

The Metabolism of 3,4-Benzpyrene in Mice and Rats

II. The Identification of the Isolated Products as 8-Hydroxy-3,4-Benzpyrene and 3,4-Benzpyrene-5,8-Quinone*

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The present work deals with the identification of the hydroxy and quinone derivatives of 3,4-benzpyrene, extracted from mouse and rat feces, as described in the preceding paper (1). In view of the labile nature of the hydroxy compound, BPX, and for other technical reasons, direct comparisons between these products and synthetic compounds were not actively pursued. Instead, the products from the feces and some synthetic derivatives of benzpyrene were converted into methoxy and other derivatives, and comparisons were made of their crystallographic character and their fluorescence and absorption spectra.

SYNTHETIC CONVERSION OF THE ISOLATED PRODUCTS INTO OTHER DERIVATIVES

Hydroxy derivatives.—Attempts were made to convert the red quinone derivative isolated from the feces (1) into a hydroxy derivative, by reduction with Zn and H₂SO₄. A greenish yellow fluorescent product was obtained, which was strongly adsorbed on alumina. This could not be isolated, however, owing to its great instability in air, especially in the alkaline state, when it was almost instantaneously reconverted into the red compound. The same occurred on reduction of the mixture of synthetic 5,8- and 5,10-quinones, prepared from benzpyrene by the method of Vollmann and his collaborators (6). Catalytic hydrogenation (with Adams' catalyst and H₂ at atmospheric pressure) yielded similar unstable fluorescent products.

These results were compatible with the view that, on reduction, both the red product from the feces and the synthetic quinones were converted into dihydroxy derivatives of benzpyrene.

Acetoxy derivatives.—Reduction of the red product from the feces and of the synthetic quinones in the

presence of acetic anhydride and pyridine, yielded the more stable acetoxy compounds, but these also were not sufficiently stable to provide good yields of pure crystalline products with the small amounts of material available. Sufficient amounts of the crude products were obtained, however, for spectrographic analysis of their fluorescence.

Methoxy derivatives.—The production of methoxy derivatives of benzpyrene by methylation of the metabolic hydroxy compound (BPX), and by reductive methylation of the red product from the feces and the synthetic quinones, proved to be the most satisfactory approach in the elucidation of the structures of the substances concerned, the derivatives being fairly stable and yielding readily crystalline products.

For the methylation of BPX, the crude fluorescent fraction from the feces, at the stage after evaporation *in vacuo* of the methanolic extract (1), was dissolved in excess of dimethyl sulfate, and heated with concentrated aqueous NaOH for a short time; then it was well cooled and treated with methanol to facilitate the destruction of the excess of dimethyl sulfate by the alkali present. After further heating to complete the reaction, the mixture was diluted with water and the product transferred to benzene. The benzene extract was washed repeatedly with water, dried with anhydrous Na₂SO₄, and passed through a column of alumina. The methoxy compound passed through into the eluate, while unchanged BPX and other impurities remained adsorbed.

The benzene eluate exhibited an intense blue fluorescence similar to that of BPX but, unlike the latter, the fluorescence remained unchanged on addition of alkali.

The solution was evaporated to dryness and sublimed in high vacuum. The main fraction distilled at about 110° C., and this was recrystallized from amyl acetate. A second fraction, coming over at about 150° C., was present in too small an amount for crystallization.

For reductive methylation of the red product from

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the feces, the substance was dissolved in dimethyl sulfate and boiled with concentrated aqueous NaOH and powdered zinc, until the mixture changed to a greenish yellow color, care being taken that sufficient NaOH was always present to maintain an alkaline reaction. The subsequent procedure was the same as in the case of the methylation of BPX, and on sublimation in high vacuum the greater part came over at about 150° C. (From subsequent comparisons it became clear that the second fraction of the methylated BPX, mentioned previously, was identical with the present product, its presence being due to a slow transformation of BPX into the quinone during the process of purification.)

The mixture of 5,8- and 5,10-quinones, obtained synthetically by the oxidation of benzpyrene with chromic acid (6), was treated in the same way as the red product from the feces, and on sublimation in high vacuum of the resulting methoxy compounds, two fractions were obtained—one at about 100° C. and the other at about 150° C. Both were further purified by repeated crystallization. Subsequently small samples of the individual quinones (5,8- and 5,10- respectively) became available,¹ and these were separately converted into the corresponding methoxy compounds. By this means it became possible to establish that the fraction of the mixture coming over at about 150° C. was mainly the 5,8- derivative, and the one at about 100° C. the 5,10- derivative.

