Vitamin E and hepatotoxic agents

2*. Lipid peroxidation and poisoning with orotic acid, ethanol and thioacetamide in rats

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1. Fatty liver was induced in 4-month-old male rats by oral dosing with ethanol. The marked increase in liver fat was not accompanied by a rise in lipid peroxides.

2. Homogenates were prepared from the livers of vitamin E-deficient rats and incubated with ethanol. In the concentration range of 10–50 μl/3 ml, ethanol increased the production of malondialdehyde. Methanol, which is not a hepatotoxin, showed a similar effect at 10–35 μl/3 ml homogenate. These findings indicate that the pro-oxidative effect of alcohols in vitro is unrelated to their hepatotoxic action in vivo.

3. Fatty liver was induced in 3·5-month-old, vitamin E-deficient male rats by oral dosing with ethanol. The effect of pretreatment with vitamin E and N,N′-diphenyl-*p*-phenylenediamine (DPPD) was studied. D-α-Tocopheryl acetate, given as three doses of 350 mg/kg at 48, 24 and 2 h before the ethanol, failed to decrease the fat accumulation and seemed rather to increase the fat content of the liver. DPPD, given as three doses of 600 mg/kg at similar intervals before the ethanol dose, reduced the fat content of the liver almost to normal.

4. Weanling rats of both sexes were given a vitamin E-deficient diet containing 1 % orotic acid for 15 days to induce fatty liver. Dietary supplements of D-α-tocopheryl acetate (500 ppm), selenium (1 ppm) or DPPD (100 ppm) did not reduce the lipid accumulation. Lipid peroxides and malondialdehyde levels were lower in the livers of animals treated with orotic acid than in controls, regardless of the presence of vitamin E.

5. Liver necrosis was produced in 9-week-old female vitamin E-deficient rats by the intraperitoneal injection of 200 mg thioacetamide. Promethazine hydrochloride (Phenergan), given intraperitoneally as two doses (25 mg/kg at the same time as the thioacetamide and 12·5 mg/kg 6 h later), markedly reduced the necrosis. D-α-Tocopheryl acetate, given as two oral doses of 1000 mg/kg 48 h and 24 h before the thioacetamide, tended to exacerbate the necrosis.

6. The results are discussed in relation to the question of lipid peroxidation as a cause of hepatotoxicity.

In the first paper of this series direct evidence for the participation of lipid peroxidation in the mechanism of CCl_4 hepatotoxicity (Ghoshal & Recknagel, 1965) was sought and found to be lacking. It has been suggested by other workers that a number of other agents are hepatotoxic because of their ability to initiate chains of free-radical catalysed lipid peroxidation. In this paper we report on an examination of some of these substances and on the action of vitamin E.

EXPERIMENTAL AND RESULTS

Methods

Animals and diets. Rats of the Norwegian hooded strain were used. Two vitamin E-deficient diets were used. Diet G 15 F was that of Cawthorne, Diplock, Muthy,

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Bunyan, Murrell & Green (1967). Diet G 10 F was similar, with the casein reduced from 15 to 10%, the replacement being sucrose.

Total lipids. The lipids from 2 g of liver were extracted as described by Diplock, Cawthorne, Murrell, Green & Bunyan (1968) and dried to constant weight at 105°.

Neutral triglycerides. The lipid obtained as above was dissolved in 5 ml diethyl ether and passed through a column of 1 g silicic acid (Mallinckrodt). The column was eluted with a further 20 ml ether and the eluate evaporated to dryness to give the neutral tryglyceride fraction. Triglycerides were determined by the colorimetric method of Van Handel & Zilversmit (1957).

Lipid peroxides. The micro-iodimetric method of Bunyan, Murrell, Green & Diplock (1967) was used.

Malondialdehyde (MDA). The liver, excised immediately after death, was homogenized in Tris-HCl buffer, pH 7·4, according to the general methods described previously (Bunyan, Green, Diplock & Robinson, 1967). The method of Placer, Cushman & Johnson (1966) was used to determine in vivo MDA at zero time. MDA produced in vitro was measured after 1 h incubation as described by Bunyan, Diplock, Edwin & Green (1962).

