Humes *et al.* [18] reported that benoxaprofen did not reduce LTC_4 synthesis by zymosan-stimulated mouse macrophages. Since LTC_4 is also a product of 5′-lipoxygenase activity these data do not support the idea that benoxaprofen actually inhibits the enzyme. In preliminary experiments we have demonstrated that benoxaprofen is approximately 100 times less active than BW755C in inhibiting LTB_4-synthesis by human PMN stimulated with serum treated zymosan (unpublished data). The latter data is consistent with the failure of benoxaprofen to reduce LTB_4-synthesis *in vivo* observed in the present study.

In conclusion, benoxaprofen failed to reduce *in vivo* synthesis of LTB_4 in a model of acute inflammation. Therefore, this observation casts doubt on the hypothesis that the reported clinical benefit afforded by benoxaprofen is due to inhibition of 5′-lipoxygenase, although caution should be exercised in extrapolating from data derived in an animal model of acute inflammation to chronic clinical disease. Benoxaprofen inhibited the cyclo-oxygenase and inhibited cell accumulation by a mechanism which is unknown and these activities probably contribute to the drug's reported clinical success. The toxic effects of benoxaprofen, which necessitated its withdrawal from clinical use, probably cannot be attributed to inhibition of 5′-lipoxygenase and therefore inhibition of the latter enzyme remains an attractive approach to novel anti-inflammatory therapy.

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Effect of disulfiram on rat liver cholesterol 7α-hydroxylase

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The major pathway for cholesterol degradation is the biosynthesis of bile acids in the liver. The first and rate-limiting step is the 7α-hydroxylation of cholesterol [1]. This hydroxylation is catalyzed by a microsomal monooxygenase system involving a cytochrome P-450 and NADPH-cytochrome P-450 reductase [2]. It has been reported that treatment of alcoholics with disulfiram (Antabus^R) can result in increased serum cholesterol levels [3]. It should therefore be of interest to study possible correlations between the effect of disulfiram on serum cholesterol concentration and on the 7α-hydroxylation of cholesterol. The present communication reports such a study with a purified cholesterol 7α-hydroxylase from rat liver.

Materials and methods

[4-^{14}C]-Cholesterol (61 Ci/mol) was obtained from the Radiochemical Centre, Amersham, England. 5β[7β-^{3}H]-Cholestane-3α,7α-diol (500 Ci/mol) was prepared as described previously [4]. Disulfiram (Antabus^R) was obtained from Dumex Läkemedel AB, Helsingborg, Sweden and from Sigma. Nyco-test^R Kolesterol was obtained from Nyegaard & Co AB, Stockholm, Sweden.

Male rats of the Sprague–Dawley strain weighing about 250 g were used. Rats used for serum cholesterol determinations were given disulfiram orally, 40–400 mg/kg body weight daily, for 3 weeks. Rats used for cytochrome P-450 preparation were treated with cholestyramine 3% (w/w) in the diet for one week. In the preparation of enzyme fractions dithiothreitol was excluded from all buffers. Cytochrome P-450 was prepared from rat liver microsomes as described previously [4, 5] except that the microsomes were not subjected to the slow freezing and thawing procedure and that the CM-Sephadex chromatography was omitted. The final cytochrome P-450 fraction was treated on a

hydroxylapatite column in order to remove detergent [6]. The preparation contained 6 nmol of cytochrome P-450/mg of protein. Cytochrome P-450 was determined as described by Omura and Sato [7] and protein as described by Lowry *et al.* [8]. NADPH-cytochrome P-450 reductase was prepared from phenobarbital-treated rats as described by Yasukochi and Masters [9]. The preparation had a specific activity of 50 units/mg of protein and was homogeneous upon gel electrophoresis. Liver cholesterol, extracted by the Folch procedure [10], and serum cholesterol were determined with Nyco-test^R Kolesterol as described by Allain *et al.* [11].

