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CARBON TETRACHLORIDE INDUCED LIVER ALTERATIONS IN RATS PRETREATED WITH *N,N'*-DIPHENYL-*p*-PHENYLENEDIAMINE

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Abstract—Glucose-6-phosphatase (G6P-ase) activity of liver microsomes was decreased 15 min following carbon tetrachloride administration, but it was unaffected at 5 min. At the latter time, however, lipid peroxidation was already detectable in liver microsomal lipids. Treatment of rats with *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) prior to CCl_4 poisoning did not prevent the peroxidation of microsomal lipids induced *in vivo* by carbon tetrachloride. Nor did it suppress the depression of G6P-ase. Malonic dialdehyde (MDA) production by liver homogenates or the microsome plus supernatant fraction was decreased by the addition of DPPD *in vitro* even at concentrations of $1{\cdot}5 \times 10^{-5}$ and $1{\cdot}5 \times 10^{-6}$ M. The *in vitro* pro-oxidant effect of CCl_4 was suppressed only when DPPD was present at the concentration of $1{\cdot}5 \times 10^{-4}$ M. Pretreatment of rats with DPPD significantly decreased the liver steatosis induced by carbon tetrachloride but it was ineffective on the fall in the plasma triglyceride level caused by the halogenated hydrocarbon. The effect of DPPD on the liver steatosis was manifested both when DPPD was administered in oil and when it was given as an aqueous suspension. Only in the former instance was DPPD found in appreciable amounts in the microsomal lipids, while in the latter case it occurred only in traces in the microsomal fraction. It is suggested that the ability of DPPD to decrease CCl_4-induced liver triglyceride accumulation is not related to an action of DPPD on the membranes of the endoplasmic reticulum of the liver cell.

IT HAS been known for many years that the administration of some antioxidants, such as α-tocopherol (vit. E), *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) and others, prior to carbon tetrachloride poisoning, affords protection against liver fat infiltration and necrosis.[1–5] In recent years a great deal of experimental evidence has shown that a peroxidation process involving the structural lipids of the liver cell occurs after CCl_4 intoxication.[6–11] It has also been suggested that this reaction may be a key event in the chemical pathology of carbon tetrachloride intoxication.[12]

Since antioxidants inhibit lipid peroxidation *in vitro*, it has been assumed that the protection afforded by these substances against some hepatotoxic effects of carbon tetrachloride could be due to an inhibition of the peroxidation of liver lipids induced *in vivo* by CCl_4.

In an early report, Cignoli and Castro[13] observed that administration of DPPD to rats prior to carbon tetrachloride intoxication does not prevent the depression of liver glucose-6-phosphatase (G6P-ase) activity after CCl_4 poisoning. They concluded that the loss of G6P-ase following carbon tetrachloride intoxication is not mediated through lipoperoxidation. However it was not investigated whether the administration of DPPD to the whole animal prevents the peroxidation of liver lipids after carbon tetrachloride intoxication.

In the course of investigations undertaken to determine whether the lipoperoxidation process is related to the hepatic changes that occur after CCl_4 poisoning, the effect of antioxidant pretreatment on CCl_4-induced lipoperoxidation and liver damage has been studied. Part of the results concerned with the effect of vit.E pretreatment have been previously reported.[14]

The present paper shows that pretreatment of rats with DPPD does not prevent the peroxidation of microsomal lipids induced *in vivo* by carbon tetrachloride and that the loss of G6P-ase occurs after the onset of this deteriorative process.

Furthermore the effect of DPPD in decreasing the liver steatosis caused by CCl_4 does not seem to be related to an action of DPPD on the membranes of the endoplasmic reticulum.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 180–250 g were used. They were maintained on a semisynthetic diet without preservative compounds (Piccioni, Brescia, Italy). The rats were starved for 14 hr before CCl_4 administration. Undiluted CCl_4 was administered by gastric intubation in a dose of 0·25 ml/100 g body wt. DPPD (Schuchardt, München, Germany) suspended in olive oil was administered intraperitoneally in three doses of 60 mg/100 g body wt each, 48 hr, 24 hr and 10 min before CCl_4 dosing. Control rats received olive oil intraperitoneally. In some experiments (that will be indicated in the Results section) DPPD was given as an aqueous suspension in order to avoid the oil vehicle in the peritoneum. The suspension was made in 2·5% acacia gum in water containing 0·25% Tween 80.[15] Control rats received the vehicle without DPPD.

