11. The Rate of Disappearance of 3:4-Benzpyrene from the Mouse after Subcutaneous and Intraperitoneal Injection

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The continuous disappearance of carcinogenic polycyclic hydrocarbons from the site of injection [Chalmers, 1934; Berenblum & Kendal, 1934; 1936; Lorenz & Shear, 1936; Hieger, 1936; Peacock, 1936], the absence of their accumulation, to any significant extent, in other parts of the body or in the excreta [Berenblum & Kendal, 1936; Chalmers & Kirby, 1940], and the demonstration of derivatives in the excreta [Chalmers & Peacock, 1936; Chalmers, 1938; 1940; Dobriner *et al.* 1939; Boyland *et al.* 1941], constitute convincing evidence of the ability of the body to metabolize such hydrocarbons. It is important to consider, therefore, whether carcinogenesis is dependent on the presence of the unchanged hydrocarbons themselves, or whether it is due to the action of some of their metabolic products.

Some indication regarding this problem may be gathered from a correlation, under varying biological conditions, of the carcinogenicity of a hydrocarbon after a single injection and its persistence in an unchanged form at the site of injection. A close correlation between the two would suggest that the hydrocarbon was itself responsible for the neoplastic change, while an inverse relationship would imply its preliminary conversion into another compound as a necessary step in the carcinogenic process. Investigations on these lines have already been attempted by several workers.

Following intraperitoneal injection of 1:2:5:6-dibenzanthracene into mice, both the rate of disappearance and the efficacy of carcinogenic action were found to depend on the medium in which the hydrocarbon had been injected [Berenblum & Kendal, 1936]. After a single injection of 2 mg. in lard, almost complete disappearance occurred in about 6 weeks, whereas after injection of the same amount as a colloidal solution in water, about 25% was still present in the animal after as long as 24 weeks. Of the two, the colloidal solution appeared to be the more effective in producing tumours, though the data for this were rather scanty. That the solvent can strongly influence carcinogenesis has been shown also by Peacock [1935], Shear [1936], Oberling *et al.* [1939], Shimkin & Andervont [1940] and others. Peacock & Beck [1938] studied the carcinogenicity and the time of disappearance of 3:4-benzpyrene after subcutaneous injection in mice, using the benzpyrene either powdered or dissolved in ether, mouse fat, total mouse lipoids, olive oil or a mixture of olive oil and paraffin. They claimed to have found a close parallel between carcinogenicity and persistence of the hydrocarbon at the site of injection. However, their criterion of persistence of unchanged benzpyrene was based on the naked-eye appearance of a violent fluorescence of the tissues in ultraviolet light. This method was found to be unreliable by the present authors, since, as will be shown below, a blue fluorescence, due to an impurity present in variable amounts in different samples of benzpyrene, can sometimes be mistaken for the violet fluorescence of benzpyrene and can persist at the site of injection long after the complete disappearance of the benzpyrene itself.

Another line of approach, adopted by Hieger [1936], was to compare the rates of disappearance of three hydrocarbons possessing different carcinogenic potencies (1:2-benzanthracene, 1:2:5:6-dibenzanthracene and 3:4-benzpyrene). He also found a correla-

tion between carcinogenicity and persistence in the tissues; the differences in the rates of disappearance were not very striking.

The present investigations were designed to study the problem from yet a third angle, which involved a comparison of the carcinogenicity and the rate of disappearance of a given carcinogen (3:4-benzpyrene) dissolved in a given medium (sesame oil), but injected at different sites in the body (subcutaneously and intraperitoneally respectively).

EXPERIMENTAL

All the experiments were carried out on white mice which received a single injection of benzpyrene in sesame oil. Altogether 350 mice were used; these were of a fairly homogeneous (though not genetically pure) strain, bred in this laboratory over a period of many years. In about half, the injections were intraperitoneal, in the others, subcutaneous. While the volume injected was always the same (0.5 ml.), the dose of benzpyrene varied in different groups (20 μ g., 200 μ g., 2 mg. and 10 mg. respectively). Some of the mice were killed at intervals for quantitative estimation of benzpyrene in the whole animal and some for quantitative estimations in separate organs and tissues; others were kept for observation of tumour production (up to 8 months). The benzpyrene contents were determined by the method of Berenblum & Schoental [1942].

