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METABOLISM OF SPIROHYDANTOIN MUSTARD IN THE MOUSE

ISOLATION OF AN ALKYLATING, MUTAGENIC METABOLITE*†

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Abstract—The metabolism of spirohydantoin mustard (SHM), a central nervous system-directed nitrogen mustard with reported anticancer activity, was studied using both the *Salmonella*/mammalian microsome mutagenicity assay and radiolabeled drug. SHM had little or no mutagenic activity by itself but was metabolized to a mutagen(s) in the presence of mouse postmitochondrial liver fraction (9000 *g* supernatant, S9). Metabolism was NADPH-dependent and was enhanced with phenobarbital-induced S9. Both SHM and mutagen(s) were extractable in chloroform. Studies using [^{14}C]SHM, uniformly labeled on the bis(2-chloroethyl)amino group, and thin-layer chromatography (TLC) of chloroform extracts of liver S9 incubation mixtures indicated the formation of a single major metabolite fraction that contained a direct-acting mutagen. Chloroform extracts of both blood and brain from BDF1 mice injected i.p. with SHM (60 mg/kg) were found to be mutagenic in the absence of S9. Also, TLC of chloroform extracts of brain taken 15 min after i.p. injection of [^{14}C]SHM suggested the presence of SHM and the mutagenic metabolite. These results suggest that the mutagenic metabolite may have a significant role in the mechanism of action of SHM.

Spirohydantoin mustard (SHM, NSC-172112) is a rationally designed, central nervous system-directed nitrogen mustard with reported activity against intracranial murine ependymoblastoma [1]. Its synthesis was prompted by reports that 5,5-diphenylhydantoin was able to pass the blood–brain barrier [2] and tended to localize in brain tumors [3]. SHM has passed the National Cancer Institute decision network 2B, and preclinical toxicology studies have been initiated.

We have used the *Salmonella* mutagenesis assay [4] in conjunction with radiolabeled drug to study the mutagenicity of SHM and its metabolism to mutagenic products in the mouse. The mutagenicity assay is based upon the ability of a chemical to revert specially constructed mutant strains of *Salmonella typhimurium* from a histidine growth requirement back to prototrophy. With the introduction of a liver activation system into the assay, certain compounds are metabolized to active mutagens [5], thus demonstrating the potential of the assay for studies of drug metabolism.

MATERIALS AND METHODS

Chemicals. Spirohydantoin mustard, 3-[2-bis(2-chloroethyl)aminoethyl]5,5-pentamethylenehydantoin, was obtained from the Division of Cancer Treatment, National Cancer Institute, NIH (Bethesda, MD). [^{14}C]SHM (13.5 mCi/mmole), uniformly labeled on the bis(2-chloroethyl)amino group, was synthesized by Research Triangle Institute (Research Triangle Park, NC) and obtained from Dr. J. A. R. Mead, Division of Cancer Treatment, NCI. Radiochemical purity was 96–98% as determined by TLC. Glucose-6-phosphate (G-6-P) and NADP were purchased from the Sigma Chemical Co. (St. Louis, MO). SHM was dissolved in either dimethyl sulfoxide (DMSO, Eastman spectro grade) or chloroform (Fisher certified A.C.S.).

Liver enzyme system. The postmitochondrial liver fraction (9000 *g*; S9) was prepared from BDF1 mice of mixed sexes and weights. Some animals were treated with 0.1% sodium phenobarbital in their drinking water for 5 days prior to being killed. All animals were fasted for 12–13 hr prior to sacrifice at which time they were anesthetized and bled by cardiac puncture. Livers were removed aseptically, washed with cold 0.15 M KCl, and weighed. The livers were transferred to 0.15 M KCl (3 ml/g liver), minced, homogenized, and centrifuged at 9000 *g* for 20 min at 5°. All experiments were done using freshly prepared S9.

The enzyme reaction mixture (S9 mixture) contained (per ml) 8 μmoles $MgCl_2$, 33 μmoles KCl, 5 μmoles G-6-P, 4 μmoles NADP, 100 μmoles phosphate buffer (pH 7.4), and S9 (0.1 to 0.3 ml). For routine mutagenicity assays, 0.5 ml of the mixture was incorporated into the top agar of the assay plates (see below).

