Effects of drugs and sterols on cholesterol 7α-hydroxylase activity in rat liver microsomes

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Abstract This study examined the effects of various drugs and sterols on the rate of 7α-hydroxycholesterol synthesis in isolated rat liver microsomes. Cholesterol 7α-hydroxylase activity was significantly inhibited by proadifen (98%), metyrapone (67%), and aminoglutethimide (45%) at concentrations of 1 mM and by ascorbic acid (40%) at a concentration of 10 mM. Cimetidine had no significant effect. The activity of cholesterol 7α-hydroxylase was also significantly inhibited by 7β-hydroxycholesterol (38%) and 7-ketocholesterol (35%) at concentrations of 1 μM, and by 10 μM 7α-hydroxycholesterol (35%), 25α-hydroxycholesterol (32%), and 5-cholenic acid-3β-ol (27%). Two bile acids, cholate and lithocholate, as well as a geometric isomer of cholesterol, coprostanol, had little influence on 7α-hydroxylase activity at concentrations of 10 μM. The inhibitory effect of metyrapone was additive with that of either 7β-hydroxycholesterol or proadifen; the effects of 7β-hydroxycholesterol and proadifen were not additive. These results suggest that proadifen and 7β-hydroxycholesterol interact with the same enzyme site while metyrapone binds at a different location. Proadifen inhibited 7α-hydroxylase irreversibly, while kinetic studies demonstrated noncompetitive inhibition by metyrapone (K_I = 0.55 mM) and competitive inhibition by 7β-hydroxycholesterol (K_I = 2.4 μM). The inhibition of 7α-hydroxylase activity by metyrapone and aminoglutethimide, drugs used to manage patients with excessive cortisol production, suggests that such treatment may also alter bile acid synthesis.—**Schwartz, M. A., and S. Margolis.** Effects of drugs and sterols on cholesterol 7α-hydroxylase activity in rat liver microsomes. *J. Lipid Res.* 1983. **24:** 28–33.

Supplementary key words proadifen • metyrapone • aminoglutethimide • ascorbic acid • 7α-hydroxycholesterol • 7β-hydroxycholesterol • 7-keto-cholesterol • 25-hydroxycholesterol • 5-cholenic acid-3β-ol

Cholesterol 7α-hydroxylase, a liver microsomal enzyme, catalyzes the rate-determining step in the synthesis of bile acids (1). One of the components of this mixed function oxidase is a cytochrome P-450 which binds both substrate and oxygen (1). Although multiple forms of cytochrome P-450 participate in the metabolism of various drugs by liver microsomes (2), several groups have reported the partial purification of a cytochrome P-450 with increased specificity for 7α-hydroxylation of cholesterol (3, 4). Metyrapone (5, 6), proadifen (SKF-525A) (7), and cimetidine (8) inhibit drug oxidation by liver microsomes. Moreover, metyrapone (9, 10), as well as aminoglutethimide (10, 11) and ascorbic acid (10, 12), inhibit mixed function oxidases that hydroxylate steroids in other rat tissues (adrenal cortex and testes). Since these agents have not been demonstrated to affect hepatic sterol metabolism, we investigated their effects on 7α-hydroxylase activity in rat liver microsomes.

Shefer, Hauser, and Mosbach (13) used different assay conditions than ours to show inhibition of cholesterol 7α-hydroxylase activity by some cholesterol derivatives and suggested the possibility of feedback regulation of bile acid synthesis. Therefore, we reexamined the inhibitory effects of several of the same steroid compounds as well as others. Preliminary experiments were performed to test the effects of pairs of inhibitory drugs and of these drugs in combination with inhibitory sterols in order to compare their sites of action. Conclusions drawn from these experiments were further supported by kinetic studies.

MATERIALS AND METHODS

Chemicals

[4-^{14}C]Cholesterol (59.4 mCi/mmol) was obtained from New England Nuclear, Boston, MA. L-Ascorbic acid, cholesterol, cholic, and lithocholic acids (sodium salts), Coomassie Blue G 250, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glutathione, β-mercaptoethylamine, NADP, rhodamine B, sucrose, Trizma Base, and Tween 80 were purchased from Sigma Chemical Co., St. Louis, MO. Betafluor was a product of National Diagnostics, Sommerville, NJ. EDTA, solvents (reagent grade), and other chemicals were obtained from J. T. Baker Chemical Co., Phillipsburg, NJ. TLC plates (0.25 mm, silica gel 60) were purchased from MCB Manufacturing, Cincinnati, OH.