ANALYSIS

Samples of the methoxy compounds derived from the methylation of BPX and reductive methylation of the mixture of synthetic quinones, were submitted for quantitative analysis in order to determine the number of methoxy groups present.

Analysis (Strauss and Weiler).—

Methoxybenzpyrene from BPX (major fraction coming over at about 110° C.)

Sample 1 = 9.88 per cent methoxy

Sample 2 = 8.02 “ “ “

(Monomethoxybenzpyrene requires 11.0 per cent methoxy.)

Methoxybenzpyrene from the mixed synthetic quinones

Sample 1 = 17.1 per cent methoxy

Sample 2 = 18.5 “ “ “

(Dimethoxybenzpyrene requires 19.9 per cent methoxy.)

¹ The separate samples of 5,8- and 5,10-quinones were kindly supplied to us by Dr. F. L. Warren, of the Chester Beatty Research Institute of the Royal Cancer Hospital. They were prepared by oxidation of benzpyrene with chromic acid, and separated through their acetyl derivatives, according to the method of Vollmann and his collaborators (6).

FLUORESCENCE SPECTRA

The distinctive fluorescence spectra of benzpyrene and of many of its derivatives can be of considerable help in distinguishing the various compounds, even in the presence of many impurities.

In the present work a semi-quantitative method of determining relative fluorescence intensity distribution in the wave length region 3,800–5,400 Å afforded a rapid and sensitive means of characterizing benzpyrene and its derivatives. A series of photographs of the fluorescence spectrum of the test substance in solution of graded concentration enabled curves of approximate intensity distribution to be drawn. The method provides a much better representation than can be got from a single exposure. Examples of such curves are given in Fig. 1.

The results obtained from a study of the fluorescence spectra of the different products and derivatives in benzene may be summarized as follows:

1. BPX (Fig. 1, a) gave three major bands, the strongest being preceded by some fluorescence around 4,150–4,250 Å. In addition, two bands of variable intensity were observed, one at about 3,900 Å and the other in the region of 5,400 Å. The former may possibly have been due to impurities; the latter (probably extending further towards the long wave length when photographed on plates more sensitive to red) was weak when the BPX was dissolved in a neutral solvent (benzene) and very intense when in the presence of alkali (*e.g.* in benzene-methanol-ammonia mixture). This would account for the change in naked eye fluorescence from blue to green on addition of alkali.

2. The fluorescence bands of methylated BPX were practically identical with those of BPX itself in benzene, except that the band at the long wave length did not increase in intensity on addition of alkali.

3. In the case of the dimethoxybenzpyrenes, obtained by reductive methylation of the synthetic quinones, the bands for the 5,8- were different from those of the 5,10- (Fig. 1, b and c), while those of the mixture appeared as a composite picture of the two (Fig. 1, d).

4. The bands of the methoxy compound obtained by reductive methylation of the red product from the feces (Fig. 1, e) were indistinguishable from those of the synthetic 5,8- compound (Fig. 1, b).

5. The methoxy compound obtained by oxidizing BPX to the quinone followed by reductive methylation, exhibited bands which were similar to (b) and (e) rather than to (d) of Fig. 1. It is clear from this that the oxidation product was a single quinone (5,8-) and not a mixture.