Some experiments with SHM involved incubation of the S9 mixture and drug prior to mutagenicity

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assay or chromatography. For this purpose, the reaction mixture (1.8 ml or 7.8 ml) was incubated at 37° for 3 min prior to the addition of 0.2 ml of DMSO containing SHM. Incubation was continued for appropriate time intervals. Controls included incubation in the absence of NADP and incubation in the absence of drug but with the appropriate DMSO addition. Reactions were stopped by the addition of 4 parts of cold chloroform to 1 part of reaction mixture. Each preparation was vortexed, and the chloroform layer was retained. A second chloroform extraction was performed on the aqueous phase, and the two extracts were pooled and evaporated to dryness in a Buchler Evapo-mix evaporator. Extracts were then lyophilized to remove the residual DMSO. The dried extracts were dissolved in chloroform and stored at −20° for chromatography. For mutagenicity assays, a portion of each chloroform extract was evaporated to dryness under a stream of nitrogen and the residue dissolved in DMSO.

Mutagenicity assay. The mutagenicity assay, using *S. typhimurium* strain TA1535 which was supplied by Dr. B. N. Ames (University of California, Berkeley), was performed as described by Ames *et al.* [4], with the exception that the base-agar medium contained 0.1% NH_4Cl, 0.73% K_2HPO_4, 0.3% KH_2PO_4, 0.05% sodium citrate, 0.012% $MgSO_4$, 0.4% glucose, and 1.5% Bacto agar. Twenty milliliters of this medium was added to each assay plate (15 × 100 mm). Samples to be assayed were incorporated into 2 ml of top agar (0.6% Difco agar, 0.5% NaCl, 0.05 mM L-histidine, 0.05 mM biotin) at 45°, together with 0.1 ml of cell culture (approximately 2×10^8 viable cells). S9 mixture (0.5 ml) was also added to the top agar when appropriate. The contents were mixed and poured onto the surface of the base-agar medium. The assay plates were incubated at 37° for 48 hr, at which time the number of histidine (*his*$^+$)-revertant colonies appearing on each plate was scored. Experimental controls included assay plates containing solvent or cyclophosphamide (50 μg/plate), which was used as a positive control for metabolic activation. Appropriate sterility checks were performed for each assay component. The number of spontaneous revertants appearing on the diluent control plates was subtracted from the counts appearing on the test plates, unless otherwise noted.

Thin-layer chromatography. ^{14}C-Labeled components in the chloroform extracts of S9 mixtures or tissues were chromatographed on heat-activated Analtech silica gel G plates (0.25 mm thickness). Solvent systems included chloroform–methanol (95:5), chloroform–acetone (1:1), and toluene–acetone (8:2). Separated components were detected on the chromatograms with iodine vapors; alkylating activity was detected with 4-(*p*-nitrobenzyl)pyridine (NBP) as described elsewhere [6]; radiolabeled components were detected with a Packard model 7720/21 radiochromatogram scanner. Radioactivity on the chromatograms was quantitated by transfer of the silica gel directly to scintillation vials to which was added 1 ml of methanol. Samples were analyzed for radioactivity with a Packard Tri-carb model 3315 liquid scintillation spectrometer.

Radiolabeled fractions were recovered from TLC plates by extraction of the silica gel with chloroform–methanol (1:1) or acetone. The extracts were evaporated to dryness under N_2 and the residues dissolved in chloroform. These were stored at −20° in sealed vials. Portions of the chloroform solutions were transferred to DMSO (see above) prior to mutagenicity assay.

In vivo studies. Male BDF1 mice (20 g) were given single i.p. doses (60 mg/kg) of either [^{14}C]SHM (about 6 μCi/mouse) or unlabeled SHM dissolved in DMSO. Each animal received 0.1 ml of DMSO. At appropriate time intervals, groups of either three or ten mice were anesthetized with carbon dioxide and bled by cardiac puncture using heparinized syringes, and the blood was pooled in tubes held in ice. The brain was excised, blotted on saline-saturated gauze pads, weighed, and homogenized in 4 parts of cold 0.9% saline. Blood samples and brain homogenates were extracted with chloroform and analyzed for mutagenic activity or radioactivity as described above. Components in the pooled chloroform extracts of brain removed 15 min after injection of [^{14}C]SHM were also separated by TLC, and the fractions were analyzed for radioactivity.

RESULTS

Mutagenicity studies. Results of the effects of mouse liver S9 and SHM concentrations on the mutagenic response of strain TA1535 are presented in Fig. 1. SHM had little or no mutagenic (revertant) activity by itself but was metabolized to a mutagen(s) in the presence of S9 (Fig. 1B). A direct relationship was observed between S9 concentration and mutagenic response at a constant SHM concentration (Fig. 1A). The activation of SHM was found to be enhanced with phenobarbital-induced S9. No mutagenic activity was observed when NADP was absent from the S9 mixture. This indicated a requirement for NADPH formed by the activity of endogenous glucose-6-phosphate dehydrogenase.

