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BIOSYNTHESIS AND TRANSFORMATION OF 20α, 21-DIHYDROXYCHOLESTEROL BY RAT ADRENAL PREPARATIONS

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SUMMARY

The biosynthesis of [^{3}H]-20α, 21 dihydroxycholestderol from [^{3}H]-20α-hydroxycholesterol and its transformation to [^{3}H]-21-hydroxypregnenolone by rat adrenal preparations has been demonstrated. 20α-Hydroxycholesterol was transformed to 20α, 21-dihydroxycholesterol by microsomal preparations in the presence of NADPH and 20α-21-dihydroxycholesterol was metabolized to 21-hydroxypregnenolone by mitochondrial preparations in the presence of a NADPH-generating-system. Comparison of the Michaelis–Menten-Kinetics of the steps "20α, 21-dihydroxycholesterol → 21-hydroxycholesterol" and "20α-hydroxycholesterol → pregnenolone" revealed that both compounds behaved as analogue substrates of the desmolase complex. The data are taken as further evidence for the participation of an alternative pathway via the sequence "21-hydroxysterol → 21-hydroxypregnenolone → deoxycorticosterone" in rat adrenal corticosteroid biosynthesis.

INTRODUCTION

It is generally accepted that the main route of corticosteroid biosynthesis proceeds via a fixed sequence of enzymatic reactions with cholesterol being the central intermediate and pregnenolone the first C-21 steroid being formed [1, 2]. However, various studies have suggested the existence of alternative pathways which might participate in corticosteroid biosynthesis of several species [1, 2]. Recently, in our laboratory evidence has come up from incubation experiments with adrenal slices and [^{3}H]-acetic acid suggesting that 21-hydroxypregnenolone participates in the biosynthesis of corticosterone in rat adrenals [3]. Furthermore, our data indicated that 21-hydroxypregnenolone is not only formed from pregnenolone but also from a 21-hydroxysterol. The present paper summarizes further evidence for the existence of an alternative pathway via the sequence "21-hydroxysterol → 21-hydroxypregnenolone → deoxycorticosterone" by directly demonstrating (a) the formation of 21-hydroxypregnenolone from a 21-hydroxysterol and (b) the 21-hydroxylation of a sterol by subcellular preparations of rat adrenal glands.

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The following trivial names have been used in the text: 20α, 21-dihydroxycholesterol = 3β, 20α, 21-trihydroxy-5-cholesten; 20α-hydroxycholesterol = 3β, 20α-dihydroxy-5-cholesten; 21-hydroxypregnenolone = 3β, 21-hydroxy-5-pregnen-20-onc; pregnenolone = 3β-hydroxy-5-pregnen-20-one

MATERIALS AND METHODS

Chemicals. Progesterone, pregnenolone, 21-hydroxypregnenolone, 20α-hydroxycholesterol: originated from Ikapharm, Israel. 20α, 21-Dihydroxycholesterol was synthetized by Grignard-reaction of 1-bomo-4-methylpentane with 21-hydroxypregnenolone acetate [4]. 20α, 21-Dihydroxycholesterol was purified by silica gel column chromatography with the solvent system isoamylacetate–ethylacetate–methanol (14:5:1, by vol.) and by recrystallization from aqueous methanol. Melting point (175–176°C) and gas chromatographic characteristics of the product were identical to those of authentic 20α, 21-dihydroxycholesterol, kindly supplied by Dr. M. Gut (Shrewsbury, MA, U.S.A.).

7-[^{3}H]-20α-Hydroxycholesterol (25 Ci/mmol, 4-[^{14}C]-pregnenolone (52.8 mCi/mmol): originated from New England Nuclear, U.S.A. [^{3}H]-20α, 21-Dihydroxycholesterol (2.2 Ci/mmol) was labelled by catalytic exchange (New England Nuclear, U.S.A.). The preparation of 4-[^{14}C]-21-hydroxypregnenolone from 4-[^{14}C]-pregnenolone will be described elsewhere (Kaufmann, Sinterhauf, and Lommer, to be published). In short, the method was similar to the steroid-21-hydroxylation-assay described in this paper. All radioactive compounds were stored in ethanol solution at −18°C. They were routinely examined for radiochemical homogeneity by radioactivity scanning after chromatography in the systems used for isolation.

