Characterization of the Lipoidal Derivatives of Pregnenolone Prepared by Incubation of the Steroid with Adrenal Mitochondria*

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Synthia Mellon-Nussbaum, Laura Ponticorvo, and Seymour Lieberman

From the Departments of Biochemistry and of Obstetrics and Gynecology and the International Institute for the Study of Human Reproduction, The College of Physicians and Surgeons, Columbia University, New York, New York, 10032

Using mass spectrometric, radioisotopic, chromatographic and chemical techniques, five fatty acid esters of 3β-hydroxy-5-pregnen-20-one (pregnenolone) have been identified as components of the lipoidal derivatives biosynthesized in vitro with bovine adrenal mitochondria. The five compounds are: pregnenolone arachidonate, pregnenolone linoleate, pregnenolone oleate, pregnenolone palmitate, and pregnenolone stearate. The distribution of the fatty acids among these five esters is different from the previously reported (Cmeli, S. H. W., and Ley, H. (1977) Comp. Biochem. Physiol. 56B, 287-270) fatty acid composition of these organelles.

The existence of nonpolar derivatives of steroids in steroidogenic tissue has recently been reported (1-3). These "lipoidal" derivatives are less polar than are the free steroids that are liberated from them by treatment with alkali. Three steroids, pregnenolone, 17-hydroxypregnenolone, and dehydroisoandrosterone, have been found to occur in bovine adrenals in these forms. When [3H]pregnenolone is incubated with adrenal mitochondria, a material (PL) less polar than pregnenolone is produced (2, 3) which is further resolved, by partition chromatography on celite, into two components, dubbed PL1 and PL2. In this paper our attempts to identify these two materials are described.

The two products, PL1 and PL2, were found to be mixtures. The structures of the lipoidal components of these mixtures were determined by examining the products formed when 6 mg of tritiated pregnenolone was incubated with adrenal mitochondria. Following the incubation, the tritiated lipoidal materials were recovered by partition between isooctane and a mixture of methanol and water (9:1). The aqueous methanol extract contains free and sulfated steroids while the hydrocarbon extract contains the nonpolar lipidic derivatives. To the isooctane extract were added traces of [14C]-cholesterol propionate and [14C]-tripalmitin. Chromatographic analysis of this mixture on silica gel succeeded in separating cleanly the 14C-labeled materials from the tritiated PL. This chromatographic procedure, therefore, was assumed to have freed the sample of lipoidal derivatives from contaminants such as cholesterol esters or triglycerides. The lipoidal derivatives of [3H]pregnenolone were then resolved into PL1 and PL2 by partition chromatography as previously described (2). After the products were rechromatographed a second time on celite, each was further purified by high performance liquid chromatography to yield PL4 and PL6.

Samples of PL1 and PL6 obtained in this manner then were analyzed by the following procedures: (a) mass spectrometric (MS) analysis, (b) MS analysis of the methoxime derivatives, (c) gas chromatographic/mass spectrometric (GC/MS) analysis of the fatty acids (as methyl esters) and of the pregnenolone (as its trimethylsilyl, Me3Si, derivative) obtained after transesterification by DCl in methanol and (d) gas chromatographic analysis of PL4 and PL6 and comparison of their profiles with those of authentic fatty acid esters of pregnenolone.

EXPERIMENTAL PROCEDURES

Labeled Substrate—7α-[3H]Pregnenolone (17.2 Ci/mmol) was purchased from New England Nuclear Corp. and was purified by partition chromatography on celite (Johnson-Manville No. 545) according to the procedure of Siiteri (4). The system used for the purification was isooctane/methanol/water (10:5:1).

Fatty Acid Esters of Pregnenolone. The following compounds were synthesized: pregnenolone palmitate, pregnenolone stearate, pregnenolone oleate, pregnenolone linoleate, and pregnenolone arachidonate. The general method of synthesis was as follows: 100 mg of unlabeled pregnenolone (recrystallized once from methanol) was dissolved in 1 ml of dry pyridine, to which an excess (0.2 ml) of the appropriate fatty acid chloride (Nu-Chlck Prep., Elysian, MN) was added at 0°C. The reaction mixture was allowed to stand at room temperature overnight. The solution then was treated with 25 ml of 2% HCl and the mixture was extracted with 75 ml of ether. The ether was washed with 10% solution of sodium bicarbonate and then with water until neutral. After evaporation of the ether, the pregnenolone fatty acid esters were dissolved in benzene and the solution was percolated through a 10-g silica gel column (ICN Pharmaceuticals, Inc.). The esters obtained in this way were purified by high performance liquid chromatography (Waters Associates, model ALC-100) using a Bondapak-C18/Corsil II column (3 feet × ½ inch) with acetonitrile as the mobile phase. The flow rate was 1.0 ml/min and the pregnenolone esters were eluted within 30 min. The appropriate eluates were evaporated to dryness and stored in hexane at 4°C.