Microsomes were isolated from 10% liver homogenates in ice-cold 0·25 M sucrose, 0·003 M EDTA, according to the scheme of de Duve *et al.* [16]

Diene conjugation absorption. The extraction of microsomal lipids as well as the spectrophotometric analysis over the u.v. range was performed essentially according to the method of Recknagel and Ghoshal[9] and of Rao and Recknagel,[10] with minor modifications. The amount of lipids present in the chloroform extract was determined by a colorimetric method.[17] After evaporation of the solvent, the lipids were redissolved in cyclohexane to give a concentration of 1 mg/ml. The recorded optical densities were further adjusted to this concentration by rechecking the amount of lipids[17] in the cyclohexane solution.

Malonic dialdehyde (MDA) production. Six % (w/v) liver homogenates in ice-cold 0·15 M KCl were prepared. The microsome plus supernatant fraction was obtained by centrifuging the homogenate at 2700 , for 10 min.[18] Aliquots of 2·5 ml (equivalent to 150 mg wet tissue) of liver preparation (whole homogenate or microsome plus supernatant fraction) were incubated with 1·5 ml of 0·15 M potassium phosphate buffer, pH 7·4. The incubation was carried out at 37°C in air, in a Dubnoff shaker and stopped by the addition of ice-cold trichloracetic acid.

When DPPD was employed *in vitro*, it was added to the incubation mixture in 0·2 ml of 0·15 M potassium phosphate buffer, pH 7·4, containing 0·25% acacia gum and 0·025% Tween 80. The vehicle only was added to the control flasks. The concentrations of DPPD used ($1·5 \times 10^{-6}$ M, $1·5 \times 10^{-5}$ M and $1·5 \times 10^{-4}$ M) were suggested by the results of the determination of DPPD in liver micosomes of rats treated *in vivo* with this substance (see the Results section). The stimulatory

effect of CCl_4 *in vitro* on lipid peroxidation was obtained as previously reported.[6,7] The TPNH generating system was avoided in these experiments, since it depressed the peroxidation in the samples in which CCl_4 is not present (see also Glende and Recknagel)[18] thus rendering the antioxidant effect of DPPD less clear in such samples.

The amount of MDA formed was determined as previously reported, [6,7,19] using a MDA standard produced by the acid hydrolysis of 1,1,3,3-tetraethoxypropane. It has been demonstrated by one of us[19] that no MDA breakdown occurs under these experimental conditions and that the concentration of MDA in the medium at the end of incubation reflects MDA formation.

G6P-ase assay. Microsomal G6P-ase activity was assayed according to de Duve *et al.*[16] Inorganic phosphorus was determined by the reduction of phosphomolibdate with the aminonaphtholsulfonic acid reagent of Fiske and Subbarow,[20] as reported by Harper.[21]

Determination of DPPD and of α-tocopherol. The concentration of DPPD and that of α-tocopherol in liver microsomes was determined according to the methods of Csallany and Draper[22] and of Bieri and Prival,[23] respectively. As regards the determination of DPPD, extreme care was taken to clean the liver sections of DPPD adsorbed to the capsule. The microsomes were washed once in the medium used for the homogenization of the liver and the bottom of the final microsomal pellet was avoided. These precautions were necessary to avoid, as far as possible, taking the particles of DPPD that were seen free in the homogenate with the microsomal pellet, and so overestimating the concentration of DPPD really occurring in the membranes of the endoplasmic reticulum.

Protein was measured according to the method of Lowry *et al.*[24]

Liver and plasma triglycerides were measured by the method of Van Handel and Zilversmit.[25]

RESULTS

Decrease of G6P-ase after carbon tetrachloride administration. The time course of the decrease in G6P-ase activity of liver microsomes following CCl_4 intoxication is given in Table 1. The activity was significantly decreased 15 min after CCl_4 dosing, but it was unaffected at 5 min.

Lipid peroxidation following carbon tetrachloride intoxication. Five min after poisoning peroxidation already occurred in microsomal lipids, as shown by the appearance of diene conjugation absorption (Fig. 1a). Therefore the time sequence of the two phenomena, G6P-ase impairment and lipid peroxidation, does not exclude the possibility that the loss of G6P-ase activity is mediated through peroxidation.

The early appearance of diene conjugation absorption in liver microsomal lipids, observed above, fully confirmed the results of Rao and Recknagel.[10] Furthermore MDA production by either whole homogenate or microsome plus supernatant fraction of the liver from rats intoxicated with carbon tetrachloride and killed 5 min later, was increased as compared to controls (Table 2). Therefore the increase in MDA production, previously reported to occur 1 and 2 hr after CCl_4 poisoning,[6,7] seems to be related to a real peroxidation process occurring in the living cell, since the reaction is increased at the same early time after poisoning at which conjugated dienes were detected (5 min).

