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Carcinogenicity of 1-Hydroxy-3-methylcholanthrene and Its Electrophilic Sulfate Ester 1-Sulfooxy-3-methylcholanthrene in Sprague-Dawley Rats

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Previous experiments have demonstrated that the carcinogen 1-hydroxy-3-methylcholanthrene is a metabolite of 3-methylcholanthrene. 1-Sulfooxy-3-methylcholanthrene, prepared by chemical synthesis from 1-hydroxy-3-methylcholanthrene, was shown to be a direct acting electrophilic mutagen and DNA damaging agent. These results imply that 1-hydroxy-3-methylcholanthrene could be metabolically activated to an ultimate electrophilic and carcinogenic form of 1-hydroxy-3-methylcholanthrene and 3-methylcholanthrene in a reaction catalyzed by 3′-phosphoadenosine-5′-phosphosulfate-dependent sulfotransferase activity. 1-Hydroxy-3-methylcholanthrene and its aralkylating reactive ester, 1-sulfooxy-3-methylcholanthrene, were individually administered to groups of 12 female Sprague-Dawley rats at a 0.2 μmol dose, three times weekly, for 20 doses. 1-Sulfooxy-3-methylcholanthrene induced sarcomas at the site of injection in 8 of 12 rats (66%) by 52 weeks, whereas 1-hydroxy-3-methylcholanthrene induced sarcomas at the site of injection in 5 of 12 rats (42%) by 52 weeks. These results, taken together with the results of previous experiments, strongly support the hypothesis that the activated electrophilic mutagen 1-sulfooxy-3-methylcholanthrene plays a major role as an ultimate electrophilic and carcinogenic form of 1-hydroxy-3-methylcholanthrene, a major metabolite of 3-methylcholanthrene. © 1998 Academic Press

In 1970, Flesher and Sydnor formulated a hypothesis to account for the metabolic activation, DNA-damage, and most, if not all, of the complete carcinogenicity of 7-hydroxymethyl-12-methylbenz[a]anthracene and 7,12-dimethylbenz[a]anthracene (DMBA). According to this hypothesis, the first step in carcinogenesis by DMBA is hydroxylation of the 7-methyl group [1]. The second is the formation of an electrophilic ester of the 7-hydroxymethyl metabolite (e.g. sulfate ester) which would be expected to be a good leaving group, and which would generate a highly reactive carbonium ion. The carbonium ion would then be expected to react with critical nucleophiles in induce a chain of cellular events which result in cancer [2, 3]. The above hypothesis for alkyl-substituted PAH was later extended to include methylation at reactive meso-anthracenic centers for electrophilic substitution as an initial step for unsubstituted polycyclic aromatic hydrocarbons which exhibit strong complete carcinogenicity [4–6]. Thus, complete carcinogenesis by both unsubstituted and alkyl-substituted PAH, as postulated by this unified hypothesis, is dependent primarily upon the occurrence of a chain of specific biochemical substitution reactions leading ultimately to DNA-damaging aralkylating agents. A scheme for the substituted carcinogen 3-methylcholanthrene and its 1-hydroxy metabolite to an aralkylating sulfate ester, as predicted by the unified hypothesis, is shown in Figure 1.

In perfect agreement with the unified hypothesis, previous experiments have demonstrated that 3-methylcholanthrene (MC) is chemically and metabolically hydroxylated at the C-1 position to form 1-hydroxyMC as a major product [8–10]. Esterification, by formation of a sulfuric acid ester, of the benzylic 1-hydroxyMC metabolite would be expected to generate a highly reactive carbonium ion capable of reacting with cellular DNA to induce mutagenesis and carcinogenesis. This prediction is further strengthened by the fact that PAH having secondary benzylic hydroxyl groups are activated by sulfotransferase to electrophilic mutagens that bind covalently to DNA [11–14].

