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THE PRESERVATION, COLOURING AND FLAVOURING OF FOODS

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Azo-dyes and Experimental Liver Tumours

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Introduction

Several hundreds of cancer-producing chemical compounds are now known. By far the largest group, which contains all the very potent carcinogens, consists of compounds, mostly polycyclic aromatic hydrocarbons and simple derivatives of these, which give tumours at the site of application. Malignant tumours, resulting from prolonged contact of the tissues with the chemical compound, may be induced in many different tissues and organs. Repeated application to the skin gives epitheliomas, subcutaneous injection (in solution or in the solid state) gives tumours of connective tissue (sarcomas), and by suitable application cancer may be induced in the lung, mammary gland, liver, kidney, spleen, brain, prostate and uterus, and in bone and in other tissues also. Several different species are susceptible, e.g. rats, mice, guinea-pigs, rabbits, hamsters and fowls. Different species and different organs vary very widely in their response to a particular compound, and different compounds also show considerable variation in their action. These variations are, on the whole, quantitative rather than qualitative. Yet, while many derivatives of 1:2-benzanthracene are equally effective in producing both skin tumours and subcutaneous tumours in mice, some of them give tumours more rapidly by the injection technique. On the other hand, derivatives of 3:4-benzphenanthrene have proved much more effective when applied to the skin (Badger, Cook, Hewett, Kennaway, Kennaway & Martin, 1942). All the evidence is consistent with the view that these polycyclic hydrocarbons act directly on the cells.

A second group of compounds having cancer-producing properties comprises the oestrogenic substances, both natural and artificial. These compounds do not in general produce tumours at the site of application, but their administration by any route to female mice results in a large increase in the incidence of mammary carcinoma. It now

* See notice on p. 306.

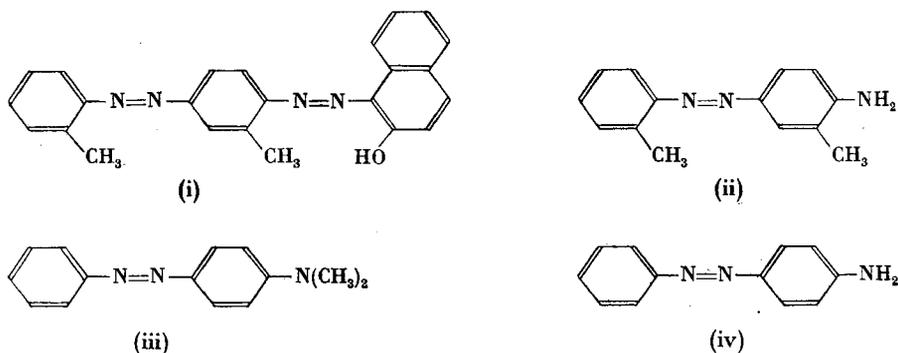
seems to be established that these substances have no intrinsic carcinogenic activity, and that the production of tumours is an indirect result of their stimulation of growth and development of mammary tissue. By administration of oestrogens to guinea-pigs, fibroid growths, probably non-malignant, may be produced in the uterus (Lipschütz, Vargas, Jedlicky & Bellolio, 1940; Lipschütz & Vargas, 1941).

A third group of carcinogenic chemical compounds consists of azo-compounds which have a specific effect in producing liver tumours in rats and mice. It is with this group that the present paper deals. Whatever the route of administration, these compounds do not in general produce tumours at the site of application, but only in the liver. Superficially this action resembles that of the oestrogens, and it might be suggested that the action of the azo-compound on the liver may be one of preparation for the influence of some other factor. On the other hand, the physiological action of oestrogens on mammary tissue has no analogy in any physiological action of azo-compounds on liver, and in the latter case it is more probable that the carcinogenic action is due to the formation of biologically active substances by biochemical transformation in the liver. It is remarkable, however, that, although the carcinogenic polycyclic hydrocarbons will produce liver tumours when applied directly to the liver (but see Dunning, Curtis & Segaloff, 1946), for the most part they do not do so when administered by other routes, in spite of the fact that considerable metabolic conversion must occur in the liver.

