Incidence of Mouse Bladder Tumors Following Implantation of Paraffin Pellets Containing Certain Tryptophan Metabolites*

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SUMMARY

Pellets of paraffin wax implanted into the lumen of the bladder of 38 mice induced one carcinoma. An equally low incidence of carcinomas was induced with paraffin wax pellets containing 15-20 per cent suspensions of 8-hydroxyquinoline or quinaldic acid, 8-hydroxyquinolinal acid, xanthurenic acid, the 8-methyl ether of xanthurenic acid, 4,8-quinolinediol, L-kynurenine sulfate, 3-hydroxy-L-kynurenine, and 3-hydroxyanthranilic acid, all metabolites of tryptophan. The inability to produce pellets of uniform composition containing these test substances was demonstrated. Paper chromatographic methods revealed that most of these compounds remained in the paraffin vehicle in vivo for as long as 342 days. It was concluded that paraffin is not a satisfactory vehicle in which to test these urinary tryptophan metabolites for bladder carcinogenicity.

A paraffin wax pellet containing a suspected oncogenic substance has been implanted into the lumen of the bladder of the mouse as a test for carcinogenic activity (1-3, 5, 9, 14). Bonser et al. (3) demonstrated the local bladder activity of 2-amino-1-naphthol hydrochloride, 1-phenylazo-2-naphthol, 1-amino-2-naphthol hydrochloride, and 3-hydroxy-4-aminodiphenyl sulfate when suspended in this vehicle, while observing a very low incidence of bladder carcinomas induced by the paraffin alone. Boyland and Watson (5) reported that 3-hydroxyanthranilic acid was carcinogenic for the mouse bladder when suspended in paraffin wax, though later they were unable to confirm this observation (1). 3-Hydroxy-DL-kynurenine, 2-amino-3-hydroxyacetophenone, and 3-hydroxyanthranilic acid, urinary metabolites of tryptophan, and 8-hydroxyquinoline were found to induce a significant incidence of benign and malignant bladder tumors in mice when suspended in cholesterol (1). However, Clayson et al. (9) were unable to confirm the activity of 3-hydroxyanthranilic acid and 8-hydroxyquinoline when tested in the same vehicle. More recently Bonser et al. (2) have used pellets of crushed paraffin wax as a vehicle in which test substances have been incorporated. With this technic the pellets are prepared in a pellet press rather than by melting the paraffin. Several compounds, including 20-methylcholanthrene and certain 2-naphthylamine derivatives, were found to be carcinogenic when this crushed paraffin vehicle was used.

Conflicting published data suggested that additional attempts should be made to test tryptophan metabolites for bladder carcinogenicity. The incidence of bladder tumors obtained in mice following the implantation of paraffin pellets containing certain tryptophan metabolites or 8-hydroxyquinoline is presented in this report.

MATERIALS AND METHODS

Preparation of chemicals.—Bioloid Embedding Paraffin1 (m.p., 53°-55°C.) was used without further purification. 8-Hydroxyquinoline, quinaldic acid, and 3-hydroxyanthranilic acid were available commercially and were recrystallized prior to use. The method of Furst and Olsen (12) was used to prepare xanthurenic acid. L-Kynurenine sulfate was synthesized by the method of Warnell and Berg (19). 8-Hydroxyquinolinal acid was prepared as described by Takahashi and Price (18). The 8-methyl ether of xanthurenic acid was synthesized as described by Price and Dodge (17), and 4,8-quinolinediol was prepared from xanthurenic acid by refluxing in Dowtherm. 3-Hydroxy-L-kynurenine was isolated from human urine as described by Brown (6).

1 Obtained from the Will Corporation, Rochester 3, N. Y.
Table 1

Incidence of Changes in Mouse Bladders and Survival of Mice Following Implantation of Various Compounds into the Bladder

<table>
<thead>
<tr>
<th>Compound</th>
<th>Per cent of calculated content found</th>
<th>No. mice given in plants</th>
<th>No. mice which died or were killed (days)</th>
<th>Squamous metaplasia</th>
<th>Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Average*</td>
<td>175-250</td>
<td>251-325</td>
<td>326-400</td>
</tr>
<tr>
<td>Paraffin alone</td>
<td></td>
<td>47</td>
<td>9</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td></td>
<td>35</td>
<td>2</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Quinaldic acid</td>
<td></td>
<td>31</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>8-Hydroxyquinolalic acid</td>
<td></td>
<td>74</td>
<td>3</td>
<td>1</td>
<td>3†</td>
</tr>
<tr>
<td>Xanthurenic acid</td>
<td></td>
<td>20</td>
<td>2</td>
<td>8</td>
<td>9†</td>
</tr>
<tr>
<td>8-Methyl ether of xanthurenic acid</td>
<td></td>
<td>24</td>
<td>2†</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>4,8-Quinolinediol</td>
<td></td>
<td>30</td>
<td>6</td>
<td>2†</td>
<td>4</td>
</tr>
<tr>
<td>L-Kynurenine sulfate</td>
<td></td>
<td>112</td>
<td>3</td>
<td>2†</td>
<td>4</td>
</tr>
<tr>
<td>3-Hydroxy-L-kynurenine</td>
<td></td>
<td>32</td>
<td>3</td>
<td>4†</td>
<td>5</td>
</tr>
<tr>
<td>3-Hydroxyanthranilic acid</td>
<td></td>
<td>80</td>
<td>32</td>
<td>7</td>
<td>5†</td>
</tr>
</tbody>
</table>

