Synthesis of 27-hydroxycholesterol in rat liver mitochondria: HPLC assay and marked activation by exogenous cholesterol

Barbara Petrack and Brian J. Latario

Research Department, Pharmaceuticals Division, CIBA-GEIGY Corporation, Summit, NJ 07901

Abstract Sterol 27-hydroxylase, the mitochondrial enzyme that catalyzes the first step in oxidation of the sterol side chain in hepatic bile acid synthesis, also catalyzes the synthesis of 27-hydroxycholesterol from cholesterol. We have developed a high performance liquid chromatography (HPLC) assay for this enzyme, using either endogenous or exogenous cholesterol as substrate and cholesterol oxidase to convert 27-hydroxycholesterol to 4-cholesten-27-hydroxy-3-one. The α,β-unsaturated ketone product was separated by normal phase HPLC and quantitated via absorption at 240 nm. Addition of cholesterol dissolved in 2-hydroxypropyl-β-cyclodextrin to the assay mixture raised the enzyme activity of rat liver mitochondria more than 10-fold. 2-Hydroxypropyl-β-cyclodextrin itself was partially effective, apparently by making more endogenous cholesterol accessible to the enzyme. Availability of cholesterol to the enzyme limits synthesis of 27-hydroxycholesterol in rat liver. Using our assay to simultaneously determine the activities of cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase in rat liver homogenates, we demonstrated that the two enzymes are separately regulated. — **Petrack, B., and B. J. Latario.** Synthesis of 27-hydroxycholesterol in rat liver mitochondria: HPLC assay and marked activation by exogenous cholesterol. *J. Lipid Res.* 1993. **34:** 643–649.

Supplementary key words cholesterol 27-hydroxylase • sterol 27-hydroxylase • 26-hydroxycholesterol • cholesterol solubilization • 2-hydroxypropyl-β-cyclodextrin • cholesterol 7α-hydroxylase

Sterol 27-hydroxylase[1], a mitochondrial cytochrome P-450 enzyme, catalyzes the first step in cleaving the cholesterol side-chain in hepatic bile acid synthesis (1). The cDNA encoding this enzyme has been cloned (2, 3). In addition to the liver, its mRNA was also found in nonhepatic, cholesterol-synthesizing tissues, e.g., duodenum, adrenal, and lung (3), suggesting that sterol 27-hydroxylase elicits some function(s) other than its role in bile acid synthesis. The enzyme, which exhibits wide substrate specificity, catalyzes 27-hydroxylation of various potential bile acid intermediates (4–6). Cholesterol itself is oxidized to 27-hydroxycholesterol (27-HC), which in the liver is further metabolized to bile acids (7–10). In nonhepatic tissues, 27-HC may contribute to maintaining cholesterol homeostasis, as the oxysterol can inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (11) and down-regulate LDL receptors (12), via interaction with an oxysterol binding protein (13). The potential role of 27-HC in metabolism was reviewed by Javitt (10) and more recently by Björkhem (14).

Studies of patients with the inborn metabolic disease cerebrotendinous xanthomatosis (CTX) further support the importance of sterol 27-hydroxylase. CTX patients have reduced levels of bile acid synthesis and accumulate cholesterol and cholestanol in the brain and other tissues, resulting in neurological problems and premature atherosclerosis (15, 16). Considerable evidence indicates that such patients lack functional mitochondrial sterol 27-hydroxylase (17, 18); Cali et al. (19) have recently demonstrated different point mutations in the sterol 27-hydroxylase genes of two CTX patients.

Previously reported assays of sterol 27-hydroxylase used either radioactive substrates (2, 4–6, 20) or isotope dilution mass spectrometry (9, 21). We now report a simple and reliable HPLC assay for this enzyme in rat liver mitochondria using either endogenous or exogenous cholesterol as substrate. Our assay for sterol 27-hydroxylase coupled to cholesterol oxidase (22) is similar to that reported for cholesterol 7α-hydroxylase (23, 24) and can be used to simultaneously assay both enzymes in rat liver homogenates. Addition of cholesterol, dissolved in 2-hydroxypropyl-β-cyclodextrin (β-CD), increased enzyme activity more than 10-fold, demonstrating that the availability of cholesterol to the mitochondrial enzyme limits synthesis of 27-HC in rat liver.