$NADP^+$, NADPH, glucose-6-phosphate, and glucose-6-phosphate-dehydrogenase: originated from Boehringer, Mannheim, Germany. All other reagents and solvents were obtained from Merck A.G., Germany.

Animals. Male Sprague–Dawley rats weighing 200–300 g were used.

Preparation of mitochondrial and microsomal fractions. Rat adrenal glands were homogenized in 2 vol. of ice cold 0.25 M sucrose in phosphate buffer (0.06 M, pH 7.4) using a Potter–Elvehjem homogenizer with Teflon pestle. The homogenate was centrifuged for 15 min at 4°C and 800 *g* and the sediment discarded. The supernatant was filtered through gauze and centrifuged for 20 min at 4°C and 14,000 *g*. The pellet was resuspended in the sucrose–phosphate buffer and centrifuged for 20 min at 4°C and 9,000 *g*. The sediment will be referred to as mitochondrial preparation. The 14,000 *g* supernatant was centrifuged for 60 min at 4°C and 105,000 *g*. The sediment will be referred to as microsomal preparation. Pellets were resuspended in phosphate buffer to an approximate protein concentration of 10 mg/ml. Protein contents were determined by the method of Lowry *et al.*[5]. The two cell fractions were examined for cross-contamination by means of electron microscopy kindly carried out by Prof. Langner, Pathologisches Institut, Johannes-Guttenberg-Universität, Mainz [6]. The mitochondrial preparation showed a small contamination with microsomes whereas a contamination of the microsomal-preparation was not observed.

Microsomal preparations were prepared directly before use. The mitochondrial preparations were either used directly or acetone dried before use. Acetone drying was done as described in [7]. 2 ml aliquots of the mitochondrial preparation were poured into 20 ml of acetone (prechilled to −18°C) under heavy stirring. The precipitate was collected over a Buchner funnel and washed with prechilled acetone and ether. The powder was dried at 4°C and stored at −18°C in a desiccator.

Incubations. Incubations were performed in a Dubnoff metabolic shaker at 37°C under air. Steroid-21-hydroxylation was carried out in a medium consisting of 80 parts of 10% albumine in 0.15 M NaCl (pH 7.4), 16 parts of 0.06 M phosphate buffer (pH 7.4), 0.8 parts of 0.11 M $MgCl_2$, and 3.2 parts of 0.15 M KCl [8] and containing 2 mol/ml NADPH. Steroid side chain cleavage was carried out in a medium consisting of 75 parts of 0.06 M phosphate buffer (pH 7.4) and 25 parts of 5 mM $MgSO_4$ and containing 2 mol/ml $NADP^+$, 20 mol/ml glucose-6-phosphate and 0.6 IU/ml glucose-6-phosphate-dehydrogenase as a NADPH-generating-system. The steroids were dissolved in the medium by means of propyleneglycol. The incubations were initiated by the addition of the enzyme preparations.

Isolation of the steroids. The incubations were terminated by adding 3 ml of chloroform. Metabolites were extracted three times with 3 ml of chloroform. The combined chloroform phases were washed with distilled water and the solvent evaporated under a gentle stream of air. Steroids were purified by thin layer chromatography on silica gel (Macherey-Nagel) with different internal and external standards as described in [3]. The following solvent systems were used: toluene–methanol (92:8, by vol., system I), ethylacetate–benzene–n-heptane (1:1:1, by vol., system II), petroleumether–diisopropylether–acetic acid (15:35:1, by vol., system III). Radioactive zones were detected by scanning and eluted with a mixture of chloroform and methanol. The radioactivity of the compounds isolated was determined by liquid scintillation counting as described in [3]. Metabolites were identified by recrystallization to constant specific radioactivity. Approximately 30 mg of authentic material were mixed with the radioactive compound and the mixture was dissolved in 1 ml of solvent. Masses were determined by gas–liquid–chromatography using 3% OV1 on 60–80 mesh Chromosorb W (Varian) at 250°C with a Varian Aerograph Model 2100.

