141. THE ELIMINATION OF 3:4-BENZPYRENE FROM THE ANIMAL BODY AFTER SUBCUTANEOUS INJECTION

2. CHANGED BENZPYRENE

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AFTER the subcutaneous injection of 3:4-benzpyrene in the rat, two fluorescent elimination products have been detected in the faeces and urine: unchanged benzpyrene and a derivative of benzpyrene. In previous communications, the fluorescent derivative of benzpyrene extracted from bile was called 'BPX' and that from faeces 'BPF'. The fluorescence spectra of unpurified extracts of 'BPX' and 'BPF' showed two bands in the blue-violet region of the spectrum, but there was a slight shift in the position of the bands. However, after purification by solution in NaOH and adsorption on alumina, concentrates of 'BPX' and 'BPF' showed the same fluorescence spectrum, and it appeared that the substances were identical. The expression 'BPX' is therefore used in this communication to denote the fluorescent derivative of benzpyrene extracted from the faeces as well as from the bile.

In a quantitative experiment [Chalmers & Kirby, 1940] it was found that approximately 1% of the injected benzpyrene was eliminated unchanged in the faeces of the rat, while only a trace was eliminated in the urine. The method of fluorescence spectrum analysis used in the estimation of benzpyrene was not so suitable for the estimation of 'BPX', which shows two wide bands in the blue-violet region of the spectrum, in contrast to benzpyrene, which shows a number of sharp bands. Consequently, there is no quantitative evidence of the relative amounts of 'BPX' eliminated in the urine and faeces, but the qualitative evidence indicates that only a small fraction of the injected benzpyrene is eliminated as 'BPX', and that 'BPX' is eliminated in relatively larger amounts in the faeces than in the urine. A more detailed study of the properties of 'BPX' has been made, and the results of this work are presented here.

EXPERIMENTAL

Rats were injected subcutaneously with a 1% solution of the hydrocarbon in olive oil. The animals were kept in metabolism cages, and the faeces and urine collected separately. Most of the animals developed tumours at the site of injection and were thereupon killed. Nephritis and lung abscesses found in some of the injected animals, and at first thought to be due to the injections, were also found to a similar extent in control animals of the same stock. The livers of injected animals examined *post mortem*, appeared normal, although subsequent histological examination revealed toxic changes.

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The method of extraction of the fluorescent material from the faeces was as described by Chalmers [1940] and Chalmers & Kirby [1940]. The 'BPX' was separated from unchanged benzpyrene by solution in dilute NaOH, and by chromatographic adsorption on alumina. A concentrate of 'BPX' was sublimed at 150–160° at 0.01 mm. pressure, when a small quantity of yellow needle-shaped crystals was obtained, together with a fluid distillate. The fluid distillate, which was probably a decomposition product of 'BPX', could not be separated from the crystalline sublimate under the conditions used: a better yield of crystalline material would possibly result from the use of a higher vacuum.

Melting point of 'BPX'. Only a small quantity of 'BPX' was available for examination, and it was therefore necessary to use a micro-M.P. apparatus. The crystals on a microscope cover glass were placed on a metal disk floating on a bath of Wood's metal, and the M.P. observed through a microscope. The apparatus was tested with a number of substances of known M.P., and the values found were correct $\pm 1^\circ$. A sample of 4'-hydroxy-3:4-benzpyrene (prepared by Prof. Cook) thus examined, softened at 214° and melted sharply at 217°, in agreement with the recorded value. With substances such as 'BPX' and 6-hydroxy-3:4-benzpyrene, which sinter and decompose on melting, in order to reduce the effects of sintering on the M.P. determination the bath of Wood's metal was allowed to reach a temperature of about 20° less than the expected value before the compound was placed on the apparatus.

The M.P. of the crystals of 'BPX', 190–196° decomp., corresponded with that of 6-hydroxybenzpyrene recorded by Fieser & Johnson [1940]. A sample of their compound, however, examined in our apparatus gave a lower value, probably due to the instability of the substance. A further investigation of the M.P. of 'BPX' and 6-hydroxybenzpyrene is recorded in the section on the crystallographic examination, by one of us (D. C.), and it is shown that these substances behave differently.

Fluorescence spectrum. The fluorescence spectra of 'BPX' and the hydroxybenzpyrenes in ethanol solution are as follows:

	Fluorescence spectrum bands Å.	
'BPX'	(4250–4400) (4500–4650)	Two bands moderately well defined
6-Hydroxy-BP	(4200–4350) (4400–4650)	Two bands less well defined than 'BPX'
4'-Hydroxy-BP	(4150–4700)	One wide band

In fluorescence spectrum, therefore, as well as in M.P., 'BPX' is not identical with 6-hydroxy- or 4'-hydroxy-benzpyrene.

Solubility of 'BPX' and hydroxybenzpyrenes.