Jeong et al. recently demonstrated the direct mutagenicity and electrophilicity of chemically synthesized 1-sulfooxyMC [15]. The mutagenicity of 1-sulfooxyMC was markedly enhanced by acetate ion and to a lesser

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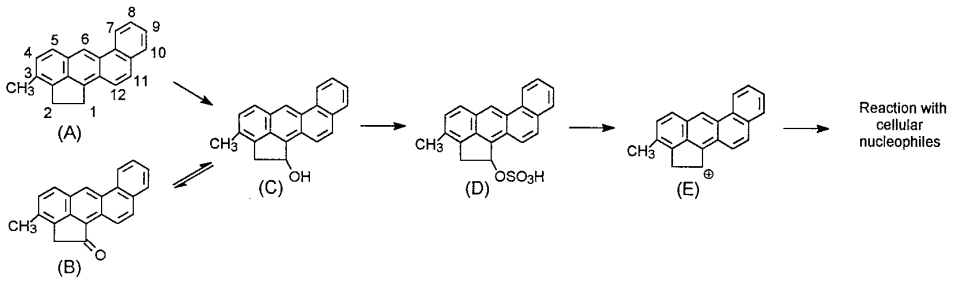

FIG. 1. Scheme for the proposed substitution reactions, *in vivo*, of the carcinogens 3-methylcholanthrene (A) and 1-keto-3-methylcholanthrene (B) to the intermediary metabolite 1-hydroxy-3-methylcholanthrene (C) and its ultimate electrophilic carcinogenic form, 1-sulfooxy-3-methylcholanthrene (D). Loss of the sulfate as a leaving group results in a reactive electrophilic cation (E) which could bind critical cellular nucleophiles.

extent by chloride ion added to the medium. The interaction of 1-sulfooxyMC with added acetate or chloride forms aralkylating agents which are more lipophilic and therefore more readily cross the bacterial cell membrane of *S. typhimurium* TA98 and TA100 to enhance mutagenic activity [15].

These results strongly support the hypothesis that 1-sulfooxyMC is an ultimate electrophilic and carcinogenic metabolite of 1-hydroxyMC and MC. However, further studies are needed, in a complete carcinogenesis model, to establish 1-sulfooxyMC as an ultimate electrophilic and carcinogenic form of 1-hydroxyMC. To test the hypothesis that the electrophilic sulfate ester of 1-hydroxyMC is a complete carcinogenic form of 1-hydroxyMC, the carcinogenic activities of 1-hydroxyMC and its electrophilic sulfate ester derivative have been investigated by repeated subcutaneous injection in female Sprague-Dawley rats.

MATERIALS AND METHODS

Chemicals. 1-Hydroxy-3-methylcholanthrene was synthesized following the method of Fieser and Hershberg [10]. Briefly, a solution of lead tetraacetate (3.4 g) in glacial acetic acid (100 ml) was added dropwise over one hour to a solution of 3-methylcholanthrene (2 g, 7.46 mmol) in 200 ml benzene, while cooling on an ice-bath. The resulting orange solution was reduced under vacuum to one-third the original volume. The addition of ice cold water (75 ml) resulted in the precipitation of a product containing both 1-keto-3-methylcholanthrene and 1-acetoxy-3-methylcholanthrene. This product was collected by vacuum filtration, washed twice with ice cold water (50 ml), and dried under vacuum at 50°C. The precipitated solid, dissolved in acetone (150 ml), was treated with decolorizing carbon and concentrated under reduced pressure to a volume of 100 ml. Standing overnight yielded crystalline 1-keto-3-methylcholanthrene (0.64 g, 2.27 mmol, crude mp 253°C). The acetone mother liquors remaining after filtration of the ketone product were combined and concentrated under reduced pressure. Two additions and evaporations of ligroin (10 ml) resulted in crystallization of 1-acetoxy-3-methylcholanthrene. The crude acetoxy compound was collected by filtration and recrystallized from acetone-ligroin to yield 1.5 g compound (4.7 mmol, 63%, mp 178°C).

