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EFFECT OF GLUTATHIONE DEPLETION ON AMINOPYRINE AND FORMALDEHYDE METABOLISM*

HARSHIKA S. BHATT, STEPHEN B. LOBER† and BURTON COMBES‡
Liver Unit, Department of Internal Medicine, University of Texas Health Science Center at Dallas, Southwestern Medical School, Dallas, TX 75235, U.S.A.

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Abstract—In previous studies, diethylmaleate (DEM)- and phorone-induced hepatic glutathione (GSH) depletion in rats was accompanied by impaired evolution of $^{14}CO_2$ from the *N*-^{14}C-labeled methyl groups of aminopyrine, which in turn was attributed to impaired generation of formaldehyde, its subsequent oxidation to formate, or to some combination of both. In the present study, *l*-buthionine sulfoximine (BSO)-induced hepatic GSH depletion was also accompanied by decreased evolution of CO_2 from aminopyrine, but the extent of the fall in CO_2 was less than that induced by DEM or phorone, even though the decrease in hepatic GSH was comparable with all three GSH-lowering compounds. Incubation of freshly prepared normal hepatic microsomes *in vitro* with the GSH-lowering agents resulted in impaired aminopyrine-*N*-demethylase (APDM) activity with inhibition by phorone > DEM > BSO. By contrast, hepatic microsomes prepared from rats pretreated with these compounds had normal APDM activity. $^{14}CO_2$ evolution from i.p. administered [^{14}C]formaldehyde was not impaired by any of the GSH-lowering compounds. Thus, assessment of APDM activity and formaldehyde metabolism did not unequivocally establish the mechanism(s) by which CO_2 evolution from aminopyrine is depressed by DEM, phorone and BSO, although low GSH is likely to impair metabolism of formaldehyde formed in liver after demethylation of aminopyrine. Quantitative differences in the degree of depression of CO_2 evolution suggest that at least DEM and phorone exert an additional inhibitory effect by a GSH-independent mechanism. This may involve inhibition of aminopyrine-*N*-demethylase activity.

We recently reported that evolution of $^{14}CO_2$ from the [^{14}C-*methyl*]-labeled dimethylamino portion of aminopyrine was impaired in rats when their hepatic glutathione (GSH§) content was lowered by administration of either diethylmaleate (DEM) or phorone [1]. Metabolism of the ^{14}C-labeled methyl groups of aminopyrine involves cytochrome P-450 catalyzed N-demethylation with formation of formaldehyde, oxidation of formaldehyde to formate, and subsequent oxidation of formate to $^{14}CO_2$ [2–10]. Since DEM- or phorone-induced GSH depletion did not affect CO_2 evolution from administered formate or bicarbonate, it was concluded that the GSH-dependent step involves generation of formaldehyde, its subsequent oxidation to formate, or some combination of both [1]. The present studies were carried out to assess these possibilities.

DEM and phorone lower hepatic GSH by interacting with the SH group of GSH to form a GSH conjugate. It seemed possible that DEM and phorone could also interact with SH groups in proteins involved in the overall metabolism of the pertinent methyl groups of aminopyrine and lower CO_2 evolution by a GSH-independent mechanism. To gain insight into this possibility, studies were carried out in animals in whom hepatic GSH was lowered by inhibiting GSH biosynthesis with *l*-buthionine sulfoximine (BSO) [11], a compound that does not appear to interact with the SH groups of GSH. Rather, BSO is a potent and specific inhibitor of gamma-glutamyl-cysteine synthetase (EC 6.3.2.2), the enzyme that catalyzes the first step in the eventual formation of GSH.

MATERIALS AND METHODS

Experimental procedures

Aminopyrine and formaldehyde breath tests. Male Sprague–Dawley rats (Sasco Inc., Omaha, NE) weighing 150–220 g were allowed access to food and water *ad lib.* until the morning of the study. Food was then withheld throughout the study period. After any pretreatment (as indicated below), rats received an i.p. injection of a ^{14}C-labeled compound (aminopyrine, formaldehyde), and they were then placed immediately into a chamber to permit collection of exhaled ^{14}C-labeled CO_2 as described previously [1]. Exhaled CO_2 was collected at 15-min intervals, for a total of 120 min, in glass scintillation vials containing 10 ml of methanol–ethanolamine (2:1, v/v). After the addition of 10 ml of aqueous counting scintillant, trapped $^{14}CO_2$ was counted in a Packard liquid scintillation spectrometer (model 3255). Quenching was corrected for by external standardization. Recovery of ^{14}C-labeled compound as $^{14}CO_2$, peak exhalation

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‡ Correspondence should be sent to: Burton Combes, M.D., Department of Internal Medicine, UTHSCD, Southwestern Medical School, 5323 Harry Hines Boulevard, Dallas, TX 75235.

§ Abbreviations: APDM, aminopyrine-*N*-demethylase; BSO, *l*-buthionine sulfoximine; DEM, diethylmaleate; DMSO, dimethyl sulfoxide; GSH, glutathione; and K_{el}, elimination rate constant.

rate, and the elimination rate constant were calculated as described previously [1].

