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EFFECTS OF DISULFIRAM AND RELATED COMPOUNDS ON THE METABOLISM *IN VIVO* OF THE COLON CARCINOGEN, 1,2-DIMETHYLHYDRAZINE*

EMERICH S. FIALA, GEORGE BOBOTAS, CALLIE KULAKIS, LEE W. WATTENBERG and JOHN H. WEISBURGER

Naylor Dana Institute, Valhalla, NY 10595, and Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, MN 55455 (L.W.W.), U.S.A.

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Abstract—In view of the recent discovery that disulfiram (DF) and diethyldithiocarbamate (DDTC) inhibit the colon carcinogenicity of 1,2-dimethylhydrazine, a study of the effects of DF, DDTC, and the related compounds bis(ethylxanthogen) (BEX), carbon disulfide (CS_2), *sec*-butyldisulfide (SBDS), diethylamine (DEA) and triethylamine (TEA) on the metabolism of 1,2-dimethylhydrazine-[^{14}C] was undertaken. With respect to vehicle-treated control rats, DF, BEX, DDTC and CS_2 significantly increased the levels of the 1,2-dimethylhydrazine metabolite azomethane and decreased the levels of $^{14}CO_2$ in the exhaled air. These compounds significantly decreased the levels of urinary azoxymethane and methylazoxymethanol and also decreased the levels of ^{14}C in various rat organs. DEA, TEA and SBDS were ineffective in all these respects. It is concluded that DF, BEX, DDTC and CS_2 inhibit the *N*-oxidation *in vivo* of azomethane to azoxymethane, an essential step in the metabolic activation of 1,2-dimethylhydrazine. It appears likely that the effective agent in the inhibition of the *N*-oxidation of axomethane by DF, BEX and DDTC is CS_2, a metabolic product of these compounds.

1,2-Dimethylhydrazine is a powerful carcinogen with a high degree of specificity for producing cancer of the large intestine in various rodent species [1–3]. Typically, 10–16 weekly s.c. injections of this carcinogen at a dose level of *ca.* 20 mg/kg to either rats or mice will yield a near 100 per cent of colon tumors within a further period of 2–3 months. In order to exert its effects, 1,2-dimethylhydrazine must be metabolically activated. Druckrey *et al.* [1, 4] have postulated that the activation sequence is as follows: 1,2-dimethylhydrazine → azomethane → azoxymethane → methylazoxymethanol.

Methylazoxymethanol is unstable under physiological conditions and breaks down to form formaldehyde and methyldiazonium hydroxide, a powerful alkylating agent. This pathway, in fact, appears to function *in vivo*, since azomethane-[^{14}C] has been detected in considerable quantities in the exhaled air of rats given 1,2-dimethylhydrazine-[^{14}C] [5]. Also, azoxymethane-[^{14}C] and methylazoxymethanol-[^{14}C] were detected in small amounts in the urine of these animals [6].

Recently, the discovery was made that the carcinogenicity of 1,2-dimethylhydrazine can be abolished by the concomitant administration of disulfiram (DF) or diethyldithiocarbamate (DDTC) in the diet [7, 8]. Preliminary studies on the effects of DF on the metabolism of 1,2-dimethylhydrazine have shown that DF causes increased levels of azomethane in the exhaled air and decreases levels of 1,2-dimethylhydrazine metabolites in the urine of rats [9, 10].

DF, *in vivo*, is rapidly metabolized to DDTC, diethylamine (DEA) and carbon disulfide (CS_2) [11–14]. Since DDTC, CS_2 as well as DF possess considerable biological activity in that they inhibit a large number of enzymes and enzyme systems (see Discussion), it was of interest to examine and compare the effects of the DF metabolites DDTC, DEA and CS_2 with the parent compound. Also examined in this study are the DF analogues bis(ethylxanthogen) (BEX), in which the —N< of DF is replaced by an —O— group and *sec*-butyldisulfide (SBDS) which contains the —S—S— of DF but lacks the >C=S group. Since DF is a tertiary amine, triethylamine (TEA) was also tested as an additional control. The structural relationships of the compounds examined are shown in Fig. 1.

