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NATURE OF THE PROTECTION AGAINST CARBON TETRACHLORIDE-INDUCED HEPATOTOXICITY PRODUCED BY PRETREATMENT WITH DIBENAMINE [*N*-(2-CHLOROETHYL) DIBENZYLAMINE]*

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Abstract—In this paper, we assess various plausible mechanisms by which pretreatment with Dibenamine [*N*-(2-chloroethyl) dibenzylamine] hydrochloride (25 mg/kg, s.c., 48 and 24 hr before the administration of CCl_4) protects rats against the hepatotoxic effects of CCl_4. Dibenamine pretreatment did not affect significantly the absorption of CCl_4 after i.p. administration or the elimination of CCl_4 from various tissues, as evident from unchanged tissue levels. Dibenamine pretreatment decreased the accumulation of the metabolite $CHCl_3$ in liver, the isopropanol-potentiated covalent binding of a small dose of $^{14}CCl_4$ (0·1 ml/kg) and the covalent binding of a necrosis-producing dose of $^{14}CCl_4$ (1·0 ml/kg) to proteins and lipids in liver, and the diene conjugation of liver microsomal lipids induced by CCl_4. These findings suggest that Dibenamine pretreatment impairs the microsomal enzymes which catalyze the formation of free radical derivatives of CCl_4. Dibenamine protection is not correlated with its concentration in liver or with the covalent binding of Dibenamine derivatives to liver microsomal protein.

A NUMBER of investigators[1–6] have proposed that carbon tetrachloride (CCl_4) is metabolized by microsomal enzymes to the carbon trichloro-free radical ($CCl_3\cdot$) and presumably other free radical derivatives. These free radical derivatives then may bind covalently to the proteins and lipids of the hepatocytes. The binding of free radical derivatives to the double bonds of polyunsaturated fatty acids may initiate processes leading to diene conjugation in liver microsomal phospholipids[6,7] and lipoperoxidation.[8] Thus, the covalent binding of the free radical derivatives of CCl_4 to liver lipids and proteins presumably initiates the processes responsible for CCl_4 hepatotoxicity.

This laboratory has shown[9] that pretreatment with Dibenamine [*N*-(2-chloroethyl) dibenzylamine] protected rats against the hepatotoxic effects of CCl_4, as evident from reduced levels of plasma glutamic-pyruvic transaminase and liver triglycerides, from decreased liver necrosis shown by histologic grading, and from an increased LD_{50} value at 48 hr. Optimal protection against the hepatotoxic effects of CCl_4

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was obtained when a large dose of Dibenamine (10–25 mg/kg) was injected, s.c., 48 and 24 hr before the administration of CCl_4. This protection was not correlated with alpha adrenergic receptor blockade.

In this paper, we have assessed various plausible mechanisms by which Dibenamine might elicit its protective effects. This paper also includes data on the distribution of ^{14}C-Dibenamine derivatives and their covalent binding to liver lipids and protein, both total and microsomal, at a time when protection against CCl_4-induced hepatotoxicity was optimal.

MATERIALS AND METHODS

Animals and drugs. Male Sprague–Dawley rats, weighing 150–225 g, were used. They had access at all times to water and a commercial rat chow.

Dibenamine hydrochloride (Smith, Kline & French, Philadelphia, Pa.) was dissolved in acidified (0·05 N H_2SO_4) ethanol in a concentration of 50 mg/ml and diluted with saline to a concentration of 5 mg/ml, with warming in a boiling water bath. Dibenamine hydrochloride (25 mg/kg) or saline was injected, s.c., 48 and 24 hr before the i.p. injection of CCl_4 (1·0 or 2·0 ml/kg in equal volume of sesame oil).

Preparation of tissue extracts for measurements of CCl_4 and $CHCl_3$ levels. Animals were stunned by a blow on the neck and killed by exsanguination. The liver, kidney, epididymal fat pad, thigh muscles and blood were removed, and 1·0-g samples were added to 5 ml of 24 N H_2SO_4 in the outer chamber of microdiffusion cells, as recommended by Dambrauskas and Cornish.[10] The microdiffusion cells were 20-ml counting vials, containing the sulfuric acid, into which were placed open 4-ml vials containing 1 ml toluene. The caps of the counting vials were tightly screwed on and the vials were allowed to stand at room temperature overnight. The next day, the vials were shaken in a Dubnoff metabolic shaking machine for about 30 min before removal of the inner vials each containing 1 ml toluene and the extracted CCl_4 and $CHCl_3$.