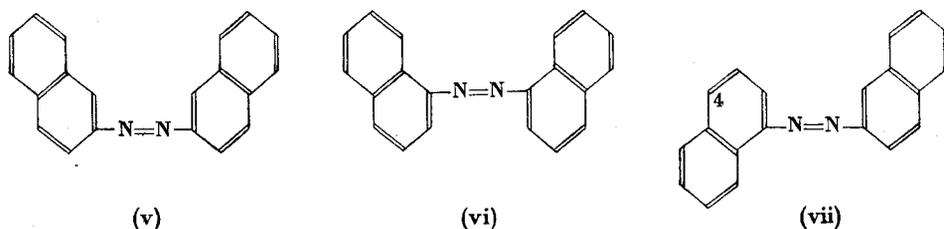
Amino-azotoluenes, Butter Yellow, and related compounds

The first description of the effect of an azo-dye in producing cellular proliferation was published 40 years ago by Fischer (1906). He showed that injection of Scarlet Red (i) into the ears of rabbits led to a proliferation somewhat akin to cancer, but the growths always receded when the treatment was stopped. These observations led to the use of Scarlet Red to accelerate wound healing. It was afterwards found that the active molecular grouping of this dyestuff was contained in the simpler 4'-amino-2:3'-azotoluene (*o*-amino-azotoluene) (ii). The first observation that addition of this latter dye to the food leads to the production of malignant liver tumours in rats was made by Yoshida (1932*a*), who later considerably extended his work (Yoshida, 1932*b*, 1933, 1934; Sasaki & Yoshida, 1935). Even more potent towards rat liver than *o*-amino-azotoluene was *p*-dimethylamino-azobenzene (iii), a dyestuff formerly used as a food colouring matter under the name of Butter Yellow. This was shown by Kinoshita (1937) to produce liver tumours in rats in a shorter time than was required by the isomeric compound (ii). Although Butter Yellow is more carcinogenic in rats than its isomeride, in mice the reverse appears to be the case (Law, 1941; Kirby, 1945*a*). The efficacy of these two compounds in producing liver tumours has been widely confirmed. They are both derived from *p*-amino-azobenzene (iv), which was reported by Sasaki & Yoshida (1935) to be inactive in both rats and mice. This finding has recently been substantiated for mice by Kirby (1945*a*), who obtained no tumours either in the liver or at any other site. In rats, however, Kirby (1944) obtained liver-cell carcinomas after oral administration of *p*-amino-azobenzene. *p*-Monomethylamino-azobenzene is just as active towards rat liver as the dimethylamino compound (Miller & Baumann, 1946).

For reasons not primarily concerned with liver cancer, Cook, Hewett, Kennaway & Kennaway (1940) carried out experiments on the action of azonaphthalenes on mice. The compounds were administered by subcutaneous injection or by addition to the food. Liver tumours, mostly of the type of cholangioma, were obtained in abundance with 2:2'-azonaphthalene (v), and also in a few of the animals which received 1:1'-azonaphthalene (vi). No activity was shown by 1:2'-azonaphthalene (vii) or its 4-amino-derivative.



Many papers have been published on the biological effects of azo-compounds since the pioneer work of Yoshida and of Kinosita. The number of compounds which have shown carcinogenic action is, however, quite small (for summaries see Shear, 1937; Cook & Kennaway, 1938; Rusch, Baumann, Miller & Kline, 1945; Miller & Baumann, 1945*b*; Kirby, 1945*a, b*).

