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THE IDENTIFICATION OF A CARCINOGENIC COMPOUND IN COAL-TAR

BY

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The historian E. A. Freeman wrote, "If you have read Mr. Froude's account of an historical event you know at any rate one way in which it did not occur," and the same comment might be made upon some descriptions of the discovery of 3:4-benzpyrene and even of those issuing from the most august sources (*Nature*, 1954). Such descriptions are either true or untrue, and one cannot attempt any allotment or reallotment of merit to the various persons concerned until the untrue versions have been eliminated.

Preliminary Survey of the Problem

Early in 1922 I was appointed to the staff, which consisted of Dr. Archibald Leitch only, of the Research Institute of the Cancer Hospital (Free) (now the Royal Marsden Hospital), London, and began an attempt to identify the cancer-producing compound in coal tar. There was nothing to guide one except evidence that it was of high boiling-point, neutral, nitrogen- and sulphur-free, and capable of forming a picrate. These properties had been ascertained by the late Dr. Bruno Bloch, a dermatologist of Zurich, in an admirable preliminary investigation (Bloch and Dreifuss, 1921). With these clues I collected from the literature of coal-tar all that was known about the presence in tar of substances which had these properties, and obtained specimens of them from various sources. In the next two years anthracene, phenanthrene, retene, fluorene, acenaphthene, chrysene, truxene, and picene were applied to the skin of mice with negative results, although in a later experiment chrysene gave us one carcinoma on the 853rd day.

A study of all that had been recorded about industrial cancers caused by various forms of tar and mineral oil suggested that exposure to temperatures above a certain level was a factor in conferring carcinogenic power upon such materials.

The information collected on this problem was published in a paper (Kennaway, 1924b), entitled "On Cancer-producing Tars and Tar-fractions," consisting of three sections: (1) The Composition and Cancer-producing Power of Lignite Tar, the Coal Tars, and Shale Oil, and the Effect of Retort Temperature upon These. (2) The Distribution of the Cancer-producing Substance among the different Fractions of Gas-Works Tar. (3) A Comparison of the Composition of Coal Tars, Lignite Tar, Shale Oil, and Petroleum, with special Reference to the High-boiling Compounds.

The Factor of Temperature

When I went to the Cancer Hospital I at once secured the help of Mr. Frank Goulden (now principal bio-

chemist at Ashford Hospital, Middlesex) as assistant, and his technical skill played a quite essential part in the advances of the ensuing years. We found that gas-works pitch, heated to 500° in an iron retort, gave a dark red strongly carcinogenic distillate, an observation which was of use to us later on. We showed that carcinogenic products could be obtained by heating to temperatures in the region of 700° to 900° in an atmosphere of hydrogen various substances—namely, (a) a non-carcinogenic sample of petroleum; (b) isoprene $\text{CH}_2:\text{C}(\text{CH}_3)\text{CH}:\text{CH}_2$, and acetylene—that is, compounds containing only carbon and hydrogen; and (c) various biological products (cholesterol; yeast; human skin, hair, and muscle).

Under these conditions molecular condensations with loss of hydrogen and production of polycyclic aromatic compounds take place, and the simultaneous acquisition of carcinogenic power suggested that the compound which we were seeking was of this class. The classical paper of Berthelot (1866), who described the production of such molecules from the simplest organic compound acetylene, was of great value to us; his very concise account of this research is even now well worth reading.* "... L'acétylène, chauffé dans une cloche courbe à une température voisine de la fusion du verre, se transforme peu à peu en polymères... après une suite fastidieuse de manipulations méthodiques... j'ai isolé... benzine, styrolène, carbures fluorescents, rétène..." The results of our experiments, which were concerned especially with questions of temperature, appeared in a paper entitled "Experiments on Cancer-Producing Substances" (Kennaway, 1925).

The Schroeter Reaction

In studying the problem of the carcinogenic effect of tar one had always in mind the possibility that these industrial cancers in man might throw light upon the far more important cancers which are caused by some internal factors. Chemical reactions which *in vitro* require the temperature of red heat, must, if they occur within the body, be brought about in some other way.

