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THE EFFECT OF DI-ISOPROPYL 1,3 DITHIOL-2-YLIDENEMALONATE (MALOTILATE) ON THE HEPATIC CHANGES INDUCED BY ETHANOL ADMINISTRATION IN THE RAT*

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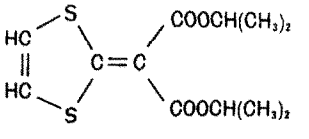
Abstract—The sulphur-containing drug, di-isopropyl-1,3-dithiol-2-ylidenemalonate (Malotilate) protects against the increase in hepatic triglyceride concentration after acute ethanol administration (either 6 g/kg p.o. or 2 g/kg i.p.) in rats. The compound had no influence on the increased hepatic NADH : NAD ratio (measured as the lactate : pyruvate and 3-hydroxybutyrate : acetoacetate ratios) after acute ethanol dosing (2 g/kg i.p.), but was found to lower hepatic acetaldehyde concentrations and prevent some of the disturbances in lipid metabolism observed in liver slices from ethanol-treated animals (e.g. decreased oxidation of [1-^{14}C]palmitate to $^{14}CO_2$) after this ethanol dose. The drug did not inhibit ethanol metabolism in this acute experiment.

Administration of Malotilate to Wistar rats (100 mg/kg/day orally) during chronic feeding of ethanol as 36% of the total calorie intake in a liquid diet, resulted in a lower intake of the alcohol-containing diet by ethanol-fed animals and reduced body weight gain in rats which received the drug, without blood ethanol levels or the ethanol intake (expressed in g/kg body weight/day) being affected. In ethanol-fed animals, Malotilate prevented the production of fatty liver and the adaptive increase in the ethanol elimination rate (EER) normally seen in ethanol-fed animals, although the drug actually caused a slight increase in EER in glucose pair-fed controls. Malotilate did not significantly decrease the degree of induction of microsomal cytochrome P-450 by ethanol, but the increase in aniline hydroxylation was much less marked in animals receiving ethanol and Malotilate, suggesting that the activity of the inducible microsomal ethanol oxidising system (MEOS) may be reduced by the compound. Determination of hepatic acetaldehyde concentrations during ethanol feeding, and during an acute ethanol challenge test following long-term ethanol treatment showed that the compound significantly lowered the level of this ethanol metabolite in the liver under both circumstances. This reduction of hepatic acetaldehyde concentrations, probably resulting in part from the reduced EER as well as increased low-K_m aldehyde dehydrogenase activities and glutathione contents seen in the livers of Malotilate-treated rats, are possible mechanisms by which the drug protects against triglyceride accumulation after ethanol administration.

Di-isopropyl 1,3-dithiol-2-ylidenemalonate (NKK-105; Malotilate) is a novel sulphur-containing drug that has been reported to exert a number of effects on hepatic functions in experimental animals (Fig. 1). Malotilate has been shown to stimulate hepatic blood flow and bile secretion and protects against the liver damage induced by agents such as allyl alcohol, bromobenzene, thioacetamide, carbon tetrachloride, paracetamol and D-galactosamine [1–3]. Stimulation of hepatic regeneration in partially hepatectomized, both cirrhotic and normal rats by the drug has been reported [4]. The compound has been shown to stimulate hepatic protein synthesis in rats [5], although the precise mechanism of its hepatoprotective effect against the liver toxins described above is not known.

The drug is currently undergoing investigation as a putative hepatoprotective agent in subjects with alcoholic liver disease. Since little data is available in the literature on the possible protective effect of Malotilate against ethanol hepatotoxicity, the current study was carried out to assess the influence of the drug on acute and chronic alcohol-induced accumulation of fat in the rat liver. In addition, the effect of Malotilate on ethanol and acetaldehyde metabolism in ethanol-fed animals was examined, with particular reference to acetaldehyde, since this



Malotilate

Diisopropyl 1,3-dithiol-2-ylidenemalonate

Fig. 1. Chemical structure of Malotilate (di-isopropyl-1,3 dithiol-2-ylidene-malonate).

* In this article, the terms ethanol and alcohol are interchangeable.

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metabolite is thought to mediate a number of changes that could contribute to the hepatotoxic effects of this alcohol [6]. In acute experiments, the effect of this drug on the hepatic redox-state (NADH:NAD ratio), proposed as a possible mechanism of lipid accumulation, was also investigated.

MATERIALS AND METHODS

Acute studies

(i) *Acute alcoholic fatty liver experiment.* Male Wistar rats (200–250 g, University of Surrey strain) were fasted overnight prior to receiving a single dose of ethanol (6 g/kg body weight) as a 50% (v/v) solution by the oral route. Control animals received an isocaloric dose of glucose by the same route. Malotilate was given (250 mg/kg i.p. in 0.5% (w/v) Gum Acacia containing 0.05% Tween 80) at 24 and 1 hr prior to ethanol administration. Sixteen hours after ethanol administration, the animals were sacrificed, the livers removed and homogenised in 0.25 M sucrose. Hepatic triglycerides were determined by adapting the method of Fletcher [7] to a 12.5% (w/v) liver homogenate.

(ii) *Effect on ethanol and acetaldehyde metabolism after acute ethanol dosing.* The effect of Malotilate pretreatment on ethanol and acetaldehyde metabolism following an acute dose of ethanol (2 g/kg i.p. as a 25% (v/v) solution) was determined in male Wistar rats following an overnight fast. Malotilate was given (100 mg/kg orally) at 24 and 1 hr prior to ethanol administration. Animals were sacrificed at either 0.75, 1.5, 3.0 or 6.0 hr after ethanol administration and the livers rapidly isolated by freeze-clamping. Blood was collected by open cardiac puncture into a heparinised syringe. Hepatic ethanol and acetaldehyde concentrations were determined as described previously [8]. Blood samples were analysed for ethanol by an enzymic method using a kit supplied by BCL (Lewes, Sussex, U.K.).