Histology. Slices, 2 mm thick, were cut from each liver, fixed in buffered formol phosphate (pH 7·4) and embedded in Paramat (G. T. Gurr Ltd, London). Sections were stained with haematoxylin and eosin. The degree of degenerative change was assessed on an arbitary scale 0, +, ++, +++, or ++++, as described by Rees, Sinha & Spector (1961).

Calcium. This was determined on a portion of liver by the method of De Loureiro & Janz (1944).

Ethanol poisoning

Administration of ethanol rapidly induces fatty liver in normal rats (Mollov & Bloch, 1956). It has been stated that vitamin E and antioxidants such as N,N′-diphenyl-*p*-phenylenediamine can markedly reduce the liver fat accumulation, and it has been suggested that the primary process involved in ethanol poisoning, as in CCl_4 poisoning, is lipid peroxidation (Di Luzio, 1964, 1966; Di Luzio & Costales, 1965; Comporti, Hartman & Di Luzio, 1967; Di Luzio & Hartman, 1967). In the following experiments we studied the relation between ethanol toxicity in the rat and lipid peroxidation.

Comporti *et al.* (1967) and Di Luzio & Hartman (1967) found that ethanol, incubated with normal liver homogenates, was a pro-oxidant, increasing the production of MDA. This effect, however, was shown only when the ethanol was in the critical concentration range of 10–50 μl/4 ml. In our experiments vitamin E-deficient female rats were used. The rats were killed and 5% (w/v) liver homogenates were prepared. A portion of each homogenate was incubated to provide the control and, to other portions, ethanol or methanol was added in concentrations similar to those used by Comporti *et al.* (1967). The results (Table 1) show that ethanol, in the range 10–50 μl/3 ml homogenate, increased MDA production during incubation for 1 h. This is in agreement with the finding of Comporti *et al.* (1967). However, methanol,

which is not a hepatotoxic agent, also increased MDA production. The critical concentration range for methanol was lower than for ethanol, in accordance with the difference in molecular weight between the two substances.

Table 1. *Effects of ethanol and methanol on the production of malondialdehyde (MDA) during incubation of rat liver homogenates in vitro*

(In each experiment a 5% (w/v) homogenate was prepared from the livers of two 7-month-old vitamin E female rats. Each result given is the mean amount, with standard deviation, of MDA produced in three 3 ml portions of the homogenate incubated in air for 1 h at 37°, with the addition of ethanol or methanol as shown)

		MDA (μg/g liver per h)		
Addition (μl)		Expt 1	Expt 2	Expt 3
None		43·4 ± 3·0	45·5 ± 2·8	36·2 ± 2·2
Ethanol	10	54·4* ± 1·4	.	.
	30	54·9* ± 3·2	.	.
	50	56·3* ± 2·2	49·6 ± 0·4	38·7 ± 6·8
	70	42·0 ± 1·0	.	.
	100	42·7 ± 2·5	.	.
Methanol	10	.	56·3† ± 5·2	35·6 ± 0
	20	.	54·4† ± 3·2	42·4† ± 1·1
	35	.	55·5† ± 11·0	38·8 ± 1·4
	50	.	41·5 ± 1·5	37·1 ± 1·4
	70	.	38·2 ± 1·4	37·1 ± 2·5

* Significantly greater than the control value ($P < 0.01$).
† Significantly greater than the control value ($P < 0.05$).

Table 2. *Effect of ethanol on triglycerides and lipid peroxides in the liver of the male rat*

(Rats, 4 months old, were starved for 18 h, given ethanol (50%, v/v, in saline) orally, and killed 24 h later. Livers were combined in pairs for each analysis)

Ethanol dose (ml/kg)	Lipid peroxides (μ-equiv./g)	Triglycerides (mg/g)
0	1·05, 1·14, 0·85	7·5, 7·3, 7·3
6	0·70	8·9, 13·3
10	1·32, 0·85	21·0, 31·0

In the second experiment (Table 2) 4-month-old male rats (weight range, 200–300 g), given an adequate diet, were used. The rats were starved for 18 h and then given ethanol (6–10 ml/kg) as a 50% (v/v) solution in normal saline by mouth. Control rats were given saline only. They were killed 24 h later and their livers analysed in pairs for neutral triglycerides and true lipid peroxides. The results show that the ethanol produced a pronounced rise in liver fat but that there was no increase in the liver peroxide value.

