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THE METABOLISM OF DIBENZO[a, h]PYRENE AND DIBENZO[a, i]PYRENE AND RELATED COMPOUNDS BY LIVER PREPARATIONS AND THEIR ENZYME-INDUCED BINDING TO CELLULAR MACROMOLECULES

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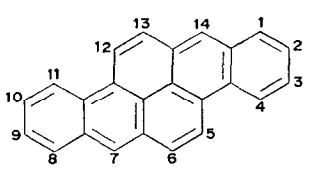
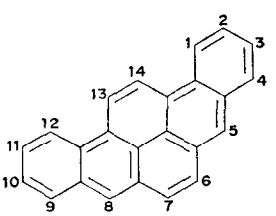
Abstract—Dibenzo[a, h]pyrene and dibenzo[a, i]pyrene are both metabolized to unidentified phenols and dihydrodiols by homogenates and microsomal fractions from the livers of rats that had been pretreated with 3-methylcholanthrene: some properties of these metabolites are described. In comparative experiments, in which the two ^{3}H-labelled dibenzopyrenes and ^{3}H-labelled benzo[a]pyrene were metabolized by microsomal fractions from the livers of normal rats, mice, hamsters and guinea-pigs, the dihydrodiols formed initially by the fractions from guinea-pigs, and to a lesser extent those formed by fractions from hamsters were further metabolized to unidentified products. Further metabolism of the dihydrodiols formed by microsomal fractions from the livers of rats and mice did not occur. Higher levels of enzyme-induced binding of all three hydrocarbons to the microsomal proteins were found in the preparations from guinea-pig livers than in the preparations from the livers of animals of the other species and in all preparations maximum levels of binding were reached after 30 min incubation. Enzyme-induced binding of the two dibenzopyrenes to DNA was observed when the ^{3}H-labelled hydrocarbons and DNA were incubated with microsomal fractions from the livers of rats that had been pretreated with 3-methylcholanthrene, but the levels of binding were low as compared with those obtained with dibenz[a, h]anthracene.

DIBENZO[a, h]]PYRENE (I) and dibenzo[a, i]pyrene (benzo[r, s, t]pentaphene) (II) are both highly active carcinogenic hydrocarbons[1,2] and dibenzo[a, i]pyrene (II) is one of the most powerful sarcomatogenic agents known.[3] Dibenzo[a, h]pyrene (I) is present in the exhaust fumes from petrol engines[4] and in the neutral fraction from cigarette smoke[5] and dibenzo[a, i]pyrene (II) is present in coal tar[6] and in tobacco smoke.[7]



(I)



(II)

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In spite of this, little is known of the metabolism of the two hydrocarbons, either in whole animals or in isolated tissues. The metabolism of dibenzo[a, h]pyrene (I) does not appear to have been studied in detail, but an early investigation[8] showed that when dibenzo[a, i]pyrene (II) was injected subcutaneously into mice, most of the hydrocarbon was recovered unchanged at the site of injection after 1 month and no metabolites were detected, either in the faeces or in the emerging tumour. Later, however, it was reported[9] that 85% of dibenzo[a, i]pyrene (II) was removed from the site of injection in two stages, an initial removal to other body sites being followed by a slower removal from these sites over a period of 10 weeks. The routes by which other polycyclic hydrocarbons are metabolized are now well established[10] and it seemed of interest to see if the hexacyclic dibenzopyrenes (I) and (II) are metabolized by similar routes. If metabolism of the dibenzopyrenes does occur, then it is possible that they also undergo the enzyme-catalyzed binding to DNA that takes place with other polycyclic hydrocarbons.

In this paper, experiments on the metabolism of the dibenzopyrene (I) and (II) by hepatic homogenates and microsomal fractions are described. The metabolism of benzo[a]pyrene, which has already been extensively studied[10-12] was reinvestigated under the same experimental conditions as those used in the study of the dibenzopyrenes (I) and (II) in order to compare the metabolism of the three hydrocarbons. The levels of binding of the benzopyrenes to DNA in the presence of a rat-liver microsomal fraction were also measured and compared with that obtained with dibenzo[a, h]anthracene under the same experimental conditions.

EXPERIMENTAL

Thin-layer chromatography (TLC). This was carried out on glass plates (20 × 20 cm) coated with layers of silica gel G (E. Merck A.-G., Darmstadt, Germany) of 0·25 mm thickness. Chromatograms were developed for 15 cm with either solvent (a), benzene, solvent (b), benzene–ethanol (19:1, v/v) or solvent (c), benzene–ethanol (9:1, v/v). Plates were examined in u.v. light while still wet and were sometimes re-examined after exposure to NH_3. Two-dimensional chromatograms were developed for 10 cm in the first direction with solvent (c), sprayed with conc. HCl and heated to 100° for 10 min before being developed in the second direction with solvent (a). In this way products arising from the decomposition of acid-labile precursors could be detected.

Ultra-violet absorption spectra. These were measured on a Unicam SP.800 recording spectrophotometer. The spectra of compounds separated by TLC were recorded after eluting with ethanol the silica gel forming the appropriate spots or bands removed from chromatograms.