Measurement of CCl_4 and $CHCl_3$. The CCl_4 and $CHCl_3$ concentrations in the toluene extracts were measured with a gas liquid chromatograph (Glowall Chromalab, model 310) equipped with a Ni^{63} ionization detector set at 240 V, d.c., in the electron capture mode. The detector temperature was 190°. The coiled glass column (2ft × 1/8in.) was filled with 30 per cent hexadecane on Chromosorb W, acid washed and DMCS treated, 45–60 mesh. The column temperature was 45° and the argon flow 60 ml/min. The injector temperature was maintained at 115°. Under these conditions, the retention time for CCl_4 was 5·2 min and that for $CHCl_3$ 2·5 min. The detector response to known quantities of $CHCl_3$ and CCl_4 was linear for amounts ranging from 0·25 to 20 ng and 0·1 to 6 ng respectively. The recoveries of known amounts of CCl_4 and $CHCl_3$, added to tissues and blood, were 89·0 per cent ± 1·47 for CCl_4 and 87·4 per cent ± 1·71 per cent for $CHCl_3$. The measurements were reproducible, with a coefficient of variation of 5·2 per cent for CCl_4 and 6·2 per cent for $CHCl_3$. No interfering peaks were observed when extracts from untreated animals were analyzed.

Preparation of microsomes. Immediately after removal from the body, each liver was washed with ice-cold saline and blotted dry. An approximately 5-g portion was weighed, cut into small pieces with a scissors, and homogenized in an appropriate ice-cold solution (3 ml solution/g liver) with a Teflon glass homogenizer. For

measurements of covalent binding to microsomal protein, we homogenized the liver in a Tris–HCl–KCl solution (1·15 per cent KCl in 0·02 M Tris buffer, pH 7·4). For measurements of diene conjugation of microsomal lipids, each liver was homogenized in a sucrose–EDTA solution (50 g sucrose, 570 mg of the tetrasodium salt of ethylenediaminetetracetic acid, and water to make 500 ml). The homogenate was centrifuged at 9000 *g* for 20 min in a refrigerated Servall centrifuge. The supernatant fraction was then centrifuged for 1 hr at 100,000 *g* in a Spinco centrifuge. The pellet of microsomes was suspended in 2·00 ml solution of the same type in which the liver had been homogenized. The total volume of the suspended microsomes was about 3·4 ml.

Preparation of a lipid extract. An aliquot (usually 2 ml) of liver homogenate (Tris–HCl–KCl) or microsomal suspension (Tris–HCl–KCl for measurements of covalent binding) was extracted with 20 vol. of chloroform–methanol (2 : 1) at room temperature for 10 min, with occasional shaking. The extract was then centrifuged at 650 *g* for 5 min. The supernatant was poured through a funnel containing a small amount of glass wool to remove any remaining suspended material. The filtrate was separated into chloroform and aqueous phases by the addition of 0·2 vol. of water, as recommended by Folch *et al.*[11] The chloroform phase or extract contained all the lipids from the liver homogenate or microsomal suspension.

Sources of radioactive compounds. $^{14}CCl_4$ (5 mCi/m-mole) was purchased from New England Nuclear (Boston, Mass.). ^{14}C-labeled Dibenamine [*N*-2-chloroethyl-1,2-^{14}C) dibenzylamine] hydrochloride (2·26 mCi/m-mole, > 98 per cent purity) was purchased from Mallinckrodt (St. Louis, Mo.). The purity of the labeled Dibenamine was confirmed by thin-layer chromatography on Silica gel "GF" plates, using the following solvent systems: (1) butanol–acetic acid–water (4 : 1 : 5), and (2) ethanol–12 M hydrochloric acid (50 ml : 4 drops).

$^{14}CCl_4$-derived radioactivity bound to lipids. Total radioactivity was determined on an aliquot (1 ml) of the chloroform extract of a homogenate or microsomal suspension from the liver of a rat which had been injected with $^{14}CCl_4$. The nonvolatile lipid radioactivity was determined on another aliquot of the chloroform phase after heating in an oven at 80° for at least 1 hr to remove all volatile radioactivity. We have confirmed the report by Reynolds[12] that heating for 1 hr at 80° is adequate to remove all volatile radioactivity. Washing a dried sample with unlabeled CCl_4 and reheating at 80° for an additional hour did not alter the radioactivity.

An aliquot (usually 5·0 ml) of the chloroform extract, containing less than 30 mg phospholipid, was added to a counting vial containing 2·0 g of reactivated "Unisil."* Fifteen ml $CHCl_3$ was added, the vial was shaken by hand several times over a 10-min period, and then the contents were allowed to settle for an additional 5 min. Fifteen ml of the $CHCl_3$ extract was then transferred to a 50-ml glass-stoppered bottle and replaced by 15 ml $CHCl_3$. The vial was shaken as before for 10 min, and the contents were again allowed to settle for 5 min before transfer of 15 ml $CHCl_3$ extract to the 50-ml bottle and replacement with 15 ml $CHCl_3$. The pooled 45 ml of $CHCl_3$ extract in the 50-ml bottle contains about 98 per cent of the neutral lipids. An aliquot of the pooled $CHCl_3$ was transferred to a counting vial for determination of the nonvolatile radioactivity of the neutral lipids.

* "Unisil" is activated silicic acid, 100–200 mesh, acid washed and controlled particle size, sold by Clarkson Chemical Co., Williamsport, Pa. Unisil was reactivated by heating overnight in an oven at 105–110°.

