PHYSICOCHEMICAL ASPECTS OF CARCINOGENIC AGENTS

A Thesis

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of the

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By

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P R E F A C E.

The author wishes to express his gratitude to Professor J.W.Cook, F.R.S., and Dr. P.R. Peacock M.B., F.R.F.P.S.G., who jointly supervised the work described in the thesis.

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The preliminary results of the chemiluminescence studies in Section I and part of the theoretical discussion in Section V have been published in a paper to Nature. The results of the extended work together with the investigations on the oxidation of 4-dimethylaminoazobenzene in Section III and a fuller discussion on the bearing of the work on the mechanism of the carcinogenic process are being prepared in parts for submission to the British Journal of Cancer. A preliminary note of the results on the oxidation of 4-dimethylaminoazobenzene has already been made in Nature. The synthetic study on the azoxy compounds in Section III is almost in the form in which it will be submitted to the Journal of the Chemical Society. The investigations on the rate of elimination of carcinogens from the animal body in Section V were conducted

jointly with Dr. P.R. Peacock and Dr. S. Beck. The results have been published in the British Journal of Cancer.

The author is indebted to Mr. J.M.L. Cameron for the microanalyses and to Mr. S. Breslin for the preparation of Plates I and III.

Research Department,
Glasgow Royal Cancer Hospital,
September, 1949.

W.A.

SUMMARY.

The theme of these investigations is the development of the concept that the proximate causal agent in chemical carcinogenesis is energy liberated during the oxidation of the substance administered. Oxidations of a wide range of carcinogens with various oxidising agents have been studied and chemiluminescence phenomena have been observed during many of the reactions. A special study of the azo-dye group of carcinogens was conducted and this shows the importance of the amino group in these compounds for the chemiluminescence effects. A close parallel has been established between carcinogenic activity and participation in chemiluminescent reactions, and there appear to be few noteworthy exceptions to this observation.

The reactions which 3:4-benzpyrene and 4-dimethylamino-azobenzene, two typical carcinogens, undergo with the Milas reagents have been studied in detail. Interesting similarities were found between these oxidations and the *in vivo* reactions of the substances. During the course of the investigation with 4-dimethylaminoazobenzene it was desirable to have samples of the azoxy compounds derived from this substance. These compounds, previously unknown, were synthesised.

The relationship between the rate of elimination of carcinogens from the animal body and the carcinogenic re-

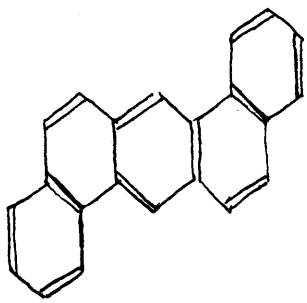
sponse has been studied in the case of 3:4-benzpyrene. The experiments show a parallel between the development of tumours and the continued presence of the carcinogen throughout the latent period.

Finally, the bearing of the work on the mechanism of the carcinogenic process is discussed. A new theory on the mode of action of carcinogens is presented. This has a wide application to chemical carcinogens and links them with the physical carcinogenic agents.

C O N T E N T S

INTRODUCTION

The production of various carcinogenic tars by Kennaway (1,2) and the synthesis by Cook (3) of homologues of 1:2-benzanthracene which had certain physical properties in common with the tars led to the demonstration that a number of pure chemical compounds, belonging to the class of polycyclic aromatic hydrocarbons, could elicit malignant neoplastic lesions when suitably applied to the skin of animals. In 1930 Kennaway and Hieger (4) showed that the property of carcinogenicity was possessed by a substance ultimately identified as 1:2:5:6-dibenzanthracene (I). This was the first pure



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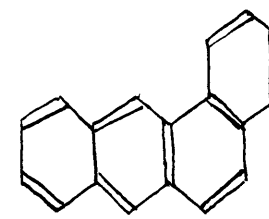
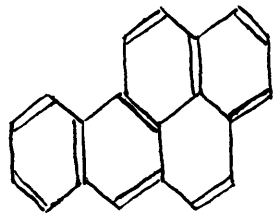


II

compound to be labelled carcinogenic. In a short time numerous investigations showed that a number of methyl derivatives of this compound and of the tetracyclic hydrocarbon, 1:2-benzanthracene (II) also had this biological property, while a strongly carcinogenic compound isolated from pitch was found

to be identical with the pentacyclic 3:4-benzpyrene (III). The idea that polycyclic hydrocarbons might arise *in vivo* from sterols prompted the preparation of 20-methylcholanthrene (IV) from desoxycholic acid and the demonstration of high carcinogenic activity associated with this structure.



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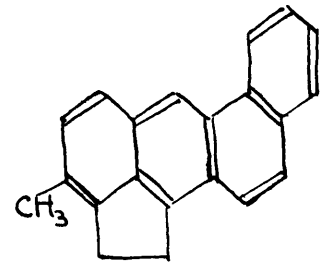
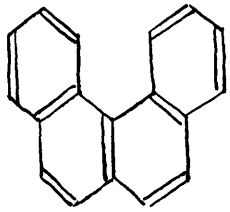


IV

These observations gave rise to numerous studies designed to discover a possible relationship between carcinogenic action and chemical constitution. Accordingly, many hundreds of substances of various chemical groups were tested and it is now proposed to indicate the different types of compounds which have been found to be carcinogenic.

Most of the carcinogenic hydrocarbons are tetracyclic or pentacyclic compounds although recently a weak activity has been demonstrated in the tricyclic compounds 1:2:3:4-tetramethylphenanthrene and 9:10-dimethylanthracene. Of the six possible tetracyclic compounds composed entirely of aromatic rings, only 3:4-benzphenanthrene (V) is active. However, many methyl derivatives of this substance and of 1:2-benzanthracene and chrysene (VI) are active compounds. Several cholanthrenes,

most conveniently considered as substituted benzanthracenes,



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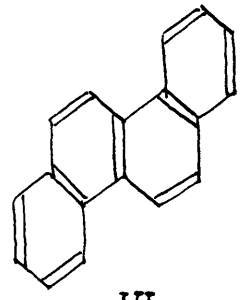
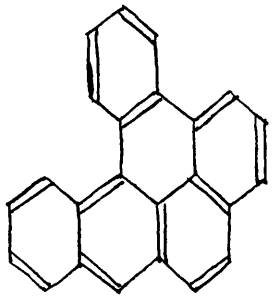


VI

are also active. In the pentacyclic group, 3:4-benzpyrene and 1:2:5:6-dibenzanthracene and certain derivatives of these are potent carcinogens while the other thirteen possible structures, although not so extensively studied, show very weak activity or are inactive. Of the hexacyclic compounds tested only 1:2:3:4-dibenzpyrene (VII) and its 7-methyl derivative, and 3:4:8:9-dibenzpyrene (VIII) are active.



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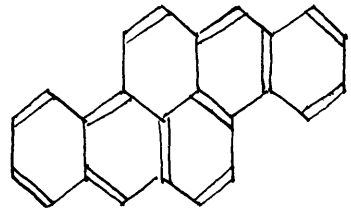
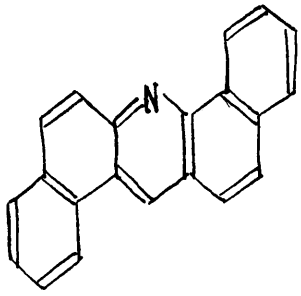


VIII

Many heterocyclic analogues of the polycyclic aromatic hydrocarbons and related fluorene derivatives have been tested and examples of active compounds are found in 1:2:5:6-dibenzacridine (IX), 1:2:5:6-dibenzfluorene (X) and 3:4:5:6-dibenz-

carbazole (XI).



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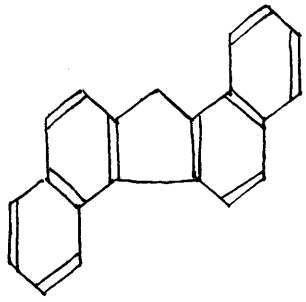
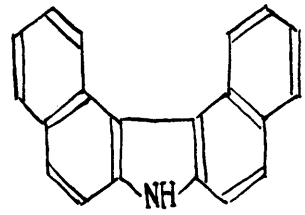
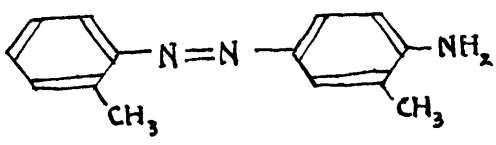


X



XI

The carcinogenic effect of the compounds listed so far is produced locally when these are applied to the skin or injected into the subcutaneous tissues of animals. Attempts to produce tumours with azo compounds were unsuccessful until Yoshida (5) produced liver tumours in rats by incorporating 4'-amino-2:3'-azotoluene (XII) in the diet over long periods. Using the same technique, Kinosita (6) obtained similar results with 4-dimethylaminoazobenzene (XIII).



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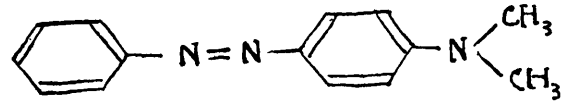
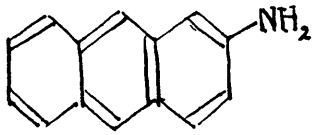


XIII

These two compounds form the basic structures of a large number of compounds of the azo-dye group of carcinogens. Another type of compound which produced multiple hepatomas in

mice is 2-aminoanthracene (XIV). Grouped with this substance



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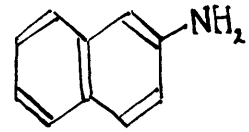

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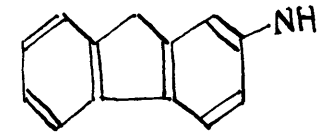
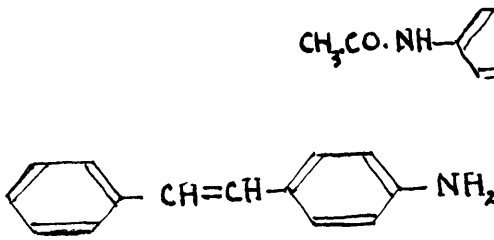
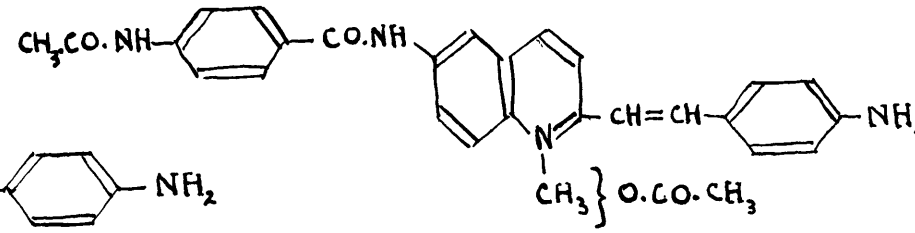


XVI

are 2-aminonaphthalene (XV) and 2-aminofluorene (XVI), the former producing bladder tumours when administered to the dog and the latter in the form of its acetyl derivative being capable of eliciting tumours in many organs of a variety of species. A different group of compounds recently discovered and having a versatile carcinogenic action are derivatives of 4-aminostilbene (XVII) and these may be compared with the compound "styryl-430" (XVIII) previously known to be carcinogenic



XVII

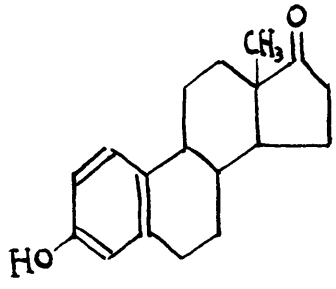


XVIII

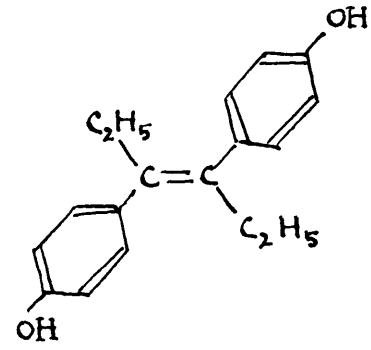
when injected into the subcutaneous tissues of mice.

Certain oestrogenic substances, occurring naturally or

synthesised in the laboratory, are capable of inducing tumours mainly in the tissues which are highly responsive to their physiological action. These substances are exemplified in oestrone (XIX) and diethylstilboestrol (XX).



XIX



XX

To this variety of chemical carcinogens may be added the salts of the radioactive elements, compounds of arsenic and zinc, the simple organic substances urethane, glucose and some nitrogen mustards, and finally hydrochloric acid and potassium hydroxide, all of which possess some carcinogenic action. Apart from these chemical carcinogens, tumours may be elicited with certain electromagnetic radiations, while other types of tumour may be propagated with preparations in which the active agent is a complex protein.

Numerous attempts have been made to find properties specific to the carcinogenic compounds. From the purely chemical standpoint, Fieser and his associates (7,8) sought to correlate the ease with which various carcinogenic hydrocarbons undergo certain substitution reactions and the mechanism of the carcinogenic

process, but the properties were not common to all the carcinogenic hydrocarbons. A similar study was carried out by Eckhardt (9) using perbenzoic acid to oxidise a number of carcinogenic and non-carcinogenic hydrocarbons and in the limited range of the experiment, a rough parallel was found between capacity for oxidation and carcinogenic activity.

Another approach was made by Clowes, Davis and Krahl (10,11) who studied the interaction of a number of polycyclic hydrocarbons with monomolecular films of sterols on water and suggested that the influence which the substances produced in these films may be related to a possible interference in vivo of the physiological function of the sterols. The idea of Schmidt (12,13) that the molecules of carcinogenic hydrocarbons possess regions of high electron density which may interact with and alter a cellular constituent, is worthy of note. Schmidt's mathematical technique with which he claimed to demonstrate the presence of regions of high electron density in certain hydrocarbons has been shown to be at fault by the Pullmans (14) who have been engaged in an extensive quantum mechanical treatment of the electron density distribution of many carcinogenic and related substances. In many cases a correlation has been found between the electron density of the phenanthrene-type double bond present in most of the carcinogenic hydrocarbons and the activity of these compounds, but this relationship is not constant. Schmidt's suggestion of the possible significance of regions of high electron density

in carcinogenic compounds has been taken up and extended by Daudel (15) although no chemical analogy is offered for the carcinogenic process envisaged. Attention has also been drawn to the shape and the size of the molecules of the polycyclic hydrocarbons and striking similarities in the carcinogenic members of the group suggest a possible relationship between these features and the biological properties, though the actual significance remains undecided.

Biochemical investigations with the polycyclic hydrocarbons have shown that they undergo hydroxylation. An interesting feature of this hydroxylation is that in a number of cases it takes place at a position in the molecule which is not the most reactive one as judged by other chemical reactions (16). It is to be emphasised that the hydroxy derivatives represent only a small fraction of the original hydrocarbon and that other metabolic reactions must occur. Metabolism studies with 4-dimethylaminoazobenzene and related compounds show that dealkylation of tertiary and secondary amine groupings occurs, together with reductive fission of the azo-linkage, and there is also evidence of direct hydroxylation of the dyestuff. Hydroxylation also occurs with 2-acetylaminofluorene and 2-aminonaphthalene. Differences in the metabolism of certain substances in different species are accompanied by differences in biological response suggesting a connection between metabolic reaction and carcinogenic action (17). On the other hand, the failure to elicit tumours

with the known or possible metabolites indicates that the carcinogenic property resides in the parent compound.

The study of the factors governing the experimental production of tumours is important since it offers an approach to the understanding of the mode of action of carcinogens. From this point of view the profound influence of the solvent used as an injection medium on the carcinogenic response in subcutaneous tissues has also been widely studied and a connection has been established between the effect of the solvent on the rate of metabolism of the carcinogen and its activity under the given conditions (18).

This introduction shows the wide range of agents to be considered in any comprehensive theory on the mode of action of carcinogens, and presents the conclusions and suggestions of some of the important researches with these agents in order to give an indication of the background to the present work. The indications that the chemical carcinogens are carcinogenic per se and do not act through their metabolic products together with the fact that many of them are compounds possessing no functional group and are comparatively inert are suggestive of a physicochemical mode of action. This view is inherent in the ideas of Schmidt and Daudel (vide supra) and is reflected in the tendency to group the chemical carcinogens with the purely physical agents rather than with the virus type of agent (19,20).

From these considerations the idea occurred to the author that the proximate carcinogenic agent may be the energy liberated during the metabolic reactions. The possibility that such energy may be in the form of electromagnetic radiation of visible and lower wave-lengths seemed worthy of investigation. It is with the development of this concept and its possible significance in chemical carcinogenesis that this thesis is mainly concerned. The major part of the work was designed to establish the extent to which the different types of carcinogens participate in reactions which are accompanied by chemiluminescence and the findings are discussed in Section I. Sections II and III are studies on the oxidation of two typical carcinogens and show the relationship between the chemiluminescent reactions and the reactions which occur with these substances in vivo. The experiments on the elimination of carcinogens from the animal body in Section IV have been included since they have a bearing on the mechanism of the carcinogenic process which is discussed in Section V.

SECTION I.

CHEMILUMINESCENCE STUDIES.

The nature of chemiluminescence. Preliminary investigations with carcinogenic compounds. - It is usual to suppose that all bodies above a temperature of absolute zero emit electromagnetic radiation. The spectral distribution of such radiation and its intensity are dependent, in the first place, on the temperature of the body. If the energy emitted at a certain wavelength greatly exceeds the value calculated for full temperature radiation, then the body is said to luminesce. Chemiluminescence is luminescence which arises from the energy of a chemical reaction. For practical purposes, a luminescent chemical reaction can be considered to emit chemiluminescence only at temperatures below about 800°K, for here temperature radiation in the red region can just be perceived. In a reaction accompanied by chemiluminescence, there are being produced molecules possessing high internal energies, and the luminescence results when such molecules pass to a lower energy level.