Materials. Dibenzo[a, h]pyrene (I) and dibenzo[a, i]pyrene (II) were purchased from Koch–Light Laboratories (Colnbrook, Bucks, England). The hydrocarbons were purified by passing their solutions in light petroleum (b.p. 80–100°) through beds of alumina (Spence type H) and recrystallizing the recovered hydrocarbons from xylene. Dibenzo[a, h]pyrene (I) formed yellow plates, m.p. 307–308°, λ_{max} at 207, 215, 237·5, 245, 253, 266·5, 283·5, 295, 308, 393, 416 and 442 nm (log ϵ 4·51, 4·56, 4·42, 4·42, 4·40, 4·39, 4·40, 4·84, 5·14, 4·05, 4·26 and 4·40 respectively). Clar[13] reports m.p. 308° for this compound. Dibenzo[a, i]pyrene (II) formed yellow needles, m.p. 278–279°, λ_{max} at 206, 211·5, 222, 233, 241, 260, 271·5, 282, 294, 314, 330, 351, 370, 392, 407 and 432 nm (log ϵ 4·51, 4·60, 4·60, 4·66, 4·97, 4·28, 4·59, 4·76, 4·94, 4·43, 4·40,

4·35, 4·74, 4·91, 3·87 and 3·47 respectively). Clar[13] reports m.p. 280° for this hydrocarbon.

^{3}H-Labelled samples of the hydrocarbons (I) and (II) were generally labelled with tritium at the Radiochemical Centre, Amersham, Bucks, to give, after purification as described above, dibenzo[a, h]pyrene (I) (sp. act. 540 mCi/m-mole) and dibenzo-[a, i]pyrene (II) (sp. act. 396 mCi/m-mole). ^{3}H-Labelled benzo[a]pyrene (sp. act. 500 mCi/m-mole) and dibenz[a, h]anthracene (sp. act. 321 mCi/m-mole) were obtained from the Radiochemical Centre.

Attempts to prepare the "K-region" *cis*-dihydrodiols of the hydrocarbons (I) and (II) proved difficult, for although solutions of the hydrocarbons (I) and (II) and OsO_4

TABLE 1. PROPERTIES OF DERIVATIVES OF DIBENZO[a, h]PYRENE AND DIBENZO[a, i]PYRENE ON THIN-LAYER CHROMATOGRAMS

Hydrocarbon	Derivative	R_F in		Fluorescence	
		Solvent (a)	Solvent (b)	Immediate	After exposure to NH_3
Dibenzo[a, h]pyrene (I)	*cis*-5,6-Dihydro-5,6-dihydroxydibenzo [a, h] pyrene	0·00	0·16	Violet	Violet
	5- or 6-Hydroxydibenzo [a, h]pyrene	0·12	0·34	Green	Orange
	Dihydrodiol (B)	0·00	0·24	Blue	Blue
	Phenol (B_1)	0·32*	—	—	—
	Dihydrodiol (C)	0·00	0·15	Blue	Blue
	Phenol (C_1)	0·21	0·58	Green	Orange
Dibenzo[a, i]pyrene (II)	Phenol (D)	0·34	0·73	Blue	Orange
	Dihydrodiol (E)	0·00	0·21	Blue	Blue
	Phenol (E_1)	0·46*	—	—	—
	Dihydrodiol (F)	0·00	0·18	Blue	Blue
	Phenol (F_1)	0·24	0·60	Blue	Green

The letters B, B_1, C, C_1, D, E, E_1, F and F_1 refer to products described in the text.
* These products were detected on chromatograms as radioactive peaks using the methods described in the text.

in benzene containing a few drops of pyridine were kept at room temperature for 28 days, there was no evidence that reaction had occurred. When the hydrocarbon (I) (285 mg) in benzene (150 mg) and pyridine (0·4 ml) was heated under reflux for 10 days with OsO_4 (300 mg), a black complex was formed. The complex was decomposed with aq. KOH and mannitol[14] to give a mixture of two products that were separated by preparative TLC in solvent (c). The product at R_f 0·31 was recrystallized from ethanol in pink crystals (5 mg) that blackened above 190° but did not melt below 360°, λ_{max} at 215, 250, 258, 267, 283·5 (inflex.), 305, 318·5 and 334 nm. The product appeared to be a dihydrodiol since it was decomposed with acid to yield a product with the chromatographic properties (see Table 1) of a phenol and since its mass spectrum showed a molecular ion of *m/e* 336 which is consistent with a dihydrodiol structure of molecular formula $C_{24}H_{16}O_2$. The dihydrodiol is therefore presumed

to be *cis*-5,6-dihydro-5,6-dihydroxydibenzo[a, h]pyrene and its derived phenol either 5- or 6-hydroxydibenzo[a, h]pyrene. The second product (R_f 0·39), which showed λ_{max} at 250, 261·5, 278, 289, 294 (inflex.), 308·5, 329, 349, 355 (inflex.), 368, 373, 387 and 394 nm, did not have the chromatographic properties of a dihydrodiol. Neither product could be further characterized.

Dibenzo[a, i]pyrene (II) failed to react with OsO_4 under any of the conditions examined.

Experiments with liver preparations. Male Chester Beatty strain rats (age 35 days), male cream hamsters (age 3 months), male CBA strain white mice (age 3 months) and male Hartley strain guinea-pigs (age 9 weeks) were used in the experiments.

