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Effects of Sex Hormones on Development of Urinary Bladder Tumours in Rats Induced by N-Butyl-N- (4-Hydroxybutyl) Nitrosamine

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Summary. Male and female Wistar strain rats were given 0.05% N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in their drinking water for 6 weeks and then water without BBN for 18 weeks. Diethylstilboestrol and testosterone were implanted subcutaneously into both intact and gonadectomised animals before or after treatment with BBN to evaluate their effects on the development of bladder tumours. - Diethylstilboestrol reduced the incidence of bladder tumours significantly in male rats. The incidence was higher in female rats after spaying and administration of testosterone after BBN treatment, than in the intact female. - These results suggest that diethylstilboestrol inhibits carcinogenesis of the urinary bladder induced by BBN and growth of bladder tumours induced by BBN, in male rats. On the other hand, testosterone seems to stimulate the growth of bladder tumours induced by BBN in female rats.

Key words: Rat urinary bladder tumour, sex hormone, gonadectomy, N-butyl-N-(4-hydroxybutyl) nitrosamine.

In general, the incidence of urinary bladder tumours is higher in men than in women. However, no clear sex difference has been demonstrated in the incidence of urinary bladder tumours in animals induced by chemical carcinogens.

Druckrey et al. (5) first reported a specific organotrophic carcinogenic effect of N butyl-N-(4-hydroxybutyl) nitrosamine (BBN) on the urinary bladder of rats. Since then many investigators have reported on the histogenesis, histology and ultrastructure of urinary bladder tumours in various animals induced by BBN (2, 10, 13, 14, 15) and the metabolism of this carcinogen has also been studied (8, 11).

We have reported previously the histogenesis of urinary bladder tumours and the inhibitory effect of diethylstilboestrol on the growth of urinary bladder tumours in male Wistar rats induced by 0.05% BBN in the drinking water (13). These results suggested that the sex and hormonal status of the animals might influence the development of urinary bladder tumours in animals induced by chemical carcinogens.

The present study was on the effects of sex hormones on the development of urinary bladder tumours in Wistar rats induced by BBN.

Material and Methods

Male and female Wistar strain rats (Fuji Animal Farm, Tokyo) weighing 140-170 g were used. The carcinogen, BBN (Izumi Chemical Co., Yokohama) was given as a 0.05% solution in the drinking water as described previously (10). All rats received commercial stock diet (Oriental MF, Oriental Yeast Co., Osaka) and water ad libitum.

Experimental Series A

A total of 129 male rats were divided into the following groups.

Group 1: Twelve rats were given drinking water

supplemented with BBN for 6 weeks and then water without BBN for 18 weeks.

Group 2: Fifteen rats were implanted with diethylstilboestrol. They were given drinking water with BBN for 6 weeks and then water without BBN for 18 weeks.

Group 3: Twelve rats were orchiectomized and given drinking water with BBN for 6 weeks, and then water without BBN for 18 weeks.

Group 4: Twelve rats were orchiectomized and implanted with diethylstilboestrol. They were given drinking water with BBN for 6 weeks and then water without BBN for 18 weeks.

Group 5: Eighteen rats were given drinking water supplemented with BBN for 6 weeks. After treatment with BBN they were implanted with diethylstilboestrol and given water without BBN for 18 weeks.

Group 6: Twelve rats were given drinking water supplemented with BBN for 6 weeks. Then they were orchiectomized and given water without BBN for 18 weeks.

Group 7: Twelve rats were given drinking water supplemented with BBN for 6 weeks. Then they were orchiectomized, implanted with diethylstilboestrol and given water without BBN for 18 weeks.

Group 8: Nine rats were implanted with diethylstilboestrol, and given drinking water without BBN for 24 weeks.

Group 9: Nine rats were orchiectomized and given water without BBN for 24 weeks.

Group 10: Nine rats were orchiectomized and implanted with diethylstilboestrol. Then, they were given water without BBN for 24 weeks.

