Vitamin E and hepatotoxic agents

3.* Vitamin E, synthetic antioxidants and carbon tetrachloride toxicity in the rat

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1. The acute toxicity of orally administered CCl_4 and its subacute toxicity (liver necrosis and hepatic fat accumulation) were studied in young adult male and female rats. CCl_4 was more toxic in males than in females. The protective effects of vitamin E (D-α-tocopheryl acetate) and three synthetic antioxidants, DPPD (*N,N'*-diphenyl-*p*-phenylenediamine), ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) and BHT (butylated hydroxytoluene) were studied.

2. Three daily oral doses (450 mg/kg) of vitamin E given before CCl_4 increased survival in male rats but not consistently in females. Single oral doses (450 mg/kg) given previously at times between 24 and 72 h were also protective in males, but slightly decreased survival in females. Single intraperitoneal doses (500 mg/kg) given to female rats 1–48 h before CCl_4 had no effect on survival. None of these treatments with vitamin E significantly decreased CCl_4-induced hepatic triglyceride accumulation. A large oral dose (2000 mg/kg) 6 h before CCl_4 not only significantly increased hepatic fat accumulation but also increased mortality.

3. Three daily doses (600 mg/kg) of DPPD or a single oral dose given 72 h before CCl_4 increased survival in male and female rats. When single doses of DPPD were given 6–48 h before CCl_4 they had no effect on survival. In contrast, DPPD usually significantly decreased the CCl_4-induced hepatic triglyceride rise when given orally 6–72 h before CCl_4. Single intraperitoneal doses (100–1500 mg/kg) of DPPD, given 1–48 h before CCl_4, decreased the hepatic triglyceride rise. Multiple doses of DPPD decreased CCl_4-induced liver necrosis, but single doses were generally less effective.

4. Three daily oral doses (300–500 mg/kg) of ethoxyquin given before CCl_4 were highly effective in preventing mortality, liver necrosis and the rise in hepatic triglycerides. Single oral doses (500 mg/kg) given 72 or 48 h before CCl_4 produced the same effect, but these single doses 24 or 6 h before CCl_4 were without effect. The effective treatments usually increased liver weight markedly. Intraperitoneal treatment with ethoxyquin also substantially reduced the toxicity of CCl_4. Ethoxyquin was the most effective of all four treatments studied, and livers from animals given this substance were often nearly normal in histological appearance.

5. The activity of oral doses (400–600 mg/kg) of BHT, given 48 h or more before CCl_4 was, in general, similar to that of ethoxyquin, but less marked. This substance also caused a large increase in liver weight after 48 h. Oral doses given 6–24 h before CCl_4 increased the CCl_4-induced triglyceride rise still further. Intraperitoneal doses of BHT were ineffective against acute toxicity, liver necrosis or the triglyceride rise.

6. Concentrations of α-tocopherol and the three synthetic antioxidants were measured in liver in many of the experiments. Very high hepatic concentrations of α-tocopherol could be obtained without affecting either the acute or subacute toxicity of CCl_4. Ethoxyquin and BHT were rapidly eliminated from the liver after oral dosage, and when maximum concentrations were reached (24 h or less after administration) they were without protective effect. In contrast, when ethoxyquin and BHT were most active (48–96 h after administration) they could not be found in appreciable concentration in the liver.

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7. These observations suggest that there is no single mode of action of the synthetic antioxidants. The protective effects of the latter are not due to their antioxidant action but to indirect mechanisms. In an attempt to understand these mechanisms, the effect of vitamin E and antioxidants on a microsomal mixed-function oxidase (the drug processing enzyme, aminopirene demethylase) was studied. Treatments that protected rats against CCl_4 toxicity were, in general, associated with an increase in enzyme activity. Ethoxyquin was also found to prevent the potentiating effects of phenobarbitone and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane (DDT) on CCl_4 toxicity, while allowing drug processing enzyme activities (hexobarbitone oxidase and BHT oxidase) to remain at raised levels. It is suggested that ethoxyquin and BHT may protect against CCl_4 poisoning by inducing the synthesis of enzymes that are associated either with the metabolism of CCl_4 through a non-toxic pathway or the removal of its toxic metabolite. DPPD may act partly in another manner, because its effects against mortality and hepatic fat accumulation are not parallel and also because it is effective within 6 h of administration. The slight activity of vitamin E against CCl_4 poisoning, although possibly partly connected with its effect on drug processing enzymes, may also involve other mechanisms, perhaps by ameliorating anoxia and increasing cellular regeneration.

In the first paper of this series (Green, Bunyan, Cawthorne & Diplock, 1969) evidence was presented that lipid peroxidation was not causally involved in the hepatotoxicity of CCl_4 in the rat and, therefore, that the protective action of vitamin E, if any, was probably not attributable to an antioxidant effect. This paper describes a study of the protective effects of vitamin E and three synthetic antioxidants on the toxic effects of CCl_4, with special reference to necrosis and fatty accumulation in the liver and mortality. The results assist in the understanding of the mode of action of these substances and the biological role of vitamin E.

EXPERIMENTAL

Animals and diets

Male and female caesarian-derived albino rats of the CSE and Wistar strains were obtained from Scientific Products Farm, Canterbury. They weighed approximately 150–220 g and were allowed 4 d in the laboratory before being used. They were given the stock diet FFG (Dixon and Sons, Ware, Herts) and allocated to groups at random with equal weight distribution in the groups. Vitamin E-deficient rats were of the hooded strain from our own colony and were reared on diet G 15 F (Cawthorne, Diplock, Muthy, Bunyan, Murrell & Green, 1967). The 3% casein diet was diet G 15 F with the casein reduced and sugar correspondingly increased and with the addition of 70 ppm vitamin E.

Materials

CCl_4 was laboratory reagent grade. Vitamin E refers to D-α-tocopheryl acetate. *N*,*N*-Diphenyl-*p*-phenylenediamine (DPPD) and butylated hydroxytoluene (BHT) were normal laboratory grade. Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) was a gift from Monsanto Chemicals Ltd (Ruabon, Denbighshire). It was distilled at reduced pressure before use. Ionox 100 (2,6-di-*tert*.-butyl-4-hydroxymethylphenol) was a gift from Shell Chemicals Ltd, London. Aminopyrine and 4-aminoantipyrine were obtained from Kodak Ltd (Liverpool) and $NADPH_2$ from Sigma (London) Chemical Co. Ltd.

Animal experiments

Experiments were done to study, first, the effects of vitamin E and synthetic antioxidants on the acute toxicity of CCl_4 and, secondly, their effects on hepatic fat accumulation and necrotic degeneration induced by subacute doses.

Acute toxicity. Test groups to be given CCl_4 and protective supplements contained at least eight to ten rats, sometimes more. Control groups given only CCl_4 usually contained fifteen to twenty rats. Preliminary tests were made to ensure that the protective supplements at the doses given were themselves without toxicity. Each dose of supplement was administered in 0·5–1·0 ml methyl oleate solution or suspension, either orally or intraperitoneally. The CCl_4 was dissolved in an equal volume of liquid paraffin BP and given orally under light ether anaesthesia. Mortality was assessed after 72 h, but most rats died between 24 and 48 h.

Subacute toxicity. Groups of six rats were used. CCl_4 was given orally at 2·0 ml/kg, irrespective of sex. Supplements and CCl_4 were given as described above. The rats were killed by cervical dislocation 24 h after the CCl_4 dose. The liver was removed at once and weighed and a 2 g sample was taken for neutral triglyceride determination. A small piece was fixed in formol-saline for histological examination (Green *et al.* 1969). Levels of vitamin E and synthetic antioxidants were determined in the rest of the liver. In some experiments these analyses were done on the livers of rats killed at the same time (0 h) as the test groups were given the CCl_4 dose. Samples of adipose tissue were sometimes taken and analysed for the synthetic antioxidant used in that particular test.

Assessment of degenerative changes (necrosis) in the liver

The degree of necrotic change in the liver was measured by histological examination. Four grades were recognized (Pl. 1): zero (0); slight (+), involving hydropic degeneration of cells with loss of stainable cytoplasm and shrinkage of the nucleus; moderate (++), involving necrosis of a single cell or small group of hepatocytes, principally in the centrilobular areas but occasionally in the mid-zonal region, together with hydropic degeneration in some cells; severe (+++), involving necrosis of the entire centrilobular region with an outer zone of hydropic degeneration.

Analytical methods

Triglycerides. Lipid was extracted from liver by application of the methods of Folch, Lees & Stanley (1957) and Bligh & Dyer (1959), as described by Diplock, Cawthorne, Murrell, Green & Bunyan (1968). The lipid was dissolved in diethyl ether (5 ml) and passed through a 1 g silicic acid (Mallinckrodt) column, which absorbed the polar lipids. The neutral lipids were eluted with ether (25 ml) and, after evaporation of solvent, dried to constant weight at 105°. Analysis of this fraction by the method of Van Handel & Zilversmit (1957) showed it to consist mainly of triglycerides.

α-Tocopherol. α-Tocopherol in liver was measured as described by Diplock, Edwin, Bunyan & Green (1961).

DPPD. Liver was saponified in the presence of pyrogallol and the non-saponifiable extract was obtained by the method of Mervyn & Morton (1959). The DPPD was then

measured colorimetrically, by the method of Csallany & Draper (1960). These authors found that DPPD could not be quantitatively recovered after saponification of the crude lipid extract of liver because of its sensitivity to oxidation. However, the method of Mervyn & Morton (1959), which is suitable for other labile substances such as α-tocopherol, ubiquinone and vitamin A, was shown to give quantitative recoveries of DPPD added to liver samples.

BHT. This was determined by the method of Daniel & Gage (1965).

Ethoxyquin. Liver (2 g) was homogenized with acetone (38 ml) for 1 min. After centrifugation 30 ml of the supernatant fraction was removed and added to 25 ml light petroleum (boiling range, 40–60°). The ethoxyquin was extracted from this solution twice with 10 ml 2 N-HCl, and the combined aqueous phase was neutralized to pH 9 with 2 N-NaOH. This was then re-extracted with 3×25 ml ether, which had been distilled immediately before to remove antioxidants. The ether solution was washed twice with H_2O and evaporated to dryness under reduced pressure. The residue was dissolved in ethanol and ethoxyquin was measured by the Emmerie–Engel ($FeCl_3$-α,α'-dipyridyl) reaction as normally used for vitamin E, but allowing 25 min for optimum development of the colour. Blank values were obtained at the same time from the liver of control rats not given ethoxyquin and were subtracted from test values. Recovery of added ethoxyquin (25 μg/2 g liver) was 80%.

Drug processing enzymes

Livers were excised immediately after death and portions were homogenized in a glass homogenizer tube using a Teflon pestle. The homogenizer was pre-cooled to 0° and thereafter kept in ice-water. The tissue was homogenized for 1 min by passing the tube up and down several times while the pestle was rotated at about 1000 rev/min. All incubations were done in 25 ml conical flasks, shaken at 100 excursions/min at 37°. Preliminary tests showed that the enzymic reactions were linear for at least 15 min under these conditions.