In the third experiment the effects of α-tocopherol and DPPD on ethanol-induced fatty liver were studied. Weanling male rats were given the vitamin E-deficient diet, G 15 F, until they were 3·5 months old. They were then divided at random into six groups of seven. Groups 1 and 2 acted as unsupplemented controls, groups 3 and 4

were given three oral doses of D-α-tocopheryl acetate (350 mg/kg at 48, 24 and 2 h before the ethanol dose); groups 5 and 6 were given oral doses of DPPD (600 mg/kg) at the same times. Groups 2, 4, and 6 were given ethanol (10 ml/kg) orally as a 66% (v/v) solution in normal saline; groups 1, 3 and 5 received an equal volume of saline. All the rats were starved for 18 h before the ethanol or saline was administered and for 1 h afterwards. They were killed 24 h after the ethanol dose and their livers were analysed. The rats in groups 2 and 4 slept for 5–6 h after ethanol administration, but it was noticed that DPPD prolonged the sleeping time, the rats in group 6 remaining asleep for the whole 24 h afterwards (one rat died).

The results are shown in Table 3. Ethanol increased the triglyceride levels in the liver about threefold. α-Tocopherol supplementation did not affect this. DPPD, however, almost entirely eliminated the accumulation of fat.

Table 3. *Effects of α-tocopherol and N,N'-diphenyl-*p*-phenylenediamine (DPPD) on ethanol-induced fatty liver in the rat*

(Six groups of seven 3·5-month-old vitamin E-deficient male rats were used. The rats were killed 24 h after the ethanol dose. Livers were analysed individually. Results are given as means with standard deviations)

Supplement	Ethanol treatment*	Rat wt (g)	Liver wt (g)	Liver triglycerides (mg/g)
None	−	198 ± 26	6·9 ± 1·1	10·9 ± 2·8
None	+	217 ± 24	5·9 ± 0·6	39·7 ± 21·5
Vitamin E†	−	234 ± 36	8·6 ± 1·6	15·8 ± 6·9
Vitamin E†	+	205 ± 24	6·3 ± 0·6	37·8 ± 11·8
DPPD‡	−	211 ± 20	7·7 ± 1·3	13·7 ± 6·2
DPPD‡§	+	214 ± 27	8·1 ± 2·9	17·0 ± 8·3‖

* +, Ethanol given orally at 10 ml/kg as 66% (v/v) solution in normal saline; −, normal saline given orally.

† D-α-Tocopheryl acetate as three doses of 350 mg/kg at 48, 24 and 2 h before the ethanol dose.

‡ Three doses of 600 mg DPPD/kg at 48, 24 and 2 h before the ethanol dose.

§ One rat died and was not analysed.

‖ Significantly lower than the value for rats given ethanol and no other treatment ($P < 0·01$).

Orotic acid poisoning

Feeding rats on orotic acid causes fatty liver. Kinsella (1967*a*, *b*), in his study of this condition, extracted the liver lipids and measured their 'zero-time' MDA content, taking this value as a measure of 'peroxides' in the liver. We followed his procedure (Kinsella, 1966), taking precautions to avoid oxidation of the lipids during handling, and measured true lipid peroxides by the micro-iodimetric method of Bunyan, Murrell *et al.* (1967). In addition, we compared the amounts of MDA in these lipid extracts with the amounts found in whole liver homogenates at zero time. We found four times as much MDA in the whole homogenate as in the lipid extract and therefore used the former to provide estimates of the amounts of water-soluble MDA present in the liver in vivo.