The list of chemiluminescent reactions collected by Trautz (21) testifies to the widespread occurrence of the phenomenon, although a number of the effects recorded were observed only at high temperatures (500°-600°K). Furthermore Drew (22) has pointed out that, with few exceptions, the chemiluminescence of organic substances in solution appears to be confined to

oxidation reactions. The low intensity of the emission from these oxidations has prevented the determination of its spectral distribution in the majority of the reactions. Observations made on the brighter reactions have shown that the spectra are of a continuous nature resembling in some cases the fluorescence spectra of the substances (22). It is possible that the radiation extends into the ultraviolet and failure to detect it in this region may be due to reabsorption by the reaction mixture (23). The emission of ultraviolet radiation by chemical reactions is now being studied by employing sensitive photoelectric counters and recordings have been made of radiation extending into the ultraviolet as far as 2000 Å (24).

Among the chemiluminescent reactions described by Trautz (21) are oxidations with chlorine or bromine water of anthracene, phenanthrene and chrysene dissolved in hot ethanol saturated with potassium hydroxide. These reactions were repeated and other hydrocarbons were also oxidised under the same conditions. The compounds tested were naphthalene, 1:2:5:6-dibenzanthracene, 9:10-dimethyl-1:2 -benzanthracene, 20-methylcholanthrene, and 3:4-benzpyrene; in each case a luminescence was observed. The possibility that the momentary emission of radiation observed in these cases might be a crystalloluminescence is excluded, since control precipitation of the substances from alcoholic alkali solution by the addition of water produced no luminescence. It is likely that the oxidising agent in these reactions was hypochlorite, as the effects could be reproduced by addition

of a solution of sodium hypochlorite to alcoholic solutions of the substances.

Attempts were now made to produce chemiluminescence with perbenzoic acid, which Eckhardt (9) has employed to oxidise a number of polycyclic aromatic hydrocarbons (see Introduction). Of a number of hydrocarbons tested, only anthracene and 20-methylcholanthrene in concentrated solutions gave a chemiluminescence. However, after consideration of the factors governing the production of chemiluminescence, a further set of experiments was designed with a more concentrated reagent, and chemiluminescence was observed with numerous substances; this is discussed later.

Trautz (21) has also described luminescent reactions on the addition of hydrogen peroxide to various substances, and Biswas and Dhar (25), using hydrogen peroxide catalysed with ferrous sulphate, obtained effects from many dye-stuffs. Similar techniques were used for the oxidation of polycyclic aromatic hydrocarbons and azo-dyes, but no chemiluminescence was observed. Since the Milas reagents (26) (hydrogen peroxide in tert. butyl alcohol, and osmium tetroxide in tert. butyl alcohol as catalyst) are capable of hydroxylating unsaturated compounds and can even convert benzene to phenol and naphthalene to naphthols (27), experiments were conducted with these. Chemiluminescence was observed with various carcinogenic and related compounds of the hydrocarbon and azo-dye groups and with other nitrogen-containing carcinogenic compounds. These

observations were more satisfactory than the previous ones, since the reaction mixture was homogeneous and the luminescence persistent; the reactions also had points of similarity with reactions occurring in the animal body (see Sections II and III).

Details of these preliminary experiments with views on their possible significance have been published (28).

Chemiluminescent reactions with carcinogens and related compounds. - The work was now extended and directed towards assessing the probability that oxidations of carcinogens in the animal body are accompanied by the emission of radiation. Sections II and III of the thesis were designed mainly to discover how far the reactions, which the hydrocarbons and the azo-dyes undergo with the Milas reagents, are comparable with the in vivo reactions judged from the point of view of the products which are formed in the different processes. In the present investigation as wide a range of chemical carcinogens as were available were tested for chemiluminescence with different oxidising agents. In this way it was hoped to gain information which would show whether the 'ability to participate in chemiluminescent oxidations' can be considered a property of these substances.

Certain limitations were imposed on this method of attack by the physical properties of the materials. It was necessary, for example, to avoid strongly colouredreagents which tend to

mask weak chemiluminescence. Oxidising agents requiring an aqueous medium were also undesirable. The Milas reagents were admirably suited to the research and a strong solution of perbenzoic acid in chloroform also proved satisfactory. Attempts to produce chemiluminescence by passing oxygen into hot alcoholic solutions of different carcinogens were unsuccessful but the technique of Biswas and Dhar (25) of passing ozone through alcoholic solutions yielded many positive results.

The observations made with the different substances during oxidation with (1) the Milas reagents, (2) perbenzoic acid, and (3) ozone, are described in accompanying tables. The experiments were applied to representatives of the groups of carcinogens outlined in the Introduction. In Tables I and II are given the results of the investigations with aromatic hydrocarbons, heterocyclic analogues of these, aromatic amines and members of the stilbene group of carcinogens and related compounds. The azo-dye group of compounds showed no sign of reaction with perbenzoic acid or ozone, and in numerous tests under various conditions no luminescence was observed with these reagents. The effects which they yield with the Milas reagents are discussed in subsequent sections.

The intensities of the luminescence effects are conditioned mainly by the strength of the reagents, the concentration of the solutions, the colour of the solutions, and the temperature of the reaction mixture. For each set of experiments reagent concentration and temperature are the same, but some difference

in solution concentration and colour were unavoidable, and where these are important to the evaluation of the results mention is made.

Among the aromatic hydrocarbons, the simpler substances show only a slight tendency to give chemiluminescence effects. Benzene, subjected to rigorous testing, did not give any reaction, and the only positive result with naphthalene was obtained using the Milas reagents with the concentration of material fifty times that used with the other substances. Weak effects were obtained with anthracene and phenanthrene using the Milas reagents or ozone, but the reaction between phenanthrene and the Milas reagents required a more concentrated solution. The tetracyclic and pentacyclic compounds and their heterocyclic analogues all gave luminescence effects with two or three of the reagents; one exception was 1:2:5:6-dibenzacridine which gave only a faint reaction with the Milas reagents. It should be noted, however, that in all the tests with this compound and with the majority of compounds of this group giving very faint or negative effects, the substances have a low solubility in the reagents. An exception to this generalisation is found in the reaction of 3:4-benzphenanthrene with the Milas reagents, for with this substance a more concentrated solution than the standard was required to produce the weak effect.

Most of the reactions with the aromatic amines were accompanied by strong luminescence effects and although aniline

showed no effects with perbenzoic acid and ozone, a positive result was obtained using the Milas reagents. The acetyl derivatives of these compounds gave negative or weaker results. Most of the reactions with the stilbene group were accompanied by chemiluminescence. The complete lack of reaction with stilbene itself shows the importance to the phenomenon of the amino group in the other compounds.

It may be concluded that most of the compounds listed in Tables I and II are readily oxidisable and that in the majority of the reactions chemiluminescence phenomena can be observed. It is interesting to note that, with the potent carcinogens 3:4-benzpyrene, 20-methylcholanthrene and 9:10-dimethyl-1:2-benzanthracene, all the reactions give a positive effect and it should be added that the oxidations of these substances with hypochlorite were also chemiluminescent (vide supra). It is further significant that all the carcinogenic substances tested in these groups of compounds give positive effects with the Milas reagents. The tables also show clearly that the ability to take part in chemiluminescent reactions is possessed by non-carcinogenic substances related to these compounds.

Chemiluminescent reactions with azo compounds. - In most of the above reactions where a luminescence effect was obtained, colour changes in the reaction mixtures gave evidence of oxidation of the substance, while with negative tests no such colour changes were observed. This suggests that the absence of

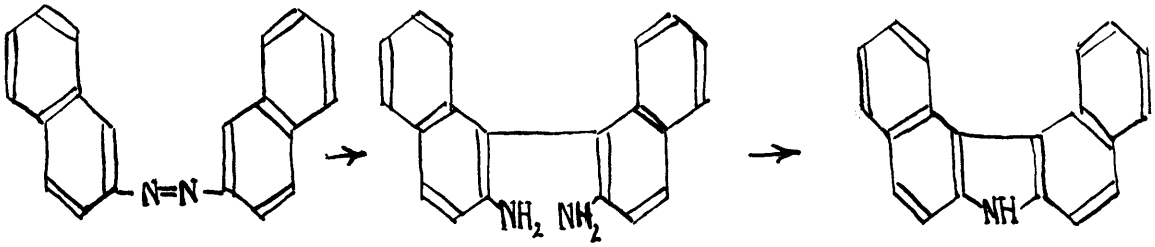
chemiluminescence may be related to lack of reaction. An interesting exception was found in the reaction of aniline with perbenzoic acid where a colour change to bright green indicated the formation of nitrosobenzene; in this case no chemiluminescence was observed. Since the test of the same substance with the Milas reagents was positive, it will be seen that this luminescence effect is dependent on a particular type of interaction.

The absence of effects with the azo compounds on treatment with ozone and perbenzoic acid may be related to a lack of reaction with these reagents, since no colour changes were observed in the solutions. The reactions conducted with the Milas reagents are described in Table III. All the carcinogenic compounds derived from Butter Yellow (N:N-dimethyl-4-aminoazobenzene) yielded positive results, as did the strongly carcinogenic 4'-amino-2:3'-azotoluene. As with the previous groups of substances, non-carcinogenic members of the series also gave luminescent reactions.

The series of closely-related derivatives of Butter Yellow provided an excellent basis for determining whether there is any relationship between the carcinogenic potency of the substance and the intensity of the luminescence. Careful consideration was given to this point and it was found that no such relationship exists. Differences in the intensity of the effects can be adequately explained by the differences in the colours of the reaction mixtures. The influence of the solution colour on the luminescence effect was striking with the three compounds

possessing primary amine groups; it was necessary in these cases to employ solutions less concentrated than the standard in order to prevent masking effects from the dark coloured reaction products.

Two azo compounds recognised as carcinogens failed to give positive tests. The first of these is 2:2'-azonaphthalene (A) which produces liver tumours in mice. However, the interesting studies of Cook, Kennaway and Kennaway (29) showed that 2:2'-diamino-1:1'-dinaphthyl (B), which would be expected on reduction of the azo compound to the hydrazo compound with subsequent rearrangement, was a more potent hepatic carcinogen than the azo compound; it is further pointed out (30) that the diamino compound readily deaminates to the potent carcinogen 3:4:5:6-dibenzcarbazole (C).



A B C

The assumption that similar reactions occur in the animal body is not unreasonable in the light of metabolic studies conducted with other azo compounds. It is therefore significant in this connection that 3:4:5:6-dibenzcarbazole gives positive lumines-

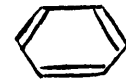
cence tests with the three oxidising agents used (Table I, No.19).

The second substance is 4-hydroxyazobenzene which has induced papilloma in the rat's stomach. We may also consider here two other substances. 2:3'-azotoluene has produced bladder tumours in the rat, and 4'-hydroxy-2':3'-azotoluene was shown to be weakly carcinogenic for the liver of the mouse. The last two substances have not been subjected to test for chemiluminescence but the experiments conducted on other structures (vide infra) strongly suggest that such tests would be negative. The possibility that the activity of compounds of this type is due to the formation of carcinogenic reduction products deserves consideration, since reductive fission has been established as a main reaction in the metabolism of many azo compounds. In this respect it is significant that the three substances possess only a weak activity. It is also noteworthy that aniline has caused the formation of papillomas in the bladder of the rabbit and recently White and her associates (31) have found a low incidence of liver tumours in rats fed with aniline. Of interest, therefore, are the chemiluminescence effects with aniline and the toluidines (Table IV). The further possibility that these azo compounds form ortho dihydroxy derivatives has to be considered, for it has been demonstrated that benzene gives rise to catechol in rabbits and dogs. The chemiluminescence results with catechol and 3:4-dihydroxyazobenzene (Tables III and IV) are therefore worthy of note.

Detailed information on the metabolism of Butter Yellow is available. The main reactions are demethylation of the tertiary amine grouping in two stages, hydroxylation, and reductive fission of the azo linkage to yield anilines. Studies designed to test the carcinogenic potency of the known and possible metabolites show that a high potency is possessed only by the parent compound and its N-monomethyl demethylation product (32). These investigations, however, do not necessarily eliminate the metabolites as responsible for the activity of the parent compound. In this connection, the positive results (Table III) with the N-monomethyl compound and primary amine derived from Butter Yellow, with the hydroxyaminoazo compounds, and the azoxy compounds which may also be considered as possible metabolites, lead to the interesting conclusion that the ability of Butter Yellow to take part in a chemiluminescent reaction is shared by many of its metabolic products. The positive luminescence tests with the anilines (Table IV) further demonstrate this observation.

The importance of the amine nitrogen in the chemiluminescence effects obtained with the azo compounds. - The importance of the amino radical in the chemiluminescence effects with the azo group of compounds is shown by its presence in all the substances giving a positive test and in the failure of azobenzene to give a reaction. To substantiate these observations, azobenzene was tested under various conditions and failed in all

tests to yield a chemiluminescent reaction. Experiments were then designed to gain information on the role of the amino group in the phenomena, since the occurrence of an amino group in the majority of azo-dye carcinogens suggests that it is also important to the carcinogenic property. The results with the various substances during reaction with the Milas reagents are shown in Table IV. Throughout these tests, comparable reaction conditions were maintained, and careful note made of a possible masking of any luminescence by strongly coloured solutions.

The positive results with aniline and N-alkyl substituted anilines demonstrate that the  residue of the parent azo compounds is not essential to the chemiluminescence process. On the other hand the absence of any effect with benzene examined under the same conditions as azobenzene confirmed the importance of the amine nitrogen to the reaction. The question now arose whether the aromatic nature of the compounds was essential to the action. To this end a series of simple aliphatic amines and cyclohexylamine were tested; trimethylamine was subjected to thorough testing since it represents the terminal moiety of the Butter Yellow molecule. In all these reactions evolution of heat and gases occurred showing that chemical reaction was taking place, but no luminescence effects were observed. These findings, summarised in Fig.1, clearly demonstrate that the amino nitrogen and the aromatic nature of the azobenzene residue are of primary importance in

the chemiluminescence effects obtained in the reactions of the azo compounds with the Milas reagents.

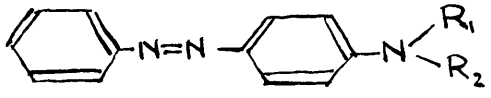
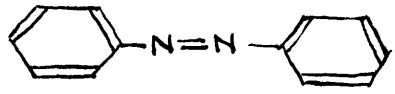
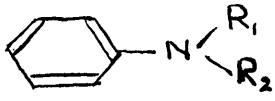
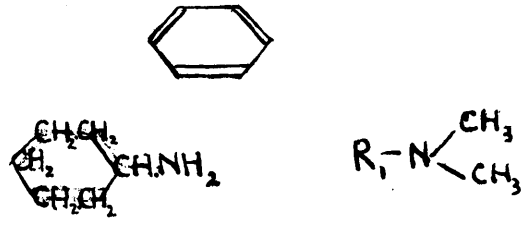
Positive Reactions	Negative Reactions
	
	

Fig. 1. — R_1 and R_2 = H or CH_3.

The luminescence is produced during the interaction of hydrogen peroxide, in the presence of osmic acid, with some region in these structures. The next set of reactions was contrived to discover whether the function of the amine grouping is of a directive nature, activating the aromatic residue, or whether it is important by virtue of providing a point for direct reaction with the peroxide. The decision between these alternatives is not straightforward, for while it is

known that the dimethylamino radical is one of the most powerful of activating groups, a study of the products of the reaction with Butter Yellow (see Section III) demonstrated that much of the action with the peroxide takes place at the amino group.

The tests conducted with phenol, anisole, toluene and chlorobenzene, all of which possess an o, p-directing group, were negative. While this observation favours the view that the directive influence of the $-N\begin{matrix}R_1\\R_2\end{matrix}$ group is not of great importance in the action, it must be remembered that the directive power of the other groups is of a lower order. The tests with di- and tri-substituted benzenes are more enlightening. If the directive influence is the main feature of the action, one should expect a positive result from resorcinol and m-cresol where there is a superposition of the directive effects from the two groups (Fig. 2(a)).

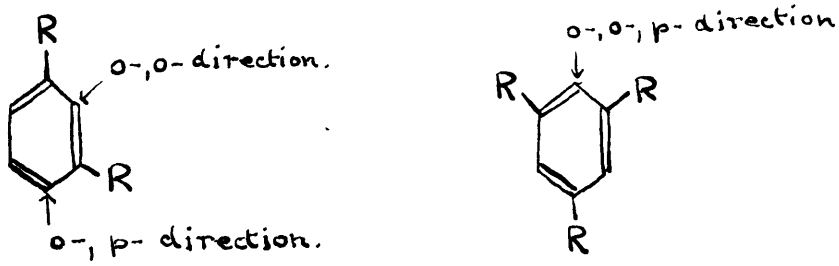


Fig. 2.

However, of the reactions conducted with the three cresols and the three dihydroxy derivatives of benzene, the only one to yield a chemiluminescence was that with the o-substituted compound, catechol, which gave a very bright effect. An even brighter effect was obtained with pyrogallol which has hydroxyl groups in the 1-, 2-, 3-, positions. The positive result with phloroglucinol (1:3:5-trihydroxybenzene)' is worthy of note since the groups here are meta to each other; the uncertain nature of the configuration of this molecule however under the conditions of the experiment make the observation less useful to the present investigation. More significant is the absence of an effect with o-dimethoxybenzene, the phenolic ether derived from catechol. This observation provides strong confirmation of the view that the positive reactions with catechol and pyrogallol are due to direct interaction of the peroxide with the hydroxyl groups. Furthermore, the highly reactive hydrocarbon mesitylene, with three points in the molecule under the influence of two ortho groups and one para group all facilitating substitution (see Fig.2 (b), failed to give a luminescence effect. Again, in a carefully conducted set of reactions, no enhancing effect was obtained of the luminescence observed with dimethylaniline or aniline, by nuclear substitution with o,-p-directing methyl groups (Table IV, reactions 27-32).