Aminopyrine demethylase. The homogenate, 20% (w/v) in ice-cold 0·09 M-KH_2PO_4 buffer, pH 7·4, was filtered through two layers of surgical gauze and incubated for 15 min at 37° in the presence of 0·7 mM-aminopyrine, 3·3 mM-$MgCl_2$, 0·21 mM-$NADPH_2$ and 0·09 M-KH_2PO_4 buffer, pH 7·4. The reaction was stopped by addition of an equal volume of 10% (w/v) trichloroacetic acid solution and the mixture was cooled and filtered. The amount of 4-aminoantipyrine produced was determined by the method of Gilbert & Golberg (1965). Enzyme activity is reported as μmoles 4-aminoantipyrine produced/g liver per h.

BHT oxidase. A 20% (w/v) homogenate was prepared in 0·25 M-sucrose, 0·1 M-sodium phosphate, 0·001 M-EDTA buffer, pH 7·4. After filtering through two layers of surgical gauze, the homogenate was centrifuged at 19000 *g* for 10 min and the supernatant fraction was used for enzyme assay. The enzyme incubation medium contained 83·3 mM-KH_2PO_4, pH 7·4, 3·3 mM-$MgCl_2$, 0·21 mM-$NADPH_2$, 0·05 mM-BHT and the enzyme from 200 mg liver, all in 3 ml. Incubations were carried out as described for aminopyrine demethylase except that the incubation time was 10 min. The reaction product, 2,6-di-*tert.*-butyl-4-hydroxymethylphenol, was measured as

described by Gilbert & Golberg (1967) except that the concentration of the Gibbs' reagent was increased to 10 mg/100 ml. Enzyme activity is reported as μmoles 2,6-di-*tert.*-butyl-4-hydroxymethylphenol produced/g liver per h.

Hexobarbitone oxidase. The 19000 *g* supernatant fraction from a 15% (w/v) homogenate was prepared as described for BHT oxidase. The enzyme incubation medium and assay were as described by Gilbert & Golberg (1965) except that each incubation contained the enzyme from 150 mg liver and the strength of the pH 11 buffer was reduced to 0·5 M. Enzyme activity is reported as μmoles hexobarbitone metabolized/g liver per h.

Statistical evaluation of results

Where possible, the usual analysis of variance technique was applied to the hepatic triglyceride and liver weight results for each experiment. However, where variance was heterogeneous, the *d*-test of Bailey (1959) was applied. Triglyceride results for CCl_4-treated rats were considered separately from those for the controls not given CCl_4, because of the consistently lower variance of the latter values. Each value for CCl_4-treated rats given vitamin E or a synthetic antioxidant was then compared with the corresponding value for the CCl_4-treated controls. No differences were found among the triglyceride values for control rats not given CCl_4. The rises in triglycerides induced by treatment with CCl_4 were clearly highly significant in all experiments, but for simplicity this fact is not noted in the tables. Values for liver weight, expressed as a percentage of body-weight, of control and CCl_4-treated rats were considered together. In the acute toxicity experiments survival rates were analysed by the χ^2 test on a 1-tailed basis since the low survival rates of the rats given CCl_4 only were expected.

RESULTS

Experiments on vitamin E

Expt 1. A preliminary test demonstrated a marked sex difference in the toxicity of CCl_4 in rats. This was noticed also by György, Seifter, Tomarelli & Goldblatt (1946). The LD_{80} was about 3·5–5·0 mg/kg in males and about 8·5 ml/kg in females. Doses approximating to these amounts were used in all the experiments on acute toxicity. Expt 1 examined the effect of dietary vitamin E on mortality in male and female rats. Groups of vitamin E-deficient rats of various ages were given dietary vitamin E (70 ppm) for lengths of time varying from about 1 to 5 months and then dosed with CCl_4. Survival rate is shown in Table 1. No significant effect of vitamin E was found in males. When the survival figures for all the tests with females were combined, vitamin E showed a significant effect in decreasing mortality ($P < 0{\cdot}01$).

Expt 2. Gallagher (1961) reported that a single 95 mg dose of vitamin E, given intraperitoneally 40 h before an oral dose of CCl_4, gave complete protection from death in female rats; but the dose was ineffective given at later times, and smaller (19 mg) doses over the previous 72 h were also relatively inactive. In Expt 2, some groups of female rats, given a stock diet, were given 8·5 ml CCl_4/kg orally; some groups were given D-α-tocopheryl acetate (100 mg) intraperitoneally 72, 48, 24 or 1 h previously; other groups were given D-α-tocopherol (100 mg) at these times. Controls were given

no vitamin E. Survival rate is shown in Table 2. There was no consistent effect of vitamin E. One dose of D-α-tocopheryl acetate did not increase survival whatever the time of administration, but two doses (at 24 and 48 h) somewhat increased survival, although not significantly. D-α-Tocopherol increased survival when given 24 h before CCl_4 but significantly decreased survival when given 1 h previously. These effects are discussed further in relation to tocopherol levels in the liver (see Expt 4) on p. 363.

Table 1. *Expt* 1. *Effect of dietary vitamin E on the acute toxicity of CCl_4 in male and female rats*

(The vitamin E-deficient diet was G 15 F (see p. 358))

Test	Sex	Age (months)	Dietary treatment	CCl_4 dose (ml/kg)	Survival*
1	♀	6	Supplemented†	7·0	3/10
		6	Deficient	7·0	0/10
2	♀	6	Supplemented†	8·0	1/9
		6	Deficient	8·0	0/9
3	♀	3–14	Supplemented‡	8·0	5/18§
		3–14	Deficient	8·0	0/18
4	♂	6	Supplemented‡	3·5	5/22
		6	Deficient	3·5	6/22
5	♂	2·5	Supplemented‡	4·0	2/10
		2·5	Deficient	4·0	2/10

* No. of survivors 72 h after CCl_4/total no. in group.
† Given 70 ppm dietary D-α-tocopheryl acetate from weaning.
‡ Given 70 ppm dietary D-α-tocopheryl acetate for 6 weeks before CCl_4 dose.
§ Significantly greater than for deficient rats ($P < 0.05$).

Table 2. *Expt* 2. *Effect of intraperitoneal doses of vitamin E on the acute toxicity of CCl_4 in female rats*

(The rats, wt range 154–169 g, were given a stock diet. They were given 8·5 ml CCl_4/kg orally after preliminary intraperitoneal injections of D-α-tocopheryl acetate (100 mg) or D-α-tocopherol (150 mg) at the times shown)

		Survival* when vitamin E was given at stated time before CCl_4				
Test	Treatment	72 h	48 h	24 h	1 h	48 and 24 h
1	Control	3/20	11/36	9/26	9/26	2/27
	D-α-Tocopheryl acetate	0/7	10/26	5/18	4/18	9/25†
2	Control	—	9/26	9/26	9/26	—
	D-α-Tocopherol	—	4/15	13/17‡	1/19§	—

* No. of survivors at 72 h/total no. in group.
† Two 100 mg doses of D-α-tocopheryl acetate.
‡ Significantly greater than for undosed controls ($P < 0.01$).
§ Significantly less than for undosed controls ($P < 0.05$).

Expt 3. In this experiment we have grouped a number of tests in which the effect of oral doses of vitamin E on mortality was examined. Details of the dosing and the results are given in Table 3. In male rats, three doses of 450 mg vitamin E/kg, given 72, 48 and 24 h before the CCl_4, significantly increased survival. Single doses given 72,

48 or 24 h previously also gave considerable protection. Each separate effect was non-significant, perhaps because of the unexpectedly low mortality in controls in this series, but the effect was significant when the three tests were combined. The effect of vitamin E in females was evidently much less. In one test, three doses of 450 mg/kg given 72, 48 and 24 h before CCl_4 were protective, but this effect was not confirmed in two other similar trials with multiple doses. In other tests, single doses of vitamin E up to 2000 mg/kg were given 6, 24, 48 or 72 h before CCl_4 and were without effect, except in one test in which the vitamin E was given 6 h previously, when survival was significantly decreased. Examination of the combined results for single doses suggested that in female rats vitamin E slightly increased mortality due to CCl_4 ($P < 0.01$).

Table 3. *Expt* 3. *Effect of oral doses of vitamin E on the acute toxicity of CCl_4 in male and female rats*

(The rats, wt range 160–220 g, were given a stock diet. Males were given 3·5 ml CCl_4/kg and females 8·5 ml/kg orally, after preliminary doses of D-α-tocopheryl acetate, as described below)

		Vitamin E given		Survival*	
Test	Sex	Amount of each dose (mg/kg)	Time of each dose before CCl_4 (h)	Controls	Vitamin E-treated rats
1	♂	450	72, 48 and 24	13/35	24/24†
2	♂	450	72	12/20	8/10
3	♂	450	48	12/20	10/10
4	♂	450	24	12/20	9/10
5	♀	450	72, 48 and 24	8/34	21/30†
6	♀	450	72, 48 and 24	5/14	3/9
7	♀	450	96, 72, 48 and 24	3/20	0/9
8	♀	450	72	11/31	6/20
9	♀	450	48	11/30	3/19
10	♀	2000	48	6/17	3/10
11	♀	450	24	16/44	6/29
12	♀	2000	24	11/31	3/19
13	♀	450	6	16/44	4/27‡
14	♀	2000	6	6/17	2/8

* No. of survivors 72 h after CCl_4/total no. in group.
† Significantly greater than the survival of rats not given vitamin E ($P < 0.001$).
‡ Significantly less than the survival of rats not given vitamin E ($P < 0.05$).

Expt 4. Eight tests were done to investigate the effects of oral and intraperitoneal doses of vitamin E on the subacute toxicity of CCl_4 in male and female rats. Some groups were given vitamin E in single or multiple doses before CCl_4, as shown in Table 4. In six of the eight tests, two control groups, one untreated and one given only CCl_4, were included. In tests 4 and 6 a CCl_4-treated control group only was included. In some tests additional control groups given vitamin E but not CCl_4 were included and these clearly showed that vitamin E itself did not affect liver triglycerides. It was, therefore, not considered necessary to include such control groups in all the tests. All the rats given CCl_4 were killed 24 h later and a portion of each liver was examined histologically. The remainder of the liver was analysed for triglyceride content and, sometimes, α-tocopherol, as shown in Table 4.

