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BIOOXIDATION OF 7,12-DIMETHYLBENZ[*a*]ANTHRACENE IN RAT SUBCUTANEOUS TISSUE

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Abstract—The present study demonstrates the biooxidation of 7,12-dimethylbenz[*a*]anthracene to the corresponding hydroxyalkyl metabolites, 7-hydroxymethylbenz[*a*]anthracene, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, and 7,12-dihydroxymethylbenz[*a*]anthracene in the dorsal subcutaneous tissue of the rat, *in vivo*, a tissue highly susceptible to the carcinogenic action of 7,12-dimethylbenz[*a*]anthracene.

In earlier studies, a unified hypothesis was advanced that the chemical or biochemical introduction of an alkyl group at the meso-anthracenic reactive centers of polynuclear aromatic hydrocarbons is a necessary structural requirement for the attainment of carcinogenic activity, at least for compounds that are strong complete carcinogens [1–3]. Therefore, this hypothesis predicts that all complete preprocarcingens of the aromatic type ArX (X = H) would be expected to undergo a biochemical substitution reaction leading to the biosynthesis of procarcinogens of the aromatic type $ArCH_2X$ (X = H). This prediction was confirmed by recent observations that weakly carcinogenic benz[*a*]anthracene, moderately carcinogenic 7-methylbenz[*a*]anthracene, and 12-methylbenz[*a*]anthracene undergo a bioalkylation substitution reaction in rat liver cytosol preparations fortified with *S*-adenosyl-L-methionine at the meso-anthracenic reactive centers, or L-region, to yield the metabolite 7,12-dimethylbenz[*a*]anthracene [4], one of the most potent carcinogenic hydrocarbons known. In subsequent studies it was found that 7,12-dimethylbenz[*a*]anthracene was oxidized in rat liver cytosol preparations to give 7-hydroxymethylbenz[*a*]anthracene, 7-hydroxymethyl-12-methylbenz[*a*]anthracene and 7,12-dihydroxymethylbenz[*a*]anthracene, indicating that the methyl groups are the most reactive centers of the molecule [5]. The latter metabolite is probably only weakly active whereas the mono-hydroxymethyl metabolites exhibit pronounced carcinogenic activity by subcutaneous injection [6, 7]. The hydroxymethyl group is suitable for conjugation with substances normally present in the animal, and strong evidence for the biosynthesis of a benzylic electrophilic sulfate ester metabolite of 7-hydroxymethyl-12-methylbenz[*a*]anthracene has been presented recently [8, 9]. The present experiments demonstrate the formation of carcinogenic metabolites of 7,12-dimethylbenz[*a*]anthracene at the site of subcutaneous injection. These observations suggest that the first step in the metabolic activation of 7,12-dimethylbenz[*a*]anthracene, in subcutis tissue, is hydroxylation of the highly reactive methyl groups.

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MATERIALS AND METHODS

Chemicals. 7,12-Dimethylbenz[*a*]anthracene and benz[*a*]anthracene were purchased from Eastman Organic Chemicals (Rochester, NY). 7-Methylbenz[*a*]anthracene was purchased from Schuchardt Chemicals (Munich, F.R.G.). 12-Methylbenz[*a*]anthracene [10], 7-hydroxymethylbenz[*a*]anthracene [11], 7,12-dihydroxymethylbenz[*a*]anthracene [12], 7-hydroxymethyl-12-methylbenz[*a*]anthracene [13], 7-formylbenz[*a*]anthracene [14], and 7-formyl-12-methylbenz[*a*]anthracene [15] were prepared by previously published methods. All other reagents and chemicals were of the highest grade available. All hydrocarbons were found to be greater than 99% pure by high performance liquid chromatography (HPLC) and gas chromatographic and mass spectral (GC/MS) analysis.

Animals. Male Sprague–Dawley rats; weighing 150 g, were purchased from Harlan Sprague–Dawley (Indianapolis, IN). Rats were maintained in polyurethane cages and provided with food (Purina rat chow) and water *ad lib*.

