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REGULATION OF BILE ACID SYNTHESIS
Isolation and Characterization of Microsomal Phosphatases

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SUMMARY: In preliminary experiments, we have shown that rat liver microsomes possess phosphatase activity which was inhibited in the presence of sodium fluoride. We have now separated six microsomal phosphatase fractions appearing to be isoenzymes. They all possess different kinetic constants and are not equally inhibited by tartrate and fluoride ions, inhibitors of phosphatase activity. One phosphatase fraction, in fact, is almost completely unaffected by fluoride ion. More pertinent to our interest, these isoenzymes exhibit differing abilities to modulate the activities of hydroxymethylglutaryl CoA reductase, acyl-CoA:cholesterol O-acetyltransferase, and cholesterol 7α-hydroxylase. Interaction of four of the fractions with rat liver microsomes resulted in a decrease in cholesterol 7 α-hydroxylase activity; two were without effect.

Cholesterol 7 α-hydroxylase, a membrane-bound microsomal mixed function oxidase, is the rate-limiting enzyme in bile acid biosynthesis (1). Sanghvi et al. (2) have proposed that this enzyme might be subject to short-term regulation by phosphorylation/dephosphorylation, with the active form being the phosphorylated species. This proposal has been supported by Goodwin et al. (3), and more recently by Tang and Chiang (4), who used a system reconstructed from highly purified cytochrome P-450 and NADPH cytochrome P-450 reductase.

Gil et al. and Sitges et al. (5,6) have shown that rat liver microsomes have separable phosphatases capable of activating HMG-CoA reductase, the major regulator of cholesterol biosynthesis. Whether they also affect cholesterol 7α-hydroxylase and ACAT activities is not known.

Abbrevations: Hydroxymethylglutaryl CoA reductase: HMG-CoA reductase
Acyl-CoA:cholesterol O-acetyltransferase: ACAT
4-Methylumbelliferyl phosphatase: 4 MU phosphate

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In the present study, we have separated six microsomal phosphatase fractions and describe here some of their kinetic properties which suggest them to be distinct moieties. Studies of interaction of these phosphatases with HMG-CoA reductase, ACAT, and cholesterol 7α-hydroxylase as substrates are also presented.

Methods

Materials. Male Sprague-Dawley rats (150-220 g) which had been maintained on a strict reverse light/dark cycle (1500-0300 h light) for two weeks before sacrifice were used throughout. All chemicals were of highest purity grade available and were obtained from Fisher, Sigma and Pharmacia. The basic buffer system throughout these studies was 25 mM potassium phosphate, 5 mM dithiothreitol, 1 mM disodium ethylenediamine, tetraacetate, pH 7.4. Additions were made to this buffer where indicated.

Enzyme assays. For convenience, the routine assay of phosphatase activity in the isolated fractions from rat liver microsomes and preliminary characterization of these fractions was done using 4-methylumbelliferyl phosphate as substrate, as described by Peters et al. (7). HMG-CoA reductase activity was determined by measuring the conversion of [^{3}H] HMG-CoA to [^{3}H] mevalonic acid as described by Sanghvi et al. (8). The mevalonolactone was isolated using thin-layer chromatography according to Sanghvi and Parikh (9). ACAT activity was measured by the procedure of Gavey et al. (10, 11) using [1-^{14}C] oleoyl-CoA as substrate. Cholesterol 7α-hydroxylase activity was determined by the GC/MS procedure of Sanghvi et al. (12, 13). Protein determinations were by the method of Bradford (14), using bovine γ-globulin as standard.

Extraction of microsomal phosphatase. Rat liver microsomes (microsomal fraction) were prepared as described previously (12). The microsomal pellet was suspended in the phosphate buffer containing 2% sodium cholate, stirred for 1 hour at 4°C, and then centrifuged at 300,000 x g for 20 minutes. The supernatant was dialyzed against buffer, 1:40, with three changes of buffer over an 18-hour period and was then centrifuged at 300,000 x g for 20 minutes. The supernatant thus obtained was loaded on a DEAE A-50 Sephadex column, 15 cm x 5 cm, which had previously been equilibrated with the buffer. The column was washed with buffer and 7 ml-fractions were collected and analyzed for protein and phosphatase activity. When phosphatase activity returned to base line, an 800-ml linear KCl gradient of 0.0 to 1.0 M was applied to the column, followed by an additional 100 ml of 1.0 M KCl. Fractions containing phosphatase activity were combined.

The fraction not retained on DEAE (breakthrough peak) was concentrated by ammonium sulfate precipitation at 70% saturation. The precipitate was dissolved in a minimal amount of buffer, dialyzed 1:10 (v/v), with two changes of buffer over a 16-hour period, and was then applied to a G-200 Sephadex column, 2.6 cm x 90 cm, which had been equilibrated with buffer. Fractions (6 ml) were collected and phosphatase activity determined.