Glutathione depletion. Hepatic GSH was lowered by i.p. administration of one of three compounds. DEM (430 μmol/100 g body weight, mixed 1:1 with corn oil) and phorone (180 μmol/100 g body weight, delivered in 100 μl corn oil) lower GSH by trapping it as a GSH conjugate with the respective compound. Previous studies in this laboratory demonstrated a rapid fall in GSH of approximately 80% within 15 min of administration of these doses of DEM [12], or phorone [1], with subsequent depression of this magnitude maintained for at least 2–3 hr. [^{14}C]Formaldehyde was administered 30 min after DEM or phorone in the present studies for performance of a 2-hr formaldehyde breath test. A comparable volume of corn oil was given i.p. to rats serving as controls in these experiments.

GSH was also lowered by i.p. administration of BSO, an inhibitor of GSH biosynthesis [11]. In initial studies, we assessed the effect of various doses of BSO [administered in 1.0 ml of isotonic saline (pH 8.5) per 100 g body weight] on hepatic GSH. Maximal depression of GSH was observed 4 hr later with persistence of low values for at least 4 hr with a BSO dose of 800 μmol/100 g body weight. In subsequent studies, BSO was given i.p.; 4 hr later either [^{14}C]aminopyrine or [^{14}C]formaldehyde was administered i.p. and 2-hr breath tests were carried out. Animals serving as controls received saline instead of BSO i.p. but otherwise were handled in the same way.

At the completion of the respective breath tests, animals were killed (approximately 150–160 min after administration of DEM and phorone, and 360–370 min after administration of BSO), and their livers were removed for determination of GSH content. GSH was measured by the dithionitrobenzene method of Beutler *et al.* [13].

Measurement of aminopyrine-N-demethylase activity. The effects of DEM, phorone and BSO on microsomal aminopyrine-*N*-demethylase (APDM) activity were assessed in two ways. The first involved giving the respective GSH-lowering compound to rats *in vivo* as described above. Animals were killed 45 min after administration of DEM or phorone, and 240 min after BSO. The livers were removed and microsomes were isolated by standard methods [14]. It took approximately 2 hr to prepare the microsomes. Microsomal APDM activity was then assessed by a modification of the method of Cochin and Axelrod [15] utilizing the Nash procedure [16] to measure the amount of formaldehyde formed during N-demethylation of aminopyrine. In the second method, DEM, phorone or BSO was added directly *in vitro* to microsomes prepared from normal rat liver. Microsomes (approximately 3 mg protein in 150 μl) added to 2.7 ml of 100 mM Tris–HCl, pH 7.4, buffer were mixed with the respective compounds. To simulate the duration of exposure of microsomes to these agents *in vivo*, mixtures containing DEM and phorone were allowed to incubate at room temperature for 30 min, and BSO for 240 min, prior to ADPM assay, which was initiated by addition of 1 mM NADPH. Globules of DEM and phorone were detected in the incubation mixtures. Thus, microsomes were exposed to lower concentrations of these compounds than added. Solubilizing agents such as DMSO and methyl alcohol were not used since they increased blank readings and interfered with the assay. Microsomal protein was measured by a biuret method [17].

Tissue distribution of ^{14}C of formaldehyde, formate and aminopyrine. Various doses of ^{14}C-labeled compound were administered i.p. or i.v. to rats. The animals were anesthetized at intervals with ether, their abdomens were opened, and they were exsanguinated by withdrawing as much blood as possible from the aorta. Various organs were removed, weighed, and washed in saline, and duplicate or triplicate aliquots of tissue were placed in 20-ml glass scintillation vials with 300 μl of 1.0 N NaOH and 3 glass beads (3 mm size). Vials were shaken on a multi-tube vortex (SMI) unit until there was complete solubilization of tissue. Once solubilized (approximately 24 hr), 400 μl of 1 N HCl was added to the digested tissue, followed by thorough mixing, addition of 10 ml of RIA-Solve II, and further shaking. Additional aliquots of 1 N HCl were added when necessary to clear the scintillant. Counting vials were precooled for at least 60 min before counting in the Packard liquid scintillation spectrometer. Radioactivity per g tissue, or per 100 g body weight, was then calculated. No correction was made for the radioactivity contained in the small amounts of blood remaining in the tissues of these exsanguinated animals.

Effect of S-methylcysteine on $^{14}CO_2$ evolution from ^{14}C-labeled aminopyrine, formaldehyde and formate. Kornbrust and Bus [18] found that CO_2 evolution from the methyl group of CH_3Cl is inhibited when animals are pretreated with *S*-methylcysteine. In the present studies, *S*-methylcysteine in saline was administered subcutaneously twice, as described by Kornbrust and Bus, in doses of 400 mg/kg, 30 min and immediately before the i.p. administration of the respective labeled compounds. Control rats received comparable volumes of saline s.c. The rats were then placed immediately in the apparatus for collection of expired $^{14}CO_2$.

