CLXXVII. FURTHER EVIDENCE REGARDING THE ELIMINATION OF CERTAIN POLYCYCLIC HYDROCARBONS FROM THE ANIMAL BODY.

By JAMES GOWANS CHALMERS AND
PHILIP REDINGTON PEACOCK.

From the Research Department, Glasgow Royal Cancer Hospital.

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RECENT fluorescence studies [Peacock, 1936], in which fluorescent samples of bile were obtained after intravenous injection of colloidal solutions of certain polycyclic aromatic hydrocarbons (1:2-benzpyrene, methylcholanthrene and anthracene) into fowls, rabbits, guinea-pigs and mice, raised the question of the presence of these compounds in such samples of bile and in the rest of the animal.

Extraction of 1:2:5:6-dibenzanthracene had already been considered. In a previous communication [Chalmers, 1934] the quantity of dibenzanthracene in the breast muscles of fowls, at short intervals after local injection of the hydrocarbon dissolved in chicken fat or in egg-yolk fat, was determined spectrographically by absorption analysis of extracts in a fat solvent, and it was found that this hydrocarbon rapidly disappeared from the site of injection. Consideration of the possible excretion of unchanged 1:2:5:6-dibenzanthracene was deferred, as the extracts of excreta were unsuitable for absorption spectrography, owing to the presence of substances showing a general absorption in the ultra-violet. Although the methods of extraction were improved, the extracts were still unsuitable for absorption spectrum analysis. The contaminants however did not significantly mask the fluorescence spectrum of 1:2:5:6-dibenzanthracene, and subsequent analysis failed to reveal the presence of this substance in extracts of excreta. The fluorescence spectrum of 1:2:5:6-dibenzanthracene has already been described [Hieger, 1930]. Fluorescence methods were also used by Berenblum & Kendal [1936], who reported that no 1:2:5:6-dibenzanthracene could be detected in the excreta of mice injected intraperitoneally with the compound dissolved in lard or in colloidal solution. Lorenz & Shear [1936] have described a method of separating 1:2:5:6-dibenzanthracene from spectroscopically interfering substances in mouse extracts by the selective action of solvents on the unsaponifiable fraction. The colouring matter was then removed by chilling and absorption on Al_2O_3 . In the preparation of 1:2:5:6-dibenzanthracene extracts for spectrography, as carried out by Berenblum & Kendal [1936], mice were killed and boiled under reflux condensers with alcoholic potash and subsequently extracted with benzene. The fluorescence of the benzene solution was then examined. In the extraction of 1:2:5:6-dibenzanthracene from excreta and from chick embryos, as will be seen, the ether extract after saponification with alcoholic potash required further treatment before fluorescence analysis was practicable. This further treatment involved the elimination of colouring matter by adsorption from a light petroleum solution of the material left after saponification and the removal of cholesterol. This method was applied to the other hydrocarbons under examination.

The evidence of chemical change of dibenzanthracene, after intramuscular injection, rendered it important to determine whether other polycyclic aromatic

hydrocarbons (carcinogens and non-carcinogens) were altered in a similar way. At the same time, it was hoped to get an indication of the effect of the solvent on the rate of chemical change. Experiments on the production of sarcoma in fowls indicate that the solvent plays an important part in the induction of tumour growth [Peacock, 1935]. The results of these experiments hitherto may be summarized, as follows:

1:2:5:6-Dibenzanthracene in lard gave 16 tumours in 31 birds, in 1-4 years
 1:2:5:6-Dibenzanthracene in egg-yolk fat gave 3 tumours in 12 birds, in 1 year
 1:2:5:6-Dibenzanthracene in egg-yolk fat gave 0 tumours in 10 birds, in 1-3 years
 1:2:5:6-Dibenzanthracene in chicken fat gave 0 tumours in 12 birds, in 2 years

Somewhat similar results were obtained by Watson [1935], who found that pinene tar, mixed with paraffin wax, produced sarcoma in 3 out of 12 rats, whilst the same tar, mixed with rat tissue extract, failed to produce tumours in a similar group of animals.

Methods of extraction.

