Vitamin E and hepatotoxic agents

1. Carbon tetrachloride and lipid peroxidation in the rat

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1. It has been suggested that carbon tetrachloride damages rat liver by accelerating processes of lipid peroxidation at subcellular sites and that the protective action of vitamin E is due to its functioning as an antioxidant in vivo. Direct evidence for these mechanisms in vivo has been sought and is critically examined.

2. The increased production of malondialdehyde by rat liver microsomal fractions during incubation with CCl_4 was shown to be a function of the vitamin E status of the rat and of an in vitro reaction, which could not be correlated with the hepatotoxic action of CCl_4.

3. Evidence for the production of lipid peroxides by CCl_4 in the livers of vitamin E-deficient and vitamin E-supplemented rats was sought (1) by measurement of ultraviolet spectral changes ('diene' formation) and (2) by direct micro-iodimetric determination of the peroxide. No differences in peroxide content were found between CCl_4-treated and control rats, nor were the spectrophotometric changes in the ultraviolet region related to the presence of vitamin E.

4. The effect of CCl_4 (2·0 ml/kg orally) on ATP levels in rat liver was studied at intervals from 3 to 68 h. The primary lesion leading to necrosis and fat accumulation after CCl_4 treatment occurred many hours before the eventual slight decline in ATP. Although the levels of ATP were somewhat higher in vitamin E-deficient rats, vitamin E did not prevent the slight decline in ATP that took place. Since ATP is known to be highly sensitive to peroxidation, the results suggest that lipid peroxidation is not the primary event in CCl_4 poisoning.

5. The effect of CCl_4 on the metabolism of [^{14}C]D-α-tocopherol in the rat was studied. A single intraperitoneal dose of CCl_4 (2·0 ml/kg) did not increase the destruction of α-tocopherol in the liver or carcass after 24 h. Three smaller daily doses of CCl_4 (0·25 ml/kg) also did not increase α-tocopherol catabolism; on the contrary, significantly more α-tocopherol was found in the livers of rats treated with CCl_4. These results suggest that CCl_4 does not increase lipid peroxidation in vivo.

It has been known for many years that vitamin E is able, in addition to its normal nutritional role, to protect the rat against a number of hepatotoxic agents. The way in which vitamin E protects against these agents is, in the main, not known; but usually its protective action is not unique, being shared by a variety of other substances. Because vitamin E and several of these other substances are antioxidants, it has been suggested that they protect against hepatotoxins by an antioxidant action in vivo; and hence, by the logic of the argument, the hepatotoxins must be assumed to exert their effects by pro-oxidant activity.

In this paper we report results of studying one such hepatotoxic agent, carbon tetrachloride. Possible mechanisms of CCl_4 toxicity have recently been reviewed by Magee (1966) and Recknagel (1967). There have been suggestions, starting with

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that of Butler (1961), that CCl_4 may produce in the liver free radicals that might attack sensitive subcellular sites. Ghoshal & Recknagel (1965) and Recknagel & Ghoshal (1966) developed this concept, suggesting that CCl_4 can initiate chains of peroxidation in the lipids of microsomes and mitochondria, and Slater (1966) has put forward a similar hypothesis. In the present work we have examined the evidence for the involvement of lipid peroxidation in CCl_4 poisoning.

EXPERIMENTAL AND RESULTS

Methods

Animals. Male and female rats of the Norwegian hooded strain were used.

Diets. The vitamin E-deficient diet was the 15% casein–5% lard diet, G 15 F, of Cawthorne, Diplock, Muthy, Bunyan, Murrell & Green (1967).

Malondialdehyde (MDA) in rat liver. Rats were killed by breaking their necks, and their livers were removed and placed at once into ice-cold buffer solution (0·112 M-NaCl and 0·05 M-NaH_2PO_4, pH 5·6) as described by Ghoshal & Recknagel (1965). Homogenates (2·5%, w/v, in buffer) were prepared as previously described (Bunyan, Green, Diplock & Robinson, 1967). After centrifuging at 4° for 12 min at 9000 *g*, the supernatant fraction (free of nuclei and mitochondria, but containing the microsomal fraction) was removed. Portions (6 ml) were incubated at 37° in 50 ml conical flasks, with shaking in air at one hundred excursions/min for various periods of time. To each flask was added 6 ml 35% (w/v) trichloroacetic acid and, after filtration, 3 ml filtrate were mixed with 3 ml 0·5% thiobarbituric acid solution. The solutions were heated at 100° for 10 min, cooled in ice-water for 3 min, and their optical densities were measured at 520 nm. A standard curve was obtained using malondialdehyde bis-bisulphite.