The CCl_4 usually, but not always, caused a rise in liver weight, and a significant rise

Table 4. *Expt* 4. *Liver necrosis and hepatic triglycerides in male and female rats treated with CCl_4 and vitamin E*

(The rats, wt range 170–200 g, were given a stock diet. The CCl_4 dose was 2·0 ml/kg, given orally, for both sexes. CCl_4-treated rats were killed 24 h after the CCl_4 dose. Untreated control rats were killed at the same time as the others received CCl_4. Each group contained six rats, except for a few groups that contained four or five. Liver wt, degree of liver degeneration and triglyceride concentrations were measured in individual rats. Livers were combined in pairs for tocopherol analysis. Results are given as means with standard deviations. The degree of liver degeneration was assessed as zero (o), slight (+), moderate (++) or severe (+++), as described on p. 359. No necrosis (o) was found in the livers of rats not given CCl_4 (not recorded below))

		Vitamin E given		Liver						
				Wt as % of body wt		Triglycerides (mg/g)		α-Tocopherol (μg/g)		
Test	Sex	Amount of each dose (mg/kg)	Time of each dose before CCl_4 given at o h (h)	Controls at o h	CCl_4-treated at +24 h	Controls at o h	CCl_4-treated at +24 h	Controls at o h	CCl_4-treated at +24 h	Necrosis CCl_4-treated at +24 h
1	♂	None	.	3·6±0·3	4·3±0·2	15·5±5·8	45·5±9·1	.	.	.
		450*	72, 48 and 24	.	5·4±0·5†	.	39·0±8·8	135±50	39±14	.
2	♂	None	.	.	.	25·7±5·7	48·4±6·7	2·9±1·2	1·8±0·4	+++
		450*	72	3·8±0·3	5·6±0·5	24·2±3·6	54·7±12·7	6·0±3·0	21±10	++
		450*	48	4·0±0·7	4·7±0·4	23·1±2·4	54·5±9·4	91±17	16±12	+++
		450*	24	4·0±0·2	4·8±0·6	25·2±3·7	49·6±12·2	167±50	39±8	+++
		450*	6	3·9±0·2	5·0±0·7	23·4±3·7	44·9±5·6	27±3	13±8	+++
3	♀	None	.	3·7±0·2	4·2±0·1	22·4±6·7	82·6±24·0	13±2	15±1	+++
		450*	72, 48 and 24	3·6±0·5	4·9±0·6†	29·5±4·8	73·9±34·9	313±93	72±9	+++
		450*	24	4·6±0·1†	4·3±0·3	20·4±3·4	72·8±18·6	117±36	66±24	++
		450*	6	3·9±0·1	4·2±0·4	20·8±5·5	97·0±13·1	141±60	70±16	+++
4	♀	None	.	.	4·0±0·2	.	49·7±19·0	.	.	+++
		450*	72	3·6±0·1	3·8±0·3	.	67·7±19·2	5±6	.	+++
5	♀	None	.	2·8±0·1	4·4±0·4	20·1±5·7	90·4±20·6	.	.	+++
		1000*	96, 72, 48 and 24	.	4·1±0·3‡	.	69·3±18·3‡	.	.	+++
6	♀	None	.	.	4·0±0·2	.	49·7±19·0	.	.	+++
		2000*	48	4·0±0·4	3·8±0·3	.	63·9±30·6	210±168	.	+++
		2000*	24	4·4±0·6	4·2±0·4	.	52·7±21·7	499±143	.	+++
		2000*	6	3·5±0·4	4·0±0·2	13·8±4·5	105·3±17·7†	105±57	.	+++
7	♂	None	.	3·6±0·3	4·3±0·2	15·5±5·8	45·5±9·1	.	.	.
		500§	48, 24 and o	.	4·8±0·5	.	41·6±12·7	.	370±118	.
8	♀	None	.	3·8±0·2	3·9±0·3	.	82·4±26·1	9±5	.	+++
		500§	48	4·0±0·3	3·9±0·2	.	101·5±26·2	100±73	.	+++
		500§	24	3·9±0·4	4·1±0·4	.	58·4±17·5‡	64±39	.	+++
		500§	1	3·7±0·2	3·9±0·2	.	84·2±18·2	103±127	.	+++
		500§‖	48	3·7±0·2	3·8±0·1	.	68·8±21·3	104±79	.	+++
		500§‖	24	3·8±0·4	3·7±0·3	.	50·7±19·6¶	62±33	.	+++
		500§‖	1	3·6±0·3	3·9±0·3	.	71·3±14·2	24±10	.	+++

* Given orally.
† Significantly different from the value for the corresponding control rats ($P < 0·001$).
‡ Significantly different from the value for the corresponding control rats ($P < 0·05$).
§ Given intraperitoneally.
‖ These groups received D-α-tocopherol; all the others received D-α-tocopheryl acetate.
¶ Significantly different from the value for the corresponding control rats ($P < 0·01$).

in triglycerides was always produced. Vitamin E did not usually affect liver weight significantly in CCl_4-treated or control animals, nor did it, in general, have any significant effect on the triglyceride rise when given in single or multiple oral doses at any time before the CCl_4. Single intraperitoneal doses of vitamin E (acetate or free alcohol form) given 24 h before CCl_4 significantly inhibited the rise in triglycerides. The additional groups of rats treated with vitamin E only were, as shown in Table 4, killed at the same time as the other groups of rats were given the CCl_4 dose (0 h) and their livers were analysed for α-tocopherol and triglycerides to determine the levels existing at that time. The liver tocopherol levels after vitamin E administration were, of course, dependent on the dosing regimen, but varied considerably. In general, they reached a maximum between 6 and 24 h after oral dosage and then declined. After intraperitoneal dosage, liver levels rose after 1 h. Wiss, Bunnell & Gloor (1962) gave much smaller doses of radioactive vitamin E to rats and found that retention of the radioactive label in the liver was maximal 4 h after dosage. In view of the failure of vitamin E, in tests 1–5, to prevent the rise in triglycerides, in test 6 we gave 2000 mg vitamin E/kg at various times before CCl_4. Extremely high liver levels of α-tocopherol were obtained, but rather than preventing the CCl_4-induced triglyceride rise, these doses of vitamin E increased it, especially when given 6 h before the CCl_4 ($P < 0{\cdot}001$). Table 4 also shows the results of histological examination. Vitamin E had little or no effect on CCl_4-induced liver necrosis, at whatever time or by whichever route it was given.

Experiments on DPPD

Expt 5. In this experiment (Table 5) we did nine tests on the effect of DPPD, given at various times, on the acute toxicity of CCl_4 in male and female rats. In confirmation of the results of Gallagher (1961) a single dose of 500 mg DPPD/kg given intraperitoneally 1 h before CCl_4 significantly increased survival in female rats; but, in our experiments, this dose had no significant effect when given 24 or 48 h previously. A single oral dose of 600 mg DPPD/kg, given 72 h before CCl_4, significantly increased survival in female rats, but oral dosage 48, 24 and 6 h before CCl_4 produced diminishing effects on mortality. When multiple oral doses, beginning 72 h before CCl_4, were given, survival was significantly increased in male and female rats.

Expt 6. In this experiment we carried out eight tests on the effect of DPPD on CCl_4-induced hepatic triglyceride rise in male and female rats (Table 6). Treatment with CCl_4 usually increased liver weight and always significantly increased liver triglyceride content. DPPD at 600 mg/kg had little effect on liver weight. In general, this dose reduced the hepatic triglyceride rise over the whole range of dosing regimens studied, being effective from 6 to 72 h before CCl_4, and as Table 6 shows, many of the reductions were significant. An exceptionally high dose of DPPD (1250 mg/kg) given intraperitoneally 1 h before CCl_4 also significantly decreased the triglyceride rise. There was some evidence that a lower dose (100 mg/kg), given intraperitoneally 48 or 24 h before CCl_4, was more effective in preventing the rise in triglycerides than the 600 mg/kg dose usually given. Table 6 also shows the results of histological examination. Three oral doses of DPPD, given 72, 48 and 24 h before CCl_4, substantially improved the histological picture, reducing the necrosis from severe (+ + +) to slight

(+). Single oral doses were, in general, only moderately effective. We found single intraperitoneal doses of DPPD to be without substantial effect on the necrosis at whatever time they were given.

Hepatic DPPD concentrations were measured in groups of rats at 0 h or at 24 h after CCl_4 administration (Table 6). In general, intraperitoneal doses induced higher levels in the liver than did oral doses. Orally administered DPPD appeared in the liver more quickly than did vitamin E, levels 6 h after dosage being higher than 24 h afterwards.

Table 5. *Expt* 5. *Effects of oral and intraperitoneal doses of* N,N′-*diphenyl*-p-*phenylenediamine* (*DPPD*) *on the acute toxicity of* CCl_4 *in male and female rats*

(The rats, wt range 160–220 g, were given a stock diet. Males were given 3·5 ml CCl_4/kg and females 8·5 ml/kg orally, after preliminary doses of DPPD, as described below)

		DPPD given		Survival*	
Test	Sex	Amount of each dose (mg/kg)	Time of each dose before CCl_4 (h)	Controls	DPPD-treated rats
1	♂	600†	72, 48 and 24	17/35	20/20‡
2	♀	600†	72, 48 and 24	4/34	11/17‡
3	♀	600†	72	5/40	9/18§
4	♀	600†	48	7/33	6/19
5	♀	600†	24	7/33	3/19
6	♀	600†	6	7/43	3/28
7	♀	500‖	48	1/18	1/8
8	♀	500‖	24	1/18	1/9
9	♀	500‖	1	2/20	6/10§

* No. of survivors 72 h after CCl_4/total no. in group.
† Given orally.
‡ Significantly greater than the survival of rats not given DPPD ($P < 0.001$).
§ Significantly greater than the survival of rats not given DPPD ($P < 0.01$).
‖ Given intraperitoneally.

Experiments on ethoxyquin

Expt 7. In this experiment (Table 7) we carried out nine tests on the effect of ethoxyquin on the acute toxicity of CCl_4 in male and female rats. The effects of this substance were remarkably well defined. Oral doses (500 mg/kg), given 6 or 24 h before CCl_4, had no effect on survival; but, given 48 or 72 h previously, they were highly protective. Multiple doses (300 mg/kg) at 72, 48 and 24 h were also highly protective in male and female rats. Single intraperitoneal doses of ethoxyquin significantly increased survival even when given as late as 1 h before CCl_4, but not more than 50% survival could be obtained in rats given ethoxyquin by this route at times from 1 to 48 h before CCl_4.

Expt 8. Five tests were made on the effect of oral and intraperitoneal doses of ethoxyquin on the CCl_4-induced triglyceride rise and liver necrosis (Table 8). Treatment with CCl_4 increased liver weight and significantly increased liver triglycerides. Ethoxyquin, given by either route, produced even larger increases in liver weight in CCl_4-treated and control rats. Both oral and intraperitoneal doses of ethoxyquin, when

Table 6. *Expt* 6. *Liver necrosis and hepatic triglycerides in male and female rats treated with CCl_4 and* N,N′-*diphenyl*-p-*phenylenediamine* (*DPPD*)

(The rats, wt range 140–200 g, were given a stock diet. The CCl_4 dose was 2·0 ml/kg, given orally, for both sexes. CCl_4-treated rats were killed at 24 h after the CCl_4 dose. Some of the untreated control rats were killed at the same time as the others received CCl_4, but some were killed 24 h later, as indicated below. Each group contained six rats. Liver wt, degree of liver degeneration and triglyceride concentrations were measured in individual rats. Livers were combined in pairs for DPPD analysis. Results are given as means with standard deviations. The degree of liver degeneration was assessed as zero (0), slight (+), moderate (++) or severe (+++), as described on p. 359. No necrosis (0) was found in the livers of rats not given CCl_4 (not recorded below))

