The Side-Chain Cleavage of Cholesterol and Cholesterol Sulfate by Enzymes from Bovine Adrenocortical Mitochondria*

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ABSTRACT: The requirements for the conversion of cholesterol into pregnenolone and of cholesterol sulfate into pregnenolone sulfate (called side-chain cleavage), by fractions from bovine adrenocortical mitochondria, have been examined and found to be the same for both substrates. The requirements are reduced triphosphopyridine nucleotide, a reduced triphosphopyridine nucleotide–cytochrome P-450 reductase, nonheme iron, cytochrome P-450, and molecular oxygen. It is likely that some additional factor(s) may be required. Optimal conditions for side-chain cleavage were obtained with both substrates and the rates of cleavage with the two substrates were compared using three solvents or suspending agents, namely, *N,N*-dimethylformamide, bovine serum albumin, and Tween-80. Values for apparent K_m and V_{max} with standard errors were calculated by least-squares fit. The apparent K_m for cholesterol sulfate (18 μM) is less than that for cholesterol (40–50 μM). Anomalous results were observed when cholesterol sulfate was suspended in Tween-80 and when cholesterol was added in *N,N*-dimethylformamide, probably as the result of inadequate saturation of the enzyme. Values for V_{max} with both substrates were of the same general order (1.5–5.9 mμ-moles/min per mg of protein). The sum of the cleavage of the two substrates incubated separately, approximately equals the total cleavage of both substrates incubated together. These observations suggest that under optimal conditions the two substrates are cleaved at about the same rate and that cholesterol and cholesterol sulfate are cleaved by separate enzymes.

An increasing body of evidence supports the view that certain steroid hormones are secreted by normal endocrine organs as sulfates (Baulieu, 1962; Roberts *et al.*, 1964; Wallace and Silberman, 1964) and that in some cases synthesis may proceed through sulfated intermediates (Calvin and Lieberman, 1964; Killinger and Solomon, 1965) presumably using the same enzymes as the free steroid intermediates, although the possibility of a second family of enzymes reserved for the sulfate pathway(s) cannot at present be excluded. The isolation of cholesterol sulfate from the adrenal cortex (Drayer *et al.*, 1964) and from plasma (Drayer and Lieberman, 1965), together with the discovery that the testis is capable of incorporating acetate-^{14}C into cholesterol-^{14}C sulfate (Young and Hall, 1968a), raised the possibility that steroid sulfates may arise from cholesterol sulfate—either cholesterol sulfate from plasma or that synthesized within the endocrine gland in question. This possibility rests, by analogy, upon the generally accepted view that unesterified steroids are derived from unesterified cholesterol (Samuels, 1961).

The first steps in steroid synthesis from cholesterol involve cleavage of the cholesterol side chain in the mitochondria of steroid-forming organs to give rise to pregnenolone (Halkerston *et al.*, 1961; Tamaoki and Pincus, 1961). One report suggested that mitochondrial preparations from bovine adrenal cortex may be capable of converting cholesterol sulfate into pregnenolone[1] sulfate (Raggatt and Whitehouse, 1966). A subsequent report showed that sonicate of bovine adrenocortical mitochondria is capable of making this conversion (Roberts *et al.*, 1967); the product, pregnenolone sulfate, was unequivocally identified and it was established that conversion takes place without cleavage of the sulfate ester bond (Roberts *et al.*, 1967).

The conversion of cholesterol into pregnenolone is a complex process which is known to require the following cofactors: TPNH, molecular oxygen, and a system of electron transport consisting of TPNH diaphorase, nonheme iron, and cytochrome P-450 (Simpson and Boyd, 1966, 1967; Bryson and Sweat, 1968). These electron carriers transport electrons from TPNH to molecular oxygen and the activated oxygen is believed to hydroxylate the side chain of cholesterol at the point of cleavage (Constantopoulos and Tchen, 1961; Shimizu *et al.*, 1962). It was decided to determine whether the conversion of cholesterol sulfate into pregnenolone sulfate requires the same cofactors and to compare the relative rates of side-chain cleavage of the two substrates (cholesterol and cholesterol sulfate) using the same enzyme preparation from bovine adrenal cortex. Some of the findings reported here have been published in preliminary form (Young and Hall, 1968b).

Experimental Procedure

Preparation of Mitochondrial Enzymes. Bovine adrenals were obtained from a slaughterhouse and medullae were removed from cortices. Adrenocortical mitochondria were prepared according to the method of Omura *et al.* (1966). For the preparation of a fraction containing cytochrome P-450, the method of the same authors was followed exactly (Omura *et al.*, 1966); this fraction, although not highly purified, will be referred to here as cytochrome P-450. It was

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[1] The following trivial name is used: pregnenolone, 3β-hydroxypregn-5-en-20-one.

found in preliminary studies that yields of nonheme iron obtained by this method were low and therefore a second batch of adrenals was used to prepare nonheme iron and TPNH–cytochrome P-450 reductase (a flavoprotein referred to here as TPNH diaphorase); these fractions were prepared by the method of Kimura and Suzuki (1967). The following represent approximate yields: 120 adrenals gave 600 g of adrenal cortex from which 400 mg of protein of the cytochrome P-450 fraction was obtained. The same weight of adrenal cortex gave 20 g of acetone-dried powder from which 30 mg of protein of nonheme iron of specific activity 200 mμmoles of cytochrome C reduced per min mg of protein (see below) and 70 mg of protein of the diaphorase of specific activity 100 mμmoles of phenol–indo-2,6-dichlorophenol reduced per min mg of protein (see below). Nonheme iron was lyophilized and stored at −10° without significant loss of activity for at least 2 months. Diaphorase and cytochrome P-450 were frozen and stored at −10° without loss of activity for at least 2 months.

Incubation was performed in air in erlenmeyer flasks (25 ml) to which the following substances were added: sodium chloride (144 μmoles), potassium chloride (100 μmoles), magnesium chloride (10 μmoles), glycylglycine buffer (pH 7.4; 23 μmoles), bovine serum albumin (purified by the method of Chen (1967) to remove fatty acids) (20 mg), TPN^+ (1.45 μmoles), glucose 6-phosphate (2.72 μmoles), and glucose 6-phosphate dehydrogenase (0.5 Kornberg unit). The three enzymes were added separately (cytochrome P-450, nonheme iron, and diaphorase–a combination referred to hereafter as the reconstituted side-chain cleavage system). The final volume was made to 2.3 ml with 0.25 M sucrose and the final pH was 7.4. The substrates cholesterol-7α-^{3}H and cholesterol-7α-^{3}H sulfate were suspended or dissolved in one of the following compounds: *N,N*-dimethylformamide, Tween-80 (Hall, 1966), or bovine serum albumin (purified by the method of Chen). The order of additions to the flasks was as follows: salts, buffer, albumin, sucrose, enzymes, substrate; finally the reaction was started by addition of a mixture containing glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and TPN^+.

Incubation was performed for the times shown with constant agitation at 37° and was stopped by placing the flasks in ice and immediately adding methanol when the substrate was cholesterol-^{3}H sulfate or methylene chloride when the substrate was cholesterol-^{3}H.

The protein content of the enzyme fractions was determined by the method of Lowry *et al.* (1951).