Lipid peroxides. These were determined in liver by the method of Bunyan, Murrell, Green & Diplock (1967).

ATP. This was determined by the method of Lamprecht & Trautschold (1963), who emphasize that ATP is extremely labile in tissues after death and that precautions must be taken to obtain meaningful values. Young (1966) has shown that ATP levels decline within a few seconds of excising the liver, and he recommended that the liver be frozen *in situ* before removal. His procedure was essentially followed. The rats were anaesthetized with Nembutal, the body wall was incised, and a lobe of the liver was frozen immediately between two specially shaped aluminium blocks, which were cooled to −70° and attached to tongs. The piece of frozen tissue was cut away from the remainder of the liver and transferred to a beaker containing CO_2 powder. It was weighed at once and then ground with more CO_2 powder. Perchloric acid was then added as described by Lamprecht & Trautschold (1963). The recovery of known amounts of ATP added at the grinding stage and put through the whole assay procedure was found to be quantitative.

Total liver lipids. A 2 g sample of liver was extracted as described by Diplock, Green, Edwin & Bunyan (1960) and lipid was determined by weight.

Histology. Paraffin wax sections were stained with haematoxylin and eosin.

Radioactivity experiments. [^{14}C]D-α-tocopherol was prepared and administered as described by Green, Diplock, Bunyan, McHale & Muthy (1967) and its determination in tissues was as described by them. During these experiments rats were housed individually in tubular cages to prevent coprophagy.

Vitamin A. This was determined on the non-saponifiable extracts of liver by the usual $SbCl_3$ spectrophotometric method.

Ultraviolet spectra of microsomal lipids. The method of Recknagel & Ghoshal (1966) was used to prepare microsomal lipids from rat liver. Their ultraviolet spectra and the difference spectra between control and CCl_4-treated rats were also measured by the method of Recknagel & Ghoshal (1966).

Production of malondialdehyde (MDA) by rat liver microsomal preparations in vitro

Priest, Smuckler, Iseri & Benditt (1962) could find no increase in rat hepatic MDA 3 h after CCl_4 poisoning. Ghoshal & Recknagel (1965) stated that this was probably because MDA can be metabolized by liver mitochondria. They found that the microsome plus supernatant fraction of normal rat liver produced more MDA on incubation in vitro with CCl_4 than control preparations without CCl_4 and claimed this as evidence that CCl_4 could accelerate lipid peroxidation in vivo.

In our experiments, the effect of CCl_4 on the production of MDA in vitro was studied in 5-month-old rats of both sexes. Liver homogenates were incubated with and without CCl_4 addition, according to the general procedure of Ghoshal & Recknagel (1965). The volume of CCl_4 added varied between 1 and 20 μl. MDA was measured at several times during the incubation. Twenty-one experiments were done on vitamin E-deficient rats and on similar rats previously given varying amounts of vitamin E for different times. The results were variable but fell into three general patterns, which are best illustrated graphically (Fig. 1). Curves of type A were always found with preparations from the livers of adult vitamin E-deficient rats, which were not studied by Ghoshal & Recknagel (1965). In these livers the production of MDA was rapid and was unaffected by CCl_4. When adult rats that had been given the vitamin E-deficient diet supplemented with 70 ppm D-α-tocopheryl acetate from weaning were used, curves of type C were always obtained. In these, the rate of MDA production in vitro was much slower, as was to be expected, and no pro-oxidant effect of CCl_4 was observed; indeed, generally the addition of CCl_4 to such liver homogenates led to a slightly diminished production of MDA. When vitamin E-deficient rats were given fairly small doses of α-tocopherol, of the order of 400 μg, 24 h before being killed, then curves of type B were sometimes, but not always, obtained. These correspond to those depicted by Ghoshal & Recknagel (1965) and demonstrate a pro-oxidant effect of CCl_4 in vitro.