Calculations and statistics

All values presented in the tables, unless indicated otherwise, are mean ± SD. $^{14}CO_2$ exhalation appeared to fall exponentially from 60 to 120 min after i.p. administration of the [^{14}C]formaldehyde and [^{14}C]aminopyrine. An elimination rate constant (K_{el}) was calculated for each rat by a least-square regression analysis of the logarithm of $^{14}CO_2$ collected with respect to time over the 60- to 120-min interval. The significance of the difference of mean data was calculated by Student's *t*-test. Due to the small sample size, Welch's approximation to the Student's *t*-test based on non-parametric analysis was used [19]. Statistical analyses were performed using the ISP programs on a DEC-10 system at the Medical Computing Resources Center, UTHSCD.

Chemicals

Unlabeled aminopyrine (4-dimethylamino-antipyrine), ethanolamine (2-aminoethanol), glutathione, D-glucose-6-phosphate, glucose-6-phosphate

dehydrogenase-Type VII (G-6-PDH), *S*-methyl-*l*-cysteine, sucrose, Tris, barium hydroxide, zinc sulfate, ammonium acetate and acetylacetone were from the Sigma Chemical Co., St. Louis, MO. NADP was obtained from P-L Biochemicals, Inc., Milwaukee, WI. Phorone (diisopropylidene acetone), unlabeled formaldehyde and DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] were obtained from the Aldrich Chemical Co., Inc., Milwaukee, WI. DEM was from Matheson, Coleman & Bell, Cincinnati, OH. *l*-Buthionine-*S*,*R*-sulfoximine (BSO) was from the Chemical Dynamics Corp., South Plainfield, NJ. [^{14}C]Formaldehyde (40 mCi/mmol) was obtained from ICN Chemical and Radioisotope Division, Irvine, CA. Dimethylamino[^{14}C]aminopyrine (114 mCi/mmol), sodium [^{14}C]formate (59 mCi/mmol), and aqueous counting scintillant (ACS) were from Amersham, Arlington Heights, IL. RIA-Solve II was obtained from the RPI Corp., Mount Prospect, IL.

RESULTS

Studies with l-buthionine-S,R-sulfoximine (BSO)

Effects on hepatic GSH. Single doses of BSO (200, 400 or 800 μmol per 100 g body weight) were administered i.p. at time 0. Values for hepatic GSH are presented in Table 1. The greatest fall in GSH was documented with the 800 μmol dose. GSH reached a low point at 4 hr and persisted at this low level for the ensuing 4 hr. Lower doses of BSO resulted both in less of a fall and in a slower rate of decline in hepatic GSH. The BSO effect was no longer apparent at 24 hr. Hepatic GSH in saline control animals was now approximately half of initial values, presumably the result of 24 hr of starvation. Similar GSH values were also present in the animals treated earlier with BSO.

Effects on aminopyrine breath test. In these studies, 800 μmol BSO per 100 g body weight was given i.p. Four hours later, [^{14}C]aminopyrine (75 μmol/100 g body weight) was administered and a 2-hr breath test carried out. The data presented in Table 2 indicate that the peak exhalation rate and cumulative exhalation of CO_2 as well as the percent recovery of administered aminopyrine dose as exhaled CO_2 were decreased significantly in rats pretreated with BSO. However, the degree of inhibition of CO_2 evolution in BSO-pretreated rats was not as great as that observed in our earlier studies with DEM and phorone [1] even though all three compounds lowered hepatic GSH to a comparable extent. This suggested that DEM and phorone might influence evolution of CO_2 from aminopyrine by additional mechanisms that are GSH-independent.

Effects of DEM, phorone and BSO on aminopyrine-N-demethylase activity (APDM)

In vitro *addition of compounds to microsomes.* Microsomes freshly prepared from normal rats were incubated with DEM, phorone or an equivalent volume of buffer for 30 min prior to assay. Both DEM and phorone induced significant decreases in APDM activity (Fig. 1A). Addition of BSO *in vitro* for 4 hr to freshly prepared microsomes from control rats led

Table 1. Effect of *l*-buthionine-*S*,*R*-sulfoximine (BSO) on hepatic GSH content*

BSO dose in saline (μmol/100 g body wt)	Hepatic GSH (μmol/100 g body wt)								
	Time in hours after intraperitoneal injection of BSO								
	0	2	3	4	5	6	7	8	24
Saline control	24.1 ± 3.2	26.8 ± 4.8	21.7 ± 3.7	21.2 ± 2.5	19.8 ± 2.1	17.9 ± 1.1	20.5 ± 1.4	20.3 ± 3.0	11.3 ± 1.5
200		11.8 ± 4.7		14.6 ± 1.8		8.4 ± 1.9		9.1 ± 1.7	10.6 ± 0.8
		P = 0.02		P = 0.02		P = 0.004		P = 0.01	P = 0.5
400		15.3 ± 3.3		11.9 ± 1.1		7.3 ± 0.3		9.0 ± 2.7	10.8 ± 1.5
		P = 0.03		P = 0.01		P = 0.002		P = 0.01	P = 0.7
800		15.6 ± 6.3	14.0 ± 2.9	6.1 ± 0.4	5.6 ± 0.5	6.8 ± 0.5	6.7 ± 0.7	6.6 ± 0.2	11.5 ± 0.9
		P = 0.08	P = 0.05	P < 0.01	P = 0.005	P < 0.001	P < 0.001	P = 0.02	P = 0.9

* Data are expressed as mean ± SD; three to four rats were used for each dose at each time period. P values indicate significance of difference from saline control values within a given time period.