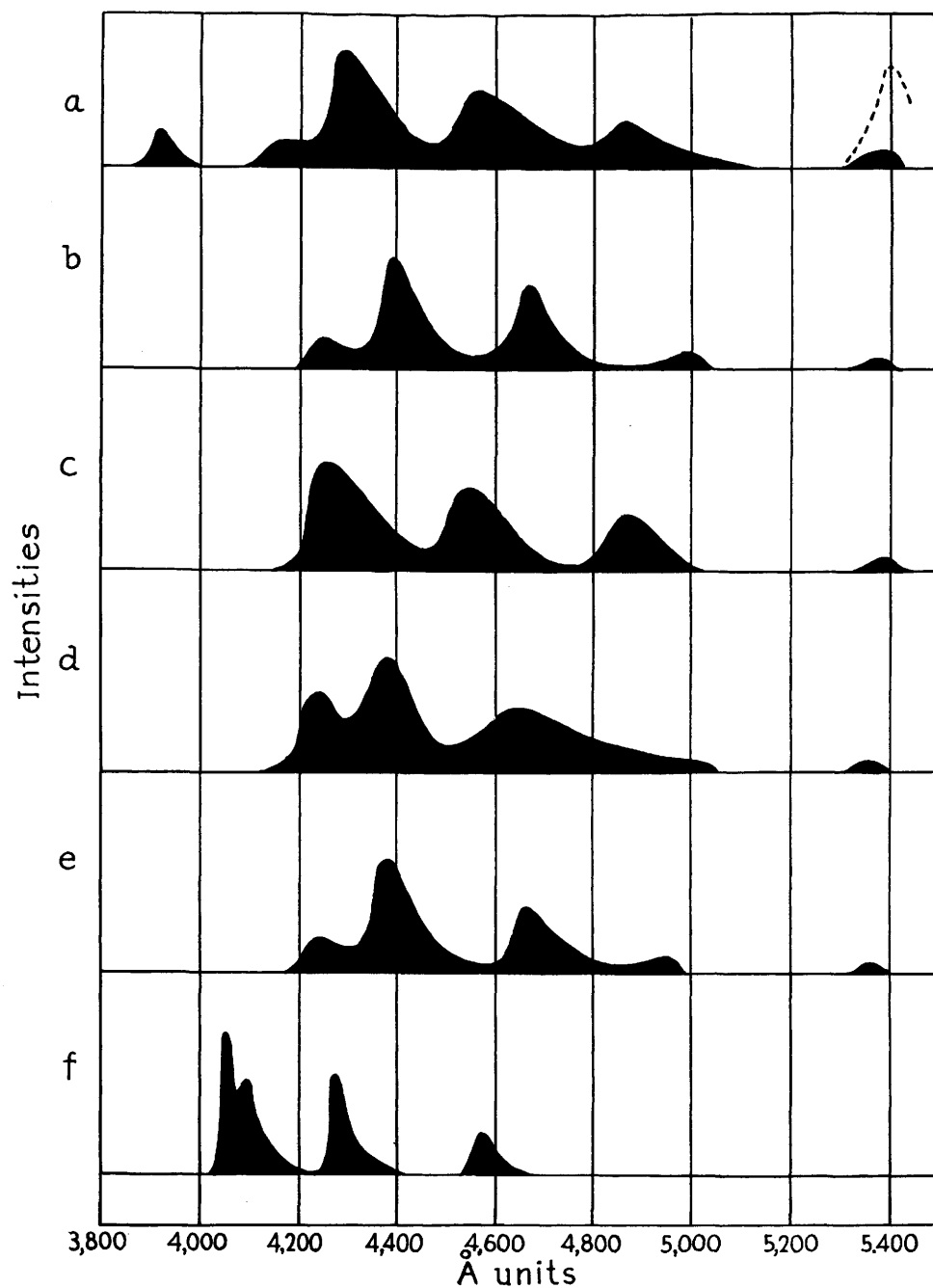


FIG. 1.—Semi-quantitative representations of fluorescence intensity distribution.
 a = Metabolic hydroxy derivative (BPX).
 b = Synthetic 5,8-dimethoxy-3,4-benzpyrene.
 c = Synthetic 5,10-dimethoxy-3,4-benzpyrene.
 d = Mixture of synthetic 5,8- and 5,10-dimethoxy-3,4-benzpyrene.
 e = Reductive methylation product of red substance from the feces.
 f = Benzpyrene.

ABSORPTION SPECTRA

Preliminary investigations were made with the method previously described by one of the authors (5). This method was found useful for rapidly comparing absorption band systems of a crude mixture with those of purified reference substances. When purified products were ultimately obtained from the crude mixtures, final checking was made by comparing plots of absorption curves obtained with the

benzpyrene (BPX) in both ethanol and hexane. In Fig. 2, the effect of methylation of the metabolic hydroxybenzpyrene on the absorption spectrum is shown.

From Figs. 2 and 3 it can be seen that definition of absorption band systems increases from the hydroxy in ethanol, to hydroxy in hexane, to methoxy in hexane. This is an added reason for using the methoxy derivative in hexane as a standard reference. The low solubility of these compounds in hexane has been given as a reason for not using this solvent (4). If, however,

