Absorption of $^{14}$C-labeled 3-Hydroxy-l-kynurenine and 3-Hydroxyanthranilic Acid from the Mouse Urinary Bladder under Carcinogenic Conditions

GEORGE T. BRYAN, C. R. MORRIS, AND R. R. BROWN

The Division of Clinical Oncology, University of Wisconsin Medical School, Madison, Wisconsin

SUMMARY

Cholesterol pellets containing $^{14}$C-labeled 3-hydroxy-l-kynurenine (HKYN) or 3-hydroxyanthranilic acid (HAA) were introduced into the mouse urinary bladder under conditions identical with those employed to assess bladder carcinogenicity. In 2–6 days 17–55% of the HKYN and in 2 to 12 days 12–27% of the HAA were eluted from the pellets. In both cases about 55% of eluted $^{14}$C appeared in respiratory CO$_2$, and about 30% was recovered in the feces and urine. The urinary bladder was found to retain much greater quantities of $^{14}$C after exposure to HKYN and HAA than did the kidneys, liver, heart and lungs, or carcass.

The bladder compared favorably with the i.p. space as an absorptive organ for HKYN and HAA. In 24 hr, 40% of the HKYN and 65% of the HAA instilled intraperitoneally (i.p.) into the bladder were metabolized to respiratory $^{14}$CO$_2$. In the same period, 64% of the HKYN and 59% of the HAA injected i.p. was converted to $^{14}$CO$_2$. The carcass contained 49% of the $^{14}$C from HKYN, 22% of the $^{14}$C from HAA injected i.p., and 19% from HKYN and HAA injected i.p. These data demonstrate that chemicals tested for carcinogenicity by implantation into the bladder may be absorbed by the bladder and subjected to metabolism by other organs.

MATERIALS AND METHODS

The development of the technic (18) of assessing the bladder carcinogenicity of a chemical by suspending it in a vehicle, compressing the mixture into a small pellet, and surgically inserting the pellet into the lumen of the urinary bladder of the mouse has provided the stimulus for the employment of this method by many investigators (1–3, 6, 9–11, 17). As the pellet is constantly bathed with fresh urine, the test chemical slowly diffuses out of the vehicle and comes into immediate contact with the bladder mucosa (18). It was suggested (12) that the principal advantage of this method over the standard methods of testing carcinogens was that the test substance would not be subjected to metabolism by the liver or other organs of the host. Presumably, the test compound would not be metabolized by or absorbed through the bladder wall to a significant extent. Thus, testing compounds by the bladder implantation technic would make it unlikely that metabolites of the chemical under test would be formed in large amounts (4–6).

With the use of this method, it was demonstrated (11) that 5 urinary metabolites of the essential amino acid, tryptophan, possessed carcinogenic activity for the mouse bladder when suspended in a cholesterol vehicle, and further, it was observed (8) that urine very rapidly eluted several of these active metabolites from the pellets in vivo. As 2 of these bladder carcinogens, HKYN and HAA, were available labeled with $^{14}$C, it seemed desirable to extend these studies (8–11) in an attempt to determine the fate of these chemicals during the period of acute, maximal exposure of the mouse bladder, under conditions identical with those used in the carcinogenicity experiments (8, 9, 11).

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paratus designed to keep intercontamination minimal. At
the completion of the collection, the cage floor, collection
apparatus, etc. were washed with distilled water, and the
washings were added to the urine, which was made up to
a standard volume. The feces were blended into a ho-
mogeneous suspension in distilled water. The respira-
tory CO$_2$ was collected in glass towers containing 2.5 $\times$ NaOH,
and a water aspirator was used to ensure a constant flow
rate of air through the closed system. At various times
during the pellet experiment, and at the completion of the
i.u. and i.p. injection experiments, the animals were sacri-
fied by ether anesthesia. The urinary bladder, and in
some cases the kidneys, liver, heart, and lungs, was dis-
sected carefully from the carcass; washed with distilled
water to remove hair, blood, urine, or other debris; quickly
frozen with liquid N$_2$; pulverized in a cooled (—196°C)
stainless steel block and cylinder; and suspended in dis-
tilled water. The carcass was mixed with a measured
volume of water in a Waring Blender, then filtered through
gauze to remove fragments of bone, tail, hair, etc. The
residue was washed thoroughly, and the washings were
added to the homogeneous suspension. The urine present
in the bladder and all washings from the bladder were
added to the previous urine collections.

