Biochem. J. (1985) 232, 877–882 (Printed in Great Britain)

The effect of Methylene Blue on the hepatocellular redox state and liver lipid content during chronic ethanol feeding in the rat

Peter R. RYLE,*§ Jagadish CHAKRABORTY† and Allan D. THOMSON‡

Departments of *Chemical Pathology and ‡Gastroenterology, Greenwich District Hospital, London SE10 9HE, U.K., and †Clinical Biochemistry Division, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, U.K.

Feeding of ethanol in a liquid diet to male Wistar rats caused decreases in the hepatic cytosolic and mitochondrial [NAD^+]/[NADH] ratios. This redox-state change was attenuated after 16 days of feeding ethanol as 36% of the total energy intake. Supplementation of the ethanol-containing liquid diet with Methylene Blue largely prevented the ethanol-induced redox state changes, but did not significantly decrease the severity of the hepatic lipid accumulation that resulted from ethanol ingestion. Methylene Blue did not affect body-weight gain, ethanol intake or serum ethanol concentrations in ethanol-fed rats, nor did the compound influence the hepatic redox state or liver lipid content of appropriate pair-fed control animals. These findings suggest that the altered hepatic redox state that results from ethanol oxidation is not primarily responsible for the production of fatty liver after long-term ethanol feeding in the rat.

INTRODUCTION

Long-term feeding of ethanol produces fatty infiltration of the liver in the rat even when this alcohol is given as part of a nutritionally adequate liquid diet (De Carli & Lieber, 1967). The mechanisms underlying this lesion are still far from clear. One of the most commonly quoted mechanisms that could account for this hepatic steatosis is the change in the hepatocellular redox state ([NADH]/[NAD^+] ratio) that results from ethanol oxidation by alcohol dehydrogenase and aldehyde dehydrogenase in the liver. This increased [NADH]/[NAD^+] ratio could influence intermediary metabolism in a number of ways to increase the hepatic triacylglycerol content. Firstly, the cytosolic redox pair [dihydroxyacetone phosphate]/[glycerol 3-phosphate] becomes shifted towards its more reduced side, so that glycerol 3-phosphate availability is increased for esterification of fatty acids into triacylglycerols (Nikkila & Ojala, 1963). Secondly, transhydrogenation of reducing equivalents from NADH may increase hepatic NADPH concentrations, so that fatty acid synthesis is increased (Lieber & Schmid, 1961). Thirdly, ethanol inhibits fatty acid oxidation *in vitro* and *in vivo*, possibly as a consequence of the redox-state changes limiting NAD availability for the dehydrogenases in the tricarboxylic acid cycle, so that complete oxidation of fatty acids to CO_2 is impaired (Leiber & Schmid, 1961; Ontko, 1973; Blomstrand *et al.*, 1973). A combination of these factors might favour esterification of fatty acids into triacylglycerol to produce fatty liver. Most of the accumulated hepatic lipid in alcoholic rats and humans is in the form of triacylglycerol (De Carli & Lieber, 1967; Cairns & Peters, 1983). There is little convincing experimental evidence, however, that this redox-state change is directly responsible for the production of fatty liver in ethanol-fed rats. Some agents, such as antioxidants, which do not influence the ethanol-induced redox-state change, will protect rats against the fatty liver induced by both acute and chronic ethanol feeding (Hartman & Di Luzio, 1968; Rossiter & Slater, 1973; Ryle *et al.*, 1985*a*). Some attempt was made by Lieber & De Carli (1966) to assess the role of the redox state in chronic ethanol-induced fatty liver by supplementing the ethanol-containing liquid diets with the hydrogen acceptors Methylene Blue and menadione. However, no redox-state measurements were made in that study, and the effect of these compounds alone on liver lipid content was not fully reported. Thus the aim of the present study was to assess in detail the influence that the hydrogen acceptor Methylene Blue has on the hepatocellular redox state during chronic ethanol feeding in the rat, and to determine whether any amelioration of the ethanol-induced redox-state changes by this compound might be associated with a significant decrease in fatty infiltration of the liver.

MATERIALS AND METHODS

Chemicals

All reagents were of the highest purity commercially available. Absolute ethanol was supplied by James Burroughs Ltd., London SE11 5DF, U.K.