MATERIALS AND METHODS

Chemicals. 1,2-Dimethylhydrazine, DF, CS_2, SBDS and TEA were obtained from Aldrich Chemical Co. (Metuchen, NJ). DEA was obtained from Fisher Scientific Co. (Springfield, NJ). BEX was synthesized by the method of Losse and Wottgen [15].† The sodium salt of DDTC was obtained from Eastman (Rochester, NY). ^{14}C-labeled, 1,2-dimethylhydrazine was obtained from New England Nuclear Corp., and its radiochemical purity was verified by thin-layer chromatography prior to use [16].

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† BEX is currently under study for its capacity to inhibit the carcinogenic effects of 1,2-dimethylhydrazine and methylazoxymethanol acetate in mice (L. A. Watterberg, A. Fladmoe and L. Lam, work in progress).

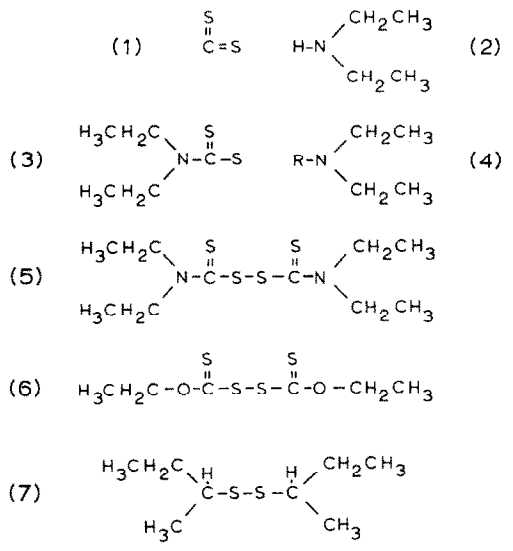

Fig. 1. Structural formulas of the compounds examined for their effects on the metabolism of 1,2-dimethylhydrazine-[^{14}C]: (1) carbon disulfide; (2) diethylamine; (3) diethyldithiocarbamate; (4) triethylamine (R = —CH_2CH_3); (5) disulfiram; (6) bis(ethylxanthogen); and (7) *sec*-butyl-disulfide.

Animals. Male F344 rats, mean body weight 240 g, obtained from Charles River Breeding Laboratories (Wilmington, MA), were maintained on Purina Lab Chow and water. The animals were housed in plastic cages under conditions of constant temperature and humidity with a light–dark cycle of 12 hr.

Administration of compounds. After an overnight fast with water available *ad lib.*, DF, DDTC or SBDS were administered, by stomach tube, as suspensions in 1.5 ml of 4% starch [17]. BEX, DEA, TEA and CS_2 were administered, by the same route, as solutions in corn oil. Control animals received 1.5 ml of the appropriate vehicle. Except for DF which was given at a dose of 3.4 m-moles/kg, all other compounds were given at a dose level of 1.1 m-moles/kg. BEX, in addition, was given at a level of 0.82 m-mole/kg in some experiments.

Administration of 1,2-*dimethylhydrazine*-[^{14}C]. Two hr after the oral administration of the test compounds or vehicle (controls), the rats were injected s.c. with 21 mg (calculated as free base)/kg of 1,2-dimethylhydrazine-[^{14}C] in 1.5 ml of aqueous solution, pH 6.4, containing ethylenediaminetetra-acetic acid (7 mg/100 ml). The amount of radioactivity used per rat was 10 μCi.

