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PREVENTION BY CYSTAMINE OF LIVER NECROSIS AND EARLY BIOCHEMICAL ALTERATIONS INDUCED BY CARBON TETRACHLORIDE

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Abstract—The effect of the previous administration of cystamine on the hepatotoxicity of orally and intraperitoneally administered carbon tetrachloride (CCl_4) was investigated. Cystamine significantly prevented the necrosis and fatty infiltration, as well as the depression of glucose 6-phosphatase and ethylmorphine *N*-demethylase activities caused by oral CCl_4 administration. These results are due to a delay in the gastrointestinal absorption of CCl_4 caused by cystamine. However, cystamine also prevented necrosis and fatty infiltration when CCl_4 was given intraperitoneally. Moreover, it also significantly blocked the irreversible binding of ^{14}C from $^{14}CCl_4$ to liver microsomal lipids and the depression of glucose 6-phosphatase in spite of the fact that the microsomal lipid peroxidation process was not inhibited and that CCl_4 levels in the livers of cystamine-treated and untreated rats were similar. The destruction of ethylmorphine *N*-demethylase could not be prevented by cystamine when CCl_4 was given intraperitoneally. The results are discussed in relation to the postulated mechanistical similarity between CCl_4 liver injury and damage from ionizing radiation.

EXPERIMENTS from several laboratories as well as our own results suggest that carbon tetrachloride (CCl_4) hepatotoxicity is not simply due to a solvent action on the liver structural components but more likely caused by the free radicals presumably arising during a CCl_4 activation step mediated by the drug-metabolizing enzymes located in the endoplasmic reticulum.[1–6,‡]

According to several authors,[1,3,7] these free radicals would later initiate a process of lipid peroxidation. The $CCl_3\cdot$ and $CCl\cdot$ free radicals generated during the initial activation step, as well as the peroxyl free radicals produced later during lipid peroxidation, were considered to be responsible for the initial alterations finally leading to necrosis.[3]

As Recknagel pointed out,[3] this mechanism of hepatotoxicity is remarkably similar to that postulated to explain the deleterious action of ionizing radiation. This similarity would raise the possibility that agents which are effective against ionizing radiation damage, like cystamine,[8] could also be useful in preventing CCl_4 liver injury.

In this work we investigate this possibility.

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EXPERIMENTAL

Chemicals. Cystamine dihydrochloride was purchased from T. Schuchardt (Germany); $^{14}CCl_4$ (27·5 mc/m-mole) was obtained from the Radiochemical Center, Amersham (England). All the other chemicals employed were reagent grade.

Treatment of animals. Male albino rats (170–260 g) were used in these experiments. Food was withdrawn 12–14 hr before cystamine administration. Cystamine was given orally as a water solution (240 mg/ml) at a dose of 600 mg/kg 30 min or 3 hr before CCl_4 administration. Control animals received an equivalent amount of water.

Carbon tetrachloride was given either orally undiluted at a dose of 2·5 ml/kg or i.p. as a 20% (v/v) solution in corn oil at a dose of 5 ml of solution/kg.

In the later case control rats received the equivalent amount of corn oil i.p. The animals were sacrificed by cervical dislocation at different times after CCl_4 administration. Their livers were rapidly removed, weighed and processed.

Whenever blood samples were taken, the animals were kept under light ether anaesthesia and blood was collected from the inferior vena cava with a heparinized syringe.

Enzymatic and chemical determinations. The microsomal fraction for determination of ethylmorphine metabolism was isolated by the procedure described by Castro and Gillette.[9] The isolation of microsomes for studies on glucose 6-phosphatase, lipid peroxidation *in vivo* and irreversible binding of ^{14}C was carried out as follows: 8 g of liver was homogenized in 16 ml of ice-cold 0·3 M sucrose–3 mM EDTA with a Teflon-glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min. The supernatant fraction was then centrifuged for 1 hr at 105,000 *g* in a Spinco model L preparative ultracentrifuge. The microsomal pellet was suspended in 2 ml of the sucrose–EDTA solution.

Ethylmorphine *N*-demethylase activity (EM-ase) in 9000 *g* supernatant or in microsomal suspensions was measured according to the procedure described by Castro and Gillette[9] except that ethylmorphine concentration in the incubation mixture was 10 mM. Activity is expressed in millimicromoles of formaldehyde formed in 15 min at 37° per milligram of protein.