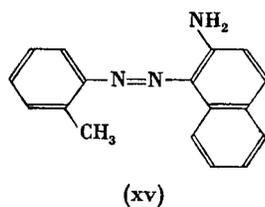
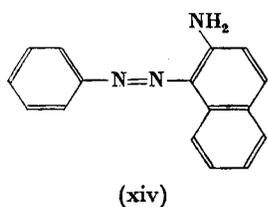
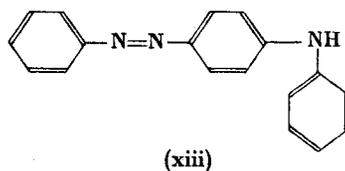
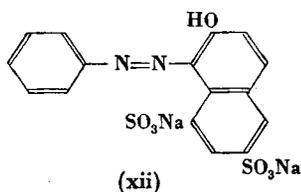
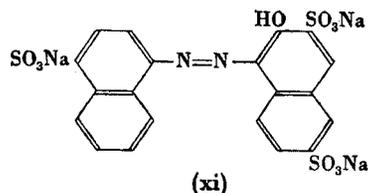
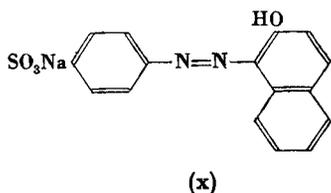
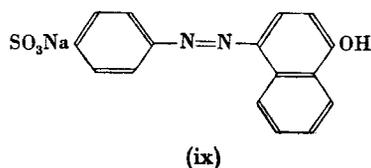
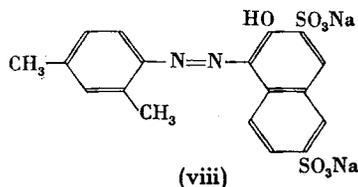


Azo-dyes used as food-colouring matters

Fresh foodstuffs in their natural condition are usually attractive in appearance as well as in taste. With modern methods of processing and preservation the appearance is apt to suffer, so artificial colouring matters are often added. In this respect, the name Butter Yellow which is assigned to *p*-dimethylamino-azobenzene suggests alarming possibilities. Fortunately, however, this azo-dye is probably no longer used as a food-colouring matter, certainly not in Great Britain. Even if it were added to butter, so little is required that it is very unlikely that the amount consumed would lead to any adverse effect on the liver or other organs. In order to obtain liver tumours in mice and rats very considerable total amounts of the dye must be administered. Thus Kirby (1945*b*), in discussing the inactivity of *p*-dimethylamino-azobenzene reported in some strains of mice, refers to positive results in experiments in which each

mouse received subcutaneously a total amount of dye of the order of 200 mg. On a weight for weight basis the corresponding amount for an average man would be in the region of 700 g. Moreover, there is no evidence that this dye can produce liver tumours in man.

Since azo-dyes are used as food colours, it was considered of interest to make biological tests with some of these, and results of experiments by Prof. E. L. Kennaway and myself have been published (Cook *et al.* 1940; Badger *et al.* 1942). The com-

