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ETHANOL METABOLISM IN THE VITAMIN C DEFICIENT GUINEA-PIG

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Abstract—Vitamin C deficiency, although reducing microsomal aniline hydroxylase and NADPH oxidase activities and decreasing the amount of cytochrome P-450, had no effect on the rate *in vivo* of ethanol elimination. Ethanol given as a daily oral dose of 2·5 g/kg for 14 days did not induce microsomal enzyme activities or increase the concentration of microsomal electron transport components. Drugs that are metabolized by the microsomal drug metabolizing system have increased plasma half lifes in scorbutic guinea-pigs, but no decrease in ethanol disappearance from the blood in the scorbutic animals used in the present study was noted. It is concluded that when ADH activity is normal, no further systems are required for ethanol metabolism.

Ethanol metabolism is regulated by both the redox state of the cytoplasm and the activity of alcohol dehydrogenase (ADH) (alcohol–NAD oxidoreductase EC 1.1.1.1) [1], the initial and rate limiting enzyme in the major pathway of ethanol metabolism [2].

A microsomal ethanol oxidizing system (MEOS) similar to the hepatic microsomal drug metabolizing system (MDMS) has been demonstrated *in vitro* [3]. MEOS can oxidize ethanol to acetaldehyde in the presence of NADPH and O_2. The significance *in vivo* of MEOS on the rate of ethanol clearance has been widely studied using inhibitors and inducers of MDMS [4–6], although several disadvantages of these methods have become apparent. The injection of SKF 525-A, an inhibitor of MDMS, caused a delay in the absorption of ethanol [4], and inducers such as phenobarbital and barbital inhibit ADH and prevent the redox state change after ethanol administration [5, 6].

The exact nature of MEOS activity has been elucidated using inhibitors to differentiate between the various enzymatic reactions involved [7]. Growing evidence now indicates that MEOS is not a separate enzyme system, but involves NADPH oxidase producing H_2O_2; ethanol is then oxidised by means of H_2O_2 and catalase. Unfortunately the various enzyme inhibitors used are not entirely specific, and some doubt on the nature and mechanism of MEOS activity still remains.

The guinea-pig is a useful animal model for the assessment of the significance *in vivo* of MEOS, as the plasma half-life of a large number of drugs is increased in the vitamin C-deficient (scorbutic) guinea-pig [8]. This is due to decreased microsomal drug hydroxylase activities and decreased amounts of microsomal electron transport components [9, 10]. Phenobarbital pretreatment returns the decreased microsomal drug hydroxylase activities to normal in the scorbutic guinea-pig, indicating that the enzyme protein synthesising mechanism in scorbutic guinea-pigs is operable [10].

Previous experimental work on the significance *in vivo* of MEOS has the disadvantage of non-specific effects introduced by the use of inhibitors and inducers of MDMS. In this study these non-specific effects have been eliminated, and experiments were carried out to assess the effect of ascorbic acid deficiency and chronic ethanol treatment on the hepatic redox state and ADH activity. The effects of decreased microsomal electron transport components and decreased enzyme activities on the rate *in vivo* of ethanol elimination, and the ability of ethanol to induce the decreased microsomal enzyme activities of the scorbutic guinea-pig were also investigated.

MATERIALS AND METHODS

Materials. Enzymes and coenzymes were obtained from the Boehringer Corporation (London) Ltd. Chemicals and substrates were obtained from British Drug Houses.

Animal treatment. Male albino guinea-pigs, weighing 200–250 g were fed on a vitamin C-deficient diet for 14 days. The ethanol pretreated groups were intubated daily with 2·5 g ethanol/kg body wt as a 50% (w/v) solution. Control groups received isocaloric quantities of glucose. Half the ethanol and glucose groups received a daily supplement of 50 mg of ascorbic acid dissolved in their respective intubation solutions. These animals were the normal controls for the scorbutic groups.

Vitamin C-deficient diet. The diet was based on that of Woodruff *et al.* [11] and had the following percentage composition, oat flakes 39; dried skimmed milk 30; wheat bran 20; vegetable oil 8; cod liver oil 2; and NaCl 1. This was supplemented with 0·5 g MgO and 0·5 g salt mixture/100 g diet.

The salt mixture contained (g), $CaCO_3$ 60; K_2HPO_4 64·5; NaCl 33·5; $MgSO_4$ 20·4; $CaHPO_4$ 15; ferric citrate 5·5; $MnSO_4 . 4H_2O$ 1·0; KI 0·16; $CuSO_4 . 5H_2O$ 0·06; and $ZnCl_2$ 0·05.