The reference methoximes of the purified pregnenolone esters were prepared by the same procedure as that described below for the unknowns, PL4 and PL6.

Preparation of Lyophilized Bovine Adrenal Mitochondria—Bovine adrenals, packed in ice at the slaughterhouse, were delivered to the laboratory where they were defatted and demedullated. The cortical tissue was homogenized in 4 volumes of 0.25 M sucrose (w/v), first by two 30-s bursts in a Waring Blender and then by five passes with a glass Potter-Elvehjem homogenizer. The suspension was centrifuged for 15 min at 350,000 g and the pellet was discarded. The
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The sample of PL was further purified on 150 g of celite, using a reverse phase system consisting of methanol/1-propanol/water/tol- 
une/isooctane (4:1:0.5:1.3). The respective fractions were obtained.

The first, PLr, was eluted in the third holdback volume and the second, PL', was eluted in the fourth holdback volume. Each fraction contained half of the radioactive material applied to the column, i.e. each contained 1390 nmol of steroid. Both PLr and PL' were separately rechromatographed on celite using the same system; in each case one radioactive material exhibiting its characteristic chromatographic behavior was recovered. The samples of PLr and PL' were further purified by high performance liquid chromatography using a polyamide liquid chromatography column (4 mm × 30 cm) with acetonitrile/tetrahydrofuran/water (2:1:1) as eluant and a flow rate of 1.0 ml/min. One-milliliter fractions were collected. PLr and PL' were also redistilled. Benzene/ethyl acetate (95:5), benzene, and finally benzene/ethyl acetate (95:5). The [14C]-cholesterol propionate was eluted with isooctane/benzene (60:40, 55:45, 50:50, 45:55, 40:60, 35:65, 30:70, 25:75, 20:80, 15:85, 10:90, 5:95), benzene, and finally benzene/ethyl acetate (95:5). Thus, the PLr obtained was devoid of radioactivity; it contained 1.9 × 10⁶ cpm of tritium, corresponding to 2.78 μmol of sterol.

S. Melton-Nussbaum and R. Hochberg, manuscript in preparation.

*Quantities are estimated from the specific activity (6.85 × 10⁵ cpm of ²H/μmol) of the radioactive pregnenolone used as substrate.
were volatilized. The spectrum of the steroidal components of 380°C.

The carrier gas was helium at 35 ml/min. About 2 nmol of PLI in 3

m/e 300 to 320°C at 2°C/min. Similarly, about 2 nmol of PLI, in 1 µl of

mol. However, the isolated chemical ionization spectrum (not shown) of

m/e 679) and pregnenolone arachidonate (m/e 600).

GC/MS Analysis of the Cleaveage Products from PLI—The

GC analysis of the methyl esters formed by treating PLI with

BCl in methanol showed that the mixture consisted primarily of

two fatty acid methyl esters. On the column used, authentic

methyl linolate has a retention time (Rt) of 10.4 min and

methyl arachidonate emerges at 16.8 min. The methyl esters

cleaved from PLI emerged at these times and MS analysis

supported the identifications. Quantification of the GC pattern

by triangulation indicated that methyl linolate constituted

26% of the total fatty acids and methyl arachidonate 67%.

Three small peaks which emerged at 4.8, 7.6, and 8.8 min

consisted of the methyl esters of palmitic (4%), stearic (2%)

and oleic acids (2%). Because of its polarity, the steroidal

mole of PLI was retained on the column and was unidentifiable.

