(DTNB) and RNAase A were obtained from the Sigma Chemical Co. (St. Louis, MO); DL-buthionine-*SR*-sulfoximine was from the Chemical Dynamics Corp. (South Plainfield, NJ), and 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) were from Eastman Kodak (Rochester, NY). All other reagents were obtained either from Sigma or Fisher Scientific (Fair Lawn, NJ). [^{14}C]Dimethylnitrosamine was synthesized by the nitrosation of [^{14}C]dimethylamine (54 mCi/mmole, New England Nuclear, Boston, MA) according to the method of Dutton and Heath [15].

Animals. Adult male 200–230 g Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were used for all experiments. The animals were kept on a normal diet until the day of the experiment, and pretreatments were initiated always at the same time each day (9:00 a.m.) in order to avoid complications due to the circadian variations in GSH levels [16].

BSO treatment. A subcutaneous injection of 4 mmoles BSO/kg (0.89 g/kg; 0.2 M solution in saline) was given to the rats in the back [12]. Controls received a weight-adjusted volume of saline alone. This dose was chosen in order to obtain an approximately 80% decrease in hepatic and renal GSH [12]. The animals were killed at various times after drug administration, according to the experimental protocol. Livers and kidneys were excised, frozen in liquid nitrogen, and stored at −70° until GSH assay or DNA isolation.

GSH assay. The organs were rapidly thawed and homogenized in ice-cold 5% trichloroacetic acid (TCA) and centrifuged at low speed for 5 min. Aliquots of protein-free supernatant fraction were used for the measurement of GSH using the colorimetric DTNB assay described by Ellman [17].

To assess the possible effect of freezing of the organs on GSH levels, the assay was also performed on fresh liver and kidney from control animals. We found that freezing of the organs did not alter the assessed GSH concentrations: 4.3 and 3.0 μmoles/g liver and kidney, respectively, relative to 4.7 and 2.4 μmoles/g in fresh tissues.

GSH *S*-transferase was measured according to the method described by Habig *et al.* [18], using CDNB and DCNB as substrates. Protein determination in the liver cytosolic and microsomal fractions was carried out according to Lowry *et al.* [19] using bovine serum albumin as a standard.

DMN treatment. The effect of increasing doses of DMN on hepatic and renal GSH was studied. Groups of five rats each were intraperitoneally injected with 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 mg/kg of the carcinogen, dissolved in a volume of 0.5 ml saline/0.1 kg body weight.

DNA methylation. The rats were administered 4 mmoles BSO/kg by s.c. injection 8 hr before being killed and [^{14}C]DMN (250 μg/kg, ~40 μCi i.p.) 4 hr before sacrifice. A control group received saline alone and 4 hr later the same [^{14}C]DMN regimen as in the BSO-treated group.

DNA isolation and HPLC analysis. DNA was isolated from individual livers and pooled kidneys by phenol extraction using the modified Kirby procedure [10], and depurinated by heating at 70° for 40 min in 0.1 M HCl immediately prior to HPLC analysis. The purines in each sample were resolved using a Waters Associates (Milford, MA) μBondapak C18 column (300 × 3.9 mm, 10 μm) eluted isocratically with 10 mM ammonium formate buffer, pH 3.8, room temperature, at a flow rate of 1 ml/min. The retention times for guanine, 7-methylguanine and O^6-methylguanine under these conditions were 9, 21 and 51 min respectively; adenine and 3-methyladenine coeluted at 13 min. Fractions were collected for the yield assessment of ^{14}C-methyl-radiolabeled 7-methylguanine and O^6-methylguanine utilizing liquid scintillation counting. The guanine yield was determined by monitoring the elution profile of a diluted sample at 254 nm and by comparing the guanine peak height with a standard curve generated utilizing guanine samples of known concentration.

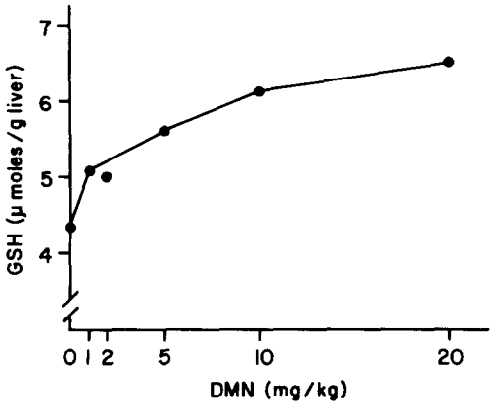

Fig. 1. Liver GSH levels 4 hr after DMN administration. Values are mean of five animals.

Statistical analysis. Statistical analysis was performed using the unpaired Student's *t*-test.

RESULTS

As a prelude to our experiments designed to test the effects of GSH modulation on DMN-derived tissue DNA alkylation yields, we checked whether DMN administration itself perturbed hepatic and renal GSH levels. We found that at 4 hr after compound administration, DMN doses as low as 1 mg/kg resulted in marked increases in GSH levels in the liver and the kidney (Figs. 1 and 2). At the highest DMN dose considered, 20 mg/kg, a GSH increase of approximately 50% was observed in both organs relative to controls ($P < 0.001$). We do not know the mechanism of this DMN-induced change in GSH levels. It is clear, however, that high doses of the compound markedly and quickly altered cellular metabolism. DMN doses of 500 μg/kg and lower did not appear to exert any effect on liver or kidney GSH levels (Table 1). We chose a DMN dose of 250 μg/kg for our subsequent work.

