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Biochemistry

Precursors of the neurosteroids

(pregnenolone/dehydroepiandrosterone/hydroperoxide)

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ABSTRACT **In addition to the neurosteroids pregnenolone and dehydroepiandrosterone, organic solvent extracts of rat brains contain related compounds that react with various reagents to yield additional amounts of these ketosteroids. Among the chemicals producing these increments are triethylamine, HCl, $FeCl_3$, and $Pb(OAc)_4$. Most revealing is the action of $FeSO_4$ on these extracts. This reducing agent also converts components of the extract into the two neurosteroids, suggesting the presence of sterol hydroperoxides or peroxides in brain. The clues provided by this study indicate that the chemical nature of the steroidal constituents existing in extracts of mammalian brains remains to be determined. It is likely that their association with neurological functions will be better understood when the structures of these substances are established.**

In a recent paper (1), estimates of the concentrations of five steroids and their conjugates in mammalian brains were recorded. Also described were the techniques used for the separation, identification, and quantification of these so-called neurosteroids. An unusual feature of the reported findings was the relatively high concentrations of steroidal conjugates, which were designated "sulfolipids." Following from our previous experience with analogous conjugates of cholesterol (2), we found that there appear to exist in mammalian brain nonpolar conjugates of neurosteroids, particularly pregnenolone (3β-hydroxy-5-pregnen-20-one) and dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one). These, when treated with an organic base such as triethylamine or pyridine, were converted to a steroid sulfate that could be identified and quantified (after acid hydrolysis) as the free steroid. Some of these conjugates appeared to be present in relatively high concentrations in brain, suggesting that these conjugates play a physiological role in the nervous system.

The experiments described in this paper bear on these substances. More importantly, the results contribute to the question of the origin of the neurosteroids. Baulieu and coworkers (3) and Papadopoulos *et al.* (4) have shown that pregnenolone can be formed from cholesterol by enzymes present in oligodendrocytes and in glial cells of the brain. Moreover, Mellon *et al.* (5) have found a mRNA in rat brain that encodes a side chain-cleaving cytochrome P450 (P450scc) that catalyzes the cleavage of six carbon atoms from the side chain of cholesterol in the course of pregnenolone formation. Thus, it is generally assumed that the biosynthesis of pregnenolone in the brain utilizes the same precursor (cholesterol) and the same process (cytochrome P450scc) by which the C_{21}-steroids are made in the steroid-producing endocrine glands (adrenals, testes, etc). The evidence presented in this paper indicates that neurosteroids may also arise by alternate routes, one of which could use preformed, labile peroxides as proximal precursors. Since the quantities of neurosteroids isolated from brain extracts may depend on conditions created, possibly inadvertently, by the researcher during the isolation procedures, it may be difficult to establish a correlation of concentrations of these substances as they are estimated in extracts of excised brains with biochemical or behavioral phenomena. This caveat also applies to other biological experiments in which pregnenolone levels are measured in brain either by radioimmunoassay or gas chromatography/mass spectroscopy (GC/MS).

This paper presents evidence that suggests that extracts of rat brains contain compounds (still to be characterized) that, upon treatment with a variety of reagents not known to cleave lipoidal or sulfate conjugates, liberate pregnenolone and dehydroepiandrosterone. This evidence is based on experiments that compare the concentrations of these steroids found in the treated aliquots with those measured in untreated samples. Invariably the former concentrations were considerably greater than those in the controls; thus, it is likely that compounds like the 20-hydroperoxide and the 17-hydroperoxide derivatives of cholesterol and perhaps uncharacterized conjugates are present in mammalian brain.

MATERIALS AND METHODS

All solvents used were HPLC grade (J. T. Baker). [21-$^2H_3$]-Pregnenolone and (20*S*)-20-hydroxy[^{3}H]cholesterol were prepared as described (6, 7). System A for Celite was isooctane/90% MeOH, 1:1 (vol/vol). System B was MeOH/1-propanol/water/isooctane/toluene, 4:1:1.5:2:2 (vol/vol). HPLC was done on a silica microcolumn with 4% (vol/vol) isopropanol in hexane as eluent. The procedures for the isolation, purification, and analysis (GC/MS) of pregnenolone and dehydroepiandrosterone have been described (1).