The lack of reaction with nitrobenzene and benzoic acid was expected, for the functional groups neither activate the

molecule nor do they provide a direct point for oxidation. Negative results with benzaldehyde and nitrosobenzene however indicate that a particular type of oxidation is essential to the chemiluminescence effect since both aldehyde and nitroso groups offer an oxidisable point to the reagent.

Attention was redirected to azobenzene derivatives. The negative tests with the mono-hydroxy, methyl- and nitro-compounds together with the bright reaction from 3:4-dihydroxy-azobenzene (Table III) compare well with the results obtained with the benzene derivatives.

The total evidence shows that the ability of the azo-dye groups of carcinogens and related non-carcinogens to take part in chemiluminescent reactions with the Milas reagents is dependent on their aromatic character and on the presence of an amino group which appears to be important by virtue of providing a point for attack to the reagent.

An application to carcinogenic mixtures in which the active agent is unknown. - The carcinogenic activity of various heated fats and lipoids (33) is of interest because of its bearing on the problem of human cancer. The nature of the active agent is unknown and the failure so far to demonstrate the presence of any known carcinogen suggests that it may be a carcinogenic substance of another chemical group. Samples of heated cottonseed oil, which has induced stomach tumours in the mouse (34), and various fractions of the heated oil, corres-

ponding to saponifiable and non-saponifiable parts, were available. On conducting chemiluminescence tests with the Milas reagents, positive results were obtained with the heated oil and with many of the fractions of the heated oil in both the non-saponifiable and saponifiable fractions. In strictly comparable tests no effects were obtained with the unheated oil or the fractions of this related to those of the heated material. A positive test was obtained with the unsaponifiable material from the unheated oil, but since this is only a trace constituent in the original oil and is not comparable with the non-saponifiable part of the heated oil, it has little significance to the investigation.

The nature of the material in the heated oil responsible for the chemiluminescent action is of much interest. The bright effect with the saponifiable fraction is associated with a substance with acidic or phenolic properties. In the luminescence studies with the Milas reagents only aromatic compounds have shown luminescent effects and this finding was substantiated by numerous other reactions with simple aliphatic substances. The possibility that aromatisation has occurred in the heating of the oil is being considered.

For the present work the important point is the demonstration that this type of carcinogen also participates in a chemiluminescent oxidation.

Carcinogenic compounds which have not been tested for chemi-

luminescence. - The question as to what extent the chemiluminescence phenomena have been associated with the vast range of substances with carcinogenic properties deserves consideration.

Although only a small fraction of the carcinogenic members of the polycyclic aromatic hydrocarbon series has been considered, it should be noted that many of these substances are derivatives of the basic structures which have been tested. It is also significant that all the substances of this group which were tested, participated at least in one luminescent reaction. A similar statement applies to the other groups of carcinogens tested. For the few exceptions in the azo-dye group of compounds there is good evidence that the compounds may form metabolites which take part in chemiluminescent reactions. It is interesting to note that all the major groups of chemical carcinogens are involved in these studies. The isolated groups which are not included are now considered.

Firstly, there is a range of oestrogenic and androgenic compounds associated with the induction of tumours at various sites (35). The large majority of these lesions, however, have features which distinguish them from those elicited by other carcinogenic chemicals. For example, the mammary cancer induced by application of oestrogenic compounds in mice is dependent also on the presence of a non-genic transmissible factor which may be a virus. Further, the effects of the compounds are largely limited to tissues which are

highly responsive to their physiological action and, apart from the breast cancer, most of the effects are reversible on cessation of oestrogen supply. The induction of subcutaneous sarcomata with the sex hormones appears to provide a parallel to the effects obtained with other carcinogenic compounds. The techniques employed in producing these tumours however leave the results open to debate. All of the tumours resulted from repeated injection, in some cases daily injection, of the material over several months. In some tests the vehicle used is suspect, and in this connection it is of interest that the substances have failed to manifest any carcinogenic action when solid pellets of the pure material have been used, or when applied to the skin. Risk of contamination from other carcinogens as pointed out by the author (36) must be taken into account, especially in experiments employing multiple treatments. Against this background it is to be admitted that preliminary experiments with steroid hormones on treatment with the Milas reagent failed to give chemiluminescence effects, although a positive result was obtained with stilboestrol. Finally, the suggestions of Cook (37) on the possibility of conversion of sterols and bile acids to polycyclic aromatic hydrocarbons within the animal body supported by examples of aromatisation of other alicyclic compounds (38) should also be borne in mind when assessing the significance of tumour-induction with these compounds.

The salts of the radioactive elements do not affect the

issue since their effects are well explained by their emission of carcinogenic radiations. The remaining inorganic materials to be considered are salts of zinc and arsenic, potassium hydroxide and hydrochloric acid. The main effect with zinc salts is the induction of teratoma in fowls but there is evidence that the action of the compounds is in the nature of a partial destruction of the testis; partial castration of the birds leads to similar results. It is therefore doubtful whether the effect of these compounds is to be regarded as the result of a specific carcinogenic action (39). The bulk of evidence pointing to arsenic as a carcinogen derives from clinical studies of workers exposed to the substance and in all of the cases other substances are suspect (40). There is some evidence of arsenic compounds being involved in the induction of neoplasia in tissues treated medicinally during long periods, but on the other hand the experimental induction of tumours with arsenic compounds is indeterminate. The possibility of a chemiluminescence occurring during the oxidation of arsenious compounds has been considered, but a few preliminary experiments failed to substantiate this view. To postulate that chemiluminescence phenomena may be involved in the carcinogenic action ascribed to hydrochloric acid and potassium hydroxide seems unnecessary since it overlooks the profound effect of hydrogen and hydroxyl ions, on all of the structural and functional components of cells.

A variety of tumours has been obtained recently with

certain aromatic nitrogen mustards and some with aliphatic nitrogen mustards (41). These compounds have not yet been tested for chemiluminescence but on the basis of the previous experiments positive results might be expected from the aromatic compounds and negative results from the aliphatic compounds. Urethane which is associated with a high incidence of lung adenomata in mice fails to give a chemiluminescence effect with the Milas reagents. These examples seem to be the only noteworthy exceptions to the generalisation that carcinogenic chemicals participate in chemiluminescent reactions.

The remaining anomalous example of a chemical carcinogen is found in the simple organic molecule, glucose. This however does not constitute an exception to the above generalisation, since Radziszewski (42), in 1877, described a chemiluminescence effect during the oxidation of glucose with air in alcoholic alkali solution.

TABLE I: AROMATIC HYDROCARBONS AND HETEROCYCLIC ANALOGUES.

No.	SUBSTANCE	LUMINESCENCE EFFECTS			CARCINOGENIC ACTIVITY*
		Milas Reagents	Perbenzoic Acid	Ozone	
1.	Benzene	Negative	Negative	Negative	?
2.	Naphthalene	Very faint†	Negative	Negative	-
3.	Anthracene	Faint	Negative	Faint	-
4.	Phenanthrene	Faint†	Negative	Faint	-
5.	Naphthacene	Faint△	Negative△	Bright	-
6.	1:2-benzanthracene	Faint△	Negative	Medium	-
7.	9:10-dimethyl-1:2-benzanthracene	Bright	Very faint	Bright	+
8.	2':6-dimethyl-1:2-benzanthracene	Very faint△	Medium	Medium	-
9.	2':7-dimethyl-1:2-benzanthracene	Very faint△	Negative△	Medium	-
10.	Chrysene	Negative△	Medium△	Faint△	-
11.	3:4-benzphenanthrene	Very faint†	Negative	Medium	+
12.	Fluoranthene	Very faint	Medium	Medium	-
13.	1:2:5:6-dibenzanthracene	Very faint△	Negative△	Bright	+
14.	3:4-benzpyrene	Medium	Faint	Medium	+
15.	Fluorene	Negative	Medium	Faint	-
16.	Cholanthrene	Faint△	Negative	Medium	+

TABLE I (cont.)

No.	SUBSTANCE	LUMINESCENCE EFFECTS			CARCINOGENIC ACTIVITY*
		Milas Reagents	Perbenzoic Acid	Ozone	
17.	20-methylcholanthrene	Medium	Faint	Medium	+
18.	Carbazole	Bright	Medium	Medium	-
19.	3:4:5:6-dibenzcarbazole	Bright	Medium	Bright	+
20.	N-methyl-3:4:5:6-dibenzcarbazole	Bright	Medium	Bright	+
21.	N-ethyl-3:4:5:6-dibenzcarbazole	Bright	Medium	Bright	+
22.	7-methyl-1:2-benzacridine	Faint	Bright	Medium	-
23.	9-piperonyl-9:10-dihydro-1:2:7:8-dibenzacridine	Faint	Medium	Bright	?
24.	1:2:5:6-dibenzacridine	Faint△	Negative△	Negative△	+

* + Substance is carcinogenic. Substances with only weak activity are included.
- Substance has been tested biologically and is inactive. It should be noted that some of the substances have not been rigorously tested.
? Substance has not been tested biologically, or the test was inconclusive.

△ Substance only slightly soluble in reagent.

† Concentration of solution higher than in other tests.

TABLE II: AROMATIC AMINES. STILBENE DERIVATIVES AND ANALOGUES.

No.	SUBSTANCE	LUMINESCENCE EFFECTS.			CARCINOGENIC ACTIVITY*
		Milas Reagents	Perbenzoic Acid	Ozone	
1.	Aniline	Very faint	Negative	Negative	+
2.	Acetanilide	Negative	Negative	Negative	?
3.	2-aminonaphthalene	Bright	Bright	Negative	+
4.	2-acetylaminonaphthalene	Negative	Negative	Negative	?
5.	2-aminoanthracene	Bright	Bright	Medium	+
6.	2-aminofluorene	Bright	Bright	Bright	+
7.	2-acetylaminofluorene	Very faint	Negative	Medium	+
8.	Stilbene	Negative	Negative	Negative	-
9.	4-aminostilbene	Bright	Bright	Very faint	+
10.	4-dimethylaminostilbene	Bright	Bright	Medium	+
11.	2-(4-aminostyryl)-6-(4-acetylamino-benzoylamino)-quinoline methoacetate	Bright	Negative△	Faint	+
12.	2-(4-acetylaminostyryl)-6-(4-amino-benzoylamino)-quinoline methoacetate	Bright	Bright	Medium	-

*, △ See Table I.

TABLE III: CARCINOGENIC AZO COMPOUNDS AND RELATED SUBSTANCES, (Reactions with Milas Reagents).

No.	SUBSTANCE	LUMINESCENCE EFFECT	CARCINOGENIC ACTIVITY*
1.	Azobenzene	Negative	-
2.	4-aminoazobenzene	Medium	+
3.	N-methyl-4-aminoazobenzene	Medium	+
4.	N:N-dimethyl-4-aminoazobenzene	Medium	+
5.	2-methyl-4-dmaab	Bright	-
6.	2'-methyl-4-dmaab	Very faint	+
7.	3'-methyl-4-dmaab	Bright	+
8.	4'-methyl-4-dmaab	Bright	+
9.	2:2'-dimethyl-4-dmaab	Medium	?
10.	2:3'-dimethyl-4-dmaab	Bright	?
11.	2:4'-dimethyl-4-dmaab	Bright	+
12.	N:N-diethyl-4-aminoazobenzene	Medium	-
13.	4'-amino-2:3'-azotoluene	Faint	+
14.	2'-amino-4:5'-azotoluene	Faint	-
15.	2:2'-azonaphthalene	Negative	+
16.	4-hydroxyazobenzene	Negative	+
17.	4-methylazobenzene	Negative	?
18.	4-nitroazobenzene	Negative	?
19.	3:4-dihydroxyazobenzene	Bright	?
20.	4'-hydroxy-4-dmaab	Bright	-
21.	4'-hydroxy-4-aminoazobenzene	Medium	-
22.	α-4-dimethylaminoazoxybenzene	Medium	?
23.	β-4-dimethylaminoazoxybenzene	Faint	?

* See Table I.

Note: 4-dmaab is a contraction for N:N-dimethyl-4-aminoazobenzene.

TABLE IV: REACTIONS OF MILAS REAGENTS WITH BENZENE DERIVATIVES AND SOME ALIPHATIC AMINES.

No.	SUBSTANCE	LUMINESCENCE EFFECT	No.	SUBSTANCE	LUMINESCENCE EFFECT
1.	N:N-dimethylaniline	Medium	19.	Hydroquinone	Negative
2.	N-methylaniline	Medium	20.	o-Cresol	Negative
3.	N:N-diethylaniline	Medium	21.	m-Cresol	Negative
4.	N-ethylaniline	Medium	22.	p-Cresol	Negative
5.	Aniline	Faint	23.	Pyrogallol	Very bright
6.	Benzene	Negative	24.	Phloroglucinol	Medium
7.	Trimethylamine	Negative	25.	Mesitylene	Negative
8.	Dimethylamine	Negative	26.	o-Dimethoxybenzene	Negative
9.	Ethylamine	Negative	27.	N:N-dimethyl-o-toluidine	Negative
10.	n-Propylamine	Negative	28.	N:N-dimethyl-m-toluidine	Very faint
11.	n-Butylamine	Negative	29.	N:N-dimethyl-p-toluidine	Faint
12.	Cyclohexylamine	Negative	30.	o-Toluidine	Very faint
13.	Phenol	Negative	31.	m-Toluidine	Faint
14.	Anisole	Negative	32.	p-Toluidine	Medium
15.	Toluene	Negative	33.	Nitrobenzene	Negative
16.	Chlorobenzene	Negative	34.	Benzoic acid	Negative
17.	Catechol	Very bright	35.	Benzaldehyde	Negative
18.	Resorcinol	Negative	36.	Nitrosobenzene	Negative

EXPERIMENTAL.

Detection of Chemiluminescence.

In all the experiments the radiation was detected by direct observation. To accomplish this it was necessary for the observer to adapt his eyes in a completely dark room for 20 minutes prior to conducting the test. Many of the effects could be seen with a shorter period of adaptation, but this period was always used in order to standardise the recordings; longer periods of adaptation made only slight differences in the observed intensities. At the commencement of each set of experiments a control reaction of known luminescence intensity was observed to ensure that the eyes were light-adapted. The very faint reactions were best observed by holding the reaction tubes close to the eyes and employing side vision. All the effects recorded were quite definite and the possibility that the faintest of effects were merely 'mouches volantes' is excluded. In the studies where the relative intensities of the luminescence effects were important to the discussion, as in the series of Butter Yellow derivatives, each reaction was compared directly with the same arbitrarily chosen standard reaction.

Source and Purity of Materials.

A number of the hydrocarbons and related compounds were samples presented to the Cancer Hospital, principally by Ppofessor J.W.Cook, F.R.S.; others were supplied by Messrs

Light & Co.Ltd., London, and Messrs Ward, Blenkinsop & Co.Ltd., London. A number of the azo compounds were synthesised in the Cancer Hospital by Dr. A.H.M.Kirby. The majority of the other compounds were supplied by Messrs British Drug Houses Ltd., Dorset. The following compounds were prepared by the author: nitrosobenzene; 4-methylazobenzene; 4-nitroazobenzene; 3:4-dihydroxyazobenzene; 4'-hydroxy-N:N-dimethyl-4-aminoazobenzene; 4'-hydroxy-4-aminoazobenzene; α- and β-4-dimethylaminoazoxybenzene.

A commercial sample of anthracene labelled 'Purified', which gave a positive test with the perbenzoic acid reagent, failed to give the same effect after it had been purified by chromatography. This observation emphasised the necessity of employing carefully purified samples of the substances to be tested. All the azo compounds were subjected to purification by chromatographic adsorption on alumina or silica columns and a similar procedure was applied to many of the hydrocarbons and related compounds. Most of the compounds supplied by Messrs British Drug Houses Ltd. were Analar grade, but where positive effects were observed and the purity of the material was at all suspect, it was purified by standard methods.

Reactions with Hypochlorite.

In the reactions with chlorine water, the solutions were prepared by warming the powdered substances with ethanol, previously saturated in the cold with potassium hydroxide, and

filtering to remove undissolved material. To a measured volume of the solution an equal volume of 0.5% chlorine water was added; both solutions were heated to 60-70° prior to mixing. In the experiments with hypochlorite, an alkaline solution of sodium hypochlorite (6.5% NaClO, 8% NaOH), prepared by passing chlorine gas through concentrated caustic soda solution, was added to an equal volume of a saturated solution of the substance in ethanol. The solutions were heated as before. Adequate controls on the reagents showed that the luminescence effects were due to the oxidation of the hydrocarbons. All the effects were momentary emissions of green or greenish-white radiation.

Reactions with Perbenzoic Acid.

The chloroform solutions of perbenzoic acid were prepared from benzoyl peroxide (43). In the early experiments the concentration of the acid was 1% and equal volumes of this solution and a strong solution of the substance in chloroform were mixed at 40-50°. In all the reactions listed in Tables I and II an 8.5% solution of the acid was employed. This solution is stable for several weeks, if stored in a refrigerator.

The effects obtained with this reagent increase with increasing concentration of the substance under test. The technique employed in all tests was to dissolve the test substance (10 mg.) in chloroform (0.5 c.c.) and add the reagent

(1 c.c.) to this solution; the solutions were heated previously to 40-45°. In those cases where the substance did not completely dissolve, the suspension was employed. The duration of the luminescence varied from a few seconds to several minutes and was green or greenish-white in appearance.

Reactions with the Milas Reagents.