Effect of carbon tetrachloride on true lipid peroxides in rat liver

Although Ghoshal & Recknagel (1965) claimed that their in vitro studies gave evidence that CCl_4 produced lipid peroxidation in vivo, Recknagel & Ghoshal (1966)

were unable to show that the 'zero-time' MDA level in rat liver was increased by giving the rats CCl_4 from 15 min to 25 h before they were killed. They postulated, therefore, that the 'organic peroxides may have relatively short metabolic lifetimes' and that the MDA produced in the injured cells might be metabolized rapidly. However, our previous studies have shown that lipid peroxides are not necessarily

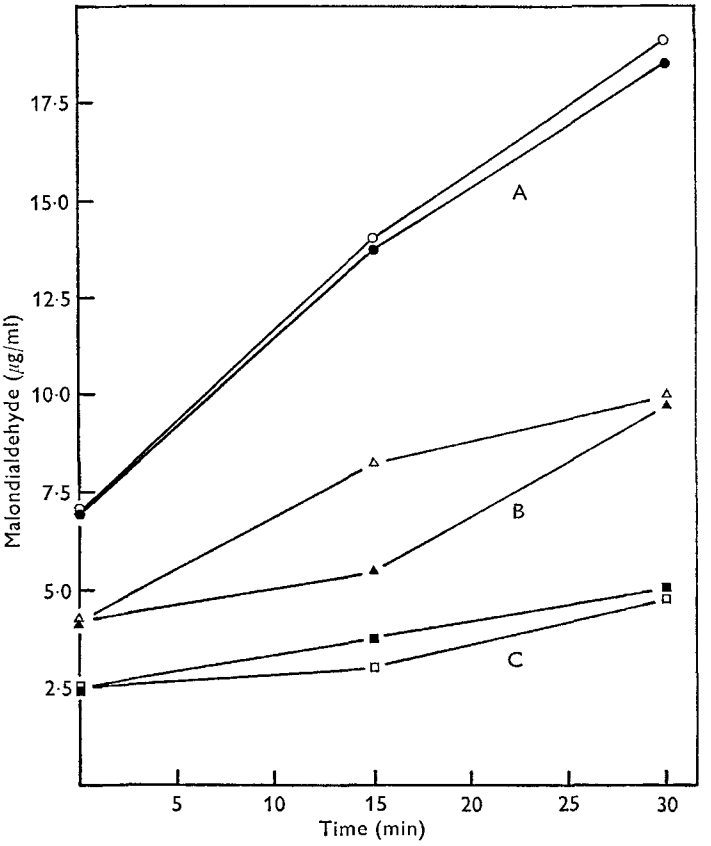

Fig. 1. Malondialdehyde production in vitro by liver microsome preparations from (1) vitamin E-deficient rats (●), (2) similar rats supplemented with 400 μg D-α-tocopheryl acetate 24 h before death (▲) and (3) rats given a dietary supplement of 70 ppm D-α-tocopheryl acetate from weaning (■). The same microsome preparations were also incubated with the addition of 10 μl carbon tetrachloride and are shown as ○, △ and □ respectively.

short-lived in vivo, and a half-life of about 6 days was found in adipose tissue (Bunyan, Green, Murrell, Diplock & Cawthorne, 1968). Such peroxides are, in fact, readily measured (Bunyan, Murrell *et al.* 1967). Accordingly, we have examined the livers of CCl_4-treated rats for true lipid peroxides.

Female rats that had received the vitamin E-deficient diet for 1 year were used. Some were given an intraperitoneal injection of 1·5 ml CCl_4/kg as a 50% (v/v) solution in arachis oil. Controls were given arachis oil. Lipid peroxides were measured in the liver immediately after CCl_4 administration and 2 h and 20 h afterwards. At the

same time, MDA production in whole liver homogenates was measured after incubation for 1 h. Table 1 gives the results. There was no significant rise in lipid peroxide due to CCl_4. In agreement with Recknagel & Ghoshal (1966), we found little change in MDA production by liver 2 h after CCl_4 administration and a greatly decreased production of MDA by liver 20 h afterwards.