Some irregularities in the results (especially in the earlier experiments) were traced to faults in the method of injection. The most serious source of error was an escape of the oily solutions through the needle puncture. It occurred most often after subcutaneous injections made through the skin of the groin into the axilla, but was practically eliminated by injecting through the skin of the mid-dorsal region into the subcutaneous tissues of the sacral region. As a further precaution, the skin punctures were sealed with collodion. In the case of intraperitoneal injections, such surface leakage was avoided by injecting at an angle, taking advantage of the valvular action of the abdominal coats, and by sealing the puncture with collodion. Here, however, unduly high values were sometimes obtained, owing to leakage from the peritoneal cavity into the subcutaneous tissues along the track of the needle. This could usually be detected by the presence of a palpable nodule in the abdominal wall. Animals in which either of these sources of error could be traced were discarded.

The disappearance of benzpyrene from whole mice

The time curves for the persistence of unchanged benzpyrene in whole mice after subcutaneous and intraperitoneal injections are shown in Fig. 1; the amounts of benzpyrene are plotted on a logarithmic scale. For the sake of clarity, the values obtained with injections of 20 μ g. are not recorded in Fig. 1. The curves for these were essentially the same in type as those for the larger quantities.

From consideration of these curves, and from autopsy examinations at various stages of the experiment, the following conclusions may be drawn.

(1) Benzpyrene in sesame oil disappears from the body about 15 times more rapidly when injected intraperitoneally than when injected subcutaneously.

(2) The logarithms of the values for benzpyrene persisting in the animals, plotted against time, follow approximately parallel straight lines for each site of injection, indicating that, for any one site of injection, the rate of disappearance is proportional to the concentration, and therefore, that the time taken for a given percentage to disappear is constant over a very wide range of concentration.

(3) None of the mice injected intraperitoneally developed tumours after 8 months, whereas in the case of subcutaneous injections, sarcomata developed at the site of injection in several animals of each group, even among those receiving the lowest concentration of benzpyrene (20 μ g.).

(4) The tissues at the site of injection of benzpyrene revealed, in ultra-violet light, a gradual change from the characteristic violet fluorescence of benzpyrene to a milky blue fluorescence which persisted for many months after the complete disappearance of benzpyrene from the body. This was found to be due to an impurity, originally present in the benzpyrene* in small amounts. It was found possible to separate this impurity from the original benzpyrene, as well as from the tissues, by differential adsorption on alumina [Berenblum & Schoental, 1942]. The purified benzpyrene disappeared from the tissues after injection without leaving a residual fluorescence. The presence in crystalline

Fig. 1. Values for benzpyrene contents of whole mice, plotted on a logarithmic scale, against time, after: intraperitoneal injection of 10,000 $\mu\text{g.}$ = ●; intraperitoneal injection of 2,000 $\mu\text{g.}$ = ○; intraperitoneal injection of 200 $\mu\text{g.}$ = ◐; subcutaneous injection of 10,000 $\mu\text{g.}$ = ×; subcutaneous injection of 2,000 $\mu\text{g.}$ = △; subcutaneous injection of 200 $\mu\text{g.}$ = □.

benzpyrene of this impurity has not previously been detected, no doubt because its properties are in many respects similar to those of benzpyrene itself. It differs from the latter, however, in its slightly greater adsorbability on alumina from light petroleum, in its diffuse fluorescence spectrum (4000–5000 $\text{\AA.}$, with maximum intensity at about 4150–4600 $\text{\AA.}$, instead of the three separate bands of benzpyrene), and in its persistence in the animal body long after all the benzpyrene has disappeared.