Table 2. Effect of BSO on aminopyrine exhaled as CO_2*

BSO dose (μmol/100 g body wt)	N	Peak exhalation rate (μmol/100 g/15 min)	Median peak time (min)	Cumulative exhalation (μmol/100 g/0–120 min)	% Recovery of administered dose (0–120 min)	K_{el}† (min^{-1})	Hepatic GSH at end of study (μmol/100 g body wt)
Saline control	20	2.8 ± 0.4	30	15.6 ± 1.9	20.8 ± 2.6	0.011 ± 0.002	14.3 ± 2.8
800	19	2.4 ± 0.5	30	13.6 ± 2.0	18.1 ± 2.6	0.010 ± 0.002	4.4 ± 1.1
		P = 0.009		P = 0.003	P = 0.003	P > 0.1	P < 0.001

* Aminopyrine dose was 0.25 μCi in 75 μmol aminopyrine/100 g body wt. Values are means ± SD.
† K_{el} = elimination rate constant.

to a smaller yet significant decrease at the $P < 0.001$ level in enzymic activity (Fig. 1B).

In vivo *administration of compounds.* Data from rats pretreated with the respective compounds *in vivo* prior to isolation of microsomes are presented in Table 3A for DEM and phorone. DEM and phorone had been administered 45 min prior to killing the animals. Small decreases in APDM activity were measured in DEM- and phorone-treated animals, but these were statistically significant only when expressed per mg microsomal protein, not when expressed per g liver or per 100 g body weight. No change in APDM activity was noted in microsomes harvested from rats given BSO 4 hr previously (Table 3B).

Effects of DEM, phorone and BSO on $^{14}CO_2$ from ^{14}C-labeled formaldehyde

This series of experiments was performed to determine whether the compounds used to lower GSH in liver had any effects on the evolution of CO_2 from formaldehyde. Initially, characteristics of $^{14}CO_2$ evolution were established in control rats.

$^{14}CO_2$ from formaldehyde in control rats. Doses of ^{14}C-labeled formaldehyde up to 200 μmol/100 g body weight were administered i.p. Characteristics of $^{14}CO_2$ exhalation are presented in Table 4. $^{14}CO_2$ was detected in the first 15-min collection period, peaked at progressively increasing times with the higher doses, and then appeared to decline exponentially from 60 to 120 min. A little over half of the administered ^{14}C was recovered as $^{14}CO_2$ in the 120-min collection period with doses of 25 μmol and greater. The findings are very similar to those reported earlier from our laboratory for normal rats given similar i.p. doses of formate [1].

Effects of GSH-lowering compounds. Virtually none of the parameters of $^{14}CO_2$ evolution were affected by DEM with a dose of formaldehyde of 100 μmol/100 g body weight; by phorone with a formaldehyde dose of 50 μmol/100 g body weight; and by BSO with a formaldehyde dose of 50 μmol/100 g body weight (Table 5).

Effect of S-methylcysteine on $^{14}CO_2$ evolution from ^{14}C-labeled aminopyrine, formaldehyde and formate

Pretreatment of rats with *S*-methylcysteine had no effect on $^{14}CO_2$ evolution from any of the radioactive compounds administered intraperitoneally with overall doses of aminopyrine of 75 μmol/100 g rat, and of formaldehyde and formate of 50 μmol/100 g rat (data not shown).

Tissue distribution of ^{14}C of formaldehyde, formate and aminopyrine

Organ distribution of ^{14}C-radioactivity during the 2 hr after i.p. administration of a trace dose of formaldehyde is shown in Fig. 2. It is evident that formaldehyde is distributed widely in many tissues. A clear pattern of distribution is evident even though only single animals were studied at each interval. Major portions of the radioactivity are present in skeletal muscle, small intestine and liver. In other studies in which similar doses of formaldehyde were given i.v., small intestine and liver also contained