Preparations of hepatic homogenates and microsomal fractions. Homogenates were prepared from the livers of rats whose hepatic enzyme levels had been raised by treatment 48 hr previously with 3-methylcholanthrene (5 mg) in arachis oil (0·5 ml), administered by intraperitoneal injection. Homogenates were prepared from liver (40 g) in 0·1 M–phosphate buffer, pH 7·4, as previously described.[15] Glucose 6-phosphate (G 6-P) (250 mg) and $NADP^+$ (34 mg) [both obtained from Boehringer Corp. (London) Ltd., London, England] were added to each incubation mixture.

Microsomal fractions were prepared as previously described[16] from the livers of normal rats, from those of the pre-treated rats described above and from those of mice, hamsters and guinea-pigs. At least two animals were used in the preparation of each microsomal fraction. In the large scale experiments described below microsomal fractions obtained from liver (40 g) obtained from pretreated rats were suspended in 0·1 M–phosphate buffer (100 ml) containing G 6-P (500 mg), $NADP^+$ (68 mg), $MgCl_2$ (25 mg) and G 6-P dehydrogenase (Boehringer Corp. Ltd.) (7 units). The conditions used in other experiments with microsomal fractions are indicated below.

Qualitative experiments with the dibenzopyrenes (I) *and* (II). Homogenates and microsomal fractions prepared from rat liver (40 g) as described above were incubated at 37° for 3 min and either dibenzo[a, h]pyrene (I) or dibenzo[a, i]pyrene (II) (10 μmoles) in ethanol (3 ml) added. In some experiments the corresponding ^{3}H-labelled hydrocarbon (6 μmoles) was also added. The incubations were continued for 30 min and the mixtures extracted with equal volumes of ethyl acetate. The extracts were dried over Na_2SO_4 and the solvent removed under reduced pressure. The residues were applied to the base lines of thin-layer chromatograms that were developed in solvent (c). The wet chromatograms were examined in u.v. light and the fluorescent bands marked off. The chromatogram was then divided into a number of approximately equal fractions marked parallel with the fluorescent bands. The fractions were removed and the product present in each fraction eluted from the silica gel with ethanol (5 ml). When ^{3}H-labelled hydrocarbons were used, portions (0·1 ml) of each extract were removed and the radioactivity present determined by liquid scintillation counting. The u.v. spectra of products present in the extracts were also measured and fractions that appeared from radioactivity and spectral measurements to contain metabolic products were examined as described below.

When the unlabelled dibenzopyrenes (I) and (II) were used as substrates, the fractions containing metabolites were evaporated and the residues retained for use as chromatographic standards in experiments described below. In addition to the hydrocarbons (I) and (II), benzo[a]pyrene was also incubated in these systems to obtain metabolites for use as chromatographic standards. Control experiments, in

which liver preparations that had been boiled and cooled were used, were also carried out.

Quantitative experiments with the dibenzopyrenes (I) *and* (II). (a) *Time-course studies on dihydrodiol formation and protein binding.* Microsomal fractions, each prepared from the livers (1 g) of animals of the species described above, were suspended in 0·1 M–phosphate buffer (10 ml), pH 7·4, containing G 6-P (12·5 mg), $NADP^+$ (1·7 mg), $MgCl_2$ (2·5 mg) and G 6-P dehydrogenase (2·8 units) and the mixtures, contained in 25 ml conical flasks, were incubated at 37° in a metabolic shaker (H. Mickle, Gomershall, Surrey, England). In each experiment with a microsomal fraction, eight such flasks were prepared and to each was added, with shaking, a solution of 16 nmoles of either ^{3}H-labelled dibenzo[a, h]pyrene (I), dibenzo[a, i]-pyrene (II) or benzo[a]pyrene in ethanol (0·2 ml). One flask was immediately removed from the metabolic shaker and others were removed at 5, 10, 15, 20, 30, 60 and 90 min after the addition of the hydrocarbon. The contents of each flask were extracted twice with ethyl acetate (50 ml) and the combined extracts dried over Na_2SO_4 (15 g) and the solvent removed under reduced pressure. The residues were applied to the base lines of thin-layer chromatograms that were developed for 15 cm in solvent (c) alongside the appropriate chromatographic standards obtained as described above. The developed chromatograms were each divided horizontally into 20 equal fractions and the silica gel from each fraction removed and added to scintillation fluid (10 ml) in a counting vial and the radioactivity in each fraction determined. From these measurements the total amount of the dihydrodiols formed in each incubation mixture was calculated. The amounts of protein present in representative samples of hepatic microsomal fractions from each species were determined separately using a modification of the biuret method[17] with bovine plasma albumin as standard. The total amount of dihydrodiol formed per mg of protein in each incubation was then calculated.

The aqueous layers remaining after extraction of the incubation mixtures in the above experiments were centrifuged at 1480 *g* for 10 min. The supernatant was discarded and the microsomal protein pellet washed twice with acetone (10 ml). The pellet was dried in air and dissolved in tetraethylammonium hydroxide (1 ml of solution for each 10 mg of protein) and the radioactivity present in duplicate portions (0·1 ml) of the solutions was determined by liquid scintillation counting.

All the incubations described above were carried out in duplicate and the results shown in Figs. 5–10 are the means of the two determinations. Control experiments, in which the ^{3}H-labelled hydrocarbons were incubated with microsomal fractions that had been boiled and cooled, were also carried out.