Initial treatment. In early experiments, the following methods for extraction of fats, were tried: (1) treatment with acetone (a better solvent for 1:2:5:6-dibenzanthracene than alcohol) and (2) desiccation, followed by extraction with a fat solvent. In later experiments, however, treatment with strong caustic potash, as recommended by Berenblum & Kendal [1936], was adopted. This resulted in a considerable saving of time, as it provided an admirable preparation for the second stage.

Saponification. Care was taken to use specially purified reagents for this process and to reduce the saponification time to a minimum, as, otherwise, the action of heat on the alcoholic potash solution gave rise to the formation of resinous material which showed a blue fluorescence. The alcohol was purified by treatment with silver nitrate [Palmer, 1922]. After the initial treatment, as above, the extract only required dilution to 25-30% caustic potash and the addition of alcohol to be ready for saponification. The unsaponifiable material was extracted with ether in the usual way, one saponification usually being sufficient though, on occasion, a second treatment was required.

Precipitation with digitonin. The unsaponifiable fraction, which was obtained as a yellow solid, was dissolved in 95% alcohol and an excess of digitonin (1% in 90% alcohol) was added at the boiling-point. A good separation of the cholesterol was obtained by allowing the digitonide to precipitate out overnight, but satisfactory and quicker results were obtained by making use of the insolubility of the digitonide in water and acetone [Yasuda, 1931]. The ether extract of the diluted acetone solution was well washed with water to remove the excess of digitonin. Cholesterol was not removed except in the experiments with 1:2:5:6-dibenzanthracene embryos and excreta. The appreciable amount of cholesterol present in the chick embryos interfered with the examination of a final extract of less than 5 ml.

Treatment with adsorbents. Preliminary experiments were carried out with the pigment of egg-yolk fat. A certain amount of the colouring matter was eliminated along with the fat during the extraction of the unsaponifiable fraction. It was found that light petroleum was more effective than ether, alcohol, benzene or chloroform as a solvent from which to adsorb egg-yolk pigment. A number of adsorbents were tried: some adsorbed the hydrocarbon itself and were consequently valueless; of the others, fuller's earth was the most generally useful. A single treatment with fuller's earth, as a rule, removed most of the pigments from chick embryos and from excreta.

Excreta experiments.

1:2:5:6-Dibenzanthracene and 1:2-benzpyrene. Two fowls were injected intramuscularly with 1:2:5:6-dibenzanthracene (4 ml. of 0.2% solution in chicken fat), and two with 1:2-benzpyrene (1 ml. and 4 ml. of 0.2% solution in chicken fat), and the excreta were collected separately, over 2 weeks. According to previous work, if these compounds were eliminated unchanged, they should be detectable in the excreta during this period. The initial treatment in each case consisted of repeated extraction with acetone. 0.2 mg. of the corresponding hydrocarbon was added to one-half of the acetone extract, in each case, and thereafter the two fractions were treated alike. The residue, after evaporation of the acetone, was extracted by the method described above. The final extracts of excreta showed a slight blue fluorescence in the ultraviolet beam, but spectrographic examination failed to show the fluorescence bands of the appropriate hydrocarbon. Bands were however seen in the extracts to which the compound had been added as described.

Chick embryo experiments.

Intramuscular injection of 1:2:5:6-dibenzanthracene and 1:2-benzpyrene. Difficulties in the extraction of excreta from individual animals are obviated by the use of chick embryos, which may be regarded as a "closed" system. It was found best to open a window in the shell and membranes the day before injecting the embryo, rather than to attempt the whole procedure at one operation. In this way, it was possible to be sure that the embryo had survived the inevitable damage to the amniotic vessels and consequent slight haemorrhage. 15-18-day-old embryos were injected intramuscularly with 0.1 ml. of fatty or colloidal solution of the hydrocarbon under test, and the embryos were subsequently killed and extracted at short intervals. For purposes of this experiment, the chicks were not allowed to hatch out. It may be mentioned, however, that some chicks injected *in ovo* have been allowed to hatch out, and these showed no obvious abnormality.

1:2:5:6-Dibenzanthracene. The results of these injections were unsatisfactory, since the small quantity of the compound which could be injected into the embryo (0.1 ml. of 0.2% solution in fat) did not give a sufficiently luminous fluorescence spectrum to permit of comparative tests of much accuracy, but it was found that the substance was altered within the embryo a few days after intramuscular injection.

