Appendix: Statistical Analysis

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By analysis of variance, the differences from litter to litter of the animals in Exp. 6 (*c*) were tested and found not to be significant. Hence it is justifiable to estimate the standard errors of mean responses or of slope from the variance in growth of animals (not littermates) on the same dose.

	b	*σb*	Ratio
Average increase in response (i.e. body-weight increase/week) when dose is doubled	4·26	1·12	3·80
Average increase in response when dose is increased tenfold	14·16	3·70	3·80

The standard error is based on 28 degrees of freedom and the slopes are therefore highly significant. The curve relating the logarithm of the dose to the response shown by rate of weight increase shows no significant departure from linearity.

Ignoring the addition to the error of any estimation due to the error of the estimate of slope, which may, of course, be considerable, the limits of error for one estimation with ten rats on the standard and ten on the test substance would be:

$$p = 0·99 \qquad 64 \text{ to } 157\%$$
$$p = 0·95 \qquad 55 \text{ to } 181\%$$

This basis is comparable with that used in the British Pharmacopoeia Commission Report on the Accuracy of Biological Assays [1936]* and the accuracy found with that usually obtained in biological assays.

For Exps. 6 (*a*) and 6 (*b*) the results are as follows:

Exp.	Slope *b*	*σb*	Ratio	Limits of error (*p* = 0·99) for 10 rats on each dose
6 (*a*)	4·30	0·76	5·7	61 to 164%
6 (*b*)	4·19	1·02	4·1	64 to 157%

The errors in the result are increased if the period of dosing is reduced from 3 to 4 weeks, although the results are still significant.

* *Gen. Med. Coun. Brit. Pharm. Com. Report*, p. 10 [1936].

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Intermediate Stages in the Metabolic Conversion of Benzpyrene to 8-Hydroxy-benzpyrene in Mice

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Chalmers & Crowfoot [1941] showed that after administration of benzpyrene to rats and mice a small amount of monohydroxybenzpyrene could be isolated from the faeces. Berenblum, Crowfoot, Holiday & Schoental [1943] identified this as 8-hydroxy-3:4-benzpyrene, and established the presence of 3:4-benzpyrene-5:8-quinone in the faeces. Intermediate metabolites, 'BPX' and 'BPF' could be demonstrated by a blue fluorescence of the bile [Peacock, 1936, 1940] and a blue-green fluorescence of the faeces [Chalmers, 1938] respectively, both distinguishable from the violet fluorescence of the parent hydrocarbon in organic solvents. Doniach, Mottram & Weigert [1943 *a, b*] found that the blue 'BPX' fluorescence changed to the blue-green 'BPF' fluorescence after the contents of the small

intestine had passed through the ileo-caecal valve. Furthermore, evidence was given that a blue fluorescence with a spectrum similar to that of 'BPX' appeared in the kidney cortex, liver and lung of mice after intravenous inoculation of, or feeding with, benzpyrene, and in the skin after painting with it. The benzpyrene compounds to which the specific fluorescence was due (called collectively 'tissue-BP-blue') were produced locally in the specific organs and in the painted area of the skin. A blue-green fluorescence with a spectrum similar to that of 'BPF' was sometimes seen in the lungs of rabbits.

The typical fluorescence spectrum of 'bile-BPX' and 'tissue-BP-blue' with maxima near 450 and 425 mμ, and that of 'BPF' with maxima near 465 and 440 mμ, were seen on examination of the original tissues and of their unpurified extracts in ultra-violet light. It has now been possible, after a simplified chromatographic purification of the fluorescent compounds in these extracts, to study in greater detail the course of benzpyrene metabolism.

