82. ELIMINATION OF 3:4-BENZPYRENE FROM THE RAT¹

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IN a previous communication [Chalmers, 1938] spectrographic evidence was given to show that intravenously injected 3:4-benzpyrene was eliminated via the bile as a fluorescent derivative in the faeces of the mouse. In the present investigation, a further study of the elimination of this carcinogen from the animal body has been made. Rats were used instead of mice to obtain larger quantities of the fluorescent material, and the urine was examined as well as the faeces for fluorescent derivatives of 3:4-benzpyrene. The fluorescence of the extracts made in the course of the investigation was taken as an indication of the presence of elimination products, the initial visual observation in the ultra-violet beam being confirmed, if necessary, by spectrographic analysis¹.

EXPERIMENTAL

The rats were injected intravenously in the tail vein with a colloidal solution of 3:4-benzpyrene and kept in metabolism cages in which the faeces and urine could be collected separately.

In the first instance, a series of 12 young rats were injected with 1 ml. of 0.03 % colloid on alternate days. This was about the maximum quantity of colloid which could be injected into small rats and, in subsequent experiments, larger animals were used.

In a series of 21 adult rats, an attempt was made to inject each rat with 2-3 ml. of 0.03 % 3:4-benzpyrene colloid on alternate days, but, owing to the difficulty of injecting the animals, occasional irregularities occurred. The quantities of colloid injected are given in Table 1. The maximum amount of hydrocarbon injected into any rat was 39 mg., and 11 of the rats were under examination for 10 months, or more.

Although 3:4-benzpyrene proved to be toxic in the rat, the animals tolerated the quantity injected well and, in certain cases where the administration of the colloid was stopped owing to difficulties in making the injections, the animals survived for 14 months.

It is interesting to note that, in this investigation, the intravenous injection of 3:4-benzpyrene did not produce lung tumours. Previous work [Chalmers, 1938] has shown that after the intravenous injection of a colloidal solution of this carcinogen the lungs of a mouse showed a number of fluorescent spots which proved to be particles of the unchanged hydrocarbon. The same deposition takes place in the rat, and it appears that no lung tumours were found in the experiments described because: (1) the rat lung was not susceptible to the

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Table 1

No. of Exp.	Rat no.	Time under examination* months	Benzpyrene colloid injected intravenously ml.	Benzpyrene mg.	Time from last injection until death months
1	4 A	18	51	15	14
2	5 A	18	50	15	14
3	1 A	17	130	39	4
4	2 A	16	117	35	3
5	9 A	16	92	28	7
6	10 A	13	96	29	3
7	11 A	13	85	25	4
8	18 A	13	75	23	10
9	17 A	12	75	23	8
10	16 A	10	75	23	5
11	21 A	10	61	18	7
12	12 A	9	99	30	†
13	6 A	7	49	15	3
14	7 A	5	24	7	4
15	3 A	3	25	8	0
16	8 A	3	24	7	2
17	13 A	3	53	15	0
18	14 A	3	62	19	0
19	15 A	3	77	23	0
20	19 A	3	49	15	0
21	20 A	3	61	18	0

* The time under examination was the time from the first injection of colloid until the death of the animal. Some of the rats survived for more than a year after the last injection, and the time of survival did not appear to bear any definite relationship to the quantity of colloid injected.

† Accidentally discarded.

carcinogenic action of 3:4-benzpyrene, or (2) the 3:4-benzpyrene did not come into contact with the tissues of the lung for a sufficiently long time or in high enough concentration.

Although no malignant change was observed, other pathological changes were found in the lungs of the animals. In some cases, there was evidence of congestion, and, in others, there were regions of bronchiectasis, with areas of squamous metaplasia, showing considerable irregular epithelial hyperplasia and marked keratinization. Histological examination of the kidneys of the rats which died or were killed during the investigation revealed in almost all cases some degree of nephritis, the condition varying from simple congestion to acute glomerular nephritis. The toxic action of 3:4-benzpyrene on the kidney must be discounted to some extent by the fact that gelatin, which was used as a protective colloid, caused haematuria in rats on injection even of a 0.05% solution. Colloidal solutions of 3:4-benzpyrene produced haematuria in the absence of gelatin, so, evidently, the hydrocarbon had some toxic effect. Post-mortem examination of the spleen in the experiments described did not show any significant pathological change.