1:2-Benzpyrene. The extracts of embryos injected with this substance gave a much brighter fluorescence than that obtained in corresponding experiments with 1:2:5:6-dibenzanthracene. The results of injections of 1:2-benzpyrene dissolved in egg-yolk fat are shown in Pl. II, fig. 1, from which it will be seen that most of the hydrocarbon was eliminated 90 hours after injection.

Intravenous injections of colloidal hydrocarbons.

Methods of preparing colloidal solutions of hydrocarbons have been recorded [Berenblum, 1932; Boyland, 1932; Boyland & Burrows, 1935]. The colloids used in the present experiments were prepared by precipitation of the hydrocarbon from solution in acetone, which was then removed by dialysis.

Technique of intravenous injection of embryo. 14-18-day-old embryos were selected for injection, since they are sufficiently large to be handled without receiving gross damage and yet are mobile within the shell. The egg was transferred from the incubator to a sterile dissecting chamber, without turning. The

surface of the shell was swabbed with spirit-flavine (acriflavine, 1 part; water, 1000 parts; methylated spirit, 2000 parts), which dries rapidly. A window about 1 cm. square was cut in the shell with a sterile ampoule saw mounted in a handle, and the shell membrane was cut away with small sterile scissors, leaving the amniotic membrane exposed. Intravenous injection was sometimes made into an amniotic blood vessel, but, as a rule, the amniotic membrane was ruptured and the injection was made into the vitelline vein which is easily picked up as it lies on the yolk sac. The latter can be handled with small forceps, if necessary, to bring the vein into position for injection. No. 20 conjunctival needles were used for the injections and, although there was always a little haemorrhage, this was rarely severe. It was found advisable to wait for a few seconds after completing the injection before withdrawing the needle, to avoid possible loss of colloid. The window was then closed with sterile cellophane fixed to the shell with paraffin wax; this gave a free view of the embryo, which could thereafter be observed at frequent intervals without disturbance.

Elimination of 1:2-benzpyrene colloid after intravenous injection. The results of this experiment are illustrated in Pl. II, fig. 2, the explanation of which is given below in tabular form:

Time after injection	Colloid %	Volume injected ml.	Concentration of extract	Remarks on fluorescence spectrum
0	0.03	0.1	1.0	—
17 hours	0.03	0.1	1.0	No bands seen
0	—	—	0.025 (standard)	—
30 min.	0.003	0.3	1.0	—
20 min.	0.003	0.3	1.0	—
0	—	—	0.1 (standard)	—
6 hours	0.03	0.3	1.0	Intensity of bands greatly reduced
5 hours 30 min.	0.03	0.3	1.0	—
1 hour 30 min.	0.03	0.3	1.0	—
1 hour	0.03	0.3	1.0	—
40 min.	0.03	0.3	1.0	—
0	0.03	0.3	1.0 (standard)	—

Chick embryos killed shortly after intravenous injections of 0.3 ml. of 0.03 % 1:2-benzpyrene colloid showed highly fluorescent body fat when examined in the ultraviolet beam, this fluorescence being due to the dissolved 1:2-benzpyrene, as proved spectrographically.

It was noticed that the gall bladder of embryonic chicks showed no fluorescence similar to that shown by adult fowls and mammals. This appears to be due to the passive role of the embryonic gall bladder which, though filled with bile, does not seem to contract *in ovo*. This point is of some interest, and the view expressed is borne out by the following experiments in 1-3-day-old chicks.

Chick experiments. Incubator-hatched chicks are normally kept in the incubator for 2 or 3 days after hatching, during which time they have no access to food and are still nourished by their egg-yolk sacs which have retracted into the peritoneal cavity. Such chicks behave like embryonic chicks in that their bile is not rendered fluorescent by intravenous injection of 1:2-benzpyrene colloid. On the other hand, hen-hatched chicks, which had access to food, were found to behave like adult fowls in that their bile was rapidly rendered fluorescent by intravenous injection of the colloid. The passage of food from the gizzard to the duodenum apparently initiates the contraction of the gall bladder, which refills with fluorescent hepatic bile.