Metabolism of 7,12-*Dimethylbenz*[a]*anthracene in the dorsal subcutaneous tissue of the rat, in vivo*. 7,12-Dimethylbenz[*a*]anthracene, 0.4 μmol, in 200 μl sesame oil was injected into the dorsal subcutaneous tissue of rats. Twenty-four hours later, the animals were killed by cervical dislocation, and the tissue in contact with the hydrocarbon (0.2 to 0.4 g) was excised after locating the site by brief exposure to UV light. The tissue was minced with scissors and homogenized in 70% acetone using a Brinkmann polytron tissue homogenizer. The tissue homogenate was extracted twice with ethyl acetate and washed with water. The organic phase was removed and evaporated to dryness under nitrogen. The residue

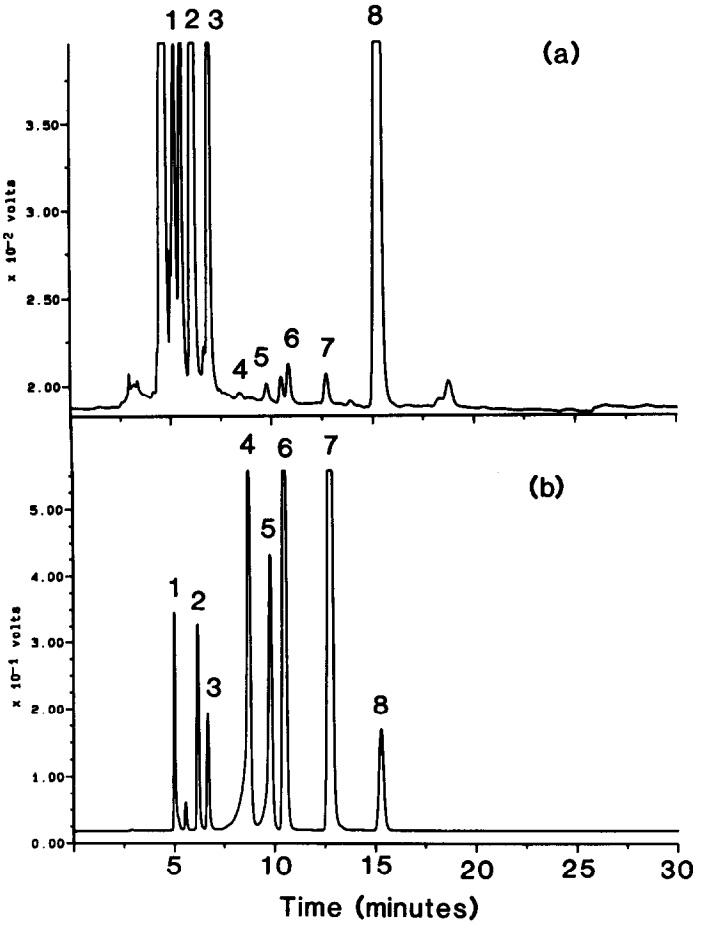

Fig. 1. (a) Typical HPLC of the ethyl acetate extractable products of the metabolism of 7,12-dimethylbenz[*a*]anthracene in the dorsal subcutaneous tissue of the rat *in vivo*. Products were found to be indistinguishable from the authentic meso-position derivatives of 7,12-dimethylbenz[*a*]anthracene shown in (b). Peak 1, 7,12-dihydroxymethylbenz[*a*]anthracene; peak 2, 7-hydroxymethylbenz[*a*]anthracene; peak 3, 7-hydroxymethyl-12-methylbenz[*a*]anthracene; peak 4, 7-formylbenz[*a*]anthracene; peak 5, 7-formyl-12-methylbenz[*a*]anthracene; peak 6, benz[*a*]anthracene; peak 7, 7-methylbenz[*a*]anthracene; and peak 8, 7,12-dimethylbenz[*a*]anthracene.

Table 1. Comparison of retention times of metabolites of 7,12-dimethylbenz[*a*]anthracene and authentic meso-position derivatives of 7,12-dimethylbenz[*a*]anthracene by gas chromatographic analysis

Compound	Retention time (min) (metabolite)	Retention time (min) (standard)	*m/z* (parent ion)
Benz[*a*]anthracene	19.97	19.98	228
12-Methylbenz[*a*]anthracene	21.48	21.50	242
7-Methylbenz[*a*]anthracene	21.82	21.93	242
7,12-Dimethylbenz[*a*]anthracene	23.12	23.21	256
7-Formylbenz[*a*]anthracene	23.51	23.53	256
7-Hydroxymethylbenz[*a*]anthracene	24.29	24.23	258
7-Formyl-12-methylbenz[*a*]anthracene	25.43	24.46	270
7-Hydroxymethyl-12-methylbenz[*a*]anthracene	26.15	26.10	272
7,12-Dihydroxymethylbenz[*a*]anthracene	30.59	30.66	288

Retention time refers to the time between the injection of the sample on a 25 m × 0.2 mm fused silica capillary column and the detection of the peak by the mass spectrometer.