TABLE 1. GLUCOSE-6-PHOSPHATASE ACTIVITY OF RAT LIVER MICROSOMES AT DIFFERENT TIMES FOLLOWING CCl_4 POISONING

Treatment	Time after CCl_4	No. of rats	G6P-ase activity*
None	5 min	5	4·52 ± 0·09
CCl_4	5 min	5	4·48 ± 0·20
None	15 min	10	4·21 ± 0·19
CCl_4	15 min	10	3·68 ± 0·18†
None	4 hr	3	4·23 ± 0·24
CCl_4	4 hr	3	2·27 ± 0·36‡

Values are expressed as means ± S.E.M.
* G6P-ase activity is expressed as μmole of inorganic phosphorus produced in 15 min/mg of microsomal protein;
† Statistically different from control ($P < 0·05$);
‡ Statistically different from control ($P < 0·01$).

Diene conjugation absorption found in liver microsomal lipids 1 hr after CCl_4 poisoning (Fig. 2a) is similar to that found at 5 min. In agreement with Recknagel and Ghoshal,[9] the difference spectrum is decreased 4 hr after the intoxication (Fig. 3a).

Effect of DPPD pretreatment on lipid peroxidation induced by CCl_4 in vivo. Administration of DPPD to rats prior to CCl_4 dosing did not prevent at all the spectral changes induced by the halogenated hydrocarbon in liver microsomal

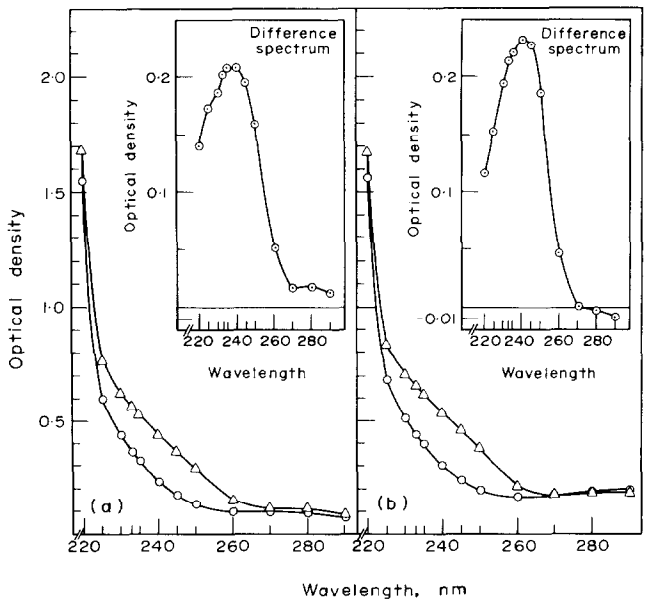

FIG. 1. Diene conjugation absorption in rat liver microsomal lipids, 5 min after carbon tetrachloride administration. (○) Control rats; (△) CCl_4 poisoned rats. (a) Rats not pretreated with DPPD. (b) Rats pretreated with DPPD.

TABLE 2. MALONIC DIALDEHYDE PRODUCTION BY RAT LIVER PREPARATION, 5 MIN AFTER CARBON TETRACHLORIDE ADMINISTRATION

Treatment	Liver preparation	Malonic dialdehyde (μg/g protein)		
		Incubation time		
		30 min	60 min	120 min
Controls	Homogenate		50·9 ± 4·2 (9)	124·5 ± 17·6 (9)
CCl_4	Homogenate		79·4 ± 19·5 (9)	150·8 ± 22·3 (9)
Controls	Microsomes + supernatant	49·4 ± 8·3 (9)	167·1 ± 16·9 (15)	392·0 ± 31·6 (6)
CCl_4	Microsomes + supernatant	79·5 ± 6·8 (9)*	210·4 ± 10·4 (15)†	418·6 ± 27·4 (6)

Values are expressed as means ± S.E.M. The number of rats are in parentheses.
* Statistically different from control ($P < 0.02$).
† Statistically different from control ($P < 0.05$).

lipids 5 min, 1 and 4 hr after the intoxication (Figs. 1b, 2b and 3b). Diene conjugation absorption was also found in DPPD pretreated rats 12 and 24 hr following CCl_4 poisoning. No apparent difference was seen between pretreated and non-pretreated animals, thus excluding the possibility of an earlier cessation of the peroxidative decomposition of microsomal lipids due to DPPD pretreatment. Results similar to those reported above were found when DPPD was administered

