THE PRESENT POSITION OF EXPERIMENTAL INVESTIGATIONS INTO THE AETIOLOGY OF BLADDER CANCER

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On an aetiological basis, human bladder cancers may be subdivided into three classes. Firstly there is the relatively small but well defined industrial group consisting of workpeople who have been engaged in the manufacture, purification and use of certain dyestuffs and intermediates. Case *et al.*[1] found 455 examples of such tumours which arose in the British chemical industry before 1952. It has been estimated that some 2,500 instances of this industrial disease have been reported in the world literature, but an unspecified and possibly considerable number may have escaped reporting. In addition, there are workmen in the industry who have received an exposure to the noxious substances but have not yet developed tumours. The second category of human bladder tumours arise in association with bilharzial infestation, which is believed by many workers to be a causal factor. The third and by far the largest group of human bladder cancers are of unknown origin. Many speculations have been advanced about the manner of their occurrence, with more or less factual backing. For example, they are said to be associated with excessive cigarette smoking, with the incorporation of certain dyes into hair oil, with the abnormal metabolism of tryptophan, etc.

Industrial Bladder Cancer: Six Carcinogens

The industrial tumours, despite their relatively small number, have been the starting point for a great deal of experimental work. Careful observations by many factory medical officers, culminating in the field survey of the British chemical industry by Case, demonstrated that 5 compounds were responsible for the majority of the human bladder tumours in the industry. These were 1- and 2-naphthylamines, benzidine, auramine and magenta. A sixth compound, 4-aminodiphenyl, was shown by Melick *et al.*,[2] in the USA, to be responsible for industrial bladder cancer as well.

Before they could be adequately investigated it was necessary to show that the industrial chemicals could reproduce the human disease in the experimental animal. Before 1937 attempts to do this were unsuccessful, owing to failure to choose the right species, to administer enough of the chemical, and to keep the animals alive long enough for them to develop tumours. In 1938 Hueper *et al.*[3] showed that 2-naphthylamine given to dogs, first by subcutaneous injection and then by feeding, induced tumours of the same nature as those found in the exposed human. The latent period of these lesions was about 2 years. This observation has been repeated on several occasions. Subsequently, other chemicals of a similar nature to 2-naphthylamine (the aromatic amines) have been shown to induce cancer in a variety of tissues (not only bladder tissues) of most of the commonly used experimental species. 2-Acetaminofluorene possessed properties which rendered it specially suitable for use as an insecticide, but, fortunately, before it was allowed into general agricultural use it was tested for long-term toxic effects in the rat. It has since been shown to induce tumours in one or more tissues of most species; it has, in fact, become one of the standard carcinogens used in cancer research. These observations imply that the aromatic amines may induce tumours in tissues other than the bladder in man.

The first point that had to be settled experimentally was whether the industrial amines induced the bladder tumours themselves or whether some impurity in them was responsible. There is no doubt that 2-naphthylamine contains traces of impurities which, from their molecular configuration, might induce cancer. However, whereas Hueper used the commercial chemical in his classic ex-

periments with 2-naphthylamine,[3] Bonser[4] used a partially purified chemical and obtained an essentially similar result. Bonser *et al.*[5] repeated the experiment again, using a sample of 2-naphthylamine that had been purified by one of the most rigorous means available, and again induced bladder cancers with a latent period of from 2 to 5 years. There can thus be little doubt that in the case of 2-naphthylamine, at least, the amine itself and not some impurity in it is responsible for the tumours.

The second question to be decided was whether the amine itself or some product derived from it in the body (a metabolite) is responsible for the observed tumours. The fact that amine tumours in man are found in the bladder and that many cancers in the experimental animal are located along the routes of excretion (i.e. the urinary and gastro-intestinal tracts, the liver, the bile ducts and, in the rat, the acoustic duct) suggests that they are converted into carcinogens by the body. Directly acting carcinogens would be expected to attack the tissues along their routes of entry into the body (such as the skin, alimentary tract or lungs) and only in exceptional circumstances is this behaviour observed with the aromatic amines. An experiment using the technique of bladder implantation confirms the impression that the aromatic amines are not direct carcinogens even to the bladder epithelium of the mouse. In this technique, the material under test is incorporated into an inert vehicle such as paraffin wax or cholesterol and implanted as a small pellet by surgical operation into the lumen of the mouse bladder. The mice are usually allowed to live for 40 weeks after the operation and are then killed and the bladders distended. The method gives cancers of the bladder epithelium with substances known to be directly carcinogenic and negative results with compounds whose carcinogenicity has not been established or is not suspected. When the aromatic amines themselves were tested in this way, a slightly higher incidence of cancers than found with the vehicle alone was usually recorded, but this was far below the level of statistical significance.

