Biochemical Pharmacology, Vol. 37, No. 2, pp. 333–338, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

INTERVENTION IN FREE RADICAL MEDIATED HEPATOTOXICITY AND LIPID PEROXIDATION BY INDOLE-3-CARBINOL

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(*Received* 29 *December* 1986; *accepted* 28 *May* 1987)

Abstract—The cytoprotective effect of the natural dietary constituent indole-3-carbinol (I-3-C) on carbon tetrachloride (CCl_4) mediated hepatotoxicity in mice was examined. I-3-C pretreatment by gavage 1 hr prior to intraperitoneal injection of CCl_4 produced a 63% decrease in CCl_4-mediated centrolobular necrosis and a related 60% decrease in plasma alanine aminotransferase activity (a marker of liver necrosis). Since the toxicological effects of CCl_4 are mediated by radical species generated during reductive metabolism by cytochrome P-450, we examined the potential ability of I-3-C to scavenge reactive radicals. Three systems were used to evaluate the ability of I-3-C to intervene in free radical mediated lipid peroxidation. These systems consisted of the following: (1) phospholipid dissolved in chlorobenzene, with peroxidation initiated by the thermal and photo decomposition of azo-bisisobutyronitrile (AIBN); (2) sonicated phospholipid vesicles in phosphate buffer (pH 7.4), with peroxidation initiated by ferrous/ascorbate; and (3) mouse liver microsomes containing an NADPH-regenerating system, with peroxidation initiated with CCl_4. Lipid peroxidation was measured in these three systems as thiobarbituric-reacting material. In the AIBN and ferrous/ascorbate systems, I-3-C inhibited lipid peroxidation, with greater inhibition under conditions of low rates of free radical generation. I-3-C was not as effective an antioxidant as butylated hydroxytoluene (BHT) or tocopherol, but it inhibited peroxidation in a dose–response manner. I-3-C was most effective as a radical scavenger in the microsomal CCl_4-initiated system by inhibiting lipid peroxidation in a dose-dependent fashion, with 50% inhibition at 35–40 μM I-3-C. This concentration is about one-third of the concentration of I-3-C achieved in liver after treatment of mice by gavage with 50 mg I-3-C/kg body weight. These data suggest that I-3-C may be a natural antioxidant in the human diet and, as such, may intervene in toxicological or carcinogenic processes that are mediated by radical mechanisms.

Free radicals mediate both toxicological [1] and carcinogenic [2, 3] responses to many chemicals, such as paraquat [4], acetaminophen [5], and carbon tetrachloride [6, 7]. Potential target molecules for such radicals include thiol groups, enzymes, amino acids, nucleotides, and unsaturated fatty acids. In the presence of oxygen, radical attack on unsaturated fatty acids results in lipid peroxidation, the extent of which is easily estimated. The toxicological consequences of lipid peroxidation *per se* are uncertain [1]. However, lipid peroxidation may provide a semi-quantitative indicator of the generation of highly reactive free radical species. Less reactive free radicals, such as tocopherol [8] and ascorbate [9], may act as antioxidants, although ascorbate also may be a pro-oxidant [10], depending on the conditions. Reduced glutathione may also be involved in scavenging free radicals in the cell perhaps, in part, via a GSH-dependent microsomal protein [11]. In a preliminary report, indole-3-carbinol (I-3-C‡) was shown to protect against hepatotoxicity mediated by carbon tetrachloride (CCl_4) [12]. Herein we confirm this finding and examine the possibility that such protection is mediated by the ability of I-3-C to scavenge reactive free radical metabolites that would otherwise lead to lipid peroxidation and cell necrosis. The chemoprotective properties of I-3-C are important, since this compound is present in the human diet as a conjugated precursor. I-3-C is generated by the hydrolysis of the precursor glucobrassicin, an indolylmethylglucosinolate present in cruciferous vegetables of the genus *Brassica* [13, 14].

