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COUNTERACTION BY SULPHYDRYL COMPOUNDS OF THE ENZYMIC CONVERSION OF AND THE METABOLIC LESIONS PRODUCED BY TWO CARCINOGENIC N-NITROSODIALKYLAMINES IN RAT LIVER

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Abstract—(1) The *in vivo* and *in vitro* effects of various sulphydryl compounds on the metabolic conversion (N-dealkylation) of the N-nitrosodialkylamines, DMNA* and DENA, to their toxic and suspected carcinogenic derivatives by rat liver microsomes, were studied. MAB and EAB were included for comparison. Subcutaneous administration of excess cysteine to rats during 2 days leads to a decrease in the activity of the N-demethylating but not of the N-de-ethylating enzymes of the microsomes. Addition of cysteine and cysteamine, but not of reduced glutathione, to normal liver microsomes causes an inhibition of both types of enzymes, the N-de-ethylating being less susceptible than the N-demethylating enzymes. The latter finding may explain the differential effect observed after subcutaneous administration of cysteine.

(2) The inhibitions of the *in vitro* amino acid incorporation into liver microsomes following administration of DMNA, DENA or CB 2446 are either completely prevented or reduced following injection of cysteamine, the degree of the latter effect being dependent on the route of application and the dose of cysteamine, and on the nature of the alkyl groups of the nitrosamines. Intravenously administered cysteine is less effective than cysteamine. The N-dealkylating enzymes of liver microsomes of rats treated with the cysteamine regimen which prevented the inhibition of amino acid incorporation by the nitrosamines, are not reduced in activity.

(3) The glycogenolysis produced in liver by the intravenous administration of cysteamine and cysteine is counteracted by DMNA and DENA.

(4) The results suggest that a mutual elimination of sulphydryl compounds and nitrosamine metabolites may occur in the livers of rats receiving dialkylnitrosamines and cysteamine or cysteine.

THE hepatocarcinogenic N-nitrosodialkylamines, DMNA* and DENA, are dealkylated by enzymes of the drug-metabolizing type, located in the microsomal membranes of rat liver, under formation of formaldehyde and acetaldehyde, respectively. N-nitrosomonalkylamines (=alkyldiazohydroxides), thus arising under aqueous conditions, yield alkyldiazonium ions which react as alkylcarbonium ions with nucleophilic components of the liver cells.[1, 2] The lesions induced by the nitrosamines in liver consist of an inhibition of microsomal protein synthesis, loss of glycogen, fatty degeneration and marked changes in the fine structure of smooth and rough endoplasmic reticulum.[1–6]

* Abbreviations used: DMNA = dimethylnitrosamine; DENA = diethylnitrosamine; MAB = 4-monomethylaminoazobenzene; EAB = 4-monoethylaminoazobenzene.

Previous work has shown[2] that subcutaneous administration of excess cysteine counteracted the metabolic lesions produced by DMNA but not those by DENA. The results did not favour the hypothesis that the alkylating agents derived from the N-nitrosodialkylamines *in situ* reacted with the administered SH-groups or that the latter protected tissue SH-groups against interaction with the former. Moreover, the lack of effect of cysteine in the case of DENA and the protective effect of cysteine towards DMNA could be explained by the finding that cysteine administration led to an inhibition of the microsomal N-demethylating but not of the N-de-ethylating euzyme. The mechanism underlying the differential effect of cysteine administration on the two enzymes has now been studied in more detail. The present paper contains the results of these experiments and gives a further account of our finding[1] that administration of cysteamine (β-mercaptoethylamine) may prevent the inhibition of amino acid incorporation into the microsomal proteins produced by both DMNA and DENA. Included are the results of similar experiments carried out with N,N′-dinitroso-N,N′-dimethyl-1:6-diaminohexane (CB2446) which after enzymic N-demethylation may give rise to a bifunctional alkylating agent.

MATERIALS AND METHODS

Adult male R and U×R rats, weighing from 275–325 g, were used. Unless mentioned in the text, the methods employed in the present investigation were similar to those described previously.[2] Intravenous injection of DMNA (100 mg/kg), DENA (200 mg/kg) and sulphydryl compounds was in the tail vein.