		DPPD given		Liver						
				Wt as % of body-wt		Triglycerides (mg/g)		DPPD (μg/g)		
Test	Sex	Amount of each dose (mg/kg)	Time of each dose before CCl_4 given at 0 h (h)	Controls	CCl_4-treated	Controls	CCl_4-treated	Controls	CCl_4-treated	Necrosis CCl_4-treated
1*	♂	None	.	4·2±0·5	5·3±0·5	10·9±1·6	25·3±7·7	.	.	.
		600†	72, 48 and 24	.	5·7±0·4	.	32·9±6·0	.	.	.
2*	♂	None	.	3·6±0·3	4·3±0·2	15·5±5·8	45·5±9·1	.	.	.
		600†	72, 48 and 24	.	5·4±0·2‡	.	33·8±4·0§	.	17±4	.
		600‖	48, 24 and 0	.	5·3±0·4¶	.	23·4±5·8‡	.	105±12	.
3*	♀	None	.	4·7±0·6	4·3±0·8	6·5±1·5	34·9±8·3	.	.	+ + +
		600†	72, 48 and 24	.	6·3±0·7‡	.	24·5±5·7	.	.	+
4**	♀	None	.	3·8±0·4	4·0±0·6	12·8±1·7	93·7±26·6	.	.	+ + +
		600†	72, 48 and 24	4·6±0·3	4·9±0·6‡	14·7±1·8	64·2±27·0	22±3	.	+
		600‖	72	4·1±0·4	4·1±0·3	11·3±1·9	67·3±24·0	8±2	.	+ + +
		600‖	1	3·9±0·7	3·7±0·3	12·6±3·7	62·0±19·0	23±9	.	+ + +
5**	♀	None	.	5·2±0·8	5·3±0·3	6·5±1·1	40·7±5·5	.	.	+ + +
		600†	48	5·4±0·6	5·5±0·5	6·7±1·8	20·9±4·6‡	14±3	16±2	+ +
		600†	24	5·0±0·6	5·5±0·3	6·7±1·3	26·3±7·0¶	32±2	35±7	+ +
		600†	6	5·0±0·7	5·5±0·3	7·4±1·1	21·5±10·0†	74±31	75±27	+
6**	♀	None	.	.	4·5±0·4	20·1±5·7	90·4±20·6	.	.	+ + +
		600†	72	.	4·1±0·3	.	63·1±9·1	.	.	+ + +
		600†	48	.	4·4±0·3	.	52·7±14·2‡	.	.	+ + +
		600†	24	.	4·5±0·3	.	46·6±12·1‡	.	.	+ + +
		600†	6	.	4·3±0·4	.	50·1±13·5‡	.	.	+ + +
		100‖	48	4·9±0·1	4·7±0·2	.	34·1±7·6‡	21±2	.	+ + +
		100‖	24	4·1±0·4	4·3±0·3	.	38·4±14·0‡	22±4	.	+ + +
		1250‖	1	4·3±0·6	4·4±0·2	.	52·7±13·9¶	27±6	.	+ + +
7*	♀	None	.	.	3·6±0·5	21·2±4·8	85·0±32·4	.	.	.
		600‖	48	.	4·3±0·2§	.	50·0±9·9¶	.	.	.
		600‖	24	.	4·2±0·6§	.	55·0±30·3§	.	.	.
		600‖	1	.	3·6±0·2	.	82·6±16·0	.	.	.
8**	♀	600‖	48	3·7±0·3	4·7±0·5	10·9±1·9	32·1±7·2	128±25	.	.
		600‖	24	3·4±0·2	4·4±0·4	10·3±1·0	32·9±12·1	139±20	.	.
		600‖	1	3·6±0·2	3·9±0·2	12·8±1·9	59·3±14·7	70±34	.	.

* Control rats were killed at +24 h.
† Given orally.
‡ Significantly different from the value for the corresponding control rats ($P < 0·001$).
§ Significantly different from the value for the corresponding control rats ($P < 0·05$).
‖ Given intraperitoneally.
¶ Significantly different from the value for the corresponding control rats ($P < 0·01$).
** Control rats were killed at 0 h.

given at least 48 h before CCl_4, inhibited the rise in hepatic triglycerides, often to a highly significant degree. However, oral doses given 24 h before CCl_4 had much less effect (two negative results out of three), and at 6 h ethoxyquin had no effect. The intraperitoneal dose of ethoxyquin was effective at 24 h but not at 1 h. Table 8 also shows the results of histological examination. Single or multiple oral doses of ethoxyquin were highly effective in preventing liver necrosis and, when given 24–72 h before CCl_4, fairly consistently reduced the lesions from severe (+ + +) to slight (+). Even when given 6 h before CCl_4, ethoxyquin reduced the lesions from severe (+ + +) to moderate (+ +). Intraperitoneal doses were, on the whole, less effective than oral doses in reducing the lesions. Ethoxyquin in liver was measured at 0 h and at 24 h after CCl_4 administration. It was absorbed rapidly into the liver and then excreted rapidly, as found by Wiss *et al.* (1962). Considerable amounts were found 6 and 24 h after oral and intraperitoneal dosage. However, negligible hepatic levels were found 48 and 72 h after oral dosage.

Table 7. *Expt* 7. *Effects of oral and intraperitoneal doses of* 6-*ethoxy*-1,2-*dihydro*-2,2,4-*trimethylquinoline* (*ethoxyquin*) *on the acute toxicity of* CCl_4 *in male and female rats*

(The rats, wt range 160–220 g, were given a stock diet. Males were given 3·5 ml CCl_4/kg and females 8·5 ml/kg orally, after preliminary doses of ethoxyquin, as described below)

		Ethoxyquin given		Survival*	
Test	Sex	Amount of each dose (mg/kg)	Time of each dose before CCl_4 (h)	Controls	Ethoxyquin-treated rats
1	♂	300†	72, 48 and 24	17/32	20/20‡
2	♀	300†	72, 48 and 24	6/27	19/20‡
3	♀	500†	72	5/40	16/19‡
4	♀	500†	48	3/20	9/9‡
5	♀	500†	24	5/40	4/17
6	♀	500†	6	2/20	0/10
7	♀	500§	48	1/18	4/10‖
8	♀	500§	24	4/38	9/20¶
9	♀	500§	1	6/58	11/24‡

* No. of survivors 72 h after CCl_4/total no. in group.
† Given orally.
‡ Significantly greater than the survival of rats not given ethoxyquin ($P < 0{\cdot}001$).
§ Given intraperitoneally.
‖ Significantly greater than the survival of rats not given ethoxyquin ($P < 0{\cdot}05$).
¶ Significantly greater than the survival of rats not given ethoxyquin ($P < 0{\cdot}01$).

Expt 9. Since hardly any ethoxyquin could be detected in the livers of animals 48 h after oral administration, an experiment was carried out in which groups of female rats were given this antioxidant by some of the dosing regimens already used, and the substance was measured in the abdominal adipose tissue. The results (Table 9) again demonstrate the remarkable effect of ethoxyquin on liver size and show that very high levels of ethoxyquin can be found in adipose tissue 24 h after dosing. However, 72 h afterwards most of the ethoxyquin had disappeared from the adipose tissue, although the level in this tissue was still several times higher than in liver at a comparable time.

Table 8. *Expt* 8. *Liver necrosis and hepatic triglycerides in male and female rats treated with CCl_4 and 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin)*

(The rats, wt range 140–250 g, were given a stock diet. The CCl_4 dose was 2·0 ml/kg, given orally, for both sexes. CCl_4-treated rats were killed 24 h after the CCl_4 dose. Some of the untreated control rats were killed at the same time as the others received CCl_4, but some were killed 24 h later, as indicated below. Each group contained six rats. Liver wt, degree of liver degeneration and triglyceride concentrations were measured in individual rats. Livers were combined in pairs for ethoxyquin analysis. Results are given as means with standard deviations. The degree of liver degeneration was assessed as zero (0), slight (+), moderate (++) and severe (+++), as described on p. 359. No necrosis (0) was found in the livers of rats not given CCl_4 (not recorded below))

		Ethoxyquin given		Liver						
				Wt as % of body-wt		Triglycerides (mg/g)		Ethoxyquin (μg/g)		
Test	Sex	Amount of each dose (mg/kg)	Time of each dose before CCl_4 given at 0 h (h)	Controls	CCl_4-treated	Controls	CCl_4-treated	Controls	CCl_4-treated	Necrosis CCl_4-treated
1*	♂	None	.	3·6±0·3	4·3±0·2	15·5±5·8	45·5±9·1	.	.	.
		300†	72, 48 and 24	.	6·5±0·6	.	25·7±6·3‡	.	< 5	.
2§	♀	None	.	3·9±0·5	4·4±0·4	13·7±3·8	59·3±17·8	.	.	+++
		300†	72, 48 and 24	4·4±0·3	6·8±0·8‡	16·3±3·5	25·3±7·9‖	< 5	< 5	+
		500†	72, 48 and 24	6·5±1·2‡	8·3±0·4‡	13·5±3·2	38·9±13·8¶	< 5	< 5	+
		500†	72	4·1±0·2	5·5±0·6‖	13·0±3·5	32·8±7·7‖	< 5	< 5	+
		500†	48	4·3±0·4	5·5±0·8‖	11·4±2·6	33·8±5·8¶	< 5	< 5	+
		500†	24	4·5±0·2	5·6±0·7‖	12·3±3·6	59·1±17·6	18±7	59±30	+
		500†	6	3·7±0·3	4·9±0·4	14·0±4·0	54·6±31·0	17±7	62±67	++
3*	♀	None	.	4·2±0·1	4·7±0·3	13·0±1·9	66·2±7·0	.	.	.
		300†	72, 48 and 24	6·5±1·1‖	6·9±0·6‡	14·7±2·9	29·7±3·0‡	.	.	.
		600†	48	6·2±0·6‖	6·9±1·2‖	13·1±2·9	20·8±3·1‡	.	.	+
		600†	24	4·8±0·4‖	6·5±0·8‖	12·9±2·1	27·4±7·9‡	.	.	++
4*	♀	None	.	2·7±0·2	3·8±0·3	20·9±3·2	107·0±32·7	.	.	.
		500†	72, 48 and 24	.	5·9±0·7	.	72·2±20·1¶	.	.	.
		300†	72, 48 and 24	.	5·8±0·5	.	54·0±14·4‖	.	.	.
		500†	72	.	4·2±0·2	.	70·7±18·2¶	.	.	.
		500†	48	.	5·0±0·5	.	42·2±7·8‡	.	.	.
		500†	24	.	4·7±0·5	.	91·9±49·1	.	.	.
		500†	6	.	4·0±0·4	.	103·3±36·4	.	.	.
5*	♀	None	.	3·7±0·3	4·1±0·3	12·6±0·5	60·0±16·7	.	.	+++
		500**	48	5·7±0·7‡	5·3±0·7‡	14·1±1·8	25·3±6·6‡	.	.	++
		500**	24	4·4±0·2¶	6·1±0·9‡	11·3±0·9	46·4±11·5¶	40±47	.	++
		500**	1	4·0±0·3	4·7±0·2	14·4±1·3	55·2±7·1	325±32	.	+++

* Control rats were killed at +24 h.
† Given orally.
‡ Significantly different from the value for the corresponding control rats ($P < 0·001$).
§ Control rats were killed at 0 h.
‖ Significantly different from the value for the corresponding control rats ($P < 0·01$).
¶ Significantly different from the value for the corresponding control rats ($P < 0·05$).
** Given intraperitoneally.