It must be concluded, therefore, that the aromatic amines do not act as direct carcinogens and that they require to be converted into the active form by metabolism. There are today two hypotheses regarding the nature of the active metabolites of the aromatic amines. The remainder of this report is devoted to discussing the present available evidence about these hypotheses. It is thought that either the amine must be converted to the ortho hydroxyamine or to an arylhydroxylamine. The structure of these 2-naphthylamine metabolites is shown in Fig. 1.

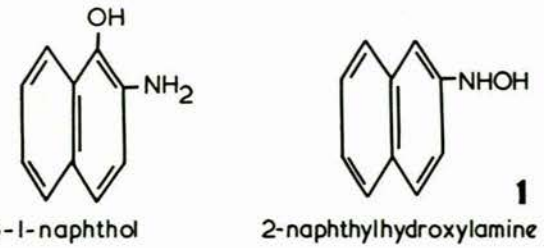

Fig. 1. The structure of 2-amino-1-naphthyl (ortho hydroxyamine) and 2-naphthylhydroxylamine (an arylhydroxylamine).

Ortho Hydroxyamines

The ortho hydroxyamines came into prominence in 1951, when Bonser and her associates[6] demonstrated the following data:

1. That there was an association between the amount of a dose of 2-naphthylamine converted to 2-amino-1-naphthol derivatives by the dog, rat, mouse and rabbit and the carcinogenic activity of the amine to these species. In the dog, 40 - 70% of the amine was converted to these derivatives and bladder cancers were induced in 2 - 5 years. In the mouse the percentage conversion was about 15% and liver tumours only arose after as long as 80 weeks, which is a very considerable proportion of the normal lifespan of this species. In the rat and rabbit, 6 - 9% and 2% respectively of the derivatives were formed and tumour incidence was low. In the rat only benign papillomas of the bladder were found at 2 years, while in the rabbit after 5 years only possibly early precancerous changes were observed in the bladder.

2. This apparent correlation was supported by the observation that when the technique of bladder implantation was used, 2-amino-1-naphthol hydrochloride proved to be carcinogenic to the mouse-bladder epithelium.

3. The third piece of evidence that appeared to support the idea that 2-naphthylamine was carcinogenic by virtue of its conversion to this metabolite was that the derivatives of 2-amino-1-naphthol were greatly concentrated in the urine relative to the plasma (200 : 1). In other words, in the dog which developed bladder tumours, the effective metabolite reached the bladder in far higher concentration by way of the urine than it reached any other tissue by way of the blood stream.

On the basis of these results with 2-naphthylamine and 2-amino-1-naphthol hydrochloride, Clayson[7] in 1953 suggested that other amines might also be carcinogenic because they were converted *in vivo* into ortho hydroxyamines. A considerable volume of work both in Leeds and elsewhere has been devoted to testing this idea. This work has followed three lines: (*a*) the attempted demonstration that ortho hydroxyamines other than 2-amino-1-naphthol were carcinogenic; (*b*) the demonstration that these metabolites were produced from the amines *in vivo* and (*c*) attempts to demonstrate that these derivatives were concentrated at and reacted with the tissues at the site of election of tumours. Some of the results obtained support the idea that the ortho hydroxyamine is important; other evidence does not.

