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The Fluorimetric Estimation of 3:4-Benzpyrene in Whole Mice

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Previous methods for the quantitative estimation of carcinogenic hydrocarbons in animal tissues are based on spectrophotometric measurements of either the absorption spectrum (Chalmers, 1934; Jones, 1942; Lorenz & Shear, 1936; Lorenz & Shimkin, 1941; Lorenz & Stewart, 1940), or the fluorescence spectrum (Berenblum & Kendal, 1936; Berenblum & Schoental, 1942; Chalmers, 1938, 1940; Chalmers & Peacock, 1936; Hieger, 1936; Peacock, 1936). Although the greater simplicity of a fluorimetric estimation would result in a saving of time and money, its unspecificity, due to the presence of fluorescent impurities, was usually regarded as too great an obstacle. The present paper records methods of extraction and purification of 3:4-benzpyrene (henceforth called benzpyrene), as developed in this laboratory, and examines the possibilities and limitations of making allowance, in fluorimetric measurements, for the remaining impurities on a statistical basis.

In the course of investigations into the effect of lipid solvents on the carcinogenic activity of benzpyrene it was decided to measure the rate of elimination of the hydrocarbon from the animal body after subcutaneous injection. As previously emphasized (Dickens & Weil-Malherbe, 1942), small doses of carcinogen should be employed in experiments of this kind, since otherwise any cocarcinogenic or anticarcinogenic properties of the solvent are liable to be smothered. Benzpyrene is particularly favourable, as it combines, relative to 20-methylcholanthrene and 1:2:5:6-dibenzanthracene, the highest fluorescence intensity with the lowest carcinogenic potency after subcutaneous injection (Bryan & Shimkin, 1943). A single subcutaneous injection of 0.3 mg. benzpyrene was found satisfactory in our experiments. The method has therefore been devised for a maximum amount of 0.3 mg. benzpyrene; it is, however, easily adapted to the estimation of larger amounts by modifications of the procedure of chromatographic purification, which will be discussed.

SURVEY OF METHOD

The following three steps which are in principle, though not in detail, the essence of some previous methods, precede the actual estimation. (1) Lysis of the tissue in hot alcoholic NaOH. (2) Extraction of the hydrocarbon, together with non-saponifiable matter from the tissues, from alkaline 50 % ethanol by light petroleum. (3) Purification of the petroleum extract by concentration and cooling and then by chromatographic adsorption and fractional elution (so-called 'liquid chromatography'). Benzpyrene is first adsorbed on a column of silica gel from light petroleum and eluted by light petroleum-benzene mixture (4 : 1). It is then reabsorbed from this mixture on a column of alumina. After development by the same mixture it is eluted by benzene. (4) The concentration of benzpyrene is determined fluorimetrically.

DESCRIPTION OF METHOD

Reagents

Purification of solvents

Ethanol, 95% (v/v). Industrial methylated alcohol is mixed with 10N-H₂SO₄ (40 ml./l.) and rectified slowly through an efficient fractionating column (Clarke & Rahrs, 1923). This is followed by refluxing for 12 hr. over AgNO₃ (8 g./l.) + KOH (8 g./l.) and another rectification.

Light petroleum. The commercial product (b.p. 40–60°) is purified by the following successive extractions, each carried out with vigorous shaking for several minutes in a separator: nitrating mixture (125 ml. conc. H₂SO₄ + 100 ml. conc. HNO₃) (4 times), fuming H₂SO₄ (3 times), water and 25% NaOH. It is finally dried over CaCl₂ and rectified.

Benzene. Extraction of benzene (British Drug Houses Ltd., A.R.) with conc. H₂SO₄ (3–4 times), followed by washing with water and alkali, drying and rectification.

Ethanol may be recovered by fractional distillation, but before being used again it should be purified by the process described. The same applies to light petroleum recovered from light petroleum-benzene mixtures.