Experiments on BHT

Expt 10. In this experiment we carried out thirteen tests on the effect of BHT on the acute toxicity of CCl_4 in male and female rats (Table 10). Significant increases in survival were found when the BHT was given orally at 600 mg/kg at least 48 h before CCl_4, and three daily doses (400 mg/kg) worked as well but not better than a single dose 72 h previously. A single 400 mg/kg dose, however, given 48 h before CCl_4 had no effect on survival. Single oral 600 mg/kg doses 6 or 24 h before CCl_4 also had no

Table 9. *Expt* 9. *Concentrations of 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin) in adipose tissue of female rats at various times after oral doses*

(The rats, wt range 170–220 g, were given a stock diet. Each group contained six rats, which were analysed individually. Results are given as means with standard deviations)

Ethoxyquin given			
Amount of each dose (mg/kg)	Time of each dose before death (h)	Liver wt as % of body-wt	Ethoxyquin concentration in adipose tissue (μg/g)
None	.	3·0 ± 0·2	.
500	72, 48, 24	5·2 ± 0·3	555 ± 105
300	72, 48, 24	5·1 ± 0·5	155 ± 75
500	72	4·6 ± 0·4	27 ± 15
500	48	4·5 ± 0·2	148 ± 48
500	24	4·0 ± 0·2	565 ± 314

Table 10. *Expt* 10. *Effects of oral and intraperitoneal doses of butylated hydroxytoluene (BHT) on the acute toxicity of CCl_4 in male and female rats*

(The rats, wt range 160–220 g, were given a stock diet. Males were given 3·5 ml CCl_4/kg and females 8·5 ml/kg orally, after preliminary doses of BHT, as described below)

		BHT given		Survival*	
Test	Sex	Amount of each dose (mg/kg)	Time of each dose before CCl_4 (h)	Controls	BHT-treated rats
1	♂	400†	72, 48 and 24	17/35	17/19‡
2	♂	600†	72	4/15	10/10§
3	♂	600†	48	9/28	13/19‖
4	♂	600†	24	10/27	5/17
5	♀	400†	72, 48 and 24	4/34	10/16‡
6	♀	600†	72	1/14	6/9§
7	♀	400†	48	0/10	2/10
8	♀	600†	48	1/14	7/8‡
9	♀	600†	24	1/24	0/18
10	♀	600†	6	3/20	0/17
11	♀	500¶	48	1/18	1/10
12	♀	500¶	24	1/18	0/10
13	♀	500¶	1	7/28	7/20

* No. of survivors 72 h after CCl_4/total no. in group.
† Given orally.
‡ Significantly greater than the survival of rats not given BHT ($P < 0.001$).
§ Significantly greater than the survival of rats not given BHT ($P < 0.01$).
‖ Significantly greater than the survival of rats not given BHT ($P < 0.05$).
¶ Given intraperitoneally.

Table 11. *Expt* 11. *Liver necrosis and hepatic triglycerides in male and female rats treated with CCl_4 and butylated hydroxytoluene (BHT)*

(The rats, wt range 140–250 g, were given a stock diet. The CCl_4 dose was 2·0 ml/kg, given orally, for both sexes. CCl_4-treated rats were killed 24 h after the CCl_4 dose. Some of the untreated rats were killed at the same time as the others received CCl_4, but some were killed 24 h later, as indicated below. Each group contained six rats. Liver wt, degree of liver degeneration and triglyceride concentrations were measured in individual rats. Livers were combined in pairs for BHT analysis. Results are given as means with standard deviations. The degree of liver degeneration was assessed as zero (0), slight (+), moderate (+ +) or severe (+ + +), as described on p. 359. No necrosis (0) was found in the livers of rats not given CCl_4 (not recorded below)

		BHT given		Liver				
				Wt as % of body-wt		Triglycerides (mg/g)		
Test	Sex	Amount of each dose (mg/kg)	Time of each dose before CCl_4 given at 0 h (h)	Controls	CCl_4-treated	Controls	CCl_4-treated	Necrosis CCl_4-treated
1	♂	None	.	3·6±0·3	4·3±0·2	15·5±5·8	45·5±9·1	.
		400*	72, 48 and 24	.	7·2±0·6†	.	33·8±7·5‡	.
2	♀	None	.	2·7±0·2	3·8±0·3	20·9±3·2	107·0±32·7	+ + +
		400*	72, 48 and 24	.	5·8±0·5	.	73·6±32·2	+
3	♀	None	.	.	4·6±0·1	.	65·0±12·0	.
		600*	96	.	4·9±0·4	.	35·1±4·3†	.
		600*	72	.	4·7±0·1	.	40·2±10·6§	.
4	♀	None	.	2·8±0·1	4·5±0·4	20·1±5·7	90·4±20·6	+ + +
		400*	96	.	4·3±0·1	.	64·7±13·9‡	+ + +
		400*	72	.	4·7±0·5	.	59·4±9·1§	+ + +
5	♀	None	.	4·5±0·5	4·8±0·5	12·3±2·6	42·3±8·4	+ + +
		400*	48	5·0±0·5‡	5·2±0·6	10·9±1·7	40·4±14·9	+ +
		400*	24	4·5±0·3	5·6±0·3§	10·7±1·2	85·1±25·6†	+ +
		400*	6	4·0±0·5	5·1±0·5	15·7±5·7	80·9±21·7§	+ + +
6	♀	None	.	.	3·6±0·5	21·2±4·8	85·0±32·4	+ + +
		600‖	48	.	4·0±0·4	.	76·8±28·5	.
		600‖	24	.	3·9±0·3	.	88·7±14·0	.
		600‖	1	.	3·9±0·3	.	120·2±34·6§	+ + +
7	♀	None	.	2·8±0·1	4·5±0·4	20·1±5·7	90·4±20·6	.
		400*¶	72	.	4·1±0·2‡	.	95·6±29·2	.
		400*¶	24	.	4·5±0·2	.	82·8±27·1	.

* Given orally.
† Significantly different from the value for the corresponding rats not given BHT ($P < 0·001$).
‡ Significantly different from the value for the corresponding rats not given BHT ($P < 0·05$).
§ Significantly different from the value for the corresponding rats not given BHT ($P < 0·01$).
‖ Given intraperitoneally.
¶ 2:6-Di-*tert.*-butyl-4-hydroxymethylphenol.

effect on survival. BHT was completely ineffective when given intraperitoneally 1, 24 or 48 h before CCl_4.

Expt 11. Six tests were carried out to study the effect of BHT on the CCl_4-induced triglyceride rise and liver necrosis (Table 11). Groups of rats were given oral doses of BHT at different times. Single doses somewhat increased liver size after 24 h, but the effect was not comparable in magnitude to that of ethoxyquin. Multiple doses, however, led to a large increase in liver size. Given 72 h or even 96 h before CCl_4, single doses of 400–600 mg BHT/kg significantly inhibited the triglyceride rise in male and

Table 12. *Expt* 12. *Effects of vitamin E and synthetic antioxidants on aminopyrine demethylase*

(Female rats, wt range 160–230 g, were given a stock diet. Livers were combined in pairs for enzyme assay. Results are given as means with standard deviations and the number of analyses is shown in parentheses)

		Doses given		
Test	Substance*	Amount of each dose (mg/kg)	Time of each dose before death (h)	Aminopyrine demethylase activity in liver (μmole†/g h)
1	None	.	.	0·15 ± 0·08 (2)
	Vitamin E	450‡	72, 48 and 24	0·14 ± 0·09 (2)
	DPPD	600‡	72, 48 and 24	0·50 ± 0·27 (2)
	Ethoxyquin	600‡	72, 48 and 24	0·28 ± 0·09 (2)
	BHT	600‡	72, 48 and 24	0·35 ± 0·07 (2)
2	None	.	.	0·16 ± 0·02 (3)
	Vitamin E	450‡	48	0·28 ± 0·07 (3)
	Vitamin E	450‡	24	0·22 ± 0·08 (2)
	Vitamin E	450‡	6	0·20 ± 0·03 (3)
3	None	.	.	0·19 ± 0·07 (2)
	Vitamin E	450‡	48	0·28 ± 0·13 (2)
	Vitamin E	450‡	24	0·27 ± 0·05 (2)
	Vitamin E	450‡	6	0·21 ± 0·08 (2)
	Vitamin E	450‡	72, 48 and 24	0·34 ± 0·10 (2)
4	None	.	.	0·28 ± 0·12 (3)
	DPPD	600‡	72	0·38 ± 0·20 (3)
	DPPD	600‡	24	0·51 ± 0·07 (3)
	DPPD	600§	1	0·22 ± 0·07 (3)
	BHT	600§	1	0·27 ± 0·02 (3)
5	None	.	.	0·20 ± 0·09 (2)
	DPPD	600‡	48	0·31 ± 0·13 (2)
	DPPD	600‡	24	0·20 ± 0·08 (2)
	DPPD	600‡	6	0·05 ± 0·02 (2)
6	None	.	.	0·22 ± 0·01 (2)
	Ethoxyquin	300‡	72, 48 and 24	0·66 ± 0·25 (2)
	Ethoxyquin	600‡	48	0·54 ± 0·11 (2)
	Ethoxyquin	600‡	6	0·12 ± 0·17 (2)
7	None	.	.	0·24 ± 0·05 (2)
	BHT	400‡	48	0·30 (1)
	BHT	400‡	24	0·41 (1)
	BHT	400‡	6	0·24 ± 0·06 (2)

* Vitamin E = D-α-tocopheryl acetate; DPPD = *N*,*N*′-diphenyl-*p*-phenylenediamine; ethoxyquin = 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline; BHT, butylated hydroxytoluene.

† μmoles 4-aminoantipyrine formed from 4-aminopyrine.

‡ Given orally.

§ Given intraperitoneally.

female rats. Multiple doses given daily for 3 d before CCl_4 were also effective. Given 48 h before CCl_4, a single dose of 400 mg/kg was without effect on triglyceride rise. The most remarkable feature of these tests was observed when BHT was given only 24 or 6 h before CCl_4. These doses led to a highly significant increase in the triglyceride rise, although BHT itself was without effect in control animals. Single intraperitoneal doses of BHT given 1–48 h before CCl_4 did not prevent the triglyceride rise. There was an indication, rather, that, like oral doses, they could increase the level of hepatic triglycerides when given only a few hours before CCl_4.

Table 11 also shows the results of histological examination. BHT was only moderately effective in inhibiting liver necrosis. Single oral doses 96 and 72 h before CCl_4 had no effect, but given 48 and 24 h previously reduced the lesions from severe (+ + +) to moderate (+ +). Given 6 h before CCl_4, oral BHT was without effect.

A number of liver samples from the various tests were analysed for the presence of BHT. Negligible amounts (< 1 μg/g) were found 24 h after the antioxidant was administered (these are not shown in Table 11). After 6 h, 7 μg/g liver were found. Some analyses of adipose tissue were also made. At 24 h after a single oral dose of 400 mg BHT/kg, about 200 μg/g tissue were found, and 48 h after the dose 100 μg/g tissue were found. It was considered possible that 24 h or more after BHT dosage a metabolite might be present in the liver at a higher concentration than the BHT itself and that this might exert a protective effect. Accordingly, the activity of 2,6-di-*tert.*-butyl-4-hydroxymethylphenol (IONOX 100), which is the known hepatic metabolite (Gilbert & Golberg, 1967), was examined. This substance at 400 mg/kg, given 24 or 72 h before CCl_4, was found to have no effect on the hepatic triglyceride rise, although it is known to be a powerful antioxidant in vitro.

