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A study of the effect of D- and L-triiodothyronine on bile acid excretion of rats*

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SUMMARY

The effect of D- and L-triiodothyronine on bile acid patterns in rats was studied by means of gas-liquid chromatography. Treatment with either D- or L-triiodothyronine caused a relative decrease of cholate and a relative increase of chenodesoxycholate. This is in gross agreement with the results obtained by means of paper chromatography recently reported by Strand. In addition, the present study shows that the above treatment caused a relative increase in a bile acid component suspected to be a metabolite of chenodesoxycholic acid. Preliminary data tend to indicate that intestinal absorption of cholesterol was poorer in D-triiodothyronine-treated rats than in control animals.

The influence of thyroid activity on the excretion of bile acids has been studied, particularly with regard to its relationship to cholesterol metabolism. Thompson and Vars (1), using a photometric method for the assay of bile acids, observed a lowering of biliary excretion of cholate in thyroid-fed, bile-fistula rats. By means of paper chromatography, Eriksson (2) showed that the thyroid-induced decrease in cholate in rat fistula bile was accompanied by a marked increase in chenodesoxycholate. Recently Strand (3), employing a similar technique, demonstrated the same type of changes in rats as a result of treatment with D(−)3:5:3′-triiodothyronine (D-T_3) and L(+)3:5:3′-triiodothyronine (L-T_3).

The recent development of gas-liquid chromatography (GLC) has provided a means for more critical investigations of mixtures of sterols and bile acids (4–8). Blomstrand (9) used this technique to ascertain patterns of bile acids in bile from cholelithiasis patients. The present report describes a gas-liquid chromatographic study of the changes in patterns of bile acids in rats following subcutaneous treatment with D-T_3 and L-T_3.

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EXPERIMENTAL METHODS

Treatment of Animals. Since it was the ultimate purpose of the present study to correlate changes in patterns of bile acids with cholesterol metabolism, a daily dose of 60 μg/kg of D-T_3 was chosen. This dose previously was shown to elicit a lowering of plasma cholesterol and a slight calorigenic effect in rats (10). Although definite changes in bile acids were observed initially in some rats at this dose level, a larger dose (500 μg/kg) was selected in order to insure a more consistent and marked response.

Adult male rats from the Carworth Farms (CFN strain), weighing 250–280 g, were used. Ten rats received daily subcutaneous injections of 500 μg/kg of D-T_3 in alkaline saline (pH 8.5–9.5) for 7 days prior to cannulation of the bile duct. A second group of 10 rats served as controls and received daily injections of the alkaline saline vehicle. The treated animals showed no apparent signs of toxicity and had an average daily weight gain of 4.1 g as compared to 4.9 g for the controls. On the 8th day of treatment, five rats, selected from each group on the basis of the average gain for the group, were anesthetized and their bile ducts cannulated with PE-10 polyethylene tubing. The animals were maintained on Purina Laboratory Chow and a 0.9% saline solution containing 5% glucose. Daily treatment with D-T_3 was continued during bile collection.

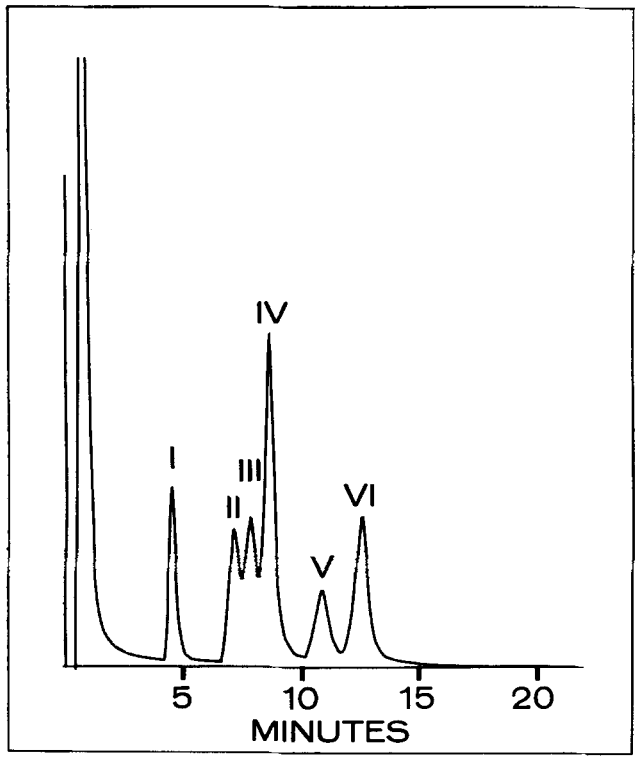

FIG. 1. GLC separation of bile acids as their methyl esters: I = lithocholate, II = desoxycholate, III = chenodesoxycholate, IV = hyodesoxycholate, V = dehydrocholate, and VI = cholate.