Isolation and Measurement of Pregnenolone-^{3}H Sulfate. To the incubation medium with 10 ml of methanol to stop the reaction, 500 μg of authentic pregnenolone sulfate was added as carrier and the mixture was poured through Celite (5 g). The Celite was washed with methanol (100 ml) and the filtrate was taken to dryness. The sample was then applied to two successive Celite columns. Column A was prepared from 7.5 g of Celite and developed with isooctane–ethyl acetate–*t*-butyl alcohol–methanol–ammonium hydroxide (1 M) (4:1:2:2:3, v/v) (Roberts *et al.*, 1967). Cholesterol sulfate was eluted in holdback volumes 3–5 and pregnenolone sulfate was eluted in methanol (75 ml). The methanol fraction was dried and applied to column B, made of 10 g of Celite, and developed with isooctane–*t*-butyl alcohol–ammonium hydroxide (1 M) (3:5:5, v/v) (Calvin *et al.*, 1963). Pregnenolone sulfate was eluted in holdback volumes 5–8. In column B the eluate was collected in fractions equal in volume to one-third of the holdback volume. Steroid sulfates were localized by means of the Vlitos reaction (Roy, 1956). The tritium content of pregnenolone-^{3}H sulfate was measured by liquid scintillation spectrometry performed upon aliquots (one-half) of the fractions in which this substance was eluted from column B. Recovery of pregnenolone sulfate through this procedure was measured by means of the Vlitos reaction performed upon aliquots (one-fifth) of the same eluate (Roy, 1956). Recoveries through this procedure were 60–80% based upon 50 determinations.

Isolation and Measurement of Pregnenolone-^{3}H. The methods for the isolation and measurement of pregnenolone-^{3}H and for the determination of recovery in each sample have been presented in detail elsewhere (Hall, 1967a).

Isolation of Cholesterol. Cholesterol was isolated preparatory to gas chromatography by extraction with methylene chloride, by paper chromatography in ligroin–propylene glycol, and finally by thin-layer chromatography (Simpson and Boyd, 1966).

Determination of Radiochemical Purity. PREGNENOLONE-^{3}H SULFATE. In order to demonstrate the identity and radiochemical purity of pregnenolone-^{3}H sulfate three procedures were used. Certain samples isolated following incubation of cholesterol-^{3}H sulfate with the reconstituted side-chain cleavage system were recrystallized after addition of authentic carrier (48 μmoles). The relevant methods are reported elsewhere (Young and Hall, 1968a,b). Other samples of pregnenolone-^{3}H sulfate were cleaved either by solvolysis (Burstein and Lieberman, 1958) or by means of a commercial steroid sulfatase. Pregnenolone-^{3}H was either recrystallized after addition of carrier pregnenolone or subjected to countercurrent distribution in the system cyclohexane–ethyl acetate (80:20, v/v)–ethanol–water (60:40, v/v), including statistical evaluation of the distribution of mass and radioactivity according to the method of Baggett and Engel (1957). The methods have been reported elsewhere (Hall, 1966; Hall and Koritz, 1965).

PREGNENOLONE-^{3}H. The method of recrystallizing pregnenolone-^{3}H after addition of carrier and of determining specific activities have been published (Hall, 1966, 1967a; Hall and Koritz, 1965).

Incubation with Carbon Monoxide and Monochromatic Light. Monochromatic light was generated with a xenon lamp (1600 W), collimated, focused, and passed through a monochromatic filter using an apparatus described by Inglis and Lennox (1965) and resembling very closely the system described by Omura *et al.* (1965) in similar studies with adrenal microsomal hydroxylations. Incubation was performed in Warburg flasks with cholesterol-^{3}H sulfate in the side arm. The reaction was started by tipping the contents of the side arms into the flasks. Where appropriate flasks were darkened by means of aluminum foil. The gas phase (combinations of O_2, N_2, and CO) was bubbled through the contents of the flasks for 2 min before incubation was commenced (Young and Hall, 1968b).

Measurement of Electron Transport. The activity of TPNH diaphorase was measured by the reduction of phenol–indo-2,6-dichlorophenol in the presence of TPNH. The method of Omura *et al.* (1966) was used with the single modification that the concentration of the dye was 0.016 mg/ml as a final concentration in the cuvet in our studies. The activity of non-

TABLE I: Recrystallization of Pregnenolone-^{3}H Sulfate and Pregnenolone-^{3}H.[a]

Pregnenolone-^{3}H Sulfate				Pregnenolone-^{3}H			
		Sp Act. (dpm/μmole)				Sp Act. (dpm/μmole)	
Recrystzn	Solvent	Mother Liquors	Crystals	Recrystzn	Solvent	Mother Liquors	Crystals
After addition of carrier			4460	After addition of carrier			1450
First	Methanol–diethyl ether	4620	4500	First	Chloroform–hexane	1520	1490
Second	Methanol–ethyl acetate	4550	4580	Second	Ethyl acetate–hexane	1440	1400
Third	Ethanol–water–diethyl ether	4580	4530	Third	Benzene–heptane	1460	1420
Fourth	Aqueous acetone	4550	4460	Fourth	Toluene–hexane	1410	1470

[a] A sample of pregnenolone-^{3}H sulfate was isolated following incubation of the reconstituted side-chain cleavage system for 10 min with cholesterol-^{3}H sulfate as described in the text. Extracts were pooled to provide approximately 220,000 dpm and 48 μmoles of authentic carrier pregnenolone sulfate was added. The mixture was recrystallized from the solvents shown and the specific activities of crystals and mother liquors were determined after each crystallization. The sample of pregnenolone-^{3}H was prepared by solvolysis of a second sample of pregnenolone-^{3}H sulfate representing approximately 18,500 dpm pooled from extracts following incubation as before. The specific activity of the pregnenolone-^{3}H sulfate was 75,000 dpm/μmole and of the pregnenolone-^{3}H following solvolysis was 74,000 dpm/μmole. The pregnenolone-^{3}H was purified by paper chromatography (Hall, 1967a). After addition of 50 μmoles of authentic pregnenolone the mixture was recrystallized from the solvent systems shown. In each case specific activities were measured as described under Experimental Procedure.

heme iron was measured by the reduction of cytochrome C in the presence of TPNH and TPNH diaphorase exactly as described by the same authors (Omura *et al.*, 1966). The aliquot of diaphorase in each cuvet was adjusted to give the equivalent of 3.25 mμmoles of phenol-indo-2,6-dichlorophenol reduced per min.

Liquid Scintillation Spectrometry. The relevant methods have been published previously. Efficiency for counting tritium was 30–40%. Values in the text expressed as dpm refer to readings which have been corrected to 100% efficiency and corrected for losses during isolation of the compound in question. In the accompanying experiments the expression no conversion refers to conditions under which less than 100 dpm of product (pregnenolone-^{3}H sulfate or pregnenolone-^{3}H) was found.

Gas–Liquid Partition Chromatography. Cholesterol and pregnenolone were measured by gas–liquid partition chromatography on SE-30 by methods described previously (Hall, 1963). Aliquots of samples were mixed with a known concentration of progesterone and applied to the column in the solid phase on stainless steel beads from which the organic solvent in which the sample and standard were applied had been evaporated. The amount of steroid was calculated from the areas under the peaks and duplicate determinations agreed within 5%. For the measurement of pregnenolone column temperature was 220° and the retention time of this steroid was 6.5 min. For cholesterol the retention time was 18 min at 220°. The minimal amount of steroid which could be detected by the methods used was 0.01 μg in the whole extract. Values for both steroids were corrected for losses during isolation on the basis of the recovery of known amounts of cholesterol-^{3}H or pregnenolone-^{3}H (approximately 100,000 dpm) added to the samples before extraction. Values for recovery of both steroids were between 35 and 42%.