Abbreviations: 27-HC, 27-hydroxycholesterol; 7α-HC, 7α-hydroxycholesterol; 7β-HC, 7β-hydroxycholesterol; 25-HC, 25-hydroxycholesterol; β-CD, 2-hydroxypropyl-β-cyclodextrin; HPLC, high performance liquid chromatography.

[1]The name sterol 26-hydroxylase has been changed to sterol 27-hydroxylase because the stereochemistry is now firmly established. Likewise, 26-HC is now called 27-HC.

EXPERIMENTAL PROCEDURES

Materials

We thank Dr. N. Javitt (New York University Medical Center, New York) for a sample of 27-HC and Drs. J. Parry and P. Magnus (University of Texas, Austin) for 4-cholesten-7α-hydroxy-3-one. Some 27-HC was obtained from Research Plus, (Bayonne, NJ). The following materials were purchased: cholesterol, 7α-hydroxycholesterol (7α-HC) and 7β-hydroxycholesterol (7β-HC) from Steraloids (Wilton, NH); 25-hydroxycholesterol (25-HC), cholesterol oxidase, NADPH, negative liposome kit (L4262), Triton WR 1339, Triton X-100, isocitrate dehydrogenase, testosterone propionate from Sigma (St. Louis, MO); sodium cholate, Ultrol grade, from Calbiochem (San Diego, CA); dodecane from Aldrich (Milwaukee, WI); Spherisorb silica columns from Alltech Associates Inc. (Deerfield, IL); HPLC grade hexane and isopropanol from Fisher Scientific (Fairlawn, NJ); Protein Assay Kit I, from Bio-Rad (Richmond, CA); and β-CD, as a 45% aqueous solution from Pharmatec, (Alachua, FL).

Enzyme preparations

Mitochondria were prepared as described (25). Briefly, livers taken from Sprague-Dawley male rats (Taconic Farms [(Tac:N)SD]sBR, weighing 250–300 g) were homogenized in buffer (0.20 M sucrose/10 mM HEPES, pH 7.4). The homogenate was centrifuged for 10 min at 2000 *g;* the supernatant was then centrifuged for 10 min at 9000 *g.* The pellet was washed twice, suspended in homogenizing buffer, and aliquots were stored at −70°C. Frozen preparations retained full enzyme activity for at least a month.

