Lipids of Bovine Adrenal Plasma Membranes*

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SUMMARY

The lipid composition of a membrane fraction from bovine adrenal cortex was determined. This preparation has the capacity to bind adrenocorticotropic hormone and is enriched in adenylate cyclase and 5'-nucleotidase. The adrenal plasma membranes have a significantly higher lipid content (54.8%) than bovine liver plasma membranes and a surprisingly low proportion of this lipid is cholesterol (4.2%). The phospholipids comprise 76.4% of the total lipids and their composition is very similar to that of bovine liver membranes with the exception of sphingomyelin which comprises only 4.5% of the phospholipids in the adrenal preparation.

The association between proteins and lipids is of critical importance for the biological properties of plasma membranes. Adrenal cortical cells contain an ACTH-sensitive adenylate cyclase system which is associated with the plasma membrane. In connection with studies on the nature of this system information was required regarding the chemical nature of the lipids of adrenal plasma membranes. Such information is of considerable importance since proteins isolated from these membranes (ACTH receptors, adenylate cyclase, etc.) may lose their biological characteristics when separated from the lipids but may regain these properties on recombination. This property of membrane-associated proteins is amply documented in the literature (1).

A procedure for the isolation from bovine adrenal cortex of a preparation enriched in plasma membranes has been described (2). In the preceding paper (3) the adenylate cyclase system of this preparation has been characterized; the present communication is concerned with its lipid composition.

MATERIALS AND METHODS

All solvents were reagent grade and were redistilled prior to use. Sphingomyelin (from bovine brain), lysolecithin (from egg lecithin, grade II), phosphatidylethanolamine (bovine brain type V), L-a-lecithin (egg yolk type III-E), cholesterol, behenic acid, palmitic acid, cholesteryl palmitate, and triolein were purchased from Sigma. Phosphatidylserine was obtained from Schwarz-Mann, N. Y., and cardiolipin (beef heart), phosphatidic acid (sodium salt), and sucrose from General Biochemicals. Silicic acid (100 mesh) was purchased from Mallinckrodt, aluminum oxide (activity grade I, nonalkaline) from Woelm-Eschwege, Germany, and Silica Gel H without CaSO4 binder was obtained from Brinkmann Instruments, Inc., Westbury, N. Y. Florisil (60 to 100 mesh) was purchased from Fisher Scientific Co.

Preparation of Subcellular Particles—The procedure for isolation of bovine adrenal cortical membranes has been described previously (2). The plasma membrane suspension obtained by zonal centrifugation was diluted with an equal volume of water and the suspension was centrifuged for 1 hour at 100,000 × g. The membrane pellet was resuspended twice in water and centrifuged for 1 hour at 100,000 × g. The final pellet was suspended in 6 ml of water to give a final concentration of about 4 mg of protein per ml. Protein was determined by the method of Lowry et al. (4) using bovine serum albumin as standard. Plasma membranes were solubilized in 0.1 N sodium hydroxide containing sodium lauryl sulfate (0.75 mg per ml).

Lipid Extraction—Lipids were extracted with chloroform-methanol essentially according to the method of Folch et al. (5) as modified by Radin (6) and Rouxer and Fleischer (7). An aliquot of plasma membrane suspension (usually 3 to 5 ml) was extracted at 4° with 15 to 33 volumes of chloroform-methanol (2:1) for periods varying from 1 to 7 days. Nonlipid material was removed by washing the extract with 0.03% MgCl2 (0.2 volume).

Thin-Layer Chromatography—For single dimension chromatography, Silica Gel H (without CaSO4 binder) was used. To minimize diffusion of lipids during two dimensional chromatography, it was advisable to add magnesium silicate (final concentration, 10%) to the silica gel. Thin layer plates, 0.25 to 0.5 mm thickness, were air dried at reaction temperature, prewashed in chloroform-methanol (2:1, v/v), activated at 100 to 120° for 1 to 2 hours, and stored in a desiccator over anhydrous CaSO4 (drierite) prior to use.

Solvent systems used for thin layer chromatography are designated as follows: System A, chloroform-methanol-acetic acid-water (25:15:4:2, v/v) (8); System B, chloroform-methanol-28% aqueous ammonia (65:25:4.5, v/v) (9); System C, chloroform-acetone-methanol-acetic acid-water (3:4:1:0.5, v/v) (8); System D, chloroform-methanol-water (65:25:4, v/v) (7); System E, 1-butanol-acetic acid-water (60:20:20, v/v) (7); System F, disopropyl ether-acetic acid (96:4, v/v) (10); System G, petroleum ether-diethyl ether-acetic acid (90:10:1, v/v) (10); System H, petroleum ether-diethyl ether-acetic acid (70:30:1, v/v) (11); System I, petroleum ether-diethyl ether-acetic acid (80:20:1, v/v) (11); System J, diethyl ether-hexane (9:4, v/v) (11).