Adsorbents

Silica gel (British Drug Houses Ltd.). The gel is ground in a mortar and the 100–160-mesh fraction is screened out, care being taken to remove the dust, which would clog the adsorption column. The 60–100-mesh fraction is usable too and may be collected separately. Small batches of the adsorbent are dried at 110° for 24 hr., cooled *in vacuo* over P_2O_5 and stored in a glass-stoppered bottle, which is itself kept in a desiccator over P_2O_5 .

Aluminium oxide (British Drug Houses Ltd., 'for chromatographic adsorption analysis') is dried at 300° for 3 hr. in small batches and cooled and stored like the silica gel.

Reproducible results can be obtained only if the adsorbents are standardized. The standardization must be repeated with every new sample, as products of different origin may show appreciable differences in adsorbing qualities. The method will be described later.

Adsorbents, alumina in particular, are extremely sensitive to atmospheric moisture, exposure to which entails a progressive reduction of adsorbing power. Once dried, a batch of adsorbent should therefore be protected from moisture and any unavoidable exposure reduced to a minimum.

Lubricants

The use of vaseline and other fluorescent lubricants on separator taps, etc., is to be strictly avoided. Suitable lubricants are lard, provided freshly greased taps are washed out with chloroform before use, and a mixture of graphite (previously extracted with hot benzene) and glycerol in such proportions that a thick, but smooth paste results. Contact with rubber is undesirable. The use of all-glass apparatus, without lubrication of joints, is recommended.

Hydrolysis of tissues and extraction of benzpyrene

The dead mouse is placed in a 300 ml. flask and 150 ml. 10% NaOH in ethanol are added. The latter is prepared by dissolving 1.2 parts of NaOH in 1 part of water and adding 11 parts of purified 95% ethanol (v/v). The contents of the flask are brought to boiling on a water-bath and refluxed for a further $\frac{1}{2}$ hr. While still hot the solution is decanted from the skeleton into a 500 ml. flask. The flask and insoluble residues are rinsed three times with 5 ml. ethanol. After the addition of 150 ml. of water the cooled mixture is extracted in a separator with four successive lots of 50 ml. commercial light petroleum, b.p. 40–60°. (The purified solvent need not be used at this stage.) The combined extracts are dried over 5 g. $CaCl_2$ for about 2 hr. and then decanted into a 300 ml. distillation flask, followed by three washings with 10 ml. of light petroleum. If the extract or the washings or both are very cloudy, it is preferable to centrifuge them, though a moderate degree of turbidity is of no account. The petroleum extract is now evaporated to dryness, first under slightly reduced pressure and finally in the full vacuum of the water pump. The residue is taken up in purified light petroleum, added in lots of about 2 ml. at a time, and rinsed into a 10 ml. centrifuge tube until a volume of 6–8 ml. is reached. The extract is chilled in a freezing mixture for 20 min., being repeatedly stirred with a glass rod to assist crystallization. Finally, the solid matter is centrifuged off and washed three times with 0.5 ml. of chilled purified light petroleum.

Chromatographic purification

A series of thistle funnels is required with a bulb of 50 mm. diam. (approx.) and a neck which makes an airtight fit with a rubber bung. The stem of the funnel is 25 cm. long and has a 5–6 mm. bore. At a distance of 17 cm. from the bulb there is a constriction, on top of which is placed a small plug of glass-wool. The funnel is mounted on a small suction flask. The correct amount of adsorbent is measured out with a calibra'ed test tube, 10 × 75 mm., and poured into the funnel.

The filtrations are carried out under pressure, preferably from a N_2 cylinder, to avoid losses by evaporation. The pressure is applied to the top of the thistle funnels and is so regulated that the rate of filtration does not exceed 20–30 drops/min.

The clear petroleum solution is first passed through a column of 1.5 g. silica gel, followed by three washings of 2 ml. of purified light petroleum. The filtrate is rejected. 25 ml. light petroleum-benzene mixture 4 : 1 (both solvents purified) are passed through the column. The entire filtrate is then passed through a second column of 2 g. of alumina, followed by a further 20 ml. petroleum-benzene mixture, part of which is used to rinse out the flask previously used. The combined filtrates are rejected. Finally 15 ml. benzene (purified) are passed through the alumina column. The solution is now ready for fluorimetric estimation.