Bile was collected in ethanol for periods of 0–6, 6–24, and 24–48 hr.

In a second type of experiment, rats were not pretreated but, rather, their bile ducts were cannulated and control bile was collected for 2 days prior to treatment. Starting on the third day, the animals received subcutaneous injections of 70 μg/kg of L-T_3 daily for 4 days; the drug was then withdrawn. Daily collection of fistula bile was continued throughout the experiment.

Extraction of Bile Acids. Bile was deproteinized and the bile acids were extracted by an adaptation of the procedure reported by Portman, *et al.* (11). Bile proteins were precipitated with 5 volumes of 95% ethanol and removed by centrifugation.[1] The protein precipitates were washed with additional ethanol to extract any residual bile acids. The volume of the combined supernatant and washings was reduced to approximately one-third in a flash evaporator. Aliquots from the concentrated solutions were extracted twice with equal volumes of petroleum ether (bp 30–60°) to remove neutral lipids. These aliquots were then acidified with 3 N HCl and further extracted with petroleum ether to remove fatty acids. The acidic alcohol phase was then evaporated under reduced pressure. The residues were dissolved in 5% NaOH and hydrolyzed in sealed thick-walled Carius tubes at 120° for 3 hr (12). The hydrolysates were cooled, acidified with 3 N HCl, and extracted twice with equal volumes of ethyl ether. The ether extracts were washed to remove chlorides and dried with anhydrous Na_2SO_4. The bile acids, in ether,[2] were then methylated with freshly prepared diazomethane (13). The reaction was allowed to proceed until a persistent excess of the reagent was attained. Ether was then removed by evaporation under nitrogen. Spot checks of such reaction mixtures by thin-layer chromatography indicated that methylation was practically complete.

Gas-Liquid Chromatography. The methyl esters of the bile acids were taken up in acetone and chromatographed in a Barber-Colman Model 10 gas chromatograph equipped with a sapphire-insulated Sr^{90} ionization detector.[3] The cell was operated at 1000 volts and the amplifier at 1×10^{-7} amp. A 4-ft x 2.5-mm i.d. column packed with 0.72% General Electric silicone rubber SE-52[4] coated on washed Gas-chrom (100–140 mesh) was used (6). The flash heater was maintained at 312°, the column at 250°, and the detector at 283°; the argon flow rate was 17 ml/min, the gas pressure 30 psi.

Since unpublished results from previous experiments in this laboratory indicated that treatment with D-T_3 tended to produce only slight changes in total production of bile acids, the emphasis in this study was placed on the change in pattern of bile acids. No attempt was made to measure the total production of bile acids. Furthermore, aliquots used for gas chromatographic analysis did not necessarily represent the same portions of the total bile samples excreted during any particular collection period. The areas associated with different peaks on the gas-chromatograph recording were estimated by the method of Bartlet and Smith (14). The authors are cognizant of the fact that the gas chromatographic responses differ with individual bile acids and do not necessarily bear a simple linear relationship to the quantities of bile acids being chromatographed. In agreement with the findings of Sweeley and Chang (15) and Bloomfield (8), the authors

[1] This procedure appeared to be adequate for the extraction of bile acids and elimination of interference from bile proteins. In work subsequent to this study, precipitation of protein was facilitated by the use of 5 volumes of absolute ethanol, and warming.

[2] In subsequent work, methylation was facilitated by the use of a mixture of ethyl ether–methanol 9:1 as suggested by Schlenk and Gellerman (27).

[3] Barber-Colman Catalog No. A-4147B.