In 1923 I read a paper by G. Schroeter (1920) describing the production at 30° to 40° from tetralin (tetrahydronaphthalene), by the action of 1 to 1.5% aluminium chloride, of a number of more complex compounds, of which benzene, octahydroanthracene, octahydrophenanthrene, α -phenyl- δ -2-tetralyl-butane, 2:6'-ditetralyl, and dodecahydrotriphenylene were identified. These low-temperature products seemed to be worth

*The abiding value of Berthelot's work has been evident to us again now, thirty years later, in relation to carcinogenic effects of tobacco smoke, in which acetylene is present.

testing for carcinogenic power, and within the next two years we obtained, with preparations made by Schroeter's method and by many modifications of it, 110 cancers in 496 mice. This was a very lucky shot, for, although the carcinogenic compound in the mixture has never been identified (though it is in all probability 3:4-benzpyrene), the result was of unexpected value to us at a rather later stage.

In 1924 Mr. I. Hieger came to us from Professor Jack Drummond's laboratory at University College, London, as a voluntary worker, and during that year and the next, when he received a grant from the British Empire Cancer Campaign, worked upon the effect of copper salts upon the growth of carcinoma, the action of x rays upon cholesterol, and the amount of glutathione in tumours, and he collaborated with me in the study of the Schroeter reaction.

We introduced a number of variations into the technique for obtaining "Schroeter" preparations, such as altering the proportion of AlCl_3 to tetralin, changing the temperature of the reaction, using tetralin of different degrees of purity, fractionating the product by distillation at low pressure, and using different concentrations for the tests on mice.

The mixture of compounds in "Schroeter" is certainly complex, but it can hardly be as complex as tar; this slight simplification, and the fact that a cancer-producing substance could now be prepared without recourse to pyrolysis or even the use of temperatures above body temperature, was encouraging to us.

The work would have stopped there except for one vitally important feature of the Schroeter products: they were vividly fluorescent not only in ultra-violet light but even in ordinary daylight. Numerous entries in our laboratory note-books show how often we were struck by the beautiful blue-violet fluorescence of the distilled Schroeter fractions.

Application of Fluorescence Spectroscopy

Dr. W. V. Mayneord (later Professor of Physics as applied to Medicine, Institute of Cancer Research) came to the radiological department of the Cancer Hospital as physicist on a part-time basis in the latter part of 1926, and became a full-time member of the staff in the following year. We had then in the laboratory various higher-boiling fractions of coal-tar, such as green oil and anthracene oil, and the various "Schroeter" preparations; and the conspicuous fluorescence of these materials caused him, without any encouragement from me, to examine this property spectroscopically.

He found, early in 1927, that the Schroeter products showed two very distinct bands in the blue and violet regions of the spectrum. He photographed over 100 fluorescence spectra of the various non-carcinogenic hydrocarbons and of the carcinogenic preparations, mentioned above, which we had acquired in the preceding five years, and found that the same bands could be picked out in the spectrum of a majority of the latter group. Hieger then joined in the work, and they examined all the tars made in the laboratory from acetylene, human tissues, and other materials, shale oil, pitch distillate, and various extracts and fractions of coal-tar. None of them showed these bands as distinctly as did Schroeter preparations; the fact that the spectrum appeared in this carcinogenic mixture which was produced at a low temperature, and had nothing to do with any form of tar or mineral oil, provided very strong evidence that the two properties were in some way associated.

They next tried to find some hint of the source of these specific "cancer bands" by examining the fluorescence spectrum of as many hydrocarbons, of types present in tar, as were available, without success.

Hieger described the joint work of Mayneord and himself in his reports to the British Empire Cancer Campaign for 1927 and 1928. These statements are of interest, as they show how the problem presented itself at a time when the later developments were all unknown.

