CORRELATION BETWEEN THE CHEMICAL INDUCTION OF HYPERPLASIA AND OF MALIGNANCY IN THE BLADDER EPITHELIUM

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Received for publication December 30, 1964

4-ETHYLSULPHONYLNAPHTHALENE-1-SULPHONAMIDE (HPA) induces hyperplasia of the bladder epithelium in the rat and mouse (Paget, 1958; Sen Gupta, 1962). The hyperplasia arises within forty-eight hours of the first administration of the chemical (Bonser and Clayson, 1964) and with repeated dosing is still present after forty weeks (Sen Gupta, 1962). Carcinomas of the bladder were found in Ab × IF mice receiving the chemical for up to sixty-five weeks and were more prevalent in female than in male mice (Bonser and Clayson, 1964).

The discovery of the carcinogenic action of HPA on mouse bladder epithelium was unusual in so far as the tests were undertaken following observation of the induction of hyperplasia. Other bladder carcinogens were found, more or less by chance, as a result of their action over long periods in man or experimental animals and their early effects have largely been ignored. The present report is an attempt to remedy this omission.

Relatively few chemicals are known to induce bladder cancer in the experimental animal (Boyland, 1963). In some cases the absence of tumours may have been due to a failure to examine this organ with sufficient care. Therefore some hitherto unpublished long term tests in which the bladder has been adequately examined, are included in this paper in order to strengthen the comparison between the early effects of a chemical and the formation of tumours.

MATERIALS AND METHODS

Mice of strains CBA and IF, and C57 × IF and Ab × IF F1 hybrids were bred in the laboratory. Stock albino mice were obtained from a dealer. Stock albino rats were from the Sheffield colony, Slonaker rats from the Cancer Research Department of the University of Nottingham and Sprague Dawley rats from a dealer.

Mice, rats and hamsters were fed on a pelleted diet (Oxo 41B) and water *ad libitum*. Dogs received one of the commercially available tinned dog foods, hound meal and water.

Wherever possible, chemicals were obtained commercially. Others were prepared in the laboratory by standard methods. Dr. A. L. Walpole is thanked for a gift of 4′-fluoro-4-aminodiphenyl, Dr. B. Silvestrini for a sample of oxolamine (3-phenyl-5β-diethylaminoethyl-1 : 2 : 4-oxadiazole) and Dr. C. Hackman

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for gifts of 2-aminodiphenylene oxide (2-aminodibenzofuran) and 3-methoxy-2-aminodiphenylene oxide (3-methoxy-2-aminodibenzofuran). Details of the administration of the chemicals in the carcinogenicity tests are given in Table I. Treatment was commenced when the mice were about ten weeks old.

In short term tests chemicals were usually administered by stomach tube under light ether anaesthesia six times weekly. Details of solvents and dosage are given in Tables V to IX. In calculating the amount of a chemical consumed in the diet, it was assumed that a mouse eats approximately 4 g. daily (Tannenbaum, 1942).

The preparation of bladder tissues for microscopical examination has been described by Sen Gupta (1962). One section from each half of the bisected bladder of each mouse, rat or hamster was examined for tumours or for hyperplasia.

RESULTS

Carcinogenicity tests

The chemicals used in the carcinogenicity tests differed widely in their toxicity and this dictated the variation in the total dose administered (Table I). 1-Phenylazo-2-naphthol was well tolerated at a level of 0·1 per cent in the diet by

TABLE I.—*Method of Administration and Amount of Chemical given in Long-term Experiments*

Compound	Type of mouse	Route of administration	Concentration	Duration (months)	Estimated total dose (mg.)
4-Acetamido-stilbene	Ab × IF	Diet	0·03 per cent (intermittently)	12	330
4-Aminodi-phenyl	Ab × IF	Stomach tube	0·2 ml. of 0·25 per cent solution in arachis oil. (2 × weekly)	9	38
4-Aminostil-bene	Ab × IF	Diet	0·03 per cent (intermittently)	18	440
4′-Fluoro-4-aminodi-phenyl	CBA	Stomach tube	0·05 ml. of 1 per cent in arachis oil (2 × weekly)	6	13
1-Phenylazo-2-naphthol	CBA stock	Diet	0·1 per cent	12	1400

both stock and CBA mice. 4-Aminodiphenyl and 4′-fluoro-4-aminodiphenyl were given by stomach tube because the volatility of the former chemical made it hazardous to include in the diet and the latter was available only in limited amounts. It was found possible to give 0·5 mg. of 4-aminodiphenyl twice weekly for 9 months, but 4′-fluoro-4-aminodiphenyl could only be given in half this quantity for 6 months.