Determination of expired azomethane-[^{14}C] *and* $^{14}CO_2$. The procedures used were exactly as described previously [5]. Briefly, immediately after dosing with 1,2-dimethylhydrazine-[^{14}C], the rats were placed in glass metabolism cages. Air from the cage was passed through three gas washers in series. The first and third gas washer in the train contained, respectively, ethanol cooled to −72° and 1 N H_2SO_4, to trap azomethane-[^{14}C]. The contents of the ethanol gas washer were changed hourly for the first 6 hr. The second gas washer in the train contained 1 N NaOH to trap $^{14}CO_2$. This gas washer, as well as the one containing 1 N H_2SO_4, was sampled every hr and the contents were renewed after 6 hr.

Determination of radioactivity. The ^{14}C content of urine was determined by dissolving duplicate 10-μl aliquots directly in Scintisol (Isolab Incorp., Akron, OH). Selected rat organs, obtained 24 hr after the administration of 1,2-dimethylhydrazine-[^{14}C] were weighed, homogenized in 0.25 M sucrose and aliquots were solubilized in NCS (Amersham Searle Corp.). In the case of colons, only the mucosal tissue layer was used. This was obtained by cleansing the colons with cold saline, slitting the organ longitudinally and scraping the mucosal layer with a spatula on a glass plate cooled to 0°.

The solubilized homogenates were dissolved in a toluene PPO–POPOP* mixture. Radioactivity was determined by liquid scintillation counting with appropriate quench corrections established by the external standard-channels ratio method.

Urinary metabolites of 1,2-*dimethylhydrazine*-[^{14}C]. Urine samples were collected and frozen as soon after excretion as possible. All samples were analyzed within 48 hr after collection. As determined in separate runs, no appreciable deterioration of 1,2-dimethylhydrazine metabolites occurred within this period when the urines were stored at −20°. Aliquots (100 μl) of thawed, centrifuged urine samples were analyzed by high pressure liquid chromatography on Aminex A-27 (acetate$^-$) columns as described previously [6] but with the following modifications. Two main columns, each $\frac{3}{8}$ × 24 in., in series, were preceded by a short ($\frac{3}{8}$ × 2$\frac{1}{4}$ in.) removable precolumn. All three columns were packed with Aminex-27 resin in the acetate form. The inclusion of a removable precolumn resulted in a greatly increased working life of the main columns, and the use of two rather than one main separating column, as in our original procedure, gave greater resolution, especially of the early eluting peaks. The columns were eluted at 1 ml/min with 0.01 M sodium acetate, pH 5.6. Fractions of 1 ml were collected directly in Scintisol. When present, urinary azoxymethane and methylazoxymethanol were verified by comparison of elution volumes with standard compounds and by rechromatography on μBondapak C_{18} [6].

RESULTS

Control animals receiving either corn oil or 4% starch 2 hr prior to the administration of 1,2-dimethylhydrazine-[^{14}C] exhale approximately 14 per cent of the radioactivity in the form of azomethane-[^{14}C]. Dosing with either SBDS, DEA or TEA does not significantly affect the amount of azomethane-[^{14}C] exhaled. In contrast, pretreatment of animals with DF, DDTC, BEX or CS_2 increases the amount of azomethane-[^{14}C] 2- to 3-fold. Approximately 13–16 per cent of the 1,2-dimethylhydrazine-[^{14}C] dose is exhaled by the control animals as $^{14}CO_2$. Again, pretreatment with SBDS, DEA or TEA has no significant effect on $^{14}CO_2$ excretion. Pretreatment with DF, DDTC, BEX and CS_2, however, causes an inhibition of approximately 65–80 per cent. These data are shown in Table 1.

* PPO = 2,5-diphenyloxazole; and POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene.