Changes in the liver were not so marked as in the kidney or lung, although, presumably, the chemical change of the hydrocarbon takes place chiefly in this organ. In view of these observations on the effect of intravenously injected 3:4-benzpyrene on the organism of the rat, a further study of this toxic action is being made.

Fluorescent derivative in faeces

Extraction. The faeces collected after the intravenous injection of 3:4-benzpyrene colloid were extracted with absolute alcohol under continuous refluxing for 4 hr. and, after grinding up with sand, for a further 4 hr. The

alcoholic extracts obtained in this way were pooled until 2-3 l. of highly fluorescent solution had been collected. The combined alcoholic extracts were then dried over Na_2SO_4 and evaporated to dryness in N_2 under reduced pressure. A solution of the residue in 2*N* NaOH was chilled at 0° and filtered to remove fat. Since there was usually a large amount of fat present, this process was repeated several times to obtain as good a separation of the fluorescent material as possible. The combined alkaline extracts were acidified with conc. HCl and extracted with benzene. This benzene solution was dried over Na_2SO_4 and concentrated to a suitable volume for further purification of the fluorescent substance by chromatographic adsorption on aluminium oxide. An estimate of the volume required was made by the colour of the solution, the initial adjustments being made by trial and error.

Chromatographic adsorption. The adsorption tower consisted of a straight glass tube with a constriction near one end to hold a Seitz filter pad in position. When the tube was more than 1.25 cm. in diameter, a Gooch filter plate was used to support the filter pad.

The adsorption of the fluorescent material was carried out in two stages. In the first stage, most of the associated colouring matter was removed from the fluorescent substance, but a second treatment was usually required to ensure a complete separation.

In the first stage, the dark brown, violet-fluorescent benzene solution was filtered through a 25 × 2.25 cm. tower of alumina. A 10-15 cm. grey zone, with a violet fluorescence, and bounded below by a dark brown ring, was formed. On development of the chromatogram with ether, a yellow zone with a yellow-green fluorescence was formed under the green zone. The yellow zone was then detached from the rest of the column and eluted with a benzene + methyl alcohol mixture.

The eluate of the yellow zone was evaporated to dryness in N_2 under reduced pressure, and the residue dissolved in benzene. Then the solution, which was much lighter in colour than the original benzene extract, was filtered through a 20 × 1.25 cm. adsorption tower of alumina. At the top of the column a 3-5 cm. grey zone with a violet fluorescence formed, and immediately underneath there was a brown ring. On development of the chromatogram with a mixture of benzene + ethyl alcohol, a brown zone descended the column and was washed into the filtrate, leaving a yellow zone with a yellow-green fluorescence on the tower. This yellow zone was then eluted with the benzene + methyl alcohol mixture, giving a solution with a yellow colour and a blue-violet fluorescence, even in daylight.

On occasion, it was possible to combine the two stages of the chromatographic adsorption. After the development of the chromatogram with ether, the upper grey zone, which contained little fluorescent material, was removed with a spatula without disturbing the rest of the column, which was then developed with the benzene + ethyl alcohol mixture. The yellow zone formed was eluted, as before.

An experiment was made with normal rat faeces, which were extracted in a similar manner to the fluorescent faeces, but the resultant chromatogram did not show a yellow-green fluorescent zone.

Fluorescence spectrum. The apparatus used for photographing the fluorescence spectrum was the same as that already described [Chalmers & Peacock, 1936]. An alcoholic solution from the eluate of the yellow zone showed fluorescence spectrum bands at (4280-4430) and (4570-4750) Å. As a result of the present investigation, it appears that the fluorescent elimination product of 3:4-benzpyrene found in the faeces of the rat consists of one fraction, showing

the fluorescence spectrum bands recorded, and not two fractions, as described in the preliminary experiments for mice [Chalmers, 1938]. The preliminary experiments were made with the relatively small amounts of fluorescent material obtained from mouse faeces, and the concentrates examined spectrographically were not so highly purified as those which have now been prepared. It seems probable, although the derivatives have not been isolated, that the same fluorescent substance is eliminated in the faeces of the rat and of the mouse after intravenous injection of 3:4-benzpyrene in these animals.

