10. The Quantitative Estimation of 3:4-Benzpyrene in Whole Animals, their Tissues and Excreta

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An accurate study of the rate of disappearance of 3:4-benzpyrene in the animal body requires a very sensitive method for the estimation of the unchanged hydrocarbon in whole animals, separate tissues, tissue fluids and excreta. The technique involved includes (a) a method of quantitative extraction, (b) a means of freeing the extract from interfering substances and (c) the actual method of estimation. The existing methods for (a) and (c) are satisfactory and may be summarized as follows.

Quantitative extraction of benzpyrene from tissues. If the tissue is homogeneous, it may be desiccated, ground to a fine powder and exhaustively extracted with an organic solvent [Chalmers, 1934; Berenblum & Kendal, 1934]. With more complex tissues or whole animals, preliminary hydrolysis with alcoholic KOH is necessary, followed by desiccation and extraction as above, or more simply, by addition of a known volume of benzene, acidification and removal of the ethanol by repeated washing with dilute acid [Berenblum & Kendal, 1936]. Acidification is essential if loss of the hydrocarbon in the aqueous ethanol washings is to be avoided. Since in the latter or 'wet' method of extraction the amount of benzene added is known, and the transfer of the hydrocarbon from the water-acid-ethanol to the benzene phase is quantitative, the amount of the hydrocarbon in the original tissue can be estimated by determining its concentration in a small sample of the benzene extract after purification. Thus, recovery of the whole extract is unnecessary by the 'wet' method.

Theoretically, any organic solvent may be used provided that the extraction is exhaustive. Desirable qualities are: high solubility for the hydrocarbon; low solubility for the impurities; absence of troublesome emulsions when shaken with aqueous solutions; non-miscibility with water (if the 'wet' method is employed); suitability for subsequent chromatographic adsorption. Benzene seems to satisfy these requirements best.

Estimation of the hydrocarbon in the purified extract. Two spectrographic methods are available: (1) comparison of the intensities of the fluorescence bands with those of a number of known standards photographed on the same plate [Berenblum & Kendal, 1936]; (2) comparison of the intensities of the absorption bands [Chalmers, 1934; Lorenz & Shear, 1936]. Though originally designed for the estimation of 1:2:5:6-dibenzanthracene, both methods are equally applicable to benzpyrene. The fluorescence technique is, however, the simpler of the two and was therefore used in the present investigation, more especially since the intensities of the fluorescence bands for benzpyrene are considerably stronger than those for dibenzanthracene and the sensitivity, therefore, is correspondingly greater.

Purification of the crude extract. The main difficulties in all previous methods are met with during the stage of purification, since this requires the removal of all interfering substances without loss of the hydrocarbon. The interfering substances are of two kinds, those which produce diffuse fluorescence in the region of the specific bands of the hydro-

carbon, and those which absorb light in that region. The methods of purification previously adopted may be summarized as follows.

(1) *Washing with acid.* This represents the first stage in the purification by the 'wet' method, and serves the double purpose of washing out the ethanol and of removing alkaline impurities.

(2) *Washing with alkali.* Provided that all the ethanol has already been removed by acid washing, shaking with a solution of Na_2CO_3 removes the bulk of the impurities from the benzene extract without any loss of hydrocarbon. In the original method [Berenblum & Kendal, 1936] the washing was repeated till all the soaps in the emulsified benzene layer were removed.

The objections to this procedure are: (a) the prolonged washing is tedious; (b) the amount of alkali needed is very great; (c) with extracts from fatty tissues the emulsification is often very troublesome; (d) with extracts from non-fatty tissues the final solution may remain darkly coloured.

(3) *Chilling with CO_2 snow.* This procedure was used by Lorenz & Shear [1936] for the removal of fats and other impurities. It is not as efficient as washing with alkali.