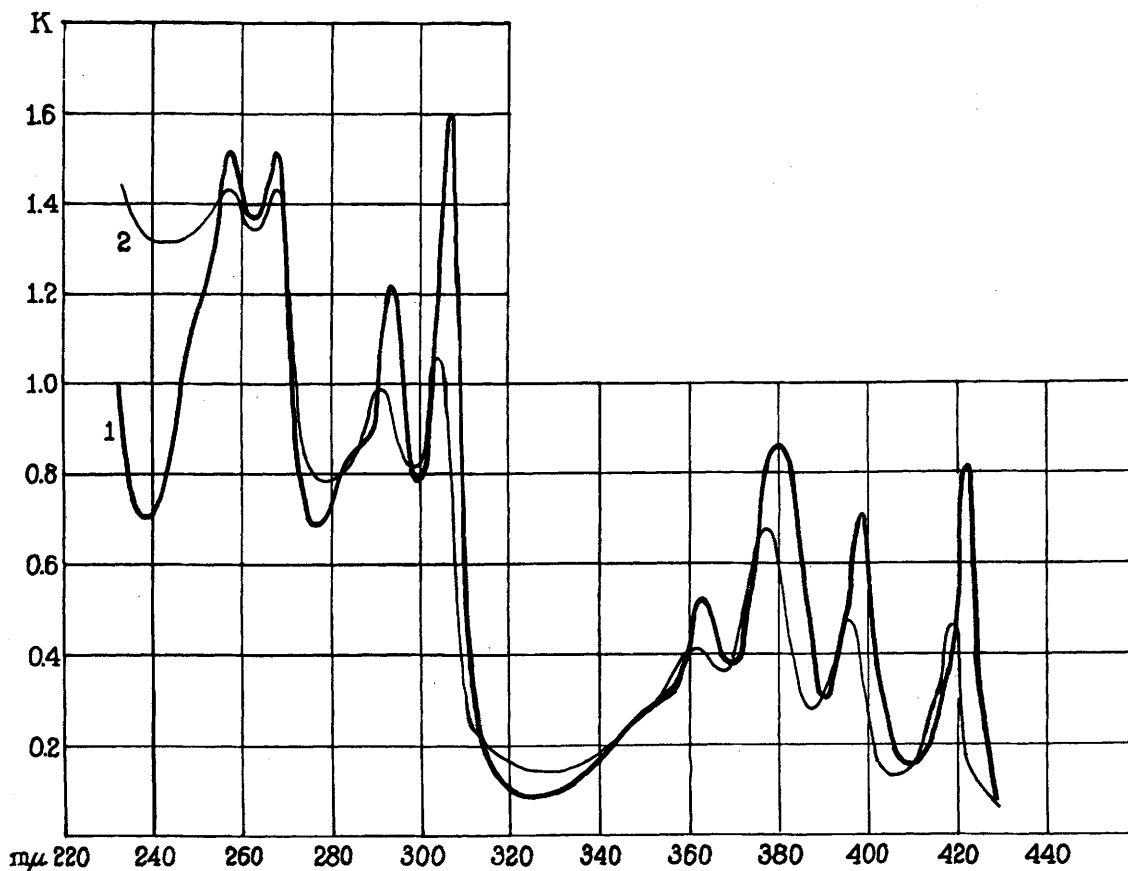


FIG. 2.—Absorption spectra.
1. Methoxy derivative of BPX in hexane.
2. BPX in hexane.

Spekker photometer. The spectrograph was a Hilger A 316 with flat focal plane adaptation. The cells used were 5 cm. long.

Identity of absorption of the methoxy compound derived from the red product from the feces, with that of synthetic 5,8-methoxy-3,4-benzpyrene, is shown in Fig. 4. By contrast, the nonidentity with 5,10-dimethoxy-3,4-benzpyrene is shown in the same figure. In Fig. 3 is reproduced the curve of 5-hydroxy-3,4-benzpyrene in ethanol, transformed from the paper of Fieser and Hershberg (4). This obviously is signally different from that of the metabolic hydroxy-

a long cell is used (5 to 10 cm.) very low dilutions, of the order of 1×10^{-6} molar, may be accurately measured in the case of unsaturated polycyclic hydrocarbons.

CRYSTALLOGRAPHIC MEASUREMENTS

Crystallographic measurements were made on several preparations of different degrees of purity of crystalline methylated BPX and also of the methoxy derivatives obtained by reductive methylation of both the synthetic quinones and the red product from the feces. In these, three crystallographic species only

could be distinguished: methylated BPX, 5,8-dimethoxy-3,4-benzpyrene, and 5,10-dimethoxy-3,4-benzpyrene (Table I and Fig. 5).

MORPHOLOGICAL AND OPTICAL EXAMINATION

The crystals of 5,10-dimethoxy-3,4-benzpyrene are lath-shaped plates with straight extinction, distinct in form from the crystals of 5,8-dimethoxy-3,4-benzpyrene

the reductive methylation derivative of the red product from the feces were indistinguishable from those of 5,8-dimethoxy-3,4-benzpyrene.

