

Metabolism and Macromolecular Binding of Benzo(a)pyrene and Its Non-carcinogenic Isomer Benzo(e)pyrene in Cell Culture

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The carcinogenicity of the polycyclic aromatic hydrocarbon benzo(a)pyrene [B(a)P] is thought to result from the metabolic formation of a highly reactive intermediate, 7,8-dihydrodiol-9,10-oxy-B(a)P (Fig. 1) which subsequently interacts with cellular macromolecules to produce neoplasia (10,12,14,21,23,26,30,31). Evidence has been presented implicating similar "Bay-region" diol-epoxides as the ultimate carcinogenic forms of benz(a)anthracene (22,27), 7-methyl-benz(a)anthracene (3), dibenz(a,h)anthracene (29), and chrysene (28). Benzo(e)pyrene [B(e)P], on the other hand, is relatively inert when tested for carcinogenicity on mouse skin (16, 25) and rat trachea (24) and when tested for mutagenicity in a mammalian system (6). B(e)P contains two (equivalent) Bay regions (Fig. 1) and quantum mechanical model calculations suggest that 9,10-dihydrodiol-11,12-oxy-B(e)P can easily form a reactive bay-region-adjacent carbonium ion (11). In order to explain the noncarcinogenicity of B(e)P in the context of the bay region theory, we have studied the metabolism of B(e)P by rat liver microsomes and by cultured hamster embryo fibroblasts. In both systems, the major metabolic pathways involve the K-region of B(e)P, with little or no attack on the isolated Benzo ring. These results suggest that the lack of carcinogenicity of B(e)P may reflect its preferred mode of interaction with the cellular metabolic machinery.

MATERIALS AND METHODS

Chemicals

Tritium-labeled B(a)P (Amersham, Arlington Heights, IL, ≥ 25 Ci/mmol) was diluted with unlabeled B(a)P (Aldrich, Milwaukee, WI, gold label) to a specific activity of 3.15 Ci/mmol, and stored at -20°C dissolved in dimethylsulfoxide (DMSO), at 0.9 mM. A stock solution of tritium-labeled B(e)P (Midwest Research Institute, NCI Contract No. N01-CP-33387, 5.02 Ci/mmol) was prepared and stored similarly, but at a final specific activity of 2.51 Ci/mmol. Ultrapure guanidine HCl was obtained from Schwarz/Mann (Orangeburg, NY); density gradient grade Cs_2SO_4 was from Atomergic Chemetals (Carle Place, NY). Glucurase (a preparation of β -glucuronidase containing very low aryl sulfatase activity) was obtained from Sigma Chemical Co. (St. Louis, MO).

Cells and Labeling

Hamster embryo fibroblasts (HEF) were grown in 100 mm dishes as described (20). All experiments were performed on confluent tertiary cultures which had been plated at a 1:2 dilution three days prior to the beginning of the labeling period. Medium was replaced with 10 ml of fresh medium containing labeled PAH at a final concentration of 4 μM and incubation continued for 24 hr. The final concentration of DMSO was 0.44%, a level which gives no measurable cytotoxicity at 24 hr.

Analysis of Extracellular Metabolites

After 24 hr incubation of PAH with cells, the extracellular medium was aspirated, the cells were gently washed with 5 ml phosphate buffered saline, and the wash and medium combined. Organic solvent-soluble metabolites were prepared by twice extracting the medium with 2.5 vol ethyl acetate. The distribution of ethyl acetate-soluble metabolites within the medium was

analyzed by high-pressure liquid chromatography (HPLC) with a Spectra Physics 3500-B chromatograph fitted with a 1 m Zorbax ODS column (Dupont Inst., Wilmington, DE) and eluted with a linear 30-70% methanol and water gradient as previously described (18).

The aqueous phase was re-extracted twice with 2.5 vol ethyl acetate. Metabolites which had been conjugated to glucuronic acid were released by a 16 hr treatment at 37°C with β -glucuronidase as described previously (4). The oxygenated metabolites released from the glucuronide conjugates were extracted twice with 2.5 vol ethyl acetate and analyzed by HPLC.

Subcellular Fractionation

The washed cell monolayers were harvested after a 15 min incubation at 37°C with 0.05% trypsin, 0.02% ethylene diaminetetraacetic acid (EDTA) in phosphate buffered saline, then washed twice at 0-4°C with buffer containing 10 mM NaCl, 10 mM Tris (pH 7.0), 1.5 mM MgCl₂ and resuspended in the same buffer. The solution was adjusted to 0.5% Triton X-100 and 0.5% Na deoxycholate and lysis was completed by 10-15 strokes of a motor-driven Potter-Elvehjem homogenizer.

Nuclei were deposited by centrifugation at 1000 x g. Cytoplasmic organic solvent soluble metabolites were determined by ethyl acetate extraction of the supernatant followed by HPLC. The nuclei were freed of residual cytoplasm by repeating the homogenization step.