The following vitamin supplement was also added/100 g diet—nicotinamide 20 mg; calcium pantothenic 3 mg; thiamine hydrochloride 2 mg; riboflavin 2 mg; folic acid 2 mg; and pyridoxine hydrochloride 1 mg.

Each animal was also given a weekly supplement of 0·05 ml cod liver oil by intubation and 10 g of hay [12].

Redox state changes. The animals were fasted overnight (18 hr) on the 14th day and injected i.p. with 1·5 g ethanol/kg body wt as a 30% (w/v) solution in isotonic saline. Control animals received the same volume of isotonic saline. After 30 min the animals were killed by cervical dislocation and their livers rapidly removed

and freeze clamped. Metabolites were extracted by the method of Williamson *et al.* [13]. Lactate and pyruvate were assayed enzymatically by the methods of Hohorst [14] and Mellanby *et al.* [15] respectively.

Ethanol metabolism in vivo. The rate of elimination of ethanol from the blood was determined in a separate group of animals treated as above, except that after an overnight fast all the animals were injected with ethanol. Twenty μl blood samples were removed from the ear vein at half-hour intervals for 3 hr, and blood ethanol was measured by an internal standard gas–liquid chromatography method [16]. The rates of ethanol elimination from the blood, the amount of ethanol metabolized/kg body wt per hr, C_0 (theoretical ethanol concentration at zero times, assuming complete absorption and uniform distribution), and r(fraction of body mass in which ethanol is equilibrated with the blood) were determined by the method of Widmark as described by Khanna and Kalant [4].

Preparation of guinea-pig liver microsomes. Animals were treated as described above for 14 days but received no ethanol for 24 hr prior to sacrifice. The animals were killed by cervical dislocation and their livers perfused *in situ* with ice-cold isotonic saline; all further procedures were carried out at 4°. The livers were quickly removed, blotted and weighed, and 1 in 4 homogenates in 1·15% KCl were prepared using a Potter–Elvehjem homogenizer. The homogenate was spun for 10 min at 900 *g* and the supernatant produced spun at 20,000 *g* for 15 min. The microsome containing supernatant was spun for 60 min at 105,000 *g*, the microsomal pellet produced was washed, resuspended in 1·15% KCl and respun for a further 60 min at 105,000 *g*. The resulting washed microsomal pellet was suspended in 1·15% KCl to give a concentration of 6 mg protein/ml.

Aniline hydroxylase. Microsomal aniline hydroxylase activity was determined by the method of Holtzman and Gillette [17]. The activity was expressed as nmoles *p*-aminophenol produced/mg microsomal protein per hr at 37°.

Cytochrome P-450. The quantity of microsomal cytochrome P-450 was determined by the method of Omura and Sato [18]. The amount of cytochrome P-450 was expressed as nmoles/mg microsomal protein.

Cytochrome b_5. The quantity of microsomal cytochrome b_5 was determined by the method of Omura and Sato [18]. The amount of cytochrome b_5 was expressed as $\Delta E_{423nm-500nm}$/mg microsomal protein.

MEOS activity. The MEOS activity was determined by the method of Leiber and De Carli [3]. The activity was expressed as nmoles acetaldehyde produced/min per mg microsomal protein.

NADPH oxidase activity. NADPH oxidase activity was measured spectrophotometrically in microsomal preparations by determining the rate of disappearance of NADPH at 340 nm as described by Gillette *et al.* [19]. The specific activity was expressed as nmoles NADPH oxidized/min per mg microsomal protein.

Catalase activity. Microsomal catalase activity was measured using the oxygen electrode method of Goldstein [20]. The specific activity was expressed as μmoles O_2 evolved/min per mg microsomal protein.

ADH activity. Homogenates of liver 1-in-10 in 0·25 M sucrose containing 1% Triton X-100 [21] were centrifuged at 10,000 *g* for 15 min, and the supernatant spun for a further 60 min at 100,000 *g*. This supernatant was used for ADH determination as described by Hillbom and Pikkarainen [22]. The specific activity of ADH was expressed as units/g protein. (One enzyme unit is equal to 1 μmole of NADH produced per min at 25°).

Ascorbic acid levels. Total liver ascorbic acid was measured by the method of Bessey *et al.* [23] on deproteinized liver homogenates in 5% trichloracetic acid. Ascorbic acid concentration was expressed as μg ascorbic acid/g wet wt liver.

Protein was determined by the method of Lowry *et al.* [24].