The concentration of benzpyrene in circulating blood and separate organs

Previous data on the distribution of benzpyrene in separate tissues and organs after local injection, are scanty and vague, owing to the limitations imposed by the inadequate methods of estimation then available. The high sensitivity of the new method [Berenblum & Schoental, 1942] has enabled more accurate studies to be made.

After intraperitoneal injection of 10 mg. of benzpyrene the hydrocarbon could be detected in the blood. Blood was collected from the severed blood vessels of the neck of anaesthetized mice. In some, 5 ml. citrate saline were slowly injected into the tail vein while the blood was dripping from the vessels of the neck, though even this procedure did not of course allow the whole of the blood in the body to be collected.

* Three different samples of benzpyrene were used in these investigations: (1) from L. Light and Co. Ltd., (2) from Hoffmann-La Roche and Co. Ltd., and (3) from Hoffmann-La Roche and Co. Ltd., obtained through the British Empire Cancer Campaign. The concentration of the impurity mentioned above differed in all three samples.

Table 1. *Benzpyrene content of the blood of mice after intraperitoneal injection of 10 mg. of the hydrocarbon in 0.5 ml. of sesame oil*

Time after injection	Benzpyrene in blood, $\mu\text{g./mouse}$	Benzpyrene in whole mouse, $\mu\text{g. (average)}$
1 hr.	0.6, 0.4	10,000
24 "	0.8, 0.4, 1.0	8,000
30 "	0.3, 0.25	7,500
54 "	0.5, 0.6, 0.4, 0.5	5,000
5 days	0.2, 0.6	2,000
7 "	0.2, 0.3, 0.25, 0.2	700
11 "	0.08	200
16 "	<0.02	50

Table 2. *Benzpyrene contents of separate tissues of mice injected subcutaneously with 10 mg. of the hydrocarbon in 0.5 ml. of sesame oil*

Tissue	$\mu\text{g. benzpyrene}$	
	After 9 days	After 33 days
Blood	0.05	0.04, 0.07
Spleen	0.02	0.05, 0.02
Kidneys	0.06	0.03, 0.03
Lungs	0.04	0.08, 0.08, 0.05
Brain	0.12	0.05, 0.09
Liver	0.20	0.07, 0.30, 0.06
Gall bladder (containing bile)	—	0.07, 0.04
Whole mouse	9000	7000

Noteworthy features of the results of this experiment (Table 1) are the following: (a) benzpyrene was detectable in the blood as early as 1 hr. after injection; (b) the highest value recorded was $1\mu\text{g.}$ (in less than 2 ml. of blood), though, from tests *in vitro* the solubility of benzpyrene in mouse serum was found to be of the order of $40\mu\text{g./ml.}$; (c) with the fall in the total benzpyrene content of the whole mouse, there was a perceptible decrease in the concentration in the blood.

The concentration of benzpyrene in separate organs was studied in mice receiving subcutaneous injection of 10 mg. of the hydrocarbon, the intraperitoneal series not being suitable owing to gross contamination of the surfaces of the abdominal organs with benzpyrene. The values obtained for the organs tested were all very low (Table 2), both after 9 and 33 days from the time of injection. The similarities in the values after 9 and 33 days indicate that there is no significant accumulation of benzpyrene in these organs. The values for blood were also low and decidedly lower than those found in the intraperitoneal series at the stage when the benzpyrene content of the whole animal was comparable.

The elimination of unchanged benzpyrene from the body

The rate of excretion of unchanged benzpyrene was investigated by determining its concentration in the faeces during the first 12 days after both subcutaneous and intraperitoneal injection of 10 mg. The average values were $0.6\mu\text{g./mouse}$ per day for the 12 days in the case of subcutaneous injection, and $4\mu\text{g.}$ in the case of intraperitoneal injection. In the former case, the excretion per day remained fairly constant, the amount excreted representing about 0.35% of the amount which disappeared from the animal; in the latter case, the rate of excretion was high in the first few days and fell off rapidly (as would be expected in view of the rapidly diminishing quantity of benzpyrene remaining in the body), the amount excreted representing about 0.6% of the amount which disappeared from the animal during that period. The difference between the percentages of unchanged benzpyrene excreted in the two series (0.35 and 0.6% respectively) is probably not significant, in view of the error entailed in estimating such small amounts of benzpyrene.