(b) *Binding of hydrocarbons to DNA in the presence of microsomal fractions.* These were carried out essentially as described by Grover and Sims[18] with the hepatic microsomal fraction from rats that had been pretreated with 3-methylcholanthrene using ^{3}H-labelled dibenzo[a, h]pyrene (I), dibenzo[a, i]pyrene (II) and dibenz[a, h]-anthracene as substrates and in the presence of DNA. The isolation and purification of the DNA and the determination of the bound radioactivity was carried out as described.[18] The binding of the hydrocarbons to microsomal protein was also determined using the method described above.

RESULTS

The metabolism of dibenzo[a, h]*pyrene* (I) *by rat liver homogenates and microsomal fractions.* When the ethyl acetate-soluble products of the metabolism of the hydrocarbon (I) by either rat liver homogenates or microsomal fractions were examined by TLC, four fluorescent bands were detected. Using the ^{3}H-labelled hydrocarbon diluted with cold carrier hydrocarbon as substrate, it was found that the regions on the chromatograms of high radioactivity corresponded with the positions of the fluorescent bands seen when the chromatograms were inspected in u.v. light. The chromatographic profile obtained in this experiment is shown in Fig. 1.

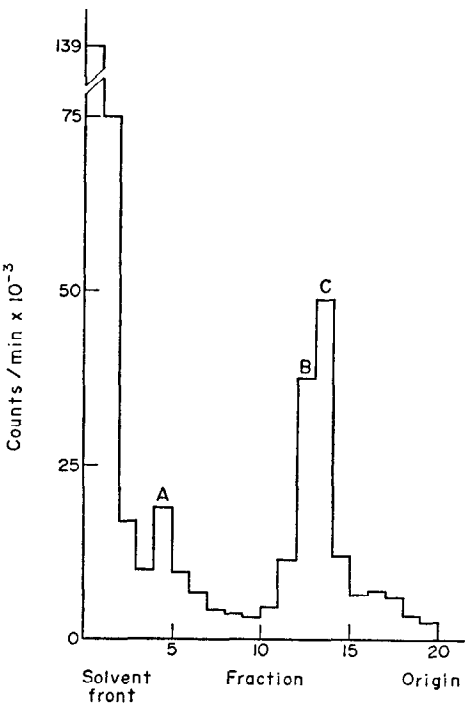

FIG. 1. The metabolism of ^{3}H-labelled dibenzo[a, h]pyrene (I) by a rat-liver microsomal fraction. The incubation was carried out as described in the text and the ethyl acetate-soluble products chromatographed on a silica gel plate in benzene–ethanol (9:1, v/v). The plate was divided into twenty fractions and the radioactivity in each fraction determined. Other properties of the metabolites A, B and C are described in the text.

The fastest-moving band contained a compound whose chromatographic properties and u.v. absorption spectrum were identical with dibenzo[a, h]pyrene. The second band contained a minor product (A) that appeared to be phenolic since its green fluorescence became orange when the chromatogram was exposed to NH_3. The third and fourth bands contained blue fluorescent products that appeared to be dihydrodiols (B) and (C). When the product (B) was examined on a two-dimensional chromatogram, a phenolic product arising from its decomposition could not be readily detected. However, in experiments with the ^{3}H-labelled hydrocarbon it was possible to show that there was a peak of radioactivity (B_1) in that region of the chromatogram

where a phenol was expected. The product (C) was decomposed by acid when examined on a two-dimensional chromatogram to yield a product (C_1) that appeared to be a phenol since it formed a spot with a green fluorescence in u.v. light that became orange when the chromatogram was exposed to ammonia. In experiments with the ^{3}H-labelled hydrocarbon (I), a peak of radioactivity was detected on the chromatogram corresponding to the fluorescent product (C_1). Neither of these dihydrodiols was chromatographically or spectroscopically identical with the dihydrodiol, presumably the "K-region" dihydrodiol, obtained as described above in the oxidation of the hydrocarbon (I) with OsO_4. The chromatographic properties of the products (B), (C) and (C_1) are shown in Table 1, and the u.v. absorption spectra of products (B) and (C) in Fig. 2.

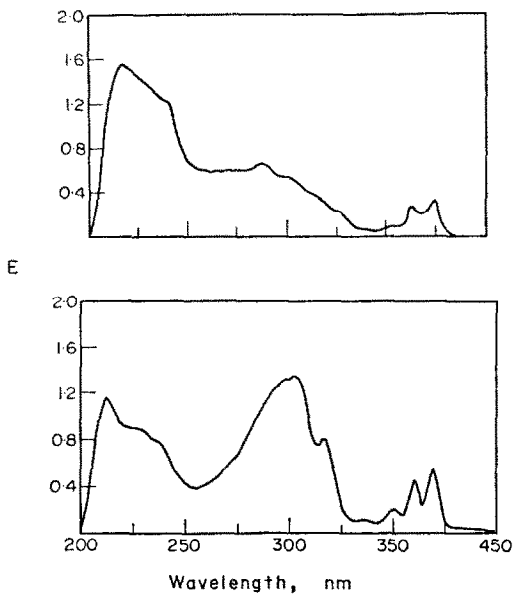

FIG. 2. The u.v. absorption spectra of the dihydrodiols B and C obtained as metabolites of dibenzo[a, h]pyrene (I) by a rat-liver microsomal fraction. Measurements were made as described in the text.

None of the above products was formed when the hydrocarbon (I) was incubated with liver preparations that had been boiled and cooled, showing that the products were formed by enzymic reactions.

