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BIOALKYLATION OF BENZ[*a*]ANTHRACENE, 7-METHYLBENZ[*a*]ANTHRACENE, AND 12-METHYLBENZ[*a*]ANTHRACENE IN RAT LUNG CYTOSOL PREPARATIONS

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Abstract—Benz[*a*]anthracene (BA) and the monomethyl *meso*-anthracenic or L-region derivatives 7-methylbenz[*a*]anthracene (7-methylBA) and 12-methylbenz[*a*]anthracene (12-methylBA) underwent a bioalkylation substitution reaction in rat lung ctyosol preparations, fortified with *S*-adenosyl-L-methionine to form the more potent carcinogen 7,12-dimethylbenz[*a*]anthracene. The methyl groups of the highly reactive L-region methylated metabolites also underwent enzymatic hydroxylation in rat lung cytosol preparations to yield the corresponding hydroxymethyl derivatives, 7-hydroxymethylbenz[*a*]anthracene, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, and 7,12-dihydroxymethylbenz-[*a*]anthracene. The biooxidation reaction took place enzymatically, and exclusively, or nearly so, at the reactive methyl groups attached to the *meso* positions or L-region of the hydrocarbon. Bioalkylation and biooxidation reactions did not occur when the hydrocarbons were incubated with a boiled cytosol preparation, indicating the need for enzymatic activation of the L-region methyl groups. Also, the bioalkylation reaction did not occur in the absence of *S*-adenosyl-L-methionine. Furthermore, the *S*-adenosyl-L-methionine-dependent reaction was inhibited by *S*-adenosyl-L-homocysteine, suggesting that the reaction is catalyzed by a cytosolic *S*-adenosyl-L-methionine-dependent methyltransferase.

The majority of the investigations of the metabolism of polynuclear aromatic hydrocarbons has been carried out in rat liver preparations [1–3], although the liver is not normally regarded as a target organ for hydrocarbon carcinogenesis. However, many polynuclear aromatic hydrocarbons are active in the induction of tumors in the pulmonary tissues of the rat [4] and other species [5]. Therefore, it becomes important to understand the metabolic activation of these compounds in lung tissue to those reactive metabolites responsible for the formation of pulmonary tumors.

In early studies concerning the metabolism of polynuclear aromatic hydrocarbons in rat lung microsomes or in rat lung homogenates, Grover *et al.* [6, 7] found that benz[*a*]anthracene was metabolized to two dihydrodiols, 5,6-dihydro-5,6-dihydroxybenz-[*a*]anthracene and 8,9-dihydro-8,9-dihydroxybenz-[*a*]anthracene. In the same studies, 7-methylbenz[*a*]-anthracene was found to be metabolized in these systems to the corresponding 3,4-dihydro-3,4-dihydroxy-7-methylbenz[*a*]anthracene as well as 8,9-dihydro-8,9-dihydroxy-7-methylbenz[*a*]-anthracene. An additional metabolite of 7-methylbenz-[*a*]anthracene was also detected and identified as 7-hydroxymethylbenz[*a*]anthracene [6, 7].

In studies conducted in our laboratory of a possible relationship between complete carcinogenic activity and biochemical reactions of polynuclear aromatic hydrocarbons, the importance of substitution reactions at the *meso*-anthracenic reactive centers or L-region was emphasized [8–17]. Studies of structure–activity relationships, known to exist in this class of compounds, clearly demonstrated that the introduction of a methyl group in the chemically reactive *meso*-anthracenic position(s) or L-region enhances carcinogenic activity [18]. Therefore, a biochemical reaction that introduces a methyl group in a *meso*-anthracenic position would be expected to contribute to the attainment of strong carcinogenic activity [16, 19].