Methylene Blue was obtained from Sigma Chemical Co., Poole, Dorset, U.K.; 'Complan' (natural flavour) was from Glaxo–Farley Foods, Plymouth, Devon, U.K.; casein (white, soluble) was supplied by BDH Chemicals, Poole, Dorset, U.K. All enzymes and cofactors for metabolite assays were from Sigma, except for 3-hydroxybutyrate dehydrogenase (Grade II), which was supplied by BCL Ltd., Lewes, Sussex, U.K., and 'Orovite-7', which was obtained from Bencard Ltd., Brentford, Middx., U.K.

Animals and diets

Male Wistar Albino rats (130–160 g initially; University of Surrey strain) were used. They were housed

§ To whom correspondence should be sent.

individually in wire-bottomed cages in a room maintained at 22 °C. Lighting was provided between 07:00 and 20:00 h daily. For the first 4 days of the experiment all animals received a control liquid diet containing no ethanol that comprised 121 g of 'Complan'/l, 98 g of glucose/l, 10 g of casein/l and 5 g of commercial vitamin mixture ('Orovite-7')/l. This diet contained 65%, 19% and 16% of its energy as carbohydrate, fat and protein respectively. The nutrient content of this diet was very similar to that of the nutritionally adequate liquid diet described by De Carli & Lieber (1967). For the next 6 days of the study (days 5–11), ethanol was introduced into the diet at a concentration of 3.2% (v/v), replacing an isoenergetic amount of glucose, appropriate control animals being pair-fed along with the ethanol-fed rats on a control diet in which the ethanol-derived energy was replaced by glucose. This ethanol concentration gave a diet in which 20% of the total energy was derived from this alcohol. For the remainder of the study (days 12–30) ethanol was given as 36% of the total energy intake by incorporating it into the liquid diet at a concentration of 5.6% (v/v), control animals being pair-fed throughout as described above. One group each of the ethanol-fed and the pair-fed control animals received liquid diets which contained Methylene Blue at a concentration of 130 mg/l between days 5 and 30 of the study. The average daily intake of Methylene Blue by this method of administration was 42 mg/day per kg. All diets were prepared fresh daily and the dietary intakes recorded daily throughout. Body weights of the animals were recorded every 2 days.

Assessment of the hepatic redox state

On days 12 and 27 of the study, three animals from each treatment group were killed at midnight so as to assess the hepatic redox state at a time when the animals were maximally intoxicated from ingestion of the ethanol-containing liquid diet. Portions of liver tissue (about 2 g wet wt.) were freeze-clamped *in situ* within 10–12 s of death, and blood samples were collected from the necks of the animals for ethanol analysis. Remaining liver tissue from each animal was homogenized in 0.25 M-sucrose and assayed for triacylglycerol as described under 'Liver lipid analysis'. Frozen liver samples were ground to a powder under liquid N_2 in a pestle and mortar, and approx. 800–1000 mg of powdered tissue was extracted with 5.0 ml of ice-cold 0.6 M-$HClO_4$, centrifuged (20000 *g*-min) in the cold, the residue re-extracted with 2.0 ml of 0.3 M-$HClO_4$ and the extract re-centrifuged. The combined supernatants were neutralized to pH 5–6 with 5 M-K_2CO_3, with a pH-meter. The clear extract, obtained after centrifuging away the $KClO_4$ precipitate, was treated with Florisil (0.1 g/ml of extract) as described by Williamson *et al.* (1967). Lactate, pyruvate, 3-hydroxybutyrate and acetoacetate were determined by the methods of Gutmann & Wahlefeld (1974), Czok & Lamprecht (1974), Williamson & Mellanby (1974) and Mellanby & Williamson (1974) respectively. Glycerol 3-phosphate was determined by the method of Michal & Lang (1974) and ATP was assayed with kits supplied by BCL. Cytosolic and mitochondrial free [NAD^+]/[NADH] ratios were calculated from the [lactate]/[pyruvate] and [3-hydroxybutyrate]/[acetoacetate] ratios respectively, assuming equilibrium constants for rat liver lactate dehydrogenase and 3-hydroxybutyrate dehydrogenase of 1.11×10^{-4} mM and 4.93×10^{-2} mM respectively (Williamson *et al.*, 1967).