Absorption spectrum. The absorption spectrum of various alcoholic contents in the fluorescent material have been examined. One of the solutions showed selective absorption in the far ultraviolet region of the spectrum, but this result appears to have been due to the presence of spectroscopically interfering substances, since subsequent examination of other concentrates has shown only general absorption.

Instability to light. A sample of the alcoholic concentrate of the fluorescent substance derived from rat faeces was sealed in a 20 cm. length of capillary tubing in the absence of O_2 and then exposed to bright sunlight for a few hours. At the end of this time the intensity of fluorescence was compared with that of a control kept in the dark, and there was a significant reduction in the intensity of the former. The fluorescent material was also found to be photo-labile when exposed to the light in the laboratory, but in this case the reduction in the intensity of the fluorescence did not take place so quickly, showing that the instability was directly related to the intensity of light to which the fluorescent substance was exposed.

Stability to the presence of oxygen and to heat. Samples of the fluorescent material were sealed in quill tubing in the presence of O_2 and of N_2 , and stored in the dark overnight. No diminution in the intensity of fluorescence of the samples was observed.

A sample of the alcoholic concentrate was sealed in a capillary tube and immersed in a closed metal water bath, which was heated to 100° for 2 hr. There was no diminution in the intensity of fluorescence of this sample, compared with that of an unheated sample stored in the dark.

Fluorescent material from urine

Extraction. In a preliminary experiment, the rat urine collected after the intravenous injection of 3:4-benzpyrene was acidified with HCl and extracted successively with ether, chloroform and amyl alcohol. The extracts were compared with similar extracts of normal rat urine, and it was found that the ether and amyl alcohol extracts were yellow-brown in colour and had a violet fluorescence in the ultraviolet beam not given by normal samples. The chloroform solution, however, did not appear to contain an extract, fluorescent or otherwise, which might have indicated the presence of an elimination product conjugated with mercapturic acid.

Ether-soluble fraction. The urine collected daily was made slightly acid with HCl, and extracted with ether in a continuous extractor for 6 hr. The ether extracts were pooled until about 2 l. had accumulated, and then, after washing with water, the extract was dried over anhydrous Na_2SO_4 and the ether evaporated off in an atmosphere of N_2 , under reduced pressure. On acidification with HCl of a solution of the residue in 2N NaOH, a dark-coloured precipitate was formed. An attempt was made to isolate the fluorescent material in the precipitate by chromatographic adsorption on alumina, but it appeared to be present only in small quantity.

Amyl alcohol-soluble fraction. After removal of the ether-soluble fraction, the urine, which was still violet-fluorescent, was extracted with amyl alcohol in a separating funnel. The amyl alcohol solution was dried over Na_2SO_4 , and evaporated to small volume in N_2 under reduced pressure. Then the fluorescent material was extracted from the amyl alcohol by treatment with 2N NaOH and, on acidification of this alkaline solution, a dark-coloured precipitate was formed. The fluorescent material, however, in this fraction also appeared to be present only in small quantity. The alkaline solution gave a positive test for glucuronic acid, with naphthoresorcinol.

DISCUSSION

3:4-Benzpyrene, after intravenous injection into the rat, was eliminated in the faeces as a fluorescent derivative, soluble in NaOH. The derivative had a blue-violet fluorescence in organic solvents, and a yellow-green fluorescence in alkaline solution. An alcoholic solution showed two diffuse fluorescence bands in the blue-violet region of the spectrum. The extraction of the substance was complicated by its photo-lability, and, as far as possible, all operations were carried out in the dark or in diffuse daylight.

The quantity of 3:4-benzpyrene injected in these experiments was about the maximum which could be tolerated by the rat, but the amount of fluorescent elimination products obtained from the urine and faeces was insufficient to isolate. (In a further series of experiments, a 1 % solution of 3:4-benzpyrene in olive oil was injected subcutaneously into rats, and in this way a large amount of the hydrocarbon has been converted into fluorescent derivatives. The fluorescent derivative eliminated in the faeces after subcutaneous injection is similar in fluorescence and in solubility in NaOH to the derivative obtained after intravenous injection. A report of this further investigation will be presented at a later date.)