Three peaks, A, B and C, eluting from the DEAE column, were individually combined and dialyzed against the buffer, 1:25, with two buffer changes over a 16-hour period, and then applied individually to a G-200 Sephadex column, 2.6 cm x 90 cm, which was topped with a DEAE A-50 Sephadex layer of 2.0 cm x 2.6 cm. Phosphatases retained on the DEAE Sephadex layer were eluted with 30 ml buffer containing 1.0 M KCl and the elution was continued with buffer. Phosphatase activity in all fractions was measured and those forming peaks of activity were combined. These peaks were concentrated to a volume of 2.0 ml using vacuum dialysis.

Effect of isolated phosphatase fractions on microsomal HMG-CoA reductase, cholesterol 7α-hydroxylase, and ACAT activities. Microsomes (0.3 - 0.5 mg protein) were preincubated in the presence of a phosphatase fraction for 60 minutes at 37°. The

activities of HMG-CoA reductase, cholesterol 7α-hydroxylase, and ACAT were then determined after the addition of appropriate cofactors and substrates and initiation of the reaction, and compared to control incubations in the absence of any added phosphatase.

Results and Discussion

Separation of phosphatase isoenzymes. Chromatography on DEAE Sephadex resulted in four peaks of phosphatase activity labeled BT, A, B, C in Fig. 1a. All were individually subjected to chromatography on Sephadex G-200 where BT, A and C gave single symmetrical peaks of activity. Chromatography of B (Fig. 1b) on Sephadex produced three peaks of phosphatase activity labeled B1, B2, B3).

Kinetic studies. Substrate saturation studies were performed on the purified fractions BT, A, B1, B2 and B3 phosphatases, using 4-methylumbelliferyl phosphate as the substrate. Kinetic constants determined from these experiments are listed in Table 1. The K_m values for the BT and B3 fractions are very similar (0.25 and 0.3 mM, respectively), while the values for the A, B1 and B2 fractions are 6- to 14-fold greater. These differences suggest that the isolated fractions represent distinct isoenzyme moieties.

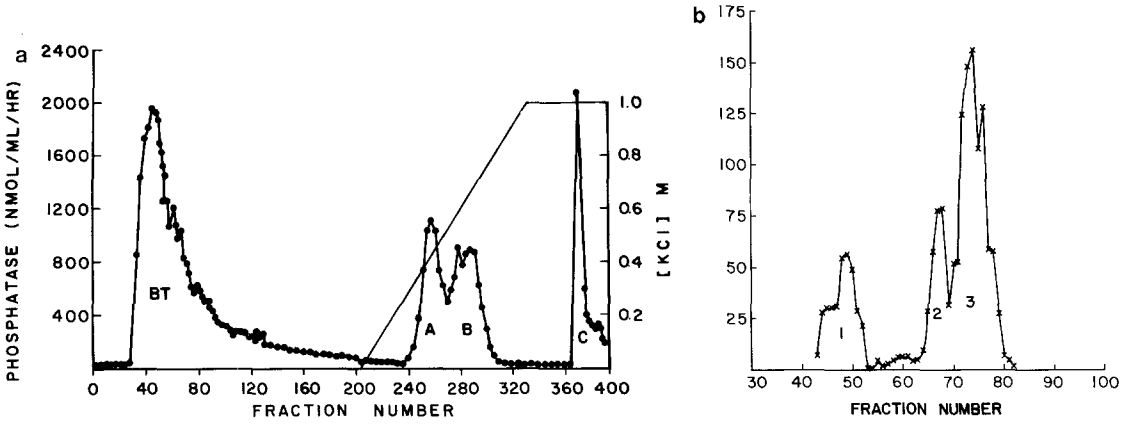

Fig. 1a. DEAE Sephadex chromatography of dialyzed microsomal cholate extract. A DEAE column (50 x 150 mm) was equilibrated with 25 mM phosphate buffer, pH 7.4. Sample was loaded and the column washed with the same buffer. At fraction 80, a linear 0-1 M KCl gradient in the same buffer was applied. The column was finally eluted with 1 M KCl in buffer. Fractions of approximately 7 ml were collected and assayed for phosphatase activity. The breakthrough (BT) was combined and concentrated by precipitation with ammonium sulfate. Fractions A, B and C were individually combined and dialyzed against 25 mM phosphate buffer.

Fig. 1b. Sephadex G-200 chromatography of DEAE fraction B. Fraction B was loaded on a Sephadex G-200 column equipped with a DEAE Sephadex plug. The sample was eluted from the plug with 1 M KCl in 25 mM phosphate buffer, pH 7.4, and the column eluted with the buffer alone. Fractions were collected and analyzed for phosphatase activity. Fractions containing activity, B1, B2 and B3 were combined and assayed.

Table 1. Kinetic Parameters

Phosphatase Fraction	Km (mM)	V (nmol/hr/mg Pro)	% Inhibition at 10 mM Inhibitor	
			Tartrate	NaF
BT	0.25	427	97	84
A	2.2	3880	100	89
B1	3.5	4730	81	89
B2	1.4	795	70	63
B3	0.23	1350	52	15

Kinetic constants were determined from the slope and intercept of double reciprocal plots of velocity vs substrate concentrations using 4-methylumbelliferyl phosphate as substrate.

