COFACTOR REQUIREMENTS FOR THE CONVERSION OF CHOLESTEROL SULFATE TO PREGNENOLONE SULFATE BY A SUBMITOCHONDRIAL SYSTEM FROM BOVINE ADRENAL CORTEX.

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Received May 20, 1968

It now appears that adrenocortical steroid hormones may be synthesised and secreted as sulfates (Baulieu, 1962; Roberts et al., 1964). Consistent with this idea is the recent demonstration by two groups of workers that acetone powders (Raggatt and Whitehouse, 1966) and sonicates (Roberts et al., 1967) of mitochondria from bovine adrenal cortex are capable of converting cholesterol sulfate to pregnenolone sulfate. It is evidently possible for the entire biosynthetic pathway from cholesterol to the secreted hormones to proceed with intermediates in the form of steroid sulfates. These observations raise the important question of whether or not the mechanism by which the side-chain of cholesterol sulfate is removed to give pregnenolone sulfate, is the same as that involved in the case of the free steroids. The present studies suggest that this is the case, since cytochrome P-450, TPNH, molecular oxygen and a TPNH-cytochrome P-450 reductase are required for side-chain cleavage of cholesterol sulfate.

Experimental Procedure

Two fractions were prepared from bovine adrenocortical mitochondria exactly as described by Omura et al. (1966). Briefly, these fractions are prepared by sonication and high-speed centrifugation of a suspension of mitochondria in water. One fraction, called S_2, has been shown to contain a TPNH-cytochrome P-450 reductase and non-heme iron; the second fraction

contains cytochrome P-450 (Omura et al., 1966). The fractions were stored at -10°.

Incubation at 37° was performed in Warburg flasks with constant agitation as described by Omura et al. (1966). These conditions are referred to here as the complete system under standard conditions. When incubation was performed in light of various wave-lengths, the method described by Omura et al. (1965) was used.

Pregnenolone-^{3}H sulfate was extracted and isolated by a procedure described in detail by Drayer and Lieberman (1967). The tritium content of pregnenolone-^{3}H sulfate was measured by methods reported elsewhere (Hall, 1966). Recrystallization and determination of specific activities of pregnenolone-^{3}H sulfate were performed by methods reported for cholesterol-^{14}C sulfate (Young and Hall, 1968).

Cholesterol-7α-^{3}H (New England Nuclear Corporation Lot No. 184-53) was purified as described elsewhere (Hall and Koritz, 1964). The purified compound was then converted to the sulfate (Young and Hall, 1968). The sulfate was purified by column chromatography on aluminium oxide and then on celite. Following purification, a sample of cholesterol-7α-^{3}H sulfate (ammonium salt) was recrystallized from four different solvent systems after addition of cholesterol sulfate (10 mg). The specific activity of crystals and mother liquors remained constant throughout this procedure, thereby demonstrating the radiochemical purity of the cholesterol-7α-^{3}H sulfate used as substrate in these studies. The preparation and purity of the cholesterol sulfate (unlabeled) used has been described in detail (Young and Hall, 1968).

Results

Table I shows that when a sample of pregnenolone-^{3}H sulfate isolated following incubation of submitochondrial fractions with cholesterol-7α-^{3}H sulfate was recrystallized from different solvent systems, the specific

activities of crystals and mother liquors remained unchanged. When another sample of this substance was cleaved by solvolysis (Burstein and Lieberman, 1958), the free pregnenolone-^{3}H could also be recrystallized without change in specific activity (data not shown).

When pregnenolone-^{3}H (1 μg : 1.3 x 10^6 dpm) was incubated in three experiments using the same fractions and additions as those used with cholesterol-7α-^{3}H sulfate, no pregnenolone-^{3}H sulfate (< 100 dpm per flask) was isolated. It is therefore unlikely that these submitochondrial fractions contained sufficient sulfatase to cleave cholesterol sulfate or sufficient sulfokinase to convert pregnenolone to the sulfate. This point is of some importance since the same fractions have been shown to convert cholesterol to pregnenolone (Hall, 1967).

The time course of the conversion of cholesterol-^{3}H sulfate to pregnenolone-^{3}H sulfate under standard conditions is shown in Figure 1. It will be seen that the rate of conversion is linear for 15 or 20 minutes but declines thereafter.

