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Effect of 1-benzyl-2-thio-5,6-dihydrouracil on hepatic metabolic activity in the rat

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IT HAS BEEN demonstrated in the rat that 1-benzyl-2-thio-5,6-dihydrouracil (CL 8099) decreased the size of the epididymal fat pads, increased thyroid weight and elevated the liver weight to body weight ratio* and also stimulated hexobarbital metabolism.† These effects have not been previously demonstrated for this type of chemical structure and prompted speculation about possible alterations in metabolic activity produced by this compound. Since its influence on thyroid weight and hexobarbital metabolism is similar to that described for *o,p'*DDD,[1,2] the possibility that CL 8099 is a stimulator of microsomal enzyme activity was investigated.[3] This study is also an attempt to further characterize microsomal metabolic reactions.

MATERIALS AND METHODS

Treatment of animals. Male albino Wistar strain rats from Royal–Hart Farms were given Purina chow and water *ad lib.* Animals were dosed orally with CL 8099 (50 mg/kg/day) for 2 days, while controls received only the vehicle, CMC (0·5% carboxymethylcellulose, 0·4% polysorbate 80, 0·9% NaCl).

Preparation of liver 9000 *g supernatant.* The animals were decapitated and exsanguinated 24 hr after the last dose. The livers were immediately removed, rinsed in cold water, blotted once and then homogenized in 3 vol. of cold 0·15 M KCl solution with a motor-driven Potter–Elvehjem Teflon homogenizer. The liver homogenate was centrifuged at 9000 *g* in a refrigerated Servall centrifuge for 20 min and the supernatant was decanted and stored at −20° until used (storing did not exceed 5 days.‡

Preparation of microsomes. Microsomes were prepared by centrifuging the 9000 *g* supernatant at 105,000 *g* for 1 hr and suspending the pellet in sufficient 0·15 M KCl to yield microsomes equivalent to 100 mg of original liver per ml. Protein was determined by an automated biuret procedure based on the description of Gornall *et al.*[4]

Cortisol metabolism. 4-^{14}C-labeled cortisol, 1·4 μmole, was incubated with 1 ml liver 9000 *g* supernatant and an NADPH-generating system in a final volume of 5 ml at 37° for 20 min, as previously described.[2] The mixture was extracted with ethyl acetate and the components were separated by descending chromatography. The cortisol area was visualized under short-wave u.v. light. The chromatograms were then cut into segments and the radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer. The extent of metabolism (μmoles/g liver) was calculated from the per cent of counts in the different radioactive zones.[2]

N-*demethylation. p*-Chloro-*N*-methylaniline, 3·0 μmole (PCMA), was incubated with 0·2 ml of the 9000 *g* liver supernatant in a medium containing an NADPH-generating system in a 2·0 ml final volume, with shaking in air at 37° for 20 min, as previously described.[5] At the end of incubation the enzymatic conversion of PCMA to *p*-chloroaniline (PCA)§ was assayed by the addition of *p*-dimethylaminobenzaldehyde and measurement of absorbance of the colored solution at 445 mμ.[5] To prevent opalescent solutions, the modification suggested by these authors was routinely used here.

Lipid peroxidation. Lipid peroxidase activity was determined by incubating the liver supernatant at 37° for 30 min as described by Hochstein and Ernster,[6] and then using thiobarbituric acid to assay the malonyl dialdehyde produced as a result of lipid peroxidation.[7]

* C. R. Boshart and S. Riggi, personal communication.

† D. Kupfer and L. Peets, unpublished data.

‡ Within this period the enzyme activities described below did not change significantly.

§ PCMA and PCA may be currently obtained from Calbiochem, Los Angeles, Calif.

RESULTS

Cortisol metabolism. Two radioactive chromatographic zones (both more polar than cortisol), in addition to a zone containing residual unreacted cortisol, were present. The two polar zones are presumably composed of ring A reduced metabolites, since these areas do not absorb short-wave u.v. light on paper and the eluted substances do not absorb light at 240 mμ in methanolic solution. These metabolites possess an intact α-ketolic side chain, since they reduce blue tetrazolium. It is assumed that the less polar metabolites (polar metabolite II) are reduced products resulting from Δ^4-3 keto reduction and the more polar ones (polar metabolite I), corresponding to Zone A in paper chromatographic analysis,[2] are the result of the hydroxylation of the reduced metabolites.

Table 1 demonstrates a significant increase in polar metabolite I formation in 80-g rats after treatment, with no change in substrate metabolism or polar metabolite II formation. Although the levels of substrate metabolism and of metabolite I formation were higher in 200-g control rats than in immature animals, CL 8099 had no effect in the older rats.