Isolation and Analysis of Phospholipids—An appropriate aliquot (a volume containing at least 10 µg of phosphorus) was applied as a single spot to a thin layer chromatography plate and developed in the first dimension with Solvent System B and the second dimension with System C. Alternatively, System D was used for the first dimension and System E for the second. Since excess moisture causes the phospholipids to migrate too fast at the expense of resolution, it was necessary to dry the plates over CaSO4 before development in the second dimension. Phospholipids were identified using group-specific spray reagents (ninhydrin, modified Dragendorff (12), and modified Schiff reagent (13)) and by chromatography with authentic reference lipids.

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† Author to whom correspondence should be addressed.
‡ The abbreviation used is: ACTH, adrenocorticotropic hormone.
For quantitative estimation of individual phospholipids the thin layer plates were exposed to iodine vapor, the stained areas were marked, the iodine was allowed to evaporate, and appropriate areas were removed by suction. The phospholipids were eluted from the silica gel with chloroform-methanol (2:1; 5 to 10 ml), Solvent System A (5 to 8 ml), methanol (5 ml), and finally chloroform-methanol (2:1; 5 ml). The eluates were evaporated to dryness under nitrogen at 40°C. Phosphorus determinations were performed according to the method of Bartlett (14) after digestion with 10% H2SO4 and hydrogen peroxide. Prior digestion with 70% HClO4 was performed in some cases to ensure liberation of phosphonate phosphorus (15) but the results obtained by both methods were identical. Total phosphorus represents the phosphorus contained in the original lipid extract.

Isolation and Analysis of Neutral and Glycolipids—Neutral and glycolipids were isolated by chromatography of the lipid extracts on florid columns according to the procedure of Dittmer and Wells (16). An aliquot of the Folch extract was applied to a florid column equilibrated with chloroform (ratio of column weight to lipid weight, 33:1). Neutral lipids were eluted with chloroform, glycolipids with chloroform-methanol (2:1). The effluents were evaporated to dryness under nitrogen and the respective residue weights were used to determine the percentage contribution of these lipid classes to the total lipid dry weight.

In some cases neutral lipids were separated from phospholipids by column chromatography of the lipid extract on silicic acid (100 mesh) essentially as described by Dittmer and Wells (16). Prior to chromatography the silicic acid was activated by heating to 120°C for 3 hours. The effluent was evaporated to dryness under nitrogen and the residue was used to determine the percentage contribution of these lipid classes to the total lipid dry weight.

For determination of total cholesterol, aliquots of the lipid extract were saponified and extracted according to the procedure of Meldolesi et al. (18) and cholesterol was determined colorimetrically as described by Bowman and Wolf (10).

The separation of neutral lipids was accomplished by single dimension two-step development thin layer chromatography (10) using System F for the first elution and System G, H, or I for the second solvent. Excellent resolution was also obtained using a combination of System F followed by System J. The lipid extract (1 to 1.5 ml) was applied to the plate as a band. Using these systems the separation of cholesterol, cholesterol esters, mono-, di-, triglycerides, and fatty acids was possible. The classes of neutral lipids were identified by comparison with authentic reference standards. The positions occupied by mono- and diglycerides were estimated from the results of Skipski et al. (10) and generous sections of the supporting medium were removed in order to assure complete recovery of these two lipids. Visualization of the lipids was achieved by exposure to iodine vapor. Rhodamine G6 (0.05%) solution (in ethanol) was used to detect co-chromatographed standards. Individual lipid bands were scraped from the plate and added to activated aluminum oxide columns (0.8 x 2 cm) which previously had been washed with chloroform-methanol (2:1) and diethyl ether. Cholesterol, cholesterol esters, mono-, and triglycerides were eluted with diethyl ether (20 ml) followed by chloroform-methanol (4:1; 10 ml). Fatty acids and diglycerides were eluted with diethyl ether only. Solvent was removed by evaporation under nitrogen at 30°C.

Cholesterol ester samples were saponified according to the method of Abell et al. (20).