X-RAY CRYSTALLOGRAPHIC MEASUREMENTS

X-ray measurements were made on: (a) a sample of small good crystals of methylated BPX, and (b) crystals of 5,8-dimethoxy-3,4-benzpyrene isolated from

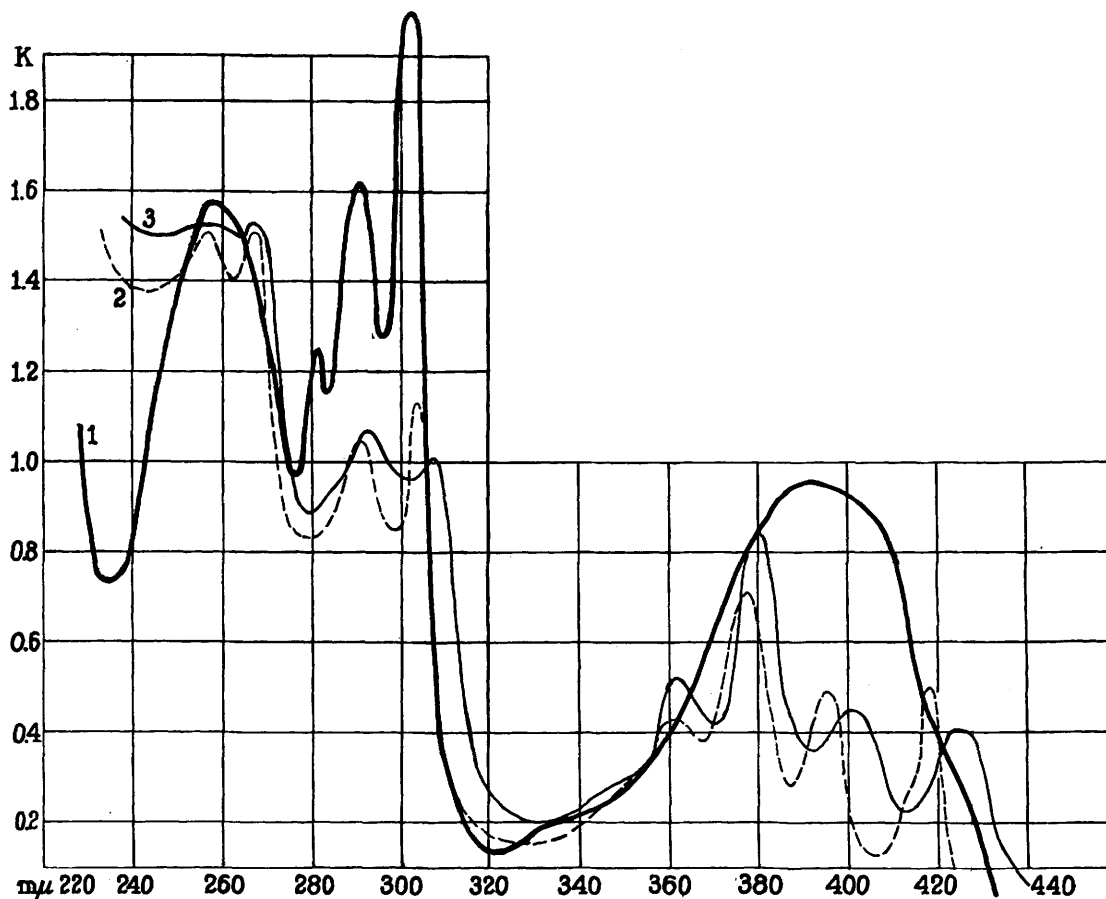


FIG. 3.—Absorption spectra.
1. 5-Hydroxybenzpyrene in ethanol (Fieser).
2. BPX in hexane.
3. BPX in ethanol.

and methylated BPX. A small proportion of crystals similar to these, and probably to be identified with them, occurred in the preparation of the 5,8- fraction from the synthetic mixture obtained by fractional sublimation.

Methylated BPX and 5,8-dimethoxybenzpyrene crystallize in fine needles, which can be distinguished by morphological and optical examination, as indicated in Table I and Fig. 5. They are, however, so similar that some doubt was felt about their nonidentity until x-ray measurements were carried out. The crystals of

the mixed methylated quinone preparation. Photographs were later obtained from the dimethoxybenzpyrene prepared from the red product of the feces, and these proved to be identical with those of the synthetic 5,8- compound.