Ab × IF mice tolerated 0·03 per cent of 4-aminostilbene and 4-acetamido-stilbene in the diet for about 20 weeks. The mice then developed paralysis of the hind legs and became progressively more emaciated. Partial recovery occurred when the animals were fed on the basal diet for one month and thereafter the chemical and basal diets were alternated weekly. The mice retained their emaciated appearance and often developed corneal opacities. It was suspected that the symptoms might be the result of peripheral neuritis, but histological examination failed to confirm this.

Morphological changes in the bladder epithelium

Bladders were examined histologically in mice surviving for 50 or more weeks in the following instances :— Ab × IF controls (38 mice) ; 4-aminodiphenyl (15 mice) ; 4-aminostilbene (23 mice) ; 4-acetamidostilbene (32 mice) ; 4′-fluoro-4-aminodiphenyl (20 mice), and 1-phenylazo-2-naphthol (28 mice).

Hyperplastic bladder epithelium, considered to be unrelated to the treatment, was occasionally observed in untreated Ab × IF mice and in those fed 4-aminostilbene or 4-acetamidostilbene. With 4-aminodiphenyl one male Ab × IF mouse at 80 weeks and four at 98 weeks had hyperplastic bladder epithelium. A male mouse at 96 weeks appeared on naked eye examination to have a tumour of the bladder and a female mouse at 97 weeks and a male at 98 weeks had Grade II carcinomas (i.e. the tumours were invading the muscular wall). The induction of two confirmed carcinomas of the bladder in 12 mice surviving to 90 weeks is suggestive, but not conclusive evidence of carcinogenic activity.

Tumours of the liver

Livers of Ab × IF mice developed hepatomas without treatment (Table II). The incidence was 40 per cent in males and 14 per cent in females and was not significantly affected by administration of 4-aminodiphenyl. 4-Aminostilbene and 4-acetamidostilbene, however, depressed the incidence of hepatomas in males to 9 and 6 per cent, respectively, and in females to zero.

The untreated CBA mice include those described by Williams and Bonser (1962). The additional 15 male and 18 female mice (Table III) have not appreciably altered the overall incidence of hepatomas. Feeding 1-phenylazo-2-naphthol in the diet did not materially affect the incidence of hepatomas, but the limited dose of 4′-fluoro-4-aminodiphenyl (13 mg.) raised the incidence from 12 to 49 per cent in males and from 4 to 63 per cent in females. In 3 of the male mice cholangiomas as well as hepatomas were present. Centrilobular necrosis was found in 4 of 6 female mice killed between 16 and 30 weeks and in another at 83 weeks.

4′-Fluoro-4-aminodiphenyl was also administered to 50 male and 31 female IF mice. Only 13 males and 3 females survived to between 50 and 80 weeks. The 3 hepatomas observed (one in a male at 62 weeks and two in females at 54 weeks) are probably significant because no hepatomas were seen at post mortem in 63 male and 133 female breeding IF mice surviving from 50 to 111 weeks. From the above data 4′-fluoro-4-aminodiphenyl is judged to be carcinogenic in CBA and IF mice.

The untreated stock mice are those described by Clayson and Ashton (1963). The addition of 1-phenylazo-2-naphthol to the diet slightly depressed the incidence of hepatomas in the males.

Intestinal tumours

The ileo-caecal junction was examined histologically in the majority of mice as tumours were found at this site in earlier work (Bonser, Clayson and Jull, 1956). One benign caecal papilloma was found in an Ab × IF mouse which had been treated with 4-aminostilbene for 104 weeks.

The untreated stock mice developed caecal adenomas, usually associated with cysts, in 7 out of 49 instances and one male mouse had a carcinoma in this region.

TABLE II.—*The Incidence of Hepatomas in Ab × IF Mice With and Without Chemical Treatment*

Chemical	Sex	Number of hepatomas in mice dying within stated period of weeks after birth (controls) or start of treatment								Total	Incidence (per cent)	Number of livers	
		50–59	60–69	70–79	80–89	90–99	100–109	110–119	120–129			Examined micro-scopically	Not examined micro-scopically
None	M.	0/1	0/1	—	3/8	2/5	1/3	5/11	1/1	12/30	40	27	3
	F.	—	—	—	—	0/1	0/1	3/17	1/9	4/28	14	26	2
4-Aminodiphenyl	M.	0/1	0/1	0/2	0/2	7/11*	—	—	—	7/17	41	16	1
	F.	—	0/1	0/1	—	0/3	—	—	—	0/5	0	5	0
4-Aminostilbene	M.	0/5	0/2	0/2	0/2	2/11	—	—	—	2/22	9	17	5
	F.	0/11	0/1	0/2	0/3	—	0/2	—	—	0/19	0	18	1
4-Acetamidostilbene	M.	0/3	0/2	0/2	0/8	1/3	1/16	—	—	2/34	6	31	3
	F.	0/4	—	—	0/3	0/2	0/4	—	—	0/13	0	8	5

* Three bladder tumours were observed in these mice.