(iii) *Effect of Malotilate on the hepatic redox-state and fatty acid oxidation by rat liver slices following acute ethanol administration.* Male Wistar albino rats (University of Surrey strain, 280–350 g) were used. They were maintained on a standard laboratory chow and water diet and starved for 24 hr prior to ethanol administration. Malotilate was given (100 mg/kg orally) as a suspension in 5% (w/v) arabic gum at 24 and 1 hr prior to ethanol administration. Appropriate controls were sham-dosed with similar volumes of the vehicle substance at the same time points.

Ethanol was given (2 g/kg i.p.) as a 25% (v/v) solution in saline, controls receiving a similar volume of saline by the same route. Ninety minutes later, animals were sacrificed by cervical dislocation, the abdomen rapidly opened, a portion of liver rapidly excised and immediately freeze-clamped between aluminium blocks which had been precooled in liquid nitrogen. A small blood sample was then collected from the necks of the animals and the remainder of the liver excised to prepare liver slices.

Slices were cut in ice-cold saline using a tissue grafting blade and approximately 80–150 mg of slices (wet weight) were weighed into 25 ml Ehrlenmeyer flasks (with a centre well) containing 3.5 ml Krebs–Henseleit buffer (pH = 7.4, calcium free). Following a 5 min pre-incubation of the slices at 37° in a shaking water bath, [1-^{14}C] palmitate (spec. act. = 51 mCi/nmol), bound to fatty acid-free bovine serum albumin was added to the incubation medium, final concentrations in the medium being albumin 0.25% w/v, and palmitate 0.33 μCi/ml; 60 μM. After addition of radiolabelled substrate, flasks were stoppered with a rubber septum cap and incubated for 90 min at 37° in a shaking water bath. Incubations were terminated by the injection of 0.5 ml 1 M citric acid through the septum cap into the outer wells of the flasks. Hyamine 10X (0.5 ml, 10% v/v in methanol) was then injected into the centre wells and the flasks reincubated for a further 45 min to trap the evolved CO_2. The hyamine was then removed into 10 ml NE260 liquid scintillation cocktail, the centre wells washed three times with 1.0 ml portions of scintillant, and the combined hyamine solution and washings counted. In some incubations, total carbon dioxide production was determined by trapping in 0.3 N barium hydroxide, then titrating to neutrality with 0.179 N HCl in the presence of thymolphathlein indicator [9]. The liver slices and incubation medium remaining in the outer wells of the flasks were homogenised and stored at −20°. Lipids were extracted from the incubation medium, and the various lipid classes separated by thin-layer chromatography on Silca Gel 60 TLC plates using hexane : diethyl ether : acetic acid (70:30:1) as the mobile phase. Spots were visualised in a tank containing iodine vapour and the spots corresponding to triglycerides scraped off and counted. Liver tissue remaining after preparation of liver slices was homogenised in 0.25 M sucrose and subsequently assayed for triglyceride content as described by Fletcher [10].

The freeze-clamped liver sample was crushed in a mortar with frequent additions of liquid nitrogen. Approximately 1 g powdered tissue was then allowed to thaw into 5.0 ml ice-cold 0.6 N perchloric acid in a preweighed homogeniser tube. After homogenisation in the cold, the tube and contents were reweighed to determine the exact amount of tissue added. After centrifugation in the cold (3000 *g* for 10 min), the supernatant was removed, the residue extracted with 2.0 ml ice-cold 0.3 N perchloric acid and re-centrifuged. The combined supernatants were then neutralised to pH 5–6 with 5 M K_2CO_3 using a pH meter. After centrifuging away the potassium perchlorate precipitate, the volume of the extract was determined and 1.5 ml used immediately for ATP and α-glycerophosphate assays. Concentrations of these and other hepatic substrates were determined by standard enzymic methods, as described previously [8].

Chronic studies

(i) *Animals and diet.* Male Wistar rats (University of Surrey strain; 230–250 g initially) were used. They were randomly allocated to one of four treatment groups (N = 15 rats per group), two groups receiving a liquid diet regime which contained a final level of 36% of the total caloric intake as ethanol, the other two groups being pair-fed with the ethanol-fed rats a liquid diet in which the ethanol calories were replaced by glucose. Details of the liquid diet method

of ethanol-feeding have been given previously [7]. The total period of liquid diet feeding was 35 days, the highest ethanol intake (36% of total calories) being introduced on day 14 of the study. Dietary intakes were recorded daily, and body weights of the animals determined every two days.

(ii) *Drug treatment.* Crystalline Malotilate was synthesised by Nihon Nohyaku Co Ltd. (Osaka, Japan) and was suspended in 5% (w/v) arabic gum prior to dosing. The drug was given (100 mg/kg orally) once daily throughout the liquid diet feeding period (days 1–35 of the study) to one of the ethanol-fed animal groups and one of the pair-fed control groups. Animals in the other ethanol-fed and control pair-fed groups were sham-dosed daily with equivalent volumes of arabic gum.

(iii) *Assessment of hepatic acetaldehyde concentrations and ethanol elimination rates.* One week before the end of the study (day 29), five animals from each of the ethanol-fed groups were sacrificed at midnight, so as to determine hepatic acetaldehyde and blood ethanol concentrations at a time point when animals are maximally intoxicated from intake of the ethanol-containing liquid diet. Animals were sacrificed by cervical dislocation and a portion of liver rapidly frozen *in situ* within 10–12 sec of death using aluminium tongs which had been precooled in liquid nitrogen. Hepatic acetaldehyde concentrations were determined in these freeze-clamped samples. A sample of blood was collected from the neck of the animals for ethanol analysis.