EXPERIMENTAL

Mice which had been inoculated in the tail vein with 1 ml. of a coarse suspension of 0·05 % benzpyrene in saline were killed after 3–7 hr., and the gall-bladder and digestive tract removed. The small intestine, which fluoresced blue, and the large intestine (caecum + colon + faeces), which fluoresced blue-green, were rinsed superficially with distilled H₂O, minced, and each shaken mechanically with about 3 ml. of acetone. The bile from the opened gall-bladder was shaken in a small tube with 1 ml. of acetone. These operations were carried out ·as quickly as possible after death. The solids were centrifuged down and the acetone extracts transferred to benzene by adding to the clear acetone solutions about 3 ml. of benzene, and washing two or three times in a tubular separating funnel with N/100 H₂SO₄. The benzene solutions were then separated and dried over anhydrous Na₂SO₄.

The dried benzene solutions were poured on to columns of alumina 7–10 mm. in diam. and 3–4 cm. long, and the chromatograms developed under gravity with benzene for about 1 hr. The progress of adsorption and the behaviour of the fluorescent zones on development could easily be followed in the ultra-violet light of a G.E.C. Osira lamp with a Wood glass bulb. The fluorescent zones and rings on the Al₂O₃ were spectrographed separately with the illuminating device described by Doniach et al. [1943a], which was particularly well suited for making 'optical sections' at any place on the column. The various portions were then cut and eluted with absolute ethanol.

RESULTS

Bile and small intestine. A bluish violet-fluorescent adsorbate with a banded fluorescence spectrum was strongly fixed at the top of the column, and did not move down on development. Its ethanol eluate fluoresced blue, with essentially the same spectrum as the adsorbate. From the spectro-

grams graphs were recorded, using Goldberg's [1910] densograph, which served as a primitive microphotometer [Weigert & Mottram, 1940]. The graphs (Fig. 1) for the adsorbates (a) and the ethanol eluates (b) are almost identical except for a slight displacement towards longer wave-lengths for the
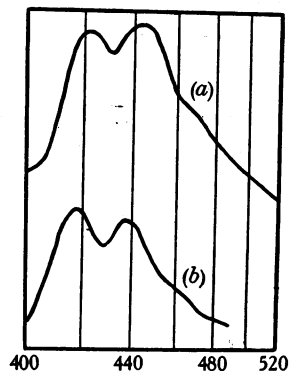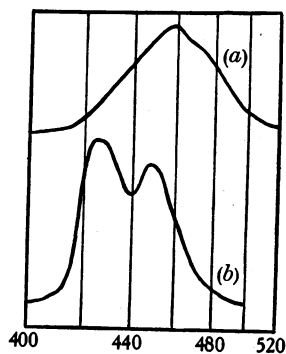

Fig. 1.

Fig. 2.

Figs. 1, 2. Photometric records of fluorescence spectrograms of mouse tissue extracts after chromatography. Abscissae: wave-length in mμ (prismatic spectrum). Ordinates: density (arbitrary scale).

Fig. 1. Bile. (a) Blue-fluorescent adsorbate. (b) Blue-fluorescent eluate in ethanol.

Fig. 2. Large intestine. (a) Green-fluorescent adsorbate. (b) Blue-fluorescent eluate in ethanol.

adsorbate. The photometric record for the adsorbate (a) shows two maxima, at 422 and 443 mμ, and an inflexion at about 472 mμ, which appears as a weak secondary band in the spectrum itself. The corresponding wave-lengths for the ethanol eluate (b) are 417, 438 and 470 mμ respectively.

Large intestine. A bright green-fluorescent ring moved slowly down; its spectrum showed one broad maximum at about 460 mμ, but no bands (Fig. 2a). The ethanol eluate fluoresced whitish blue with a banded spectrum. The photometric record (Fig. 2b)

shows two maxima, at 425 and 449 mμ, but no inflexion. Sometimes a faint blue-fluorescent adsorbate remained fixed at the surface.

Influence of storage of material before extraction. When the extraction was not carried out immediately after killing the mouse, a green-fluorescent chromatographic zone also appeared with extracts from bile and small intestine. Therefore, in another set of experiments, the diluted bile and the minced small and large intestines were each divided into two portions immediately after death, one of which was stored in ice for 3 days, while the other was extracted at once. The benzene extracts of the latter were kept over anhydrous Na_2SO_4 for 3 days until the others were ready, when they were all chromatographed together side by side in pairs. With the material stored before extraction, the blue fluorescence at the surface was greatly reduced in the case of the bile and small intestine, while a new green zone appeared and moved slowly down on development. This zone showed, both as adsorbate and as eluate, the spectrographic properties described above for the large intestine. In the case of the large intestine the phenomena were unaltered by postponement of the extraction. The change from the blue- to the green-fluorescing adsorbate with bile and small intestine was greatly accelerated when the specimens were stored at room temperature, or at 37°, before extraction.