1-Acetoxy-3-methylcholanthrene (0.5 g, 1.5 mmol) added to 100 ml methanol containing potassium hydroxide (0.5 g) was refluxed for approximately 1 hr. The reaction mixture, which contained needles of the alcohol, was cooled on ice then filtered. Yellow needles of 1-hydroxy-3-methylcholanthrene were washed sequentially with 1:1 methanol:water, 0.1M HCl, and ice cold water before drying under vacuum. Recrystallization from acetone-ligroin yielded 0.3 g (1 mmol, 66%) of pale yellow needles of 1-hydroxy-3-methylcholanthrene, mp 209°C (lit. mp 209-211°C [10]).

1-Sulfooxy-3-methylcholanthrene was synthesized as a sodium salt by an adapted procedure of Surh et al. for the synthesis of 6-sulfooxymethylbenzo[a]pyrene [16]. Specifically, to a solution of 1-hydroxy-3-methylcholanthrene (95 mg, 0.33 mmol) in 5 ml ice cold dimethylformamide (DMF), was added a 5-fold molar excess of dicyclohexylcarbodiimide (0.3438g, 1.665 mmol) in solution with 5 ml ice cold DMF. This was followed by slow addition of 1.5-fold molar excess of sulfuric acid (28 μl of 18 M, 0.5 mmol) in 1 ml ice cold DMF. The mixture was stirred for 1 hr on dry ice/acetone bath. The supernatant was collected by centrifugation, and brought to neutrality by dropwise addition of ice cold 1M methanolic NaOH. This was evaporated under reduced pressure, the subsequent residue was dissolved in 1 ml ice cold DMF:ethanol (1:1 v/v). The sulfate ester was then precipitated as the sodium salt upon the slow addition of 10 ml ice cold ether (10 volume excess), followed by collection via centrifugation, and drying under reduced pressure.

Instrumentation. Purity of the test compounds was assessed by TLC on Whatman reverse-phase KC18 plates with 9:1 methanol:water development (1-sulfooxyMC R_f=0.92, 1-hydroxyMC R_f = 0.64), and HPLC on a Waters system equipped with reverse-phase ODS column (5 μ, 4.6 mm X 25 cm) with UV and fluorescence detection. The chromatogram was developed with 90% methanol at 1 ml/min (1-sulfooxyMC R_t = 3.3 mins, 1-hydroxyMC R_t = 4.5 mins). The purity of each compound was greater than 97% by HPLC.

Animals. Female Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) as 21 day old weanlings. Rats were acclimatized to the animal room for 1 week before use in the determination of carcinogenic activity. All animals were housed in polyethylene cages with wood chip bedding, 3 rats per cage, in a temperature-controlled animal room with an alternating light-dark cycle of 12 hr while given Purina rat chow and tap water *ad libitum*.

Determination of complete carcinogenicity. Complete carcinogenicity tests were modeled after those employed previously [2]. The compounds to be tested were analyzed for purity by HPLC, reprecipitated as needed to maintain purity, then dissolved to desired concentration in 9:1 sesame oil: dimethyl sulfoxide (DMSO) (0.2 μmol/0.1 ml). Briefly, a group of twelve rats administered 1-hydroxyMC or 1-sulfooxyMC was tested against vehicle-treated and vehicle non-treated control groups. The compounds were administered to their respective twelve-rat group by subcutaneous injection (dorsal subcutis) three times per week for 20 doses resulting in a 4 μmol total dose. 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, while another control group of

