Oxidation of Cholesterol and Cholesterol Analogues by Mitochondrial Preparations of Steroid-Hormone-Producing Tissue

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The rate of oxidation of cholesterol and its analogues to pregnenolone (3 β -hydroxypregn-5-en-20-one) by various mitochondrial preparations was measured. Sterols with the cholest-5-en-3 β -ol ring system and saturated side chains of different lengths were converted into pregnenolone at rates similar to that of cholesterol. This marked lack of mitochondrial specificity towards the steroid side chain is in direct contrast with the rat liver microsomal cholesterol 7 α -hydroxylase, which has a high specificity for the side chain. Steroids that retain the ring system, but contain hydroxyl groups at various points in the side chain, are converted into pregnenolone at rates three to eight times higher than is cholesterol. The results are discussed with reference to current ideas on the mechanism of the side-chain cleavage of cholesterol.

Cholesterol is oxidized in mammalian tissues by two main routes. In liver endoplasmic reticulum, hydroxylation at C-7 occurs as the first step in bile acid synthesis from cholesterol. In steroid-hormone-producing tissue, pregnenolone (3 β -hydroxypregn-5-en-20-one) is formed by the cleavage of the cholesterol side chain by a mitochondrial oxidizing system. Both the microsomal and the mitochondrial systems are mixed-function oxidases and require O₂, NADPH and cytochrome *P*-450 (Boyd *et al.*, 1972). The nature of the two oxidations is very different. In the microsomal system a single hydroxylation takes place, whereas in the mitochondrial system, formally at least, two hydroxylations followed by an oxidative cleavage of a C-C bond are required. The measured stoichiometry of the cholesterol side-chain-cleavage reaction in a partially purified system is in agreement with these assumptions (Shikita & Hall, 1974).

Clearly, although the components of these two cholesterol-oxidizing systems are similar in many respects, they must show great differences in their sterol-binding sites, which are presumably associated with the cytochrome *P*-450. Since one of the systems acts on the cholesterol side chain and the other at a position remote from the side chain, we have attempted to use cholesterol analogues with different side chains to define further the binding site for cholesterol in the microsomal and mitochondrial systems.

In the present paper we describe experiments with several preparations from steroid-hormone-producing tissues and from two groups of cholesterol analogues. The first group contained sterols

with the cholest-5-en-3 β -ol ring system but whose side chains were varied in length (Table 1). The second group also retained the cholest-5-en-3 β -ol ring structure, but the side chains were substituted with hydroxyl groups in various positions. These two series of analogues were incubated with mitochondrial preparations from pig testis and rat adrenals (as examples of steroid-hormone-producing tissue with different functions) and also with an acetone-dried-powder preparation from bovine adrenal-cortex mitochondria, a preparation frequently used in studies of the cholesterol side-chain-cleavage reaction. This is a system from which endogenous cholesterol has been removed. The rate of pregnenolone production in each case was measured by radioimmunoassay.

A preliminary account of some of this work has already been published (Arthur *et al.*, 1975).

Materials and Methods

Cholesterol analogues: C₂₇ series

Reagents used were of standard commercial grade and were purified as required. All synthetic intermediates were characterized by i.r., n.m.r. (nuclear-magnetic-resonance) and mass spectroscopy and purity was confirmed by t.l.c. and g.l.c.

Desmosterol (VII; see Table 1) was obtained from Organon Laboratories (Newhouse, Strathclyde, Scotland, U.K.); β -sitosterol (VI) was from Koch-Light Laboratories (Colnbrook, Bucks., U.K.); pregn-5-en-3 β -ol (I) was synthesized as described by Brown & Boyd (1974). Cholest-5-ene-3 β ,20 α -diol (XII) was synthesized by the method of Petrow &

Stuart-Webb (1956). Cholest-5-ene-3 β ,24-diol (VIII), cholest-5-ene-3 β ,25-diol (IX) and cholest-5-ene-3 β ,26-diol (X) were prepared by Dr. S. A. M. Ali (Ali, 1968).

C₂₂ series

3 β -Hydroxy-23,24-bisnor-5-cholenic acid methyl ester. The ester was prepared from the corresponding

acid (Steraloids Inc., Pawling, NY, U.S.A.), by treatment of the acid with diazomethane (Fernholz, 1933). The product was crystallized from methanol [m.p. 141–142°C, literature value 140°C (Fernholz, 1933)].

3 β -Triphenylmethoxy-23,24-bisnor-5-cholenic acid methyl ester. The above methyl ester (360mg) was

Table 1. Rates of production of pregnenolone for various steroid substrates with mitochondrial preparations

Absolute rates and rates relative to cholesterol (C₂₇) are given. In Expts. 1, 2 and 3, the rate is expressed in pmol of pregnenolone produced/min per mg of protein (mean of two independent determinations). In Expt. 4, the rate is expressed in nmol of pregnenolone produced/min per mg of protein. Values in parentheses are the numbers of independent determinations. *P* values (Student's *t* test) for each rate compared with the endogenous rates and cholesterol rates are shown.