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‡ Abbreviations: AIBN, 2,2′azobis(2-methyl propionitrile) or azobisisobutyronitrile; BHT, butylated hydroxytoluene; GSH, reduced glutathione; I-3-C, indole-3-carbinol; KP_i, potassium phosphate buffer; MDA, malonaldehyde bis-(dimethylacetal); CCl_4, carbon tetrachloride; and TBA, 2-thiobarbituric acid.

MATERIALS AND METHODS

Chemicals. Semi-purified asolectin (soybean phospholipids) was obtained from Associated Concentrates, Woodside, Long Island, NY, and was further purified by precipitation from a chloroform solution with acetone. This procedure was repeated three times to devoid the phospholipids of contaminating tocopherols. The purified phospholipid was dried in a desiccator; chloroform solutions of the phospholipid were stored at −20° under argon. I-3-C, CCl_4 and malonaldehyde bis-(dimethylacetal) (MDA) were obtained from the Aldrich Chemical Co., Milwaukee, WI, whereas TBA, BHT, indole, alpha-tocopherol and ascorbic acid were purchased from the Sigma Chemical Co., St. Louis, MO. Ascorbic acid was purified by repeated extractions with ethanol followed by an acetone extraction; it

was dried under vacuum. AIBN was from the Eastman Kodak Co., Rochester, NY. Aquasol-2 LSC and [carbinol-^{14}C]-I-3-C at 10.0 mCi/mmol (>99% radiochemical purity) were from New England Nuclear, Boston, MA. Bicinchoninic acid protein assay reagent was purchased from the Pierce Chemical Co., Rockford, IL. All other chemicals were of reagent grade or better and were used without further purification.

Animals. Male ICR Swiss mice, about 30 g, were allowed water and Teklad Standard Rodent Diet (Teklad Mills, Madison, WI) *ad lib.* until 12 hr before the start of the experiment. A light–dark photocycle of 12 hr was maintained, and the bedding consisted of hardwood chips. Animals were treated by gavage (feeding needle PS20; Popper & Sons, Inc., New Hyde Park, NY) with corn oil alone (about 1.5 μl/g body wt) or I-3-C in corn oil vehicle. Prior to use, the corn oil was extracted with methanol to remove tocopherols and then was rendered peroxide free by passage through a ferrous Dowex AG 1-X8 column [15]. One hour after gavage, mice were injected intraperitoneally with corn oil (2 μl/g body weight) or CCl_4 in corn oil vehicle (24 mg CCl_4/kg body weight). Twenty-four hours after receiving CCl_4, mice were anesthetized with 65 mg pentobarbital per kg body weight by i.p. injection. Blood samples were removed from the left ventricle and centrifuged to obtain serum for immediate evaluation of alanine transaminase activity [16]. Livers were perfused *in situ* via the hepatic vein with 0.9% (w/v) NaCl at room temperature and then were removed to ice-cold saline. Gall bladders were dissected away, and a section of the medial lobe was placed in 10% phosphate-buffered (pH 7.0) formalin. Paraffin sections (4 μm) were stained with hematoxylin and eosin for histological evaluation. Slides were coded so the pathologist could not distinguish the treatment groups on that basis.

Mouse liver microsomes were prepared from untreated animals that had been fasted overnight, as previously described [17].

Pharmacokinetics. For the pharmacokinetic analysis of I-3-C uptake and distribution, we utilized carbinol-radiolabeled [^{14}C]-I-3-C. Mice were treated by gavage with 50 mg I-3-C plus 100 μCi [^{14}C]-I-3-C/kg body weight, in methanol-extracted corn oil vehicle. At various times animals were killed and blood and liver samples were obtained. A 0.5-ml aliquot of whole blood or a 0.5-g sample of minced liver was added to 3 ml of 70% perchloric acid, covered, and incubated at 70° for 5 hr. The sample was cooled to 0°, and 3 ml of 30% hydrogen peroxide was added slowly with swirling. The sample was allowed to digest and bleach at 70° overnight, and then 1 ml was added to 15 ml Aquasol-2 scintillation fluid. Radioactivity was determined in a Packard Tri-Carb 460 CD Liquid Scintillation System, which was programmed to convert cpm to dpm on the basis of a set of known quenched standards.