Ethanol elimination rates were determined on the last day of the study. Diet was removed at 0700 hr and the animals maintained in the fasted state for 11 hr to allow any residual ethanol to be metabolised. At about 1800 hr on the same day, a blood sample was removed from the tails of the animals for ethanol analysis, and an acute dose of ethanol was then given (2 g/kg i.p. as a 20% (w/v) solution in saline). Further blood samples were then taken at 1.0, 2.0 and 3.0 hr after this ethanol dose to estimate the ethanol elimination rate. At 3.0 hr after ethanol, the animals were sacrificed, and a liver sample removed for acetaldehyde determination as described above.

Ethanol and acetaldehyde were determined by head-space gas chromatography, all experimental details, and calculation methods for the ethanol elimination rate being identical to those described previously [8].

(iv) *Assessment of hepatic changes induced by ethanol feeding.* On the last day of the study, five animals from each treatment group which were not being used for ethanol elimination studies were anaesthetised with diethyl ether at approx. 0900 hr, blood removed by closed cardiac puncture and the livers removed into ice-cold 0.25 M sucrose. Blood was allowed to clot and was centrifuged to prepare the serum. Livers were rinsed in 0.25 M sucrose, blotted, weighed and portions removed into formal-saline for histological assessment using haematoxylin and eosin and Oil Red 'O' staining methods. Remaining liver tissue was homogenised in 0.25 M sucrose to give a 50% (w/v) homogenate. A portion of homogenate was immediately frozen in liquid nitrogen for the determination of hepatic reduced glutathione content using the method of Bernt and Bergmeyer [11]. Liver total lipid and triglyceride contents were assayed by the application of colorimetric methods to 12.5% (w/v) dilutions of these homogenates [10, 12]. Liver protein was determined by the method of Lowry using bovine serum albumin as the standard [13].

(v) *Tissue preparation for enzyme assays.* A portion of 50% (w/v) homogenate was diluted with 0.25 M sucrose containing 1 mM EDTA and 6% (w/v) sodium deoxycholate to give a 25% (w/v) homogenate. This was centrifuged for 45 min at 40,000 *g* in a Beckman JM-21M centrifuge and 50 μl aliquots of the supernatant used for determination of total liver aldehyde dehydrogenase activity.

A further portion of 50% (w/v) homogenate was diluted with 0.25 M sucrose buffered with 10 mM Tris (pH = 7.4) to give a 10% (w/v) homogenate. This was then centrifuged at 17,000 *g* for 20 min in a Beckman JM-21M centrifuge. The supernatant was then centrifuged at 100,000 *g* for one hour in a Beckman L8-70 ultracentrifuge to yield a microsomal pellet. Fifty-microlitre portions of the post-microsomal supernatant were used for alcohol dehydrogenase assays. The microsomal pellet was resuspended in 50 mM Tris (pH = 7.4) made up in 20% (v/v) glycerol containing 0.1 mM EDTA. Microsomal aniline hydroxylase activity was determined by the method of Chhabra *et al.* [14]. Microsomal cytochrome P-450 and cytochrome b_5 contents were determined as described by Omura and Sato [15]. Microsomal protein was measured by the Lowry procedure using bovine serum albumin as the reference standard [13].

(vi) *Enzyme assays.* Aldehyde dehydrogenase (ALDH) activity was determined by a modification of the method of Tottmar *et al.* [16]. The final assay incubation mixture, 2.0 ml, contained: 50 μl sample, 50 mM potassium phosphate (pH = 7.4); 3 μM rotenone; 1 mM pyrazole; 1.2 mM $MgCl_2$; 0.5 mM NAD and either 50 μM or 5 mM acetaldehyde. Activities were determined at 37° at 340 nm and the two different acetaldehyde concentrations were employed to differentiate between the low-K_m and high-K_m aldehyde dehydrogenase activities in rat liver.

Alcohol dehydrogenase (ADH) activity was determined at 37° in a final reaction volume of 2.0 ml containing: 50 μl sample, 58.8 mM potassium dihydrogen phosphate (pH = 7.4); 0.18 mM NADH and 8 mM acetaldehyde [17].

(vii) *Serum analyses.* Triglyceride, cholesterol, albumin concentrations and glutamate dehydrogenase (GLDH) activities were determined using kits supplied by BCL Ltd., Lewes, Sussex, U.K. Serum ethanol was determined by gas chromatography as described for liver and blood ethanol analyses [8].

(viii) *Statistical analysis.* All results are given as means ± SD. Statistical significance of differences between mean values for the various treatment groups was determined using Student's *t*-test for paired data.

RESULTS

(i) *Acute studies*

Administration of a single large acute dose of

Table 1. Effect of Malotilate on acute alcoholic fatty liver in the rat

Group	Hepatic triglyceride 16 hr after ethanol (mg/g liver)
A. Glucose	5.0 ± 1.0
B. Glucose + Malotilate	5.9 ± 1.2
C. Ethanol	29.1 ± 8.0*
D. Ethanol + Malotilate	10.9 ± 4.9**

* $P < 0.001$ vs A.
** $P < 0.001$ vs C.
Ethanol was given at a dose of 6 g/kg by gastric tube. Malotilate was given (250 mg/kg i.p.) at 24 and 1 hr prior to ethanol. N = 6 rats per group. Results are shown as means ± SD.

ethanol (6 g/kg) caused a 480% increase in hepatic triglyceride measured 16 hr after dosing. Pretreatment of animals with Malotilate largely prevented this fatty infiltration of the liver, the extent of the increase in hepatic triglycerides being reduced to 120% in the ethanol–Malotilate treated animals (Table 1).