Whenever a particularly bright green-fluorescent zone appeared, a red-fluorescing diffuse zone could be seen under it on the column; it moved down more quickly than the green zone, and could be eluted with chloroform. Neither the red adsorbate nor its eluate in chloroform showed a banded fluorescence spectrum.

The effect of preservation of the intestines by formaldehyde was also investigated. The digestive tracts were kept for periods up to 2 months in formol-saline (containing 4% H.CHO) at room temperature. Acetone extracts of the minced small intestines which had been transferred to benzene gave only the blue fluorescence at the top of the chromatographic column, while those of the large intestine gave the green-fluorescent zone which moved down slowly on development.

Properties of the different fluorescent substances. The solubility of the blue-fluorescent adsorbate in ethanol seemed to be distinctly lower than that of the green-fluorescent adsorbate. This was in agreement with the observed fact that the green zone moved slowly down on development with benzene, while the blue-fluorescent substance stayed at the top. In $N/100$ NaOH the blue-fluorescent adsorbate on the Al_2O_3 dissolved to give a solution with a banded blue fluorescence spectrum, while the alkali solution of the green adsorbate fluoresced green with a single broad maximum.

When the separated blue-fluorescent Al_2O_3 zone was dried and heated in an evacuated tube to 100–200°, the fluorescence changed from blue through white to yellow-green, and the fluorescence spectrum (which in the case of the original adsorbate was banded) changed to a spectrum with one broad maximum. An ethanol extract after this transformation fluoresced whitish blue and had the same banded spectrum as the eluate of the green zone. When the ethanol extract was transferred to benzene and again poured on to a column of Al_2O_3, a single green-fluorescent ring appeared and moved slowly down on development.

The ethanol eluate of the blue-fluorescent adsorbate from the small intestine of a single mouse contained a quantity of material sufficient to record some absorption bands. With the eluate in a quartz tube, 4 cm. long and 4 mm. diam., narrow bands appeared at 366–372, 377–382, 386–393 and 405–412 mμ. The small intestine of another mouse was kept for 1 day at 37° before extraction with acetone, when the blue-fluorescent derivative was completely transformed into the derivative which gave a green-fluorescent adsorbate on alumina. Its eluate in ethanol in a tube 1 cm. long and 4 mm. diam. showed quite different and wider bands at 358–364, 375–384, 392–407 and 415–433 mμ, with maxima at 362, 381, 400 and 422 mμ respectively.

The minced kidneys, livers and lungs of benzpyrene-treated mice were extracted with acetone immediately after killing, and the extracts transferred to benzene and chromatographed. The excess of dissolved unchanged benzpyrene, which was particularly great in the lung, was first washed out with benzene, after which development was continued for about 1 hr. Bright blue-fluorescent adsorbates remained on the column in the case of liver and kidney, and traces in the case of lung. The fluorescence spectra of these adsorbates and of their eluates were exactly the same as in the case of bile and small intestine. However, in contrast to them, the blue-fluorescent zones were not sharp but extended diffusely (about 1 cm. with kidney and about 2 cm. with liver) into the column. The lungs of three mice yielded a green-fluorescent adsorbate, which behaved like that from the large intestine.

Postponement of the extraction of kidney and liver had an effect on the chromatographic phenomena similar to that observed with bile and with small intestine. For instance, when the organs had been left for 1 day at 37° in saline, a narrow green ring moved slowly through the diffuse blue zone down the column. The rate of this movement was, in the case of liver, about twice that for kidney, but the fluorescence spectra of these green rings as adsorbates and as eluates in ethanol were the same as with the large intestine. The green zones did not appear if the organs were stored in formol-saline.