Coprostanol, 5-cholenic acid-3β-ol, 7-ketocholesterol, 7α-, 7β-, and 25α-hydroxycholesterol were from Steraloids, Wilton, NH. Metyrapone was purchased from Aldrich Chemical Co., Milwaukee, WI. Aminoglutethimide was a gift of CIBA Pharmaceutical Co.,

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Summit, NJ. Hydrochlorides of cimetidine and proadifen (SKF-525A) were kindly supplied by Smith, Kline and French Labs, Philadelphia, PA.

Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratory, Wilmington, MA) had continuous access to water and Purina Rat Chow. Rats were exposed to a reversed light cycle (light from 4 PM to 4 AM and dark from 4 AM to 4 PM) for at least 1 week to ensure maximum enzyme activity at the time of killing (9–10 AM).

Preparation of microsomes

Endoplasmic reticulum was isolated as previously described (14) with the following exceptions. Livers were homogenized in buffer containing 225 mM sucrose, 25 mM Tris (pH 7.8), 5 mM glutathione, and 50 mM NaCl. Microsomes were washed once and resuspended and assayed in buffer containing 100 mM sucrose, 40 mM potassium phosphate (pH 7.2), and 50 mM KCl.

Liver microsomes, prepared as needed from 12 rats, were used in the cholesterol 7α-hydroxylase assay within 2 weeks after isolation. Microsomes were stored in multiple vials at −20°C and were thawed only once before use. Hydroxylase activity of frozen microsomes declined no more than 30% during storage. In most cases, when inhibition experiments with a particular drug or steroid compound were repeated, microsomes from different rats were employed; in no instance is all the data for an inhibitor based on results from one rat. The microsomes in the combined inhibitor study were obtained from one rat. A single batch of microsomes isolated from the livers of two rats was used in the kinetic studies.

Assay and isolation of product

Cholesterol 7α-hydroxylase activity was measured with minor modifications of the method by Goodwin, Cooper, and Margolis (15). Briefly, all incubations included 0.4 to 0.6 mg of microsomal protein, an NADP regenerating system, and 10 mM β-mercaptoethylamine in a final volume of 0.5 ml of assay buffer.

In the percent inhibition studies and combined inhibitor studies, washed microsomes were incubated with 10 μM purified [4-^{14}C]cholesterol, previously diluted to a specific activity of 15.2 mCi/mmol with unlabeled cholesterol, dissolved with 0.6 mg of Tween 80 detergent in assay buffer. In the kinetic studies the cholesterol concentration was varied from 12.5 to 225 μM; label was diluted to a specific activity of either 15.2 or 1.5 mCi/mmol; and substrate at all concentrations was dissolved with 1.0 mg of Tween 80. Except where otherwise noted, test drugs were added to the suspension buffer while sterols and bile acids were added to the mixture of labeled cholesterol and Tween 80.

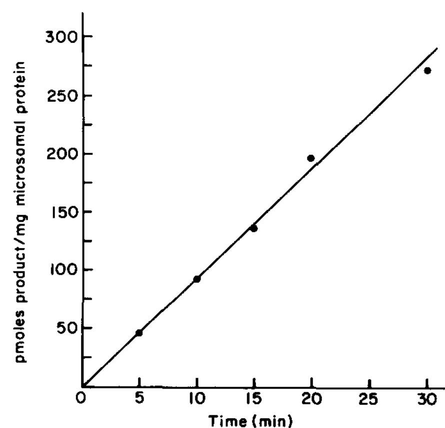

Fig. 1. Time course of cholesterol 7α-hydroxylase activity. Incubations were terminated at the indicated times using the assay conditions described in the Methods section. Each time point is the mean of triplicate assays. In this and subsequent figures pmoles product represents the amount of product formed from added substrate, calculated from the specific activity of the added cholesterol.

Blank values, determined by duplicating all assay conditions in the absence of NADP, were subtracted before comparing enzyme activities. Blank values averaged approximately 4% of values obtained in the presence of NADP.

Microsomal protein concentrations were determined by the method of Bradford (16) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

As shown in **Fig. 1,** the activity of cholesterol 7α-hydroxylase was linear for at least 30 min in the assay system used in these studies. A 30-min incubation period was employed in all subsequent experiments in order to test the effects of inhibitors on the initial rate of the reaction and at the same time to assure the formation of a sufficient quantity of product.