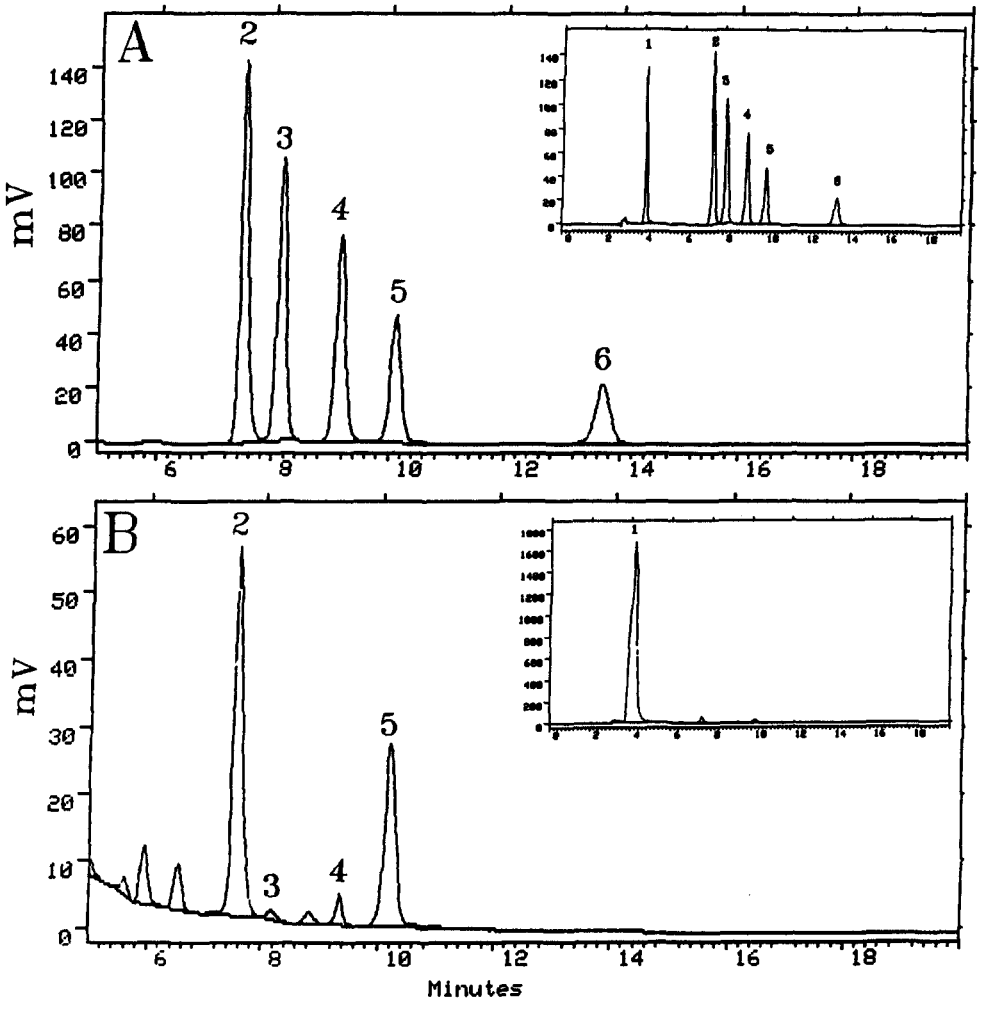

Fig. 1. Normal phase chromatography of α,β-unsaturated ketones generated by incubating with cholesterol oxidase. A: extracted standards; B: metabolites extracted from a mitochondrial assay; details are described in Experimental Procedures. The peaks are the ketones derived from: cholesterol (1), 7α-HC (3), 25-HC (4), 27-HC (5), and 7β-HC (6); testosterone propionate (2) may be used (without oxidation) as an internal standard to monitor extraction. *HPLC profiles begin at 5 min, eliminating huge cholesterol peak at 3.9 min and magnifying other peaks;* inserts are complete HPLC chromatograms.

Solubilization of cholesterol

Cholesterol (up to 15.6 mg, purified by HPLC) was dissolved in β-CD (1 ml of 45%) by initially preparing a paste in a minimal volume of β-CD with a glass rod (to wet the sterol), then adding the remaining β-CD.

Assay for sterol 27-hydroxylase

For routine assays, mitochondria (200–400 μg protein) were incubated at 37°C for 15 min in a total volume of 500 μl containing 100 mM phosphate, pH 7.5, 1 mM DTT, 0.2 mM EDTA, 1.2 mM NADPH, 5.0 mM D,L-trisodium isocitrate, and 0.2 units isocitrate dehydrogenase. The hydroxylase reaction was initiated with isocitrate/NADPH and it was stopped with 50 μl of 40% sodium cholate. Appropriate blanks were prepared by adding cholate before mitochondria. Cholesterol oxidase (2 units) was added and the tubes were incubated for 20 min at 37°C to generate the α,β,unsaturated ketones. The reaction was terminated with 1.5 ml methanol; 20 μl of testosterone propionate (5 μg/ml hexane) was added as internal standard, followed by 0.5 ml saturated KCl, and the mixture was extracted twice with 3.0 ml hexane. The organic layer was evaporated under nitrogen; the residue was dissolved in 100 μl of 5% isopropanol in dodecane. An aliquot of 50 μl was analyzed via normal phase HPLC on an Alltech spherisorb silica column (4.6 × 250 mm), using an isocratic mobile phase of hexane–isopropanol 95:5 and a flow rate of 1 ml per min; absorbance was monitored at 240 nm. To obtain maximum resolution, the silica column was initially washed briefly with methanol. Although NADPH and isocitrate dehydrogenase do not penetrate intact mitochondria, we routinely added these components to assay total enzyme activity. More than 95% of the product was routinely recovered in the extraction procedure. All assays were carried out in duplicate (including blanks), which usually agreed within 5%. Each experiment shown was repeated at least 3 times with essentially the same results.