Ethanol determination

Blood samples were allowed to clot and serum was prepared. Portions (50 μl) of serum were placed in a glass vial containing 1.0 ml of propan-1-ol internal-standard solution (0.5 mg/ml) and 0.1 ml of $HClO_4$ (60%, v/v). The vials were sealed with Teflon-lined rubber septum caps and heated in a water bath at 60 °C for 30 min. Then 2.0 ml of head space was removed with a gas syringe and injected into a Hewlett–Packard 5710A gas–liquid chromatograph fitted with a 1.8×4 mm (internal diameter) glass column packed with Poropak Q (50–80 mesh). Running conditions for the gas chromatograph were: column temp. 150 °C, flame ionization detector temp. 250 °C, injection port temp. 200 °C, nitrogen carrier gas flow 30 ml/min, hydrogen flow 30 ml/min and air flow 200 ml/min. The retention times for ethanol and propan-1-ol were 98 and 260 s respectively. The gas chromatograph was calibrated daily with freshly prepared aqueous solutions of ethanol, the peak-height-ratios method being used to calculate the concentrations present in the samples.

Liver lipid analysis

On day 30 of the experiment, the remaining animals in each group were anaesthetized with diethyl ether, blood was collected by closed cardiac puncture and serum prepared. Livers were rapidly excised, rinsed in ice-cold 0.25 M-sucrose containing 3 mM-EDTA, blotted and weighed, and a portion was frozen in liquid N_2 for the determination of reduced glutathione by the methylglyoxal/glyoxylase I method of Bernt & Bergmeyer (1974). Further portions of tissue were fixed in formal/saline for histological examination by using haematoxylin + eosin and Oil Red 'O' staining methods. Remaining liver tissue was homogenized in 0.25 M-sucrose and assayed for total lipid and triacylglycerol content by the application of colorimetric serum methods to a 12.5% (w/v) dilution of the homogenate (Fletcher, 1968; Woodman & Price, 1972).

Serum was assayed for ethanol as described above, and the glutamate dehydrogenase activity was determined with kits supplied by BCL.

Statistical significance of differences between means for the various treatment groups was assessed by Student's *t* test for paired data.

RESULTS

Various biochemical components associated with the hepatic redox state were determined during chronic ethanol feeding, and the results are shown in Table 1. At the beginning of administration of 36% of the total energy as ethanol (day 12), ethanol-fed animals showed 154% and 249% increases in the [lactate]/[pyruvate] and [3-hydroxybutyrate]/[acetoacetate] ratios respectively compared with their pair-fed controls. These changes were associated with 62% and 71% decreases in the cytosolic and mitochondrial [NAD^+]/[NADH] ratios respectively. Ethanol caused a 65% increase in the hepatic glycerol 3-phosphate concentration, but did not affect hepatic ATP concentrations at this time point. Methylene Blue alone did not affect any of these parameters in pair-fed controls at this stage of the experiment, but significantly reversed some of the redox-state changes observed in ethanol-fed animals. Partial correction of the [lactate]/[pyruvate] ratio and cytosolic [NAD^+]/[NADH]

Table 1. Effect of Methylene Blue supplementation of ethanol-containing liquid diets on the hepatocellular redox state during chronic ethanol feeding

Rats were fed with ethanol as part of a liquid diet in which 36% of the total energy was derived from ethanol. Pair-fed controls received diet in which glucose replaced the ethanol energy. Animals were killed at midnight on either day 12 or day 27 of the study, and freeze-clamped liver samples were analysed for metabolites as described in the Materials and methods section. Results are shown as means ± S.D. ($n = 3$ rats per group): *$P < 0.001$ versus (A); **$P < 0.01$ versus (A); †$P < 0.001$ versus (C); ††$P < 0.01$ versus (C). Abbreviation: MB, Methylene Blue.