TABLE III.—*The Incidence of Hepatomas in CBA Mice With and Without Chemical Treatment*

Chemicals	Sex	Number of hepatomas in mice dying within stated periods of weeks after birth (controls) or start of treatment								Total	Incidence (per cent)
		50–59	60–69	70–79	80–89	90–99	100–109	110–119	120+		
None	M.	—	0/2	0/3	1/10	0/4	2/8	3/21	0/2	6/50	12
	F.	0/1	0/2	0/3	1/18	1/10	0/13	1/20	0/6	3/73	4
4-Amino-4′-fluorodiphenyl	M.	0/5	1/3	5/8	4/6	0/1	8/14	—	—	18/37	49
	F.	1/2	1/4	2/4	6/6	6/9	9*/14	0/1	—	25/40	63
1-Phenylazo-2-naphthol	M.	0/1	0/4	0/3	1/5	0/1	0/4	—	—	1/18	5
	F.	0/5	0/1	0/2	0/4	0/8	0/6	—	—	0/26	0

* Three mice had both cholangioma and hepatoma.

Stock mice treated with 1-phenylazo-2-naphthol developed adenomas, often associated with cysts, in 6 out of 23 cases.

Tumours of other tissues

These were apparently unrelated to the treatment. Four untreated female Ab × IF mice had breast carcinomas, one had a probable sarcoma and one a leiomyosarcoma of the cervix, one a myoma and one a haemangiomatous polyp of the uterus, 4 had cystic ovaries and one a granulosa cell tumour. In the male there were two certain and one probable sarcomas (of the epididymis, the shoulder and the back). There were also two papillomas of the stomach and two instances of leukaemia, one of each being in a female and the other in a male. As would be expected with an A strain parent, lung adenomas were frequent.

Similar tumours were observed distributed at random in the chemically treated Ab × IF mice. The only features of interest were one renal carcinoma in a male 90 weeks after the start of treatment with 4-acetamidostilbene and four granulosa cell tumours of the ovary 89 to 104 weeks after the start of treatment with 4-aminostilbene.

CBA mice were relatively free from spontaneous tumours other than of the liver. Both the control and the chemically treated groups developed a low incidence of granulosa cell tumours of the ovary. There were two reticulum cell tumours of the ovary in the group treated with 1-phenylazo-2-naphthol and a probable similar tumour with 4'-fluoro-4-aminodiphenyl. Five mice receiving 1-phenylazo-2-naphthol had leukaemia.

The female stock mice used developed mammary carcinomas, and ovarian cysts and tumours, without chemical treatment.

TABLE IV.—*The Incidence of Hepatomas in Stock Mice With and Without Chemical Treatment*

Compound	Sex	Number of hepatomas in mice dying within stated period of weeks after birth (controls) or start of treatment					Total	Incidence (per cent)
		50–59	60–69	70–79	80–89	90–99		
None	M	—	1/6	3/8	0/4	—	4/18	22
	F.	0/1	0/3	0/11	0/14	0/2	0/31	0
1-Phenylazo-2-naphthol	M.	0/3	0/2	1/4	0/7	—	1/16	6
	F.	—	0/2	0/6	0*/11	—	0/19	0

* One mouse with reticulum cell sarcoma.

Short Term Tests

The results of the short term tests are summarised in Tables V to IX. In considering these results it was decided to use a numerical formulation in order to assess the fine differences which occur in individual bladders. Five points were scored for a hyperplastic bladder epithelium in which there were foci, large areas or the whole of the epithelium composed of six to eight cell layers ; three points were scored for a mildly hyperplastic bladder epithelium with three to five cell layers, and one point for doubtful hyperplasia, that is to say for a bladder epithelium which was not normal but had not progressed to the mildly hyperplastic stage.