RESULTS AND DISCUSSION

Inhibition by SH-compounds of the N-dealkylation of N-nitrosodialkylamines and 4-monoalkylaminoazobenzenes by liver microsomes

N-Dealkylation of the N-nitrosodialkylamines, DMNA and DENA, and of the 4-monoalkylaminoazobenzenes, MAB and EAB, was studied using the combined microsomal-soluble fraction from 600 mg of fresh weight of rat liver in a total of 5 ml medium fortified with cofactors.[2] The soluble fraction was included to furnish the glucose-6-phosphate dehydrogenase required for the generation of reduced triphosphopyridine nucleotide. The soluble fraction could be replaced by the addition of crystalline dehydrogenase to the microsomes (105,000 × *g* pellet). Neither the soluble fraction nor the microsomes were active when incubated singly.

In vivo *administration of cysteine and cysteamine.* Subcutaneous administration of excess cysteine (150 mg) twice daily for 2 days led to an inhibition of the demethylation of DMNA and MAB but not of the de-ethylation of DENA by microsomes isolated from the rat liver on the third day.[2] While confirming these results, Table 1 shows that the de-ethylation of another N-ethyl containing substrate, EAB, was also unaffected by the cysteine treatment. Cysteine thus appears to exert *in vivo* a differential effect on the two types of N-dealkylating enzymes.

It is also shown in Table 1 that administration of cysteamine did not lead to an inhibition of the N-dealkylation of any of the four substrates. In these experiments 12·5 mg cysteamine was administered intraperitoneally followed after 1 hr by another 10 mg dose intravenously (regimen *a*); the animals were killed 4 hr after the latter injection.

In vitro *addition of* SH-*compounds.* Reduced glutathione (20 μmole/flask) showed no inhibitory effect on the N-dealkylation of DMNA, DENA, MAB and EAB by the liver microsomes of normal rats (Table 2). By contrast, cysteamine and cysteine (20 μmole/flask) appeared to be markedly and about equally inhibitory to the N-dealkylation of all four substrates.

TABLE 1. EFFECT OF CYSTEINE AND CYSTEAMINE ADMINISTRATION ON N-DEALKYLATION BY LIVER MICROSOMES

(Typical results are listed. The modes of administration of cysteine and cysteamine are mentioned in the text. For further data regarding the effect of cysteine administration on the N-dealkylation of MAB, DMNA and DENA, and methods compare ref. 2. Aldehyde production by microsomes from 1 g of fresh weight of liver.)

Treatment	Micromoles formaldehyde or acetaldehyde produced from			
	MAB	EAB	DMNA	DENA
—(Control)	1·10	0·80	0·35	0·45
Cysteine (subcut.)	0·55	0·85	0·13	0·45
—(Control)	1·00	0·77	0·40	0·50
Cysteamine (regimen *a*)	1·00	0·80	0·41	0·52

TABLE 2. *In vitro* EFFECT OF CYSTEINE, CYSTEAMINE AND REDUCED GLUTATHIONE ON N-DEALKYLATION BY LIVER MICROSOMES

(20 μmole of the SH-compounds were added *to* 5 ml medium. Listed are the mean values with standard deviation and in parentheses the number of experiments. Aldehyde production by microsomes from 1 g of fresh weight of liver.)

Addition	Micromoles formaldehyde or acetaldehyde produced from			
	MAB	EAB	DMNA	DENA
—	1·05 ± 0·13 (11)	0·65 ± 0·03 (4)	0·39 ± 0·05 (3)	0·51 ± 0·02 (4)
Cysteine	0·53 ± 0·09 (7)	0·40 ± 0·02 (4)	0·18 ± 0·02 (3)	0·22 ± 0·01 (4)
Cysteamine	0·55 ± 0·06 (6)	0·36 ± 0·01 (4)	0·17 ± 0·02 (3)	0·23 ± 0·02 (4)
Glutathione	0·93 ± 0·08 (5)	0·71 ± 0·03 (4)	0·37 ± 0·03 (3)	0·53 ± 0·03 (4)