Assays. For the cell-free lipid peroxidation assay, an aliquot of chloroform containing phospholipid was mixed with 40 vol. acetone. Following centrifugation, the supernatant solution was decanted, and the phospholipid was dried under vacuum. For the chlorobenzene lipid peroxidation system (Figs. 2–4), lipid was dissolved in chlorobenzene. To 1 ml of the stopped reaction mixture containing BHT, described in the legend to Fig. 2, was added 1 ml of 5% trichloroacetic acid (TCA) and 0.6 ml TBA reagent. The mixture was heated to 100° for 30 min. The upper aqueous phase (containing TBA-reactive material) was extracted with 4 vol. dichloromethane in order to remove non-specific chromophores that are produced by indole compound oxidation. The TBA reactive species are not extracted by this procedure. Xylene may be used in place of dichloromethane. Absorbances were measured at 532 nm (maximal absorbance) and 600 nm (turbidity correction); the resulting difference in absorbance at these two wavelengths was compared to that observed for MDA standards. Finally, values obtained for the lipid peroxidation assay performed in the presence of 10 μl of 0.5 M BHT were subtracted from values obtained for the assay performed in the absence of BHT, in order to take into account any non-specific effects of the putative antioxidants.

For the ferrous/ascorbate lipid peroxidation system, 6.25 ml of 0.1 M potassium phosphate buffer (KP_i), pH 7.4, was added to 12.5 mg dried phospholipids. After flushing with argon for 2 min, the suspension was sealed with five layers of Parafilm and sonicated in a bath sonicator (Laboratory Supplies Co., Hicksville, NY) at full power for about 60 sec or until the suspension was translucent. The resulting phospholipid vesicles were added to the reaction mixture described in the legend to Fig. 5. After stopping the reaction by the addition of 10 μl of 0.2 M BHT in dimethyl sulfoxide (DMSO), 0.1 vol. of 50% (w/v) TCA and 0.6 vol. of 1% (w/v) TBA in 0.28% (w/v) NaOH were added, and the mixture was incubated at 100° for 30 min. The mixture was cooled to room temperature and extracted with dichloromethane; reaction products were quantified by the same methods as described for the chlorobenzene assay system.

CCl_4-mediated lipid peroxidation. To correlate *in vivo* protection by I-3-C against CCl_4 hepatotoxicity and antioxidant potential of I-3-C, we tested the ability of I-3-C to inhibit CCl_4-initiated lipid peroxidation in mouse liver microsomes. The reaction was performed as described in the legend to Fig. 6.

RESULTS

Hepatotoxicity. Livers from mice treated with corn oil vehicle or I-3-C in vehicle showed no necrosis, whereas treatment with CCl_4 produced large areas of centrolobular necrosis, with loss of cell structure in these regions (Fig. 1). No toxicity was observed in the periportal region of the liver. In contrast, the extent of cell necrosis was reduced 63% when I-3-C was administered 1 hr prior to CCl_4. Furthermore, while hepatocytes stained lightly in necrotic regions after I-3-C plus CCl_4 treatment, general cell structure was preserved and amorphous regions were not observed. Parallel with alterations in liver necrosis were the levels of plasma alanine aminotransferase (ALT), an enzyme commonly used to assess liver damage. CCl_4-elevated levels of ALT were reduced 60% by prior treatment with I-3-C.