Table 1. Effects of pretreatment with DF and related compounds on the 24-hr excretion of radioactivity derived from 1,2-dimethylhydrazine-[^{14}C] in the expired air and in the urine of CDF rats*

Compound	No. of animals	Mol. wt	Dose (mg/kg)	% Dose radioactivity (±S. E. M.) Expired azomethane	Expired CO_2	Urine
DF	3	296.5	1000	36 ± 2	2.1 ± 0.3	13 ± 1
DDTC†	3	225.3	250	35 ± 1	2.6 ± 0.4	10 ± 3
CS_2	3	76.1	85	44 ± 1	2.8 ± 0.3	11 ± 4
BEX	2	242.0	198	27 ± 3	5.2 ± 1.6	12 ± 3
BEX	3		269	45 ± 1	2.8 ± 0.7	20 ± 2
SBDS	3	178.4	198	14 ± 1	13.0 ± 1.0	17 ± 1
DEA	2	73.1	73	16 ± 1	17.0 ± 1.0	18 ± 1
TEA	3	101.2	112	17 ± 2	16.0 ± 3.0	18 ± 1
Corn oil	2		1.5 ml	14 ± 1	13.0 ± 2.0	17 ± 1
4% Starch	2		1.5 ml	15 ± 1	16.0 ± 1.0	17 ± 1

* DF, DDTC and SBDS were given by stomach tube as suspensions in 1.5 ml of 4% starch solution. BEX, DEA, TEA and CS_2 were administered by the same route as solutions in 1.5 ml corn oil. Two hr later, 1,2-dimethylhydrazine-[^{14}C] was given s.c. at 21 mg/kg.

† The trihydrate of the sodium salt of DDTC was used.

The time courses of the appearance of exhaled azomethane-[^{14}C] and $^{14}CO_2$ derived from 1,2-dimethylhydrazine-[^{14}C] in animals pretreated with SBDS and CS_2 are shown in Fig. 2. While only these two compounds were selected for this illustration, the time courses for DEA, TEA, 4% starch controls and the corn oil controls were essentially identical to the SBDS-treated animals and overlap the respective curves for azomethane-[^{14}C]. Similarly, the effects of DF, DDTC and BEX were essentially identical to those obtained with CS_2.

Considerable quantitative variations in the 24-hr urinary output of ^{14}C derived from 1,2-dimethylhydrazine-[^{14}C] were observed among individual rats in each group pretreated with a given test compound as well as between the different groups (Table 1). When analyzed by high pressure liquid chromatography, however, the urines could be divided into two classes with respect to 1,2-dimethylhydrazine metabolites. In one class, characteristic of animals pretreated with SBDS, DEA, TEA, corn oil or 4 per cent starch, a complex pattern was observed which included non-metabolized 1,2-dimethylhydrazine-[^{14}C], azoxymethane-[^{14}C] and methylazoxymethanol-[^{14}C]. In the second class, characteristic of animals pretreated with DF, DDTC, BEX and CS_2, greatly decreased levels of 1,2-dimethylhydrazine metabolites were present in the 24-hr urines. In particular, azoxymethane and methylazoxymethanol were greatly reduced or completely absent. Figure 3 shows the urinary high pressure liquid chromatographic elution patterns for animals receiving SBDS and corn oil. These patterns are essentially identical to those obtained from the urines of animals receiving DEA, TEA and 4% starch. Also shown in Fig. 3 are elution patterns of urines from animals pretreated with CS_2 and BEX. These patterns are also characteristic of urines from animals pretreated with DF and DDTC.

The effects of pretreatment with the various test compounds on the ^{14}C content of selected organs 24 hr after the administration of 1,2-dimethylhydrazine-[^{14}C] is shown in Table 2. The data show that less ^{14}C is present in the organs of rats pretreated with DF, DDTC, BEX or CS_2 than in the corresponding organs of rats pretreated with SBDS, DEA, TEA or control vehicles. For the liver, the difference is from 69–70 per cent. For the kidneys, it is 43–59 per cent; for the lungs, 49–62 per cent; for the spleen, 42–62 per cent; and for the colon mucosa, 54–70 per cent.

