The Biochemistry of Benzpyrene
III. The Quantitative Estimation of the Metabolites.*

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INTRODUCTION
The first two papers of this series gave evidence for the chemical changes undergone by 3,4-benzpyrene in the tissues of the mouse. A number of metabolites were described and provisionally named, X₁, X₂, F₁, and F₂. Of these, the chemical nature is known only of F₂, this being, in the case of mice, 8-hydroxybenzpyrene. Furthermore, it was shown that the X-derivatives are the first formed. It has since been shown that these are formed at the site of application of the benzpyrene (1).

In order to further the studies of these phenomena, an improved technic for the separation of the metabolites has been evolved and a system for their quantitative estimation developed.

THE SEPARATION OF THE METABOLITES
Throughout the method to be described allowance must be made for the relative instability of the metabolites and the fact that normal tissue components are liable to interfere in the process. Previously, a method for separation by partition chromatography has been described (2). As, however, the removal of the adsorbed material from the adsorbate cannot be carried out satisfactorily, this method has been discarded, and attention concentrated on the separation of the metabolites by way of their different solubilities in varying solvents. This method, with the improvements noted below has been found satisfactory.

Ultimately all preparations are destined for examination by ultraviolet light. Therefore, the reagents used must be checked for fluorescence before use. In actual practice, it has been found that even the best commercial reagents are rarely free from fluorescence due to impurities, and purification methods have had to be developed.

**Extraction of tissues.**—In the case of painted skins, it has been found advantageous to remove any loosely adherent benzpyrene by preliminary washings with benzene. Thereafter, the technic is the same for all tissues. After mincing very finely, small quantities not exceeding 1 gm. being used, the tissues are repeatedly extracted with 7 to 10 cc. lots of 70 per cent acetone (70 cc. acetone, 30 cc. glass-distilled water). The 70 per cent acetone is found to give a better extraction than pure acetone. Extractions are continued until both acetone and the tissue fail to fluoresce under the ultraviolet lamp.

The acetone extracts are pooled and evaporated under reduced pressure in the vessels described previously (3). To prevent loss from frothing during this process, 0.5 to 1.0 cc. of petrol ether (b.p. above 120° C.) is added. Thereafter the procedure is that described in Fig. 1.

The only point needing special mention is the fact that F₁ and F₂ still require to be separated from benzpyrene by chromatographic methods, since, unlike the X metabolites, no suitable solvents have been found to separate them. The choice of method followed must be determined by the experiment in hand.

Using these methods, solutions of the metabolites are obtained, and after clearing by centrifugation are ready for quantitative estimation.

THE OPTICAL ESTIMATION OF THE METABOLITES
Although the following methods are based on the quantitative extinction spectra of purified substances, the amounts recovered from mouse tissues are rarely sufficient to allow the production of absorption spectograms which can be directly estimated by photometric methods. Therefore, the indirect method of spectro-fluorometry has been used. Such a method has its limitations, but if these are borne in mind, the results are generally of the same order of precision as those in biological experiments.

No special experimental method can be recommended for the fluorescence spectrophotometry, as it depends upon the laboratory facilities available. Whatever method is used must be the subject of a period of trial and error until reliable results can be consistently obtained.

The problem under study is concerned with 5

*NOTE: Because of accelerated production schedule, proof of Dr. Weigert's paper has not been read in his laboratory.
†The Editors of Cancer Research announce with sincere regret that Dr. Weigert died April 13, 1947. The present paper was prepared for publication shortly before his death.
different compounds, all of which emit blue fluorescent light with only slightly different spectral band structures. Furthermore, all compounds are liable to be contaminated with fluorescent cell constituents. This excludes the sensitive but unspecific photoelectric comparison of the fluorescence intensities, and leaves, as the more reliable method, the match between a fluorescence spectrum of a derivative under standard conditions, and the fluorescence spectrum of the same derivative after its separation by the previously outlined method.

The matching may be done by photoelectric microphotometry or by direct visual comparison of the spectrograms. Although less sensitive, the visual method is the quicker. Furthermore, it has an added advantage in that the spectrograms usually exhibit a slight uniform background besides the normal bands, and the error due to this can be more easily reduced by visual examination than by microphotometry. The background itself can be reduced considerably by exciting the fluorescence, not with the strong ultraviolet Hg line at 365 m\(\mu\), but with the weaker violet lines after filtering out the 365 m\(\mu\) line. The use of the weaker violet lines does, however, involve considerable extension of exposure times to produce good spectrograms.