The " Hyperplastic Index " was defined as $20N/n$ where N is the total number of points scored in a group of animals and n is the number of animals in the group. This index can have a range of values from 0 to 100 and as a result of our experience we suggest that when a section from each half of the bisected bladder is examined, an index of more than 50 indicates that the administered chemical induces hyperplasia, an index of under 30 indicates inactivity, while the interim between 30 and 50 represents an indecisive result. The latter category was infrequent.

4-Ethylsulphonylnaphthalene-1-sulphonamide (HPA) and 2-acetamidofluorene are the only unequivocal bladder carcinogens in the mouse (Armstrong and Bonser, 1947 ; Bonser and Clayson, 1964). Both compounds have high hyperplastic indices whether given by stomach tube or fed in the diet (Table V). 4-Aminodiphenyl has been shown to induce two bladder carcinomas in 12 surviving mice after a long latent period and is thus probably a bladder carcinogen in this species. It has hyperplastic activity when given by stomach tube but not when incorporated in the diet.

Large numbers of chemicals have been tested for carcinogenic acitivity in the mouse and cancer of the bladder has not been reported. In order to obviate the possibility of the inclusion of bladder carcinogens among compounds thought not to induce cancer of the bladder, it was decided to use only those compounds tested in Leeds where the bladders were examined to establish the absence of cancer. 2-Naphthylamine (Bonser, Clayson, Jull and Pyrah, 1956), benzidine, 1-methoxy-2-naphthylamine (Bonser, Clayson and Jull, 1956), auramine (Williams and Bonser, 1962), 1-naphthylamine (Clayson and Ashton, 1963), 4-aminostilbene (this paper) and *o*-aminoazotoluene (Bonser and Clayson, unpublished results) are not carcinogenic to the bladder and with the exception of *o*-aminoazotoluene have low hyperplastic indices (Table VI). The latter chemical had an index of 42 when given at a level of 1 mg. daily. The toxic nature of this treatment led to the death of all the animals by 6 weeks. Administration of the chemical on alternate days reduced the hyperplastic index to 31, a value indicating that *o*-aminoazotoluene is probably not without some degree of hyperplastic activity in the bladder epithelium. When the chemical was given in the diet rather than by stomach tube much less hyperplastic activity was exhibited, a result which parallels that obtained with 4-aminodiphenyl (Table V).

It was next decided to investigate a group of chemicals of which the carcinogenic activity in the mouse bladder is not known (Table VII). The first three of these induce cancer of the bladder in the rat. 2-Aminodiphenylene oxide is a weak and 3-methoxy-2-aminodiphenylene oxide a more potent bladder carcinogen in the rat (Hackmann, 1959). The former has a high hyperplastic index in the C57 × IF mouse and the latter an equivocal index. Di-*n*-butylnitrosamine induced tumours of the bladder in six of sixteen rats (Druckrey, Preussmann, Schmähl and Müller, 1962). It induces hyperplasia in the mouse. Oxolamine (3-phenyl-5β-diethylaminoethyl-1 : 2 : 4-oxadiazole) induced hyperplasia of the bladder when given in high doses to the dog and rat but not to the mouse (Silvestrini, Bignami, Garan and Pozzatti, 1963). It has also been claimed to induce bladder tumours in the two former species (Barron, 1963).* In the present experi-

* One of us (G. M. B.) has seen Dr. Barron's material and is unable to agree that he has obtained frank carcinomas of the bladder by the administration of oxolamine. The lesions in the dog and the rat appear to be precancerous and might have progressed to frank carcinoma *if* the animals had been permitted to live for more than 12 months.

TABLE V.—*Incidence of Hyperplasia of the Bladder Epithelium in Male Mice Treated for Short Periods with Bladder Carcinogens*

Compound	Vehicle	Dose (mg./day)	Type of mouse	Hyperplasia in mice killed after stated period of weeks*									Hyperplastic Index
				1	2	3	4	5	6	8	9	11	
4-Ethylsulphonyl-naphthalene-1-sulphonamide	Arachis oil	1	C57 × IF	1/1	$\frac{1M+1}{3}$	—	1/1	—	2/2	—	—	—	80
	Diet (0·01 per cent)	0·4	Ab	—	2/3	$\frac{2M+1}{3}$	2/3	—	1/1	—	—	1/1	75
2-Acetamido-fluorene	Diet (0·03 per cent)	1·2	C57 × IF	0/2	$\frac{1M+1}{2}$	—	2/2	—	1*M*/2	—	2/2	2/2	68
	Arachis oil	1	C57 × IF	1*D*/2	$\frac{1D+1}{2}$	—	2/2	—	$\frac{1D+1}{2}$	—	—	—	58
4-Aminodiphenyl	Arachis oil	0·5	C57 × IF	—	2/2	—	1/1	—	—	—	—	—	100
	Arachis oil	0·5	C57 × IF	1/3	$\frac{2M+1}{3}$	—	3*M*/3	—	3/3	—	—	—	67
	Diet (0·01 per cent)	0·4	C57 × IF	—	—	—	—	—	0/3	0/3	—	—	0
	Diet† (0·015 per cent)	0·6	C57 × IF	—	—	—	$\frac{1M}{4}$	—	$\frac{1D}{5}$	—	—	—	9

M = mild hyperplasia.
D = doubtful hyperplasia.