Group 11: Nine rats were given drinking water without BBN for 24 weeks.

Experimental Series B

A total of 120 female rats divided into the following groups:

Group 1: Twelve rats were given drinking water supplemented with BBN for 6 weeks and then water without BBN for 18 weeks.

Group 2: Twelve rats were implanted with testosterone. They were given drinking water with BBN for 6 weeks and then water without BBN for 18 weeks.

Group 3: Twelve rats were spayed and given drinking water with BBN for 6 weeks, and then water without BBN for 18 weeks.

Group 4: Twelve rats were spayed and implanted with testosterone. They were given drinking water with BBN for 6 weeks and then water without BBN for 18 weeks.

Group 5: Twelve rats were given drinking water supplemented with BBN for 6 weeks. Then they were implanted with testosterone and given water without BBN for 18 weeks.

Group 6: Twelve rats were given drinking water supplemented with BBN for 18 weeks. Then they were spayed and given water without BBN for 18 weeks.

Group 7: Twelve rats were given drinking water supplemented with BBN for 6 weeks. Then they were spayed and implanted with testosterone and given water without BBN for 18 weeks.

Group 8: Nine rats were implanted with testosterone. Then they were given drinking water without BBN for 24 weeks.

Group 9: Nine rats were spayed and then given water without BBN for 24 weeks.

Group 10: Nine rats were spayed and implanted with testosterone, and were given water without BBN for 24 weeks.

Group 11: Nine rats were given drinking water without BBN for 24 weeks.

Gonadectomy was carried out one week before the experiment was started or after treatment with BBN for 6 weeks. In castration of male rats the testes and epididymis were excised after laparotomy under ether anaesthesia. In spaying of female rats the ovaries and uterus were excised after laparotomy under ether anaesthesia.

Hormones were implanted as subcutaneous, sterile implants into the interscapular region under ether anaesthesia. They consisted of 1 part of diethylstilboestrol (Merck AG. Darmstadt) or 1 part of testosterone (Schering AG. Berlin) and 1 part of cholesterol. Each animal received 50 mg of hormone.

Rats were housed three to a cage in wire cages in an air conditioned room at 24 °C, and weighed once a week. Animals that died within 20 weeks of the start of the experiment were excluded.

At the end of the experiment animals were sacrified with ether and carefully examined. The carcass, liver, both kidneys, spleen and testes were weighed and samples were taken for histological studies. The urinary bladder was punctured at the urethro-vesical junction and 0.5 ml of 10% buffered formaldehyde solution was injected into the bladder.

After fixation for 1 day, the bladder was carefully examined for tumours. All tissues were routinely stained with Hematoxylin and Eosin, van Gieson, PAS and Mallory stains.

Results

Histological findings and the incidence of tumours in the bladder are shown in Tables 1 and 2.

Abnormal epithelial patterns were classified into 3 types, hyperplasia (Fig. 1), papilloma (Fig. 2) and cancer (Fig. 3) as described previously (10, 14, 15). Areas of squamous metaplasia in the cancer tissue and invasive growth of cancer cells into the urinary bladder wall (Fig. 4) were noted. No remarkable changes were seen in the liver, kidney, lung or gastrointestinal tract.

Table 1. Effect of diethylstilboestrol and castration on development of urinary bladder tumours in male rats given 0.05% BBN in the drinking water for 6 weeks and then water without BBN for 18 weeks