Apart from the uniform background which detracts from the specificity of the spectra, another serious source of error must be considered. Some preparations containing the X metabolites, especially those from mouse skins, exhibit on long exposures a band spectrum similar to that of the X metabolites. That this alien “S” spectrum—origin unknown—is due to some normal tissue component and not to any benzpyrene derivative is shown by the fact that it can be obtained from extracts of untreated skin. Instead of the almost uniform width of the 2-3 X bands, the violet “S” band is narrow and the “blue” band much wider. Any spectrogram showing this narrow violet band is useless for fluorimetric purposes. The system is therefore limited in its sensitivity to about 0.002 \(\mu\)g/cc.cm by the presence of this unknown tissue component.

The method adopted was the visual matching of an unknown fluorescence spectrum, exposed for time \(t\) seconds with one of a series of fluorescence spectrograms of a standard solution of the same compound, at graded exposures to seconds. If the volume of the solution under test is \(V\) cc. and the standard contains \(c\) mgm./cc., the amount of metabolite is calculated as:

\[
\frac{c \times V \times t}{E} \text{ gm.}
\]

where \(E\) is the maximal extinction.

For these results to be reliable, the fundamental conditions of fluorometry must be fulfilled; i.e., the optical conditions must remain unchanged and the test solutions be so diluted that the fluorescent intensity be practically uniform throughout the whole layer of liquid.

The standard solutions of known concentration \(c\) were made up from preparations obtained by chromatography, as described in Part I of this series (2). As the chemical identities of the derivatives are unknown, the estimations of the standard solutions have been done by absorption spectro-photometry. If volume \(V\) of the solution is used in a tube length \(L\), and \(E\) is the maximal extinction in the near ultraviolet whilst \(C\) is a special constant for each derivative, the amount, \(N\), present in the solution is given by the expression:

\[
N = \frac{V E}{L C} \text{ mgm.}
\]

The numerical values of \(C\) were the same as previously adopted (2); i.e., for benzpyrene 120 at 365 m\(\mu\), for \(X_1\) and \(X_2\), 85 at 380 m\(\mu\) and for \(F_1\) and \(F_2\), 100 at 380 m\(\mu\). Additionally, the two maxima in the violet may be used for the separate estimation of \(F_1\) and \(F_5\). In this case, the values for \(C\) are: \(F_1\)=65 at 118 m\(\mu\), and \(F_2\)=40 at 425 m\(\mu\).

This last equation may be used in cases where the less sensitive but more direct absorption spectrophotometric method is applicable. Examples of such cases are:

- The estimation of the sum of the \(X_1\) and \(X_2\) metabolites in the extracts of intestines prior to separation.
- The total estimation of \(F_1\) and \(F_2\) in xylene extracts of feces, when negligible amounts of unchanged benzpyrene are present.
- The method of absorption spectrophotometry is generally useful for the estimation of residual, unchanged benzpyrene in the carcass. But in all cases where the amounts involved are too small for absorption spectrophotometry recourse must be made to the fluorimetric method.

DISCUSSION

The methods developed for the quantitative estimation of the various intermediate products of the metabolic conversion of benzpyrene are the result of a number of compromises. Two main factors limit the systems:

- The necessity to use physical methods for the separation of the metabolites—imposed by the ignorance of the exact chemical nature of the substances in question.
- The fact that the benzpyrene derivatives are rela-
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tively unstable and can, therefore, be obtained only in small quantities.
Both of these factors reduce the precision of the methods. Nevertheless, the order of quantitative results obtained under these conditions must be regarded as reliable and as such is of value in the general approach to the problem of the metabolism of benzpyrene.

SUMMARY
1. An improved technic, founded upon physical properties of the substances concerned, for the separation of the metabolic products of 3,4-benzpyrene is described.
2. A fluorimetric method for the quantitative estimation of these products is outlined.
3. Consideration is given to the conditions under which absorption spectrophotometry can be used for quantitative estimations.
4. The limitations of the systems considered are discussed.

REFERENCES

TREATED TISSUE
Wash with benzene
MINCED TISSUES
Fully extracted with 70% acetone
ACETONE EXTRACT
Add Pet. ether and drive off acetone
WATER PHASE
Saturate with Ammon. Sulphate and extract with xylene

Fig. 1.—Chart showing method of extraction of metabolites. Decision as to whether the whole or part of the scheme should be used must depend on the experiment in hand.
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