Table 1. *Effect of carbon tetrachloride on lipid peroxides and malondialdehyde (MDA) production in rat liver*

(Vitamin E-deficient female rats, 1 year old, were given 1·5 ml CCl_4/kg intraperitoneally and their livers were examined 0, 2 and 20 h later. Results are given as means with standard deviations, with the no. of analyses, each on the combined tissues of two rats, in parentheses)

Time after dose (h)	Lipid peroxides (μ-equiv./g)	MDA production* (μg/g)
0	0·61 ± 0·28 (3)	25 ± 2·7 (3)
2	0·86 (1)	32 (1)
20	0·91 ± 0·28 (4)	8·2 ± 2·1 (4)

* Measured after incubation of whole liver homogenate for 1 h.

'Diene conjugation' in subcellular components of rat liver after carbon tetrachloride poisoning

Recknagel & Ghoshal (1966) obtained the microsomal and mitochondrial lipids from the livers of rats poisoned with CCl_4 and compared them spectrophotometrically with the lipids of control rats. They found a difference spectrum, with a low intensity peak in the 235 nm region, which they ascribed to a conjugated diene peroxide, presumed to have been formed by the peroxidation of membrane structural fatty acids initiated by free radicals formed from CCl_4. They used male rats given adequate diets. We have repeated their work, studying only microsomal lipids, but using rats given diets with and without vitamin E. With 5-month-old vitamin E-deficient male rats, we observed a difference spectrum similar to that described by Recknagel & Ghoshal (1966), with an absorption peak in the 238 nm region, but we also observed differences in the spectrum at 217–220 nm. When similar rats reared on the same diet supplemented with 70 ppm D-α-tocopheryl acetate were examined, the same difference spectrum between CCl_4-treated and control rats was observed. When 1-year-old male rats were given three successive 100 mg doses of vitamin E, 72 h, 48 h and 24 h before poisoning, a difference spectrum, somewhat reduced in intensity, was still observed. However, with 5-month-old female rats, a difference spectrum was not found irrespective of whether they had received vitamin E previously. When vitamin E-deficient 5-month-old male rats were given 2·0 ml/kg of fluorotrichloromethane (CCl_3F), which is not a hepatotoxic agent (Slater, 1965), no difference spectrum was found.

Effects of carbon tetrachloride and vitamin E on liver ATP levels

CCl_4 poisoning produces both necrosis and fat accumulation in rat liver. For a number of reasons, which are dealt with in the discussion, we thought it of interest

to examine the effects of CCl_4 and vitamin E on liver ATP levels during the development of the toxicity. The precise time-scale at which these events occur in the liver might give information as to the nature of the primary metabolic lesion. Thus, in rats poisoned with ethionine, liver ATP levels have been found (Farber, Shull, Villa-Trevino, Lombardi & Thomas, 1964) to fall to 20% of normal within 2 h, which is before any changes in lipid metabolism can be observed. In our experiment, the effects of CCl_4 and vitamin E were studied at intervals up to 68 h.

Table 2. *Effect of vitamin E on lipid and ATP levels in livers of rats poisoned with carbon tetrachloride*

(Vitamin E-deficient female rats*, 5 months old, were given 2·0 ml CCl_4/kg orally, and some groups were given 70 mg D-α-tocopheryl acetate daily for 8 days previously. Livers were analysed either singly or in pairs. Each result is given as the mean of three such analyses with the standard deviation)

		Vitamin E-deficient rats		Vitamin E-supplemented rats	
No. of rats	Time after CCl_4 (h)	ATP (μmoles/liver per 100 g rat wt)	Lipid (mg/liver per 100 g rat wt)	ATP (μmoles/liver per 100 g rat wt)	Lipid (mg/liver per 100 g rat wt)
6	0	16·2±2·0	152±19	18·6±1·9	167±33
6	3	17·3±0·4	253±33	22·6±1·8	267±41
6	6	14·5±0·8	308±27	20·7±3·7	333±40
6	12	13·9±1·7	387±74	18·1±1·5	369±55
3	16	14·2±1·9	381±108	18·1±2·9	367±78
3	20	13·4±2·9	504±84	—	353±15
6	24	—	597±86	—	505±44
3	30	12·4±2·6	582±48	15·3±0·9	392±37
3	68	19·6±5·6	378±64	20·7±3·2	481±93

* Wt range, 90–153 g.