RESULTS

Redox state changes in chronic ethanol treated scorbutic and normal guinea-pigs. The well documented cytoplasmic redox state shift after ethanol administration has been demonstrated in the present work (Table 1) for all the animals injected with ethanol, compared with their saline injected controls. There is a small but significant decrease in the redox state shift with ethanol in both the ethanol pretreated normal and scorbutic animals, compared with their respective untreated controls ($P < 0·05$). No statistical difference can be seen between the redox states of the

Table 1. Cytoplasmic redox states and calculated free NADH/free NAD^+ ratios* with and without ethanol§

Groups	Injection	Lactate	Pyruvate	Lactate/pyruvate	Free NADH/free NAD^+ $\times 10^4$
Scorbutic	Saline	780 ± 150	39 ± 5	21·4 ± 2·5	23·8 ± 2·8
Scorbutic	Ethanol	1106 ± 163	28 ± 6	†38·9 ± 2·6	43·2 ± 2·9
Scorbutic + chronic ethanol	Saline	793 ± 155	38 ± 5	19·6 ± 2·7	20·7 ± 3·0
Scorbutic + chronic ethanol	Ethanol	1024 ± 159	26 ± 4	†34·4 ± 3·0	38·2 ± 3·2
Normal	Saline	750 ± 149	33 ± 5	21·1 ± 2·4	24·7 ± 2·7
Normal	Ethanol	1044 ± 161	24 ± 4	‡40·1 ± 2·5	44·5 ± 2·8
Normal + chronic ethanol	Saline	712 ± 140	31 ± 5	21·4 ± 1·9	23·8 ± 2·2
Normal + chronic ethanol	Ethanol	1012 ± 158	25 ± 5	‡36·1 ± 2·8	40·2 ± 3·1

* Calculated from the lactate/pyruvate ratio and using the value of $1·11 \times 10^{-4}$ for the equilibrium constant of lactate dehydrogenase [11].

† and ‡ differ by $P < 0·05$.

§ Metabolite concentrations are nmoles/g wet wt liver. Each figure is the mean ± S.D. of six animals.

Table 2. Effect of chronic ethanol treatment and vitamin C-deficiency on body weight, liver weight, ADH activity and liver ascorbic acid content of guinea-pigs.*

	Normal	Scorbutic	Normal + chronic ethanol	Scorbutic + chronic ethanol
Terminal body wt g ± S.D.	286 ± 17	273 ± 32	266 ± 29	269 ± 33
Liver wt g ± S.D.	11·0 ± 1·0	10·2 ± 1·4	10·0 ± 0·8	10·5 ± 1·8
Liver wt/body wt	0·038 ± 0·002	0·037 ± 0·003	0·037 ± 0·003	0·039 ± 0·003
ADH activity (U/g protein)	9·6 ± 1·5	9·9 ± 1·4	9·8 ± 0·9	10·4 ± 1·7
Liver ascorbic acid (μg/g wet wt)	164 ± 23	46 ± 7	159 ± 19	42 ± 5

* Each figure represents the mean of six animals ± S.D.

scorbutic groups and their corresponding normal controls.

ADH activity and liver ascorbic acid content in chronic ethanol treated scorbutic and normal guinea-pigs. No difference in terminal body wt, liver wt, liver/body wt or ADH activity was found between any of the groups of animals studied (Table 2). The liver ascorbic acid content was decreased to 30 per cent of normal values in both the scorbutic and chronic ethanol treated scorbutic groups, although this did not produce any changes in the other parameters measured in Table 2.

Effect of ascorbic acid deficiency and chronic ethanol treatment on microsomal enzyme activities, electron transport components and protein content. Aniline hydroxylase and NADPH oxidase activities were significantly decreased in the scorbutic groups compared to their normal controls. Catalase and MEOS activities

Table 3. Effect of chronic ethanol treatment and vitamin C deficiency on microsomal enzyme activities, electron transport components, and protein content*

	Normal	Scorbutic	Normal + chronic ethanol	Scorbutic + chronic ethanol
Aniline hydroxylase (nmoles *p*-aminophenol produced/mg microsomal protein per hr.)	58 ± 7	45 ± 5†	61 ± 8	46 ± 7†
NADPH oxidase (nmoles NADPH oxidised/min per mg microsomal protein)	8·3 ± 1·3	6·1 ± 1·3‡	8·2 ± 1·4	5·9 ± 1·4‡
Catalase (μmoles O_2 evolved/min per mg microsomal protein)	9·2 ± 1·2	9·5 ± 1·5	9·3 ± 1·8	9·1 ± 1·9
MEOS (nmoles acetaldehyde produced/min per mg microsomal protein)	1·60 ± 0·22	1·60 ± 0·28	1·61 ± 0·26	1·60 ± 0·20
Cytochrome P-450 (nmoles/mg microsomal protein)	0·934 ± 0·073	0·709 ± 0·073§	0·963 ± 0·092	0·709 ± 0·086§
Cytochrome b_5 ($\Delta E_{423-500nm}$/mg microsomal protein)	0·073 ± 0·009	0·078 ± 0·008	0·070 ± 0·006	0·070 ± 0·009
Microsomal protein content (mg/g wet wt liver)	9·90 ± 0·50	8·78 ± 0·68†	9·65 ± 0·71	8·31 ± 0·77‡

*Each figure represents the mean ± S.D. of six animals.
Significance: † $P < 0·02$ ‡ $P < 0·05$ § $P < 0·005$ with respect to normals.

