

K-Region and Non-K-Region Metabolism of Benzo(a)pyrene by Rat Liver Microsomes

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SUMMARY

The metabolism of benzo(a)pyrene (BP) by liver microsomes from normal and 3-methylcholanthrene (MC)-treated rats was quantitatively analyzed by a double label method using BP-³H and BP-¹⁴C. Qualitatively, the metabolism of BP by both microsomal preparations was similar. The identified metabolites were 7,8-dihydro-7,8-dihydroxy-BP, 4,5-dihydro-4,5-dihydroxy-BP, 9,10-dihydro-9,10-dihydroxy-BP, 3-hydroxy-BP, 9-hydroxy-BP, and BP-1,6-quinone and BP-3,6-quinone.

The quantitative analysis by the double labeling method showed the profile of BP metabolites produced by rat liver microsomes from normal and MC-induced rats. The ratio of metabolites from induced microsomes to that from normal microsomes was greatest for the 7,8-dihydro-7,8-dihydroxy and 9,10-dihydro-9,10-dihydroxy metabolites, less for the phenols and K-region metabolites, and least for the quinones. Similar ratios were obtained when microsomes from MC-treated rats were diluted except that dilution reduced the relative amount of phenol formation. These results suggest that higher enzyme activity favors the formation of the non-K-region diols and phenols relative to the K-region diol.

INTRODUCTION

In 1957, Conney *et al.* (2) characterized a number of metabolites of BP³ formed *in vitro*. The metabolites of BP formed by rat liver homogenates and their methylated derivatives were isolated by silica and alumina column chromatography and were recognized as 1-HO-BP, 3-HO-BP, and F₁ [the metabolite that was reported by Weigert and Mottram (34, 35) as major metabolites] and small quantities of BP-3,6-quinone, BP-1,6-quinone, and 3,6-dihydroxy-BP. Ten years later, Sims (26) analyzed the BP metabolites formed by liver homogenates from MC-treated rats. The metabolites were separated on silica gel TLC and identified by R_F value, UV spectra, and the

fluorescence in UV. He found in addition to 3-HO-BP and BP-1,6-quinone and BP-3,6-quinone 2 previously unrecognized dihydrodihydroxy compounds, 1,2-dihydro-1,2-dihydroxy-BP and 7,8-dihydro-7,8-dihydroxy-BP. The latter was subsequently identified as the 9,10-dihydro-9,10-dihydroxy-BP (27). Very recently, the 1,2-dihydro-1,2-dihydroxy-BP was identified as the 7,8-dihydro-7,8-dihydroxy-BP (33). In 1970 Sims (29) carried out qualitative and quantitative studies on the metabolism of various polycyclic hydrocarbons by liver homogenates and microsomes from control and MC-treated rats. He was particularly interested in the relation between carcinogenic potency and metabolism of the hydrocarbon in the K region.

In this report, we investigated the metabolism of BP in liver microsomes from normal and MC-treated rats using BP-³H and BP-¹⁴C. The K-region metabolite of BP, which was previously unrecognized, was detected and identified. These results are in accord with recent reports of metabolism at the K region (13, 23, 32). The double labeling method was also used to analyze the spectrum of metabolites of BP produced by normal microsomes and microsomes from MC-induced rats.

MATERIALS AND METHODS

Chemicals. BP and MC were purchased from Eastman Organic Chemicals, Rochester, N. Y. Both compounds were purified by column chromatography using silica gel as the adsorbent and benzene:hexane (1:1) as the eluent. They were then recrystallized just prior to use, BP from absolute ethanol and MC from benzene and ether. NADPH was obtained from Calbiochem, San Diego, Calif.

Radiochemicals. BP-³H (13 Ci/mmol) and BP-¹⁴C (21 mCi/mmol) were purchased from Amersham/Searle, Arlington Heights, Ill. Each was purified by TLC with silica gel using benzene:hexane (1:15). The BP-³H was diluted with nonradioactive purified BP just prior to use. The assays contained BP-³H (specific activity, 370 or 479 mCi/mmol), 5 × 10⁻⁵ M in methanol, or BP-¹⁴C (specific activity, 21 mCi/mmol), 5 × 10⁻⁵ M in methanol.

Preparation of Microsomes. Adult male Sprague-Dawley rats weighing 160 to 180 g were obtained from the NIH Animal Supply. Forty hr prior to sacrifice, the control

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³ The abbreviations used are: BP, benzo(a)pyrene; 1-HO-BP, 1-hydroxy-BP; 3-HO-BP, 3-hydroxy-BP; MC, 3-methylcholanthrene; TLC, thin-layer chromatography; MC-microsomes, liver microsomes from MC-treated rats; 9-HO-BP, 9-hydroxy-BP; 6-HO-BP, 6-hydroxy-BP.