The metabolism of dibenzo[a, i]*pyrene* (II) *by rat liver homogenates and microsomal fractions.* The ethyl acetate-soluble products of the metabolism of the hydrocarbon (II) by either rat liver homogenate or microsomal fractions were similar and contained products that formed four fluorescent bands on thin-layer chromatograms. The chromatographic profile, obtained when the ^{3}H-labelled hydrocarbon was used as substrate is shown in Fig. 3. The peaks of radioactivity obtained corresponded with the position of the fluorescent bands.

The fastest moving band was identified as unchanged hydrocarbon (II) by its chromatographic properties and u.v. absorption spectrum. The second band contained a product (D) with the expected chromatographic properties of a phenol,

TABLE 2. REACTION OF POLYCYCLIC HYDROCARBONS WITH DNA AND PROTEIN IN THE PRESENCE OF RAT LIVER MICROSOMAL FRACTIONS

Hydrocarbon	Reaction with DNA (μmoles/g atom of DNA P*)	Reaction with microsomal protein (pmoles/mg protein)
Dibenzo[a, h]pyrene	0·08	25·1
Dibenzo[a, i]pyrene	0·05	32·1
Dibenz[a, h]anthracene	0·21	97·2

Reactions were carried out as described in the text.
* Calculated on a basis of 8% phosphorus.

λ_{max} at 242, 275 (inflexion), 285, 296, 319 (inflexion), 335 (inflexion), 360, 378, 400, 410 and 546 nm. When the chromatograms were examined in u.v. light the product showed a blue fluorescence which changed to orange when the chromatograms were exposed to NH_3. Two dihydrodiols (E) and (F) appeared to be present. On acid-treated two-dimensional chromatograms, the product (E) gave rise to a product (E_1)

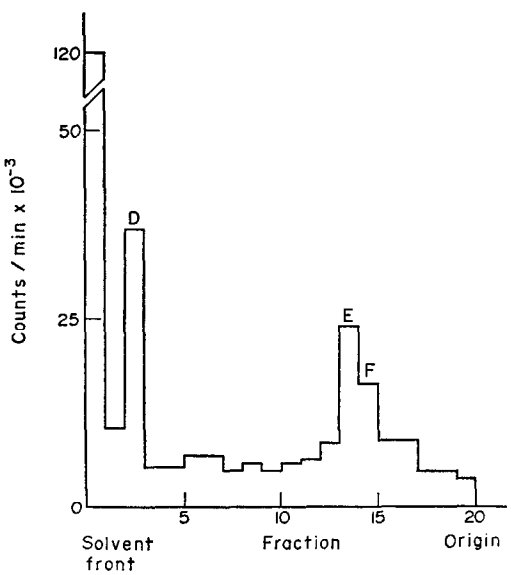

FIG. 3. The metabolism of ^{3}H-labelled dibenzo[a, i]pyrene (II) by a rat-liver microsomal fraction. The experiment was carried out as described in Fig. 1. Other properties of the metabolites D, E and F are described in the text.

that had the chromatographic properties of a phenol and that could sometimes be detected by the pink fluorescence produced when the chromatograms were exposed to NH_3 and examined in u.v. light. When the ^{3}H-labelled hydrocarbon was used as substrate, a peak of radioactivity was detected in this region of the chromatograms. The product (F) appeared to be a dihydrodiol because it gave rise to product (F_1) with the chromatographic properties of a phenol after treatment with acid on two-

dimensional chromatograms. The phenol formed a blue fluorescent spot in u.v. light that changed to a green fluorescence when the chromatograms were exposed to NH_3. When the ^3H-labelled hydrocarbon was used as substrate, a peak of radioactivity was present on the chromatogram in the same position as the phenol.

The chromatographic properties of the products (D), (E), (E_1), (F) and (F_1) are shown in Table 1 and the u.v. absorption spectra of (E) and (F) in Fig. 4. The u.v. spectra of products (E) and (F) did not resemble that of 6,7-dihydrodibenzo[a, i]-pyrene[8] so that it is unlikely that either of the products is the 6,7-dihydrodiol. None of these products was formed in control experiments in which liver preparations that had been boiled and cooled were used.

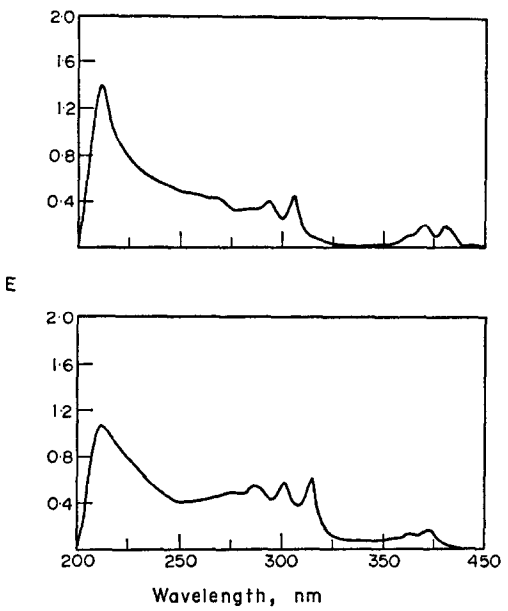

FIG. 4. The u.v. absorption spectra of the dihydrodiols E and F obtained as metabolites of dibenzo-[a, i]pyrene (II) by a rat-liver microsomal fraction. Measurements were made as described in the text.

