Obligatory Role of the Δ^5-Bond of Cholesterol for Steroid Formation by Adrenal Preparations (35290)

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Dihydrocholesterol[1] occurs as a normal constituent of the nonsaponifiable fraction in all mammalian tissue studied thus far (1). It is similar in many respects to cholesterol. Both are readily absorbed and largely esterified in the process (2). Like cholesterol, feeding of dihydrocholesterol to rabbits produces atheromatous lesions, fatty livers, and bile stones (3, 4). In rabbits and guinea pigs, relatively high proportions (10%) of dihydrocholesterol occur in the adrenal sterol fraction (5, 6). When dihydrocholesterol is added to the diet of rats, the total beta-hydroxy sterol level of the adrenal gland rises to more than double the control level and this increase is due entirely to dihydrocholesterol deposition without cholesterol displacement (7). Its high concentration and ready deposition in the adrenal along with its structural similarity to cholesterol posed the question of the significance of adrenal dihydrocholesterol and whether it too, like cholesterol, could serve as a precursor for the synthesis of adrenal steroids. To investigate this hypothesis, radioactive dihydrocholesterol was incubated with various adrenal preparations; *i.e.*, homogenates of adrenals from hogs, normal rats, and rats maintained on a diet containing 1% dihydrocholesterol and suspensions of acetone powder preparations of beef adrenal mitochondria.

Materials and Methods. Glands. Adrenal glands were obtained from female rats of the Wistar strain, 6 to 10 months of age, maintained on Purina rat pellets. In one experiment, the rats were maintained for 7 to 11 weeks on a diet of ground Purina rat pellets to which was added 1% dihydrocholesterol. Hog and beef adrenal glands were obtained at a slaughterhouse, immediately cooled on ice, trimmed of fat, and frozen. Glands frozen several days to 11 weeks were used.

Whole adrenal homogenates. Adrenals (pooled from 3 to 9 rats or minced from hog) were homogenized in Krebs–Ringer bicarbonate buffer (8), 100 mg/ml. For each sample, 1 ml of homogenate was added to a small Erlenmeyer flask containing 1 mg of reduced sodium nicotinamide-adenine dinucleotide phosphate (NADP) and 3 mg of glucose 6-phosphate. As a control, 1 ml of homogenate was heated in boiling water for 10 min and transferred to a flask with cofactors. The mixture was made to a volume of 3 ml with additional buffer and 0.3 μCi (2.3 μg) of 4-^{14}C-dihydrocholesterol or 0.05 μCi (0.4 μg) of 4-^{14}C-cholesterol[2] was added in 0.2 ml of propylene glycol. All samples were incubated for 2 hr in a Dubnoff shaker under an atmosphere of 95% O_2 and 5% CO_2 at 37.5°. After incubation, the contents of each flask were quickly frozen by immersion in a Dry Ice–acetone bath.

Acetone powders of adrenal mitochondria. Acetone-dried preparations were prepared from mitochondria separated by differential centrifugation of beef adrenal glands homogenized in 0.44 *M* sucrose by the method of Halkerston *et al.* (9). The equivalent of 5 g of adrenal cortex tissue (110.5 mg of powder) was incubated as a suspension in 5 ml of 0.33 *M* phosphate buffer (pH 7.4) containing 7.7 mg of glucose 6-phosphate, 1 mg of

[1] The trivial name of dihydrocholesterol has been employed for 5α-cholestan-3β-ol.

[2] New England Nuclear Corporation.

sodium NADP, and 2 units of glucose-6-phosphate dehydrogenase. Addition of radioactive sterol (in 0.075 ml of propylene glycol) and incubation conditions were the same as with whole adrenal homogenates.