Studies on the interaction of Malotilate with ethanol and acetaldehyde metabolism following acute ethanol administration demonstrated that the drug lowers the hepatic acetaldehyde concentration during ethanol intoxication (Table 2). Measurement of hepatic and blood ethanol concentrations in the same experiment demonstrated that the drug does not achieve this through inhibition of ethanol metabolism. In fact there is evidence that the drug may slightly enhance the ethanol elimination rate, as blood and liver ethanol were significantly lower 6 hr after ethanol dosing in the Malotilate-treated animals (Table 3).

Studies on the ability of liver slices, taken from rats 90 min after the same acute ethanol dose, to oxidise palmitate showed that ethanol alone caused a 39% inhibition in this oxidation, a parallel decrease being seen in total carbon dioxide production, indicating this was not simply due to dilution of the labelled palmitate in the slices from ethanol-treated rats. Pre-treatment of ethanol-dosed animals with Malotilate largely prevented this reduction in fatty acid oxidation. There was a 93% increase in radioactivity incorporated into triglycerides in the liver slices from ethanol-treated rats, this increased esterification of fatty acids being prevented by Malotilate pre-treatment (Table 4). Studies on the hepatic redox-state made in the same experiment showed that ethanol caused large increases in the hepatic lactate : pyruvate, 3-hydroxybutyrate : acetoacetate ratios and α-glycerophosphate concentrations. Malotilate did not affect these ethanol-induced changes in the hepatic redox-state, although the drug did

Table 2. Effect of Malotilate on hepatic acetaldehyde following acute ethanol administration

	Hepatic acetaldehyde (nmol/g liver)		
Time after ethanol (hr)	Ethanol only	P	Ethanol and Malotilate
0.75	49.8 ± 9.9	0.01	17.2 ± 4.3
1.5	24.0 ± 6.0	0.02	8.2 ± 2.7
3.0	19.8 ± 8.3	N.S.	12.1 ± 1.8
6.0	16.3 ± 3.9	0.02	7.4 ± 1.8

Ethanol was given at a dose of 2 g/kg (i.p.). Malotilate was given (100 mg/kg p.o.) 24 and 1 hr before ethanol. Results are shown as means ± SD. N = 4 rats per group and time point studied. N.S. = Not significant.

Table 3. Effect of Malotilate on blood and liver ethanol concentrations following acute ethanol administration

Time after ethanol (hr)	0.75	1.5	3.0	6.0
Blood ethanol (mmol/l)				
Ethanol only	50.0 ± 2.0	49.1 ± 2.6	35.0 ± 2.0	21.1 ± 2.2
Ethanol + Malotilate	48.3 ± 3.5	42.6 ± 4.6	32.8 ± 1.7	13.7 ± 2.2*
Hepatic ethanol (μmol/g)				
Ethanol only	39.6 ± 1.8	41.5 ± 2.1	27.6 ± 2.9	14.5 ± 1.4
Ethanol + Malotilate	34.7 ± 2.1*	38.6 ± 0.7	25.2 ± 1.5	11.0 ± 1.0*

* $P < 0.005$ vs ethanol only group.
Expression of results and other experimental details as in Table 2.

Table 4. Effect of malotilate on CO_2 production from palmitic acid by liver slices from ethanol-treated rats

	Saline			Ethanol		
	A. Control		B. Malotilate	C. Control		D. Malotilate
$^{14}CO_2$ production from ^{14}C-palmitate (dpm/g tissue/90 min)	33042 ± 583	N.S.	30580 ± 6131	20284 ± 2275†	$P < 0.005$	29980 ± 5587†
Radioactivity incorporated into triglycerides (dpm × 10^{-3}/g tissue/90 mins)	99.8 ± 25.3	N.S.	100.5 ± 30.6	192.8 ± 38.9**	$P < 0.025$	81.1 ± 19.0
Total CO_2 evolved (μmol/g tissue/90 min)	112.1 ± 10.4	N.S.	102.5 ± 8.4	65.9 ± 4.1†	$P < 0.05$	108.3 ± 21.7*
Specific activity CO_2 (dpm/μmol evolved in palmitate incubations)	344.2 ± 39.1	N.S.	333.3 ± 53.7	372.6 ± 72.3	N.S.	325.8 ± 46.4

Measurements were made on liver slices from rats 90 min after i.p. injection of ethanol (2 g/kg) or an equivalent volume of saline. Slices (80–150 mg) were incubated in 3.5 ml Krebs–Henseleit buffer containing 0.3 μCi/ml albumin-bound 1-^{14}C palmitate. Final concentration of substrate was 600 μM. After 90 min incubations at 37° in a shaking water bath, 0.5 ml 1 M citric acid was injected into the outer wells of the flasks and CO_2 collected into hyamine 10X. Malotilate was given (100 mg/kg orally on each occasion) in 5% (w/v) arabic gum at 24 and 1 hr prior to ethanol or saline administration. Values are shown as means ± SD. N = 5 animals per treatment group.

N.S. = not significant.

† $P < 0.005$ vs A.

* $P < 0.05$ vs C *and* n.s. vs A.

** $P < 0.05$ vs A.

reverse the increase in hepatic triglycerides observed after this ethanol dose (Table 5).

