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7-Sulfooxymethyl-12-methylbenz[a]anthracene Is an Exceptionally Reactive Electrophilic Mutagen and Ultimate Carcinogen[1]

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The hypothesis was tested that an ultimate carcinogen of 7-hydroxymethyl-12-methylbenz[a]anthracene (HMBA), a major metabolite of 7,12-dimethylbenz[a]-anthracene (DMBA), is a benzylic carbonium ion generated from an exceptionally reactive aralkylating metabolite, such as an electrophilic sulfate ester. In conformity with this hypothesis, sarcomas were rapidly induced in rats following repeated subcutaneous injection of HMBA (67%) or its electrophilic sulfate ester, sodium 7-sulfooxymethyl-12-methylbenz[a]anthracene (SMBA) (100%). It would appear from the results summarized here that the search for a carcinogenic metabolite of DMBA has been successful. In addition, an aralkylating electrophilic mutagen and carcinogen has been prepared from HMBA, which is itself either an ultimate carcinogen or a direct precursor of an ultimate carcinogen, i.e., a benzylic carbonium ion.

Previous experiments have established that 7,12-dimethylbenz[a]anthracene (DMBA) is metabolized to 7-hydroxymethyl-12-methylbenz[a]anthracene (HMBA) in rat liver homogenates (1,2,3) and in rat mammary gland (3). The metabolite was also found to be a potent complete carcinogen (4,5). In 1967, Flesher et al. suggested that mammary cancer induced by DMBA in female Sprague-Dawley rats was mediated by HMBA (2). Earlier, Boyland et al. had suggested that HMBA might play a role in carcinogenesis by DMBA, but commented that this is not certain (4). This was the first instance of a hydrocarbon metabolite which demonstrated strong complete carcinogenic and toxic properties (6). Studies of its distribution showed that far less of HMBA than DMBA reached the mammary gland and other fatty tissue (7). This was in accordance with the finding of its lower carcinogenicity for mammary tissue.

The hypothesis was tested that a highly reactive carbonium ion intermediate is an ultimate carcinogen of HMBA. The hypothesis states that HMBA, a major metabolite of DMBA, is metabolically activated by forming an aralkylating metabolite, bearing a good leaving group (e.g, sulfate ester), which would be expected to generate a highly reactive benzylic carbonium ion in an S_N1 reaction. The carbonium ion would be expected to react with critical nucleophiles to initiate a chain of cellular events which result in cancer (5,8). However, the data could also be accounted for by assuming an S_N2 reaction.

In support of the aralkylating metabolite hypothesis, HMBA and related compounds were shown to induce sarcomata at the site of repeated s.c. injection: 7-iodomethyl-, 7-bromomethyl-, 7-chloromethyl-, 7-benzoyloxymethyl-, 7-acetoxymethyl-, and 7-formyl-12-methylBA. Only DMBA and HMBA induced breast cancer in addition to subcutaneous sarcomas. The high degree of complete carcinogenicity of the most active compounds, considered together with their chemical structure, suggested that conversion to highly reactive electrophilic metabolites or electrophilic intermediates must occur *in vivo* (5,8).

Buu-Hoi et al. studied 14 halogenated aralkylating derivatives for their actions on DNA template activity in a DNA-dependent RNA polymerase system. They reported that a perfect correlation exists between the inhibitory effect of the aralkylating derivatives on RNA synthesis and the carcinogenic potencies of the corresponding non-halogenated hydrocarbons. The halomethyl aralkylating derivatives were considered to be closely related to the active forms of the parent compounds, and to be able to easily generate benzylic carbonium ions, in support of the hypothesis (9).

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Further support for the hypothesis of aralkylating metabolites as electrophilic mutagens and ultimate carcinogens of HMBA was provided by the observation that HMBA underwent reaction with calf thymus DNA in the presence, but not in the absence, of a 3′-phosphoadenosine-5′phosphosulphate (PAPS) generating system (10). However, owing to the complexity of the cytosolic enzyme system and its possible capacity for generating a phosphate ester, the biosynthesis of 7-hydroxymethyl sulfate ester (SMBA) was not clearly established. Nevertheless, a reaction between HMBA and DNA did appear to be partially dependent on the presence of sulfate ion (10). Subsequently, Watabe et al. convincingly demonstrated that sulfotransferase activity in rat liver cytosols fortified with HMBA and PAPS catalyzes the synthesis of the electrophilic mutagen, SMBA (11). They have repeatedly emphasized the importance of this pathway in carcinogenesis of HMBA (11-14). Furthermore, both HMBA and SMBA gave the same aralkyl-DNA covalently bound products *in vitro* and *in vivo* (12,15).