* Numerator = number of mice with hyperplasia. Denominator = number of mice.

† The chemical-containing diet was administered as a porridge.

TABLE VI.—*Incidence of Hyperplasia of the Bladder Epithelium in Mice Treated for Short Periods with Chemicals Not Known to Induce Bladder Cancer in the Species*

Compound	Vehicle	Dose (mg./day)	Type of mouse	Sex	Hyperplasia in mice killed after stated period of weeks* 1	2	4	6	7	8	9	Hyperplastic Index
4-Aminostilbene	Arachis oil	1	C57×IF	M.	—	$\frac{1D}{2}$	0/2	0/1	—	—	—	4
	,,	1	C57×IF	M.	0/3	$\frac{2M}{3}$	0/3	0/3	—	—	—	10
Benzidine	Arachis oil	1	C57×IF	M.	0/2	$\frac{2D}{2}$	0/2	0/2	—	—	—	5
	,,	1	C57×IF	M.	0/3	0/3	0/3	0/3	—	—	—	0
2-Naphthylamine	Arachis oil	1	C57×IF	M.	0/2	0/2	1*M*/2	0/2	—	—	—	8
	,,	1	Ab×IF	M.+F.	—	0/2	1*D*/4	1*D*/2	—	—	—	5
	,,	1	C57×IF	M.	1/5	—	1*M*/3	0/3	—	—	—	15
1-Naphthylamine	Arachis oil	1	C57×IF	M.	0/3	0/3	0/3	0/2	—	—	—	0
o-Aminoazotoluene	Arachis oil	1	C57×IF	M.	1*M*/2	0/2	$\frac{2+2M\dagger}{5}$	—	—	—	—	42
	,,	1‡	C57+IF	M.	—	—	—	—	—	$\frac{2M+1}{7}$	—	31
	In diet (0·1 per cent)	4	C57×IF	M.	—	—	—	—	1*M*/3	—	—	20
		4	Ab	M.	—	—	—	—	1*M*/7	—	—	8
Auramine	Arachis oil	1	C57×IF	M.	—	2*M*/3	1*M*/3	0/3	—	—	0/3	15
1-Methoxy-2-naphthylamine	Arachis oil	1	C57×IF	M.	—	0/3	0/4	0/2	—	$\frac{1D+1M}{4}$	—	6

Key. M = Mild hyperplasia. D = doubtful hyperplasia.

* Numerator = number of mice with hyperplasia. Denominator = number of mice.

† Chemical toxic, no survivors.

‡ Alternate days.

TABLE VII.—*Incidence of Hyperplasia of the Bladder Epithelium in Male C57 × IF Mice Treated with Various Chemicals*

Compound	Vehicle	Dose (mg./day)	Hyperplasia in mice killed at stated period of weeks* 1	2	3	4	5	6	7	8	Hyperplastic Index
2-Aminodiphenylene oxide	Arachis oil	0·1	0/1	—	4/4	—	—	—	—	—	80
	,,	0·1	$\frac{1D+1M}{3}$	$\frac{3M}{3}$	—	$\frac{1M+2}{3}$	—	3/3	—	—	69
3-Methoxy-2-aminodiphenylene oxide	Arachis oil	1	—	—	2D/4	—	—	—	—	—	10
	,,	1	0/2	$\frac{1M}{2}$	—	3/3	$\frac{1M}{3}$	—	—	—	42
Di-*n*-butylnitrosamine	Arachis oil	1	$\frac{2M+1}{3}$	1/1	$\frac{1M}{2}$	—	—	$\frac{2M}{2}$	—	$\frac{1M+1}{2}$	66
Oxolamine	Arachis oil	10†	—	0/2	—	0/4	—	—	—	—	0
	Water	8	$\frac{1D}{2}$	0/2	—	0/3	—	0/3	—	—	2
5-Chloro-*o*-toluidine	Arachis oil	1	$\frac{1M}{2}$	0/3	—	$\frac{1M}{2}$	—	0/3	—	$\frac{1D}{3}$	12
4-Dimethylaminoazobenzene	Arachis oil	1	$\frac{1M}{3}$	0/3	—	0/3	—	$\frac{1M}{3}$	—	—	10
3-Hydroxyanthranilic acid	Aqueous teepol	2	—	0/3	—	0/2	0/2	—	—	—	0
Teepol	Water	—	—	0/3	—	0/3	—	1/3	$\frac{1M}{3}$	—	13
20-Methylcholanthrene	Arachis oil	1	0/3	0/3	—	0/3	—	$\frac{1D}{3}$	—	—	2