(4) *Treatment with digitonin.* This troublesome and expensive procedure for the removal of cholesterol, adopted by Chalmers [1936], seems unnecessary, since most of the cholesterol can be removed by adsorption on alumina (see below), while small amounts which may remain do not interfere with fluorescence spectrography.

(5) *Adsorption of impurities on alumina.* According to the method of Lorenz & Shear [1936], the extract is shaken with aluminium oxide for the further removal of impurities. The method, though effective for the removal of impurities, leads to inaccuracies, owing to some adsorption of the hydrocarbon itself on the alumina.

In short, none of the methods of purification so far described is ideal, while the most efficient one is very troublesome to carry out for large numbers of tests. Moreover, in certain cases there is a persistence of diffuse fluorescence in spite of all the above treatments. As the result of the following experiments, most of the difficulties have been overcome and a method has been devised which combines high sensitivity with relative simplicity of procedure.

EXPERIMENTAL

Alkaline washing of the crude benzene extract

By varying the concentration and amount of Na_2CO_3 used, it was found possible to control to some extent the tendency for persistent emulsification. Tests on extracts from animals and tissues containing varying amounts of fat, elicited the following information.

(1) The more persistent the emulsification, the smaller were the amounts of impurities in the resulting benzene extract.

(2) Extracts from tissues with a low fat content, which normally yield deeply coloured solutions after alkaline washing, could be made to yield almost colourless solutions by the preliminary addition of stearic acid.

(3) The purification of the crude benzene extracts was no more complete when the soaps were removed by prolonged alkaline washing than when removed by centrifuging the emulsion after only a single washing.

From these results, it is evident (a) that emulsification is beneficial, in that impurities are carried down with the soaps, (b) that when emulsification is inadequate; it is advantageous to produce it artificially by addition of stearic acid and vigorous shaking, and (c) that prolonged alkaline washing (as used in the original method of Berenblum & Kendal) is unnecessary, since the soaps can be removed more simply and with equally good results by centrifuging.

With these modifications in the technique, almost colourless extracts could now consistently be obtained, though some impurities still remained.

Adsorption on alumina

Since benzpyrene is less readily adsorbed on alumina than is dibenzanthracene [Winterstein & Vetter, 1934], experiments were undertaken to determine whether it was possible to include treatment with alumina as an additional method of purification for benzpyrene, without causing a significant loss of the hydrocarbon.

Benzene extracts with known concentrations of benzpyrene were passed through columns of alumina (1 cm. wide, 4 cm. high), and successive samples of the filtrate were tested for the hydrocarbon. While the first few drops contained no benzpyrene, the amounts in the subsequent fractions increased progressively until, after 8–10 ml. had passed through, the benzpyrene content in the filtrate was the same as in the original solution. Thus, under standard conditions, using benzene as the solvent, an equilibrium is soon reached between the benzpyrene in solution and that adsorbed, while most of the impurities remain strongly adsorbed. The method can therefore safely be used for purification, provided that the first 10 ml. are discarded. Using narrower columns, the amount to be discarded can, of course, be correspondingly reduced.

Removal of the residual diffuse fluorescence

When the benzpyrene content of a mouse is very low, so that concentration of the final extract is necessary for its detection spectrographically, a diffuse fluorescence is often observed in the blue region of the spectrum, and this may interfere with the accurate estimation of the benzpyrene. This diffuse fluorescence was found to be due to an impurity present in variable amounts in different samples of benzpyrene, which persists in the tissues long after the benzpyrene has disappeared [Berenblum & Schoental, 1942]. It can be separated from benzpyrene by differential adsorption, for while neither is adsorbed to any significant extent on alumina from benzene, both are strongly adsorbed from light petroleum. If such a column is developed with a mixture of 1 part of benzene and 2–3 parts of light petroleum, the benzpyrene alone passes through into the eluate.

