STEROL METABOLISM. XVI. CHOLESTEROL 20α-HYDROPEROXIDE AS AN INTERMEDIATE IN PREGNENOLONE BIOSYNTHESIS FROM CHOLESTEROL

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SUMMARY

Incubation of adrenal cortex mitochondrial enzymes with cholesterol 20α-hydroperoxide, 20α,22R-dihydroxycholesterol, 20α-hydroxycholesterol, or pregnenolone prior to incubation with cholesterol-1,2-^{3}H inhibits incorporation of tritium into pregnenolone and other steroids. By means of added carrier sterols it was demonstrated that both cholesterol 20α-hydroperoxide and 20α,22R-dihydroxycholesterol contained radioactivity after incubation. The combination of reaction inhibition and of incorporation of tritium into cholesterol 20α-hydroperoxide and 20α,22R-dihydroxycholesterol suggest that both be important intermediates in the biosynthesis of pregnenolone from cholesterol.

We have demonstrated that cholesterol 20α-hydroperoxide is rearranged by adrenal cortex mitochondrial enzymes to 20α,22R-dihydroxycholesterol and 20α,21-dihydroxycholesterol and have suggested that cholesterol 20α-hydroperoxide may be an initial intermediate in the cleavage of the cholesterol side-chain to pregnenolone (1). In order to support this contention we have attempted to demonstrate the presence of cholesterol 20α-hydroperoxide in adrenal cortex mitochondrial incubations of radioactive cholesterol and to examine its role as an inhibitor of the side-chain cleavage reaction.

EXPERIMENTAL

Acetone-dried bovine adrenal cortex mitochondrial enzyme preparations were prepared as previously described (1). Incubations were conducted using the enzyme (10 mg of S-1 or P-2 protein in 0.02M phosphate buffer, pH 7.5) supplemented with an NADPH-generating system consisting of 0.1 mmoles $MgCl_2$, 7 μmoles NADPH, 200 μmoles glucose 6-phosphate, and 1 unit glucose 6-phosphate dehydrogenase, all

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in a total of 5 ml of solution. The preparation was preincubated for 5 min at 37° as such and with several test sterols pregnenolone, 20α,22R-dihydroxycholesterol, cholesterol 20α-hydroperoxide, and 20α-hydroxycholesterol. After preincubation 40 µl of an ethanol solution of radiochemically pure cholesterol-1,2-^{3}H (400 µg containing 2 x 10^{5} cpm, previously purified via thin-layer chromatography, column chromatography on Sephadex LH-20, and multiple recrystallization from methanol) was added by a micro syringe and the incubation was continued at 37° for 45 min. To the incubation mixture was added 100 ml of methylene chloride containing 200 µg each of the following carrier sterols: pregnenolone, cholesterol 20α-hydroperoxide, 20α,22R-dihydroxycholesterol, 20α,21-dihydroxycholesterol, progesterone. The mixture was shaken and the methylene chloride layer was filtered through Whatman phase separating filter paper, concentrated under vacuum, and applied as a 2 cm wide streak to a 20 cm x 20 cm chromatoplate of Silica Gel HF_{254} (E.Merch GmbH., Darmstadt) 0.25 mm thick. The several carrier sterols were spotted on one edge of the plate. The chromatoplate was irrigated with benzene-ethyl acetate (3:2), the progesterone zone located under 254 nm untraviolet light, and a line was marked just under the progesterone band. The edge bearing the reference sterols was visualized by spraying with 50% sulfuric acid and warmed at 110° briefly to develop the characteristic colors. A line was marked above the cholesterol band and below the 20α,21-dihydroxycholesterol band, thus giving two major zones, Zone I containing 20α,21-dihydroxycholesterol, 20α,22R-dihydroxycholesterol, and pregnenolone, and Zone II containing progesterone, 20α-hydroxycholesterol, cholesterol 20α-hydroperoxide and cholesterol (Fig. 1).

Each zone was scraped from the chromatoplate and the mixed sterols were extracted with chloroform-methanol (2:1). Each mixed sterol fraction was chromatographed on a 1 cm x 60 cm column of Sephadex LH-20 irrigated with methylene chloride (2). Twenty fractions of 4 ml were taken. This system gives baseline separations of most of the sterols of interest. A 100 µl volume from each fraction was analyzed by thin-layer chromatography with benzene-ethyl acetate (3:2), and the remainder of the sample was evaporated under vacuum, dissolved in 10 ml of toluene containing