Glucose 6-phosphatase activity (G6P-ase) in 1200 *g* supernatant or in microsomal suspensions was measured according to the procedure described by Harper.[10] Activity is expressed in micrograms of inorganic phosphorus produced in 15 min per milligram of protein at 37°.

The quantitative estimation of the lipid peroxidation *in vivo* was determined by diene conjugate ultraviolet absorption of lipid extracts of the microsomal fractions, as described by Klaassen and Plaa.[11] The results are expressed as the change in absorbance at 243 mμ × 1000 for a solution having 1 mg of microsomal lipid per milliliter.

The incorporation *in vivo* of ^{14}C into microsomal lipids was determined as follows: $^{14}CCl_4$ was dissolved in corn oil to give a solution containing 2·3 mμmoles/ml (1,400,000 dis./min/ml) and given i.p. to 12–14 hr fasted male albino rats at a dose of 5 ml/kg of final solution. After isolation of microsomes as described above, an aliquot of 0·5 ml of the microsomal suspension was taken and added to 9·5 ml of a chloroform–methanol (2 : 1) mixture. The mixture was shaken and after 2 or 3 min it was centrifuged at about 3000 *g* for 10 min. The supernatant layer was transferred to a graduated centrifuge tube and brought to a volume of 10 ml with chloroform–methanol mixture. Two ml of distilled water was added, the two phases were thor-

oughly mixed and then centrifuged 5 min at 3000 *g*. The upper phase was aspirated off and discarded. The interphase was washed twice with 0·5 ml of the upper phase of a mixture obtained by mixing 25 ml of water and 95 ml of the chloroform–methanol mixture and shaking. The lower phase was evaporated in a tared scintillation vial under a nitrogen atmosphere at 40°. After addition of a few drops of non-labeled CCl_4 to the residue, the sample was dried again at 40° and weighed. Then the residue was dissolved in 10 ml of 5% 2,5-diphenyloxazole (PPO) in toluene and counted in a Beckman LS-133 Liquid Scintillation Counter. The counts were quench-corrected by the channels ratio method and the background subtracted. Results are given in dis./min per 100 mg of microsomal lipid.

Carbon tetrachloride concentrations in liver were estimated according to the procedure described by Recknagel and Litteria.[12] In the experiment with labeled $^{14}CCl_4$ the latter procedure was followed up to the microdiffusion step; then the $^{14}CCl_4$ collected in the toluene of the center well of the cell was transferred to a scintillation counting vial and counted as mentioned above.

Protein concentrations were estimated either by the procedure described by Lowry *et al.*[13] or by the biuret method.[14]

NADP-linked isocitric dehydrogenase activity in plasma (ICDH) was measured according to Sterkel *et al.*[15] Activity is given in units; one unit is the amount of enzyme producing 1 mμmole of NADPH per milliliter of plasma per hour at 25°.

Histological techniques. After removing the liver, small portions from the left and central lobes were immediately fixed in Bouin's solution, embedded in paraffin and stained with hematoxylin-eosin and Mallory's trichrome stain.

Frozen sections of liver tissue fixed in calcium-formalin were stained with Sudan black to show lipid.

Statistics. The significance of the difference between two mean values was assessed by the Student's *t*-test[16] and significance of differences involving more than two mean values was assessed by analysis of variance according to the method of Brownlee.[17]

RESULTS

Effect of cystamine on CCl_4 oral hepatotoxicity. Table 1 shows that cystamine prevents the increase in liver weight and the efflux of ICDH activity from liver to plasma caused by the oral administration of CCl_4. In accordance with these results, the histological inspection of the liver showed that cystamine protected it against the necrosis and fatty infiltration caused by CCl_4 24 hr after its oral administration. Cystamine by itself does not alter the normal architectural pattern of the liver (Figs. 1 and 2).

The prior treatment with cystamine largely prevents not only the depression of G6P-ase activity but also the destruction of the EM-ase caused by CCl_4 3 hr after its oral administration (Table 1).

The previous treatment with cystamine apparently delays the gastrointestinal absorption of CCl_4 as can be seen in Fig. 3 in which the liver content of CCl_4 was determined 1–24 hr after its oral administration to control or to cystamine-treated rats.