Day of study	Measurement	Group (A) Control	(B) Control + MB	(C) Ethanol	(D) Ethanol + MB
12	Liver				
	ATP (nmol/g)	2991 ± 187	3062 ± 90	2857 ± 350	2967 ± 221
	Glycerol 3-phosphate (nmol/g)	189 ± 21	207 ± 31	311 ± 27*	205 ± 39†
	[Lactate]/[pyruvate]	12.4 ± 3.9	17.1 ± 4.1	31.5 ± 4.0*	21.8 ± 3.8†
	[3-Hydroxybutyrate]/[acetoacetate]	2.34 ± 1.10	1.48 ± 0.82	8.17 ± 2.60*	3.03 ± 0.80†
	Cytosolic [NAD+]/[NADH]	726 ± 228	527 ± 126	286 ± 36*	413 ± 72†
	Mitochondrial [NAD+]/[NADH]	8.67 ± 4.08	13.7 ± 7.6	2.48 ± 0.79*	6.69 ± 1.77†
	Serum ethanol (mg/100 ml)	—	—	145 ± 34	182 ± 90
27	Liver				
	ATP (nmol/g)	3405 ± 211	2899 ± 197**	3509 ± 545	2664 ± 303††
	Glycerol 3-phosphate (nmol/g)	212 ± 28	193 ± 17	290 ± 17*	200 ± 20†
	[Lactate]/[pyruvate]	16.8 ± 2.4	15.4 ± 2.7	26.8 ± 3.4**	21.4 ± 3.7
	[3-Hydroxybutyrate]/[acetoacetate]	2.39 ± 0.70	1.79 ± 0.49	3.30 ± 0.45	2.11 ± 0.67††
	Cytosolic [NAD+]/[NADH]	536 ± 77	585 ± 103	336 ± 43**	421 ± 73
	Mitochondrial [NAD+]/[NADH]	8.49 ± 2.49	11.3 ± 3.09	6.15 ± 0.84	9.61 ± 3.05††
	Serum ethanol (mg/100 ml)	—	—	190 ± 52	202 ± 39

Table 2. Effect of Methylene Blue supplementation of ethanol-containing liquid diets on the hepatic concentrations of redox-pair metabolites

Experimental details and expression of results are as given in the legend to Table 1. Abbreviation: MB, Methylene Blue. All values are in nmol/g wet wt. of tissue ($n = 3$ rats per group): *$P < 0.001$ versus (A); †$P < 0.001$ versus (C).

Day of study	Concn. in liver	Group (A) Control	(B) Control + MB	(C) Ethanol	(D) Ethanol + MB
12	Lactate	1544 ± 301	2859 ± 504*	736 ± 102*	1009 ± 139
	Pyruvate	125 ± 21	167 ± 31	23 ± 9*	46 ± 13†
	3-Hydroxybutyrate	119 ± 57	144 ± 37	385 ± 93*	242 ± 40†
	Acetoacetate	51 ± 20	98 ± 19*	47 ± 14	80 ± 17†
27	Lactate	2639 ± 325	2014 ± 393	662 ± 98*	828 ± 107
	Pyruvate	157 ± 14	131 ± 29	25 ± 11*	39 ± 12
	3-Hydroxybutyrate	123 ± 20	159 ± 23	207 ± 31*	176 ± 29
	Acetoacetate	51 ± 14	89 ± 17	63 ± 12	83 ± 17

ratio was achieved by Methylene Blue, whereas the [3-hydroxybutyrate]/[acetoacetate] ratio, mitochondrial [NAD+]/[NADH] ratio and glycerol 3-phosphate concentrations were returned virtually to control values. After 16 days of ethanol feeding (i.e. day 27 of the study), the redox-state changes observed in the ethanol-fed rats were less marked than at day 12, the increases in the [lactate]/[pyruvate] and [3-hydroxybutyrate]/[acetoacetate] ratios being 59% and 38% when ethanol-fed rats were compared with their pair-fed controls. The glycerol 3-phosphate concentration was increased by 37% in ethanol-treated rats compared with controls. The ethanol-induced increase in the [3-hydroxybutyrate]/[acetoacetate] ratio was not statistically significant at this time point, possibly as a result of the small number of animals studied. Once again, Methylene Blue corrected these redox-state changes in ethanol-fed animals, although the effect on the [lactate]/[pyruvate] ratio was not statistically significant. Ethanol alone did not affect hepatic ATP concentrations at day 27, whereas Methylene Blue caused a slight decrease in it in both controls and ethanol-fed animals. Serum ethanol concentrations were unaffected by Methylene Blue on both day 12 and 27 of the study. Hepatic concentrations of individual redox-pair metabolites during the study are shown in Table 2. Methylene Blue alone did increase lactate and acetoacetate concentrations on day 12 of the study, although no effect was observed on day 27. Hepatic lactate and pyruvate concentrations were consistently lower in the ethanol-fed animals. Methylene Blue corrected the [lactate]/[pyruvate]

Table 3. Effect of Methylene Blue supplementation of ethanol-containing liquid diets on hepatic triacylglycerol content during chronic ethanol feeding

Determinations were made on liver tissue taken at the same time as freeze-clamped samples for redox-state measurements were prepared as described in the legend to Table 1. Abbreviation: MB, Methylene Blue. Results are shown as means ± S.D. ($n = 3$ rats per group): *$P < 0.001$ versus (A) and versus day-12 value for the same group.