However, when the cleavage products were treated with

the silylating reagent, GC/MS analysis of the products disclosed

that pregnenolone was the sole steroidal component of

PLI. The same fatty acid methyl esters were identified as

before. As far as the steroid component was concerned, two

silylated materials were detected. One had a retention time of

28 min and the other, 32 min. Since both fractions gave mass

spectra that were identical, the products were considered to be

isomers formed during the BCl treatment. This was confirmed by

subjecting pregnenolone to the transesterification and silylation processes. When pregnenolone (or one of its

synthetic esters) was treated with BCl in methanol and the products silylated, two MeSi derivatives were recovered by

GC analysis. Both had the same mass spectrum and it seems reasonable to assume that one product is the MeSi derivative of

pregnenolone and the other, the MeSi derivative of 17-

isopregnenolone (6). When pregnenolone was silylated, without

prior treatment with BCl, one MeSi product resulted. It emerged from the column 20 min later.

Gas Chromatographic Analysis of Synthetic Esters of

Pregnenolone and of PLI—Synthetic pregnenolone esters

(pregnenolone palmitate, pregnenolone stearate, pregnenolone oleate, pregnenolone linoleate, and pregnenolone arachidonate) were studied by high temperature gas chromatography. On short columns (0.3 or 0.5 m) it was possible to separate the esters into three classes: the C14, the C16, and the C18 fatty acid esters of pregnenolone. Within each class, however, the esters of the saturated fatty acids were not resolved from those of the unsaturated analogues. The gas chromatographic profile of PLI shown in Fig. 2 shows clearly that the two major classes of pregnenolone esters are pregnenolone-C16 (70%, R1 = 12 min) and pregnenolone-C18 (25%, R1 = 10.4 min). The minor component emerging first (R1 = 7.5 min) consisted of

**RESULTS**

**Characterization of PLI**

Mass Spectral Analysis of PLI and its Methoxime—The
electron impact mass spectrum of PLI is depicted in Fig. 1A.

As the temperature of the probe was being increased some

nonsteroidal materials (as judged from their mass spectra) were

volatilized. The spectrum of the steroidal components of

PLI shown in Fig. 1A evolved when the temperature was

above 320°C. Two distinct molecular ions: one at m/e 578

(M1) corresponding to pregnenolone linoleate (M1 = 578) and

the other at m/e 602 (M1) corresponding to pregnenolone arachidonate (M1 = 602) are displayed. Thus the molecular

weights of two lipidic derivatives were obtained by direct

spectral analysis. The fragment at m/e 298, which is the base

peak, is formed by the loss of the fatty acid moiety (M -

RCOOH). Masses at m/e 283, 281, and 255 represent other fragments of the steroid moiety. The low intensity peaks at m/e 280 and m/e 303 and 304 correspond to the ionized fatty acids (C17H31COOH+, M1 = 280; C18H33COO+ and C18H35COOH+, M1 = 304).

In Fig. 1B, the electron impact mass spectrum of the methoxime derivative of PLI is shown. It displays two quasimolecular ions, one at m/e 608, corresponding to the methoxime of pregnenolone linolate (M1 + 1)+ and another at m/e 632 corresponding to the methoxime of pregnenolone arachidonate (M1 + 1)+. That these were (M1 + 1)+ ions was confirmed by MS analysis of authentic pregnenolone arachidonate methoxime and pregnenolone linolate methoxime employing the same conditions (EI at 25 eV) as those used for PLI methoxi-

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Fig. 2. Gas chromatographic profile of the pregnenolone esters in PLI. Conditions: glass column (2 mm x 0.5 m) packed with 3% SP-2100 on 100/120 Supelcoport; carrier gas, helium at 35 ml/min; injector temperature, 320°C; detector temperature, 320°C; column temperature, programmed 290/320°C at 2°C/min. Retention times for authentic pregnenolone esters on this column were 7.6 min for C16 esters, 10.4 min for C18 esters, and 12 min for C20 esters.

the Cα fatty acid ester (5%). Thus, the evidence gathered by MS, GC/MS, and GC analysis indicated that PLI consists primarily of two pregnenolone esters, pregnenolone arachidonate and pregnenolone linoleate, together with minor amounts of pregnenolone palmitate, pregnenolone stearate, and pregnenolone oleate.