Effect of cystamine on the liver damage caused by intraperitoneal administration of CCl_4. Cystamine is also effective in preventing the liver damage caused by i.p.

TABLE 1. ORAL CCl_4 HEPATOTOXICITY IN RATS PREVIOUSLY TREATED WITH CYSTAMINE

Treatment*	ICDH activity† (unit/ml ± S.D.)	Liver weight† (g/100 g body wt ± S.D.)	G6P-ase activity† (μg inorganic phosphorus/mg/ 15 min ± S.D.)	EM-ase activity† (mμmoles CH_2O/mg/ 15 min ± S.D.)
Control	133 ± 41	2·63 ± 0·07	19·87 ± 1·37	9·75 ± 1·40
CCl_4	113,000 ± 33,800‡	5·39 ± 0·29‡	9·00 ± 2·23‡	1·92 ± 0·43‡
Cystamine	118 ± 37	3·18 ± 0·19	20·39 ± 0·98	8·30 ± 1·32
Cystamine + CCl_4	300 ± 165‡	3·02 ± 0.50§	17·68 ± 1·46‡	7·22 ± 2·18§

*CCl_4 was given orally, undiluted, at dose of 2·5 ml/kg to rats fasted for 12–14 hr. Cystamine was given orally dissolved in distilled water at a dose of 600 mg/kg, 30 min before CCl_4. Controls received distilled water. The animals were killed 24 hr after CCl_4 administration for studies on ICDH activity and liver weight and 3 hr after CCl_4 for those on EM-ase and G6P-ase activities.

† The data presented are from one of two similar experiments (ICDH activity, liver weight/100 g body weight and G6P-ase activity in 1200 *g* supernatant; six rats/group) or of three similar experiments (EM-ase in 9000 *g* supernatant; seven rats/group); mg = mg of protein. The P value for the significance of the overall effect of the prior treatment with cystamine obtained by analysis of variance was $P < 0·001$ for all the parameters measured. The P value for the significance of the intrinsic ability of cystamine to modify liver weight/100 g body weight was $P < 0·001$.

‡ $P < 0·05$ when compared to its respective control.

§ $P > 0·05$ when compared to its respective control.

administration of CCl_4. This protective effect is clearly shown in Table 2 in which the effects on liver weight and ICDH activity in plasma are shown.

These results were confirmed by the histological examination of the liver for necrosis and fatty infiltration (Figs. 4 and 5).

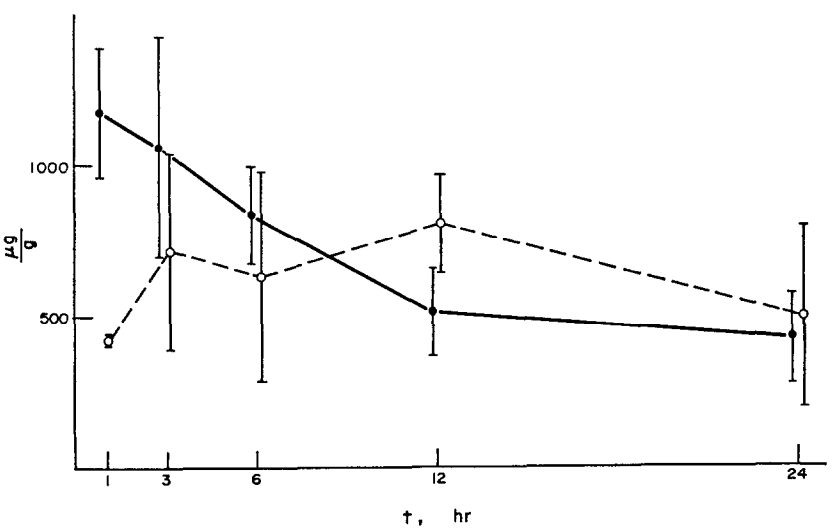

FIG. 3. CCl_4 concentrations in liver at different times after oral administration. CCl_4 (●——●) and cystamine + CCl_4 (○——○) were given as indicated in Table 1. Results in both groups were significantly different at 1 hr ($P < 0·01$), at 3 and 12 hr ($P < 0·05$) and non-significant at 6 and 24 hr ($P > 0·05$). Ten animals were used per group. Vertical bars represent S.D.

