenosyl-L-[*methyl*-$^3$H]methionine; (b) incubation with 150 $\mu$M *S*-adenosyl-L-[*methyl*-$^3$H]methionine. Incubation time, 60 min.

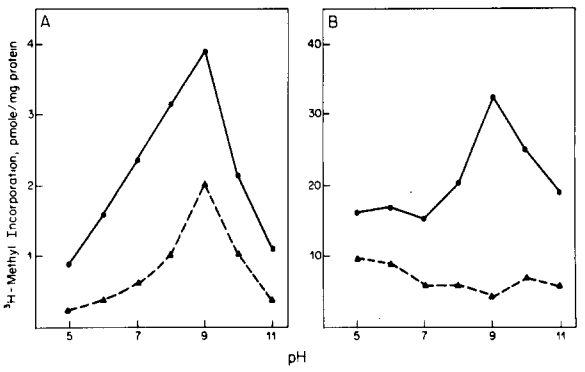


Fig. 2. Phospholipid methyltransferase activity of rat aorta microsomes at various pH levels. The ordinate is pmol [$^3$H]methyl incorporated into phospholipids per mg of microsomal protein. Tris-acetate buffer was employed between pH 5 and pH 8. Tris-glycylglycine buffer was employed between pH 9 and pH 11. Microsomal protein (200 $\mu$g) incubated for 60 min. (A) 1 $\mu$M *S*-adenosyl-L-methionine; (B) 150 $\mu$M *S*-adenosyl-L-methionine. ▲———▲, PME formed ●———●, PDE + PC formed.

Methylation of rat aorta microsomal phospholipids was measured between pH 5 and pH 11. The microsomes were incubated for 60 mins with 1 $\mu$M and 100 $\mu$M *S*-adenosyl-L-methionine (Fig. 2). The optimal activity was at pH 9 with both levels of *S*-adenosyl-L-methionine. At pH 9 at the high *S*-adenosyl-L-methionine level, product formation was greatly enhanced and the product accumulated shifted from PME to PC. Magnesium (5 mM) was routinely included in the assays, but omission of magnesium at low or high *S*-adenosyl-L-methionine levels did not affect the methylation activity.

*Effect of exogenous phospholipids.*

Phosphatidylethanolamine, PME and PDE (50 $\mu$g and 500 $\mu$g) were added exogenously to aorta microsomes incubated with 100 $\mu$M *S*-adenosyl-L-methionine (Table I). The addition of exogenous phospholipid enhanced the phospholipid methylation activity. The enhancement was principally an increase in the methylation specific for the substrate that was added. The addition of 50 $\mu$g substrate resulted in a 2-fold increase in methyl group incorporation. Addition of 500 $\mu$g PE re-

TABLE I

EFFECT OF EXOGENOUSLY ADDED PHOSPHOLIPIDS ON [$^3$H]METHYL INCORPORATION INTO *N*-METHYL DERIVATIVES OF PHOSPHATIDYLETHANOLAMINE

Aortic microsomes (0.3–0.4 mg protein) were incubated with phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME), and phosphatidyldimethylethanolamine (PDE) at indicated concentration for 60 min. The phospholipids were suspended in 50 mM Tris-glycylglycine (pH 8.2) buffer/0.05% Triton X-100, by sonication at 20°C for 30 min. Methylation was carried out for 60 min by the addition of 100 $\mu$M *S*-adenosyl-L-[*methyl*-$^3$H]methionine and the reaction was terminated by addition of 10% trichloroacetic acid. Phospholipids were extracted and separated. Values are mean ± S.E. of three experiments. Control values are absolute in pmol [$^3$H]methyl/mg protein per 60 min. All other values are ratios of experimental: control. The methylation product specific for the added exogenous substrate is in italics. 50 $\mu$g exogenous phospholipid represents a final concentration of 1.33 mM.

| Additions | Total incorporation | PME | PDE | PC |
|---|---|---|---|---|
| None (control) | 25.47 ± 1.89 | 6.68 ± 0.09 | 7.57 ± 0.84 | 9.89 ± 0.92 |
| Triton X-100 (0.05%) | 0.8 ± 0.05 | 0.79 ± 0.02 | 0.9 ± 0.5 | 0.84 ± 0.02 |
| PE (50 $\mu$g) | 1.65 ± 0.06 | *2.21* ± 0.08 | 1.85 ± 0.1 | 1.33 ± 0.11 |
| PME (50 $\mu$g) | 1.36 ± 0.04 | 0.96 ± 0.08 | *2.28* ± 0.02 | 1.11 ± 0.07 |
| PDE (50 $\mu$g) | 1.14 ± 0.05 | 0.65 ± 0.04 | 0.82 ± 0.07 | *1.86* ± 0.08 |
| PE (500 $\mu$g) | 3.87 ± 0.09 | *6.00* ± 0.02 | 4.40 ± 0.09 | 2.50 ± 0.02 |
| PME (500 $\mu$g) | 3.77 ± 0.04 | 2.25 ± 0.01 | *7.02* ± 0.15 | 2.94 ± 0.03 |
| PDE (500 $\mu$g) | 1.78 ± 0.01 | 1.14 ± 0.04 | 1.47 ± 0.01 | *2.70* ± 0.03 |