"Spectroscopic Studies on Substances Present in Carcinogenic Tars"

"These experiments are being carried on in collaboration with Mr. W. V. Mayneord, Physicist at the Cancer Hospital. Recent researches on tars obtained from acetylene indicate that the carcinogenic power of coal-tar resides in compounds consisting solely of carbon and hydrogen similar possibly to hydrocarbons like anthracene. Anthracene and its associated hydrocarbons are characterized physically by their property of fluorescing with an intense blue or violet colour when exposed in crystalline form or in solution to ultra-violet rays such as are generated by the ordinary quartz mercury-vapour lamp.

"In this investigation the hydrocarbon was illuminated by means of a quartz lamp and a photograph taken of the spectrum of the fluorescent light. Each one of a large number of hydrocarbons, of which about 14 were synthesized in this laboratory, was photographed in this way and the spectra were compared. It now seems practically certain that each type of hydrocarbon and hydrocarbon derivative has its own characteristic fluorescence spectrum. This remarkable specificity could possibly assist in the exploration of hydrocarbons, present in tar fractions, which have hitherto not been isolated. These ill-defined substances may be related to or identified with the cancer-producing factor, since the components of tar which are crystalline and easily removed have been shown to be non-carcinogenic.

"Again, the fluorescence spectrum can be made an instrument for investigating the mixtures obtained in our attempts to synthesize carcinogenic substances by methods not involving high temperature. The spectra could thus serve as delicate tests for judging when the methods of the experiments were successful in imitating high-temperature reactions by revealing the appearance of the types of hydrocarbon present in coal-tar."

A year later the report for 1928 is slightly more encouraging.

"The Use of the Spectroscope in the Study of Cancer-producing Tars (In collaboration with Mr. W. V. Mayneord, Physicist at the Cancer Hospital.)"

"An investigation of the optical properties of the hydrocarbon class of compounds to which belong the cancer-producing factor found in tars. Tar is an exceedingly complicated mixture, and the isolation of the cancer-producing factor will prove a very difficult achievement if reliance is placed on chemical methods alone. Spectroscopic analysis has supplied some evidence of the existence of at least one new compound in carcinogenic tars. A large number of hydrocarbons have now been synthesized in the laboratory in order that their spectra may be examined and compared. Progress is somewhat slow, since many of the compounds required have not been described in chemical literature. There is a prospect that the examination of a sufficient series of spectra of old and new compounds may enable one to define the characteristic spectra of certain classes of bodies, and then to detect their existence in very small amounts in complex mixtures such as tar."

Professor Mayneord has given me a copy of his contribution to an informal congress convened by the British Empire Cancer Campaign on December 13, 1932. He begins:

"It is certainly a curious state of affairs which causes a physicist to take part in a discussion on 'biochemical' research in relation to cancer, perhaps more so when he is the first member of a team of workers to speak on this subject." (This introductory statement certainly "dates," for in the next 23 years cancer research has passed more and more into the hands of physicists.) He goes on to say that, in view of the unavoidable slowness of the mouse-painting test for carcinogenic power "... we tried to imagine a method which should (a) tell us something of the structure of the compound undergoing test, or at least serve as a method of identification; (b) be rapid in use as well as simple; and (c) be capable of detecting very small quantities of the substance. The last desideratum suggests optical methods, since, as is well known, many substances such as anthracene can be detected in one part in a million by their absorption of U.V. light and fluorescence. We tried, therefore, commencing in 1927, to measure the U.V. absorption of some of the mixtures known to be carcinogenic." When "... a number of substances had been examined in this way there did appear to be some correlation between the type of spectra obtained and the carcino-

genic power of the material. The carcinogenic mixtures yield in a number of cases a certain type of spectrum—namely, 3 bands on the U.V. limits of visibility at about 4,000 Å. At first an invariable relationship between carcinogenic activity and spectral type was suspected, but more extensive investigation shows that, though a useful indication, the two phenomena are not invariably linked together, or, stated otherwise, we have not yet found a feature of the fluorescent mixture common to all carcinogenic compounds.