Sodium fluoride and L (+) tartaric acid, which are known phosphatase inhibitors, were investigated for their effect on these microsomal phosphatases and the data are shown in Table 1. At a concentration of 10 mM for both inhibitors, phosphatase A and BT activities were inhibited almost totally, while B3 is much less inhibited by tartrate and was relatively unaffected by sodium fluoride. Fractions B1 and B2 exhibit intermediate levels of response to these inhibitors.

Effect of phosphatase fractions on cholesterol 7 α -hydroxylase, HMG-CoA reductase and ACAT activities. The ability of the phosphatase fractions to modify the activities of the three enzymes of cholesterol metabolism, cholesterol 7 α-hydroxylase, HMG-CoA reductase and ACAT, was examined using rat liver microsomes as a source of the three enzymes. Preincubation of microsomes was carried out at 37°C in the presence and absence of an added phosphatase fraction. The amount of phosphatase added was expressed in terms of 4 MU phosphatase units of activity, and the results of these experiments are presented as the number of 4 MU phosphatase units calculated to be necessary to produce either a 100% activation (HMG-CoA) or a 50% loss (ACAT and cholesterol 7α-hydroxylase) of activity (Table 2). The following observations appear pertinent concerning the data in Table 2:

(a) Levels of microsomal phosphatase activities required to stimulate or inhibit substrate enzyme activities are:

Table 2. Phosphatase necessary to produce 50% inhibition or 100% activation*

Fraction	HMG-CoA Reductase	ACAT	Cholesterol 7α-Hydroxylase
BT	2500	8750	NC[a]
A	200	860	1150
B1	NC[b]	5000	1600
B2	38	1880	326
B3	14	ND[c]	NC[d]
C	260	1070	790

* Number of phosphatase units calculated to be necessary to produce 100% activation (HMG-CoA reductase) or 50% inactivation (ACAT and cholesterol 7α-hydroxylase) relative to control enzyme activities.
[a]NC No change at 2500 units
[b]NC No change at 600 units
[c]ND Not determined
[d]NC No change at 1300 units

ACAT ▷ Cholesterol 7α-hydroxylase ▷ HMG-CoA reductase

(b) B3 phosphatase is most effective with HMG-CoA reductase, requiring only 14 units of phosphatase for a doubling of activity, but is without effect on cholesterol 7α-hydroxylase. This provides a clear cut segregation of substrate specificity.

(c) B1 phosphatase is most effective with cholesterol 7α-hydroxylase but has no effect on HMG-CoA reductase.

(d) BT fraction, which has the major proportion of microsomal phosphatase activity, has no effect on cholesterol 7 α-hydroxylase; but also, relatively much larger amounts of this fraction are required to alter HMG-CoA reductase and ACAT activities.

These observations demonstrate that these rat liver microsomal phosphatase fractions are able to modulate the activities of cholesterol 7α-hydroxylase, ACAT and HMG-CoA reductase in vitro and, secondly, that they exhibit distinct separation of substrate specificities. Throughout these studies we have been impressed by the extremely small amounts of phosphatase which elicit increases of several fold in HMG-CoA reductase activity. Comparatively, changes in ACAT and cholesterol 7α-hydroxylase activities are

more modest. It may be that relatively exaggerated responses of HMG-CoA reductase are dictated by the cellular need for cholesterol in several biochemical pathways. In this respect, ACAT and cholesterol 7α-hydroxylase are single pathway enzymes.

Regulation of HMG-CoA reductase activity by means of phosphorylation/dephosphorylation is well established. The fact that the phosphatase fractions studied in these experiments are able to bring about very significant changes in its activity strongly imply that they are phosphoprotein phosphatases. There is also data suggesting that ACAT is regulated by means of changes in its state of phosphorylation, with the phosphorylated species being the active moiety (11). Recent investigations on the mechanism of regulation of cholesterol 7α-hydroxylase activity also suggest regulation by phosphorylation/dephosphorylation (2,3,15). It is not known if this is a direct or an indirect effect.

More recently (4), investigations which utilized a reconstituted cholesterol 7α-hydroxylating system consisting of purified cytochrome P-450 and cytochrome C reductase have shown that its activity could be modulated by phosphorylation/dephosphorylation when the system included a phospholipid. Specifically, the reconstituted enzyme system lost its 7α-hydroxylating ability by incubations with E. coli alkaline phosphatase, but this ability was restored in the presence of phosphate and a cAMP-dependent kinase. The specific role of added phospholipid in the system which allows this modulation to occur is not clear.

The data in this paper which shows the presence in microsomes of several phosphatases with varying susceptibilities to different inhibitors and possessing distinct substrate specificities further adds to the notion that like HMG-CoA reductase and ACAT, cholesterol 7α-hydroxylase activity may also be regulated by phosphorylation/dephosphorylation.

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