(ii) *Chronic study*

Body weight changes during the study are shown in Fig. 2. Malotilate had no effect on weight gain in control or ethanol-fed animals during the initial stages of the experiment, but once ethanol was introduced at a level of 36% of the total calorie intake (day 14), weight gain in the ethanol-fed animals was reduced, particularly in the Malotilate-treated animals. Malotilate caused a 18% reduction in liquid diet intake in ethanol-fed animals during this part of the experiment, but in view of the lower body

Table 5. Effect of malotilate on hepatic redox-state, triglyceride concentration and blood ethanol levels in ethanol-treated rats

	Saline			Ethanol		
	A. Control		B. Malotilate	C. Control		D. Malotilate
Liver						
ATP	3726 ± 363	N.S.	3887 ± 66	3536 ± 26†	N.S.	4010 ± 302†
α-Glycerophosphate	144 ± 19	N.S.	113 ± 27	585 ± 28*	N.S.	580 ± 150*
Lactate	653 ± 194	N.S.	543 ± 91	972 ± 62**	$P < 0.05$	797 ± 41†
Pyruvate	42.5 ± 9.1	N.S.	52.7 ± 5.0	29.6 ± 3.5**	N.S.	28.6 ± 3.2**
3-Hydroxybutyrate	1173 ± 69	$P < 0.05$	904 ± 139	1323 ± 104**	N.S.	1248 ± 42†
Acetoacetate	458 ± 33	N.S.	484 ± 31	300 ± 35*	$P < 0.05$	243 ± 27*
Lactate:pyruvate	13.2 ± 4.0	N.S.	10.3 ± 1.4	32.1 ± 5.5*	N.S.	28.0 ± 2.3*
3-Hydroxybutyrate: Acetoacetate	2.60 ± 0.30	N.S.	1.89 ± 0.41	4.43 ± 0.18*	N.S.	5.15 ± 0.57*
Liver triglyceride (mg/g)	12.2 ± 4.4	N.S.	10.7 ± 1.9	21.7 ± 4.9**	$P < 0.05$	13.4 ± 1.4†
Blood ethanol (mmol/l)	—		—	53.0 ± 2.2	$P < 0.05$	48.0 ± 2.6

All metabolite concentrations are expressed as nmol/g liver.

Measurements were made on freeze-clamped liver samples taken 90 minutes after i.p. injection of ethanol (2 g/kg) or an equivalent volume of saline. Malotilate was given (100 mg/kg orally) in 5% (w/v) arabic gum at 24 and 1 hr prior to ethanol or saline administration. Values are shown as means ± SD. N = 5 animals per treatment group.

N.S. = Not significant.

† N.S. vs A.

* $P < 0.001$ vs A.

** $P < 0.05$ vs A.

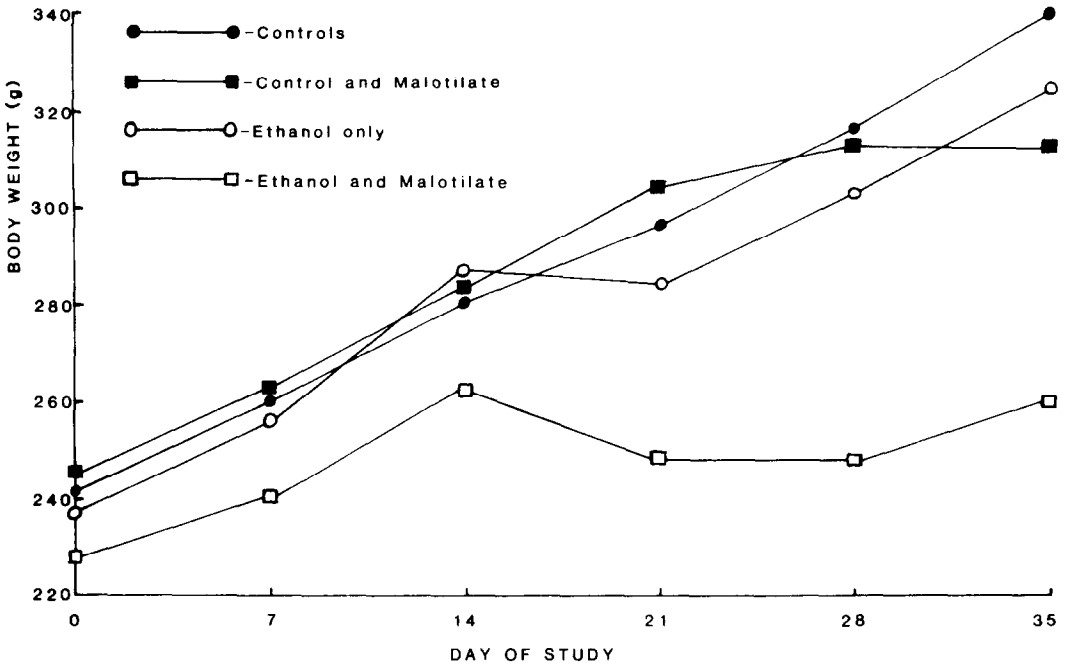

Fig. 2. Body weights during chronic ethanol feeding study.

weight of the drug-treated animals, ethanol intake, when expressed in g/kg body wt/day was not affected. Malotilate caused an increase in relative liver weights, this effect being significant in the control animals (Table 6).

Liver acetaldehyde measurements made during ethanol feeding on day 29 of the study showed that Malotilate almost halved the hepatic concentration of this ethanol metabolite in ethanol-fed rats, in the presence of comparable blood and liver ethanol levels. Acute ethanol challenge studies made on day 35 of the study demonstrated that Malotilate caused a 24% increase in the ethanol elimination rate in pair-fed control animals, whilst causing a dramatic decrease in the liver acetaldehyde concentration measured 3 hr after ethanol dosing. Ethanol feeding caused a 48% increase in the ethanol elimination rate when values were compared with those from pair-fed control animals, hepatic acetaldehyde concentrations, after acute ethanol dosing also being greatly reduced by chronic ethanol intake. This adaptive metabolic response to ethanol was not present in ethanol-fed rats that had also received Malotilate, ethanol elimination rates being comparable or somewhat less than those in non-drug treated pair-fed controls. Malotilate did significantly lower hepatic acetaldehyde concentrations after acute ethanol challenge in ethanol-fed rats (Table 7).