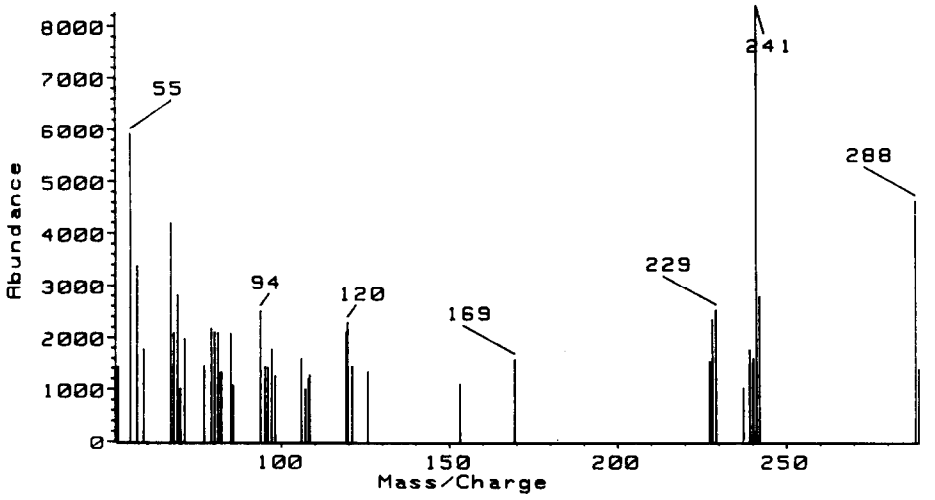

Fig. 2. Mass spectral profile of the metabolite identified by HPLC and GC as 7,12-dihydroxymethylbenz[*a*]anthracene. The metabolite yielded a parent molecular ion of *m/z* 288 and a corresponding fragmentation pattern that were found to be indistinguishable from the authentic compound.