The solutions of hydrogen peroxide in tert. butyl alcohol were prepared by the method of Milas and Sussman (26); the alcohol was distilled before use. The solutions are stable for several months if stored in a refrigerator. Difficulty was found in obtaining consistent results with the catalyst solution, osmium tetroxide in tert. butyl alcohol, until the following technique was used. The alcohol was dried over magnesium wire and distilled. Osmium tetroxide was dissolved in this to give a 0.5% solution which was maintained at 40° for four days. This yielded a black sol which was stored at the same temperature, and gave constant results over many months.

The tests were made by dissolving the substance in the peroxide reagent and adding the solution to the catalyst. Using different types of compound it was found that the intensity of the chemiluminescence increased with increasing concentration of substance, temperature and concentration of peroxide reagent. No advantage was obtained by using a catalyst solution more concentrated than 0.5%. Optimum

results were given by addition of 4 parts of peroxide reagent to 1 part of catalyst solution and this arrangement was used throughout the tests. The most suitable concentration of the substance in the reagent was found to be 0.1%, and when the substance was not completely soluble to this extent suspensions were used. This value was varied in the case of some azo-dyes which produce a masking effect at this concentration, in cases of very faint chemiluminescence and in comparison experiments between azo-compounds and benzene derivatives where equimolar solutions were desirable. In the early experiments a 6% peroxide reagent was used but for the tests described in Tables I to IV an 8% solution was employed. In the rigorous testing of azobenzene, benzene and trimethylamine solutions ranging from 6% to 16%, H_2O_2 were utilised. The solutions of solid materials were prepared by powdering the material and shaking a weighed quantity with the reagent; slight heating was used if required. Liquid substances were measured with a micropipette. Gases were bubbled through the reagent and the concentrated solutions estimated and diluted as required. Careful note was taken of the colour of the solutions before and immediately after the test and where these features influence the intensity of any effect which is important to the discussion, reference is made in the text.

Reactions in Tables I and II were conducted at 60-65°. Reactions in Tables III and IV were conducted at 37° except

numbers 13 to 23, Table III and numbers 7 to 36, Table IV, which were conducted at 60-65°. A number of the tests recorded at the higher temperature were repeated at 37°. It was found that only the "bright" and "medium" effects observed at 60-65° could still be observed, but with diminished intensity. The effects were green or greenish-white radiation except that from pyrogallol which was blue. The duration of the luminescence varied in the different reactions from a few seconds to several minutes and was largely controlled by the colour of the reaction products. Many controls were carried out on all the reagents but these always gave negative results.

Reactions with Oxygen and Ozone.

The negative tests conducted with oxygen were made by passing oxygen from a cylinder through boiling solutions of the substances in absolute ethanol. The substances tested were: 3:4-benzpyrene; 9:10-dimethyl-1:2-benzanthracene; 4-dimethylaminostilbene; 4-dimethylaminoazobenzene. The concentration of the solutions was 0.2%.

For the production of ozone used in the investigations a 'Siemens tube' was constructed and the electrical discharge effected by an induction coil supplied with 4 volts. Oxygen from a cylinder was dried in a calcium chloride tube prior to ozonisation. The concentration of ozone in the exit gases was not estimated but was sufficient to cause a rapid 'tailing' effect in mercury and give an immediate colour reaction with

moist starch-iodide paper. The whole apparatus was housed in a light-tight enclosure to avoid confusion between the radiation from the tube and induction coil and weak chemi-luminescence effects.

Ethanol was the most suitable solvent for these reactions although effects were obtained using glacial acetic acid, ethyl acetate, carbon tetrachloride and benzene; all these solvents are generally employed in the formation of ozonides. The alcohol used was freed from aldehyde, dried over activated aluminium amalgam and distilled. The tests were conducted with 0.5% solutions or suspensions of the various substances, by bubbling the ozone through the solutions. The observations were made at 60-65°. In the cases where the solutions were saturated, the fall in temperature caused by the introduction of the ozone led to crystallisation of some material. Lest the corresponding luminescence should be a crystallo-luminescence, duplicate tests were made of these reactions passing only oxygen through the solution. In all examples the luminescence was due to interaction of the compound with ozone. The nature of the radiation was similar to that obtained with the other reagents.

Attempts to Increase the Intensities of the Luminescence Effects.

A few attempts were made to photograph the radiation from some of the brighter reactions, using sensitive emulsions. It became obvious however that this would only be practicable

if the intensities of the effects were greatly increased. The intensities were increased by adjusting the concentration of the reagents and increasing the reaction temperature, but the effects were still too weak to be recorded.

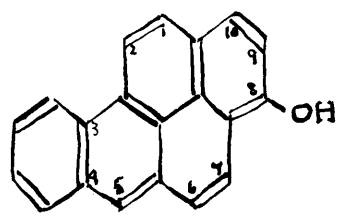
Increases in chemiluminescence intensity have been produced by adsorbing substances on alumina or silica gels (44), or on silicic acid (25). Adaptations of these procedures were not successful with the substances in the present investigation. The observations of Biswas and Dhar (25) when oxidising various dye-stuffs with ozone showed that on mixing a dye-stuff which gave a weak luminescence effect with one giving a strong effect there was produced a luminescence much brighter than with either of the substances separately. This method failed to give similar results when applied to the reactions of the polycyclic hydrocarbons with ozone.

SECTION II.

OXIDATIONS OF 3:4-BENZPYRENE.

It was established in Section I that chemiluminescence is produced during the oxidation of various polycyclic aromatic hydrocarbons with different oxidising agents. In order to form an opinion on whether the oxidations which occur with these substances in the animal body may also be accompanied by chemiluminescence, a comparative study of the reaction products of the chemical oxidations with those of the biological reaction was carried out with 3:4-benzpyrene.

Benzpyrene was chosen since numerous biological studies have been conducted with it, and much attention has been directed to the formation of 8-hydroxybenzpyrene (I), (45, 46)



I

in various animals. In this metabolic reaction, as with other polycyclic aromatic hydrocarbons studied, hydroxylation has been effected at a different position in the molecule from that usually attacked (position 5, with benzpyrene).

Preliminary experiments were conducted in an attempt to simulate this effect in a chemical oxidation of benzpyrene.

It was observed during the chemiluminescent oxidation with sodium hypochlorite of benzpyrene, solubilised in water with caffeine, that the bright violet fluorescence of the benzpyrene solution was changed to a green fluorescence. Extraction of the altered material showed that it possessed a blue fluorescence in benzene solution; this gave a two-banded fluorescence spectrum similar to that described by Chalmers (45) for a hydroxybenzpyrene isolated from the rat. For direct comparison a fraction containing 8-hydroxybenzpyrene was prepared from the faeces of rats fed with benzpyrene. This material and the fraction obtained from the hypochlorite oxidation were similar in their chromatographic behaviour and in their solubility and fluorescence properties. Methylation of the two fractions allowed their purification by chromatography. This showed that the fraction from the hypochlorite oxidation, corresponding with the two-banded fluorescence spectrum, was present only in minute quantity. Its spectrum was similar to, but not identical with, that of 8-methoxybenzpyrene. Furthermore, an absorption spectrum of the material showed none of the fine structure characteristic of 8-methoxybenzpyrene. Thus while the fluorescence spectrum indicated the presence of a methoxybenzpyrene, no further evidence was obtained to support the view. It should also be noted that this spectrum differs in the position of the

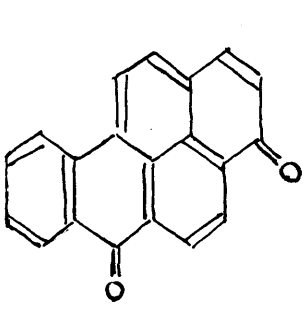
bands from those recorded by Berenblum and Schoental (47) for 5- and 10-methoxybenzpyrene.

Although this particular reaction was unsuccessful, the idea of forming a complex with another molecule prior to oxidation seemed worthy of further investigation, since it has been suggested (48) that a process of this type may operate in the biological reaction. The strongly coloured complex which benzpyrene forms with trinitrobenzene (49) seemed to offer an approach to the problem. It was decided to use this complex in conjunction with osmium tetroxide which Cook and Schoental (50) have shown to be capable of hydroxylating many polycyclic aromatic hydrocarbons including benzpyrene. A study of the reactions of benzpyrene with osmium tetroxide in presence and in absence of trinitrobenzene showed that the trinitrobenzene had no significant influence on the course of the reaction. The products isolated in both cases were those obtained by Cook and Schoental. Further investigations on similar lines were prevented by the difficulty of obtaining adequate supplies of starting materials.

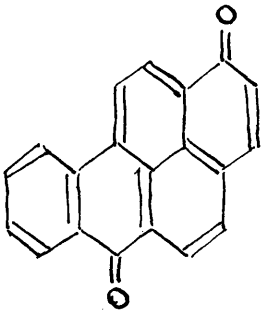
The production of dihydrodiols during the reactions of various hydrocarbons <u>in vivo</u> suggests the action of a hydrogen peroxide system (51); on this basis the chemiluminescent reactions of the hydrocarbons with the Milas reagents which incorporate hydrogen peroxide, have a point of similarity to the biological processes. The reaction of 3:4-benzpyrene with the Milas-reagents is discussed below, in relation to

the biological oxidations which occur with this substance.

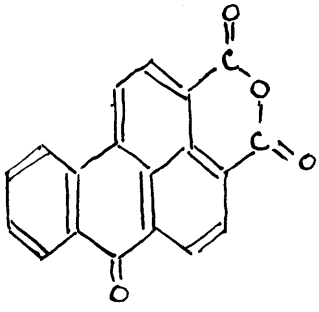
Two main products isolated from the reaction of benzpyrene with the Milas reagents were identified as 3:4-benzpyrene-5:8-quinone (II) and 3:4-benzpyrene-5:10-quinone (III).



II



III



IV

A fraction was also obtained which may be the anhydride of benzanthrone-peri-dicarboxylic acid (IV). A large portion of the reaction products was acidic. A fluorescent material obtained from this had a fluorescence spectrum similar to the spectrum of the 'fraction V' isolated by Berenblum and Schoental (52) from rats and rabbits injected with benzpyrene. These authors regard this fraction as a metabolite of benzpyrene possibly formed by breakdown of the molecule. It is also of interest that Peacock and Chalmers (unpublished work) isolated a fraction with a similar fluorescence spectrum, from the bile of goats fed with benzpyrene.

Under the conditions employed in the reaction with the Milas reagents no dihydrodiols or hydroxyl derivatives of

benzpyrene were detected although the isolation of the quinones suggests the intermediary formation of hydroxy compounds.

From various metabolism studies on benzpyrene there have been isolated the 8- and 10-hydroxybenzpyrenes (45,46,52), the 5:8- and 5:10-quinones (52), and the 'fraction V' of Berenblum and Schoental. The evidence for the formation of a dihydrodiol of benzpyrene is not conclusive (53), although the 8- and 10-hydroxy compounds may arise via 'perhydroxylation' of the 8-9 and 9-10 bonds with subsequent dehydration. The formation in the chemical oxidation of the quinones of benzpyrene together with the similarity between one of the reaction products and 'fraction V' shows some relationship to the course of the biological reaction.

The individual importance of the various metabolites produced from hydrocarbons to the carcinogenic process has not yet been elucidated. In the cases so far tested the metabolites are inactive compounds or possess only slight activity. With benzpyrene, the quinones are inactive; the hydroxyl derivatives have not been adequately tested, but it has been shown that 8-methoxybenzpyrene is strongly carcinogenic. It is of interest in this connection that chemiluminescence tests with the quinones of benzpyrene were negative but that a bright effect was obtained from a mixture of methoxybenzpyrenes (believed to be the 6- and 7-derivatives) prepared in the osmium tetroxide oxidation of benzpyrene (vide supra).

In conclusion, it may be stated that the evidence adduced supports the view that reactions of the hydrocarbons in the animal body may also be accompanied by chemiluminescence. Further study however to gain information on the particular reaction responsible for the luminescence is desirable.

EXPERIMENTAL.

Oxidation with Sodium Hypochlorite:

The alkaline solution of sodium hypochlorite (6% NaClO, 10% NaOH) was prepared by passing chlorine gas through a concentrated solution of sodium hydroxide. Equal volumes (1 litre) of this solution and a solution of 3:4-benzpyrene* (0.01%) in aqueous caffeine solution (4%) were mixed at 70°. The mixture was maintained at this temperature for 30 minutes and allowed to stand at room temperature overnight. The green fluorescent solution was acidified with hydrochloric acid and extracted with ether. On removal of the ether there remained a semi-crystalline mass melting 150°-170°. The solubility of this material in alkali to give a strong green fluorescent material, and the formation of a strongly adsorbed green fluorescent zone on an alumina column were in accord with the properties described by Chalmers (45) for a hydroxy-benzpyrene isolated from the faeces of rats fed with benzpyrene; the fluorescence spectrum shown on Plate I (Spectrum (a)) is also similar to that described by Chalmers for the hydroxy-metabolite. A spectrum of benzpyrene is shown for comparison (Spectrum (b)). A crude preparation of 8-hydroxy-benzpyrene (54) was obtained from the faeces of rats fed with benzene dissolved in milk and the spectrum of this (Spectrum (c)) shows a similarity to that of the material from the

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* The benzpyrene used in all of this work was supplied by Messrs Ward, Blenkinsop & Co.Ltd., London. It was purified by chromatography on alumina, using light petroleum (b.p. 60-80°) as solvent and eluent.

hypochlorite oxidation. The fraction from the oxidation and the crude fraction from the faeces containing the 8-hydroxy compound were methylated by treatment with diazomethane (55) in ethereal solution at room temperature for 24 hours. This allowed further purification of the fractions for the methyl products formed blue fluorescent zones on an alumina column, which were readily eluted by benzene. From the chromatography of the methylated oxidation product only 2 mg. of semi-solid material which possessed a two-banded spectrum was obtained. The fluorescence spectrum of this methylated fraction (Spectrum (d)) is similar to that of 8-methoxybenzpyrene (Spectrum (e)) but is displaced to longer wavelengths. The absorption spectrum of the methylated material in hexane had no fine structure.

No detectable quantities of benzpyrene quinones were formed in the reaction.

Oxidation with Osmium Tetroxide:

A preliminary oxidation of benzpyrene with osmium tetroxide in the presence of pyridine was carried out by the method described by Cook and Schoental (50). The benzpyrene-osmate-pyridine complex was converted to the dihydrodiol and thence to the mixture of hydroxybenzpyrenes (probably the 6- and 7-hydroxy compounds). The spectrum of this mixture is shown (Spectrum (f)). The corresponding mixture of methoxy compounds was prepared by treatment with diazomethane. A

partial separation of these was effected by chromatography on alumina using light petroleum (b.p. 60°-80°) - benzene mixtures as solvents and eluents. The fraction eluted first gave Spectrum (g) and the more strongly adsorbed material gave Spectrum (h).

The oxidation was now repeated incorporating s-trinitrobenzene in the reaction mixture. Twice the theoretical amount of the polynitro compound required to form the complex with benzpyrene (49) was added to the solution of benzpyrene in benzene, which became red. The other reagents were added as before. The reaction was conducted at 40°, instead of at room temperature, and 4 times the previous volume of benzene was employed, to prevent separation of the benzpyrene-trinitrobenzene complex. The course of this reaction was similar to that of the reaction without the trinitrobenzene, though the yield of methoxy compounds was slightly reduced. The fluorescence spectra of the various related parts were identical.

Oxidation with the Milas Reagents:

The Milas reagents were prepared by the same methods used in the chemiluminescence studies. A 6% peroxide reagent and a 0.5% catalyst solution were used. Benzpyrene (0.15 g.) was dissolved in tert. butyl alcohol-hydrogen peroxide (150 c.c.) with slight heating, and the catalyst (7.5 ml.) added. The reaction mixture was maintained at

37° for 48 hours. It was then diluted with water (1.5 litres) and extracted with ether (Extract A) till colourless and non-fluorescent. The ether was extracted with sodium hydroxide (2N) which became green-fluorescent. The alkaline extract was acidified with hydrochloric acid and extracted with ether (Extract B). This extract B was taken to dryness and yielded a brown solid (40 mg.). A fluorescence spectrum of this is shown on the plate (Spectrum (i)). Attempts to methylate this material with diazomethane and purify the product by chromatography were unsuccessful.

The ether extract A, taken to dryness, yielded a red-brown solid (0.1 g.). This was dissolved in acetic acid (4 c.c.) containing a few drops of concentrated hydrochloric acid, and the mixture boiled for a few minutes to convert any dihydrodiol present to hydroxyl derivatives. The absence of hydroxybenzpyrenes was demonstrated by failure to obtain a fluorescent alkali-soluble fraction from the dehydration mixture. The bulk of the material was recovered from the acetic acid solution and chromatographed on alumina using a 50:50-benzene:light petroleum (b.p. 60°-80°) mixture as solvent and eluent. The main zones which formed were a violet fluorescent zone which moved quickly down the column and two strongly adsorbed yellow and red zones which were closely associated and eluted together by washing the column with chloroform. The violet fluorescent zone gave rise to unchanged benzpyrene (45 mg.). The yellow and red zones

yielded a red-brown material (40 mg.) and it seemed likely that this comprised the two quinones of benzpyrene.

A mixture of the quinones of benzpyrene was prepared by oxidation of benzpyrene with chromic acid, as described by Vollmann et al. (56). This mixture was passed on to a silica column from benzene. The material spread over the column in a diffuse zone which consisted of 3 main bands; the lower band was yellow, the middle yellow-orange, and the upper orange-red. The lower band was eluted and rechromatographed. The middle and upper bands were separated by cutting the column and washing the materials from the silica with chloroform. The separated materials were subjected to 3 other similar chromatographic procedures. The m.p. 293°-294° of the orange-yellow material from the middle band compared well with that of 3:4-benzpyrene-5:10-quinone, m.p. 295°. The m.p. 241°-243° of the orange-red material from the upper band also compared well with that of 3:4-benzpyrene-5:8-quinone, m.p. 245°. The colour reactions of these products with sulphuric acid were also in accord with those recorded for the quinones. The material from the lower band was only present in small quantity but the view that it was the anhydride of benzanthrone-peri-dicarboxylic acid was suggested by the fact that Vollmann et al. isolated some of this substance from the reaction with chromic acid. The colour reaction of the material with concentrated sulphuric acid (deep red coloration) was in accord with that recorded for the anhydride (m.p. 364°),

although the melting point of the material was not sharp (310°-325°).