These studies are consistent with the unified hypothesis which predicts that the chemical or biochemical introduction of an alkyl group into unsubstituted polynuclear aromatic hydrocarbons is a structural requirement for the attainment of strong complete carcinogenic activity [8, 10, 13]. Here we report that the weak carcinogen benz[*a*]anthracene (BA†) underwent bioalkylation in rat lung cytosol preparations fortified with *S*-adenosyl-L-methionine to form the moderately active monomethyl metabolites 7-methylbenz[*a*]anthracene (7-methylBA) and 12-methylbenz[*a*]anthracene (12-methylBA). These metabolites were substrates for further bioalkylation reactions to form the strong carcinogen 7,12-dimethylbenz[*a*]anthracene (7,12-dimethylBA). In addition, these methylated L-region metabolites were oxidized in preparations of rat lung cytosol to the corresponding carcinogenic hydroxymethyl and formyl metabolites.

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† Abbreviations: BA, benz[*a*]anthracene; 7-methylBA, 7-methylbenz[*a*]anthracene; 12-methylBA, 12-methylbenz-[*a*]anthracene; and 7,12-dimethylBA, 7,12-dimethylbenz-[*a*]anthracene.

MATERIALS AND METHODS

Chemicals. Benz[*a*]anthracene was purchased from the Aldrich Chemical Co., Milwaukee, WI, U.S.A.; 7-methylbenz[*a*]anthracene from the Schuchardt Chemical Co., Munich, West Germany; and 7,12-dimethylbenz[*a*]anthracene from Eastman Organic Chemicals, Rochester, NY, U.S.A. 12-Methylbenz[*a*]anthracene [20], 7,12-dihydroxymethylbenz[*a*]anthracene [21], 7-hydroxymethylbenz[*a*]anthracene [22], 7-hydroxymethyl-12-methylbenz[*a*]anthracene [21], and 7-formylbenz[*a*]anthracene [23] were synthesized according to previously published methods.

S-Adenosyl-L-methionine, iodide salt, and *S*-adenosyol-L-homocysteine were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. All compounds were of the highest grade commercially available. The hydrocarbons were found to be greater than 99% pure by HPLC and gas chromatography/mass spectroscopy (GC/MS) analysis.

Animals. Male Sprague–Dawley rats, weighing 150 g, were used in the experiments. The animals were housed in polyurethane cages and provided with food and water *ad lib.*

Rat liver cytosol preparations. Animals were killed by cervical dislocation. The lungs were quickly excised and rinsed in 0.1 M potassium phosphate buffer, pH 7.4, to remove excess blood. The tissue was then weighed and homogenized in ice-cold 0.1 M potassium phosphate buffer, pH 7.4, using a Brinkmann Polytron homogenizer. The homogenate was diluted to 0.33 g wet weight tissue/mL and centrifuged at 9000 *g* for 30 min at 5°. The supernatant was collected and centrifuged for 60 min at 105,000 *g* to give the cytosolic preparations.

In vitro *incubations with rat lung cytosol preparations.* Incubations of the hydrocarbon with rat lung cytosol preparations were performed in air at 37° for 1 hr using a Dubnoff light-protected shaker incubator. Incubations contained 200 nmol of the hydrocarbon in 200 μL ethanol, 3 μmol magnesium chloride, 20 μmol potassium phosphate buffer, pH 7.4, 500 nmol of *S*-adenosyl-L-methionine, and 200 μL of rat lung cytosol preparation (4–6 mg protein) in a total volume of 1.1 mL. Control reactions were performed in the absence of rat lung cytosol and in the presence of a boiled rat lung cytosol preparation as well as in the presence of 500 nmol of the potent inhibitor of methyltransferase-mediated reactions, *S*-adenosyl-L-homocysteine. The reactions were initiated by the addition of the rat lung cytosol. After 1 hr the incubations were stopped by the addition of 1 vol. of cold acetone, and the resulting mixture was extracted twice with ethyl acetate. The ethyl acetate extracts were combined, washed with water, evaporated under nitrogen, and stored at −20° until analysis by HPLC and GC/MS.

