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1-Sulfooxymethylpyrene Is an Electrophilic Mutagen and Ultimate Carcinogen of 1-Methyl- and 1-Hydroxymethylpyrene

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1-Hydroxymethylpyrene and 1-sulfooxymethylpyrene were tested for complete carcinogenic activity by repeated s.c. injection in groups of 12 female Sprague-Dawley rats, respectively. A dose of 0.2 μmol of either 1-sulfooxymethylpyrene or 1-hydroxymethylpyrene was administered every other weekday for 20 doses (i.e., total dose 4 μmol) to each of 12 rats, beginning at 30 days of age. Once a week the rats were weighed then palpated for the appearance of tumors. Tumor-bearing rats were sacrificed 20-50 days after the appearance of first palpable tumor. By 52 weeks, 1-sulfooxymethylpyrene had induced sarcomas at the site of injection in 58% of the rats with an average induction time of 33 weeks. By contrast, 20, 0.2 μmol doses of 1-hydroxymethylpyrene failed to induce tumors at the site of injection in a group of 12 rats. Similarly, neither of the two control groups produced tumors. The present experiment, together with previous data, strongly supports the hypothesis that 1-sulfooxymethylpyrene is either itself an ultimate carcinogen or a direct precursor of an ultimate carcinogen, the highly reactive benzylic carbonium ion, which reacts with DNA to form aralkyl-DNA adducts in a chain of events leading to malignant growth.

In the early 1970s Flesher and Sydnor proposed an activation pathway for the induction of cancer based on the observation that polycyclic aromatic hydrocarbons (PAHs) methylated at specific positions had enhanced carcinogenic activity, and on the assumption that the ultimate carcinogen could be a benzylic carbocation, derived from reactive hydroxymethyl ester metabolites [1,2]. As opposed to the popular diol-epoxide pathway, this pathway proposed the formation of a reactive carbocation via initial bioalkylation at a reactive meso-anthracenic center with subsequent hydroxylation followed by conjugation with a good leaving group, e.g. sulfate, phosphate or acetate. Evidence demonstrating the existence of the metabolic reactions required this chain of substitution reactions is substantial and continues to grow. For instance, bioalkylation studies by Flesher et al. indicate that methyl substitution occurs *in vivo* for carcinogenic PAHs at the meso-anthracenic positions, whereas methylation does not readily occur for those PAHs devoid of such positions [3-6]. Microsomal enzymes responsible for hydroxylation of the methyl groups have been characterized by various groups in attempts to explain the carcinogenicity of DMBA [7,8]. Finally, hepatic sulfotransferase enzymes have been shown to catalyze the formation of PAH sulfate esters *in vivo* by Watabe et al. [9] and more recently by Surh et al. [10,11].

A series of hydroxymethyl PAH and their sulfooxymethyl derivatives are now being tested in our lab for complete carcinogenicity. 1-Sulfooxymethylpyrene (SMP) is an interesting example of an aralkylating ultimate carcinogen because its unsubstituted parent PAH, pyrene, is mutagenically and carcinogenically inert [9]. However, 1-methylpyrene (MP), a constituent of cigarette smoke, diesel soot, mineral oil, and coal, has been shown to produce mutation in the Ames assay [12] and hepatomas in the livers of newborn male mice [13]. Similarly, 1-

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hydroxymethylpyrene (HMP), is a mutagen capable of forming DNA adducts, both *in vitro* and *in vivo*, when incubated in the presence of cytosol fortified with the sulfo-group donor, 3′-phosphoadenosine-5′-phosphosulfate (PAPS) [14]. Additionally, HMP is a weak tumor initiator in the two-stage mouse skin test [14]. Finally, synthetic SMP, a strong direct mutagen toward *S. typhimurium*, is a modest tumor initiator in the two-stage mouse skin test and forms DNA adducts readily *in vivo* and *in vitro* [14].

These observations strongly support the foregoing hypothesis, but do not establish whether SMP, an exceptionally reactive electrophilic mutagen, is a comparatively active ultimate carcinogen. Therefore, the present study was undertaken to determine the extent to which SMP could account for the complete carcinogenic activity of HMP and MP.

