Detection of bovine adrenal cortex of a lipoidal substance that yields pregnenolone upon treatment with alkali

(lipoidal steroid/steroid biosynthesis)

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ABSTRACT  Bovine adrenal cortical tissue contains a lipoidal derivative of pregnenolone (3β-hydroxy-preg-5-en-20-one) from which the free steroid can be liberated by treatment with alkali. Evidence for the presence of such an entity comes from examination of a nonpolar extract of tissue from which pregnenolone and its sulfate had been removed by chromatography. Treatment of the nonpolar fraction with alkali followed by exhaustive chromatographic analysis led to the detection of pregnenolone. The steroid was identified by both gas chromatography/mass spectrometry and double isotope procedures. Quantitative analysis indicated that the three forms of pregnenolone are present in bovine adrenal cortical tissue in the following amounts (μg/kg): lipoidal derivative, 290; free steroid, 435; and sulfate, 65. Because the only known metabolic function of pregnenolone is to serve as a precursor of the steroid hormones, these findings have far-reaching implications for steroid hormone biochemistry.

This paper describes experiments that indicate that there is present in bovine adrenal cortices a lipoidal substance that yields pregnenolone (3β-hydroxy-preg-5-en-20-one) upon treatment with alkali. Adrenals have long been known to contain pregnenolone and pregnenolone sulfate, but we now present conclusive evidence for the existence of a nonpolar steroid derivative which, upon alkaline treatment, leads to the liberation of pregnenolone. This C21-steroid, as is well known, serves as a common precursor for all the steroid hormones. Although it is not now possible to assess what significance the lipoidal derivative of pregnenolone has, its existence is noteworthy and accounts for the fact that this report is being made before its exact structure has been determined.

We were led to search for such a lipoidal form of pregnenolone by the recent observation (1) that acyl esters of cholesterol, as well as some inorganic esters of that sterol, such as the nitrate or phosphate, can serve as substrates for the cholesterol side-chain cleavage enzyme present in adrenal mitochondria. When cholesterol nitrate, cholesterol acetate, or cholesterol caproate was used as substrate for this enzyme, the corresponding pregnenolone ester was found to be the product. These results suggested that the side-chain cleavage enzyme was unable to distinguish between cholesterol derivatives that differ from each other by the presence of various carbonyl and inorganic acid esters at C-3.

These findings recalled some observations that were made almost a decade ago and whose full significance still remains unexplained. During a search for intermediates that might be involved in the conversion of cholesterol into pregnenolone, we reported the isolation of (20S)-20-hydroxycholesterol from adrenal extracts (2). In that study, fractions that were assumed to contain the fatty acid esters of cholesterol were processed in a manner that would permit us to explore the possibility that an esterified form of (20S)-20-hydroxycholesterol was also present in these extracts. The appropriate nonpolar fraction (devoid of any free sterols) was treated overnight with 1% methanolic KOH. From the saponifiable fraction, an amount of 20α-hydroxycholesterol was isolated that was about twice that isolated from the unesterified fraction (70 μg/kg as opposed to 37 μg/kg).

Because these results suggested the possibility that nonpolar forms of steroid hormone intermediates might be present in extracts of the adrenal cortex and because the presence of such compounds might have significant consequences for many facets of steroid hormone biochemistry, an effort to detect such molecules was instituted. The bulk of the cholesterol esters are undoubtedly irrelevant for the process of steroidogenesis and thus it seemed more prudent to concentrate the search on an analogous product of this process, the pregnenolone derivative.

It was reasonable to expect that the number of derivatives of pregnenolone less polar than the parent steroid would be limited, perhaps to one. Certainly, the number of such forms would be far fewer than the number of cholesterol esters that are known to occur in mammalian tissues. The isolation of pregnenolone after alkaline treatment of a fraction less polar than the C21-hydroxyketone, itself, would be a novel finding and would constitute proof of the existence of such a derivative.

MATERIALS AND METHODS

Chromatographic Procedures. Celite column chromatography was performed by the method described by Siiteri (3). The solvent systems used for celite chromatography were: system 1, isooctane/methanol/H2O (10:9.1, vol/vol); system 2 (reverse phase), methanol/n-propanol/H2O/isooctane/toluene (4.0:7.5:2:2.2, vol/vol); and system 3, isooctane/ethyl acetate/n-butanol/methanol/1 M NH4OH (2.7:4:2:2:3, vol/vol).