Incubations with cholesterol were performed at 37° for 20 min with 0.1–0.5 nmol of cytochrome P-450, 1 unit of NADPH-cytochrome P-450 reductase, 25 μg of dilauroylglycero-3-phosphorylcholine and 1.2 μmol of NADPH in a total volume of 1 ml of 50 mM Tris–acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Cholesterol, 25 nmol in 25 μl of acetone, was added to the incubation mixture with 0.6 mg of Triton X-100 in 25 μl of water. Incubations with 5β-cholestane-3α,7α-diol, 100 nmol in 25 μl of acetone, were performed as described for cholesterol with the exception that 150 mM potassium phosphate buffer was used instead of the Tris–acetate buffer and that Triton X-100 was omitted. When the effect of disulfiram on hydroxylase activities was studied disulfiram dissolved in 10 μl of acetone was added to the cytochrome P-450. The same amount of acetone was added in control incubations. Cytochrome P-450 and disulfiram were then preincubated for 2 min. In the reactivation studies dithiothreitol was added to the mixture of cytochrome P-450 and disulfiram after the preincubation and a second preincubation was performed for 2 min. Incubations were then performed as described above. The incubations were terminated and the incubation mixtures were analyzed as described previously [4].

Results

Table 1 shows the effect on serum and liver cholesterol concentrations as well as on cholesterol 7α-hydroxylase activity by treatment with increasing concentrations of disulfiram. A dose-related increase of serum and liver concentrations was observed, which was parallelled by a dose-related decrease in microsomal 7α-hydroxylase activity. Incubation of microsomes from disulfiram-treated rats with 10 mM of dithiothreitol resulted in a reactivation of the 7α-hydroxylase activity to 85% of the activity in microsomes from control rats. Figure 1 shows the effect of disulfiram *in vitro* on 7α-hydroxylation of cholesterol and on 12α-hydroxylation of 5β-cholestane-3α,7α-diol catalyzed by a purified cytochrome P-450 fraction. The cholesterol 7α-hydroxylase activity was completely inhibited at a disulfiram concentration of 10 μM. The 12α-hydroxylase activity

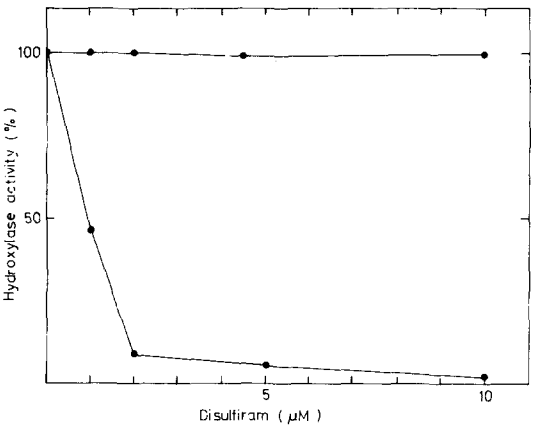

Fig. 1. Effect of disulfiram on 7α-hydroxylation of cholesterol and 12α-hydroxylation of 5β-cholestane-3α,7α-diol catalyzed by a purified cytochrome P-450 fraction from rat liver microsomes. Incubation procedures were as described under Material and Methods. One hundred percent corresponds to 160 pmol 7α-hydroxylated product formed/nmol cytochrome P-450 × min and 90 pmol 12α-hydroxylated product formed/nmol cytochrome P-450 × min, respectively.

was not affected at the disulfiram concentrations studied. Figure 2 shows that the inactivation of cholesterol 7α-hydroxylase activity by preincubation of the cytochrome P-450 fraction with disulfiram can be reversed by a second preincubation with dithiothreitol. Maximal reactivation was observed with 3 mM dithiothreitol. The cholesterol 7α-hydroxylase activity of the reactivated cytochrome P-450 fraction was as high as the original activity.

Discussion

Studies with microsomes [12] and with purified cytochrome P-450 have shown that cholesterol 7α-hydroxylase is dependent on reduced sulfhydryl groups for catalytic activity [13]. Sulfhydryl compounds such as dithiothreitol and reduced glutathione activate the purified system whereas glutathione disulfide inactivates [14]. It should be mentioned that another important hydroxylation in bile acid biosynthesis, the 12α-hydroxylation of 5β-cholestane-3α,7α-diol, is not affected by reduced or oxidized sulfhydryl compounds [15].