sulted in a 6-fold increase in the methyl group incorporation into the PME product. Addition of 500 $\mu$g PME resulted in a 7-fold increase in the methyl group incorporation into the PDE product. However, the addition of 500 $\mu$g PDE resulted in only a 2.7-fold increase in the formation of PC. With the addition of 500 $\mu$g exogenous PDE as substrate, the enhanced conversion of the exogenous PDE to PC appeared to be much slower than the other steps.

*Rat aorta microsomes treated with trypsin or phospholipase C*

In order to determine the accessibility of methylating enzymes and phospholipids to exogenous enzymes, the microsomes were exposed to trypsin or to phospholipase C before and after exposure to taurocholate. The taurocholate served to open sealed microsomal vesicles and to expose the internal surface to enzymatic attack. Trypsin reduced the phospholipid methylation activity to about the same extent both in sealed and in open microsomal vesicles (Table II). Thin-layer chromatographic analysis of product formation indicated a marked decrease in the percentage of methyl groups found in PME with a corresponding increase in the percentage of the methyl groups in PDE and PC.

Microsomes from Sprague-Dawley rat aorta were first incubated with *S*-adenosyl-L-[*methyl*-$^3$H]methionine to label the membrane phospholipids. The microsomes were then incubated with phospholipase C for 5 mins. In the sealed microsomal vesicles there was a partial hydrolysis of PC and PME, but under the specific experimental conditions that were employed there was virtually no hydrolysis of PDE (Table III). With microsomes treated with taurocholate, all three substrates underwent partial hydrolysis. When microsomes were incubated with the same level of phospholipase C for 30 min, all three methylated substrates were completely hydrolyzed. PDE appears to be less accessible to the phospholipase C from the outer surface.

*Inhibitors of phospholipid methyltransferase activity*

The effect of *S*-adenosyl-L-homocysteine and of homocysteinethiolactone on phospholipid methylation in aorta microsomes was determined. The chromatographic profile of methylated phospholipids when microsomes were incubated with 2 $\mu$M *S*-adenosyl-L-[*methyl*-$^3$H]methionine in the presence and absence of 2 mM inhibitor compound is presented in Fig. 3. Both *S*-adenosyl-L-methionine and L-homocysteinethiolactone inhibited phospholipid methylation (90% and 77%,

TABLE II

EFFECT OF TRYPSIN ON THE INCORPORATION OF [$^3$H]METHYL GROUPS FROM *S*-ADENOSYL-L-METHIONINE INTO PHOSPHOLIPIDS OF RAT AORTA MICROSOMES

Microsomes (0.5 mg) in 0.05 M Tris-HCl buffer (pH 7.4)/0.25 M sucrose were treated with 0.4% taurocholate at 2°C for 30 min. Trypsin (0.2 mg/mg microsomal protein) was added and the samples were incubated at 37°C for 30 min. At the end of the incubation, trypsin inhibitor (0.2 mg/mg protein) was added. Methylation was started by incubating the membranes with *S*-adenosyl-L-[*methyl*-$^3$H]methionine (2 μM) at pH 8.2. Phospholipids were extracted and separated as described in Methods. Results are an average of three determinations ± S.E.

| | pmol [$^3$H]methyl incorporated per mg protein in total phospholipids | % reduction in level of incorporation | % [$^3$H]methyl in phospholipid product |
|---|---|---|---|
| Untreated sealed vesicles | 3.15 ± 0.02 | | PC 40 ± 1<br>PME 42 ± 2 |
| Sealed vesicles treated with trypsin | 1.55 ± 0.04 | 49 ± 1.3% | PC 48 ± 1.3<br>PME 19 ± 0.6 |
| Vesicles opened with taurocholate | 3.43 ± 0.03 | | PC 40 ± 4.1<br>PME 36 ± 4.4 |
| Vesicles opened with taurocholate and treated with trypsin | 1.44 ± 0.05 | 42 ± 1.5% | PC 46 ± 1.4<br>PME 20 ± 2.6 |

respectively). When the microsomes were incubated with 100 μM *S*-adenosyl-L-[*methyl*-$^3$H] methionine, the inhibition was greatly reduced. *S*-Adenosyl-L-homocysteine then inhibited phos-

