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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*†

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From previous metabolic studies, it is known that conversion of carcinogenic hydrocarbons into hydroxy derivatives can occur in the animal body. In the case of 3,4-benzpyrene, a fluorescent substance (BPX), discovered by Peacock and Chalmers in the bile (24) and the feces (14, 10, 11), is considered by Chalmers and Crowfoot (12) to be a monohydroxy derivative, while in the case of 1,2,5,6-dibenzanthracene, a dihydroxy derivative has been isolated from the excreta by several workers (7, 8, 18, 19). The position of the hydroxy group in the benzpyrene derivative was not established by previous workers. The dibenzanthracene derivative was identified as 4',8'-dihydroxy-1,2,5,6-dibenzanthracene in mice and rats (9, 19), but has not yet been identified in rabbits (7, 8, 18, 19). Metabolic derivatives of cholanthrene and 20-methylcholanthrene have so far not been isolated, but the detection of alkali-soluble fluorescent products in the bile of animals injected with these hydrocarbons (15), suggests that a similar metabolic mechanism is probably involved. No metabolic products of carcinogenic hydrocarbons other than hydroxy derivatives have so far been described.

The present work deals with a reinvestigation, on a more extensive scale, of the problem of the metabolism of benzpyrene, made possible by improving the conditions determining the production of the metabolic products, and by simplifying the method of their extraction. The identification of these products is considered in a separate communication (4).

FACTORS AFFECTING THE EXCRETION OF METABOLIC PRODUCTS OF 3,4-BENZPYRENE

In view of the small amount of BPX produced by the animal under previous conditions, a reinvestigation of the factors influencing production was necessary.

In the case of intravenous injection of a colloidal solution of benzpyrene, the low yield of BPX in the excreta is obviously due to the small dose which can be introduced by the intravenous route. In the case of subcutaneous injection, by which large amounts of benzpyrene in oil can be introduced into the animal, the low yield of BPX is attributable to the very slow rate of metabolic change. This was demonstrated by the present authors in a previous communication (5), when it was found that the rate of disappearance of benzpyrene is about 15 times greater when injected intraperitoneally than when injected subcutaneously, and that this disparity is due primarily to a difference in the rate of transfer of the unchanged benzpyrene from the site of injection to the site of metabolic transformation and elimination. Since the increase in the rate of transfer, in the case of intraperitoneal injection, is not accompanied by any significant accumulation elsewhere in the body, or by any significant increase in excretion, of the unchanged benzpyrene, it follows that the tissues in which the metabolic change occurs are capable of coping with all the benzpyrene brought to them.

From this, it was to be expected that the feces of animals injected *intraperitoneally* with *large* doses of benzyprene, would serve as rich sources of metabolic products of this hydrocarbon. This was in fact found to be the case.

The amounts injected were about 40 mgm. of benzpyrene in 2 ml. of sesame oil in rats, and about 10 mgm. in 0.5 ml. of sesame oil in mice. (Of the two, the mouse is the more efficient in metabolizing benzpyrene, probably owing to the larger surface area of its peritoneal cavity in relation to its body size.) The animals were kept in darkened cages, and the feces collected at 3 to 5 day intervals for 10 to 15 days. The feces were subsequently ground to a fine powder and kept in a desiccator over H_2SO_4.

* A preliminary note on this work has been published (6).

† Because of the difficulties of international communication the proof sent to the authors did not come back.

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Extraction and Isolation of Metabolic Products of 3,4-Benzpyrene from the Feces

Extraction of the feces with boiling ethanol under continual reflux, as recommended by Chalmers (11), was found to be wasteful, owing to considerable decomposition of the labile metabolic products. The subsequent procedure recommended for the purification of BPX, involving evaporation in N_2, solution in NaOH, repeated chilling, and removal of insoluble matter, followed by acidification and transference to benzene, before chromatographic adsorption, was found to be both wasteful and unduly complicated for large scale work.

The method described below, though theoretically less efficient for quantitative extraction, yet in the end led to reasonably good yields as destruction of the labile products was greatly minimized. In view of the marked photosensitivity of BPX, the various stages of purification were carried out in subdued light or, whenever possible, in total darkness.

The powdered feces were packed into a tall glass column, having a constriction below plugged with a wedge of cotton wool, and cold benzene was allowed to percolate slowly for long periods, the progress of extraction being judged by the intensity of the fluorescence of the extract. The benzene extract was then passed through short, wide columns of aluminium oxide [1] for chromatographic separation. A brownish grey zone was adsorbed at the top; this was followed by an intermediate zone (ill defined in daylight at this stage, but easily recognizable in ultraviolet light by its strong yellow fluorescence); and below this was a deep red zone, slowly travelling down the column. On developing the column with chloroform, the red zone was eluted; while the intermediate zone containing the BPX travelled down slowly and became distinct from the upper zone as a light greenish yellow zone with intense yellow fluorescence in ultraviolet light. This zone was cut and eluted with methanol containing about 1 per cent ammonia. The methanolic extract was evaporated *in vacuo,* yielding a yellow material that exhibited an intense green fluorescence in alkaline solutions and a strong blue fluorescence when dissolved in neutral organic solvents.