Experimental Group	Effective No. of rats	Total tumours	Incidence of changes in urinary bladder (%) Hyperplasia	Papilloma	Cancer	Invasion	Metaplasia	P-value
1 BBN	10	6(60.0)	9(90.0)	6(60.0)	2(20.0)	2(20.0)	1(10.0)	-
2 BBN + Diethylstilboestrol	14	1(7.1)	4(28.6)	1(7.1)	0 -	0 -	0 -	0.025
3 BBN + Castration	10	5(50.0)	7(70.0)	5(50.0)	2(20.0)	1(10.0)	1(10.0)	N.S.
4 BBN + Diethylstilboestrol and castration	12	0 -	1(8.3)	0 -	0 -	0 -	0 -	0.01
5 BBN → Diethylstilboestrol	17	1(5.9)	7(41.2)	1(5.9)	0 -	0 -	0 -	0.005
6 BBN → Castration	11	4(36.4)	7(63.7)	4(36.4)	3(27.3)	2(18.2)	1(9.1)	N.S.
7 BBN → Diethylstilboestrol and castration	11	1(9.1)	2(18.2)	1(9.1)	0 -	0 -	0 -	0.025
8 Diethylstilboestrol	9	0	0	0	0	0	0	
9 Castration	9	0	0	0	0	0	0	
10 Diethylstilboestrol and castration	9	0	0	0	0	0	0	
11 Control	9	0	0	0	0	0	0	

P-value: Significance of difference in the incidence of tumours from that in intact male rats in group 1

N.S. : no significant difference

Table 2. Effect of testosterone and spaying on development of urinary bladder tumours in female rats given 0.05% BBN in the drinking water for 6 weeks and then water without BBN for 18 weeks

Experimental Group	Effective No. of rats	Total tumours	Incidence of changes in urinary bladder (%) Hyperplasia	Papilloma	Cancer	Invasion	Metaplasia	P-value
1 BBN	11	2(18.2)	5(45.5)	2(18.2)	1(9.1)	0 -	1(9.1)	0.1[a]
2 BBN + Testosterone	11	4(36.4)	7(63.6)	4(36.4)	3(27.3)	3(27.3)	2(18.2)	N.S.
3 BBN + Spaying	10	3(30.0)	6(60.0)	3(30.0)	1(10.0)	1(10.0)	0 -	N.S.
4 BBN + Testosterone and Spaying	10	5(50.0)	9(90.0)	5(50.0)	3(30.0)	2(20.0)	2(20.0)	N.S.
5 BBN→ Testosterone	11	6(54.5)	8(72.7)	6(54.5)	4(36.4)	1(9.0)	3(27.3)	0.2
6 BBN→ Spaying	12	3(25.0)	6(50.0)	3(25.0)	1(8.3)	0 -	1(8.3)	N.S.
7 BBN→ Testosterone and Spaying	11	8(72.7)	10(90.9)	8(72.7)	6(54.5)	5(45.5)	3(27.3)	0.05
8 Testosterone	9	0	0	0	0	0	0	
9 Spaying	9	0	0	0	0	0	0	
10 Testosterone and spaying	9	0	0	0	0	0	0	
11 Control	9	0	0	0	0	0	0	

P-value: Significance of difference in the incidence of tumours from that in intact female rats in group 1

N.S. : no significant difference

a: Significance of difference in the incidence of tumours from that in intact male rats in group 1 of series A