Weanling rats (weight range 49–60 g) of both sexes were allocated at random to eight groups. They were given the vitamin E-deficient diet, G 10 F. The groups received additional dietary supplements of orotic acid, vitamin E, sodium selenite and DPPD,

as shown in Table 4. After 15 days on the experimental diets the rats were killed by asphyxiation with nitrogen and their livers were analysed for total lipids, lipid peroxides and 'zero-time' MDA. The livers were combined in pairs, one from each sex, for analysis.

The results are shown in Table 4. Orotic acid produced a marked increase in the liver lipids and this increase was unaffected by vitamin E, Se, or DPPD. The latter three supplements, in the rats without orotic acid, also produced a smaller but significant rise in the liver lipids. We have observed this on other occasions with this vitamin E-deficient diet. Orotic acid increased neither true lipid peroxides nor MDA in the liver lipids; indeed, the values were generally lower in the orotic acid-supplemented animals.

Table 4. *Effect of vitamin E, selenium and N,N'-diphenyl*-p-*phenylenediamine (DPPD) on orotic acid-induced fatty liver in the rat*

(Weanling rats of both sexes, in eight groups, were given a vitamin E-deficient diet, with and without 1 % orotic acid and the supplements shown. They were killed after 15 days and their livers analysed in pairs (one rat of each sex). Results, where applicable, are given as means with standard deviations)

Dietary supplement	Orotic* acid	Liver wt† (g)	Lipid† (mg/g)	Lipid peroxide (μ-equiv./g)	Malondialdehyde (μg/g)
None	−	2·94 ± 0·75 (8)	35·6 ± 5·6 (8)	0·37, 1·26	0·57, 0·45
None	+	3·56 ± 0·82 (8)	155·9 ± 39·3 (8)	0·58, 0·62	0·30, 0·30
Vitamin E‡	−	2·51 ± 0·76 (10)	65·1 ± 25·3 (10)	1·02, 1·30	0·42, 0·55
Vitamin E‡	+	4·00 ± 1·17 (10)	136·1 ± 34·1 (10)	0·46, 0·46	0·30, 0·38
Selenium§	−	2·96 ± 0·64 (6)	54·6 ± 10·1 (6)		
Selenium§	+	4·71 ± 0·99 (6)	196·0 ± 53·2 (6)		
DPPD‖	−	3·29 ± 0·43 (6)	51·1 ± 7·2 (6)		
DPPD‖	+	4·6 ± 1·11 (6)	191·9 ± 89·9 (6)		

* +, diet with 1 % orotic acid; −, diet without orotic acid.
† No. of rats shown in parentheses. ‡ DL-α-Tocopheryl acetate, 500 ppm.
§ Se, 1 ppm, as sodium selenite. ‖ 100 ppm.

Thioacetamide poisoning

Thioacetamide does not cause fat accumulation in the liver but produces a centrilobular necrotic degeneration, accompanied by raised liver calcium levels and leakage of hepatic enzymes. Gallagher, Gupta, Judah & Rees (1956) and Rees *et al.* (1961) showed that promethazine hydrochloride (Phenergan; May & Baker Ltd) and other antihistamines, given in large doses immediately before the thioacetamide, considerably reduced the necrotic process, calcium levels and leakage of enzymes. Antihistamines are also active against CCl_4-induced liver necrosis, and Slater (1966), who considers that the mechanism of CCl_4 toxicity is 'lipid peroxidation', suggests that they act as 'antioxidants', inhibiting free radical-producing systems at lipid interfaces. It was, therefore, of some interest to examine the effect of vitamin E on thioacetamide-induced liver necrosis.