The vial containing the 2·0 g "Unisil," with the adsorbed phospholipid, was dried first at room temperature in the hood and then at 80° in an oven for 1 hr. We then added to the vial 0·5 ml methanol, 0·1 ml NCS solubilizer,* and 15 ml BBOT solution,† and the radioactivity was determined in a liquid scintillation counter.

Covalent binding in vivo *of* ^{14}C*-labeled* CCl_4 *or Dibenamine to liver protein.* Covalent binding *in vivo* was measured by a procedure developed in our laboratory[13] on homogenates or suspensions of microsomes isolated from livers of rats killed at various times after the i.p. injection of ^{14}C-labeled CCl_4 or Dibenamine. An aliquot (0·5 ml) of liver homogenate or microsomal suspension was transferred to a glass-stoppered centrifuge tube and the protein was precipitated by the addition of 5 ml ice-cold 10 per cent TCA. The mixture was centrifuged at 650 *g* for 5 min, the supernatant aspirated, and the precipitate was resuspended in 5 ml of 10 per cent TCA. The sample was centrifuged again, and the washing with 10 per cent TCA was repeated two more times. The pellet was then suspended in 5 ml of methanol–ether (3 : 1), heated at 60° for 5 min, and centrifuged. Washing with other 5-ml aliquots of the hot methanol–ether mixture was repeated ten times to remove radioactivity that was not covalently bound to protein. After aspiration of the final supernatant, which contained no radioactivity, the protein pellet was dissolved in 2·0 ml of 1·0 N NaOH. The protein content was determined by the method of Lowry *et al.*[14] The radioactivity of a suitable aliquot of the NaOH solution containing the protein was determined in a Packard scintillation counter. Binding was calculated from these measurements either as nmoles of CCl_4/mg protein or as nmoles of CCl_4/g liver.

Measurement of diene conjugation of microsomal lipids. Lipids were extracted with chloroform–methanol (2 : 1) from microsomes isolated from liver which had been homogenized in a sucrose–EDTA solution, as described in a preceding section. The chloroform phase was separated from the aqueous phase by the addition of 0·2 vol. of water. An aliquot (10 ml) of the chloroform extract was transferred to a weighed 13 ml glass-stoppered centrifuge tube. The chloroform was evaporated under nitrogen at 50° in a water bath. The lipid was weighed and dissolved in cyclohexane at an approximate concentration of 2 mg lipid/ml. The optical densities of the cyclohexane solution were measured against a cyclohexane blank at wavelengths from 220 to 300 nm in cells with a 1-cm path length.[7] Diene conjugation is expressed as E_{240}/m-mole phospholipid.[6] The phospholipid content of a lipid extract was determined by measurement of the phosphorus content by the procedure of Bartlett.[15]

Measurement of Dibenamine-derived radioactivity. An approximately 0·2-g portion of each tissue was placed in a pre-weighed counting vial and reweighed. To each vial was added 0·10 ml water and 2·00 ml NCS solubilizer. The vials were capped and kept at 50° in an oven overnight. The next day to each vial was added 50 μl glacial acetic acid and 15 ml BBOT solution and the total radioactivity was determined (see Table 4).

In the experiment (see Fig. 4) in which we measured the change with time of total Dibenamine-derived radioactivity in liver and radioactivity in liver present as Dibenamine, an aliquot of liver (3·5 g) was homogenized in pH 3·0 citrate buffer. The

* NCS solubilizer, a quaternary ammonium base in toluene solution, sold by Amersham/Searle.

† Composition (% by weight): 52·15 toluene, 39·35 methycellosolve, 8·10 napthalene and 0·40 BBOT [2·5-bis-2-(5-tert-butylbenzoxazolyl)-thiophene].

radioactivity of an aliquot of this homogenate gave total radioactivity. The homogenate was then extracted with heptane. The radioactivity extracted into the heptane represented radioactivity present as Dibenamine.[16]

To determine the covalent binding of Dibenamine derivatives to lipids, an aliquot of liver was extracted with chloroform–methanol (2 : 1) and the phases were separated by the addition of water, as previously described. The chloroform layer, which contained the lipids, was evaporated under nitrogen to a volume of 4 ml and then shaken with 8 ml of 2·5 N HCl, to remove any noncovalently bound Dibenamine. The washing of the chloroform phase with 2·5 N HCl was repeated. Any remaining radioactivity in the chloroform extract represented radioactivity covalently bound to lipids.

RESULTS

CCl_4 and $CHCl_3$ levels and the effects of Dibenamine pretreatment. During the first 6 hr after the i.p. administration of CCl_4, the levels of CCl_4 in fat, liver, kidney, muscle and blood were approximately the same in rats pretreated with saline or Dibenamine (Figs. 1 and 2). Concentrations in fat were approximately 100 times those in blood. At 2 hr after the administration of CCl_4, the concentrations in liver were about 10 times the concentrations in blood and the concentrations in kidney and muscle were two to three times blood levels. From 12 to 24 hr, the concentrations in liver, kidney and muscle were approximately the same as that in blood, but the concentration in fat was still about 100 times the level in blood.