The quantitative estimation of benzpyrene in faeces presented special difficulties. (a) Owing to the low water content of faeces as compared with tissues and the presence of cellulose and other undigested residues from the food, hydrolysis with alcoholic KOH is insufficient to reduce the material to a condition suitable for extraction. (b) The high concentration of pigments in the faeces, including certain metabolic products of benzpyrene, produces coloured extracts. (c) Certain factors in the diet of the animals can influence the results of quantitative estimations.

The substitution of aqueous for alcoholic KOH was found to result in more effective breaking up of the faecal particles, and, though some of the material remained undissolved, this did not interfere with the quantitative extraction of benzpyrene. The pigments derived from the faeces of rats or mice were found to be sufficiently strongly adsorbed on alumina to yield colourless filtrates free from non-specific fluorescence, provided that the column was tall enough.

The factor of diet was found to be seriously disturbing in two different respects. Irregular, but consistently low, values for benzpyrene were found in faeces from control mice or rats after known amounts of benzpyrene had been added to them. This was traced to the presence of adsorbent carbon, derived from black biscuits ('Spratt's ovals') given as part of the diet, the carbon having acted as a strong adsorbing agent for the hydrocarbon. After eliminating the black biscuits from the diet, accurate values for benzpyrene were obtained. In the case of faeces from rabbits or guinea-pigs, the benzene extracts remained deeply coloured even after passing through alumina. The pigments responsible for this, presumably derived from the green vegetables in the diet, could be removed by

adsorption on alumina from light petroleum followed by elution of the benzpyrene with a mixture of light petroleum and benzene, as used for the removal of the fluorescent substances occasionally present in tissues (see above).

METHOD

For whole mice

The animal is boiled for 2–3 hr. with 70–100 ml. ethanol and 8–12 ml. sat. aqueous KOH under a reflux condenser. 50 ml. benzene are then added and the boiling is continued for a few minutes. After cooling, the solution is transferred to a separating funnel, while the sediment (mainly bones) is dissolved in 25–50 ml. conc. HCl which are added to the contents of the separating funnel. About 50 ml. water are also added and the mixture is shaken and allowed to separate. The acid-aqueous-ethanol phase is discarded and the benzene phase washed with a further 100–150 ml. water, the aqueous phase being again discarded.

About 100 ml. water are again added, the mixture is vigorously shaken, and a sat. solution of Na_2CO_3 is added, a few ml. at a time, with vigorous shaking. At first, a fine milky emulsion develops, but with further addition of alkali the colour changes to a greenish brown and the emulsion becomes coarser, till a distinct flocculation occurs and the mixture separates into two layers, a flocculated emulsion in the upper layer and an aqueous layer below. Usually 25–35 ml. of alkali are required, depending on the amount of fat originally present and on the amount of acid remaining from inefficient separation during the previous treatment. The aqueous phase is discarded and the emulsified upper layer is centrifuged for 5–10 min. Most of the clear benzene extract, which separates as an almost colourless solution, is decanted, dried by shaking with anhydrous Na_2SO_4 and passed through a column of alumina 1 cm. wide and 4 cm. high. The first 10 ml. of filtrate are discarded and the remainder kept for quantitative analysis by fluorescence spectrography, as described by Berenblum & Kendal [1936]. The fluorescence spectrographic apparatus used was similar to that described by Hieger [1930], with certain minor improvements in design suggested by Dr Hieger.

By this method, the fluorescence bands of the test solution are photographed on the same plate as those of a number of standard solutions of benzpyrene in benzene, and the intensities compared by eye. The standard solutions used ranged between 0.005 and 0.15 $\mu\text{g.}/\text{ml.}$, so that the test solutions were diluted or concentrated to bring them within this range; the amount of dilution or concentration was taken into account in the final calculation. With the optical arrangements used, the limit of sensitivity for benzpyrene (using 50 ml. of benzene in the original extraction, and concentrating the purified extract

5 times) was $\frac{50 \times 0.005}{5} = 0.05 \mu\text{g.}/\text{mouse.}$

If, as mentioned previously, the final extract has a diffuse fluorescence, the following further treatment is necessary. An aliquot part (20 ml.) of the purified extract is evaporated to dryness, taken up in excess of light petroleum (b.p. 40–60°) and passed through a column of alumina on which both the benzpyrene and the impurity are adsorbed. The column is then developed with a mixture of 1 part of benzene and 2–3 parts of light petroleum until all the benzpyrene is eluted (as judged by ultraviolet light, the benzpyrene passing down as a violet fluorescence, while the impurity remains on the column as a bluish-white fluorescence). The eluate is then evaporated to dryness and taken up again in 20 ml. of benzene.