Both the formation of the metabolite, a direct-acting mutagen, and the chemical degradation of SHM could be followed using selective assay conditions (Fig. 2). The results suggested that SHM, which is not a direct-acting mutagen, was rapidly converted (apparent half-life = 9 min) to nonmutagenic products when the S9 mixture was deficient in NADP (curve C). With adequate NADP present, however, mutagenic activity increased with time for 10 min (curve B). This was followed by a decay in activity with an apparent half-life of 34 min. Curve A (Fig. 2) represents the contribution of both metabolite and SHM to the mutagenic response. Subtraction of curve B from curve A results in values similar to those for curve C (data not shown).

Thin-layer chromatography. Isolation of the mutagenic metabolite(s) of SHM from the S9 mixture was attempted using [^{14}C]SHM together with TLC. Radiochromatogram scans of chloroform extracts of mixtures incubated under various conditions are presented in Fig. 3. The radioactive peak that appeared at an R_f of 0.73 was confirmed to be SHM by using both an authentic standard and the mutagenicity assay. Incubation of SHM with an S9 mixture without NADP resulted in the appearance of at least four radiolabeled components in addition to SHM (Fig.

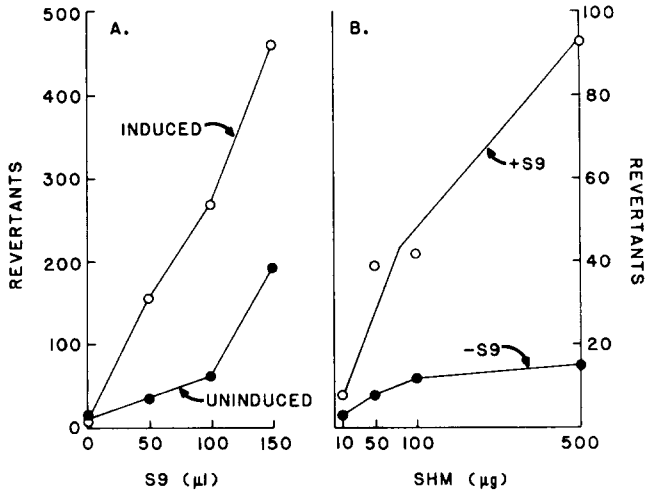

Fig. 1. (A) Effect of phenobarbital-induced and uninduced mouse liver S9 on the induction of *his*⁻ revertants by SHM (50 μg/plate). (B) Dose response of *S. typhimurium* TA1535 to SHM in the presence and absence of mouse liver S9 (50 μl/plate, uninduced). All assays were done in triplicate.

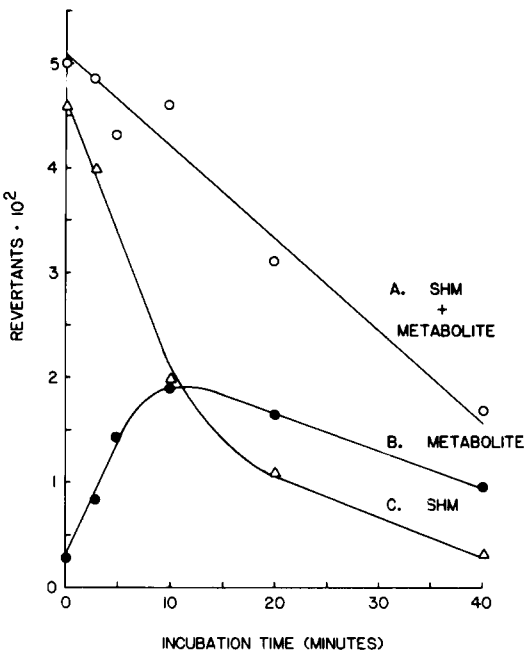

Fig. 2. Kinetics of the *in vitro* activation of SHM. S9 mixture, 8 ml/tube, was prepared with and without NADP using phenobarbital-induced S9 as described in Materials and Methods. Reactions were initiated by the addition of SHM (400 μg/ml). Incubation temperature was 37°. At selected time intervals, duplicate 1-ml samples were taken and immediately extracted with chilled chloroform. The chloroform-extracted material was transferred into DMSO and assayed for mutagenic activity. Curve A: mutagenic activity of extracts of a complete S9 mixture; assays were done in the presence of S9. Curve B: activity of extracts of a complete S9 mixture; assays were done in the absence of S9 to detect the mutagenic metabolite(s). Curve C: activity of extracts from an NADP-deficient S9 mixture; assays were done in the presence of S9 to detect SHM.

