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9-Sulfooxymethylanthracene Is an Ultimate Electrophilic and Carcinogenic Form of 9-Hydroxymethylanthracene

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The role of electrophilic hydroxymethyl sulfate esters in the metabolic activation, DNA-damage, mutagenicity, and complete carcinogenicity of polycyclic aromatic hydrocarbons has been elucidated considerably in recent years. The observations are in agreement with a unified hypothesis which predicts that electrophilic hydroxymethyl sulfate esters and closely related aralkylating agents are major ultimate carcinogenic forms of most, if not all, carcinogenic alkyl-substituted and even unsubstituted carcinogenic polycyclic aromatic hydrocarbons. The common final step in a chain of enzymatic substitution reactions is the formation of an aralkylating agent bearing a good leaving group. Activation of hydroxymethyl derivatives, including 9-hydroxymethylanthracene, to electrophilic mutagens has been shown to be catalyzed by 3′-phosphoadenosine-5′-phosphosulfate-dependent sulfotransferase activity. Recent studies, in a complete carcinogenic model, demonstrate that a number of sulfuric acid ester derivatives are more potent than their hydroxymethyl precursors by repeated subcutaneous injection in female Sprague-Dawley rats. In this paper, these observations have been extended to include 9-sulfooxymethylanthracene as an ultimate electrophilic and carcinogenic form of 9-hydroxymethylanthracene.

Primary and secondary benzylic alcohols derived from polycyclic aromatic hydrocarbons (PAH) can be metabolized to electrophilic hydroxyalkyl sulfate esters and closely related aralkylating agents. Reactivity toward DNA and mutagenic activity, *in vitro,* have been demonstrated for numerous metabolically and synthetically generated sulfuric acid esters (reviewed in 1). However, more *in vivo* data are required on the biological potency of this class of metabolically produced aralkylating agents in animals to clearly identify such aralkylating agents as ultimate electrophilic and carcinogenic forms of PAH. In the present experiments, we compared the complete carcinogenic activity of 9-hydroxymethylanthracene and its electrophilic sulfuric acid ester derivative by subcutaneous injection in female Sprague-Dawley rats. We have investigated 9-hydroxymethylanthracene as a model compound because 1) it can be activated to a potent electrophilic mutagen in the presence of liver cytosol sulfotransferase activity and 3′-phosphoadenosine-5′-phosphosulfate (2), and 2) it cannot be metabolized to vicinal bay-region dihydrodiol-epoxides, but it could be formed from 9-methylanthracene radical- cation. The prediction that the aralkylating agent 9-sulfooxymethylanthracene would be an ultimate electrophilic and carcinogenic form of 9-hydroxymethylanthracene was fully realized.

MATERIALS AND METHODS

9-Hydroxymethylanthracene. Using conditions similar to those previously described for the reduction of 6-formylbenzo[a]pyrene to 6-hydroxymethylbenzo[a]pyrene, 9-anthraldehyde (8 g, 38.8 mmol, purchased from Aldrich Chemicals) and $NaBH_4$ (1.64 g, 43.2 mmol) in 250 ml ethanol was heated to reflux for 2 hr in an oil bath (3). The solution was treated with acetic acid and the solvent removed completely under reduced pressure. The solid was washed with water, filtered, and recrystallized from benzene (5.318 g, 25.57 mmol) for a 72% yield with mp 159-160°C, lit. mp 161-162°C (4). Thin-layer chromatography on Whatman KC-18 reverse-phase plates gave a single spot (R_f = 0.555) in a 9:1 methanol:water solvent system. Reverse-phase HPLC on a Waters system fitted with ODS column and eluted with isocratic 9:1 methanol:water resulted in a single peak at R_t = 7.06 min.

9-Sulfooxymethylanthracene. The compound was prepared following the method developed by Horn et al. for the synthesis of 1-sulfooxymethylpyrene (5). Briefly, 9-hydroxymethylanthracene (69 mg, 0.33 mmol) in 5 ml ice cold dimethylformamide (DMF) and 5-fold molar excess of dicyclohexylcarbodiimide (DCC) (0.3438 g, 1.665 mmol) in 5 ml ice-cold DMF was reacted at 0°C with stirring for one hour after the slow addition of 1.5-fold molar excess of sulfuric acid in 1 ml ice cold DMF. The sulfuric acid ester contained in the supernatant and subsequent washings, was collected by centrifugation, and neutralized with 1M methanolic NaOH. The solution was then taken to near dryness under reduced pressure. The resulting