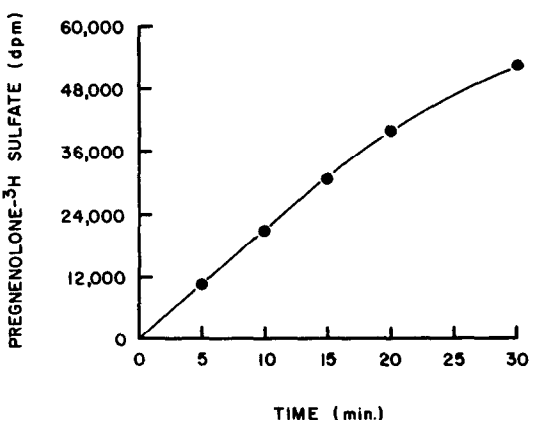

Figure 1: The conversion of cholesterol-7α-^{3}H sulfate (0.04 μg : 2.5 x 10^6 dpm/flask) to pregnenolone-^{3}H sulfate by submitochondrial fractions of bovine adrenocortical mitochondria. Each flask also contained S_2 (8 mg protein) and the cytochrome P-450 fraction (2 mg protein).

Table I

Recrystallization of Pregnenolone-^{3}H Sulfate

Recrystallization	Solvent	Specific Activity (dpm/mg) Crystals	Mother Liquors
After addition of carrier:		980	
1st	methanol/ diethyl ether	980	900
2nd	methanol/ethyl acetate	950	905
3rd	ethanol/water/ diethyl ether	985	940
4th	water/acetone/ ethyl acetate	960	930

Table II

The Conversion of Cholesterol-7α-^{3}H Sulfate to Pregnenolone-^{3}H Sulfate : Effect of Omission of Various Components of the Complete System

Additions	Pregnenolone-^{3}H Sulfate (dpm)
Complete system	30,000
Zero Time	<100
Heated Enzyme	<100
- TPNH	<200
- Cytochrome P-450	1,000
- S_2	3,000
Anaerobic	1,000

Table II shows that the cholesterol-^{3}H sulfate contained no pregnenolone-^{3}H sulfate (heated enzyme) and that conversion is time dependent (no conversion at zero time). Moreover omission (singly) of TPN^{+}, of S_2,

of oxygen or of cytochrome P-450 prevented conversion of cholesterol sulfate to pregnenolone sulfate (Table II).

The need for cytochrome P-450 in this conversion is further shown in Table III where it will be seen that conversion is inhibited by carbon monoxide and that this inhibition is reversed by light of wave-length 450 mμ (± 10 mμ) but not by light at 500 mμ(± 10 mμ).

Table III

The Influence of Carbon Monoxide on the Conversion of Cholesterol-^{3}H Sulfate to Pregnenolone-^{3}H Sulfate by a Submitochondrial System

Gas Mixture	Light	Pregnenolone-^{3}H Sulfate (dpm)
Air	White	120,000
Air	White	114,000
CO (5%)	Dark	36,000
CO (5%)	Dark	30,000
CO (5%)	450 mμ	60,000
CO (5%)	450 mμ	81,000
CO (5%)	500 mμ	24,000
CO (5%)	500 mμ	30,000

Cholesterol-^{3}H sulfate was incubated with a submitochondrial system from bovine adrenal cortex and pregnenolone-^{3}H sulfate was isolated and measured as described under Experimental Procedure. Incubation was performed under standard conditions in daylight or in black flasks or in the presence of monochromatic light as shown. CO (5%) refers to the following gas mixture : N_2 : 85; O_2 : 10 and CO : 5. Each flask contained S_2 (15 mg protein) and the cytochrome P-450 fraction (4 mg protein).

Discussion

These experiments confirm the observations of Raggatt and Whitehouse (1966) and those of Roberts *et al.* (1967) that submitochondrial fractions from bovine adrenal cortex are capable of converting cholesterol sulfate to pregnenolone sulfate. The present studies demonstrate that the rate of

conversion is linear for 15 or 20 minutes and thereafter declines. Further exploration of the enzyme systems involved should therefore make use of incubation times less than 15 minutes.

The most important observation to arise from these experiments is the demonstration that cytochrome P-450 is required for the side-chain cleavage of cholesterol sulfate. Simpson and Boyd (1967) showed that this compound was necessary for side-chain cleavage of free cholesterol. Inhibition of the cleavage reaction by carbon monoxide and the specific reversal by light of wave-length 450 mμ (Omura et al., 1965) demonstrates unequivocally the need for cytochrome P-450 in this system.

It also appears that a fraction (S_2) known to contain TPNH-cytochrome P-450 reductase and non-heme iron (Omura et al., 1966), is necessary for side-chain cleavage of cholesterol sulfate. Finally TPNH and molecular oxygen are also required for these reactions. These observations suggest that the conversion of cholesterol sulfate to pregnenolone sulfate may follow the same general mechanism as cleavage of the free steroid. The requirements for TPNH and oxygen suggest that one or more hydroxylation reactions are required. It may turn out that the same hydroxycholesterol intermediates (20α and 20α,22ξ) occur in the sulfate pathway. The question which arises from these considerations is whether or not the sulfate pathway uses the same enzymes as the free sterol or whether there is more than one enzyme system present in the submitochondrial fractions used here.

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