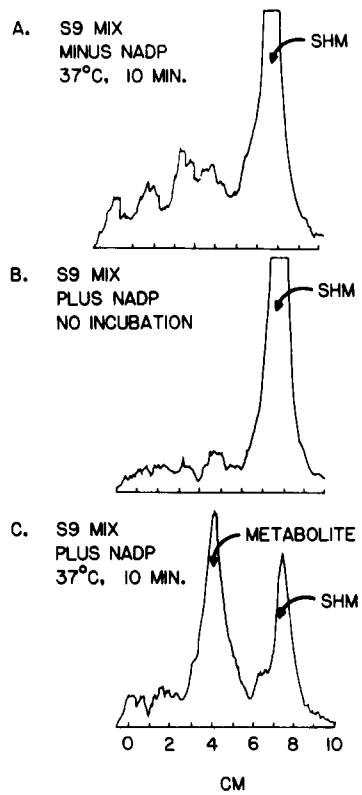

Fig. 3. Radiochromatogram scans of chloroform extracts of S9 reaction mixtures. S9 mixture, in 2-ml volumes, was prepared with and without NADP using phenobarbital-induced S9 as described in Materials and Methods. Reactions were initiated by the addition of [^{14}C]SHM (2 nCi/μg, 200 μg/ml). Incubation temperature was 37°. Key: (A) chloroform extract of an S9 mixture (NADP-deficient) after a 10-min incubation; (B) chloroform extract of a complete S9 mixture that was extracted without incubation; and (C) chloroform extract of a complete S9 mixture after a 10-min incubation. The solvent system was chloroform–methanol (95:5). Equivalent amounts of radioactivity were applied to each TLC plate.

Table 1. ^{14}C-Recovery and mutagenic activity of chloroform extracts of S9 reaction mixtures and their TLC fractions*

Sample†	Relative radioactivity‡	Mutagenesis assay: SHM equivalents§ (μg)	Mutagenesis assay: *His*⁻ revertants −S9	Mutagenesis assay: *His*⁻ revertants +S9
(A) $CHCl_3$ extract	1.0 (0.27)	21	8	91
R_f 0.43 fraction	0.23	NT‖	NT	NT
R_f 0.73 fraction	0.51	NT	NT	NT
(B) $CHCl_3$ extract	1.0 (0.96)	38	11	329
R_f 0.73 fraction	0.98	NT	NT	NT
(C) $CHCl_3$ extract	1.0 (0.26)	21	89	151
R_f 0.43 fraction	0.55	12	62	75
R_f 0.73 fraction	0.28	7	9	44
SHM (standard)		50	23	570
DMSO (diluent)			16	11

* S9 mixtures were prepared and handled as described for Fig. 3.

† Samples A, B and C are as described in Fig. 3. The fractions refer to material recovered from sections of the chromatograms that are indicated by the R_f values: R_f 0.73 = SHM; R_f 0.43 = metabolite.

‡ Ratio of the radioactivity recovered, from the indicated section of the chromatogram, to the total radioactivity applied. Values in parentheses are the ratios of the radioactivity recovered in $CHCl_3$ extracts of the S9 mixtures to total radioactivity.

§ Estimates of concentration per assay plate were based upon radioactivity.

‖ NT = not tested.

3A). Optimal conditions for the formation of the mutagenic metabolite(s) resulted in the appearance of a single major peak ($R_f = 0.43$) in addition to SHM (Fig. 3C). Scan B was the reaction control.

Quantitative results on ^{14}C-recovery and mutagenic activity of chloroform extracts of the above S9 mixtures and their resulting TLC fractions are presented in Table 1. The chloroform extracts and TLC fractions are those referred to in Fig. 3. The percentages of chloroform-extractable radioactivity for samples A (S9 mix, NADP-deficient, 10-min incubation), B (S9 mix, complete, no incubation), and C (S9 mix, complete, 10-min incubation) were 27, 96, and 26% respectively. The major metabolite fraction ($R_f = 0.43$, sample C) accounted for 55% of the chloroform-extractable radioactivity. The same region of the chromatogram from sample A accounted for 23% of the chloroform-extractable activity. Mutagenicity assay of the fraction containing the metabolite ($R_f = 0.43$, sample C) in the absence of S9 yielded an increase in the number of *his*⁺ revertants over those for the DMSO control. The incorporation of S9 into the assay did not greatly change the revertant count, suggesting that this fraction contained a direct-acting mutagen. Samples A and B were mutagenic only when assayed with S9, indicating the absence of direct-acting mutagens. Reassay of the metabolite fraction ($R_f = 0.43$) using an independently purified preparation confirmed the initial mutagenicity test results (data not shown). Both the mutagenic metabolite and SHM possessed alkylating activity as evidenced by their reaction with NBP.