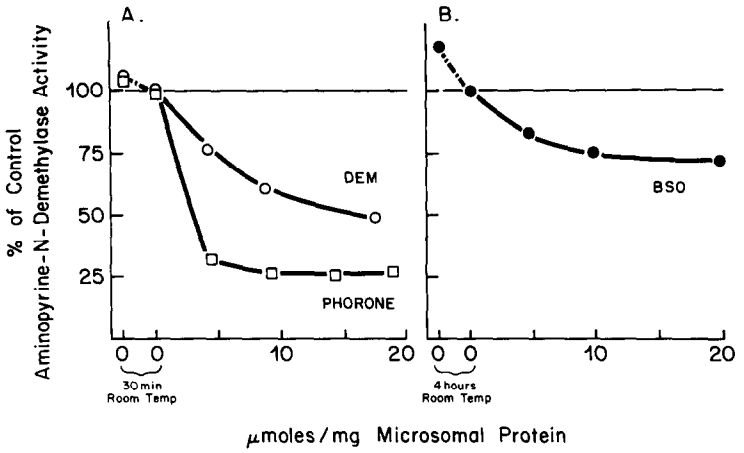

Fig. 1. Effects of DEM, phorone and BSO on aminopyrine-*N*-demethylase activity. Microsomes freshly prepared from normal rats were incubated at room temperature with various amounts of a GSH-lowering agent. APDM activity was then assayed and expressed as percent of activity found in microsomes incubated for a comparable period of time with an equivalent volume of buffer. In panel A, DEM, phorone or buffer alone was incubated for 30 min with microsomes (N = 9); in panel B, BSO or buffer alone was incubated for 240 min prior to the APDM assay (N = 8). The decreases in APDM activity induced by each concentration of the respective compounds were statistically significant ($P < 0.001$). DEM, phorone and BSO *per se* did not interfere with the Nash reaction. Incubation without a GSH-lowering agent (control microsomes) led to a 4–5% decrease in APDM activity in microsomes incubated for 30 min and to a 16% decrease in microsomes incubated for 240 min. The absolute values for APDM activity in nmoles formaldehyde per mg microsomal protein per minute in control microsomes after these periods of incubation at room temperature and plotted as 100% activity in the figure were (mean ± SD) 5.67 ± 0.9, 6.93 ± 1.2 and 6.58 ± 1.1 in the DEM, phorone and BSO experiments respectively.

comparable amounts of radioactivity (data not shown), implying that radioactivity in these organs was not simply due to adsorption of i.p. administered radioactivity. The fate of i.p. administered trace doses of $H^{14}CHO$ in the composite organs shown in Fig. 2, in respiratory $^{14}CO_2$ and, by difference, in the peritoneal cavity and remaining carcass (skin, bones, bone marrow) is shown in Fig. 3.

Distribution at 2 hr of larger doses of i.p. administered formaldehyde is summarized in Table 6. Similar data for animals given i.p. formate or aminopyrine are also presented for comparison. Data for formaldehyde and formate are very similar to each other, whereas relatively more of the label of aminopyrine is contained in muscle and less in expired CO_2.

DISCUSSION

In the present study, evolution of $^{14}CO_2$ from the [^{14}C-*methyl*]-labeled dimethylamino portion of aminopyrine was depressed in rats when their hepatic GSH content was lowered by administration of BSO, an inhibitor of GSH biosynthesis. Qualitatively these findings are comparable to what we reported earlier when hepatic GSH was lowered by administration

Table 3. Effects of DEM, phorone and BSO administered *in vivo* on hepatic microsomal aminopyrine-*N*-demethylase activity*

Treatment	N	Aminopyrine *N*-demethylase activity nmole formaldehyde per min per			Microsomal protein mg protein per	
		mg protein	g liver	100 g body wt	g liver	100 g body wt
(A) Corn oil	7	5.4 ± 0.3	149.1 ± 15.0	746.8 ± 60.3	27.5 ± 1.5	138.6 ± 6.4
DEM	7	4.5 ± 0.5	142.0 ± 10.8	701.1 ± 95.7	31.9 ± 2.2	151.8 ± 14.8
		P = 0.002	P = 0.3	P = 0.3	P = 0.001	P = 0.06
Phorone	7	4.7 ± 0.4	139.1 ± 12.8	704.6 ± 95.3	29.7 ± 1.8	148.7 ± 16.3
		P = 0.003	P = 0.2	P = 0.3	P = 0.03	P = 0.2
(B) Saline	6	5.3 ± 0.9	176.7 ± 39.9	870.3 ± 153.5	33.2 ± 3.7	164.5 ± 15.8
BSO in saline	6	5.2 ± 0.6	161.2 ± 25.0	793.4 ± 126.7	31.1 ± 3.3	153.7 ± 13.1
		P = 0.8	P = 0.4	P = 0.4	P = 0.3	P = 0.2

* P values indicate significance of differences from respective controls, i.e. corn oil in A, and saline in B. Values are means ± SD. Doses: DEM, 430 μmol/100 g body wt; phorone, 180 μmol/100 g body wt; and BSO, 800 μmol/100 g body wt.

Table 4. Formaldehyde exhaled as CO_2

Formaldehyde dose (μmol/100 g body wt)	N	Peak exhalation rate (μmol/100 g/15 min)	Median peak time (min)	Cumulative exhalation (μmol/100 g/0–120 min)	% Recovery of administered dose (0–120 min)	K_{el}† (min^{-1})
0.006*	3	0.0009 ± 0.00005	15	0.003 ± 0.00003	46.6 ± 0.4	0.020 ± 0.002
25	3	3.9 ± 0.5	15	14.1 ± 1.2	56.3 ± 4.7	0.023 ± 0.002
50	3	7.1 ± 0.3	30	27.0 ± 1.3	53.9 ± 2.6	0.020 ± 0.002
100	3	11.6 ± 0.8	45	57.0 ± 2.2	57.0 ± 2.2	0.024 ± 0.003
200	2	19.6	45	118.2	59.1	0.012

* Each rat received 0.25 μCi [^{14}C]formaldehyde in 0.006 μmol formaldehyde/100 g body wt plus the stated unlabeled dose of formaldehyde. Values are means ± SD, except for the 200 μmol dose where values are presented as an average of two.