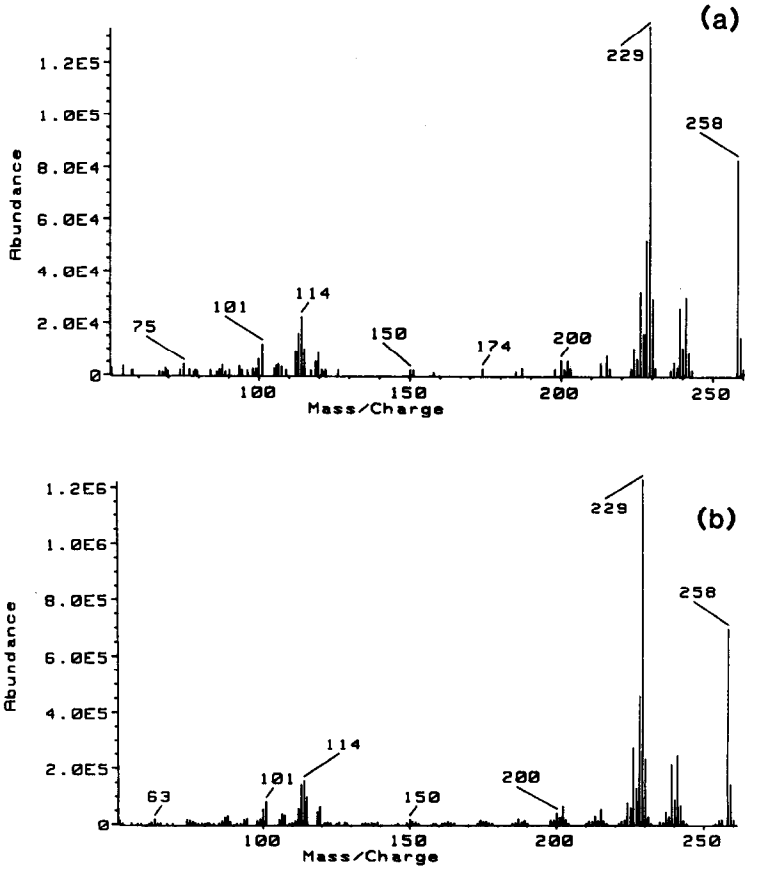

Fig. 3. (a) Mass spectral profile of the metabolite identified by HPLC and GC as 7-hydroxymethylbenz[*a*]anthracene. The metabolite gave a parent molecular ion of *m/z* 258 and other ions that were found to be indistinguishable from the authentic compound shown in (b). E = exponential value (E5 = 10^5).

was stored under nitrogen at −20° until analysis by HPLC and GC/MS.

Analysis of metabolites by HPLC. The residue was dissolved in 200 μl of methylene chloride and 2-μl aliquots were analyzed by reverse-phase HPLC. A 25 cm × 10 mm C_{18} column, packed with ultrasphere ODS, 5 μm, eluted with methanol, 100%, temperature 20°, at a flow rate of 2.5 ml/min, was connected to a Waters M6000 solvent pump. Ultraviolet absorbance was recorded at 254 nm using a Waters M440 absorbance detector. Chromatographic profiles were analyzed with a Waters Maxima 820 software program in an IBM AT computer. Metabolic profiles were compared to the retention times of authentic standards of various 7,12-dimethylbenz[*a*]anthracene derivatives. Quantitation of the metabolites was accomplished by comparison of peak areas of known concentrations of the various derivatives of 7,12-dimethylbenz[*a*]anthracene.

Analysis of metabolites by GC/MS. Analysis of metabolites was accomplished using a Hewlett–Packard 5890 Gas chromatograph/mass spectrometer. A 1–2 μl portion of the extracted sample in methylene chloride was introduced into the gas chromatographic column using a splitless injection technique. The gas chromatographic column was a 25 m × 0.2 mm fused silica capillary column. The gas chromatographic oven was programmed to increase oven temperature from 70° to 250° at a rate of 8°/min. Molecular ions were generated by electron impact, operated at 1800 eV. Retention times on the column as well as mass fragmentation patterns of the various metabolites were compared to the retention times and fragmentation patterns of authentic derivatives of 7,12-dimethylbenz[*a*]anthracene.

RESULTS

The present experiments demonstrate that 7,12-dimethylbenz[*a*]anthracene undergoes biooxidation reactions in the dorsal subcutaneous tissue of the rat, *in vivo*, to give metabolites which were found by HPLC and GC/MS analysis to be indistinguishable from the authentic meso-hydroxymethylated derivatives. A typical high pressure liquid chromatogram of the metabolites of 7,12-dimethylbenz[*a*]anthracene in the dorsal subcutaneous tissue of the rat is shown in Fig. 1a. The chromatogram reveals the presence of several metabolites of 7,12-dimethylbenz[*a*]anthracene which were found to be indistinguishable from the authentic standards of various meso-position derivatives shown in Fig. 1b.