Day of study	Group...	Hepatic triacylglycerol (mg/g)			
		(A) Control	(B) Control+MB	(C) Ethanol	(D) Ethanol+MB
12		11.0±3.4	13.0±2.0	15.5±5.3	20.9±8.5
27		13.5±4.0	16.7±4.0	50.3±11.1*	46.9±8.7*

Table 4. Effect of Methylene Blue supplementation of ethanol-containing liquid diets on the hepatic lipid content after chronic ethanol feeding

Rats were given ethanol as part of a liquid diet in which 36% of the total energy was derived from ethanol. Pair-fed controls received diet in which glucose replaced the ethanol energy. Methylene Blue was added to the diets at a concentration of 130 mg/l, to give an average daily intake of 42 mg/day per kg. Animals were killed after 20 days of receiving 36% of their energy as ethanol (i.e. on day 30 of the study). Abbreviation: MB, Methylene Blue. Results are shown as means ± S.D. ($n = 6$ rats per group): *$P < 0.025$ versus (A); **$P < 0.001$ versus (A); †$P < 0.005$ versus (A).

Measurement	Group			
	(A) Control	(B) Control+MB	(C) Ethanol	(D) Ethanol+MB
Change in body wt. during study (g)	96.4±8.8	90.0±3.0	80.0±22.0	83.6±9.5
Ethanol intake (g/day per kg)	—	—	15.1±3.9	14.8±2.4
Liver wt. (g/100 g body wt.)	3.29±0.11	3.53±0.44	3.64±0.35*	3.73±0.14**
Liver				
Total lipids (mg/g)	48.0±10.7	52.5±8.4	90.7±10.8**	79.1±11.9**
Triacylglycerol (mg/g)	15.6±8.6	19.8±6.2	46.1±13.3**	38.5±6.7**
Reduced glutathione (μmol/g)	6.11±0.44	5.46±0.28	4.21±0.81†	4.58±1.10
Serum ethanol (mg/100 ml)	—	—	168±83	158±61
Serum glutamate dehydrogenase (units/l)	2.7±1.5	1.4±1.1	9.4±5.8*	9.3±5.5*

and [3-hydroxybutyrate]/[acetoacetate] ratios in ethanol-fed animals on day 12 through increases in hepatic pyruvate and acetoacetate concentrations, and by decreasing the 3-hydroxybutyrate concentration. Ethanol feeding caused significant increases in hepatic 3-hydroxybutyrate concentrations at both time points studied.

Hepatic triacylglycerol contents at these time points in the study are shown in Table 3. On day 12 there was no significant differences in triacylglycerol contents between any of the groups, whereas on day 27 there was significant increase in the ethanol-fed rats, irrespective of whether they had received Methylene Blue supplements. Hepatic lipid, body weight, ethanol intake and other determinations made at the end of the study are shown in Table 4. There was no difference in body-weight gain between any of the groups, and Methylene Blue did not affect ethanol intake. Ethanol feeding caused slight enlargement of the livers, which was not prevented by Methylene Blue supplementation. Ethanol feeding caused 89% and 196% increases respectively in the total hepatic lipid and hepatic triacylglycerol concentrations, which were not significantly influenced by Methylene Blue. Ethanol caused a slight decrease in the reduced glutathione content of the liver but, again, this was not affected by Methylene Blue. Serum ethanol concentrations and the elevated serum glutamate dehydrogenase activities observed in ethanol-fed animals were also not affected by Methylene Blue.

Histological examination of liver tissue revealed the presence of the characteristic centrilobular fatty infiltration of alcoholic steatosis in all the ethanol-fed animals, the extent or intralobular distribution of the accumulated fat not being affected by Methylene Blue supplementation.