MATERIALS

1-pyrenecarboxaldehyde was obtained from Aldrich Chemicals. HMP was synthesized following the adapted procedure of Flesher et al. [15]. Briefly, 1-pyrenecarboxaldehyde (8g, 34.7 mmol) dissolved in 550 mls ethanol was reduced with $NaBH_4$ (1.5g, 38.6 mmol) by reaction at reflux for 2 hrs. The reaction mixture was quenched with glacial acetic acid, then evaporated under reduced pressure. The residual white crystals of HMP were washed with water, filtered, and recrystallized from benzene (yield=79%, m.p. 116-119°C).

The sulfate ester was generated by the method of Surh et al., from the reaction of HMP with sulfuric acid and dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF) for 1 hour at 0°C [16]. The DCC urea precipitate was removed by centrifugation. The acid form of the sulfate ester, located in the supernatant was then neutralized with 1M methanolic NaOH. After evaporation under reduced pressure, the residue was dissolved in 1:1 DMF:ethanol. Upon the addition of 10 volumes ether, the sulfate ester precipitated out as the sodium salt (yield = 85%). The product was examined by reverse-phase TLC on Whatman KC18 plates in 9:1 methanol:water. SMP migrated as a single blue spot (Rf = 0.846).

23 day old female Sprague-Dawley weanlings were obtained from Harlan Sprague-Dawley (Madison, WI), housed in polyethylene cages with wood chip bedding, fed Purina rat chow and water ad libitum, then quarantined for one week prior to start experiment.

Method for determination of complete carcinogenicity. HMP and SMP were assayed for purity both prior to and during the injections. Each compound gave a single peak on a Waters reverse-phase HPLC fitted with a ODS column and an isocratic solvent system of 9:1 methanol:water (HMP Rt = 8.3min, or SMP Rt = 3.1min). Twelve rats, 30 days of age, were injected subcutaneously every other weekday with 0.2 μmol of either the sulfate or hydroxymethyl compound, in 0.1ml of 9:1 sesame oil:dimethylsulfoxide (DMSO), for 20 doses resulting in a total dose of 4 μmol. This schedule was chosen to reduce the anticipated toxicity of the reactive sulfate ester.

Two groups of twelve rats each served as controls. One group was administered vehicle only, in a manner similar to that already described, that is 0.1ml of 9:1 sesame oil:DMSO injected every other weekday for a total of twenty injections. Another control group was left untreated to check for spontaneous tumor development within the female Sprague-Dawley rat model. All rats were weighed and palpated once a week. Tumor-bearing rats were sacrificed 20-50 days after appearance of first palpable tumor. These tumors were fixed in neutral formalin for histological inspection of tumor type. The experiment was terminated at 52 weeks.

RESULTS

As shown in Fig. 1, of the four groups (SMP-treated, HMP-treated, vehicle-treated control, and untreated control) only the SMP treated group had sarcomas develop at the site of repeated injection. Seven of the twelve rats (58%) administered 4 μmol SMP, developed sarcoma at the site of repeated injection. Average induction time for these sarcomas was 33 weeks. Additionally, one rat, without sarcoma at the site of injection, developed a fibroadenoma.

A similar regime of HMP administered to a group of twelve rats, failed to induce sarcoma at the site of repeated injection (0%) at this dose. However, two rats did develop fibroadenomas. Neither control group, had any type of tumor formation at the end of the 52 week period.

DISCUSSION

Diol-epoxide derivatives are generally considered to be the principal ultimate carcinogens of the majority of polynuclear aromatic hydrocarbons. However, pyrene, 1-methylpyrene, 1-hydroxymethylpyrene and 1-sulfooxymethylpyrene do not possess a terminal benzo ring with