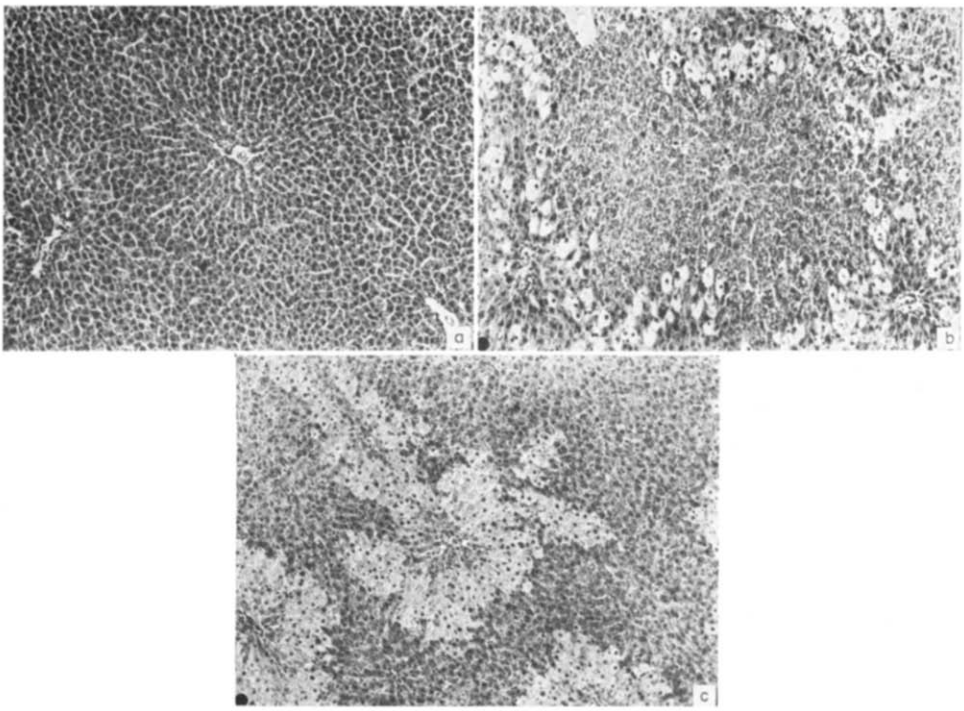

FIG. 1. Liver sections taken from rats which had received: (a) cystamine 24 hr before. The liver structure is essentially normal; (b) oral CCl_4 24 hr before. Note the striking centro-lobular necrosis and ballooning; (c) cystamine and oral CCl_4 24 hr before. The liver cells of the central areas are swollen and the periportal zones are well preserved (hematoxylin and eosin, × 100).

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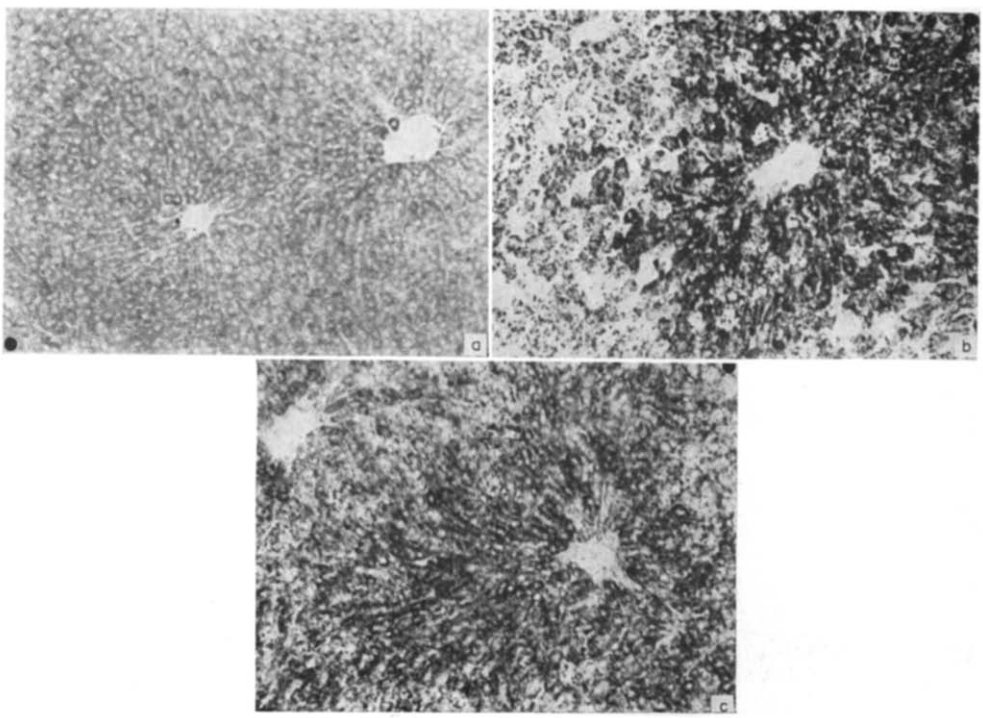