(Endogenous)		Bovine adrenal-cortex acetone-dried powder (Expt. 1)		Pig testes (Expt. 3)		Rat adrenal (Expt. 4)		
		Rate	Relative rate	Rate	Relative rate	Rate	<i>P</i> ('endo- genous')	<i>P</i> ('chole- sterol')
Non-polar group		0	0	150	0.35	1.96 (12)		0.49
C ₂₁ (I)		Not detected		360	0.54	—	—	—
C ₂₂ (II)		95	0.95	—	—	4.11 (4)	<0.02	1.04
C ₂₄ (III)		74	0.74	960	2.23	3.74 (4)	<0.01	0.96
C ₂₆ (IV)		130	1.3	680	1.58	3.86 (4)	<0.01	0.97
C ₂₇ (V)		100	1.00	430	1.00	3.97 (10)	<0.001	1.00
C ₂₉ (VI)		—	—	420	0.98	—	—	—
C ₂₆ (IV)		Expt. 2 50 1.00		—	—	—	—	—
C ₂₇ (V)		50	1.00	—	—	—	—	—
Demosterol (VII)		200	4.00	2130	4.95	7.81 (4)	<0.001	<0.01

(continued on facing page)

Table 1—continued

Polar group		Bovine adrenal-cortex acetone-dried powder (Expt. 2)		Pig testes (Expt. 3)		Rat adrenal (Expt. 4)			
		Rate	Relative rate	Rate	Relative rate	Rate	<i>P</i> ('endo- genous')	<i>P</i> ('chole- sterol')	Relative rate
20 α -Hydroxy- C ₂₇ (XI)		160	3.20	3770	8.77	15.60 (5)	<0.001	<0.01	3.93
C ₂₆ (XII)		140	2.80	—	—	—	—	—	—
24-Hydroxy- C ₂₇ (VIII)		120	2.40	3290	7.65	19.1 (4)	<0.001	<0.001	4.81
25-Hydroxy- C ₂₇ (IX)		130	2.60	2040	4.74	15.1 (4)	<0.001	<0.001	3.80
26-Hydroxy- C ₂₇ (X)		130	2.60	2830	6.58	11.5 (8)	<0.001	<0.001	2.90

refluxed in dry pyridine (2ml) with triphenylmethyl chloride (280mg) for 6h. The pyridine was evaporated, water (10ml) was added, and the mixture heated on a water bath (90°C) for 1h. The mixture was cooled and extracted with dichloromethane (3×15ml), and the dried organic extracts were evaporated. Chromatography on silica gel (20g) with dichloromethane yielded the required product, which was recrystallized from methanol (360mg, 65% yield, m.p. 162–163°C, M^+ 602).

3 β -Triphenylmethoxy-23,24-bisnor-5-cholen-22-ol. The above ester (200mg) was treated with LiAlH₄ (20mg) in dry tetrahydrofuran. Work-up as described by Fieser & Fieser (1967) afforded the required alcohol (180mg, 95% yield, m.p. 185–186°C from methanol, M^+ 574).

3 β -Triphenylmethoxy-23,24-bisnor-5-chole. The above alcohol (45mg) was caused to react with

toluene-*p*-sulphonyl chloride (45mg) in dry pyridine (2ml) at 4°C for 16h. The mixture was poured into ice/10% (v/v) HCl (20ml) and the resulting white solid filtered off and dried. This material was reduced without further purification by treatment with LiAlH₄ (20mg) in dry tetrahydrofuran (20ml). Isolation was as described previously, and purification by preparative t.l.c. gave the required steroid (22mg, 51% yield, m.p. 194–195°C from methanol, M^+ 558).

23,24-Bisnor-5-chole-3 β -ol (II). The above steroid was dissolved in dry benzene, and the solution cooled. Trifluoroacetic acid (3 drops) was added, and the mixture kept below 10°C for 15min. The solvent was evaporated, and the resulting mixture separated by preparative t.l.c. with chloroform/ethyl acetate (2:1, v/v) to give the required sterol (12mg, 96% yield, m.p. 134–135°C from methanol, M^+ 318).

C₂₄ series

5-Cholen-3 β -ol (III) was prepared from 3 β -hydroxy-5-cholenic acid methyl ester (Organon) in exactly the same way as the C₂₂ series. The physical characteristics of the products were: 3 β -triphenylmethoxy-5-cholenic acid methyl ester, m.p. 151–152°C from ethanol/methanol, M^+ 630; 3 β -triphenylmethoxy-5-cholen-24-ol, m.p. 117–119°C from methanol, M^+ 602; 3 β -triphenylmethoxy-5-cholene, m.p. 160–161°C from ethanol/ether, M^+ 586; and 5-cholen-3 β -ol (III), m.p. 129–130°C from methanol, M^+ 344.