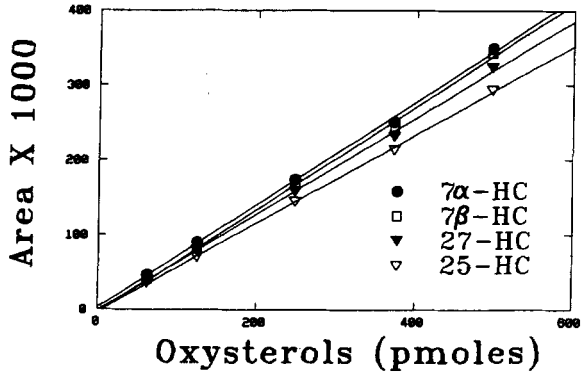

Fig. 2. HPLC calibration curve of standards incubated with cholesterol oxidase.

Assay for cholesterol and protein

Cholesterol was determined by an enzymatic, fluorometric assay of isopropanol extracts of the mitochondrial pellet (26). Mitochondrial protein, solubilized in 0.025% SDS, was assayed by the Bradford procedure (27) with bovine gamma globulin standards in SDS using the Coomassie dye Bio-Rad Protein Assay Kit.

RESULTS AND DISCUSSION

Fig. 1A profiles the normal phase HPLC separation of α,β-unsaturated ketones generated by incubating cholesterol and the oxysterols 7α-HC, 7β-HC, 25-HC, and 27-HC with cholesterol oxidase. The peak corresponding to cholesterol (at 3.9 min) was omitted from HPLC printouts to magnify the relatively small oxysterol peaks observed in routine assays; the insert shows the complete chromatogram. Fig. 1B demonstrates that in a typical assay of rat liver mitochondria, 27-HC and small amounts of 25-HC were the only observed reaction products; the traces of 7α-HC were equivalent to blanks and no 7β-HC was formed. We did not detect any further oxidation of 27-HC to the cholestanoic acid, as reported with assays carried out under different conditions (longer incubations and 5β-cholestane-3α,7α,12α-triol as substrate (2, 28). In contrast to mitochondria, rat liver microsomes synthesized 7α-HC but no 27-HC (data not shown), confirming reports that microsomal sterol 27-hydroxylase cannot use cholesterol as substrate (1). Similar calibration curves were obtained for quantitating the different oxysterols that were tested (**Fig. 2**); as little as 10 pmol can be detected.

It has been reported that cholesterol, added in acetone, liposomes, or detergent dispersions, increases the activity of other enzymes using cholesterol as substrate, e.g., cholesterol 7α-hydroxylase (29, 30) and acyl-CoA:cholesterol acyltransferase (31). As shown in **Table 1,** however, such preparations either inhibited or only slightly enhanced sterol 27-hydroxylase activity. Recently, β-CD, a cyclic oligosaccharide of seven glucopyranose units, has been used to deliver hydrophobic drugs to target sites both in vitro and in vivo; it forms aqueous-soluble inclusion complexes with many hydrophobic drugs, including bile acids and sterols (32–34). As shown in Table 1, adding cholesterol dissolved in β-CD to the mitochondrial assays raised 27-HC synthesis more than 10-fold. β-CD alone increased enzyme activity but to a lesser extent. **Table 2** demonstrates that the increase was enzyme-dependent; it required the isocitrate/NADPH system and was inhibited by cyclosporin, a reported inhibitor of cholesterol 27-hydroxylation (20); all of the activity was eliminated either by boiling the mitochondria or exposing them to carbon monoxide.

TABLE 1. Effects of exogenous cholesterol on the synthesis of 27-hydroxycholesterol in rat liver mitochondria

Cholesterol Vehicle	27-Hydroxycholesterol Synthesis	
	Exogenous Cholesterol Added	
	190 nmol	20 nmol
	pmol/mg/min	
Control (endogenous cholesterol)	(8.6)	
Acetone (1.7%)	9.0	7.7
Liposomes[a]	10.4	6.8
Triton WR-1339[b]	11.4	4.6
Triton X-100	11.1	
2-Hydroxypropyl-β-cyclodextrin (0.9%)	113.0	90.0

[a] Negative liposome kit contained L-α-phosphatidylcholine–dicetyl phosphate–cholesterol 7:2:1 in chloroform. The solvent was evaporated and the liposomes were reconstituted in assay buffer.
[b] Cholesterol was dissolved in Triton WR-1339 as described by Billheimer, Tavani, and Nes (31).

