XXXVI. THE ELIMINATION OF 3:4-BENZPYRENE AND OTHER POLYCYCLIC HYDROCARBONS FROM THE MOUSE

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It has been shown that, after the intravenous injection of the carcinogenic hydrocarbons, 3:4-benzpyrene and methylcholanthrene, and of the non-carcinogenic hydrocarbon, anthracene, abnormally fluorescent bile is produced in rabbits, guinea-pigs, fowls and mice [Peacock, 1936]. So far, the fluorescent compounds present in such samples of bile have not been isolated, but spectrographic analysis appears to indicate the presence of derivatives of the injected aromatic hydrocarbons. The fluorescent substance, "BPX", found in the bile after the injection of 3:4-benzpyrene, has a characteristic fluorescence spectrum, and this property was utilized in the following attempt to trace the subsequent fate of this substance in the animal body.

Objections to the use of fluorescence spectrum analysis as a means of following the fate of 3:4-benzpyrene in the animal organism have been raised by Sannié [1936] who obtained fluorescence curves of 0.5, 0.05 and 0.005 g. 3:4-benzpyrene per l. hexane, by examination of the fluorescence spectra with a microphotometer, and showed that "the fluorescence spectrum of benzpyrene varied markedly with concentration". He also indicated that the fluorescence spectrum of benzpyrene varied markedly with solvent, and quoted the finding of Winterstein *et al.* [1934], that the fluorescence of anthracene is extinguished by adding 1/30,000th part of naphthacene, and concluded that it was impossible to ascertain the fate of carcinogenic hydrocarbons in tissues solely from fluorescence data. Substances, such as naphthacene, may interfere with spectrum analysis made during the extraction of anthracene or 3:4-benzpyrene from coal tar, and similar experiments, but, presumably, such substances are not present in normal tissues. Auto-absorption or re-absorption of the fluorescence bands in strong solution may take place, as suggested by Sannié, but as Hieger [1937] has pointed out this property does not prevent quantitative fluorescence analysis being made by the method of comparison of the fluorescence of extracts with the fluorescence of standards photographed on the same plate. This method was first described by Berenblum & Kendal [1936] and has been used in this laboratory, Chalmers & Peacock [1936]. It should be noted that in these experiments each extract was made in a similar manner. Conclusions have not been drawn from small changes in the intensity of fluorescence but from changes readily seen even on visual examination of the fluorescent solutions.

Effects of solvents on the fluorescence spectra of polycyclic hydrocarbons

An examination of the effects of various solvents on the fluorescence spectra of 3:4-benzpyrene, 1:2:5:6-dibenzanthracene, methylcholanthrene and anthracene has been made, and the spectra of two of these compounds in a number of

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solvents are given (Figs. 1 and 2). The positions of the maximum of the first prominent band of the fluorescence spectrum of 3:4-benzpyrene in various solvents are given below:

Solvent	Maximum of band (approximate) Å.
Alcohol	4030
Ether	4030
Light petroleum	4030
Acetone	4040
Chloroform	4050
Benzene	4050

It will be seen from the figures that the general character of the fluorescence spectrum is unchanged in the various solvents but that there is a regular shift in the position of the bands, as exemplified in the case of the first prominent band. In acetone, there is a shift of 10 Å. in the position of the fluorescence spectrum compared with alcohol, ether and light petroleum, while, in chloroform and benzene, there is a further shift of 10 Å. towards the longer wavelengths.

The fluorescence spectrum of anthracene showed a similar shift in the position of the fluorescence bands in various solvents, as illustrated, and the fluorescence spectra of 1:2:5:6-dibenzanthracene and methylcholanthrene in the solvents examined (alcohol, light petroleum, chloroform and benzene) also exhibited a similar shift in corresponding solvents. Apart from this definite shift in the position of the bands, however, the character of the fluorescence spectra of the four hydrocarbons remained unaltered.

