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

## I. UNCHANGED BENZPYRENE

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Evidence has already been presented [Chalmers, 1940] that 3:4-benzpyrene after intravenous injection of its collodial solution is eliminated from the rat as a derivative having a blue fluorescence in the ultra-violet beam. Spectrographic examination showed that this fluorescence was characterized by two diffuse bands in the blue region of the spectrum. It appears that this fluorescent derivative of benzpyrene is eliminated in the bile and subsequently in the faeces, and to a much smaller extent in the urine. In order to obtain a sufficient quantity of the substance for complete identification, recourse was had to subcutaneous injections of benzpyrene, using a 1% solution in olive oil; by this means much larger quantities of the hydrocarbon could be made available for metabolism than was possible by the intravenous route. That the same derivative was obtained in the excreta as with the former method of injection was shown by the similarity of the fluorescence spectrum in organic solvents, the solubility in NaOH solution and the selective adsorption on alumina.

### *Qualitative elimination*

The urine and faeces collected separately from the rats, which were kept in metabolism cages, were extracted by the method already described [Chalmers, 1940]. In this method, after removal of fatty material, the fluorescent substance was separated from other benzene-soluble constituents of the urine or faeces by chromatographic adsorption on alumina. When a benzene solution obtained from fluorescent urine was passed through a tower of alumina, it was observed that the filtrate, though colourless in daylight, had a violet fluorescence in the ultra-violet beam. The fluorescent derivative of benzpyrene is strongly adsorbed on alumina from benzene solution, and it therefore appeared that the filtrate contained a second fluorescent fraction. Spectrographic examination of this filtrate revealed the presence of the characteristic fluorescence spectrum bands of benzpyrene.

Although it seemed unlikely that the result could be explained by the accidental contamination of the urine with benzpyrene from the hair of recently injected animals, it was thought advisable to examine the fluorescent elimination products obtained from a source not liable to such contamination. An operation for the daily collection of bile has been devised by Dr P. R. Peacock, and will be described elsewhere in detail. It consists essentially of the introduction into the gall-bladder of a glass cannula connected by narrow rubber tubing to a rubber balloon of about 25 ml. capacity, situated in the subcutaneous tissues. From this balloon, bile can be aspirated at intervals, through the skin,

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by means of a syringe and small hypodermic needle. Fowl bile collected in this way after daily intravenous injections of 5 ml. of 0·03% benzpyrene colloid was extracted, after acidification with HCl, with *n*-butyl alcohol. The solution was evaporated to dryness in $N_2$ under reduced pressure and taken up in benzene. The procedure was then the same as for the urine extracts. Spectrographic analysis revealed the presence of benzpyrene, thus establishing qualitatively the elimination in fowl bile of unchanged benzpyrene after intravenous injection. Subsequently benzpyrene was detected by the same method in an extract of fowl excreta, after the subcutaneous injection of the hydrocarbon. The elimination of unchanged benzpyrene in the excreta of two fowls injected intravenously with benzpyrene colloid has been reported by Peacock [1936]. This carcinogen is therefore, to some extent, eliminated unchanged by the fowl after intravenous or subcutaneous injection.

The original method of extracting the fluorescent rat faeces was unsuitable for the isolation of unchanged benzpyrene, since it involved passage through 2*N* NaOH to remove fat, and consequent disappearance of any benzpyrene present. It was found possible to remove most of the non-fluorescent material by extracting with warm acetone the residue obtained after evaporating the original alcoholic extract to dryness. The acetone solution was filtered after thorough chilling in the refrigerator to about 4°. Two further extractions yielded practically all the fluorescent material, and the combined extracts were transferred to benzene solution and subjected to chromatographic analysis. Two fluorescent zones could be seen on the chromatogram: an upper, having a greenish-yellow fluorescence, and a lower, having a violet fluorescence. The initial yellow filtrate had a faint green fluorescence, and was discarded. The chromatogram was developed with ether, which rapidly eluted the violet-fluorescent zone. The presence of benzpyrene in this eluate was detected by fluorescence spectrum analysis.