The crystal structures of methylated BPX and 5,8-dimethoxybenzpyrene are similar and are of an interesting and complex triclinic variety. The simplest unit cell contains four molecules, but for ease of measurement the structure has been referred to a larger unit, in which the angles between crystal axes

approximate to right angles and the a and c axes are practically indistinguishable in length. The near identity in the two cell dimensions extends to the intensities of corresponding x-ray reflections, and suggests that the arrangement of the molecules in the b plane has symmetry which is destroyed in the crystal structure as a whole by staggering of the planes.

The unit cell dimensions may be given as shown in Table II. The cell dimensions are probably correct to

widen the permitted limits for the measured molecular weight. But the good agreement between $M_{calc.}$ and $M_{theor.}$ proves clearly that methylated BPX is a monomethoxybenzpyrene, while it confirms the dimethoxy character of the quinone derivative.

The fact that the crystal structures of methylated BPX and 5,8-dimethoxybenzpyrene are so similar is presumably correlated with the presence of the methoxy group in methylated BPX at the same posi-

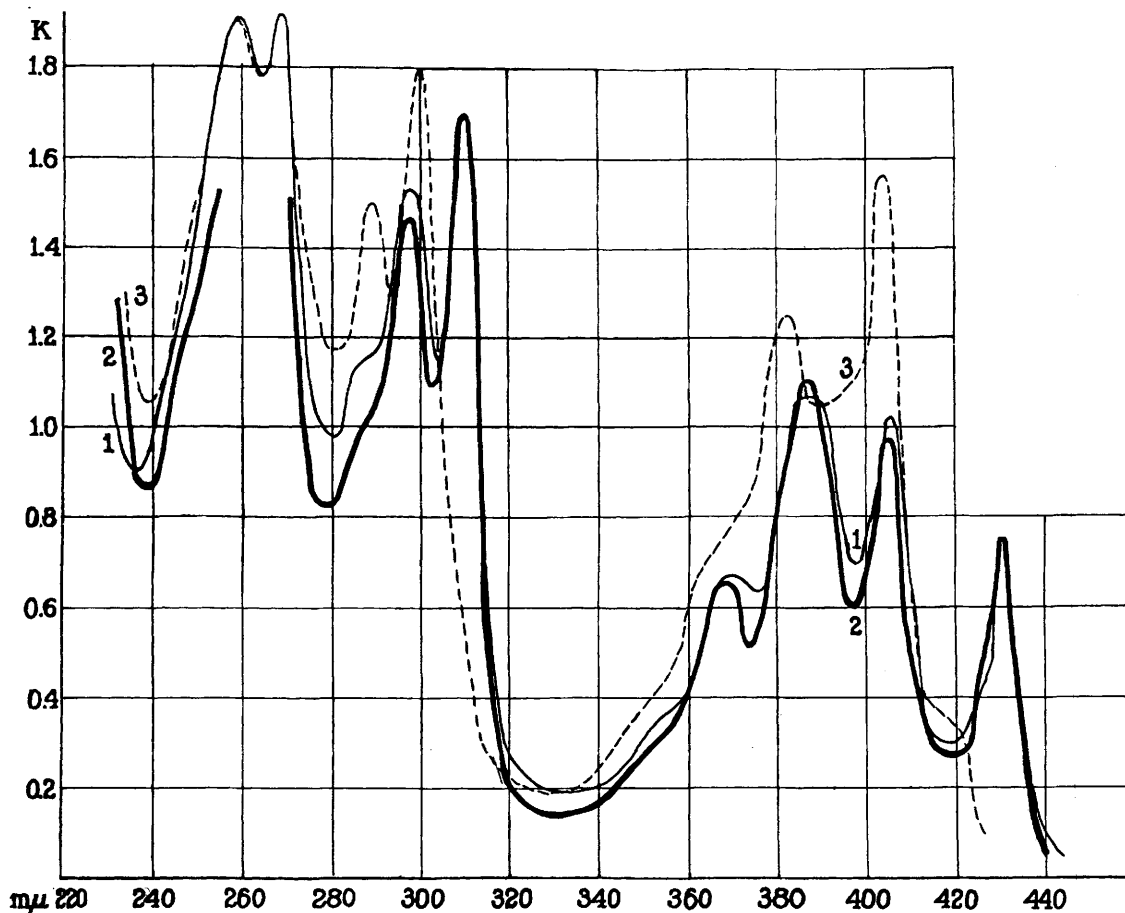


FIG. 4.—Absorption spectra.