*The effect of sodium deoxycholate on the fluorescence spectra
of polycyclic hydrocarbons*

Since the fluorescent substance found in the bile after the injection of 3:4-benzpyrene has been characterized by its fluorescence spectrum, the following tests were made to determine whether or not benzpyrene itself could be detected in the presence of bile salts by fluorescence analysis. At the same time, similar tests were made with 1:2:5:6-dibenzanthracene, methylcholanthrene and anthracene. Winterstein & Vetter [1934] recorded the fact that 3:4-benzpyrene dissolved in aqueous sodium deoxycholate to the extent of 2 mg. per ml. Fieser & Newman [1935] found that 1:2:5:6-dibenzanthracene and methylcholanthrene, and other aromatic hydrocarbons gave pure choleic acids. In their experiments, the hydrocarbon and 10–15 parts of deoxycholic acid were dissolved in hot absolute alcohol, and the character of the crystals which appeared on slow cooling at various stages of concentration indicated whether a choleic acid had been produced, or not. Some hydrocarbons, including anthracene, crystallized unchanged from the alcoholic solution, and the formation of a choleic acid was determined by the relative solubilities of the components in the solvent used. Lorenz [1935] photographed the absorption spectra of the hydrocarbon choleic acids made by Fieser & Newman, and found that these spectra and those of the unchanged hydrocarbons were identical, showing that the choleic acids were completely dissociated.

In the present instance, small quantities of the four hydrocarbons, together with 10–15 parts of sodium deoxycholate, were dissolved in acetone. In each case, on evaporation to dryness the solid residue had a violet fluorescence in the ultraviolet beam which was quite distinct from the normal yellow fluorescence

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Figs. 1 and 2. Effect of solvent on the fluorescence spectra of polycyclic hydrocarbons.
Figs. 3—7. Fluorescence spectra of extracts after intravenous polycyclic hydrocarbons.

of the unchanged hydrocarbon. The violet fluorescence of the residue appeared to be due to a solid solution of the hydrocarbon in sodium deoxycholate. It is interesting to note that all the four hydrocarbons tested gave this solid solution in sodium deoxycholate, whereas Fieser & Newman obtained choleic acids with 1:2:5:6-dibenzanthracene and methylcholanthrene, but not with anthracene. Reverting to the present experiment, the residue was then taken up in a small quantity of water and filtered. The aqueous filtrates of 3:4-benzpyrene and methylcholanthrene were clear, and those of 1:2:5:6-dibenzanthracene and anthracene cloudy; the clarity, or otherwise, of the solution depended on two factors: (1) the relative amount of bile salt present, and (2) the dilution, as will be shown later. In each case the fluorescence spectrum of the 3:4-benzpyrene in the filtered aqueous extract was similar to that of the unchanged hydrocarbon. On shaking the aqueous solution with ether in which bile salts are insoluble, practically all the fluorescent material was transferred to the ether layer.

3:4-Benzpyrene in aqueous sodium deoxycholate

Sodium deoxycholate in alcohol 10% ml.	3:4-Benz- pyrene in alcohol 0.25% ml.	Water ml.	Appearance of mixture	
			Normal light	Ultraviolet beam
1	1	2	Yellow and cloudy	Yellow
2	1	2	Yellow and slightly cloudy	Yellowish violet
4	1	2	Yellow and clear	Pale violet

In the above experiments, the solutions of sodium deoxycholate and benzpyrene were mixed and evaporated to dryness. Then the residue was taken up in 2 ml. water, and the results of the visual and ultraviolet examination of the extract are recorded. An increase in the relative amount of sodium deoxycholate, which itself has little fluorescence, resulted in an increase in the violet fluorescence of the aqueous extract. On dilution with water, the clear pale violet fluorescent solution obtained in the last experiment became cloudy and yellow fluorescent.

Aqueous suspensions of polycyclic hydrocarbons

Incidentally, since 3:4-benzpyrene formed an aqueous solution in the presence of bile salt, the possibility of injecting animals with benzpyrene dissolved or suspended in this medium was considered. Sodium deoxycholate being rather toxic, tests were made with the much less toxic compound, sodium dehydrocholate [Horrall, 1931]. 25 ml. of 0.1% 3:4-benzpyrene in alcohol were added to 1 g. sodium dehydrocholate, and, on evaporation to dryness, the residue was taken up in 50 ml. water, forming a yellow fluorescent suspension. This suspension contained 0.05% 3:4-benzpyrene and 2% sodium dehydrocholate. It could be filtered free from gross particles and boiled for sterilization and, although the solid precipitated out on standing overnight, it remained in suspension for 1-2 hr. A larger amount of 3:4-benzpyrene could be held in suspension by using a larger quantity of bile salt, and, consequently, at the expense of an increase in toxicity. Suspensions of the other hydrocarbons were made in a similar manner, and in each case a greater concentration of the hydrocarbon could be obtained than in the corresponding colloidal suspension. As a rule, the experiments described have been carried out with colloidal suspensions which, although less concentrated than the sodium dehydrocholate suspensions, do not contain relatively large quantities of bile salt. The presence of the bile salt is a disadvantage in that an extraneous factor is introduced into the experiment and that the resultant suspension is somewhat toxic. The suspensions were not

highly toxic, since the following successful intravenous injections were made during the course of experiments on the fate of polycyclic hydrocarbons in the animal body.