Extraction, purification, and assay. Incubates were thawed and extracted three times with 20 ml of methylene chloride to remove steriods. The incubate was then poured into 100 ml of acetone–alcohol (v/v), brought to a boil, cooled, filtered, and combined with the methylene chloride extracts. The combined extracts were evaporated to dryness and made up to a volume of 25 ml with acetone:alcohol (v/v). Aliquots of these extracts were evaporated to dryness and acetylated overnight with 1 ml each of pyridine and acetic anhydride. The acetylated samples were dried under a stream of air, dissolved in 0.2 ml of chloroform containing 80 μg each of dihydrocholesterol acetate and cholesterol acetate, and applied in lanes to thin-layer chromatography plates coated 0.4 mm thick with walter–silver nitrate–silica gel G (20:4:10) as described by Chattopadhyay and Mosbach (10). Plates were developed in a Sandwich Apparatus[3] in the cold (5°) with freshly distilled methylene dichloride which was allowed to rise 15 cm above the origin. Steroid acetates were located in the zone extending 3 to 5 cm above the origin. In runs with standards, the acetates of corticosterone, the major corticosteroid produced in incubations of quartered rat adrenals (11), cortisol or 5α-pregnane-3β-ol-20-one, the steroid which would be formed by cleavage of the side chain of dihydrocholesterol, were retained within this area. Cholesterol acetate (av R_f 0.31) and dihydrocholesterol acetate (av R_f 0.60) separated into bands on the plate. When ^{14}C-cholesterol used as a substrate in this study was acetylated and applied to a plate, 94 to 95% of the radioactivity was present in the cholesterol acetate band after development, while 96 to 98% of ^{14}C-dihydrocholesterol acetate occurred in its band. Sterol acetates were detected on the plate by spraying lightly with 2,-7-dichlorofluorescein (0.04% in methanol) and viewing under ultraviolet light, and the thin-layer plate was divided into 5 or 6 appropriate areas. The adsorbent in each area, including that containing steroid or sterol acetate, was sucked into a glass extractor fitted with a sintered glass disk (12) and eluted with 8 ml of chloroform drawn through the extractor. Aliquots of samples to be counted were taken to dryness in 25-ml glass counting vials and dissolved in 15 ml of scintillation fluid containing 3 g of 2, 5-diphenyloxazole and 0.1 g of 1, 4-bis[2(4-methyl-5-phenyloxazolyl) benzene/liter of toluene. Radioactivity was measured in a Model 314 ES Packard Tri-Carb liquid scintillation spectrophotometer (75% efficiency for counting ^{14}C) to a counting error $\pm$ 5%. Results were calculated as percentage of total radioactivity appearing in the two sterol acetate and steroid acetate areas.

Results. The results presented in Table I show that normal rat adrenal homogenates, which are capable of converting 25 to 40% of added radioactive cholesterol to labeled steroids, do not convert radioactive dihydrocholesterol to labeled steriods to any significant extent (less than 3.5%). Moreover, it can be seen that even adrenal homogenates from rats which had been fed dihydrocholesterol, thus "loading" the gland with the sterol, also failed to effect a significant conversion of the radioactive substrate to labeled steroids. In addition, when radioactive cholesterol in tracer amounts was incubated with homogenates of these dihydrocholesterol-loaded adrenals, the conversion to labeled steroids was greatly reduced (3.4%) compared to the conversion by normal rat adrenal homogenates (above 25%). Although the concentration of cholesterol in the adrenals of rats fed dihydrocholesterol was essentially the same as that in the adrenals of normal rats, (see footnote *b*, Table I) the total amount of cholesterol per adrenal more than doubled (from 0.67 to 1.68 mg).

In Table II, the data indicate that even the essentially cholesterol-free acetone powder preparations of beef adrenal mitochondria, which have a high capacity (79.1%) to convert added radioactive cholesterol to la-

[3] Kontes Glass Company, Vineland, New Jersey.

TABLE I. Formation of Steroid from Radioactive Sterol by Adrenal Homogenates.