TABLE III

HYDROLYSIS BY PHOSPHOLIPASE C OF LABELLED PHOSPHOLIPIDS OF AORTIC MICROSOMES VESICLES OPENED WITH TAUROCHOLATE

Microsomes (0.4 mg) were incubated with *S*-adenosyl-L-[*methyl*-$^3$H]methionine (1 μM) in 50 mM Tris-glycylglycine buffer (pH 8.2) for 60 min at 37°C. The incorporation was stopped by addition of *S*-adenosylhomocysteine. The suspension was treated with 0.4% taurocholate by standing on ice for 30 min. Samples were again incubated with or without 10 units of phospholipase C for 5 min at 37°C and the reaction was stopped by adding 1 ml 20% trichloroacetic acid. Phospholipids were extracted and separated as described in Methods. Results given are mean of three determinations.

| | % hydrolysis | | |
|---|---|---|---|
| | PC | PDE | PME |
| Closed vesicles | 30.8 ± 2.0 | 2.8 ± 3.24 | 45.8 ± 2.8 |
| Open vesicles | 20.1 ± 0.5 | 56.7 ± 4.5 | 37.9 ± 3.4 |

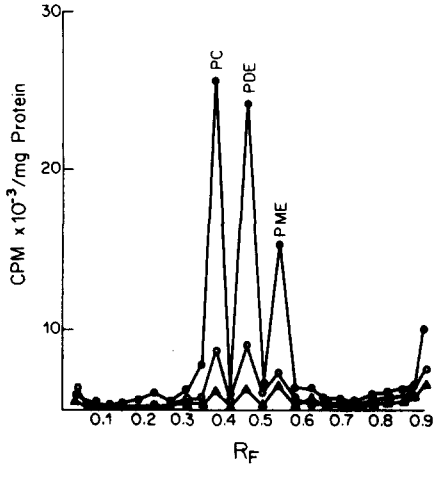


Fig. 3. Chromatographic profile of (*methyl*-$^3$H)-labeled phospholipids separated by thin-layer chromatography after the incubation of Sprague-Dawley rat aorta microsomes in the presence and the absence of inhibitors of phospholipid methylation. The ordinate is [$^3$H]methyl (cpm × $10^{-3}$) incorporated into phospholipids per mg of microsomal protein. The concentration of *S*-adenosyl-L-[*methyl*-$^3$H]methionine was 2 μM. The incubation time was 60 min. ●———●, no inhibitor; ○———○, 2 mM L-homocysteinethiolactone; ▲———▲, 2 mM *S*-adenosyl-L-homocysteine.

pholipid methylation only 38% and the L-homocysteinethiolactone inhibited phospholipid methylation only 14%.

*Phospholipid methylation in isolated rat aorta strips incubated with L-[methyl-$^3$H]methionine*

Isolated aorta strips were studied to determine whether phospholipid methylation would be detected in the intact aorta. The aorta strips were incubated with tracer levels of L-[methyl-$^3$H]methionine. This tissue was then homogenized and the phospholipids were separated by thin-layer chromatography. Methionine levels in the incubation were varied. [$^3$H]Methyl from methionine was incorporated into PME, PDE and PC. The incorporation increased in linear fashion as the methionine level increased from 0.2 to 6 $\mu$M (Fig. 4). The relative amounts of PME, PDE and PC formed were approximately similar to that found for microsomes that were incubated with 2 $\mu$M *S*-adenosyl-L-methionine (Fig. 3). Incubation of aorta strips with 2 mM L-homocysteinethiolactone inhibited the phospholipid methylation. Five paired aorta strips incubated in the presence of the L-homocysteinethiolactone had phospholipid methylation activity levels 42 $\pm$ 3% of the control incubation.

