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Effect of the Urine and Calculus Formation on the Incidence of Bladder Tumors in Rats Implanted with Paraffin Wax Pellets[1]

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SUMMARY

A double pouch of bladder was formed by divison of the bladder of inbred Fischer 344 rats and implantation of paraffin pellets into each pouch. The upper pouch could be in contact with or isolated from the urinary stream, while the lower pouch was always in contact with the urine. No tumors formed in 99 isolated upper pouches, while 49 formed in 119 communicating upper pouches in contact with the urine.

The presence of 20% 3-hydroxyanthranilic acid in the pellets made no change in the incidence of tumors. When the urine was in contact with the pouch, stone formation was evident in one-half of the pouches and, in those pouches with stone formation, the incidence of tumors approximately doubled.

The technique allows comparison of the effect of the pellet in the presence or absence of urine. The data suggest that the urine may play an as yet undefined role in the production of these tumors.

INTRODUCTION

An underlying assumption of much work with experimental bladder tumors is the so-called urogenous or carrier theory. According to this hypothesis, bladder tumors are caused by carcinogens excreted by the kidney which are then carried by the urine to the bladder where they act on the urothelium to product the tumors. This hypothesis explains the clinical behavior of these tumors, which are multiple in both time and space [that is, they occur (or recur) over long periods of time and in different areas of the bladder].

Support for this mechanism comes from the experiments of Scott and Boyd (13) and of McDonald and Lund (9); both groups worked with dogs fed β-naphthylamine. Scott diverted the urine to the sigmoid colon and found that this prevented the occurrence of bladder tumors. McDonald isolated the top one-half of the bladder from the urinary stream by closing the bladder around its midportion and found tumors in the intact bottom one-half of the bladder but found none in the isolated top one-half, which had not been exposed to urine.

The urogenous theory has been used to explain the mechanism of carcinogenesis by endogenous tryptophan metabolites, and it has been assumed, in experiments with the bladder pellet technique, that the tumors produced are the result of the release of the proximate carcinogen from the pellet.

The experiments described below were conducted to ascertain whether urine has a role in bladder carcinogenesis beyond that of being simply a carrier for known carcinogens.

MATERIALS AND METHODS

Pellets were made of either plain paraffin (Parawax) or 20% 3-OHAA[3] in paraffin. They were produced from powdered wax or a wax-3-OHAA mixture in a Calton tablet press and measured 2 mm thick by 4 mm in diameter and weighed 30 ± 2 mg.

3-OHAA was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and its identity was confirmed by melting-point determination and comparison of the absorption spectra with a sample synthesized by Method I of Nyc and Mitchell (11).

Weanling male Fischer 344 rats were used. The dome of the bladder was opened and 2 pellets were inserted. The bladder was closed with a 5-0 silk suture-ligature, and a 2nd suture-ligature was placed around the midportion of the bladder between the 2 pellets. Thus, 2 pouches were formed, each containing a pellet; the lower pouch was a functioning small bladder and the upper pouch was a normal bladder with no access to urine, filled in most instances with a clear serous fluid. The latter were called isolated upper pouches.

In about one-third of the bladders, the dividing suture eroded through the bladder wall, leaving the bladder in an hourglass shape with upper and lower pouches in communication and with urine having access to the upper pouch. These were called communicating upper pouches. In another one-third of the bladders, the dividing suture eroded completely through the bladder wall so that the pellets were no longer confined to either the upper or lower pouch, and the bladder essentially reverted to a cavity with a single lumen. These were called single pouches.

Rats surviving for over 40 weeks were included in the experiment. Those living at the end of 1 year were killed with sodium pentothal administered i.p. The lower portion of the bladder was distended with formalin through a 25-gauge needle, and we watched the upper pouch to determine whether a functional communication was present. The bladder

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[3] The abbreviation used is: 3-OHAA, 3-hydroxyanthranilic acid.

neck was then tied off, and the distended bladder was opened in the midline and examined under a dissecting microscope for the presence and location of the pellets and for the presence or absence of stones, tumors, and worms (*T. crassicauda*). Since there were no worms present in any of these animals, they will not be mentioned further. If the pellets were not present, the animals were discarded.