As shown in **Table 1,** aminoglutethimide and metyrapone inhibited 7α-hydroxylase activity by 45 and 67%, respectively, at concentrations of 1 mM, and by 27 and 19%, respectively, at concentrations of 0.1 mM. The effects of these agents on the rate-limiting step in bile acid synthesis is noteworthy for two reasons: *1*) both drugs are used to treat hyperadrenalcorticosteroidism, and *2*) we are unaware of other evidence indicating an inhibitory action of aminoglutethimide on cytochrome P-450 enzymes in rat liver. Ascorbic acid demonstrated comparable inhibitory effects only at concentrations one order of magnitude greater (40% at 10 mM and 18% at 1.0 mM) than metyrapone and aminoglutethimide. Proadifen was the most potent drug tested; it inhibited 7α-hydroxylase by 98 and 55% at concentrations of 1.0

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TABLE 1. Inhibition of cholesterol 7α-hydroxylase by aminoglutethimide, ascorbic acid, cimetidine, metyrapone, and proadifen (SKF-525A)

Addition	% Inhibition[a]		
	10 *mM*	1.0 *mM*	0.1 *mM*
Aminoglutethimide		45 ± 8.7 (3)[b]	27 ± 0.71 (2)[b]
Ascorbic acid	40 ± 8.2 (3)[b]	18 ± 8.1 (2)	
Cimetidine		−6.4 ± 17 (3)	9.1 ± 6.7 (2)
Metyrapone		67 ± 6.9 (3)[c]	19 ± 4.1 (3)[b]
Proadifen		98 ± 0.58 (3)[c]	55 ± 1.4 (2)[b]

[a] Each experiment was performed with triplicate incubations. The number of independent experiments is given in parentheses. All inhibitions are expressed as percent of product in control incubations and are given as the mean of independent experiments ±S.E. Control activities averaged 340 pmoles product per mg microsomal protein per 30 min.
[b] Significantly different from control with $P < 0.01$.
[c] Significantly different from control with $P < 0.005$.

and 0.1 mM, respectively. In separate experiments (data not shown), microsomes were incubated with 1.0 μM proadifen for 30 min, reisolated by centrifugation from a large volume of buffer, and resuspended. These reisolated microsomes demonstrated no cholesterol 7α-hydroxylase activity, supporting the finding that proadifen forms an irreversible inhibitory complex with cytochrome P-450 (17). Cimetidine, a histamine H_2-receptor antagonist, commonly used in the treatment of peptic ulcers, had no significant effect on cholesterol 7α-hydroxylase activity.

As shown in **Table 2,** 7β-hydroxy- and 7-ketocholesterol inhibited 7α-hydroxylase by 38 and 35%, respectively, at concentrations of 1 μM. 7α-Hydroxycholesterol, the enzyme product, was less potent but showed a substantial inhibition (35%) at 10 μM. No attempt was made to measure the extent of interconversion of these 7-substituted cholesterols during the assay. Since Aringer and Eneroth (18), employing similar assay conditions, detected substantial conversion of 7-ketocholesterol to the 7β-hydroxy derivative, it is possible that our observed inhibitions with 7-ketocholesterol were in part due to the presence of newly formed 7β-hydroxycholesterol.

TABLE 2. Inhibition of cholesterol 7α-hydroxylase by selected sterols and bile acids

Addition	% Inhibition[a]	
	10 *μM*	1.0 *μM*
7α-Hydroxycholesterol	35 ± 4.9 (3)[b]	6.3 ± 5.7 (3)
7β-Hydroxycholesterol	85 ± 3.0 (3)[c]	38 ± 2.9 (3)[d]
7-Ketocholesterol	83 ± 7.0 (3)[c]	35 ± 1.2 (3)[d]
25α-Hydroxycholesterol	32 ± 1.0 (3)[c]	13 ± 9.5 (3)
5-Cholenic acid-3β-ol	27 ± 6.0 (3)[b]	

No significant inhibition was found with 10 μM coprostanol (3), 10 μM sodium cholate (3), or 10 μM lithocholic acid (2). The number of independent experiments is given in parenthesis. Inhibitors were added with [4-^{14}C]cholesterol substrate solubilized with Tween 80.
[a] Percent inhibitions are expressed as in Table 1. Control activities averaged 280 pmoles product per mg protein per 30 min.
[b] Significantly different from control with $P < 0.025$.
[c] Significantly different from control with $P < 0.005$.
[d] Significantly different from control with $P < 0.001$.