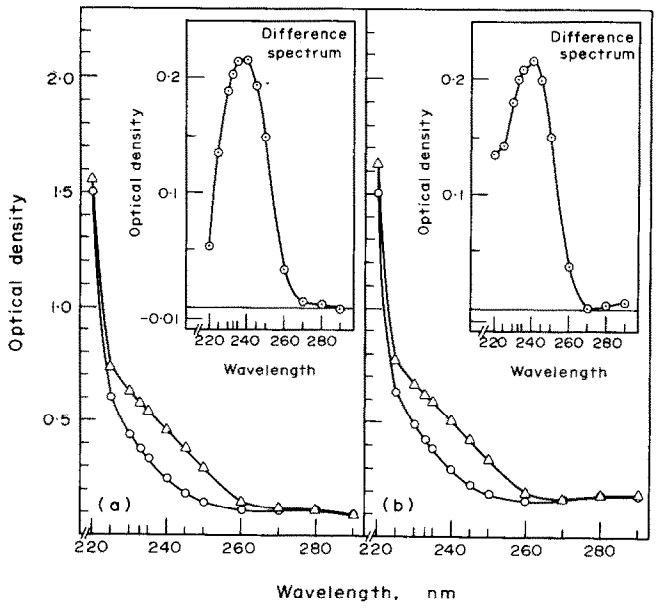

FIG. 2. Diene conjugation absorption in rat liver microsomal lipids, 1 hr after carbon tetrachloride administration. (○) Control rats; (△) CCl_4 poisoned rats. (a) Rats not pretreated with DPPD. (b) Rats pretreated with DPPD.

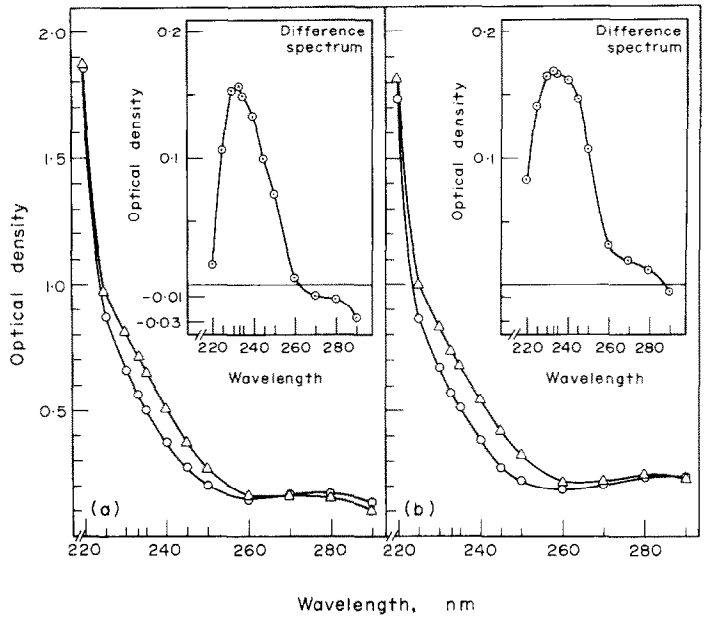

FIG. 3. Diene conjugation absorption in rat liver microsomal lipids, 4 hr after carbon tetrachloride administration. (○) Control rats; (△) CCl_4 poisoned rats. (a) Rats not pretreated with DPPD. (b) Rats pretreated with DPPD.

as an aqueous suspension (see Materials and Methods), or when a double dose of DPPD (120 mg/100 g body wt, 48 hr, 24 hr and 10 min before CCl_4 poisoning) was given to rats. Therefore this antioxidant is ineffective in modifying the lipid peroxidation induced by carbon tetrachloride *in vivo*.

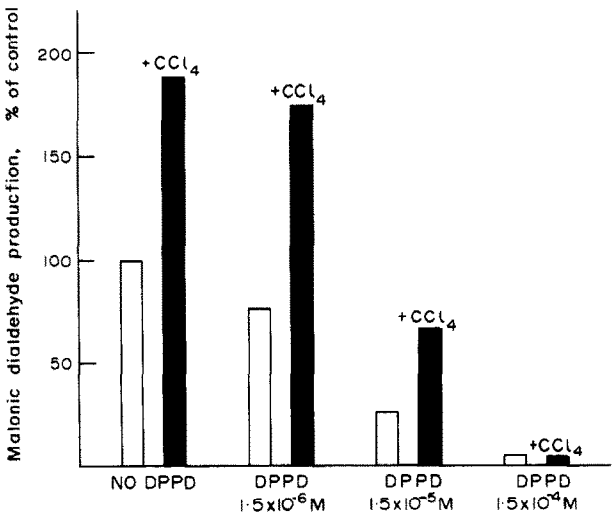

FIG. 4. Effect of different concentrations of DPPD on malonic dialdehyde production by liver homogenates, either in the presence or in the absence of CCl_4. The experimental conditions are reported in Materials and Methods. Incubation time: 60 min.