These observations strongly support the aralkylating metabolite hypothesis, but do not establish whether SMBA, an exceptionally reactive electrophilic mutagen, is a potent ultimate carcinogen. The present study was undertaken to determine whether the electrophilic mutagen SMBA is a potent ultimate carcinogen. In addition, the experiments in this report were undertaken to determine the role of SMBA in the metabolic activation and carcinogenicity of HMBA.

MATERIALS AND METHODS

Chemicals. 7-Hydroxymethyl-12-methylbenz[a]anthracene (HMBA) was prepared as previously described by Flesher et al. with m.p.160-2°C (2). Analysis of purity yeilded a single peak on reverse-phase HPLC with 9:1 methanol:water elution (R_t=0.43min) and single spot on reverse-phase TLC on Whatman KC 18 plates in 9:1 methanol:water (R_f=10.6).

7-Sulfooxymethyl-12-methylbenz[a]anthracene (SMBA) was prepared from HMBA and sulfuric acid by the dicyclohexylcarbodiimine method essentially as described for the synthesis of the electrophilic sulfuric acid ester of 6-hydroxymethylbenzo[a]pyrene (16). The sulfuric acid ester of 7-hydroxymethyl-12-methylbenz[a]anthracene was neutralized with methanolic NaOH and isolated as a sodium salt by the addition of 10 volumes of dry ether to a solution of the compound in dimethylformamide:ethanol (1:1) to precipitate the product. The product was collected by centrifugation and dried under reduced pressure. Purity was greater than 96% by reverse phase HPLC (methanol:water, 9:1), retention time 3.4 min and gave essentially a single spot (R_f 0.86) by reverse phase TLC carried out on Whatman KC 18 plates in methanol:water (9:1).

Determination of complete carcinogenicity. Groups of twelve, 23 day old Female Sprague Dawley rats, purchased from Harlan Sprague-Dawley (Indianapolis, IN), were acclimatized to the animal room for 1 week prior to the experiments. All 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*. One control group was administered sesame oil:DMSO alone (9:1). The other control group was untreated. The metabolites to be tested were checked for purity by HPLC, then made up to the desired concentration (0.2 μmol/0.1ml) in 9/1 sesame oil/DMSO and stored in dark bottles until used for injection. The metabolites were administered by subcutaneous injection (dorsal subcutis) of 0.2 μmol, 3 times per week for 20 doses. The initial dose was administered when the rats were 30 days of age. All animals were weighed once each week and examined for the presence of tumors. Twenty to fifty 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 microscopic examination. Tumor-negative animals were observed for 52 weeks prior to autopsy (5).

RESULTS

As shown in Fig. 1, HMBA and SMBA induced sarcomas at the site of repeated subcutaneous injection, in a remarkably short time. SMBA was more rapid in inducing sarcomas at the site of subcutaneous injection than HMBA, but by 32 weeks both compounds had induced sarcomas in a high percentage of the animals tested. No tumors were found in a control group given sesame oil:DMSO (9:1) alone or in an untreated control group. Under these conditions, SMBA is at least as potent as HMBA.

A total dose of 4 μmol SMBA administered at the site of subcutaneous injection in female Sprague Dawley rats was the same total dose of DMBA administered by Surh et al. (17) and the resulting incidence of sarcomas were similar, suggesting that SMBA could mediate most, if not all, of the complete carcinogenicity of DMBA by subcutaneous injection. It appears that the strong toxicity of SMBA noted by Surh et al. (17) was alleviated by dividing the total dose into 20 doses. We found no evidence of SMBA toxicity under these conditions.