Table 1. Effect of disulfiram treatment on cholesterol concentrations in serum and liver tissue and microsomal cholesterol 7α-hydroxylase activity

	Cholesterol concentration		
	Serum	Liver	Cholesterol 7α-hydroxylase activity in microsomes (pmoles/nmole P-450 × min)
	(mM)	(mg/g wet weight)	
Control	1.5 ± 0.1	1.2 ± 0.5	35 ± 5
Disulfiram-treated			
40 mg/kg body weight	2.4 ± 0.2	1.2 ± 0.3	28 ± 3
120 mg/kg body weight	2.8 ± 0.3	1.6 ± 0.3	25 ± 4
240 mg/kg body weight	2.9 ± 0.2	1.8 ± 0.3	23 ± 4
400 mg/kg body weight	2.9 ± 0.4	1.8 ± 0.4	21 ± 4

Separate determinations were performed using five rats in each group. The results are given as the means ± S.D. of the means.

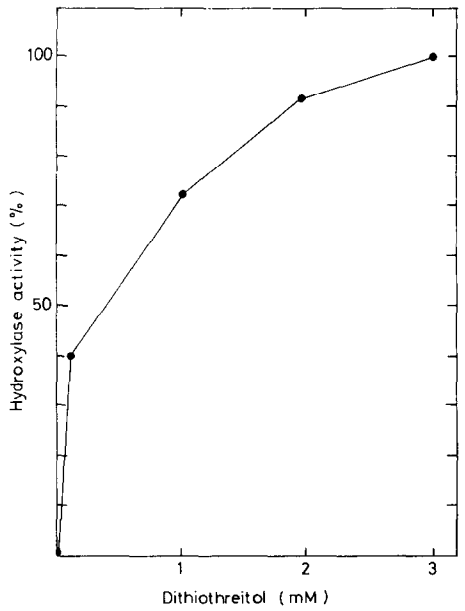

Fig. 2. Reactivation of cholesterol 7α-hydroxylase activity by dithiothreitol. Cholesterol 7α-hydroxylase activity was first inhibited by preincubation of the cytochrome P-450 fraction with 10 μM of disulfiram. The inactivated cytochrome P-450 was then reactivated by increasing concentrations of dithiothreitol in a second preincubation. Incubation procedures were as described under Materials and Methods. One hundred percent corresponds to 160 pmol 7α-hydroxylated product formed/nmol cytochrome P-450 × min.

In the present investigation the effect of the disulfide containing drug disulfiram on cholesterol 7α-hydroxylase activity was studied with a cytochrome P-450 fraction that catalyzed both 7α-hydroxylation of cholesterol and 12α-hydroxylation. The finding that 7α-hydroxylation of cholesterol but not 12α-hydroxylation of 5β-cholestane-3α,7α-diol was inhibited by disulfiram indicates that disulfiram interacts with essential sulfhydryl groups in the cholesterol 7α-hydroxylating cytochrome P-450. This interpretation is further supported by the fact that the inhibition could be reversed by dithiothreitol. In this connection, it should be of interest to point out that it has been proposed that disulfiram inhibits aldehyde dehydrogenase by covalent modification of enzymic sulfhydryl groups [16].

The present results showing that disulfiram is a potent inhibitor of purified rat liver cholesterol 7α-hydroxylase indicate that the increased serum cholesterol concentration observed after treatment with disulfiram can be due to an inhibition of cholesterol 7α-hydroxylase. The dose-related increase in liver tissue cholesterol shows that the increased serum cholesterol levels is not a consequence of a change in the relative distribution between liver and blood cholesterol.

In summary, the effect of disulfiram (Antabus^R) on serum cholesterol concentrations and on cholesterol 7α-hydroxylation, the rate-limiting step in bile acid biosynthesis, was studied in the rat. Treatment with disulfiram resulted in a marked increase in serum cholesterol concentration. Disulfiram effectively inhibited the activity of a purified, reconstituted cholesterol 7α-hydroxylase system from rat liver microsomes. The inhibition could be completely reversed by the sulfhydryl reducing agent dithiothreitol, indicating that disulfiram acts through its disulfide on the cholesterol 7α-hydroxylase. It is suggested that the effect of disulfiram on serum cholesterol level is related to this action.

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