"Mr. Hieger during the last year or two has examined hundreds of mixtures, and will in a few minutes describe a recent example of the use of the method. The type of spectrum emitted, however, indicated that investigation of pure polycyclic aromatic hydrocarbons would be well worth while, and the spectra, therefore, led to the extension of the work which will be described by my colleagues." He then describes the technique of absorption spectroscopy and its use in identifying the compound isolated from pitch with the 3:4-benzpyrene synthesized by Dr. J. W. Cook.

When Hieger and I (1930) published the first results he possessed more than 700 photographs of fluorescence spectra, of which 70 were reproduced in his paper; we had tested 61 more or less pure compounds in addition to all our Schroeter preparations and had used several thousands of mice.

Mayneord appeared as co-author of the above reports and of three papers mentioned below, but by an inexcusable oversight his introduction of fluorescence spectroscopy was not acknowledged by me (Kennaway, 1930) or by Hieger (1930) when we reported our work on cancer-producing substances. The very serious character of this omission was made plain by Hieger (1937): "The position of the carcinogenic hydrocarbons in cancer research has now been established. That these remarkable compounds are available is due to the fruitful pioneer work of Mayneord on the fluorescence spectrum of tar. . . . Mayneord made the fundamental observation—namely, that the same characteristic bands were to be found in the spectra of tars and other carcinogenic substances such as 'Schroeter mixture.'"

Interest in some peculiar features of the fluorescence spectrum of anthracene led Hieger (in March, 1928) to consult the author of a standard book on that hydrocarbon, *Anthracene and Anthraquinone*, Dr. E. de Barry Barnett, head of the department of organic chemistry at the Sir John Cass College, and he received from Dr. J. W. Cook, who was then Barnett's assistant, some samples of polycyclic hydrocarbons for fluorescence spectroscopy.

Very fortunately, among these samples was one of 1:2-benzanthracene. The fluorescence spectrum of this hydrocarbon was found to be so similar to that of Schroeter as to leave no doubt that one was dealing with closely allied compounds; the two spectra are practically identical, but that of the hydrocarbon lies in a region of longer wavelength.

The next move was clear; some derivative of 1:2-benzanthracene must be sought which by reason of intramolecular addition would slow the vibration of the fluorescence and so cause a shift to longer wavelength. Hieger attempted, but with little success, to synthesize simple derivatives such as the 3-methyl compound, which, being a heavier molecule, might have a spectrum of the required wavelength.

The First Synthetic Carcinogens

Early in 1929 I read in *British Chemical Abstracts* of the synthesis by Clar (1929), then working in Dresden, of 1:2:5:6-dibenzanthracene. I translated the paper and Goulden prepared the compound, and also its 3'-methyl derivative by the method of Fieser and Dietz (1929). Both proved to be carcinogenic when applied to the skin of the mouse. The fluorescence spectrum was found to consist of the Schroeter bands in a position intermediate between those of Schroeter and those of 1:2-benzanthracene. These were the first synthetic carcinogens; later on these compounds were prepared in a very highly purified form by J. W. Cook (see below). Without the clue of the fluorescence spectrum there would have been nothing whatever to direct one's attention to those particular papers in the *Berichte* among the immense number produced by organic chemists.

Clar and his fellow-workers (1929) published three more papers on the synthesis of polycyclic hydrocarbons, and during the summer of 1929 Goulden prepared by their methods the following materials:

- (1) 1:2:3:4-Dibenzanthracene.
- (2) Mixture of isomers of (1), probably naphtho-2':3':1:2-phenanthrene and naphtho-2':3':2:3-phenanthrene.
- (3) Naphtho-2':3':1:2-anthracene.
- (4) 7:7'-Dimethyl (naphtho-2':3':1:2-anthracene).
- (5) Anthraceno-2':1':1:2-anthracene.
- (6) Anthraceno-1':2':1:2-anthracene.

They were not all highly purified, a process which may require as many as 20 recrystallizations of a single compound (Clar), but they sufficed for preliminary tests on mice for carcinogenic power, which was shown by three of them—namely, 1:2:5:6-dibenzanthracene, its 3'-methyl derivative, and 1:2:3:4-dibenzanthracene.