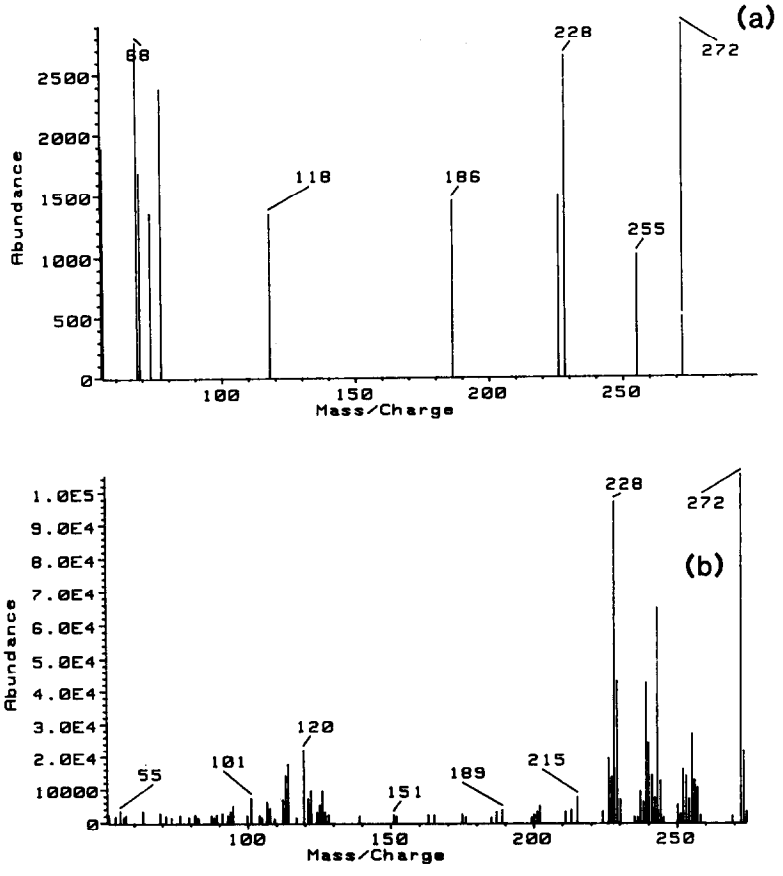

Fig. 4. (a) Mass spectral characterization of the metabolite identified by HPLC and GC analysis as 7-hydroxymethyl-12-methylbenz[*a*]anthracene. The metabolite gave a parent molecular ion of *m/z* 272 and other ions that were found to be indistinguishable from the authentic standard (b). E = exponential value (E4 = 10^4).

7,12-Dimethylbenz[a]anthracene (peak 8) was found to undergo a biooxidation reaction in the dorsal subcutaneous tissue to yield, 7,12-dihydroxymethylbenz[a]anthracene (peak 1), 7-hydroxymethylbenz[a]anthracene (peak 2), and 7-hydroxymethyl-12-methylbenz[a]anthracene (peak 3). Additionally, it was found that the substrate, 7,12-dimethylbenz[a]anthracene, underwent dealkylation reactions to yield 7-methylbenz[a]anthracene and 12-methylbenz[a]anthracene (peak 7), and benz[a]anthracene (peak 6) as metabolites. The further oxidation of the hydroxymethyl metabolites of 7,12-dimethylbenz[a]anthracene was also detected. Both 7-formylbenz[a]anthracene (peak 4) as well as 7-formyl-12-methylbenz[a]anthracene (peak 5) were found to be indistinguishable by HPLC from the authentic compounds. Further confirmation of the identity of the various metabolites of 7,12-dimethylbenz[a]anthracene, formed in rat subcutaneous tissue, was provided by gas chromatographic and mass spectral analysis. Table 1 shows the comparison of gas chromatographic retention times between authentic meso-position derivatives of 7,12-dimethylbenz[a]anthracene and its metabolites obtained in rat subcutaneous tissue, *in vivo*. The identities of the various metabolites 7,12-dihydroxymethylbenz[a]anthracene (Fig. 2); 7-hydroxymethylbenz[a]anthracene (Fig. 3); 7-hydroxymethyl-12-methylbenz[a]anthracene (Fig. 4); 7-formylbenz[a]anthracene (Fig. 5); 7-formyl-12-methylbenz[a]anthracene (Fig. 6); and 7-methylbenz[a]anthracene and 12-methylbenz[a]anthracene (Fig. 7) was confirmed by mass spectral analysis.

Quantitation of the metabolites of 7,12-dimethylbenz[a]anthracene 24 hr after injection of 0.4 μmol into the dorsal subcutaneous tissue of rats revealed that in the samples analyzed there were approximately 1441 pmol of hydrocarbons detected, of which 89% was 7,12-dimethylbenz[a]anthracene and 11% was metabolites. Of the approximately 161 pmol of metabolites found, 3.5% was benz[a]anthracene, 15.6% the 7-methyl- and 12-methylbenz[a]anthracenes, 38.0% was 7-hydroxymethylbenz[a]anthracene, 33% was 7-hydroxymethyl-12-methylbenz[a]-anthracene, 1% was the 7,12-dihydroxymethylbenz[a]anthracene, 1% was 7-formylbenz[a]anthracene, and 7.7% was 7-formyl-12-methylbenz[a]-anthracene.