We have found it convenient to run four experiments simultaneously up to this stage.

Fluorimetric estimation

In this laboratory two fluorimeters are in use: a direct-reading apparatus built locally to our own specifications and the Spekker fluorimeter (Adam Hilger, Ltd.) which is a null-point instrument. The first-mentioned apparatus has been described in previous publications (Weil-Malherbe, 1943, 1944). Fig. 1 shows the tubes used with this apparatus. They contain 5 ml. solution of suitable concentration, i.e. a total of not more than 3 μ g. benzpyrene. A preliminary dilution of the final benzene eluate may be necessary. If the total amount of benzpyrene is less than 1–2 μ g. the benzene solution is concentrated *in vacuo* to less than 5 ml., made up to exactly 5 ml. and the total used for the estimation. A stream of O_2 -free N_2 , saturated with benzene, is passed through the solution for 2 min. before a reading is taken. A reference curve, which in this range of concentrations is practically linear, is established with standard solutions of known amounts of benzpyrene, treated in the same way. Every reading is corrected for a zero reading obtained with pure solvent.

For use with the Spekker fluorimeter special containers had to be made (Fig. 2), as the containers originally supplied

Fig. 1. Tube for use in the direct-reading fluorimeter.

were not suitable for gassing; moreover, 20 ml. of solution were required to fill them, which caused a corresponding loss of sensitivity. The present container (Fig. 2, 2) (supplied by Tintometer, Ltd., Salisbury) is a fused glass cell with two parallel circular faces, of 35 mm. internal diam. and 10 mm. depth. Its capacity is 10 ml. The cell is provided with a gas inlet tube (3) and a two-bulb splash head (4).

Fig. 2. Container with adaptor and gassing arrangement as used in the Spekker fluorimeter. For key to figures see text.

It is mounted on a brass adaptor without bottom (1) which fits exactly into the cell slide of the apparatus. Back-diffusion of O_2 is prevented by the mercury seal (5) made out of a fritted glass micro-filter tube. It is necessary to provide for equalization of pressure before taking a reading, as gas in the gas inlet tube causes total reflexion and prevents the ultra-violet light from penetrating into the solution beyond. If the gas inlet tube is filled with solution, fluorescence is evenly distributed within the range of the beam of ultra-violet radiation. O_2 -free N_2 , saturated with benzene, is bubbled through the solution for an appropriate time (3–5 min.) with tap 8 closed. Before a reading is taken the tap is opened, which causes the solution to fill the gas inlet tube while the gas stream escapes through the short-cut. The box which encloses the photoelectric cell and the container of the fluorescent solution must be enlarged to accommodate the modified vessel. This is done by unscrewing the lid with its hinges and fitting a brass extension of 95 mm. height to the back wall. A similar extension of the same height is fitted to the lower end of the three sides of the lid, which is then remounted on top of the extended back wall. The two gas-leading tubes (6 and 7) enter the box through holes in the extension of the back wall.

Solutions of quinine sulphate in $N-H_2SO_4$ are used as standards of fluorescence, a solution containing $3\mu g./ml.$, if 10 ml. of the unknown solution contain 0.5 – $2\mu g.$ benzpyrene, and a solution containing $10\mu g./ml.$, if 10 ml. of the unknown solution contain 2– $10\mu g.$ benzpyrene. Stronger

solutions of benzpyrene should be suitably diluted. The vessel used for the standard solution is an ordinary rectangular glass cell, and the measurement is carried out without preliminary gassing. Calibration curves are established for both concentrations of standard solution with known amounts of benzpyrene and the results plotted on semi-logarithmic graph paper. The resulting curves are linear.

The colour filters between fluorescing solution and photo-cell are the same as those used with the direct reading apparatus (Weil-Malherbe, 1944), viz. Wratten filters nos. 35 + 43, with a transmission maximum at about $400m\mu$.

Calculation of results

Two corrections are applied to the final result, after allowance has been made for the sample used: first an amount of $0.5\mu g.$ is subtracted to allow for the blank; the remainder is then multiplied by a factor 1.145 to allow for a fairly constant loss, which amounts to 13% on the average.