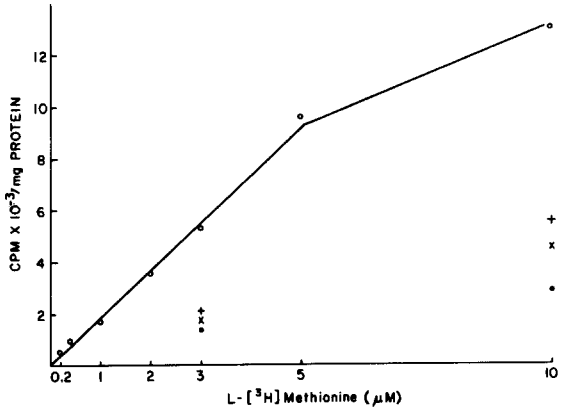


Fig. 4. The incorporation of [$^3$H]methyl into phospholipids when strips of aorta from Sprague-Dawley rats are incubated with designated levels of L-[*methyl*-$^3$H]methionine in Krebs-Ringer bicarbonate (pH 7.4) for 60 min. The ordinate is [$^3$H]methyl (cpm$\times 10^{-3}$) incorporated into phospholipids per mg of tissue protein. O——O total phospholipids; +, PC; $\times$, PDE; ●, PME. Values are the mean of three incubations. S.E. is about 7% of mean values.

*Phospholipid methylation in aorta microsomes from spontaneously hypertensive rats*

Microsomal membranes from SHR (genetically hypertensive rat strain) and WKY (normotensive control rat strain) aortas were incubated at 37°C with 2 $\mu$M *S*-adenosyl-L-[*methyl*-$^3$H]methionine for 60 min. Incorporation of methyl into total phospholipids was 3.75 $\pm$ 0.1 pmol/mg protein for WKY ($n = 6$) and 5.45 $\pm$ 0.16 pmol/mg protein for SHR ($n = 7$). In three SHR and three WKY preparations, incorporation into total phospholipids was measured at selected time intervals over a period of 60 min. The increase in incorporation of methyl groups with time and the greater incorporation in the SHR microsomes is shown in Figs. 5 and 6.

SHR and WKY rat aorta microsomes were layered on sucrose density gradients to determine whether differences in the amount or the distribution of selected marker activities would help to explain the enhanced methylation activity of the SHR microsomes (Fig. 7). The plasma membrane marker enzyme, 5′-nucleotidase, was found principally at the top of the gradient and the activity was similar in WKY and SHR aorta microsomes. Membrane protein content was greater in the lower fractions of the gradient with no obvious difference between SHR and WKY. ATP-dependent

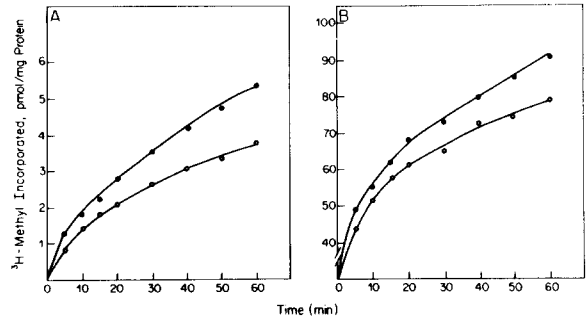


Fig. 5. The time-course of methylated phospholipid synthesis in aorta microsomes from WKY and SHR rats. The ordinate is [$^3$H]methyl groups incorporated into the phospholipids (pmol/mg microsomal protein). The microsomal protein (200 $\mu$g) was incubated for designated time periods with (A) 2 $\mu$M *S*-adenosyl-L-[*methyl*-$^3$H]methionine and (B) 100 $\mu$M *S*-adenosyl-L-[*methyl*-$^3$H]methionine. The figure represents the mean of three preparations of SHR and WKY microsomes. ●——●, SHR; O——O, WKY. S.E. about 8% of mean for each point.

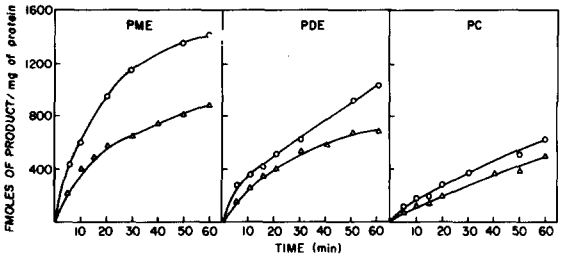


Fig. 6. The time-course of methylated phospholipid synthesis in the aorta microsomes from WKY (△) and SHR (○) rats. The ordinate is fmol of phospholipid product formed per mg of microsomal protein. The microsomal protein (200 μg) was incubated with 2 μM *S*-adenosyl-L-[*methyl*-$^3$H]methionine for designated time periods. The figure represents the mean of three preparations of SHR and WKY microsomes.