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group received injections i.p. of 0.5 ml corn oil while the rats to be induced were treated with 0.5 ml corn oil containing 5 mg MC. The 2 main lobes of the liver from each rat were used for the microsome preparation. Two g from each lobe were homogenized in 10 ml 0.25 M sucrose: 0.05 M Tris buffer, pH 7.5, using a Potter-Elvehjem glass-glass homogenizer (20 strokes). The homogenates were centrifuged at $750 \times g$ for 15 min, and the supernatant was centrifuged at $10,000 \times g$ for 15 min. The microsomes were obtained by centrifugation of the supernatant at $105,000 \times g$ for 60 min. The microsomal pellets were homogenized gently in a small volume of sucrose:Tris buffer and frozen at -70° until ready for use.

Metabolism of BP. For the microsome-catalyzed reaction the following assay was performed. Each flask contained, in a total volume of 1 ml, 50 μ moles of Tris-chloride buffer (pH 7.5), 0.36 μ mole of NADPH, 3 μ moles of $MgCl_2$, 0.10 ml of the microsomal preparation (containing 100 to 150 μ g of protein), and 50 nmoles of BP (added in 0.04 ml methanol just prior to incubation). In all cases 2 experiments, each in duplicate, were run simultaneously, one using BP labeled with ^{14}C (specific activity, 21 mCi/mmole) and the other using BP generally labeled with tritium (specific activity, 370 or 479 mCi/mole) to provide exact conditions suitable for comparison. The reaction was started by the addition of substrate. The flasks were gently shaken at 37° for 20 min in air. The reaction was stopped by the addition of 1.0 ml cold acetone. The flasks were placed in ice and 2.0 ml of cold ethyl acetate were added. The contents of duplicate flasks were pooled into glass-stoppered tubes and the organic soluble components were extracted by gentle shaking for 2 min. The organic layer was then removed and dried by the addition of anhydrous Na_2SO_4 . One-ml samples from each of the 2 experiments were pooled and evaporated to a very small volume (20 to 50 μ l). The total residue was spotted on thin-layer plates and the pattern was developed in 2 dimensions.

TLC. Camag, Inc., Milwaukee, Wis., supplied the thin-layer plates (200 x 200 mm) precoated with starch-bound silica gel, 250 μ m thick. The plates were dried in an oven for 30 to 45 min at 110° just prior to use. The metabolite mixture was spotted and the pattern developed in the 1st dimension using 5% methanol in benzene for the separation of metabolites. This was followed immediately by chromatography in the 2nd dimension using benzene:hexane (1:15) for the removal of the unreacted BP. The chromatograms were inspected in UV (2537 \AA). Fractions from the plate were removed by scraping with a steel spatula 2 mm wide. The radioactivity in each fraction was measured by liquid scintillation counting.

Radioactivity Assays. The silica gel powder scraped from the plate was placed into the counting vial directly and counted in 10 ml of toluene solution of Liquifluor (New England Nuclear, Boston, Mass.). A Beckman Model LS-100 liquid scintillation counter was used for measurement of radioactivity. The background for 3H was 60 cpm and for ^{14}C was 7 cpm.

Computer Program. The following data were given to the computer: cpm 3H and cpm ^{14}C in each fraction

scraped from the plate, the background and counting efficiency for each isotope, and the ^{14}C cross-over ratio. The program was designed to make the calculation:

$$Pmoles = cpm / (efficiency \times specific\ activity \times 2.22)$$

and to correct for background and cross-over at each point. The response was dpm, the dpm 3H :dpm ^{14}C ratio, percentage of 3H and percentage of ^{14}C in each fraction and pmoles 3H and pmoles ^{14}C at each point. Since the entire plate was scraped, the percentage of total recovery of radioactivity from the chromatography was calculated.

RESULTS

Separation of BP- 3H and BP- ^{14}C Metabolites Formed by Microsomes from MC-treated Rats.

The metabolism of BP by MC-microsomes was investigated using BP- 3H and BP- ^{14}C . Chart 1 shows that 6 peaks were detected. These peaks have been designated as M-1 to M-6, which represent 6 metabolite areas on the chromatogram. The metabolic pattern obtained from the BP- ^{14}C and BP- 3H were qualitatively similar. However, the ratios of the cpm of 3H and ^{14}C in metabolite areas M-1, M-2, and M-3 regions differ somewhat from that in the M-4, M-5, and M-6 regions of metabolites. This suggests that BP- 3H may be specifically detritiated in certain regions when incubated with microsomes (N. Kinoshita and H. V. Gelboin, manuscript in preparation). Table 1 shows the R_F values and qualitative fluorescence of the various metabolite areas (designated M-1 to M-6) formed by microsomes from MC-treated rats.