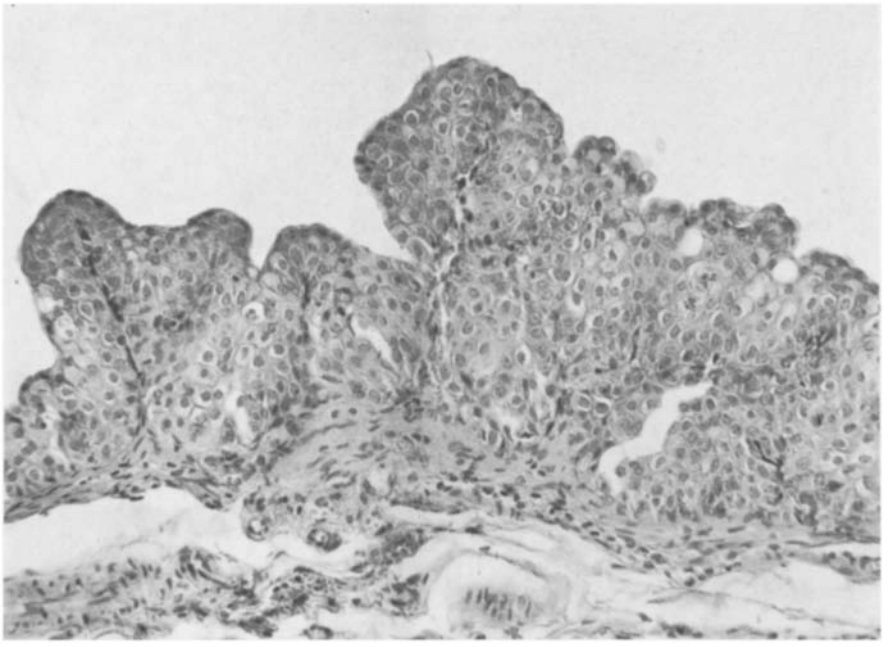

Fig. 1. Hyperplasia and slight papillomatous growth of bladder epithelium in a male rat given water with BBN for 6 weeks, and then water without BBN for 18 weeks. H-E. x 90

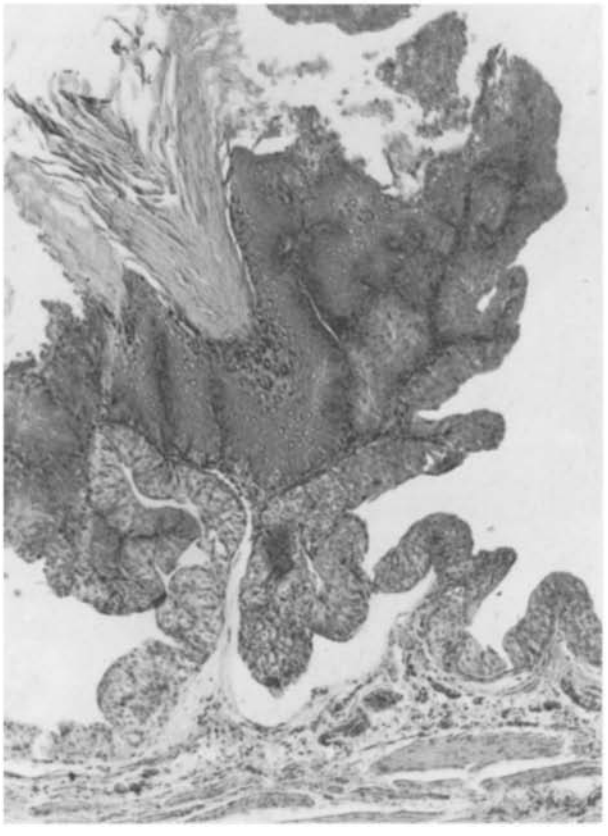

Fig. 2. Transitional cell papilloma and squamous metaplasia of the urinary bladder in a female rat after BBN treatment for 6 weeks, and then spaying and implantation of a testosterone pellet. H.E. x 36