RESULTS

(1) *Side chain cleavage of 20α, 21-dihydroxycholesterol by mitochondrial preparations*

Formation of 21-hydroxypregnenolone. The freshly prepared mitochondrial preparations (final protein concentration 2 mg/ml) was incubated with 10 μCi of [^{3}H]-20α, 21-dihydroxycholesterol in the presence of the NADPH-generating-system in a total volume of 5 ml for 90 min. Approximately 70% of the added radioactivity were recovered by extraction of the material with chloroform. After chromatography in system III five main radioactive zones could be determined on the radiochromatogram, one zone corresponding to 21-hydroxypregnenolone and another one to 20α, 21-dihydroxycholesterol. The remaining three zones were not further identified. The radioactive material corresponding to 21-hydroxypregnenolone was acetylated with acetic anhydrid in pyridine and afterwards rechromatographed in system II. Two radioactive zones were detected, one of which corresponded to 21-hydroxypregnenolone acetate. The material was mixed with authentic 21-hydroxypregnenolone acetate and recrystallized to constant specific radioactivity and thus the formation of 21-hydroxypregnenolone from 20α, 21-dihydroxycholesterol by rat adrenal mitochondrial preparations was established (Table 1).

In order to gather further information as to whether the formation of 21-hydroxypregnenolone from a 21-hydroxysterol might be of any physiological relevance, the side chain cleavage of 20α, 21-dihydroxycholesterol was compared quantitatively with that of 20α-hydroxycholesterol, a compound known to be the physiological precursor of pregnenolone [9].

Comparative studies on the side chain cleavage of 20α-hydroxycholesterol and 20α, 21-dihydroxycholesterol. Comparative kinetic studies on the side cleavage of 20α, 21-dihydroxycholesterol and 20α-hydroxycholesterol were carried out with acetone-dried preparations of rat adrenal mitochondria since no loss of the desmolase activity had been observed with

Table 1. Identification of [^{3}H]-21-hydroxypregnenolone by recrystallization to constant specific radioactivity of [^{3}H]-21-hydroxypregnenolone acetate

Step of purification	Solvent	Specific radioactivity (c.p.m./mg)
System III		9260
1st Crystallization	Methanol/H_2O	9300
2nd Crystallization	Acetone/hexane	9020
3rd Crystallization	Acetone/hexane	9030

these preparations. The samples were chromatographed separately in system III. The radioactive materials of the zones corresponding to 21-hydroxypregnenolone or pregnenolone, respectively, were acetylated with acetic anhydride in pyridine and afterwards rechromatographed in system II. The formation of [^{3}H]-21-hydroxypregnenolone or [^{3}H]-pregnenolone, respectively, was determined by liquid scintillation counting of the radioactive materials, and corrected for losses by means of ^{14}C-labelled internal standards [3]. Since [^{3}H]-20α, 21-dihydroxycholesterol had been tritiated by catalytic exchange, the radioactivity data of [^{3}H]-21-hydroxypregnenolone had to be corrected due to the cleavage of [^{3}H]-20α, 21-dihydroxycholesterol into [^{3}H]-21-hydroxypregnenolone and [^{3}H]-isocapronic acid. It was assumed that [^{3}H]-20α, 21-dihydroxycholesterol was randomly labelled and the data were corrected by multiplication with the factor 1.43.