Analysis of products by high performance liquid chromatography. The residue was dissolved in 200 μL methylene chloride and 2-μL aliquots were analyzed by reverse-phase HPLC. A 25 cm × 10.0 mm column, packed with Ultrasphere ODS 5 μm, was eluted with 100% methanol, temperature 20°, at a flow rate of 2.5 mL/min. Ultraviolet absorbance was monitored at 254 nm using a Waters M440 absorbance detector, connected to a M6000 solvent pump. Chromatographic data were analyzed with the Waters 820 Maxima software program in an IBM/AT computer. Metabolic profiles were compared to the retention times of authentic standards of various hydrocarbon derivatives. Identities of the various metabolites were confirmed by gas chromatographic and mass spectral analysis. Quantitation of metabolites was accomplished by measurement of peak areas associated with various concentrations of

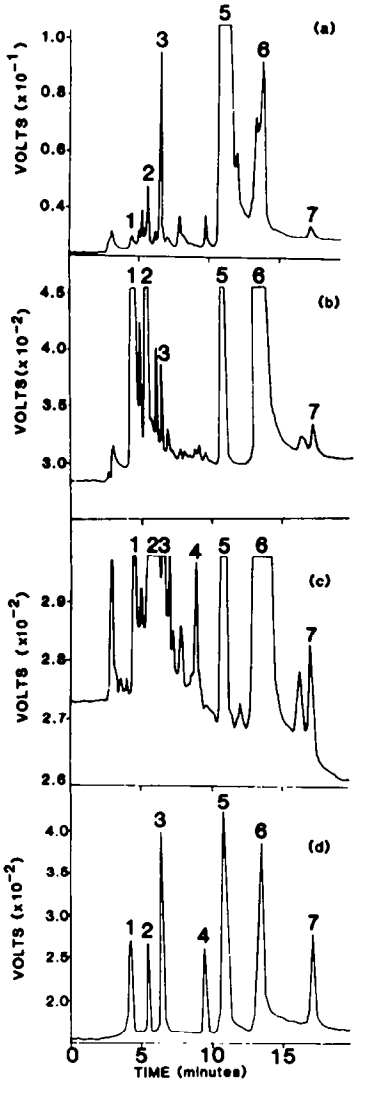

Fig. 1. Typical high pressure liquid chromatograms of (a) the products obtained from the reaction of benz[*a*]anthracene (BA) and rat lung cytosol preparations fortified with *S*-adenosyl-L-methionine, (b) the products of the reaction of 7-methylBA and rat lung cytosol preparations, (c) the products of the reaction of 12-methylBA and rat lung cytosol preparations, and (d) authentic standards of *meso*-anthracenic derivatives of BA. Peak 1, 7,12-dihydroxymethylBA; peak 2, 7-hydroxymethylBA; peak 3, 7-hydroxymethyl-12-methylBA; peak 4, 7-formylBA; peak 5, BA; peak 6, monomethylBA; and peak 7, 7,12-dimethylBA.

Table 1. Comparison of the retention times of benz[*a*]anthracene metabolites detected by gas chromatography with the retention times of authentic standards

Compound	Metabolite retention time (min)	Standard retention time (min)	*m/z*
Benz[*a*]anthracene (BA)	19.95	19.99	228
12-MethylBA	21.48	21.50	242
7-MethylBA	21.87	21.92	242
7,12-DimethylBA	23.23	23.30	256
7-FormylBA	23.52	23.57	256
7-HydroxymethylBA	24.29	24.32	258
7-Formyl-12-methylBA	24.44	24.46	270
7-Hydroxymethyl-12-methylBA	26.19	26.23	272
7,12-DihydroxymethylBA	30.64	30.72	288

Retention time refers to the time between injection of the sample on a 25 m × 0.25 mm fused silica capillary column and the detection of the peak by the mass spectrometer.

authentic standards of benz[*a*]anthracene derivatives by HPLC and GC/MS.

