142. THE EXCRETION OF DERIVATIVES OF CERTAIN CARCINOGENIC AND NON-CARCINOGENIC HYDROCARBONS IN FOWL BILE

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THE fate of 3:4-benzpyrene after injection (subcutaneously and intravenously) in experimental animals has been described in previous publications from this Laboratory. A fluorescent derivative 'BPX' is excreted by the liver and may be recovered either from fistula bile or from the faeces [Peacock, 1936; Chalmers, 1938]. Subsequent fluorescence spectrum analysis has shown that about 1 % of a quantity of benzpyrene injected subcutaneously in the rat was eliminated unchanged in the faeces, while only a trace was eliminated in the urine [Chalmers & Kirby, 1940]. Apart from this 1 % of unchanged benzpyrene, the proportion excreted as 'BPX', or other derivatives, is not yet determined. Attempts to recover the fluorescent derivative 'BPX' have resulted in the isolation of minute amounts of a crystalline substance, whose properties suggest that it may be a monohydroxybenzpyrene [Chalmers & Crowfoot, 1941]. Earlier experiments with 1:2:5:6-dibenzanthracene had given no evidence of its excretion in the bile, either unchanged or as a fluorescent derivative, but the intravenous injection of methylcholanthrene and of anthracene gave rise to fluorescent substances in the bile [Chalmers & Peacock, 1936]. As will be shown in this communication, a derivative of dibenzanthracene with a faint blue fluorescence is excreted in the bile, but its detection by direct fluoroscopy is difficult.

In view of the finding that benzpyrene was partly excreted unchanged, investigation of a number of other hydrocarbons, both carcinogenic and non-carcinogenic (see Table 1), was undertaken by the method of sampling bile from fowls with enclosed cholecystostomy fistulae [Peacock, 1940].

EXPERIMENTAL

Methods. Colloidal 0·03–0·05 % solutions of each hydrocarbon were prepared, and 5–6 ml. were injected intravenously into fowls with previously prepared biliary fistulae. It was found that the majority of the hydrocarbons tested gave rise to fluorescence of the bile within a few hours of the intravenous injection. For comparative purposes, solutions of the hydrocarbons in fowl serum and bile were tested, as described below, and in Table 1 the results of these tests are compared with the appearances of the fistula bile.

Bile was collected until the samples no longer showed any fluorescence in the ultra-violet beam (usually 1–2 days), in which time about 20 ml. of bile were obtained. The bile was extracted by a method similar to that used in the extraction of benzpyrene from the urine and faeces of rats [Chalmers, 1940; Chalmers & Kirby, 1940]. The bile was acidified with dilute HCl and extracted

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with ether, and the ether extract was then washed with dilute NaOH and water. Subsequent reference will be made to the examination of the alkali-soluble fraction. The ether extract was dried over Na_2SO_4, and the solvent evaporated in N_2 under reduced pressure. A benzene solution of the residue was filtered

Table 1. *Comparison of fluorescence of hydrocarbon solutions and fistula bile*

Hydrocarbon	Fluorescence of fowl bile + hydrocarbon, *in vitro*	Fluorescence of fowl bile after injection
Carcinogenic:		
1. 3:4-Benzphenanthrene	Blue-violet	—
2. 3:4-Benzpyrene	Violet	Blue-violet
3. 1:2:5:6-Dibenzanthracene	—	Faint blue
4. Cholanthrene	Violet	Blue
5. Methylcholanthrene	Violet	Blue
Non-carcinogenic:		
6. Anthracene	—	Faint green
7. Phenanthrene	—	—
8. Pyrene	Green-blue	Blue
9. 2′:6-Dimethyl-1:2-benzanthracene	—	Blue
10. 2′:7-Dimethyl-1:2-benzanthracene	—	Blue
11. Fluoranthene	Green-blue	Blue

through a 3–5 cm. column of alumina, to remove the bile pigments, giving a colourless filtrate: a small column was sufficient to remove the colouring matter. The alumina was washed first with benzene to remove any extraneous fluorescent material. The chromatogram filtrate which should contain any hydrocarbons present was evaporated to dryness and the residue dissolved in a small quantity of ethanol or benzene, for spectrographic analysis.

After intravenous injection in the fowl, benzpyrene was the only hydrocarbon examined which appeared unchanged in the bile. In view of the fact that benzpyrene is intensely fluorescent, it was possible that fluorescence spectrum analysis could detect smaller quantities of this compound in bile extracts than of other less intensely fluorescent compounds. To meet this possibility, extracts were made of normal bile to which 1 % or less of the quantity of 1:2:5:6-dibenzanthracene, methylcholanthrene and anthracene used for intravenous injection had been added, and it was found that in each case the fluorescence spectrum of the hydrocarbon in question could be detected. It may therefore be concluded that less than 1 % of the quantity of these hydrocarbons injected intravenously was eliminated unchanged in fowl bile.