1 μCi of [^{3}H]-20α, 21-dihydroxycholesterol or [^{3}H]-20α-hydroxycholesterol, respectively, were incubated with the acetone-dried mitochondrial preparation (final concentration 2.2 mg/ml) in the presence of the NADPH-generating-system and 1 ml aliquots were removed at different time points (Fig. 1). Although

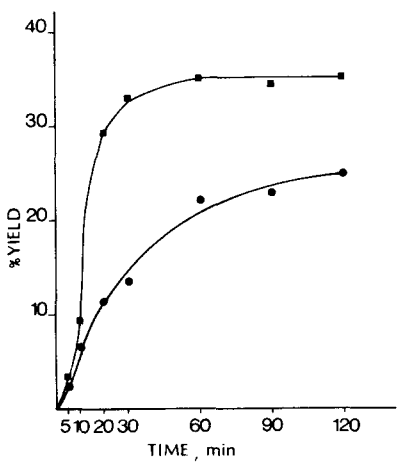

Fig. 1. Time course of the desmolase reaction. [^{3}H]-20α, 21-Dihydroxycholesterol or [^{3}H]-20α-hydroxycholesterol were incubated with the acetone-dried mitochondrial preparation of rat adrenals and the formation of [^{3}H]-21-hydroxypregnenolone (●——●) or [^{3}H]-pregnenolone (■——■), respectively, determined after different times.

the formation of [^{3}H]-pregnenolone was somewhat greater and faster than that of [^{3}H]- 21-hydroxypregnenolone, the initial transformations of both substrates differed only slightly. Furthermore, after an incubation time of 5 min the formation of products other than [^{3}H]-pregnenolone and [^{3}H]-21-hydroxypregnenolone was not demonstrable. Therefore, an incubation time of 5 min was chosen for the determination of the apparent K_M-values of 20α-hydroxycholesterol and 20α, 21-dihydroxycholesterol in the following experiment.

[^{3}H]-20α, 21-Dihydroxycholesterol and [^{3}H]-20α-hydroxycholesterol were diluted with authentic material to give a final specific activity of 0.22 Ci/nmol. Different concentrations of each substrate were incubated with the acetone dried mitochondrial preparation (final protein concentration 1.6 mg/ml) in the presence of the NADPH-generating-system for 5 min. In an additional test sample unlabelled 20α, 21-dihydroxycholesterol (10 μM) was incubated together with ^{3}H-20α-hydroxycholesterol.

Apparent K_M-values were measured by the graphical method of Lineweaver and Burk (Fig. 2). As can be seen from Table 2, the apparent K_M-values for both substrates were in the same order of magnitude. When the transformation of [^{3}H]-20α-hydroxycholesterol was inhibited by 20α, 21-dihydroxycholesterol the slope was increased, whereas the ordinate intercept remained constant, indicating the competitive nature of the inhibition (Fig. 2).

These data demonstrate that 20α, 21-dihydroxycholesterol and 20α-hydroxycholesterol are analogue substrates of the mitochondrial desmolase complex with nearly identical affinities, suggesting that the side chain cleavage of a 21-hydroxysterol might be of importance for rat adrenal corticosteroid biosynthesis. That the intermediate 20α, 21-dihydroxycholesterol can be formed from a physiologically occurring precursor, as well, will be shown in the next experiment.

Table 2. Apparent K_M-values for 20α-hydroxycholesterol and 20α, 21-dihydroxycholesterol

Substrate	K_M
20α-Hydroxycholesterol	1.7×10^{-5} M
20α, 21-Dihydroxycholesterol	1.8×10^{-5} M