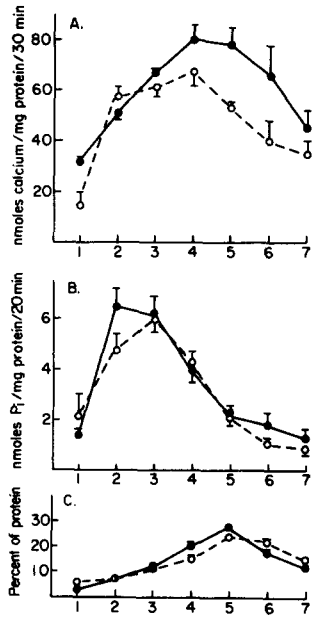


Fig. 7. Sucrose density gradient fractionation of SHR and WKY rat aorta microsomes analyzed for protein, ATP-dependent calcium sequestration and 5'-nucleotidase (a plasma membrane marker enzyme). The detailed procedure for assay of calcium sequestration and the 20%–70% (w/v) sucrose gradient is described in Ref. 36. Fraction 1 is the top and fraction 7 the bottom of the gradient. The results are the mean values of five gradients. (A) ATP-dependent calcium uptake; (B) 5'-nucleotidase; (C) protein.

calcium sequestration was greater in the lower gradient fractions. A decreased calcium sequestration activity in the SHR microsomes was found in the lower fractions of the gradient. Decreased calcium sequestration activity has been previously encountered in SHR cardiac and aorta tissue microsomes [32,36,39].

*Fluorescence polarization, fluorescence anisotropy and lipid order parameter of aorta microsomes*

The steady-state fluorescence polarization and fluorescence anisotropy for diphenylhexatriene-labeled rat aorta microsomes are given in Table IV. There is a significant decrease in fluorescence polarization in the SHR microsomes compared with the WKY controls. An empirical relation between steady-state fluorescence anisotropy and the limiting fluorescence anisotropy was developed by Van Blitterswijk et al. [20]. This relation was employed to determine the lipid structural order parameter in the aorta microsomes (Table IV). The value was significantly decreased in SHR microsomes compared with the WKY controls and com-

TABLE IV

STEADY-STATE FLUORESCENCE POLARIZATION, FLUORESCENCE ANISOTROPY, LIPID ORDER PARAMETER AND MICROVISCOSITY OF AORTIC MICROSOMES

The values represent the mean ± S.E. The number of animals is given in parentheses. $P_{DPH}$ is the fluorescence polarization $((I_{vv} - I_{vh})/(I_{vv} + I_{vh}))$; $r_s$ is the fluorescence anisotropy $(2P/(3-P))$; $r_\infty$ is the limiting anisotropy $(4/3r_s - 0.10)$; $S_{DPH}$ the lipid order paramater $((r_\infty/r_0)^{1/2}$ (where $r_0 = 0.40)$); and $\eta$, the apparent microviscosity in centipoise.

| | $P_{DPH}$ | $r_s$ | $r_\infty$ | $S_{DPH}$ | $\eta$ |
|---|---|---|---|---|---|
| Sprague-Dawley (42) | 0.2659 ± 0.001 | 0.1936 ± 0.002 | 0.1611 ± 0.001 | 0.6325 ± 0.001 | 222 ± 4 |
| WKY (7) | 0.2631 ± 0.001 | 0.1923 ± 0.003 | 0.1564 ± 0.002 | 0.6253 ± 0.002 | 217 ± 6 |
| SHR (7) | 0.2372 ± 0.002 | 0.1717 ± 0.001 | 0.1275 ± 0.001 | 0.5621 ± 0.002 | 180 ± 8 |
| | | | | $p < 0.01$ | $p < 0.01$ |

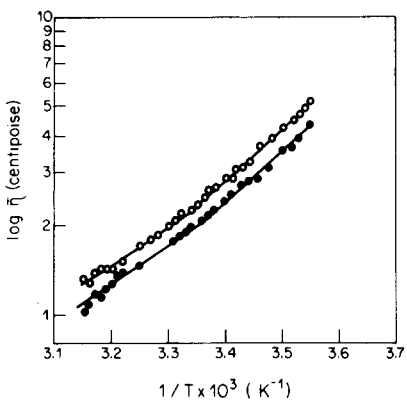


Fig. 8. Apparent microviscosity of SHR (●) and WKY (○) rat aorta microsomes plotted against the reciprocal of the absolute temperature.

pared with microsomes from Sprague-Dawley rat aorta. This signified an enhancement of membrane fluidity. The fluorescence measurements were also employed to calculate an apparent microviscosity in centipoise utilizing the procedure of Shinitzky and Barenholz [21]. This again expressed an enhanced fluidity of the SHR microsomes. When polarized fluorescence was measured for WKY and SHR microsomes at several temperatures and the apparent microviscosity was determined, the decreased microviscosity of the SHR microsomes held for the entire temperature range (Fig. 8).