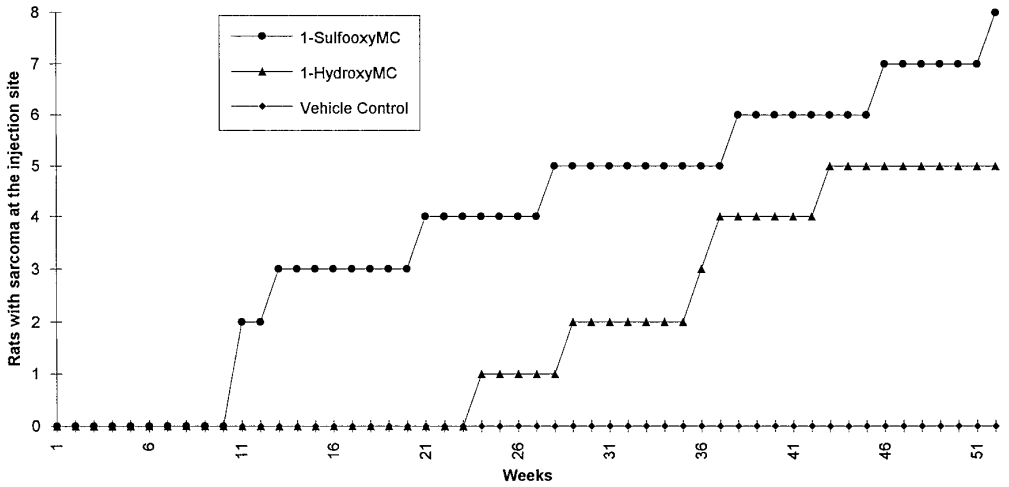

FIG. 2. By 52 weeks a total dose of 4 μmol of 1-sulfooxy-3-methylcholanthrene (1-SulfooxyMC) induced sarcomas at the site of injection in 8 of 12 rats (66%). A total dose of 4 μmol 1-hydroxy-3-methylcholanthrene (1-HydroxyMC) induced sarcomas in only 5 of 12 rats (42%). Neither the vehicle treated nor the untreated control group had sarcomas develop within the 52 week experimental time period (untreated control not shown).

twelve rats was untreated. All animals were weighed once each week and examined for the presence of palpable 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

The complete carcinogenic activity of 1-hydroxyMC, a major metabolite of MC, and the synthetic sulfate ester of 1-hydroxyMC were determined by repeated subcutaneous injection of 0.2 μmol of test compound three times each week for 20 doses in groups of 12 female Sprague-Dawley rats, age 30 days. As shown in Figure 2, two rats treated with the metabolite 1-hydroxyMC had sarcomas by 29 weeks whereas two rats treated with its synthetic sulfate ester had sarcomas by 11 weeks. By 52 weeks 5 rats treated with the metabolite 1-hydroxyMC had sarcomas (42%), whereas 8 rats treated with its synthetic sulfate ester had sarcomas (66%). A total dose of 4 μmol of 1-sulfooxyMC or 1-hydroxyMC induced sarcomas at the site of repeated subcutaneous injection with a latent period to the appearance of the first tumor of 11 and 24 weeks, respectively. Neither control group had sarcoma develop within the 52 week experimental period.

These results clearly demonstrate that 1-sulfooxyMC is more carcinogenic than 1-hydroxyMC. It was therefore concluded that the highly reactive sulfate ester of 1-hydroxyMC accounts for most, if not all, of the complete carcinogenic activity of the metabolite 1-hydroxyMC in a very satisfactory manner, and therefore, at least some of the complete carcinogenic activity of MC.

DISCUSSION

The unified hypothesis of polycyclic aromatic hydrocarbon carcinogenesis predicts that the electrophilic hydroxyalkyl sulfate ester 1-sulfooxyMC would be a highly reactive carcinogenic aralkylating agent and, if formed *in vivo*, an ultimate electrophilic and carcinogenic form of MC owing to the fact that 1-hydroxyMC is a well-known carcinogenic metabolite of MC [2, 4, 8, 17–19].

The unified hypothesis was formulated to incorporate both unsubstituted and alkyl-substituted PAH into a chain of specific biochemical substitution reactions leading ultimately to highly reactive DNA damaging aralkylating agents. According to the unified hypothesis, carcinogenesis depends only on the ability of the administered PAH to undergo one, two or three steps in a chain of specific biochemical substitution reactions, but is independent of the ability to undergo some form of addition reaction.