The rate of 27-hydroxylation depended on the amount of exogenous cholesterol added (**Fig. 3A**), reaching a maximum at 190 nmol. Fig. 3B shows that in assays using endogenous cholesterol, β-CD exhibited an optimum between 0.45 and 0.9%, inhibiting at higher concentrations; adding exogenous cholesterol further enhanced activity. These data suggest that β-CD alone, up to optimum concentrations, raises activity by carrying more endogenous cholesterol to the enzyme, whereas at higher concentrations it may dilute the endogenous cholesterol in the vicinity of the enzyme. The experiment summarized in **Fig. 4** supports this suggestion. Treating mitochondria with β-CD, followed by washing, removed both cholesterol and enzyme activity; activity was restored by adding exogenous cholesterol dissolved in β-CD but not by adding β-CD alone. The β-CD extraction rendered 27-HC synthesis almost completely dependent on exogenous cholesterol, suggesting that β-CD acts on cholesterol delivery to the enzyme, rather than by altering the enzyme per se. β-CD either improves aqueous dispersion of the sterol or it may possibly mimic an endogenous cholesterol transport system. Sterol 27-hydroxylase resides in the inner mitochondrial membranes (35) and the availability of cholesterol apparently limits 27-HC synthesis in rat liver.

The enzymatic activity was linear for at least 30 min in the presence but not in the absence of exogenous cholesterol (**Fig. 5A**), reflecting limiting substrate. The reaction rate was proportional to mitochondrial protein in the assay (Fig. 5B). The variability of enzyme activity in rat liver mitochondria was evaluated by assaying individual mitochondria preparations from nine rats. The mean specific activities ± SE of the nine preparations were 7.5 ± 0.22 and 107.6 ± 5.9 pmol 27-HC/min/per mg protein, without and with exogenous cholesterol in β-CD, respectively. These values are similar to those reported for rat liver mitochondria and solubilized mitochondria, respectively, using radioactive cholesterol as substrate (4), supporting the suggestion that β-CD acts by delivering cholesterol.

Both cholesterol 27- and 7α-hydroxylating enzymes can be assayed simultaneously in rat liver homogenates with

TABLE 2. Requirements for β-CD stimulation of 27-hydroxycholesterol synthesis by rat liver mitochondria

Treatment	Synthesized 27-Hydroxycholesterol		
	Endogenous Cholesterol No β-CD	Endogenous Cholesterol + β-CD	+ Exogenous Cholesterol + β-CD
		pmol/mg/min	
Control	5.2	58.2	82.1
Boiled mitochondria	0	0	0
+ *Cyclosporin* (20 μM)[a]	0	0	0
Control	11.1	50.3	99.0
No NADPH or isocitrate	0	0	2.8
+ Carbon monoxide[b]	0	0	0

β-CD = 0.9%; cholesterol = 190 nmol.
[a] Cyclosporin was dissolved in 1% dimethylsulfoxide; the vehicle did not affect activity.
[b] Mitochondria were pretreated with carbon monoxide, which was continued during incubation.

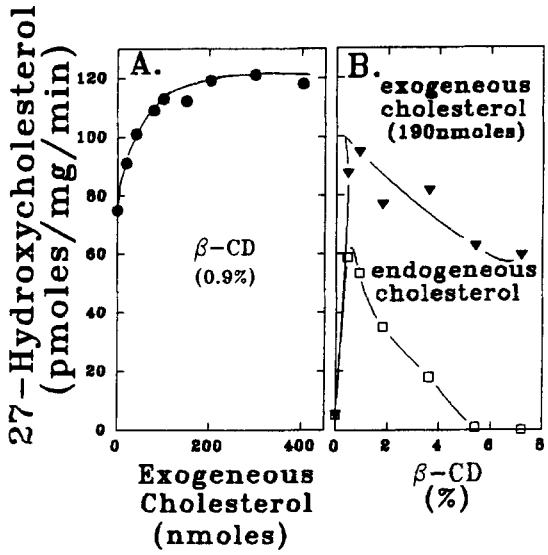

Fig. 3. Effects of varying exogenous cholesterol and β-CD concentration on 27-HC synthesis in rat liver mitochondria. A: increasing exogenous cholesterol in 0.9% β-CD. B: increasing β-CD concentration either with endogenous cholesterol or with 190 nmol of exogenous cholesterol in 0.9% β-CD.