Chromatography on LH-20 Sephadex columns (300 x 22 mm) (Pharmacia) was done with system 4, isooctane/ethyl acetate/methanol (6:1.1, vol/vol). With this system, fractions of 8 ml were collected and pregnenolone was eluted in fractions 14-16. High pressure liquid chromatography was performed on a Waters high pressure liquid chromatograph ALC-100 using a 0.9 x 1.6 mm C18 Corasil column (Waters) with system 5, acetonitrile/H2O (55:45, vol/vol) at a flow rate of 1 ml/min. Fractions of 1 ml were collected and pregnenolone was eluted in fractions 8-9.

Radiochemical Procedures. Radioactive tracers were purchased from New England Nuclear Corp. [7α-3H]Pregnenolone (25 Ci/mmol) was purified as described (3). From this tracer, [7α-3H]pregnenolone sulfate was prepared and purified as described (5). [1-14C]Acetic anhydride (10 Ci/mol) was used as supplied without further purification. Radioactivity of samples was measured in a Packard model 3255 liquid scintil-
Table 1. Radiochemical analysis

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Crystallization data of pregnenolone acetate* (3H dpm/14C dpm)†</th>
<th>Pregnenolone isolated (µg/210 g adrenal)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$X_1$</td>
<td>$ML_1$</td>
</tr>
<tr>
<td>I Free</td>
<td>15.2</td>
<td>16.0</td>
</tr>
<tr>
<td>VII (Sulfate)</td>
<td>1.8</td>
<td>1.0</td>
</tr>
<tr>
<td>V (Lipoidal derivative)</td>
<td>20.0</td>
<td>12.2</td>
</tr>
<tr>
<td>VI (Lipoidal derivative)</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>VIa (Control: 1 day)</td>
<td>34.0</td>
<td>2.7</td>
</tr>
<tr>
<td>VIb (Control: 4 weeks)</td>
<td>14.8</td>
<td>2.6</td>
</tr>
<tr>
<td>Vlc (Control: alkali-treated)</td>
<td>11.7</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* $X_n$: crystallization product of the nth crystallization. $ML_n$: residue left in the mother liquor of the nth crystallization.
† The amounts of $^{14}$C in all but one sample were in the range of 125-300 cpm. In the one exception, sample $X_1$ of fraction VIa (which contained the least amount of pregnenolone), only 20 cpm of $^{14}$C were available.
‡ The values for fractions V, VI, VIa, VIb, and Vlc are uncorrected for losses that occurred prior to the addition of the tritiated internal standard.

The specific activity of the $[1^4$C]acetate anhydride, 10 Ci/mol, was verified by acetylation 4 µg of pregnenolone to which 741,000 dpm (4.3 ng) of $[7a-3H]$pregnenolone had been added. The $3H/14C$ ratio of pregnenolone acetate, purified as described above, was 5.6. From this value it was determined that 1 µg of pregnenolone was equivalent to 33,080 dpm of $14C, 741,000 dpm/(5.6 × 4 µg); the expected value was 54,810. This experimentally determined specific activity was used in the following equation to calculate the amount of pregnenolone found in the various adrenal functions: $\mu g = a/bcd$, where a = dpm of tritiated internal standard, b = $3H/14C$ ratio of recrystallized pregnenolone acetate (see below), c = 33,080, the specific activity of $14C$-acetate anhydride in dpm/µg of pregnenolone, and d = fraction of the sample to which the tritiated internal standard had been added.