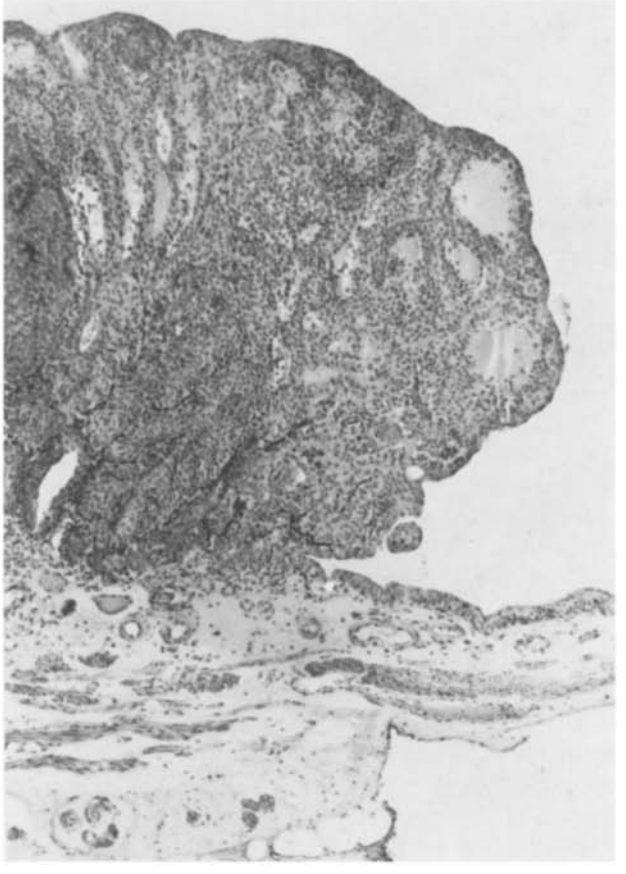

Fig. 3. Transitional cell carcinoma of the urinary bladder in a female rat after BBN treatment for 6 weeks, and then spaying and implantation of a testosterone pellet. H.E. x 34

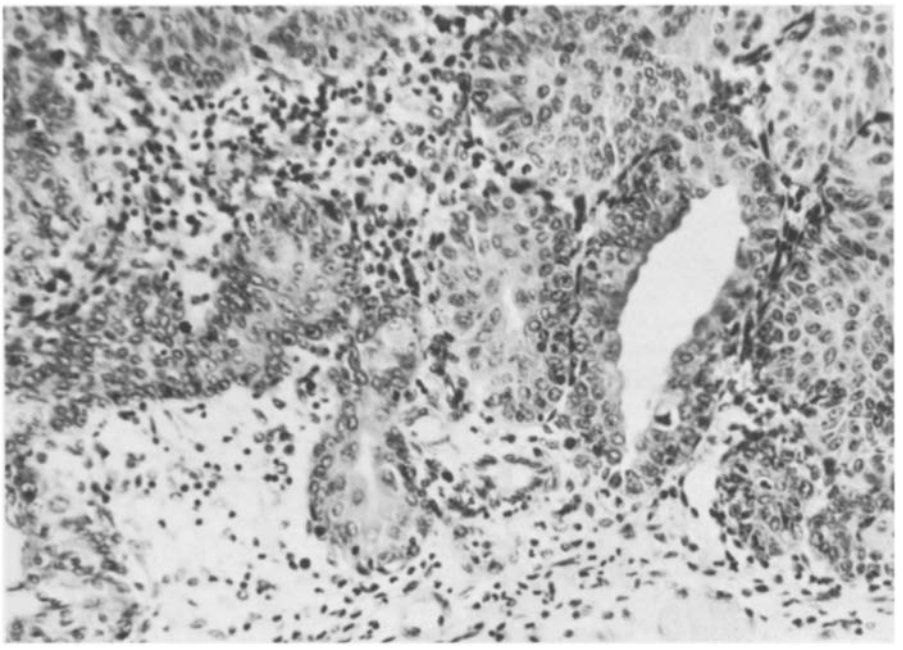

Fig. 4. Infiltrative growth of cancer cells in the submucosal layer of the bladder wall of a rat after BBN treatment for 6 weeks, and then spaying and implantation of a testosterone pellet. H.E. x 90

Experimental Series A

The average body weights of all groups increased throughout the experiment, though weight gains in groups 2, 4, 5, 7, 8 and 10 of series A (implanted with diethylstilboestrol) were slightly less. All rats in groups 2, 5 and 8 (with implanted diethylstilboestrol) showed atrophy of the testes. Histological findings in the urinary bladder in each group are summarized in Table 1.

Group 1. Hyperplasia of the urinary bladder epithelium was found in 9 rats (90.0%), papilloma in 6 rats (60.0%), and cancer in 2 (20.0%) of the 10 rats in this group. Muscular invasion of the urinary bladder wall by tumour was seen in 2 rats (20.0%) while squamous metaplasia developed in 1 rat (10.0%).