FIG. 2. Liver sections taken from rats which had received: (a) cystamine 24 hr before. The appearance is essentially normal; (b) oral CCl_4 24 hr before. Note the fatty infiltration in the central and periportal zones; (c) cystamine and oral CCl_4 24 hr before. There is a decrease in the fatty infiltration produced by CCl_4 (Sudan black, $\times$ 150).

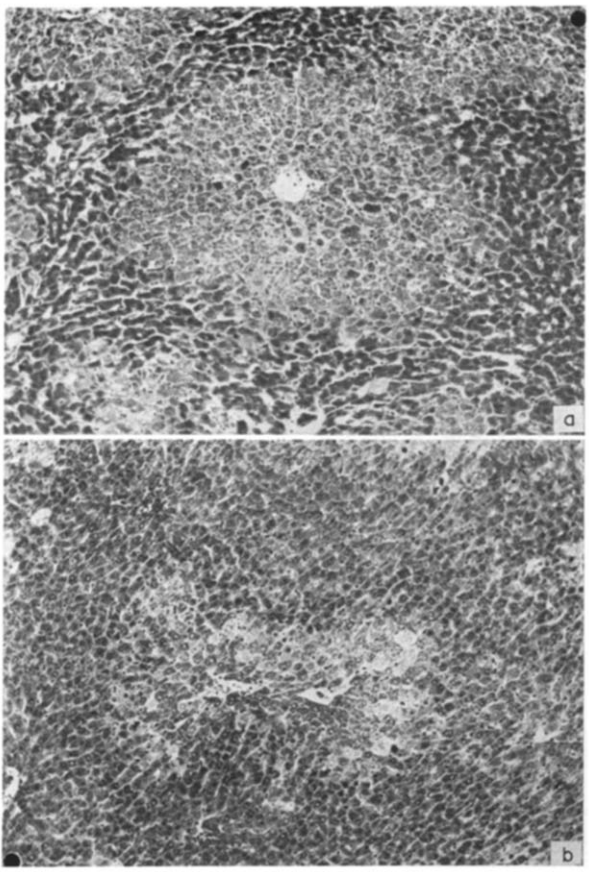

FIG. 4. Liver sections taken from rats which had received: (a) CCl_4 i.p. 24 hr before. A striking centrolobular necrosis is observed; (b) cystamine and CCl_4 i.p. 24 hr before. The necrotic areas are reduced (hematoxylin and eosin, × 100).