In vivo *studies*. Preliminary studies were performed to determine whether or not the mutagenic metabolite of SHM was formed *in vivo*. Three groups of ten mice were injected i.p. with 60 mg SHM/kg and killed 5 min after treatment. Chloroform extracts of pooled samples of blood or brain from each group were assayed for mutagenicity in the presence and absence of S9. The number of *his*⁺-induced revertants was 18 ± 7.5 (S.D.) per ml of blood and 27 ± 11 per g of brain in the absence of S9, and 51 ± 10 per ml of blood and 37 ± 10 per g of brain in the presence of S9. These results indicated the presence of a direct-acting mutagen in addition to SHM in these tissues 5 min after treatment.

Mice were also injected with [^{14}C]SHM and killed 15 min after treatment. The brains were excised, homogenized, extracted with chloroform, and the chloroform extracts pooled. The pooled extract contained 30% of the total radioactivity. Radioactive components in the pooled chloroform extract were separated by TLC, and the fractions were analyzed for radioactivity (Table 2). The fraction at R_f 0.43

Table 2. Relative levels of [^{14}C]SHM in TLC fractions of a 15-min brain extract*

R_f fraction	cpm	Relative %
0	122	5.8
0.22	127	6.0
0.43	1094	52
0.58	420	20
0.77	339	16

* Pooled chloroform extracts of brain from mice injected with [^{14}C]SHM at 60 mg/kg were chromatographed on a preparative silica gel G plate in acetone–chloroform (1 : 1). Fractions (0.5 cm) of gel were mixed with 1 ml of methanol and assayed for radioactivity as described.

corresponded to that of the mutagenic metabolite and represented 52% of the total extracted radioactivity. Further confirmation that this component (R_f 0.43) was identical to the mutagenic metabolite was obtained by isolation of the fraction followed by co-chromatography with the *in vitro*-isolated metabolite in two solvent systems (data not shown). The component at R_f 0.77 corresponded to SHM and represented 16% of the total radioactivity. Another major component (R_f 0.58) represented 20% of the total radioactivity but was not further identified.

DISCUSSION

SHM was designed to have an optimal partition coefficient for passage across the blood–brain barrier [1]. Preliminary studies by Plowman *et al.* [7] have indicated that low concentrations of SHM enter the cerebral spinal fluid of dogs after i.v. administration. Plowman and Adamson [8] later reported that, in rats and dogs, the amount of [^{14}C]SHM equivalents in brain tissue 4 hr after i.v. administration was equivalent to, and greater than, the total and unbound SHM equivalents in plasma respectively.

Results of the present study show that SHM, although a nitrogen mustard, is not mutagenic by itself but must first be metabolized before mutagenic activity is observed. The metabolic requirements for NADPH and liver S9 suggest that a microsomal mixed function oxidase is involved in the activation of SHM. In these respects, the requirement of metabolic activation of SHM to produce a mutagen(s) resembles that for cyclophosphamide, which also requires metabolic activation in the mutagenicity test system used here [9].

Chromatography of extracts from liver S9 mixtures that were incubated in the presence of [^{14}C]SHM indicated the formation of a single, major nonpolar metabolite (Fig. 3). Mutagenesis assays of the isolated metabolite fraction demonstrated this component to be a direct-acting mutagen (Table 1). Since the aqueous phase, obtained after chloroform extraction of the S9 reaction mixtures, contained no detectable mutagenic activity, it is likely that the chloroform-extractable metabolite is the mutagen assayed in crude extracts.

From the above discussion, it appears reasonable to propose that SHM is metabolized *in vivo* to a direct-acting mutagen. Results of *in vivo* studies with SHM-treated mice suggested that the mutagenic metabolite, along with SHM, passes the blood–brain barrier in measurable amounts. Since the mutagenic metabolite, like SHM, is an alkylating agent, it is possible that it may be a dominant mediator of the reported antitumor activity of SHM.

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