Shefer, Hauser, and Mosbach (13) reported similar inhibitory effects of various sterols on the 7α-hydroxylation of labeled cholesterol and cholestanol, but their studies differed from ours in several important respects. Their concentrations of sterols and bile acid were approximately 40 times greater than the ones used in the present experiments. As a result, their ratio of inhibitory sterol to added substrate was 2:1, while ratios of 1:1 and 1:10 were used in our experiments. Therefore, the present results demonstrate more clearly the competitive potency of these sterol inhibitors. In addition, their studies were carried out in rats fed cholestyramine to induce increased 7α-hydroxylase activity (1, 13).

We also considered the possibility that the added sterols produced an apparent inhibition by altering the form of the Tween 80-cholesterol micelle rather than by exerting direct effects on the enzyme. The following experiment was performed to examine this question. Both 7β-hydroxycholesterol and sodium cholate were added to the assay mixture in two different ways: *1*) the sterol or bile acid was solubilized with the cholesterol substrate in Tween 80, and *2*) the sterol or bile acid was dissolved in a separate solution containing 10% (0.06 mg) of the standard amount of Tween 80 and added to the suspension buffer. Identical inhibitions of 40% were observed with 1 μM 7β-hydroxycholesterol under both conditions. If the 7β-hydroxycholesterol had merely interfered with the availability of substrate to the enzyme by altering the micellar structure of the detergent-cholesterol suspension, little or no inhibition would be expected when the 7β-hydroxycholesterol was added in micelles separated from the cholesterol micelles. Additionally, sodium cholate caused no appreciable inhibition when added under either of the above conditions

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or when added to the buffer alone (data not shown). These experiments demonstrate that the effect of 7β-hydroxycholesterol on enzyme activity is not influenced by its method of addition.

As shown in Table 2, 1 μM 7α-hydroxycholesterol inhibited 7α-hydroxylase by only 6%. Based on the specific activity of the cholesterol substrate and the cholesterol content of the microsomes, it can be estimated that the maximal amount of 7α-hydroxycholesterol formed during our incubations (1 nmole) produced a final product concentration of about 2 μM. Therefore, in agreement with the linear time course (Fig. 1), the amount of product formed during the assay was probably too small to inhibit enzyme activity significantly. Although 7β-hydroxy- and 7-ketocholesterol were potent inhibitors at a low concentration (1 μM), these sterols probably were not inhibitory during the assay since it is estimated that the combined final concentrations of the two did not exceed 0.1 μM. It is unlikely that these sterols play a direct physiological role as product inhibitors because their production was the same whether NADPH was included in the assays or not (data not shown). However, the possibility of a regulatory function for these 7-modified sterols can not be dismissed without further information on the extent of formation and accumulation of 7β-hydroxy- and 7-ketocholesterol in vivo. It is worth noting that rats synthesize bile acids that contain 7β-hydroxy groups (19); however, no studies have been carried out to determine whether intermediates in the biosynthesis of these bile acids can bind to and inhibit 7α-hydroxylase activity. Interestingly, in rat hepatocytes, 7α-hydroxy- and 7-ketocholesterol inhibit 3-hydroxy-3-methyl glutaryl CoA reductase (HMG CoA reductase) (20), the rate-limiting enzyme in cholesterogenesis (21). Therefore, it is possible that accumulated 7α-hydroxycholesterol, and less likely 7-ketocholesterol, may inhibit both 7α-hydroxylase and HMG CoA reductase activity, thereby decreasing the overall production of bile acids in vivo.

The reduction of 7α-hydroxylase activity by low concentrations (10 μM) of 25-hydroxycholesterol and 5-cholenic acid-3β-ol (Table 2) demonstrates that a substitution at the 7-position is not required for an inhibitory effect on the enzyme; however, the mechanism of inhibition is unclear. Boyd et al. (22) demonstrated that cholesterol analogs with shortened or altered (β-sitosterol) side chains could not act as substrates for 7α-hydroxylase and proposed a specific apolar binding site on the enzyme for the iso-octane side chain of cholesterol. The mechanism of inhibition by 25-hydroxycholesterol and 5-cholenic acid-3β-ol must be ascertained, and demonstrated to be competitive, in order to disprove the presence of an apolar side-chain binding site for substrate.