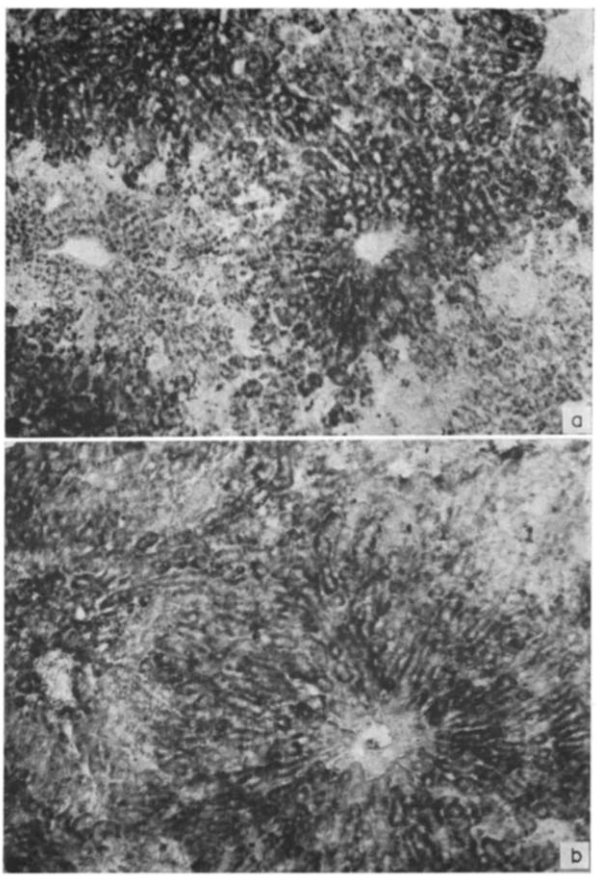

FIG. 5. Liver sections taken from rats which had received: (a) CCl_4 i.p. 24 hr before; (b) cystamine and CCl_4 i.p. 24 hr before. Cystamine decreased the fatty infiltration produced by CCl_4 (Sudan black, × 150).

TABLE 2. EFFECT OF INTRAPERITONEAL CCl_4 24 hr AFTER ADMINISTRATION ON PLASMA ISOCITRIC DEHYDROGENASE (ICDH) AND LIVER WEIGHT IN RATS PREVIOUSLY TREATED WITH CYSTAMINE

Treatment*	ICDH activity† (unit/ml ± S.D.)	Liver weight† (g/100 g body wt. ± S.D.)
Control	187 ± 96	3·10 ± 0·20
CCl_4	111,600 ± 34,880‡	4·56 ± 0·19‡
Cystamine	168 ± 72	3·39 ± 0·25
Cystamine + CCl_4	7200 ± 6550‡	4·22 ± 0·18‡

* CCl_4 was given i.p. as a 20% (v/v) solution in corn oil at a dose of 5 ml of solution/kg. Control rats received corn oil i.p. Cystamine was given orally dissolved in distilled water at a dose of 600 mg/kg 30 min before CCl_4. Controls received distilled water; six animals were used per group.

† The P value for the significance of the overall effect of the prior treatment with cystamine obtained by analysis of variance was $P < 0·001$ for ICDH activity and $P < 0·005$ for liver weight/100 g body weight.

‡ $P < 0·05$ when compared to its respective control.

As can be observed, cystamine also largely prevents the decrease in microsomal G6P-ase activity produced when CCl_4 is administered i.p. (Table 3).

In contrast to the results found when CCl_4 was given orally, in the case of the i.p. administration of CCl_4 cystamine was not able to prevent the destruction of EM-ase whether given either half an hour or 3 hr before CCl_4 (Table 4).

The degree of lipid peroxidation, as measured by the diene conjugation technique,[11] was found to be increased not only in the CCl_4-treated rats but also in those animals treated with both CCl_4 and cystamine (Table 5).

In one out of four of these experiments we also found a small but statistically significant increase in lipid peroxidation caused by cystamine itself; however, in the other

TABLE 3. EFFECT OF INTRAPERITONEAL CCl_4 3 hr AFTER ADMINISTRATION ON GLUCOSE 6-PHOSPHATASE ACTIVITY IN RATS PREVIOUSLY TREATED WITH CYSTAMINE

Treatment*	G6P-ase activity† (μg inorganic phosphorus/15 min/ mg microsomal protein/37° ± S.D.)
Control	81·6 ± 3·1
CCl_4	37·9 ± 5·3‡
Cystamine	83·3 ± 15·1
Cystamine + CCl_4	70·0 ± 11·3‡

* CCl_4 and cystamine were given as indicated in Table 2.

† The data presented are of one of two similar experiments using microsomal suspensions with five animals per group each. The P value for tne significance of the overall effect of the prior treatment with cystamine obtained by analysis of variance was $P < 0·005$.

‡ $P < 0·05$ when compared to its respective control.