Effect of DPPD in vitro *on lipid peroxidation.* As shown in Fig. 4, MDA production by liver homogenates was decreased by the addition of DPPD *in vitro* even at concentrations of $1{\cdot}5 \times 10^{-6}$ M and $1{\cdot}5 \times 10^{-5}$ M. In both cases, however, the *in vitro* pro-oxidant effect of CCl_4 was still evident and it was practically unaffected by the lower concentration of DPPD ($1{\cdot}5 \times 10^{-6}$ M). Only when the concentration of DPPD was increased to $1{\cdot}5 \times 10^{-4}$ M, was the peroxidation suppressed even in the presence of CCl_4. Results similar to those reported in Fig. 4 were obtained by using the microsome plus supernatant fraction.

In the experiments carried out with a concentration of $1{\cdot}5 \times 10^{-6}$ M DPPD, 1·6 μg DPPD were available for an aliquot of liver preparation corresponding to 150 mg wet liver. It must be pointed out that this concentration is the same as that found (see below) in an equivalent amount of liver microsomes of rats pretreated with DPPD suspended in olive oil (1·6 μg DPPD per microsomes equivalent to 150 mg liver). Even with a concentration of DPPD tenfold higher ($1{\cdot}5 \times 10^{-5}$ M) the amount of MDA formed was higher in the presence of CCl_4 and therefore it can be concluded that "physiological" amounts of DPPD, when tested *in vitro*, are not enough to suppress the stimulating effect of CCl_4 on liver lipid peroxidation.

TABLE 3. EFFECT OF PRIOR TREATMENT WITH DPPD ON GLUCOSE-6-PHOSPHATASE ACTIVITY OF RAT LIVER MICROSOMES 4 HR AFTER CCl_4 POISONING

Treatment	No. of rats	G6P-ase activity*
Control	7	4·22 ± 0·14 (a)
CCl_4	7	2·21 ± 0·17 (b)
DPPD	8	4·31 ± 0·12 (c)
DPPD + CCl_4	8	2·95 ± 0·11 (d)

Values are expressed as means ± S.E.M.

* G6P-ase activity is expressed in μmole of inorganic phosphorus produced in 15 min/mg of microsomal protein.

Statistical significance of the difference, P-value for b–a: $P<0{\cdot}001$, d c: $P<0{\cdot}001$, d–b: $P<0{\cdot}02$.

Effect of DPPD pretreatment on the impairment of G6P-ase by carbon tetrachloride. As shown in Table 3, CCl_4 administration resulted in a 47 per cent decrease in liver microsomal G6P-ase activity 4 hr after poisoning. Prior treatment of rats with DPPD did not suppress the depression of G6P-ase, although the impairment is somewhat less severe in the DPPD-pretreated rats than in non-pretreated ones.

Effect of DPPD pretreatment on liver and plasma triglyceride concentration after CCl_4 poisoning. The failure of DPPD in preventing the lipid peroxidation induced *in vivo* by CCl_4, prompted us to investigate the nature of the well known protection afforded by DPPD against CCl_4 induced liver steatosis. In this set of experiments, DPPD was administered both in oil, as usual, and as an aqueous suspension (see Materials and Methods) since the administration of the oil vehicle in the peritoneum caused an abnormal hypertriglyceridemia and therefore the plasma triglyceride values may be unreliable under this experimental condition.

Carbon tetrachloride administration resulted in a threefold increase in the liver triglyceride level 4 hr after poisoning (Table 4). The liver steatosis was significantly