Isopycnic Separation

The nuclear pellet was lysed and chromatin sheared by resuspension in 6 M guanidine-HCl, 10 mM EDTA (pH 7.0) and sonication. The lysate was extracted three times at room temperature with 3 vol ethyl acetate (water-saturated), and residual ethyl acetate was removed with a stream of N₂. The volume was adjusted to 2.2 ml with the same solution and layered over a 2.8 ml cushion of 2.2 M Cs₂SO₄, 10 mM EDTA (pH 7.0), 1.26 M DMSO in a 1/2" x 2" polyallomer

tube. Centrifugation was carried out in a swinging bucket rotor (SW 50.1) at 35,000 rpm (147,000 x g max) at 20°C for 40 \pm 2 hr. The resulting gradients were fractionated by piercing the tubes near the bottom and collecting 5 or 6 drop fractions. Nucleic acids were monitored by A_{260} , proteins by reaction with fluorescamine. Density was determined by weighing 10 μ l aliquots in glass capillaries. Bound hydrocarbon was determined by liquid scintillation counting using a Searle Mark III equipped with dpm facility. Using the known specific activity, dpm were converted to pmol hydrocarbon.

RESULTS AND DISCUSSION

Microsomal Metabolism of B(e)P

In experiments carried out in collaboration with the laboratories of D. Jerina (NIH, Bethesda, MD) and W. Levin (Hoffman-La Roche, Nutley, NJ) microsomes were prepared from the livers of rats which had been pretreated with methylcholanthrene (MC), phenobarbital (PB) or left untreated. The metabolites formed from 3 H-B(e)P after incubation with the three sets of microsomes, as assayed by HPLC, are shown in Fig. 2. The mobilities of a set of synthetic standards are also indicated in the top panel. For all three sets of microsomes, the major metabolite co-chromatographed with the synthetic K-region dihydrodiol, 4,5-dihydrodiol-B(e)P. The identification of this metabolite as the K-region dihydrodiol was confirmed by UV absorption spectra (Fig. 3) and by fluorescence spectra (data not shown). Of particular interest was the absence of an identifiable peak which chromatographed with the same mobility as 9,10-dihydrodiol-B(e)P. This was true even in the incubation with MC-induced microsomes, where non-K-region metabolism should be favored (13,15).

This finding suggested the possibility of a metabolic explanation for the relative inactivity of B(e)P as a carcinogen (16,24,25). If vicinal diol-

epoxides are indeed the ultimate carcinogens, then the major metabolite of B(e)P, 4,5-dihydrodiol-B(e)P is a carcinogenic "dead-end", since formation of a vicinal diol-epoxide in the K-region is impossible. The lack of carcinogenicity of B(e)P would therefore be due to the lack of formation of 9,10-dihydrodiol-B(e)P, the obligate precursor to the bay region diol epoxide 9,10-dihydrodiol-11,12-oxy-B(e)P. However, since previous work from this laboratory (20,17) has shown that in vitro metabolism is often very different from in vivo metabolism and since the liver is not a target for PAH carcinogenesis it was necessary to further test this suggestion in a situation which more closely resembled that found in vivo.

Metabolism of B(e)P by Hamster Embryo Fibroblasts

For these studies we chose tertiary cultures of HEF. These cells contain the full array of metabolic enzymes in an intact, non-disrupted orientation and can be transformed in culture by treatment with carcinogenic PAHs (9). We incubated confluent cultures for 24 hr in medium containing ^3H -B(e)P at a concentration of 4 μM ; this concentration was chosen to minimize cytotoxicity. The time period was chosen because we had previously found that the binding of ^3H -B(a)P to nuclear DNA reached a plateau level between 18 and 24 hr of incubation (unpublished data). Ethyl acetate-soluble metabolites found in the extracellular medium are shown in Fig. 4A and those found in the cytoplasmic fraction are shown in Fig. 4B. Chromatograms of the small amount of ethyl acetate-soluble radioactivity found in the nuclear fraction (0.1-0.2% of the total input) demonstrated only unmetabolized B(e)P (data not shown). As seen previously in the microsomal incubations, the major metabolite of B(e)P formed by HEF was the K-region dihydrodiol. This metabolite was found in both the extracellular medium (Fig. 4A) and in the cytoplasm (Fig. 4B). Neither

of these fractions contained demonstrable amounts of the non-K-region, 9,10-dihydrodiol-B(e)P, confirming the microsomal data. However, since intact cell systems generally produce appreciable amounts of water-soluble metabolites, primarily glucuronide conjugates (1), it was possible that the HEF cultures formed 9,10-dihydrodiol-B(e)P but rapidly conjugated it to the corresponding glucuronide. To test this, the extracellular water-soluble metabolites were hydrolysed with β -glucuronidase and then analyzed by HPLC. As shown in Fig. 5, we detected by this method 4,5-dihydrodiol-B(e)P, and a phenolic derivative which co-chromatographs with 4-hydroxy-B(e)P but no 9,10-dihydrodiol-B(e)P. Table 1 summarizes the overall recovery of metabolites in the various fractions we have assayed. It is clear that at least in terms of the dihydrodiols, K-region metabolism is favored to the virtual exclusion of metabolism at the 9,10 position. The small amount of radioactivity (0.64 - 1.07% of total metabolites) found in the 9,10-dihydrodiol-B(e)P region of the chromatogram may be due in part to triols, tetrols and other unidentified metabolites which contaminate this region, and thus represents an upper limit to 9,10-dihydrodiol-B(e)P formation in these cells.