DISCUSSION

It is now generally accepted that electrophilic hydroxymethyl sulfate ester metabolites play a role in mutagenesis and carcinogenesis by some polynuclear aromatic hydrocarbons (18-22). Additionally, recent work identified 1-sulfooxymethylpyrene as an electrophilic mutagen and ultimate carcinogen (23). We define the ultimate carcinogens, or activated forms of PAH, as exceptionally reactive electrophilic metabolites, or reactive intermediates, that are capable of forming covalent bonds with critical cellular components and causing cancer in whole animals. An ultimate carcinogen is an activated metabolite of a proximate carcinogen.

An example of a hydroxymethyl hydrocarbon that is activated to one or more DNA reactive electrophilic metabolites or reactive intermediates is HMBA, a major metabolite of DMBA. Previous studies on the mutagenicity and carcinogenicity of known or potential metabolites of HMBA and their covalent binding to DNA strongly support the hypothesis that an ultimate carcinogen of HMBA is an aralkylating metabolite capable

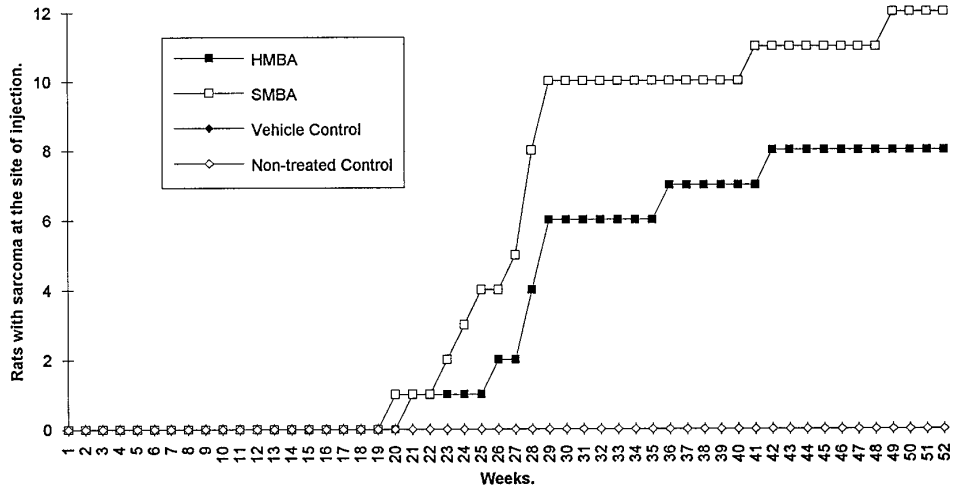
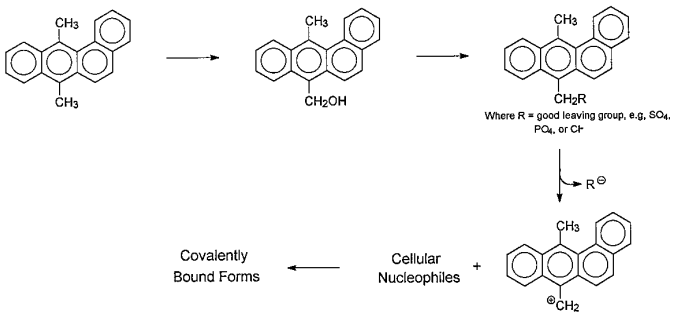


FIG. 1. By 52 weeks, a total dose of 4μmol SMBA induced sarcomas at the site of injection in 12 out of 12 rats with an average induction time of 29 weeks. A total dose of 4μmol HMBA induced sarcomas in only 9 out of 12 rats with an average induction time of 30 weeks. Neither the vehicle (9:1 sesame oil:DMSO) treated, nor the untreated control group had sarcoma induced at the site of s.c. injection.

of generating a benzylic carbonium ion (5,10-15,24). See Scheme 1.

That SMBA is an exceptionally reactive electrophilic mutagen and potent ultimate carcinogen has been firmly established. SMBA was more potent than the strong carcinogen HMBA by repeated subcutaneous injection in female Sprague-Dawley rats. Therefore, we conclude that SMBA is an exceptionally reactive activated metabolite of HMBA that accounts for most, if not all, of the complete carcinogenicity of HMBA in a very satisfactory manner in this tumor model. Since HMBA is a major metabolite of DMBA and SMBA is a major metabolite of HMBA, it seems likely that SMBA also accounts for at least a small fraction of the complete carcinogenicity of DMBA.