Group 2. Among the 14 rats, hyperplasia of the urinary bladder epithelium was seen in 4 rats (28.6%) and papilloma in only one (7.1%). No rats developed cancer of the urinary bladder and no invasion of the bladder wall was seen. The incidence of tumours in this group (7.1%) was significantly lower than that in group 1 (60.0%) ($P < 0.025$).

Group 3. Seven of 10 rats (70.0%) in this group developed hyperplasia of the urinary bladder epithelium, 5 (50.0%) developed papilloma and 2 (20.0%) developed cancer.

One of the rats (10.0%) showed muscular invasion of the bladder wall by tumour cells and one rat (10.0%) showed squamous metaplasia. The incidence of bladder tumours was not significantly different from that in the intact males of group 1.

Group 4. Hyperplasia of the urinary bladder epithelium was seen in only one of 12 rats (8.3%). No rats developed papilloma or cancer of the urinary bladder. The incidence of tumours in this group was significantly lower than that in group 1 ($P < 0.001$).

Group 5. Seven of 17 rats (41.2%) developed hyperplasia of the bladder epithelium. Papilloma was found in only one (5.9%). No rats developed cancer of the urinary bladder. The incidence of tumours was significantly lower than that in group 1 ($P < 0.005$%).

Group 6. Hyperplasia of the urinary bladder epithelium was seen in 7 of 11 rats (63.7%), papilloma in 4 (36.4%), and cancer in 3 (27.3%). Two of the rats (18.2%) showed muscular invasion of the bladder wall by tumour and one rat (9.1%) showed squamous metaplasia. The incidence of tumours was not significantly different from that in group 1.

Group 7. Hyperplasia of the urinary bladder epithelium developed in 2 of 11 rats (18.2%) and papilloma in only one (9.1%). No rats developed cancer of the urinary bladder. The incidence of tumours in this group was significantly lower than that in group 1 ($P < 0.0025$).

Group 8, 9, 10 and 11. No rats developed hyperplasia or tumour of the bladder epithelium. No histological abnomalities were observed.

Experimental Series B

The average body weight of all groups increased throughout the experiment. Histological findings in the urinary bladder in each group are summarized in Table 2.

Group 1. Hyperplasia of the urinary bladder epithelium developed in 5 of 11 rats (45.5%), papilloma in 2 (18.2%) and cancer in one rat (9.1%). One rat (9.1%) showed squamous metaplasia, but no rats showed invasive growth of cancer cells into the urinary bladder wall. The incidence of tumours in this group tended to be lower ($P < 0.1$), than that in group 1 in series A.

Group 2. Seven of 11 rats (63.6%) developed hyperplasia of the urinary bladder epithelium. Papilloma was found in 4 rats (36.4%) and cancer in 3 rats (27.3%). Three rats (27.3%) showed muscular invasion of the bladder wall by tumour cells and 2 rats developed squamous metaplasia (18.2%). The incidence of bladder tumours was not significantly different from that in the intact female rats of group 1.

Group 3. Hyperplasia of the urinary bladder epithelium was seen in 6 of 10 rats (60.0%), papilloma in 3 (30.0%), and cancer in one rat (10.0%). One rat (10.0%) showed muscular invasion of the bladder wall by tumour cells. The incidence of bladder tumours was not significantly different from that in the intact female rats of group 1.

Group 4. Nine of 10 rats (90.0%) developed hyperplasia of the urinary bladder epithelium. Papilloma was found in 5 rats (50.0%) and cancer in 3 rats (30.0%). Two rats (20.0%) showed muscular invasion of the bladder wall by tumour and 2 rats (20.0%) developed squamous metaplasia. The incidence of bladder tumours was not significantly different from that of the intact female rats of group 1.

Group 5. Hyperplasia of the bladder epithelium was seen in 8 of 11 rats (72.7%), papilloma in 6 (54.5%), and cancer in 4 (36.4%). One of the rats (9.1%) showed muscular invasion of the bladder wall by tumour and squamous metaplasia developed in 3 rats (27.3%). There was no significant difference in the incidence of bladder tumours from that of the intact female rats of group 1 ($P < 0.2$).