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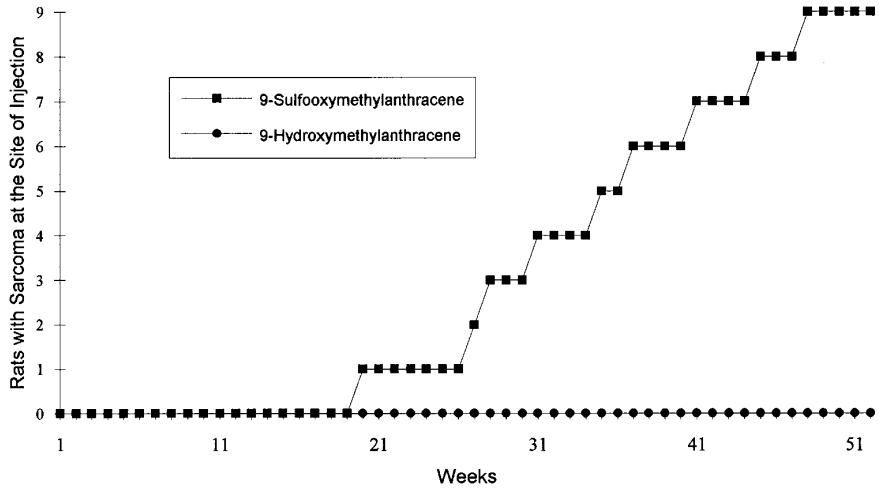


FIG. 1. By 52 weeks, a total dose of 4 μmol 9-sulfooxymethylanthracene induced sarcomas at the site of injection in 9 of 12 female Sprague-Dawley rats. A total dose of 4 μmol 9-hydroxymethylanthracene failed to induce tumors within the 52 week experimental period. Likewise, neither the vehicle treated nor the non-treated controls had sarcoma develop by 52 weeks.

residue was dissolved in 1.5 ml ice cold DMF:ethanol (1:1). Addition of 10 volumes ice-cold ether (15 ml) precipitated the sulfate ester as a sodium salt, which was collected by centrifugation and dried under reduced pressure (yield 76%). Thin-layer chromatography on Whatman KC18 reverse-phase plates gave a single spot (R_f = 0.855) in a 9:1 methanol:water solvent system. Reverse-phase HPLC on a Waters system fitted with ODS column and eluted with isocratic 9:1 methanol:water resulted in a single peak at R_t = 3.6 min. The sulfate ester was stored at -70°C.

Determination of complete carcinogenicity. Twenty-one day old, female, Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, In.). All animals were acclimatized to the animal room for one week prior to the experiment. Throughout the experiment animals were housed in cages with wood chips for bedding, 3 rats per cage, in a temperature-controlled animal room with an alternating light-dark cycle of 12 hours while given Purina rat chow and tap water *ad libitum* (6).

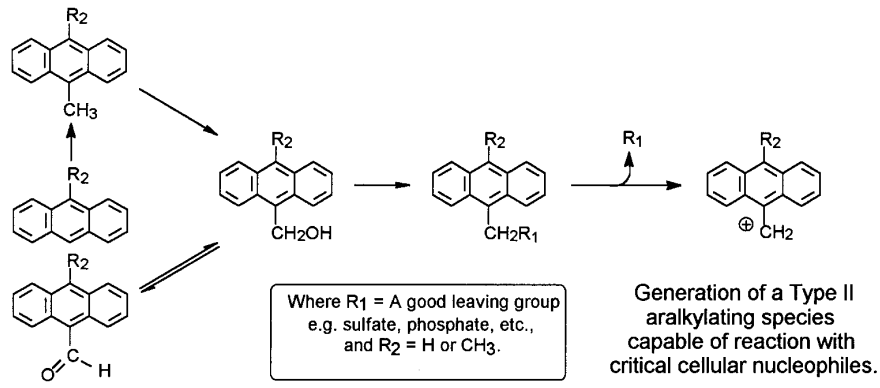
The metabolites to be tested were analyzed for purity by HPLC, reprecipitated as needed to maintain purity, and then made to desired concentration in 9:1 sesame oil:DMSO (0.2 μmol/0.1ml). The metabolites were administered in 0.2 μmol doses to their respective rat group by subcutaneous injection (dorsal subcutis) three times per week for 20 doses resulting in a total dose of 4 μmol. The initial dose was administered when rats were thirty days of age. In addition, one control group of twelve rats was administered vehicle only, i.e. sesame oil:DMSO (9:1), while another control group of twelve rats remained untreated. All animals were weighed once each week and examined for the presence of tumors. Ten to thirty days after the appearance of a palpable tumor, the animal was sacrificed and all grossly pathological tissue was removed, fixed in 10% neutral formalin, and prepared for histological examination of tumor type. Tumor-negative animals were observed for 52 weeks prior to autopsy.