The present results with cysteine *in vitro* are in accord with the former ones obtained after *in vivo* administration only as regards the N-methyl substrates. In order to account for the discrepancy that existed between the *in vivo* and the *in vitro* effects of cysteine on the N-de-ethylation, the possibility was considered and studied that the N-de-ethylating enzyme was less susceptible than the N-demethylating enzyme to cysteine and that this difference was obscured in the above *in vitro* experiments by the amount of cysteine being added. Table 3 shows that 6–8 μmole cysteine/flask still inhibited the N-demethylating enzyme while having no effect on the N-de-ethylating enzymes. These results might indicate that the activity of the N-dealkylating enzymes, following the *in vivo* administration of cysteine, is governed by the amount of cysteine reaching the liver, this being insufficient for inhibition of the N-de-ethylases but sufficient for that of the N-demethylases.

The difference between the *in vivo* and *in vitro* effects of cysteamine is attributed to the short duration of the *in vivo* treatment (5 hr) with a relatively small dose.

Possible mechanism by which SH-*compounds inhibit N-dealkylating enzymes.* The finding that reduced glutathione does not inhibit microsomal N-dealkylation indicates that the inhibition produced by cysteine and cysteamine is not due to the presence of

TABLE 3. *In vitro* EFFECT OF CYSTEINE ON N-DEALKYLATION BY LIVER MICROSOMES

(Listed are the average values of two closely agreeing experiments carried out with 8 μmole cysteine in the case of the monoalkylaminoazobenzenes and with 6 μmole cysteine in the case of the N-nitrosodialkylamines. Aldehyde production by microsomes from 1 g of fresh weight of liver.)

Cysteine	Micromoles formaldehyde or acetaldehyde produced from MAB	EAB	DMNA	DENA
absent	0·92	0·66	0·33	0·47
present	0·61	0·69	0·18	0·46

a SH-group *per se*. Provided that the lack of effect of glutathione does not result from its inability, for steric reasons, to reach the enzymic site, the following conclusions may be drawn. First, the SH-compounds are not inhibitory by trapping the N-hydroxyalkyl (-alkylol) intermediates of the enzymic N-dealkylating reaction, although evidence for this type of reaction has previously been presented by Mueller and Miller[7] using glutathione, 3′-methyl-4-monomethylaminoazobenzene and liver homogenate. Secondly, the SH-compounds do not trap either aldehyde released from the former intermediate or the hydroxyl donor, such as [HO—OH], generated by reduced triphosphopyridine nucleotide, oxygen and enzyme. Thirdly, the possibility that added SH-groups might interfere with the proper function of enzyme SH-groups—the microsomal degradation of DMNA by the combined microsomes-soluble fraction of liver is inhibited by SH reagents[8]—may be discarded.

The lack of effect of glutathione and the marked inhibitory effects of cysteine and cysteamine on N-dealkylation *in vitro* correspond to the relatively poor protection afforded by the former and the much more favourable effects shown by the latter compounds against ionizing radiation.[9, 10] According to Knoblock and Purdy[9] there may exist a correlation between radiation prophylaxis produced by a sulphydryl compound of the type under discussion and the measure of chelate formation of the latter with Cu^{2+}. Since several lines of evidence point to the presence of a metal as an active constituent of the drug-metabolizing enzymes,[11, 12] the chelating power of the various suphydryl compounds (glutathione $\ll$cysteine$<$ cysteamine) might also underly their *in vitro* inhibitory effect on the N-dealkylating enzymes of the microsomes. However, the finding that cysteine and cysteamine (10–20 μmole/flask) are about equally inhibitory to the latter enzymes argues against this interpretation. It will be evident that the decreased activity of the N-demethylase of liver microsomes isolated from rats following subcutaneous administration of excess cysteine must be due to a change in the enzyme, elaborated by cysteine *in vivo* and persisting under the *in vitro* conditions of the enzyme assay, since it is unlikely that under the latter conditions the inhibition is due to cysteine still being present in the cell-free preparation.