Adrenal Extracts. Fresh bovine cortical tissue, 210 g, was homogenized in 660 ml of methanol. Water (200 ml) and then chloroform (330 ml) were added to the homogenate, after which the insoluble material was removed by filtration through a Büchner funnel using course filter paper that had been layered with a mat of glass wool. The insoluble residue was resuspended in 600 ml of 1:1 mixture of chloroform and methanol. The suspension was stirred for 3 hr and then the insoluble material was again separated by filtration. It was resuspended in 600 ml of chloroform/methanol (1:1) and kept at room temperature overnight. After filtration the undissolved solid was again macerated in 300 ml of chloroform and collected by filtration. All four filtrates were combined (volume about 3 liters) and to the combined extract was added 900 ml of water and 48.4 × 10$^6$ dpm (276 ng) of $[7a-3H]$pregnenolone dissolved in a few drops of methanol. After the mixture was shaken vigorously in a separatory funnel, the two layers were separated. The top aqueous layer (extract b) was reserved for the isolation of pregnenolone sulfate. The bottom organic layer (extract a) contained all the $[3H]$pregnenolone added as tracer. The extract was taken to dryness under reduced pressure on a flash evaporator. In order to remove traces of residual solvent, we added 300 ml of isooctane to the residue and then removed this solvent by vacuum distillation. After this procedure was repeated once again, a waxy residue weighing 10 g was obtained. This material was chromatographed on a 1.25-kg Celite column (550 × 100
mm) in system 1. All the tritiated tracer pregnenolone was found in hlv 4-5 (2 liters = 1 hlv). The tritium-containing eluates were combined and evaporated to dryness. The residue (fraction I) weighed 1 g and contained 44.7 X 10^6 dpm of ^3H. The polar material was washed from the column with 8 liters of methanol (fraction II). By this means the free pregnenolone (fraction I) present in the adrenal was separated from those constituents that were less polar (fraction II) and from those that were more polar (fraction III) than the free steroid.

The eluates of the chromatogram containing the nonpolar material (fraction II), i.e., those eluted in the first hlv, were combined and evaporated to dryness under reduced pressure. Fraction II weighed 6 g. Not more than 96,000 dpm of ^[3H]pregnenolone was found in this fraction, i.e., at most 0.2% of the added radioactive tracer pregnenolone remained.

An additional sample of ^[3H]pregnenolone, 12.4 X 10^6 dpm, was added to fraction II and the mixture was chromatographed on a cellulose column (500 g) in system 2. In this reverse phase chromatographic system, pregnenolone eluted at the solvent front and pregnenolone acetate is eluted in hlv 3-4. The first 30 fractions (fraction IV) of the chromatogram (40 fractions of 20 ml/fraction constituted 1 hlv) were combined and found to contain 12.3 X 10^6 dpm of ^3H. The eluates comprising hlv 2-9 were united and evaporated to dryness. The residue (fraction V) weighed 500 mg and was devoid of any radioactivity. In order to obtain the least polar material, we washed the column with 5 liters of benzene. After evaporation of the solvent, the resulting oil, fraction VI, weighed 6 g. It, too, was devoid of radioactivity.

Free Pregnenolone. The procedure used to purify free pregnenolone (fraction I) was the same as that used for the sample isolated after solvolysis from the sulfate fraction (VII) and for those samples (V and VI) derived from the lipoidal derivative. This procedure (procedure A) consisted of acetylation of the steroid and purification of its 3-acetate by celite chromatography with system 2. Then the acetate (eluted in hlv 2-3) was saponified and the recovered hydroxyketone was purified by three successive chromatograms on celite with system 1 and one LH-20 Sephadex column (system 4), and finally by high pressure liquid chromatography with system 5. The samples obtained by this procedure were each divided in half; one portion was converted into its trimethylsilyl derivative, which was analyzed by gas chromatography/mass spectrometry, while the other half was quantified by the technique using ^[14]C-acetic anhydride as described above. In each case, (pregnenolone obtained from fractions I, V, VI, Vla, Vlb, VIc, and VII), the trimethylsilyl ether of the natural material emerged from the gas chromatogram at the same time (28 min) as the ether of authentic pregnenolone. Moreover, the mass spectrum of every sample of the material isolated by the above procedure was characteristic of that of pregnenolone. Another proof of identity was obtained by radiochemical means. The constancy of ^3H/^14C ratios of the recrystallized samples of pregnenolone acetate provided a sensitive index of the identity of the naturally occurring substance with the added radioactive pregnenolone.

The quantitative results obtained by the ^[14]C-acetate procedure are given in Table 1.

