CARBON TETRACHLORIDE TARGET LIPIDS IN RAT LIVER MICROSOMES. EFFECT OF CYSTAMINE ADMINISTRATION ON THEIR PATTERN OF LABELING BY $^{14}CCl_4$

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Summary

^{14}C from $^{14}CCl_4$ irreversibly binds to lipids from the smooth (SER) and rough (RER) endoplasmic reticulum. Most of the label is associated with the phospholipid fraction (> 95%). Prior cystamine administration decreased the extent of that binding but does not change its pattern of distribution. About the half of the label in phospholipids is in the phosphatidylcholine fraction; the other half is distributed similarly among lysophosphatidylcholine, sphingomyelin and phosphatidylethanolamine, while only a very minor fraction is associated with diphosphatidyl glycerol. No differences were found in the pattern of labeling of phospholipids in SER and RER.

Reynolds (1) and Reynolds and Yee (2) reported that ^{14}C and ^{36}Cl from $^{14}C^{36}Cl_4$ irreversibly bind to liver lipids, particularly to those from the microsomal fraction. These authors found that ^{14}C from this chlorocarbon preferentially labels cholesterol esters, triglycerides, fatty acids and to a lesser extent phospholipids. In contrast, more recent studies by Gordis (3) evidenced that more than the 65% of the ^{14}C label from $^{14}C^{36}Cl_4$ is associated with the phospholipid fraction. In this study, we have attempted to check under our usual experimental conditions one of these observations is confirmed and in addition to test: a) whether the reduction in the extent of the irreversible binding of $^{14}CCl_4$ to microsomal lipids accomplished by the previous administration of cystamine (4,5,6) was either associated with a particular class of lipid or was due to a uniform decrease in the labeling by $^{14}CCl_4$ in all the lipid moieties, b) if the pattern of labeling was different in the smooth (SER) and rough (RER) endoplasmic reticulum, c) if there occurs a different labeling of the different phospholipids in SER and RER, d) if phosphatidylcholine which is known to be involved as a lipid factor in the microsomal drug hydroxylating system (7,8) is an important CCl_4 target lipid.

Material and Methods

$^{14}CCl_4$ (27.5 mCi/mM), was obtained from the Radiochemical Center, Amersham England. All other chemicals were reagent grade. Sprague-Dawley male rats (200-280 g) were used in these experiments. Food was withdrawn 12-14 hr before $^{14}CCl_4$ administration. Cystamine was orally given as a water solution (240 mg/ml) at a dose of 600 mg/kg 30 min. before $^{14}CCl_4$. The $^{14}CCl_4$ was dissolved in

olive oil to give a solution producing about 6 x 10^6 dpm/ml. This solution was given i.p. at a dose of 5 ml of solution/kg. Controls received the equivalent amount of water or olive oil. The animals were sacrificed by decapitation 3 hr after $^{14}CCl_4$ administration. The preparation of the microsomal fraction as well as the isolation of the labeled lipids from microsomes was previously described (4,6). The rough and smooth fractions of the endoplasmic reticulum were isolated according to Ragnotti et al. (9).

The separation of the different types of lipids and phospholipids was accomplished by TLC on silica gel G 20 x 20 cm glass plates according to Tuna and Mangold (10) and Wagner et al. (11) respectively. Typical separations achieved by the use of the above mentioned procedures are depicted in figs. 1 and 2. The plates were prepared in a Desaga Apparatus (Germany) and were 500 microns thick in silica gel G (Merck, Darmsdadt). About 20 mg of lipid were deposited in each plate. The different lipids were evidenced by exposing the plates to iodine vapor and by spraying other part of the plates with 2'7'dichlorofluorescein. More specific tests were employed for discrimination among different classes of lipids or phospholipids, for example: Acetic anhydride - sulphuric acid (Liebermann - Bouchard test) for cholesterol and cholesterol esters; Ninhydrin solution for phosphatidylethanolamine; Dragendorff reagent for phosphatidylcholine (iodobismutate), these tests were described in detail by Stahl (12); Phospholipid phosphorus was evidenced according to J. Dittmer and R. Lester (13). Pure standards of each lipid class were run simmultaneously. After removal of the iodine from the developed TLC plates the marked spots were quantitatively transferred to scintillation counting vials and counted as previously stated by Stahl (12).

Results

Distribution of the label from $^{14}CCl_4$ among the different classes of lipids in microsomes and the effect of cystamine on it. As shown in Table 1 more than 95% of the ^{14}C from $^{14}CCl_4$ in microsomal lipids is irreversibly bound to the phospholipid fraction (PL), while the rest of the label is distributed among triglycerides (TG), diglycerides (DG), free fatty acids (FFA) cholesterol (Ch), cholesterol esters (ChE), and monoglycerides (MG).

A dose of cystamine which is able to reduce the extent of the total label to microsomal lipids in a 56.3 per cent did not modify the pattern of distribution of the label to a significant extent.

Distribution of the label from $^{14}CCl_4$ among the different classes of lipids from the rough (RER) and smooth (SER) endoplasmic reticulum.