Table 4. Effect of intraperitoneal CCl_4 3 hr after administration on ethylmorphine *N*-demethylase activity in rats previously treated with cystamine

Treatment*	EM-ase activity† (mμmoles formaldehyde/15 min/37°/ mg microsomal protein ± S.D.) Cystamine 30 min before CCl_4	Cystamine 3 hr before CCl_4
Control	68·2 ± 20·2	61·3 ± 7·2
CCl_4	39·9 ± 14·9‡	25·5 ± 4·0‡
Cystamine	77·1 ± 14·4	68·0 ± 13·0
Cystamine + CCl_4	43·3 ± 13·1‡	38·0 ± 4·8‡

* CCl_4 and cystamine were given at the same doses as indicated in Table 2.

† The data presented are of one of two similar experiments using microsomal suspensions with five rats per group each. The P values for the significance of the overall effect of the prior treatment with cystamine obtained by analysis of variance was $P > 0·1$ for both experiments

‡ $P < 0·05$ when compared to controls.

three, the values in the cystamine-treated group were consistently slightly higher than those of the controls but the difference was not significant.

The previous administration of cystamine decreased by about 40 per cent the irreversible binding of ^{14}C from $^{14}CCl_4$ to microsomal lipids (Table 6), in spite of the fact that the levels of free $^{14}CCl_4$ present in the livers of cystamine-treated rats were not lower but slightly higher than those found in the livers of untreated rats (Table 6).

Table 5. Effect of the previous treatment with cystamine on the microsomal lipid peroxidation *in vivo* induced by intraperitoneal CCl_4 3 hr after administration

Treatment*	Lipid peroxidation *in vivo* ± S.D.†
Control	353 ± 23
CCl_4	463 ± 24‡
Cystamine	377 ± 35
Cystamine + CCl_4	462 ± 33‡

* CCl_4 and cystamine were given as indicated in Table 2.

† The lipid peroxidation value is expressed as Δ absorbance at 243 mμ × 1000 for a solution having 1 mg of microsomal lipid per ml. The data presented are of one of four similar experiments with five rats per group each. The P value for the significance of the overall effect of the prior treatment with cystamine obtained by analysis of variance was $P > 0·1$.

‡ $P < 0·05$ when compared to its respective control.

TABLE 6. EFFECT OF THE PREVIOUS TREATMENT WITH CYSTAMINE ON THE IRREVERSIBLE BINDING OF ^{14}C TO MICROSOMAL LIPIDS AND ON THE $^{14}CCl_4$ LEVELS IN LIVERS OF RATS 3 hr AFTER ITS ADMINISTRATION

Treatment*	dis./min/g/100 mg microsomal lipids ± S.D.† (10 rats/group)	dis./min/g liver wt. ± S.D.† (five rats/group)
$^{14}CCl_4$	9602 ± 2247	488 ± 161
$^{14}CCl_4$ + cystamine	5542 ± 1179‡	663 ± 54‡

* $^{14}CCl_4$ was given i.p. as a corn oil solution, 2·3 mμmoles /ml of solution (1,400,000 dis./min/ml) at a dose of 5 ml/kg to 12–14 hr fasted rats. Cystamine was given as in Table 2. The animals were sacrificed 3 hr after $^{14}CCl_4$ administration and processed as indicated in Methods.

† The data presented are of one of four similar experiments.

‡ $P < 0{\cdot}05$ when compared to its control.

The difference between the amounts of CCl_4 in the livers of cystamine-treated and non-treated rats after i.p. administration of CCl_4 was found not to be significant (Fig. 6).

DISCUSSION

In agreement with the postulated similarity between the mechanism of CCl_4-produced liver necrosis and the mechanism of radiation damage,[3] cystamine, a well-known antidote against radiation injury, also prevented the CCl_4-induced liver necrosis. We selected orally administered cystamine because of its long-term effect as an antidote[8] since CCl_4, in contrast to usual radiation treatments, acts on the body over a period of several hours (Figs. 3 and 6). This preventive effect was clearly observed not only