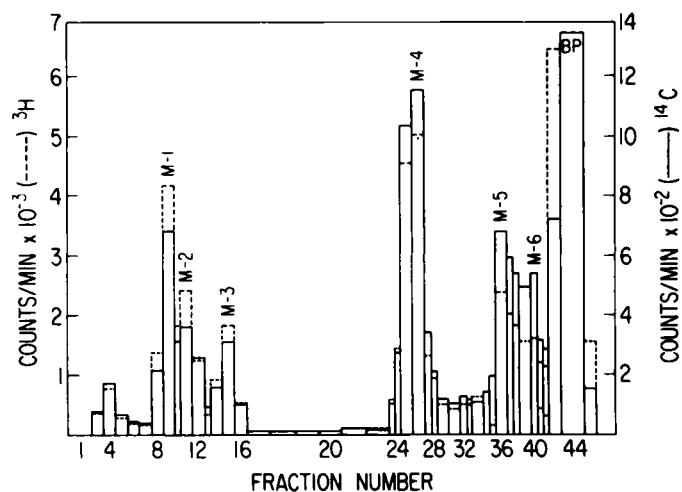


Chart 1. The metabolism of BP by liver microsomes from MC-treated rats. Incubations with microsomes using BP- 3H (370 mCi/mmole) and BP- ^{14}C (21 mCi/mmole) were performed by the procedure described in the text. One ml of the ethyl acetate-extractable products from each of the incubations was mixed and evaporated. The residual material was chromatographed in 1 dimension as described in "Materials and Methods." The radioactivity assay is described in the "Materials and Methods." - - -, BP- 3H and its derivatives; —, BP- ^{14}C and its derivatives.

Identification of Metabolites of BP

Non-K-Region Dihydrodiols. The production of relatively large quantities of BP metabolites was accomplished by using nonradioactive BP and combining many flasks. The partially isolated metabolites were further purified by TLC and column chromatography with silica gel. Chart 2 shows the UV spectral analysis of Fractions M-1, M-2, and M-3. The UV spectrum of M-1 was in complete agreement with that of 9,10-dihydro-9,10-dihydroxy-BP reported by Sims (29). The UV spectrum of M-2 was in essential agreement with that of 7,8-dihydro-7,8-dihydroxy-BP reported by Waterfall and Sims (33).

K-Region 4,5-Dihydroxy 4,5-Dihydrodiol. M-3 (Charts 1 and 2) was first detected by its radioactivity. Subsequent examination of the UV spectrum of M-3 showed it to be different from any spectrum of reported metabolites. The spectrum of M-3 was indistinguishable from the spectrum of chrysene (Chart 3) but very different from that of pyrene (Chart 3). Moreover M-3 moved on TLC in the same region as the other dihydrodihydroxy derivatives. The only positions on the BP that could be hydroxylated to give a diol and yield a chrysene-like nucleus are the 4,5 positions. If metabolism occurred at other positions the chrysene nucleus would no longer be intact or if it occurred at the 7,8 or 9,10 positions a pyrene-like nucleus would be expected. The M-3 metabolite is not the 11,12-dihydro-11,12-dihydroxy-BP since the formation of this diol would result in a benz(a)anthracene nucleus. The spectrum of the latter is quite different from that of chrysene or of M-3. The benz(a)anthracene exhibits peaks at about 287 and 276 nm whereas the M-3 and chrysene peaks are approximately at 273 and 263 nm. Thus, the character of the chrysene-like spectrum and the TLC evidence for a diol type compound indicates that the M-3 metabolite is 4,5-dihydroxy-4,5-dihydrobenzopyrene. Lijinsky and Zechmeister (18) reported that chemically synthesized 4,5-dihydro-BP has the UV absorption spectrum of chrysene, and Sims (26) reported that the UV spectrum of chemically synthesized *cis*-4,5-dihydroxy-BP resembled that of a chrysene. Thus our evidence and the reported literature spectra indicate that the M-3 is a K-region metabolite.

Phenol Metabolites

Chart 4 shows that the UV spectrum of M-4 is very similar to that of 3-HO-BP, but the excitation spectrum of M-4 at 560 nm of fluorescence in 1 N NaOH was slightly different from pure 3-HO-BP (Chart 5). Separating the contaminating component in this fraction from 3-HO-BP on TLC was unsuccessful. M-4 was then further purified by silica gel column chromatography. Chart 6 shows the UV spectra of the 2 compounds obtained from M-4 by column chromatography. The faster moving compound (Chart 6a) was indistinguishable from 3-HO-BP (Chart 4). The absorption maximum in the spectrum of the slower moving compound (Chart 6b) was practically indistinguishable from that of 9-HO-BP reported by Sims (27). The fluorescence spectrum analysis and excitation spectra

Table 1
R_F values on TLC of metabolites formed from BP
R_F values shown are the mean values from 5 experiments. Methods are identical to those in Chart 1.