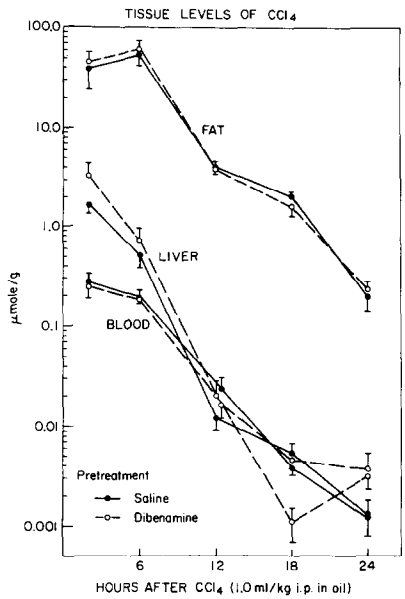

FIG. 1. CCl_4 concentrations in fat, liver and blood during the first 24 hr after i.p. administration of CCl_4 (1·0 ml/kg in an equal volume of sesame oil). Solid circles, solid lines represent levels in rats pretreated with saline. Open circles, dashed lines represent levels in rats pretreated with Dibenamine hydrochloride (25 mg/kg, s.c., 48 and 24 hr before the administration of CCl_4). Each point is the mean of determinations on six to eight rats ± S. E. Dibenamine pretreatment did not affect the tissue levels of CCl_4.

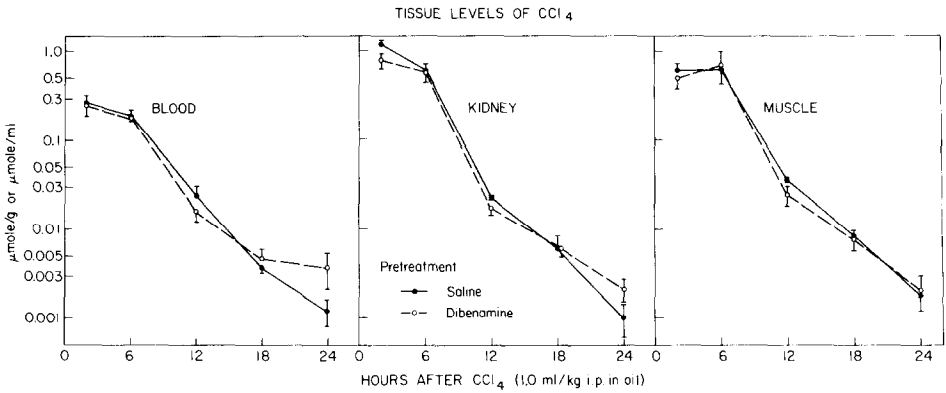

FIG. 2. CCl_4 concentrations in blood, kidney and muscle during the first 24 hr after i.p. administration of CCl_4 (1·0 ml/kg in an equal volume of sesame oil). Solid circles, solid lines represent levels in rats pretreated with saline. Open circles, dashed lines represent levels in rats pretreated with Dibenamine hydrochloride (25 mg/kg, s.c., 48 and 24 hr before the administration of CCl_4). Compare with Fig. 1. Although levels in liver, kidney and muscle tended to be higher at 2 and 6 hr than levels in blood, the levels in these tissues were approximately the same as in blood at 12–24 hr. Each point is the mean of determinations on six rats ± S. E.

Only the liver contained measurable chloroform levels (Fig. 3). At 2 hr, in rats pretreated with saline, concentrations of CCl_4 in liver were about 22 times the concentration of its metabolite, $CHCl_3$ (cf. Figs. 1 and 3). Concentrations of CCl_4 in liver decreased more rapidly than did the $CHCl_3$ concentrations. Thus, the half-life for CCl_4 in liver was less than 2 hr, in contrast to an apparent half-life of 3–4 hr for $CHCl_3$. At 12 hr, $CHCl_3$ concentrations in liver were approximately equal to the CCl_4 levels.

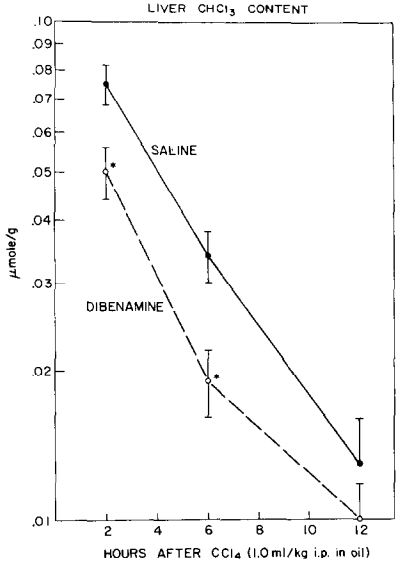

FIG. 3. Chloroform concentrations in liver during the first 12 hr after i.p. administration of CCl_4 (1.0 ml/kg in an equal volume of sesame oil). Symbols as in Fig. 1. Note that chloroform levels at 2 and 6 hr were significantly lower ($P < 0.05$) in Dibenamine-pretreated rats than in saline-pretreated rats.