Weanling female rats were given the vitamin E-deficient diet for 4 months. They were then divided randomly into eighteen groups, each containing three or six rats. Some groups (Table 2) were given eight consecutive daily doses of 70 mg D-α-tocopheryl acetate. Some supplemented and control groups were then given a single oral dose of 2·0 ml CCl_4/kg, as a 30% (v/v) solution in liquid paraffin. Because of the amount of time taken by each analysis, the experimental scheme was spread over several weeks, and control groups were examined at the start and end of the experiment. Previous pilot tests on similar control animals had already shown that little variation occurred in either liver ATP or liver lipid levels over several weeks.

At intervals from 3 to 68 h after CCl_4 dosage, rats were taken singly or in pairs and anaesthetized with Nembutal. The abdominal cavity was opened and a lobe of the liver was removed with the special tongs maintained at −70°. This lobe was immediately assayed for ATP. The remainder of the liver was excised, examined histologically, and its total lipid content was measured. Three analyses were carried out in each group. The results are shown in Table 2, all the measurements being expressed in terms of whole liver per 100 g rat (Slater & Sawyer, 1966).

Fat accumulation in the liver was clearly apparent in deficient and supplemented rats 3 h after CCl_4 dosing and continued to increase up to 24 h. Degenerative changes

were histologically apparent after 6 h, and by 16 h the reticulate structure of the liver was disorganized. The necrotic and fatty area increased up to 24 h until it involved the whole lobule and there was marked phagocytosis in the centrilobular area. By 68 h, regeneration of the liver was apparent. Vitamin E supplementation did not prevent the accumulation of lipid (Table 2) and had no effect on the necrotic process. Liver ATP levels in the deficient rats were slightly lower than in the supplemented rats throughout the experiment. The general trend in both groups was for ATP levels to rise at 3 h, to fall slightly until 30 h and then to return to normal or higher values.

Effect of carbon tetrachloride on the metabolism of [^{14}C]D-α-*tocopherol*

Expt 1. Male rats were given the vitamin E-deficient diet from weaning for 5 months. They were then divided randomly into four groups of nine and given a single oral dose of 52·50 μg (6412 dps (disintegrations/sec)) [^{14}C]D-α-tocopherol. After 24 h, the rats in groups 2 and 4 were given intraperitoneally 2·0 ml CCl_4/kg. Groups 1 and 3 acted as controls, receiving no injection. The rats in groups 1 and 2 were killed 3 h, and those in groups 3 and 4 24 h, after the injection. Livers and carcasses from each group were combined in lots of three and stored at $-20°$ until analysed for [^{14}C]α-tocopherol and its fat-soluble metabolites. Livers were analysed, in addition, for vitamin A. At 24 h, the livers of the rats given CCl_4 showed visible signs of fatty infiltration and, on histological examination, necrotic degeneration was seen.

Table 3. *Expt* 1. *Effect of carbon tetrachloride on the metabolism of* α-*tocopherol in the vitamin E-deficient rat*

(Male rats, in four groups of nine, were given the deficient diet for 5 months and then given 52·50 μg (6412 dps) [^{14}C]D-α-tocopherol. Groups 2 and 4 were given a single intraperitoneal dose of CCl_4 (2·0 ml/kg). Groups 1 and 2 were killed after 3 h and groups 3 and 4 after 24 h. Each analysis was carried out on the combined tissues of three rats, and there were three such analyses for each group. Results are given as means with standard deviations)