Female rats were given the vitamin E-deficient diet, G 15 F, until they were 9 weeks old. They were divided at random into three groups of six and then given 200 mg/kg thioacetamide by intraperitoneal injection. Group 1 acted as controls; group 2 was

given two oral doses of D-α-tocopheryl acetate (1000 mg/kg) at 48 and 24 h before the thioacetamide; group 3 was given two interaperitoneal doses of Phenergan, one of 25 mg/kg at the same time as the thioacetamide, the other of 12·5 mg/kg 6 h later. All the rats were killed 24 h after the thioacetamide dose. Their livers were examined histologically and their calcium content was measured. The results (Table 5) show that thioacetamide induced necrosis and raised liver calcium levels. Phenergan markedly reduced the liver necrosis, but vitamin E had no effect. Neither vitamin E nor Phenergan lowered the calcium levels.

Table 5. *Effects of vitamin E and promethazine hydrochloride (Phenergan) on thioacetamide-induced liver necrosis in the rat*

(Three groups of six 9-week-old vitamin E-deficient female rats were given thioacetamide, 200 mg/kg intraperitoneally, and killed 24 h later. One group was treated with vitamin E, another with Phenergan. Calcium analyses were carried out on individual livers and the results are given as means with standard deviations)

Treatment	Degree of necrosis*	Calcium† (μg/ liver)
None	++++(1); +++(1); ++(3); +(1)	446 ± 32
Vitamin E‡	++++(3); +++(2); +(1)	364 ± 137
Phenergan§	+++(1); +(5)	483 ± 88

* Degree of degenerative change assessed on an arbitrary scale of 0, +, ++, +++, and ++++, with the number of rats shown in parentheses.

† The normal calcium level found in similar rats was about 70 μg/g.

‡ D-α-Tocopheryl acetate, 1000 mg/kg, given orally at 48 and 24 h before the thioacetamide dose.

§ Intraperitoneal doses of 25 mg/kg at the same time as the thioacetamide dose and 12·5 mg/kg at 6 h afterwards.

DISCUSSION

Butler (1961) suggested that CCl_4 was hepatotoxic because it could produce, in the liver, free radicals, which would be highly destructive towards certain intracellular compounds. His original concept did not include the idea of lipid peroxidation, with which, indeed, it is not logically connected. The connexion between the two concepts arises because vitamin E and certain antioxidant substances have been shown to reduce the toxic effects of CCl_4. Slater (1966) and Recknagel (1967) have suggested that the CCl_4–vitamin E relationship in vivo is simply that of pro-oxidant and antioxidant, and in the preceding paper of this series (Green, Bunyan, Cawthorne & Diplock, 1969) we have discussed these views.

Attempts have been made to accommodate other examples of hepatotoxicity and 'antioxidant' protection into the 'lipid peroxidation' framework. Thus, Di Luzio and his colleagues have suggested that the mechanism of ethanol toxicity involves lipid peroxidation. Di Luzio (1964, 1966) and Di Luzio & Costales (1965) showed that ethanol-induced fatty liver was markedly reduced by DPPD, given intraperitoneally or orally, and by oral administration of an antioxidant mixture containing butylated hydroxytoluene. Di Luzio & Costales (1965) found that α-tocopherol, given intraperitoneally, was also effective, although apparently less so than DPPD. Comporti *et al.* (1967) and Di Luzio & Hartman (1967) found that liver homogenates from rats treated with ethanol produced more malondialdehyde (MDA) in vitro than control

homogenates and that addition of ethanol in vitro also increased MDA production in homogenates. They considered these findings to demonstrate that ethanol could increase lipid peroxidation in the liver.

In our experiments we confirmed that ethanol, added to liver homogenates in vitro, increased the production of MDA. However, in similar experiments we found that methanol also increased MDA production. Methanol, unlike ethanol, does not produce fatty liver in rats (Eger & Zundorf, 1953). It would appear that the pro-oxidant effect of these alcohols in vitro has no relation to the problem of their hepatotoxicity.

Two additional points about the results of Comporti *et al.* (1967) can be commented on. The increased MDA values they found with liver homogenates from rats treated with ethanol were not adjusted for the large increase in liver fat. If these MDA values are adjusted to a g-lipid basis, the pro-oxidant effect of ethanol disappears. There is also an incongruity between the findings of Comporti *et al.* (1967) on ethanol and those of Recknagel & Ghoshal (1966) on CCl_4. Using conditions essentially similar to those of Comporti *et al.* (1967), Recknagel & Ghoshal (1966) found that the postulated 'pro-oxidant' effect of CCl_4 on the liver in vivo could not, in fact, be demonstrated by an increase in the production of MDA in vitro. They suggested that this was because 'liver can further metabolize the TBA-positive products of lipoperoxidation'.