The processing of fraction I is given in detail and is illustrative of the work-up of all the other samples containing pregnenolone. Fraction I was acetylated with acetic anhydride and pyridine and the product was chromatographed on a 150-g celite column in system 2. Those fractions containing tritium (hlv 2-3) were combined and evaporated to dryness. The residue, weighing 84 mg, was found to contain 39.7 X 10^6 dpm of ^3H. This acetylated product was saponified overnight in 100 ml of 2% methanolic KOH solution at room temperature. The next day 100 ml of water and enough acetic acid to adjust the pH to 7 were added. The methanol was removed under reduced pressure and the resulting aqueous solution was extracted with three volumes of ethyl acetate. The organic extract was washed with 0.5 M NaOH solution, with 1 M HCl solution, and then with water until neutral. The washed extract was evaporated under reduced pressure, leaving a residue weighing 35 mg and containing 39.7 X 10^6 dpm of ^3H. The product was chromatographed on a LH-20 Sephadex column in system 4. The pregnenolone fraction contained 28.9 X 10^6 dpm of ^3H and was rechromatographed by high pressure liquid chromatography on a C18 Corasil column in system 5. The recovered tritiated pregnenolone contained 25.4 X 10^6 dpm of ^3H. Half of this sample was converted into its trimethylsilyl derivative, and this was analyzed by gas chromatography/mass spectrometry. The other half of the sample from the high-pressure liquid chromatography column was quantified by the acetylation procedure described above. The results are given in Table 1.

Pregnenolone Sulfates. Fraction III was evaporated to dryness, yielding 4 g of a dark oil. This was dissolved in a small volume of methanol and added to the original aqueous layer (extract b). The methanol was removed under reduced pressure and the remaining aqueous solution (approximately 1.5 liters) was saturated with solid NaCl. The resulting mixture was divided into five equal aliquots, each of which was extracted with the same 2-liter portion of tetrahydrofuran (H4furan). Five milliliters of concentrated NH4OH was added to the H4furan, followed by 0.74 X 10^6 dpm (4 ng) of ^[3H]pregnenolone sulfate. The H4furan was evaporated under reduced pressure, yielding a dry residue weighing 3 g. The sulfates in this residue were converted into their pyridinium salts by the addition of 400 ml of 0.5M aqueous solution of pyridinium sulfate. The pyridinium salts were extracted into chloroform with two 400-ml portions of that solvent. The chloroform extract was evaporated to dryness under reduced pressure, yielding an oil containing 0.61 X 10^6 dpm of ^3H and weighing 1.6 g. The oil was dissolved in 450 ml of benzene and this solution was extracted with 450 ml of 1 M NaOH, a process which converted the pyridinium salts of the sulfates into their water-soluble sodium salts. Unexpectedly, only 0.16 X 10^6 dpm of ^3H was found in the aqueous alkali. Consequently, the benzene layer was extracted twice more with water, by which means an additional 0.23 X 10^6 dpm of ^3H was recovered. The three aqueous extracts were combined, neutralized with acetic acid, and made 20% wt/vol with solid NaCl. The sulfates were extracted into H4furan as described above. Evaporation of the organic phase left a flaky residue which was leached three times with 10-ml portions of methanol. The combined methanol extracts were evaporated to dryness, leaving a solid weighing 1.1 g. It contained 0.36 X 10^6 dpm of ^3H. This residue was chromatographed on a 250-g celite column in system 3, where the tritiated carrier is eluted in hlv 4-5. Those fractions containing ^3H (0.34 X 10^6 dpm) were combined and evaporated, and the residue was solvolyzed by treatment with 100 ml of H4furan containing 0.09 ml of 70% perchloric acid (8). After the sample remained for 2 days at room temperature, 3 ml of NH4OH in 10 ml of methanol was added to the H4furan solution. The mixture was evaporated to dryness and the residue was dissolved in 250 ml of ethyl acetate. The solution was washed with 50 ml of 4% Na2CO3 solution and then with water until neutral. The ethyl acetate was evaporated under reduced pressure, yielding 12 mg of oil containing 0.32 X 10^6 dpm of ^3H. The pregnenolone present in the residue was purified and quantified by procedure A outlined above. The quantitative results are recorded in Table 1.
Lipoidal Derivative of Pregnenolone. Fractions V and VI, devoid of radioactivity derived from the added tracer $[3H]$-pregnenolone, contained adrenal constituents less polar than that steroid. The fractions were examined for the presence of a nonpolar form of pregnenolone in the following way: 50% of each fraction was treated with a 2% methanolic KOH solution (100 ml for fraction V and 200 ml for fraction VI). The alkaline solutions were kept at room temperature overnight, after which 2.42 $\times 10^6$ dpm (14 ng) of $[3H]$pregnenolone was added to each. Both fractions were worked up by procedure A described above.