DISCUSSION

Two different kinds of fluorescence phenomena can be distinguished by chromatography in extracts of various tissues and of the bile of mice after their treatment with benzpyrene. The prototypes are, on the one hand the bile, where Peacock [1936] discovered the blue-fluorescent 'BPX', and on the other hand the large intestine and faeces, where Chalmers [1938] established the presence of the blue-green-fluorescent 'BPF'. Since it is obvious that the fluorescent zones on the Al_2O_3 contain the bulk of the fluorescent compounds in bile and faeces, it is suggested that the term [BPX] should be used for the type of the compound from bile which gives after chromatography a *banded blue* fluorescence spectrum as adsorbate and *the same* banded spectrum as eluate (Fig. 1 *a*, *b*) and the term [BPF] should be used for the type of the compound from the large intestine and faeces which gives after chromatography a *non-banded green* fluorescence spectrum as adsorbate and a *banded whitish blue* spectrum as eluate (Fig. 2 *a*, *b*).

The approximate fluorescence maxima of the four compounds before chromatography (inverted commas) and after chromatography (square brackets) may be summarized as follows:

Before	After
'BPX': 425, 450 mμ	[BPX]: 417, 438, 470 mμ
'BPF': 440, 465 mμ	[BPF]: 425, 449 mμ

[BPX] is more strongly adsorbed on Al_2O_3 and less soluble than [BPF]. They are chemically to be distinguished from each other, because their fluorescence spectra, their absorption spectra and their chromatographic behaviour are different.

The nature of [BPX] and of [BPF] has not yet been ascertained because our adsorbates contained only minute amounts. However, the fluorescence and absorption spectra of [BPX] and [BPF] and their chromatographic behaviour lead to some tentative conclusions:

(*a*) [BPF] is closely related to 8-hydroxybenzpyrene prepared by Berenblum & Schoental [1943] from faecal extracts which had been concentrated by chromatography followed by sublimation *in vacuo*. These authors were in fact working with [BPF].

(*b*) Slight differences in the rate at which the green zone moved down the chromatographic column were seen when [BPF] was prepared from different sources. Hence, [BPF] probably comprises a group of complexes which can contain various cell constituents in combination with 8-hydroxybenzpyrene.

(*c*) This combination must be a loose one, because the absorption maxima of the eluate of [BPF] in ethanol (362, 381, 400 and 422 mμ) correspond closely with the absorption maxima (362, 380, 401 and 424 mμ) read from the graph of the absorption spectrum of pure 8-hydroxybenzpyrene in ethanol published by Berenblum, Crowfoot, Holiday & Schoental [1943].

(*d*) The marked difference between the fluorescence spectra of the adsorbate of [BPF] on Al_2O_3 and of its eluate in ethanol shows that its hydroxybenzpyrene component is directly affected by the adsorption.

(*e*) [BPX] too must be considered as a group of complexes, made up of benzpyrene or a derivative and various cell constituents as carrier molecules. This follows from the different chromatographic behaviour of [BPX] from various sources.

(*f*) The fact that the same banded fluorescence spectrum is shown by all the members of the [BPX] family, as adsorbates and as eluates, indicates that the benzpyrene 'nucleus' is the same in all cases and is not affected by the adsorption. Hence the strong adsorption to the top of the column is due to the adsorbing properties of the carrier molecules.*

(*g*) The absorption bands of the ethanol eluate from [BPX] at 366–372, 377–382, 386–393 and 405–412 mμ coincide neither with those of 8-hydroxybenzpyrene nor with those of any other known benzpyrene derivative. Therefore, nothing definite can be said at present about the chemical nature of the benzpyrene derivative in the complex. However, the observed easy transformation of 'BPX' into 'BPF' in the living mouse after passage through the ileo-caecal valve or post mortem by autolysis, and of [BPX] into [BPF] *in vitro* by heat, shows that 'BPX' and [BPX] are chemical precursors of 'BPF' and [BPF] respectively.
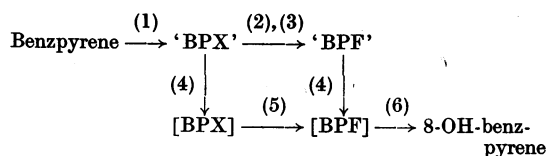
The limits of the stability of [BPX] have not yet been established: it is stable in tissues in the form of 'BPX' over long periods of time in the presence of formalin, and visible and near ultra-violet light do not alter [BPX] to a perceptible degree, either as adsorbate or as eluate.