DISCUSSION

Sarcomagenic activity in mice and rats by subcutaneous injection has been demonstrated repeat-

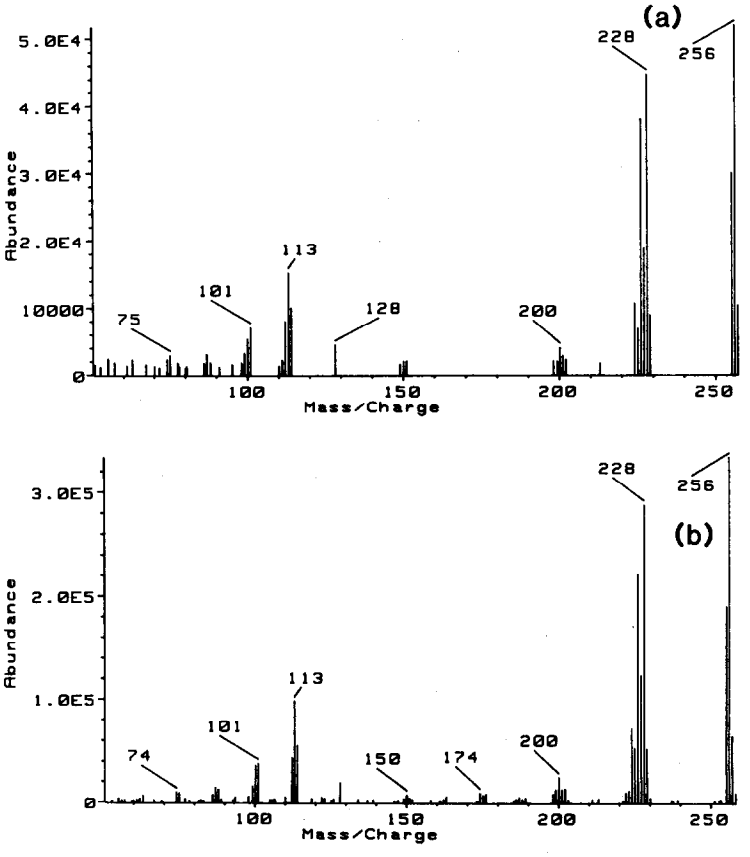

Fig. 5. (a) Mass spectral characterization of the metabolite identified by HPLC and GC as 7-formylbenz[a]anthracene. The metabolite yielded a parent molecular ion of *m/z* 256 and a corresponding fragmentation pattern that was indistinguishable from authentic 7-formylbenz[a]anthracene shown in (b). E = exponential value (E4 = 10^4).