Administration of BSO at 4 mmoles/kg caused significant GSH depletion in the liver and kidney, reaching a maximum decrease between 4 and 8 hr post-treatment (Fig. 3). During this period the GSH levels were 25–30% of control values. This degree

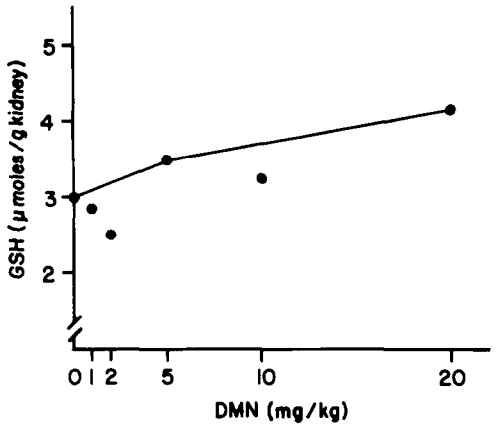

Fig. 2. Kidney GSH levels 4 hr after i.p. injection of DMN. Values are mean of five individual estimations.

Table 1. Liver and kidney GSH levels in the rat 4 hr after i.p. admininstration of DMN

DMN (mg/kg)	GSH (μmoles/g tissue)	
	Liver	Kidney
	4.3 ± 0.4	3.0 ± 0.3
0.05	4.4 ± 0.8	2.7 ± 0.3
0.1	4.6 ± 0.7	2.8 ± 0.2
0.5	4.0 ± 0.6	2.7 ± 0.3

Controls received saline alone. Values represent mean of five rats ± SD.

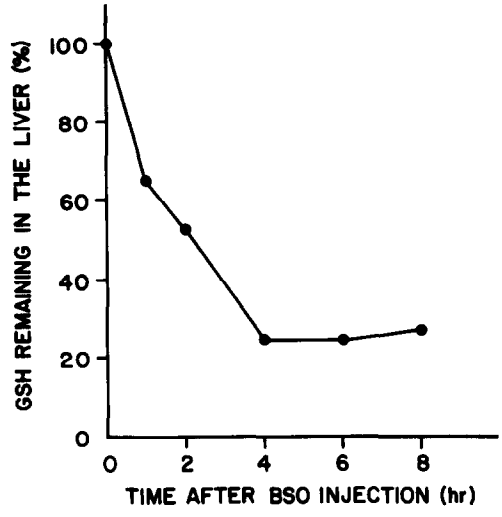

Fig. 3. GSH remaining in the rat liver, expressed as a percentage of the controls, at times after BSO administration (4 mmoles/kg, s.c.). Control GSH value was 4.3 μmoles/g liver.

of depletion parallels that found in the livers of BSO-treated mice and rats [12] and hamsters (D. Jensen and G. Stelman, unpublished observations). Based on these results we established the protocol of dosing our rats with DMN 4 hr after BSO treatment and then killing the animals 4 hr later. In control experiments we could detect no changes in total microsomal or cytosolic protein levels 4 hr after BSO treatment and no changes in cytosolic glutathione *S*-transferase specific activity relative to controls (Table 2).

In our experiments we used the 7-methylguanine/guanine yield as an indicator of the DNA methylation produced by DMN treatment. Removal of this adduct from liver DNA has been shown by Pegg *et al.* [11] to be negligible during the first 3 hr after doses of DMN comparable to that utilized in this work. Under our conditions, BSO pretreatment did not cause any significant change in DMN-induced DNA alkylation in the liver and kidney relative to control animals (Table 3, Fig. 4). As a check, we found that the GSH levels in the organs reserved for DNA isolation (i.e. 8 hr after BSO administration) were approximately those assumed at the time of DMN dosing. Our liver DNA 7-methylguanine/guanine yields were consistent with those reported by Pegg [20]; the yield of this adduct in the kidney relative to the liver was about 0.14 in both BSO-treated and control animals. As expected considering the low DMN dose used in these experiments and the time between compound administration and sacrifice [11], no significant amounts of radioactivity eluted at the O^6-methylguanine retention time in the analysis of any of our DNA hydrolysates.