The removal of a metal from the enzyme *in situ* might account for the latter situation. However, we have been unable to restore the N-demethylase activity of isolated liver microsomes from cysteine-treated rats to the normal level by addition of Fe^{2+} or Cu^{2+} (0·5–2 μmole/flask) *in vitro*.

Thus, the possibility remains that the *in vivo* effect of cysteine on the N-demethylase is an indirect one, that cannot be compared with the *in vitro* effect. In this connexion it is of interest that administration of excess cysteine has been found to affect the endoplasmic reticulum of liver cells.[13]

Counteraction by SH-*compounds of the inhibition of microsomal amino acid incorporation produced by DMNA, DENA and CB*2446

In vivo *administration of cysteamine.* (a) Rats were treated with cysteamine according to regimen *a* (indicated above). DMNA (100 mg/kg) or DENA (200 mg/kg) was administered together with the second, intravenous, dose of cysteamine, the rats being killed 4 hr later. Table 4 shows that the inhibitory effects of DMNA and DENA on the *in vitro* incorporation of leucine-1-^{14}C into the proteins of the microsomal-soluble fraction isolated from the rat livers (9000 × *g* supernatant[2]) were completely abolished under these conditions. When the intraperitoneal injection of cysteamine was omitted and only 10 mg cysteamine was administered intravenously (regimen *b*) together with the nitrosamines, the inhibitory effect of DENA was still completely prevented, but that of DMNA was only partly reduced. Intraperitoneal injection of 10 mg cysteamine was without any significant effect.

Since, as illustrated in Table 1, the N-dealkylation of DMNA and DENA by the liver microsomes is not inhibited following the administration of cysteamine, the present results obtained with regimen *a* are taken to mean that the SH-group of cysteamine reacts with the alkyldiazonium or alkylcarbonium ions (compare (1)) derived from the nitrosamines *in situ*, under formation of harmless products. Further indirect evidence for this conclusion will be presented below. The results obtained with regimen *b* show that apparently less cysteamine SH-groups are required for the elimination of the toxic derivative of DENA than for that of DMNA. This in is accord with smaller toxicity of DENA as compared with that of DMNA as regards the inhibition of amino acid incorporation, glycogenolysis and the LD_{50}.[2]

(b) Four hours after injection of 75 and 125 mg CB2446/kg, an average inhibition of amino acid incorporation of, respectively, 47 (range: 42–52) and 67 (65–70) per cent was obtained. The inhibition produced by 75 mg CB2446/kg was counteracted by cysteamine (Table 4) to about the same extent as that produced by 100 mg DMNA/kg. The rate of N-demethylation of CB2446 and DMNA by the liver microsomes was found to be the same. Roughly speaking, CB2446 and DMNA appear to be about equally inhibitory on a weight basis, corresponding to a molar ratio of 1:2·6 and a N—N=O ratio of 2:2·6. This indicates that $^{+}H_3C—(CH_2)_4—CH_3^{+}$, produced from CB2446 *in situ*, is but little more inhibitory to microsomal amino acid incorporation than CH_3^{+}. Since there is, however, no evidence that the bifunctional agent is actually formed—either only one group of the molecule may be converted or hydrolysis at one part may precede the conversion of the other group—the present findings are not necessarily in disaccord with the well-known fact that bifunctional alkylating agents have a much more pronounced effect on tumour growth than monofunctional ones.