RESULTS

As shown in Figure 1, only the 9-sulfooxymethylanthracene treated group developed sarcomas at the site of repeated subcutaneous injection. Nine of twelve rats (75%) administered a total dose of 4 μmol 9-sulfooxymethylanthracene were tumor bearing by 48 weeks, with the first tumor appearing 20 weeks after the initial 0.2 μmol dose. The observation period for the appearance of tumors continued for 52 weeks. 9-Hydroxymethylanthracene, administered in the same manner and dose to a group of twelve rats, failed to induce tumors. Similarly, no tumors were observed in either the untreated or vehicle-treated control groups. Clearly, 9-sulfooxymethylanthracene has been identified as an ultimate electrophilic and carcinogenic form of 9-hydroxymethylanthracene.

DISCUSSION

The metabolic activation/inactivation, and chemical reactivity of unsubstituted carcinogenic PAH, and derivatives of aromatic type ArX, have been considered to be fundamentally different from carcinogenic methyl-substituted PAH, and their derivatives of aromatic type $ArCH_2X$ (7, 8). Brookes and Dipple proposed two types of electrophilic intermediates could be derived from carcinogenic hydrocarbons. Type I could be derived from the meso-phenanthrenic region of unsubstituted hydrocarbons, whereas Type II could be derived from methyl-substituted hydrocarbons, in which the meso-anthracenic region was the site of substitution (9). However, the mechanisms by which these possible electrophilic intermediates were derived was not clear. To provide a connecting link between aromatic types ArX and $ArCH_2X$, Flesher and Sydnor formulated a unified hypothesis which states that the complete carcinogenicity of unsubstituted PAH and their meso-region derivatives is dependent primarily upon the occurrence of a chain of *in vivo* substitution reactions leading ultimately to highly reactive electrophilic and carcinogenic forms capable of generating aralkylating intermediates of Type II. The unified hypothesis was originally derived from the results of



SCHEME 1. Scheme to account for the metabolic activation and carcinogenicity of 9-hydroxymethylanthracene and metabolically related compounds, as predicted by the unified hypothesis.

metabolism and complete carcinogenicity studies of compounds formed by substitution of the active meso-region of benz[a]anthracene and benzo[a]pyrene, with methyl, hydroxymethyl, chloromethyl, formyl and related groups (3,6,10,11,12). According to this hypothesis the first step in carcinogenesis by unsubstituted PAH, such as anthracene, is methyl-substitution in the electron-dense meso-region (10). The second step, and the point at which methyl-substituted PAH such as 7,12-dimethylbenz[a]-anthracene might enter into this activation pathway, is hydroxylation of the methyl group (11). The third step, and the point where hydroxymethyl-PAH or reduced formyl-PAH enter, is the formation of a derivative (e.g. sulfate ester) bearing a good leaving group to generate a highly reactive carbonium ion. The carbonium ion would be expected to react with critical nucleophiles to start a chain of cellular events which result in cancer (6). See Scheme 1.

Although the carcinogenicity of anthracene has been disputed, high doses of the carefully purified compound were reported to be carcinogenic by subcutaneous injection in rats (13). The pathways by which anthracene and its meso-region derivatives could be activated/inactivated are of particular interest because the anthracene ring system is present in most, if not all, of the most potent carcinogenic polynuclear aromatic hydrocarbons, and because the unified hypothesis predicts the presence of an active meso-region as a structural requirement for the attainment of complete carcinogenic activity (14).

In support of this unified hypothesis, methylation of numerous unsubstituted PAH at meso-regions generally enhances both carcinogenic and mutagenic activities. Although it was found that 9-methylanthracene is formed *in vivo* in rat subcutaneous tissue (15), neither anthracene nor 9-methylanthracene appear to act as tumor initiating agents on mouse skin, whereas, 9,10-dimethylanthracene was significantly tumorigenic (16). This could be explained by the fact that 9-methylanthracene was metabolically demethylated to anthraquinone (17). Other studies demonstrate that meso-region methylation of anthracene, benz[a]anthracene, dibenz[a,h]anthracene and benzo[a]pyrene is catalyzed by S-adenosyl-L-methionine-dependent methyltransferase activity in rat liver cytosol (14, 18, 19).