† K_{el} = elimination rate constant.

of DEM or phorone. However, the degree of impairment induced by BSO was less than that seen with DEM or phorone. Thus, $^{14}CO_2$ evolution was depressed approximately 12.8% after BSO; in our earlier studies [1] it was lowered by 22.5% after DEM, and by 19.4% after phorone with a dose of 90 μmol/100 g body weight and 45.0% with a dose of 180 μmol/100 g body weight. These differences in the extent of depression of evolution of CO_2 are not explained by differences in degree of hepatic GSH depletion induced by these compounds. Moreover, although we did not measure intracellular pools of GSH, it is unlikely that differences in degree of depletion of these GSH pools account for our findings. Approximately 85–90% of the GSH in liver is contained in cytosol, the other 10–15% in mitochondria [20–22]. DEM, phorone and BSO primarily depress cytosolic GSH. Mitochondrial GSH is less depressed proportionately, but is probably lowered to a similar extent by all three compounds [23–25]. Thus, the current findings with BSO support our earlier conclusion that hepatic GSH is important for evolution of CO_2 from aminopyrine. In addition, however, they suggest that the agents (at least DEM and phorone) used to lower hepatic GSH may also contribute to impaired evolution of CO_2 by a GSH-independent mechanism.

On the basis of our earlier studies, we had concluded that DEM and phorone depress CO_2 evolution from aminopyrine by interfering with either or both the generation of formaldehyde from the N-methyl groups of aminopyrine or the subsequent oxidation of formaldehyde to formate. Evolution of CO_2 from administered formate was not affected by DEM or phorone. In the present studies, we designed experiments to assess the effects of DEM,

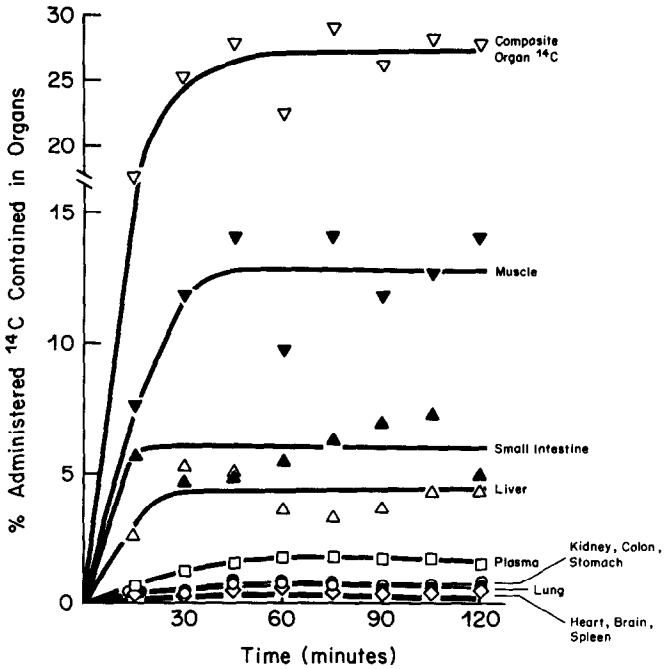

Fig. 2. Organ distribution of ^{14}C after i.p. administration of trace doses of formaldehyde, presented as percent of administered ^{14}C. Composite organ ^{14}C is the sum of the individual data for each of the organs listed in the figure.

Table 5. Effects of DEM, phorone and BSO on formaldehyde exhaled as CO_2*

Treatment	Formaldehyde dose (μmol/100 g body wt)	N	Peak exhalation rate (μmol/100 g/15 min)	Median peak time (min)	Cumulative exhalation (μmol/100 g/0–120 min)	% Recovery of administered dose (0–120 min)	K_{el}† (min^{-1})
Corn oil	100	6	10.6 ± 1.6	30	50.7 ± 4.4	50.7 ± 4.4	0.028 ± 0.005
DEM	100	6	11.2 ± 1.7	30	49.7 ± 7.0	49.7 ± 7.0	0.024 ± 0.003
Corn oil	50	4	5.4 ± 0.7	30	24.0 ± 0.8	48.0 ± 1.7	0.024 ± 0.006
Phorone	50	5	5.3 ± 0.3	30	22.9 ± 1.0	45.8 ± 2.0	0.023 ± 0.005
Saline	50	7	6.3 ± 1.1	30	25.8 ± 3.7	51.5 ± 7.4	0.025 ± 0.005
BSO	50	7	6.0 ± 0.9	45	25.6 ± 1.3	51.2 ± 2.7	0.025 ± 0.003

* Each rat received 0.25 μCi [^{14}C]formaldehyde in 0.006 μmol formaldehyde plus the stated unlabeled dose of formaldehyde per 100 g body weight. Values are means ± SD. Doses: DEM, 430 μmol/100 g body wt; phorone, 180 μmol/100 g body wt; and BSO, 800 μmol/100 g body wt.
† K_{el} = elimination rate constant.

phorone and BSO on formaldehyde metabolism and on generation of formaldehyde from aminopyrine.