Experiments on drug processing enzymes

Expt 12. McLean & McLean (1966) found that treatments that increased or decreased the toxicity of CCl_4 in rats correspondingly increased or decreased the activity of certain microsomal mixed-function oxidases, which are concerned with the metabolism of drugs by the liver. They suggested that the activity of these enzymes was closely connected with the activation of CCl_4 to a toxic intermediate. Carpenter (1967) found that in vitamin E deficiency the activity of two such processing enzymes, codeine demethylase and aminopyrine demethylase, was substantially reduced. However, vitamin E protects against certain aspects of CCl_4 poisoning. Moreover, BHT, which is highly protective against CCl_4 poisoning, not only induces the synthesis of drug processing enzymes (Gilbert & Golberg, 1965), but is also metabolized by one (Gilbert & Golberg, 1967). In an attempt to understand the two conflicting ideas, we studied the effects of vitamin E and synthetic antioxidants on aminopyrine demethylase.

Female albino rats were given the doses of vitamin E and antioxidants shown in Table 12 and killed at times varying from 1 to 72 h afterwards. Their livers were examined for enzyme activity. The general effect of vitamin E was to produce small rises in aminopyrine demethylase when given 24–72 h before death. At 6 h before death it had little effect. Consideration of all the results for vitamin E together showed

Table 13. *Expt* 13. *The effects of phenobarbitone,* 1,1,1-*trichloro*-2,2-*bis*(p-*chlorophenyl*)*ethane* (*DDT*) *and* 6-*ethoxy*-1,2-*dihydro*-2,2,4-*trimethylquinoline* (*ethoxyquin*) *on* CCl_4 *toxicity and drug processing enzymes*

(In test 1, male rats (wt range 182–264 g) were given the pre-treatment indicated below and a stock diet. Phenobarbitone (40 mg/kg) was given intraperitoneally 72, 48 and 24 h before CCl_4. Ethoxyquin (150 mg/kg) was given orally at the same time as the phenobarbitone. In test 2, male rats (wt range 162–174 g) received the 3 % casein diet (see p. 358) for 8 days before CCl_4. DDT was given as a single 100 mg/kg subcutaneous injection 1 week before the CCl_4 dose. Ethoxyquin was given as a single oral dose (500 mg/kg) 48 h before CCl_4. Drug processing enzymes were measured in the livers of rats given the pre-treatments but killed at the time the other rats received CCl_4. Enzyme activities are given as means with standard deviations and the number of individual livers assayed is given in parentheses)

					Hexobarbitone oxidase		Butylated hydroxytoluene (BHT) oxidase	
Test	Pre-treatment	CCl_4 (ml/kg)	Survival*	LD_{50}† (ml/kg)	μmoles/g per h	Significance of difference from the value for rats given no pre-treatment (P)	μmoles/g per h	Significance of difference from the value for rats given no pre-treatment (P)
1	None	3·0 4·5 6·75	5/5 4/5 1/5	*ca.* 5·3	16·1±0·9 (2)	.	0·14±0·02 (2)	.
	Phenobarbitone	0·7 1·4 2·8	3/5 1/5 0/5	*ca.* 0·9	19·9±0·6 (3)	0·05	0·71±0·07 (3)	0·001
	Phenobarbitone + ethoxyquin	1·4 2·8 5·6	3/5 5/5‡ 2/5	3–4	21·3±1·5 (3)	0·01	0·77±0·02 (3)	0·001
2	None	6·0 7·8 10·1 13·2	4/4 4/6 0/5 0/5	*ca.* 8·2	0·0±0·0 (3)	.	0·01±0·02 (3)	.
	DDT	2·0 3·0 4·5 6·75	1/6 2/6 0/6 0/6	1–2	2·3±0·4 (4)	0·01	0·15±0·02 (4)	0·05
	DDT + ethoxyquin	3·0 4·4 6·75 10·0	4/5 6/7§ 5/5§ 1/5	5–6	7·3±1·0 (4)‖	0·01	0·31±0·08 (4)¶	0·01
	Ethoxyquin	.	.	.	5·6±2·9 (4)	0·01	0·24±0·10 (3)	0·01

* No. of survivors 72 h after CCl_4/total no. in group.
† Estimated graphically.
‡ Significantly greater than the survival rate of rats given the same doses of CCl_4 and phenobarbitone but not ethoxyquin ($P < 0·01$).
§ Significantly greater than the survival rate of rats given the same doses of CCl_4 and DDT but not ethoxyquin ($P < 0·01$).
‖ Significantly greater than the value for rats given DDT but not ethoxyquin ($P < 0·001$).
¶ Significantly greater than the value for rats given DDT but not ethoxyquin ($P < 0·01$).

that it significantly raised aminopyrine demethylase activity ($P < 0.05$). None of the effects of the synthetic antioxidants were statistically significant. In general, however, DPPD, ethoxyquin and BHT also tended to raise aminopyrine demethylase activity when given 24 h or more before death.

Expt 13. McLean & McLean (1966) showed that two drug-processing enzyme inducers, phenobarbitone and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), markedly increased the toxicity of CCl_4 in rats. However, in Expt 12 ethoxyquin, which markedly reduces CCl_4 toxicity, was also found to increase processing enzyme activity to a slight extent. In Expt 13, therefore, we did two tests to see whether ethoxyquin would reverse the potentiating effects of phenobarbitone and DDT on CCl_4 toxicity (Table 13). In test 1, male rats in groups of five were given either phenobarbitone (40 mg/kg intraperitoneally) or phenobarbitone together with ethoxyquin (150 mg/kg orally) daily for 3 d before receiving graded doses of CCl_4. Phenobarbitone increased the toxicity of CCl_4, as found by McLean & McLean (1966), but administration of ethoxyquin partly reversed this effect. Pretreatment with phenobarbitone increased the levels of hexobarbitone oxidase and BHT oxidase existing in the liver at the time of CCl_4 treatment, but ethoxyquin did not inhibit this rise. In test 1, we were unable to give the normal 300 mg/kg protective dose of ethoxyquin because of toxic effects when jointly administered with phenobarbitone; therefore, in test 2, we used DDT (100 mg/kg subcutaneously) as the potentiator because this could be given as a single dose 5 d before the ethoxyquin. In addition, in order to reduce the basal level of drug-processing enzymes we gave all the animals in this test a 3% casein diet (McLean & McLean, 1966). This diet also decreased the toxicity of CCl_4 considerably from that found in previous experiments. DDT markedly increased the toxicity of CCl_4, and ethoxyquin (500 mg/kg), given as a single oral dose 48 h before the CCl_4, completely reversed this effect. No hexobarbitone oxidase or BHT oxidase could be found in livers of control rats given only the 3% casein diet. However, DDT and ethoxyquin both induced the synthesis of the two enzymes and, given together, their joint effect was approximately the summation of their separate effects.

DISCUSSION

Vitamin E

Hove (1948) first discovered the protective effect of vitamin E against CCl_4 toxicity. He gave young male rats 1 mg/d for 21–70 d and found this gave good protection against mortality caused by 2 ml CCl_4/kg given intraperitoneally. Hove & Hardin (1951*a*) found that 25 mg α-tocopherol but not 5 mg would markedly increase the survival of CCl_4-treated rats, indicating that normally 'adequate' nutritional levels of vitamin E were insufficient to protect. Hove & Hardin (1951*b*), in confirmation, noted that vitamin E, given as a dietary supplement at 0·01% for 56 d, did not prevent fatty livers in rats given weekly subcutaneous 0·05 ml doses of CCl_4. Moretti (1952) allowed normal rats to inhale CCl_4 vapour for 10 min twice daily for up to 75 d; he found that vitamin E (8 mg/d) lowered mortality and delayed but did not prevent liver necrosis and fat accumulation. Krone (1952) gave normal female albino rats 100 mg

vitamin E daily for 16–18 d and then a single oral dose of 0·5 ml CCl_4. Vitamin E had no effect on the necrotic degeneration, but reduced the fat accumulation and promoted liver regeneration. Hartmann, Hertel, Schulze & Wellmer (1952) gave normal rats subcutaneous doses of 5 ml CCl_4/kg daily for 9 consecutive d. When 10 mg vitamin E per d were also given, liver fat accumulation was only marginally reduced. Butturini, Mancini, Baronchelli & Casa (1955) observed that α-tocopherol given prophylactically was protective against the 'necrotic and glycogenolytic components' of CCl_4 toxicity, but less effective against the 'steatotic component'. If injected together with the CCl_4, vitamin E increased the fat accumulation at first, but after a few days increased liver regeneration also. In contrast, Tamura, Tsuchiya, Harada, Kuroiwa & Kitani (1959) gave normal male albino rats 0·1 ml CCl_4 subcutaneously and found that vitamin E, given at 10 mg/d for 3 d previously, significantly inhibited the rise in hepatic fatty acids.

Gallagher (1961) gave normal female rats oral CCl_4 (4 ml/kg; approximately LD_{100}) and found that a single intraperitoneal 95 mg dose of vitamin E protected completely against death, provided it was given at least 40 h before the CCl_4. The same amount, given as four consecutive daily doses before the CCl_4, had only a marginal effect. Two 95 mg doses of vitamin E, at 24 and 6 h before CCl_4, reduced mortality by only 40%. Eliseo & Pesaresi (1963) gave normal rats CCl_4 intraperitoneally (2·5 ml/kg). Vitamin E, given as a single 100 mg intramuscular dose 48 h before the CCl_4, reduced the liver damage and also the fat accumulation. Sunho, Blasek & Babala (1963), however, found that vitamin E appeared to increase hepatic lipids in rats given 0·5 ml CCl_4 orally. Petzold & Weber (1963) gave normal rats 0·25 ml CCl_4/kg subcutaneously. Vitamin E (0·2 mg/100 g daily for 20 d after CCl_4) did not affect the onset of either necrosis or fatty accumulation but markedly decreased the time taken for complete regeneration of the liver. El-Kateb, Soliman, Elwi & Kamel (1965) gave normal mice graded subcutaneous doses of CCl_4 (8–74 ml/kg; LD_{10}–LD_{85}). They found no protection against mortality or liver damage with three 33 mg doses of tocopherol given intramuscularly for 3 successive d before the CCl_4 but some protection with a single 100 mg dose 3 d before. Di Luzio (1967) found that vitamin E had no effect on the hepatic triglyceride rise when given intravenously (50 mg/kg) at the same time as CCl_4 but significantly reduced it if given 24 h previously. McLean (1967) found little or no effect of oral vitamin E on liver necrosis or fat accumulation. Meldolesi (1968) gave starved rats 2·5 ml CCl_4/kg orally and found that three successive intraperitoneal doses of vitamin E, given 72, 48 and 26 h previously, markedly decreased liver necrosis. Blendermann & Friedman (1968) found that vitamin E had no effect on a number of CCl_4-induced alterations of carbohydrate and fat metabolism in rat liver.