Metabolite	<i>R_F</i> values	Fluorescence in UV	Authentic samples
M-1	0.08	Violet	
M-2	0.12	Violet	
M-3	0.18	Nondetectable	
M-4	0.48	Violet	3-HO-BP
M-5	0.66	Orange	BP-3,6-quinone
M-6	0.72	Yellow	BP-1,6-quinone
BP	0.87	Violet	BP

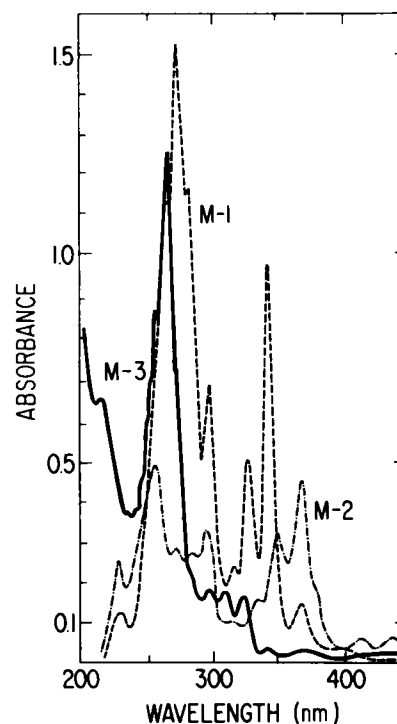


Chart 2. Uv absorption spectra of the dihydrodihydroxy products. Nonradioactive BP was incubated with microsomes from MC-treated rats by methods described in the text. The total ethyl acetate-soluble metabolites from 25 flasks were concentrated and spotted on TLC plates a 15-cm-long line, which was 3.5 cm from the bottom of the plate and developed with 5% methanol in benzene. Metabolite bands were eluted with 1 ml of ethanol. The eluate was rechromatographed by the same method described above. The main metabolite band was scraped and eluted again with 1 ml of ethanol. The ethanol solution was centrifuged for 10 min at 10,000 rpm at 0°. The solutions were analyzed spectrally in a Cary Model 14 recording spectrophotometer. - - - -, M-1; —, M-2; —, M-3.

of both compounds were not similar (Chart 7). The faster-moving compound (Chart 7A) had the character of 3-HO-BP (Chart 5) and the slower-moving compound (Chart 7b) resembled 9-HO-BP, which was reported by Sims (26) to be formed from the acid decomposition of 9,10-dihydro-9,10-dihydroxy-BP. It is thus clear that M-4 is a mixture of a relatively large proportion of 3-HO-BP and a smaller amount of 9-HO-BP.

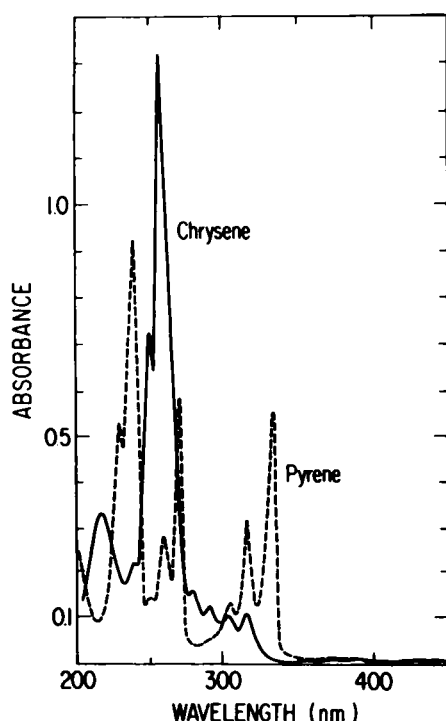


Chart 3. UV spectra of chrysene and pyrene. Chrysene and pyrene were purified by silica gel column chromatography with a mixture of hexane and benzene. An ethanol solution at a concentration of 1×10^{-5} M was measured. - - - -, pyrene; —, chrysene.

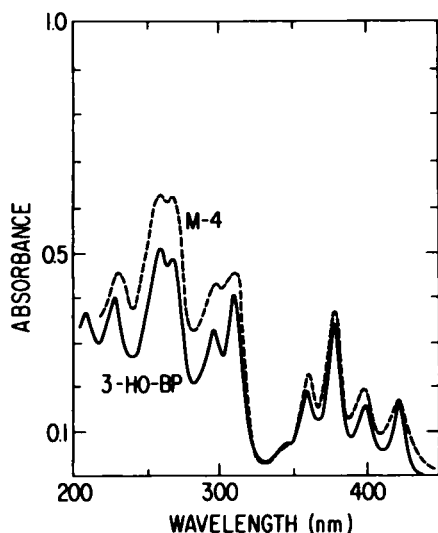


Chart 4. UV spectra of the phenol product and 3-HO-BP. The UV spectra of an ethanol solution of Fraction M-4 (....) and obtained from the experiment shown in Chart 2, that of an authentic sample of 3-HO-BP (1×10^{-5} M) (—).