In contrast to this, metabolism of B(a)P by HEF produces large amounts of the non-K-region dihydrodiols, 7,8-dihydrodiol- and 9,10-dihydrodiol-B(a)P (Fig. 6), but little or no K-region dihydrodiol which in this system chromatographs at approximately fraction 40. In addition, no dihydrodiols of B(a)P are conjugated to glucuronides by HEF (Fig. 7). In terms of the phenols, it is likely that the P1 and P2 regions of the chromatogram (Fig. 4) contain a number of different phenolic metabolites of B(e)P in analogy to the clustering of the 12 monohydroxy-derivatives of B(a)P which are incompletely resolved in a single chromatographic run (7,19). It thus remains possible that HEF are capable of forming 9,10-hydroxy-B(e)P but that this compound is very unstable, isomerizing to 9-hydroxy- and/or 10-hydroxy-B(e)P before it can be converted to the dihydrodiol by epoxide hydrase.

Binding to Nuclear Macromolecules

However, 9,10-oxy-B(e)P would be expected to have reasonably good alkylating ability as would the K-region oxide, 4,5-oxy-B(e)P. Considering the relative inactivity of B(e)P as a carcinogen it was of interest to determine if this was correlated with a low macromolecular binding. We chose to concentrate on nuclear macromolecules which we prepared by a novel method involving denaturation in 6 M guanidine followed by isopycnic separation in Cs_2SO_4 . To demonstrate the separation which could be obtained with this method nuclei were labeled with specific precursors of RNA, DNA and protein and then subjected to isopycnic centrifugation as described in Materials and Methods. In Fig. 8A, the distribution of radioactivity after labeling RNA with ^3H -uridine and DNA with ^{14}C -thymidine is shown. The labeled RNA and DNA separated into 2 bands at densities of 1.65 g/cc and 1.44 g/cc, respectively; these bands coincided with peaks of A_{260} (data not shown). Less than 2% of the ^3H -radioactivity was found in the DNA band and less than 0.1% of the ^{14}C -radioactivity was found in the RNA band. Figure 8B shows the distribution of radioactivity after labeling protein with ^3H -leucine and DNA with ^{14}C -thymidine. The ^3H -leucine was found in a broad band with densities less than about 1.30 g/cc. Determination of protein by reaction with fluorescamine yielded a profile which paralleled the radioactivity (data not shown) and similar results were obtained when calf thymus histones (Sigma, St. Louis, MO) were applied to an isopycnic gradient. Less than 1% of the ^3H -radioactivity was found in the DNA band and less than 2% of the ^{14}C -radioactivity was found in the protein band.

In Fig. 9, nuclei from cells labeled with ^3H -B(a)P (Fig. 9A), or with ^3H -B(e)P (Fig. 9B) were analyzed on isopycnic gradients. To permit comparisons, a fivefold scale difference has been introduced between the left and right sides of each panel. With B(a)P (Fig. 9A) large peaks of radioactivity were found to band coincidentally with the RNA, DNA and protein bands. With B(e)P (Fig. 9B) the hydrocarbon peaks were much more modest, but are consistently found in coincidence with the macromolecular bands. Table 2 gives the calculated specific activities attained in 3 experiments with B(e)P. For comparison, the average specific activities attained in a series of experiments with B(a)P were: RNA, 55.9 pmol/mg; DNA, 40.4 pmol/mg; protein, 169 pmol/mg. Thus, the binding of B(e)P to nuclear macromolecules is 80-150-fold lower than the binding of B(a)P.

SUMMARY

In summary, a plausible explanation for the difference in carcinogenicity between B(a)P and B(e)P is to be found in their respective metabolic fates in either microsomal incubations or HEF. With B(a)P the major site of metabolic attack is the isolated benzo ring, resulting in the formation of the non-K-region dihydrodiols, 7,8-dihydrodiol-B(a)P and 9,10-dihydrodiol-B(a)P. In particular, the 7,8-dihydrodiol, which is the precursor for the presumed ultimate carcinogen 7,8-dihydrodiol-9,10-oxy-B(a)P, is not conjugated to the corresponding glucuronide but is selectively retained by the cells (MacLeod, Cohen and Selkirk, submitted for publication). This favors formation of the bay region diol epoxide and correlates with a relatively high level of binding to nuclear macromolecules. With B(e)P, the major attack is the K-region, producing 4,5-dihydrodiol-B(e)P which is found intracellularly, extracellularly and as the glucuronide conjugate. It has not been possible to demonstrate formation of 9,10-dihydrodiol-B(e)P in any of the fractions examined. This absence of the precursor for bay region diol epoxide formation correlates with a very low level of binding of B(e)P to nuclear macromolecules.

Our data are in accord with predictions of the bay region theory of chemical carcinogenesis and reduce the importance of K-region derivatives in the induction of neoplasia. The targets for reaction with the diol epoxide of B(a)P which are important in the production of cancer remain unknown as do the various steps in this pathway. We expect that continued study of a variety of non-carcinogenic PAHs will provide us with cases in which the "block" occurs at later steps in this pathway. This will allow a dissection of this multi-step process in a manner analogous to that used so successfully in elucidating cellular metabolic pathways by biochemical genetics (2,5,8).

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TABLE 1. DISTRIBUTION OF METABOLITES OF B(e)P FORMED BY HEF.