It is clear from these reports that there is no general agreement on the ability of vitamin E to influence CCl_4 toxicity or on the conditions under which it might do so. Some of the disagreements might be attributed to the many differences in the experimental methods of various workers, who have used both sexes of rat (although they have different susceptibilities to CCl_4), both vitamin E-deficient and normal stock diets, starved and fed animals, several different routes of CCl_4 and vitamin E administration, and vastly different dosage regimens. We have investigated some of these variables

and compared their effects. To simplify the experiments, we have given CCl_4 by the oral route only. We first compared CCl_4 toxicity in vitamin E-deficient and vitamin E-supplemented rats that had been given dietary vitamin E for long periods (Table 1). Considering all the results for females together, there appeared to be a slight but significant protective effect of vitamin E, but there was no effect in males. Livers of rats given the vitamin E-supplemented diet used in these experiments were found to contain about 30 μg α-tocopherol/g. This level, although adequate for the rat's normal needs, was insufficient to prevent more than one-quarter of the deaths following CCl_4 treatment. The best-known report on the effects of vitamin E is that of Gallagher (1961), who found a marked reduction in mortality when female rats were given large intraperitoneal doses of α-tocopheryl acetate before the CCl_4. However, we were unable to confirm his results. We tested, in addition, α-tocopherol, which had no effect when given 48 h before CCl_4, increased survival when given 24 h before, but decreased survival when given 1 h previously. It can be seen that 24–48 h after intraperitoneal injection of vitamin E, concentrations of approximately 60–100 μg/g can be found in the liver (Table 4) but even these high levels do not appear consistently to reduce CCl_4 toxicity. Several other studies (Table 3) on survival, in which vitamin E was given by the oral route, were made. A considerable protective effect was found in male rats, but no consistent effect was found in females, even with multiple oral doses. There was no indication in these experiments that either the time of dosage with vitamin E or its concentration in the liver (Table 4) was critical. CCl_4-induced fatty liver was also studied, since Butturini *et al.* (1955) had distinguished between the effects on fat accumulation and necrosis. Multiple 450 mg/kg doses of vitamin E for 3 d before CCl_4 only slightly diminished the triglyceride rise, but there was a suggestion that higher or more prolonged dosage had a slightly greater effect. Single oral doses of 450 mg/kg had virtually no effect in males, at whatever time the doses were given. These single doses seemed to be slightly more effective in lowering triglycerides in female rats, but never produced a significant decrease. When the vitamin E dose was raised to 2000 mg/kg (Table 4) and given 6 h before CCl_4, a remarkable increase in hepatic triglycerides was produced in CCl_4-treated rats. Hepatic tocopherol concentrations in such rats were 105 μg/g, but after 24 h reached 499 μg/g. This unusual effect of vitamin E is clearly not directly related to its level in the liver and other causes must be looked for. Intraperitoneal doses of vitamin E had no consistent effect in lowering triglycerides in CCl_4-treated rats, even though substantial levels of vitamin E in the liver were achieved.

To summarize, therefore, vitamin E appears to have some protective effect against the acute toxicity of CCl_4, although this is inconsistent and is influenced by sex, and time and level of dosage. We were unable to confirm the highly protective effects found for intraperitoneal doses of vitamin E by Gallagher (1961), but, for some reason, the LD_{100} of CCl_4 in his female rats were only half ours and approximated to the LD_{100} we found for male rats. We did not examine the effect of intraperitoneal vitamin E in male rats. Vitamin E, we found, hardly affected the CCl_4-induced hepatic triglyceride rise and sometimes made it worse, in confirmation of Butturini *et al.* (1955). Although we sometimes found that vitamin E reduced necrosis, this also

appeared to depend on the conditions of dosage and the route of administration. Our results thus reflect the contradictory reports in the literature.

Synthetic antioxidants

DPPD has particular relevance to the vitamin E problem. It is well absorbed and is probably the most active vitamin E-like or 'tocopheromimetic' substance in most species (Green & Bunyan, 1969). Single oral doses of DPPD were found to give significant protection against mortality due to CCl_4 in both males and female rats, provided the dose was given at least 72 h before CCl_4; but, as comparison of Tables 5 and 6 shows, the effect on survival varies inversely with the concentration of DPPD in the liver. In agreement with Gallagher (1961) we found that a single intraperitoneal dose of DPPD, given 1 h before CCl_4, significantly protected female rats, but unlike him we could find no effect with DPPD given 1 or 2 d earlier. The effect of oral doses of DPPD on the hepatic triglyceride rise was clearly the reverse of its effect on mortality. It was most effective when given 6–48 h before CCl_4, and partly effective at 72 h. Multiple doses seemed less effective than single doses against fat accumulation, but the reverse was true for liver necrosis. Ethoxyquin was clearly the most effective of the substances examined, both in the range and magnitude of its effects. Single oral doses in both sexes consistently increased survival, if they were given at least 48 h (cf. DPPD) before the CCl_4. Multiple doses were equally effective, and 100% survival was often obtained. Intraperitoneal doses also increased survival significantly, but only up to 50%, even though ethoxyquin given by this route is rapidly taken up into the liver and maintained at a fairly high level for 24 h (Table 8). Ethoxyquin markedly reduced the triglyceride rise when it was given orally at least 48 h before the CCl_4, and multiple doses were also highly effective. However, as with vitamin E, a lower dose (300 mg/kg) could be more effective than a higher dose (500 mg/kg), and single doses tended to reduce triglycerides more than multiple doses. Ethoxyquin was exceptional in being able to reduce liver triglyceride levels in CCl_4-treated rats nearly to the normal base-line levels of untreated controls. The effect of an oral dose of ethoxyquin on the liver triglyceride rise was much weaker when it was given only 24 h before CCl_4, and a time delay somewhere between 24 and 48 h seemed necessary for activity. Livers of rats given ethoxyquin were almost normal histologically, with only a few signs of necrosis. At the times when ethoxyquin produced its most marked effects on hepatic triglycerides very little of the antioxidant could be found in the liver (Table 8). Although ethoxyquin is rapidly absorbed (Wiss *et al.* 1962) and high levels occur in adipose tissue 24 h after oral dosing (Table 9), the levels are much lower 72 h after dosing, when the protective effects of ethoxyquin are maximal. The significance of these results is discussed further in the final section below.

The effects of BHT were different from those of either DPPD or ethoxyquin. Like ethoxyquin, it significantly increased survival in male and female rats if given orally at least 48 h before CCl_4, but the magnitude of its effects was not so great. In further contrast to ethoxyquin, intraperitoneal doses of BHT were without effect on survival. A single dose, given 96 or 72 h before CCl_4, significantly lowered liver triglycerides; but the effect was not as great as that obtained with ethoxyquin, and the triglyceride

levels still remained elevated in these BHT-treated rats. Given orally 48 h before CCl_4, BHT had no effect on triglyceride levels. Given 24 or 6 h previously, BHT led to a remarkable rise in the hepatic triglyceride level of CCl_4-treated rats. This is shown clearly in Table 11. We were unable to detect BHT in the livers of animals 24 h after dosage and clearly its effect in the liver must be indirect. It can, however, be measured in adipose tissue up to 48 h after dosage. Intraperitoneal doses of BHT were without consistent effect on liver triglycerides in CCl_4-treated animals. Single doses of BHT had no effect on liver necrosis, in contrast again with the effect of ethoxyquin. It is clear that DPPD, ethoxyquin and BHT affect CCl_4 toxicity in a manner different from that of vitamin E and there is evidence also that the three antioxidants produce their effects by differing mechanisms.

Carbon tetrachloride toxicity, the biological antioxidant theory and the function of vitamin E

The mode of action of CCl_4 at the molecular level is still controversial. It is currently believed that it is metabolized to an intermediate that is the true hepatotoxic agent. Oberling & Rouiller (1956) demonstrated early pathological involvement of the endoplasmic reticulum and suggested that CCl_4 is activated by a microsomal process. Butler (1961) showed that CCl_4 was metabolized to chloroform in vivo and suggested that $CCl_3^{\cdot}$ radicals were formed by homolytic cleavage and that these could alkylate the sulphydryl groups of enzymes. Rubinstein & Kanics (1964), however, have shown that the homolytic nonenzymic reaction of CCl_4 with glutathione is of little significance in the metabolism of CCl_4. Cessi, Columbini & Mameli (1966) suggest that ionic intermediates such as carbonyl chloride might be toxic metabolites. Hove (1948) first proposed that CCl_4 was a 'pro-oxidant' and that vitamin E protected because it was an 'antioxidant'. Several other substances, such as DPPD (Gallagher, 1961), antihistamines (Rees, Sinha & Spector, 1961), and selenium (Fodor & Kemeny, 1965) can also moderate CCl_4 toxicity. All these substances have come to be regarded, *ipso facto* as 'antioxidants' (McLean, 1960; Gallagher, 1962). Recknagel & Ghoshal (1966) suggested that the primary biochemical lesion involved in CCl_4 toxicity is lipid peroxidation. Following Butler (1961), they consider that CCl_4 is converted, by a microsomal process, into free radicals, which then catalyse chains of autoxidation in the structural lipids of microsomes and mitochondria, resulting in fatty accumulation and cellular necrosis. Slater (1966) has also postulated that CCl_4 is converted in the microsomes to $CCl_3^{\cdot}$ radicals that initiate lipid peroxidation, which, he considers, may promote necrosis by a 'diffusion' process and fat accumulation by a 'localized' process. He proposes five ways in which substances or treatments may affect the overall toxic process: (1) blockage of the endogenous radical-initiating system; (2) removal of the formed $CCl_3^{\cdot}$ radicals; (3) competition with CCl_4 in its reaction with the endogenous radical chain, e.g. by O_2; (4) removal of the free radical that activates the homolytic fission of CCl_4 by increasing the rate of the endogenous reaction, e.g. by O_2; (5) unspecific action, e.g. adrenalectomy. The proposals of Recknagel & Ghoshal (1966) and Slater (1966) with respect to lipid peroxidation, however, conflict with several known facts. These proposals require, for example, that there be one primary 'activation' step

common to the eventual processes of necrosis and fat accumulation. It has, however, been shown that many so-called 'antioxidants' like the antihistamines and the cationic surfactants protect against liver necrosis but are without effect on the fatty accumulation (Rees *et al.* 1961; Bangham, Rees & Shotlander, 1962; Fox, Dinman & Frajola, 1962). Alexander, Scheig & Klatskin (1967), in contrast, found that L-asparagine fully protected against the CCl_4-induced hepatic triglyceride rise but did not prevent liver necrosis. There is other evidence that a single pro-oxidant mechanism is not causally involved in CCl_4 toxicity. Smuckler, Arrhenius & Hultin (1967) found no connexion between lipid peroxidation and hepatic microsomal function in the early stages of CCl_4 damage. Castro, Sasame, Sussman & Gillette (1968) showed that, although DPPD moderated CCl_4-induced liver necrosis, it did not prevent impairment of the microsomal cytochrome P-450 nor of the microsomal drug processing enzyme, ethylmorphine demethylase. On the other hand, they found that SKF 525-A prevented necrosis and the impairment of cytochrome P-450 and ethylmorphine demethylase, but had no effect of hepatic fat accumulation. Dingell & Heimberg (1968) also studied the inhibition by CCl_4 of hexobarbitone oxidase and aminopyrine demethylase and found that neither α-tocopherol nor DPPD protected against these effects. Sasame, Castro & Gillette (1968) concluded that the early impairment of microsomal enzymes by CCl_4 'is not mediated by lipid peroxidation'. Alpers, Solin & Isselbacher (1968) have shown that CCl_4-induced lipid peroxidation does not affect protein synthesis by isolated hepatic microsomes. Furthermore, DPPD and α-tocopherol did not prevent the decline of hepatic protein synthesis in CCl_4-treated rats, although DPPD reduced the hepatic triglyceride rise.