DISCUSSION

The results of this study show that supplementation of ethanol-containing liquid diets with Methylene Blue can largely prevent the hepatocellular redox-state changes induced by chronic ethanol feeding in the rat. These redox-state changes are, however, less marked than those that are often observed after acute ethanol administration to naive rats, and they become attenuated after long-term feeding, confirming previous reports (Domschke *et al.*,

1974; Saluspuro *et al.*, 1981; Ryle *et al.*, 1985*b*). It has been suggested that one mechanism underlying this redox-state attenuation is enhanced Na^+/K^+-activated-ATPase activity and associated oxidative capacity after chronic ethanol feeding (Bernstein *et al.*, 1973). In our study, this attenuation of ethanol-induced redox-state changes between day 12 and day 27 occurred in spite of an increase in ethanol consumption by the animals in the intervening period, and the fact that serum ethanol concentrations were slightly higher at the later time point. The mechanism by which Methylene Blue corrects the ethanol-induced redox-state changes is thought to be through a non-enzymic oxidation of NADH. This may, in turn, affect availability of NADH for mitochondrial NADH oxidation and oxidative phosphorylation. This is demonstrated to some extent by the slightly lower ATP concentrations found on day 27 in the Methylene Blue treated animals. This decrease was not sufficient, however, to cause any evidence of deranged hepatocyte function, for example in the form of increased liver lipid contents or serum glutamate dehydrogenase activities, in control animals. Another possible consequence of the action of Methylene Blue might be decreased reduced glutathione concentrations as a result of lower NADPH concentrations to act as a cofactor for glutathione reductase. This, in turn, could possibly affect the susceptibility of hepatocytes to injury from toxins such as ethanol. Acute injections of Methylene Blue can cause hepatic glutathione depletion in mice (Hrushesky *et al.*, 1985); however, in the present study there was no evidence of Methylene Blue supplementation of the diet affecting hepatic reduced glutathione concentrations in either controls or ethanol-fed animals.

The concentrations of the individual redox-pair metabolites (Table 2) show that the lower carbohydrate content of the ethanol-containing diet causes decreases in the hepatic lactate and pyruvate concentrations as compared with the values obtained in pair-fed controls. Under these circumstances, there may be more dependence on fatty acid oxidation for hepatic energy production, hence ketone-body concentrations may increase simultaneously, provided that β-oxidation of fatty acids to C_2 fragments is not inhibited by ethanol. In this study, 3-hydroxybutyrate concentrations were higher in ethanol-fed animals, but probably only as a consequence of the increased mitochondrial [NADH]/[NAD^+] ratio. Overall, taking this into account, the ketone-body concentrations were comparable between the control and ethanol-fed rats, indicating that β-oxidation itself may not be affected by ethanol feeding. However, the complete oxidation of fatty acids to CO_2 may be impaired at the tricarboxylic acid-cycle stage, and thus be an important factor in the production of steatosis, as previously proposed by Lieber & Schmid (1961). This may arise after chronic ethanol intake through irreversible injury to the mitochondria (Cederbaum & Rubin, 1975), this being indicated in the present study by the elevated serum glutamate dehydrogenase activities in ethanol-fed animals, this enzyme being localized in the mitochondrial matrix.

The fact that Methylene Blue only caused partial correction of the cytosolic [NAD^+]/[NADH] ratio in ethanol-fed animals might indicate that the decrease in this ratio was a contributory factor in the production of steatosis. However, probably the most important consequence of the decrease in this ratio, which in turn might influence triacylglycerol synthesis, is the increase in glycerol 3-phosphate concentrations during ethanol feeding. However, concentrations of this metabolite were restored almost to control values by Methylene Blue without hepatic lipid content being decreased. Thus the true role of the altered cytosolic redox state in the production of fatty liver is still somewhat uncertain. The investigation of hepatic lipid concentrations clearly shows that, despite amelioration of the ethanol-induced redox-state changes, Methylene Blue does not prevent the fatty infiltration of the liver after chronic ethanol intake.