BP-Quinones

The 2 products, M-5 and M-6, were indistinguishable on TLC from authentic BP-3, 6-quinone and BP-1, 6-quinone (a generous gift of Dr. Hans Falk) and gave characteristic red and yellow spots, respectively. The red M-5 is BP-3, 6-quinone and appears to be the major product.

When Are the Quinones Formed?

BP-1, 6-quinone and BP-3, 6-quinone were reported as the only recognizable products when 6-HO-BP was incubated with liver homogenates (26) and 3-HO-BP is also readily oxidized in air to the 3, 6-quinone (26). We therefore investigated the origin of the quinones that were detected on TLC. Two kinds of metabolic extracts were prepared for TLC; 1 was composed of all the metabolites that were extracted by organic solvents after the incubation,

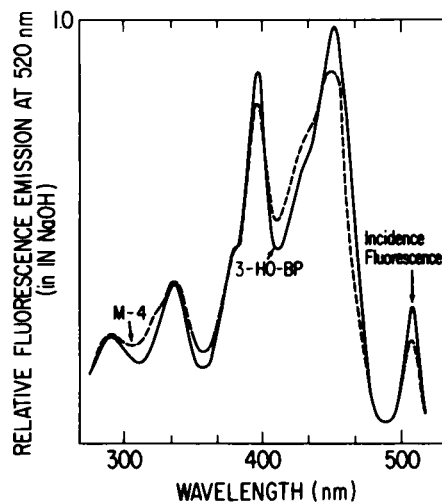


Chart 5. Excitation spectra of the phenol product and 3-HO-BP. A small amount of ethanol solution (Chart 4) was placed into 1 N NaOH aqueous solution, and the excitation spectrum at 520 nm of fluorescence was measured. - - - -, M-4; —, 3-HO-BP.

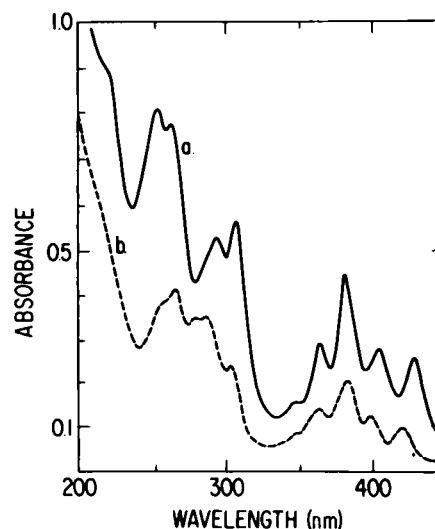


Chart 6. UV spectra of 2 compounds obtained from the phenol product. Fraction M-4 from the experiment shown in Chart 2 was concentrated under vacuum and applied to a column (5 x 500 mm) of silica gel for chromatography and developed by benzene. Two bands were inspected by UV and cut from column. Each compound was eluted by 1 ml of ethanol. The solution was centrifuges at 10,000 rpm for 10 min and the UV spectrum was measured. a (—), faster moving metabolite, probably 3-HO-BP; b (- - -), slow moving metabolite, probably 9-HO-BP.

and the 2nd was an identical extract from which the phenols were first removed by extraction with alkali. Alkali treatment of phenols may produce quinones. Chart 8 shows that the majority of the quinones detected on TLC were produced during the incubation and were not a result of treatment with the alkali. We cannot be sure, however, whether the quinones were formed by enzymatic reaction or a post-enzymatic reaction from the phenols. Further investigation is required to resolve this problem.

Differences in BP Metabolism between Liver Microsomes from Control and MC-treated Rats

The double labeling method using both BP- ^3H and BP- ^{14}C was carried out to determine possible differences in the metabolism of BP by different microsome preparations. BP- ^3H with a high specific activity was the substrate for control microsomes with low enzyme activity and BP- ^{14}C having low specific activity for microsomes with high activity from MC-treated rats. Ethyl acetate-soluble metabolites obtained from incubation of BP with either microsome preparation were mixed and developed by 2-dimensional TLC. Each area containing ^3H and ^{14}C was counted by a liquid scintillation counter. The pmoles of metabolite per 0.1 mg of protein of microsomes were calculated and drawn by a computer (Chart 9). The qualitative profile of metabolites of BP obtained from each microsomal preparation was similar. The relative quantities of each metabolite formed by the different microsomal preparations were different (Chart 9). In another experiment we examined the effect of dilution of the MC-microsomes, *i.e.*, reducing the enzyme to substrate ratio, on the pattern of metabolites formed. Table 2 shows the amount of each metabolite in 2 experiments. MC treatment or induction of the enzyme system causes a large increase in the metabolism of BP. This increase is not uniform. Table 2, Experiment 1, shows that the increase in metabolite formation was greatest for non-K-region diol