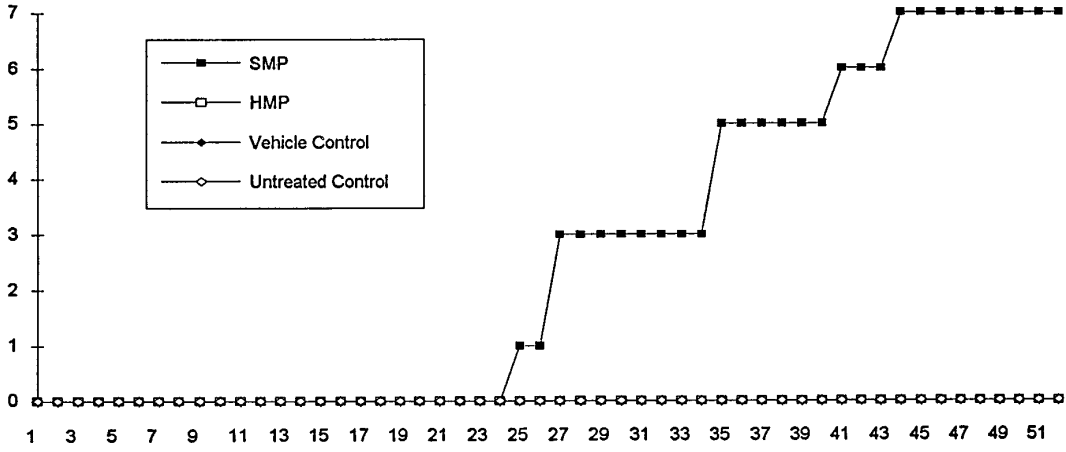

FIG. 1. Rats with sarcoma at the site of injection (Y-axis) vs weeks (X-axis).

four adjacent unsubstituted positions and therefore the formation of a bay-region diol-epoxide metabolite is not possible. Nevertheless, 1-methylpyrene, 1-hydroxymethylpyrene and 1-sulfooxymethylpyrene all possess some carcinogenic or tumor-initiating activity; with MP and HMP possessing weak activity, while SMP has comparatively stronger activity.

It is now generally accepted that hydroxymethyl sulfate ester metabolites play a role in mutagenesis and carcinogenesis of some PAHs [10]. The role of these sulfate esters, as defined in the aralkylating hydrocarbon hypothesis, is as exceptionally reactive ultimate carcinogen metabolites which are direct precursors to the highly reactive benzylic carbonium ion that reacts with DNA forming aralkyl-DNA adducts to initiate a chain of events leading to cancer (Figure 2). If this hypothesis is correct, it would be expected that the 1-sulfooxymethylpyrene would be of comparable, if not greater, potency to its metabolic precursors, 1-hydroxymethylpyrene and 1-methylpyrene, as a mutagen, as an electrophile in DNA adduct formation, and more importantly, as a complete carcinogen.

As mentioned previously, pyrene itself is both mutagenically and carcinogenically inert, with no evidence to date of DNA adduct formation. This is not surprising, since pyrene possesses neither a reactive meso-anthracenic nucleus to undergo bioalkylation, nor a bay-region to undergo diol-epoxide formation, it therefore possesses no obvious way to enter into reactions which, in theory, lead to cancer.

However, 1-methylpyrene, found within the environment can, in theory, enter into the chain of substitution reactions required by the aralkylating hydrocarbon pathway (12). Indeed MP, as previously mentioned, has been shown to be mutagenic in the Ames assay [12] and to possess weak carcinogenic activity in newborn male mice [13]. Notably, of the three possible pyrene monomethyl derivatives, only 1-MPs mutagenicity is profoundly increased when incu-

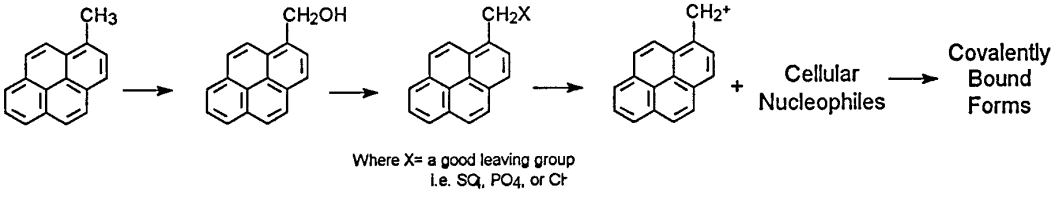

FIG. 2. Schematic for the metabolic activation of 1-methylpyrene.

bated *in vitro* with S-9 rat liver cytosol [12]. Unfortunately, it appears that no experimental data exist which determine whether 1-MP forms aralkyl-DNA adducts.