Synthetic Chemistry and Cancer Research

Dr. J. W. Cook* came to us in October, 1929, from the Scientific Staff of the Chemical Research Laboratory of the Department of Scientific and Industrial Research, and undertook the synthesis of numerous compounds, most of them previously unknown, to be examined for carcinogenic activity. He soon had under test the 3'-methyl-, 10-benzyl-, 6-phenyl-, and 9:10-diphenyl- derivatives of 1:2-benzanthracene, and many other compounds (1- and 2-phenyl anthracene, acenaphthanthracene and its 10-benzyl derivative, 2:3-benzo-8:9 (1':2'-naphtho)-chrysene, 2:3:8:9-dinaphtho-1:2'-chrysene, and 1:2:3:4:5:6-tribenzanthracene. By the end of 1930 he had synthesized 60 compounds previously unknown, and we had 146 series of mice exposed to these and many other compounds.

A preliminary statement on these results, by J. W. Cook, I. Hieger, E. L. Kennaway, and W. V. Mayneord, entitled "The Production of Cancer by Pure Hydrocarbons, Part I," was communicated to the Royal Society by Sir Henry Dale and read by Mayneord and Cook at a meeting on June 30, 1932, and a fuller account was published by the same four authors (Cook *et al.*, 1932).

At the meeting of the Royal Society we reported that "1:2:5:6-dibenzanthracene shows undiminished carcinogenic power when very highly purified; it has been proved to be active in nine different media and has produced cancer of the skin in a concentration of 0.003% in benzene."

Mayneord and Roe (1936) examined the fluorescence spectrum of 3:4-benzpyrene in greater detail, using a spectrograph of higher dispersion than that used in the earlier observations, and they made (1935, 1937) an elaborate study of the ultra-violet absorption spectra of over 30 polycyclic aromatic compounds, supplied by J. W. Cook, measured at temperatures down to -120° . They contributed a section on ultra-violet absorption spectra to the paper by Cook *et al.* (1934) on "The Constitution of Diel's Hydrocarbon 'C₂₈H₂₄' from Cholesterol," and to another on "Polycyclic Aromatic Hydrocarbons, Part XIII: Condensed Fluorene Derivatives" (Cook *et al.*, 1935).

The Fractionation of Two Tons of Pitch

Early in 1930 Hieger attacked the isolation of a carcinogen in gas-works tar by extraction of our "pitch distillate" with different solvents, and showed that the "Schroeter" fluorescence spectrum bands could be sharpened, or rather that the accompanying fluorescent substances which gave a foggy background to the bands could be removed. Cook had found that vacuum distillation of crude pitch was impracticable owing to frothing. At this stage we were greatly helped by the Gas Light and Coke Company; through the kindness of Mr. W. Gordon Adam arrangements were made for the distillation at their Becton works of two tons of pitch and for a preliminary extraction

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with alcohol. The soft reddish-black waxy extract was sent to our laboratory, where it was fractionally distilled at low pressure, differentially extracted, picrated, and crystallized. The whole process required some hundreds of test-tube pilot tests followed by preparation on a larger scale, designed to produce enough material for tests on mice. The two guides used throughout the work were, of course, the fluorescence spectrum and the assay of carcinogenic potency by application to the skin of mice. The various fractions showed a correspondence between the intensity of the Schroeter bands and the carcinogenic potency. By the autumn of 1931 about 7 g. of a yellow crystalline powder melting at 116° had been accumulated, which showed both high carcinogenic potency and powerful fluorescence in the spectral position required. The process by which this compound was tracked down is illustrated by a fine series of photographs of spectra in Hieger's later paper (1937). Without the clue provided by Mayneord's introduction of the fluorescence spectrum the testing for carcinogenic power upon mice of every crystalline fraction which one could isolate from pitch would have been an almost endless task, and even when a pure crystalline carcinogen had been isolated there would have been nothing to direct one's attention to the benzanthracene type of molecular structure. The fluorescence spectrum was the single thread that led all through the labyrinth.*

Another batch of this crystalline material was fractionated further, in collaboration with C. L. Hewett, who joined the staff in October, 1931, under the direction of J. W. Cook, and several other compounds (2:3-benzcarbazole, chrysene, perylene) were separated from the final fractions of crystals melting at 160°. This product was taken over by Cook for identification.