There were no significant differences in hepatic alcohol dehydrogenase (ADH) activities between any of the treatment groups at the end of the study. Ethanol and Malotilate, alone or in combination, caused significant increases in the aldehyde dehydrogenase (ALDH) activities measured at an acetaldehyde concentration of 50 μM. Ethanol and Malotilate individually also caused slight increases in ALDH activity measured with an acetaldehyde concentration of 5 mM in the assay procedure. However, ethanol and Malotilate in combination did not

Table 6. Effect of malotilate on dietary intake and liver weights in chronic ethanol feeding study

Treatment	Liver weight (% body wt.)	Liq. diet intake (ml/day)	Ethanol intake (g/kg body wt.day)
A. Control	3.08 ± 0.14	—	—
B. Control + Malotilate	3.76 ± 0.15*	—	—
C. Ethanol	3.39 ± 0.40	78.8 ± 13.3	12.2 ± 1.8
D. Ethanol + Malotilate	3.86 ± 0.26	64.5 ± 11.2	11.9 ± 2.1

Malotilate (100 mg/kg orally) was given once daily during a 35-day liquid diet feeding regime in which ethanol was given at a final level of 36% of the total caloric intake (days 14–35). Controls were pair-fed diet in which the ethanol calories were replaced by glucose. Dietary and ethanol intakes given were calculated from values obtained during the period of ethanol intake as 36% of the total calories (days 14–35; N = 10 rats per group). Liver weights given were determined in five animals per group at sacrifice on day 35.

* $P < 0.005$ vs A.

Table 7. Effect of Malotilate on liver acetaldehyde and blood ethanol concentrations during ethanol feeding and after acute ethanol challenge

	Treatment			
	A. Control	B. Control + Malotilate	C. Ethanol	D. Ethanol + Malotilate
Measurements at 0000 hr on Day 29:				
Liver acetaldehyde (nmol/g)	—	—	47.4 ± 10.8	26.1 ± 6.3*
Liver ethanol (μmol/g)	—	—	41.9 ± 5.7	53.2 ± 6.7
Blood ethanol (mmol/l)	—	—	53.7 ± 11.3	65.4 ± 7.6
Measurements after acute ethanol challenge (day 35):				
Ethanol elimination rate (mmol/l/hr)	5.45 ± 0.78	6.83 ± 0.43†	8.13 ± 2.37†	4.39 ± 0.67††
Blood ethanol 3 hr after dosing (mmol/l)	41.1 ± 1.7	37.4 ± 0.87†	35.9 ± 1.7§	43.9 ± 2.0*
Liver acetaldehyde 3 hr after dosing (nmol/g)	79.0 ± 26.8	8.9 ± 1.7**	18.5 ± 4.0**	8.3 ± 2.4*

Details of chronic ethanol feeding and drug treatment are given in the legend to Table 6. Acute ethanol dose (day 35) was 2.0 g/kg given intraperitoneally, and elimination rates were determined from tail blood ethanol concentrations determined hourly for the first 3 hr after this dose. N = 5 rats per group for all measurements.

* $P < 0.001$ vs C.
† $P < 0.05$ vs A.
†† $P < 0.02$ vs C and N.S. vs A.
§ $P < 0.01$ vs. A.
** $P < 0.001$ vs A.
N.S. = Not significant.

cause a significant increase in ALDH activity measured at this substrate concentration, when comparisons were made with the control group.

Ethanol feeding induced microsomal cytochrome P-450 and caused a 220% increase in microsomal aniline hydroxylase activity. Malotilate did not significantly affect the degree of cytochrome P-450 induction in ethanol-fed animals, but the increase in aniline hydroxylase activity was reduced to 75% over control values in animals that received Malotilate in combination with ethanol (Table 8).

Ethanol feeding induced a fatty liver as evidenced by increased hepatic total lipid and triglyceride contents. Malotilate significantly decreased the

Table 8. Effect of Malotilate on hepatic alcohol and aldehyde dehydrogenase activities and hepatic microsomal cytochrome concentrations after chronic ethanol feeding

	Treatment			
Parameter	A. Control	B. Control + Malotilate	C. Ethanol	D. Ethanol + Malotilate
Liver				
Alcohol dehydrogenase (μmol/min/g liver)	5.82 ± 0.19	5.52 ± 0.75	6.90 ± 0.92	5.38 ± 1.25
Aldehyde dehydrogenase (μmol/min/g liver)				
(a) 50 μM Acetaldehyde	3.16 ± 0.94	6.50 ± 0.43*	6.59 ± 0.88*	7.75 ± 0.55
(b) 5 mM Acetaldehyde	6.59 ± 0.35§	7.65 ± 0.29†	7.34 ± 0.16†	6.97 ± 0.25
Microsomal cytochrome P-450 (nmol/mg mic. prot)	0.87 ± 0.14	1.00 ± 0.11	1.48 ± 0.07*	1.34 ± 0.09
Microsomal cytochrome b_5 (nmol/mg mic. prot)	0.40 ± 0.05	0.64 ± 0.02*	0.54 ± 0.13	0.71 ± 0.08††
Microsomal protein (mg/g liver)	17.9 ± 1.4	20.3 ± 1.6	18.6 ± 1.2	19.2 ± 1.4
Microsomal aniline hydroxylase (nmol/min/mg mic. protein)	0.72 ± 0.06	0.80 ± 0.16	2.32 ± 0.22*	1.26 ± 0.13**

Values were obtained from 5 animals in each treatment group on day 35 of the study. Experimental details are as given in the legends to Table 6.

* $P < 0.001$ vs A.
† $P < 0.05$ vs A.
§ $P < 0.001$ vs 50 μM values for same group.
†† $P < 0.05$ vs C.
** $P < 0.001$ vs C.