Chemicals. Cholesterol sulfate was prepared, purified, and identified by methods already published. Cholesterol-^{3}H sulfate was prepared from cholesterol-^{3}H purchased from Nuclear-Chicago (lot no. 321-168; 25 mCi/μmoles) and purified before use by paper chromatography (Hall and Koritz, 1964). Sulfation was performed as for unlabeled cholesterol (Young and Hall, 1968a). The pyridinium salt resulting from this procedure was dissolved in 17 ml of benzene–chloroform (1:1, v/v) and applied to a column of aluminum oxide (7 g). The column was developed with the following solvents: chloroform (10 ml), chloroform-methanol (3:5, v/v, 10 ml), and methanol (10 ml) followed by 1 M ammonium hydroxide–methanol (1:2, v/v, 25 ml). The last fraction contained cholesterol-^{3}H sulfate contaminated by approximately 2% of the total amount of cholesterol-^{3}H remaining unconjugated after sulfation. This procedure of chromatography on aluminum oxide was repeated exactly as before with fresh aluminum oxide to remove the remaining cholesterol-^{3}H. The eluate containing cholesterol-^{3}H sulfate was taken to dryness and purified by column chromatography on Celite using column A followed by column B (see above). In column A cholesterol-^{3}H sulfate was eluted in holdback volumes 4 and 5 and in column B in holdback volumes 2 and 3.

The radiochemical purity of the cholesterol-^{3}H sulfate was demonstrated by recrystallization of a sample (approximately 16,000 dpm) after addition of carrier cholesterol sulfate (31 μmoles) from four different solvent systems. The initial specific activity was 520 dpm/μmole and at the end of four recrystallizations it was 535 dpm/μmole. The specific activities of all samples of crystals and mother liquors fell within 5%

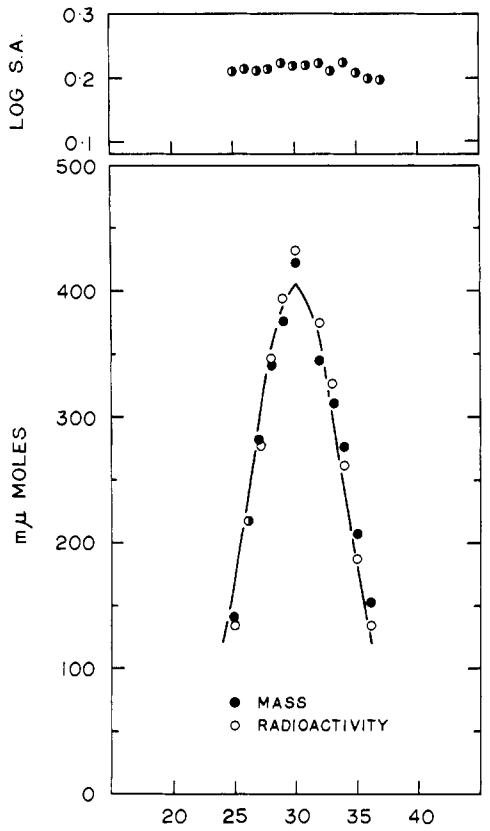


FIGURE 1: Countercurrent distribution (59 transfers) of pregnenolone-^{3}H in the system cyclohexane–ethyl acetate (80:20, v/v)–ethanol–water (60:40, v/v). Samples of pregnenolone-^{3}H sulfate isolated following incubation of the reconstituted side-chain cleavage system with cholesterol-^{3}H sulfate were pooled and the specific activity was determined. The pooled sample was cleaved by solvolysis and the pregnenolone-^{3}H resulting from cleavage was purified by paper chromatography and thin-layer chromatography (Hall, 1967a). After addition of pregnenolone (4.1 μmoles) the sample was subjected to countercurrent distribution and aliquots of solvents from tubes in which pregnenolone was expected to occur were analyzed for mass and radioactivity by published methods (Hall, 1966, 1967a,b). The method of statistical analysis of the data in order to compare the distribution of mass and radioactivity follows the procedure of Baggett and Engel (1957) who also described the rationale for plotting log 10 specific activities. Values on the ordinate are mass (millimicromoles) and radioactivity divided by mean specific activity (also expressed in millimicromoles). Mean specific activity was calculated from the specific activities of tubes in the peak region. The normal distribution curve was constructed with the following parameters: $K = 1.03$ and standard deviation $= \pm 3.83$. Log specific activity of pregnenolone-^{3}H (disintegrations per minute per millimicromoles) is plotted above the distribution curve.

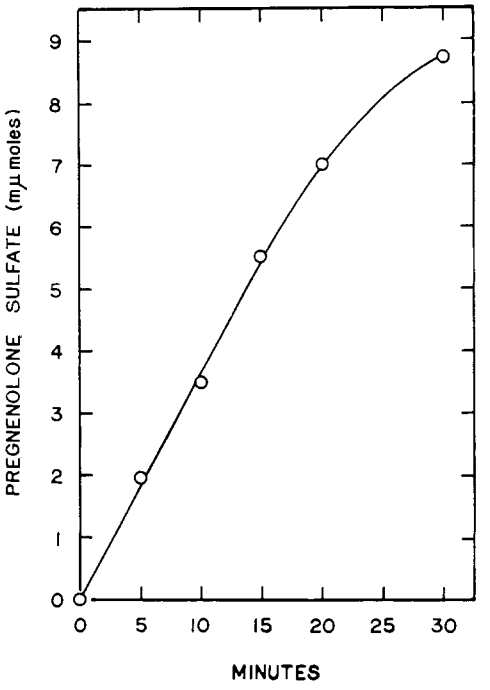


FIGURE 2: The influence of time on the conversion of cholesterol-7α-^{3}H sulfate into pregnenolone-^{3}H sulfate by the reconstituted enzyme system. Cholesterol-7α-H sulfate (3×10^6 dpm; 21 mμmoles/flask) was dissolved in *N,N*-dimethylformamide (0.2 ml) and incubated with a reconstituted enzyme system for the times shown. Previous experience has shown that the reconstituted system used in the experiment was optimal for cleavage of cholesterol-^{3}H sulfate. Pregnenolone-^{3}H sulfate was extracted and measured by the procedures described in the Experimental Section of this paper.

of each other. Specific activities were measured as described elsewhere (Young and Hall, 1968a).

Pregnenolone-7α-^{3}H was obtained from The Radiochemical Center, Amersham, England (TRA 157 Batch 3; 0.45 mCi/μmole). This compound was purified as described elsewhere (Hall *et al.*, 1963). Celite 545 was obtained from Johns Manville and was purified and prepared for column chromatography by the method of Gurpide *et al.* (1962). Neutral aluminum oxide was purchased from Merck (Darmstadt, Germany). Phenol–indo-2,6-dichlorophenol was from British Drug Houses Limited (Poole, England) and cytochrome C (lot no. 15B-7000) from Sigma Chemical Co. Steroid sulfatase was obtained from Calbiochem (lot no. 109422).