		% of Total Metabolites			
		Extracellular Organic Soluble	Extracellular Glucuronide	Intracellular Organic Soluble	Total
Expt. 1 -	4,5-dihydrodiol	22.5	2.64	0.14	25.2
	9,10-dihydrodiol	1.07	nd ^a	nd	1.07
	monohydroxy compounds ^b	7.09	15.7	1.04	23.8
Expt. 2	4,5-dihydrodiol	23.9	7.35	1.10	32.4
	9,10-dihydrodiol	0.63	nd	0.01	0.64
	monohydroxy compounds	3.91	9.63	1.06	14.6

^and - not detectable

^bregions of the appropriate chromatograms corresponding to regions P1 and P2 in Fig. 2 were summed.

TABLE 2. Binding of ^3H -B(e)P to Nuclear Macromolecules

	Specific Activity (pmol/mg)		
	RNA	DNA	Protein
Experiment 1	0.16	0.22	1.94
Experiment 2	0.20	0.19	1.63
Experiment 3	0.49	0.30	2.32
Average	0.28	0.24	1.96

FIGURE LEGENDS

Fig. 1 Structures of B(a)P and B(e)P, showing the locations of the K-regions and bay-regions.

Fig. 2. Pattern of B(e)P metabolites formed in incubations with liver microsomes from rats which were (A) untreated; (B) pretreated for three days with phenobarbital (75 ng/kg/day); or (C) pretreated for three days with methylcholanthrene (25 ng/kg/day). In each case, 100 nmol 3 H-B(e)P were incubated 10 min at 37° with from 0.1 to 1.0 mg microsomal protein in a total volume of 2.0 ml of a solution containing 0.1 M sodium phosphate (pH 7.4), 3 mM $MgCl_2$, 0.5 mM NADPH and 5% acetone. Reactions were terminated by extraction with 2.0 ml acetone and 4.0 ml ethyl acetate and organic soluble metabolites were analyzed by HPLC. The relative mobilities of a series of synthetic standards are also shown in panel (A) and the regions of the chromatogram which contain the various metabolites are designated in panel (C) as D1, D2, D3, Q, P1 and P2.

Fig. 3. Comparison of the ultraviolet absorbance spectra of (A) synthetic 4,5-dihydro-B(e)P and (B) the metabolite isolated from the chromatograph in region D2. Spectra were recorded with a Cary 118-C spectrophotometer.

Fig. 4. Pattern of B(e)P metabolites formed in a 24 hr incubation with HEF and found (A) in the extracellular medium or (B) in the cytoplasmic fraction.

Fig. 5. Identification by HPLC of B(e)P metabolites conjugated to glucuronides after a 24 hr incubation with HEF.

Fig. 6. Pattern of B(a)P metabolites formed in a 24 hr incubation with HEF and found (A) in the extracellular medium, or (B) in the cytoplasmic fraction.

Fig. 7. Identification by HPLC of B(a)P metabolites conjugated to glucuronides after a 24 hr incubation with HEF.

Fig. 8. Separation of nuclear macromolecules by isopycnic centrifugation in Cs_2SO_4 , guanidine-HCl gradients.

Fig. 9. Isopycnic separation of nuclear macromolecules labeled in culture with (A) ^3H -B(a)P or (B) ^3H -B(e)P. Note the fivefold differences in the size of the aliquots of each fraction which were analyzed from the top (right) and bottom (left) of the gradients.

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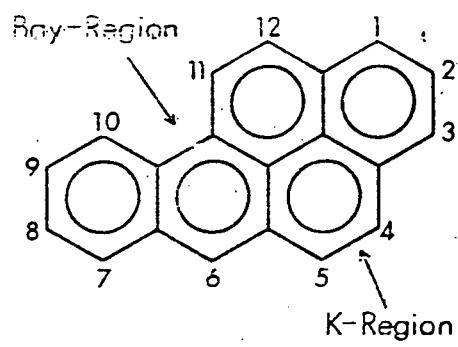
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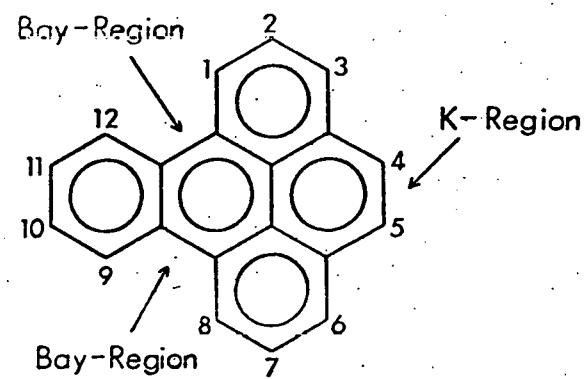
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FIGURE 1