*Quantitative elimination*

In order to find what fraction of the injected benzpyrene was eliminated unchanged from the rat, three adult rats were injected subcutaneously, each with 2 ml. of a 1% solution of benzpyrene in olive oil. The animals were housed in a metabolism cage, and the urine and faeces collected separately in the usual way. Ethanol extracts of samples of the faeces were examined each day for the presence of fluorescent elimination products. The intensity of the fluorescence of the samples decreased progressively until, after about one month, the samples were practically non-fluorescent. There were by this time no palpable signs of residual injections to be found. It was concluded that most of the benzpyrene had been eliminated, although examination in the ultra-violet beam of animals injected subcutaneously one month previously with a solution of benzpyrene in olive oil, showed that the tissue at the site of injection was intensely fluorescent, indicating that some of the benzpyrene had been encapsulated there.

The pooled samples of the urine and faeces, respectively, were extracted by the modified method already indicated. In the case of the faecal extract, on filtration of the benzene solution through an alumina tower a wide zone, having a greenish-yellow fluorescence, was formed at the top of the tower, and another wide zone, having a violet fluorescence, was formed lower down. The initial yellow filtrate which was non-fluorescent was discarded. The filtrate from development with ether, containing the eluate of the violet-fluorescent zone, was collected. This filtrate had a pale yellow colour in daylight, and an intense violet fluorescence in the ultra-violet beam.

In the case of the urine extract, on filtration of the benzene solution through an alumina tower a wide zone containing a yellow-fluorescent material was formed at the top, but there was no obvious violet-fluorescent zone below. However, on development of the chromatogram with ether, a colourless filtrate with a violet fluorescence was obtained.

*Spectrographic analysis.* The apparatus used for recording of fluorescence spectra has been described [Chalmers & Peacock, 1936]. Ilford Iso-zenith photographic plates were used. Quantitative estimation of the amount of benzpyrene present was made by comparison of the intensity of fluorescence of the solutions of unknown strength with that of standard solutions photographed on the same plate, as described by Berenblum & Kendal [1936]. It was found that there was a recognizable variation in the intensity of fluorescence of 0·1, 0·2, and 0·3 mg./100 ml. solutions of benzpyrene in ethanol under the conditions used, and comparisons of ethanol solutions of the recovered benzpyrene with these standards have been made in the experiments described here. It was found that with solutions of benzpyrene containing more than 0·3 mg./100 ml. the intensity of fluorescence did not appear to increase so markedly with increase in concentration, and such solutions were therefore less suitable as standards.

The violet-fluorescent filtrate from the chromatogram of the faecal extract described above was taken to dryness. A solution of the residue in ethanol was then prepared for spectrographic analysis. Solvent was added until the solution had a fluorescence of intensity comparable on visual examination with that of the standards. On spectrographic examination, it was found that the faecal extract had a fluorescence corresponding to a total benzpyrene content of 0·4–0·6 mg. Since, in all, 60 mg. of benzpyrene were injected, the recovery represented about 1 % of the amount administered.

The fluorescence spectrum of the urine extract revealed the presence of only a trace of benzpyrene.

*Efficiency of the method of extraction.* In order to establish the efficiency of the method of extracting the benzpyrene from the faeces of the rat, a sample was taken from the pooled ethanol extracts of the faeces collected daily from a series of rats injected subcutaneously with benzpyrene in olive oil. The sample was divided into two fractions, A and B. To A were added 2 mg. benzpyrene; fraction B served as a control. Both fractions were then extracted by the acetone method already described. On spectrographic analysis, it was found that the extract from fraction A contained approximately 2 mg. benzpyrene, while the control extract from B contained about 0·2 mg. Hence it follows that 90 % of the added benzpyrene was recovered by this method of extraction, and that the method could therefore be relied on to yield reasonably quantitative data.