Inhibition of lipid peroxidation in the non-enzy-

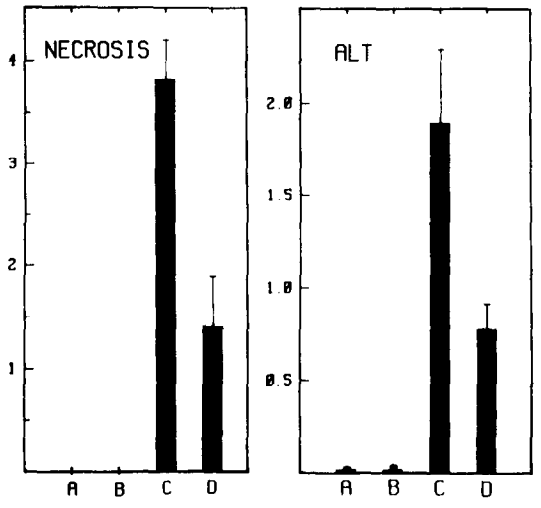

Fig. 1. Effects of CCl_4 and I-3-C on liver necrosis and serum enzyme levels. Animals were treated as described in Materials and Methods. The four treatment groups were: (A) corn oil gavage/corn oil i.p.; (B) I-3-C gavage/corn oil i.p.; (C) corn oil gavage/CCl_4 i.p.; and (D) I-3-C gavage/CCl_4 i.p. The scale for centrozonal liver necrosis was: (0) none; (3) moderate; and (5) severe. ALT (plasma alanine aminotransferase) was calculated as μmol/min · ml plasma. The results are expressed as the average of four animals in each group ± standard errors. For each animal, several sections were prepared, and about fifteen central vein regions were examined.

matic systems. To validate our test systems for the study of lipid peroxidation, we utilized the conventional radical chain-breaking antioxidants, alpha-tocopherol and BHT, as positive controls. In the chlorobenzene system, these compounds produced the same degree of inhibition of lipid peroxidation, measured as TBA reactive substances (Fig. 2). Since there are many products of lipid peroxidation and TBA reacts with many compounds other than MDA [18], we calculated our results as TBA reactive MDA equivalents. The percent inhibition at lower tocopherol and BHT concentrations was about 1.2% per μM inhibitor. I-3-C also inhibited the formation of TBA reactive substances at about 0.37% inhibition per μM I-3-C. Therefore, under these assay conditions, I-3-C was about 30% as effective an antioxidant as tocopherol or BHT. In contrast, indole (Fig. 2) and anthracene (data not shown) did not inhibit lipid peroxidation even at concentrations of 200 μM.

The efficiency of I-3-C in preventing free radical mediated lipid peroxidation was found to be inversely related to the rate of generation of radical species. In the AIBN-initiated system, the rate of free radical generation by homolytic cleavage (thermal decomposition) of AIBN is proportional to AIBN concentration at any given temperature [19]. By altering AIBN concentration, the efficiency of I-3-C as an antioxidant could be modified (Fig. 3), i.e. the lower the rate of chain initiation, the greater the protectivity by I-3-C. These data (Fig. 3) also suggest the possibility of two components to the I-3-C effect. At less than 10 μM I-3-C, peroxidation was exquisitely sensitive to the rate of radical generation. At 50 mM AIBN there was little (if any) I-3-C effect, while at 3 mM AIBN lipid peroxidation was inhibited 24% by 2 μM I-3-C. Above 10 μM I-3-C, the rate of radical generation did not appear to affect the antioxidant efficacy of I-3-C as reflected by the similar slopes of the curves in Fig. 3. We do not attach relevance to the slight inhibition by 2 μM I-3-C of