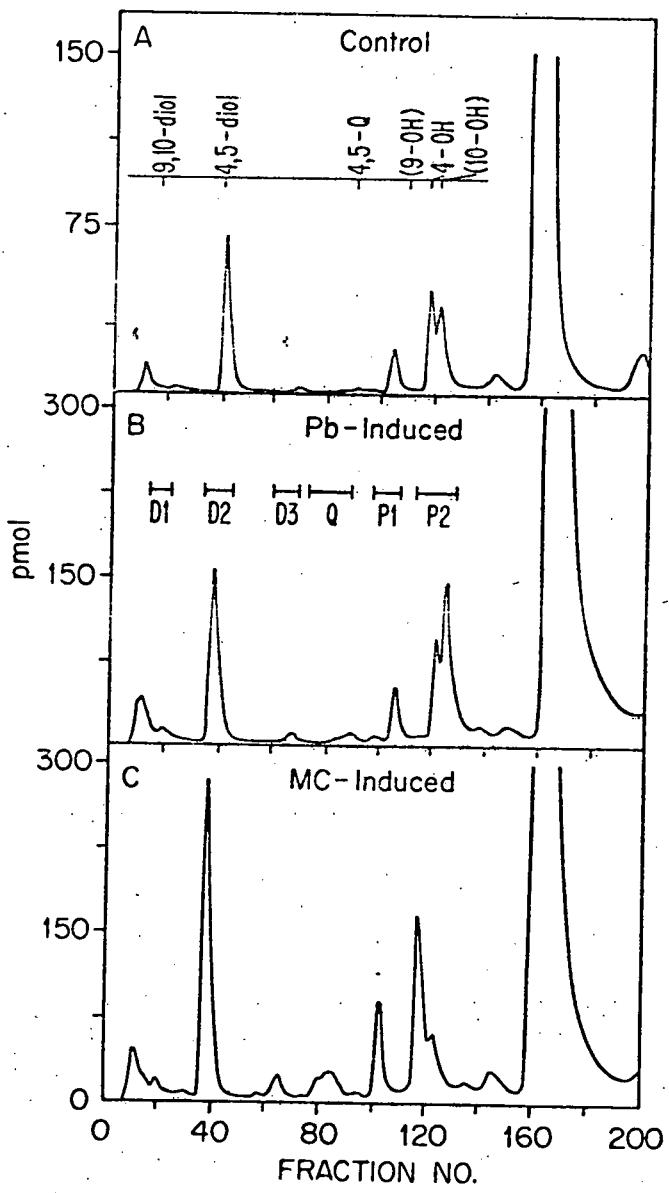
Benzo(a)pyrene



Benzo(e)pyrene

20

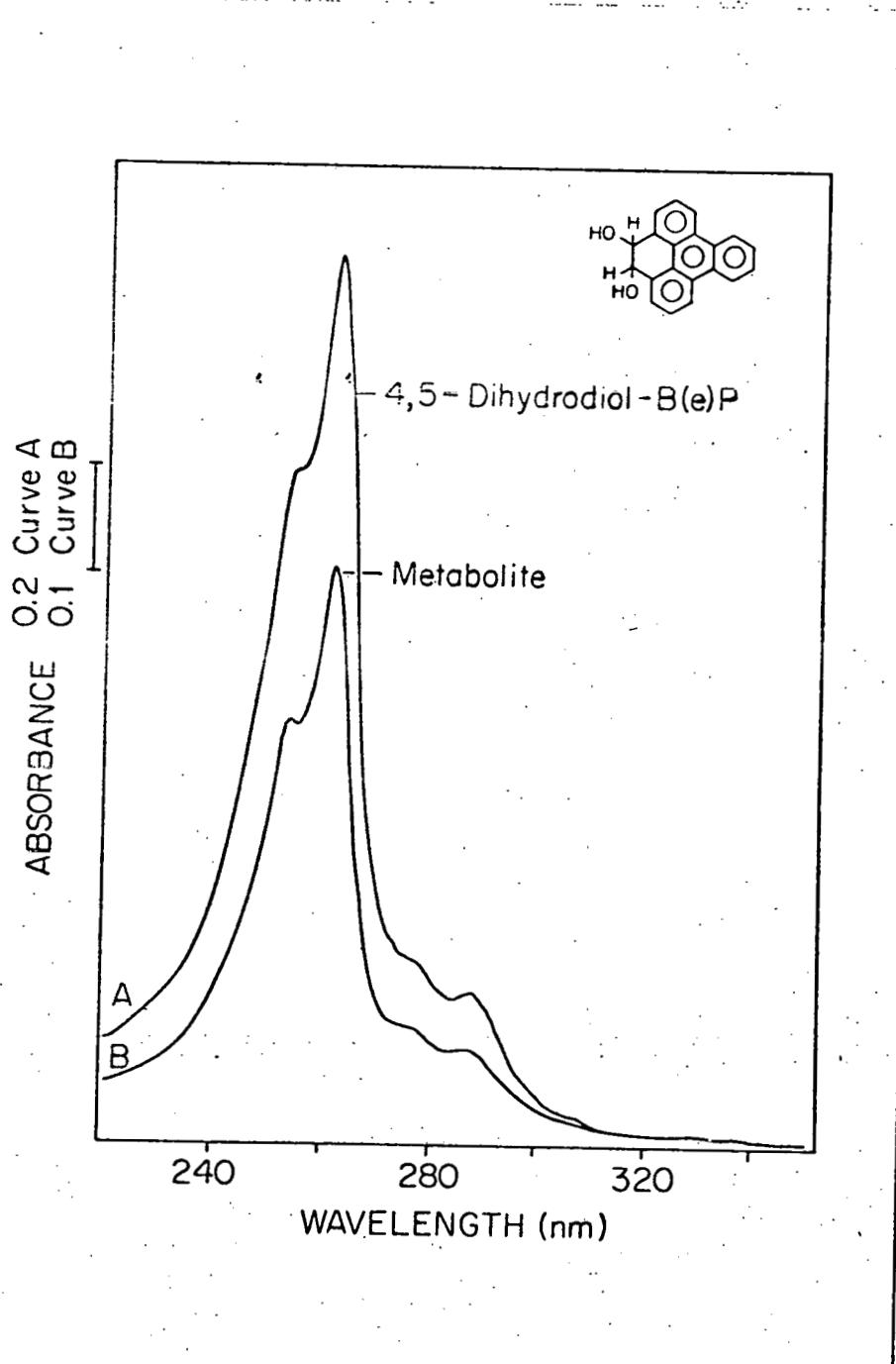