Excluding the last-mentioned procedure, which is only rarely necessary, the whole purification takes about 1 hr., and, with sufficient glassware and apparatus for serial work, it is possible for one person to carry out more than 20 complete analyses per day.

* 'Aluminium oxide (for adsorption purposes)', British Drug Houses, Ltd.

For separate tissues

The method for separate tissues or blood is similar to that described for whole mice, though smaller amounts of reagents can be used when the amount of tissue is less. The only difference arises from the fact that most tissues contain proportionately less fat than the whole mouse, so that adequate emulsification must be artificially produced by addition of a few mg. of stearic acid before the alkaline washing. The amount necessary varies with different tissues, and a preliminary trial with a sample of the normal tissue is advisable. By reducing the amount of benzene from 50 to 20 ml., the limit of sensitivity of the method was found to be $0.02\mu\text{g.}$

For mouse or rat faeces

(*Note.* The diet of the experimental animals should contain no activated carbon or charcoal, and green vegetables should be excluded.) 1 g. of faeces is boiled, with occasional agitation, for about 1 hr. with 10–15 ml. 10 % aqueous KOH; 30–40 ml. ethanol are added and the boiling is continued for a further 1 hr. After the addition of 25 ml. benzene and further boiling for 5–10 min., the mixture is allowed to cool and acidified with conc. HCl, and the supernatant fluid is decanted into a separating funnel. The residue in the flask is washed with a small amount of ethanol and the washing added to the remainder. A little water is then added in the separating funnel and the mixture shaken and allowed to separate. After discarding the aqueous phase, the washing and separation are repeated. The benzene layer is then shaken with about 50 ml. water and small additions of a few ml. of sat. aqueous Na_2CO_3 and centrifuged. The separated benzene phase is dried with anhydrous Na_2SO_4 and passed through a column of alumina. The first 10 ml. of filtrate are discarded and the remainder kept for spectrographic analysis.

Accuracy of the method

Numerous tests were carried out to determine the accuracy and reliability of the method, and the range of concentration of benzpyrene over which the method is effective. Different amounts of benzpyrene, ranging from $1\mu\text{g.}$ to 10 mg., were added either directly to the vessel in which a whole mouse was being hydrolysed, or injected into mice immediately before killing them. The recovery of benzpyrene, as estimated spectrographically, was consistently satisfactory over the whole range of concentrations tested, the maximum error observed not exceeding $\pm 20\%$. Since the error due to visual comparisons of the intensities of the fluorescence bands, and to variations in the intensity of the source of light during exposure, may account for at least $\pm 15\%$, it is evident that no significant loss of benzpyrene occurs during the stage of purification. Similar tests were carried out with faeces, the results being also satisfactory.

SUMMARY

A method is described for the quantitative estimation of 3:4-benzpyrene in whole mice, separate tissues, tissue fluids and excreta, based on the 'wet' method of extraction and spectrographic analysis of the fluorescence, originally described by Berenblum & Kendal [1936] for 1:2:5:6-dibenzanthracene.

The essential changes in the method are concerned with the purification of the extract for the removal of interfering substances; these result in a simplification of the procedure and a greater sensitivity.

The limit of sensitivity of the present method was found to be in the region of $0.05\mu\text{g./}$ mouse, while the accuracy was of the order of $\pm 20\%$ over a range of 1–10,000 $\mu\text{g./}$ mouse.

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