Quantitative aspects of the metabolism of dibenzo[a, h]*pyrene* (I), *dibenzo*[a, i]-*pyrene* (II) *and benzo*[a]*pyrene*. The results of time-course experiments in which the three hydrocarbons were incubated for various times with liver microsomal fractions from animals of different species and the amounts of dihydrodiols measured are shown in Figs. 5–7. All the hydrocarbons gave essentially the same results. With microsomal fractions from the livers of rats and mice each hydrocarbon yielded dihydrodiols, and the amounts of these present in the reaction mixtures reached maximal values after incubation times of about 20 min and thereafter remained constant. With the microsomal fraction from the liver of guinea-pigs, however, the amounts of dihydrodiols present reached maximal values after about 10 min of incubation and then decreased. The microsomal fraction from the livers of hamsters was intermediate in its action, for with dibenzo]a, h]pyrene (I) and benzo[a]pyrene the amounts of dihydrodiols present in the reaction mixture reached maximal values

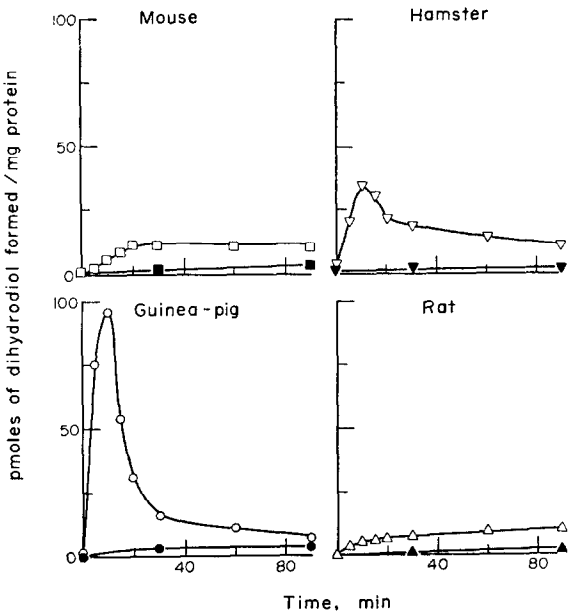

FIG. 5. Time-course studies on the formation of dihydrodiols from dibenzo[a, h]pyrene (I) by microsomal fractions from the livers of mice, hamsters, guinea-pigs and rats. The open and closed symbols show the values obtained when fresh and boiled microsomal fractions respectively were used. The incubations were carried out as described in the text.

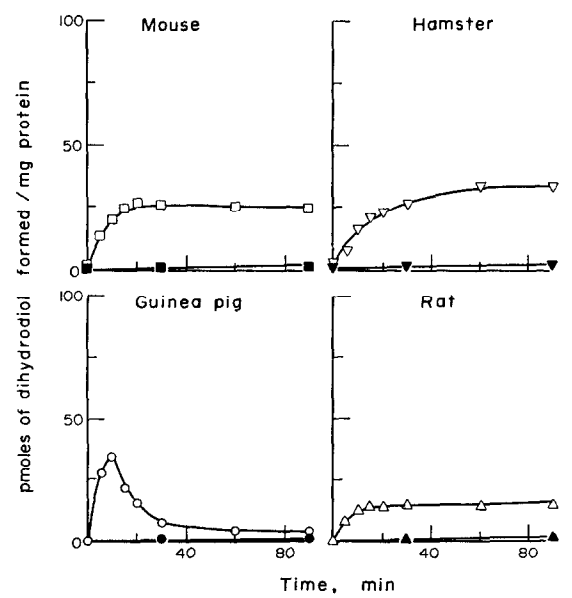

FIG. 6. Time-course studies on the formation of dihydrodiols from dibenzo[a, i]pyrene (II) by microsomal fractions from the livers of mice, hamsters, guinea-pigs and rats. The symbols are the same as those in Fig. 5 and incubations were carried out as described in the text.

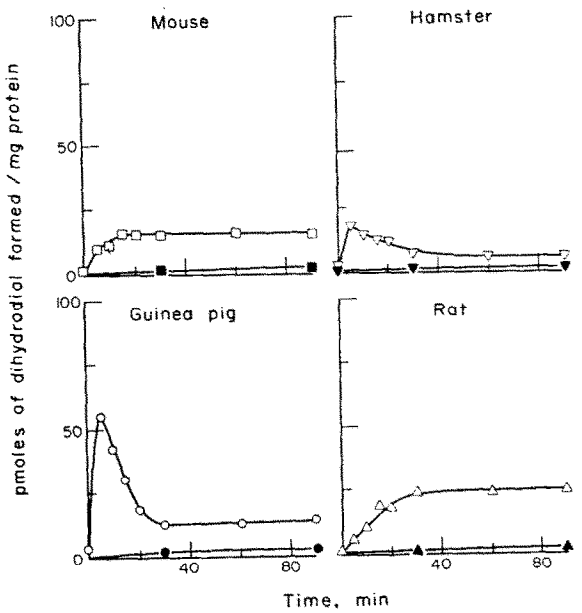

FIG. 7. Time-course studies on the formation of dihydrodiols from benzo[a]pyrene by microsomal fractions from the livers of mice, hamsters, guinea-pigs and rats. The symbols are the same as those in Fig. 5 and incubations were carried out as described in the text.