Characterization of PLII

Mass Spectral Analysis of PLII and its Methoxime—The isobutane chemical ionization spectrum of PLII is shown in Fig. 3A. Three quasimolecular ions were observed: one (M + 1)+ at m/e 555 corresponds to pregnenolone palmitate (M, = 554), another at m/e 581 corresponds to pregnenolone oleate (M, = 580), and a third at m/e 583 corresponds to pregnenolone stearate (M, = 582). The base peak at m/e 299 represents the steroid fragment left after the loss of the fatty acid moieties [(M + 1)+ - (RCOOH)]. The isobutane chemical ionization spectrum of the methoxime of PLII (Fig. 3B) shows the expected shift of 29 atomic mass units for each of the three quasimolecular ions, m/e 584 for pregnenolone palmitate methoxime (M, + 1)+, m/e 610 for pregnenolone oleate methoxime (M, + 1)+, and m/e 612 for pregnenolone stearate methoxime (M, + 1)+. Loss of the fatty acid moieties gives rise to the base peak at m/e 328, which is 29 atomic mass units greater than the base peak of PLII, displayed in Fig. 3A.

Fig. 3. Mass spectra of PLII. Panel A displays the CI spectrum of PLII using isobutane as the reagent gas. Panel B displays the CI spectrum of the methoxime derivative of PLII. Both spectra were recorded at a source temperature of 200°C and a probe temperature between 320 and 380°C.

Gas Chromatography-Mass Spectral Analysis of the Cleavage Products of PLII—The GC/MS analysis of the methyl esters of the fatty acids derived from PLII by transesterification indicated that the most abundant product was methyl oleate (Rt = 8.8 min). It constituted about 82% of the total fatty acids. Methyl palmitate (Rt = 4.8 min) constituted about 12% and methyl stearate (Rt = 7.6 min) about 6%. All three methyl esters exhibited retention times and mass spectra that were identical with those of authentic samples. The only steroid detected after silylation was the trimethylsilyl ether of pregnenolone. The isomer, 17α-pregnenolone, was also detected.

Gas Chromatographic Analysis of PLII—The high-temperature gas chromatographic profile of the sample of PLII is depicted in Fig. 4. Only two classes of pregnenolone esters, the C16 esters (Rt = 4.5 min) and the C18 esters (Rt = 6.5 min) were found, the latter being the most abundant. From the peak areas, it was estimated that they were present in a ratio of 1:7. This estimate is in general agreement with that made...
by analysis of the methyl esters resulting from the transesterification process.

**Analysis of Authentic Esters**—To substantiate these interpretations, authentic pregnenolone esters (pregnenolone palmitate, pregnenolone stearate, pregnenolone oleate, pregnenolone linoleate, and pregnenolone arachidonate) and their methoxime derivatives were analyzed by electron impact ionization (EI) and by chemical ionization (Cl-isobutane) mass spectrometry. Each ester gave an EI spectrum with a weak molecular ion (M') and a base peak at m/e 298 (M – RCOOH). The methoximes all gave EI spectra at 25 eV that exhibited quasimolecular ions (M + 1)+ and a base peak at m/e 328 [(M + 1) + (RCOOH)]. In addition, the methoximes displayed a fragment at (M + 1 – OCH3). With EI at 75 eV molecular ions were unstable and often could not be observed.

Chemical ionization analysis of the esters gave intense quasimolecular ions (M + 1)+ and a base peak at m/e 298, which corresponds to the steroid fragment after the loss of the fatty acid moiety [(M + 1)+ – (RCOOH)]. Chemical ionization analysis of the methoxime derivatives of the esters also gave intense quasimolecular ions (M + 1)+ with the base peak at m/e 328. As a representative example, the mass spectra of pregnenolone arachidonate and its methoxime derivative are presented in Fig. 5. These were obtained under conditions comparable to those used for the analyses of PL-I and PL-II. In Fig. 5A the EI spectrum at 25 eV of the pregnenolone arachidonate (Mr = 602) displays a molecular ion at m/e 602, a base peak at m/e 298 (M – 304), and fragments at m/e 303 and 304 which correspond to the arachidonate moiety. In Fig. 5B the spectrum of the methoxime derivative (Mr = 631) obtained under the same conditions (EI at 25 eV) shows an intense (M + 1)+ at m/e 632, the base peak at m/e 328 (M + 1 – 304), and a fragment at m/e 601 (M + 1 – OCH3). The Cl-isobutane spectrum of pregnenolone arachidonate shown in Fig. 5C displays an (M + 1)+ at m/e 603, the base peak at m/e 299 (M + 1 – 304), as well as a fragment at m/e 305 corresponding to the protonated fatty acid moiety (304 + 1).