Analysis of metabolites by gas chromatography and mass spectroscopy. The analysis of metabolites was accomplished using a Hewlett–Packard 5890 gas chromatograph attached to a Hewlett–Packard 5970 mass selective detector (GC/MS). Samples were fractionated by preparative HPLC prior to analysis by GC/MS. Samples of metabolites were dissolved in 100 μL of methylene chloride and 1–2 μL was injected into the GC for analysis. The gas chromatographic column was a 25 m × 0.2 mm fused silica capillary column. The gas chromatographic oven was temperature programmed from 70 to 250° at a rate of 8° per min. The instrument was set to operate by electron impact at 70 eV. Data obtained from each analysis included retention time on the column and mass fragmentation patterns of each metabolite or authentic standard.

RESULTS

The present experiments demonstrated that BA, 7-methylBA, and 12-methylBA undergo a bioalkylation substitution reaction in rat lung cytosol preparations fortified with *S*-adenosyl-L-methionine to yield methyl-substituted metabolites.

Control reactions were performed in the presence of a boiled rat lung cytosol preparation, in the absence of added rat lung cytosol and in the presence of *S*-adenosyl-L-methionine, a potent inhibitor of methyltransferase-mediated reactions. All three control reactions demonstrated that no detectable bioalkylation substitution or biooxidation reactions occurred, demonstrating the enzymatic nature of the reaction as well as its dependence on *S*-adenosyl-L-methionine.

When BA (Fig. 1a), 7-methylBA (Fig. 1b), or 12-methylBA (Fig. 1c) was incubated with rat lung cytosol preparations fortified with *S*-adenosyl-L-methionine, metabolites were found by HPLC analysis that were indistinguishable from the authentic *meso*-position derivatives of BA shown in Fig. 1d. When BA was used as a substrate in the reaction, a methyl group was introduced in the most reactive *meso*-anthracenic position to yield 7-methylBA (peak 6). Table 1 shows the comparison of retention times of the various metabolites in rat lung cytosol compared to the retention times of the various *meso*-position standards. The identity of the 7-methyl-substituted metabolite of BA was confirmed by mass spectroscopy. The metabolite yielded a molecular ion of *m/z* 242 and a corresponding fragmentation pattern (Fig. 2a) that was found to be identical to the molecular ion and the fragmentation pattern of authentic 7-methylBA (Fig. 2b) and was clearly distinguishable from the mass spectrum of authentic 12-methylBA shown in Fig. 2c. Further bioalkylation of the monomethyl-substituted metabolites was found to occur yielding the potent carcinogen 7,12-dimethylBA as a metabolite (peak 7). In addition to the bioalkylation reaction, oxidation of the hydrocarbon was observed. Oxidation of the hydrocarbon was found to yield products that were indistinguishable by HPLC from 7,12-dihydroxymethylBA (peak 1). 7-hydroxymethylBA (peak 2), and 7-hydroxymethyl-12-methylBA (peak 3).

When 7-methylBA (peak 6, Fig. 1b) was used as a substrate in the reaction with rat lung cytosol, a bioalkylation product was detected that was indistinguishable from 7,12-dimethylBA (peak 7). The most pronounced reaction was the dealkylation of 7-methylBA to yield BA (peak 5). This metabolite yielded a parent molecular ion of *m/z* 228 and other accompanying ions that were identical to authentic BA. In addition to the bioalkylation and dealkylation reactions of 7-methylBA, its methylated metabolite, 7,12-dimethylBA, was found to also undergo bio-oxidation reactions to give products indistinguishable from 7,12-dihydroxymethylBA (peak 1), 7-hydroxymethylBA (peak 2) and 7-hydroxymethyl-12-methylBA (peak 3). Similar results were obtained when the compound 12-methylBA (Fig. 1c, peak 6) was used as a substrate in the reactions with rat lung cytosol preparations. When 12-methylBA was used as a substrate, a dealkylation reaction was also found to occur, giving BA as a metabolite (peak 5). The metabolite, identified as BA by HPLC, yielded a