The reddish-brown material (40 mg.) from the oxidation with the Milas reagents was treated in the same way as the quinone mixture from the chromic acid oxidation. The appearance of the column was identical to that described above and the three products were isolated as before. The identity of the middle and upper bands with the 5:10- and 5:8-quinones respectively was shown by melting point and mixed melting point determinations, and by the reactions with sulphuric acid. The lower band yielded a fraction, melting 298°-318°, which gave a deep red coloration with concentrated sulphuric acid, and may be the anhydride referred to above.

Recording of Fluorescence and Absorption Spectra:

All fluorescence spectra were made with benzene solutions of the materials, to obviate solvent effects in the recordings. The fluorescence was excited by radiation from a G.E.C. Osira lamp, focused by a condensing lens on to the solutions, which were contained in a small glass cell placed close to the slit of the spectrograph. The instrument was a Hilger Medium Quartz Spectrograph and the slit width used throughout was 0.1 mm. Ilford Iso Zenith plates were used to record the spectra; the exposures varied from 1 to 10 minutes. The benzene used as solvent was freed from fluorescent impurities before use.

For absorption spectra the spectrograph was fitted with

a Hilger 'Spekker' photometer, using a condensed spark between tungsten steel electrodes as light source. Ilford Process plates were used in these measurements, and the readings were made by visual inspection. The hexane used in the measurements was a special grade for absorption spectrophotometry, supplied by Messrs British Drug Houses Ltd., Dorset.

PLATE I.

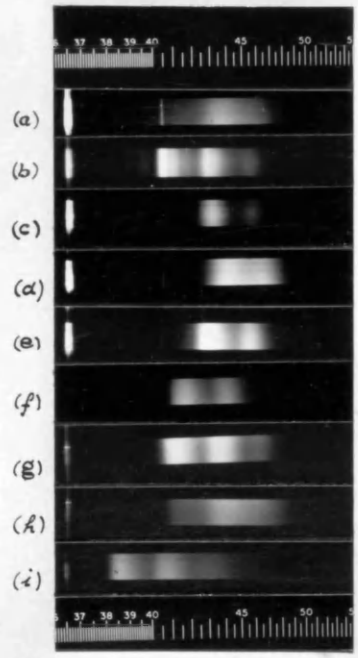

Fig. 3 — Spectra of 3 : 4 - benzpyrene

and some fluorescent oxidation products.

SECTION III.

CONTRIBUTIONS TO THE CHEMISTRY OF N:N-DIMETHYL-4-AMINOAZOBENZENE.

In this Section a similar study to that conducted with 3:4-benzpyrene in Section II was designed with a typical carcinogen of the azo-dye group. The course of the chemiluminescent reaction of 4-dimethylaminoazobenzene with the Milas reagents (see Section I) was investigated to discover whether there is any relationship between this reaction and the reactions which occur with this substance in the animal body. The experimental work involved a detailed study of the products formed in the chemical oxidation and to aid in their identification a number of substances reckoned as possible reaction products were collected as reference compounds. It seemed likely that the azoxy compounds derived from 4-dimethylaminoazobenzene would be formed, and since these were unknown* their synthesis was attempted. This work is reported below prior to the oxidation studies.

1. The Synthesis of the Azoxy Compounds derived from N:N-dimethyl-4-aminoazobenzene.

The methods available for the preparation of unsymmetrical azoxy compounds are (1) the direct oxidation of the corresponding azo compounds and (2) the condensation of N-aryl-hydroxyl-

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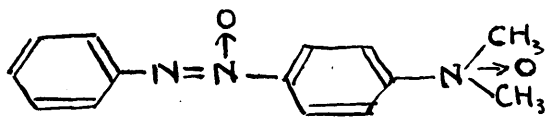
* See note on errors in the literature (p.59).

amines and aryl-nitroso compounds.

The first of these methods was used by Angeli (57) in the oxidation of 4-dimethylaminoazobenzene with hydrogen peroxide (30%) in glacial acetic acid, the usual reagent for the preparation of azoxy compounds. However, the dimethylamino group present in this compound is also susceptible and the main reaction product obtained was an amine oxide (I).



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The second method has not been used in the case of the azoxy compounds in question*, and indeed it has been applied successfully in only a very few cases, for whilst differently substituted N-aryl-hydroxylamines and aryl-nitroso compounds may be used, symmetrical azoxy compounds generally result, by a process of mutual oxidation and reduction:

$$2R.NHOH + 2R'.NO \rightarrow R.\overset{\uparrow O}{N}=N.R + R'.\overset{\uparrow O}{N}=N.R' + 2H_2O.$$

A survey of the literature on this type of reaction indicated that the absence of the unsymmetrical compounds as products may be due to the reaction conditions employed. It has been shown (58,59) that the presence of alkali greatly increases the rate of formation of azoxy compounds from hydroxylamine derivatives and nitroso compounds. However,

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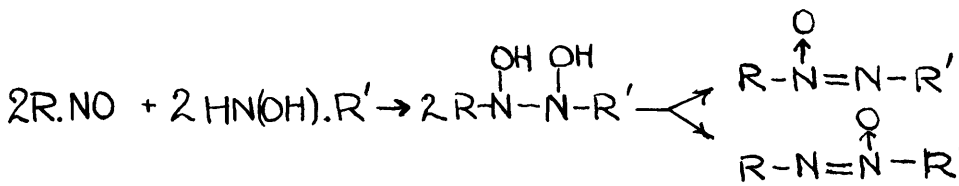
* See note on errors in the literature (p.59).

these studies were only applied to reactions of hydroxylamine derivatives and nitroso compounds with the same aryl residue, and it seems significant that with compounds possessing different aryl residues (60,61,62,63), the reactions were conducted in neutral solution. The condensations leading to azoxy compounds have been represented (63) by a scheme similar to that employed for the aldol type condensation:

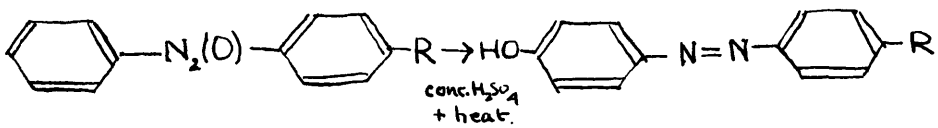


On this basis the influence of the alkali on the reaction rate would have a similar explanation to the catalysing influence of bases in other types of condensation (64).

N-phenylhydroxylamine was condensed with p-nitrosodimethylaniline in alcoholic solution in the presence of alkali. The crude reaction product subjected to chromatographic analysis yielded two pale orange crystalline substances, (1) m.p.122°, (2) m.p. 126°. The separation of the two substances on the column is quite definite and the melting points are strongly depressed in a mixture so that there is no doubt that they are distinct chemical compounds. Their basic nature is shown by their solubility in warm dilute hydrochloric acid and white crystalline hydrochlorides may be formed if special precautions are taken to exclude moisture. The method of preparation made it possible that the products were isomeric azoxy compounds derived from 4-dimethylaminoazobenzene and this was

confirmed by the analyses. The substances were characterised as their picrates.

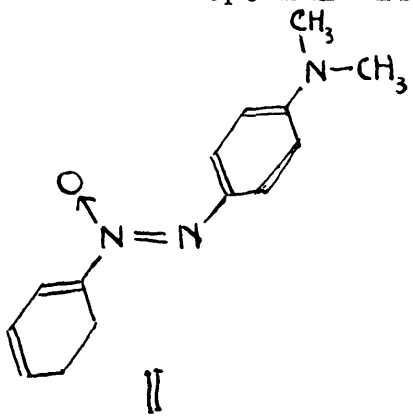
The weaker colour of the substances in comparison with the azo compound is in accord with the general experience for colour relationships of azoxy and azo compounds. Reduction of both substances to 4-dimethylaminoazobenzene provided further confirmation of the structures assigned to them. The little understood Wallach transformation of para substituted azoxy compounds to phenols by heating with concentrated sulphuric acid is a general reaction:



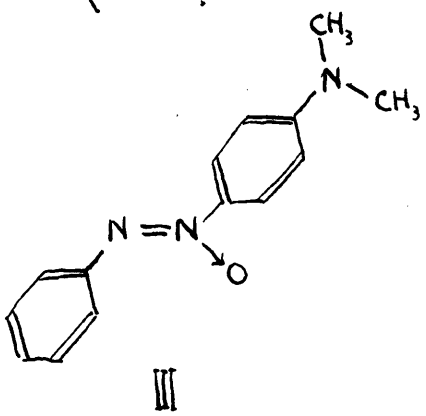
Subjected to this treatment, the two isomers yielded 4'-hydroxy-4-dimethylaminoazobenzene almost quantitatively. It is noteworthy that both isomers gave rise to the same phenol. Most reactions of this type have been applied to only one compound of an isomeric pair, although transformations of both to the same phenol are not unknown (65).

The absorption spectra of the substances in ethanol show a similar relationship to the spectrum of 4-dimethylaminoazobenzene, as was found by Szegö (66) for numerous pairs of unsymmetrical isomeric azoxy compounds and their corresponding azo compounds. These results provide good evidence that the substance with m.p. 126° is the α-isomer (II) and that

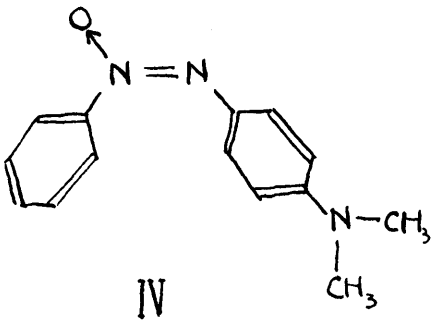
the substance m.p. 122° is the related β-isomer (III).



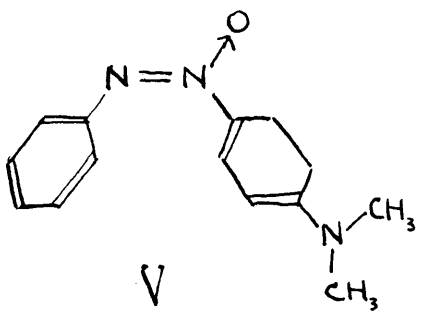
II



III



IV



V

The spectra are also in accord with the opinion that both compounds possess the trans-azo configuration since spectra of cis-azoxy compounds, while similar in structure to those of the related azo compounds, have greatly reduced absorption intensities (63). Also, the compounds were stable at temperatures well above their melting point. Attempts to produce the cis-azoxy compounds (IV and V) by the methods employed by Müller (63) were unsucdessful.

A study of the conditions for the formation of the trans-isomers showed that the reactions proceeded at room temperature and that the presence of alkali was essential. Under these conditions no detectable quantities of azoxybenzene or N:N:N':N'-

tetramethyl-4:4'-diaminoazoxybenzene were obtained. The reaction seems to be the first of its type in which the two possible unsymmetrical isomeric trans-azoxy compounds have been isolated. All these features of the reaction are in keeping with the theoretical scheme postulating the intermediary formation of a dihydroxy-azo compound. It is to be emphasised however that a dihydroxy compound of this type has never been isolated from nitroso-hydroxylamine condensations and the scheme may be an over-simplification. The results suggest that the failure of previous attempts to synthesise unsymmetrical azoxy compounds by this method may be related to the absence of a condensing agent in the reaction mixture. It is to be noted, however, that Bamberger and Bernays (67) were able to isolate some unsymmetrical 4-hydroxyazoxybenzene from a neutral reaction of p-nitrosophenol with N-phenylhydroxylamine, although azoxybenzene, and possibly 4:4'-dihydroxyazoxybenzene, was also produced. This indicates that the directive influence of the hydroxyl group in the nitroso compound favours the aldol type condensation and suggests that the ease of formation of the azoxy compounds in the present study is partially related to the powerful directive influence of the dimethylamino group in p-nitrosodimethylaniline.

From the biological point of view the synthesis of the azoxy compounds derived from 4-dimethylaminoazobenzene is of interest in that it will allow the testing of the substances for carcinogenic activity and will aid their detection if

present in reactions occurring in the animal body. As possible metabolites of the azo compound their testing is essential to the studies of Miller and Miller (32) which suggest that the dye molecule is active **per se** or through the N-monomethyl compound and not by virtue of conversion to any other of the known or possible metabolites. A tentative forecast of inactivity of the substances may be made on the basis of experiments with one of the azoxy-compounds of the other potent hepatic carcinogen, 4'-amino-2:3'-azotoluene; in this case the only biological response was some bile duct proliferation (68).

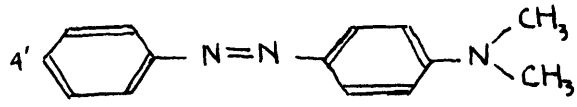
Errors in the literature. (1) In Chemical Abstracts, 1936, 30, 9996 and ibid, 2934[5] a substance is listed as 'aniline, N:N-dimethyl-phenylazoxy' with molecular formula $C_{14}H_{15}N_3O$. This is in reference to a paper by Bigiavi, D., and Albanese, C., Gazz.chim.Ital., 1935, 65, 773. The compound used by these authors, however, was an amine oxide and should be designated 'aniline oxide, N:N-dimethyl-phenylazoxy' with molecular formula $C_{14}H_{15}N_3O_2$

(2) In a review on azoxy compounds Bigelow, H.E., Chem. Reviews, 1931, 9, 157, refers to a paper by Fischer, O., and Wacker, L., Ber.d.deutsch.chem.Gesellsch., 1888, 21, 2609, and states that these authors condensed p-nitrosodimethylaniline with phenylhydroxylamine in absolute alcohol and obtained the symmetrical p-azoxydimethylaniline. The reaction actually conducted by Fischer and Wacker was a condensation

of p-nitrosodimethylaniline with phenylhydrazine in absolute ether.

2. Oxidations of N:N-dimethyl-4-aminoazobenzene.

(a) Oxidation with the Milas reagents. - Previous studies with the Milas reagents have been mainly concerned with hydroxylation of ethylenic compounds (26) but it has also been shown that they can convert aromatic hydrocarbons to phenolic derivatives (27). The latter observations indicated the possibility that 4-dimethylaminoazobenzene (Butter Yellow) (VI) in the presence of these reagents would be converted to a



VI

phenolic derivative. Since there is strong evidence that hydroxylation of Butter Yellow at the 4'- position occurs to some extent in the rat (69,70), there was a possible connection between the chemiluminescent oxidation with the Milas reagents and the in vivo reaction. However, a preliminary study (71) of the reaction products from the oxidation of Butter Yellow with these reagents revealed that much of the action was taking place at the dimethylamino group with the removal of the methyl groups and further oxidation to the nitro compound. This uncommon type of reaction gave an added significance to

the investigations since demethylation is one of the principal metabolic reactions which occurs with Butter Yellow (72). A further investigation of the course of the reaction was then designed.

It was found that the main product of the reaction was N-methyl-4-aminoazobenzene and that small quantities of 4-aminoazobenzene and 4-nitroazobenzene were produced. It has also been established that hydroxylation of the dye occurs to a small extent and some evidence has been adduced of the presence of 4'-hydroxy-4-dimethylaminoazobenzene in minute amount in the phenolic fraction. There is strong evidence that the amine oxide of 4-dimethylaminoazobenzene was also present in the reaction products but no trace of the azoxy compounds was found. The chromatographic technique used extensively in the separations showed that many other coloured fractions were present but all of these were in small quantity and were not identified; they included basic, phenolic and amphoteric fractions. The investigations were not extended to the isolation of colourless products although since only 75 per cent of the starting weight was recovered it is probable that some fission of the molecule to simpler products took place. A semi-quantitative evaluation of the course of the reaction was made possible by the methods employed; this is summarised in Fig.4.

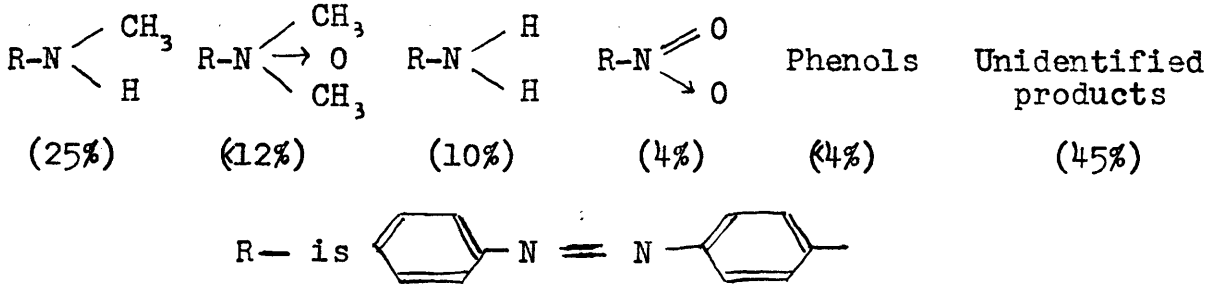


Fig.4 - Products of Reaction of 4-dimethylaminoazobenzene with the Milas Reagents at 37° for 48 hours.

Note: The products are shown as percentages of the total weight of 4-dimethylaminoazobenzene which was altered in the reaction.