The pattern of distribution of the label of the lipids by $^{14}CCl_4$ in RER and SER is quite similar and only minor differences between them were found.

TABLE 1. Pattern of distribution of the irreversible bound ^{14}C from $^{14}CCl_4$ among the different microsomal lipid fractions and the effect of the prior cystamine administration on it*

Lipid fraction	% of total label in each fraction mean ± sd (6 animals/group)		
	CONTROL	CYSTAMINE	P
PL	97.53 ± 1.18	94.54 ± 2.50	= 0.05
MG	0.46 ± 0.94	1.21 ± 1.64	> 0.04
DG	0.11 ± 0.19	0.03 ± 0.08	> 0.4
Ch	0.61 ± 0.67	0.65 ± 0.42	> 0.4
FFA	0.27 ± 0.15	0.56 ± 0.58	> 0.2
TG	0.89 ± 0.20	2.16 ± 1.01	< 0.02
ChE	0.04 ± 0.06	0.10 ± 0.25	> 0.5

* Cystamine p o was administered 30 min before $^{14}CCl_4$ ip. Controls received the equivalent amount of $^{14}CCl_4$. The animals were sacrificed 3 hr after $^{14}CCl_4$ administration. Their liver microsomal lipids were isolated and separated by TLC as shown in Fig 1. After identification of the different lipids, the radioactivity associated with each fraction was counted. (see Methods for details). Total label in controls was 3830 ± 700 dpm/mg lipid and 1676 ± 540 dpm/mg lipid in the cystamine treated group. The extent of the label in the cystamine group was 43.7% of that in the control group ($P < 0.001$).

(See Table 2). About the half of the label in the phospholipids from both, SER and RER is associated with phosphatidylcholine (PC). The other half of the label is fundamentally distributed in similar proportions among lysophosphatidylcholine (LPC), sphingomyeline (SPH) and phosphatidylglycerol (DPG). No important differences were found in the pattern of the labeling of the phospholipids from SER and RER.

Discussion

The present observations on the distribution of ^{14}C label from $^{14}CCl_4$ among different classes of lipids from rat liver microsomes show that under our experimental conditions, the label was found preferentially associated with the phospholipid fraction. Similar type of results were obtained when lipids were isolated from either SER or RER instead than from total microsomal fraction. The prior treatment of the animals with cystamine, which is

TABLE 2. Pattern of distribution of the irreversible bound ^{14}C from $^{14}CCl_4$ among the different lipid fractions of RER and SER *

Lipid fraction	% of total label in each fraction	
	RER	SER
PL	97.97	96.41
MG	-----	-----
DG	0.64	1.22
Ch	0.50	0.30
FFA	0.18	0.71
TG	0.61	1.34
ChE	0.10	0.02

* $^{14}CCl_4$ was administered ip as described en Methods. The animals were sacrificed 3 hours after $^{14}CCl_4$ administration. The liver RER and SER was obtained and their lipids were isolated and separated by TLC as shown the Fig 1. Lipids were identified and their radioactivity was measured by liquid scintillation counting (see methods for details). The samples were a pool of the lipids from 8 animals in each group. The ratio between total label SER/RER is 1.3.

known to decrease the extent of the irreversible binding of $^{14}CCl_4$ to microsomal lipids (4,5,6) does not change fundamentally the pattern of distribution of the label among the different lipid fractions. This effect of cystamine is compatible with our previous assumption that this compound behaves in that form because it inhibits the CCl_4 activation step to $\cdot CCl_3$, with the consequent reduction in the amount of free radicals produced and not because it gives some complex with the target lipids with a resulting shielding of them against the $\cdot CCl_3$ free radicals (6). In effect, a similar percentual shielding of the different lipids by cystamine involves the assumption that cystamine binds to and that it has a similar affinity for each one of the structurally different types of lipid and this is something which is only hardly conceivable.

The preferential irreversible binding of $^{14}CCl_4$ to phospholipids as well as its decrease caused by the previous administration of cystamine to the rats is a very interesting feature, since in addition, we previously reported that cystamine partially prevents the early loss of glucose 6 phosphatase

TABLE 3. Pattern of distribution of the irreversible bound ^{14}C from $^{14}CCl_4$ among the different phospholipid fractions of RER and SER *

Phospholipid fraction	% of the total label in phospholipids found in each fraction (mean ± sd)		
	RER	SER	P
LPC	17,64 ± 2.34	15.07 ± 2.70	> 0.2
SPh	12.32 ± 2.60	18.50 ± 0.40	< 0.02
PC	51.63 ± 0.28	49.01 ± 3.59	> 0.2
PE	16.48 ± 1.06	16.02 ± 1.02	> 0.2
DPG	1.94 ± 1.07	1.50 ± 1.50	> 0.2

* $^{14}CCl_4$ administration, separation of RER and SER as in Table 2. Phospholipids were separated by TLC as shown in Fig 2 and after identification as described in Methods, their radioactivity was measured by liquid scintillation counting (see methods for details).
LPC (Lysophosphatidylcholine); SPh (Sphingomyeline); PC (Phosphotidylcholine); PE (Phosphatidylethanolamine); DPG (Diphosphatidylglycerol).
The reported values were obtained from 3 samples, each one being a pool of the lipids from either the RER or SER of 3 animals.