Strips of aorta were incubated in Krebs-Ringer Bicarbonate for 30 min with 2 mM methionine or with 2 mM homocysteinethiolactone and the microsomes were isolated. The polarized fluorescence change indicated that methionine enhanced membrane fluidity and homocysteinethiolactone decreased membrane fluidity. The control membrane microviscosity in centipoise was $220 \pm 5$ ($n = 17$). Methionine lowered the value to $184 \pm 7$ cP ($n = 11$) and homocysteine increased the value to $272 \pm 12$ cP ($n = 6$).

## Discussion

Microsomal membranes, prepared from rat aorta and then analyzed for marker activity, consist of membrane vesicles which derive from sarcoplasmic reticulum and plasma membrane [36]. In the present study, aorta microsomes were found to methylate endogenous phosphatidylethanolamine in the presence of SAM and strips of aorta were seen to methylate endogenous phosphatidylethanolamine when incubated with L-[*methyl*-$^3$H] methionine. The evidence for the participation of more than one enzyme in the methylation sequence is much weaker than that obtained in other systems such as erythrocytes and liver [2,3]. In those two membrane system, the pH activity curve at high and low levels of *S*-adenosyl-L-methionine clearly separated the enzyme activity forming PME from that forming PC. The significance of two separate enzymes is related to the translocation of the methylated products [2], the role of intermediate products in membrane fluidity [4,42] and the asymmetrical distribution of phosphatidylethanolamine and phosphatidylcholine in the membrane [23–25].

Data obtained by treating the microsomes with trypsin and then measuring the phospholipid methylation activity of the aorta microsomes may suggest the presence of two methylating enzymes. Trypsin reduced the phospholipid methylating activity of rat aorta microsomes whether or not microsomal vesicles were opened up with taurocholate to provide access for trypsin to the luminal surface. Trypsin treatment reduced the formation of PME to a greater extent than it reduced the formation of PC. This suggested that trypsin may act at the outer surface of the microsomes and inhibit the first methylating step and that a second enzymes is more deeply positioned in the membrane.

Treating the microsomal vesicles with phospholipase C for 5 min after incubating the microsomes with *S*-adenosyl-L-[*methyl*-$^3$H]methionine suggested asymmetrical phospholipid distribution in the aorta microsomes. In sealed microsomal vesicles, a low level of phospholipase C partially degraded PME and PC but had virtually no effect on PDE. After taurocholate treatment of the microsomes there was extensive degradation of PDE. The PDE appeared to be less accessible to the phospholipase from the outer surface of the microsomal membrane. In the red blood cell [2,4] and in liver microsomes [38] PME and some PDE appeared to be inaccessible to degradation by phospholipase C.

The aorta microsomes appear to methylate ex-

ogenously added phospholipids. Exogenous phosphatidylethanolamine (500 μg) increased the methylation of phosphatidylethanolamine 6-fold and exogenous PME (500 ug) increased the methylation of PME 7-fold. With the addition of 500 μM PDE, the formation of PC from PDE appeared to be rate limiting. The methyl group incorporation was enhanced only 2.7-fold when the PDE was added.

The methylation of exogenous phospholipid has been studied in preparations from solubilized rat liver microsomes [41]. The methylation activity is very much higher in the liver preparations. Apparent affinity of PME and PDE as exogenous substrate was determined in the liver study. The utilization of exogenous phosphatidylethanolamine appeared to be the limiting step in this liver study.

Inhibition of *S*-adenosyl-L-methionine-dependent transmethylation by *S*-adenosyl-L-homocyteine, a product of the transmethylation reaction, has been demonstrated in rat liver [26,27] and in other tissues [1,2,27]. Randon et al. [28] demonstrated that L-homocysteinethiolactone potentiates the inhibition of methyltransferase activity seen with 3-deazaadenosine [29–31]. In the present study, both *S*-adenosyl-L-homocysteine and homocysteinethiolactone inhibited phospholipid methylation in rat aorta microsomes. The inhibition of phospholipid methylation appeared to be most effective at low levels of *S*-adenosyl-L-methionine.

Evidence that phospholipid methylation is a participant in the transduction of signal across membranes has been obtained for varied tissues, including erythrocytes [7], hepatocytes [9], pituitary cells [15] and various leucocytes [8,10–14,16]. In rat aorta strips, methionine enhances and L-homocysteinethiolactone depresses the contractile response to KCl and to norepinephrine [37]. In the present study, methionine also decreases and homocysteine increases microsomal membrane microviscosity in the rat aorta. It is possible that methionine methylation activity in the rat aorta plays a role in the function of calcium channels which mediate the response to contractile agonists.