* Five pellets were analyzed from each group.
† Compounds were detected in paraffin pellets of mice dying at these times.
‡ Compounds were not detected in paraffin pellets of mice dying at these times.

Preparation of pellets.—Pellets were prepared by the method of Jull (14). Each chemical which was selected to be tested was ground to a fine powder in an agate mortar prior to being added to the paraffin. A 15 per cent suspension of quinaldic acid, or 4,8-quinolinediol, and a 20 per cent suspension of the other compounds in paraffin was prepared by melting the wax in an oven set at 60°C., adding the finely ground chemical, mixing thoroughly, and dropping the melt by means of a capillary pipette on a clean glass Petri dish. An attempt was made to obtain spheres about 3-4 mm. in diameter by molding the wax with a warm scalpel. Pellets of comparable size were also prepared from paraffin alone.

Analyses of the pellets.—Paper chromatograms were run as described by Mason and Berg (15) for the qualitative identification of the compounds remaining in the paraffin pellets removed from the mouse bladders during the carcinogenicity experiments. Methods were developed to assay the pellets in order to verify the concentration of the chemical under test in the paraffin vehicle. Ultraviolet spectrophotometric methods of analyses were used for quinaldic acid, 8-hydroxyquinoladic acid, xanthurenic acid, the 8-methyl ether of xanthurenic acid, L-kynurenine sulfate, and 3-hydroxyanthranilic acid. Pellets containing one of these compounds were dissolved in a small volume of 50 per cent CHCl₃:50 per cent of 95 per cent ethanol (v/v), and a few drops of concentrated NH₄OH were added. This solution was diluted to 25 ml. in a volumetric flask with the CHCl₃:ethanol solvent, mixed well, and a 1-ml. aliquot was diluted to an appropriate volume with 95 per cent ethanol prior to measuring the optical density in a Beckman DU spectrophotometer. A blank was prepared in the same manner, with a piece of paraffin wax of equivalent mass being dissolved instead of the pellet. The wavelength of maximal ultraviolet light absorption of the chemical was chosen for each analytical procedure with suitable consideration of the ultraviolet spectrum of paraffin to insure minimal interference from the vehicle.

Animal selection and care.—Swiss albino female mice were housed in screen-bottomed metal cages with eight or less animals in a cage. The animals were 60-90 days old at the time of surgery and were fed a stock grain diet (16) and water ad libitum. Pellets were implanted into the bladders by the surgical technic of Jull (14), as modified by Boyland and Watson (5).

Carcinogenicity studies.—Pellets were inserted into the bladders of groups of 30 to 47 animals (Table 1). The animals were allowed to survive either until 455-480 days following surgery or until fifteen or fewer animals were alive in any one group. Only animals surviving more than 175 days were evaluated for the presence of bladder carcinoma. The bladders were distended post mortem with Bouin's fixative inserted per urethram, and gross and microscopic evaluation of the lesions observed was made by the criteria of Bonser and Jull (4). Carcinomas which were not observed to invade muscle were classified as Grade I, those which were seen to invade muscle as Grade II. Only the total incidence of carcinomas was used to assess carcinogenicity. Probabilities of statistical signifi-
cance were evaluated by the exact method for $2 \times 2$ tables (11).