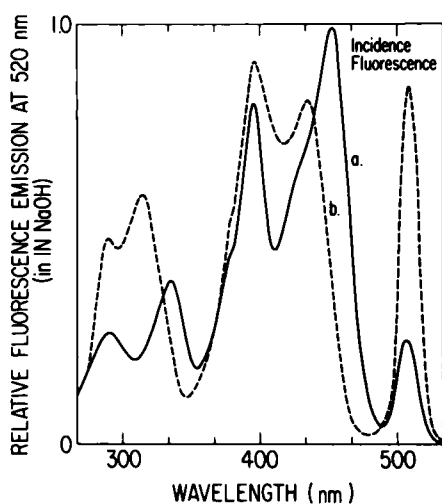


Chart 7. Excitation spectra of 2 compounds obtained from the phenol product, Fraction M-4, were measured in 1 N NaOH aqueous solution. a (—), probably 3-HO-BP; b (---), probably 9-HO-BP.

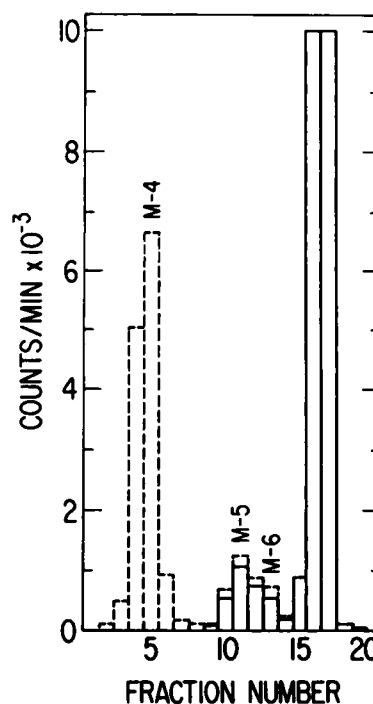


Chart 8. BP-quinones produced in incubation. Two flasks were incubated with microsomes from MC-treated rats with BP- ^3H (370 Ci/mole) as described in the text. One ml of acetone was added to each flask after 20 min of incubation and then extracted by 2 ml of hexane. The hexane layers from the 2 flasks were combined. Phenolic metabolites in 2 ml of hexane layer were extracted with 6 ml of 1 N NaOH, and the other 2 ml of hexane layer were extracted with 6 ml of water only. Each 1.5 ml of the hexane solutions was concentrated. A sample of the extracts was chromatographed using 1-dimensional TLC with 5% methanol in benzene. The region of phenols and quinones were scraped at 4-mm intervals and the radioactivity was measured, as described in the text. ---, the pattern of metabolites pretreated by water only; —, the pattern of metabolites after pretreatment by 1 N NaOH.

formation, less for phenol and K-region diol formation, and least for quinone formation. Experiment 2 shows that a reduction of enzyme activity by dilution of the microsomes results in ratios of metabolites similar to those of Experiment 1 except that phenol formation is considerably higher in the high-activity preparation. Thus it appears that high-enzyme activity in induced rats favors non-“K”-region dihydrodiol formation relative to the amount of K-region diol formed.

DISCUSSION

In 1967, Sims (26) reported that in addition to the 3-HO-BP, BP-1,6-quinone, and BP-3,6-quinone, which were well-known metabolites *in vivo* (36) and *in vitro* (27, 29), BP was also converted by rat liver homogenates into 2 previously unrecognized dihydrodihydroxy compounds at positions 1 and 2 (26, 29) and 9 and 10 (27, 29). Furthermore, he recognized that 1,2-dihydro-1,2-dihydroxy-BP yielded 1-HO-BP when treated with HCl; while 9,10-dihydro-9,10-dihydroxy-BP yielded 9-HO-BP with acid treatment (26). He therefore suggested that the 1-HO-BP, which Conney *et al.* (2) reported, might be