All solvents were redistilled from the best grades commercially available as follows: pyridine, *N,N*-dimethylformamide, and isooctane as analytical reagent grades from By-products and Chemicals Pty. Ltd. (Auburn, N. S. W., Australia), ethyl acetate and *t*-butyl alcohol as analytical reagent grades from Ajax Chemicals Ltd. (Victoria, Australia). Tween-80 was obtained from Atlas Powder Co.

Results

Identity of Reaction Products. The substance isolated following incubation of cholesterol-^{3}H sulfate with the reconstituted cleavage system was identified as pregnenolone-^{3}H sulfate by the following criteria: (i) recrystallization with carrier pregnenolone sulfate from four different solvent systems showed constant specific activities of crystals and mother liquors throughout this procedure within the limits of experimental error (Table I). (ii) Material extracted after solvolysis and after cleavage by sulfatase behaved like pregnenolone-^{3}H according to two criteria: recrystallization with authentic pregnenolone and countercurrent distribution in which mass and tritium showed the same distribution according to the analysis described by Baggett and Engel (1957) ($p < 0.01$; Figure 1).

The radiochemical purity of samples of pregnenolone-

TABLE II: Reconstitution of Side-Chain Cleavage System from Fractions of Bovine Adrenocortical Mitochondria.[a]

Additions	Pregnenolone-^{3}H (dpm)	Pregnenolone-^{3}H Sulfate (dpm)
Complete	8,200	480,000
−TPN$^+$	200	<100
−O_2	<100	<100
−Diaphorase	<100	<100
−Nonheme iron	300	600
−Cytochrome P-450	200	500
Complete		
Heated enzyme	<100	<100
Zero time	<100	<100

[a] The complete system is described under Experimental Procedure. Buffer, TPN$^+$ (1.45 μmoles), glucose 6-phosphate (2.72 μmoles), and glucose 6-phosphate dehydrogenase (0.5 Kornberg unit) were added with a buffered salt solution and either cholesterol (2 μCi; 5 mμmoles/flask) or cholesterol-^{3}H sulfate (1.5 μCi; 26 mμmoles/flask). Incubation was started by addition of the TPNH and following incubation pregnenolone-^{3}H or pregnenolone-^{3}H sulfate was isolated and measured as described under Experimental Procedure.

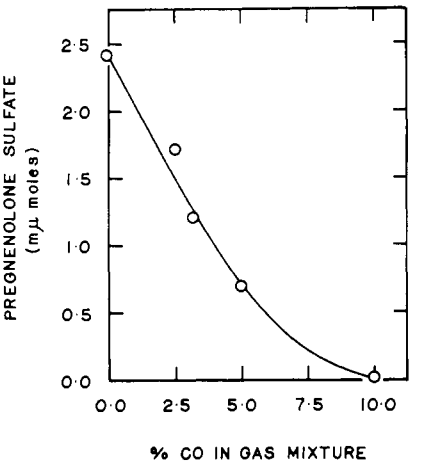


FIGURE 3: The influence of carbon monoxide upon the side-chain cleavage of cholesterol-^{3}H sulfate. Cholesterol-7α-^{3}H sulfate (3×10^6 dpm; 15 mμmoles/flask) was incubated for 10 min with the reconstituted side-chain cleavage system described under Figure 2 and the additions described in Experimental Procedure. Pregnenolone-^{3}H sulfate was isolated and measured. Incubation was performed in Warburg flasks after a preliminary period of 2 min during which the gas phase was bubbled through the contents of each flask. Incubation was started by pouring the substrate from the side arms of the flasks. The gas phase contained a fixed percentage of oxygen (10%) in all flasks; carbon monoxide in the concentrations shown and nitrogen to 100%.

^{3}H formed from cholesterol-^{3}H was similarly demonstrated by recrystallization and countercurrent but because such data have been presented on a number of previous occasions (Hall, 1967a,b; Koritz and Hall, 1964) the results are not given in detail here.

Cofactor Requirements for Side-Chain Cleavage of Cholesterol Sulfate. When cholesterol-7α-^{3}H sulfate was incubated with the complete system, salts, buffer, TPN$^+$ with a TPNH-generating system, TPNH diaphorase, nonheme iron, cytochrome P-450, bovine serum albumin, and molecular oxygen, conversion into pregnenolone sulfate was linear for 20 min and thereafter began to decline (Figure 2). Subsequent incubations were performed routinely for 10 min.

Table II shows that TPNH and oxygen are necessary for the conversion of cholesterol-^{3}H sulfate into pregnenolone-^{3}H sulfate. Moreover the three components of the reconstituted enzyme system (diaphorase, nonheme iron, and cytochrome P-450) are all essential for activity. The reaction measured is time dependent since no conversion is observed at zero time and the side-chain cleavage activity is destroyed by heating the reconstituted system at 60° for 2 min. Table II also demonstrates that conversion of cholesterol-^{3}H into pregnenolone-^{3}H shows the same requirements as those shown by the sulfates. Since bovine serum albumin was observed to stimulate side-chain cleavage with both substrates in preliminary experiments, this protein was added in the present experiments.

Side-chain cleavage of cholesterol-^{3}H sulfate by the reconstituted system is inhibited by carbon monoxide (Figure 3) and inhibition is approximately linear with respect to the concentration of carbon monoxide when the proportion of oxygen in the gas mixture remains constant.

Influence of the Composition of the Reconstituted System upon Side-Chain Cleavage of Cholesterol Sulfate and Cholesterol. On the basis of a series of preliminary experiments, the relative proportions of the three enzymes in the reconstituted system was varied in order to determine the influence of such variations upon side-chain cleavage (Figure 4). With diaphorase (4 mg of protein/flask) and cytochrome P-450 (2 mg of protein/flask; selected on the basis of previous experience), the amount of nonheme iron was varied between 0 and 1 mg of protein per flask (Figure 4A). It will be seen that maximal conversion was obtained with 0.5 mg of protein/flask. With cytochrome P-450 (2 mg of protein/flask) and nonheme iron (0.5 mg of protein/flask), the concentration of diaphorase was increased between the limits of 0 and 4 mg of protein per flask (Figure 4B). Although a clear plateau was not obtained, the rate of conversion declined with addition of more diaphorase than 2.5 mg of protein/flask. Figure 4A,B further confirms that both diaphorase and nonheme iron are necessary for side-chain cleavage (zero additions giving no cleavage in each case) and that the rate of cleavage is linear with respect to enzyme concentration within the limits defined. The amount of cytochrome P-450 was then varied through the range of 0–2.0 mg of protein/flask (Figure 4C). A clear plateau was reached at 0.7 mg of protein/flask. Thereafter for the accompanying kinetic studies the mixture of diaphorase (2.5 mg of protein/flask), nonheme iron (0.5 mg of protein/flask), and cytochrome P-450 (0.7 mg of protein/flask) was used routinely. In studies not shown here it was observed that TPN$^+$ (1.45 μmoles), glucose 6-phosphate (2.72 μmoles), and glucose 6-phosphate dehydrogenase (0.5 Kornberg unit) provided saturating levels of TPNH for at least 20 min in the side-chain cleavage system since subsequent additions of this mixture during incubation did not increase the rate of side-chain cleav-