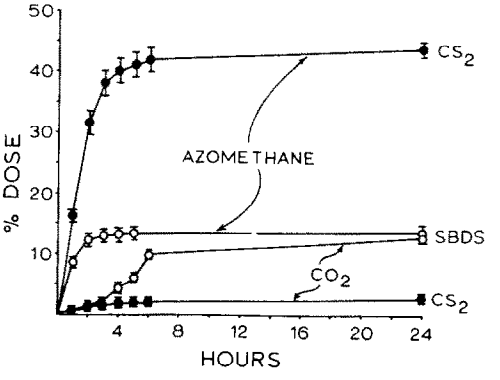

Fig. 2. Time course for the appearance of [^{14}C]azomethane and $^{14}CO_2$ in the exhaled air of CDF rats pretreated with CS_2 or SBDS and 2 hr later injected with 1,2-dimethylhydrazine-[^{14}C] (21 mg/kg). Azomethane and CO_2 were determined as described in Methods. Each point represents the mean ± S. E. of three separate experiments each using one animal.

DISCUSSION

The results presented here show that DF, DDTC, BEX and CS_2 have in common the following effects on the metabolism of 1,2-dimethylhydrazine-[^{14}C]: (1) they increase the amount of exhaled azomethane-[^{14}C]; (2) they decrease the amount of exhaled $^{14}CO_2$; (3) they reduce the levels of azoxymethane-[^{14}C] and methylazoxymethanol-[^{14}C] in the urine; and (4) they decrease ^{14}C tissue levels. The similarities in their effects suggest that these compounds share a common locus of action.

With regard to the enhanced appearance of axomethane-[^{14}C] in the exhaled air, this increase can-