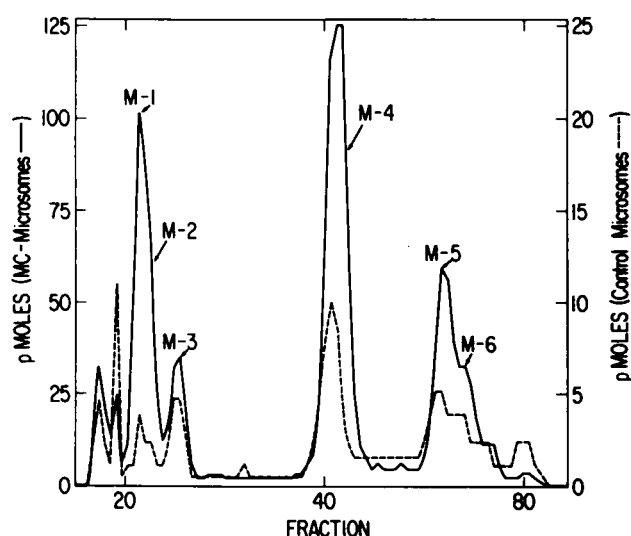


Chart 9. Metabolism of BP by microsomes from MC-treated and control rats. BP-¹⁴C (21 mCi/mmole) was incubated with MC-microsomes as described in the text, and 5.4 ml of the ethyl acetate extract obtained from 2 flasks were dried by anhydrous Na₂SO₄. BP-³H (479 mCi/mmole) was incubated with control microsomes; 5.4 ml of whole ethyl acetate extract obtained from 2 flasks were dried by anhydrous Na₂SO₄. One ml from each solution was dried and then combined. The 2 ml were reduced to about 40 μl at room temperature under vacuum. The total residue was spotted at a point 3.5 cm from the bottom and 3.5 cm from the right side on a thin-layer plate. Two-dimensional TLC was carried out as described in the text. Chromatograms were scraped at 2-mm intervals. Radioactivity ³H and ¹⁴C were counted as described in the text. The cpm obtained was converted into pmoles by computer, which was programmed for 60 cpm for ³H background and 7 cpm for ¹⁴C; 0.475 for ³H counter efficiency and 0.675 for ¹⁴C; 0.175 for the cross-over rate of ¹⁴C to ³H; 479 mCi/mmole for H metabolites and 21 mCi/mmole for ¹⁴C metabolites. Chart 9 also was made by the same computer using the scale for 100 to 125 pmoles of metabolites. —, ¹⁴C metabolites; - - - - , ³H metabolites.

produced by the dehydration of the 1,2-dihydro-1,2-dihydroxy-BP during column chromatography on silica. This possibility could also apply to the conversion of 9,10-dihydro-9,10-dihydroxy-BP into the 9-HO-BP. This phenol may correspond to the "F" metabolite reported by Weigert and Mottram (34, 35). Further studies are needed to determine the relationship between the phenols and the dihydrodihydroxy compounds. In 2 recent reports by Sims (28, 29), 9-HO-BP and the 2 quinones were not reported as BP metabolites. Our studies indicate their presence although we do not know whether they are primary metabolic products.

Our experiments clearly show the production and isolation of 6 metabolites of BP that are detected on TLC and column chromatography. The 9-HO-BP was not separable from 3-HO-BP by TLC but we found that they could be separated by column chromatography. The UV spectrum of the mixture of both phenols is indistinguishable from that of 3-HO-BP. The fluorescence spectrum analysis, however, shows slight differences. Thus it is quite possible that 9-HO-BP is a contaminant in the 3-HO-BP reported by Sims (29) since there is some tailing of 3-HO-BP on the chromatogram. Although we are not certain that quinone derivatives are direct enzymatic products, we did find that a large percentage of the quinones exists before extraction. In all experiments that we performed the quinone derivatives were detected. This is in contrast to the report of Sims (29) in which quinones were not detected although the metabolism of 11 different polycyclic aromatic hydrocarbons by rat liver homogenates and microsomes was examined.

In our experiments we failed to detect either the 1-HO-BP or the 6-HO-BP as metabolites. Conney *et al.* detected a relatively large amount of what he described as 1-HO-BP but did not find 6-HO-BP in *in vitro* experiment. *In vivo* Falk *et al.* (7) detected 6-HO-BP as a major metabo-

Table 2
Benzpyrene metabolites formed from microsomes from control and MC-treated rats

The data in Experiment 1 were obtained from that of Chart 9. The data in Experiment 2 were obtained from another experiment using the microsomes that were prepared freshly from additional animals.

Metabolite	Experiment 1						Experiment 2					
	Normal microsomes		MC-induced microsomes		MC-induced: normal		MC-induced: microsomes (diluted) ^a		MC-induced microsomes		MC-induced: diluted MC	
	pmoles ^b	%	pmoles	%	pmoles	% ^c	pmoles	%	pmoles	%	pmoles	% ^c
Total	1114	100	5383	100	4.8	1.0	1186	100	5382	100	4.5	1.0
9,10-Dihydro-9,10-dihydroxy-BP	69	6	543	10	7.9	1.7	59	5	438	8	7.4	1.6
7,8-Dihydro-7,8-dihydroxy-BP	44	4	435	8	9.9	2.0	47	4	341	6	7.3	1.5
4,5-Dihydro-4,5-dihydroxy-BP	71	6	403	7	5.7	1.2	68	6	339	6	5.0	1.0
3-HO- (and 9-HO-) BP	376	34	2158	40	5.7	1.2	389	33	2473	46	6.4	2.0
BP-3,6-quinone	132	12	616	11	4.7	0.9	133	11	578	11	4.3	1.0
BP-1,6-quinone	106	9	399	7	3.8	0.8	105	9	392	7	3.7	0.8
Unidentified	316	28	829	15	2.6	0.5	386	33	821	15	2.1	0.5

^a MC-microsomes diluted 4.5 times and thus activity reduced 4.5 times.