TABLE 4. COUNTERACTION BY CYSTEAMINE OF THE INHIBITION OF MICROSOMAL AMINO ACID INCORPORATION PRODUCED BY DMNA, DENA AND CB 2446

(Cysteamine regimens indicated in the text. R-rats were used in exp 1, 3, 4 and 6 and U × R rats in exp 2 and 5. In parentheses is listed the radioactivity (counts/min of an "infinitely" thick layer of protein) of the controls (microsomal-soluble preparations of livers of normal and cysteamine-treated rats) after incubation with 58 mμmole DL-leucine-1-^{14}C. Experiments carried out in duplicate as described previously.[2])

Nitrosamine (mg/kg)	Cysteamine (regimen)	Per cent inhibition of leucine-1-^{14}C incorporation					
		(exp. 1)	(exp. 2)	(exp. 3)	(exp. 4)	(exp. 5)	(exp. 6)
DMNA (100)	—	60 (1048 ± 22)	55 (953 ± 41)	66 (1052 ± 50)	56 (1088 ± 14)	59 (853 ± 2)	—
DMNA (100)	(*a*)	4 (940 ± 12)	9 (1127 ± 9)	0 (1015 ± 12)	—	—	—
DMNA (100)	(*b*)	47 (1261 ± 19)	—	—	34 (1121 ± 13)	46 (1054 ± 20)	—
DENA (200)	—	—	—	32	35	30	—
DENA (200)	(*a*)	—	—	0	2 (1345 ± 45)	0 (1139 ± 32)	—
DENA (200)	(*b*)	—	—	0 (1104 ± 16)	4	0	—
CB 2446 (75)	—	42	45	—	—	48	52 (1132 ± 7)
CB 2446 (75)	(*a*)	9	8	—	—	5	20 (1144 ± 4)
CB 2446 (75)	(*b*)	32	—	—	—	25	42 (1154 ± 20)

In vitro *addition of cysteamine.* Cysteamine (10–20 μmole/ml), added *in vitro* to the amino acid incorporation system of the microsomal-soluble fraction of the livers of rats treated with DMNA (4 hr), did not produce any significant reduction of the inhibition of this system (Table 5).

TABLE 5. LACK OF EFFECT OF ADDED CYSTEAMINE ON THE INHIBITION OF MICROSOMAL AMINO ACID INCORPORATION PRODUCED BY DMNA

DMNA	Cysteamine (μM/flask)	Per cent inhibition of leucine-1-^{14}C incorporation
100 mg/kg ..	—	57 (943 ± 19)
..	20	51 (945 ± 11)
160 mg/kg ..	—	70 (1102 ± 6)
..	10	65 (1070 ± 8)

In vivo *administration of cysteine.* In previous experiments[2] subcutaneous administration of excess cysteine (150 mg twice daily for 2 days) reduced the percentage inhibition of amino acid incorporation produced by DMNA but not that by DENA. In the present experiments either 75 mg cysteine was administered intraperitoneally followed after 1 hr by 50 mg cysteine intravenously (regimen *c*) plus DMNA (100 mg/kg) or DENA (200 mg/kg), or 75 mg cysteine was administered twice intravenously (regimen *d*), the second injection being accompanied by DMNA or DENA; the rats were killed 4 hr later. Table 6 shows that the inhibitory effects of both nitrosamines

TABLE 6. COUNTERACTION BY CYSTEINE OF THE INHIBITION OF MICROSOMAL AMINO ACID INCORPORATION PRODUCED BY DMNA AND DENA

(Cysteine administration according to regimen *c* (exp. 4) and *d* (exp. 1–3), mentioned in the text. Male R-rats were used in exp. 1 and 2 and U × R rats in exp. 3 and 4.)

Nitrosamine (mg/kg)	Cysteine	Per cent inhibition of leucine-1-^{14}C incorporation			
		(exp 1)	(exp 2)	(exp 3)	(exp 4)
DMNA (100)	−	65 (1120 ± 6)	—	62 (873 ± 13)	56 (930 ± 45)
DMNA (100)	+	39 (1172 ± 11)	—	44 (907 ± 4)	35 (905 ± 18)
DENA (200)	−	—	42 (1048 ± 28)	41	30
DENA (200)	+	—	23 (979 ± 16)	20	15

were reduced, that of DENA to a relatively greater extent than that of DMNA. Two conclusions can be drawn from these results. First, cysteine affords a smaller measure of protection against the toxic metabolites of the nitrosamines than does cysteamine. A possible reason for this difference is that less cysteine than cysteamine is present in the liver as a result of a difference in distribution and of SH-oxidation. Cystine, in contrast to cystamine, the oxidation product of cysteamine, does not protect against alkylating agents and radiation.[10] Secondly, the intravenous administration of cysteine protects to some extent against the inhibitory effect of DENA whereas subcutaneous administration, as applied previously, does not.