RESULTS

Pellets prepared in the same manner as those used in the carcinogenicity experiments (Table 1) were analyzed quantitatively for their content of test compound in an effort to ascertain how closely the actual content of these pellets resembled the calculated content. About 30 pellets were prepared for each one of the six compounds analyzed, and from this group five pellets were selected at random for analysis. A great variation in range of the actual content was found—e.g., xanthurenic acid. Since the chemical composition of these pellets was poorly reproducible, no effort was made to complete in vivo estimations of the rate of elution of these compounds from paraffin in a manner similar to that described for many of these same substances from a cholesterol vehicle (7). Paper chromatographic analysis of several pellets removed after variable periods of time in situ in the mouse bladders revealed the retention of detectable quantities of test compounds for as long as 342 days following surgery (Table 1). Quinclidic acid, 8-hydroxyquinclidic acid, xanthurenic acid, the 8-methyl ether of xanthurenic acid, and L-kynurenine sulfate were all present in pellets at some time between 246 and 342 days after initial exposure to urine. 3-Hydroxy-L-kynurenine was present at 76 days but could not be detected 275 days after pellet implantation. 3-Hydroxyxanthanilic acid and 4,8-quinolinediol could not be found in pellets removed at 256 days, and 247–260 days, respectively.

A total of 285 mice of the 336 initially subjected to the insertion of pellets (84.8 per cent) survived a minimum of 175 days and were suitable for evaluation (Table 1). Most groups had an average survival as long as or longer than that of the paraffin control group (366 days). The incidence of squamous metaplasia, benign tumors, and carcinomas observed within each group is shown in Table 1. One Grade I noninfiltrating carcinoma was found in the bladders of the 38 mice exposed to paraffin. Bonser et al. (3) reported two carcinomas in 56 mice exposed to paraffin pellets, and Chalmers and Pullinger (8) observed no carcinomas in 46 mice exposed to the bladder implantation of paraffin. The highest incidence of carcinomas was provided by the L-kynurenine sulfate group, where two of the 22 animals examined had bladder carcinoma. This incidence, however, was not significantly different from that of the control group. Benign tumors and squamous metaplasia were rarely encountered. Hyperplastic and inflammatory changes in the mucosa and bladder wall were also notable by their absence.

DISCUSSION

The paper chromatographic evidence obtained suggests that many of the compounds suspended in paraffin, and tested for carcinogenic activity in this experiment, remain for prolonged periods of time in this vehicle after insertion into the mouse bladders. The failure to detect evidence of 4,8-quinolinediol, 3-hydroxy-L-kynurenine, or 3-hydroxyxanthanilic acid in pellets after about 250 days in vivo may indicate that the pellets analyzed in this manner initially contained very low concentrations of these compounds, or that these substances were leached out of the bladder more rapidly than some of the other compounds. The demonstration of the presence of 3-hydroxy-L-kynurenine in a pellet after 76 days in vivo supports the latter possibility. Chalmers and Pullinger (8) demonstrated a variable rate of disappearance of three steroids from paraffin pellets in vivo. Allen et al. (1) found that 3-hydroxanthanilic acid was leached out of a paraffin vehicle placed in a water solution that was changed daily much more slowly than it disappeared from a cholesterol base under similar conditions. The rate of elution in vivo of xanthurenic acid, the 8-methyl ether of xanthurenic acid, 8-hydroxyquinclidic acid, 4,8-quinolinediol, 3-hydroxy-L-kynurenine, and 3-hydroxyxanthanilic acid, when suspended in a cholesterol vehicle, was determined to be much more rapid (7) than the available evidence would suggest is the case for these same compounds when present in paraffin. The apparently prolonged retention of these compounds in paraffin may prevent an adequate exposure of the mouse bladder to these test substances.

The low incidence of bladder tumors observed in the present series may mean that none of these compounds is significantly more active as mouse bladder carcinogens than the paraffin vehicle alone; or it may suggest that, because of technical difficulties, an inadequate dose of any one of these compounds was presented to the bladder mucosa. The latter possibility seems more probable than the former. Hoch-Ligeti (13) described the induction of carcinoma of the uterus in rats treated intravaginally with a contraceptive cream containing 8-hydroxyquinoline, and Ehrhart and co-workers (10) demonstrated the leukemogenic effect of 3-hydroxyxanthanilic acid administered to mice. 3-Hydroxy-DL-kynurenine, 3-hydroxyxanthanilic acid, and 8-hydroxyquinoline were found to induce a significant number of benign and malignant lesions when suspended in cholesterol (1), but, when tested in paraffin, 3-hydroxyxanthanilic acid produced only a very low, insignificant incidence of benign and malignant tumors. The demonstrations (Table 1) that the compounds under study were retained in vivo in the paraffin for long periods of time and that pellets containing widely varying quantities of test substances were probably introduced into the mouse bladders suggest that paraffin wax is not the ideal vehicle in which to test these compounds for bladder carcinogenicity, in spite of the low incidence of carcinomas induced by the paraffin alone. It therefore appears reasonable to conclude that the failures experienced here were partly caused by the technical difficulties described. For the testing of the tryptophan metabolites media should be sought that would allow the introduction of a pellet of greater uniform chemical composition into the bladder and that would favor a more rapid egress of the test compound from the pellet into the urine.

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