Of fundamental historical importance is the fact that the first strong inference for a mechanism of carcinogenesis, *in vivo*, of substituted carcinogens such as MC and 7-methylbenz[a]anthracene was based on the chemical hydroxylation of the hydrocarbon with lead tetraacetate, which introduces the hydroxy group as an acetic acid ester. Thus, Fieser suggested that MC, administered to a test animal, might undergo some form of biochemical substitution reaction, e.g., hydroxylation of the activated C-1 methylene group in MC as a critical step in a chain of events leading ultimately to carcinogenesis [20, 21].

Shear and Leiter compared the carcinogenicity of the potential metabolites 1-hydroxyMC and the related 1-ketoMC with MC by subcutaneous injection in mice.

The results demonstrated that 1-hydroxyMC and 1-ketoMC were not more carcinogenic than MC [18]. On the basis of these observations Shear and Leiter concluded that the potencies of the oxygen-containing derivatives of MC do not furnish any support for the hypothesis that oxidation of MC may be an important step in the mechanism of carcinogenesis by MC. They also concluded that, if any such oxidation occurs, it probably does not involve either 1-hydroxyMC or 1-ketoMC.

Fieser's suggestion that the first step in the metabolic activation of a carcinogen consists in the introduction of a simple group such as hydroxyl, sulfhydryl or amino was largely discounted by Wood and Fieser [22] since such simple derivatives as were investigated did not show enhanced carcinogenic activity. Furthermore, these investigators thought it unlikely that a benzo[a]-pyrene derivative of aromatic type ArX could function in the same manner as the structurally different type $ArCH_2X$ derived from substituted hydrocarbons such as 7-methylbenz[a]anthracene and MC.

Although the biological tests of Shear and Leiter did not appear to support Fieser's strong inference, Sims found that 1-hydroxyMC and 2-hydroxyMC were indeed major metabolites of MC incubated with rat-liver preparations [8, 9]. The hydroxy compounds were readily oxidized to the ketones and the ketones reduced to the hydroxy compounds by rat-liver homogenates [8]. When Sims tested the metabolites of MC for carcinogenic activity by subcutaneous injection into C57 black mice, the results demonstrated that both 1- and 2-hydroxyMC were strongly carcinogenic, but the latent periods of the tumors induced by the 2-hydroxy compound were shorter than those of the tumors produced by the 1-hydroxy compound. Similarly, 2-ketoMC appeared to be more active than the 1-ketone [17]. Sims concluded that if the carcinogenic activity of MC involves the metabolic formation of 1- and 2-hydroxy derivatives or ketones, then MC must operate by a mechanism different from that of 7-methylBA and DMBA metabolized to hydroxymethyl derivatives since the hydroxymethyl metabolites tested did not appear to be as active as the hydrocarbon from which it was derived [17]. Boyland and Sims suggested that either the hydrocarbons are active without being metabolized, that the active metabolic species have not yet been identified or that because of solubility and other factors the intermediates are less able to enter the cell or are removed more rapidly from the injection sites than are the parent hydrocarbons [23].

Thus, the studies of Boyland and Sims suggested that if oxidation of 7-methylBA or DMBA is involved in the mechanism of carcinogenesis, it does not appear to involve the formation of hydroxymethyl derivatives [23]. However, this tentative conclusion is based on the unproven assumption that a metabolite must be more carcinogenic than the parent hydrocarbon to be identified as an active metabolite. Furthermore, if more than one type of active metabolite exists it seems unlikely that any of the carcinogenic metabolites identified could be responsible for most, if not all, of the complete carcinogenicity of the parent hydrocarbon. The unified hypothesis predicts a mechanism of complete carcinogenesis an the nature of the ultimate carcinogen. It does not predict that hydroxymethyl metabolites must be more carcinogenic than the parent hydrocarbons to play a major role in carcinogenesis. The unified hypothesis is not disproved by the fact that hydroxymethyl metabolites or their synthetic sulfate esters may not appear to be more carcinogenic than the 7-methylBA or DMBA.