FIGURE 2



2

21

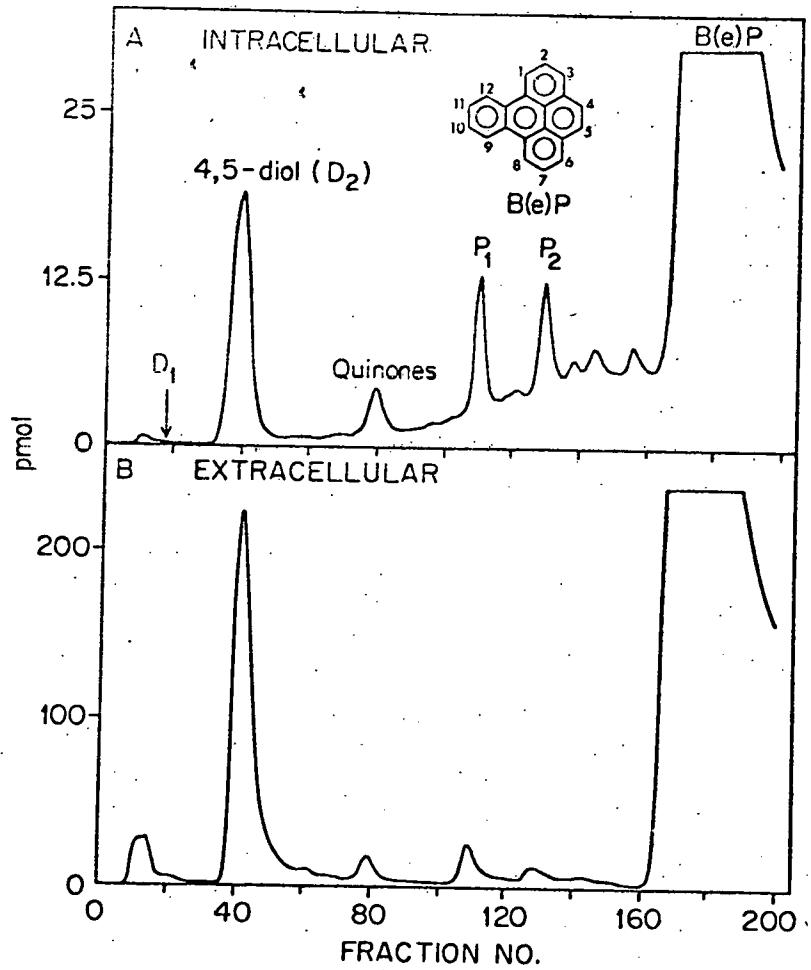
FIGURE 3



3

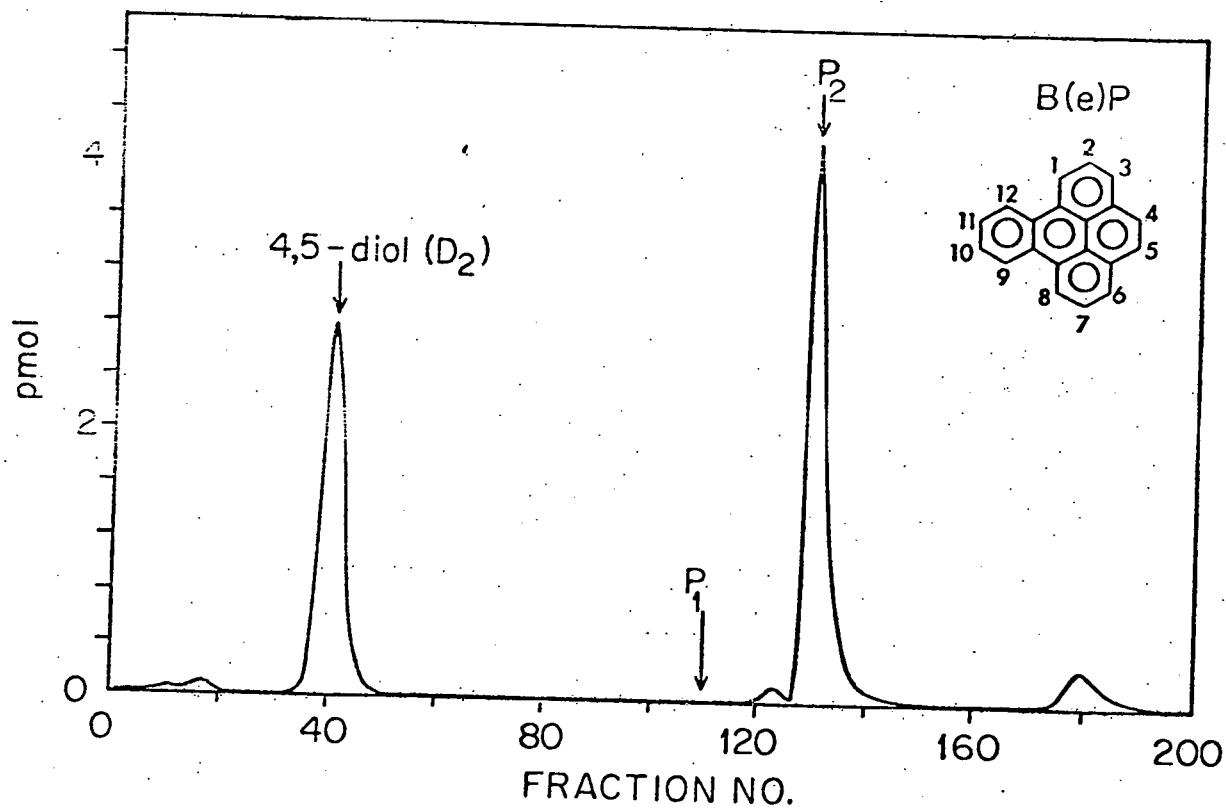
22