Solvent	'BPX'	6-Hydroxybenzpyrene	4'-Hydroxybenzpyrene
2N NaOH	s-cold (green fluor.-soln.)	s-cold (red fluor. soln.)	s-cold (red fluor.-soln.)
N Na ₂ CO ₃	s-cold	i-cold, s-hot	i-cold, s-hot
N NaHCO ₃	i-cold, s-hot	i-hot	i-hot

For comparison the solubilities of 'BPX' and the known hydroxybenzpyrenes are tabulated above. The solubility of the substances was indicated by the fluorescence of the solutions in the ultraviolet beam. It will be seen that 'BPX' is slightly more acidic than the other compounds, but this difference may be due to the presence of the fluid distillate, associated with the crystals examined. It is therefore likely that 'BPX' is phenolic.

*Crystallographic examination of 'BPX', 6-hydroxybenzpyrene
and 4'-hydroxybenzpyrene*

In an attempt to obtain additional evidence on the structure of 'BPX', samples of the sublimed substance were examined crystallographically. The examination was first undertaken to check the identity or otherwise of the preparation with 6-hydroxybenzpyrene and comparative observations were later made also on 4'-hydroxybenzpyrene.

All the preparations were available as very small samples of crystals. These were studied first under the microscope and subsequently X-ray measurements were made on 'BPX' alone.

Morphological and optical examination. The observations made on the optic and morphological character of the different crystals are tabulated below and further summarized by the diagram (Fig. 1). Optic figures were observed only in the case of preparations 1 and 3.

	Morphology	Optics
1. 'BPX'	Yellow laths on (001), elongated along [010]. Marked cleavage $\parallel$ [010]	Extinction straight, $\alpha \parallel$ [010], β probably $\perp$ (001)
2. 6-Hydroxybenzpyrene:		
(a) From toluene	Fine yellow needles	Extinction oblique, 'slow' direction 47° to needle axis
(b) Sublimed	Very small laths	Extinction straight, 'slow' direction $\parallel$ needle axis
3. 4'-Hydroxybenzpyrene	Yellow laths	Extinction straight, $\beta \parallel$ needle axis

It is clear from these data that the four preparations described are crystallographically different. Presumably 2(a) and 2(b) are polymorphic modifications of 6-hydroxybenzpyrene 2, of which 2(a) might contain solvent of crystallization. Since 1 and 2(b) were prepared in the same way, by sublimation, it

Fig. 1. Diagram to illustrate the optic and morphological character of crystals of (1) 'BPX'; (2a) 6-hydroxy-3:4-benzpyrene from toluene; (2b) 6-hydroxy-3:4-benzpyrene, sublimed; (3) 4'-hydroxy-3:4-benzpyrene. The extinction directions are shown by the arrows.

seemed unlikely that the 'BPX' crystals were also a polymorphic modification of 6-hydroxybenzpyrene, but to check this point mixed melting experiments were carried out under the microscope.

The melting and recrystallization were observed by using a hot wire stage [Bernal & Crowfoot, 1933]. A few crystals of 'BPX' were first melted on a cover slip by sliding this gradually towards a hot wire. On withdrawing the wire a short distance 'BPX' recrystallized in thin leafy plates, optically and morphologically identical with the original crystals. The process could be repeated with very little apparent decomposition of the preparation provided that the melting was carried out quickly and was not prolonged. The sample was then

melted and seeded with a crystal of 6-hydroxybenzpyrene, 2(a) (from toluene). 'BPX' crystals reformed on cooling and there was no growth of crystals of type 2(a).

Samples of both preparations of 6-hydroxybenzpyrene were then melted similarly. The melts rapidly discoloured and no recrystallization occurred on cooling either alone or after seeding with crystals of 'BPX'. It was clear also that the crystals melted at a lower temperature than 'BPX'.

X-ray crystallographic examination of 'BPX'. The fact that so far only a small quantity of very small crystals of 'BPX' has been available for study has limited the amount of information obtainable by the usual routine X-ray methods. It is not possible at present to attempt anything in the way of an X-ray analysis of the molecular structure, but an approximate measure of the mol. weight and some indication of the sort of molecular structure present can be gained even from the very limited observations possible.

The actual crystals used were approximately $1/10 \times 1/50 \times 1/250$ mm. in linear dimensions and were inclined to be bent. X-ray photography was therefore difficult, but photographs were obtained of three crystals with exposure times of 24 hr. for 15° oscillations. The photographs show few X-ray reflexions, and those tending to be blurred and drawn out owing to imperfections in the crystals. In the circumstances it is possible that the true unit cell is some multiple of that deduced from the present observations and that the space group indicated now is only a pseudo space group. The crystal symmetry may, in fact, not be orthorhombic but only monoclinic. But since even a determination of the pseudo cell and space group should provide useful information the measurements are recorded as follows:

Unit cell dimensions $a = 8.22 \text{ \AA}$.