However, with the onslaught of accumulating facts, the unified hypothesis like every hypothesis and conclusion is subject to denial. For example, according to Baird, Dipple, Grover, Sims and Brookes, if DNA is the critical target with which carcinogenic metabolites must react in the process of carcinogenesis, then the ultimate electrophilic and carcinogenic metabolite of 7-methylbenz[a]anthracene could not be an ester of 7-hydroxymethylbenz[a]anthracene [24]. Their conclusion was based on experiments which demonstrated that after enzymatic conversion to deoxyribonucleosides, the reaction products of the aralkylating agent 7-bromomethylbenz[a]anthracene with DNA are chromatographically different from products obtained from the DNA of mouse embryo cells in culture that had been treated with 7-methylbenz[a]-anthracene [reviewed in 25].

However, this conclusion must be rechecked because no evidence of sulfotransferase activity in mouse embryo cells in culture was demonstrated for 7-hydroxymethylbenz[a]anthracene. In the absence of sulfotransferase activity in mouse embryo cells in culture one would hardly expect to find evidence for the formation of an electrophilic hydroxymethyl sulfate ester capable of reacting with cellular DNA in a manner analogous to the reaction with DNA of the aralkylating agent 7-bromomethylbenz[a]anthracene. Clearly, the DNA-binding results of adding 7-methylbenz[a]-anthracene derivatives to mouse embryo cells in culture could not disprove the electrophilic hydroxyalkyl sulfate ester hypothesis. Actually, the hypothesis is enormously strengthened by the fact that 7-sulfooxymethylBA has been unequivocally identified as an ultimate electrophilic, mutagenic and carcinogenic form of 7-hydroxymethylBA, a carcinogenic metabolite of 7-methylBA [26]. Also, our observations demonstrate that MC was metabolized to 1-hydroxyMC, 1-ketoMC and cholanthrene in rat liver cytosol preparations [27] and in human bone marrow preparations [28]. Clearly, 1-hydroxyMC and 1-ketoMC have been identified as carcinogenic metabolites of MC.

In studies of a chemical model for one-electron oxidation of MC [29] Fried demonstrated that one-electron

oxidants give rise to 1-hydroxyMC and 1-ketoMC. The products can be readily rationalized on the basis of a radical cation intermediate which can react with water in the aqueous acetone medium. In a similar manner Fried and Schumm demonstrated that one-electron oxidation of DMBA produced products analogous to those described for MC [29, 30]. Clearly, the products of chemical one-electron oxidation of MC and DMBA, if formed metabolically, are the same metabolites required by the electrophilic hydroxyalkyl sulfate ester hypothesis. However, it appears that Cavalieri et al. do not consider 1-hydroxyMC or the related ketone to be products, formed metabolically, by one-electron oxidation of MC. In fact, Cavalieri et al. predict that if one-electron oxidation is the activating pathway, the 1-substituted MC metabolites should be devoid of carcinogenic activity [31]. Thus, according to Cavalieri et al. the carcinogenic activity of 1-hydroxyMC cannot be attributed to activation by one electron oxidation, but could be activated by sulfate ester formation as has been conclusively demonstrated for 6-hydroxymethylbenzo[a]pyrene [16, 32].

The overall usefulness of the electrophilic hydroxyalkyl sulfate ester hypothesis could not be better illustrated than by the many instances in which the hypothesis has been confirmed by the results of biological testing. Recent studies have clearly demonstrated that hydroxymethyl sulfate esters play a major role as ultimate electrophilic and carcinogenic forms of 1-hydroxymethylpyrene, 7-hydroxymethyl-12-methylbenz[a]anthracene, 7-hydroxymethylBA, 6-hydroxymethylbenzo[a]pyrene, and 9-hydroxymethylanthracene [26,32-34 and unpublished data].