In vitro or *in vivo*, oxidation to hydroxymethyl metabolites has been shown to occur for a variety methyl-substituted compounds, including 9-methylanthracene (15, 17). Numerous hydroxyalkyl derivatives of polycyclic aromatic hydrocarbons can be metabolized or chemically transformed to aralkylating agents, e.g. hydroxyalkyl sulfate esters, acetate esters, phosphate esters, chlorides, bromides and iodides, which act as electrophilic mutagens and ultimate carcinogens and which are capable of forming aralkyl-DNA covalently bound products (1,2,4,6,14,20,21). Numerous hydroxymethyl derivatives tested for mutagenicity in *Salmonella typhimurium* TA98 in the presence of liver cytosolic sulfotransferase activity from adult male or female rats and 3′-phosphoadenosine-5′-phosphosulfate cofactor were activated to electrophilic mutagens. These hydroxymethyl derivatives included 1-hydroxymethylpyrene, 9-hydroxymethylanthracene, 9-hydroxymethyl-10-methylanthracene, 7-hydroxymethyl-12-methylbenz[a]anthracene, 6-hydroxymethylbenzo[a]pyrene and 6-hydroxymethylanthanthrene (2). The major sulfotransferase enzyme from the liver of adult female rats was later purified and identified as hydroxysteroid sulfotransferase a. Stable expression of the STa-1 or STa-2 isoform enhanced cytotoxicity of 1-hydroxymethylpyrene, 9-hydroxymethylanthracene and 6-hydroxymethylbenzo[a]pyrene in Chinese hamster V79 cells as compared with sulfotranferase-deficient control cells (22).

It should be noted that four of the hydroxymethyl derivatives, 1-hydroxymethylpyrene, 9-hydroxymethylanthracene, 9-hydroxymethyl-10-methylanthracene and 6-hydroxymethylanthanthrene, do not possess bay-regions. Clearly, bay-region diol-epoxide activation is excluded for these hydroxymethyl derivatives and related compounds which do not possess bay-regions.

Recently, a series of hydroxymethyl sulfate ester derivatives have been identified as ultimate electrophilic and carcinogenic forms of the hydroxymethyl-PAH, including 1-hydroxymethylpyrene (5), 7-hydroxymethylbenz[a]-anthracene (23), 7-hydroxymethyl-12-methylbenz[a]-anthracene (24), 6-hydroxymethylbenzo[a]pyrene (25), and 1-hydroxy-3-methylcholanthrene (26). In the present study we found similar ultimate electrophilic and carcinogenic activity by subcutaneous injection in female Sprague-Dawley rats for 9-sulfooxymethylanthracene which, like 1-sulfooxymethylpyrene, does not possess a bay-region. Although no carcinogenic activity was found for 9-hydroxymethylanthracene at this relatively low dose, larger doses would presumably be carcinogenic if 9-hydroxymethylanthracene can be metabolized sufficiently to 9-sulfooxymethylanthracene *in vivo*.

Boyland and Levi found dihydroxydihydroanthracene in the urine of rabbits fed anthracene (27). Since the unified hypothesis excludes the addition reactions, which destroy aromatic character, as activating reactions in carcinogenesis, dihydroxydihydroanthracene is predicted to be carcinogenically inert. It also excludes quinones formed from anthracene radical-cation in aqueous media (16, 28). On the other hand, it does not exclude metabolic one-electron oxidation to an electrophilic radical-cation intermediate or to the formation in aqueous media of a hydroxymethyl derivative from 9-methylanthracene (16, 28). The unified hypothesis could be disproved if it could be shown that some other electrophilic mutagen and ultimate carcinogen accounts for more of the complete carcinogenic activity of anthracene, 9-methylanthracene, and 9-hydroxymethylanthracene than the electrophilic ester 9-sulfooxymethylanthracene.

SUMMARY AND CONCLUSIONS

1. The present concept that complete polycyclic hydrocarbon carcinogenesis is dependent primarily on the occurrence of biochemical substitution reactions leading ultimately to electrophilic hydroxymethyl sulfate esters and related aralkylating agents, is about as satisfactory a hypothesis as can be formulated on the basis of the facts available.

2. Sulfate conjugation of intermediary hydroxyalkyl metabolites, catalyzed by sulfotransferase activity, is a final common step in the metabolic activation of most, if not all, carcinogenic PAH.

3. Mechanisms of carcinogenesis based on addition reactions of the administered hydrocarbon, *in vivo*, are not derived from tests for carcinogenicity that could account for the strong complete carcinogenicity of the parent hydrocarbon.

4. One-electron oxidation produces electrophilic radical-cation intermediates from methyl-substituted derivatives which in aqueous medium react with water to generate oxygen-containing carcinogenic derivatives. These derivatives fit into our present concept of a chain of substitution reactions leading ultimately to carcinogenesis.

5. The hypothesis that hydroxyalkyl sulfate esters play a major role in polycyclic hydrocarbon carcinogenesis could be disproved if it could be shown that some other electrophilic mutagen and ultimate carcinogen accounts for more of the complete carcinogenic activity of PAH and their intermediary hydroxyalkyl metabolites than hydroxyalkyl sulfate esters and closely related aralkylating agents.

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