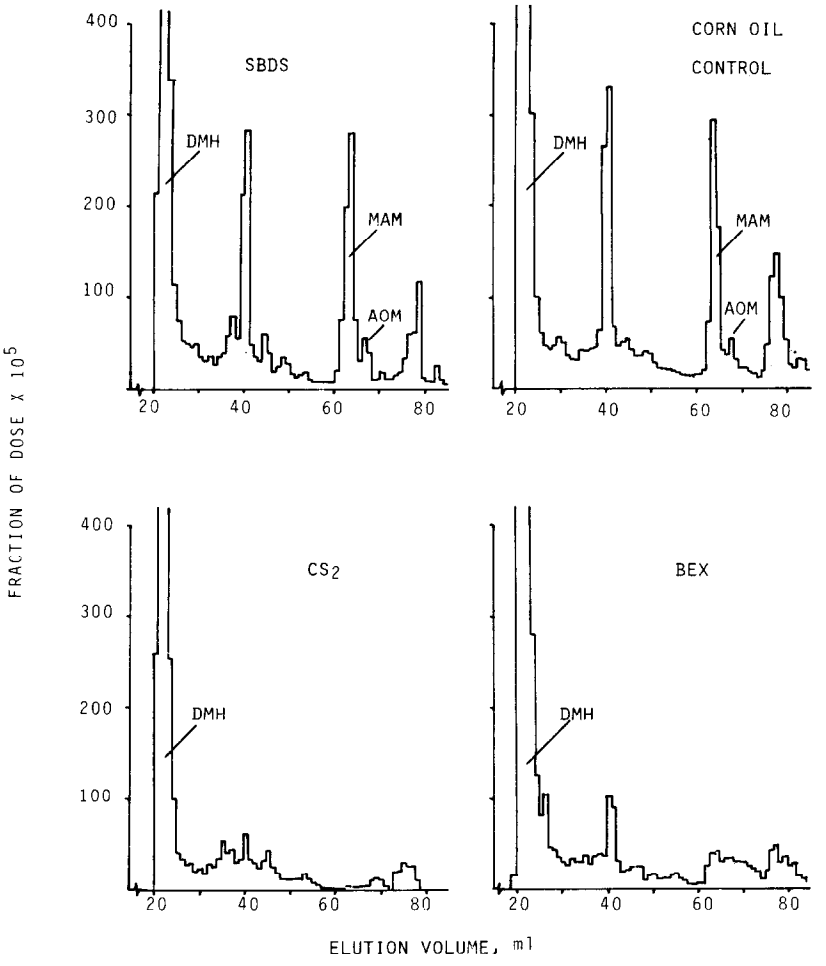

Fig. 3. High pressure liquid chromatographic profiles of 24-hr urines of CDF rats pretreated with SBDS, corn oil vehicle, CS_2 or BEX and 2 hr later with 1,2-dimethylhydrazine (21 mg/kg). The large early eluting peak (21–25 ml) is due largely to non-metabolized 1,2-dimethylhydrazine-[^{14}C] (DMH). Methylazoxymethanol-[^{14}C] (MAM) and azoxymethane-[^{14}C] (AOM) elute at approximately 61–65 ml and approximately 66–69 ml respectively. The other peaks in the profiles have not as yet been identified. Profiles for SBDS and corn oil vehicle control are essentially identical to those obtained from rats pretreated with DEA, TEA or 4% starch. Profiles for CS_2 and BEX are essentially identical to those obtained after pretreatment with DSF or DDTC. For technical details, see Methods.

Table 2. Tissue distribution of radioactivity derived from 1,2-dimethylhydrazine-[^{14}C] in animals pretreated with DF and related compounds*

Compound	No. of animals	Liver	Kidney	Lung	Spleen	Colon mucosa
DF	3	0.09 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
DDTC	3	0.06 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
CS_2	3	0.08 ± 0.01	0.08 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.01
BEX†	2	0.15 ± 0.07	0.10 ± 0.02	0.05 ± 0.02	0.06 ± 0.02	0.07 ± 0.02
BEX‡	3	0.09 ± 0.01	0.10 ± 0.01	0.05 ± 0.01	0.04 ± 0.02	0.05 ± 0.01
SBDS	3	0.37 ± 0.07	0.19 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.15 ± 0.02
DEA	2	0.35 ± 0.19	0.21 ± 0.01	0.11 ± 0.01	0.13 ± 0.03	0.22 ± 0.08
TEA	3	0.35 ± 0.04	0.18 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.16 ± 0.02
Corn oil	2	0.38 ± 0.02	0.18 ± 0.01	0.10 ± 0.01	0.10 ± 0.09	0.15 ± 0.22
4% Starch	2	0.39 ± 0.09	0.16 ± 0.03	0.13 ± 0.09	0.08 ± 0.06	0.13 ± 0.09

* The same animals used to obtain the data in Table 1 were killed 24 hr after the administration of 1,2-dimethylhydrazine-[^{14}C]. Tissues were excised, homogenized and the ^{14}C content was determined as described in Methods. Triplicate determinations of ^{14}C on each tissue homogenate were done. Values represent per cent dose ^{14}C/g wet weight tissue.

† BEX at 198 mg/kg.

‡ BEX at 269 mg/kg.

not be accounted for by increased oxidation of 1,2-dimethylhydrazine. Such an increase in oxidation would lead to a greater availability of substrate for conversion to azoxymethane, methylazoxymethanol and further products. This, in turn, would lead to an increased excretion of these metabolites in the urine, and to increased tissue binding of ^{14}C. Even if the metabolic enzymes were saturated, these parameters could in no way be of lesser magnitude than in the control animals. But this is in contradiction to the observed results. Thus, a more plausible explanation for the effects of these compounds is that their primary effect is to block the *N*-oxidation of azomethane to azoxymethane. This mechanism fits the observed effects since such a block would not only cause azomethane to accumulate and be excreted in greater amounts in the expired air, but also results in decreased urinary excretion of metabolites and decreased tissue binding.

With regard to the production of $^{14}CO_2$ from 1,2-dimethylhydrazine-[^{14}C] by control rats and rats pretreated with DEA, TEA or SBDS, much of the $^{14}CO_2$ exhaled is presumably derived from the oxidation of formaldehyde-[^{14}C] [18]. The formation of formaldehyde *in vitro* has been observed during the oxidative demethylation of 1,2-dimethylhydrazine [19] and also of azoxymethane [4]. The spontaneous breakdown of methylazoxymethanol also leads to formaldehyde as one of the products [20]. Unfortunately, not enough data are as yet available to permit the evaluation of the relative importance of these pathways *in vivo*.