C₂₆ series

26-Norcholest-5-ene-3 β ,25-diol 3 β -acetate. 25-Oxo-26-norcholest-5-en-3 β -ol (Organon) was reduced with NaBH₄ in methanol to give the required diol monoacetate (m.p. 129–130°C) in quantitative yield.

26-Norcholest-5-en-3 β -ol (IV). The above diol monoacetate was treated with toluene-*p*-sulphonyl chloride as described above, and the resulting tosylate reduced with LiAlH₄ to give the required sterol (m.p. 127–129°C, M^+ 372).

Mitochondrial preparations and incubations

Three mitochondrial preparations were used. Female rates of the Wistar strain were obtained from the Small Animal Breeding Station, University of Edinburgh. Pig testes were obtained from routine castrations at local pig farms, and bovine adrenal glands were obtained from the Edinburgh slaughterhouse. A rat adrenal-mitochondrial preparation, a pig testicular-mitochondrial preparation and an acetone-dried powder of bovine adrenal-cortex mitochondria were prepared from these tissues by techniques previously described (Simpson & Boyd, 1967; Simpson *et al.*, 1972).

Incubations were carried out at 37°C in a medium containing 250mM-sucrose, 20mM-KCl, 15mM-NADP⁺ and 0.1% (w/v) bovine serum albumin [type F; Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.]. Cholesterol side-chain-cleavage activity was initiated in the incubations with DL-isocitrate (final concn, 20mM), and steroids were added in 10 μ l of ethanol from stock solutions made up to 4mg/ml, a concentration that was sufficient to saturate the enzyme. Cyanoketone (2 α -cyano-4,4,17 α -trimethyl-17 β -hydroxy-androst-5-en-3-one) was included in the incubations to prevent the further metabolism of the pregnenolone produced by the side-chain cleavage reaction (Simpson *et al.*, 1972). Incubations were 1ml in volume and contained 1–2mg of protein. The reactions were stopped by the addition of 5ml of methanol. [7-³H]Pregnenolone (New England Nuclear Corp., Boston, MA, U.S.A.) was added as a recovery marker. After the addition of 5ml of chloroform and 2ml of water, the aqueous

phase and the protein were removed, and the organic phase evaporated to dryness. The residue was dissolved in 5ml of ethanol, and 2.5ml of the solution was counted for the recovery of radioactivity. Pregnenolone was determined in 10 μ l of the dissolved extract by radioimmunoassay by using an antiserum raised to the conjugate of pregnenolone and bovine serum albumin (Abraham *et al.*, 1973). Protein was determined by the method of Lowry *et al.* (1951).

Results and Discussion

The results for the various preparations are collected in Table 1, and a typical time-course for the reaction is shown in Fig. 1. In the present mitochondrial study, cholesterol was the poorest substrate, in contrast with the liver microsomal cholesterol 7 α -hydroxylase, where cholesterol is hydroxylated at C-7 to the greatest extent (Arthur *et al.*, 1975). The rates obtained from all the tissues, although differing in the absolute values, clearly fall into two groups, which are significantly different. Sterols with the non-polar side chains are cleaved at rates that are comparable with the rate of cholesterol side-chain cleavage. The sterols with polar side chains are cleaved at rates that are consistently higher than

Fig. 1. Time-course of pregnenolone production by an acetone-dried powder of bovine adrenal-cortex mitochondria

Incubations were carried out in duplicate as described in the Materials and Methods section. Pregnenolone production from: endogenous sterols, ▲; C₂₂ sterol (II), ■; C₂₄ sterol (III), □; C₂₆ sterol (IV), ●; C₂₇ sterol (V, cholesterol), ○.

that of cholesterol. This is particularly clear with the pig testis mitochondria.

It is clear from these results and from earlier studies (Luttrell *et al.*, 1972; Tait, 1972; Hochberg *et al.*, 1974) that the cholesterol side-chain-cleavage system is capable of cleaving a wide variety of substrates with the formation of pregnenolone. One of the difficulties in interpreting these and earlier results is clearly the heterogeneous nature of the preparations used. The significance of the different rates observed for the two groups of substrate is difficult to establish in an unpurified system. However, the results reported here suggest that, in the present systems, a step other than the side-chain cleavage of the substrates could be rate-determining, and support the hypothesis that, under certain conditions, the rate-determining step in mitochondrial cholesterol-side-chain cleavage is the transport of cholesterol within the mitochondrion itself (Boyd *et al.*, 1975).

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