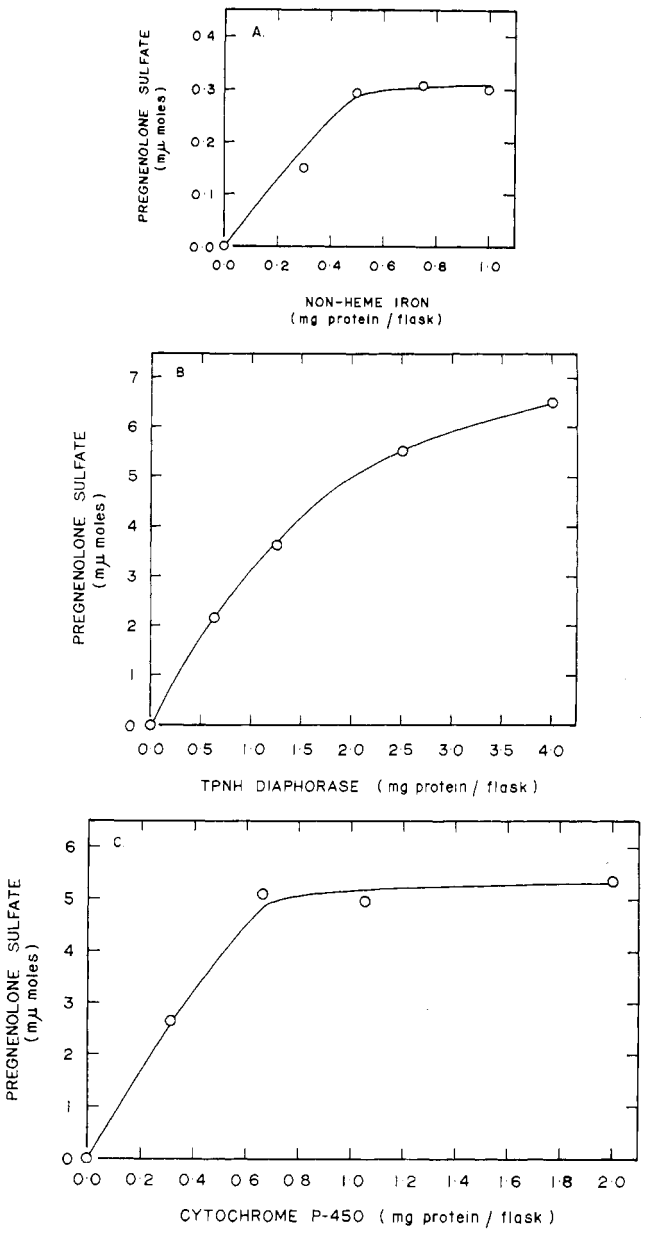


FIGURE 4: The composition of the reconstituted side-chain cleavage system in relation to the extent of conversion of cholesterol-7α-^{3}H sulfate into pregnenolone-^{3}H sulfate. In part A the reconstituted system consisted of diaphorase (4 mg of protein); cytochrome P-450 (2 mg of protein) and the amounts of nonheme iron shown, together with those additions required to make the complete system (Experimental Procedure). In part B the reconstituted system consisted of cytochrome P-450 (2 mg of protein), nonheme iron (0.5 mg of protein), and the amounts of diaphorase shown. In part C the reconstituted system consisted of diaphorase (2.5 mg of protein), nonheme iron (0.5 mg of protein), and the amounts of cytochrome P-450 shown. In each case pregnenolone-^{3}H sulfate was isolated and measured as described in Experimental Procedure. The cytochrome P-450 used in part A was a different preparation from that used in parts B and C.

age. This system was then used in the accompanying kinetic studies. Finally, similar studies showed that the above conditions were optimal with cholesterol-7α-^{3}H as substrate in that further additions of any of the various components of the system did not increase side-chain cleavage of cholesterol-^{3}H.

TABLE III: Conversion of Endogenous Cholesterol into Pregnenolone by Reconstituted Enzyme System from Adrenocortical Mitochondria.[a]

Duration of Incubn (min)	Cholesterol (mμmoles)	Pregnenolone (mμmoles)
0	23.2	0.1
5	21.3	2.4
10	17.2	6.1
15	9.4	13.1
30	7.6	17.4

[a] Samples of the enzyme system composed of diaphorase (2 mg of protein), nonheme iron (1 mg of protein), and cytochrome P-450 (0.5 mg of protein) together with the additions described above were incubated for the times shown. Following incubation cholesterol and pregnenolone were isolated from each sample and measured by gas chromatography (Experimental Procedure). Values expressed in millimicromoles have been corrected for losses during recovery.

Characteristics of the Reconstituted Enzyme System. (i) In eight experiments the reconstituted system was incubated with pregnenolone-7α-^{3}H (4×10^7 dpm; 19 mμmoles/flask); no pregnenolone-^{3}H sulfate (<100 dpm/flask) was found. Similarly the system failed to convert cholesterol-^{3}H into cholesterol-^{3}H sulfate. (ii) In a number of studies using a less purified enzyme system than that used for the studies described above, it was found that pregnenolone-^{3}H sulfate was not converted into pregnenolone-^{3}H and cholesterol-^{3}H sulfate was not converted into cholesterol-^{3}H. (iii) The reconstituted system is free of esterase activity and cholesterol esters constitute <10% of total endogenous cholesterol. (iv) The less purified system when incubated with pregnenolone-^{3}H sulfate failed to form cholesterol-^{3}H sulfate and on incubation with pregnenolone-^{3}H failed to form cholesterol-^{3}H. These observations support the claim (Roberts *et al.*, 1967) that cholesterol sulfate is converted into pregnenolone sulfate in the present system without cleavage of the ester bond, since neither sulfatase nor sulfokinase appear to be present. (v) Samples of the reconstituted system and the various additions described above were incubated without exogenous substrate for various times and following incubation cholesterol and pregnenolone were extracted and purified by paper and thin-layer chromatography. The purified extracts were examined by gas chromatography on SE-30 (Table III). It will be seen that the rate of decrease in levels of endogenous cholesterol is approximately linear and approximately equal to the production of pregnenolone with time. It is clear that 1 mole of cholesterol gives 1 mole of pregnenolone, that most of the endogenous cholesterol is accessible to the side-chain cleavage system, that no significant accumulation of biosynthetic intermediates occurs and that no other metabolic pathway is available to endogenous cholesterol in the reconstituted enzyme system.