RESULTS AND DISCUSSION

Meaningful lipid data on subcellular organelles depends on the availability of homogeneous preparations and such preparations are difficult to obtain. The homogeneity of the adrenocortical plasma membrane preparation (2) whose lipid spectrum is described in this communication was assessed by electron microscopy and by determination of the specific activity of the membrane marker enzymes 5'-nucleotidase and adenylate cyclase and the mitochondrial marker cytochrome oxidase. The highest specific activity of the membrane markers was observed in the lightest fractions from the zonal centrifugation. When these fractions were incubated with the presence of 5'-AMP and Pb(NO3)2 and prepared for electron microscopy, precipitates of lead phosphate were concentrated both in large vesicles and membranes with free ends. Electron microscopic evaluation revealed very few mitochondria in the membrane fraction. A comparison of the cytochrome oxidase activity of various fractions from the zonal centrifugation was in agreement with the results of the electron microscopic findings. The specific activity of cytochrome oxidase in the mitochondrial fractions was approximately 48 units (1 unit corresponds to oxidation of 1 nmol of reduced cytochrome c per min per mg of protein); the low density fractions containing mainly membranes exhibited an activity of only 1 to 4 units. Some contamination of the plasma membrane fraction with mitochondria is suggested by the presence of phospholipid phosphorus in an area of the chromatogram usually assigned to cardiolipin. If this does indeed represent cardiolipin the value is higher than would be expected based on the degree of mitochondrial contamination estimated by electron microscopic cytological techniques or by cytochrome oxidase assays.

The lipid composition of plasma membranes is highly variable and depends not only on the species but on the tissue source (21). The gross lipid composition of the bovine adrenocortical membrane preparations shows (Table I) that the preparation contains 38.6% protein and 54.8% lipid. The largest proportion (76.4%) of the lipids is present in the phospholipid fraction. The high lipid and low cholesterol content are noteworthy features of the adrenal membranes. The ratio of milligrams of lipid to milligrams of protein for bovine liver membranes is 0.54 (22) and for the adrenal preparation this value is 1.42. The high lipid content of the adrenal membranes reflects itself in the low buoyant density (1.14) of these particles. The high lipid to protein ratio might be attributed to the fact that the adrenal membrane preparation contains very few desmosomes and tight junctions which are known to be enriched with respect to protein.

Early concepts of plasma membrane structure depicted a phospholipid-cholesterol complex involving a molecule of each as a basic structural unit. Such an arrangement probably was suggested by the rather high ratio of cholesterol to phospholipid found in myelin and red cell ghosts, two of the earliest membranes investigated. A high proportion of cholesterol appears to be a characteristic feature of many plasma membranes (21). This is not found with the adrenal membranes whose cholesterol content represent only 4.2% of the total lipid. The bulk (39.9%) of the cholesterol is present in the free form in agreement with previous.

### Table I

<table>
<thead>
<tr>
<th>Class</th>
<th>Dry weight</th>
<th>Lipid</th>
</tr>
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<tbody>
<tr>
<td>Protein</td>
<td>38.6</td>
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</tr>
<tr>
<td>Lipid</td>
<td>54.8</td>
<td>100</td>
</tr>
<tr>
<td>Phospholipid</td>
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<td>76.4</td>
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<tr>
<td>Neutral Lipid</td>
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<td>11.5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>Free</td>
<td></td>
<td>3.9</td>
</tr>
<tr>
<td>Esterified</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Glycerides</td>
<td></td>
<td>2.3*</td>
</tr>
<tr>
<td>Glycolipids</td>
<td></td>
<td>4.4</td>
</tr>
<tr>
<td>Unidentified</td>
<td></td>
<td>7.7</td>
</tr>
</tbody>
</table>

a Calculated from lipid phosphorus using a factor of 25.
b Calculated as cholesteryl palmitate.
c Calculated as triolein. Because of the low glyceride content of the adrenal plasma membranes, insufficient amounts of di- and monoglycerides were available for quantitation.
Nonlipid material
Phospholipid
Total lipid...

is not sphingomyelin but phosphatidyl serine and the sphingo-
serine content the distribution of phospholipids in beef adrenal
The third most prevalent phospholipid in the adrenal prepara-
tion and beef liver plasma membranes is rather similar (Table III).