Three grams of lyophilized rat brain (corresponding to 15 g wet weight) was extracted twice with 25 ml of chloroform/methanol, 3:2 (vol/vol). After removal of the solvent, the residue was divided into two parts. Aliquots of one part (part A), representing the whole brain, were treated with various reagents as indicated in Table 1.

The second part was separated into its ketonic and nonketonic (part B) components by treatment with polystyrenebenzyloxime resin to remove carbonyl compounds (8), including the endogenous ketosteroids and their derivatives. Sample B was dissolved in 20 ml of benzene containing 2 ml of acetic acid and a bolus of 200,000 dpm of tritiated pregnenolone. The oxime resin (100 mg) was added, and the suspension was stirred for 2 hr at room temperature. A check of the tritium content of the supernatant revealed that >90% of the tritiated steroid had bound to the resin. The reaction was allowed to proceed to

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Abbreviation: P450scc, side chain-cleaving P450.

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Table 1. Concentrations [ng/g (wet weight of tissue) of pregnenolone (P) and dehydroepiandrosterone (D) in an extract of rat brain after various treatments

Treatment	Whole-brain extract A		Nonketonic extract B	
	P	D	P	D
1. None	60	1.5	0	0
2. Heat (in benzene at 60°C for 2 hr)	—	—	38	0
3. Et_3N	155	8	70	*
4. Imidazole (3 mg in 1.5 ml of benzene)	162	10	150	14
5. $FeSO_4$ (100 mM in H_2O)	368	*	256	*
6. $FeSO_4$ (100 mM in H_2O followed by Et_3N	448	*	ND	ND
7. Et_3N followed by $FeSO_4$	ND	ND	315	*
8. Lead tetraacetate (10 mg in 1.5 ml of benzene)	320	*	240	*
9. HCl [100 μl of conc HCl in 1.5 ml of benzene/acetone, 9:1 (vol/vol)]	ND	ND	120	7
10. $FeCl_3$ (100 mM in H_2O)	503	*	ND	ND
11. Iodine in dioxane	ND	ND	0	0
12. Sodium methylate in methanol	ND	ND	0	0

The concentrations for all samples are given as the averages from replicate determinations. For samples 3B, 4B, and 8B, $n = 10$; for samples 5B and 9B, $n = 4$; for the remainder, $n = 2$. The values are precise to between 15% and 20%. Et_3N, triethylamine; ND, not determined; *, identity established by mass spectroscopy but the amount was too small to quantify accurately.

completion overnight. Control experiments showed that fatty acid esters and sulfates of the steroids also bound completely to the resin under these conditions. The resin was removed by filtration and washed with ethyl acetate several times. The filtrate, which was devoid of radioactivity, was neutralized and taken to dryness. The residue constituted the nonketonic fraction of part B. Aliquots of this fraction were treated with the reagents indicated in Table 1. All reactions were carried out at 60°C for 2 hr.

After each treatment, a neutral organic soluble fraction was obtained by extraction into ethyl acetate. Each fraction was labeled with 100,000 dpm of [^{3}H]pregnenolone (as a marker for chromatography) and 100 ng of [21-$^2H_3$]pregnenolone (internal standard for GC/MS). After purification by solid-phase extraction with C_{18}-modified silica and then by HPLC as described (1), each sample was converted to its silyl derivative by treatment with Tri-Sil/bovine serum albumin (Pierce). Quantification was achieved by calculating the areas under the base peaks for the trimethylsilyl derivatives of [21-$^2H_3$]pregnenolone, pregnenolone, and dehydroepiandrosterone (m/e 301, 298, and 304, respectively). The results given in Table 1 are expressed in ng/g (wet weight) of brain tissue.