Our work confirms that necrosis, fat accumulation and death are caused by processes that are separable and may be different in origin. Thus, vitamin E can increase survival without materially affecting the hepatic lesions, whilst DPPD increases survival and inhibits fat accumulation. In contrast, BHT affects fat accumulation and survival, although in a non-parallel fashion, but has little effect on liver necrosis. Only with ethoxyquin do the ameliorative effects on survival, fat accumulation and necrosis appear to parallel each other. Although it is possible that these substances might operate at later stages of the development of toxicity via different mechanisms, the findings suggest that there is no single primary step subject to 'antioxidant control'. Our results show that the effects of the four substances on CCl_4 toxicity cannot be correlated with their concentrations in the liver. Thus, vitamin E did not reduce the hepatic lesions under conditions in which it reached a high level in the liver. The effects of ethoxyquin and BHT were maximal when these substances were hardly detectable in the liver and they clearly must function by indirect mechanisms that take about 48 h to operate. The ineffectiveness of the hepatic metabolite of BHT, which is itself a powerful antioxidant in vitro, reinforces this concept. It is to be noted, moreover, that BHT, which is highly effective against CCl_4 toxicity, is inactive against most vitamin E deficiency diseases in animals. McLean & McLean (1965) and Seawright & McLean (1967) have suggested that the toxicity of CCl_4 and the protection conferred by some treatments could be understood in terms of the activity of microsomal drug processing enzymes. Thus, feeding rats on a pro-

tein-free diet reduced these enzymes and increased resistance to CCl_4 poisoning. Administration of phenobarbitone and DDT increased enzyme synthesis and restored the sensitivity to CCl_4. These authors considered that such enzymes might be concerned with the activation of CCl_4 to a toxic metabolite. However, the relation between enzyme activity and CCl_4 toxicity is not simple. Thus McLean & McLean (1966) showed that starvation increased the toxicity of CCl_4 in male rats, but Kato & Gillette (1965) found that starved male rats had decreased aminopyrine demethylase and hexobarbitone oxidase activity. Moreover, our experiments show that DPPD, ethoxyquin and BHT, all protected against some aspects of CCl_4 poisoning, and vitamin E did so to some extent; but all four substances tended to increase hepatic aminopyrine demethylase activity (Table 12). Furthermore, ethoxyquin protected against CCl_4 toxicity in rats that had been previously sensitized by the processing enzyme inducer, DDT, although the antioxidant raised processing enzyme activity as much as did the inducer (Table 13). It may be noted that Carpenter (1967) and Weber (1969) found more drug processing enzyme activity in vitamin E-supplemented than in vitamin E-deficient rat liver. If the role of the synthetic antioxidants and vitamin E is to be explained in terms of the drug processing enzyme concept, it appears necessary to postulate two specific metabolic steps, one toxifying and the other detoxifying CCl_4. The resultant toxicity under a given set of circumstances will depend on the balance of these two processes. Any substrate for such processing enzymes as might be involved in CCl_4 metabolism could affect this balance by direct competition. In addition, certain substances might act as enzyme inducers and, depending whether either the toxifying or detoxifying enzymes were mainly affected, would increase or decrease toxicity. The effects of ethoxyquin and BHT seem explicable in these terms. Both substances markedly increase liver weight, and both require about 48 h for their activity to be developed. These effects are consistent with the idea of enzyme induction. Furthermore, with both substances, there was a marked tendency for single doses to be more effective than multiple doses against fatty accumulation. This would be expected if the first dose induced enzyme synthesis, and later doses then competed with CCl_4 for these enzymes. The fact that very high doses of vitamin E and BHT, expecially if given fairly soon before the CCl_4, markedly increase hepatic triglyceride accumulation also suggests that these substances may compete for processes concerned in the detoxication of CCl_4. It may be noted (Table 8) that ethoxyquin, which is metabolized very rapidly, is found at a considerably lower level in the liver 24 h after three daily doses than after a single dose. This again suggests that the first dose induces enzyme synthesis and that the later doses are then metabolized faster. Vitamin E might also affect enzyme synthesis, although more weakly. There is certainly some evidence that a time lag of 24–48 h is necessary before its weak activity against the hepatic lesions is manifested, and Gallagher (1961) found that even intraperitoneal doses of vitamin E had to be given more than 40 h before CCl_4, if they were to increase survival. However, unlike ethoxyquin and BHT, vitamin E has little effect on liver size and does not prevent fat accumulation. Its weak activity may be due to other mechanisms. Some of the differences in the reported effects of vitamin E on CCl_4 toxicity might be attributable to the fact that the levels of drug processing enzymes

are readily affected by sex and by dietary and environmental conditions, such as dietary protein and fat, cage bedding and insecticides. McLean (1967) has shown that fasting affects the protective effect of oral vitamin E and this factor may account for the unusually great protective effect found by Meldolesi (1968). Effects on drug processing enzymes are not the only means by which substances can reduce CCl_4 toxicity. Although the protective effect of O_2 (Glynn & Himsworth, 1948) could be due to a competitive reaction between it and an endogenous microsomal process (Slater, 1966), the recent work of Rapin, Got, Le Gall & Goulon (1967) suggests that O_2 might affect the regeneration of liver by reducing anoxia after CCl_4. Some of the effect of vitamin E on survival may be due to similar causes and this is supported by the work of Butturini *et al.* (1955), Petzold & Weber (1963) and Maros, Fodor, Kovacs & Katonai (1966). Our results suggest also that intraperitoneal injections of antioxidants and vitamin E may function, in part at least, by mechanisms different from those of oral doses, perhaps by stimulating the adrenals. This idea was originally suggested by Judah, Ahmed & McLean (1963) and is supported by McLean (1967).

The action of DPPD seems more complex than that of ethoxyquin or BHT. It is difficult to attribute it solely to an effect on processing enzymes, for it can reduce hepatic triglycerides when given 6 h before the CCl_4. There have been several reports that DPPD and α-tocopherol have qualitatively different effects in vivo. Thus, DPPD was found to potentiate hypervitaminosis A in rats given 3×10^6 USP units vitamin A palmitate/kg, whereas α-tocopherol did not (Cox, Deuel & Ershoff, 1957). DPPD, unlike α-tocopherol, is poorly active against exudative diathesis and muscular dystrophy in the chick (Scott, 1962). The haematological response to DPPD in the vitamin E-deficient rhesus monkey is also different from that of α-tocopherol (Fitch & Dinning, 1963). Bunyan, Cawthorne, Diplock & Green (1969) found DPPD highly effective in preventing hepatic triglyceride accumulation in rats after ethanol poisoning, whereas α-tocopherol was practically inactive. Roberts & DeLuca (1969) found that DPPD, but not vitamin E, would inhibit the decarboxylation of retinoic acid in vivo. Aware of the difficulty in accommodating their finding within the concept of in vivo lipid peroxidation, they considered that 'as an antioxidant, DPPD has a stronger, more direct effect than α-tocopherol'—a conclusion difficult to reconcile with the comparative activities of the two substances in vitamin E deficiency diseases. Our results suggest that the observed differences between the activities of DPPD and α-tocopherol may reside in a real difference in function, due partly perhaps to different effects on hepatic enzyme synthesis. Two main conclusions emerge from the discussion. First, synthetic antioxidants have now been found to exert a function in vivo not shared by vitamin E; and, secondly, it has been shown that this function cannot be attributed to their antioxidant action. The many instances in which vitamin E and synthetic antioxidants function similarly in animals have been regarded as strong grounds for believing that the vitamin itself must function as an antioxidant in vivo. The force of this argument would now appear to be considerably weakened.

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EXPLANATION OF PLATE

(*a*) Liver from a rat given orally 500 mg ethoxyquin/kg 48 h before CCl_4, showing slight (grade +) necrotic degeneration. There is hydropic degeneration and vacuolation.

(*b*) Liver from a rat given orally 500 mg ethoxyquin/kg 6 h before CCl_4, showing moderate (grade + +) necrotic degeneration. There is an area of focal necrosis, hydropic degeneration and vacuolation.

(*c*) Liver from a rat given CCl_4 (2 ml/kg) without pre-treatment, showing severe centrilobular necrosis with hydropic degeneration (grade + + +).

(Magnification × 170.)

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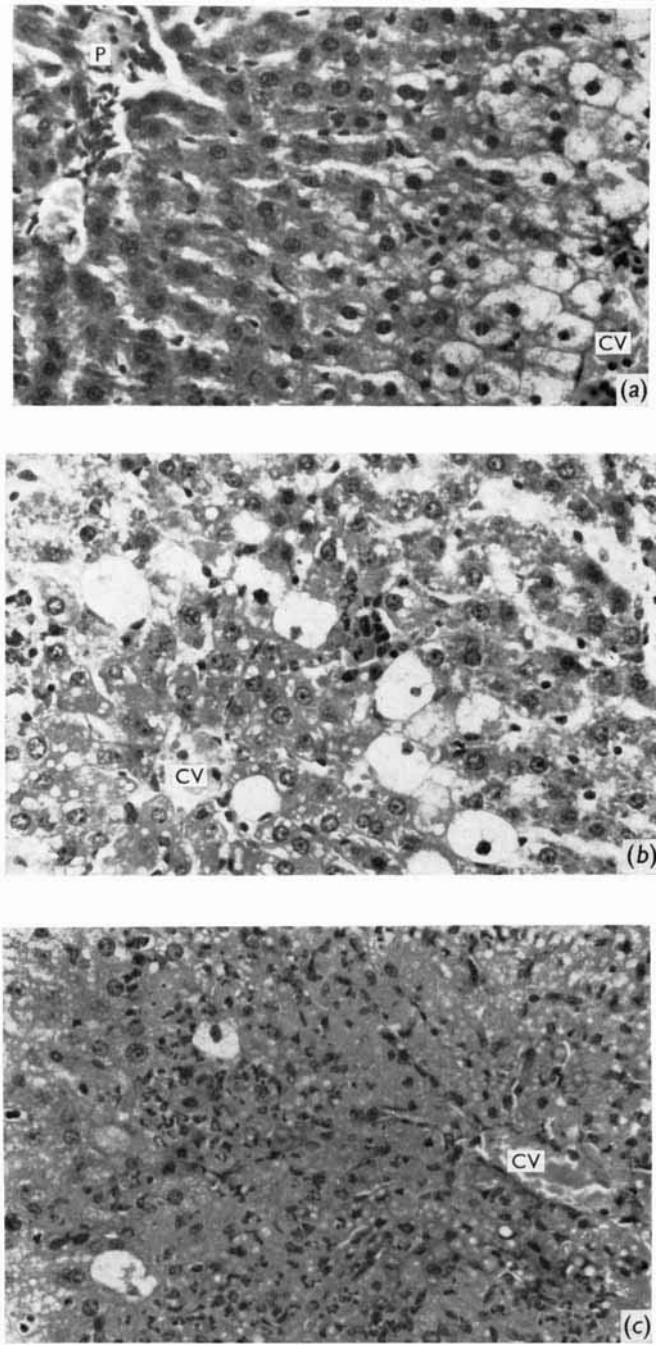

(*Facing p.* 384)