Another aliquot (25%, 1.5 g) of fraction VI was treated as a control (VIa). The sample was dissolved in 100 ml of methanol and kept, without alkali, overnight at room temperature. Tracer $[3H]$pregnenolone, 1.029 $\times 10^6$ dpm (6 ng), was added and the solvents were evaporated. The residue was chromatographed on celite in system 1. The nonpolar material (containing the fraction in the first hbv at room temperature, after which solvent was washed with extract) was eluted three times with 100 ml of ethyl acetate. The residue chromatographed on celite in system 1. The nonpolar material (containing the still-intact lipoidal derivative) was eluted in the first hbv of the chromatogram and the pregnenolone-containing fraction was eluted in hbv 4-5. The nonpolar fraction (VIb) was redissolved in methanol containing 1.029 $\times 10^6$ dpm (6 ng) of $[3H]$pregnenolone and kept at room temperature for 4 weeks. The residue chromatographed on celite in system 1. The nonpolar fraction in the first hbv (VIc, 0.6 g) was dissolved in methanol containing 1.029 $\times 10^6$ dpm and 100 ml of water were added. The methanol was removed under reduced pressure and the residue chromatographed on celite in system 1. The nonpolar fraction in the first hbv (VIc, 0.6 g) was dissolved in methanol containing 1.029 $\times 10^6$ dpm and 100 ml of water were added. The methanol was removed under reduced pressure and the aqueous mixture was extracted three times with 100 ml of ethyl acetate. The organic solvent was washed with $\text{H}_2\text{O}$ until neutral and evaporated and the residue was chromatographed on celite in system 1. The pregnenolone fractions (hbv 4-5) from each of the above chromatograms were purified and quantified by procedure A outlined above.

From the data recorded in Table 1 it was possible to establish that 210 g of beef adrenal cortex tissue contains 91.4 $\mu$g of pregnenolone, 13.5 $\mu$g of pregnenolone sulfate, and 60.4 $\mu$g (V + VI, corrected) of the lipoidal derivative of pregnenolone. Because a known amount of tracer $[3H]$pregnenolone had been added to the original extract of the adrenal tissue, it was possible to correct the value for free pregnenolone for procedural losses. The tritiated internal standards for the sulfate and the lipoidal derivatives were not added at the beginning of the extraction procedure and consequently the values calculated for these substances are minimal estimates uncorrected for such losses. However, the quantity of lipoidal derivative has been corrected for those fractions removed (10%) for measurement of radioactivity prior to the addition of $[3H]$pregnenolone. From the control, VIa, the sample kept in methanol at room temperature for 1 day, 3.7 $\mu$g of pregnenolone was isolated. From the control VIb, which had been kept for 4 weeks, an additional 8.4 $\mu$g of the steroid was recovered. The nonpolar residue (VIc), remaining after the removal of the liberated pregnenolone in VIb, was treated with alkali, and an additional 12.3 $\mu$g of pregnenolone was obtained. When this value is corrected for experimental losses (VIc weighed 60% less than VI), the amount of pregnenolone recovered (VIa + VIb + VIc) was approximately what was expected (VI).

As mentioned before, the identity of all samples of pregnenolone isolated in this study was established by gas chromatography/mass spectroscopy, as well as by the isotope data.