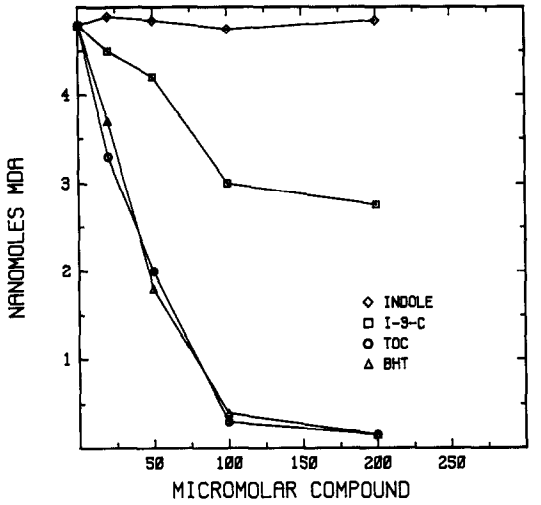

Fig. 2. Effects of various compounds on AIBN-initiated lipid peroxidation. The reaction mixture contained 200 μg/ml phospholipid and the various compounds as indicated in the figure (toc = alpha-tocopheral), plus or minus 10 μl of 0.5 M BHT in DMSO, all in 2.5 ml chlorobenzene. The reaction was initiated with 10 mM AIBN and performed at 37° under white fluorescent lighting. At 2 hr the reactions were terminated with 10 μl of 0.5 M BHT in DMSO. TBA reactive material was determined as described in Materials and Methods. The results shown are the average values for two experiments.

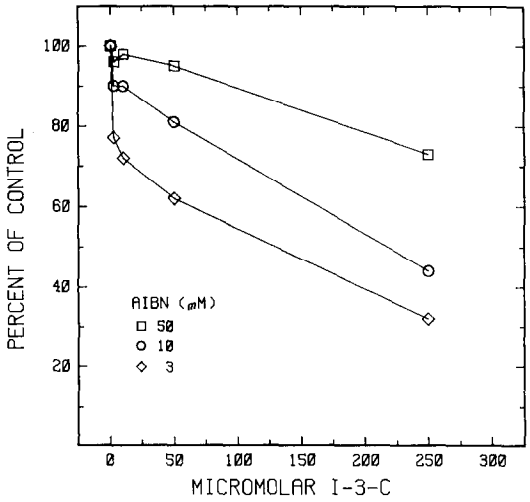

Fig. 3. Effect of AIBN concentration on I-3-C inhibition of lipid peroxidation. The reaction conditions are described in the legend to Fig. 2. Results shown are the average values for two experiments.

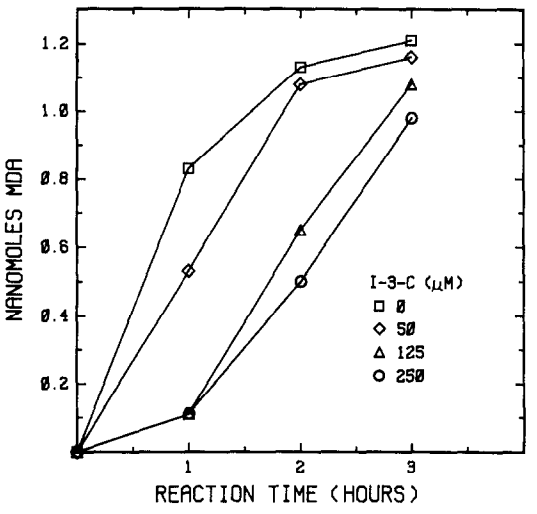

Fig. 4. Time course of AIBN-initiated lipid peroxidation at different I-3-C concentrations. The assay conditions are described in the legend to Fig. 2. The average values from two experiments are shown.

lipid peroxidation initiated by 50 mM AIBN in this system.

When AIBN-initiated lipid peroxidation was monitored over time, it was observed (Fig. 4) that I-3-C was inhibitory at early time points, but it lost efficiency as the peroxidation assay proceeded. These data would be consistent with the consumption of I-3-C in the course of its acting as an antioxidant, similar to the antioxidant action of tocopherol and ascorbate.

In order to examine the potential antioxidation by I-3-C in an aqueous system, ferrous ion was used in conjunction with micromolar concentrations of ascorbate (Fig. 5). At these concentrations, lipid peroxidation was directly related to ascorbate concentration. At higher (millimolar) ascorbate concentrations, ascorbate may inhibit peroxidation (data not shown, and [20]). Therefore, ascorbate concentrations were maintained below 400 μM. Within this ascorbate concentration range, an I-3-C dose-dependent decrease in ferrous/ascorbate mediated lipid peroxidation was observed (Fig. 5). This effect was especially evident at 10–100 μM ascorbate. At higher ascorbate concentrations, antioxidation was evident only at 250 μM I-3-C.

It should be noted that, in the two nonenzymatic assay systems, it was necessary to extract tocopherol from the phospholipids with acetone; without this process, peroxidation of lipids did not occur (data not shown).