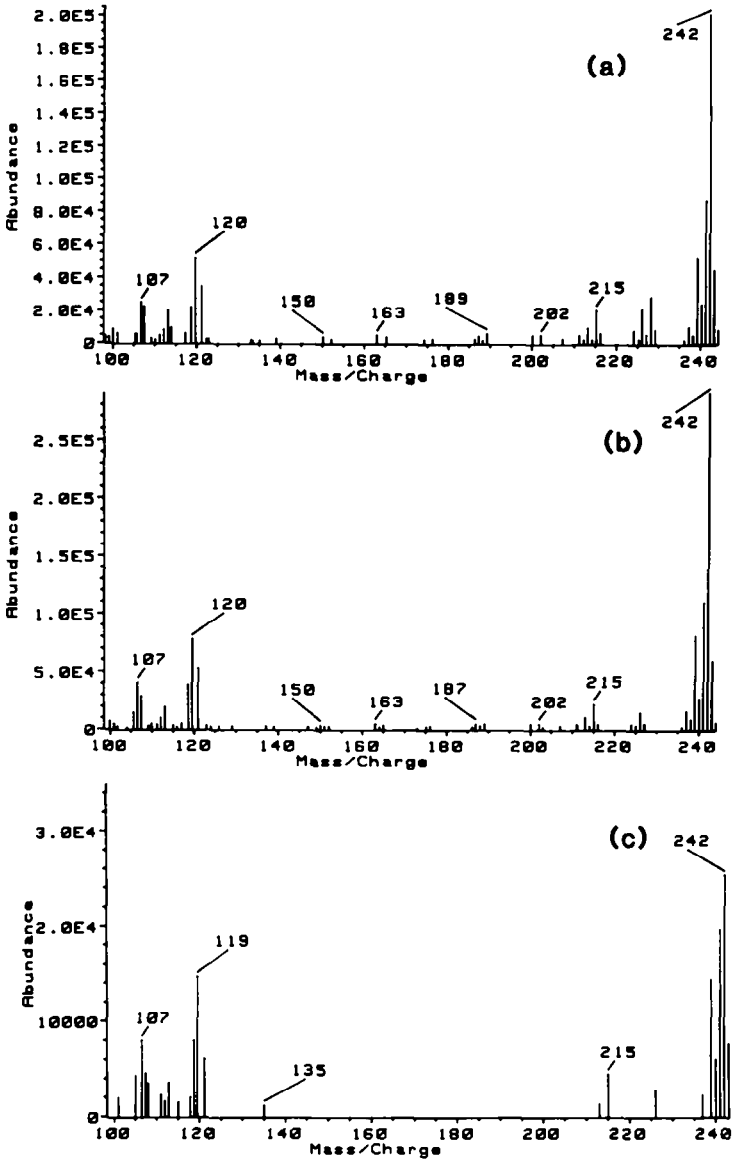

Fig. 2. (a) Mass spectrum of the metabolite of BA in preparations of rat lung cytosol and identified by HPLC and GC as 7-methylBA. The metabolite yielded a parent molecular ion of m/z 242 and a corresponding fragmentation pattern that was indistinguishable from authentic 7-methylBA shown in (b) and was clearly distinguishable from the spectrum of authentic 12-methylBA shown in (c).