An attempt was made early in the experiments to distend the isolated upper pouches with formalin. This was not successful, since they appeared to be relatively less distensible than the intact bladder and leaked most of the extra volume out through the needle puncture hole. However, since the isolated pouches were uniformly filled with serous fluid and distended to between 4 to 15 mm in diameter with the mucosa lying flat, histological interpretation was not complicated by infolding artifacts.

Paraffin sections stained with hematoxylin and eosin were taken through each pouch to include the base of any tumor, and the histology was interpreted independently by 2 of the investigators, starting with criteria similar to those of Bonser and Jull (3). If a tumor was seen on initial examination with a dissecting microscope and was not found on histological review, we took additional sections from the block to see whether a tumor had been missed. The final interpretation of record, however, was the histological one.

The distinction between hyperplasia and papillomas could be made with consistency. The distinction between papillomas and early noninvasive carcinoma could not be made with consistency between reviewers, or even by the same reviewer looking at the sections on different occasions. In this series, no tumors were seen that invaded muscle. All lesions were therefore classed as either normal (or hyperplasia) or as tumor (papilloma or carcinoma).

The data for each rat were coded and punched on Hollerith cards for storage and initial evaluation by a package statistical program (XTAB). All the data presented were then confirmed by use of the card sorter and counter.

RESULTS

In this study, 320 rats were included: 102 had single pouches, 119 had communicating upper pouches, and 99 had isolated upper pouches. The effect of isolating the bladder mucosa from the urine was dramatic (Chart 1). No tumors developed in the 99 isolated upper pouches while, in the communicating upper pouches exposed to urine, there were 49 tumors (41%) ($p < 0.001$).

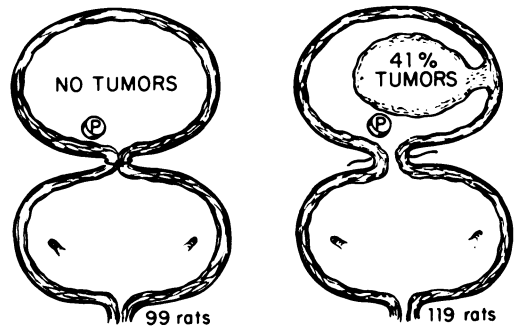

Chart 1. Incidence of tumors in the upper bladder pouch when it is isolated from the urine (*left*) and communicating with the urine (*right*). *P*, pellet.

All tumors (on careful examination with a dissecting microscope) appeared to be benign and confined to the bladder with no muscle invasion; most were composed of well-differentiated transitional cells and had a narrow base or a stalk (Figs. 1 to 4), but some had undergone squamous metaplasia. Some of these tumors appeared to extend into the submucosa, or at least the basement membrane was not identifiable, but epithelial cells were not found in or beyond muscle except in association with remnants of the silk ligature, and these were interpreted as a surgical artifact.

The presence of stones had a marked effect on the incidence of tumors in all pouches (Table 1). In the single pouches, the tumor incidence increased from 59% without stones to 85% with stones. In the lower bladder pouches, it went from 33% without stones to 63% with stones, while in the upper communicating pouches, the incidence rose from 27% without stones to 66% with stones.

The inclusion of 20% 3-OHAA in the pellet made no consistent difference in the tumor incidence within various types of pouch (Table 2). A breakdown of these figures by stone formation showed no difference in stone formation with the addition of 3-OHAA.

DISCUSSION

The unique feature of these experiments is that in this preparation no tumors were produced without the presence of both urine and a foreign body. The role of the foreign body has been described as cocarcinogenic (2), as promoting (6), as an inducer of mitoses (8), and as a "stimulus to neoplasia in an already rapidly proliferating epithelium" (12). The role of the urine beyond that of being a carrier has not been investigated.

The fact that all of these lesions were papillomas makes somewhat controversial the definition of their neoplastic nature. Recent work by Taranger *et al.* (14) in which the same rat bladder preparation with a single pouch was used showed that invasive carcinomas have a tumor tissue type-specific antigen in common with that seen in the papillomas. This common antigen suggests that the papillomas are closely related to the carcinomas and have some neoplastic attributes.