^b Pmoles formed per mg protein per 20 min incubation.

^c Relative ratio of metabolites with total set at 1.0.

lite but did not detect 1-HO-BP. Our experimental conditions are too dissimilar from the latter to permit a discussion of the different data obtained by those groups. Chart 8 shows that the amount of quinones did not increase after extraction of the metabolites by organic solvent from the incubation mixtures. We believe that if the 6-HO-BP is indeed produced as a metabolite, it may be converted into the quinone immediately. An interesting study of Nagata *et al.* (20) has indicated that the 6-oxo radical is produced by liver homogenates. From examination of both metabolites and authentic samples on chromatograms we have the impression that 9-HO-BP is more stable than 3-HO-BP and that 6-HO-BP is more labile than 3-HO-BP. 6-HO-BP may be one of the most unstable metabolites of BP. Quinones might indeed be direct enzyme products as well as nonenzymatic products since the 3,6-quinone was produced to a greater extent with active enzyme than with heat-inactivated enzyme in the presence of added 3-HO-BP (unpublished data).

The earlier reports of Sims (26, 28, 29) failed to detect K-region metabolites suggesting an absence of metabolism at this position. Very recently, however, the Sims group (13, 30), Raha (23), and Wang *et al.* (32) all have reported metabolites of the K region. Falk *et al.* reported the presence of a K-region metabolite *in vivo*. The UV spectrum of this metabolite, however, shows an intact BP nucleus that indicates that this compound is not the K-region 4,5-dihydro-4,5-dihydroxy-BP. When the double bond at the K region is saturated, the UV spectrum would resemble that of chrysene. One of the metabolites with the chromatographic character of a dihydrodiol we find to have the UV spectrum of chrysene (Charts 2 and 3; Table 1). We therefore believe that this is a positive identification of the metabolite as 4,5-dihydro-4,5-dihydroxy-BP.

Qualitatively, there was no difference in BP metabolism by liver microsomes from control or MC-treated rats. Sims (29) reported similar results. However, by quantitative analysis, Sims (29) reported that the relative increase in the amounts of each metabolite of BP formed from control and induced microsomes was similar. In our results, however, using the double labeling method with BP-³H and BP-¹⁴C, we found a considerable difference in the quantitative profile of metabolites produced by microsomes from control and MC-treated rats. The stimulation of the metabolism by MC-treated animals was greatest for 7,8-dihydro-7,8-dihydroxy-BP and 9,10-dihydro-9,10-dihydroxy-BP, less for phenol and "K" region derivatives, and least for quinones. This difference appears to be due in part to enzyme amount rather than enzyme character. A 5-fold dilution of the high-activity MC-microsomes yields a microsome preparation that metabolizes the benzpyrene similar to that for the control microsomes except that there was relatively less phenol formed in these low-activity microsomes.

If the K region is the site most closely joined to carcinogenicity, our results would indicate that increasing the level of enzyme by induction of the enzyme system would reduce the ratio of active carcinogen formation relative to other metabolites. Thus with high enzyme levels the

ratio of K region to other metabolites is reduced. Since induction does, however, also increase the absolute amount of K-region metabolism we are still confronted with the problem of whether induction would enhance or reduce carcinogenicity. This may be a function not only of enzyme amount but also of substrate amount and flux, tissue or cell site, subsequent enzymes in the pathway, and a host of other factors. A number of studies have indicated that aryl hydrocarbon hydroxylase is involved not only in detoxification (36) but also in the conversion of polycyclic hydrocarbons to toxic and carcinogenic metabolites (9-12, 16, 17).

Recent studies have suggested that some epoxide derivatives of the polycyclic hydrocarbons may be the proximate carcinogenic forms of polycyclic hydrocarbons. Microsomal metabolism results in epoxide formation (25). Some epoxides may bind to DNA (14) and some epoxides are more active than the parent hydrocarbon in the transformation of cells *in vitro* (15). It has been suggested that the dihydrodihydroxy derivatives may be products derived from epoxide intermediates (1). This was shown to be the case for the conversion of 1,2-epoxy-1,2,3,4-tetrahydronaphthalene to the dihydrodiol (1). It is also of interest to note the significant differences in the enzymatic (2, 7, 21, 26, 28, 29, 36) and the chemical oxidation of BP (3-5, 8, 18, 19, 24, 31).

The detection of the "K"-region metabolite of benzpyrene, a most ubiquitous carcinogenic hydrocarbon, may be of great importance in view of the "K"-region theory of polycyclic hydrocarbon carcinogenesis. An understanding of the molecular factors governing BP oxidation at various sites of the molecule may be necessary fully to understand polycyclic hydrocarbon carcinogenesis.

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