molecular ion of m/z 228, consistent with BA. In addition, bioalkylation of 12-methylBA was found to yield 7,12-dimethylBA (peak 7). The identity of the metabolite of 12-methylBA identified by HPLC as 7,12-dimethylBA was confirmed by gas chromatographic/mass spectral analysis. The metabolite gave a molecular ion of m/z 256 and other accompanying ions (Fig. 3b) that were consistent with authentic 7,12-dimethylBA shown in Fig. 3a. The metabolism of 12-methylBA in rat lung cytosol gave rise to several polar products. Identities were established by HPLC and confirmed by gas chromatographic and mass spectral analysis for the formation of 7,12-dihydroxymethylBA, m/z 288, (peak 1); a hydroxymethylBA, m/z 258, (peak 2), which is presumably 12-hydroxymethylBA; 7-hydroxymethyl-12-methylBA, m/z 272, (peak 3); and a formylBA, m/z 256, (peak 4), which is presumably 12-formylBA. The identities of the remaining metabolites are under investigation. A proposed scheme for the bioalkylation and biooxidation of BA and its *meso*-methylated metabolites is presented in Fig. 4.

Quantitation of the metabolites of BA formed in a 60-min incubation demonstrated that from 200 nmol of substrate, 9 nmol (4.5%) of metabolites was found. Methyl-substituted metabolites accounted for 4.6 nmol (51.1%) of the 9 nmol and hydroxymethyl-

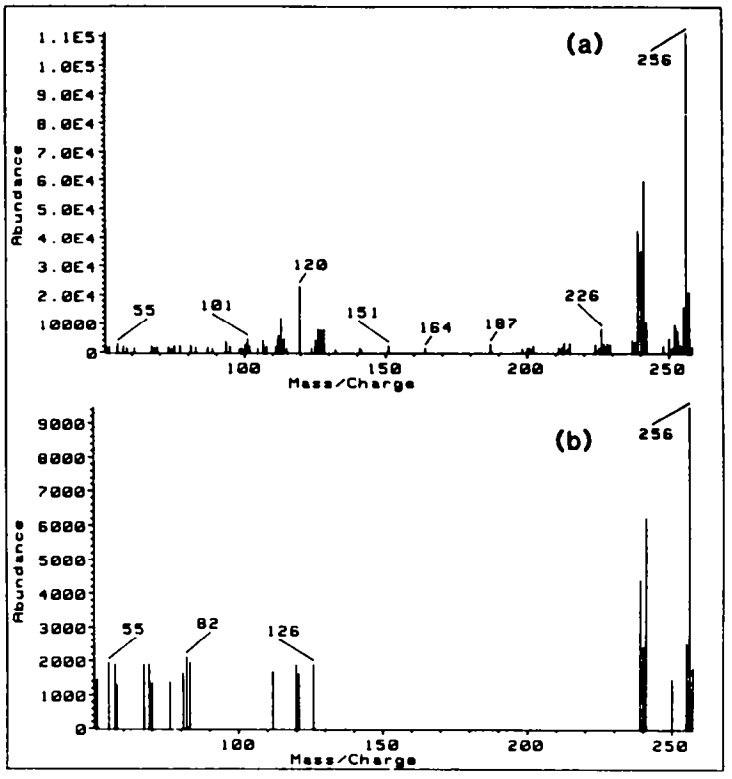

Fig. 3. (a) Mass spectral profile of authentic 7,12-dimethylbenz[*a*]anthracene (7,12-dimethylBA). The mass spectrum shows the characteristic molecular ion of *m/z* 256 and other accompanying ions that were found to be indistinguishable from the molecular ions of the metabolite of 12-methylBA in preparations of rat lung and identified by HPLC as 7,12-dimethylBA shown in (b).