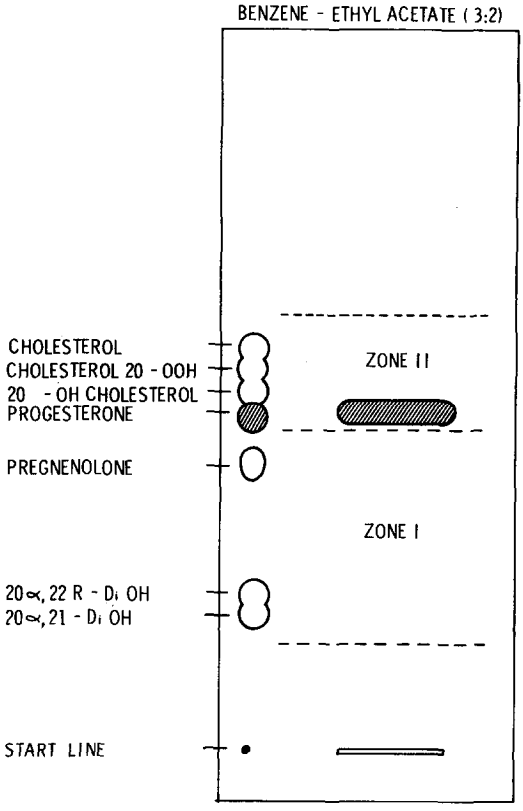

Fig. 1. Thin-layer chromatogram of total steroids recovered after incubation with cholesterol-1,2-^{3}H.

5% POP and the tritium content was determined using a Beckman Model LS-200 liquid scintillation counting system (5% precision, 50% efficiency for tritium).

A plot of the radioactivity eluted from the Sephadex LH-20 column for the uninhibited whole system (Table I, No. 1) and for that inhibited by cholesterol 20α-hydroperoxide (Table I, No. 4) is presented in Fig. 2 (Zone I steroids) and Fig. 3 (Zone II steroids). Total radioactivity in each identified steroid is summarized for all experiments in Table I. These results suggest that in the uninhibited whole system pregnenolone and progesterone are the major products and that both putative intermediates cholesterol 20α-hydroperoxide and 20α, 22R-dihydroxycholesterol have significant radioactivity. Attempted recovery of the radioactive cholesterol 20α-hydroperoxide after scintillation counting was not successful because of extensive decomposition of the hydroperoxide.

TABLE I

No.	Enzyme	NADPH System	Preincubation (5 min) with	Tritium Found in Each Steroid, % of Tritium Added				
				Cholesterol	Cholesterol 20α-Hydroperoxide	20α,22R-Dihydroxy-Cholesterol	Pregnenolone	Progesterone
1.	S-1	+	-	78.9	0.1	0.3	13.8	6.1
2.	S-1	+	Pregnonolone (500 μg)	90.5	*	0.05	8.0	0.8
3.	S-1	+	20α,22R-Dihydroxy-cholesterol (160 μg)	92.7	*	0.05	6.5	0.3
4.	S-1	+	Cholesterol 20α-hydroperoxide (500 μg)	97.5	*	0.02	2.3	**
5.	S-1	+	20α-Hydroxy-cholesterol (500 μg)	97.7	*	0.01	1.7	**
6.	S-1	-	-	99.9	*	*	**	**
7.	P-2	-	-	99.9	*	*	**	**

* Not detected at 0.01%
** Not detected at 0.1%

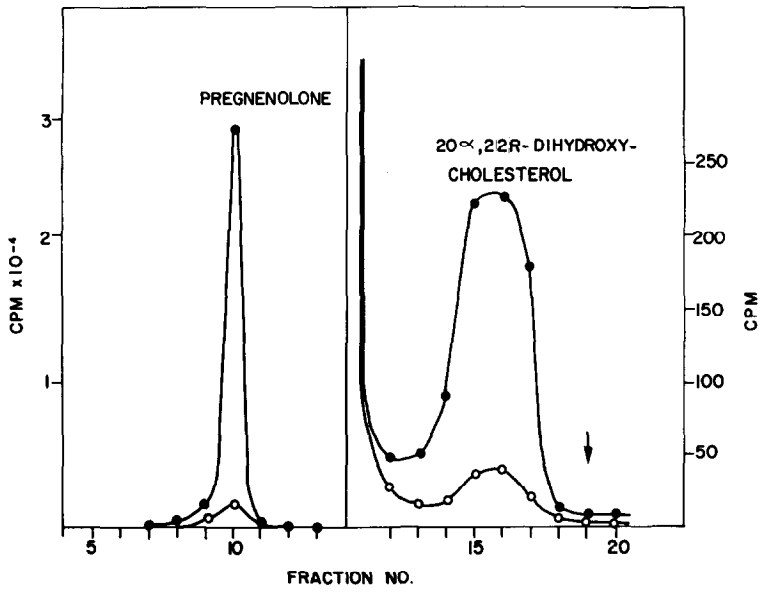

Fig. 2. Column chromatogram on Sephadex LH-20 of Zone I steroids (20α, 22R- and 20α, 21-dihydroxycholesterols and pregnenolone). The position of 20α, 21-dihydroxycholesterol is marked by an arrow. Solid circles represent uninhibited whole system; open circles are for the system inhibited by cholesterol 20α-hydroperoxide.