The increase in tumor formation with the presence of stones has previously been noted. Mobley *et al.* (10) sacrificed their animals at 36 weeks and found concretions in 50% of their animals. They found that the stones were associated with epithelial changes 29 to 45% of the time, while animals with no stones had these changes only 6% of the time.

There are at least 2 explanations for the difference in tumor growth with stone formation. Using the bladder pellet technique, Clayson and Pringle (8) showed that an increase in the number of mitoses may be associated with the increase in tumors. A further increase could be expected with the irritation produced by stones. Ball *et al.* (2) produced tumors in the mouse bladder and noted the presence of stones, but Roe (12), in an accompanying paper, specifically mentions that they did not attempt to correlate the increased incidence of tumors with stones. A 2nd possible explanation might be

Table 1

The effect of urine and calculus formation on the incidence of bladder tumors in rats implanted with paraffin wax pellets

Group	No. of rats surviving 12 months	Overall incidence of bladder tumors/ total no. of rats	%	Incidence of bladder tumors in rats without stones/ total no. without stones	%	Incidence of bladder tumors in rats with stones/total no. with stones	%
A. Single bladder pouches	102	79/102	78	17/29	59	62/73	85
B. Lower bladder pouches (single and communicating	218	108/218	50	32/98	33	76/120	63
C. Communicating upper bladder pouches	119	49/119	41	20/75	27	29/44	66
D. Isolated upper bladder pouches	99	0/99	0	0/99	0		

that urinary tract infections were causing the tumors and also causing the stones. Clinically the relationship of infection to stone growth is well known and in the rat has been demonstrated by Vermeulen and Goetz (15). Routine cultures were not obtained in our series, so we can only speculate on this possibility.

The possibility that all animals with tumors had stones at one time, but that some animals passed them, cannot be completely ruled out by these experiments. However, the initial stone usually formed on the surface of the pellet, and we lost almost no pellets. Therefore, we think it unlikely that an animal would form a stone which would then completely disappear without removing the pellets. There were, of course, no stones in the isolated pouches, but when the absence of tumors in these pouches (0 of 99) was compared with the incidence of tumors in communicating upper pouches with no stones (20 of 75), the difference was still highly significant ($p < 0.001$).

The incidence of tumors without stones was very similar in both upper and lower communicating pouches, confirming that these were comparable preparations and suggesting that there was no inherent difference in the tumor susceptibility of the dome *versus* the floor of the bladder. The incidence of tumors in the single pouches was greater but was still in the same order of magnitude, if one considers the greater area of mucosa exposed and calculates a tumor-per-area ratio.

The role of 3-OHAA in these experiments should be minimized, and our data should not be extrapolated to suggest that 3-OHAA is never active in the bladder.

We did not measure the elution of 3-OHAA from these pellets but, in unpublished work (W. H. Chapman, D. Kirchheim, and J. W. McRoberts) with the isolated mouse bladder pouch preparation (7), 3-OHAA was eluted from paraffin pellets so that one-half was gone in 16 weeks ($t_{½}$ = 112 days).

This rate was the same whether the pellets were in intact bladders or in the closed bladder pouch. Comparative data are available from Bryan (4), who found no detectable levels of 3-OHAA in paraffin pellets after 256 days in the mouse bladder. Bryan (4) and Allen *et al.* (1) noted no increased incidence of tumors with 3-OHAA in paraffin over that with the control paraffin pellets. This is in contrast to the data with cholesterol pellets: both Bryan and Allen *et al.* found tumors developing with 3-OHAA in cholesterol pellets. Bryan found the $t_{½}$ of 3-OHAA *in vivo* in cholesterol to be 5.3 days. Allen *et al.* found that, *in vitro*, the $t_{½}$ of 3-OHAA in cholesterol was 15 days, while only 6% of the chemical was eluted from paraffin in 10 days. It thus seems possible that in our experiment the 3-OHAA was not released from paraffin pellets at a rate sufficient to cause an increase in incidence of tumors.

Table 2

Effect of 20% 3-OHAA in pellet on the incidence of bladder tumors

No differences are significant at the 0.05% level.