TABLE 4. EFFECT OF PRIOR TREATMENT WITH DPPD ON LIVER AND PLASMA TRIGLYCERIDE CONCENTRATION 4 HR AFTER CCl_4 POISONING

Treatment	Liver wt (g/100 g body wt)	Liver triglycerides (mg/100 g body wt)	Plasma triglycerides (mg/100 ml)
Exp. 1 Control	3·07 ± 0·10	7·93 ± 0·82 (a)	51·85 ± 2·32 (e)
CCl_4	3·59 ± 0·22	23·06 ± 2·83 (b)	22·01 ± 5·59 (f)
DPPD	3·59 ± 0·12	9·72 ± 1·26 (c)	56·67 ± 4·31 (g)
DPPD + CCl_4	4·05 ± 0·18	14·02 ± 0·85 (d)	32·20 ± 4·08 (h)
Exp. 2 Control	3·60 ± 0·13	10·37 ± 1·25 (i)	26·04 ± 2·37 (m)
CCl_4	3·64 ± 0·10	30·61 ± 3·02 (j)	9·41 ± 1·67 (n)
DPPD	3·40 ± 0·12	7·55 ± 0·75 (k)	48·42 ± 3·14 (o)
DPPD + CCl_4	3·43 ± 0·12	19·73 ± 4·03 (l)	18·12 ± 1·51 (p)

Exp. 1: DPPD was given in oil as usual; control rats received olive oil only.

Exp. 2: DPPD was given as an aqueous suspension (see Materials and Methods); control rats received the aqueous vehicle only.

Results are given as means of 10 animals for Exp. 1 (6 only for plasma triglycerides) and 13 animals for Exp. 2.

Statistical significance of the difference. P value for b–a: $P<0·001$; d–c: $P<0·01$; d–b: $P<0·01$; f–e: $P<0·001$; h–g: $P<0·001$; h–f: N.S.; j–i: $P<0·001$; l–k: $P<0·01$; k–i: N.S.; l–j: $P<0·05$; n–m: $P<0·001$; p–o: $P<0·001$; o–m: $P<0·001$; p–n: $P<0·001$.

Mean plasma triglyceride value for 11 untreated starved controls: 27·79 ± 2·12.

reduced in DPPD-pretreated rats. The plasma triglyceride level was decreased by nearly 60 per cent in the rats intoxicated with the halogenated hydrocarbon. CCl_4 poisoning resulted in a decrease in plasma triglycerides also in the rats given DPPD. Even if the plasma triglyceride level in rats given DPPD as an aqueous suspension and then carbon tetrachloride is significantly higher than the plasma triglyceride level in rats which received CCl_4 alone, account must be taken of the fact that treating the rats with DPPD as an aqueous suspension resulted by itself in a marked increase in plasma triglycerides.

TABLE 5. EFFECT OF PRIOR TREATMENT WITH DPPD ON LIVER AND PLASMA TRIGLYCERIDE CONCENTRATION 24 HR AFTER CCl_4 POISONING

Treatment	Liver wt (g/100 g body wt)	Liver triglycerides (mg/100 g body wt)	Plasma triglycerides (mg/100 ml)
Exp. 1 Control	3·80 ± 0·16	9·57 ± 1·84 (6) (a)	51·53 ± 2·73 (11) (e)
CCl_4	5·33 ± 0·23	146·53 ± 22·95 (5) (b)	30·46 ± 3·09 (11) (f)
DPPD	4·38 ± 0·07	10·76 ± 1·25 (10) (c)	56·75 ± 3·88 (13) (g)
DPPD + CCl_4	5·71 ± 0·15	75·60 ± 8·30 (11) (d)	40·27 ± 3·80 (16) (h)
Exp. 2 Control	3·23 ± 0·09	10·56 ± 1·95 (6) (i)	39·24 ± 4·77 (6) (m)
CCl_4	4·92 ± 0·13	120·09 ± 15·38 (6) (j)	17·58 ± 1·78 (6) (n)
DPPD	3·47 ± 0·11	11·85 ± 1·48 (6) (k)	74·56 ± 8·22 (6) (o)
DPPD + CCl_4	4·09 ± 0·20	34·72 ± 8·70 (6) (l)	21·80 ± 3·53 (6) (p)

Exp. 1: DPPD was given in oil as usual; control rats received olive oil only.

Exp. 2: DPPD was given as an aqueous suspension (see Materials and Methods); control rats received the aqueous vehicle only.

Results are given as means ± S.E.M. The number of rats is reported in brackets.

Statistical significance of the difference. P value for b–a: $P<0·001$; d–c: $P<0·001$; d–b: $P<0·01$; f–e: $P<0·001$; h–g: $P<0·01$; h–f: N.S.; j–i: $P<0·001$; l–k: $P<0·05$; l–j: $P<0·01$; n–m: $P<0·001$; p–o: $P<0·001$; o–m: $P<0·01$; p–n: N.S.

Twenty-four hr after CCl_4 poisoning the liver triglyceride accumulation was strikingly reduced in DPPD pretreated rats (Table 5). A higher degree of protection was afforded by DPPD given as an aqueous suspension than by DPPD given in oil. Hypertriglyceridemia was observed again in the rats to which DPPD was administered as an aqueous suspension. The CCl_4-induced fall in the plasma triglyceride level occurred in both non-pretreated and pretreated animals.