Mouse experiments.

To test the mechanism of hydrocarbon elimination on mammals comparable in weight with chicks, mice were injected in the tail vein with colloidal solutions of 1:2:5:6-dibenzanthracene, 1:2-benzpyrene, methylcholanthrene and anthracene. All except 1:2:5:6-dibenzanthracene mice showed the presence of a fluorescent substance in the bile 1–2 hours after injection. In making extracts of whole mice, initial treatment with caustic potash was followed by saponification. An almost colourless extract of the unsaponifiable fraction was obtained, and the fluorescent spectrum of this fraction was examined without further treatment.

Methylcholanthrene. The extract of an animal killed 3 hours after injection of 0.5 ml. of 0.01 % colloid did not show the fluorescence spectrum bands shown by the extract of a mouse killed immediately after injection.

Anthracene. Mice were injected intravenously with 0.5 ml. of 0.6 % colloidal solution and the animals killed at intervals after injection. The fluorescent spectra of the extracts of the whole mice are shown (Pl. II, fig. 3). It is just possible that the general fluorescence masks the anthracene spectrum in the early extracts, but in the 17-hour extract there is no evidence of the presence of the hydrocarbon.

Notes on fluorescence analysis.

The fluorescence spectra were recorded on a Hilger E 3 quartz spectrograph.¹ The source of ultraviolet rays was a Kelvin, Bottomley and Baird quartz mercury lamp, which was fitted with a Wood's glass filter, and the solutions were examined in a small flat-sided quartz cell fitted with a flat vita-glass cover to prevent loss of evaporation.

Pl. II, fig. 4 gives comparative spectra for the three carcinogens under test, and shows that the fluorescence of 1:2:5:6-dibenzanthracene is much less intense than that of methylcholanthrene or 1:2-benzpyrene.

In the course of the experiments carried out in this laboratory, the shift in the fluorescence bands of 1:2-benzpyrene with different solvents, described by Sannié [1936], has been observed, the bands in alcohol and light petroleum solutions being displaced about 50 Å. towards the ultraviolet region, relative to the bands in chloroform and benzene. Similar displacement in the fluorescence bands of 1:2:5:6-dibenzanthracene and methylcholanthrene in these solvents has also been noted. The importance of specifying the solvents used in such experiments is therefore obvious.

DISCUSSION.

The dosage of colloid, per body weight used in the case of small animals represented 5–10 times that employed in the case of large animals; comparable doses, in the case of fowls and rabbits, would have involved injection of about 50 ml. colloid. Equivalent dosage for small animals was therefore obtained by injecting a certain number with diluted colloid, which gave similar but less marked results. There seems to be no reason to doubt that essentially the same mechanism was involved in all the animals tested.

Benzpyrene, methylcholanthrene and anthracene appeared to be eliminated in the same way, but, as the fluorescence of benzpyrene and of its derivatives is much more brilliant and therefore more easily detected than that of the other hydrocarbons, most of the work has been done with this substance.

The course of events, after intravenous injection of 1:2-benzpyrene colloid, is substantially the same in rabbits, guinea-pigs, mice, fowls and chicks and may be summarized as follows:

¹ Purchased with a grant from the British Cancer Campaign.

0-15 min. after injection. Fresh blood films examined by ultraviolet illumination show particles of yellow fluorescence in Brownian movement, the corpuscles appearing as dark non-fluorescent bodies. Naked eye examination shows that the animal's body fat is beginning to dissolve the benzpyrene, thereby acquiring a violet fluorescence.

15-30 min. after injection. Blood films show greatly reduced numbers of fluorescent particles which are larger and no longer show Brownian movement. They are independent of the blood cells. At this time, body fat shows violet fluorescence, proved spectrographically to be due to unaltered benzpyrene. Frozen sections of liver, examined by ultraviolet illumination, show lavender-coloured fluorescent droplets in the liver cells. (Morphologically similar droplets in controls show greenish fluorescence.)

30 min. to 2 hours after injection. Blood films show no fluorescence; body fat fluorescence diminishes; fluorescent bile appears in the gall bladder, reaching a maximum at from 1 to 2 hours. This fluorescence is due to a water-soluble derivative, or derivatives, which does not show fluorescent bands of benzpyrene.