Further purification of this material by repeated chromatography proved unsatisfactory owing to serious losses, partly through incomplete elution from the alumina but mainly through decomposition during manipulation. However, on sublimation in high vacuum, by use of the micro-still of Strain and Allen (28) and a mercury vapor pump, a pale yellow material containing needle-shaped crystals was obtained, which appeared identical with the "crystalline BPX" of Chalmers and Crowfoot (12).

The red fraction, eluted from the alumina column with chloroform, was further investigated. Since this fraction was not found in extracts of normal feces it seemed reasonable to suppose that it, too, might be a derivative of benzpyrene.

For its purification, the chloroform eluate was evaporated to dryness, taken up in light petroleum, in which it was only sparingly soluble, and passed through another column of alumina, on which it was strongly adsorbed. The column was washed with large quantities of light petroleum, which eluted any unchanged benzpyrene present as well as large amounts of a colorless crystallizable material, probably a sterol. The red zone was then cut and eluted with chloroform. The procedure was repeated, and on evaporation of the final chloroform eluate, rosettes of deep brownish red crystals separated out. This material was further purified by high vacuum sublimation and recrystallization from glacial acetic acid.

This crystalline red product was found to be readily soluble in chloroform and hot glacial acetic acid, moderately soluble in benzene, ethanol, and acetone, sparingly soluble in light petroleum, and insoluble in water at any pH.

In solution it is yellow in color with no discernible fluorescence. It is not readily destroyed by irradiation with ultraviolet light and in many other ways is more stable than BPX. With concentrated H_2SO_4 it gives an olive green coloration. In all these respects its behavior is similar to that of the 3,4-benzpyrene-5,8-quinone, as synthesized by Vollmann and his associates (30), and this was further supported by the fact that a mixture of the two could not be separated by chromatography.

Evidence Concerning the Extent of Metabolic Transformation

In view of the instability of BPX, quantitative estimations of its concentration in the excreta are unreliable. Approximate data could still be of value, however, if they could indicate whether the excretion of BPX could account for the greater part of the benzpyrene that disappears from the body, or whether, as might be inferred from the observations of Chalmers and Crowfoot (12), it represents only a small fraction of the loss.

Such approximate values were obtained by matching the intensities of fluorescence of the crude benzene extracts of mouse feces, in various dilutions, with known standards. It was found that feces collected during the first 3 days after intraperitoneal injection of 10 mgm. of benzpyrene contained about 1 to 1.5 mgm. of fluorescent material. Since about 6 mgm. of

[1] "Aluminium oxide for adsorption purposes," British Drug Houses, Ltd.

the 10 mgm. injected had disappeared from the body during these 3 days, while less than 1 per cent of this was excreted as unchanged benzpyrene (5), it may be concluded that one-sixth to one-fourth of the amount lost could be accounted for as fluorescent metabolic products in the feces. The amounts of fluorescent metabolic products excreted in the urine were not estimated.

Carcinogenicity of Metabolic Products of 3,4-Benzpyrene

The small amounts of metabolic products available, and their instability on keeping, prevented large scale experiments on carcinogenesis by skin painting. Tests by subcutaneous injection seemed more feasible, and these were therefore undertaken on groups of 10 mice, both with respect to the metabolic hydroxybenzpyrene (BPX) and the quinone derivative obtained from the feces.

Crude preparations of these products were used, dissolved or suspended in sesame oil. Single injections of 0.5 ml. were given subcutaneously in the sacral region. From approximate estimations of the crude materials used, the amounts of pure BPX and quinone respectively appeared to be of the order of 2 mgm. per mouse.

The animals tolerated the treatment well, most of them remaining alive after 7 months. Some were killed for autopsy and chemical examination between the 7th and 10th months, while 3 of the BPX series and 4 of the quinone series were still alive after 10 months, when they were killed. There was evidence of persistence of the injected substance *in situ* in both series, though in the animals injected with the quinone, a changed product—salmon pink in color, and strongly adsorbed on alumina—was also present. Its nature has not yet been determined.

In the quinone series, none of the animals developed any tumors. In the BPX series, one mouse, killed after 10 months, had a small nodule attached to the thin capsule around the injected material, which proved histologically to be a spindle cell sarcoma; another of this series had a typical mammary carcinoma away from the site of injection (left axilla), which was presumed to have developed spontaneously; the other mice of this series were free from tumors.