[4] SE-52 contains 5 moles % diphenylsiloxane units in a copolymer with dimethylsiloxane.

also observed that the detector (Sr^{90}) response decreased as the polarity of bile acids increased, for example, from lithocholate to chenodesoxycholate to cholate. Hence the ratio of the areas measured for two bile acids, such as cholate and chenodesoxycholate, does not represent directly the ratio of the absolute quantities of these two bile acids. Nevertheless, in the present report, the ratios of the estimated areas serve to demonstrate alterations in the relative amounts of bile acids as a result of D- and L-T_3 treatment. The direct quantification of bile acids by gas-liquid chromatography is currently under investigation with the aim of applying it to similar studies of bile acid in the future.

RESULTS AND DISCUSSION

Fig. 1 illustrates the separation of six reference bile acids as their methyl esters. It has been found that the resolution of the dihydroxy bile acids can be improved by decreasing the column temperature; however, some skewing of the cholic acid peak is then encountered.

Representative chromatograms of the methyl esters of bile acids from controls and animals treated with D-T_3 prior to, and following, cannulation of the bile duct are shown in Fig. 2. The presence of peaks with the same retention times as those of reference desoxycholate, chenodesoxycholate, hyodesoxycholate, and cholate, respectively (Fig. 1), was confirmed by gas-liquid chromatography using QF-1[5] and a modification of SE-52[6] separately as stationary phases. Further confirmation was obtained by thin-layer chromatography on silica gel with a toluene–methanol–ethyl acetate 85:10:5 solvent system. In the D-T_3-treated rats, there appeared to be a slight trend toward a relative increase in chenodesoxycholate (peak III) during the 0- to 6-hr period. In later collection periods, as the residual effects of the enterohepatic circulation wore off, a progressive relative increase in chenodesoxycholate occurred in D-T_3-treated animals, resulting in a marked decrease in the peak area ratio cholate/chenodesoxycholate (VI/III). A pronounced reversal of this ratio was observed in the treated rats during the 24- to 48-hr period when compared to the controls. The above shift in ratio is in agreement with the observations made by other investigators using various thyromimetic preparations. Thus Strand (3) showed that treatment

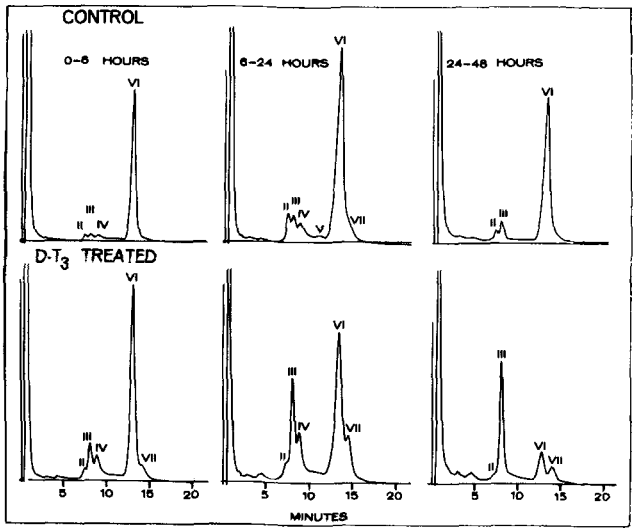

FIG. 2. Patterns of bile acids of control and D-T_3-treated rats. The designations of the peaks of methyl esters of bile acids correspond to those in Fig. 1; in addition, VII = component X.

of both male and female rats with multiple daily doses of either 40 μg D-T_3/kg, 40 μg L-T_3/kg, or 400 μg D-T_3/kg, increased the level of chenodesoxycholate in fistula bile from a normal value of 30% to 60–80% of the total bile acids. Besides a shift in relation to the level of cholate, this represented a quantitative increase of chenodesoxycholate since he found that total bile acid excretion in his treated groups was roughly the same as in his control groups.

In addition to the above findings, an unidentified component, designated X, was noted in the present study, particularly in the bile of the D-T_3-treated animals (peak VII). With D-T_3 treatment, this component also increased concomitantly with a decrease in cholate. Comparative chromatographic investigations, using three different stationary phases (QF-1[5], SE-52, and a modification of SE-52[6]), showed component X to have the same retention time as α-muricholic acid, also referred to as Acid II (3α, 6β, 7α-trihydroxycholanic acid). The results also indicated that component X was most probably not one of the epimeric 3,6,7-trihydroxycholanic acids, namely, hyocholic, β-muricholic, and ω-muricholic acids. Acid II has been reported by Matschiner and Hsia and their coworkers (16, 17) to be a constituent of rat bile and a metabolite of chenodesoxycholate. Further attempts to identify this component are in progress.