Animal	Volume of suspension injected	Sodium dehydrocholate in suspension g.
1 rabbit	6 ml. of 0.075% 3:4-benzpyrene	0.2
1 fowl	4 ml. of 0.075% methylcholanthrene	0.2
3 fowls	5 ml. of 0.05% 3:4-benzpyrene	0.1
3 fowls	5 ml. of 0.08% anthracene	0.1
5 mice	0.5 ml. of 0.05% 3:4-benzpyrene	0.01
5 mice	0.5 ml. of 0.08% anthracene	0.01

All these animals survived the injection for more than 2 days. The injection of 0.2 and 0.5 ml. of an anthracene suspension containing 0.01 g. sodium dehydrocholate proved fatal in two mice, but larger quantities have been injected without any apparent ill effects and it is probable that these fatalities were due to emboli caused by solid particles.

General methods for elimination experiments

Mice were injected intravenously with small quantities of 3:4-benzpyrene, methylcholanthrene and anthracene colloids and the faeces and urine collected for 24 hr. Then the animals were reinjected with a similar quantity of colloid and killed 1 hr. later, when it was known that the fluorescence of the bile in the gall-bladder would be at about maximum intensity. The small quantity of bile in the mouse gall-bladder proved sufficient for fluorescence analysis. Alcoholic extracts of the bile, urine and faeces were made and examined spectrographically. The extracts were examined in a small quartz cell which was attached to the slit of a Hilger E 3 quartz spectrograph by a wire support. In one or two cases, where the volume of the extract was very small, the examination was made in a capillary tube. A Kelvin, Bottomley and Baird quartz mercury vapour lamp, fitted with a Wood's glass filter, provided the source of ultraviolet illumination.

Elimination of 3:4-benzpyrene (Fig. 3). In this experiment, a mouse was injected intravenously with 1.0 ml. of a 0.03% 3:4-benzpyrene colloid.

Spectrum I. Fluorescence of alcoholic extract of bile—diffuse bands at (4100–4250) and (4350–4500) Å.

Spectrum II. Fluorescence of alcoholic extract of lungs—benzpyrene bands.

On examination of the mouse *post mortem*, the lungs showed a number of yellow fluorescent spots which proved to be particles of the unchanged hydrocarbon. The same effect was observed after the injection of methylcholanthrene and anthracene.

Spectrum III. Fluorescence of alcoholic extract of urine—general fluorescence.

Spectrum IV. Fluorescence of alcoholic extract of normal urine—general fluorescence.

No significant change in the fluorescence of urine after the injection of 3:4-benzpyrene was noted. Normal mouse urine has a greyish blue fluorescence which would be sufficient to mask the presence of benzpyrene derivatives having a weak-banded or general fluorescence.

Elimination of 3:4-benzpyrene (Fig. 4). In this experiment, a mouse was injected intravenously with 0.4 ml. of a 0.03% 3:4-benzpyrene colloid.

Spectrum I. Fluorescence of alcoholic extract of bile—diffuse bands at (4150–4300) and (4350–4500) Å.

Spectrum II. Fluorescence of alcoholic extract of faeces—diffuse bands at (4300–4400) and (4500–4600) Å.

An extract of faeces collected after the first 24 hr. did not show fluorescence bands.

To confirm the presence of an alcohol-soluble substance showing banded fluorescence in the faeces after 3:4-benzpyrene injection and to test the effect of varying the quantity of injection, a series of experiments was carried out with injections of 0.2, 0.4, 0.6, 0.8 and 1.0 ml. of a 0.03 % colloid. In each case the alcoholic extract of a 24-hr. specimen of faeces showed fluorescent bands in the blue-violet region of the spectrum, as described above.