TABLE 3. Effect of various combinations of inhibitors on the activity of cholesterol 7α-hydroxylase

Experiment	Additions	Concentration	Inhibition
		μM	%
1	7β-Hydroxycholesterol	0.5	28
	Metyrapone	100	23
	7β-Hydroxycholesterol	0.5	57
	+ Metyrapone	100	
2	7β-Hydroxycholesterol	0.5	22
	Proadifen	50	26
	Metyrapone	100	18
	7β-Hydroxycholesterol	0.5	19
	+ Proadifen	50	
	Proadifen	50	45
	+ Metyrapone	100	

7β-Hydroxycholesterol was added to the incubation assay along with substrate cholesterol (10 μM) in Tween 80. Metyrapone and proadifen were added in assay buffer. All incubations were carried out in triplicate. Control values averaged 280 and 275 pmoles product per mg microsomal protein per 30 min for experiments 1 and 2, respectively.

The inhibition by 5-cholenic acid-3β-ol is particularly interesting since the other 24-carboxylated cholesterol derivatives tested, cholic and lithocholic acid, were not inhibitory. Besides having opposite orientations of the 3-hydroxy group, 5-cholenic acid-3β-ol and lithocholic acid (3α-hydroxy substituted) differ only in their A/B ring fusion. The ring orientation of cholesterol and 5-cholenic acid-3β-ol are geometrically similar to that of cholestanol, which has *trans* A/B fusion, and differs from coprostanol (5β-cholestan-3β-ol), lithocholic, and other bile acids that have *cis* A/B ring fusion. Therefore, it was not surprising that coprostanol produced no significant inhibition at the concentration tested (10 μM). The 7α-hydroxylase enzyme may have low affinity for a steroid nucleus that has *cis* A/B ring fusion and/or is 3α-hydroxy substituted. The need to add larger amounts of such steroid compounds to obtain significant interactions with the enzyme may explain the results of Shefer et al. (13), who detected inhibition of 7α-hydroxylase activity when they used 40 times the lithocholic acid concentration (10 μM) employed in the present studies. Conversely, a compound with the same ring orientation and ring substitutions as cholesterol (such as 5-cholenic acid-3β-ol) may have higher affinity for the enzyme. However, this conclusion needs to be substantiated through K_I determination of several steroids with the required ring features.

In an initial experiment to compare the sites of action of the sterol inhibitor 7β-hydroxycholesterol and the two most potent drugs tested, metyrapone and proadifen, we assayed the effects of various combinations of these compounds (**Table 3**). When metyrapone was added in combination with either 7β-hydroxycholesterol or proadifen, the inhibitory effects were roughly

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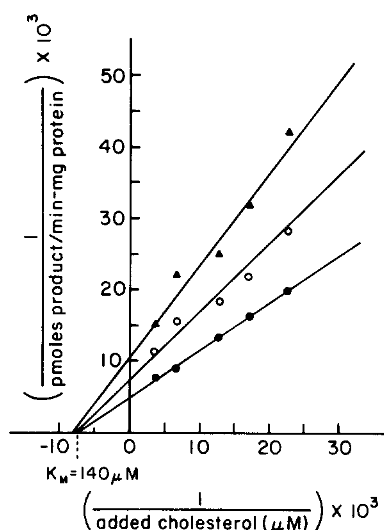

Fig. 2. Lineweaver-Burk plots showing the inhibitory effects of metyrapone on cholesterol 7α-hydroxylase activity. Reciprocal reaction velocity was plotted against reciprocal concentration of added [4-^{14}C]cholesterol in the presence of 0.75 μM (▲), 0.25 μM (○), or no (●) metyrapone.

the sums of each agent assayed separately (Table 3). When proadifen and 7β-hydroxycholesterol were incubated together, their combined effects (19% inhibition) were approximately equal to their separate inhibitory effects (27% and 22%, respectively). These results suggested that proadifen and the sterol inhibitor interact with the same enzyme site while metyrapone probably has a different site of action.

Since proadifen had been established as an irreversible inhibitor of 7α-hydroxylase (see above), no kinetic studies with it were attempted. Kinetic studies performed with metyrapone and 7β-hydroxycholesterol

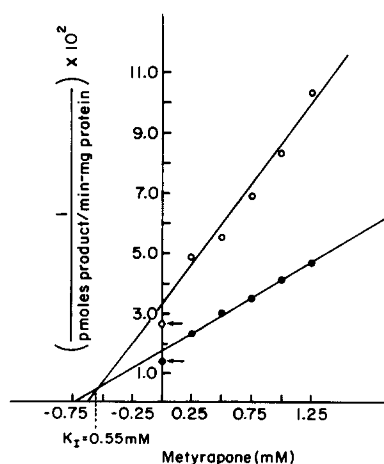

Fig. 3. Dixon plot of the inhibition of cholesterol 7α-hydroxylase by metyrapone. Reciprocal reaction velocity was plotted against metyrapone concentration in the presence of 50 μM (●) or 25 μM (○) added [4-^{14}C]cholesterol. Arrows mark the reciprocal of reaction velocity in the absence of inhibitor.