HMP, shown by Rice et al. [12] to be a metabolite of MP, is not a direct mutagen. However, when incubated in rat liver cytosol fortified with the sulfo-group donor, PAPS, HMP's mutagenicity is significantly enhanced to levels above those shown for MP [14,12]. HMP also possesses weak tumor initiating activity, as determined by the formation of papillomas in the two-stage mouse skin test [14], and is expected to have weak carcinogenic activity, although the dose of HMP administered in the present experiment was evidently insufficient to induce sarcoma. Additionally, when given i.p. in male Sprague-Dawley rats, HMP produces the same *in vivo* DNA adducts as are produced when HMP is reacted *in vitro* with calf thymus DNA, rat liver cytosol, and PAPS [14].

SMP, on the other hand, is a direct mutagen, a stronger tumor initiator in the two-stage mouse skin test than HMP and, as indicated by the data presented here, a moderately potent ultimate carcinogen. SMP yielded higher levels of the same adducts than HMP, both when it was reacted directly with DNA *in vitro,* and when injected i.p. in male rats for *in vivo* study [14]. Interestingly, the mutagenicity of SMP is strongly enhanced by anions such as acetate, chloride, or bromide [17-19]. These experiments indicate that electrophilic metabolites such as SMP may be converted to a secondary electrophilic aralkylating metabolites such as 1-chloromethylpyrene [CMP]. However, Surh et al. suggest that the increase of CMP mutagenicity relative to SMP is most probably due to their differences in lipophilicity since both SMP and CMP have relatively equal activity in producing benzylic DNA adducts *in vitro* [10].

Surh et al. found the aralkyl-DNA adducts produced in the livers of rats treated with tritium-labeled HMP accounted for 60-70% of bound radioactivity [14]. The major adducts from the reaction of SMP with DNA *in vitro,* as determined by post-labeling and autoradiography [20] confirm the findings of Surh et al. who used labeled compounds and HPLC analysis. The remaining 30-40% are thought to possibly arise from a 4,5 K-region epoxide [14]. Studies of the carcinogenic, and mutagenic nature of this epoxide are lacking, but could provide an additional means of testing the hypothesis. One electron oxidation to a radical-cation also seems unlikely since hydroxymethyl metabolites have not been shown to arise by this mechanism nor have hydroxymethyl metabolites been shown to be activated by one-electron oxidation.

Results from this experiment indicate that SMP fulfills all criteria required for an ultimate carcinogen; that is this highly reactive electrophilic ester metabolite is an active form of the parent PAH, pyrene, capable both of direct covalent binding to critical cellular macromolecules such as DNA, and of inducing cancer (sarcoma) in experimental animals when administered alone. In addition, it is evident that the electrophilic sulfate ester metabolite accounts for the complete carcinogenicity of 1-hydroxymethylpyrene and 1-methylpyrene in a satisfactory manner. Of note is the argument that, due to the highly reactive nature of such an ester it is anticipated that it should indeed be carcinogenic when delivered directly to test animals, and the data generated is therefore the product of circular reasoning. Two points of interest serve as a rebuttal to this argument. First, the trans-diolepoxide metabolites, although considered to be highly reactive, mutagenic, and tumorigenic have not proven to be as potent carcinogens as would have been expected, when delivered to whole animals. Secondly, as Harvey has postulated, the sulfate esters may be considered as relatively unimportant as active metabolites due to their rapid intracellular destruction by indiscriminate reaction with proteins and other cellular nucleophiles [21].

It appears, therefore, that the aralkylating hydrocarbon pathway may be the only mechanism capable of generating an ultimate carcinogen from 1-methylpyrene and 1-hydroxymethylpyrene. Additionally, we postulate that all aralkylating PAH, bearing a good leaving group, would be expected to easily generate benzylic carbonium ions that react with DNA to initiate the chain of cellular events which result in cancer. The expectation that the exceptionally reactive

electrophilic metabolite SMP would be a potent ultimate carcinogen was fully realized in accordance with the hypothesis that a chain of substitution reactions leading eventually to benzylic electrophilic metabolites, constitutes an important pathway in the mutagenesis and carcinogenesis for a majority of PAH.

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