It therefore appears that 3:4-benzpyrene is partly eliminated in the faeces as an alcohol-soluble substance, showing fluorescence bands at (4300–4400) and (4500–4600) Å.

Fractionation of alcoholic extract of faeces (Fig. 5). An attempt was made to fractionate the fluorescent substance in the alcoholic extract of the pooled faeces from six mice, each of which was injected with 0.6 ml. 3:4-benzpyrene colloid. The extract was diluted and treated with ether. The bulk of the fluorescent substance was soluble in ether, and the ether-insoluble fraction proved to be soluble in amyl alcohol. Fluorescence spectra of the ether-soluble and ether-insoluble fractions are shown. It should be noted that the ether-insoluble fraction—called “BPF (1)” —having bands at (4100–4200) and (4300–4450) Å., similar to “BPX”, was present in much smaller quantity than the ether-soluble fraction, called “BPF (2)”, having bands at (4300–4400) and (4550–4650) Å., similar to the initial alcoholic extract of faeces, and therefore had no appreciable influence on the spectrum of the original extract (see Fig. 4), which was made from a relatively small volume of faeces.

The ether-soluble fraction was obtained as a light yellow coloured solution which had a bright violet fluorescence, even in ordinary light. It was unstable in air, changing to a non-fluorescent substance and, consequently, all manipulations were carried out, as far as possible, in an atmosphere of nitrogen. On hydrolysis of this ether-soluble material, the unsaponifiable fraction contained a compound showing a banded fluorescence similar to that of the parent substance but with a slight shift in the position of the bands.

Elimination of methylcholanthrene (Fig. 6). In this experiment a mouse was injected intravenously with 0.4 ml. of a 0.075 % methylcholanthrene suspension.

Spectrum I. Fluorescence of alcoholic extract of faeces—general fluorescence.

Spectrum II. Fluorescence of alcoholic extract of urine—general fluorescence.

Spectrum III. Fluorescence of alcoholic extract of bile—general fluorescence.

Spectrum IV. Fluorescence of alcoholic extract of lungs—banded fluorescence of methylcholanthrene.

The spectrum of the material extracted from the lungs was that of the unchanged hydrocarbon, and the spectrum of the bile having a general fluorescence was presumably that of a derivative. Since the bile substance had no characteristic fluorescence spectrum, it was impossible to obtain any definite indication of its subsequent fate in the mouse by fluorescence analysis.

The quantity of fluorescent bile produced by the mouse in this experiment was small, and the spectrographic examination was carried out in a capillary tube. A larger quantity of bile was produced by the intravenous injection of a fowl with 4 ml. of a 0.075 % suspension of methylcholanthrene in aqueous sodium dehydrocholate and, on examination of an alcoholic extract, a general fluorescence in the blue-violet region of the spectrum was observed, similar to that given by the extract of mouse bile. Fluorescent bile produced in the mouse and in the fowl after the intravenous injection of 3:4-benzpyrene colloid, showed

the same banded fluorescence which has been described above in the case of the mouse.

The experiment on the elimination of methylcholanthrene has been repeated on a series of eight mice injected intravenously with 0.4, 0.5, 0.9, 1.0, 1.0, 1.0, 1.0 and 1.2 ml., respectively, of a 0.03% colloid. Methylcholanthrene colloid was used instead of the suspension in aqueous sodium dehydrocholate so that the experiment would be comparable with that on 3:4-benzpyrene. Alcoholic extracts of 24-hr. specimens of urine and faeces were fractionated with ether and amyl alcohol. The fluorescence spectra of the various extracts showed a general fluorescence, except in the case of the ether extract of faeces, which showed a very faint band with a maximum at about 4300 Å. The intensity of this band, however, was small compared with the intensity of the fluorescence bands of an ether extract of faeces after 3:4-benzpyrene.

The elimination of anthracene (Fig. 7). Experiments with anthracene gave similar results to those with methylcholanthrene. In the experiment illustrated, a mouse was injected intravenously with 0.2 ml. of a 0.06% anthracene suspension in aqueous sodium dehydrocholate. Alcoholic extracts of the bile, urine and faeces showed a general fluorescence in the blue-violet region of the spectrum.

As in the methylcholanthrene experiment, a larger quantity of bile was produced by the intravenous injection of a fowl with 4 ml. of a 0.06% suspension of anthracene in aqueous sodium dehydrocholate. The bile collected *post mortem* was extracted with alcohol, and the fluorescence spectrum of this extract showed a general fluorescence in the blue-violet region of the spectrum, similar to that of the mouse bile.