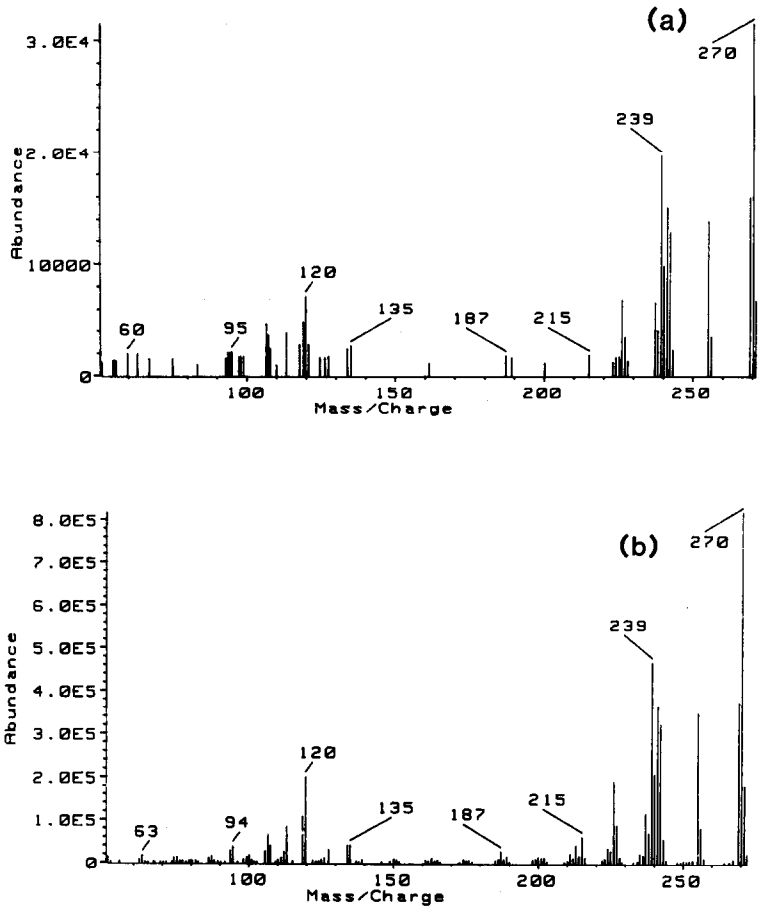

Fig. 6. (a) Mass spectral characterization of the metabolite identified by HPLC and GC as 7-formyl-12-methylbenz[*a*]anthracene. The metabolite yielded a parent molecular ion of m/z 270 and other ions that were found to be indistinguishable from authentic standards of 7-formyl-12-methylbenz[*a*]anthracene (b). E = exponential value (E4 = 10^4).

edly for the potent carcinogen 7,12-dimethylbenz[*a*]anthracene [16, 17]. However, the biochemical reactions of compounds of this type in subcutaneous tissue have not been investigated. The metabolism of 7,12-dimethylbenz[*a*]anthracene in this tissue clearly demonstrates that the oxidation of carcinogenic aromatic hydrocarbons can occur in a tissue which is highly susceptible to the carcinogenic action of certain polynuclear aromatic hydrocarbons and their derivatives. These biooxidation reactions give rise to the meso-anthracenic hydroxyalkyl metabolites of 7,12-dimethylbenz[*a*]anthracene, including the potent carcinogenic metabolites, 7-hydroxymethylbenz[*a*]anthracene and 7-hydroxymethyl-12-methylbenz[*a*]anthracene. A scheme for the metabolic activation of the 7,12-dimethylbenz[*a*]anthracene is presented in Fig. 8.

In 1938, Fieser and Hershberg [18] postulated that polynuclear aromatic hydrocarbons, administered to an experimental animal, may undergo some form of substitution reaction, possibly a hydroxylation, and that this constitutes an important step in a complicated chain of events leading eventually to carcinogenesis. In 1940, Shear and Leiter [7] demonstrated that 7-hydroxymethylbenz[*a*]anthracene, 7-acetoxymethylbenz[*a*]anthracene and 7-formylbenz[*a*]anthracene were highly carcinogenic in mice when administered by subcutaneous injection. In 1965, Boyland *et al.* demonstrated that 7-hydroxymethyl-12-methylbenz[*a*]anthracene induced cancer in mice and rats [19] and that 7-hydroxymethyl-12-methylbenz[*a*]anthracene was a metabolite of 7,12-dimethylbenz[*a*]anthracene [20]. Subsequent studies revealed that various derivatives of 7,12-dimethylbenz[*a*]anthracene, including 7-hydroxymethyl-12-methylbenz[*a*]anthracene, 7-formylbenz[*a*]anthracene, and 7-acetoxymethyl-12-methylbenz[*a*]anthracene were carcinogenic in rats by subcutaneous injection [6]. These results, taken together with earlier data on the bioalkylation of benz[*a*]anthracene, 7-methylbenz[*a*]anthracene, and 12-methylbenz[*a*]anthracene to form 7,12-dimethylbenz[*a*]anthracene, provide clear evidence that the most reactive centers of dimethylbenz[*a*]anthracene are the methyl groups and the meso-anthracenic centers to which they are attached. The results presented here are consistent with the hypothesis of Fieser that the function of the alkyl group is not to