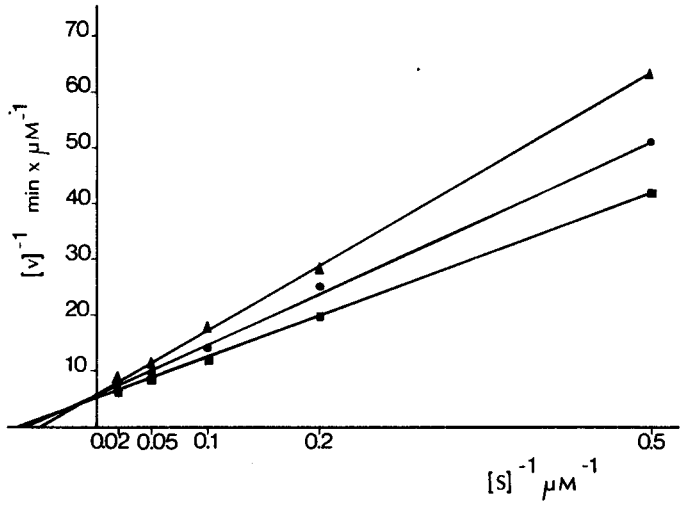

Fig. 2. Activity of the desmolase reaction in dependence of the substrate concentration. Lineweaver-Burk-plot. Different concentrations of [^{3}H]-20α, 21-dihydroxycholesterol or [^{3}H]-20α-hydroxycholesterol were incubated with the acetone-dried mitochondrial preparation of rat adrenals and the formation of [^{3}H]-21-hydroxypregnenolone (●——●) or [^{3}H]-pregnenolone (■——■), respectively, determined. ▲——▲: competitive inhibition of the formation of [^{3}H]-pregnenolone by 20α, 21-dihydroxycholesterol (10 μM).

(2) *21-Hydroxylation of 20-hydroxycholesterol by microsomal preparations*

The freshly prepared microsomal preparation (final protein concentration 2.3 mg/ml) was incubated with 20 μCi of [^{3}H]-20α-hydroxycholesterol in the presence of NADPH in a total volume of 5 ml for 90 min. Approximately 85% of the added radioactivity were recovered by extraction of the material with chloroform. After chromatography in system I the radiochromatogram showed four radioactive zones, one zone corresponding to 20α, 21-dihydroxycholesterol and another one to 20α-hydroxycholesterol. The remaining two zones were not further identified. After rechromatography of the radioactive material corresponding to 20α, 21-dihydroxycholesterol in system III two radioactive zones were detected, one of which corresponded to 20α, 21-dihydroxycholesterol. This material was acetylated with acetic anhydride in pyridine and afterwards chromatographed in system II. It was finally identified as 20α, 21-dihydroxycholesterol acetate by recrystallization to constant specific activity and thus the formation of 20α-21-dihydroxycholesterol from 20α-hydroxycholesterol by rat adrenal microsomal preparations was established (Table 3).

DISCUSSION

In the present study we could demonstrate (a) the side chain cleavage of [^{3}H]-20α, 21-dihydroxycholesterol by mitochondrial preparations, and (b) the 21-hydroxylation of [^{3}H]-20α-hydroxycholesterol by microsomal preparations of rat adrenals.

It is known that pregnenolone is formed from 20α-hydroxycholesterol by hydroxylation of C-22, and consecutive cleavage between C-20 and C-22 [10] and it is most probable that the side-chain-cleavage of 20α, 21-dihydroxycholesterol proceeds via the same sequence of reactions. Therefore, in the kinetic studies described only the end product of a complex series of reactions could be measured and no conclusions could be made on the individual rates of the reactions. However, it was possible to determine the apparent K_M-values of the overall reactions, allowing

Table 3. Identification of [^{3}H]-20α, 21-dihydroxycholesterol by recrystallization to constant specific radioactivity of [^{3}H]-20α, 21-dihydroxycholesterol acetate

Step of purification	Solvent	Specific radioactivity (c.p.m./mg)
System II		2840
1st Crystallization	Methanol/H_2O	2530
2nd Crystallization	Acetone/hexane	1050
3rd Crystallization	Acetone/hexane	980
4th Crystallization	Methanol/H_2O	1070

the comparison of the two closely related transformations "20α-hydroxycholesterol → pregnenolone" and "20α, 21-dihydroxycholesterol → 21-hydroxypregenenolone". This comparison revealed that both substrates, the 21-hydroxysterol as well as the 21-deoxysterol, were analogue substrates of the desmolase-complex with nearly identical affinities.