DISCUSSION

We were unable to demonstrate any change in DNA alkylation yield over a range in GSH levels which is likely to exceed *in vivo* fluctuations. Our finding that GSH is apparently ineffective as a scavenger of the methylating species generated by DMN metabolism is in accord with several published observations. Craddock [21] found that the levels of *S*-methylcysteine (a supposed catabolite of *S*-methylglutathione) in the acid-soluble fraction of liver homogenates from DMN-treated rats are a very small percentage (0.08%) of the total acid-soluble thiol. Further, the *S*-methylcysteine detected in the urine of those animals represented a vanishingly small percentage (0.002%) of the accounted for DMN dose. Consistent with these results is the recent observation of Chung *et al.* [22] that less than 0.001 to 0.004% of the DMN added to mouse hepatic 10,000 *g* supernatant containing cofactors is converted to *S*-methylglutathione; this corresponds to less than 3% of the DMN metabolized under the assay conditions. Also, it has been found recently that GSH alone or in the presence of cytosol failed to modulate the methylation of added DNA by dimethylnitrosamine incubated with hamster liver microsomes.* Finally, our result appears to be in accord with the observation of Jensen and Magee [23] that high levels of added cysteine have little or no effect on the *in vitro* DNA methylation yields produced by methylnitrosourea or 1-methyl-2-nitro-1-nitrosoguanidine, compounds that are believed to decompose to generate the same DNA-modifying fragment as produced by the breakdown of α-hydroxylated DMN [7, 9].

Seemingly contrary to our findings are the recent studies of Frei *et al.* [24] which have been interpreted

* Prasanna *et al.* manuscript submitted for publication.

Table 2. Liver microsomal and cytosolic protein levels and GSH *S*-transferase activity in rats 4 hr after BSO treatment

	Protein (mg/g liver)		GSH *S*-transferase activity (cytosolic fraction)	
	Microsomal fraction	Cytosolic fraction (105,000 *g* supernatant)	nmoles CDNB conjugated/ min/mg protein	nmoles DCNB conjugated/ min/mg protein
Control	19.2 ± 5.3	44.8 ± 7.4	1848.6 ± 578	403.7
BSO, 4 hr	20 ± 4.3	40.7 ± 5.7	1682.5 ± 466	393

Values are mean ± SD of three different animals. BSO was administered s.c. at a dose of 4 mmoles/kg body weight. No SD for DCNB because data insufficient.

Table 3. Liver and kidney DNA methylation generated by DMN treatment (250 μg/kg) in control and BSO pretreated rats

	(μmoles 7-Methylguanine/mole guanine)	
	Liver	Kidney
DMN (control)	82.9 ± 27.5 (5)	11.8
BSO + DMN	83.5 ± 27.5 (6)	11.7

Details of the treatment are given in Materials and Methods. Values represent mean ± SD. The numbers of animals utilized are given in parentheses. Kidneys of each group were pooled together.

as indicating that variations in GSH levels have a pronounced effect on DNA methylation by DMN in the rat. In this work pretreatment with disulfiram was used to enhance GSH levels and diethylmaleate to reduce these levels. In addition, a high dose level (10 mg/kg) of DMN was utilized. Since both of these pretreatment regimens are known to alter the activity of drug-metabolizing systems [14, 25–27] and high DMN doses affected the basic cell metabolism (this work), an alteration in DMN activation rather than a GSH-scavenging effect may thus have been responsible for the observed differences in DNA alkylation yield. Four-hour pretreatment of rats with 4 mmoles/kg BSO had no effect on the rate and yield of radiolabeled carbon dioxide generated after dosing the animals with 250 μg/kg [^{14}C]DMN (L. Myers and P. Magee, unpublished observations). This result and our finding that there was no difference in organ DNA 7-methylguanine/guanine yield are consistent with the view that neither BSO itself nor lower than normal GSH levels (over the 4-hr intervals of the present experiments) has any effect on DMN metabolism at the low dose considered here.

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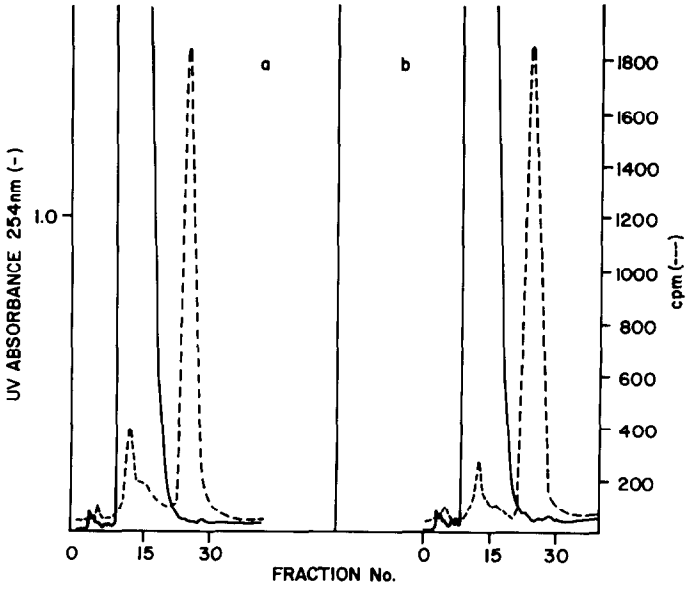

Fig. 4. HPLC chromatogram of liver DNA hydrolysates from a control (a) and from a BSO-pretreated rat (b). The radioactive peak at 21 min corresponds to the fraction containing 7-methylguanine, as assessed by elution of authentic marker. HPLC details are given in Materials and Methods.

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