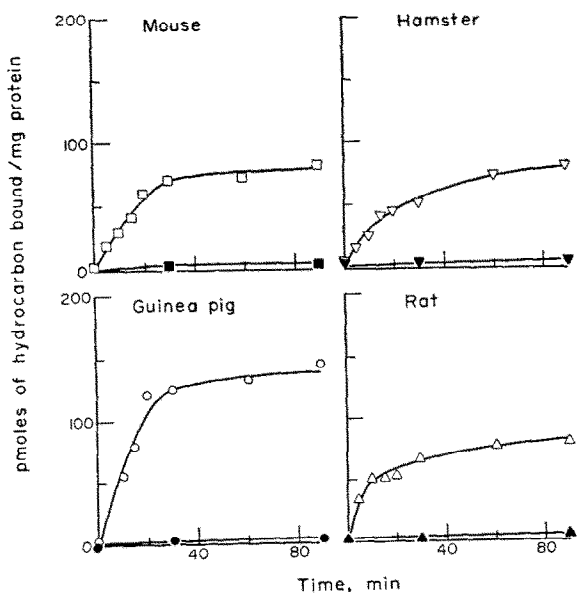

FIG. 8. Time-course studies on the binding of dibenzo[a, h]pyrene (I) to the protein of microsomal fractions from the livers of mice, hamsters, guinea-pigs and rats. The symbols are the same as those in Fig. 5 and incubations and measurements were made as described in the text.

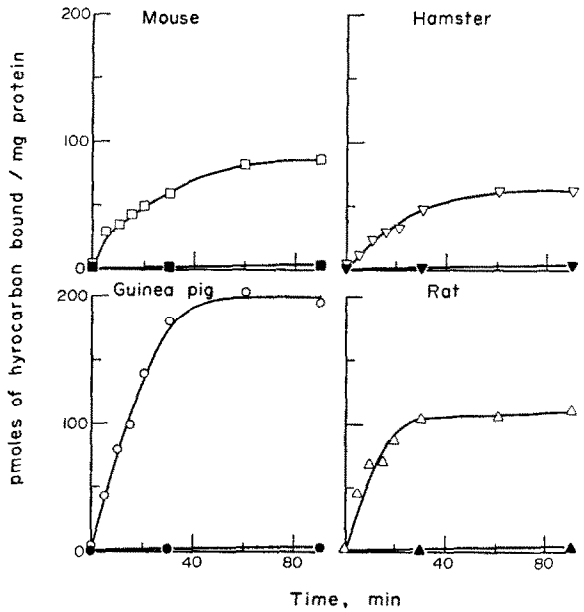

FIG. 9. Time-course studies on the binding of dibenzo[a, i]pyrene (II) to the protein of microsomal fractions from the livers of mice, hamsters, guinea-pigs and rats. The symbols are the same as those in Fig. 5 and incubations and measurements were made as described in the text.

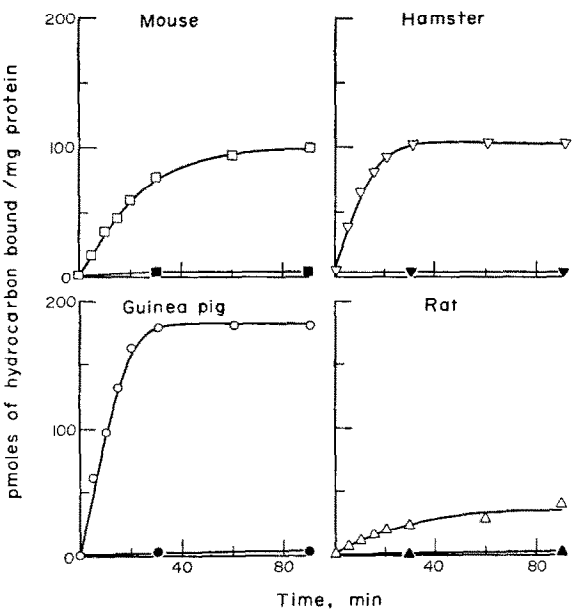

FIG. 10. Time-course studies on the binding of benzo[a]pyrene to the protein of microsomal fractions from the livers of mice, hamsters, guinea-pigs and rats. The symbols are the same as those in Fig. 5.

after 10 min and then decreased, whereas with dibenzo[a, i]pyrene (II) the amounts of dihydrodiols present did not decrease but became constant after about 30 min incubation. In the control experiments, using boiled homogenates, dihydrodiols were not formed.

In the experiments on the time-course of the binding of the hydrocarbons to microsomal proteins, the results of which are shown in Figs. 8–10, the three hydrocarbons reached their maximal levels of binding to the proteins of the microsomal fractions of the livers of all the animal species examined after 30–40 min incubation time. The highest levels of binding were obtained with those from guinea-pigs. The binding was considered to be the result of some metabolic activation process since little or no binding occurred when boiled microsomal fractions were used.

The results obtained when some ^{3}H-labelled hydrocarbons were incubated with a rat liver microsomal fraction in the presence of added DNA are shown in Table 3. The levels of binding to DNA obtained with the dibenzopyrenes (I) and (II) were lower than that obtained with the strong carcinogen, dibenz[a, h]anthracene. In the experiments in which the cofactors were omitted from the incubation mixtures, no significant levels of binding, either to DNA or to the microsomal protein were observed.