Our data confirm earlier reports in which the desmolase-complex was shown to be of limited substrate specificity [9]. Furthermore, the naturally occurring derivatives of cholesterol with hydroxy-functions at C-20 and/or C-22 are even more efficiently transformed to pregnenolone than cholesterol itself [10, 11, 12]. Our data extend these results by demonstrating that the presence of a hydroxy-function at C-21 does not reduce the rate of the side-chain-cleavage system, as well. This observation is in agreement with the findings published by Burstein *et al.*[13]. Using bovine adrenocortical preparations these authors found that 20α, 21-dihydroxycholesterol is a more potent inhibitor of the desmolase-reaction than cholesterol itself. Thus, it appears probable that the side-chain cleavage of 20α, 21-dihydroxycholesterol might be of physiological relevance.

From their extensive studies on the 21-hydroxylation of corticosteroids carried out with partial purified enzyme preparations from bovine adrenocortical microsomes Cooper *et al.*[8] have proposed a high specificity of the 21-hydroxylase-system to the 3-keto-4-ene-group. Nevertheless, the formation of 21-hydroxypregnenolone has been demonstrated in various laboratories by using adrenal tissue from different species [3, 14–17], and Mackler *et al.*[18] found that the microsomal 21-hydroxylase-system looses its capacity to transform pregnenolone during the course of purification, while the transformation of progesterone remains unaffected. Demonstrating the transformation of 20α-hydroxycholesterol to 20α, 21-dihydroxycholesterol we proved that not only pregnenolone but even a sterol can serve as substrate of the microsomal 21-hydroxylase system.

The formation of 20α, 21-dihydroxycholesterol from cholesterol-20α-hydroperoxid has been shown by Van Lier and Smith[19]. However, this reaction involved a mitochondrial enzyme and is independent of NADPH, while the 21-hydroxylation of 20α-hydroxycholesterol involves a microsomal enzyme and is NADPH-dependent. Furthermore, we failed to demonstrate any 21-hydroxylation of 20α-hydroxycholesterol by mitochondrial preparations although several attempts, including the use of pregnenolone and 21-hydroxypregnenolone as inhibitors, had been made [20]. Thus, the formation of 20α, 21-dihydroxycholesterol from cholesterol-20α-hydroperoxid seems to be mediated by a completely different reaction than the 21-hydroxylation of 20α-hydroxycholesterol described here.

While the participation of cholesterol-20α-hydroperoxid in corticosteroid-biosynthesis has not been conclusively proven, it is clear that 20α-hydroxycholesterol is a natural intermediate of the side-chain cleavage of cholesterol [9]. However, the yield of 20α, 21-dihydroxycholesterol was relatively poor as compared to the 21-hydroxylation of progesterone and even pregnenolone [21] and furthermore it is most likely that under *in vivo* conditions 20α-hydroxycholesterol remains bound to the mitochondrial desmolase-complex [22]. Therefore, the question whether the transformation of 20α-hydroxycholesterol to 20α, 21-dihydroxycholesterol is of any physiological relevance certainly needs further investigations.

In previous experiments carried out in our laboratory [3] it was found that incubation of rat adrenals with tritiated acetate resulted in specific radioactivities which were much lower in cholesterol, pregnenolone, and progesterone than in deoxycorticosterone and corticosterone. When we isolated 21-hydroxypregnenolone it exhibited specific radioactivities ten to four times higher than those of deoxycorticosterone. We, therefore, suggested the existence of an alternative pathway in which 21-hydroxypregnenolone deriving from a 21-hydroxysterol—presumably 21-hydroxydesmosterol—is the direct precursor of deoxycorticosterone.

The experiments reported here were conducted in order to further examine the existence of the proposed pathway by using 20α-hydroxycholesterol and 20α, 21-dihydroxycholesterol as representative sterols. Thus, the demonstration of the 21-hydroxylation of 20α-hydroxycholesterol and of the side chain cleavage of 20α, 21-dihydroxycholesterol provides further evidence for the existence of the suggested alternative pathway via 21-hydroxypregnenolone.

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