Key. M = mild hyperplasia.
D = doubtful hyperplasia.

* Numerator = number of mice with hyperplasia.
Denominator = number of mice.
† = female mice.

ments the absence of any hyperplastic effect in the urinary tract epithelium of the mouse has been confirmed.

5-Chloro-*o*-toluidine is responsible for inflammation and haematuria among workmen handling it in the chemical industry. It did not show a hyperplastic action in the mouse bladder when fed by stomach tube at a level of 1 mg. per day. 4-Dimethylaminoazobenzene is a weak hepatocarcinogen but has not been reported to induce bladder cancer in the mouse, although tumours of this site are evoked in the dog (Nelson and Woodard, 1953). It is without short term action on the mouse bladder. 3-Hydroxyanthranilic acid has been shown to induce carcinomas of the bladder epithelium when implanted into the lumen in cholesterol pellets (Allen, Boyland, Dukes, Horning and Watson, 1957; Clayson, Jull and Bonser, 1958; Bryan, Brown and Price, 1964). It is without hyperplastic activity when given systemically to the mouse. 20-Methylcholanthrene is not known to induce bladder cancer following systemic administration and has no hyperplastic action.

Finally it was decided to examine the reaction of species other than the mouse. The rat, hamster and dog have responded to the administration of chemicals by developing tumours of the bladder.

A group of Sheffield rats treated with 2-acetamidofluorene for 12 days failed to show any hyperplastic activity (Table VIII). This accords with the failure of 2-acetamidofluorene to induce bladder cancer in this colony (Bielschowsky, 1944). The use of Slonaker rats of which the bladders are susceptible to the chemical (Wilson, DeEds and Cox, 1941) led to a hyperplastic index of 72. No hyperplasia was observed in the bladders of rats killed up to 4 weeks (index = 0) but all of those killed at 5 and 8 weeks had mild hyperplasia (index=60). *o*-Amino-azotoluene produced a hyperplastic index of 53 in Sprague-Dawley rats. Bladder cancer was reported in rats treated with this chemical by Yoshida (1935) but his observation has not been confirmed in other laboratories.

The hamster (Table IX) develops bladder tumours under the influence of *o*-aminoazotoluene (Tomatis, Della Porta and Shubik, 1961), but not under that of 2-acetamidofluorene (Della Porta, Shubik and Scortecci, 1959). Hyperplasia was induced by both chemicals in every animal tested. On the other hand, 4-ethylsulphonylnaphthalene-1-sulphonamide (HPA) of which the carcinogenicity to this species is not known, did not induce as marked hyperplasia as either of the foregoing chemicals.

2-Naphthylamine is a potent bladder carcinogen in the dog (Bonser, Clayson, Jull and Pyrah, 1956). When the chemical (400 mg. daily) was administered to a dog for 2 weeks, extensive epithelial hyperplasia of the bladder was induced. In a second dog treated for 4 weeks there was mild patchy hyperplasia. Benzidine is at the most only weakly carcinogenic to the bladder of the dog (Bonser, 1962, 1963). When this chemical was fed to the dog (300 mg. daily) for 2 or 4 weeks, no hyperplasia of the bladder epithelium was observed.