Electron Transport by Submitochondrial Fractions. TPNH DIAPHORASE. Reduction of phenol–indo-2,6-dichlorophenol by TPNH diaphorase was measured by the method described above and the rate of reduction was linear with respect to

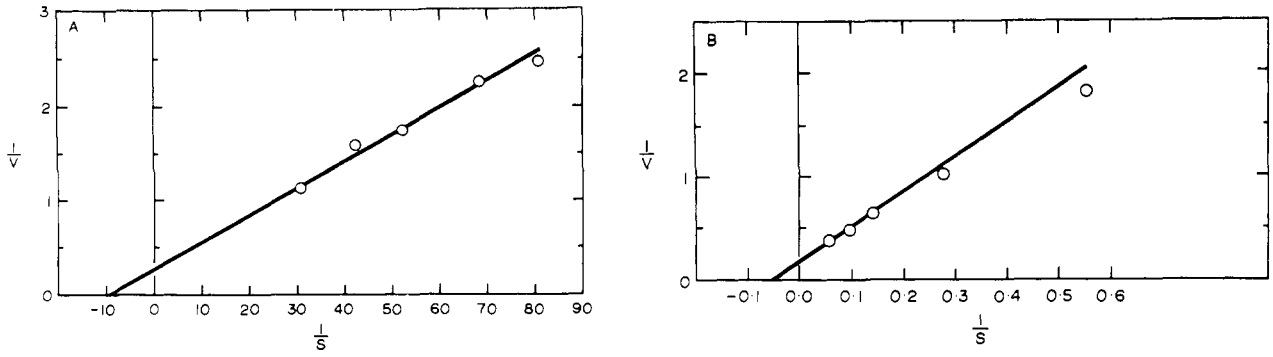


FIGURE 5: Kinetics of side-chain cleavage of cholesterol and cholesterol sulfate. The reconstituted enzyme system described above as that for routine use, together with the additions given under Experimental Procedure, was incubated with varying concentrations of cholesterol-7α-^{3}H (specific activity 4 × 10^4 dpm/mμmole) in part A and of cholesterol-7α-^{3}H sulfate (specific activity 2 × 10^5 dpm/mμmole) in part B. Pregnenolone-^{3}H and pregnenolone-^{3}H sulfate were isolated and measured as described above. The substrates were dissolved in *N,N*-dimethylformamide. Values for $1/V$ were calculated after correction for losses during recovery and for the efficiency of counting tritium (see Experimental Procedure). The method of calculating $1/V$ in the case of cholesterol involves allowance for endogenous cholesterol (see Results).

TABLE IV: Apparent Kinetic Constants for Side-Chain Cleavage of Cholesterol and Cholesterol Sulfate in Various Solvents.[a]

	Cholesterol			Cholesterol Sulfate	
Solvent	K_m (μM)	V_{max} (mμmoles/min mg of protein)	Solvent	K_m (μM)	V_{max} (mμmoles/min mg of protein)
Dimethylformamide	103.9 ± 52.6	3.6 ± 1.5	Dimethylformamide	18.3 ± 2.3	5.6 ± 0.4
Bovine serum albumin	44.2 ± 6.2	1.9 ± 0.25	Bovine serum albumin	18.6 ± 1.4	3.5 ± 0.2
Tween-80	46.7 ± 6.8	2.1 ± 0.2	Tween-80	38.8 ± 5.6	5.9 ± 0.6

[a] Incubation was performed with the reconstituted side-chain cleavage system (see Experimental Procedure) in the presence of excess TPN^+ and a reducing system. The substrates were suspended or dissolved in the solvents shown in separate flasks and the production of pregnenolone from cholesterol and of pregnenolone sulfate from cholesterol sulfate was measured as described under Experimental Procedure. A least-squares fit of the experimental data to the initial velocity equation $v = VS/K_m + S$ was made to obtain values for V and K_m together with values for the standard errors. Standard errors are shown with the two kinetic constants. One enzyme preparation was used in the experiments with cholesterol sulfate and with cholesterol suspended in *N,N*-dimethylformamide; the remaining two experiments with cholesterol employed a second enzyme preparation.

concentration of diaphorase. Diaphorase activity was also measured by reduction of cytochrome C with nonheme iron. For a fixed concentration of nonheme iron, reduction of cytochrome C was also linear with respect to concentration of diaphorase. The curves and the rates of reduction of the two compounds were similar to those reported by Omura *et al.* (1966) and for that reason are not reported here. The specific activity of the diaphorase used in studies of side-chain cleavage was 100 mμmoles of phenol–indo-2,6-dichlorophenol reduced per min mg of diaphorase protein.

NONHEME IRON. Reduction of cytochrome C by nonheme iron was measured as described above. Again for a fixed concentration of diaphorase, the rate of reduction of cytochrome C was linear with respect to concentration of nonheme iron protein. Moreover curves and rates of reduction followed values reported by Omura *et al.* (1966) very closely. The specific activity of the nonheme iron used in studies of side-chain cleavage was 200 mμmoles of cytochrome C reduced per min mg of nonheme iron protein.

Kinetics of Side-Chain Cleavage. Figure 5 shows double-reciprocal plots for the rate of side-chain cleavage of cholesterol and cholesterol sulfate when *N,N*-dimethylformamide was used to dissolve the substrates. The conditions used measure initial velocity with both substrates and values have been corrected for losses during recovery. Since no cholesterol sulfate was detected in the enzyme system used, no correction is required for endogenous substrate with the sulfate. However, the preparation contained 23.3 mμmoles of cholesterol and this amount of unlabeled substrate was considered to be available to the enzyme system (see characteristics of the system above). Substrate concentration was calculated as the amount of exogenous cholesterol-7α-^{3}H added plus 23.3 mμmoles and the amount of product formed was calculated from the tritium content of pregnenolone-^{3}H, assuming that 1 mole of cholesterol gives 1 mole of pregnenolone and that the reaction is irreversible.

The values for apparent K_m and V_{max} with the two substrates using three suspending agents or solvents are presented in Table IV. It will be seen that values for apparent K_m for cholesterol are considerably higher than those for cholesterol sul-

TABLE V: Side-Chain Cleavage of Cholesterol and Cholesterol Sulfate Separately and Together.[a]

Substrate (mμmoles)	Product (mμmoles/min mg of Protein)			
	Pregnenolone-^{3}H	Pregnenolone-^{3}H Sulfate	Mean and Range	Sum
Cholesterol-^{3}H	6.3		7.0	
	7.7		±0.7	
Cholesterol-^{3}H sulfate		14.1	15.0	
		16.0	±1.0	22.0 ± 1.7
Cholesterol-^{3}H +	8.1		9.4	
	10.7		±1.3	
cholesterol-^{3}H sulfate		11.2	10.2	
		9.2	±1.0	19.6 ± 2.3

[a] Duplicate determinations of side-chain cleavage were made for cholesterol, for cholesterol sulfate separately, and for both substrates together. The conditions used were the same as those given in Table II for the complete system.

fate. The value for cholesterol in *N,N*-dimethylformamide shows a large standard error probably resulting from inadequate saturation of the enzyme as revealed by the slope seen in double-reciprocal plots. The agreement between the other two values for this substrate suggests that the true value is at the lower level of this wide range, *i.e.*, 40–50 μM. The value for cholesterol sulfate in Tween-80 appears to be anomalous for the same reason, so that the true value is probably approximately 18 μM. On the other hand values for V_{max} are approximately the same for both substrates.

One and the same enzyme preparation was used for the kinetic studies of Table IV, with cholesterol sulfate in three suspending agents and for cholesterol with *N,N*-dimethylformamide. The remaining two studies with cholesterol employed a second preparation.

V_{max} for Two Substrates Separately and Together. Table V shows the results of an experiment in which the two substrates were added in concentrations that greatly exceeded K_m for each substrate. When incubated separately the sum of the cleavage of the substrates was 22.0 ± 1.7 mμmoles and when incubated together the total cleavage was 19.6 ± 2.3 mμmoles.