It is notable that, while the alteration of the pattern of bile acids in the D-T_3-treated group during the first 24 hr following establishment of the bile duct fistula was only slight, the relative increase of chenodesoxycholate and component X in this group became much

[5] DC QF-1 (FS-1265), a fluoro silicone manufactured by Dow Corning Corp. and supplied by Applied Science Laboratories, Inc.

[6] GE No. 191-203, kindly supplied by Dr. A. C. Martellock of the Silicone Products Dept., General Electric Co. It contains 35 moles % of diphenylsiloxane.

TABLE 1. RATIOS OF GLC PEAK AREAS FOR CHOLATE, CHENODESOXYCHOLATE, AND COMPONENT X IN BILE OF CONTROL AND D-T_3-TREATED RATS AT VARIOUS TIMES AFTER PRODUCTION OF BILE FISTULA

	Cholate/Chenodesoxycholate/Component X		
	0–6 hr	6–24 hr	24–48 hr
Control rats	9.98:1:0.94	4.12:1:0.54	14.08:1:0.00
	5.13:1:0.51	7.61:1:0.56	9.76:1:0.00
	7.78:1:0.38	5.84:1:0.74	17.32:1:0.00
	6.63:1:0.55	4.58:1:0.55	19.70:1:0.00
	6.58:1:0.53	2.34:1:0.41	8.66:1:0.00
Averages	7.24:1:0.58	4.90:1:0.56	13.90:1:0.00
D-T_3-treated rats	7.11:1:0.92	3.68:1:0.69	1.23:1:0.26
	4.82:1:0.52	2.27:1:0.65	0.46:1:0.28
	4.63:1:0.71	...	0.05:1:0.00
	3.47:1:1.67	4.64:1:1.72	...
	9.06:1:0.97	...	1.51:1:0.41
Averages	5.82:1:0.98	3.52:1:1.02	0.81:1:0.24

more pronounced in the 24-to 48-hr period (Table 1). The difference between the treated and control groups in the ratios cholate/chenodesoxycholate/component X determined by estimations of peak areas is evident despite individual variations within each group. Thus, the average ratio cholate/chenodesoxycholate/component X in this period was 0.81/1/0.24 for the treated in contrast to 13.90/1/0 for the control. The pattern for the 0- to 24-hr period was influenced by residual effects of enterohepatic circulation as evidenced by the presence of appreciable amounts of a known intestinal metabolite of cholate, namely, desoxycholate (18). Generally, this bile acid and hyodesoxycholate (peak IV) were not present in bile samples collected in later periods. It follows, therefore, that the pattern for the

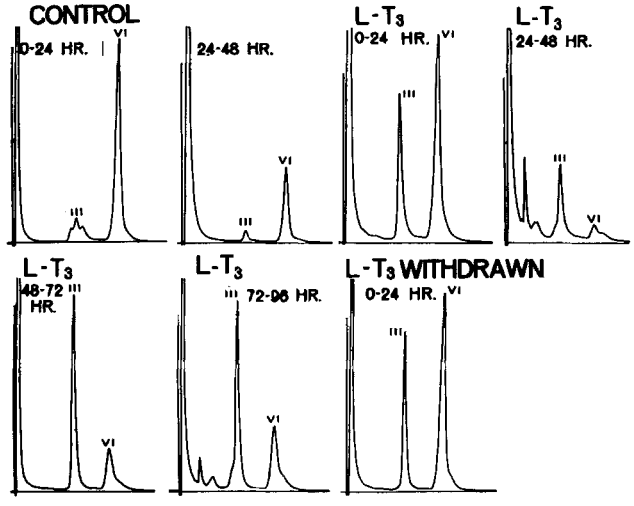

FIG. 3. Effect of L-T_3 treatment on the pattern of bile acids. III = chenodesoxycholate, VI = cholate.