The experiments here described, in conjunction with those of Doniach, Mottram & Weigert [1943 *a*, *b*], provide evidence for a tentative chart (shown on p. 501) of the various stages in the metabolic and chemical change from benzpyrene to 8-hydroxybenzpyrene.

The red-fluorescent derivative which appears on the alumina column when [BPF] is produced via (2) or (3) and (4) has chromatographic properties similar to those of the benzpyrene-5:8-quinone discovered by Berenblum & Schoental [1943] in the faeces, i.e. it moves down more quickly than [BPF]

* We owe the suggestion of the possible existence of a complex of a benzpyrene derivative with another strongly adsorbing substance to a personal communication from Dr Berenblum.

and can be eluted with chloroform. However, its red fluorescence is not in agreement with the properties of benzpyrene-5:8-quinone, according to Berenblum & Schoental.

Benzpyrene $\xrightarrow{(1)}$ 'BPX' $\xrightarrow{(2),(3)}$ 'BPF'

$\qquad\qquad$ (4) $\Big\downarrow$ $\qquad$ (4) $\Big\downarrow$

$\qquad\qquad$ [BPX] $\xrightarrow{(5)}$ [BPF] $\xrightarrow{(6)}$ 8-OH-benz-
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ pyrene

(1) *In vivo*, local production in the skin, kidney, lung and liver (excreted with the bile).

(2) *In vivo* in the large intestine and occasionally in the lung of rabbits and mice.

(3) *In vitro* by post-mortem autolysis.

(4) *In vitro* by adsorption on to alumina.

(5) *In vitro* at elevated temperature *in vacuo*.

(6) *In vitro* by sublimation *in vacuo*.

Of the various steps in the transformation of benzpyrene into 8-hydroxybenzpyrene outlined above, step (1) is the only one concerned in the problem of benzpyrene carcinogenesis, because it alone occurs in the living cell. The later steps which eventually lead to hydroxybenzpyrene are merely concerned with the removal of the hydrocarbon from the body.

SUMMARY

It has been shown by fluorescence chromatography of quite fresh extracts from organs of mice which had been treated with benzpyrene that the hydrocarbon is not metabolized directly to 8-hydroxybenzpyrene but that the transformation passes through three intermediate stages which have been studied separately. The suggested relationship of these stages has been discussed.

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Effect of Vitamin B on the Growth of Fibroblasts

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Components of the vitamin B complex have long been considered of importance in growth. In particular vitamin B_1 has been assayed by animal growth methods. The experiments described were designed to test whether such a growth-promoting effect could be demonstrated directly on cells *in vitro*. In essence the problem was approached in two ways: first, by depriving fibroblasts of components of the vitamin B complex by growing them in media obtained from pigeons suffering from acute beriberi; secondly, by adding to such deficient media appropriate amounts of vitamin B (yeast extract) and vitamin B_1 (aneurin).

METHODS

Culture material. Tissue cultures of fibroblasts were obtained from the choroidal and sclerotic layers of the eyes of 9-day chick embryos, and culture by the hanging drop technique was carried out for at least ten passages prior to each experiment. This length of cultivation has been found to produce a uniform outgrowth of fibroblasts, and any initial supply of growth-promoting substance present in the tissue has been largely exhausted [Carrel & Ebeling, 1921]. Cultures which showed excellent outgrowth were then graded for an experiment. The areas of the explants were measured, and the cultures divided into sets in such a way that the explants in any two or more sets to be compared were approximately equal in area. The selection