				Liver				Carcass	
Group	Treatment	Experimental period (h)	Rat wt (g)	Wt (g)	α-Tocopherol (dps/g)	[^{14}C]metabolites (dps/g)	Vitamin A (i.u./g)	α-Tocopherol (dps/carcass)	[^{14}C]metabolites (dps/carcass)
1	None	3	167±29	6·2±1·4	42±10	11±5	263±15	1897±430*	850±221†
2	CCl_4	3	174±28	5·8±0·3	46±15	13±3	299±24	1588±108	915±238†
3	None	24	183±29	7·0±1·0	26±5	8±2	255±40	1858±216*	583±10
4	CCl_4	24	170±34	7·3±0·4	20±4	6±0	223±25	1324±161	590±70

* Results for groups 1 and 3, taken together, were greater than those for groups 2 and 4 ($P < 0·025$).
† Results for groups 1 and 2, taken together, were greater than those for groups 3 and 4 ($P < 0·025$).

The analytical results are given in Table 3. At 3 h, no significant effect of CCl_4 on the metabolism of the radioactive tocopherol was found in liver or carcass. No effect of CCl_4 on the metabolism of α-tocopherol was found in liver after 24 h. However, analysis of variance of the results for the carcasses showed that the [^{14}C]α-tocopherol values were somewhat higher in control groups 1 and 3, considered together, than in CCl_4-treated groups 2 and 4 ($P < 0·025$). The [^{14}C]α-tocopherol values in group 1

were not significantly higher than those in group 2, and those in group 3 were not significantly higher than those in group 4. No effect of CCl_4 on vitamin A in the liver was observed.

Expt 2. In Expt 1 it was noticed that the tissues of the CCl_4-treated rats still contained substantial amounts of CCl_4 when removed from storage at −20° for analysis. Because of the possibility that some tocopherol might have been destroyed in that experiment, by CCl_4-catalysed peroxidation *post mortem*, in Expt 2 we analysed the livers and carcasses immediately after killing the animals. Otherwise the experimental conditions were as in Expt 1, but analyses were carried out only after 24 h. The results, given in Table 4, show that there was no significant effect of CCl_4 on the destruction of [^{14}C]α-tocopherol in liver or carcass. The slight effect observed in carcass in Expt 1 presumably took place *post mortem*.

Table 4. *Expt* 2. *Effect of carbon tetrachloride on the metabolism of α-tocopherol in the vitamin E-deficient rat*

(Male rats, in two groups of ten, were given the deficient diet for 4 months and then given 49·52 μg (6239 dps) [^{14}C]D-α-tocopherol. One group was given a single intraperitoneal dose of CCl_4 (2·0 ml/kg). The rats were killed after 24 h and the tissues were combined in pairs for analysis (five analyses per group). Results are given as means with standard deviations)

		Liver				Carcass	
Treatment	Rat wt (g)	Wt (g)	α-Tocopherol (dps/g)	[^{14}C]metabolites (dps/g)	Vitamin A (i.u./g)	α-Tocopherol (dps/rat)	[^{14}C]metabolites (dps/rat)
None	212±39	7·8±1·6	21±2	8·9±1·7	305±66	1178±357	1482±668
CCl_4	220±42	8·5±0·7	21±2	8·9±2·0	248±54	1420±467*	1465±873

* Significantly different from the control value ($P < 0·05$).

Table 5. *Expt* 3. *Effects of repeated small doses of carbon tetrachloride on liver lipid, vitamin A, and the metabolism of α-tocopherol in the vitamin E-deficient rat*

(Each group contained six rats, three of each sex, and received the deficient diet for 6 months. Each rat was given 112 μg (13 423 dps) [^{14}C]D-α-tocopherol. After 24 h, one group was given, daily for 3 days, 0·25 ml CCl_4/kg intraperitoneally. After 5 days, livers and carcasses were analysed in pairs (one of each sex). Results are given as means with standard deviations)

		Liver					Carcass	
Treatment	Rat wt (g)	Wt (g)	α-Tocopherol (dps/g)	[^{14}C]metabolites (dps/g)	Vitamin A (i.u./g)	Lipid (mg/g)	α-Tocopherol (dps/rat)	[^{14}C]metabolites (dps/rat)
None	215±6	7·37±2·33	50·8±11·3	31·0±8·2	763±138	31·3±2·0	4331±1267	1266±362
CCl_4	190±36	8·77±0·83	93·5±22·3*	41·2±11·5	544±81	55·7±5·0†	5124±394	1362±233

* Significantly different from the control value ($P < 0·05$).
† Significantly different from the control value ($P < 0·01$).