1. Methoxy derivative of red substance from the feces.
2. 5,8-Dimethoxy-3,4-benzpyrene.
3. 5,10-Dimethoxy-3,4-benzpyrene.

1 to 2 per cent. Since in the case of methylated BPX only small quantities of material were available, the crystal densities were determined by flotation in drops of solutions of known densities under the microscope, as in the case of BPX itself (3). Three solutions, of densities 1.332, 1.361, and 1.396, were prepared and the behavior of the crystals in these liquids is indicated in Table II. $M_{calc.}$ in these figures is derived from the best values of the unit cell dimensions and the outside limits for the density. Possible experimental errors in the x-ray measurements certainly

tion as one of those in the dimethoxy compound. That this should be the 8- rather than the 5- position is in good agreement with the observations made on BPX itself (3).

DISCUSSION

The evidence of the foregoing results leads to the following conclusions: (a) the nonidentity of methylated BPX with the methoxy compounds derived from benzpyrene-quinones; (b) the identity of the methoxy compound derived from the red product from the

feces with that from the synthetic 5,8-quinone of benzpyrene; and (c) the allocation of the hydroxy group in BPX to the 8- position.

crystal structure are all different, while both the analytical data for methoxy groups and the x-ray measurements agree in establishing that the former

TABLE I

	Morphology	Optics
1. 5,10-Dimethoxybenzpyrene	Light yellow laths, probably monoclinic or orthorhombic	Extinction straight
2. 5,8-Dimethoxybenzpyrene	Yellow needles or prisms, triclinic, showing $\{100\}$, $\{001\}$; elongated along $[010]$; frequently twinned	Extinction oblique, "slow" direction, $46-50^\circ$ to needle axis
3. Methylated BPX	Yellow needles, triclinic, showing $\{100\}$, $\{001\}$; elongated along $[010]$	Extinction oblique, "slow" direction c, 42° to needle axis

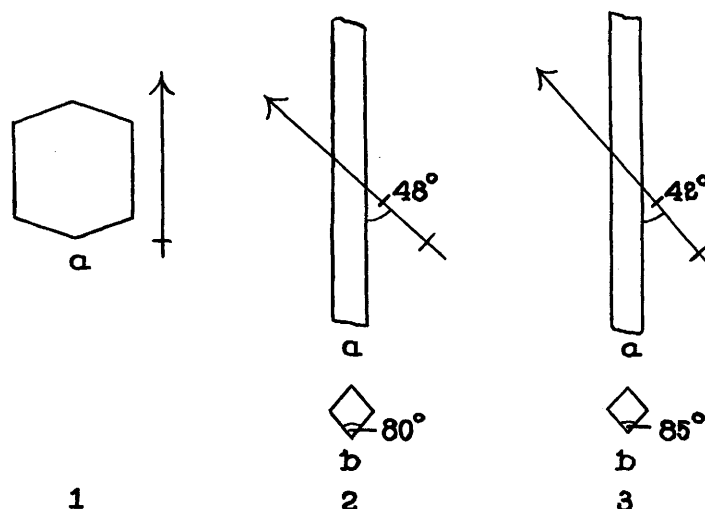


FIG. 5.—Diagram to illustrate the optic and morphological character of crystals of:

1. 5,10-Dimethoxy-3,4-benzpyrene.
2. 5,8-Dimethoxy-3,4-benzpyrene.
3. Methylated BPX.

The "slow" extinction direction in each case is shown by the arrows: (a) normal view in microscope, (b) needle cross-section.

TABLE II: UNIT CELL DIMENSIONS (X-RAY MEASUREMENTS)

	Methylated BPX	5,8-Dimethoxybenzpyrene
<i>a</i>	18.72 Å	19.0 Å
<i>c</i>	18.7 Å	19.0 Å
<i>d</i> ₍₀₁₀₎	8.01 Å	8.52 Å
β	95°	100°
	Space group $P1$ or $P\bar{1}$ <i>n</i> = 8	
ρ	1.322–1.361	1.361–1.396
<i>M</i> _{calc.}	276–286	310–318
<i>M</i> _{theor.}	282 for $C_{20}H_{11}OCH_3$	312 for $C_{20}H_{10}(OCH_3)_2$

(a) Methylated BPX clearly differs from any of the methoxy compounds derived from the synthetic quinones, since its fluorescence and absorption spectra and

is a mono- and the latter are dimethoxy derivatives of benzpyrene. From this it is evident that BPX itself is a monohydroxybenzpyrene, in confirmation of the findings of Chalmers and Crowfoot (3).