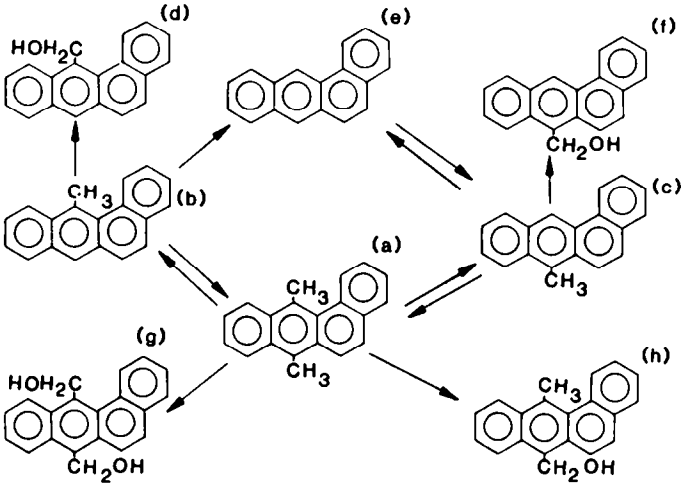

Fig. 4. Proposed scheme for the metabolism of BA and its *meso*-methylated derivatives by rat lung cytosol preparations. Key: (a) 7,12-dimethylbenz[*a*]anthracene, (b) 12-methylbenz[*a*]anthracene, (c) 7-methylbenz[*a*]anthracene, (d) 12-hydroxymethylbenz[*a*]anthracene, (e) benz[*a*]anthracene, (f) 7-hydroxymethylbenz[*a*]anthracene, (g) 7,12-dihydroxymethylbenz[*a*]anthracene, and (h) 7-hydroxymethyl-12-methylbenz[*a*]anthracene.

and formyl-substituted metabolites accounted for 4.4 nmol (48.9%). Quantitation of the metabolites of 7-methylBA formed in a 60-min incubation demonstrated that from 200 nmol of substrate, 12.5 nmol of metabolites (6.25%) was found. The 12.5 nmol of metabolites consisted of 4.2 nmol (33.6%) BA, 0.4 nmol (3.2%) 7,12-dimethylBA, and 7.9 nmol (63.2%) hydroxymethyl- and formyl-substituted BAs.

DISCUSSION

A unified hypothesis, developed in this laboratory, predicts that a bioalkylation substitution reaction takes place readily in the most reactive *meso*-anthracenic center of the molecule to yield a *meso*-anthracenic alkyl-substituted hydrocarbon as a prerequisite for carcinogenic activity [10, 13, 14]. In addition, the *meso*-anthracenic alkyl group serves as a reactive center for the further biotransformation of the hydrocarbon to hydroxyalkyl-substituted derivatives [24]. The results presented in this report, concerning the bioalkylation of BA, 7-methylBA, and 12-methylBA are consistent with this hypothesis.

Fifty years ago Fieser and his collaborators [25] recognized that methyl substitution in the *meso*-anthracenic position(s) or L-region confers strong carcinogenic activity on the parent unsubstituted hydrocarbon. Furthermore, the idea was advanced that the function of the methyl group was not to accentuate the reactivity at some other position or region of the molecule, but to serve as a reactive center for the conjugation of the hydrocarbon with substances present in the organism [19, 25].

Earlier studies from our laboratory demonstrated that anthracene [26], benz[*a*]anthracene [13], benz[*a*]pyrene [8, 11, 12], and dibenz[*a*,*h*]anthracene [15] undergo a methyl substitution reaction in rat liver cytosol preparations fortified with *S*-adenosyl-L-methionine. Further studies revealed that 7-methylBA and 12-methylBA were substrates for the biosynthesis of the potent carcinogen 7,12-dimethylBA in rat liver cytosol preparations fortified with *S*-adenosyl-L-methionine [13]. The bioalkylation of BA, 7-methylBA, and 12-methylBA in rat lung cytosol preparations fortified with *S*-adenosyl-L-methionine is consistent with the hypothesis that most, if not all, unsubstituted carcinogenic hydrocarbons undergo a bioalkylation substitution reaction as a necessary first step in the metabolic activation of the hydrocarbon. In addition, the enzymatic hydroxylation of the methyl-substituted hydrocarbon to a carcinogenic hydroxymethyl metabolite indicates that hydroxylation reactions on the methyl groups are almost certainly involved in the reactions of the hydrocarbon which lead to carcinogenesis [21, 27, 28]. Further studies will be reported as to the nature of the ultimate carcinogen of benz[*a*]anthracene and its methyl-substituted derivatives.

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