The effect of DPPD on the CCl_4-induced increase in liver weight is generally parallel to the effect of DPPD on the CCl_4-induced increase in liver triglycerides.

Concentration of DPPD in liver microsomes of DPPD pretreated rats. The concentration of DPPD in liver microsomes of pretreated rats was determined both when DPPD was administered in oil and when it was administered as an aqueous suspension. As shown in Table 6, a very small amount of DPPD was found in liver microsomes when it was given as an aqueous suspension. On the contrary, when the chemical was administered in oil, a far larger amount of DPPD was present in the liver microsomal fraction (Table 6). Under the same condition of administration, an equal concentration of DPPD was found in liver microsomes of rats treated with doses of either 60 or 120 mg/100 g body wt.

TABLE 6. CONCENTRATION OF DPPD AND α-TOCOPHEROL IN LIVER MICROSOMES OF RATS TREATED WITH DPPD AND α-TOCOPHEROL, RESPECTIVELY

Treatment with DPPD	nmoles DPPD/ mg protein
Exp. 1. DPPD 3 × (60 mg/100 g body wt)	1·7 ± 0·2 (10)
DPPD 3 × (120 mg/100 g body wt)	1·7 ± 0·1 (11)
Exp. 2. DPPD 3 × (60 mg/100 g body wt)	0·3 ± 0·05 (6)
Treatment with α-tocopherol	**nmole α-tocopherol/ mg protein**
None (control)	0·4 ± 0·1 (9)
α-Tocopherol 25 mg/100 g body wt	2·0 ± 0·4 (9)
α-Tocopherol 75 mg/100 g body wt	4·2 ± 0·6 (9)

Exp. 1: DPPD was given in oil.
Exp. 2: DPPD was given as an aqueous suspension.
DPPD was administered intraperitoneally 48 hr, 24 hr and 10 min before sacrifice.
dl-α-tocopherol acetate was administered orally 24 hr before sacrifice.
Results are expressed as means ± S.E.M. The number of rats is reported in brackets.

In a previous report[14] concerned with the effect of the pretreatment with vit.E on the peroxidation of liver microsomal lipids induced *in vivo* by CCl_4, we observed that a dose of 25 mg of α-tocopherol per 100 g body wt was ineffective in preventing the peroxidation, while a dose of 75 mg/100 g body wt greatly decreased the diene conjugation absorption of microsomal lipids. These findings were confirmed during the present experiments in which DPPD was shown to be ineffective in reducing the peroxidation elicited by carbon tetrachloride. Therefore the concentration of α-tocopherol obtained in liver microsomes of rats treated with both doses of vit.E was determined and compared to the concentration of DPPD. As can be seen (Table 6), when DPPD was given in oil, its concentration, on a molar base, was even

lower than the concentration of α-tocopherol obtained with the dose of vit.E (25 mg/100 g body wt) that was ineffective on the CCl_4-induced lipid peroxidation. When DPPD was given as an aqueous suspension, its concentration in liver microsomes was even lower than the naturally occurring concentration of vit.E.

DISCUSSION

Although microsomal G6P-ase is somewhat less impaired in DPPD pretreated, CCl_4 poisoned rats, compared with the non-pretreated ones, in essential agreement with Cignoli and Castro,[13] our results seem to indicate that the enzyme activity is markedly depressed by carbon tetrachloride poisoning even when the rats were previously given DPPD. However DPPD administration does not prevent the peroxidation of microsomal lipids induced *in vivo* by carbon tetrachloride. Furthermore lipid peroxidation occurs prior to the decrease in G6P-ase activity. Therefore a role of lipid peroxidation in the mechanism of the CCl_4-induced enzyme impairment is not ruled out by these experiments. It remains to be ascertained, however, whether G6P-ase is impaired by lipid peroxidation or rather by a direct action of $CCl_3^{\cdot}$ and $Cl^{\cdot}$ free radicals, as postulated by Castro *et al.*[26,27] under various experimental conditions.