(G6P-ase) and ethylmorphine N-demethylase (EM-ase) activity as well as the decrease in cytochrome P-450 content (4,5,6) caused by CCl_4 administration. This simultaneous effect of cystamine on the above mentioned parameters is tempting for speculation, particularly when it is very well known that both, G6P-ase and EM-ase need of a phospholipid for activity (14,15,16,17,18,19). Moreover, in the case of EM-ase that phospholipid was identified as phosphatidylcholine (7,8) which here was showed to be the main target phospholipid in rat liver microsomes. Notwithstanding, one should be aware of the fact that either G6P-ase or EM-ase or P-450 have components other than phospholipids in their molecules, which may be suitable targets for the $\cdot CCl_3$ and $\cdot Cl$ free radicals as well. As a matter of fact, P-450 itself is also a component of EM-ase activity (19) and it is also destroyed by CCl_4 (20). The irreversible binding of ^{14}C from $^{14}CCl_4$ to microsomal proteins with the consequent loss of their function; which is also known to occur (1,2) and which is also partially prevented by cystamine administration (21) may also be responsible for the prevention of either G6P-ase, EM-ase or P-450 loss of activity. Furthermore, in the case of P-450 an effect on the heme-iron moiety of its

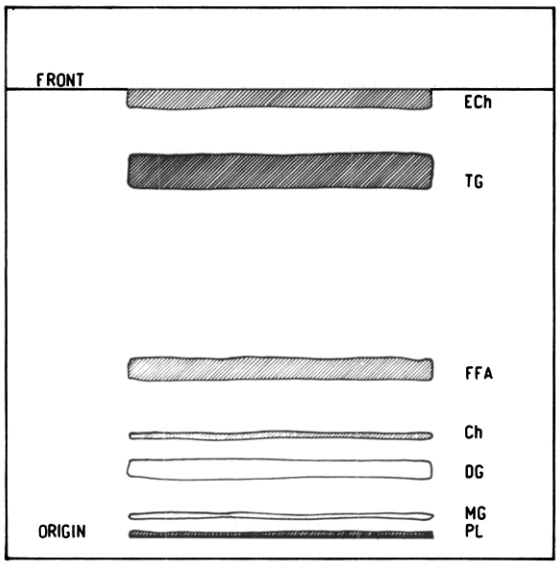

Fig. 1.

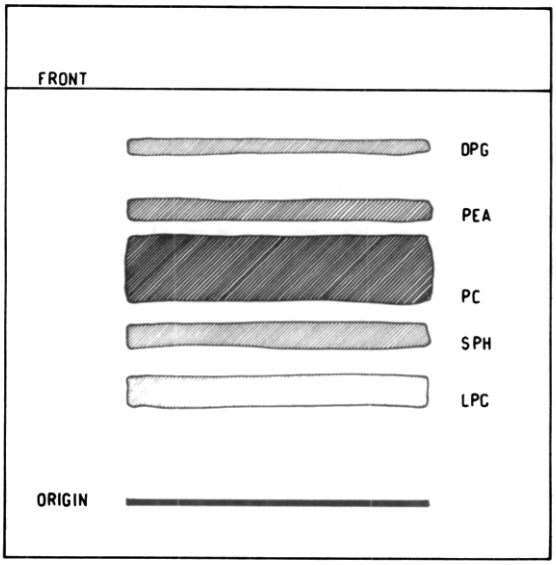

Fig. 2.

molecule with its resulting destruction is equally likely, particularly when heme breakdown products were reported to be observed after CCl_4 administration (22). In summary, the G6P-ase activity deppression observed after CCl_4 administration may be due to the irreversible binding of CCl_4 to either microsomal phospholipids or proteins. It is interesting to point out here

that other autors reported that G6P-ase activity loss after CCl_4 was "in vitro" partially restored by addition of microsomal lipids (24). The loss of EM-ase activity observed after CCl_4 administration is probably due to a combination of two deletereous actions of CCl_4. One (the most important), is the ability to destroy P-450 and the other may be as suggested by our present experiments an alteration of phosphatidylcholine, mediated by the irreversible binding of CCl_4 to its molecule. This alteration in phosphatidylcholine may hinder its postulated function in the electron transfer from NADPH to P-450 catalyzed by the P-450 reductase (7,8).

The preventive effect of cystamine against all these different deletereous effects of CCl_4 may be explained by assuming that it inhibits the CCl_4 activation step to $\cdot CCl_3$ and $\cdot Cl$ free radicals as we previously postulated (6,24,25). The decrease in the formation of free radicals, may well explain the decrease in all the alterations caused by their interaction with different target molecules (phospholipids, proteins, heme, etc).

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