## Discussion

The primary aim of a series of experiments at present in progress in this laboratory, in which rats have been injected subcutaneously with a solution of benzpyrene in olive oil at weekly intervals, has been the isolation of the fluorescent derivative of benzpyrene eliminated in the faeces and urine under such conditions. The fluorescent derivative of benzpyrene eliminated in the faeces in the course of the quantitative experiment described in this communication has been collected, and it is hoped that the amount eliminated may be estimated by fluorescence analysis. It appeared, from previous work, that a relatively large amount of the injected benzpyrene is converted into this derivative, but

about 1% of the benzpyrene injected in this quantitative experiment has been recovered unchanged in the faeces. The detection of unchanged benzpyrene in fowl excreta indicates that the elimination of a small fraction of the injected hydrocarbon unchanged after intravenous or subcutaneous injection is not peculiar to the rat.

After the subcutaneous injection of benzpyrene in the rat, it is probable that unchanged benzpyrene is slowly taken up by the blood stream and transferred to the liver, where most of it is converted into the fluorescent derivative. A small quantity of the hydrocarbon, however, is apparently eliminated from the liver unchanged or in conjugation with bile salts. The quantity of benzpyrene present at any time in the bile of the rat must be small, since, in the quantitative experiment, less than 0·2 mg. benzpyrene was eliminated per rat in one month. The fact that benzpyrene dissolves in aqueous sodium deoxycholate to the extent of 2 mg. per ml. [Winterstein & Vetter, 1934], suggests that the unchanged benzpyrene may be eliminated in the bile as a conjugate with bile salts. The presence of bile salts does not influence the fluorescence spectrum of benzpyrene [Chalmers, 1938]. This finding is in agreement with the results of Lorenz [1935], who photographed the absorption spectra of the hydrocarbon choleic acids made by Fieser & Newman [1935] and found that these spectra and those of the unchanged hydrocarbons were identical, showing that the choleic acids were completely dissociated.

In the case of the fowl, evidence has been found of the presence of a fluorescent derivative of benzpyrene and of unchanged benzpyrene in the bile after intravenous injection. In the case of the rat, it has not been practicable to examine the fluorescent bile, but the presence of the fluorescent derivative of benzpyrene and of unchanged benzpyrene in the faeces indicates that the mechanism of elimination of benzpyrene is the same. Only a trace of benzpyrene was found in the urine in the quantitative experiment, and while in the larger scale experiment the presence of benzpyrene became more evident, the elimination of benzpyrene in the urine appears to be of minor importance.

The mode of administration of the benzpyrene may have some influence on the relative amounts of the fluorescent derivative of benzpyrene and of unchanged benzpyrene eliminated. After intravenous injection of benzpyrene colloid, or subcutaneous injection of benzpyrene in olive oil, the quantity of the fluorescent derivative eliminated seems to be greater than the quantity of unchanged benzpyrene. But it has been noted that bile obtained from a fowl injected intravenously with a 0·05% solution of benzpyrene in "Emmal" oil emulsion contained a relatively larger amount of unchanged benzpyrene, while the amount of fluorescent derivative present was less than after the injection of a colloidal solution of the hydrocarbon owing, possibly, to the whole of the injected benzpyrene reaching the liver in a shorter space of time. After subsequent injection of benzpyrene colloid in the same bird, the bile contained a trace of unchanged benzpyrene and a relatively large amount of the derivative, similar to the bile obtained after the initial injection of the colloid.

## Summary

Evidence has been obtained that after the subcutaneous injection of benzpyrene in the rat, a small fraction of the hydrocarbon is eliminated unchanged in the urine and faeces. Approximately, 1% of 60 mg. benzpyrene injected subcutaneously in three rats was eliminated unchanged in the faeces, while only a trace was eliminated in the urine.

Evidence has also been obtained of the partial elimination of benzpyrene in an unchanged form in fowl bile after intravenous injection, and in fowl excreta after subcutaneous injection.

The mechanism of elimination of benzpyrene by the liver has been discussed.

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