Table 4. Ethanol metabolism *in vivo**

	C_o (mg ethanol/100 ml blood)	r	Disappearance of ethanol from blood (mg/100 ml per hr)	Ethanol metabolized (mg/kg body wt/hr)
Normal	153·3 ± 11·1	0·970 ± 0·053	29·3 ± 2·7	280·4 ± 13·2
Scorbutic	152·8 ± 10·3	0·984 ± 0·071	28·1 ± 2·6	274·5 ± 12·9
Normal + chronic ethanol	154·8 ± 7·4	0·970 ± 0·025	28·6 ± 2·2	278·8 ± 11·5
Scorbutic + chronic ethanol	152·0 ± 8·3	0·986 ± 0·063	28·4 ± 2·8	276·6 ± 12·4

* Each figure represents the mean of six animals ± S.D.

were unchanged. Although cytochrome P-450 content is significantly decreased in the scorbutic groups, there is no change in the concentration of cytochrome b_5. Microsomal protein content is decreased in the scorbutic groups compared with their controls. No evidence of microsomal enzyme induction with ethanol is evident.

Metabolism of ethanol in vivo. From Table 4 it can be seen that pretreatment of guinea-pigs with 2·5 g ethanol/kg body wt for 14 days has no effect on the rate of ethanol disappearance from the blood, or the calculated rate of ethanol metabolism. Neither does vitamin C deficiency impair ethanol metabolism *in vivo* in guinea-pigs fed a scorbutic diet for 14 days.

DISCUSSION

Drugs that are metabolized by the MDMS have an increased plasma half life in scorbutic guinea-pigs [8], but no decrease in the rate of elimination of ethanol from the blood of scorbutic animals used in the present study was observed.

An increased rate of elimination of ethanol might have been expected for the chronic ethanol treated animals, as there was a small but significant decrease in the cytoplasmic redox state shift with ethanol compared to their respective untreated controls (Table 1). The redox state is important in the regulation of ethanol elimination *in vivo* [1], although in the present work, the redox state shift is only decreased by 10 per cent of that of the untreated control animals. Hillbom [25] demonstrated an increase in ethanol elimination of 20 mg ethanol/kg body wt/hr in rats treated with promethazine which decreased the redox state shift with ethanol by 50 per cent of that of untreated control animals. The decrease in the redox state shift in the present work is probably too small to effect ethanol elimination, although it may represent metabolic adaption to ethanol via increased mitochondrial permeability to NADH after chronic ethanol treatment, which has been demonstrated by Rawat and Kuriyama [26] in chronic ethanol treated mice.

Aniline hydroxylase and NADPH oxidase activities, as well as cytochrome P-450 content were all decreased in the scorbutic animals, although no significant decreases were shown for catalase and MEOS activity. This adds further evidence of a role for catalase in MEOS activity, although NADPH oxidase and the production of H_2O_2, is considered the rate limiting step in this system [27], and may not be sufficiently reduced to effect MEOS activity. Cytochrome b_5 was not changed in the scorbutic animals which is in agreement with Wade *et al.* [28], who examined guinea-pigs after 12–18 days on a scorbutic diet.

No induction of microsomal enzymes with ethanol was evident in this study, although this has been shown by other workers in rats fed a semi-liquid diet containing large amounts of ethanol [27]. These rats received 12–14 g ethanol/kg per day which induced microsomal enzymes, as well as increasing the rate of elimination of ethanol. Induction of microsomal systems depends on the quantity of ethanol and its mode of administration. In the present study 2·5 g ethanol/kg per day was given as a single dose, and this could not be raised as the LD_{50} for a single oral dose of ethanol in guinea-pigs is 4 g/kg [29].

No evidence has been found for a role *in vivo* for MEOS in ethanol metabolism, as a reduction in microsomal enzyme activities and electron transport components had no effect on ethanol elimination *in vivo*. Therefore, when ADH activity is normal, no further systems appear to be required for ethanol metabolism. This is in agreement with other studies on the significance *in vivo* of MEOS, except that in the present work non-specific effects of inhibitors and inducers of MDMS have been avoided.

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