FIGURE 4



23

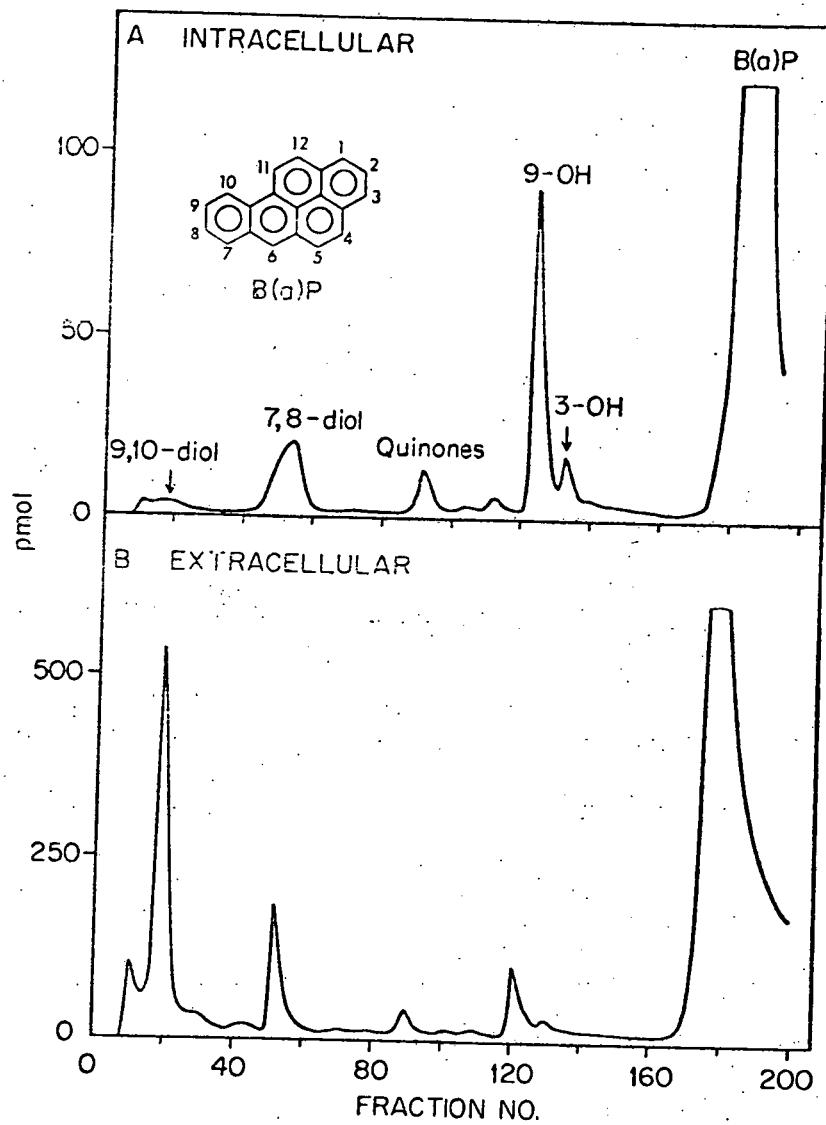
FIGURE 5

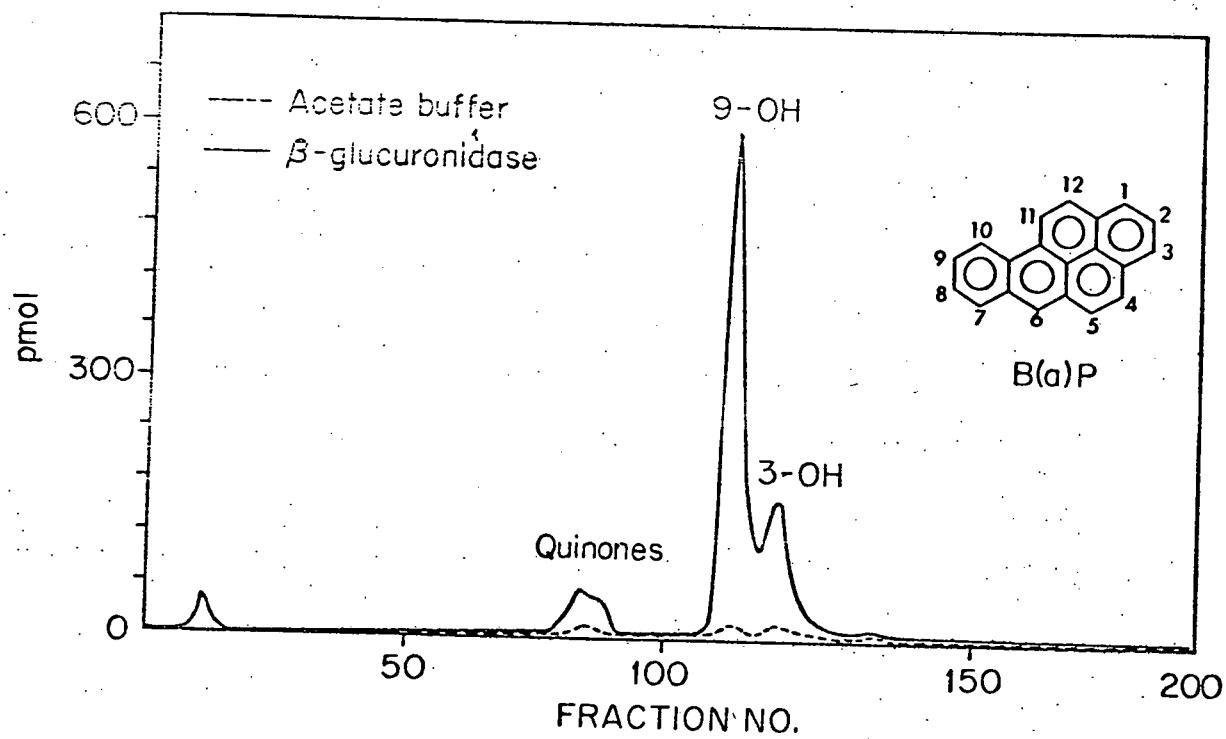