The experiment on the elimination of anthracene has been repeated on a series of seven mice, each of which was injected intravenously with a 0.03% anthracene colloid, one with 0.6 ml. and the others with 1.0 ml. The alcoholic extracts of 24 hr. specimens of faeces and urine were fractionated with ether and amyl alcohol, as in the corresponding 3:4-benzpyrene and methylcholanthrene experiments. The fluorescence spectra of the various extracts indicated the absence of compounds having a characteristic banded fluorescence spectrum from the urine and faeces of mice after the injection of anthracene colloid.

DISCUSSION

The results of the spectrographic analysis described above give a qualitative indication of the fate of 3:4-benzpyrene in the mouse after intravenous injection. The fluorescence spectrum of the bile substance, BPX, showing two diffuse bands, is different from that of 3:4-benzpyrene, which shows a number of sharp bands, and the spectrum of benzpyrene is unaltered by the addition of the bile salt, sodium deoxycholate. The difference in the fluorescence spectrum, coupled with the insolubility of "BPX" in ether, in which solvent 3:4-benzpyrene is soluble, suggests the presence of a derivative or an additive compound of benzpyrene in the "BPX" fraction.

The subsequent fate of "BPX" in the mouse has been considered, and an examination of the faeces collected after the intravenous injection of 3:4-benzpyrene colloid demonstrated the presence of two substances showing a banded fluorescence in the blue-violet region of the spectrum:

- (1) "BPF (1)", which is ether-insoluble and has a fluorescence spectrum similar to "BPX";
- (2) "BPF (2)", which is ether-soluble, and has a fluorescence spectrum similar to "BPX" but showing a shift in the position of the bands.

"BPF (1)", which was present only in small amounts, appears to be residual "BPX", and it seems to be probable that "BPX" is converted into "BPF (2)" in the intestine and finally eliminated as such.

The difference between the fluorescence spectra of "BPX" and "BPF (2)", i.e. a simple shift in the position of the bands, might be caused by spectroscopically interfering substances in the bile or faeces. An attempt to control this factor was made by adding an extract of normal mouse bile to the fluorescent extract of faeces, and an extract of normal mouse faeces to the fluorescent extract of bile. In each case alcohol was the solvent used. No change in the position of the bands, however, was caused by these additions. Solubility tests appeared to confirm the results of spectrographic analysis, "BPX" being ether-insoluble and "BPF (2)" ether-soluble, and it would therefore appear that 3:4-benzpyrene, "BPX" and "BPF (2)" are three distinct substances, although a final decision can only be made on this point after the isolation of the fluorescent compounds in question.

No significant change in the fluorescence spectrum of alcoholic extracts of faeces was found on varying the injection of 3:4-benzpyrene colloid from 0.2 to 1.0 ml., and the presence of "BPF (2)" in the faeces does not depend on the amount of injection. "BPF (1)", which is presumably present in all the samples, is either much less intensely fluorescent than "BPF (2)", or is present in much smaller amount.

Fluorescence methods did not give any definite evidence of the elimination of methylcholanthrene and anthracene from the mouse, probably owing to the fact that the fluorescent bile produced on intravenous injection of these hydrocarbons had a general and not a banded fluorescence.

No attempt has been made to account for the quantitative elimination of 3:4-benzpyrene and, although definite evidence has been obtained of its qualitative elimination in the faeces, it is possible that this is only part of the mechanism of elimination and that elimination in the urine may be an important factor. With regard to elimination of polycyclic hydrocarbons in the urine, Boyland & Levi [1935, 1936] have reported the isolation of anthracene derivatives from the urine of rats and rabbits fed on a diet containing anthracene.

SUMMARY

The fluorescence of mouse bile, faeces and urine, after the intravenous injection of 3:4-benzpyrene, methylcholanthrene and anthracene colloids is described. 3:4-Benzpyrene is eliminated in the faeces as an ether-insoluble substance, having a fluorescence spectrum similar to "BPX", and as an ether-soluble substance, having a fluorescence spectrum similar to "BPF (2)" but showing a shift in the position of the fluorescence bands of approximately 200 Å. towards the longer wave-lengths. The former substance appears to be present only in relatively small amount.

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