endogenous or exogenous cholesterol as substrate. Our assay is useful for testing effects of in vivo treatments on these enzyme activities. As shown in **Fig. 6,** fasting rats overnight markedly reduced cholesterol 7α-hydroxylase activity but did not affect synthesis of 27-hydroxycholesterol. Exogenous cholesterol increased the rate of 27-hydroxlation considerably more than 7α-hydroxylation, indicating a greater dependence of the mitochondrial enzyme on cholesterol availability. These observations demonstrate that the two enzymes are separately regulated, supporting another report (9). Bile acid synthesis involving 27-hydroxylation of cholesterol as a first step is more pronounced in humans than in rats (9). This pathway, proposed by Javitt (10) and termed the "acid pathway" by Axelson and Sjövall (9), may be responsible for up to 50% of all bile acid synthesis in human liver under basal conditions (14). Our assay should be helpful in further studies of the contribution of cholesterol 27-hydroxylation to bile acid synthesis.

Liver sterol 27-hydroxylase exhibits analogy with adrenal cholesterol side-chain cleavage enzyme. Both are cytochrome P-450-mixed function oxidases, residing in inner mitochondrial membranes linked to ferredoxin reductase electron transport systems, and both utilize cholesterol as substrate. Adrenal cholesterol side-chain cleavage enzyme catalyzes the conversion of cholesterol to pregnenalone, the rate-limiting step in ACTH-stimulated steroidogenesis (36, 37). We have modified our HPLC procedure to assay this enzyme. (Incubation with cholesterol oxidase converts pregnenalone to an α,β-unsaturated ketone that can be quantitated by our HPLC system.) Using the assay, we demonstrated that cholesterol dissolved in β-CD markedly increased pregnenalone synthesis in mitochondria from bovine adrenal cortex. It is well established that cholesterol transport to the inner mitochondrial membranes of adrenal cells controls the activity of cholesterol side-chain cleavage enzyme; the function of ACTH is to stimulate this cholesterol transport (36, 37). These data support the suggestion that β-CD acts on cholesterol delivery and raises the possibility that some regulatory hormone might also contribute to cholesterol transport in the liver.

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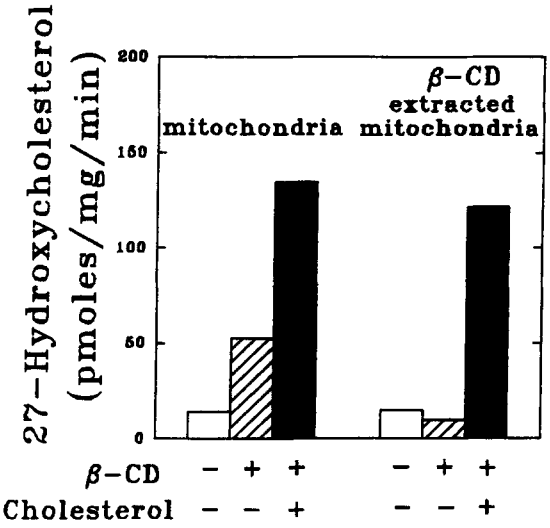

Fig. 4. Extracting mitochondria with β-CD removes cholesterol, rendering 27-HC synthesis dependent on exogenous cholesterol. Mitochondria were preincubated with β-CD for 5 min at 37°C (11 mg protein/g β-CD). The suspension was centrifuged for 20 min at 15,000 *g*, washed with cold buffer, and resuspended in its original volume. Control mitochondria were treated similarly but without β-CD. Both preparations were assayed for cholesterol, protein, and for sterol 27-hydroxylase activity with endogenous cholesterol, 0.9% β-CD, and 190 nmol of exogenous cholesterol in β-CD (0.9%). Control mitochondria contained 6.0 nmol cholesterol/mg protein, whereas the cholesterol content of the β-CD-extracted mitochondria was below the limits of detection; protein was unchanged (data not shown). The specific activities of sterol 27-hydroxylase under the different assay conditions are shown.