DISCUSSION

It is evident from the results presented in this paper that dibenzo[a, h]pyrene (I) and dibenzo[a, i]pyrene (II) are both metabolized by the mixed function oxidases of the hepatic microsomal systems to give phenols and dihydrodiols. The sites on the molecule at which oxidation occurred have not yet been identified because of the difficulty in synthesizing the appropriate reference compounds, but it is apparent that metabolism has not taken place at the "K-regions" of either hydrocarbon. This is in agreement with results found in the metabolism of other polycyclic hydrocarbons: with benzo[a]pyrene, for example, the detection of the "K-region" dihydrodiols as metabolites is difficult.[19]

Benzo[a]pyrene is converted by hepatic microsomal preparations mainly into a mixture of 3-hydroxybenzo[a]pyrene, 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene and 9,10-dihydro-9,10-dihydroxybenzo[a]pyrene,[12] and it is possible, therefore, that with the dibenzopyrenes, metabolism to form dihydrodiols takes place on bonds equivalent to the 7,8- and 9,10-bonds of benzo[a]pyrene. With dibenzo[a, h]pyrene (I), these are the 1,2- and 3,4 or the 8,9- and 10,11-bonds and with dibenzo[a, i]pyrene (II), the 1,2- and 3,4- or the 9,10- and 11,12-bonds.

The low reactivity of the dibenzopyrenes (I) and (II) towards OsO_4 was unexpected since other aromatic hydrocarbons such as benzo[a]pyrene react quantitatively at room temperature with the oxidising reagent within a few days.[14]

The results of the time-course experiments show that microsomal fractions from the livers of rats, mice, guinea-pigs and hamsters are all able to metabolize the dibenzopyrenes (I) and (II) and benzo[a]pyrene to dihydrodiols, but with the more active preparations from hamsters and, more particularly with those from guinea-pigs, further metabolism of the dihydrodiols takes place. Although in the present work no attempt was made to identify the new products, evidence has been presented[20] that with another polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene, dihydrodiols formed from this hydrocarbon by metabolism at bonds other than at that

of the "K-region" are converted by homogenates from the livers of rats that had been pretreated with 3-methylcholanthrene into water-soluble derivatives. These derivatives may be GSH-conjugates since they yield cysteine, glycine and glutamic acid after hydrolysis with sodium hydroxide.

Since dihydrodiols of the type suggested above possess olefinic double bonds it seems likely that these are the bonds that are involved in the further metabolism of the dihydrodiols. Some evidence for this is provided by the fact that 7,8-dihydrobenzo-[a]pyrene and 9,10-dihydrobenzo[a]pyrene, which are related to the two dihydrodiols formed from benzo[a]pyrene by metabolism, are themselves metabolized mainly at the 9,10- and 7,8-bond respectively to yield dihydrodiols.[12] Presumably these dihydrodiols arise through the intermediate formations of epoxides and it is of interest that of the two epoxides that are probably formed, 7,8-dihydrobenzo[a]pyrene 9,10-oxide is very active in the alkylation of 4-(*p*-nitrobenzyl)pyridine whereas the isomeric 9,10-dihydrobenzo[a]pyrene 7,8-oxide has only a low activity.[12]

The ^{3}H-labelled dibenzopyrenes (I) and (II) and benzo[a]pyrene all bind covalently with microsomal protein when they are incubated with hepatic microsomal fractions and the cofactors necessary for microsomal mixed function oxidase activity. When boiled microsomal fractions were used or when the cofactors were omitted from the incubation mixture, no protein binding was observed, which suggests that activation of the hydrocarbons by the mixed function oxidases is necessary before binding can occur. Similar results were obtained in earlier experiments[18] in which any one of a number of ^{3}H-labelled hydrocarbons, including benzo[a]pyrene, was incubated with rat-liver microsomal systems to which bovine serum albumin was added as a source of protein.

The results obtained when DNA was incubated with rat liver microsomal fractions and either of the ^{3}H-labelled dibenzo[a]pyrenes (I) or (II) also indicate that activation of the hydrocarbons by the mixed function oxidase systems is necessary for binding to the nucleic acid to occur. These results are in agreement with earlier experiments[18,21] where other aromatic hydrocarbons were used as substrate, except that the levels of binding were lower with the dibenzopyrenes (I) and (II) than with other hydrocarbons. This may be due to the low levels of metabolism observed with the dibenzopyrenes and this in turn may be due to their large molecular size and low solubility.

It seems likely that the intermediate involved in the reactions of the hydrocarbons with macromolecules are epoxides since many polycyclic hydrocarbons are now known to be metabolized into epoxides by hepatic microsomal systems.[19,22,23] Some of these epoxides react with nucleic acids and proteins, both chemically[24] and in cells in culture[25,26] and many possess biological activities in that they are mutagenic to bacteria,[27] bacteriophage[28] and mammalian cells[29] and will produce malignant transformations in rodent cells in culture.[30,31] Epoxides are converted enzymically into dihydrodiols[32,33] and although with the dibenzopyrenes (I) and (II), the metabolic formation of epoxides has yet to be proved, it is now clear that both these hydrocarbons are metabolized to dihydrodiols. They thus resemble most other polycyclic hydrocarbons, which are also metabolized to dihydrodiols, presumably through the intermediate formation of epoxides.

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