The formation of hydroxyl derivatives and demethylation products shows an interesting relationship between the reaction and the oxidation processes so far known to occur with Butter Yellow in the liver of the rat. It should be noted that reductive fission of the azo linkage also occurs to a large extent *in vivo* but Miller *et al.* (72,69) have demonstrated that the demethylation and hydroxylation processes occur largely prior to cleavage of the dye molecule. There is as yet insufficient evidence to decide which, if any, of these biological reactions are important to the carcinogenic process and which are harmless detoxications. It is significant however for the present study that interactions with the dimethylamino group of Butter Yellow occur in the animal body and lead to the same products which arise in a similar interaction with the Milas reagents. Furthermore, as demon-

strated in Section I, the chemiluminescence phenomenon associated with the chemical oxidation is probably related to this interaction. The demonstration by Kirby and Peacock (73) of a carcinogenic action associated with 4-aminoazobenzene suggests that the demethylation process is not essential to the carcinogenic action of Butter Yellow, although the possibility that biological methylation of the primary amine takes place has been considered by these authors. At any rate it has been shown that chemiluminescence also occurs during the oxidation of 4-aminoazobenzene. It must be emphasised however that there is no general relationship in the biological experiments between reactions at the amine group and carcinogenic activity. For example, dealkylation of the inactive N:N-diethyl-4-aminoazobenzene has been demonstrated by Kensler et al. (74) and it is likely that similar processes occur with other inactive derivatives of Butter Yellow. On the other hand the far-reaching experiments of Miller and Miller (75) show a striking relationship between the carcinogenic action of Butter Yellow and its ability, in association with its demethylation products and some other unidentified metabolite, to form strong unions with certain proteins of liver tissue. The response of the different tissues of various species is strictly related to the levels of bound dyes in these tissues. Furthermore, the levels of bound dyes obtained with the very weakly active 4-aminoazobenzene in comparable tissues are much smaller than with the very active dimethyl derivative.

These observations strongly suggest that ability to form an adsorption complex with certain proteins is a prerequisite for carcinogenesis with these compounds, and thus give an indication of the features which may distinguish the closely related carcinogenic and non-carcinogenic compounds. It is possible that reactions at the amine group while the dye is strongly adsorbed to protein are important to the carcinogenic process. This view is discussed more fully in Section V.

From the chemical standpoint the reaction with the Milas reagents shows a number of interesting features. The removal of alkyl groups from aromatic tertiary amines is not uncommon in <u>in vivo</u> reactions but little is known of the reaction mechanism and the process is simply referred to as 'dealkylation'. Studies with amine oxidase (77,78) show that a number of amines undergo oxidation at the carbon-nitrogen linkage in the presence of this enzyme. The experiments of Hess <u>et al.</u> (79) on cyclic $>N.CH_3$ compounds containing a ketone or aldehyde grouping (derivatives of pyrrolidine or piperidine) afford examples of intramolecular oxidative demethylation. Various amino alcohols containing contiguous hydroxyl and tertiary amino groups have been found to undergo oxidative cleavage with lead tetraacetate (80). The present experiments provide an example of oxidative demethylation of an aromatic tertiary amine, effected by hydrogen peroxide. The recent work by Rusch and Miller (76) showing that demethyl-

ation of 4-dimethylaminoazobenzene is also effected by autoxidising linoleic acid is worthy of mention. It is possible that this process occurs *via* peroxides present in the mixtures.

The unexpected absence of azoxy compounds from the reaction products emphasises the fundamentally different mode of action of hydrogen peroxide catalysed by osmium tetroxide and hydrogen peroxide in the presence of glacial acetic acid, although it is noteworthy that both oxidising agents give rise to the amine oxide (*vide infra*). The versatility of the Milas reagents is further evidenced by the isolation of the nitro and hydroxyl derivatives. The appearance of the nitro compound is possibly due to oxidation of the primary amine formed in the reaction and may arise *via* the nitroso compound. Oxidation of primary amines to nitroso and nitro compounds has been observed with numerous oxidising agents including Caro's acid, peracetic acid and aqueous sodium peroxide. It would be of interest to know whether conversion of Butter Yellow to 4-nitroazobenzene occurs in the animal body, since the formation of this compound may be connected with the chemiluminescence process.

(b) *Oxidation with peracetic acid*. - The principal action of peracetic acid (30% hydrogen peroxide in glacial acetic acid) with azo compounds is the formation of the azoxy compounds. The reagent is however also capable of converting aromatic tertiary amines to amine oxides and as mentioned

previously Angeli (57) isolated a product from the oxidation of 4-dimethylaminoazobenzene with peracetic acid containing oxygen atoms on both the azo and the tertiary nitrogen (see p.54, Structure I). The experimental details in Angeli's paper are somewhat vague, and numerous attempts to repeat his results were unsuccessful. A product similar in appearance and reaction to Angeli's oxide was always obtained but after many crystallisations it melted with decomposition over a range below that recorded by him. Conversion to the sulphate gave similar results. In each attempt it appeared that mixtures of two or more substances not easily separable were formed. Separation by chromatography was also unsuccessful for the material formed a single diffuse zone on the column.

From the oxidation of 4-dimethylaminoazobenzene with the Milas reagents a fraction had been obtained which seemed to be the amine oxide of this substance. During the removal of solvent from this material on the water bath, it decomposed with the formation of red vapours. Examination of the altered material showed that it consisted largely of the parent azo compound. These findings led to a study of the effect of heat on the mixtures formed in the oxidation with peracetic acid.

The partially purified mixture was heated to its decomposition temperature and in this region yellow vapours were thrown off. A chromatographic separation of the altered material revealed that it comprised mainly three substances; these were 4-dimethylaminoazobenzene and the recently synthe-

sised isomeric azoxy compounds derived from this substance. The previous chromatographic behaviour and other properties of the unheated material excluded the possibility that these substances were present prior to the decomposition. The only satisfactory explanation was that the product from the oxidation of the azo compound with peracetic acid contains the amine oxides of these three substances. Thus while this method of oxidation does not appear to be suitable for the preparation of the separate amine oxides, the present study provides interesting information on the course of the reaction, and of the behaviour of the compounds. A possible route to the separate preparation of the amine oxides of the azoxy compounds lies in the direct oxidation of the azoxy compounds with peracetic acid.

It may be added that the amine oxides are possible metabolites of Butter Yellow and their biological testing is important to an understanding of the carcinogenic action of this dye.

EXPERIMENTAL.

1. Synthesis of Azoxy Compounds.

Reaction of N-phenylhydroxylamine with p-nitrosodimethylaniline. - The N-phenylhydroxylamine, prepared by reduction of nitrobenzene with zinc dust and aqueous ammonium chloride, was dried quickly on porous plate and used immediately. Commercial p-nitrosodimethylaniline was purified by crystallisation from light petroleum (b.p. 60°-80°).

Solutions of the nitroso compound (1 g. in 12 c.c.) and the hydroxylamine (2 g. in 12 c.c.) in ethanol were mixed and a few drops of 1:1-aqueous potassium hydroxide added immediately, with shaking. The mixture was heated in boiling water for 10 minutes, then cooled and poured into water (400 c.c.). After standing 24 hours, the precipitated material was filtered off and dried to give a brown powder (0.6 g.). Although a similar yield was obtained when the reaction was conducted at room temperature, finer precipitates were obtained which were difficult to handle. Strong heating beyond 10 minutes also resulted in fine precipitates. In the absence of alkali only a fine tarry suspension, which was not stopped by the filter, was obtained. The yields were higher when the hydroxylamine was employed in excess of the theoretical amount.

The methods described by Müller (63) for the formation of cis-azoxy compounds from hydroxylamine derivatives and nitroso compounds were applied to the compounds under study. Only

fine tarry suspensions which passed through the filter were obtained from the diluted reaction mixtures.

The brown powder (0.6 g.) was dissolved in benzene and passed on to an alumina column (10" x 1.5") (aluminium oxide for chromatographic adsorption analysis supplied by Messrs British Drug Houses Ltd., Dorset, gave satisfactory separations). The column was developed with benzene, and the material spread into a broad orange zone which finally resolved into two bands. The two bands were eluted separately, although it was not possible to achieve a sharp separation in this first chromatogram. The two parts were subjected to two further chromatographic separations and small fractions suspected as mixtures of the two zones were discarded. Removal of the solvent from the eluate derived from the lower band yielded a pale yellow-orange crystalline solid (0.17 g.), which was an azoxy compound of 4-dimethylaminoazobenzene. Absorption spectral data suggested that it was trans-β-N:N-dimethyl-4-aminoazoxybenzene (III). After sublimation of the material at 135°/0.05 mm. and crystallisation from light petroleum (60°-80°) it formed pale yellow-orange needles, m.p. 122° (Found: C, 69.87; H, 6.22; N, 17.26. $C_{14}H_{15}ON_3$ requires C, 69.68; H, 6.27; N, 17.42%). The picrate prepared and crystallised from ethanol formed stout yellow prisms, m.p. 149°-151° (decomp.). (Found: C, 51.22; H, 3.93. $C_{14}H_{15}ON_3$, $C_6H_3O_7N_3$ requires C, 51.06; H, 3.86%). The pale orange material (0.31 g.) obtained on removal of the solvent

from the eluate derived from the upper band was also an azoxy compound of 4-dimethylaminoazobenzene. The absorption spectrum suggests that it is trans-α-N:N-dimethyl-4-aminoazoxybenzene (II). After crystallisation from light petroleum ether (b.p. 60°-80°) it formed small pale orange prisms, m.p. 126°. (Found: C, 70.00; H, 6.27; N, 16.90. $C_{14}H_{15}ON_3$ requires C, 69.68; H, 6.27; N, 17.42%). The picrate prepared and crystallised from ethanol formed fine yellow needles, m.p. 155.5°-157.5° (decomp.). (Found: C, 51.15; H, 3.89. $C_{14}H_{15}ON_3$, $C_6H_3O_7N_3$ requires C, 51.06; H, 3.86%).

The appearances of the alumina columns varied with different batches of material. They were less complex when the reaction had been carried out at room temperature. In columns corresponding to reactions conducted at raised temperatures a faint zone preceded the main orange zone and another orange zone appeared after the main zone. These extra zones yielded only a few milligrams of impure materials but their chromatographic behaviour suggests that they may be azoxybenzene and N:N:N':N'-tetramethyl-4:4'-diaminoazoxybenzene.

The two azoxy compounds (II) and (III) heated in sealed tubes at 200° for 1 hour were largely unchanged. Only the substance m.p. 126° showed slight decomposition.

Reduction of the Isomers. Reduction of the isomers was carried out with zinc dust in presence of aqueous alcoholic sodium hydroxide. The substances (0.05 g.) were suspended in ethanol (5 c.c.) and 25% aqueous sodium hydroxide (10 c.c.)

added. An excess of zinc dust was added over a period of 10 minutes with vigorous stirring. The reaction mixtures were diluted with water (100 c.c.) and extracted with light petroleum (b.p. 60°-80°), till the extract was colourless. Chromatography of each extract on alumina with light petroleum gave an orange zone which eluted slowly. Removal of the solvents from the eluates of the zones gave bright orange crystalline substances (0.02 g.) each with m.p. 115°-117°. In chromatographic behaviour, solubilities and colours with dilute hydrochloric acid these substances were identical with 4-dimethylaminoazobenzene, m.p. 117°. The identity of the materials was confirmed by mixed melting point determinations.

This method of reduction was used by Meldola and Andrews (81) for the reduction of 3:3'-diaminoazoxybenzene to the azo and hydrazo compounds.

Wallach Transformation. Samples of the isomers (0.05 g.) were dissolved in concentrated sulphuric acid (5 c.c.) and the solutions heated on the water bath for 10 minutes. The reaction mixtures cooled and stirred into water (50 c.c.), gave bright magenta-coloured solutions. These were neutralised with sodium hydroxide (2N) and fine suspensions developed which were extracted with ether. The yellow ether solutions were dried over anhydrous sodium sulphate and the solvent was then removed. Both extracts yielded bright red solids (0.045 g.) each with m.p. 202° (decomp.). In solubilities and colours with dilute acids these solids were identical with 4'-hydroxy-4-dimethyl-

aminoazobenzene, m.p. 202° (decomp.). The identity of the materials was confirmed by mixed melting point determinations.

Basic nature of the isomers. The substances were soluble in dilute acids on warming giving colourless or slightly pink solutions; this is in contrast to 4-dimethylaminoazobenzene which yields bright red solutions. It was not possible to isolate the sulphate presumably due to its ready hydrolysis in an aqueous medium. White crystalline hydrochlorides of the substances were obtained by passing dry hydrogen chloride through dry benzene solutions of the materials. These hydrochlorides were readily hydrolysed in the laboratory atmosphere and were not suitable for routine analysis. Handled with as little exposure to moisture as possible, they did not give sharp melting points even in sealed tubes. The hydrochloride of the isomer m.p. 122° decomposed over the range 140°-152° and the other material decomposed over the range 150°-165°. The picrates formed readily in ethanol and were stable (vide supra).

Absorption spectra. (For description of apparatus see p. 51). Ilford Rapid Panchromatic Process plates, which are sensitive up to 650 mμ, were used to record the spectra. The ethanol employed as solvent was purified by the method recommended by Leighton et al. (82) for optical measurements. For each compound, 0.01% solutions were used and measurements were made with different cell thicknesses to increase the accuracy of the curves. The samples of azoxy compounds were

of the same purity as those used in their elementary analysis, and the 4-dimethylaminoazobenzene, m.p. 117°, had been purified chromatographically on an alumina column using light petroleum (b.p. 60°-80°) as solvent and eluent.

The recordings are shown diagrammatically on Plate II, Fig.5 and are summarised in the following table, values of λ max. being followed in parenthesis by those of log. ε max. The values obtained for 4-dimethylaminoazobenzene are in good agreement with those recorded by Pongratz et al. (83) in 0.05% ethanol solution. There is a slight difference in the ε max. values of the shorter wavelength bands.

Azoxy compound, m.p. 122°	393 (4.47)	241 (4.17).
Azoxy compound, m.p. 126°	414 (4.53)	261 (4.16).
4-dimethylaminoazobenzene	409 (4.48)	257 (4.13).
4-dimethylaminoazobenzene *	409 (4.48)	255 (4.04).

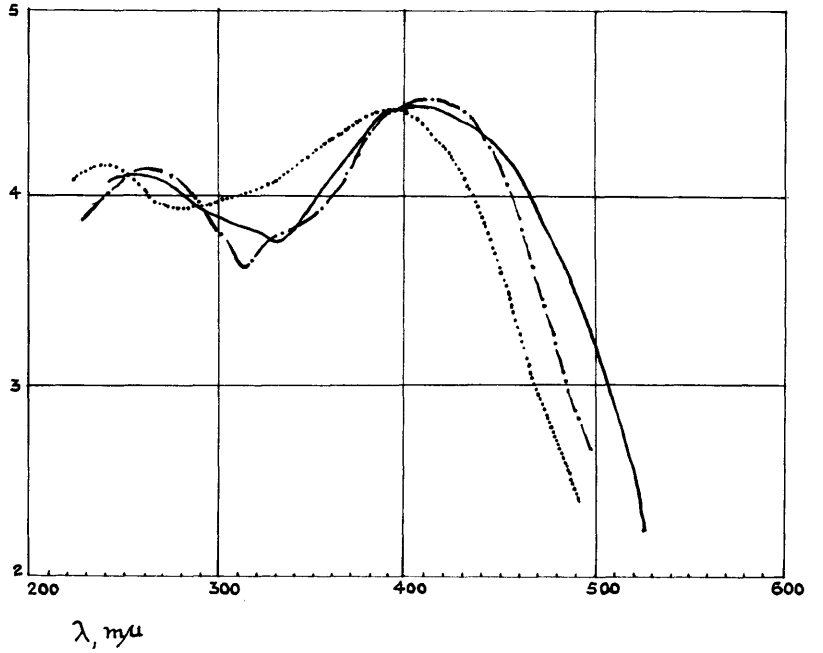
* These are values obtained by Pongratz et al.

The features significant to the present work are (1) the similarity for the three compounds of the ε max. values in the comparable bands, (2) the larger shift of the λ max. positions in the azoxy compound, m.p. 122°, relative to the azo compound, and (3) the closer relationship of the general shape of the curve of the azoxy compound m.p. 126° to that of the azo compound.

The first point is consonant with the view that the isomers possess a trans configuration, for Müller's cis azoxy compounds (63,84) have intensities about half the values of those of the

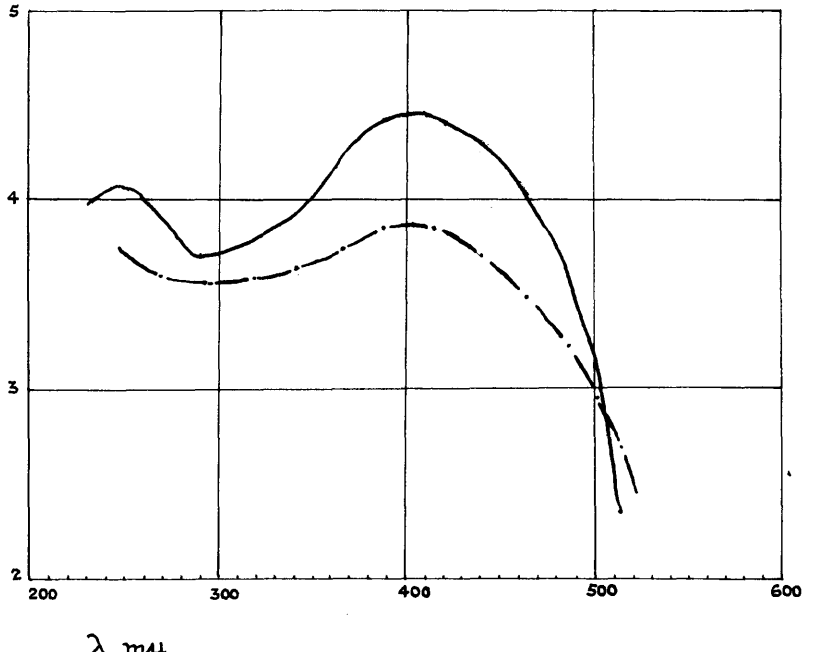
trans stereoisomers, while the trans compounds have similar values to those of the related azo compounds. The second two points together provide strong evidence that the azoxy compound m.p. 122° is the β -isomer and the other the α -isomer. For numerous pairs of isomeric azoxy compounds containing strongly directive groups, Szegö (66) showed that the compounds to which the α -structure had been assigned by chemical methods had absorption curves with λ max. closer to that of the comparable bands of the related azo compounds than had the β -isomers. The absorption bands of the β -isomers are shifted to regions of shorter wavelength, and there are differences in the shape of the curves in comparison with those of the α -azoxy and azo compounds with complete disappearance of the higher frequency band in many cases. Szegö has suggested that the absence of this band in these cases may merely be due to a hypsochromic effect causing the maximum to move to a region outwith the observable range. It is therefore of interest that the compound corresponding to the β -isomer, in the present study, shows two bands, one of which is just within the measured region. This is in accord with Szegö's suggestion for the whole absorption curve of the parent azo compound lies further towards the red end of the spectrum than do those of most of the azo compounds studied by him.