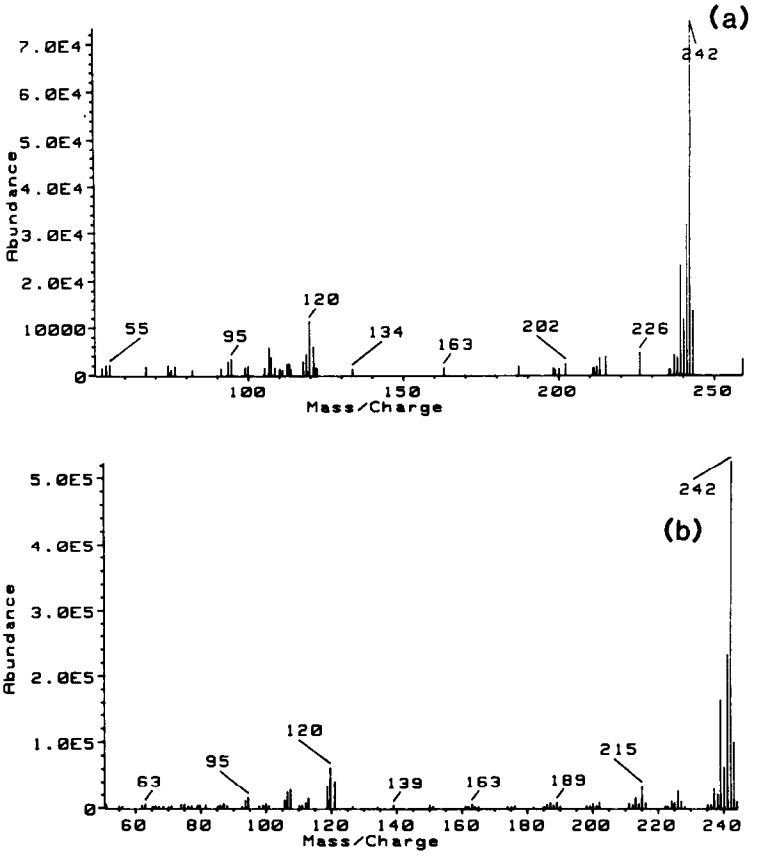

Fig. 7. Mass spectral profile of the metabolite of 7,12-dimethylbenz[*a*]anthracene in the dorsal subcutaneous tissue of the rat and identified by HPLC as a mono-methylbenz[*a*]anthracene derivative. (a) Metabolite identified by GC as 7-methylbenz[*a*]anthracene and (b) metabolite identified by GC as 12-methylbenz[*a*]anthracene. Both spectra show the parent molecular ion of m/z 242 and other accompanying ions that are characteristic of a mono-methyl substituted benz[*a*]anthracene. E = exponential value (E4 = 10^4).

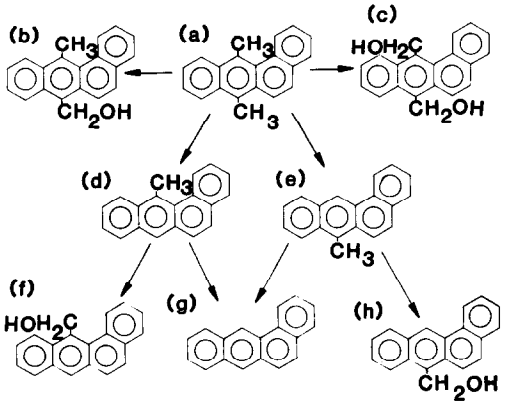

Fig. 8. Scheme for the metabolism of 7,12-dimethylbenz[*a*]anthracene in the dorsal subcutaneous tissue of the rat *in vivo*. (a) 7,12-dimethylbenz[*a*]anthracene; (b) 7-hydroxymethyl-12-methylbenz[*a*]anthracene; (c) 7,12-dihydroxymethylbenz[*a*]anthracene; (d) 12-methylbenz[*a*]anthracene; (e) 7-methylbenz[*a*]anthracene; (f) 12-hydroxymethylbenz[*a*]anthracene; (g) benz[*a*]anthracene; and (h) 7-hydroxymethylbenz[*a*]anthracene.

accentuate the reactivity of the molecule at some other position, but to serve as a reactive center for further metabolism and activation [21]. Biooxidation of the methyl groups of 7,12-dimethylbenz[*a*]-anthracene is equivalent to the chemical introduction of a hydroxyl group with lead tetraacetate. With lead tetraacetate the hydroxyl group is introduced in an acetylated condition. Fieser and Hershberg [18] suggested that a high degree of reactivity to substitution, of a type exemplified by reaction with lead tetraacetate, may be of importance in determining the carcinogenic activity of a hydrocarbon.

It remains to be demonstrated whether the biosynthesis of a benzylic electrophilic ester of hydroxymethyl metabolites takes place in subcutaneous tissue and whether an electrophilic ester metabolite is highly carcinogenic in that tissue.

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