Conversion of [^{14}C]formaldehyde to $^{14}CO_2$ in the intact rat was assessed by a formaldehyde breath test. ^{14}C-Labeled formaldehyde was given i.p. in doses equivalent to, or larger than, those that would have been generated during demethylation of aminopyrine. The characteristics of $^{14}CO_2$ evolution in expired air in control animals were found to be very similar to those we reported earlier for i.p. administered ^{14}C-labeled formate [1]. Fifty percent or more of the formaldehyde was accounted for by CO_2 in expired air at 2 hr. Peak exhalation rates and elimination rate constants of $^{14}CO_2$ were quite similar for formaldehyde and formate at i.p. doses up to 200 μmol/100 g body weight. Since formaldehyde is converted to formate prior to the evolution of CO_2, these data suggest that the conversion step proceeds rapidly and is not rate-limiting in normal rats.

DEM, phorone and BSO in doses that depressed CO_2 evolution from i.p. administered aminopyrine did not affect CO_2 evolution from i.p. administered formaldehyde (Table 5). If under these circumstances the liver is primarily responsible for conversion of formaldehyde to CO_2, our results indicate that this series of steps is not impaired by a 75–80% reduction in hepatic GSH content nor by the compounds used to achieve this degree of GSH depletion. This is somewhat surprising since the two major pathways in liver involved in conversion of formaldehyde to formate are either dependent on GSH [26–31] or are depressed by DEM and phorone [32]. On the one hand, GSH is required in at least two ways for activity of a specific cytosolic formaldehyde dehydrogenase. Thus, GSH reacts nonenzymatically with formaldehyde to form *S*-hydroxymethyl glutathione [26], the presumed substrate for this enzyme [29], and free GSH is an activator of the enzyme [30]. *S*-Formylglutathione, the product of the reaction, is then hydrolyzed to formate and GSH by a second cytosolic enzyme, *S*-formylglutathione hydrolase [11]. Although GSH is not consumed in

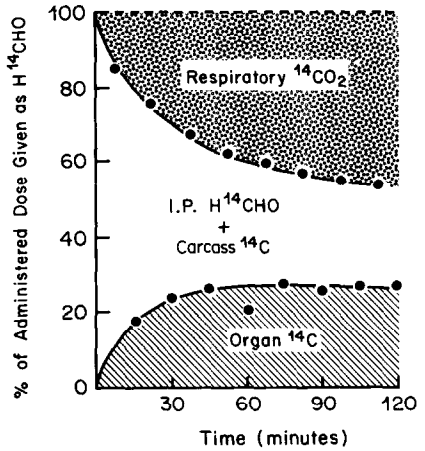

Fig. 3. Distribution of ^{14}C in composite organs, in respiratory CO_2 and, by difference, in the peritoneal cavity and the remaining carcass (skin, bone and bone marrow) at varying intervals after i.p. administration of trace doses of $H^{14}CHO$.

Table 6. Distribution of formaldehyde, formate and aminopyrine 2 hr after i.p. administration*

	Percent of administered dose						
	Dose in μmol /100 g body wt						
	Formaldehyde			Formate		Aminopyrine	
	25	50	100	25	100	25	75
Liver	5.6	4.4	4.8	3.8	3.3	6.2	3.8
Muscle	16.1	18.0	14.7	15.0	12.8	24.5	28.6
Stomach	0.4	0.5	0.4	0.3	0.3	1.7	0.9
Small intestine	3.2	3.6	3.6	2.5	2.0	2.0	1.6
Colon	1.2	1.3	1.2	1.1	0.8	1.0	1.1
Kidneys	1.1	1.2	0.8	1.0	0.6	1.0	0.6
Heart	0.1	0.1	0.1	0.1	0.1	0.1	0.2
Lung	0.2	0.3	0.2	0.2	0.1	0.3	0.2
Brain	0.2	0.2	0.2	0.1	0.1	0.3	0.5
Eyes	0.04	0.05	0.05	0.03	0.04	0.06	0.05
Respiratory CO_2	56.0	54.0	57.5	56.3	55.2	30.0	28.1
I.P. + remaining carcass	15.9	16.4	16.1	19.6	24.7	32.9	34.4

* Each animal received 1 μCi of ^{14}C-labeled trace compound plus additional non-labeled compound to provide administered dose.

the reaction, a profound decrease in cytosolic GSH might be expected to depress formaldehyde oxidation by this pathway. Formaldehyde can also be oxidized effectively in mitochondria by a low K_m aldehyde dehydrogenase [33, 34]. It has been estimated that the mitochondrial and cytosolic pathways contribute equally to the metabolism of formaldehyde in isolated rat hepatocytes [35]. In a recent publication [32], Dicker and Cederbaum demonstrated that DEM and phorone inhibit the activity of this low K_m mitochondrial aldehyde dehydrogenase when acetaldehyde is used as substrate. They further indicate in their discussion that in preliminary experiments this inhibition extends to formaldehyde oxidation.