Pouch	Paraffin		20% 3-OHAA in paraffin	
	No. of tumors/ no. of pouches	% tumors	No. of tumors/ no. of pouches	% tumors
Upper isolated	0/47	0	0/52	0
Upper communicating	35/71	49	14/48	29
Single	20/30	67	59/72	82
Lower isolated	17/45	38	24/54	44
Lower communicating	22/39	56	44/80	55
All pouches	94/232	41	141/306	46

The mechanism of the urinary effect on tumorigenesis still remains unclear. The idea that the paraffin pellet might itself be carcinogenic was discarded since it was felt that any carcinogen in the pellet would be exposed to the bladder mucosa of the isolated upper pouch at a higher concentration than in the communicating pouches, in which the carcinogen would be diluted and washed away by the urine. It is possible that urine might elute a carcinogen or cocarcinogen from the pellet which was not eluted by the fluid in the isolated pouch. This seems unlikely but could be tested by repeating these experiments with other foreign bodies.

Bryan and Springberg (5) suggested that the incidence of bladder tumors in mice with paraffin or cholesterol pellets might be due to the promoting activity of the pellet combined with the tryptophan metabolites, 3-OHAA and 3-hydroxykynurenin, present in the urine of normal mice. If 3-OHAA were the effective agent, we might have expected the pellet containing 20% 3-OHAA to produce at least some tumors in the isolated upper pouch, although the release rate was much slower than the optimum.

From our data, we conclude that there are at least 2 factors necessary for the formation of bladder tumors in our preparation. The 1st is an unidentified substance and/or factor related to urine. This could be a carcinogen or even a physical factor such as the repetitive distension and contraction of the bladder. The 2nd is the presence of a foreign body.

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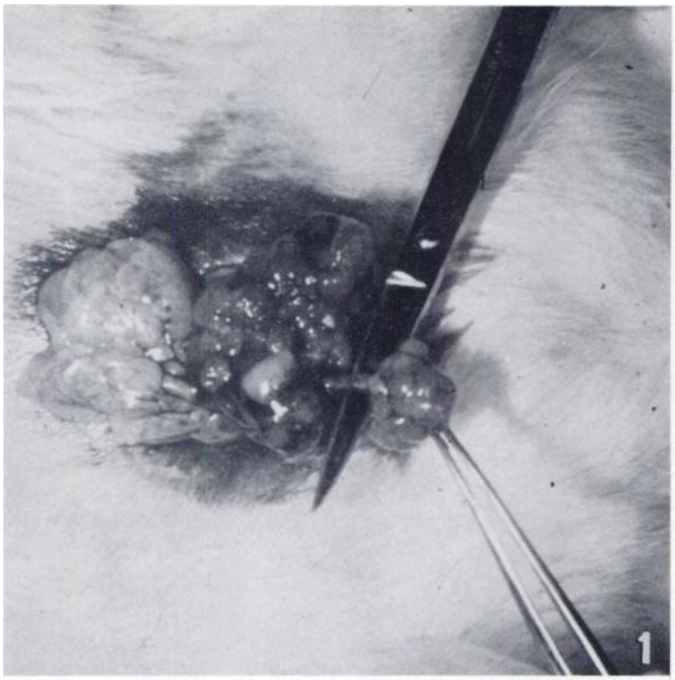