DISCUSSION OF METHOD

Extraction

Berenblum & Kendal (1936) and Berenblum & Schoental (1942) claim that dibenzanthracene and benzpyrene cannot be extracted quantitatively from an *alkaline* ethanol solution; and that quantitative extraction is possible only after acidification. No such difficulties, for which indeed no theoretical basis can be seen, were observed under our conditions, which differ from those of Berenblum *et al.* with respect to (1) dilution of the alcoholic solution with an equal volume of water before extraction and (2) the choice of light petroleum instead of benzene as solvent. It seems not unlikely that the effect ascribed by Berenblum *et al.* to acidification is in fact due to dilution with water, or is a salting-out effect.

Chromatographic purification

The method adopted is based on a detailed quantitative study of chromatography (Weil-Malherbe, 1943) aimed at finding empirical rules which would allow the prediction of the effect of varying such factors as concentration or quantity of the substance to be adsorbed, quantity or quality of the adsorbent, quality of the solvent, etc. It was shown that for the analysis of a simple chromatographic system the estimation of two parameters is sufficient, Freundlich's adsorption isotherm coefficient and the threshold volume V_t . The latter is defined as the volume of filtrate collected up to the beginning of elution, provided only one solvent is used for adsorption, development and elution. Whereas the adsorption isotherm is a constant for any given system, V_t is a variable depending on the variation of the factors mentioned and related to them by simple empirical expressions. With their aid it is possible to devise a procedure of such a nature that it is capable of coping with any specified range of concentrations. In the application of this principle to the purification of benzpyrene the upper limit of the concentration range was chosen at $300\mu g.$, as this was the dose injected. The lower limit was taken at $0.5\mu g.$, as the blank made the fluorimetric estimation of smaller amounts too uncertain. In the procedure finally adopted a wider margin of safety was chosen than that originally proposed (Weil-Malherbe, 1943). It was found that this could be done without increasing the blank in any way.

Adaptation of the procedure to different ranges of benzpyrene concentration

The exponential coefficient of Freundlich's adsorption isotherm for the system silica gel, benzpyrene, light petroleum, benzene, was found to be very nearly 1. This means that the amount adsorbed on a given amount of adsorbent is almost proportional to the concentration of the initial solution and V_t is almost independent of the concentration of the initial solution. It will therefore not be necessary to modify either the quantity of silica gel in the column or the volume of solvent used for elution, even though the range of concentration be considerably extended in either or both directions, especially as a wide margin of safety has already been allowed. The alumina system, however, is less elastic. Here V_t changes inversely with the concentration of the initial solution: increasing the latter means either reducing the volume of solvent used for development or increasing the amount of adsorbent. The empirical rules governing V_t give an idea of the magnitude of the effect to be expected. Thus, using the same constants as were employed in the numerical example on p. 310 of the paper by Weil-Malherbe (1943), we find $V_t = 56$ ml. for the case in which we pour a solution of 300 μg . benzpyrene, dissolved in 25 ml. light petroleum-benzene mixture, on to a column of 2 g. of alumina. With an increase of the quantity of benzpyrene to 1000 μg ., V_t is reduced to 43 ml. On the other hand, if we increase the amount of adsorbent to 2.5 g., V_t for 1000 μg . is the same as V_t for 300 μg . and a column of 2 g., viz. 56 ml. In this case the volume of solvent used for development need not be reduced, but account must be taken of the fact that V_t for 0.5 μg . benzpyrene is increased from 2.8 ml. benzene to 3.9 ml. benzene. So, if it is desired to maintain the lower range of concentrations at the same level as before, the volume of benzene used for the final elution must be increased in the same proportion, i.e. from 15 to 20 ml.