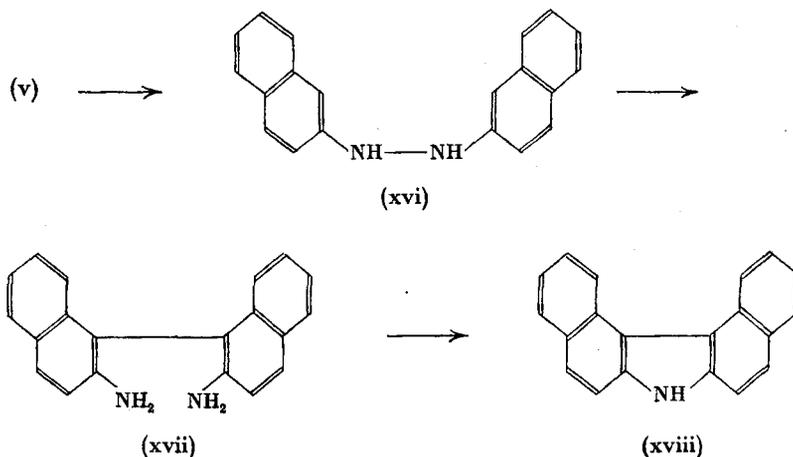


pounds tested have the structures given above (viii-xv). They were chosen for test partly on the ground that they are extensively used, and partly because some of them show structural resemblance to the azo-dyes which produce liver tumours in mice. The last two compounds (Yellow AB, xiv; Yellow OB, xv) are probably not used in Great Britain, but are included in the list of synthetic food colours permitted in the United States. These two were administered by mouth, by subcutaneous injection and by application to the skin. The others were given with the food, so that each mouse consumed weekly about 15-20 mg. of pure colouring matter. The experiments, which were of adequate duration, led to no tumours attributable to the action of the dyes.

Although, personally, I deplore the addition of artificial colouring matters to foods, I do not consider that any real case can be made for prohibiting the use of such colours on the grounds of possible carcinogenic action.

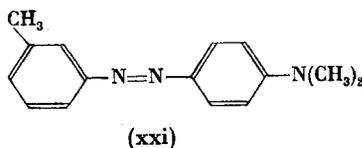
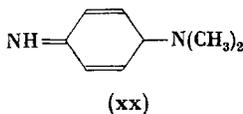
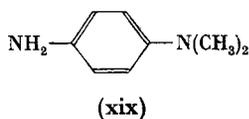
Mechanism of production of liver tumours by azo-compounds

It has been generally assumed that the production of liver tumours by feeding or injecting azo-compounds is due to their transformation in the liver into other compounds which are the true carcinogenic agents. This assumption is largely based on the specific action which these azo-compounds have on the liver. It is true that the production of sarcomas at the site of injection of oily solutions of azo-dyes has been reported by several workers, but this cannot be dissociated from the influence of fatty solvents which are themselves known to produce similar effects. In the case of 2:2'-azonaphthalene, it was suggested (Cook *et al.* 1940) that the true carcinogen, formed in the liver, is 3:4:5:6-dibenzcarbazole, which might be formed *in vivo* by the following stages, which are readily brought about in the laboratory:



In support of this view it was shown that the postulated intermediate diamine (xvii) produced liver tumours of cholangiomatous type rapidly and in abundance when administered to mice. This di-amine is readily de-aminated to 3:4:5:6-dibenzcarbazole, which had previously been shown by Boyland & Brues (1937) to produce liver tumours in mice and also local tumours at the site of application. Further support was given to this view of the mechanism of carcinogenesis by 2:2'-azonaphthalene by some experiments of Elson & Warren (1944), who injected azobenzene into rats and found that it was partly converted into benzidine. In the case of *p*-dimethylamino-azobenzene, benzidine rearrangement of the intermediate hydrazo-compound occurs in the body even more readily. While this hypothesis of a benzidine rearrangement offers an attractive interpretation of the carcinogenic action of 2:2'-azonaphthalene, it is not so obviously applicable to the azo-dyes of the type of *o*-amino-azotoluene and *p*-dimethylamino-azobenzene (see, however, Cook, 1943).

Another view of the mechanism of liver carcinogenesis by azo-dyes has been put forward by Kensler, Dexter & Rhoads (1942), who suggested that the biologically active material in the case of Butter Yellow is *NN*-dimethyl-*p*-phenylenediamine (xix), or a semi-quinone free radical (xx) formed as the first stage of its oxidation. This hypothesis is based on the observation that *p*-dimethylamino-azobenzene undergoes reductive fission in the animal body to give products which have a strong inhibitory action on important enzyme systems. However, *NN*-dimethyl-*p*-phenylenediamine has not itself been isolated from the metabolic products. The azo-dye undergoes demethylation in the body to *p*-monomethylamino-azobenzene and *p*-amino-azobenzene, for which methods of estimation have been devised (Miller & Baumann, 1945*a*). It also undergoes fission at the azo-linkage, so that *p*-phenylenediamine, *p*-aminophenol and their acetylation products have been detected in the urine (Stevenson, Dobriner & Rhoads, 1942). Reductive fission is preceded by demethylation