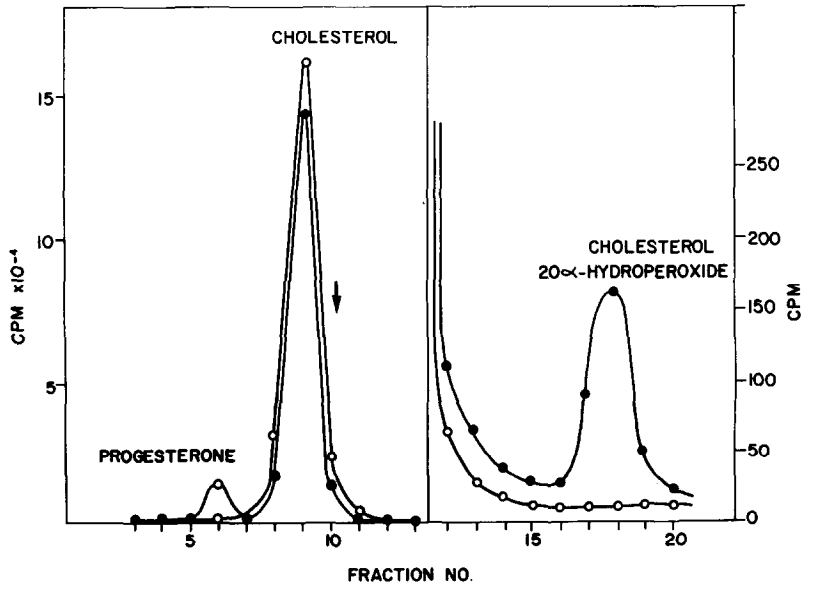

Fig. 3. Column chromatogram on Sephadex LH-20 of Zone II steroids (progesterone, cholesterol, cholesterol 20α-hydroperoxide, 20α-hydroxycholesterol). The position of 20α-hydroxycholesterol is marked by an arrow. Solid circles represent uninhibited whole system; open circles are for the system inhibited by cholesterol 20α-hydroperoxide.

Preincubation of the system with the terminal product pregnenolone, previously demonstrated to inhibit its own formation from cholesterol (3-6), or with the putative intermediate 20α-hydroxycholesterol, also shown to inhibit the reaction (4-7), completely suppresses formation of radioactive cholesterol 20α-hydroperoxide and reduces substantially the amount of radioactivity incorporated into the other steroids. We now also demonstrate that both cholesterol 20α-hydroperoxide and 20α,22R-dihydroxycholesterol inhibit the reaction in exactly the same manner (Table I).

20α,21-Dihydroxycholesterol, known to be formed in incubations of cholesterol 20α-hydroperoxide (1), was not found radioactive and is presumed not to have been formed in these experiments. 20α-Hydroxycholesterol is eluted immediately after cholesterol from the Sephadex LH-20 column, and because of the high radioactivity of cholesterol it was not possible to demonstrate labeling in 20α-hydroxycholesterol fractions directly from the column. However, 20α-hydroxycholesterol isolated therefrom by further thin-layer chromatography was not radioactive and is presumed not to have been formed in the incubations.

Omission of the NADPH-generating system from the incubation led to no detectable reaction of any sort. In these studies at no time was there detected any other radioactive or unidentified component on the several chromatograms.

These experiments suggest that both 20α,22R-dihydroxycholesterol and cholesterol 20α-hydroperoxide have been labelled as a result of their biosynthesis from cholesterol-1,2-^{3}H and that both sterols inhibit the side-chain cleavage of cholesterol to give pregnenolone. Although inhibition of the side-chain cleavage reaction cannot be used reliably as a basis for a special position as an intermediate in the bioconversion (6), the combined inhibition of the reaction and the indicated trapping of both sterols does suggest that these two sterols both share a position as intermediate in the cleavage of the cholesterol side-chain. The importance of 20α,22R-dihydroxycholesterol as such an intermediate has been supported by much other evidence (8-11).

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REFERENCES

1. van Lier, J.E., and Smith, L.L., Biochim.Biophys.Acta, in press.
2. van Lier, J.E., and Smith, L.L., J.Chromatography, 41, 37 (1969).
3. Koritz, S.B., and Hall, P.F., Biochim. Biophys. Acta, 93, 215 (1964).
4. Koritz, S.B., and Hall, P.F., Biochemistry, 3, 1298 (1964).
5. Raggatt, P.R., and Whitehouse, M.W., Biochem.J., 101, 819 (1966).
6. Simpson, E.R., and Boyd, G.S., European J.Biochem., 2, 275 (1967).
7. Hall, P.F., and Koritz, S.B., Biochim.Biophys.Acta, 93, 441 (1964).
8. Shimizu, K., Gut, M., and Dorfman, R.I., J.Biol.Chem., 237, 699 (1962).
9. Constantopoulos, G., Satoh, P.S., and Tchen, T.T., Biochem.Biophys.Research Commun., 8, 50 (1962).
10. Burstein, S., and Gut, M., Steroids, 14, 207 (1969).
11. Burstein, S., Zamoscianyk, H., Kimball, H.L., Chaudhuri, N.K., and Gut, M., Steroids 15, 13 (1970).