These considerations may seem superfluous, since it is often possible to observe the progress of development and elution by the fluorescence of benzpyrene in filtered ultra-violet light. Might it not be preferable simply to collect the fluorescent fraction of the filtrate as determined by direct observation in every individual case, rather than to maintain a fixed routine capable of dealing with a whole range of concentrations? There are three reasons in favour of the latter course. (1) In the evaluation of the fluorimetric measurement a correction is applied for the blank. It is therefore essential that the blanks of different experiments should be strictly comparable. Yet there is no guarantee that modifications of the procedure of purification will leave the blank unaltered. (2) With low concentrations of benzpyrene (<10 μg .) fluorescence becomes so weak, especially in solvents containing light petroleum, that direct observation is no longer possible. Even with higher concentrations it is very difficult to decide when the elution is complete. (3) A routine procedure has the advantage of making the analyst independent of the ultra-violet lamp. This does not mean that the procedure should not be controlled from time to time by direct observation in ultra-violet light.

Standardization of adsorbents

The procedure of chromatographic purification described has been worked out for adsorbents with strictly defined adsorbing qualities. Adsorbents of unknown quality have to be standardized before they are used in the estimation.

This is done by determining the value of k for each adsorbent, k being defined by the equation

$$k = \frac{V_t}{s^{1/\alpha}}$$

where V_t is the threshold volume in ml., s the amount of adsorbent in g. and α the exponential coefficient of Freundlich's adsorption isotherm. The equation holds only if the volume and concentration of the initial solution are kept constant. For the purpose of estimating k it is convenient to use a solution of 300 μg . benzpyrene in 1 ml., as initial solution. Strictly speaking, α should be estimated separately for every system investigated, but it will probably introduce no serious error if the values found in this laboratory are assumed, viz. $\alpha = 0.87$ for the alumina system and $\alpha = 0.96$ for the silica gel system. For testing alumina a mixture of light petroleum-benzene 4:1 is used as solvent and for silica gel 3% benzene in light petroleum (purified solvents). The experiment is done in triplicate with columns of 2 or 3 g. adsorbent. After addition of the initial solution, pure solvent is poured on, and the progress of development followed in ultra-violet light. It is not necessary to determine V_t accurately by extrapolation from the data of a complete elution curve (cf. Weil-Malherbe, 1943) for this purpose. The filtration is stopped when the first fluorescent drop leaves the column, the volume of the filtrate is measured and the mean value is used as V_t for the calculation of k . Alumina is satisfactory if its k -value is found to lie between 15 and 18, silica gel if its k -value is between 19 and 21. If the adsorbent tested has a k -value which differs considerably from these figures, the amounts used must be modified according to the equation

$$s_2 = s_1 (k_1/k_2)^\alpha,$$

where s_1 is the weight and k_1 the constant of the standard adsorbent and s_2 the weight and k_2 the constant of the adsorbent to be used.

Fluorimetric measurement

Theoretically, a null-point instrument, such as the Spekker fluorimeter, should give more accurate results, as any fluctuations in output of ultra-violet light are automatically compensated. In practice, however, the direct-reading instrument was found just as satisfactory and in many respects even preferable. Light fluctuations caused no serious trouble; moreover, they can be guarded against by use of a voltage stabilizer and by frequent reference to the standard solution. The advantages are: simple and rapid manipulation without the necessity of making meticulous adjustments; readings directly proportional to fluorescence intensity; more convenient arrangement for gassing and for insertion and change of containers; superior sensitivity owing to higher energy output of illuminating lamp, larger photo-cell and smaller volume of test solution.

The choice of the colour filter with transmission maximum at 400 $m\mu$. was actuated by the finding that it reduced the blank, expressed in units of benzpyrene, in a higher degree than did filters with transmission maximum at longer wave-lengths; specificity is thereby increased. A similar gain of specificity is achieved by carrying out the measurement in an atmosphere of pure N_2 . This is due to the fact that the blank is less subject to the quenching effect of O_2 than benzpyrene. Other advantages of eliminating O_2 are: a greater sensitivity owing to the increased fluorescence intensity, and an almost complete independence from solvent effects, i.e. variations of fluorescence intensity according to the solvent used (Weil-Malherbe & Weiss, 1942).