Dibenamine pretreatment decreased the $CHCl_3$ levels at 2 and 6 hr by 30–50 per cent, but did not appreciably affect the half-life of $CHCl_3$ in the liver (Fig. 3). Thus, pretreatment with Dibenamine apparently slows the conversion of CCl_4 to $CHCl_3$.

Isopropanol potentiation of covalent binding of $^{14}CCl_4$ to liver lipids and protein and the effect of Dibenamine pretreatment. A dose of CCl_4 too small to produce appreciable necrosis (0·1 ml = 1·03 m-mole/kg) produced relatively slight covalent binding to neutral lipids, phospholipids and protein (Table 1), as measured 2 hr after the

TABLE 1. EFFECT OF DIBENAMINE PRETREATMENT ON ISOPROPANOL-POTENTIATED COVALENT BINDING OF $^{14}CCl_4$ TO LIVER LIPIDS AND PROTEIN*

	Water orally		Isopropanol orally	
Liver component	Saline pretreatment	Dibenamine pretreatment	Saline pretreatment	Dibenamine pretreatment
Neutral lipids	1·32 ± 0·11	1·37 ± 0·11	4·90 ± 0·79†	2·22 ± 0·27‡
Phospholipids	27·1 ± 3·20	24·3 ± 5·98	84·8 ± 10·50†	47·3 ± 3·18‡
Protein	14·0 ± 0·90	10·1 ± 2·83	65·3 ± 13·29†	28·3 ± 3·64

* Effect of pretreatment with Dibenamine hydrochloride (25 mg/kg, s.c., 48 and 24 hr before CCl_4) on isopropanol-potentiated covalent binding of a small dose of $^{14}CCl_4$ (0·1 ml/kg = 1·03 m-moles/kg, i.p., in sesame oil) to liver lipids and proteins. Isopropanol (2·5 ml/kg orally as a 25 per cent solution in water) or water was administered 18 hr before the labeled CCl_4. Each value is the mean of determinations on three rats ± S. E. Values are expressed as nmoles $^{14}CCl_4$/g liver. Binding was measured 2 hr after the administration of CCl_4.

† Isopropanol significantly potentiated the binding of $^{14}CCl_4$ in saline-pretreated rats, $P < 0.025$.

‡ Dibenamine pretreatment significantly reduced the isopropanol-potentiated binding, $P < 0.05$.

administration of CCl_4. This binding was not affected by Dibenamine pretreatment.

Traiger and Plaa[17] have reported that isopropanol is the most potent among a series of aliphatic alcohols in potentiating the hepatotoxicity of a small dose of CCl_4, as evaluated by serum glutamic-pyruvic transaminase levels. Table 1 shows that isopropanol administration increased 3- to 4-fold the covalent binding *in vivo* of $^{14}CCl_4$ to liver neutral lipids, phospholipids and protein. Dibenamine pretreatment reduced this isopropanol-potentiated binding by approximately 50 per cent.

Effect of Dibenamine pretreatment on covalent binding to liver lipids and protein of a necrosis-producing dose of CCl_4. A larger dose of CCl_4 (1·0 ml/kg = 10·3 m-moles/kg, i.p.) produced much greater covalent binding to liver lipids and protein (Table 2), as measured 2 hr after the administration of CCl_4. This dose of CCl_4 consistently produces marked necrosis. The binding was slightly greater than the isopropanol-potentiated binding of the small dose of CCl_4 (Table 1).

Dibenamine pretreatment reduced significantly the binding of the necrosis-producing dose of CCl_4 to liver neutral lipids, phospholipids and protein, whether the measurements were made on total liver or on liver microsomes (Table 2). However, the covalent binding to protein/mg protein was greater in microsomes than in total liver. This is consistent with the hypothesis that CCl_4 is metabolized by microsomal enzymes and that the covalent binding represents binding of the free radical derivatives.

TABLE 2. EFFECT OF DIBENAMINE PRETREATMENT ON COVALENT BINDING TO LIVER LIPIDS AND PROTEIN OF A NECROSIS-PRODUCING DOSE OF $^{14}CCl_4$*

Liver component	Saline pretreatment	Dibenamine Pretreatment	% Decrease	P†
Total liver				
Neutral lipids				
nmoles/g liver	9·18 ± 0·48	7·53 ± 0·65	18	NS
Phospholipids				
nmoles/g liver	119·5 ± 5·09	92·38 ± 6·71	23	< 0·02
nmoles/μeq PL	2·59 ± 0·14	2·03 ± 0·13	22	< 0·02
Protein				
nmoles/g liver	70·5 ± 5·33	45·34 ± 6·46	36	< 0·02
pmoles/mg protein	306 ± 25	210 ± 22	31	< 0·05
Liver microsomes				
Neutral lipids				
nmoles/g liver	8·58 ± 0·38	5·79 ± 0·20	33	< 0·001
Phospholipids				
nmoles/g liver	52·1 ± 2·43	36·8 ± 2·51	29	< 0·01
nmoles/μeq PL	2·34 ± 0·06	1·76 ± 0·09	25	< 0·001
Protein				
nmoles/g liver	18·31 ± 1·89	12·19 ± 0·59	33	<0·001
pmoles/mg protein	593 ± 24	411 ± 25	31	< 0·001