However, it has been suggested that HMBA also could be activated, *in vivo,* by addition of water to an epoxide to form a dihydrodihydroxy metabolite as a proximate carcinogen followed by addition of a second epoxide to the terminal benzo ring to form a diol epoxide metabolite of HMBA as the ultimate carcinogen (25-27).



SCHEME 1. Scheme for the metabolic activation and carcinogenicity for HMBA, and possibly DMBA. The carbocation shown has several resonance structures which may be relevant to the carcinogenicity of the compound.

Recently, Surh et al. (17) compared the carcinogenicity of HMBA and SMBA with DMBA. They found that although SMBA was an exceptionally reactive electrophilic mutagen it did not appear to be more carcinogenic than HMBA or DMBA. This is not surprising as it is unlikely that highly polar metabolites such as HMBA and SMBA, administered to a test animal, could penetrate cell membranes as readily and rapidly as DMBA. Furthermore, Surh et al. offered several reasons for the apparent relative weakness of the aralkylating metabolite, SMBA, as an ultimate carcinogen (17):

1) It was difficult to test more than very low levels of SMBA because it was highly toxic, and
2) It was likely that the SMBA was rapidly hydrolyzed to HMBA.

In addition, Harvey has offered the hypothesis that the apparent relative weakness of hydroxymethyl sulfate esters as ultimate carcinogens may be accounted for by their exceptional chemical reactivity that is responsible for their rapid intracellular destruction by indiscriminate reactions with proteins and other cellular nucleophiles (28). However, it should be noted that if exceptional chemical reactivity destroys most of the hydroxymethyl sulfate ester, then the carcinogenic dose is much smaller than the administered dose. It seems likely therefore, that the incidence of tumors induced by the actual carcinogenic dose of the exceptionally reactive 7-hydroxymethyl sulfate ester, was far less than the administered dose. If this is the case, then the aralkylating metabolite, SMBA, is also an exceptionally potent ultimate carcinogen.

The carcinogenicity studies of Surh et al. (17) indicate that most, if not all, of the complete carcinogenic activity and toxicity of HMBA is caused by SMBA. This follows from the fact that SMBA was comparable to HMBA in several tests. A single 0.01 μmol/g body weight i.p. dose of HMBA induced hepatomas in 94% of male B6C3F1 mice whereas for the same dose of SMBA the incidence was 58%. At 0.0025 μmol/g body weight, the incidence of hepatomas was 42% and 39%, respectively. SMBA was comparable to HMBA, at three sub-carcinogenic dose levels in the two stage mouse skin model, and, at two dose levels, in inducing sarcomas at the site of s.c. injection in female Sprague-Dawley rats, but SMBA was more active than HMBA in inducing lung adenomas in female A/J mice. A total dose of 4 μmol divided into two doses each of either HMBA or SMBA induced sarcomas at the site of injection in only 2 of 12 female Sprague-Dawley rats at 16 months. Because SMBA was not, generally, more potent than HMBA or the extremely powerful carcinogen DMBA, Surh et al. reasoned that the exceptionally reactive electrophilic mutagen SMBA does not appear to play a role in carcinogenesis by either HMBA or DMBA. To further support this view the authors cited evidence for the rival diol-epoxide theory. They concluded that based on these studies, and the results in their paper, DMBA appears to be activated in vivo to a DNA-binding and presumed carcinogenic species, most probably DMBA-3,4-dihydrodiol-1,2-epoxide, without metabolism to the 7-hydroxymethyl derivative.

An opposing view is that more than one mechanism may be responsible for the metabolic activation and carcinogenicity of DMBA. Also, recent theoretical work from this laboratory predict that dihydrodiol epoxide metabolites may be relatively unimportant in the metabolic activation and carcinogenicity of a majority of PAH (29,30).