RESULTS

The blank value

Crude petroleum extracts of benzpyrene-free mice, after concentration to about 6–8 ml., are usually faintly yellow and show an intense white or yellow fluorescence. After chromatographic purification the fluorescence is a pure blue. A faint blue fluorescence is even shown by solutions from experiments without tissue. This latter value is referred to as 'reagent blank', while the value found with benzpyrene-free mice is called the 'blank'. The blank is therefore of composite nature, as both reagents and mouse contribute to it. As the accuracy and sensitivity of the method are limited by the blank, its magnitude and distribution have been studied in numerous analyses. The blank has throughout been measured in units of benzpyrene.

Reagent blank

The reagent blank was usually found to lie between 0.10 and 0.15 μg . If only that part of the analysis is carried out which starts with the chromatographic purification, the reagent blank amounts to about 0.08 μg . The origin of the other more variable part of the reagent blank could not be traced with certainty to any particular reagent or phase of analysis; an atmospheric impurity is not responsible, as shown by experiments in which air was filtered through cotton-wool during evaporations under reduced pressure, and by other experiments in which unfiltered air was sucked through benzene for about 8 hr.

The mean value of the blank

The mean blank of 54 analyses was found to be $0.50 \pm 0.01^* \mu\text{g}$., the standard deviation of a single observation (s.d.) being $0.117 \pm 0.0076^*$. To give a significant, if only qualitative, indication of the presence of benzpyrene a reading should not be less than 0.85 μg . (i.e. mean + 3 s.d.). In fact, the highest value of the blank observed was 0.74 μg ., and the lowest 0.27 μg ., both being observed only once. Of the 54 animals 32 were males and 22 females; the mean blank value for the males was 0.49 μg . (s.d. 0.107) and that for the females 0.51 μg . (s.d. 0.119). The difference does not exceed the probable error of the mean for both groups combined and is therefore not significant. If the animals were grouped according to their weights regardless of sex, a group of 24 animals weighing less than 30.0 g. (av. wt. 27.2 g.) had a mean blank of 0.46 μg . (s.d. 0.114), whereas a group of 27 animals weighing more than 30.0 g. (av. wt. 36.9 g.) had one of 0.53 μg . (s.d. 0.127). The difference between the means is significant ($P=0.03$). Thus it seems that the blank increases with the weight of the mouse.

* Probable error.

Chemical nature of the blank

Various experiments, which were carried out in an attempt to reduce the blank, also permit some—mainly negative—conclusions as to the chemical nature of the material responsible for it.

The possibility that the blank might result from autoxidation during the phase of alkaline hydrolysis was ruled out by an experiment in which the hydrolysis was carried out in an atmosphere of pure N_2 . No difference in the blank value resulted.

In order to test the homogeneity of the material responsible for the blank, residues from over 50 blank experiments were pooled and evaporated to dryness *in vacuo*. They were taken up in 10 ml. light petroleum and passed through a column of 2 g. of silica gel. A single band with grey-blue fluorescence formed, and was washed out by 25 ml. light petroleum-benzene mixture 4:1. The filtrate was now passed through a column of 2 g. of alumina. On development with more of this mixture the single band separated into two, which were at no stage completely parted, but showed a continuous transition. The lower band had a dull grey-blue fluorescence and the upper a light blue fluorescence. The eluates of the two bands were collected separately and the following results were obtained in experiments with portions of each fraction: (1) neither fraction formed an alkali-soluble semister with succinic or phthalic anhydride, which indicated the absence of alcoholic hydroxyl groups; (2) neither fraction reacted with trimethylammonium acetohydrazide chloride, which indicated the absence of carbonyl groups; (3) neither fraction reacted with digitonin, which indicated the absence of sterols of the β -type; (4) after extraction of 5 ml. samples in light petroleum by 2, 1, 1 ml. portions of conc. H_2SO_4 the fluorescence of both fractions was reduced by more than 50%; (5) the fluorescence of neither fraction is appreciably affected by shaking with oxidizing agents in alkaline medium (e.g. Na_2O_2 , alkaline KMnO_4). It is diminished to some extent by shaking with oxidizing agents in acid medium. In this respect the behaviour is similar to that of benzpyrene, though the latter is somewhat more susceptible to oxidizing agents in acid medium. The difference is, however, not sufficiently great to allow a differential estimation.