(Miller, Miller & Baumann, 1945), although it is conceivable that some of the dye also becomes reduced before undergoing demethylation. Kensler, Maghill & Sugiura (1947), who have recently reported further on the metabolism of Butter Yellow, have found that some demethylation occurs when a solution of the dye in cottonseed oil is mixed with ground brown rice. These workers also noted that liver slices destroy both *p*-amino-azobenzene and its *NN*-dimethyl derivative.

It was found by Kensler *et al.* (1942) that certain enzyme systems, namely, the yeast carboxylase-coccarboxylase system and the diphosphopyridine nucleotide enzyme system, were strongly inhibited by *p*-phenylenediamine and *NN*-dimethyl-*p*-phenylenediamine, and it was suggested that the carcinogenic process is associated with enzyme poisoning due to the formation of 'split products' of these types. Kuhn & Beinert (1943) also noted the inhibition of enzymes by *p*-phenylenediamine and its oxidation products. They stated that in the carboxylase test pure *p*-benzoquinone is more active than all the oxidation products of *p*-diamines hitherto examined. They concluded that *p*-benzoquinone is the true carcinogen, and referred to the report of Takizawa (1940) that this compound when applied to the skin of mice produced epitheliomas in a significant proportion of them. Prof. E. L. Kennaway (personal communication) has been unable to confirm this claim.

The weakness of the 'split product' theory of carcinogenesis lies in the fact that neither of the diamines (*p*-phenylenediamine or its *NN*-dimethyl derivative) has been

found to produce liver tumours when administered to experimental animals (see Rusch *et al.* 1945, who give a full discussion). In the course of an examination of a series of methyl derivatives of *p*-amino-azobenzene, Miller & Baumann (1945*b*) made the interesting observation that *m*'-methyl-*p*-dimethylamino-azobenzene (xxi) is even more carcinogenic towards rat liver than *p*-dimethylamino-azobenzene.

These two theories of carcinogenesis by azo-compounds have been discussed by Kirby (1945*b*), who suggested that metabolism of azo-compounds to compounds of benzidine type may favour bile-duct proliferation and hence the formation of cholangioma, whereas 'reductive fission' of the type postulated by Kensler *et al.* (1942) may favour hepatoma formation.

Influence of diet on formation of liver tumours

Perhaps of much greater importance than the induction of liver tumours by azo-compounds is the finding that the formation of such tumours is profoundly influenced by the nature of the diet of the test animals. The basal diet used in the early Japanese work with azo-dyes consisted of rice and carrots, to which was added a solution of the dye in olive oil. Other workers using different diets were unable to obtain liver tumours, and it eventually became apparent that the determining factor is the quality of the diet. With the basal diet of rice and carrots, which is a deficient diet, Kinoshita (1937) obtained tumours in up to 80% of the rats to which *p*-dimethylamino-azobenzene was given. The incidence of tumours was reduced when wheat, rye or millet formed the basal diet (cf. Rusch *et al.* 1945). Addition of beef liver to the rice basal diet afforded almost complete protection against liver damage and liver cancer (Nakahara, Mori & Fujiwara, 1939). Yeast had a similar effect (Ando, 1938). In mice the production of liver tumours by *o*-amino-azotoluene or 2:2'-azonaphthalene was considerably delayed by the addition to the diets of whole dried liver (Rusch *et al.* 1945).

The effect of various members of the vitamin B complex has been evaluated with purified diets, and it seems to be established that riboflavin has a protective action against liver-tumour formation. In fact, protective diets are usually rich in both protein and riboflavin. Increase in liver-tumour production by addition of biotin to the diet has been reported. A protective effect is exerted by a synthetic diet in which hydrogenated coconut oil is the source of fat. The mechanism of this protective action has been the subject of considerable study, but the results are inconclusive.