* Effect of pretreatment with Dibenamine hydrochloride (25 mg/kg, s.c., 48 and 24 hr before CCl_4) on the covalent binding of a necrosis-producing dose of $^{14}CCl_4$ (1·0 ml/kg = 10·3 m-moles/kg, i.p., in sesame oil) to liver lipids and proteins. Each value is the mean of determinations on five rats ± S. E. Values are expressed as nmoles $^{14}CCl_4$/g liver, nmoles $^{14}CCl_4$/μeq PL or pmoles $^{14}CCl_4$/mg protein, 2 hr after the administration of $^{14}CCl_4$.

† Probability level indicating that covalent binding in Dibenamine-pretreated rats is significantly less than in saline-pretreated rats. NS = not significant.

Effect of Dibenamine pretreatment on CCl_4-induced diene conjugation. Table 3 shows the effects of Dibenamine pretreatment on the diene conjugation of liver microsomal lipids from rats killed 30 min after i.p. administration of CCl_4 (2.0 ml/kg in an equal volume of sesame oil). Administration of CCl_4 increased the diene conjugation both in saline-pretreated rats and in Dibenamine-pretreated rats. However, the value of E_{240}/m-mole phospholipid in CCl_4-treated rats was significantly less after Dibenamine pretreatment than after saline pretreatment.

Distribution of radioactivity after administration of ^{14}C-Dibenamine. Table 4 gives the distribution of radioactivity in tissues of rats killed 24 hr after the second of two doses of ^{14}C-labeled Dibenamine HCl (each dose 25 mg/kg, s.c.). This time of sacrifice corresponds to the time of administration of CCl_4 in our protection experiments.[9] The highest concentration of Dibenamine-derived radioactivity was found at the site of injection. The concentration of radioactivity in the liver was almost six to eight times that in muscle (Tables 4 and 5). The concentration of radioactivity in fat was almost as great as that in liver. With the aid of a Table of Vital Statistics from the *Biochemists' Handbook*,[18] the approximate radioactivity in each tissue per rat has been calculated. The data in Table 4 suggest that the total Dibenamine-derived radioactivity in the rat at the time of optimal protection in our experiments was

TABLE 3. EFFECT OF DIBENAMINE PRETREATMENT ON CCl_4-INDUCED DIENE CONJUGATION IN LIVER MICROSOMAL LIPIDS*

Pretreatment	Treatment	E_{240}/m-mole Phospholipid
Saline	Oil	196 ± 12 (9)
Saline	CCl_4	342 ± 26 (9)†
Dibenamine	Oil	191 ± 17 (9)
Dibenamine	CCl_4	272 ± 18 (9)‡

* Effect of pretreatment with Dibenamine hydrochloride (25 mg/kg, s.c., 48 and 24 hr before CCl_4) on CCl_4-induced diene conjugation in liver microsmal lipids isolated from rats killed 30 min after CCl_4 (2·0 ml/kg, i.p., in an equal volume of sesame oil). Values are the means of determinations on the number of rats indicated in the parentheses ± S.E.

† Significantly greater than saline or Dibenamine control, $P < 0.01$.

‡ Significantly less than in rats given CCl_4 after saline pretreatment, $P < 0.05$.

less than 5 μmoles, or about 15 per cent of the administered Dibenamine. With the exception of fat, very little of the radioactivity measured in a tissue at this time of optimal protection was present as Dibenamine (Table 5).

Figure 4 shows the changes in the liver with time in total radioactivity, radioactivity covalently bound to protein, and radioactivity extracted by heptane, during the first 72 hr after the administration of a single s.c. dose of ^{14}C-Dibenamine HCl (25 mg/kg = 84·4 μmoles/kg). Total Dibenamine-derived radioactivity declined slowly, from 64 nmoles/g liver at 6 hr to 17 nmoles/g at 72 hr; the half-life of total Dibenamine-derived radioactivity was therefore about 45 hr. This radioactivity may be associated with Dibenamine itself, a Dibenamine metabolite, or a methyl or ethyl group from the labeled chloroethyl group of the injected Dibenamine. The Dibenamine-derived radioactivity which was covalently bound to liver protein was greater 18–48

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN TISSUES OF RATS KILLED 24 hr AFTER THE SECOND OF TWO DOSES OF ^{14}C-LABELED DIBENAMINE HYDROCHLORIDE (25 mg/kg, s.c., 24 hr APART)*