In the present study, phospholipid methylation activity was compared in microsomes prepared from the SHR genetic hypertensive strain rat aorta and from the WKY normotensive control strain rat aorta. The activity level was enhanced in the SHR aorta microsomes. The SHR and WKY microsomes did not appear to differ when they were examined by fractionation on a sucrose density gradient, except for diminished ATP-dependent calcium uptake in SHR microsomes. If methylated phospholipid influences the function of calcium channels and the rate of calcium entry from outside of the cell, an increased phospholipid methylation activity in the SHR strain rat aorta compared with that of its WKY control would conceivably enhance the calcium level and the contractile state in unstimulated vascular muscle.

It has also been demonstrated that prostaglandin synthesis in enhanced in SHR rat aorta [32]. Since this increased prostaglandin synthesis is derived from phospholipid precursors, it is possible that pathways of phospholipid synthesis and phopholipase degradation of phospholipids in the SHR rat aorta are augemented in a general way. Other studies with SHR rat aorta indicate a reduced contractile response of the arterial strips and impaired calcium sequestering activity in the aorta microsomes [39].

In this study, the SHR microsomes were incubated with diphenylhaxatriene and the polarized fluorescence was measured and compared with that in normotensive WKY rat aorta microsomes. The findings are compatible with increased fluidity of SHR membranes. It has been shown that PME enhances membrane fluidity [4,42]. The phospholipid methylation activity in the presence study was increased in SHR microsomes and the polarized fluorescence findings are compatible with this enhanced phospholipid methylation activity. Microsomes isolated from aorta strips incubated with homocysteinethiolactone showed a decreased fluidity. These findings are also compatible with a relationship between the formation of PME in the intact tissue and membrane fluidity. In other studies membrane fluidity changes have been related to activity levels of processes such as transport, cell fusion, lateral mobility of membrane proteins and permeability of membranes [33–35].

The possible functional significance of phospholipid methylation is also suggested by the studies of McGivney et al. [16]. In their work, calcium-dependent histamine secretion was found to be preceded by phospholipid methylation. In-

hibition of the methylation or absence of the methylation resulted in suppression of the secretion. They generalized the finding in their studies and postulated that the phospholipid methyltransferase enzymes, the calcium channel, membrane receptors and phospholipase $A_2$ are clustered in a specific domain of the membrane. Appropriate activation of membrane receptors by a suitable ligand activates phospholipid methyltransferase and results in the formation of methylated intermediates and the translocation of phospholipid. This results in an influx calcium and fluidity change. The next sequence in their proposed cascade is an activation of phospholipase $A_2$ and either secretion or contraction triggered by calcium. Products of the phospholipase such as prostaglandins and lysophospholipids may further amplify the chain of events. Their supporting evidence was also the strongest demonstration of two phospholipid-methylating enzymes. Genetic mutants (basophilic leukemic leukocytes) were obtained in which the first or the second phospholipid-methylating enzyme was absent.

Overall in this study we have obtained evidence for methylation of endogenous and exogenous phospholipids in rat aorta microsomes. We have found both increased phospholipid methylation and increased fluidity in SHR aorta microsomes. We have found that methionine increases and homocysteinethiolactone decreases microsomal membrane fluidity in the rat aorta. In related studies, methionine enhanced and homocysteinethiolactone depressed the contractile response of rat aorta to KCl and to norepinephrine. It is postulated that phospholipid methylation may play a role in calcium channel function. Increased phospholipid methylation in SHR rat aorta membranes may be associated with an increased basal calcium inflow into the vascular muscle and perhaps a greater leak of calcium from intracellular calcium sequestration sites. This would contribute to a much increased basal tone of contraction and a corresponding decreased contractile response of isolated arterial strips when they are stimulated. An apparent decrease in calcium pump activity found in the SHR rat aorta may also be a consequence of relative membrane leakiness. We also do not know the nature of the intracellular signal for release of sequestered calcium. It is possible that membrane fluidity changes may promote a slow release of sequestered calcium in the basal resting state.