The mechanism by which cysteine reduces the inhibitory effect of the nitrosamines in the present experiments is considered (compare below) to be similar to that by which cysteamine acts and to be different from that operating in the case of DMNA and subcutaneous administration of excess cysteine in our previous experiments.[2] In discussing[1] the lack of effect of subcutaneous administration of excess cysteine on the inhibition of amino acid incorporation produced by DENA, the possibility was taken into account that S-ethylcysteine formed *in situ* (DENA → $C_2H_5^+$ + $^-$S-cysteine) was inhibitory to amino acid incorporation. However, it has now been found that 200 mg S-ethylcysteine/kg applied intraperitoneally produced only an insignificant inhibition of amino acid incorporation and did not affect the glycogen content of the liver 5–10 hr after injection.

TABLE 7. COUNTERACTION BY DMNA AND DENA OF THE GLYCOGENOLYSIS PRODUCED BY THE INTRAVENOUS ADMINISTRATION OF CYSTEAMINE AND CYSTEINE IN RAT LIVER

(Cysteamine and cysteine administration as mentioned in the text. Each value represents the mean liver glycogen content (with S.D.) of seven rats (male U × R rats in the case of cysteamine and 4 R and 3 U × R rats in the case of cysteine) measured in duplicate by the anthrone method.)

Treatment	Liver glycogen (% of fresh weight of liver)	Treatment	Liver glycogen (% of fresh weight of liver)
—	7·2 ± 0·5	—	6·9 ± 0·7
Cysteamine	3·7 ± 0·3	Cysteine	4·2 ± 0·6
Cysteamine + DMNA	5·8 ± 0·4	Cysteine + DMNA	5·9 ± 0·6
Cysteamine + DENA	5·1 ± 0·4	Cysteine + DENA	6·0 ± 0·7

Counteraction by N-nitrosodialkylamines of the glycogenolysis produced by sulphydryl compounds

Various investigators[14, 15] have observed a rapid fall in the glycogen content of liver following administration of sulphydryl compounds. This phenomenon was also found in the present experiments with cysteamine and cysteine. In male R-rats the normal glycogen content of 6·9 per cent (range: 5·8–7·7, in seven experiments) dropped to 1·6 (1·1–2·0, in five experiments) and 2·6 (1·9–2·8, in five experiments) per cent after application of the cysteamine regimens *a* and *b*, respectively. Cysteamine-induced glycogenolysis in male U × R rats was somewhat less pronounced. It has previously been reported[2] that DMNA and DENA produce also a decrease in the liver glycogen content. During the experimental period of 4 hr the latter decrease is less pronounced than that elaborated by cysteamine. If the toxic derivatives of DMNA and DENA react with the SH-groups of cysteamine, as was concluded from the counteraction of the inhibition of amino acid incorporation, the glycogenolytic effect of cysteamine might in turn also be reduced, provided that the concentration of the two reactants in liver were approximately the same. The results of experiments with regimen *a* were negative; this failure is attributed to the excess cysteamine remaining after reaction of the latter with the toxic derivatives of DMNA and DENA. As judged from the amino acid incorporation experiments the latter is not the case (or less so) following application of regimen *b*. Table 7 shows that DMNA and DENA markedly reduced

the glycogenolysis produced in the livers of U × R rats by the cysteamine regimen *b*. Reproducible results were only obtained with rats fed the standard laboratory diet fortified with wheat grains and receiving the injection of cysteamine early in the morning (8 a.m.); otherwise the glycogen contents of the individual rats varied too much. Similar results were also obtained with cysteine administered according to regimens *c* and *d* to male R and U × R rats. The outcome of these experiments suggest that a mutual elimination of sulphydryl compounds and nitrosamine derivatives may take place in the livers of animals treated with the dialkylnitrosamines and sulphydryl compounds.

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