$b = 4.34$.

$c = 35.58$ (or $c \sin \beta$).

Space group Pcb .

Density Between 1.375 and 1.402, nearer 1.402.

An attempt was made to measure the density of the crystals by flotation under centrifugal force in a solution of potassium mercury iodide but it proved too difficult to distinguish the very few very small crystals in the bulk of the solution. Individual crystals were therefore observed in drops of solution of known density under the microscope and their behaviour confirmed by their subsequent transfer into the solution in bulk. The limits between which the density must lie could certainly be refined by a more careful experiment, but since these limits appear close enough to fix the mol. weight the experiment was concluded.

Molecular weight. Assuming that there are four molecules in the unit cell as required by the space group found, and using the cell dimensions given above and a value for the density of 1.39, $M = 267$. This agrees well with the value 268 calculated for a monohydroxybenzpyrene.

Even allowing a probable error of 1 % on the X-ray measurements and taking the outside limits found for the density, the mol. weight may be calculated as lying between 278 and 258. These figures would exclude the possibility that the compound is a dihydroxybenzpyrene.

Molecular structure. The crystallography of the compound is in good agreement with the molecule having an aromatic structure of roughly the dimensions calculated for a derivative of benzpyrene. The b unit cell dimension is short,

4.34 Å., and since b is optically the direction of α , the least refractive index, it is reasonable to suppose that the molecules lie with the plane of the aromatic ring system approximately in the b plane. The area defined by the lengths of a and c is roughly that required by four times the area of the benzpyrene rings suitably packed together.

There is no evidence from the crystallographic data that 'BPX' is a hydroxy-benzpyrene derivative beyond the fact that the mol. weight found is of the right order of magnitude. But if there is a hydroxyl group present, the arrangement of the molecules in the crystal must almost certainly be one in which the hydroxyl groups of neighbouring molecules are brought sufficiently close together for hydrogen bonds to be formed between them [cf. for example Robertson, 1940]. This consideration, combined with the admittedly rather doubtful space group data given above, restricts the probable position of the hydroxyl group among the number available within the benzpyrene molecule. The most likely position for the hydroxyl group would appear to be 7, 8, 9, 1', 2', or 3', with 5, 6, 10, and 4' just possible (of which 6 and 4' are excluded by the direct comparison) and 1 and 2 very unlikely.

DISCUSSION

The evidence that 'BPX' is a hydroxy derivative of benzpyrene is based on its solubility in dilute alkali. Only a small quantity of the crystalline material was available and even this was not obtained free from fluid distillate, which, as explained above, may have influenced the solubility and m.p. of the 'BPX'. It is hoped that a fuller examination of its properties may be made when more crystalline 'BPX' has been obtained.

A crystallographic examination of the 'BPX' at present available has shown that the mol. weight of the compound is of the right order of magnitude for a monohydroxy derivative of benzpyrene, assuming 'BPX' to be phenolic. In addition, consideration of the arrangement of the molecules in the crystal has indicated the most likely positions for the hydroxyl group in the molecule.

Spectrographic evidence has been obtained that benzpyrene is metabolized, at least in part, to a fluorescent derivative, by all the animals tested in this laboratory [Peacock, 1936; Chalmers & Peacock, 1936 and unpublished, and Chalmers, 1938]. In the mouse, rabbit, fowl and goat the carcinogen was introduced intravenously or subcutaneously; in the case of the rat, the hydrocarbon was also fed in the diet. The change of benzpyrene to 'BPX' was common to these different animals, and although the 'BPX' eliminated represented only a fraction of the injected benzpyrene, it is possible that 'BPX' is an intermediate compound formed in the metabolism of benzpyrene by the animal body.

The fluorescent elimination products of benzpyrene—unchanged benzpyrene and 'BPX'—do not appear to represent more than a fraction of the injected benzpyrene. Moreover, no evidence has been obtained of the elimination of benzpyrene or a derivative of benzpyrene in significant quantities conjugated with mercapturic acid or glucuronic acid. It therefore appears that most of the injected benzpyrene is eliminated as a non-fluorescent derivative or is further metabolized.

With regard to the elimination of benzpyrene in conjugation with glucuronic acid, it was found that after a subcutaneous injection of benzpyrene in the rat the urine gave a more intense glucuronic acid reaction with naphthoresorcinol than normal rat urine. Attempts to isolate the glucuronic fraction from the urine, however, were unsuccessful, and by comparison with the urine of rats fed with borneol it appeared that the glucuronic acid was present in small quantity.

SUMMARY

3:4-Benzpyrene, after subcutaneous injection in the rat, is metabolized to a fluorescent derivative which is probably a monohydroxybenzpyrene.

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