One aliquot of the nonketonic fraction, B, was labeled with 250,000 dpm of [^{3}H]cholesterol, and the mixture was chromatographed on a reverse-phase Celite column (in system B). The first 50 ml of eluant from the column containing material more polar than cholesterol (whose retention volume, R_v, is 60–85 ml) was pooled and relabeled with 250,000 dpm each of tritiated (20*S*)-20-hydroxycholesterol and tritiated pregnenolone. This mixture was rechromatographed on celite by using system A. The retention volumes of the two tracers were 85–100 ml and 105–120 ml, respectively. Each of the tritiated fractions was separately heated for 2 hr with lead tetracetate in benzene at 60°C. After workup, GC/MS analysis failed to reveal any steroid in either fraction. The nonpolar fraction, eluted earlier in 10–80 ml of eluant, was similarly treated with lead tetracetate. Both pregnenolone and dehydroepiandrosterone were identified by GC/MS in this fraction.

RESULTS

The results summarized in Table 1 show that heat or treatment with an organic base (triethylamine or imidazol) led to the isolation from brain of larger amounts of pregnenolone and dehydroepiandrosterone than could be obtained simply by extraction. More revealing were the results with the reducing agent $FeSO_4$. The specificity of this reaction (ketones produced by the action of a reducing agent) suggests that the reactant is a peroxide.

The results in Table 1 suggest that there are present in brain extracts labile constituents other than peroxides that may also be precursors of pregnenolone and dehydroepiandrosterone. The quantities of these steroids, liberated after using both $FeSO_4$ and triethylamine in tandem, first in one order and then in reverse, suggest different classes of progenitors. Additional effort will be required to define the prevailing situation accurately. Similarly, the effects of the oxidants, $FeCl_3$ and lead tetraacetate, remain to be explained.

DISCUSSION

The results of this study reveal that there exist in organic solvent extracts of rat brain constituents that are readily convertible into the neurosteroids pregnenolone and dehydroepiandrosterone. Thus, organic extracts of brain contain the two neurosteroids *per se* as well as labile compounds that, upon treatment with various reagents, react to yield additional amounts of the two ketosteroids. The structure of at least one form of these precursors is strongly suggested by the results obtained with Fe^{2+}. Fe^{2+}, a reducing agent, is known to produce carbonyl-containing compounds from hydroperoxides (9–11). Most likely this reaction occurs by one-electron reduction of the —O—O— bond of the peroxide with subsequent fragmentation by β-scission of the resulting alkoxy radical (RO·). Lability to heat, air, and acid is also a property characteristic of a precursor like the 20-hydroperoxide derivative of cholesterol, a known compound isolated by vanLier and Smith (12) from a sample of cholesterol that had been heated in air for 7 days at 100°C. The chromatographic analysis of the nonketonic fraction, B, of the organic extract is consistent with the presence of a hydroperoxide of cholesterol. The relevant constituent(s) is more polar than cholesterol and less polar than either pregnenolone or (20*S*)-20-hydroxycholesterol. In a different chromatographic system, vanLier and Smith (12) had also observed that the 20-hydroperoxide of cholesterol is less polar than 20-hydroxycholesterol. Under the conditions used in this study, lead tetraacetate does not oxidize the 20-hydroxy derivative to pregnenolone. In a previous report (13), we showed that this reaction does occur, but the reaction conditions used were more vigorous than those used in the present study. The significance of the results with the bases triethylamine and imidazole is less obvious. The findings obtained with lead tetraacetate and Fe^{3+} are also unclear. The results obtained with these reagents suggest the possibility that precursors, other than peroxides, also exist in brain extracts.

As mentioned, the 20-hydroperoxide derivative of cholesterol was isolated from heated samples of cholesterol (12), but we have made no effort to isolate such a compound from rat brains. Nevertheless, the circumstantial evidence presented in this paper clearly indicates the presence of sterol hydroperoxides or peroxides in extracts of that organ. The lability of such molecules to heat, air, and light, etc., makes it likely that they may make a delusive contribution to the concentrations of the free neurosteroids estimated either by radio-

immunoassay (14) or GC/MS (1). These facts could explain the initial difficulty we (1) and Baulieu and Robel (14) had in obtaining reproducible analyses of the steroidal components of extracts of mammalian brain. As Kharasch *et al.* (10) and Hawkins and Young (11) have shown, treatment of tertiary hydroperoxides with aqueous solutions of $FeSO_4$ gives rise to several products, only one of which may be a ketone. Thus, the amounts of cholesterol peroxides in brain tissue must be greater than is evident from the additional quantities of ketosteroids produced by the action of Fe^{2+}.