It is unnecessary to stress the importance of the finding that the cancer-producing action of certain chemical substances may be markedly inhibited by purely dietary means. The manner in which this inhibitory influence is exercised is, however, still unknown. If the effective carcinogenic agent is a metabolite of the azo-compound, then it may be that the rate or the direction of the metabolic changes is modified by variations in the diet. Thus the effect of a protective substance may be to hinder the conversion of the azo-compound into its carcinogenic derivative. On general grounds this is the most likely interpretation of the effect of diet, for if it were due to an influence on the fundamental tumour process one would expect similar sensitivity to

dietary influences to be shown in carcinogenesis by other types of chemical agents such as the polycyclic hydrocarbons. Such sensitivity has not, in fact, been observed (see e.g. Strong & Figge, 1946).

Diet in relation to human cancer

The profound influence of diet on the incidence of liver cancer due to azo-compounds in rats and mice perhaps provides some vindication of the widely held and very rational view that the onset of cancer of the internal organs in man may be influenced by the nature of the diet. Whether this is so in regard to tumours of the alimentary tract must remain an open question. The distribution of primary cancer of the liver in different races and regions of the world does, however, reinforce the view that one of the most important factors in determining this form of cancer is the nature of the diet. The literature is summarized by Rusch *et al.* (1945).

Primary cancer of the liver, although rare among Western races, is very prevalent among Orientals, and in both China and Japan and in the Philippines it takes a high place in the order of frequency of various forms of cancer. It is very suggestive that the common diet in these countries consists largely of rice and is similar to that which favours the production of cancer of the liver in laboratory animals. In South Africa primary cancer of the liver is widespread among the Bantus, although rare in the Europeans. In East Africa also, liver cancer is common among the natives. That these differences are not due to racial differences is indicated by the rareness of liver cancer among American negroes; they are almost certainly related to diet. On general grounds it seems improbable that the diets of the peoples who are unusually subject to primary liver cancer contain some general carcinogenic factor. It is much more likely that the determining factor is that such diets are deficient diets, as seems to be the case with experimental animals.

These studies on liver cancer, both experimental and statistical, suggest that renewed attention should be given to the possible influence of diet in the incidence of all forms of cancer affecting the organs of digestion and elimination.

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The Chemical Preservation, Colouring and Flavouring of Foodstuffs with Special Reference to Fruit and Vegetables

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The addition of substances to foodstuffs for the purpose of preservation, colouring and flavouring is nowadays carried out in accordance with the strict definition and limitations imposed by the Food and Drugs (Adulteration) Act of 1928 and by the Preservatives in Food Regulations of 1927. Much of the objectionable and unsuitable treatment applied in the past was apparently practised as much out of ignorance as with the object of fraud.

The ideal methods of preservation are those effecting sterilization either by heat treatment, as in canning, or by freezing. These methods are not always practicable or economic; moreover, the highly specialized plant required cannot be made available for 'on the spot' preservation of such perishable foodstuffs as fruit. In such cases chemical preservation is of importance in saving valuable foodstuffs. The Food and Drugs Act defines a 'chemical preservative' as a substance added to foodstuffs to hinder or retard undesirable changes, and specifically excludes from restriction those substances which we may class as 'traditional preservatives', e.g. salt, sugar, vinegar, saltpetre, spices, wood smoke, alcohol and hops. Barnard (1911) listed the requirements for a preservative in the form of eight points which are worth repeating: (1) the substance used must not, under reasonable circumstances, injure health; (2) it must not make possible the use of unfit raw material; (3) its use must not make possible the use of careless or imperfect methods of handling; (4) it must be non-poisonous and non-irritant; (5) it must be efficient; (6) it must not retard the action of the digestive enzymes; (7) it must not decompose within the body into substances more toxic than itself; (8) it should lend itself to simple methods of estimation in order to simplify control.