**DISCUSSION**

It is one of the noteworthy features of the study reported in this paper that the judicious combination of chromatographic, chemical, radioisotopic, and especially mass spectrometric techniques made possible the identification of five fatty acid esters of pregnenolone even though these were available in only microgram quantities. By labeling 6 mg (19 μmol) of pregnenolone with [3H]pregnenolone, it was possible to trace the extraction and purification of the steroidal esters formed by incubation of this steroid with adrenal mitochondria. The sum of the five highly purified esters was about 850 nmol. The samples of PL-I and PL-II obtained by high performance liquid chromatography analysis were not homogeneous. Moreover they contained nonsteroidal contaminants which became evident during the mass spectral analysis. As the probe temperature was slowly increased, nonsteroidal impurities were removed by volatilization at temperatures below 320°C. Between 320 and 380°C the steroidal components (pregnenolone esters or their methoximes) evolved and interpretable spectra were obtained. When synthetic pregnenolone esters (or their methoximes) were subjected to mass spectral probe analysis, there was no evidence of a "forerun" of impurities. The mass spectral analysis of PL-I and PL-II revealed the presence of mass or (M + 1) ions corresponding to various fatty acid esters of pregnenolone. Mass or (M + 1) ions corresponding to large molecular weights of 554, 578, 580, 582, and 602 suggested the identity of the products, but substantiation was secured by additional evidence. By converting a few micrograms of each product into its methoxime, products were obtained which yielded mass ions 29 atomic mass units greater than the mass ions of the underivatized esters. This shift in mass is consistent with the modification of the C-20 ketone group of the steroid from C=O to C=NOCH3. The transesterification process added further certainty to the identifications. The mass spectrum of the trimethylsilyl derivative of pregnenolone was identical with that of the authentic sample and established the identity of the steroid moiety. Pregnenolone was the only steroid detected; if others had been present in detectable amounts their presence would have become apparent at this stage. The methyl esters of the various fatty acids produced by transesterification were separated from each other by gas chromatography and were identified by both their retention times and mass spectra. Only those fatty acids were detected that were indicated by MS analysis of the intact pregnenolone esters. Finally, authentic fatty acid esters of pregnenolone were prepared and their mass spectra and retention times on gas chromatographic columns were determined. When PL-I and PL-II were chromatographed on the same columns (Figs. 2 and 4), each was resolved into its steroidal fatty acid ester components whose retention times corresponded exactly to those of the appropriate authentic samples. The mass spectra of the synthetic esters were identical to those of the corresponding biosynthesized esters. Although PL-I and PL-II were mixtures of several pregnenolone esters, the components could be clearly identified by the spectra of the mixtures.

The fatty acids found in the neutral lipids and phospholipids of bovine adrenal mitochondria have recently been characterized (5). The most abundant are listed in Table I along with the fatty acids found in the five pregnenolone esters biosynthesized by in vitro incubation with bovine adrenal mitochondria. As is evident from Table I, the fractions, PL-I and PL-II, were mixtures of pregnenolone esters. Moreover, the three esters comprising PL-II, pregnenolone palmitate, pregnenolone stearate, and pregnenolone oleate, are also present in PL-I.
TABLE I

<table>
<thead>
<tr>
<th>Fatty acid designation</th>
<th>Bovine adrenal mitochondria</th>
<th>Lipoidal derivatives biosynthesized in mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutral lipids$^a$</td>
<td>Phospholipids$^a$</td>
</tr>
<tr>
<td>$C_{16:0}$</td>
<td>18.0</td>
<td>17.1</td>
</tr>
<tr>
<td>$C_{18:0}$</td>
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<td>24.3</td>
</tr>
<tr>
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</tr>
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<td>13.4</td>
<td>9.5</td>
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<tr>
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<td>6.0</td>
<td>19.0</td>
</tr>
<tr>
<td>$C_{22:4,n6}$</td>
<td>4.5</td>
<td>6.0</td>
</tr>
</tbody>
</table>

$^a$ From Ref. 5. Small amounts of other fatty acids (less than 2%) were found in bovine adrenal mitochondria. They are omitted from this table since none was detected in either PL$_{1}$ or PL$_{2d}$.