The narrower bands of the azoxy compounds in comparison with that of 4-dimethylaminoazobenzene is also in keeping with Szegö's findings.



$Log_{10}\Sigma$

Fig. 5—— Spectral absorption curves of N : N— dimethyl— 4 — amino — azobenzene (———), the azoxy compound, m.p. 122° (--------), and the azoxy compound, m.p. 126 (-·-·-·).



$Log_{10}\Sigma$

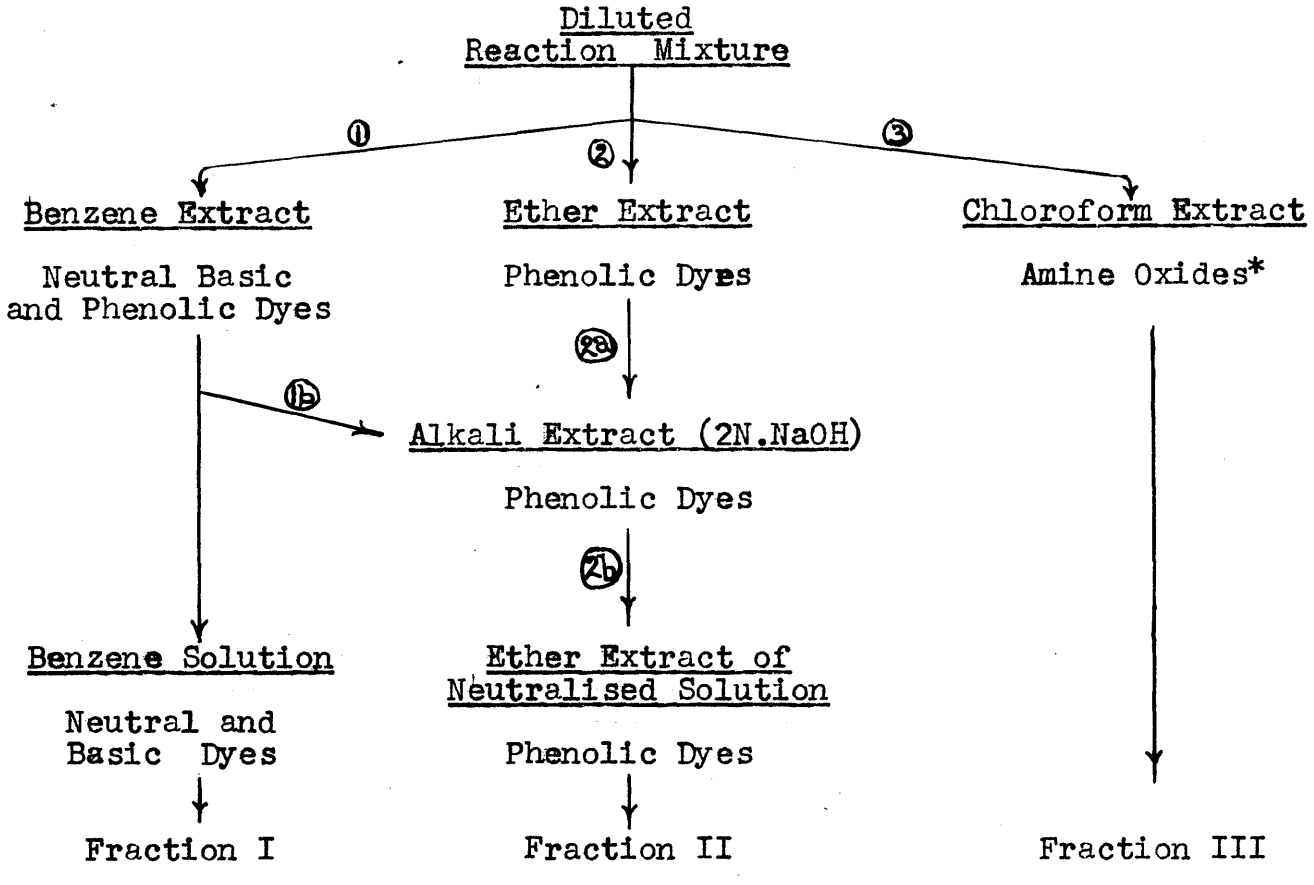
Fig. 6—— Spectral absorption curves of 4′—hydroxy—4—dimethylaminoazobenzene (———), and an oxidation product of 4—dimethylaminoazobenzene. (-·-·-·).

2. Oxidations of N:N-dimethyl-4-aminoazobenzene.

Oxidation with the Milas Reagents. - The reagents used in these oxidations were prepared as described in the chemiluminescence studies (see p.35). Throughout the investigations an 8% hydrogen peroxide-tert. butyl alcohol reagent and a 0.5% osmium tetroxide catalyst solution were employed, and the reactions were conducted at 37° in order to have the conditions comparable with those of the chemiluminescence test. The reaction products were isolated by extraction of the diluted reaction mixture with organic solvents, since the method used by Milas and Sussman (26) of distillation of the alcohol from the reaction mixture may have led to further oxidation during the concentration of unchanged peroxide. Several preliminary oxidations were carried out in which extraction was made with benzene and the extract subjected to chromatographic separations. While a large part of the identification of the products found in the reaction was accomplished in these studies, the following scheme, which was employed in a more detailed semi-quentitative investigation, records the experimental evidence for the results discussed in the text.

4-dimethylaminoazobenzene (1 g.) purified by chromatography, was dissolved in the peroxide reagent (200 c.c.) and catalyst solution (10 c.c.) added. The mixture was maintained at 37° for 48 hours, and then poured into water (1.8 litres). This was extracted by shaking successively with benzene, ether

TABLE V: SCHEME OF SEPARATION OF REACTION PRODUCTS FROM OXIDATION OF 4-DIMETHYLAMINOAZOBENZENE WITH THE MILAS REAGENTS.



*The basis for the use of chloroform to extract possible amine oxides was provided by tests conducted with the mixture of amine oxides produced in the oxidation of 4-dimethylaminoazobenzene with peracetic acid (loc.cit.).

and chloroform. The scheme of separations shown in Table V gave rise to three fractions which are discussed below. It should be noted that numerous extracts were made with each solvent and the process was continued till an extract was almost colourless. After the chloroform extraction the diluted reaction mixture was almost colourless. Before removal of the solvents from the three fractions by distillation they were dried over anhydrous sodium sulphate.

FRACTION I: Removal of the solvent left a dark brown residue (0.55 g.) which was dissolved in a 1:1 mixture of benzene and light petroleum (b.p. 60°-80°) and passed on to an alumina column (10" x 1.3"). On washing with the same solvent three main bright orange zones appeared preceded by a diffuse fawn band. By gradually increasing the proportion of benzene in the eluent four parts were eluted separately and will be referred to as A, B, C, and D, taken in order of elution. There remained on the column a diffuse band comprising six or more zones, but these could not be separated effectively and were discarded. Part A yielded a neutral pale orange substance (0.03 g.), m.p. 130°-132°. An absorption spectrum in ethanol was closely similar to that described by Pongratz (83) for 4-nitroazobenzene, m.p. 134°. A sample of the nitro compound was synthesised. Its melting point was not depressed on admixture of fraction A. Parts B (0.14 g.), C (0.206 g.) and D (0.079 g.), in colour reactions with dilute hydrochloric acid and in chromatographic behaviour were similar respectively

to unchanged 4-dimethylaminoazobenzene, 4-monomethylaminoazobenzene, and 4-aminoazobenzene. Mixed melting point determinations confirmed the identity of the similar materials. It should be pointed out that the materials were not obtained in pure form from the single chromatographic separation. Many other chromatographic separations employing different eluents were required to effect adequate removal of other materials from the main zones. Greatest difficulty was experienced with Part D which was closely associated on the column with unidentified material.

The azoxy compounds of 4-dimethylaminoazobenzene were not present in detectable quantities. A mixture of these compounds with 4-monomethylaminoazobenzene and 4-aminoazobenzene was resolvable by the chromatographic techniques employed above.

FRACTION II: Removal of the solvent left a dark brown residue (0.03 g.) which was dissolved in benzene and chromatographed on a silica column using benzene as eluent, since this technique had proved suitable for separation of phenolic derivatives of azobenzene. Many diffuse bands were formed and the numerous amphoteric fractions obtained from these each consisted of only a few milligrams of impure material. Some of these gave pink colours in dilute hydrochloric acid and it is possible that they were hydroxyl derivatives of the basic dyes present in the reaction mixture. An absorption spectrum was made of a fraction whose chromatographic behaviour and

colour in dilute acid was similar to that of 4'-hydroxy-4-dimethylaminoazobenzene. The spectrum is shown diagrammatically in Plate II, Fig.6, with the spectrum of the hydroxyaminoazo compound for comparison. Ethanol was used as solvent. The sample of the reference compound was one which had been synthesised and purified by chromatography on silica. The curves are very similar in the longer wavelength region; λ max. values of the main bands are identical (403 mμ). The disparity in the shorter wavelength region may be due to absorbing impurities.

FRACTION III: The removal of the last traces of the chloroform from this fraction by heating on the water bath resulted in its decomposition and bright red vapours were thrown off. There remained a red-brown material (0.11 g.). The solubility properties of the fraction had altered in the decomposition for it was now only sparingly soluble in water and on the other hand it was, in part, readily soluble in light petroleum (b.p. 60°80°). It was chromatographed on alumina and yielded 4-dimethylaminoazobenzene (0.03 g.). It is certain that this latter compound arose in the decomposition of Fraction III and was not merely some unchanged starting material which had escaped previous extraction, for control tests showed that complete extraction of this substance is obtained with benzene. Furthermore, in the decomposed Fraction III there was no trace of 4-monomethylaminoazobenzene or 4-aminoazobenzene, which would also have escaped extraction.

Strong confirmation that the azo compound arose from the decomposition of its amine oxide is provided elsewhere (p.65).

Oxidation with peracetic acid. 4-dimethylaminoazobenzene (3 g.) crystallised from light petroleum (b.p. 60°-80°) was suspended in glacial acetic acid (30 c.c.) and 30% hydrogen peroxide (15 c.c.) added. This mixture was allowed to stand at room temperature for 4 days. The yellow brown solution was diluted with water (50 c.c.) and dilute sulphuric acid added (100 c.c.). Orange laminae (2.5 g.) separated overnight in the cold room. These were filtered off, suspended in water and dilute sodium carbonate added to neutralise the mixture. The resulting material was crystallised once from water and twice from benzene. This gave a product which decomposed on heating, 116°-120°. Further crystallisation from benzene did not raise this decomposition temperature. The m.p. recorded by Angeli for β-4-dimethylaminoazoxybenzene oxide (I) is 127° (decomp.). The melting point of the sulphate of this he records as 156° (decomp.). The sulphate of the present material decomposed on heating at 136°-142°.

A sample of the base, m.p. 116°-120° (decomp.), (0.2 g.) was heated in a tube to 120° when a rapid darkening occurred and yellow-orange vapours were thrown off. The tarry residue was thoroughly extracted with a 1:1 mixture of benzene and light petroleum (b.p. 60°-80°) and the extract chromatographed

on alumina. Three main orange zones appeared which were eluted separately by gradually raising the propertion of benzene in the eluent. In order of elution the three zones gave rise to 4-dimethylaminoazobenzene (0.05 g.), β-4-dimethylaminoazoxybenzene (0.02 g.), and α-4-dimethylaminoazoxybenzene (0.06 g.). This identification was made by comparison of the appearance, chromatographic behaviour and solubility properties of the different materials with pure samples of these substances. The melting points of the fractions were not more than 2° below those of the related pure samples and there was no depression of the melting points in mixtures.

SECTION IV.

THE RATE OF ELIMINATION OF CARCINOGENS FROM THE ANIMAL BODY.

In contrast with the previous sections of the thesis, which represent a new approach to the problem of experimental carcinogenesis, the work described here is an extension of a series of investigations on the fate of chemical carcinogens in the animal body, conducted in the Cancer Hospital by Peacock and his colleagues over the past twenty years. The results of the present work are briefly discussed below in relation to the similar studies of other investigators. The investigations were made in collaboration with Dr. P. R. Peacock and Dr. S. Beck, who jointly conducted the biological part of the experimental work involved (85).

The detailed study of the fate of chemical carcinogens in the animal body is made difficult by the fact that small quantities of the carcinogen are sufficient to elicit tumours and because of this many of the investigations have been conducted with relatively larger quantities of the carcinogen, studying the metabolism of the substance by examination of the products in bile, urine or faeces (86). While this is an important method of attack it must be reckoned as only a preliminary approach to the problem, which must be tackled by determining the intimate metabolism of the substances in the tissues which ultimately become malignant. Much progress has

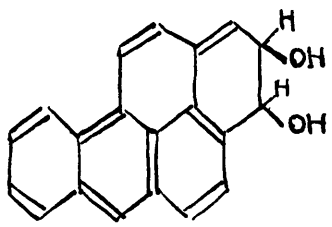
been made with the azo-dye group of carcinogens since the chemistry of these substances is relatively simple and because the liver, in which they produce their carcinogenic effect, is comparatively large and gives rise to detectable quantities of the metabolites in situ. With the hydrocarbon group where the chemistry is complex and the tumours appear on the skin or in the subcutaneous tissues, the most fruitful method has been to study the rate of elimination of the substance from the site of application. In these investigations use has always been made of the fluorescence properties of the hydrocarbons which allow their detection even when present only in minute traces. The experiments of Peacock and Beck (87), in which 3:4-benzpyrene was injected subcutaneously in various solvents into groups of mice, indicated that a rapid elimination of benzpyrene was accompanied by a low incidence of tumours, and that a high incidence of tumours was related to the ability of the solvent to retain some benzpyrene at the site of injection for several months. On the other hand, in a quantitative study of the rate of elimination of benzpyrene after subcutaneous injection in tricaprylin and other solvents into mice, Dickens (88) states that "the surprising result was obtained that the more rapid elimination of benzpyrene was associated with the higher carcinogenic activity, and slower elimination with lower activity". One of the objects of the present investigation was to attempt a reconciliation of these two

apparently contradictory observations. In the previous experiments of Peacock and Beck (87) only visual observation of the fluorescence had been made, but here it was decided to analyse the fluorescent material spectrographically in order to obviate confusion between the original substance and possible fluorescent metabolites.

A study was made of the elimination of benzpyrene dissolved in tricaprylin injected subcutaneously into 36 mice, in relation to tumour production. The technique adopted in these experiments was to examine an extract of the tissues immediately in contact with the site of injection by fluorescence spectrography to decide whether benzpyrene was present or absent in detectable quantity. It was felt that this was preferable to the method used by other investigators (88) of extracting the whole animal, since the issue is concerned with the interaction of the carcinogen and the cells which finally give rise to the tumour. The mice were killed after various times over a period of 10 months; the results of the spectrographic examination are summarised in Table VI in relation to the biological effects.

It will be seen that of 17 mice killed over a period of 6 months, 13 contained detectable amounts of the carcinogen, and that the 4 mice in which benzpyrene was absent were also free from tumours. The absence of benzpyrene from extracts of 2 tumour-bearing mice killed after a period of 7 months does not preclude the possibility that the unchanged

hydrocarbon was still present at the inception of the tumour process as it was in the other tumour-bearing animals killed after shorter periods. After this 7 month period no other tumours developed and in the remaining mice killed at 10 months no benzpyrene was detected. The spectrographic examination also showed a steady decrease in the amounts of benzpyrene present after the various times and the examinations at 5 months and after, indicated the presence of a fluorescent metabolite. This possible metabolite, if present, would not have been detected before the 5 month period, for its fluorescence intensity was much weaker than that of benzpyrene which was then present in relatively large amounts. The fluorescence spectrum of the unknown material was similar to that recorded by Weigert and Mottram (89) for fractions extracted from tissues exposed to benzpyrene. These authors regard the fractions as dihydrodiols as shown in the formula -



There was insufficient material in the present experiments to allow further investigation of these fractions.