Inhibition of CCl_4-initiated peroxidation. Since our original observations regarded the cytoprotection against CCl_4 hepatotoxicity by I-3-C, we examined lipid peroxidation in mouse liver microsomes, initiated by the free radical metabolites of CCl_4. Figure 6 shows the time course of *in vitro* CCl_4-initiated lipid peroxidation in the presence of various concentrations of I-3-C. The rate of lipid peroxidation (slopes of the lines of Fig. 6, calculated by least-squares linear regression) versus concentration of I-3-C (Fig. 7) shows a dose–effect relationship, with 50% inhibition of the rate of lipid peroxidation at

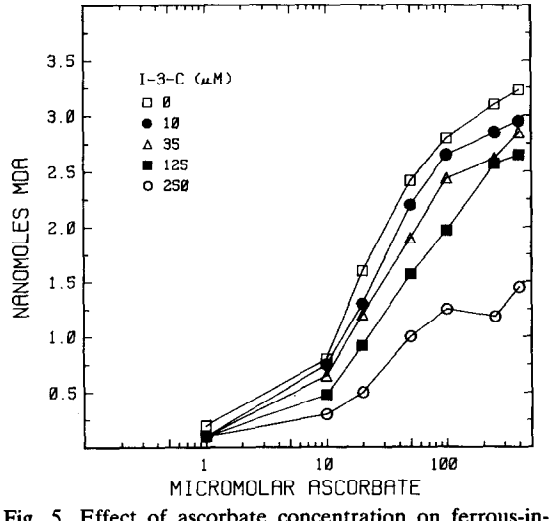

Fig. 5. Effect of ascorbate concentration on ferrous-initiated lipid peroxidation. The reaction mixture for the oxidation of phospholipid vesicles contained 200 μg/ml phospholipid in 0.1 M KP_i (pH 7.4). The reaction was initiated by the addition of ascorbic acid as shown, plus 10 μM $Fe(NH_4)_2(SO_4)_2$. The reaction continued at 37° in a shaking water bath for 30 min and was stopped by adding 10 μl of 0.5 M BHT in DMSO. TBA reactive material was determined as described in Materials and Methods. The average values for two experiments are shown.

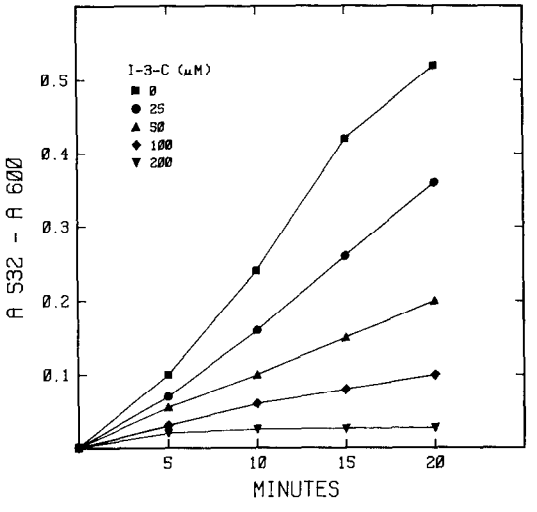

Fig. 6. Inhibition of CCl_4-mediated lipid peroxidation by I-3-C. The reaction mixture contained 1 mg/ml mouse liver microsomes, 1 mM $NADP^+$, 10 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, 10 mM $MgCl_2$, I-3-C at the indicated concentration in DMSO, and 50 mM KP_i (pH 7.4), in a final volume of 2.0 ml. The samples were preincubated for 5 min at room temperature. The reaction was initiated with 5 μl of 400 mM CCl_4 in DMSO (final CCl_4 concentration of 1 mM), and a zero time aliquot of 0.9 ml was placed into a stopping solution consisting of 0.1 ml of 50% TCA and 10 μl of 0.2 M BHT in DMSO. The lipid peroxidation reaction proceeded with shaking at 37°, and stopped at 20 min. The stopped reaction mixtures were developed with thiobarbituric acid reagent as described in Materials and Methods. Each point represents the average value from two experiments.