TABLE 1. EFFECT OF CL 8099 ON THE METABOLIC ACTIVITY OF LIVER 9000 *g* SUPERNATANT FROM MALE RATS*

Animal wt.† (g)	Experimental group‡	Cortisol metabolism (μmoles/g liver/20 min)			*N*-demethylation (μmoles PCA formed/g liver /20 min)	Lipid peroxidation (μmoles malonyl dialdehyde formed/g liver/30 min)
		Polar metabolite I formed	Polar metabolite II formed	Substrate metabolized		
80 g	Control	0·07 ± 0·01	0·76 ± 0·08	0·86 ± 0·03	1·16 ± 0·10	0·0034 ± 0·0003
	Treated	0·13 ± 0·01§	0·71 ± 0·06	0·85 ± 0·05	2·16 ± 0·12§	0·0099 ± 0·0018§
200 g	Control	0·37 ± 0·03	0·73 ± 0·11	1·19 ± 0·11	1·59 ± 0·19	0·0433 ± 0·0081
	Treated	0·44 ± 0·05	0·82 ± 0·08	1·27 ± 0·15	1·88 ± 0·17	0·0480 ± 0·0062

* Tabulated values represent the calculated mean ± S.E. for 8 animals.

† The range of weights in the two groups was 74–86 g and 187–206 g.

‡ Animals were treated with 50 mg CL 8099/kg/day for 2 days while controls received vehicle alone.

§ Significantly different from control value at $P < 0{\cdot}05$.

In examining the possibility that the observed effects were caused by the activation of the system *in vitro* by CL 8099, it was found that the addition of the compound to control supernatant in a final concentration of 10^{-4} M did not influence cortisol metabolism.

N-demethylation and lipid peroxidation. There was a marked stimulation of *N*-demethylation by CL 8099 in immature rats, with no significant effect in mature ones (Table 1). Treatment produced a significant increase in lipid peroxidase activity in immature animals, and again there was no change in mature rats.

Protein synthesis and hexobarbital sleep-time. Animals in both weight groups demonstrated higher liver weight to body weight ratios and higher liver microsomal protein levels after CL 8099 treatment (Table 2). In examining the possible stimulation of *de novo* enzyme synthesis, it was observed that the increase in liver weight, microsomal protein and *N*-demethylation produced by CL 8099 was absent after puromycin administration, and that the lowering of hexobarbital sleep-time by CL 8099 was partially blocked (Table 2). Cycloheximide was also effective in inhibiting these changes produced by CL 8099.

TABLE 2. EFFECT OF PUROMYCIN AND CYCLOHEXIMIDE ON THE METABOLIC ACTIVITY OF CL 8099 IN MALE RATS*

Treatment†	Liver wt/body wt. (%)		Liver microsomal protein (mg/100 g body wt.)		Hexobarbital sleep-time‡ (min)	*N*-demethylation (μmoles PCA formed/g liver/20 min)
	80 g	200 g	80 g	200 g	80 g	80 g
Control	5·66 ± 0·17	4·48 ± 0·08	148·8 ± 16·2	117·9 ± 9·1	90·6 ± 5·3	0·94 ± 0·10
CL 8099	7·51 ± 0·23§	6·47 ± 0·11§	232·2 ± 10·1§	155·3 ± 10·1§	21·0 ± 1·9§	1·83 ± 0·14§
Puromycin + CL 8099	5·91 ± 0·40‖	4·69 ± 0·10‖	153·7 ± 11·1‖	106·0 ± 5·1‖	65·2 ± 7·1‖	0·90 ± 0·11‖
Puromycin	5·72 ± 0·38	4·53 ± 0·21	140·7 ± 12·2	109·0 ± 4·7	96·4 ± 12·8	1·00 ± 0·07
Control	5·10 ± 0·38		170·8 ± 11·1		93·0 ± 7·21	1·25 ± 0·21
CL 8099	7·91 ± 0·31§		293·2 ± 31·6§		14·8 ± 0·95§	2·41 ± 0·26§
Cycloheximide + CL 8099	5·69 ± 0·51‖		200·2 ± 12·8‖		65·3 ± 5·11‖	0·91 ± 0·11‖
Cycloheximide	5·46 ± 0·40		160·0 ± 8·3		91·1 ± 2·32	0·76 ± 0·22

* The range of weights in the two groups was 76–83 g and 194–205 g. (Tabulated values represent the calculated mean ± S.E. for 8 animals.)

† Animals were treated orally with 50 mg CL 8099/kg/day for 2 days while controls received vehicle alone; puromycin, 100 mg/kg i.p., and cycloheximide, 50 μg i.p., were administered in saline solution 30 min before CL 8099 or alone for 2 days.