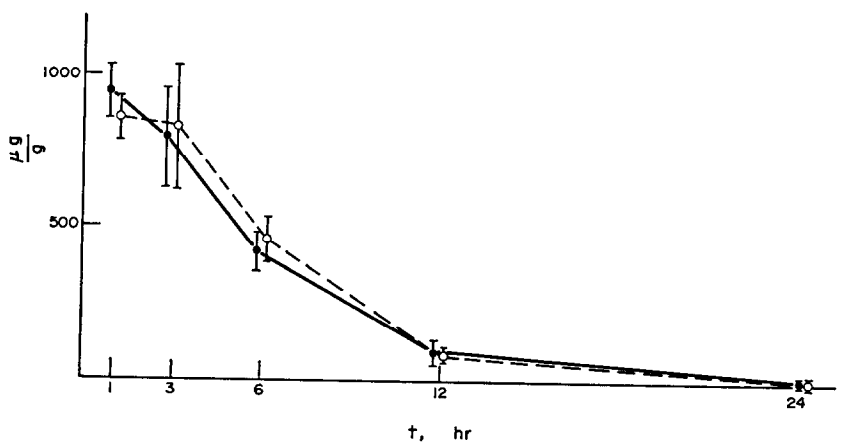

FIG. 6. CCl_4 concentrations in liver at different times after i.p. administration. CCl_4 (●——●) and cystamine+CCl_4 (○——○) were given as indicated in Table 4. Differences between both groups were non-significant at 1, 3, 6, 12 and 24 hr ($P > 0{\cdot}05$). Five animals were used per group. Vertical bars represent S.D.

when CCl_4 was given orally, but also when it was administered i.p. This fact is particularly important, since our experiments on oral CCl_4 administration have shown that cystamine delays the gastrointestinal absorption of CCl_4 and the protection could have been solely attributed to this effect.

In the case of the i.p. administration, CCl_4 concentrations in the livers of cystamine-treated and non-treated rats were similar (Fig. 6). These results would appear to argue against the delay in gastrointestinal absorption as being the unique or even the main mechanism of protection against necrosis in the case of the oral administration experiments; an alternative, although less likely possibility, is that cystamine acts by one mechanism when given orally and by another when given intraperitoneally, i.e. that cystamine protects after oral administration because of its effects on absorption and after intraperitoneal administration because of effects at the molecular level.

We also determined whether some of the molecular alterations caused by CCl_4 were prevented by previous cystamine administration. For example, prior treatment with cystamine partially prevented the depression of G6P-ase activity caused by CCl_4 after either oral or i.p. administration (Tables 1 and 3).

Since cystamine does not prevent the increase *in vivo* in microsomal lipid peroxidation caused by CCl_4 as measured by diene conjugation but inhibits by about 40 per cent the irreversible binding of $^{14}CCl_4$ to microsomal lipids, these results further strengthen our previous hypothesis that G6P-ase suppression is caused by the initial $CCl_3\cdot$ and $Cl\cdot$ free radicals* and not by the lipid peroxidation process as postulated by Ghoshal and Recknagel.[18]

In contrast, we were not able to find support in the present experiments for the hypothesis that EM-ase activity is destroyed by the initial $CCl_3\cdot$ and $Cl\cdot$ free radicals as postulated by Sasame *et al.*[6]

Apparently, the partial protection on EM-ase activity observed when CCl_4 was given orally may be almost entirely attributed to the delay in gastrointestinal absorption caused by cystamine, since a similar protective effect was not obtained when CCl_4 was given i.p., whether cystamine was administered either half an hour or 3 hr before (Table 4). This effect of cystamine on oral CCl_4 absorption is similar to that obtained by Marchand *et al.*[19] using SKF-525A.

Since it was previously postulated by several authors that activation of CCl_4 to free radicals is caused by its interaction with the drug metabolizing enzymes,[1–3] there remains the possibility that cystamine protection of EM-ase against $CCl_3\cdot$ and $Cl\cdot$ free radicals is hindered sterically because of close juxtaposition between EM-ase and the CCl_4 activation site.

The protection against necrosis here described appears to follow more closely the early decrease in the irreversible binding of ^{14}C to microsomal lipids than early changes in lipid peroxidation, suggesting that the effect of the initial $CCl_3\cdot$ and $Cl\cdot$ free radicals may be more pertinent than the lipid peroxidation to the development of necrosis. Other authors have also recently cast some doubts on the role of lipid peroxidation.[11] However, studies at times longer than 3 hr on irreversible binding and lipid peroxidation as well as histological examination of the liver at 48 and 72 hr would be necessary before a clear picture of the temporal relations involved could be obtained since, for example, cystamine may be merely delaying the lesion as in the case of hypothermia and cordotomy.[20,21]

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