Recovery of added benzpyrene

The fluorescence intensities of the blank and benzpyrene are strictly additive, as ascertained in experiments where a known amount of benzpyrene was added to a previously measured blank solution. The recovery in the method as a whole was tested by adding known amounts of benzpyrene, varying from 3 to 300 μg ., dissolved in 1 ml. of light petroleum, to the mixture of mouse and alcoholic

Table 1. *Recovery of benzpyrene added to whole mouse*

Benzpyrene added ($\mu\text{g.}$)	No. of analyses	Mean recovery of benzpyrene		Standard deviation (%)
		($\mu\text{g.}$)	(%)	
300	20	263.7	87.9	2.48
100	4	85.3	85.3	0.65
30	4	26.5	88.5	2.68
10	4	8.62	86.2	4.86
3	4	2.58	86.0	3.14
Total	36	Averages 87.36 ± 0.32		$2.82 \pm 0.22^*$

* Probable error.

NaOH before hydrolysis. Table 1 shows the results obtained. No correction for the blank was made in the analyses with 300 $\mu\text{g.}$, where such a correction would be clearly insignificant; but when smaller amounts of benzpyrene were added, 0.5 $\mu\text{g.}$ was subtracted from the recovered quantity to allow for the blank. The recovery falls short of the theoretical figure by about 13%. But this error is fairly constant within the range studied and can therefore be corrected by multiplying the result with a factor of 1.145. This procedure will introduce an error of 6.3% or more in 5% of cases or less.

Accuracy and sensitivity

When the results of the 36 analyses are corrected by the factor 1.145, the errors shown in Table 2, expressed as standard deviations of the analytical results from the theoretical values, are obtained. The accuracy of the method does not diminish appreciably with diminishing quantities of benzpyrene within the range of 3–300 $\mu\text{g.}$

Table 2. *Accuracy of benzpyrene estimations*

Benzpyrene added ($\mu\text{g.}$)	No. of analyses	Standard error (after correction for blank and recovery)	
		(%)	
300	20	± 2.8	
100	4	± 2.3	
30	4	± 3.0	
10	4	± 5.0	
3	4	± 3.4	
0.5	10	± 25.5	

The limit of sensitivity of the method is determined by the blank. When the difference between the fluorescence intensities of benzpyrene and blank becomes small, the errors due to deviations of the blank from the average value used for correction will increase. With quantities of benzpyrene $> 3 \mu\text{g.}$ this error will be insignificant compared with the other errors of the method. But with smaller amounts it assumes larger proportions and the other errors become correspondingly less important. Thus, if we take no account of the variations of recovery, the standard deviation of the mean blank (0.117 $\mu\text{g.}$) shows that in the estimation of 1 $\mu\text{g.}$ benzpyrene a

standard error of 11.7% is to be expected. For 0.5 $\mu\text{g.}$ this error will be doubled. The standard error which was actually observed in 10 analyses is very close to the expected figure (Table 2).

DISCUSSION OF RESULTS

The accuracy of the method is satisfactory if the amount of benzpyrene is not less than 1 $\mu\text{g.}$ As this is only 0.3–0.6% of the marginal dose necessary for tumour production, the sensitivity of the method is sufficient for most purposes. If it is desired to estimate smaller amounts, the spectrophotometric method must be employed which, according to Berenblum & Schoental (1942), has a lower limit of sensitivity of 0.05 $\mu\text{g.}$ benzpyrene/mouse.

If a standard of accuracy is admissible where a standard error of 25% is no serious objection, even results of 0.5 $\mu\text{g.}$ by the present method may still be valuable. Results of 0.3–0.5 $\mu\text{g.}$ benzpyrene should be regarded as qualitative only.

SUMMARY

1. A method is described for the fluorimetric estimation of 3:4-benzpyrene in mice. The range of the method extends from 0.5 to 300 $\mu\text{g.}$, but it is easily adaptable to the estimation of larger quantities.