‡ Single injection of sodium hexobarbital, 125 mg/kg i.p.

§ Significantly different from control value at $P < 0·05$.

‖ Significantly different from CL 8099 value at $P < 0·05$.

DISCUSSION

Previous work has demonstrated the stimulation of both cortisol and hexobarbital metabolism by the 9000 *g* supernatant from rat liver homogenate after *o,p'*DDD treatment.[2] The work reported here indicates that the action of CL 8099 resembles those effects. The similarity lies in the comparable stimulation of microsomal drug-metabolizing reactions and cortisol transformations. Thus CL 8099 increased polar cortisol metabolite formation, hexobarbital oxidation and *N*-demethylation by rat liver supernatant. However, limits in metabolic stimulation were apparently reached in mature animals.

The decrease in the weight of the rat epididymal fat pads produced by CL 8099 suggested a possible effect of this compound on lipid metabolism. Lipid peroxidase activity was therefore particularly appropriate as a parameter because of this possibility and because the relationship between lipid peroxidation and drug hydroxylation has been noted.[8] This relationship has also been demonstrated here by the ability of CL 8099 to stimulate these two processes and by the fact that at similar doses an analogue, 1-*p*-methyl-benzyl-2-thio-5,6-dihydrouracil, which has no effect on fat pad weight in the rat,* did not influence hexobarbital sleep, *N*-demethylation and lipid peroxidation.

The observation that puromycin and cycloheximide inhibit the CL 8099-produced increase in liver weight to body weight ratios, liver microsomal protein levels and *N*-demethylation by liver 9000 *g* supernatant, and partially inhibit the CL 8099-mediated reduction in hexobarbital sleep-time suggests that stimulation of hepatic microsomal activity by this compound may occur via a mechanism similar to that reported for other stimulators of this activity, namely, by an increase in the *de novo* synthesis of microsomal enzymes.[9, 10] Further evidence for this is the fact that the addition of CL 8099 to liver supernatant had no effect on cortisol metabolism.

The data support the concept of stimulation of metabolic reactions by the induced synthesis of hepatic microsomal enzymes.

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* C. R. Boshart and S. Riggi, personal communication.

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An interaction of disulfiram and ethanol on lipid metabolism

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IT IS well known that the administration of a single large dose of ethanol to fasted rats produces an accumulation of liver triglycerides.[1–2] Among the several factors that have been postulated to contribute to this effect, the availability of free fatty acids (FFA), as substrate for the synthesis of triglycerides seems to be an important one. In fact it has been demonstrated that the fatty acid moieties of triglycerides accumulated in the liver after ethanol administration originate from adipose tissue[3] and that an inhibition of the lipolytic activity of adipose tissue reduces the accumulation of triglycerides in the liver of ethanol treated rats.[4–5]

Disulfiram (TETD or Antabuse (R)) was introduced in 1948 in the treatment of chronic alcoholism because of its unpleasant symptoms produced in connection with ethanol.[6]

Since fatty liver has been observed in the pathogenesis of chronic alcoholism in man[7] it was of theoretical and possible practical interest to study the interaction between ethanol and disulfiram on the accumulation of liver triglycerides in rats. Overnight fasted male Sprague–Dawley rats of the average body weight 170 ± 10 g received 16 ml/kg of 50% v/v ethanol by oral route, or disulfiram 200 mg/kg by i.p. route, or both treatments. Results reported in Table 1 indicate that disulfiram given simultaneously with ethanol was able to prevent the formation of fatty liver.

TABLE 1

Treatment	Time* (hr)	Liver Triglycerides mg/100 g ± S.E.	Plasma FFA μEquiv/l ± S.E.	Adipose tissue FFA μEquiv/ g ± S.E.
Controls	—	217 ± 34	716 ± 96	10·7 ± 0·4
Disulfiram	8	234 ± 18	821 ± 40	—
Ethanol	8	1168 ± 83	621 ± 75	10·3 ± 0·6
Disulfiram + Ethanol	8	264 ± 20†	248 ± 14†	3·5 ± 0·2†
Ethanol	18	1044 ± 77	—	—
Disulfiram + Ethanol	18	312 ± 59†	—	—

* Time = Interval between treatment and sacrifice.

The rats received 16 ml (50% v/v) of ethanol/kg by stomach tube and at the same time Disulfiram i.p. in a dose of 200 mg/kg.

Disulfiram was suspended in saline (40 mg/ml) by means of a few drops of Tween 80.

Triglycerides were determined according to Van Handel and Zilversmit[12] with minor modifications. FFA in plasma and in epididymal adipose tissue were determined according to Trout *et al.*[13]

Number of animals in each group was at least 5.

† = $P < 0.01$ in respect to ethanol.