2-6 hours after injection. Fluorescence of body fat disappears and fluorescence of bile diminishes and finally returns to normal (brownish green fluorescence).

1:2:5:6-Dibenzanthracene colloid disappears from the circulating blood like the other hydrocarbons investigated, but its subsequent fate has not been demonstrated. If it forms a bile-soluble derivative, this must be of feeble fluorescence or non-fluorescent.

Recently, most of our work has been done on mice and chicks, since the saving in costly materials and the ease of housing large numbers outweigh consideration of technical difficulties. As the intravenous injection of chick embryos does not seem to be a procedure in common use, a short description of the technique has been given above, but it may be mentioned here that chick embryos are quite easily handled and injected successfully after a little practice. 1-5-day-old chicks are much more difficult to inject, and light anaesthesia is required to prevent struggling.

As far as the colloids of 1:2-benzpyrene, methylcholanthrene and anthracene are concerned, the whole process, from the moment of intravenous injection to the time of final elimination by the liver, can be followed visually by virtue of the fluorescence of these hydrocarbons and their derivatives. Blood films for examination are examined fresh and unstained under a 4 mm. objective; no special lenses are required, as the ordinary optical glass transmits the near ultraviolet rays that penetrate the Wood's glass filter and are used to excite the fluorescence. The fluorescence of subcutaneous fat can be seen through the skin, particularly in the neck region of young chicks. Examination of samples of bile, in the case of fowls, was facilitated by the formation of biliary fistulae by preliminary cholecystostomy, from which bile could be withdrawn before and at frequent intervals after intravenous injection of the colloids. Fowls tolerate such fistulae well, but rabbits and guinea-pigs do not, and in these animals it was necessary to perform laparotomy for the collection of samples, at various time intervals after intravenous injection. The subsequent fate of the bile-soluble derivatives has not been satisfactorily determined, owing to the general nature of their fluorescence spectra. In the case of mice however it is possible to trace the fluorescent cystic bile in the duodenum and in gradually diminishing intensity in the small intestine. It is possible that destruction, or reabsorption, of these fluorescent derivatives occurs in the intestine, and that this explains their absence from the faeces.

Excretion in the bile is probably common to many substances in addition to

those described, and it seems likely that any carcinogenic substance of the 1:2-benzanthracene type that might be formed in the body would be eliminated by this route. The problem of the mode of action of these carcinogens is somewhat simplified by the foregoing observations, for it seems clear that our efforts should now be concentrated on the early events of the latent period, rather than on that part of the cycle of events generally regarded as the earliest stage of malignancy, namely, the period of first demonstrable tumour growth. It is possible that the action of the chemical carcinogens is far more rapid than has been generally assumed, and that essential changes occur in the cell long before malignant multiplication commences. If this is so, it is possible that histological research may reveal hitherto unrecognized appearances characteristic of such early cytological changes. If, on the other hand, these carcinogens act as such and over a prolonged time, then the amounts required for carcinogenesis must be very minute and they must become fixed in some way at the site of injection so as to be protected from the process of elimination.

SUMMARY.

1. Methods of extraction of certain polycyclic hydrocarbons from chick embryos and from excreta are described.
2. Benzpyrene and dibenzanthracene have not been detected in the excreta of fowls injected intramuscularly with these substances in fatty solution.
3. Benzpyrene and dibenzanthracene are eliminated from chick embryos within a few days of intramuscular injection in fatty solution.
4. Benzpyrene is eliminated from chick embryos within a few hours of intravenous injection of colloidal solution.
5. Benzpyrene, methylcholanthrene and anthracene are eliminated from mice within a few hours of intravenous injection in colloidal solution.
6. The mechanism of elimination of benzpyrene, methylcholanthrene and anthracene, and the excretion of their fluorescent water-soluble derivatives in the bile and their recovery from the gall bladder, is described.
7. The absence of such fluorescent derivatives from the gall bladders of chick embryos and of 1-3-day-old incubator-hatched chicks previously injected intravenously with benzpyrene colloid is noted and explained.

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DBA denotes 1:2:5:6-dibenzanthracene
BP 1:2-benzpyrene
MeC methylcholanthrene