2. The principal steps in the method are: lysis of the tissue in hot alcoholic NaOH, extraction with light petroleum, purification by chromatographic adsorption and fractional elution, and finally photoelectric measurement of the fluorescence intensity.

3. The evaluation of results requires the application of corrections for a blank and for incomplete recovery of added benzpyrene.

4. The limit of sensitivity is determined by the blank, i.e. the fluorescence intensity of extracts from benzpyrene-free mice. The average value of 54 experiments was found to be 0.50 $\mu\text{g.}$ in terms of benzpyrene. The blank is the same in both sexes, but increases somewhat with the weight of the animal.

5. The mean recovery of 36 experiments ranging from 3 to 300 $\mu\text{g.}$ benzpyrene was found to be 87.36%.

6. The standard error of the same recovery experiments, after correction for blank and losses, varied between 2.3 and 5%. It rose to 25.5% for 0.5 $\mu\text{g.}$, as compared with a standard error of 23.4% to be expected on the basis of the standard deviation of the blank.

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A Note on the Fluorimetric Estimation of 3:4-Benzpyrene by the Method of Miller and Baumann

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After completion of the preceding paper (Weil-Malherbe, 1944*b*) a communication by Miller & Baumann (1943) on the fluorimetric estimation of carcinogenic hydrocarbons came to hand. These authors describe an ingenious method of 'two-condition fluorimetry' in which use is made of the different susceptibility to the quenching effect of O_2 of hydrocarbons on one hand and tissue material on the other (cf. Weil-Malherbe, 1944*a*). If the quenching ratios of both are known, the composition of a mixture can be deduced from the increment of fluorescence obtained on changing from an oxygenated to an oxygen-free medium.

The method has been tested in the following experiment with amounts of 0.2, 0.3 and 0.5 μg . benzpyrene added to three previously purified mouse extracts. Benzene was used as solvent and the measurements carried out first in pure N_2 and then in pure O_2 . The fluorescence ratios (cf. Miller & Baumann, 1943) were: $N=8.8$, $n=3.8$, K (μg . benzpyrene/unit of fluorescence in O_2) = 0.032.

Benzpyrene added (μg .)	Reading in N_2	Reading in O_2	Benzpyrene recovered (μg .)
0.2	227	51	0.21
0.3	218	45	0.30
0.5	272	52	0.48

These results are very satisfactory and there is no doubt that the method of Miller & Baumann will extend the range of sensitivity of the fluorimetric method considerably.

One point of Miller & Baumann's method could not be confirmed. They use crude, unpurified extracts directly for the fluorimetric analysis. These crude extracts are, at any rate as far as whole mice are concerned, entirely unsuitable for fluorimetry for the following reasons: (1) They very often contain finely suspended matter which cannot be re-

moved by centrifugation or which reappears on standing after centrifugation. This is especially true for unchilled extracts. (2) They usually contain yellow pigments of animal origin, and other material, which exert a filter effect by absorbing either the primary or the secondary light. Their fluorescence is white or yellow with hardly any blue component; thus with a violet or blue colour filter the paradoxical result is obtained that they cause a smaller reading than the purified extract. Addition of benzpyrene to these crude extracts does not result in a corresponding increase of fluorescence, as shown by the following experiment:

Extract	Dilution*	Colour	Reading before and after the addition of 1 μg . benzpyrene	
			Before	After
None	—	—	—	118
1	1 in 20	Colourless	0	71
2	1 in 2	Colourless	13	61
3	1 in 2	Yellow	0	4

* Compared with the concentration used for fluorimetry on purified extracts.

It follows (1) that yellow extracts may blot out the fluorescence of benzpyrene completely, (2) that even colourless extracts may reduce the fluorescence appreciably, and (3) that they may do so even after twentyfold dilution. It is unlikely that the technique of hydrolysis and extraction used by Miller & Baumann for large amounts of tissue, which is practically identical with that described in the preceding paper, would have yielded less impure extracts. It seems, therefore, that Miller & Baumann's method, if applied to whole mice, does not dispose of the necessity of purifying the extracts.

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