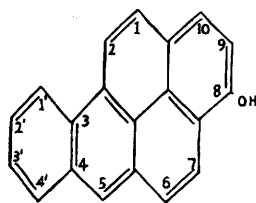
(b) The identity of the red product from the feces with 3,4-benzpyrene-5,8-quinone, already suggested by its general properties (1), is established by the identity of the fluorescence and absorption spectra and the crystal structures of their methylated derivatives, and by the demonstration that the methylated derivative of the 5,10-quinone possesses entirely different spectra and crystalline form.

(c) Since on oxidation BPX yielded a red substance which, on reductive methylation, produced a methoxy compound indistinguishable from the 5,8-methoxybenzpyrene, it may be concluded that BPX itself is either 5-hydroxy- or 8-hydroxybenzpyrene, and that

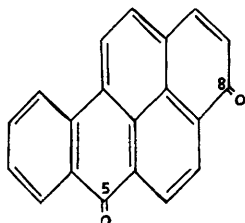
an intermediate 5,8-dihydroxybenzpyrene stage is probably involved in the conversion. (The difficulty of isolating this intermediate product is understandable, in view of the observed instability in air of the reduction products of benzpyrene-quinones.) However, 5-hydroxybenzpyrene is excluded by the pronounced difference between the absorption spectrum of synthetic 5-hydroxybenzpyrene (4) and that of BPX. Further, 5-hydroxybenzpyrene would be expected to yield on oxidation a mixture of 5,8- and 5,10-quinones but, as shown above, oxidation of BPX yields entirely 5,8-quinone. The hydroxy group in BPX is therefore allocated to the 8- position. Thus the prediction of Cason and Fieser (2), that the hydroxy group in the metabolic derivative of benzpyrene is not likely to be in the 5- position, is substantiated.

SUMMARY

A study has been made of the crystallography and the fluorescence and absorption spectra of certain derivatives of the two products of 3,4-benzpyrene iso-



8-hydroxy-3,4-benzpyrene



3,4-benzpyrene-5,8-quinone

lated from mouse and rat feces, and these data have been compared with similar data from comparable derivatives of synthetic compounds.

By these means it was possible to identify the red product with 3,4-benzpyrene-5,8-quinone, and to arrive at the conclusion that the fluorescent product BPX is 8-hydroxy-3,4-benzpyrene.

We wish to thank Mr. W. Weinstein and Mr. H. W. Wheel for valuable technical assistance.—AUTHORS.

REFERENCES

1. BERENBLUM, I., and SCHOENTAL, R. The Metabolism of 3,4-Benzpyrene in Mice and Rats. I. The Isolation of a Hydroxy and a Quinone Derivative, and a Consideration of Their Biological Significance. *Cancer Research*, **3**: 145-150. 1943.
2. CASON, J., and FIESER, L. F. Synthesis of 4',8'-Dihydroxy-1,2,5,6-Dibenzanthracene and Its Relation to Products of Metabolism of the Hydrocarbon. *J. Am. Chem. Soc.*, **62**:2681-2686. 1940.
3. CHALMERS, J. G., and CROWFOOT, D. The Elimination of 3:4-Benzpyrene from the Animal Body after Subcutaneous Injection. 2. Changed Benzpyrene. *Biochem. J.*, **35**: 1270-1275. 1941.
4. FIESER, L. F., and HERSHBERG, E. B. The Orientation of 3,4-Benzpyrene in Substitution Reactions. *J. Am. Chem. Soc.*, **61**:1565-1574. 1939.
5. HOLIDAY, E. R. A Method of Absorption Spectrography for the Detection of the Detailed Structure of Absorption Spectra. *J. Scient. Instruments*, **14**:166-172. 1937.
6. VOLLMANN, H., BECKER, H., CORELL, M., and STREECK, H. Beiträge zur Kenntniss des Pyrens und seiner Derivate. *Ann. d. Chem.*, **531**:1-159. 1937.

ADDENDUM

After this paper had been submitted for publication, direct comparisons were made between the methylated product of the metabolic hydroxy derivative of benzpyrene and 5-methoxy-3,4-benzpyrene, synthesized by the method of Fieser and Hershberg (4). The two were found to be different as regards their crystal structure and their absorption and fluorescence spectra, thus confirming the conclusions already reached.