Kornbrust and Bus [18] have reported that the methyl group of CH_3Cl is converted, at least in part, to CO_2 via an additional GSH-dependent pathway to those already described, and this pathway is also depressed by DEM. One of the features characterizing this pathway is that CO_2 evolution from CH_3Cl is depressed by administration of *S*-methylcysteine. We utilized this latter approach to assess whether the N-methyl groups of aminopyrine might be metabolized via this pathway. *S*-Methylcysteine given in a manner that Kornbrust and Bus have shown to depress CO_2 evolution from CH_3Cl had no effect in our experiments on CO_2 evolved from aminopyrine, formaldehyde or formate, findings that argue against this pathway having an important role in CO_2 evolution from aminopyrine.

Nevertheless, in a setting in which formaldehyde oxidation through two major pathways might be expected to be impaired, the finding of a normal formaldehyde breath test raises additional considerations. First, it is possible that oxidation of formaldehyde via catalase may compensate for any impairment of these other pathways. It is also possible that formaldehyde absorbed from the peritoneal cavity is not disposed of in the same manner as a comparable amount of formaldehyde generated in the liver from demethylation of aminopyrine. To gain some insight into the tissue distribution of i.p. administered formaldehyde, we gave trace doses of [^{14}C]formaldehyde i.p., killed individual animals at 15-min intervals up to 2 hr, and determined the radioactivity in a number of organs. From the data shown in Fig. 2, it is evident that formaldehyde is distributed widely. Since such data do not indicate the subsequent fate of the formaldehyde in individual tissues, i.e. whether or not converted to formate and CO_2, we did not carry out extensive distribution studies with larger doses of formaldehyde but did assess in single animals the distribution at 2 hr of larger doses of i.p. formaldehyde. These findings are summarized in Table 6. For comparison, organ content of radioactivity 2 hr after i.p. administration of formate and aminopyrine is also presented. There are similarities in organ distribution of the ^{14}C-labeled compounds. Nevertheless, we appreciate that these data do not indicate whether formaldehyde formed from aminopyrine occurs in the same sites in which the ^{14}C label of i.p. administered $H^{14}CHO$ is located. It is conceivable that extrahepatic formaldehyde is converted to CO_2. If agents such as DEM and phorone inhibit hepatic metabolism of formaldehyde, this might not be detected by a formaldehyde breath test if extrahepatic tissues account for generation of a major portion of the CO_2 exhaled after i.p. administration of formaldehyde.

In addition to examining the fate of administered formaldehyde, we also assessed whether the agents used to lower hepatic glutathione content might affect the generation of formaldehyde by inhibiting the activity of microsomal aminopyrine-*N*-demethylase. Addition of DEM or phorone to control hepatic microsomes *in vitro* for 30 min prior to assay, in order to simulate *in vivo* conditions in which CO_2 evolution from aminopyrine was already impaired 30 min after i.p. administration of these compounds, resulted in significant inhibition of APDM activity (Fig. 1A). Incubation *in vitro* of BSO with microsomes for 4 hr, again to simulate *in vivo* conditions in which the aminopyrine breath test was carried out

4 hr after i.p. administration of BSO, also lowered APDM activity but significantly less than that seen with DEM and phorone (Fig. 1B). These findings support the possibility that the GSH-lowering compounds impair generation of formaldehyde from the N-methyl groups of aminopyrine. By contrast, experiments in which the agents used to lower hepatic GSH were administered *in vivo*, the livers removed after 45 min for DEM and phorone, and after 4 hr for BSO, hepatic microsomes isolated and then APDM activity measured, revealed virtually no depression of enzymic activity. The relevance of the *in vitro* findings to the situation *in vivo* is unknown, since we do not know whether the microsomal concentration attained by these compounds is similar in the two situations. Moreover, even if DEM and phorone were to reach comparable concentrations in microsomes *in vivo* as *in vitro*, it is not known whether they might be removed from microsomes during their preparation with impaired enzymic activity being restored to normal. Such a possibility is raised by the recent demonstration of Dicker and Cederbaum [32] that DEM-induced inhibition of acetaldehyde oxidation in isolated rat hepatocytes was less in cells treated with DEM and then washed, than in cells treated with DEM and not washed.

BSO, DEM and phorone all decrease hepatic GSH content and simultaneously impair CO_2 evolution from aminopyrine. It seems likely that some of the depressed evolution of CO_2 is the consequence of low GSH by mechanisms discussed earlier. The methods used in the current study to pinpoint the site(s) of impairment in the intact animal, i.e. assessment of formaldehyde metabolism and of generation of formaldehyde, did not unequivocally resolve the mechanism, however. Quantitative differences in the extent of depression of CO_2 evolution suggest that at least DEM and phorone impair CO_2 generation from aminopyrine by a GSH-independent mechanism. *In vitro* data indicate this may involve inhibition of aminopyrine demethylase activity.

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