Although the data in the present report indicates that, SMBA accounts for the complete carcinogenicity of HMBA in a very satisfactory manner, it must be admitted that at the moment, SMBA may account for only a small fraction of the complete carcinogenicity of DMBA. Therefore, DMBA also could be activated, *in vivo*, to a DNA reactive and carcinogenic electrophile either by one-electron oxidation to a benzylic carbonium ion and/or by a series of enzymatic addition reactions to form a terminal ring triol benzylic carbonium ion, without the intervening process of metabolism to HMBA. RamaKrishna et al., have reported that 3-methylcholanthrene-induced rat liver microsomes activated DMBA mainly by one-electron oxidation to form DMBA-DNA adducts involving the 12-methyl group, whereas adducts bound to DNA through the 7-methyl group were not detected (31). However, one-electron oxidation cannot account for the formation, DNA binding, mutagenicity or carcinogenicity data for either HMBA or SMBA.

Some evidence supports the hypothesis that a diol epoxide metabolite could account for the carcinogenic activity of DMBA. The trans-3,4-dihydrodiol metabolite induced papillomas in the two stage mouse skin test and lung adenomas in new born mice (32,26). However, potent tumor-initiating activity of the trans-3,4-dihydrodiol metabolite, in the absence of evidence for a metabolite with strong promoting activity, cannot account for the complete carcinogenic activity of DMBA. Furthermore, the diol-epoxide mechanism is not derived from carcinogenicity tests of a bay-region dihydrodiolepoxide derivative that could account for most, if not all, of the strong complete carcinogenicity of DMBA. Nevertheless, one or both of these alternative mechanisms may account for most of the metabolic activation and complete carcinogenicity of DMBA.

The results of the present study, taken together with the results of previous investigations, strongly support the aralkylating metabolite hypothesis of Flesher and Sydnor for HMBA outlined above. Aralkylating hydrocarbons are a class of exceptionally reactive DNA damaging agents that are mutagenic, carcinogenic, and teratogenic. When proximate carcinogens are activated in vivo to aralkylating metabolites, ultimate carcinogens are formed. Thus, aralkylating hydrocarbons are models for ultimate carcinogens. Clearly, SMBA has been identified as an ultimate electrophilic and carcinogenic form of HMBA and possibly DMBA. Further studies are necessary to identify clearly the ultimate electrophilic and carcinogenic forms of DMBA. Also, comparative studies are needed to determine the relative potency of the potential diol epoxide metabolites and SMBA as prime candidates for the most potent ultimate carcinogen of DMBA. In this regard, the hypothesis that SMBA could account for most, if not all, of the complete carcinogenicity of HMBA/DMBA could be disproved, if it could be shown that some other electrophilic mutagen and ultimate carcinogen accounts for far more of the complete carcinogenicity of HMBA/DMBA than SMBA.

CONCLUSIONS

The present paper records our and others efforts to gain an understanding of the nature of the ultimate carcinogen of HMBA which presumably influences the flow of genetic information coded in the base sequence of DNA. The direction taken by the series of investigations discussed was greatly influenced by three factors. One was the assumption that an intermediary carcinogenic metabolite between the procarcinogen DMBA and the ultimate carcinogen must exist. The second assumes that this intermediary complete carcinogen could be isolated, identified, and synthesized. The third assumes that the ultimate carcinogen would be exceptionally DNA reactive and that metabolic precursors of the ultimate carcinogen possess no intrinsic carcino-

genic activity because they are not themselves exceptionally DNA reactive. Before the intermediary complete carcinogen could be accepted as a point of departure for a more extensive search for the ultimate carcinogen, its carcinogenicity had to be established beyond doubt. This was accomplished in previous studies and confirmed in the present study. It was further shown that the lower mammary carcinogenicity following oral administration was due to lower concentrations in tissues. Having established HMBA as a general complete carcinogen and devised a method for its synthesis, it was possible to go to the next stage of investigation and inquire into the nature of the ultimate carcinogen. This was done in terms of selective synthesis of aralkylating derivatives related to HMBA. It was possible to show that tumors were indeed rapidly formed when aralkylating ester and halide derivatives of HMBA were tested. In the present study, the expectation that the aralkylating metabolite, SMBA, would be a potent ultimate carcinogen was fully realized. It would appear from the results summarized here that the search for a carcinogenic metabolite of DMBA has been successful. In addition, an exceptionally reactive electrophilic ester, SMBA, has been synthesized, as an activated form of HMBA, which is either itself an ultimate carcinogen or a direct precursor of an ultimate carcinogen of HMBA and possibly DMBA.

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