Table 9. Effect of Malotilate on liver lipids, reduced glutathione and serum parameters after chronic ethanol feeding in the rat

Parameter	Treatment A. Control	B. Control + Malotilate	C. Ethanol	D. Ethanol + Malotilate
Total liver lipids (mg/g)	51.4 ± 5.2	51.6 ± 7.9	96.8 ± 12.3*	68.5 ± 3.2†
Liver triglycerides (mg/g)	12.9 ± 3.2	11.8 ± 3.6	55.0 ± 11.2*	29.7 ± 5.9**
Total liver protein (mg/100g b.w.)	808 ± 45	884 ± 36§	922 ± 83	1063 ± 57§§
Liver reduced gluthathione (μmol/g)	5.36 ± 0.46	8.51 ± 1.55††	4.61 ± 0.80	7.92 ± 1.06†
Serum ethanol (mmol/l)	—	—	56.1 ± 9.1	63.7 ± 14.8
Serum triglycerides (mg/100 ml)	128.1 ± 30.9	83.1 ± 20.4§	182.2 ± 79.6	189.1 ± 34.3
Serum cholesterol (mg/100 ml)	56.4 ± 8.7	72.3 ± 6.0§	90.6 ± 6.7*	106.5 ± 11.9
Serum albumin (g/l)	35.5 ± 2.9	38.2 ± 2.2	35.8 ± 2.0	33.8 ± 2.3
Serum GLDH (U/l)	8.6 ± 4.1	7.5 ± 4.0	14.3 ± 8.1	4.3 ± 3.0

Experimental details are as given in the legend to Table 6. N = 5 rats per group.
* $P < 0.001$ vs A.
† $P < 0.005$ vs C.
** $P < 0.001$ vs C.
§ $P < 0.05$ vs A.
§§ $P < 0.05$ vs C.
†† $P < 0.005$ vs A.

degree of fatty infiltration in the ethanol-fed animals as assessed both by biochemical lipid measurements and histological examination. Malotilate caused significant increases in hepatic protein and reduced glutathione content in both control and ethanol-fed animals. There was no effect of the drug on serum ethanol concentrations measured at sacrifice. Malotilate caused a slight decrease in serum triglyceride concentrations in control animals, whereas the compound alone as well as in combination with ethanol, tended to increase serum cholesterol. There was wide individual variation in serum glutamate dehydrogenase (GLDH) activity values, but ethanol feeding tended to increase serum GLDH activity, this increase being prevented when Malotilate was administered concurrently (Table 9).

DISCUSSION

The present findings show that Malotilate protects against the hepatic triglyceride accumulation observed after an acute dose of ethanol. The mechanism underlying this action of the drug does not involve inhibition of ethanol metabolism or reversal of the hepatic redox-state changes after intraperitoneal ethanol administration. However, under these conditions, Malotilate lowers liver acetaldehyde concentrations and reverses many of the ethanol-mediated derangements of lipid disposal in liver tissue. It is possible that this lowering of the hepatic acetaldehyde concentration is implicated in the recovery of mitochondrial fatty acid oxidation to the normal state, thereby correcting the ethanol-induced increase in hepatic triglyceride synthesis. It is also possible that this influence of Malotilate on hepatic acetaldehyde may explain the slight stimulating effect of the drug on ethanol oxidation in these acute experiments, in view of the regulatory role acetaldehyde can play in determining ethanol elimination under these conditions [8].

It will be evident from the results of the chronic studies (Tables 6–9) that the effects of Malotilate on hepatic functions and its interactions with ethanol metabolism and hepatotoxicity under these conditions are rather more complex. The hepatic lipid values and histological assessment clearly demonstrate that Malotilate protects against fatty liver induction during long-term ethanol feeding in the rat. There are a number of possible mechanisms indicated in the present study that could account for this effect of the drug.

One of the most striking effects of the compound is the reduction in liquid diet intake, and consequently weight gain, that Malotilate caused when ethanol was present in the diet. When ethanol intakes were corrected for body weight, there was no difference between the ethanol dose taken by drug treated animals and by animals receiving ethanol alone. The fact that the drug-treated control animals, pair-fed with those which received both Malotilate and ethanol, gained considerably more weight than the latter group adds support to the hypothesis, that, under circumstances of restricted calorie intake, ethanol calories are not equivalent to calories derived from carbohydrate [18]. This situation of dietary imbalance, with the consumption of so-called "empty calories" as ethanol, may potentiate ethanol hepatotoxicity. It could be argued that consumption of a given dose of ethanol by two groups of animals, one of which is on a restricted calorie intake, might result in exacerbation of alcoholic liver injury due to deficiencies of dietary factors that may afford some protection against ethanol hepatotoxicity [6]. In the present experiment, Malotilate was observed to reduce the liquid diet intake when ethanol was present, yet fatty liver production was inhibited by the drug, without ethanol intake (in g/kg/day) being significantly reduced. Thus, the effect of Malotilate on diet consumption by ethanol-fed rats cannot fully explain the protective effect of the compound against hepatic steatosis under these conditions.

The studies on ethanol metabolism indicate a possible explanation for Malotilate apparently lowering