There were changes in the ureters and kidneys of these dogs. The dog treated with 2-naphthylamine for 2 weeks had a unilateral hydroureter with a mild ureteritis but without any accompanying hyperplasia. At the point where this ureter passed through the bladder wall there was hyperplasia up to eight cell layers thick. The other 2-naphthylamine-treated animal showed mild inflammation at the top end of the ureter. The dog treated for 2 weeks with benzidine showed unilateral hydronephrosis and epithelial hyperplasia in the lower third of the

TABLE VIII.—*Incidence of Hyperplasia of the Bladder Epithelium in Rats Treated with Chemicals for Short Periods*

Compound	Method of administration	Dose	Type of rat	Sex	Hyperplasia in rats killed after stated period of weeks 1	2	4	5	8	Hyperplastic index
2-Acetamido-fluorene	Stomach tube	7 mg./day	Sheffield	M.	—	0*/12	—	—	—	0*
2-Acetamido-fluorene	Stomach tube	3 mg./day	Slonaker	M+F.	0/2	0/1	0/2	$\frac{2M}{2}$	$\frac{2M}{2}$	27
o-Aminoazo-toluene	Diet	0·1 per cent	Sprague-Dawley	M.	—	—	—	$\frac{2+1M}{3}$	$\frac{1M}{3}$	53

* These rats had bladder pouches and the degree of hyperplasia did not differ from that in controls.

TABLE IX.—*Incidence of Hyperplasia of the Bladder Epithelium in Hamsters treated with various Chemicals for Short Periods*

Compound	Method of administration	Duration (weeks)	Dose (per cent)	Sex	Degree of hyperplasia	Hyperplastic index	Carcinogenic activity to bladder epithelium
HPA	Diet	10	0·01	M.	Mild hyperplasia (2/3)	40	Not known
o-Aminoazo-toluene	Diet	6	0·1	M.	Marked in 2, mild in 1	87	Positive
2-Acetamido-fluorene	Diet	6	0·05	F.	Marked in 2, mild in 2	80	Negative

ureter. The epithelium at the ureteric orifice was not hyperplastic. These changes in the kidney and ureter are thought to be due to the chemical treatment.

DISCUSSION

Of five chemicals tested for carcinogenic activity in the mouse, 4-aminodiphenyl induced carcinomas of the **bladder** in low yield and with a long latent period. The total amount of chemical administered was small because of its toxicity, and in the hope of obtaining a more substantial result the experiment is being repeated in a different strain of mouse. The only other carcinogen to be demonstrated was 4′-fluoro-4-aminodiphenyl which induced **hepatomas** in CBA and in IF mice.

The failure of 1-phenylazo-2-naphthol to augment the incidence of hepatomas in CBA or stock mice was disappointing as Kirby and Peacock (1949) found that this chemical induced liver cancer in their mice. Two possibilities may explain the discrepancy. The experimental conditions were different, as Kirby and Peacock injected the chemical into their stock mice whereas in the Leeds experiment it was fed to another type of stock mouse or to CBA mice. Alternatively, Kirby and Peacock's control group may have been inadequate as the chemically treated mice developed hepatomas when they were 18 to 21 months old (15 to 18 months after the start of treatment) whereas the controls were stated to be " more than 14 months old ".

4-Aminostilbene and 4-acetamidostilbene depressed the incidence of hepatomas in Ab × IF mice. Both chemicals were toxic at the dose given and the animals

became emaciated, had hindleg paralysis and later in the experiment developed corneal opacities. Tannenbaum and Silverstone (1949) showed that both underfeeding and caloric restriction, which led to reduced body weight, depressed the incidence of spontaneous hepatomas in male C3H mice and it is probable that the diminution in the yield of spontaneous hepatomas in mice treated with the stilbene derivatives is also associated with reduced body weight.

The carcinogenicity tests extend the number of chemicals for which there is reasonable certainty of failure to induce bladder cancer in the mouse. They and other compounds tested for carcinogenic activity, mainly in Leeds, form the basis of the investigation into whether or not, after the systemic administration of a chemical, the induction of early hyperplasia and the ultimate development of carcinomas of the bladder epithelium are related. Despite the paucity of available bladder carcinogens the results in the mouse, rat, hamster and dog support such a correlation. Some of the chemicals of unknown carcinogenic activity which have been shown to induce early hyperplasia in the epithelium of the mouse bladder will therefore be tested for the former property.

The most important apparent discrepancy is the failure of the Slonaker rat to develop marked hyperplasia after the administration of 2-acetamidofluorene, a known bladder carcinogen in these animals. It was first suspected that the Slonaker rats which had been employed were not related to those used by Wilson *et al.* (1941) in their early work on the toxicity of 2-acetamidofluorene. These workers derived their rats from the colony maintained by Stanford University, California, in 1931. The British Slonaker rats are descended from those sent by Stanford University to Dr. Kenneth Baker in 1951 or 1952. He, in turn, passed them to Dr. A. L. Walpole of Imperial Chemical Industries Ltd. (Pharmaceuticals Division) who in February, 1956 sent a breeding nucleus to the Cancer Research Department of the University of Nottingham who have inbred them since then. The experiment described in this paper was performed on animals from the latter source. So far as can be ascertained the carcinogenicity of 2-acetamidofluorene has not been assayed on the Slonaker rats in use in this country. However, their bladder epithelium was more sensitive to the carcinogenic action of 3 : 2′-dimethyl-4-aminodiphenyl than that of the Wistar rat (Walpole, Williams and Roberts, 1955) and without direct evidence it would be unwise to assume that Nottingham-bred Slonaker rats are resistant to the induction of bladder tumours by 2-acetamidofluorene.