In contrast to these results, 3 groups of 20 mice, injected subcutaneously with 2.0, 0.2, and 0.02 mgm. of benzpyrene in sesame oil, yielded 90, 40, and 15 per cent of tumors respectively under comparable conditions.

DISCUSSION

Three separate problems are involved in the elucidation of the biological activities of a carcinogenic hydrocarbon such as 3,4-benzpyrene: (a) the rate of disappearance of the unchanged hydrocarbon; (b) the nature of the substances into which it is transformed in the body; and (c) the distinction between those transformed substances which may be concerned with the biological activities of the parent hydrocarbon (carcinogenesis, inhibition of tumor growth, estrogenic action, etc.), and those which are not.

The first of these problems has already been considered by the present authors in a previous publication (5), from which three significant conclusions may be drawn: First, the limiting factor that determines the rate of disappearance of benzpyrene from the mouse is the rate of diffusion of the unchanged hydrocarbon from the site of injection to the blood, and not the rate of its metabolic transformation or excretion after transference *via* the blood stream. Second, more than 99 per cent of the benzpyrene lost from the animal must be accounted for by conversion into metabolic products—a fact already observed by Chalmers and Kirby (13) with regard to rats. Third, persistence of unchanged hydrocarbon *in situ* seems to be necessary for local carcinogenic action.

Reference has already been made to the earlier work on the isolation of a metabolic product of benzpyrene (BPX) by Peacock, Chalmers, and Crowfoot (24, 14, 10, 11, 12) and to the conclusion that it was in all probability a monohydroxy derivative. The present investigation has led, in addition, to the isolation of a second derivative of benzpyrene, possessing properties similar to synthetic 3,4-benzpyrene-5,8-quinone. The identification of the red product with 3,4-benzpyrene-5,8-quinone, and the evidence for concluding that the hydroxy group in BPX occupies the 8- position, are described in a separate communication (4).

Since 8-hydroxy-3,4-benzpyrene is very labile, quantitative data concerning its excretion cannot be accurate. Approximate comparisons of the intensities of fluorescence of crude extracts of feces with those of known standards are useful, however, in that the values obtained represent minimal values. Such comparisons have shown that one-sixth to one-fourth of the benzpyrene lost from the body can be accounted for in the form of fluorescent material. Since the part excreted in the urine is not included in this, and the values for feces are too low, owing to losses through decomposition before and during extraction, it is probable that the metabolized fluorescent products in the excreta must account for a considerable fraction of the benzpyrene that had disappeared from the body, and that the very small yields of the purified fluorescent material bear little relation to the amount actually excreted. That the fluorescent products do not account for the whole of the lost benzpy-

rene is evident, however, from the presence also of a nonfluorescent benzpyrene-quinone in the feces.

Unlike the hydroxy derivative (BPX), the red quinone possesses no specific fluorescence, and its detection in the body is therefore rendered difficult. Moreover, its presence in purified extracts of tissues or feces does not necessarily prove that it is a product of body metabolism, since the conversion of BPX to the quinone may have occurred by auto-oxidation. From the evidence available, it is not possible to state whether the quinone is or is not produced metabolically.

With the isolation and identification of two derivatives of benzpyrene from the feces, and the knowledge that the two probably account for the greater part of the benzpyrene disappearing from the body, the third of the problems mentioned above becomes a matter of considerable interest; namely, whether these derivatives play any part in the biological activities of the parent hydrocarbon.

In this connection, the striking diminution in carcinogenicity of these derivatives of benzpyrene, in relation to the parent hydrocarbon, may be compared with that of other hydroxy and quinone derivatives of carcinogenic hydrocarbons described in the literature.

Of the synthetic hydroxy derivatives of 3,4-benzpyrene tested for carcinogenesis, the 4′- (1, 2, 27), 2- (20), and 5- (26) were negative, and the 6- (20) slightly positive; of the hydroxy derivatives of 1,2-benzanthracene, the 8- (20) and 10- (26, 27) were negative and the 3- (26, 27) reported as both negative and positive; while 3-hydroxy-10-methyl-1,2-benzanthracene (27) was also negative; and of the hydroxy derivatives of 20-methylcholanthrene, negative results were obtained with the 2- (27) and 3- (2, 27) and positive results with the 15- (27). Of particular interest in this connection is the fact that 4′, 8′-dihydroxy-1,2,5,6-dibenzanthracene (the metabolic derivative of the parent hydrocarbon) is also noncarcinogenic (8, 20).

Similarly, in the case of the quinone derivatives of carcinogenic hydrocarbons, 1,2-benzanthraquinone (27), 1,2,5,6-dibenzanthraquinone, and 3,4,9,10-dibenzpyrene-5,8-quinone (22) were all found to be noncarcinogenic.