The Identification and Synthesis of 3:4-Benzpyrene

The crystalline material, m.p. 160°, prepared from the distillate of the pitch, was of dubious homogeneity, and by means of a series of fractional crystallizations of the picrates Cook was able to isolate from it two essentially pure crystalline products (m.p. 176° and 187°). Analysis of these two compounds and their picrates showed them to be hydrocarbons uncommonly rich in carbon. Both of them, in fact, appeared to be isomeric with the highly condensed pentacyclic aromatic hydrocarbon perylene. The major component (m.p. 176°) showed in high dilution the characteristic fluorescence spectrum discussed above.

Consideration of these and other properties led Cook and Hewett to conclude that it might assist identification of these two hydrocarbons to prepare for comparison synthetic specimens of the then unknown pentacyclic hydrocarbons isomeric with perylene, 3:4-benzpyrene and 1:2-benzpyrene. This they were able to achieve by methods which, taken in conjunction with one another, established unequivocally the structures of the synthetic products. It was shown by direct comparison that 3:4-benzpyrene was identical with the strongly fluorescing major component of the crystals prepared from the pitch distillate. The synthetic preparation (m.p. 177°) was also highly carcinogenic. The synthetic 1:2-benzpyrene was identical with the minor component (m.p. 187°) of the original crystallate.

In 1939 the first award of the Anna Fuller Memorial Prize was made to J. W. Cook, C. L. Hewett, I. Hieger, E. L. Kennaway, and W. V. Mayneord "in recognition of their notable accomplishments in the fields of Cancer Research, specifically for the isolation and synthesis of cancer-producing hydrocarbons from coal-tar, the identification by fluorescence spectroscopy, and for the study of the biological effects of these substances."

The work described here received throughout the valuable support of the British Empire Cancer Campaign. These notes have been compiled in consultation with all five of

those—F. Goulden, I. Hieger, W. V. Mayneord, J. W. Cook, and C. L. Hewett—who took part in an investigation begun 33 years ago. We all continue to be engaged in biochemistry in more or less close relation to the study of cancer.

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HYPOPHYSECTOMY IN MAN*

FURTHER EXPERIENCES IN SEVERE DIABETES MELLITUS

BY

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Whereas the introduction of insulin in the treatment of diabetes mellitus has increased the life expectancy of juvenile diabetics, this longer survival has been accompanied by an increasing incidence of diabetic vascular complications. Once these have begun they progress, leading ultimately to blindness and death, usually from renal insufficiency or cerebral haemorrhage.

In an attempt to arrest the progress of such complications when they occur we have performed hypophysectomy (Luft *et al.*, 1952, 1953, 1954, 1955). Our purpose in doing so was to eliminate those anterior pituitary factors which animal experiments have shown to possess a diabetogenic action, as we thought that these hormones might be connected with the development and/or progression of the vascular disorders. The only hypophysectomy in a case of diabetes mellitus reported previously was that of Chabanier *et al.* (1936). However, from the data given, and especially from the fact that the post-operative daily requirement of insulin in this case was 70–80 units, we are inclined to consider the operation as incomplete.

Our first case was operated on in November, 1951, and up to date 20 patients in all have been subjected to this procedure. The operative technique has already been described in detail (Luft and Olivecrona, 1953). Of the 20 hypophysectomies, 11 have been performed since

*Hieger discovered that the active fractions all gave a deep cherry-red colour with concentrated sulphuric acid, and this test also proved quite useful in sorting out the various fractions. Of the three $C_{20}H_{12}$ hydrocarbons ultimately isolated, the active 3:4-benzpyrene was the only one to give this colour reaction.

*Based on a Ciba Foundation Lecture given by Professor Olivecrona on June 15, 1955, and a communication by Dr. Luft at the Second International Congress of Diabetes at Cambridge, July 8, 1955.