After the intravenous injection of quantities of 0·03 % benzpyrene colloid varying from 1 to 10 ml., unchanged benzpyrene could be detected in the bile. In an attempt to find if there was a threshold below which benzpyrene would not be eliminated unchanged, smaller quantities of the hydrocarbon were injected, but the method of extraction and analysis was not considered satisfactory for experiments in which quantities of less than 0·3 mg. benzpyrene were injected.

Quantitative controls were not made with the other compounds examined, but it was found that they could be recovered unchanged from solution in fowl bile *in vitro* by the same method of extraction. It may be concluded, therefore, that after intravenous injection they were not eliminated unchanged in the fowl, or that they were eliminated unchanged in relatively small amounts compared with benzpyrene.

Elimination in altered form. The alkali-soluble fraction of the fluorescent fowl bile to which reference has already been made was acidified with dilute HCl and extracted with ether. The ether extract was dried over Na_2SO_4, and the

solvent evaporated in N_2 under reduced pressure. If the benzene solution of the residue showed a fluorescence in the ultra-violet beam, the fluorescence spectrum of the solution was examined before chromatographic adsorption on alumina. If not, the solution was adsorbed on alumina in an attempt to separate any fluorescing material from the spectrographically interfering colouring matter.

Chromatographic adsorption. On filtering a benzene solution, prepared as described above, through a 5–7 cm. tower of washed alumina, it was found that in almost every case the fluorescent material in the solution was strongly adsorbed forming a fluorescent ring or zone at the top of the column (see Table 2). The initial filtrate was usually non-fluorescent. Some difficulty was found in eluting the fluorescent material from the alumina; in each case, benzene plus MeOH was the best eluant. The eluates were evaporated to dryness in N_2 under reduced pressure, and the residue dissolved in a small quantity of EtOH or benzene, depending on its solubility for fluorescence spectrum analysis. The fluorescence spectra of extracts prepared in this way compared with the fluorescence spectra of the hydrocarbons under examination are also recorded in Table 2. 3:4-Benzphenanthrene and fluoranthene showed only a general fluorescence, and consequently fluorescence spectrum analysis was not suitable for the detection of these compounds.

An examination was made of the alkali-soluble fraction of 200 ml. normal fowl bile, which were extracted by the method described above. On filtration of the benzene solution through a tower of alumina, a small pale-blue fluorescent zone was formed at the top of the column, but the filtrate of the chromatogram was non-fluorescent and the eluate of the small fluorescent zone, while showing a pale-blue fluorescence, did not exhibit a specific fluorescence spectrum.

The fluorescence spectrum examination of the derivatives of the hydro-

Table 2

Hydrocarbon	Fluorescence spectra: Hydrocarbon	Fluorescence spectra: Derivative from bile	Chromatogram
Carcinogenic:			
3:4-Benzphenanthrene	(369–431)	Nil	Nil
3:4-Benzpyrene	(403–408) (409–411) (426–434) (454–459)	(428–446) (458–468)	Greenish yellow zone
1:2:5:6-Dibenzanthracene	(394–399) (404–409) (418–423) (429–438) (444–448)	Non-specific	Green ring
Cholanthrene	(391–406) (411–426) (431–441)	Non-specific	Green ring
Methylcholanthrene	(393–404) (415–429) (444–447)	Non-specific	Tower completely blue
Non-carcinogenic:			
Anthracene	(381–391) (399–414) (424–436) (453–460)	Non-specific	Ill-defined 2–3 cm. zone
Phenanthrene	(374–384) (396–408) (418–431) (446–456)	Nil	Nil
Pyrene	(373–375) (378–380) (383–386) (389–392) (393–397)	(385–389) (392–395) (403–413) (426–436)	Blue zone
2′:6-Dimethyl-1:2-benzanthracene	(386–396) (406–421) (431–441)	(404–421) (429–451)	Blue zone
Fluoranthene	(406–486)	Non-specific	Blue zone

Wave-lengths in Å. $\times 10^{-1}$.

carbons found in the bile showed that the derivatives of pyrene and 2′:6-dimethyl-1:2-benzanthracene, like that of benzpyrene, have a banded fluorescence spectrum, while those of 1:2:5:6-dibenzanthracene, cholanthrene, methylcholanthrene, anthracene, 2′:7-dimethyl-1:2-benzanthracene and fluoranthene have a general fluorescence spectrum. The results of the fluorescence spectrum examination, however, must be regarded as tentative in view of the small quantities of fluorescent material used in the chromatographic adsorption experiments.

Solubility of hydrocarbons in fowl bile and serum. The extraction of unchanged benzpyrene from the bile raised the question of the relative solubilities of benzpyrene and the other hydrocarbons in fowl bile and serum.