We have previously criticized the use of MDA production in vitro as a measure of peroxidation in vivo (Bunyan, Murrell *et al.* 1967) and it is our opinion that neither the in vivo nor the in vitro values for MDA are relevant to the mechanism of hepatotoxicity of either ethanol or CCl_4. In confirmation, we found no rise in true lipid peroxides in the livers of rats treated with ethanol (Table 2). Furthermore, although, in agreement with Di Luzio & Costales (1965), oral doses of DPPD markedly reduced ethanol-induced fatty liver, we found vitamin E given orally to be without effect. In some other (unpublished) experiments we have found that the concentration of both α-tocopherol and DPPD in liver produced by the doses described in Table 2 is of the order of 100 μg/g, which is within the known range of efficient antioxidant action. The remarkable difference in the effectiveness of the two substances against ethanol toxicity cannot readily be explained on the basis of the 'biological antioxidant' hypothesis. Porta, Hartroft & de la Iglesia (1965) also found that vitamin E, at 150 ppm in the diet, had no effect on ethanol toxicity in the rat.

Hashimoto & Recknagel (1968), using the 'diene conjugation' method of Recknagel & Ghoshal (1966) and also, as we have done here, direct iodimetric determination of the peroxides, found no evidence of lipid peroxidation involvement in ethanol toxicity. They conclude that 'for antioxidant protection against ethanol toxicity, the question as to properties of the protective agents other than their antioxidant action must be raised, and by extension, the question can also be raised as to whether, in the case of CCl_4 poisoning, the protection afforded by antioxidants might not be possibly due to properties other than or in addition to their antioxidant action'. With this we are in agreement.

The induction of fatty liver in rats with orotic acid was found to be unaffected by dietary vitamin E, DPPD or Se. The suggestion, therefore, of Kinsella (1967*a*, *b*), on the basis of changes in the MDA level of the liver, that the primary process involved

must be lipid peroxidation would seem to carry the 'peroxidation' hypothesis of hepatotoxicity to a *reductio ad absurdum*, for it implies a type of 'peroxidation' that cannot be affected by 'antioxidants'. Our results show that there is a lower concentration of lipid peroxides in orotic acid-treated liver than in control liver, and this would be expected if the resting peroxide levels were merely diluted by the fat accumulation. Di Luzio & Hartman (1967) also suggested that antioxidants were ineffective against orotic acid-induced fatty liver, although, it should be noted, they considered that lipid peroxidation was involved in its genesis. It is, however, generally considered that orotic acid induces fatty liver by interfering with lipoprotein metabolism (Roheim, Switzer, Gerard & Eder, 1966).

Thioacetamide poisoning in rats produces liver necrosis without fatty infiltration, and it was shown by Gallagher *et al.* (1956) that promethazine hydrochloride, (Phenergan) would prevent this process. Rees *et al.* (1961) showed that Phenergan would prevent the necrosis, but not the fat accumulation, produced by CCl_4 poisoning. Slater (1966) suggested that the necrogenic action of CCl_4 was due to lipid peroxidation and that the action of Phenergan was due to its acting as an antioxidant. However, our results show that, although Phenergan was effective against thioacetamide-induced necrosis, oral administration of vitamin E was not. How, then, can this be reconciled with the view that both substances protect against the necrosis of CCl_4 poisoning by virtue of the same mechanism, i.e. the ability to form free radicals in vivo (Gallagher, 1961)?

Correlation of the evidence from experiments with these three toxic agents, ethanol, orotic acid and thioacetamide, suggests that it is highly unlikely that lipid peroxidation is the primary event leading to either necrosis or fatty infiltration. The mechanisms by which certain substances may exert protective actions need further investigation.

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