Tissue	Dibenamine-derived radioactivity (nmoles/g tissue ± S. E.)	Dibenamine-derived radioactivity (μmoles/rat)
Site of injection	478 ± 184	1·0
Plasma	45·5 ± 3·7	0·39
Liver	53·5 ± 4·3	0·36
Kidney	44·8 ± 4·2	0·05
Fat	38·3 ± 4·0	1·44
Lung	28·7 ± 2·2	0·04
Heart	17·5 ± 1·0	0·02
Brain	8·2 ± 0·7	0·02
Muscle	7·0 ± 0·5	0·60

* Values are expressed as Dibenamine equivalents in nmoles/g or μmoles/rat. The total tissue content/rat (μmoles/rat) was calculated with the aid of a Table of Vital Statistics from the *Biochemists' Handbook*.[18] The radioactivity may be associated with Dibenamine itself, Dibenamine metabolites, or a methyl or ethyl group from the labeled chloroethyl group of the injected compound. Each value is the mean of determinations on eight rats ± S. E. A total of 169 μmoles/kg (50 mg/kg = *ca.* 34 μmoles/rat) was injected.

TABLE 5. RADIOACTIVITY PRESENT AS DIBENAMINE 24 hr AFTER THE SECOND OF TWO DOSES OF ^{14}C-LABELED DIBENAMINE HYDROCHLORIDE (25 mg/kg, s.c., 24 hr APART).*

Tissue	Radioactivity	
	Total (nmoles/g tissue)	Dibenamine (nmoles/g tissue)
Liver	49·9 ± 6·20	0·15 ± 0·02
Muscle	8·4 ± 1·55	0·66 ± 0·18
Fat	37·2 ± 8·03	26·0 ± 5·9

* Each value is the mean of determinations on four rats ± S. E. Total dose of ^{14}C-Dibenamine HCl was 50 mg/kg (169 μmoles/kg).

hr after the administration of Dibenamine than at 6 or 72 hr. The radioactivity extracted by heptane largely represents the radioactivity associated with Dibenamine itself. This radioactivity present as Dibenamine declined from 0·36 nmole/g liver at 6 hr to 0·11 mole/g liver at 24 hr; this corresponds to an approximate half-life of 13 hr. At 18–48 hr, when the radioactivity covalently bound to liver protein was greatest, the radioactivity present as Dibenamine was significantly less than at 6 hr.

At a time corresponding to the time of administration of CCl_4 in our protection experiments (i.e. 24 hr after the second of two doses of Dibenamine 24 hr apart), about 33 per cent of the Dibenamine-derived radioactivity was covalently bound to

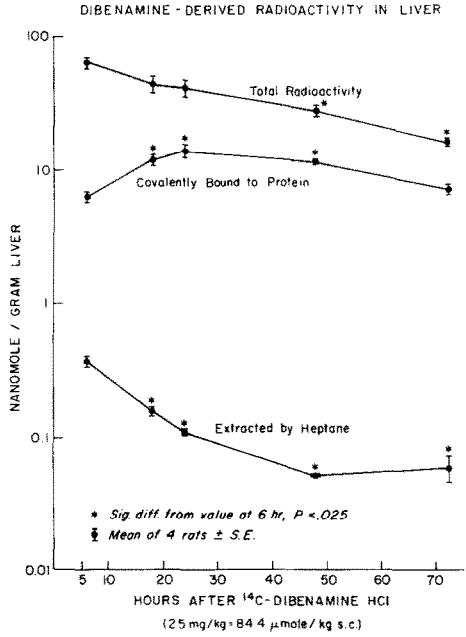

FIG. 4. Dibenamine-derived radioactivity in liver during the first 72 hr after the s.c. administration of a single dose of ^{14}C-Dibenamine [*N*-(2-chlorethyl-1,2-^{14}C) dibenzylamine] hydrochloride (25 mg/kg = 84·4 μmoles/kg). Each point is the mean of determination on four rats ± S.E. The graphs, from top to bottom, represent total Dibenamine-derived radioactivity, Dibenamine-derived radioactivity which is covalently bound to liver protein, and radioactivity which is extracted by heptane and presumably is present as Dibenamine itself.

TABLE 6. COVALENTLY BOUND RADIOACTIVITY IN LIVER 24 hr AFTER THE SECOND OF TWO DOSES OF ^{14}C-LABELED DIBENAMINE HYDROCHLORIDE (EACH DOSE 25 mg/kg, s.c., 24 hr APART)*

Liver component	Covalent binding (nmoles/g liver)	% Total radioactivity
Protein	15·7 ± 1·3	32·9
Lipids	1·5 ± 0·2	3·1
Phospholipids	1·1 ± 0·1	2·3
Neutral lipids	0·4 ± 0·1	0·8

* Total radioactivity in liver was 47·8 ± 8·0 nmoles/g. Each value is the mean of determinations on four rats ± S. E.

liver protein (Table 6). Only one-tenth as much of the total radioactivity was covalently bound to liver lipids. Moreover, even the covalent binding of Dibenamine derivatives to liver protein was low, when expressed per mg of protein (Table 7). The covalent binding to microsomal protein was only 116 pmoles/mg protein. Since the covalent binding to protein was approximately equal regardless of the liver fraction, as shown in Table 7, there was no evidence of preferential binding of Dibenamine derivatives to microsomal protein.