Although 1-sulfooxy-3-MC accounts for most, if not all, of the complete carcinogenicity of the intermediary metabolite 1-hydroxyMC, formed by chemical or biological oxidative substitution of the activated C-1 position of MC, it may not account for all of the complete carcinogenicity of MC. Therefore, MC could also be activated, *in vivo*, to a DNA damaging agent and carcinogenic electrophile by a series of oxidative addition reactions yielding a diol-epoxide without the intervening process of metabolism to 1-hydroxy- or 2-hydroxyMC. Thus, if the formation a vicinal diol-epoxide is involved in the metabolic activation of MC, then a series of enzymatic addition reactions must occur at the 7,8 and 9,10 positions of the angular ring. Experiments with MC incubated in liver microsomes of Long-Evans rats failed to clearly identify a 9,10-dihydrodiol among the metabolic products of MC [35]. These results suggest that a diol-epoxide metabolite of MC may play a relatively minor role in the metabolic activation and complete carcinogenesis of MC.

On the other hand, 1-hydroxyMC was metabolized to 1-hydroxyMC-9,10-dihydrodiol in appreciable amounts [35]. 1-HydroxyMC-9,10-dihydrodiol could be converted into the related 'bay-region' vicinal diol-epoxide, but hydroxylation of the activated C-1 position of MC could have been essential to the conversion to an electrophilic sulfate ester metabolite. In any case, it appears that 1-hydroxyMC plays a major role in the metabolic activation and carcinogenicity of MC. In a similar manner, it has been reported that the potent carcinogen 2-ketoMC, an oxidative metabolite of 2-hydroxyMC, was metabolized to 2-ketoMC-9,10-dihydrodiol by rat liver microsomes [36]. However, no evidence has been presented to demonstrate that the carcinogenicity of bay region diol-epoxide metabolites of either 1- or 2-hydroxyMC, or their related ketones, can account for the complete carcinogenic potency of MC.

The carcinogenic aralkylating agents are a class of exceptionally reactive DNA damaging agents that are themselves electrophiles, but the majority of carcinogenic PAH are not reactive *per se* and must be metabolized to ultimate electrophilic and carcinogenic forms, *in vivo*. This is in accordance with the view of Miller and Miller that the ultimate carcinogenic forms of most, if not all, chemical carcinogens are probably electrophilic reactants [37, 38].

The electrophilic aralkylating agent 1-sulfooxyMC was more potent than the strong carcinogen 1-hydroxyMC by repeated subcutaneous injection in female Sprague-Dawley rats. Therefore, we conclude that 1-sulfooxyMC is an exceptionally reactive electrophilic and carcinogenic form of 1-hydroxyMC that accounts for most, if not all, of the complete carcinogenicity of 1-hydroxyMC in a very satisfactory manner. Clearly, 1-sulfooxyMC has been identified as an electrophilic and carcinogenic form of 1-hydroxyMC.

The present concept that the mechanism of carcinogenesis by administered PAH involves one, two or three steps in a chain of biochemical substitution reactions leading ultimately to electrophilic aralkylating agents appears to be as satisfactory a hypothesis as can be formulated on the basis of the facts available. Further studies are required to identify clearly the ultimate electrophilic and carcinogenic forms of MC. MC is one of the most potent polycyclic hydrocarbon carcinogens and it could be that its biological potency is attributable to the formation of more than one electrophilic metabolite. In this regard, the hypothesis that 1-sulfooxyMC accounts for most, if not all, or the complete carcinogenicity of 1-hydroxyMC, 1-ketoMC and at least a substantial fraction of the complete carcinogenicity of MC, 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 1-hydroxyMC, 1-ketoMC and MC than the electrophilic ester 1-sulfooxyMC.

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