After pretreatment with DF, DDTC, BEX and CS_2, the amount of $^{14}CO_2$ exhaled is significantly decreased (Table 1). Such a decrease could result from decreased production of formaldehyde-[^{14}C], from a decrease in its oxidation or both. Since DF is known to inhibit aldehyde dehydrogenase *in vitro* and both DF and DDTC inhibit the enzyme *in vivo* [21, 22], it might be expected that the oxidation of formaldehyde to CO_2 by way of formate could be decreased in rats treated with these compounds. However, we known of no reports in the literature describing the inhibition of aldehyde dehydrogenase *in vivo* by either CS_2 or BEX.

The block imposed on the *N*-oxidation of azomethane to azoxymethane by DF, DDTC, BEX and CS_2 would decrease the formation of the ultimate [23] carcinogenic metabolite of 1,2-dimethylhydrazine, the methyldiazonium ion, by way of methylazoxymethanol. Thus, a block at this level would be sufficient as an explanation for the inhibition of 1,2-dimethylhydrazine carcinogenicity as observed previously for DF and DDTC [7, 8]. However, it is possible that DF, DDTC, BEX and CS_2, besides inhibiting the *N*-oxidation of azomethane, could also exert additional blocks on 1,2-dimethylhydrazine metabolism. DF, DDTC and, in particular, CS_2 have been found to inhibit the hepatic mixed function oxidase activity and to decrease liver cytochrome P-450 [24–28]. Specifically, inhibitions of *N*- and *O*-demethylations, as well as C-hydroxylations by these compounds have been demonstrated [25, 27, 29–32]. Thus, these additional blocks could consist of the inhibition of oxidative demethylations of 1,2-dimethylhydrazine and azoxymethane as well as inhibition of the hydroxylation of azoxymethane to methylazoxymethanol. We have recently obtained preliminary data indicating that the formation of methylazoxymethanol-[^{14}C] through the hydroxylation of azoxymethane-[^{14}C] is indeed depressed *in vivo* when the latter compound is administered to rats pretreated with DF or CS_2 [33]. However, it appears that this step, as well as the inhibition of demethylation of azoxymethane, is of only minor importance relative to the inhibition of *N*-oxidation of azomethane in the case when 1,2-dimethylhydrazine is administered, simply because insufficient quantities of azoxymethane are formed. This is evident from Fig. 3. Incomplete inhibition of formation of azoxymethane together with inhibition of its further metabolism would result in its appearance in the urine. The absence of discrete peaks due to azoxymethane in the urines of rats pretreated with DF, DDTC, BEX or CS_2 indicates that this is not the case.

The effects of DF, DDTC, BEX and CS_2 reported here are not obtained with the DF metabolite DEA, the tertiary amine TEA or with the disulfide analog, SBDS. This indicates that the common structural denominator of the effective compounds consists of the thiono-sulfur group. The mechanism of enzyme inhibition due to DF and BEX *in vitro* may be ascribed to the formation of mixed disulfides with protein-SH groups [21, 34, 35] and that of DDTC to metal ion chelation [36, 37]. However, in view of the effectiveness of CS_2 with regard to the results reported in the present communication, we tentatively conclude that neither of these mechanisms plays a major role in the inhibition of the metabolism or the carcinogenicity of 1,2-dimethylhydrazine. While the conversion of CS_2 to xanthates or dithiocarbamates by reaction with alcohols or amines, respectively, *in vivo* with subsequent oxidation to the disulfide form cannot be excluded, it would appear that a simpler explanation for the effects described here is that DF, DDTC and BEX are all metabolized to CS_2, and that the latter, perhaps after further metabolism to carbonyl sulfide [28, 38], represents the effective inhibitor.

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