Expt 3. Weanling rats of both sexes were given the vitamin E-deficient diet for 6 months. They were given 112 μg (13423 dps) [^{14}C]D-α-tocopherol orally and then divided into two groups of six, with equal sex distribution. After an interval of 24 h, one group was given, intraperitoneally, 0·25 ml CCl_4/kg as a 30% (v/v) solution in

methyl oleate. The other group received methyl oleate only. The rats were killed on the 5th day, and their livers and carcasses were analysed for [^{14}C]α-tocopherol and its fat-soluble metabolites. Liver was also analysed for vitamin A and total lipid.

The results are shown in Table 5. CCl_4 produced a large increase in liver lipid. It also appeared to lower the concentration of vitamin A slightly, although the effect was not found to be significant. CCl_4 did not increase the destruction of radioactive α-tocopherol. Indeed, significantly more α-tocopherol was present in the liver of rats given CCl_4 than in controls. The amount of tocopherol in the carcass was not significantly affected by CCl_4.

DISCUSSION

In our earlier papers (Green *et al.* 1967; Diplock, Bunyan, McHale & Green, 1967; Diplock, Green, Bunyan, McHale & Muthy, 1967) the role of vitamin E was discussed in relation to the general problem of 'stress' and, in particular, to the current hypothesis of 'lipid peroxidation' as a primary causal event in vitamin E-deficiency diseases. The evidence of direct investigations, described in those papers, did not support the hypothesis. Observations that vitamin E protects against the effects of stress continue to be reported, however, and these have led some workers to suggest that such stresses must involve lipid peroxidation as a common causal process.

Vitamin E protects rats against a number of hepatotoxic agents. Of these, CCl_4 is of special importance as it has been studied extensively in recent years and much is already known about the biochemical changes it produces in the liver. It has recently been claimed that lipid peroxidation is the causal mechanism of CCl_4 hepatotoxicity (Ghoshal & Recknagel, 1965; Recknagel & Ghoshal, 1966) and we have examined some of the evidence for this.

Ghoshal & Recknagel (1965) showed that CCl_4, in vitro, increased the production of MDA during the incubation of the microsome plus supernatant fraction of rat liver. We found (Fig. 1) that the demonstration of the effect was critically dependent on the vitamin E status of the animal and could not be correlated with the toxic effects of CCl_4 in vivo. No effect was discernible with the livers of adult vitamin E-deficient rats, nor with the livers of such rats supplemented from weaning with 70 ppm D-α-tocopheryl acetate in their diet. Both types of rat are, in fact, susceptible to the hepatotoxic action of CCl_4, showing typical fat accumulation and necrotic changes in their livers. We were able to demonstrate the effect of CCl_4 in vitro only by using rats previously given a small single dose of α-tocopherol. When we measured true lipid peroxide levels in livers of rats poisoned with CCl_4 we found no increase over normal. Recknagel & Ghoshal (1966) also found no increase in true lipid peroxides, but they considered this was due to the 'pronounced lability of organic peroxides in biological systems'. However, Bunyan *et al.* (1968) have shown that fatty acid peroxides have a half-life of 6 days in the adipose tissue of the rat.

Recknagel & Ghoshal (1966) found that microsomal and mitochondrial lipids from the livers of normal male rats poisoned with CCl_4 had ultraviolet spectra slightly different from those of normal lipids, and the difference spectra showed an absorption maximum near 235 nm. They suggested that this was due to the presence

of a diene, produced by the peroxidative decomposition of structural lipids. In similar experiments, but with vitamin E-deficient male rats, we confirmed their findings, although on occasion, the 'diene' absorption peak was at a wavelength greater than 240 nm. However, the 'diene' absorption peak was still evident in male rats given large oral doses of vitamin E, even though the acute toxicity of CCl_4 in such rats had been found to be considerably less than in vitamin E-deficient controls (unpublished findings). We were unable to produce the 'diene' difference spectrum satisfactorily in female vitamin E-deficient or supplemented rats.