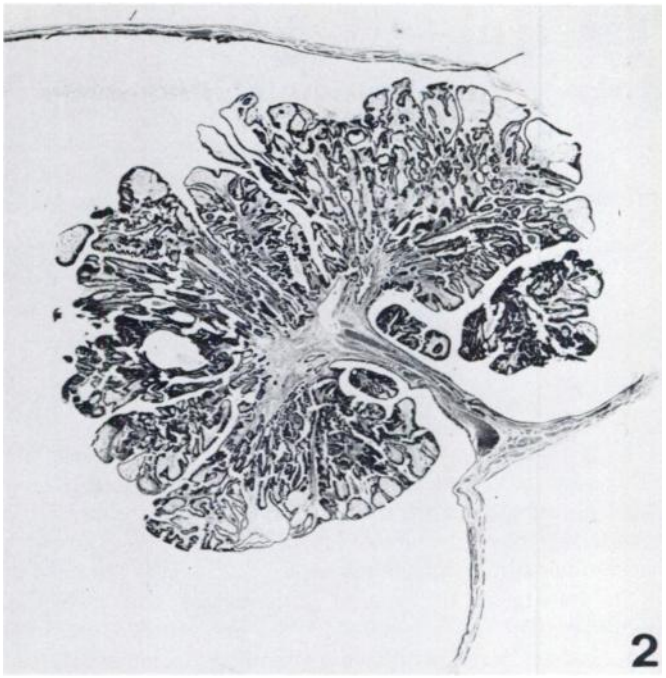

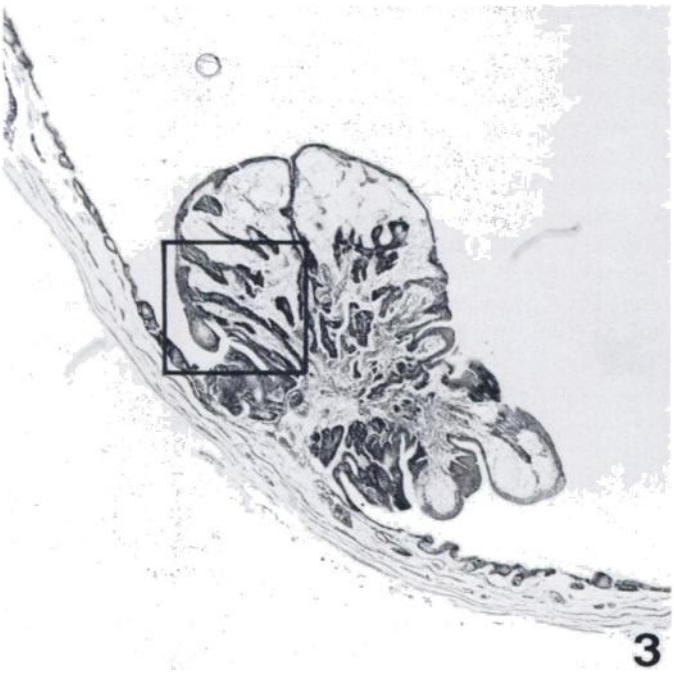

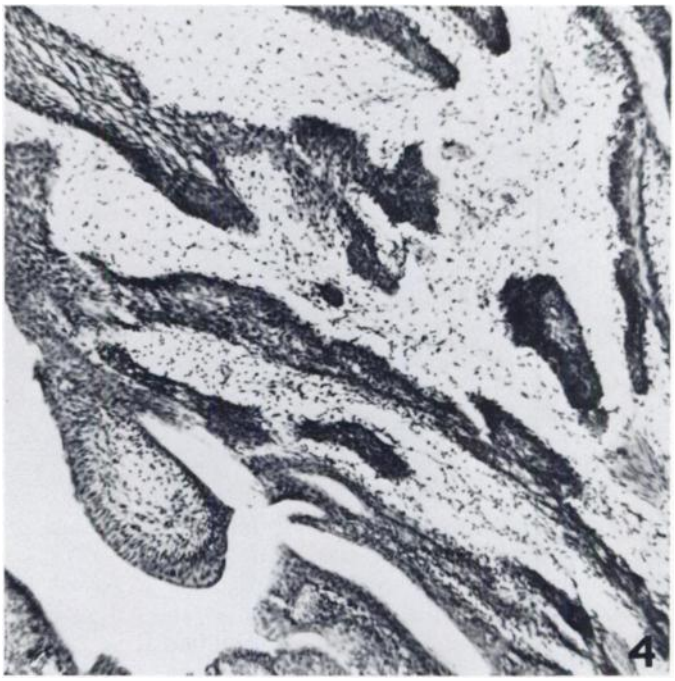

Fig. 1. Gross appearance of bladder tumor on a long stalk.
Fig. 2. Histological appearance of tumor with a stalk. × 15.
Fig. 3. Low-power photomicrograph of another tumor. × 15.
Fig. 4. High-power photomicrograph of the tumor in Fig. 3. × 90.