Substrate:		^{14}C-Dihydrocholesterol			^{14}C-Cholesterol		
		Percentage of incubated radioactivity in:					
		Steroid	Chol[a]	DH-Chol[a]	Steroid	Chol	DH-Chol
Rat[b]	Boiled control	1.3	0.7	98.0	1.9	97.6	0.5
	Samples[c]	5.0	0.6	94.4	44.0	55.4	0.6
	Minus control	3.7			42.1		
	Boiled control	1.6	0.3	98.1	1.1	97.7	1.2
	Samples	4.7	0.6	94.7	27.7	71.7	0.6
	Minus control	3.1			26.6		
Hog	Boiled control	2.7	0.9	96.4	3.0	90.6	6.4
	Samples	2.1	0.45	97.45	32.2	59.1	8.7
	Minus control	0			29.2		
Rats fed dihydrocholesterol[b]	Boiled control	0.5	3.4	96.1	3.6	95.9	0.5
	Samples	3.2	1.6	95.2	7.0	92.45	0.55
	Minus control	2.7			3.4		

[a] Chol = cholesterol; DH-Chol = dihydrocholesterol.

[b] Average weight of one adrenal: Normal rat, 29 mg; DH-Chol-fed rat, 68 mg. Sterol content, mg/100 mg adrenal: Normal rat, 2.32 mg of cholesterol, 0.21 mg of dihydrocholesterol; DH-Chol-fed rat: 2.47 mg of cholesterol, 8.34 mg of dihydrocholesterol.

[c] Average of duplicates or triplicates.

beled steroid, cannot effect the conversion of radioactive dihydrocholesterol to any appreciable degree (2.2%).

Discussion. It appears that the adrenal gland cannot utilize dihydrocholesterol as a precursor for steroid synthesis. On the other hand, while not serving as a substrate itself for steroid synthesis, high concentrations of the sterol in the adrenal appear to reduce the utilization of cholesterol for such synthesis and result in a buildup of cholesterol in the adrenal.

Competitive inhibition cannot account for the lack of conversion of dihydrocholesterol by adrenal preparations since there is insignificant conversion even by highly active adrenal mitochondria preparations depleted of cholesterol. Nor is there appreciable conversion by homogenates of adrenals which contain markedly elevated levels of dihydrocholesterol; *i.e.*, adrenals from rats fed the sterol.

Since cleavage cannot proceed in the absence of the Δ^5-bond, the enzyme system involved in the side-chain cleavage of cholesterol appears to be very specific. Had formation from dihydrocholesterol proceeded through the first step of side-chain cleavage, the expected product, 5α-pregnane-3β-ol-20-one, would have appeared in the steroid fraction. In addition, the adrenal gland apparently has no enzyme system capable of introducing a double bond into the C-5, 6 position of the steroid nucleus, for the formation of cholesterol from dihydrocholesterol would be followed by the appearance of steroids.

The inhibition of steroid synthesis from cholesterol by dihydrocholesterol in rat adrenal homogenates demonstrated in the present experiments confirms and extends the *in vivo* work of Frederickson *et al.* (13), who found the steroid level in adrenal vein blood greatly decreased in rats fed cholest-4-ene-3-one, a precursor of dihydrocholesterol.

Summary. The Δ^5-bond appears to be a very specific requirement for the formation of steroids from cholesterol. Rat and hog adrenal homogenates were incapable of converting tracer amounts of radioactive dihydrocholesterol to labeled steroids to any significant extent. This inability was not due to competitive inhibition by cholesterol since

TABLE II. Formation of Steroid from Radioactive Sterol by Acetone Powder[a] Preparations of Bovine Adrenal Mitochondria.

Substrate:	^{14}C-Dihydrocholesterol			^{14}C-Cholesterol		
	Percentage of incubated radioactivity in:					
	Steroid	Chol	DH-Chol	Steroid	Chol	DH-Chol
Boiled control	1.0	0.9	98.1	4.2	94.9	0.9
Samples[b]	3.2	0.4	96.4	83.3	13.5	3.2
Minus control	2.2			79.1		

[a] 110.5 mg of acetone powder (= 5 g of whole tissue) were incubated in each sample: cholesterol content = 0.520 mg.

[b] Average of duplicates.

cholesterol-extracted acetone powder preparations of beef adrenal mitochondria or homogenates of adrenals with high levels of dihydrocholesterol were similarly unable to effect a significant conversion. Steroid synthesis from radioactive cholesterol was substantially inhibited in homogenates of adrenals from rats fed dihydrocholesterol while the total amount of cholesterol in these adrenals was increased as compared to that of normal rats.

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