DISCUSSION

The toxicity of CCl_4 in animals can be decreased in a number of ways. For example, SKF 525-A (β-diethylaminoethyl diphenylpropyl acetate) prevents the toxicity[19] and decreases the diene conjugation [20] induced by CCl_4, perhaps by slowing its absorption from the gastrointestinal tract. [21] But unlike SKF 525-A, Dibenamine did not alter the absorption of CCl_4, nor did it affect the elimination of CCl_4 from various tissues (Figs. 1 and 2). Instead, Dibenamine pretreatment decreased the accumulation of $CHCl_3$ in liver (Fig. 3) and the covalent binding of $^{14}CCl_4$ to proteins and lipid of liver (Tables 1 and 2). Moreover Dibenamine pretreatment decreased the diene conjugation induced by CCl_4 (Table 3). Our findings thus suggest that

TABLE 7. COMPARISON OF TOTAL AND COVALENTLY BOUND RADIOACTIVITY IN LIVER PROTEIN 24 hr AFTER THE SECOND OF TWO DOSES OF ^{14}C-LABELED DIBENAMINE HYDROCHLORIDE (EACH DOSE 25 mg/kg, s.c., 24 hr APART)*

Liver fraction	Radioactivity	
	Total (pmoles/mg protein)	Covalently bound (pmoles/mg protein)
Nuclear and mitochondrial	173 ± 8·7	107 ± 3·2
Soluble	408 ± 45	114 ± 1·9
Microsomal	254 ± 29	116 ± 4·8

* Total liver radioactivity was 43·2 ± 2·3 nmoles/g liver. Each value is the mean of determinations on four rats ± S. E.

Dibenamine pretreatment impairs the microsomal enzymes which catalyze the formation of free radical derivatives of CCl_4. This could explain the reduction in covalent binding to liver lipids and proteins and the reduction in diene conjugation. Thus, the hepatotoxicity is less as the result of the reduced binding of free radical derivatives to liver phospholipids and protein. In accord with this view, we found [22] that the binding of $^{14}CCl_4$ to liver microsomal protein *in vitro* was 44 per cent less in microsomes from Dibenamine-pretreated rats than from control rats and that the *N*-demethylation of ethylmorphine and dimethylnitrosamine was inhibited by 40–50 per cent. Moreover, Dibenamine pretreatment caused a 23 per cent decrease in cytochrome P-450 in the liver microsomes (B. Stripps, I. G. Sipes, H. M. Maling and J. R. Gillette, manuscript submitted for publication).

The mechanism by which Dibenamine impairs the conversion of CCl_4 to its active metabolites, however, is still obscure. We have previously reported that Dibenamine protection against hepatotoxicity is unlikely to be associated with alpha adrenergic receptor blockade since protection is greater 24 hr after a single dose of Dibenamine than at 6 hr, when adrenergic blockade should be marked. Moreover, Dibenamine protection is definitely not correlated with the concentration of Dibenamine in liver itself, since even at 6 hr, only 0·36 nmole/g liver, less than 1 per cent of the total Dibenamine-derived radioactivity, was present as Dibenamine itself. This small amount of Dibenamine present at 6 hr in the rat is not surprising since Axelrod *et al.*[16] reported that 4 hr after the i.v. injection of a large dose of Dibenamine in the dog, the concentrations of Dibenamine in plasma were too low to be measured by their colorimetric method (less than 0·2 μg/ml = 0·8 nmole/ml). In our experiment, after 6 hr the Dibenamine content of liver continued to fall until it was less than 0·06 nmole/g from 48 to 72 hr after administration. Thus, it is evident that the protective action of Dibenamine appears after Dibenamine itself has almost completely disappeared from the body, except in fat[10] (Table 5).

The delay in the protective action of Dibenamine suggests the possibility that Dibenamine might act through an active metabolite. Indeed, at the time of optimal protection against CCl_4-induced hepatotoxicity, the liver contained as much as 40–50 nmoles/g of Dibenamine derivatives (Table 5) and about 40 per cent of these radiolabeled metabolites were covalently bound to liver proteins and lipids (Fig. 4, Tables 6 and 7). Since the covalent binding of Dibenamine derivatives to lipids was much less than it was to protein (Table 6), it might seem reasonable that the protective action of Dibenamine pretreatment was primarily associated with the covalent binding of its derivatives to microsomal protein. However, the covalent binding of Dibenamine to protein was approximately the same in all three fractions, nuclear and mitrochondrial, soluble and microsomal (Table 7) and was only slightly greater than 100 pmoles/mg of liver protein. It is therefore, unlikely that the protection against hepatotoxicity is mediated solely by the covalent binding of Dibenamine derivatives to cytochrome P-450 in liver microsomes. Whether the non-covalently bound metabolites of Dibenamine could inhibit the metabolism of CCl_4, however, remains to be determined.

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