A slight excess of the hydrocarbon was mixed with 1 ml. of normal fowl bile and 1 ml. of normal fowl serum and incubated at 37° for at least 24 hr. with occasional shaking. The solubility of the hydrocarbon in the bile or serum was determined by the fluorescence of the solution in the ultraviolet beam. The solutions were examined every few hours, and the incubation was terminated when no further evidence of solution could be detected. It may be noted here that direct visual fluoroscopy is a very convenient method for detecting the more obviously fluorescent hydrocarbons or their derivatives in bile, but that negative findings do not necessarily mean that no fluorescent substance is present, since a weak fluorescence may easily be masked by bile pigments. Finally the ultraviolet fluorescence of samples of the filtered solutions of bile and serum was compared with that of normal samples.

An estimate of the relative solubilities of the hydrocarbons in fowl bile and serum was based on: (*a*) the time taken for the bile or serum to exhibit a distinctive fluorescence due to the hydrocarbon, and (*b*) a visual comparison of the intensity of fluorescence of the saturated solution in the bile or serum after filtration from any undissolved hydrocarbon. The order of solubility in fowl serum from maximum to minimum was: (1) benzpyrene and fluoranthene; (2) 3:4-benzphenanthrene and pyrene; (3) cholanthrene, methylcholanthrene, and 2′:6-dimethyl-1:2-benzanthracene; (4) anthracene. 1:2:5:6-Dibenzanthracene, phenanthrene, 2′:7-dimethyl-1:2-benzanthracene and chrysene, which were also tested, appeared to be insoluble. Similar solubility findings were obtained with rabbit and mouse sera. This order of solubility, however, made no allowance for the relative intensity of fluorescence of solutions of the hydrocarbons of equal concentration, and in order to make allowance for this factor ether extracts of the serum saturated with the hydrocarbon were compared with fluorescent standards of the hydrocarbon in the same solvent. The results indicated that benzpyrene was more soluble than cholanthrene, methylcholanthrene, anthracene and 2′:6-dimethyl-1:2-benzanthracene in fowl serum.

Similar results were also obtained for the solubility of the hydrocarbons in fowl bile. In this case, however, probably owing to the masking effect of the bile pigments, anthracene and 2′:6-dimethyl-1:2-benzanthracene, which were slightly soluble in fowl serum, appeared to be insoluble in the bile. Otherwise, the solubilities of the hydrocarbons in fowl bile and serum were similar.

Discussion

Various carcinogenic and non-carcinogenic hydrocarbons have been compared with benzpyrene as regards their elimination in bile after intravenous injection in the fowl. Part of the injected benzpyrene could be recovered unchanged from fistula bile, the remainder being eliminated as a fluorescent

derivative, or, possibly, further metabolized. So far as could be judged, the amount of benzpyrene injected did not affect the mode of elimination. None of the other hydrocarbons tested was found to be eliminated unchanged in fowl bile. A number of the hydrocarbons, 1:2:5:6-dibenzanthracene, cholanthrene and methylcholanthrene (carcinogenic), and anthracene, pyrene, 2′:6- and 2′:7-dimethyl-1:2-benzanthracene and fluoranthene (non-carcinogenic), after intravenous injection in the fowl, gave rise to the production of fluorescent bile. Subsequent investigation of the fluorescent substances in such samples of bile showed that the fluorescence was not due to the elimination of the unchanged compound but to a derivative of the hydrocarbon analogous to 'BPX'.

Since benzpyrene was the only hydrocarbon eliminated unchanged, it was thought that the solubility of benzpyrene in fowl bile and serum, which appeared to be somewhat greater than that of the other hydrocarbons, might explain this elimination. Visual examination of the fluorescence of the hydrocarbons in fowl, rabbit and mouse sera indicated that benzpyrene was more readily soluble than the other hydrocarbons examined, and this was confirmed by estimations of the concentration of the hydrocarbons in fowl serum. However, Lorenz & Andervont [1936] found that methylcholanthrene and 1:2:5:6-dibenzanthracene have the same solubility in fowl serum, and record differences in the solubility of 1:2:5:6-dibenzanthracene in horse, fowl and dog sera. Also, Brock *et al.* [1938] have observed that benzpyrene is less soluble than 1:2:5:6-dibenzanthracene in horse serum. These results, indicating a variation in the solubility of 1:2:5:6-dibenzanthracene and in the relative solubility of hydrocarbons in sera from different species, suggest that if the partial elimination of the hydrocarbon from the animal body in an unchanged form depends on its solubility in serum, then variable results are to be expected in different species. Although in our experiments 1:2:5:6-dibenzanthracene appeared to be insoluble in fowl serum, the lipins of the serum would presumably dissolve the hydrocarbon to some extent, and variations in the lipin content of the serum may account for differing solubilities. It would also be reasonable to expect the species to affect the solubility and evidence that factors other than lipin content may influence the result is provided by our recent finding that a sample of horse serum, from which the lipin fraction had been removed by ether extraction, appeared to dissolve benzpyrene as readily as a control sample of the normal serum, and a more detailed study of the solvent action of serum is being made.