Cholesterol Sulfate in Mitochondrial System. In order to measure the levels of endogenous cholesterol sulfate in an unpurified mitochondrial enzyme system, acetone powder from 50 g of adrenocortical tissue was homogenized in phosphate buffer (0.07 M, pH 6.8) and centrifuged at 100,000*g* for 60 min. Cholesterol-^{3}H sulfate (10^4 dpm, 0.3 mμmole) was added to the supernatant fraction. This fraction was then treated as described by Drayer *et al.* (1964) in order to isolate cholesterol sulfate. The final fraction from the third Celite column (system C of Drayer *et al.* (1964)) was subjected to solvolysis (Burstein and Lieberman, 1958) and extracted into ether. One half of the final extract was examined by gas chromatography and no cholesterol (*i.e.*, <0.1 mμmole) was found. It is concluded that the amount of reconstituted enzyme added to each flask in the present studies (corresponding to approximately 20 g of adrenocortical tissue) did not contain significant amounts of endogenous cholesterol sulfate, since the reconstituted system is considerably more purified than the extract of acetone powder used here.

Side-Chain Cleavage of Cholesterol in the Presence of Cholesterol Sulfate. Table VI shows that cholesterol sulfate does not inhibit the side-chain cleavage of cholesterol, whether the concentration of substrate is low (0.1 μmole/flask, *i.e.*, approximately K_m) or high (2.6 μmoles/flask, *i.e.*, >25 × K_m).

Discussion

The present experiments demo nstrate that an enzyme system reconstituted from fractions isolated from bovine adrenocortical mitochondria, converts cholesterol into pregnenolone and cholesterol sulfate into pregnenolone sulfate without cleavage of the ester bond. These observations are consistent with previous reports showing that various preparations derived from adrenocortical mitochondria are capable of cleaving the side chain of cholesterol (Halkerston *et al.*, 1961; Tamaoki and Pincus, 1961; Raggatt and Whitehouse, 1966; Roberts *et al.*, 1967; Hall, 1967a,b; Simpson and Boyd, 1966, 1967; Bryson and Sweat, 1968). The components of the reconstituted system used here include a TPNH diaphorase and nonheme iron which are together capable of transporting electrons from TPNH to cytochrome C but which alone can neither reduce this cytochrome nor support cleavage of either substrate (Table II). The yellow diaphorase has been shown to reduce phenol–indo-2,6-dichlorophenol in the presence of TPNH and the nonheme iron has been identified by spectroscopy (Kimura and Suzuki, 1967). In addition to these factors, a fraction containing cytochrome P-450 is necessary for the cleavage reactions. The conclusion that the functional component of this fraction is cytochrome P-450 rests on the following observations. Side-chain cleavage requiring this fraction is inhibited by carbon monoxide; inhibition increasing with increasing concentration of this gas (Figure 3) and a previous report (Young and Hall, 1968b) demonstrated that inhibition by carbon monoxide is specifically reversed by exposure to light of 450-mμ wavelength. A preliminary report from another laboratory demonstrated inhibition by carbon monoxide with cholesterol as substrate (Simpson and Boyd, 1967). Moreover Omura *et al.* (1966) have demonstrated that diaphorase and nonheme iron in the presence of TPNH, reduce cytochrome P-450 prepared by the method used in these

studies. Evidently the three components mentioned above, together with TPNH and molecular oxygen, are required for side-chain cleavage of either substrate. The fact that the same requirements have been demonstrated for 11β and 18 hydroxylations in adrenocortical mitochondria (Omura *et al.*, 1966; Greengard *et al.*, 1967) supports the current hypothesis that the conversion of cholesterol into pregnenolone involves hydroxylation of the substrate. The fact that both substrates (free and sulfate) show the same requirements suggests that the mechanism of cleavage may be the same in both cases. Since side-chain cleavage is the rate-limiting step in steroid biosynthesis (Halkerston *et al.*, 1961; Hall, 1963; Koritz and Hall, 1964) and since the relative importance of biosynthetic pathways involving free and sulfated steroids remains uncertain, the kinetics of the side-chain cleavage of the two substrates becomes of considerable interest.

It would appear that values for apparent K_m are much higher for cholesterol than for the sulfate. In making these calculations it was assumed that all of the endogenous cholesterol in the preparation was available as substrate for side-chain cleavage, although approximately 31% remained after 20-min incubation (Table III). If all of this residual cholesterol was bound in some unavailable (membrane) form, values for K_m with cholesterol would be lower than with those shown in Table IV but still considerably higher than for the sulfate. In view of the large standard error in the value for apparent K_m of cholesterol in *N,N*-dimethylformamide and the good agreement between values in the other two suspending agents it is likely that the true value lies between 40 and 50 μM. Raggatt and Whitehouse (1966) found great variation in values for K_m with *N,N*-dimethylformamide. On the other hand, the value for apparent K_m for cholesterol sulfate using Tween-80 does not agree well with the values using the other two suspending agents (Table IV). Possibly the two detergents cholesterol sulfate and Tween-80 interact in such a way as to make less of the cholesterol sulfate available to the enzyme. In spite of these reservations it is likely that the apparent K_m for cholesterol (40–50 μM) is considerably higher than that for the sulfate (18 μM).

If all of the cholesterol sulfate of the adrenal cortex (1.5 mg/kg; Drayer *et al.*, 1964) is found in mitochondria and if mitochondria contain 10%[2] of the cellular water of the adrenal cortex, the concentration of this substrate available to the enzyme would be 30 μM, which exceeds the apparent K_m under present conditions. No sulfokinase was found in adrenal mitochondria (Results), so that cholesterol must be sulfated elsewhere. However, since the only known or likely function of cholesterol sulfate in the adrenal is to serve as a precursor of steroids, it is reasonable to suppose that much of it will be found in mitochondria where the first step on the pathway to steroid hormones takes place. If concentrations of 30 μM are available and if the enzyme behaves similarly *in vivo*, the possibility exists that cholesterol sulfate is cleaved under physiological conditions. Moreover it is unlikely that cholesterol sulfate is uniformly distributed throughout the mitochondrion and concentrations in the vicinity of the enzyme may be greater than 30 μM. It seems clear that cholesterol sulfate could act as an effective substrate for side-chain cleavage *in vivo*.

[2] No values appear to have been reported for the adrenal cortex but a value of 50% is given for muscle (by Kisch, 1952); muscle is unusually rich in mitochondria. The values chosen for this calculation are somewhat arbitrary but may set flexible limits which indicate that cholesterol sulfate *could* be present in mitochondria at levels approaching the apparent K_m reported here.

TABLE VI: Side-Chain Cleavage of Cholesterol in the Presence and Absence of Cholesterol Sulfate.[a]

Cholesterol (μmoles/flask)	Cholesterol Sulfate (μmoles/flask)	Pregnenolone (mμmoles/min mg of protein)
2.6		6.4
2.6		6.8
2.6	0.5	6.8
2.6	0.5	6.7
2.6	1.0	6.5
2.6	1.0	6.4
2.6	2.0	6.0
2.6	2.0	5.7
0.1		0.48
0.1		0.51
0.1	0.02	0.50
0.1	0.02	0.47
0.1	0.04	0.49
0.1	0.04	0.45

[a] Methods used are given with Table II except that the substrate was cholesterol. Pregnenolone was measured by gas chromatography. Cholesterol and cholesterol sulfate were dissolved in *N,N*-dimethylformamide.