This study suggests that the increased [NADH]/[NAD^+] ratio resulting from ethanol oxidation is not the primary mechanism by which fatty liver is induced after long-term intake, and that the metabolic disturbances resulting from the redox-state change, including increased glycerol 3-phosphate concentrations, may not be implicated in ethanol-induced steatosis. This view is supported by the finding in the present study that there was no significant increase in hepatic triacylglycerol on the day when the ethanol-induced redox-state changes were most marked (i.e. day 12), whereas triacylglycerol accumulation was observed on day 27 when the ethanol-induced redox-state changes were attenuated. It is possible that other mechanisms, such as toxic effects attributable to acetaldehyde, which include impaired mitochondrial function, impairment of hepatic protein secretion, and initiation of lipid peroxidation, are more important in producing the fatty liver that results from chronic ethanol intake (Cederbaum & Rubin, 1975; Tuma *et al.*, 1980; Muller & Sies, 1982). Of these mechanisms, lipid peroxidation may be of importance, owing to the inhibitory action of antioxidant compounds on ethanol-induced hepatic steatosis, although the mechanism of ethanol-induced lipid peroxidation is not fully understood. It may, however, involve depletion of hepatic reduced glutathione by acetaldehyde as well as specific enzymic mechanisms such as superoxide production arising from acetaldehyde metabolism by xanthine oxidase (Hartman & Di Luzio, 1968; Ryle *et al.*, 1985*a*).

We are indebted to the Medical Research Council of Great Britain and the Medical Council on Alcoholism for their continued financial support. We also thank Mr. Brian Thackeray for technical assistance and Mrs. Jean Johns for typing the manuscript.

REFERENCES

Bernstein, J., Videla, L. & Israel, Y. (1973) Biochem. J. **134**, 515–521

Bernt, E. & Bergmeyer, H. U. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 1446–1451, Academic Press, London

Blomstrand, R., Kager, L. & Lantto, O. (1973) Life Sci. **13**, 1131–1141

Cairns, S. R. & Peters, T.J. (1983) Clin. Sci. **65**, 645–652

Cederbaum, A. I. & Rubin, E. (1975) Fed. Proc. Fed. Am. Soc. Exp. Biol. **34**, 2045–2051

Czok, R. & Lamprecht, W. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. H., ed.), pp. 1643–1647, Academic Press, London

De Carli, L. M. & Lieber, C. S. (1967) J. Nutr. **91**, 331–336

Domschke, S., Domschke, W. & Lieber, C. S. (1974) Life Sci. **15**, 1327–1334

Fletcher, M. J. (1968) Clin. Chim. Acta **22**, 393–397

Gutmann, I. & Wahlefeld, A. W. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 1464–1468, Academic Press, London
Hartman, A. D. & Di Luzio, N. R. (1968) Proc. Soc. Exp. Biol. Med. **127**, 270–276
Hrushesky, W. J. M., Olshefski, R., Wood, P., Meshnick, S. & Eaton, W. J. (1985) Lancet **i**, 565–566
Lieber, C. S. & De Carli, L. M. (1966) Gastroenterology **50**, 316–322
Lieber, C. S. & Schmid, R. (1961) J. Clin. Invest. **40**, 394–399
Mellanby, J. & Williamson, D. H. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 1840–1843, Academic Press, London
Michal, G. & Lang, G. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 1415–1418, Academic Press, London
Muller, A. & Sies, H. (1982) Biochem. J. **206**, 153–156
Nikkila, E. A. & Ojala, K. (1963) Life Sci. **2**, 717–721
Ontko, J. A. (1973) J. Lipid Res. **14**, 78–86
Rossiter, P. & Slater, T. F. (1973) Biochem. Soc. Trans. **1**, 933–935
Ryle, P. R., Chakraborty, J., Shaw, G. K. & Thomson, A. D. (1985*a*) Proc. Nutr. Soc. **44**, 11A
Ryle, P. R., Chakraborty, J. & Thomson, A. D. (1985*b*) Alcohol Alcohol. **20**, 287–291
Saluspuro, M. P., Shaw, S., Jayatilleke, E., Ross, W. A. & Lieber, C. S. (1981) Hepatology **1**, 33–38
Tuma, D., Zetterman, R. K. & Sorrell, M. F. (1980) Biochem. Pharmacol. **29**, 35–38
Williamson, D. H., Lund, P. & Krebs, H. A. (1967) Biochem. J. **103**, 514–527
Williamson, D. H. & Mellanby, J. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 1836–1839, Academic Press, London
Woodman, D. D. & Price, C. P. (1972) Clin. Chim. Acta **38**, 39–43

Received 12 June 1985/6 August 1985; accepted 27 August 1985