The results also require that our previous suggestion (1)—that "sulfolipid" conjugates of pregnenolone and dehydroepiandrosterone exist—be rexamined. We reported that heating nonpolar fractions of brain extracts (from which pregnenolone and dehydroepiandrosterone had been removed) in an organic base like pyridine or triethylamine liberate, on occasion, larger amounts of these ketosteroids than were isolated as free steroids. Based on our previous experience with the lipophilic complexes of the sulfate ester of cholesterol (2), we interpreted our findings with neurosteroids (1) as indicating the existence of analogous conjugates of these steroids in extracts of brain. While our conclusions about sterol (i.e., cholesterol) sulfolipids were better founded (nonpolar extracts were converted by pyridine into sterol conjugates that behaved as sulfates), the assignment of neurosteroid sulfolipids (1) is now in question. The uncertainty arises from the fact that constituents sensitive to triethylamine are present in a nonketonic fraction (B). This observation appears to rule out conjugation only through the 3-position, where it presumably exists in the cholesterol "sulfolipid." Treatment with an organic base (without subsequent solvolysis) liberates pregnenolone from the nonketonic portion just as reaction with Fe^{2+} does. We now know that the experimental conditions used do not permit a distinction to be made between neurosteroid precursors that are sulfolipids from those that are sterol peroxides. Other than peroxides, the identities of those components of brain that yield ketosteroids upon treatment with acid or alkali or the other reagents used in this study remain to be established.

In the course of steroid hormone biosynthesis, the sterol precursor is traditionally considered to react at a side-chain carbon atom, either at C-20 or C-22 (15), with the activated oxygen–cytochrome P450scc complex. The resulting complex, which could be represented as steroid-O—O–Fe^{3+}–cytochrome P450scc, is known to fragment to the isolable C-20 ketone, pregnenolone. Although this reaction (reaction 1 in the scheme in Fig. 1) can probably proceed without the intermediacy of a stable, isolable peroxide, it is conceivable that the transient complex can serve as a precursor of an organic soluble sterol hydroperoxide, sterol cyclic peroxides, and/or other disubstituted sterol peroxides, steroid-O—O—R. (R is not H.) Thus, one possibility is that the complex could be converted into the isolable 20-hydroperoxide (compound I in the scheme in Fig. 1), the 20,22-cycloperoxide (compound II), or the 17,20-cycloperoxide (compound III) by enzymes that are specific for hormone synthesis. Possibly through the action of a different enzyme, the 17-hydroperoxide of cholesterol (compound IV) could be the product from the sterol. If the relevant enzymes in the brain are dedicated to neurosteroid production from peroxide precursors by reactions 3 or 3′ in the scheme in Fig. 1, the processes for which they served as catalysts could be considered to constitute biosynthetic pathways for the formation of C_{21} steroids and C_{19} steroids that are different from the cytochrome P450scc process usually considered to be involved in adrenal corticosteroid biosynthesis. On the other hand, the peroxidation of cholesterol (reaction 2 and 2′) may be catalyzed by enzymes that are different from the well-known cytochrome P450scc.