In view of the development of mild hyperplasia in the 2-acetamidofluorene-treated Slonaker rats killed after 5 and 8 weeks, a more plausible explanation of the discrepancy is to be found in the increasing amounts of the suspected carcinogenic metabolite of 2-acetamidofluorene, the *N*-hydroxy derivative, during continued dosing. Cramer, Miller and Miller (1960) showed that the amount of the *N*-hydroxy derivative in the urine increased from a low level to about 10 per cent of the administered dose of acetamide during the first 6 weeks of treatment. Thus, if *N*-hydroxylation is an essential step in the induction of hyperplasia as well as of bladder cancer, the delay in the appearance of hyperplasia can be explained. This speculation needs experimental investigation.

Some compounds have been found which induce early hyperplasia but are not known to be bladder carcinogens. *o*-Aminoazotoluene, for example, induces slight hyperplasia in the mouse and 2-acetamidofluorene hyperplasia in the hamster, but in neither case do the compounds induce cancer of the bladder. In

these instances the chemicals cause tumours at other sites and it is possible that the animals do not survive to develop bladder tumours.

Is long continued hyperplasia the only epithelial change necessary for the induction of cancer of the bladder? In the present experiments most of the chemicals have the ability to induce cancer in one or more tissues and therefore may apply a generalised carcinogenic stimulus throughout the body. Such a stimulus is most likely to manifest itself as a cancer in situations where there is an excessive proliferation of cells, i.e. hyperplasia. Experience in other tissues (Berenblum, 1944) indicates that there are factors in carcinogenesis other than hyperplasia.

Oxolamine (Silvestrini *et al.*, 1963) induced hyperplasia of the bladder in the rat when large single daily doses were given but not when the same dose was administered in portions during the day. Hyperplasia appeared to depend on attaining a peak concentration of the active agent, thought by the Italian workers to be diethylamine. Two similar examples have been found in the present experiments : 4-aminodiphenyl and *o*-aminoazotoluene induced hyperplasia when given to the mouse in single doses by stomach tube, but not when an equivalent dose was spread out by administration in the diet. It would help in deciding the importance of hyperplasia in the induction of cancer if the incidence of bladder tumours could be compared after the administration of 4-aminodiphenyl in the diet and by stomach tube.

The chemical and food industries are faced with the prospect of testing chemicals, which are to be used in the human environment, for carcinogenic activity. This is both time consuming and expensive. It is pertinent to ask whether the establishment of a correlation between early hyperplasia and ultimate malignancy of the bladder epithelium would in any way help in eliminating potentially dangerous compounds. From the work which has been described, it appears that there is a fair degree of correlation between the two processes. It would therefore seem feasible to argue that certain chemicals are likely to induce cancer of the bladder on the basis of preliminary toxicity studies. If similar early changes were to be established in other tissues, and this calls for much more experimental work, it is conceivable that industry would be able to regard chemicals inducing these early changes as potentially dangerous and to reject them. It would then be possible to concentrate long term carcinogenicity tests on fewer compounds which could be investigated more thoroughly.

SUMMARY

1. 4′-Fluoro-4-aminodiphenyl induced hepatomas in CBA and IF mice.

2. 4-Aminodiphenyl induced two confirmed carcinomas of the bladder in twelve mice. It is regarded as probably carcinogenic to the mouse.

3. 1-Phenylazo-2-naphthol, 4-acetamidostilbene and 4-aminostilbene were not carcinogenic but the two latter chemicals depressed the incidence of spontaneous hepatomas in Ab × IF mice. This is thought to be associated with the toxicity of these substances.

4. The ability of a number of chemicals to induce early hyperplasia of the bladder epithelium has been investigated in the mouse, rat, hamster and dog.

5. It is considered that there is a reasonable degree of correlation between early hyperplasia and the ultimate development of malignancy.

6. These results are discussed in relation to the part played by early hyperplasia

in the carcinogenic process and to the bearing it may have on the detection of potential environmental carcinogens.

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