**DISCUSSION**

By the device of adding tritiated pregnenolone to the extract of the adrenal tissue, it has been possible to show that at least 99.8% of the free steroid present in the tissue was removed from the nonpolar fraction by the first partition chromatogram. The second chromatogram, using a reverse phase system, removed all the second aliquot of tracer that had been added to the lipoidal fraction. Thus, from this evidence it appears that none of the free pregnenolone present in the original extract could have remained in the nonpolar fraction after these two chromatographic steps. The isolation of this C-21 steroid from such nonpolar extracts after treatment with alkali leads to the conclusion that pregnenolone is present in bovine adrenal cortex tissue as a lipoidal derivative. The results obtained with the controls, VIa and VIb, support this contention.

Although small amounts of pregnenolone were isolated from neutral methanolic solutions of nonpolar extracts of adrenals after these had been stored at room temperature, the fact that any was found is significant. The results seem to indicate either that the lipoidal derivative per se is unstable in methanol or that it reacts with some constituent in the nonpolar extract in a way that liberates the free steroid.

It is noteworthy that the quantities of two of the forms of pregnenolone, the free steroid and the lipoidal derivative isolated in this study, were approximately equal. Thus, if abundance were a measure of importance, it is impossible to attribute, on this basis, special significance to either form.

Because pregnenolone is, in a formal sense, a progenitor of all the steroid hormones, the demonstration of the presence in adrenal extracts of a lipoidal derivative of this C$_{21}$-steroid suggests that this entity may play a role in steroidogenesis because no metabolic function other than as a precursor for the steroid hormones has ever been associated with pregnenolone. Indeed, it is possible that the lipoidal derivative of pregnenolone is itself biosynthesized from the analogous derivative of cholesterol, whose special function is to serve as the substrate for the side-chain cleavage enzyme. While it is clear that cholesterol and many of its esters are substrates for the enzyme in vitro (1), the identity of the true precursor in vivo is less certain. It is not far-fetched to suggest that these compounds must first be converted into an active precursor, perhaps an analogous lipoidal derivative of cholesterol, before cleavage in the intact cell occurs. The notion that an "active" form of cholesterol (different from cholesterol itself) exists has long appeared in the lore of steroid hormone biochemistry (9), but this almost always referred to the sequestration of free cholesterol into some physical compartment. The possibility that some covalent derivative of cholesterol is the "active" form has rarely been considered. Now, with the discovery that a lipoidal derivative of a steroid is present in the adrenal, this alternative warrants examination.

If the derivative of pregnenolone detected in this study also serves as a substrate for subsequent enzymatic steps in biosynthetic pathways, then it might be expected that analogous derivatives of other 3β-hydroxy-Δ5-steroids, such as 17-hydroxypregnenolone (3β,17-dihydroxypregnen-5-en-20-one) or dehydroandrosterone (3β-hydroxyandrost-5-en-17-one) also exist. Of particular interest would be the lipoidal derivative of dehydroandrosterone. In spite of the fact that this C$_{19}$-steroid and its sulfate are produced by the adrenals and testes in milligram quantities daily, neither has been associated with a specific metabolic function in normal, nonpregnant individuals (10). Knowing that a lipoidal derivative of this abundantly produced steroid exists would renew interest in this question.

Whatever the nature of the derivatizing group in the lipoidal derivative of pregnenolone, it is natural to consider that it serves to "protect" the 3β-hydroxy-Δ5-grouping of that steroid from
dehydrogenation-isomerization (to progesterone) by the abundant 3β-hydroxysteroid dehydrogenase system present in the adrenal. Because the enzyme specificities of steroids with a Δ4-3-keto structure appear to differ from those with a 3β-hydroxy-Δ5-grouping (11), the preservation of the latter by derivatization may serve some special role in the biogenesis of a particular steroid hormone.

In connection with the findings reported in this paper, the work of George W. Oertel should be mentioned. During the course of a decade, Oertel and his coworkers published a series of many papers (12) that described the occurrence, composition, and metabolic conversions of a plasma-borne lipophilic conjugate of dehydroisoandrosterone. He dubbed this conjugate "sulfatidyl" because he claimed the molecule was composed of the sulfate conjugate of the steroid, glycerol, and fatty acids joined in a fashion analogous to the known phosphatides. Oertel's findings have not been confirmed by others and have been largely ignored. The relationship, if any, that exists between the substance reported by Oertel and the lipoidal derivative of pregnenolone detected in this study may become evident after elucidation of the structure of the latter.

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