Dobriner *et al.* [1939] report that 'the absorption bands of unchanged 1:2:5:6-dibenzanthracene were present in the fraction containing neutral compounds of the faeces of injected rats, mice and, rarely, of rabbits'. In our experiments, we did not detect the elimination of 1:2:5:6-dibenzanthracene in an unchanged form in fowl bile, and it is possible that this result may be explained by a difference in the metabolism of this compound in the fowl, or by the fact that we used much smaller quantities of the hydrocarbon.

The elimination of triphenylmethane from the animal body in an unchanged form has been described by Miriam *et al.* [1927]. In a feeding experiment these authors recovered 27·5 % of the hydrocarbon in the urine of rabbits, but only traces in the faeces. In the dog, 35 % of this compound was recovered unchanged from the urine and the faeces were not examined. A much larger fraction of triphenylmethane than of benzpyrene is therefore eliminated unchanged, and the mode of elimination is different, since it takes place chiefly in the urine and not in the faeces. The presence of traces of triphenylmethane in the faeces suggests that the hydrocarbon is partly excreted in the bile but re-absorption may have occurred in the bowel followed by ultimate excretion in the urine.

Considering the elimination of the hydrocarbons in altered form, pyrene and 2′:6-dimethyl-1:2-benzanthracene are excreted in fowl bile as fluorescent derivatives which show a banded fluorescence spectrum in organic solvents and are strongly adsorbed on alumina. In these properties, they resemble benzpyrene. After intravenous injection of the other hydrocarbons (1:2:5:6-dibenzanthracene, cholanthrene, methylcholanthrene, anthracene, 2′:7-dimethyl-1:2-benzanthracene and fluoranthene) benzene solutions of the alkali-soluble fraction of the fluorescent fowl bile were strongly adsorbed on alumina as fluorescent zones. The fluorescence of the bile and the adsorption on alumina constitute presumptive evidence that these hydrocarbons were partly excreted in the bile as fluorescent derivatives, although the solutions when examined either before or after chromatographic adsorption showed a general and not a specific fluorescence spectrum.

Boyland & Levi [1935; 1936] have found that when anthracene is fed to rats and rabbits different stereoisomeric forms of 1:2-dihydroxy-1:2-dihydroanthracene and their glucuronic acid derivatives are excreted in the urine. It is possible that the metabolism of anthracene to this derivative may be responsible for the fluorescence of fowl bile collected after injection of the hydrocarbon. This derivative of anthracene, however, is a neutral compound, and would not be present in the alkali-soluble fraction which was strongly adsorbed on alumina from benzene solution. This suggests the formation of another derivative of anthracene which may be peculiar to the fowl.

Boyland & Levi [1938] and Boyland *et al.* [1941] have also described the isolation of fluorescent hydroxy derivatives from the urine of rabbits fed on a diet containing 1:2:5:6-dibenzanthracene or 3:4:5:6-dibenzcarbazole, and Dobriner *et al.* [1939] have described the isolation of a hydroxy derivative of 1:2:5:6-dibenzanthracene from the 'excreta' of rabbits, rats and mice injected with this compound. 4′:8′-Dihydroxy-1:2:5:6-dibenzanthracene has been synthesized by Cason & Fieser [1940] and is considered by them to be probably identical with the rat and mouse metabolite of this carcinogen, but not with the rabbit metabolite. The metabolism of 1:2:5:6-dibenzanthracene in the fowl to a hydroxy derivative would account for the fluorescence of the bile collected after the intravenous injection of this hydrocarbon, and for the presence of a fluorescent derivative in the alkali-soluble fraction of the bile.

Summary

The elimination after intravenous injection in the fowl of ten polycyclic hydrocarbons (enumerated in Table 1) has been compared with that of 3:4-benzpyrene.

It has previously been shown that 3:4-benzpyrene is eliminated in the bile and faeces, partly unchanged and partly as a fluorescent derivative, 'BPX', which is probably a monohydroxybenzpyrene.

In the present experiments, it was found by analyses of fowl bile that:

(1) Unlike benzpyrene, none of the other hydrocarbons was eliminated unchanged in recognizable amounts.

(2) Hydrocarbons 3, 4, 5, 6, 8, 9, 10 and 11 were eliminated as fluorescent derivatives. Of these, the derivatives of hydrocarbons 8 and 9 showed a characteristic banded fluorescence spectra, analogous to that of 'BPX', while the others showed only a general fluorescence.

(3) Hydrocarbons 1 and 7 gave rise to no fluorescent derivative in the bile.

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