The first important result to arise from the investigation of the ortho hydroxyamine hypothesis was the demonstration that the various ortho hydroxyamine conjugates were not equally effective in the induction of tumours on implantation into the mouse bladder. For example, the sulphate ester (2-amino-1-naphthyl hydrogen sulphate) did not induce a significant yield of tumours when implanted. Boyland *et al.*[8] (1956) thought that this was due to the inability of mammalian sulphatases to split sulphate esters derived from ortho hydroxyamines, an idea that was confirmed experimentally. Boyland suggested that, as the glucuronide conjugate of 2-amino-1-naphthol was split by β-glucuronidase, it was this metabolite rather than the generality of derivatives of the ortho hydroxyamine which

was the intermediate in the formation of the amine-induced tumours.[9] He supported this by showing that 2-amino-1-naphthyl glucuronide, in a limited experiment using small numbers of animals, was carcinogenic on bladder implantation in mice. On testing the derivatives of 4-aminodiphenyl, the ortho hydroxyamine, 3-hydroxy-4-aminodiphenyl, was found to be active, but the amine, and the sulphuric ester of the ortho hydroxyamine were inactive. Similar results had been obtained in testing the derivatives of 2-naphthylamine.[10]

The Ortho-hydroxyamine Hypothesis

In a discussion of this length, it is necessary to pass over a considerable amount of information, some of which supported the ortho-hydroxyamine hypothesis and none of which contradicted it. In the first place, apparently contradictory evidence has been obtained in the case of 1-naphthylamine.[11] This amine has not been found to be a potent carcinogen in the experimental animal. In man the commercial chemical, which contains 5 to 10% of the potently carcinogenic 2-naphthylamine, induced tumours of the bladder if the exposure was sufficiently long. It has also been shown that the ortho hydroxyamine corresponding to 1-naphthylamine, 1-amino-2-naphthol, was carcinogenic on bladder implantation in the mouse. It was anticipated that a study of the metabolism of 1-naphthylamine would show that very small quantities of the derivatives of 1-amino-2-naphthol were produced in the body. It was found, however, that all experimental species metabolized the amine in a similar way and yet no tumours were produced in feeding dogs (or mice) with the amine itself; and that considerable amounts of the derivatives of 1-amino-2-naphthol were formed along with the derivatives of 1-amino-4-naphthol.

These are two ways in which this evidence may be fitted into the ortho-hydroxylation hypothesis. Firstly, it is possible that the carcinogenic aromatic amines are converted to a derivative that is readily degraded to an ortho hydroxyamine, whereas the non-carcinogenic amines do not produce this derivative. The phosphate derivative described in the urine of 2-naphthylamine-treated dogs[12] might be such an intermediate. Secondly, it is possible that the presence of relatively greater amounts of the derivatives of 1-amino-4-naphthol in urines containing 1-amino-2-naphthol derivatives may suppress the formation of the free ortho hydroxyamine.

A second observation which is not expected or explicable on the ortho-hydroxylation hypothesis is that neither of the ortho hydroxyamine derivatives of 2-aminofluorene is carcinogenic on bladder implantation.

Despite the above two observations there is other indirect biochemical evidence that ortho hydroxyamines may play a part in the carcinogenic process. It is now generally accepted that in chemical carcinogenesis it is necessary for the carcinogen or its active metabolite to react with the tissues of the experimental animal. It is considered that combination with proteins is the essential reaction. Gutmann *et al.*[13] showed that chemicals called quinone imines which are obtained by the enzymic oxidation of ortho hydroxyamines do, in fact, react avidly with protein. Similarly, Belman and Troll[14] showed that 2-amino-1-naphthol, the ortho hydroxyamine from 2-naphthylamine, combined by a slightly different reaction with proteins, including those of the isolated rat bladder. The real test of the ortho-hydroxylation hypothesis will come when it is possible to isolate and characterize the tissue carcinogen complexes that are formed in the experimental animal.