the animals' preference for the alcohol-containing liquid diet. Whilst Malotilate alone tends to increase ethanol metabolism, as was observed in the acute studies, in rats fed ethanol chronically, Malotilate prevents the metabolic adaptation to ethanol intake. This effect does not seem to be related to any action of the drug on the alcohol dehydrogenase pathway of ethanol metabolism, thought to be the major route of metabolism in naive animals, or to impaired acetaldehyde removal by aldehyde dehydrogenase. Neither of these enzyme activities was reduced by Malotilate, in fact the compound tends to increase the latter, particularly affecting the low-K_m ALDH activity in control animals. The lack of adaptive response is probably due to an effect of the drug on the activity of the microsomal ethanol-oxidising system (MEOS), which is induced by chronic ethanol consumption and probably accounts, in part, for the increased ethanol elimination rate after long-term ethanol intake [19, 20]. In the present study, Malotilate did not affect the extent of induction of microsomal cytochrome P-450 by ethanol, a particular isoenzyme of which is thought to be an integral part of the MEOS pathway, but the extent of the increase in aniline hydroxylase activity during ethanol feeding is much less in the drug-treated animals. The ethanol-induced form of cytochrome P-450 has a high affinity for both alcohols and aniline as substrates; thus these results suggest that whilst Malotilate does not influence induction of this cytochrome P-450 isoenzyme, there may still be functional changes due to the drug, that produce an overall impairment of the microsomal component of ethanol oxidation [21]. Alternatively, Malotilate may alter the composition of the molecular species of microsomal cytochrome P-450, as suggested by previous studies on the effects of the drug on microsomal drug oxidation described by Katoh *et al.* [22]. These workers also observed inhibition of aniline hydroxylase activity, and induction of cytochrome b_5 in the absence of any concomitant increase in cytochrome P-450 following Malotilate administration in rats. The increase in cytochrome b_5 may be thought to enhance certain cytochrome P-450-dependent drug oxidations, such as benzphetamine *N*-demethylase and *p*-nitroanisole-*O*-demethylase, if results from reconstitution studies are used as a guide [23–25]. With regard to aniline hydroxylation, the increase in cytochrome b_5 may decrease this reaction, since in reconstitution studies, incorporation of b_5 into membrane vesicles containing isolated cytochrome P-450 inhibits ethanol oxidation by this system, suggesting that cytochrome b_5 may modulate in some way the activity of the P-450 isoenzyme which carries out aniline hydroxylation and ethanol oxidation [26].

With regard to the effect of impaired ethanol oxidation during chronic ethanol intake, the results of the present study can be compared with those from studies that employed pyrazole [27, 28] and 4-methylpyrazole [29] respectively to determine the role of ethanol metabolism in the production of alcoholic fatty liver in rats. In one pyrazole study, doses of this ADH inhibitor which caused a reduction in intake of alcohol-containing liquid diets and body weight gain, as has been observed for Malotilate in the current experiments, in fact greatly increased the severity of fat accumulation in the livers of rats fed ethanol chronically. In the more recent study of Lindros *et al.* [29], a moderate level of supplementation of alcoholic liquid diets with 4-methylpyrazole, which allowed maintenance of steady blood ethanol levels throughout the day and night, without affecting body weight gain, also increased the severity of the fatty liver produced by ethanol feeding. These authors concluded that uninterrupted, continual ethanol oxidation was an important factor in chronic ethanol hepatotoxicity. Therefore, it might be expected that Malotilate would exacerbate the ethanol-induced liver injury because of its effects on ethanol oxidation seen in the present study, whereas this was not found to be the case. It is therefore possible that the protective effect of the drug concerns events that are the consequences of ethanol metabolism, that in turn are associated with the production of fatty liver.

Ethanol metabolism has two major effects in the liver that could be important in causing fat accumulation. Firstly, it increases the hepatic NADH:NAD ratio as a result of ethanol oxidation by ADH, this redox-state alteration having a number of effects on intermediary metabolism that could favour triglyceride deposition [30]. However, recent studies have shown that supplementation of alcohol-containing liquid diets with the hydrogen acceptor methylene blue, which corrected many of the ethanol-induced redox-state changes, did not prevent fatty liver induction during long-term ethanol intake in rats [7]. In addition, the acute experiments have shown Malotilate to have no influence on the hepatic redox-state change induced by acute ethanol administration [9]. Therefore, the drug does not mediate its effects through modulation of the NADH:NAD ratio. It seems much more probable that the drug exerts its protective effect through lowering hepatic acetaldehyde concentrations after acute and chronic ethanol intake.

Various mechanisms have been proposed through which hepatic lipid disposition could be altered by acetaldehyde. These include direct inhibitory effects on mitochondrial function, including impairment of fatty acid oxidation [31], covalent binding to a number of critical hepatic proteins, including microtubular protein involved in the assembly and secretion of lipoproteins [32], the formation of adducts with glutathione or glutathione precursors favouring increased lipid peroxidation [33], and direct initiation of lipid peroxidation through some specific enzymic mechanism [34]. Lipid peroxidation has been proposed as a mechanism by which ethanol induces fatty liver and hepatic damage [35]. Thus, effective removal of acetaldehyde may inhibit ethanol hepatotoxicity. Malotilate could mediate this through its effects on aldehyde dehydrogenase activity, the low-K_m (mitochondrial) activity, probably the most important in terms of removing ethanol-derived acetaldehyde *in vivo*, being increased by the drug, particularly in control animals. Reduced rates of acetaldehyde formation resulting from effects of the drug on ethanol metabolism during long-term consumption, as already discussed, probably contribute to the lowering of liver acetaldehyde concentrations by Malotilate.

Furthermore, the compound increases reduced glutathione concentrations, which will also favour acetaldehyde removal, as well as helping to maintain cellular and organelle integrity, and inhibiting increased lipid peroxidation that may occur during ethanol feeding [33]. Exogenous sulphydryl compounds that are capable of trapping acetaldehyde have been observed to protect against ethanol hepatotoxicity *in vivo* [36, 37], and the inhibitory actions of acetaldehyde on mitochondrial functions *in vitro* [38, 39].

Thus, to summarise the above findings, Malotilate prevents the metabolic adaptation to chronic ethanol intake in rats, probably through preventing increased MEOS activity. This results in a lower ethanol elimination rate in drug-treated animals after long-term ethanol feeding, resulting in lower rates of acetaldehyde formation. In the acute studies, Malotilate actually slightly enhances the ethanol elimination rate, possibly as a result of the regulatory role acetaldehyde plays in ethanol metabolism under these conditions [8]. The drug also favours acetaldehyde removal through increasing low-K_m ALDH activity and increasing hepatic reduced glutathione concentrations, the latter also helping protect against cellular damage. A combination of these factors probably accounts for the protective effect Malotilate exerts against ethanol-induced fatty liver.

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