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## References

1 Hirata, F., Viveros, O.H., Diliberto, E.M. and Axelrod, J. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1718–1721
2 Hirata, F. and Axelrod, J. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2348–2352
3 Sastry, B.V.R., Statham, C.H., Axelrod, J. and Hirata, F. (1981) Arch. Biochem. Biophys. 221, 762–773
4 Hirata, F. and Axelrod, J. (1978) Nature (London), 275, 219–220
5 Kennedy, E.G. and Weiss, S.B. (1956) J. Biol. Chem. 222, 193–214
6 Sundler, R. and Akesson, B. (1975) J. Biol. Chem. 250, 3359–3367
7 Hirata, F., Strittmater, W.J. and Axelrod, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 368–372
8 Hirata, F., Corcoran, B.A., Venkatasubramanian, K., Schiffmann, E. and Axelrod, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76 2640–2643
9 Castano, J.G., Alemany, S., Nieto, A. and Mato, J.M. (1980) J. Biol. Chem. 255, 9041–9043
10 Hirata, F., Toyoshima, S., Axelrod, J. and Waxdal, M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 862–865
11 Hirata, F., Axelrod, J. and Crews, F.T. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4813–4816
12 Ishizaka, T., Hirata, F., Ishizaka, K. and Axelrod, J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1903–1906
13 Crews, F.T., Morita, Y., McGivney, A., Hirata, F., Siraganian, R.P. and Axelrod, J. (1981) Arch. Biochem. Biophys. 212, 561–571
14 Bareis, D.L. Hirata, F., Schiffmann, E. and Axelrod, J. (1982) J. Cell Biol. 93, 690–697
15 Hook, V.Y., Heisler, S. and Axelrod, J. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6220–6224
16 McGivney, A., Morita, Y., Crews, F.T., Hirata, F., Axelrod, J. and Siraganian, R.P. (1981) Arch. Biochem. Biophys. 212, 572–580
17 Crews, F.T. Hirata, F. and Axelrod, J. (1980) Neurochem. 34, 1491–1498
18 Hotchkiss, A., Jordan, J.V., Hirata, F., Shulman, N.R. and Axelrod, J. (1981) Biochem. Pharmacol. 30, 2089–2095
19 Higgins, J.A. and Dawson, R.M.C. (1977) Biochim. Biophys. Acta 470, 342–356
20 Van Blitterswijk, W.J., Van Hoeven, R.P. and Van der Meer, B.W. (1981) Biochim. Biophys. Acta 644, 323–332
21 Shinitzky, M. and Barenholtz, Y. (1978) Biochim. Biophys. Acta 515, 367–394

22 Lowry, O.H., Rosebrough, H.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
23 Nilsson, O. and Dallner, G. (1977) J. Cell Biol. 72, 568–583
24 Nilsson, O. and Dallner, G. (1977) Biochim. Biophys. Acta 464, 453–458
25 Higgins, J. and Evans, W. (1978) Biochem. J. 174, 563–567
26 Gibson, K.D., Wilson, J.D. and Udenfriend S. (1961) J. Biol. Chem. 236, 673–679
27 Hoffman, D.R., Marion, D.W., Cornatzer, W.E. and Duerre, J.A. (1980) J. Biol. Chem. 255, 10822–10827
28 Randon, J., Lecompte, T., Chignard, M., Siess, W., Marles, G., Dray, F. and Vargaftig, B.B. (1981) Nature (London) 293, 660–662
29 Zimmerman, T.P., Schmitges, C.J., Wolberg, G., Deeprose, R.G., Duncan, G.S., Cuatrescas, P. and Ellion, G.C. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5639–5643
30 Chiang, P.K., Richards, H.H. and Cantoni, G.L. (1977) Mol. Pharmacol. 13, 939–947
31 Borchardt, R.T. (1975) Biochem. Pharmacol. 24, 1542–1544
32 Limas, C., Goldman, P. and Limas, C.J. (1981) Am. J. Physiol. 240, H33–H38.
33 Overath, P., Schairer, H.V. and Stoffel., W. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 602–612
34 Shinitzky, M. and Inbar, M. (1974) J. Mol. Biol. 85, 603–615
35 Deleers, M. and Malaise, W.J. (1980) Biomed. Res. 1, 400–404
36 Moore, L., Hurwitz, L., Davenport, G.R. and Landon, E.J. (1975) Biochim. Biophys. Acta 413, 432–443
37 Jaiswal, R.K., Sastry, B.V.R. and Landon, E.J. (1982) Fed. Proc. 42, abstr. 8076
38 Higgins, J.A. (1981) Biochim. Biophys. Acta 640, 1–15
39 Mushlin, P.S., Rama Sastry, B.V., Boerth, R.C., Surber, M.J. and Landon, E.J. (1978) J. Pharm. Exp. Ther. 207, 331–339
40 Chauhan, B. and Kalra, B. (1983) Biochim. Biophys. Acta 727, 185–195
41 Schneider, W.J. and Vance, D.E. (1979) J. Biol. Chem. 254, 3886–3891
42 Sastry, B.V.R., Statham, C.N., Meeks, R.G. and Axelrod, J. (1981) Pharmacology 23, 211–232
43 Bremer, J. and Greenberg, D.M. (1961) Biochim. Biophys. Acta 46, 205–216