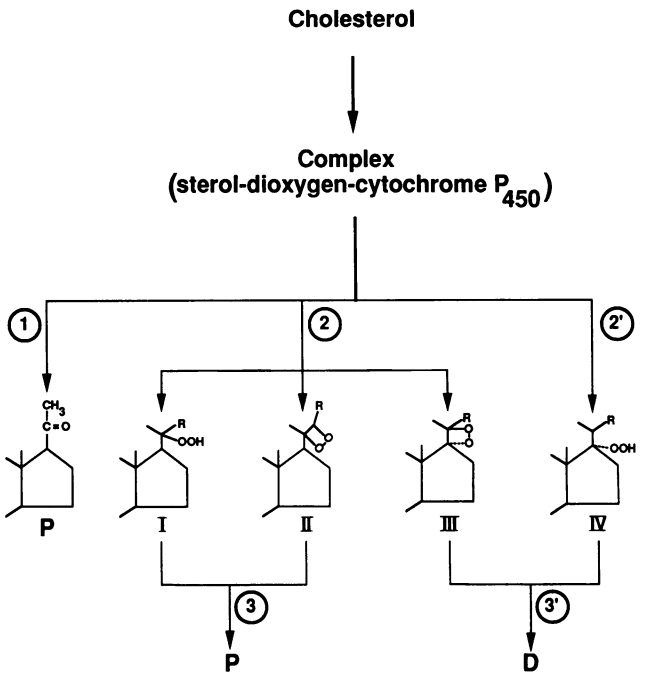

FIG. 1. Reaction 1 is assumed to be catalyzed by a specific enzyme leading to the formation of pregnenolone without the intermediacy of compounds I, II, III, or IV, all of which are soluble in organic solvents and are nonketonic. Reaction 2 (or 2′) is assumed to be catalyzed by a specific enzyme leading to the formation of the peroxides, compounds I, II, III, or IV. Reaction 3 (or 3′) is catalyzed by a specific enzyme leading to the formation of steroidal products P (3β-hydroxy-5-pregnen-20-one, pregnenolone) or D (3β-hydroxy-5-androsten-17-one, dehydroepiandrosterone).

In the formulation presented in the scheme in Fig. 1, the 20,22-dioxetane could be converted into the 20-ketosteroid pregnenolone, and in an analogous manner the 17,20-dioxetane could be the source of the 17-ketosteroid dehydroepiandrosterone.

The suggestion that an alternate pathway involving a hydroperoxide could exist for the biosynthesis of steroid hormones has already been proposed by vanLier and Smith (16). In 1970, they showed that the 20-hydroperoxide derivative of cholesterol could be converted into pregnenolone by incubation with adrenal cortex mitochondrial enzymes. That the 20,22-cyclic peroxide, the dioxetane compound II, could be involved in the formation of pregnenolone from cholesterol was also previously considered by Kraaipoel *et al.* (17). For a different facet of steroid biosynthesis, Tan and Rousseau (18) also have invoked a dioxetane species as an intermediate.

What has been shown in the present study is that species that behave like hydroperoxides or peroxides exist in extracts of rat brains. If the isolable compounds I, II, III, and IV (or related peroxides) in the scheme in Fig. 1 were formed enzymatically (reaction 2 or 2′) in the brain, and if there existed enzymes specifically dedicated to the production of pregnenolone and dehydroepiandrosterone from these peroxides (reaction 3 or 3′), then indeed an alternate pathway for steroid biosynthesis different from the traditional one would be indicated.

Two groups of investigators (4, 19) have shown that selected brain cells can convert cholesterol into pregnenolone. This conversion is presumably catalyzed by cytochrome P450scc, and Mellon and Deschepper (5) proved the existence in brain of the mRNA that encodes the cytochrome P450scc isolated from bovine adrenals. As far as is known, the intermediacy of an organic soluble sterol peroxide (or hydroperoxide) is not involved in this biosynthetic

path, which is generally considered to operate in all steroid-producing tissues. Thus, a pathway involving an isolable sterol peroxide as the proximal precursor of pregnenolone or dehydroepiandrosterone could be different from the traditional one.

That steroid hormone biosynthesis in the brain is different from that characteristic of the adrenal, testes, etc., is suggested by other observations. Although Hu *et al.* (19) were able to show the conversion of cholesterol to pregnenolone by brain cells, the investigators (20) were unable to show the conversion of pregnenolone to 17-hydroxypregnenolone or to dehydroepiandrosterone. The conversion of pregnenolone to these steroids is easily demonstrable using tissue preparations from adrenals or testes. In accord with this finding, Mellon and Deschepper (5) reported that they could not detect the mRNA that coded for P450c17 (17-hydroxylase/17,20-lyase), the cytochrome that is required for C_{19}-steroid formation from C_{21} steroids. These observations introduce the possibility that the 17-ketosteroid is made in the brain by a route different from the one ordinarily accepted for C_{19}-steroid synthesis.