The fact that the metabolites of carcinogens, and their synthetic analogues, are considerably less carcinogenic than their parent hydrocarbons confirms the conclusion already reached by indirect evidence (5) that local carcinogenesis depends on persistence of the unchanged hydrocarbon, and not on the presence of its metabolites.

The other biological activities that many of the carcinogenic hydrocarbons possess, namely inhibition of tumor growth (22, 23, 3) and estrogenic action (16, 17, 25), differ in two essential respects from carcinogenesis: (a) The amounts necessary to produce such effects are many hundred times greater than those required for carcinogenesis. (b) The effects are produced at a distance from the site of injection. The possibility that these remote effects of carcinogenic hydrocarbons are due to some of their metabolic products rather than to the parent hydrocarbons themselves must therefore be seriously considered.

Until now, the metabolic conversion of a carcinogenic hydrocarbon has only been considered as a detoxication mechanism in the *negative* sense of loss of carcinogenic propensities (19, 9). The suggestion now put forward is that the metabolites may also have *positive* action.

As regards inhibition of tumor growth, while some hydroxy and quinone derivatives of hydrocarbons have little or no inhibitory action, *e.g.* 1,2,5,6-dibenzanthraquinone (23), 1,9-benzanthrone (22), anthanthrone, pyranthrone, dibenzanthrone, *iso*-dibenzanthrone, and allomesonaphthodianthrone (3), definite inhibitory effects have been obtained with 4′-hydroxy-3,4-benzpyrene, 9,10-dihydroxy-9,10-di-N-propyl-9,10-dihydro-1,2,5,6-dibenzanthracene (22), the two dibenzpyrene-quinones, 4-methyl- and 4-aldehyde-benzanthrone, and 1,2,5,6-dibenzfluorenone (3). Thus the positions of the hydroxy and quinone groups may strongly influence inhibitory action. However, the fact that several hydroxy and quinone derivatives of carcinogenic hydrocarbons possess strong inhibitory action, while none of those so far tested possess more than slight carcinogenic action, seems significant. The fact that the experimental conditions which most favor inhibitory action (*i.e.* injection by the intraperitoneal route) are also those most effective in eliciting metabolic products, also supports the view that preliminary conversion of a carcinogen into its metabolites may play a necessary part in the inhibitory effect of the parent hydrocarbon.

Finally, in discussing the influence which the position of the hydroxy or quinone groups may have in determining inhibitory action, attention must also be drawn to the fact that the types of metabolic products may be different in different species. It has already been shown (7, 8, 18, 19, 9) that in the case of 1,2,5,6-dibenzanthracene the hydroxy derivative found in the rabbit is different from that excreted by mice and rats. Preliminary investigations by the present authors have shown that, contrary to the statement of Chalmers and Crowfoot (12), the metabolism of 3,4-benzpyrene is also different in the rabbit from that in the rat or mouse.

The available data for estrogenic activity of hydrocarbons (16, 17, 25) and their derivatives are too scanty for conclusions to be reached on the subject. It may be significant that so many of the potent estrogens are hydroxy derivatives, and that those synthetic com-

pounds that are not, are metabolized in the body into phenolic derivatives that are more active than their parent compounds (29, 21). But in order to decide whether the estrogenic effect of a carcinogen is due to its preliminary conversion into a hydroxy compound, the known metabolites would themselves have to be tested for such action.

It may be concluded, therefore, that whereas the carcinogenic action of a polycyclic hydrocarbon does not depend on conversion into hydroxy or quinone derivatives, the arguments in favor of these metabolites playing an essential part in some of the other biological activities that a carcinogen may possess (inhibitory action on tumor growth and estrogenic action) appear to be sufficiently plausible to warrant further investigation.

SUMMARY

Injection of 3,4-benzpyrene by the intraperitoneal route in mice or rats was found to be most favorable in providing high yields of metabolic products in the feces.

By a simplified method of extraction two products were isolated from the feces: a fluorescent phenolic derivative, identical with BPX of Peacock, Chalmers, and Crowfoot, and another product, not previously described, with properties similar to synthetic 3,4-benzpyrene-5,8-quinone.

From approximate estimations it seems probable that the greater part of the benzpyrene lost from the body appears in the excreta in the form of these two isolated derivatives.

The quinone was found to be noncarcinogenic after 10 months, following subcutaneous injection of about 2 mgm. in sesame oil. Similar injections of the metabolic 8-hydroxy product (BPX) yielded a sarcoma in 1 mouse of 10.

The significance of the conversion of 3,4-benzpyrene into these two derivatives, in relation to the biological activities of the parent hydrocarbon, is discussed. While these derivatives probably play no part in the carcinogenic mechanism of the parent hydrocarbon, the arguments in favor of their being concerned with other biological activities (*e.g.* inhibition of tumor growth and estrogenic action) are suggestive, and call for further investigation.

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