$^b$ Values were calculated from the equation:

$$\% = 100 \left( f_1 \cdot p_1 + f_2 \cdot p_2 + f_3 \cdot p_3 + f_4 \cdot p_4 \right)$$

where $f_1$ is percentage of acid a in PL, $f_2$ is percentage of acid a in PL$_{2d}$, $f_3$ is percentage of acid a in PL$_{2}$, $p_1$ is nmol of PL$_{1}$, and $p_2$ is nmol of PL$_{2d}$.

revealing the inadequacy of the chromatographic separation. The principal constituents of PL$_{4}$ were pregnenolone linoleate and pregnenolone arachidonate.

The last column in Table I lists the percentage of each of the five esters isolated as PL. The content of esters in PL$_{4}$ as determined from its tritium content, was 2780 nmol. Chromatographic separation divided PL equally between the two fractions, PL$_{1}$ and PL$_{2}$ (1390 nmol). In the subsequent purification of these mixtures, unequal losses were incurred; the amount of PL$_{4}$ available for GC/MS was 700 nmol, while that of PL$_{2}$ was 154 nmol. Consequently, the percentage composition of steroidal esters in PL (given in the last column of Table I) is not based on the quantities of PL$_{1}$ and PL$_{2}$.

Rather, the percentage composition of esters in PL was calculated from the equation given in the legend to Table I. This estimation was made with the assumption that the losses incurred in the purification of PL$_{1}$ (to PL$_{1}$) and PL$_{2}$ (to PL$_{2d}$) were not selective.

While it is true that the predominant fatty acids found in the mitochondrial neutral lipids and phospholipids are the same as those that occur in PL, it is apparent that the distribution of fatty acids in PL is quite different from that of the total fatty acid composition of bovine adrenal mitochondria. The principal difference occurs in the relative concentrations of the saturated and unsaturated fatty acids. The concentrations of palmitic and stearic acids are each about 20% in bovine adrenal mitochondria, whereas in PL these acids comprise less than 10% of the total. The linoleic acid content of PL (12.5%) is about the same as that in the whole mitochondria.

Most striking are the findings for oleic and arachidonic acid esters. Pregnenolone oleate is about twice as abundant in PL as are the oleic esters in adrenal mitochondria as a whole. Moreover, pregnenolone arachidonate is much more abundant in PL (33.5%) than are the corresponding esters of this acid in the neutral lipids and phospholipids of adrenal mitochondria. It is noteworthy that the unusual unsaturated acids $C_{22:4,\omega 6}$ (adrenic acid) and $C_{22:6,\omega 3}$ were present in the mitochondrial lipids but were not detected in PL.

Since the significance of the lipoidal derivatives of steroids is at present unknown, it is not possible now to understand the meaning of the distinctive distribution of the fatty acids in these steroidal esters.

Studies on cholesterol ester metabolism have revealed that three different mechanisms may be involved in cholesterol ester synthesis. Only two of these are known to occur in adrenals. Cholesterol esterification is catalyzed by a transferase enzyme, lecithin:cholesterol acyltransferase (EC 2.3.1.43) which transfers a fatty acid from the $\beta$ position of lecithin to the 3$\beta$-hydroxy group of cholesterol (7). This enzyme has been found in plasma and lymph, but it appears to be absent from adrenals. The acyl-CoA:cholesterol $\alpha$-acyltransferase (EC 2.3.1.26) is found in adrenals and utilizes a fatty acyl-CoA as the fatty acid donor (8). The pH optimum of this enzyme is near 6.6 and ATP, CoA, GSH, and MgCl$_2$ are required for activity. Since the pH optimum for the formation of PL is 5.0, no cofactors are not required for activity, 1 it does not seem that an enzyme analogous to cholesterol acyltransferase catalyzes the esterification of pregnenolone in bovine adrenal mitochondria. Rather, an enzyme resembling the cholesterol ester hydrolase (EC 3.1.1.13) (9) may be of importance in esterifying pregnenolone. This enzyme has been found in adrenals and employs unionized fatty acids as the fatty acid donor. Its pH optimum, like that for the esterification of pregnenolone, is near 5.0.

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