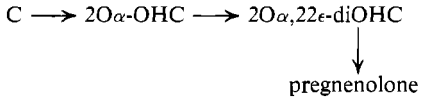
Values for apparent V_{max} with the two substrates do not appear to differ greatly. Side-chain cleavage is the rate-limiting step for the synthesis of free steroids (Halkerston *et al.*, 1961; Hall, 1963; Koritz and Hall, 1964); it may turn out that the same step is limiting for the sulfate pathway. In that case if cleavage of the two substrates occurs under optimal conditions *in vivo*, the rate-determining step of the two pathways could proceed at the same rate.

This consideration raises the important question of whether the two substrates compete for a single enzyme system or whether two enzyme systems exist. The data of Table V strongly suggest that the two substrates are not competing for a single enzyme since cleavage of the two substrates incubated together is approximately equal to the sum of cleavage of each substrate incubated separately. It is true that cholesterol has caused some inhibition of the side-chain cleavage of cholesterol sulfate (15.0 $\pm$ 1.0 *vs.* 10.2 $\pm$ 1.0; Table V), when the two substrates are present in concentrations which exceed solubility. It should be noticed that levels of endogenous cholesterol in such preparations (approximately 11 μM) are low compared with levels of exogenous cholesterol used in this study (1100 μM). However Table VI clearly shows that at high or low concentrations of cholesterol, cholesterol sulfate does not inhibit side-chain cleavage of cholesterol. These observations are incompatible with the idea of a single enzyme acting upon two competing substrates.

The fact that cholesterol causes no more than a minor degree of inhibition of the side-chain cleavage of cholesterol sulfate means that either cholesterol is a poor inhibitor of the

cleavage of the sulfate or that the enzyme system is already saturated by endogenous levels of cholesterol (C). Attempts to distinguish between these two possibilities were not successful since the kinetics of inhibition are complex and values for K_i could not be calculated.

If the pathway generally accepted for side-chain cleavage (Constantopoulos and Tchen, 1961; Shimizu *et al.*, 1962)



does in fact operate *in vivo*, side-chain cleavage may require some factor(s) in addition to the known components of the system used here (*i.e.*, TPNH diaphorase, nonheme iron, and cytochrome P-450). It is unlikely that the final step would require cytochrome P-450 and in any case it seems probable that some factors will be required to specify which carbon atoms of the substrate are hydroxylated (11, 18, 20, or 22). Presumably the three known components mentioned above are shared by the various hydroxylation systems, especially since a recent report suggests that liver microsomes can hydroxylate more than one substrate with only one cytochrome P-450 (Hildebrandt *et al.*, 1968). These considerations lead to the idea that a common system of electron transport may activate oxygen for a family of enzymes which have not so far been isolated. The nonheme iron used in these studies is highly purified (Kimura and Suzuki, 1967) and although the purity of the diaphorase has not been defined, the most likely source of these putative factors would appear to be the relatively crude preparation of cytochrome P-450.

Some comment is required concerning our values for apparent K_m which in the case of cholesterol sulfate differ considerably from the value of 500 μM reported by Raggatt and Whitehouse (1966). This value was obtained with intact mitochondria and another group of workers failed to observe side-chain cleavage with intact mitochondria (Roberts *et al.*, 1967). It is unlikely that the conditions used by Raggatt and Whitehouse were optimal if only by reason of the inevitable problem of permeability with whole mitochondria.

The conclusion that cholesterol sulfate can act as a substrate for side-chain cleavage is consistent with observations by Roberts *et al.* (1967) made with sonicates of mitochondria. The present data further suggest that the sulfate could be cleaved as rapidly as the free sterol under optimal conditions and point strongly to the existence of separate enzymes for the two substances. Further exploration of the details of side-chain cleavage appears to call for purification of the fraction containing cytochrome P-450.

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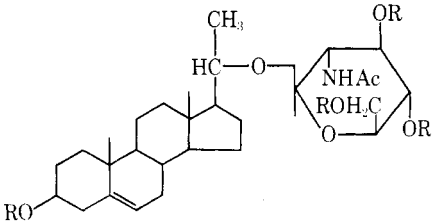
On the Configuration of Naturally Occurring Steroid *N*-Acetylglucosaminides*

Michio Matsui† and David K. Fukushima

ABSTRACT: Synthesis of 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside and the anomers of 3β-hydroxy-5α-pregnan-20α-yl 2′-acetamido-2′-deoxy-D-glucopyranoside has provided evidence for the assignment of the β configuration to C-1′ in 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-D-glucopyranoside which was recently isolated from human urine by Arcos and Lieberman.

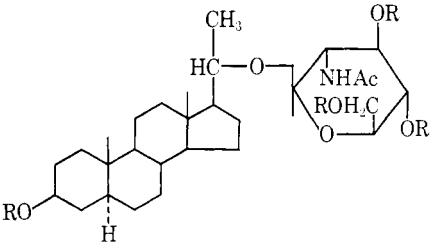
The synthesis of the anomeric pair of 2-acetamido-2-deoxy-D-glucopyranoside of C_{19} steroids conjugated at C-3 or at C-17 has been recently accomplished (Sauer *et al.*, 1969). Their physical properties were examined in order to determine whether the configuration of the glycoside linkage could be assigned from these studies. The results indicated that 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-D-glucopyranoside isolated from human urine as the 3-sulfate ester by Arcos and Lieberman (1967) was not the α anomer as assigned by these authors but had the β-glycoside linkage. In order to confirm this conclusion the synthesis of 3β-hydroxy-Δ⁵-pregnen-20α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside (Ib) was undertaken. Since the α anomer of this



Ia, R = Ac
Ib, R = H

conjugate could not be prepared by the method employed in the synthesis of C_{19} derivatives, the anomeric pair of the *N*-acetylglucosaminide of the saturated 5α-pregnane-3β,20α-diol was prepared for comparison purposes. The results firmly establish the β configuration of the *N*-acetylglucosaminide isolated from human urine.

It has been well established that the Koenigs–Knorr reaction leads almost exclusively to β-glycosides. *N*-Acetyl-β-D-glucosaminides of C_{19} steroids have recently been prepared by this method (Sauer *et al.*, 1969). Thus condensation of 5α-pregnane-3β,20α-diol 3-monoacetate and 1α-chloro-2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-D-glucopyranose with mercuric salts afforded 3β-acetoxy-5α-pregnan-20α-yl 2′-acetamido-2′-deoxy-3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranoside (IIa). Transesterification with sodium methoxide readily



IIa, R = Ac
IIb, R = H

gave 3β-hydroxy-5α-pregnan-20α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside (IIb). The β configuration of the glycoside linkage in these compounds was further established by their physical properties. The nuclear magnetic resonance spectrum (δ) of the tri-*O*-acetyl derivative IIa exhibited a doublet at 4.80, J = 8.5 cps for the anomeric H-1′ proton, and a broad multiplet at 4.17, $W_{1/2}$ = 8 cps. These values are consistent with those, 4.67–4.90 ppm, J = 8.5 cps, and 4.15–4.18 ppm, $W_{1/2}$ = 8 cps, found for the β anomer of the C_{19}-steroid conjugates. In addition, the molecular rotation difference between the conjugate and the steroid aglycone ΔM_D (β − steroid) was +9° for the free IIb and −1° for the tri-*O*-acetylated compound IIa. These values are in the range −91 to +51° calculated for the C_{19}-steroid *N*-acetyl-β-glucosaminides.

Anomerization of the tri-*O*-acetyl derivatives of the C_{19}-steroid *N*-acetyl-β-glucosaminides has been achieved in good

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