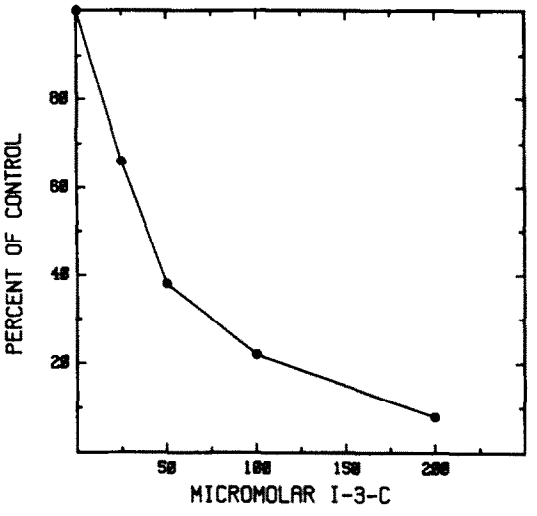

Fig. 7. Inhibition of lipid peroxidation by I-3-C. The slopes of the lines in Fig. 6 were calculated by a linear least squares regression analysis, and replotted as a percent of the 0 μM I-3-C control slope, against I-3-C concentration.

35–40 μM I-3-C. We speculate that the greater effectiveness of I-3-C in the microsomal assay system is due to enzymatic activation of I-3-C to metabolities having greater antioxidation efficacy than the parent compound. This speculation is supported by the observation that preincubation of I-3-C with NADPH-fortified liver microsomes prior to CCl_4 addition enhanced the inhibition of lipid peroxidation. This enhancement was complete within a 5-min preincubation period (Fig. 8).

To determine whether the inhibition of CCl_4-mediated lipid peroxidation could be toxicologically relevant, we performed pharmacokinetic studies. Mice treated by gavage with 50 mg [^{14}C]-I-3-C/kg body weight absorbed radiolabel rapidly, with peak blood concentrations occurring 30 min after I-3-C administration (Table 1). The I-3-C and its metabolites appeared to partition into the liver from the blood, with a partition ratio (liver/blood) of about 5. Liver concentrations of I-3-C and metabolites remained in the 100 μM range from 10 min to 2 hr after I-3-C administration.

Since the levels of hepatic GSH may be involved in the degree of the susceptibility of liver to lipid peroxidation [21, 22], we assayed for protein free sulfhydryls [23], and for glutathione *S*-transferase activity using 1,2-dichloro-4-nitrobenzene as the substrate [24]. Levels of hepatic ascorbate were determined also [25]. These parameters were found to be unaltered in mouse hepatic 14S fraction 1 hr following I-3-C treatment (data not shown).

Table 1. Concentrations of radiolabel in blood and liver after administration of [^{14}C]-I-3-C

Min after I-3-C	Radiolabel concentrations (μM)		
	Blood	Liver	Liver/Blood
10	13.5	63	4.7
20	18.8	101	5.4
30	28.3	121	4.3
60	23.5	118	5.0
120	15.0	82.5	5.5

Mice were treated by gavage with [^{14}C]-I-3-C in corn oil, as described in Materials and Methods. Mice were killed at the indicated times, dpm/g tissue were determined in whole blood and liver, and the specific radioactivity of [^{14}C]-I-3-C (345.6 dpm/nmol) was used to calculate μM tissue concentrations of radiolabel. The results shown are the average values from two experiments.