The present results suggest that DPPD does not act *in vivo* as an antioxidant against the pro-oxidant effect of carbon tetrachloride. The failure of DPPD in preventing the CCl_4-induced lipid peroxidation *in vivo* may be due to the fact that it enters the membranes of the endoplasmic reticulum in a relatively small amount even when it is given in oil, notwithstanding the large excess in which it is usually administered to rats. In fact, the concentration of DPPD obtained in rat liver microsomes by three administrations of 60 mg (0·230 mmoles) per 100 g body wt is lower than the concentration of α-tocopherol obtained with a single dose of 25 mg (0·058 mmoles) of vit.E per 100 g body wt. Furthermore the concentration of DPPD cannot be increased by increasing the dose administered to rats. On the contrary, the concentration of α-tocopherol does increase when the rats are given 75 mg (0·174 mmoles) of vit.E per 100 g body wt; in these rats the extent of the CCl_4-induced lipid peroxidation is markedly decreased, as compared to the values found for the non-pretreated controls.[14] It seems therefore that vit.E can counterbalance to some extent the pro-oxidant action of carbon tetrachloride, provided that its concentration in the cell membranes is relatively high.

It has been demonstrated[28] that vit.E is bound in a complex consisting of microsomal structural protein and lipid micelles. On the contrary, synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) although inhibiting lipid peroxidation *in vitro* even to a greater extent than vit.E. do not bind to the complex.[28] It has also been suggested[29] that lipophilic side chains of a certain length, like that of tocopherol, are necessary for the binding of the compound to subcellular structures. DPPD has no side chain and therefore it may behave like the other synthetic antioxidants. It is therefore conceivable that DPPD brings about its antioxidant effect *in vitro* (provided that its concentration is relatively high) because in such a condition the contact with the lipid structures is possible, but not *in vivo* if its binding to the cell membranes is poor.

Despite its ineffectiveness on the CCl_4-induced lipid peroxidation, pretreatment of rats with DPPD markedly reduced the fatty liver after carbon tetrachloride poisoning. However, such an effect of DPPD occurred both when the chemical was administered in oil and (to an even greater extent) when it was given as an aqueous suspension. As shown above, only in the former instance was DPPD found in appreciable amounts in liver microsomes, while in the latter case it was present in the microsomal fraction only in traces. It is therefore conceivable that DPPD decreases the CCl_4-induced liver triglyceride accumulation not by an interaction with the membranes of the endoplasmic reticulum, but rather by a non-specific mechanism involving structures which are not the primary target of the CCl_4-induced lipid peroxidation. Such structures could be inside or outside the liver parenchimal cells. It has been demonstrated[30,31] that prior reticuloendothelial blockade with trypan blue or colloidal carbon affords a striking protection against the hepatotoxic effect of carbon tetrachloride. It has also been demonstrated that many compounds injected into the peritoneum afford similar protection.[32] Since it is likely that DPPD is taken up to a great extent by the reticuloendothelial cells (the liver capsule appears thickened, infiltered with DPPD and containing a large number of macrophages), the mechanism of the protection afforded by this substance could be similar to that exhibited by the reticuloendothelial system blocking agents.

Since the level of plasma triglycerides was decreased after CCl_4 poisoning also in the rats pretreated with DPPD, it seems that the impairment of the secretory mechanism of lipoproteins from the liver cells caused by CCl_4 is not substantially affected by previous treatment with DPPD. However, Crafton and Di Luzio[33] showed that DPPD maintains a normal hepatic release of triglycerides into the plasma compartment in rats poisoned with carbon tetrachloride, as assessed by the maintainance of the post Triton hypertriglyceridemic response to normal values. In similar experiments with Triton, we obtained results essentially identical to those of Crafton and Di Luzio.[33] In our experiments, however, DPPD was administered as an aqueous suspension, i.e. under the condition in which DPPD very poorly enters the membranes of the endoplasmic reticulum. Therefore the fact that the hypertriglyceridemic response following Triton was manifested to a comparable degree in saline and CCl_4-treated rats which received DPPD, is probably not due to an action of DPPD on the structures of the endoplasmic reticulum. However, the possibility remains that DPPD is active even in minimal concentrations; but it would have to be active in amounts smaller than the naturally occurring concentration of vit.E in the liver microsomes (Table 6).

The finding that rats given DPPD as an aqueous suspension show hypertriglyceridemia could be consistent with the hypothesis that DPPD induces a decreased hydrolysis of circulating triglycerides, so decreasing the afflux of fatty acids to the liver; this could account for the less severe degree of steatosis induced by carbon tetrachloride in DPPD pretreated rats. Such an hypothesis has to be proved, and therefore the mechanism by which DPPD decreases the extent of the CCl_4-induced fatty liver is, at the present, unsolved. From the results shown above, however, it is possible to infer that such an effect of DPPD is not related to a preservation of the membranes of the endoplasmic reticulum of the liver cell from the alterations produced by carbon tetrachloride on their physiological mechanisms.

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