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24

FIGURE 6





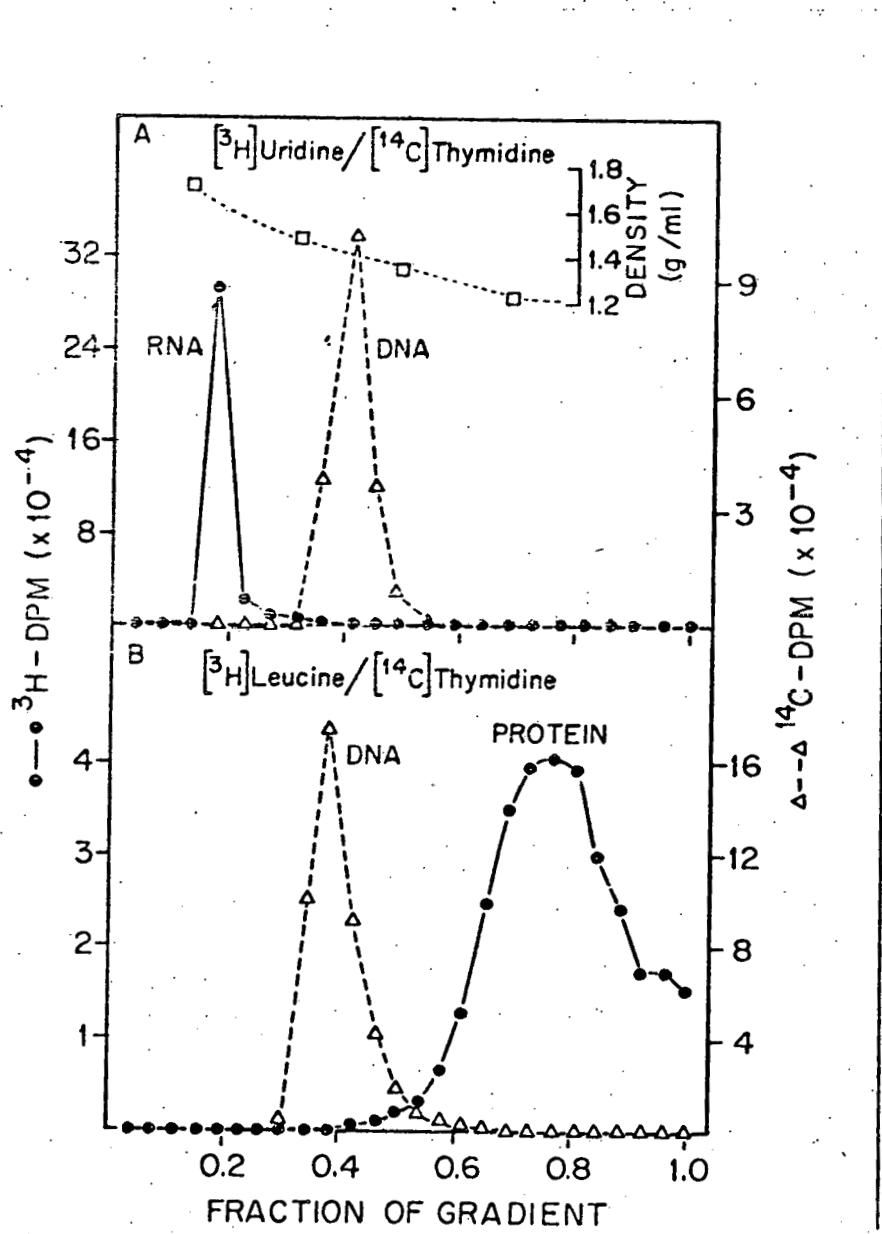
26
FIGURE 8

FIGURE 9