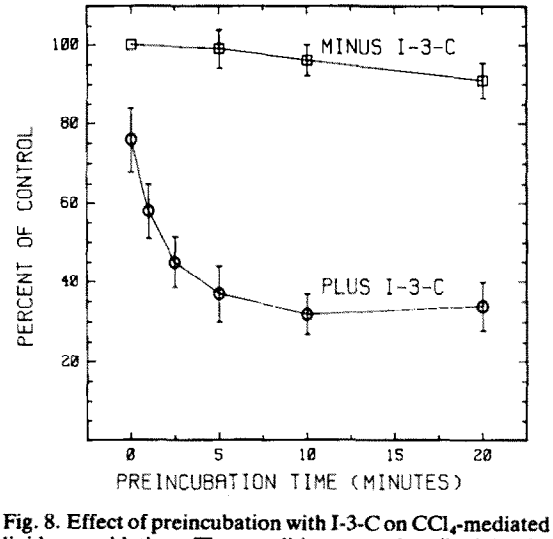

Fig. 8. Effect of preincubation with I-3-C on CCl_4-mediated lipid peroxidation. The conditions are described in the legend to Fig. 6 and in Materials and Methods. The time of preincubation with 50 μM I-3-C at room temperature prior to the addition of CCl_4 was varied. Each point represents the average value from three experiments ± standard deviation. The "minus I-3-C" value with no preincubation was taken as the 100% value.

DISCUSSION

Free radicals are involved in the mediation of various types of cellular and tissue injuries, such as cell necrosis, radiation damage, transition metal overload, inflammation and chemical carcinogenesis [1–3]. Various synthetic antioxidants have been shown to intervene in these toxicological processes and, thus, afford protection from a variety of insulting sources [26]. For CCl_4, it has been suggested that free radical metabolites generated in the course of one electron reduction [27] of the molecule initiate toxicological events which eventually lead to tissue necrosis and cell death. The CCl_4-generated radical species produce a myriad of specific cellular events, including alterations in protein turnover [28], triglyceride secretion [29], calcium homeostasis [30], lipid peroxidation [31] and covalent binding to cellular constituents [32]. The precise relationship between lipid peroxidation and cellular toxicity is not clear [1]. However, antioxidants have been shown to protect against CCl_4 toxicity *in vivo* and *in vitro* [12, 33, 34]. We have used lipid peroxidation as an indicator that free radicals had been generated and

had persisted long enough to react with other chemical or cellular components. The concentrations of I-3-C and its metabolites that are generated in liver tissue, 30 min after treatment with I-3-C, are about 3-fold higher than those concentrations of I-3-C that inhibit by 50% CCl_4-initiated lipid peroxidation *in vitro*. Thus, I-3-C and/or metabolites possess both the chemical and the pharmacokinetic properties necessary to provide a free radical trap for CCl_4 metabolites. *In vitro* (CCl_4-initiated lipid peroxidation with liver microsomes), and *in vivo* we propose that either I-3-C or an enzymatic/chemical reaction product of I-3-C is responsible for inhibiting lipid peroxidation. I-3-C has been shown previously to have no effect on hepatic cytochrome P-450 levels after 1 hr, and a slight inductive effect after 24 hr [35]. Furthermore, concentrations of 100 μM I-3-C do not inhibit cytochrome P-450-mediated mixed-function oxidations of benzo[*a*]pyrene and the low K_m *N*-nitrosodimethylamine [35, 36]. A common isozyme of cytochrome P-450 apparently catalyzes the low K_m *N*-nitrosodimethylamine demethylase and the metabolism of CCl_4 to phosgene [37]; if phosgene is a major toxic reaction product of CCl_4, then it appears that an inhibition of cytochrome P-450 by I-3-C is not the mechanism of protection against CCl_4 toxicity. Since the toxic reaction products of CCl_4 metabolism have not been identified clearly, it is difficult to rule out with certainty that an inhibition of CCl_4 activation by I-3-C is part of the mechanism of protectivity by this indole compound. However, we propose that at least part of the protection against CCl_4 hepatotoxicity afforded by I-3-C *in vivo* may be due to its ability to act as a radical scavenger, analogous to our demonstrated effects *in vitro*. The data suggest that the dietary indole, I-3-C, or metabolite of I-3-C, may act as a natural antioxidant and partially protect against free radical mediated chemical toxicity.

Acknowledgements—Supported by NIH Grants ES-03373 and CA-38277. The authors thank Nancy Knapp for typing this manuscript.

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