DISCUSSION

The present results confirm those previously reported in that there is a continuous disappearance of benzpyrene from the body, and that only a very small fraction of it is excreted unchanged in the faeces. Chalmers & Kirby [1940] examined both the urine and faeces of three rats, each of which had received 60 mg. of benzpyrene in olive oil subcutaneously; they found only traces of unchanged benzpyrene in the urine, but about 1 % in the faeces. In the present investigations on mice, only the faeces were examined; the values were 0.35 % excreted as unchanged benzpyrene in the case of subcutaneous injection and 0.6 % in the case of intraperitoneal injection. Thus, more than 99 % of the loss is due to destruction or change into a form which no longer gives the characteristic fluorescence spectrum.

The presence of unchanged benzpyrene in the blood stream 1 hr. after the time of injection, and the absence of any gradual accumulation of the hydrocarbon in the blood subsequently, indicate that there is a continuous transportation of the hydrocarbon via the blood stream. But since there is also no significant accumulation in other tissues of the body, and relatively little in the excreta, it follows that a considerable amount of chemical transformation must occur after transportation. The detection of a metabolic product in the bile (BPX) and faeces (BPF) by Peacock [1936], Chalmers & Peacock [1936] and Chalmers [1938; 1940], suggests that the liver may be concerned with this transformation.

In view of the facts that benzpyrene does not accumulate progressively in the blood even during its rapid disappearance from the body, and that the rates of disappearance from the body follow approximately parallel straight lines when plotted on a logarithmic scale, it may be concluded that the limiting factor which determines the rate of disappearance is the rate of diffusion of the hydrocarbon from the site of injection into the blood, and not the rate of chemical change after transference via the blood stream. Thus it may be deduced that the more rapid disappearance of benzpyrene from the peritoneal cavity is due in part at least to the large surface which exists there, compared with that of the single oily globule in the case of subcutaneous injection.

The original purpose of this investigation was to compare the carcinogenicity and the rate of disappearance of benzpyrene, when injected subcutaneously, with those following intraperitoneal injection, in order to derive further evidence as to whether carcinogenesis is dependent on the presence of the unchanged carcinogen or on the action of some of its metabolic products. The present results show that the site of injection from which benzpyrene disappears more slowly is that in which tumours develop more readily. This favours the view that carcinogenesis is dependent on persistence of unchanged benzpyrene.

SUMMARY

A study was made of the rate of disappearance and the carcinogenicity of 3:4-benzpyrene in mice when amounts varying from 20 to 10,000 μ g. in sesame oil were injected subcutaneously or intraperitoneally.

Benzpyrene was found to disappear about 15 times more rapidly from the peritoneal cavity than from the subcutaneous tissues.

From any one site of injection, the rate of disappearance at any given time was found to be proportional to the logarithm of the concentration, irrespective of the amount originally injected.

Tumours developed readily at the site of injection when benzpyrene persisted for a long time (subcutaneous tissues), while no tumours occurred when the disappearance was rapid (peritoneal cavity).

With the disappearance of benzpyrene, an intense milky-blue fluorescence in ultra-violet light took the place of the characteristic violet fluorescence at the site of injection and persisted for many months. This was found to be due to an impurity not readily metabolized by the tissues, present in the originally injected benzpyrene.

During the course of disappearance of benzpyrene from the body, perceptible amounts of the unchanged hydrocarbon were detected in the blood stream, separate organs and faeces. The results indicate a continuous transfer of unchanged benzpyrene through the blood stream, with subsequent rapid destruction accounting for more than 99 % of the amount which disappears from the body, less than 1 % being excreted as unchanged benzpyrene.

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