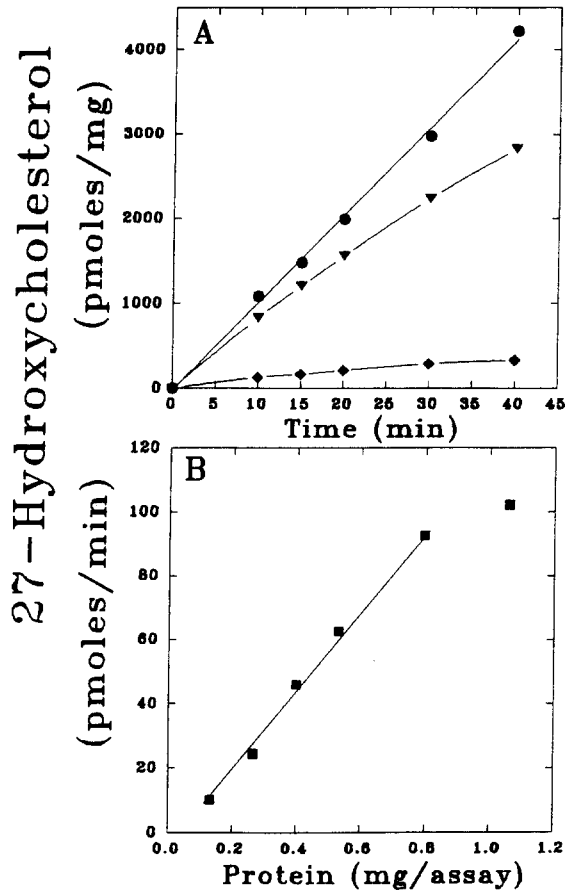

Fig. 5. Sterol 27-hydroxylase activity linearity with time and protein. A: 250 μg mitochondrial protein was incubated for various time intervals with endogenous cholesterol (♦), with 0.9% β-CD alone (▼) and with 190 nmol exogenous cholesterol in 0.9% β-CD (●). B: mitochondrial protein was varied in the presence of 190 nmol of exogenous cholesterol in 0.9% β-CD.

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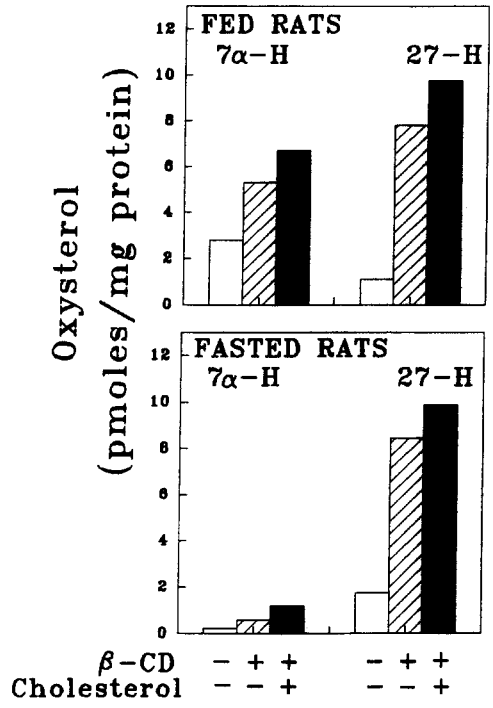

Fig. 6. Cholesterol 7α-hydroxylase and 27-hydroxylase activities in liver homogenates from fed and fasted rats. Livers from fed and fasted rats were homogenized in buffer (0.25 M sucrose–0.5 mM EDTA–10 mM phosphate, pH 7.5). Crude homogenates (5 mg protein) were assayed simultaneously for cholesterol 7α- and 27-hydroxylase activity with endogenous cholesterol, with 0.9% β-CD alone, and with 190 nmol exogenous cholesterol in 0.9% β-CD.

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