Table II
Comparison of lipid composition of rat and beef adrenals

<table>
<thead>
<tr>
<th>Classes</th>
<th>Rat</th>
<th>Beef</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>Medulla</td>
</tr>
<tr>
<td>Nonlipid material</td>
<td>61.0</td>
<td>74.8</td>
</tr>
<tr>
<td>Total lipid</td>
<td>39.0</td>
<td>16.4</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>12.9</td>
<td>13.5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td>11.2</td>
</tr>
<tr>
<td>Ester</td>
<td>18.5</td>
<td>0.98</td>
</tr>
<tr>
<td>Free</td>
<td>0.6</td>
<td>1.17</td>
</tr>
</tbody>
</table>

* From Eberhagen and Hauck (23).
† From Eberhagen and Jossiphov (22).
‡ Lipid phosphorus is converted to phospholipid using a factor of 25.
§ Cholesterol ester calculated from cholesterol using a factor of 1.7.

Table III
Comparison of phospholipid composition of bovine adrenocor-
tical and liver plasma membranes

<table>
<thead>
<tr>
<th>Phospholipid class</th>
<th>Adrenocortical plasma membranes</th>
<th>Beef liver plasma membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
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<td></td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>2.4</td>
<td>42.6</td>
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<tr>
<td>Lysophosphatidylcholine</td>
<td>3.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>21.6</td>
<td>22.4</td>
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<tr>
<td>Lysophosphatidylethanolami</td>
<td>2.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Phosphatidyserine</td>
<td>9.0</td>
<td>5.7</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>3.9</td>
<td>6.1</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>4.5</td>
<td>14.6</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>2.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Phosphatic acid</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Unidentified</td>
<td>2.9</td>
<td></td>
</tr>
</tbody>
</table>

* Expressed as per cent of phospholipid recovered from tlc. Recoveries varied from 85 to 100% of phospholipid phosphorus applied.
† Values taken from Fleischer and Fleischer (24).
‡ In all thin layer chromatography systems investigated some phosphorus containing material failed to migrate.

myelin content (4.5%) is significantly lower than is found in beef liver plasma membranes (14.5%) (24). To confirm this result, sphingomyelin was determined (25) after alkaline hydro-
dalysis (26) of the total phospholipid extract from homogenates and from the membrane fraction. It accounted for 4.1% of the phospholipid from the homogenate and 4.3% from the membranes.

The observation that the sphingomyelin content of the adrenal membrane preparation was lower than anticipated prompted a re-examination of the 5'-nucleotidase activity of these membranes. Although it was shown previously (2) that the membrane fraction was enriched in this enzyme, the concentration relative to the homogenate was not determined.

Adrenal homogenates contain considerable alkaline phosphatase activity, so that when the hydrolysis of 5'-AMP, 2'-AMP and β-glycerophosphate was compared between pH 6.1 and pH 10.7, no evidence was obtained for the specific hydrolysis of 5'-AMP (Fig. 1). In contrast, when the purified membrane preparation was investigated, specific hydrolysis of 5'-AMP was observed between pH 7.0 and pH 7.5 (Fig. 2). As judged by the hydrolysis of 2'-AMP and β-glycerophosphate, the alkaline phosphatase was not active in this pH range and was only de-
tected above pH 8.0. Alkaline phosphatase was enriched about 3.5-fold in the purified membrane fraction, compared to homog-
genates, whereas 5'-nucleotidase, at pH 7.5, was enriched about 30-fold. This latter value must be regarded as a minimum estimate, since there was no basis for an assumption that the hydrolysis of 5'-AMP at pH 7.5 by homogenates actually repre-
sented 5'-nucleotidase activity.

In view of the extensive evidence (see Ref. 2) that 5'-nucleotidase is concentrated in the plasma membranes of a variety of cell types, these results, together with our earlier observations (2) provide strong support for the conclusion that the membrane
fraction is indeed derived from the plasma membrane of the cells of the adrenal cortex.

Evaluation of the lipid content of beef liver cell fractions (24) led to the conclusion that the sphingomyelin content increased in the order nuclear envelope < endoplasmic reticulum < plasma membrane and that the sphingomyelin content of the Golgi complex is intermediate between that of the endoplasmic reticulum and plasma membrane. It is conceivable that the low sphingomyelin content of the adrenal plasma membrane fraction may reflect contamination by other cell fragments. The results presented in this communication suggest, however, that the subcellular distribution of sphingomyelin in the adrenal cortex is not the same as observed in the hepatocyte.

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Fig. 2. Effect of pH on the phosphatase activity of adrenocortical membrane fraction. Incubations were performed and Pi release was determined as described for Fig. 1. Symbols as in Fig. 1.
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