24- to 48-hr period represented more specifically the result of biosynthesis in the liver. The comparative enrichment of chenodesoxycholate in this later period suggests that this bile acid perhaps is less readily absorbed in D-T_3-treated animals than cholate from the intestine via the enterohepatic pathway. Such an alteration in bile acids could thus facilitate the fecal excretion of the bile acids and lead to an increase in the catabolism of cholesterol.

Similar changes in the relative amounts of cholate, chenodesoxycholate, and component X were observed in rats that served initially as their own control and then were treated with L-T_3 (Fig. 3). It is evident that the subcutaneous administration of L-T_3 48 hr after the preparation of the bile duct fistula resulted in a rapid change in the pattern of bile acids. A marked

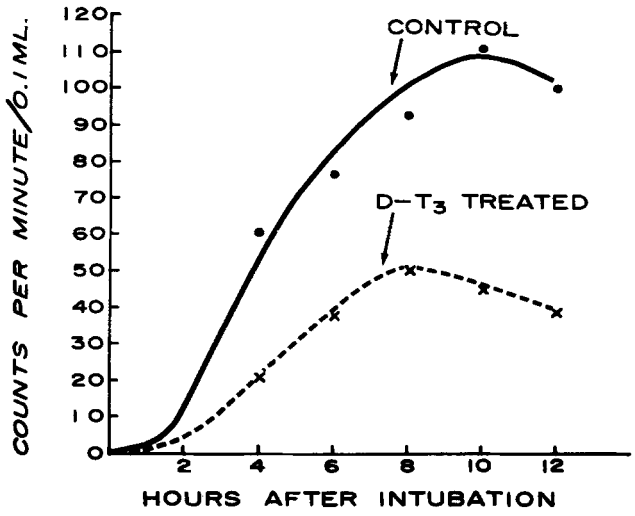

FIG. 4. Plasma-C^{14} activity in rats following gastric intubation of cholesterol-4-C^{14}. Each point represents the value of pooled plasma from the five rats used in each group.

relative increase in chenodesoxycholate was observed within 24 hr. The maximum effect of the drug appeared to be attained in 48 hr and was maintained during the remainder of the 96-hr period of treatment. It is noteworthy, on the other hand, that the bile acids tended to revert quickly to the control pattern upon withdrawal of the drug. It was also observed in such an animal that, following further rest from treatment, the administration of another thyromimetic agent produced again a decrease in the cholate/chenodesoxycholate ratio.

Bergström (18) showed that, while chenodesoxycholate (3α,7α-dihydroxycholanate) and cholate (3α) 7α,12α-trihydroxycholanate) can be formed from cholesterol through a common intermediate (3α,7α-dihydroxycoprostane), chenodesoxycholate is not a

biosynthetic precursor of cholate in the rat; i.e., 12α-hydroxylation, necessary for the formation of the latter, does not take place after oxidation of the side chain of 3α,7α-dihydroxycoprostane. The present data, as well as those of Eriksson (2) and Strand (3), lend support to the suggestion that thyromimetic agents favor the biosynthesis of chenodesoxycholate and/or inhibit that of cholate, possibly by affecting the relative rates of side-chain oxidation and 12α-hydroxylation in the formation of bile acids.

The investigations of Portman (19, 20), Beher, *et al.* (21, 22, 23), and Howe, *et al.* (24) have indicated that various bile acids can exert different effects on the metabolism of cholesterol. In a preliminary experiment to test possible effects of changes in pattern of bile acids on absorption of cholesterol, control rats and rats pretreated with D-T_3 as previously described were intubated, following a 15-hr fast, with an aqueous emulsion of cholesterol-4-C^{14} (1 μc in 5 mg) containing oleic acid (36.5 mg), serum albumin (12.5 mg), and glucose (18.8 mg), in proportions described by Swell et al. (25). Bile salts, however, were purposely omitted from the emulsion. The rate and extent of appearance of C^{14} in the plasma were markedly lower in the D-T_3-treated group (Fig. 4). While the authors are cognizant of other possible causes, this observation tends to indicate a poorer intestinal absorption of cholesterol, which they suspect might be mediated through changes in pattern of bile acids, and might contribute to the hypocholesteremic effect of D-T_3 and similar thyromimetic agents. Work is in progress to determine further the effect of changes in the pattern of bile acids and of chenodesoxycholate, in particular, on the absorption, excretion, and turnover of cholesterol.

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