Evidence was also found of the elimination of benzpyrene as a fluorescent derivative in the urine. This fluorescent substance consisted of two fractions, one soluble in ether, the other in amyl alcohol, but the separate identity of the two fractions has not been established. Both fractions showed a blue-violet fluorescence in organic solvents and were soluble in NaOH with yellow-green fluorescence. The amyl alcohol fraction gave a positive naphthoresorcinol test for glucuronic acid. The fluorescence of the combined extracts of urine was much less intense than that of the extract of faeces, and, although no definite conclusion can be made until the derivatives have been isolated, it appears that the fluorescent substance in the urine is present in much smaller quantity than that in the faeces.

If this is so, then the elimination of 3:4-benzpyrene in the bile, first described by Peacock [1936] and, subsequently, in the faeces, is the chief route of elimination of the fluorescent derivative in the rat. It is, of course, possible that this carcinogen is also converted into a non-fluorescent derivative, which may be eliminated chiefly in the urine. In respect of elimination in the bile, benzpyrene appears to differ from naphthalene. Stekol & Mann [1937] conclude that bile is not the main channel for the excretion of naphthalene in animals, in a study of the excretion of 1- α -naphthalenemercapturic acid in dogs with biliary fistula. Detoxication products of anthracene in rats and rabbits have been isolated from the urine by Boyland & Levi [1935; 1936, 1, 2]. The animals received the hydrocarbon in their diet and there is no record of an examination of the faeces. In experiments carried out in this laboratory [Chalmers & Peacock, 1936; Chalmers, 1938], in which the fluorescence of extracts of bile and of faeces

obtained after intravenous injection of 3:4-benzpyrene, methylcholanthrene and anthracene were examined spectrographically, evidence was given to show that fluorescent derivatives of these hydrocarbons were eliminated via the bile and faeces. As the fluorescence of the extracts from the animals injected with 3:4-benzpyrene was more intense and showed a banded fluorescence spectrum, most of the work so far has been done with this substance. Further investigation, however, has not altered the original hypothesis that the carcinogens 3:4-benzpyrene and methylcholanthrene are eliminated in the same way as the non-carcinogen, anthracene. The detoxication of naphthalene fed to rats [Stekol, 1937-8] and rabbits [Bourne & Young, 1934], and anthracene fed to rats and rabbits [Boylard & Levi, 1936, 2] by conjugation with mercapturic acid has been described. Consequently, it was anticipated that the detoxication of 3:4-benzpyrene would also involve this type of conjugation, but no evidence of such was found although it is possible that the methods used would not have detected the presence of small quantities of mercapturic acid.

White & White [1939] found that the growth of white rats, which was retarded by the addition of methylcholanthrene, benzpyrene and pyrene to the diet, could be restored by dietary supplements of sulphur-containing amino-acids. They concluded that the hydrocarbons caused "a specific deficiency in the sulphur-containing amino-acids, probably by imposing on the organism an increased requirement for organic sulphur in the form of cystine or methionine for detoxication mechanisms". But they also considered another possibility, that these polycyclic hydrocarbons retard cellular metabolism and that the sulphur-containing amino-acids "may be regarded as general cell metabolic stimulants, with consequent effect on the general growth of the organism".

It is possible that the mode of administration of 3:4-benzpyrene has an effect on the course of its alteration in the animal body. The mercapturic acids of the carbocyclic compounds already isolated have all been obtained from the urine of animals fed with the hydrocarbon, while, in the experiments described in this communication, the hydrocarbon has been injected intravenously. An indication of the presence or absence of the mercapturic acid derivative in the detoxication of 3:4-benzpyrene could be found by the quantitative estimation of the detoxication products, other than the mercapturic acid found in the urine and faeces, but the establishment of the identity of these products is a necessary preliminary to such an investigation.

SUMMARY

The elimination of 3:4-benzpyrene by the rat after intravenous injection has been studied. Evidence is presented to show that the hydrocarbon is eliminated as fluorescent derivatives in the urine and faeces.

The extraction of the fluorescent substance in the faeces, and its purification by chromatographic adsorption on aluminium oxide are described. It is photolabile, and soluble in NaOH, with green-yellow fluorescence. An alcoholic solution shows two diffuse fluorescence bands in the blue-violet region of the spectrum.

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