Group 6. Hyperplasia of the urinary bladder epithelium was seen in 6 of 12 rats (50.0%), papilloma in 3 (25.0%), and cancer in one (8.3%). One rat (8.3%) showed squamous metaplasia, but none showed invasive growth of cancer cells into the urinary bladder wall. The incidence of bladder tumours was not significantly different from that in group 1.

Group 7. Ten of 11 rats (90.0%) developed hyperplasia of the bladder epithelium. Papilloma was found in 8 rats (72.7%) and cancer in 6 (54.5%). Five rats (45.5%) showed muscular invasion of the bladder wall by tumour and squamous metaplasia developed in 3 rats (27.3%). The incidence of tumours in this group (72.7%) was significantly higher than that in group 1 (18.2%) ($P < 0.05$).

Group 8, 9, 10 and 11. No rats developed hyperplasia or tumour of the bladder epithelium. No histological abnormalities were observed.

Discussion

It is of interest that the incidence of bladder cancer is higher in men than in women. However bladder cancer in humans is not usually considered hormonally dependent although there are ma reports on the importance of sex and hormones in development of urinary bladder tumours in experimental animals. A number of these studies showed that the incidence of urinary bladder tumours induced by chemical carcinogens (such as 1-fluorenylacetamide, 4-aminodiphenyl, 2-aminodiphenylene oxide and BBN) are higher in male animals than in female animals (1, 2, 3, 6, 19, 20). Conversely, it has been reported that the incidence of bladder tumours is higher in female than in male animals, or is not significantly different between the two sexes (12, 18, 21). The sex difference in the incidence of bladder tumours has been explained as due to the influences of sex hormones on the metabolic pathways leading to the production of carcinogens or their precursors in the urine. Therefore, the influence of sex or hormonal status on the development of bladder tumours induced by chemical carcinogens has not been clarified.

It has been clearly demonstrated that diethylstilboestrol prevents or reduces the induction of liver tumours in male rats by 2-fluorenylacetamide (17). It was also observed that diethylstilboestrol inhibits the development of kidney tumours in male rats induced by dimethylnitrosamine (9). In addition, diethylstilboestrol has been shown to inhibit cell division of Paracentrotus lividus (4).

The present experiments show that the incidence of urinary bladder tumours in rats induced by BBN is higher in male rats (60.0%) than in female rats (18.2%) ($P < 0.1$). In experimental groups 2, 4, 5 and 7 of series A, the incidence of bladder tumours in male rats receiving diethylstilboestrol with or without castration before or after treatment with BBN for 6 weeks was significantly lower than that in the intact male rats in group 1 ($P < 0.005$-0.0025).

However, the incidence of bladder tumours in groups 3 and 7 which were castrated and treated with BBN were not significantly different from that in intact male rats in group 1. These results suggest that diethylstilboestrol inhibits bladder carcinogenesis by BBN and also inhibits the growth of bladder tumours from premalignant lesions of the bladder epithelium in male rats induced by BBN treatment for 6 weeks.

On the other hand, Beatram et al. (2) reported that male mice treated with BBN developed bladder tumours significantly earlier than female mice and concluded that potentiation of the carcinogenic response of females to BBN by testosterone could be due to alteration of a metabolic pathway. Furthermore, testosterone has been shown to stimulate a microsomal drug metabolizing enzyme system in rat liver (7, 16). In series

B in this work, we found that the incidence of bladder tumours was higher in groups 5 and 7 implanted with testosterone after BBN treatment for 6 weeks than in groups 2 and 4 implanted with testosterone before BBN treatment. These results suggest that testosterone increases the growth of tumours from premalignant lesions of the bladder epithelium induced by BBN in female rats, rather than stimulating a metabolic pathway, or having a synergistic action with BBN.

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