It would seem to follow, therefore, that although the action of CCl_4 on the microsomal lipids of liver can, under some conditions, produce changes in their spectra, the cause and nature of these changes are unclear. The suggestion by Recknagel & Ghoshal (1966) that their 'difference spectrum' method measures the extent of lipid peroxidation in vivo, in circumstances in which other known sensitive methods are inadequate, is difficult to support. They, in fact, provided statistically significant and reproducible estimates of true lipid peroxides, using conventional micro-iodimetry, in good agreement with the results for whole liver found by Bunyan, Murrell *et al.* (1967), and these values, unlike the 'diene' measurements, showed no change after CCl_4 administration, thus agreeing with our own findings (Table 1). Recknagel & Ghoshal (1966), claimed that the 'difference spectrum' method was five times as sensitive as iodimetry. This contention conflicts with their finding, by iodimetric titration, of about fourteen times as much lipid peroxide in fully peroxidized microsomes as in control non-peroxidized microsomes, whereas they found that the 'diene' conjugation method produced only a tenfold difference. The determination of peroxides by spectrophotometric iodimetry is, in fact, nearly as sensitive as the measurement of diene absorption; the respective $E^{1\%}_{cm}$ values are 560 by iodimetry and 738 at 235 nm (MacGee, 1959).

Study of the effect of CCl_4 and vitamin E on liver ATP levels (Table 2) also failed to provide evidence for CCl_4-induced peroxidation in vivo. There was no marked decline in ATP during the early stages of toxicity, when fat accumulation and necrotic change were already apparent. Although ATP levels were slightly higher in animals given vitamin E, the presence of vitamin E did not affect the slight downward trend in ATP levels during the later stages of the toxicity. Although Dianzani (1957) originally suggested that fatty liver induced by CCl_4 might result from an ATP deficiency, it is now considered (Farber *et al.* 1964; Magee, 1966) that ATP depletion is not the primary event in CCl_4 hepatoxicity. Our results confirm this point of view. Although Hyams & Isselbacher (1964) found that ATP levels were depressed in rat liver 5 h after CCl_4 administration, it is now considered (Lamprecht & Trautschold, 1963; Young, 1966) that the older methods for assaying ATP probably gave doubtful results. Recknagel (1967) considers that CCl_4 produces 'in or near the lipoidal centres of sheets of endoplasmic reticulum...so many chains of autocatalytic peroxidative breakdown of the unsaturated fatty acids, that normal levels of antioxidant protection are overwhelmed'. If this were so, a major decline in ATP could be expected to occur during the early stages of the lesion, when fatty infiltration is increasing and when protein synthesis is known to be markedly inhibited (Smuckler &

Benditt, 1965). Roubal & Tappel (1967), indeed, have found ATP to be highly sensitive towards radiation and peroxidative processes, and they consider that ATP depletion ought to be one of the major consequences of peroxidative processes in vivo, if they occur.

We also studied the metabolism of small amounts of [^{14}C]D-α-tocopherol in vitamin E-deficient rats given single and multiple doses of CCl_4. When 2·0 ml/kg was given as a single oral dose, no destruction of α-tocopherol was found 3 h or 24 h afterwards. When three successsive daily dose of 0·05 ml were given intraperitoneally, more α-tocopherol was found in the livers of the CCl_4-treated rats than in controls. This latter effect is similar to those we have previously observed with other 'stress' conditions; namely, silver-induced liver necrosis in the rat (Diplock, Green, Bunyan, McHale & Muthy, 1967), iron toxicity in the rat (Diplock, Green, Bunyan, Cawthorne & Dawson, 1967) and muscular dystrophy in the chick (Diplock, Bunyan, McHale & Green, 1967). It would seem, then, that the study of CCl_4 hepatotoxicity is no more able to provide tangible evidence of lipid peroxidation in vivo than other examples of anti-vitamin E stress.

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