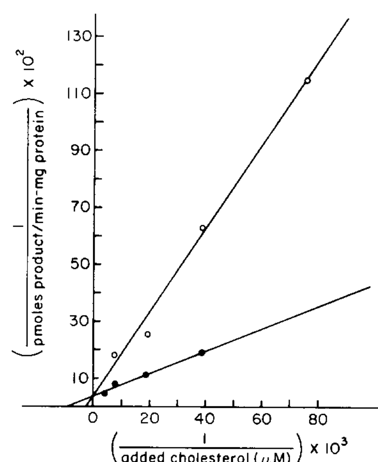

Fig. 4. Lineweaver-Burk plots showing the inhibitory effects of 7β-hydroxycholesterol on 7α-hydroxylase activity. Reciprocal reaction velocity was plotted against reciprocal concentration of added [4-^{14}C]cholesterol in the presence of 5 μM (●) or 20 μM (○) 7β-hydroxycholesterol. V_{max} = 26 pmoles product/mg protein min.

confirmed their separate mechanisms of inhibition. As shown in **Fig. 2,** the fact that the K_m value of 140 μM for 7α-hydroxylase was virtually unchanged in the presence of metyrapone demonstrates a noncompetitive mechanism of inhibition. The K_I value for metyrapone was 0.55 mM (**Fig. 3**). Although the intersection of the lines above the X-axis suggests a slight competitive component, the values obtained in the absence of inhibitor at both substrate concentrations are distinctly below the intersection point of the regression lines with the y-axis. These results indicate that the mechanism of metyrapone inhibition is predominantly noncompetitive.

From their spectroscopic work with drug-metabolizing, mixed function oxidases, Netter, Kahl, and Magnussen (5) have proposed a cytochrome P-450 model with two binding sites, one for substrate and another for molecular oxygen. For drug-metabolizing cytochrome P-450's, the evidence indicates that proadifen (5) and cimetidine (8) interact with the substrate site while metyrapone binds at the O_2 site. For cholesterol 7α-hydroxylase, although we found no inhibition with cimetidine and did no experiments to confirm the site of proadifen action, our results suggest a similar mechanism of 7α-hydroxylase inhibition by metyrapone.

Fig. 4 and **Fig. 5** clearly demonstrate competitive inhibition of 7α-hydroxylase by 7β-hydroxycholesterol. The V_{max}-value of 26 pmoles product/mg protein per min (y-intercept in Fig. 4) was independent of the presence of 7β-hydroxycholesterol, while the apparent K_m increased with increasing inhibitor concentration. In the Dixon plot of the inhibition of cholesterol 7α-hydroxylation by 7β-hydroxycholesterol (Fig. 5), the regression lines drawn for the two cholesterol concentrations intercept above the X-axis, and the reciprocal activity val-

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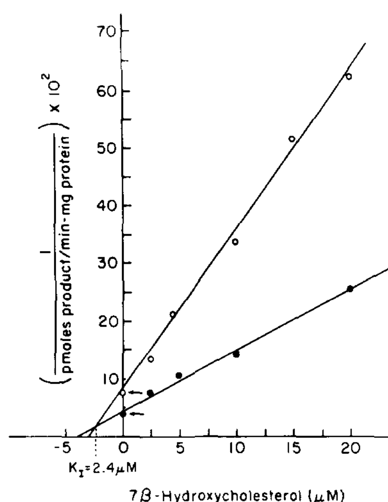

Fig. 5. Dixon plot of the inhibition of cholesterol 7α-hydroxylase by 7β-hydroxycholesterol. Reciprocal reaction velocity was plotted against 7β-hydroxycholesterol concentration in the presence of 50 μM (●) or 25 μM (○) added [4-^{14}C]cholesterol. Arrows mark the reciprocal of reaction velocity in the absence of inhibitor.

ues obtained in the absence of inhibitor were close to the y-intercepts of the respective lines. These findings indicate competitive inhibition. The low K_I value (2.4 μM), compared to the K_m value (140 μM) determined for cholesterol as substrate, demonstrated the great inhibitory effectiveness of 7β-hydroxycholesterol.

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