The Arylhydroxylamine (N-hydroxy compound) Hypothesis

It is next necessary to consider the alternative hypothesis, i.e. the metabolism of an aromatic amine to an arylhydroxylamine. This has been advanced to account for the carcinogenic activity of the aromatic amines. The Millers and their colleagues[15,16] observed a new urinary metabolite of 2-acetamidofluorene in the rat: N-hydroxy-2-acetylaminofluorene. This compound increased several-fold in amount during the induction of tumours by 2-aminofluorene. It was shown to induce tumours of a similar nature to those induced by 2-acetylaminofluorene, which was not surprising since the acetamide and its N-hydroxy derivative were interconvertible in the rat. The N-hydroxy compound also induced carcinomas of the stomach when fed to the animal and sarcomas of the peritoneum if injected by the peritoneal route. The importance of this metabolite was also indicated by the following experiment: 2-Acetamidofluorene induces many tumours of the liver when fed to the rat by itself; if, however, it is fed conjointly with 20-methylcholanthrene the yield of liver tumours is suppressed. N-hydroxy-2-acetamidofluorene also gives liver tumours when fed alone but the yield is not diminished by feeding 20-methylcholanthrene. Furthermore, 20-methylcholanthrene has been shown to inhibit the increase in output of the N-hydroxy compound when 2-acetamidofluorene is fed. It thus appears that the N-hydroxy compound is of paramount importance in the induction of tumours of the liver of the rat, and the Millers suggested that it might be the 'directly acting' (or 'proximate') carcinogen. It is interesting to observe, however, that Bradley (personal communication) failed to observe any effect of 20-methylcholanthrene on the induction of tumours of the bladder by 2-acetamidofluorene in the strain of rats that he used.

Similar N-hydroxy derivatives have been isolated from the urine of animals dosed with other aromatic amines, and the work performed so far has indicated that there is a reasonably good correlation between the carcinogenic activity of an amine and the production of this type of metabolite. Two questions must be considered: (1) Are the N-hydroxy compounds 'proximate carcinogens' or do they require further conversion to render them active? (2) Are they converted in the animal body into ortho hydroxyamines to a greater extent than are the original amines?

The latter question may be answered in the affirmative if the *in vivo* and *in vitro* reactions of these compounds are similar. The Millers[17] showed that there was a several-fold increase in the output of the ortho hydroxyamines when the N-hydroxy compound was administered to rats instead of 2-acetamidofluorene. On the other hand Boyland[18] has strongly urged that the N-hydroxy compounds react directly with the tissue.

Bonser (personal communication) tried to distinguish experimentally between the ortho-hydroxylation hypothesis

and the N-hydroxylation hypothesis. They tested 2-naphthylhydroxylamine (the N-hydroxy compound derived from 2-naphthylamine) and the phosphate ester of 2-amino-1-naphthol, which it has been suggested is the effective form of the ortho hydroxyamine, 2-amino-1-naphthol. The experiment, which it was hoped would be decisive in deciding between the two hypotheses, indicated that there might well be truth in both. This leads to the conclusion that the mechanism of induction of these tumours may differ from example to example and that each case will have to be considered on its merits.

Experimental Work in Human Bladder Cancer

Finally, it is necessary to consider the implications of the experimental work for the human disease. Allen *et al.*[9] suggested that as the essential amino acid, tryptophan, is metabolized to derivatives of a similar nature to the ortho hydroxyamines it was possible that these metabolites played some part in the induction of the human disease. It is not intended to discuss the evidence for this idea, but it is fair to comment that no convincing evidence has yet been forthcoming to substantiate its validity.

The value of the experimental approach to human bladder cancer is greatest in the industrial field. Thus far, only 6 chemicals have been firmly established as human carcinogens, but two or three score compounds of the same chemical nature have been shown to induce tumours in the experimental animal. Unless great care is taken in introducing new industrial processes it is likely that new human carcinogens will be allowed into the environment. If this occurs it will fall to the clinician to demonstrate the probable industrial origin of the induced tumours and to avoid shrugging off the tumours as merely further spontaneous cancer or allocating them to some entirely erroneous cause.

The experimental work on bladder cancer has so far concentrated on one limited class of chemicals. It is not inconceivable that other types of compound may also induce bladder tumours in man or the experimental animal. In Leeds one of the sulphonamides is being investigated which induces hyperplasia of the urinary-tract epithelium in the mouse and rat. Experiments are being conducted to see whether this chemical will either hasten the appearance of bladder tumours or increase their number in animals receiving a small carcinogenic stimulus. Fortunately this chemical has been detected before being allowed into clinical practice. Other unsuspected chemicals may, however, not be detected. This raises the question: Are there any unsuspected bladder carcinogens in our environment today? It does not seem unreasonable to suggest that some part of the spontaneous disease which is diagnosed today is, in fact, chemically induced and that possibly it could be eliminated by control of our environment. It is the clinician who can answer this speculation.

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