The results of the experiment accord well with the previous observations of Peacock and Beck (87) and can best be interpreted as showing that benzpyrene acts throughout

the latent period of carcinogenesis. The findings do not allow a decision to be made on whether the hydrocarbon acts per se or through metabolic products to induce malignancy. Dickens and Weil-Malherbe (90), on the other hand, conclude that their observations suggest that a metabolite is to be regarded as the true carcinogenic substance. The evidence on which this suggestion is based derives from an experiment using solutions of benzpyrene in tricaprylin containing 3% cholesterol injected subcutaneously into mice; the incidence of tumours was relatively high. The plot of the amount of benzpyrene remaining in animals killed at various times against the time, is assumed to show complete elimination of the carcinogen after 9 weeks. However, the graph is drawn as a straight line through widely scattered points and would not seem to justify this conclusion. Furthermore, these authors did not examine any mice killed after this period for the presence or absence of benzpyrene, and thus give no indication of any difference in the fate of the carcinogen in tumour-bearing mice and in mice without tumours. Other experiments ofDickens and Weil-Malherbe (90) seem to justify the conclusion that "slow" elimination is accompanied by a low tumour incidence. In these cases, however, it would appear that the absence of tumours is due to an encapsulation of the carcinogen to such an extent that only sub-threshold amounts are allowed to act on the neighbouring cells. Numerous examples of this type have been found in isolated tests

carried out by the author in collaboration with Dr. P. R. Peacock, where lack of response of tissues to potent carcinogens seems to be related to the formation of a capsule in which the carcinogen remains unaltered throughout its stay in the animal body.

It may be concluded that the evidence at present indicates that optimal carcinogenic action is obtained by presenting the carcinogen to the animal body in such a manner that it is supplied continuously to the neighbouring cells until these give rise to a tumour.

TABLE VI.

Duration of Experiment. (days)	No.of mice killed.	Tumour incidence.	Fluorescence at site of injection.	Spectrographic Examination.
123	12	3/12	10/12	Benzpyrene identified in the 10 fluorescent extracts, including tumour mice.
153	3	1/3	2/3	Benzpyrene in 2 fluorescent extracts, including tumour mouse; additional band suggests metabolite in both.
198	2	1/2	2/2	Benzpyrene in tumour mouse plus ? metabolite. ? Metabolite in other mouse.
232	3	2/3	1/3	? Metabolite in tumour mouse with large tumour. General fluorescence only in other tumour mouse.
307	10	0/10	0/10	

NOTE: 1 mouse died after 136 days with a tumour and persistent fluorescence at the site of injection and 1 after 165 days. These mice were not examined histologically or spectrographically. 4 other mice died early in the experiment due to ulceration and sepsis at the site of injection.

EXPERIMENTAL.

Biological Details of the Investigation.

Thirty-six mice were injected subcutaneously in the right flank, with 0.3 c.c. of a 0.1% solution of 3:4-benzpyrene, which had been purified chromatographically, in tricaprylin. The mice were killed after different periods and the subcutaneous tissues exposed and examined for the presence of fluorescent material. When no sign of distinctly fluorescent material was found, the animal was not examined further. After the third month of experiment tumours began to be clinically recognised at the sites of injection, and some of these were examined histologically. The tumours were similar to those generally found after subcutaneous injection of benzpyrene in mice.

Extraction of Fluorescent Material.

In many cases one or more small fluorescent cysts were present, and these were opened and their contents expressed into benzene (2 c.c.). In other cases numerous minute spots of brightly fluorescent material pervading a tumour, or merely a general fluorescence localised in the neighbourhood of the site of injection, were best extracted by trituration of the tissues in benzene with a glass rod, after dissection of the region bearing the fluorescent material from the rest of the animal. Where this method was unsuccessful, the tissue was hydrolysed with 10% aqueous alcoholic potash

(1:1 - H_2O:EtOH mixture), the alcohol removed, and the unsaponifiable fraction extracted with benzene which was then dried over anhydrous sodium sulphate.

The benzene was rectified before use and was free from visible fluorescence. Furthermore, to eliminate the possibility of a false positive result due to traces of benzpyrene adhering to the apparatus used in the experiments, benzene washings of each piece of chemical apparatus or other instruments used, were examined under the ultra-violet lamp to ensure that these were free from fluorescent material. The necessity for such precautions, recently noted by the author (36), arises from the difficulty of eliminating contamination by minute traces of material which are recorded by the very sensitive fluorescence method of detection. It is considered that the precautions employed exclude the possibility of any false positive result.

Fluorescence Spectra.

The spectra of the extracts in benzene were recorded as described in the experimental part of Section II (see p.51), the exposures being varied from 1-60 minutes, depending on the fluorescence intensity of the solution. In long exposures there is often a strong reflection of radiation from the exciting source, and a spectrum of the radiation from the mercury lamp used is shown on Plate III, Fig.7 (Spectrum (a)) to allow comparison with the radiation due to fluores-

cence in the other spectra.

Spectrum (b) is from one of the extracts made at 4 months and is identical with the spectrum of pure benzpyrene in benzene (see Spectrum (f)). All the extracts at this period gave identical spectra.

Spectra (c) and (d) are those of the two fluorescent extracts made at 5 months. Both spectra have the benzpyrene bands at 405–412 mμ, and the fluorescence also extends over the region 426–460 mμ, which coincides with the longer wavelength region of the benzpyrene spectrum, and also with the range of the fluorescence of certain metabolites. The interesting feature of these spectra is that they show an increase in the intensity of the fluorescence in the latter region, compared with the spectrum of pure benzpyrene. These spectra are interpreted as a mixture of the spectra of benzpyrene and another material with a spectrum similar to that of the fluorescent metabolites of benzpyrene.

Spectra (e) and (g) are of extracts from the two mice killed at 6 months. Spectrum (e) shows no sign of the strong bands of benzpyrene at 403–412 mμ, and has only a diffuse band from 430–450 mμ and may correspond to a metabolite of benzpyrene. Spectrum (g) is complex like (c) and (d) and it will be seen that these three spectra would be simulated by a superposition of the spectrum (e) and the spectrum (f) which is that of pure benzpyrene. It is suggested that these complex spectra are to be explained in this way as

mixtures of benzpyrene and one or more of its metabolites. Another spectrum, having only a diffuse band and identical to spectrum (e), was obtained from a mouse killed at 7 months after injection.

Although the experiments were not conducted quantitatively, the limit of sensitivity of the method provides some indication of the quantities of benzpyrene detected in the various animals. It is just possible to detect the fluorescence of benzpyrene in solution in benzene by visual inspection at a concentration of 1μg/100 c.c., and a solution at this concentration will give a recognisable spectrum with an exposure of 60 minutes, using the apparatus described above. Since only 2 c.c. of benzene were used in the extraction process it can be concluded that a positive spectrum of benzpyrene shows that it was present in quantities greater than 0.02μg.

There is no doubt that this limiting value was greatly exceeded in all of the extracts giving a positive spectrum of benzpyrene, for most of the spectra were obtained with short exposures and in all cases the intensity of the spectrum was greater than a control spectrum made under the limiting conditions.

PLATE III.

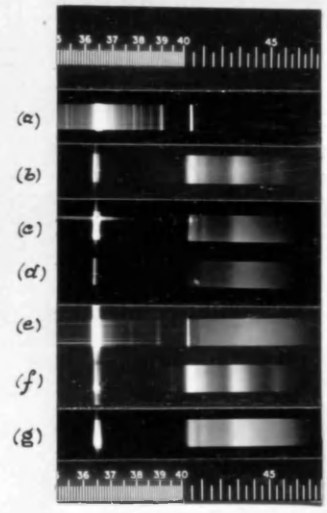


Fig. 7 —— Spectra of extracts from mice treated with 3 : 4 – benzpyrene.

SECTION V.

MODE OF ACTION OF CARCINOGENS:

A Physicochemical Approach.

The main difficulty in an approach to the understanding of the mode of action of carcinogens lies in the lack of knowledge of the vital biological changes which these substances effect and which differentiate neoplastic and normal cells. That these changes involve alterations in the cells themselves is indicated by the ability of cancer cells to retain their proliferative activity and often their identity in the course of serial transplantation and in metastases. Furthermore, after a certain stage in the experimental production of tumours the continued presence of the carcinogen is no longer necessary since permanent changes are inherited by daughter cells. These facts support the view that carcinogens effect some structural alteration and this in turn implies an alteration of cellular protein. Comparative studies (91, 92) of normal and neoplastic tissues leave no doubt of vast differences in protein chemistry, although there is little indication as to the significant alterations.

This point of view suggests that a solution of the problem of the mechanism of chemical carcinogenesis should be sought in a consideration of those properties of the carcinogens which might lead to structural alterations in cellular protein. It is then worthy of note that the effects produced by chemical carcinogens can be simulated by physical agents such as ultra-

violet rays, X-rays and the radiations emitted by radium and other radioactive elements. With these agents sufficient is known of their photolytic and ionising actions to understand how they might accomplish the degradation of some vital cellular component. In the following discussion an attempt is made to show that a similar mechanism is available to chemical carcinogens.

It has been established that many typical chemical carcinogens give rise to electromagnetic radiation in the visible region during certain oxidations. The evidence which supports the view that similar emissions of radiation may occur during the oxidations which the hydrocarbons undergo in the animal body may be summarised as follows: (1) A number of the potent carcinogens give rise to chemiluminescent reactions with several different oxidising agents; (2) there is a partial relationship between the oxidation of 3:4-benzpyrene with the Milas reagents and the *in vivo* oxidation of this substance; (3) there is a striking relationship between the oxidation of Butter Yellow with the Milas reagents and the metabolic reactions which occur with this substance in the liver of the rat; (4) the luminescence effects with the carcinogens were obtained at temperatures encountered in the animal body.

The least that these investigations demonstrate is a property common to the different groups of carcinogenic compounds and it must now be considered whether this property satisfies the concept of the carcinogenic action outlined

above. There is a clear connection with the physical type of carcinogenic agent although the energy involved in the emission of visible radiation is of a different order to that of even the lowest frequency ultra-violet radiation known to have a carcinogenic effect. It must be remembered however that although only visible radiation has been detected in the reactions this does not preclude the possibility of shorter wavelength emissions; in this connection the observations by Audubert (24) of the emission of high frequency radiation from certain chemical reactions may be quoted. For the present argument the chemiluminescence accompanying the oxidations of carcinogens may be assumed to extend over the visible region and possibly into the near ultra-violet. Against the observation that the emissions in the chemiluminescent reactions are of very low intensity must be placed the information that reactions in the animal body are generally of a heterogeneous nature and here one might expect a higher radio-chemical yield than in the corresponding homogeneous reaction, since energised molecules would be less liable to deactivation by collision (93).

Photochemical change induced by radiation of visible and longer wavelength is not unknown, and is exemplified by the photolysis of diazomethane (94) and the rearrangements produced in the nitrobenzaldehydes (95); at a higher molecular level we may note the inactivation of the enzyme urease by radiation from 750 to 1400 mμ (96). The possibility that radiation liberated from a chemiluminescent reaction occurring within a

cell could be readsorbed to produce a chemical change in a molecule of the cell structure was previously suggested by the author (28). However, the facts of optical sensitisation of silver halides (97,98) and their theoretical interpretation provide a useful analogy from which by induction we may construct a more satisfying model.

For practical purposes the sensitivity of silver halides is negligible for wavelengths greater than 500 mμ and for extension into this region they are sensitised with dyes. Thus, with the pentacarbocyanines the photographic spectrum may be extended to beyond 12,000 mμ. Studies of this phenomenon show that the dye molecules are adsorbed and oriented on the surfaces of the halide grains in the photographic emulsion. Incident radiation is then absorbed by the dye molecules which become electronically excited. In solution such an excited molecule would lose its excess energy by a deactivating collision with some other molecule, or by re-emission as fluorescence. However, the coupling forces which exist between the dye molecule and the ions of the halide lattice together with the regular internal structure of the lattice allow the excess energy to be transmitted in the form of an electron or as an exciton. The photolytic process ensues when the excitation-wave is trapped in a lattice defect.

In the case of chemical carcinogens there is strong evidence that during metabolism they are adsorbed to a cell constituent (vide infra), and the experiments described in the

present work indicate that at some stage in this metabolism there is the possibility of the formation of potential emitter molecules. The adsorbed carcinogen can be compared with the sensitising dye adsorbed to the surface of the halide grain. Then, an oxidation product of the carcinogen electronically excited via the energy changes involved in the metabolic reaction, would be comparable to a sensitising dye molecule electronically excited by virtue of absorption of radiation from an external source. The fate of the excitation energy in the photographic model has already been indicated and it is finally manifested in the appearance of the latent image. On the above basis similar possibilities obtain in the cellular model; the excess energy may be emitted as a photon, it may be converted into vibrational energy or it may be transformed into chemical energy and result in the photolysis of some cellular component. The theoretical discussion of Franck and Teller (99) on the migration of excitation energy in crystals emphasises the importance of the coupling between the particles in the crystal and the resonance caused by the identity of the crystal cells, for the transmission of the excitation wave from one crystal cell to another. In a living cell composed of many heterogeneous structures it would be expected that excitation energy created in a specific cellular component would be localised and dissipated within that structure. This conception is in excellent agreement with practical results of Miller and Miller (75) in their study of the formation of

strongly bound dye-protein complexes during carcinogenesis with 4-dimethylaminoazobenzene. Not only did these authors demonstrate a striking correlation between the ability of different tissues to form such complexes and their corresponding biological response, but they also found that the cells of the resulting cancerous tissue were free from bound dyes. Since the cancer cells were derived from cells normally containing bound dyes it would appear that the cellular components involved in the complexes have been altered.

Another interesting practical demonstration of structural alteration arising by a sensitising mechanism is afforded by the degradation of cellulose in fabrics impregnated with certain dye-stuffs. Mott (100) has pointed out that this process is probably to be explained by a mechanism similar to that operating in the phenomenon of optical sensitisation.

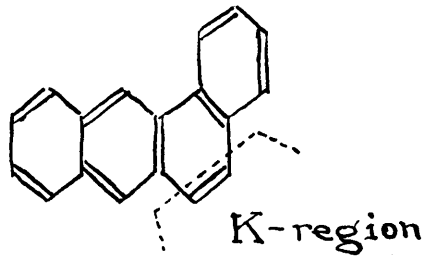
It would appear that sufficient evidence is at hand to justify the conclusion that a mechanism is available to carcinogenic substances whereby they might effect chemical change in another molecule. The efficiency of the process envisaged is unknown, since there are alternative routes for the dissipation of the reaction energy. In this connection the experiments on the elimination of carcinogens from the animal body described in the latter part of the thesis are of interest. They indicate that a prerequisite of the carcinogenic action is a continued insult to the cells by the carcinogen

over a long period, and are at least not opposed to the view that a low efficiency process may be involved.

The striking differences in carcinogenic activity of closely related substances deserve consideration in any views on the mode of action of carcinogens. The ideas so far presented leave this problem unanswered for most of the related non-carcinogenic substances also participate in chemiluminescent reactions. That the question is complex and is concerned not only with the properties of the compounds themselves but also with those of the biological material on which they produce their action, is clear from the profound differences in the response of the cells of different tissues to the same carcinogen. There are certain carcinogens, however, which in many cases have an activity of a higher order than the great majority and the early approaches to the problem of the mode of action of carcinogens (see Introduction), showing similarities in the chemical reactivity of these potent compounds, may have some significance. In this connection the ability of several of the potent carcinogens tested to participate in chemiluminescent reactions with several different oxidising agents is a further point of similarity. The recent work of the Pullmans (see Introduction) shows some correlation between carcinogenic activity of the hydrocarbons and related groups of carcinogens and the electron density of the phenanthrene-type double bond present in most of these substances. The findings have been excellently

reviewed and discussed by Badger (101) who has pointed out numerous discrepancies in the treatment. The significance of the presence of these regions of high electron density has been considered by many workers, and Daudel has constructed a theory of chemical carcinogenesis in which he postulates that molecular alterations may arise in a cellular component in the presence of carcinogens by virtue of interaction of the region of high electron density with a protein molecule. Some attempt has been made to extend the investigations to include the azo-dye (102) and the stilbene (103) groups of carcinogens in a similar theory. Preliminary observations suggest the presence of high electron density regions at the azo and the ethylenic linkages, although the relationship of the values obtained in these cases to those found with the hydrocarbon type of carcinogen is not clear. It may be noted however that profound differences are known to exist in the reactivity of azo and ethylenic bonds (104) and these facts must not be overlooked in postulating interactions of these groups with cellular constituents.

The results of the work of the Pullmans and Daudel are capable of an alternative explanation which is supported by chemical facts. A high concentration of electrons about a specific region in a molecule will confer on that region the property of greater reactivity; thus the K-regions to which the Pullmans refer in the hydrocarbons are highly reactive positions in the molecules.



The careful metabolism studies on the hydrocarbons, showing that hydroxylation occurs at positions other than the K-regions, suggest the formation of hydrocarbon-protein complexes in which the K-regions are directly involved in the linkage (16). This suggestion that the hydrocarbons are absorbed by a cellular component prior to oxidation is much favoured by the observation that, while the mechanism of enzyme catalysis is not fully understood, one feature which is accepted is the formation of an enzyme-substrate complex (105). Furthermore, evidence which shows the ability of hydrocarbons to take part in the type of complex formation envisaged is contained in the studies of Weiss on the complexes formed between the hydrocarbons and quinones (or polynitro compounds) (106), and on the salts of these hydrocarbons with different acids (perchlorate, sulphate and pyrophosphate) (107). In both types of structure there is an electron transfer from the hydrocarbon to the other component of the complex and the formation of a linkage of an ionic nature. Such an electron transfer would originate at the K-region of the hydrocarbon molecule. The suggestion may therefore

be made that the significance of the presence of regions of high electron density in carcinogenic molecules is that it provides them with a means whereby they can form a strong coupling with a cellular constituent. On this basis differences in carcinogenic potency of closely related substances are determined by the ease of formation and stability of such linkages.

The term 'carcinogenic potency' must be interpreted cautiously. Indeed, the relative potency of certain carcinogens varies from one cell type to another (101) and the theoretical approach of the French school is therefore an over-simplification. If the true carcinogen is the energy produced during some metabolic reaction all the factors which influence the amount of this energy liberated during a given time period will contribute in the determination of the carcinogenic potency of a given substance. It will be seen that the precise spatial configuration of the molecule of the carcinogen and of the cell structure and the intimate interactions of these will be the controlling factors in such a process.

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