Another relevant fact that supports the notion that C_{19}-steroid formation in the brain differs from that existing in rat adrenal or testes is our observation (unpublished) that treatment of extracts of these tissues with Fe^{2+} did not increase the estimated amount of either pregnenolone or dehydroepiandrosterone over that found in untreated extracts. In this respect, as Table 1 shows, the results with rat brain are clearly different.

Evidence for a naturally occurring biosynthetic pathway usually consists of demonstrating (*i*) the occurrence of a rational precursor in the relevant endocrine tissue and (*ii*) the existence of an enzymatic system for converting this precursor into the final hormonal product. If the sterol peroxide is a natural constituent of brain, and if the enzyme catalyzing the conversion of the peroxide to the final secretory product is specifically intended for that reaction, both of these criteria would be satisfied. The demonstration that an unnatural synthetic product like the 20-*tert*-butyl derivative of pregnenediol (21), when incubated with adrenal mitochondria, can readily serve as a precursor of pregnenolone does not necessarily connotate the existence of a biosynthetic pathway different from that which uses cholesterol as precursor. Likewise if nonspecific enzymes (i.e., catalase) catalyze the conversion of the hydroperoxides of cholesterol (or its cyclic peroxides) to the corresponding ketosteroids, this reaction by itself would not indicate a second pathway. In the scheme in Fig. 1, both of the two relevant enzyme systems [one for the production of the precursor peroxide (reaction 2) and the other for its conversion to pregnenolone or dehydroepiandrosterone (reaction 3)] are considered to be specific for the synthesis of the ketosteroids. If only one enzyme is specific for hormone synthesis, the processes using this route could be of physiological significance. If neither enzyme is specific—i.e., the hydroperoxide is formed by autooxidation—and a nonspecific enzyme catalyzes the formation of pregnenolone from the autooxidized product, the physiological effects due to that part of the ketosteroids formed through this spurious nonspecific mechanism might be inconsequential.

An issue of particular interest in this connection is the origin of the adrenal secretory product, dehydroepiandrosterone. It is customary to consider that the pathway by which this C_{19} product is biosynthesized resembles those leading to the formation of the C_{21} hormones cortisol, aldosterone, and progesterone. In this traditional scheme, the first step in the biosynthesis cleaves six carbon atoms from the side-chain of cholesterol—i.e., $C_{27} \rightarrow C_{21} + C_6$. In this process the C_{21} fragment can be considered to be an obligatory precursor of the C_{19} hormones—i.e., $C_{21} \rightarrow C_{19} + C_2$. If a pathway for the formation of dehydroepiandrosterone using as the proximal precursor either the 17-hydroperoxide or the 17,20-cyclic peroxide derivative of cholesterol exists, the side-chain cleavage product that would result from this conversion possibly contains eight carbon atoms. An intensive search for a C_8 fragment accompanying the biosynthesis of a C_{19} steroid from cholesterol was made about a quarter of century ago (22–24), but none was found. If the path cholesterol → cholesterol peroxide → dehydroepiandrosterone does exist in the brain or even in the steroid-producing endocrine glands, then it would undoubtedly be associated with its own regulatory system (trophic factors, etc.) and would represent a new aspect of steroidogenesis.

The possibility that pregnenolone and dehydroepiandrosterone and other ketosteroids play a role as neurotransmitters has recently evoked much activity (25). The results of this study suggest that other steroidal constituents exist in brain in sensible amounts. This introduces the possibility that these unidentified relatives may also serve neuronal functions. These compounds have been detected by their conversion to the two known steroids, but it remains to be determined whether they serve merely as precursors or whether they also have physiological significance.

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