An aliquot of the 2.5 $\times$ NaOH solution containing re-
spiratory $^{14}$CO$_2$ was precipitated with an excess of BaCl$_2$.
The suspension of BaCO$_3$ in 95% ethanol was diluted to a
final volume of 2 ml, and duplicate 0.5-nil aliquots were
pipetted onto tared aluminum planchets. Duplicate ali-
quets of the tissue, urine, and fecal samples were placed on
tared planchets before they were counted. The radioac-
tivity was determined in a thin window, automatic gas
averaged; those samples that did not fall within this ac-
ceptable range were reanalyzed. Since the total radioac-
tivity present in the animal and its feces, urine, and re-
spiratory CO$_2$ was measured, the % of activity present in
each sample was calculated from the total counts recov-
ered.

**Surgical introduction of pellets containing labeled com-
ounds into bladder.**—The preparation of cholesterol pellets
containing 20% (w/w) HKYN or HAA has been described
(8). Pellets weighing 18–22 mg and containing about
0.9 $\mu$g of $^{14}$C-labeled HKYN or HAA were weighed prior
to surgical introduction (1, 9, 18) into the mouse bladder,
and the total cpm in each pellet were calculated. After
the mouse was put in the metabolism cage, respiratory
CO$_2$ was collected over the periods 0–1 hr, 1–6 hr, and 6–
24 hr and for each 24-hr interval thereafter until the an-
imal was sacrificed. The cumulative % $^{14}$CO$_2$ exhaled
was calculated from the total radioactivity recovered. The
radioactivity remaining in the pellets was measured after
dissolving the pellets as described (8).

**Introduction of $^{14}$C-labeled HKYN or HAA i.u. and i.p.**—
An aqueous solution of HKYN (0.2 $\mu$ mole, 0.05 $\mu$ c, 0.05
ml) or a 4.72 $\times$ 10$^{-3}$ m phosphate buffer solution (pH 7.4)
of HAA (0.1 $\mu$ mole, 0.005 $\mu$ c, 0.05 ml) was instilled i.u.
in the bladder, or i.p.

To facilitate the i.u. introduction of HKYN or HAA an
incision was made in the midline of the lower half of the
abdomen of the mouse. The bladder and urethra were
exposed and dissected carefully away from the dorsal-
lying uterus and vagina. A portion of the urethra was
placed under tension, 2 ligatures were tied loosely around
the urethra, and the solution of the test compound was in-
jected directly into the bladder through a needle (0.05-ml
syringe with No. 705 needle, Hamilton Company, Inc.,
Whittier, California) carefully placed in the urethra and
guided into the lumen of the bladder under direct vision.
To prevent leakage of the solution during the instillation,
the ligatures were tightened around the needle. Follow-
ing injection, the ligatures were successively tightened and
tied as the needle was drawn out past them. The abdo-
men of the mouse was sutured, and the animal was placed
in the metabolism cage. No water or food was given dur-

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>% of Total cpm after Implantation of Pellets Containing 3-Hydroxy-L-kynurenine-keto-$^{14}$C</strong></td>
</tr>
<tr>
<td><strong>Sample</strong></td>
</tr>
<tr>
<td>Pellet</td>
</tr>
<tr>
<td>$^{14}$CO$_2$</td>
</tr>
<tr>
<td>Urine</td>
</tr>
<tr>
<td>Feces</td>
</tr>
<tr>
<td>Bladder</td>
</tr>
<tr>
<td>Kidneys</td>
</tr>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Lungs and heart</td>
</tr>
<tr>
<td>Carcass</td>
</tr>
</tbody>
</table>

* No urine or feces passed by animal.
TABLE 2

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Animal Days of Survival</th>
</tr>
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<tr>
<td></td>
<td>1.75</td>
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<td>Pellet</td>
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<tr>
<td>14CO2</td>
<td>6.1</td>
</tr>
<tr>
<td>Urine</td>
<td>1.0</td>
</tr>
<tr>
<td>Feces</td>
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<tr>
<td>Bladder</td>
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<tr>
<td>Kidneys</td>
<td>0.14</td>
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<tr>
<td>Liver</td>
<td>0.10</td>
</tr>
<tr>
<td>Lungs and heart</td>
<td>0.06</td>
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<tr>
<td>Carcass</td>
<td>3.5</td>
</tr>
<tr>
<td>Total cpm measured</td>
<td>575,000</td>
</tr>
<tr>
<td>Total estimated cpm</td>
<td>534,000</td>
</tr>
</tbody>
</table>

RESULTS

Surgical introduction of pellets containing labeled compounds into bladder.—The percentages of total cpm distributed in the pellet, CO2, urine, feces, carcass, and several organs of mice surviving for various periods of time after surgical introduction of pellets containing either HKYN or HAA are presented in Tables 1 and 2, respectively. The mean total cpm of HKYN recovered were 90 ± 20 (S.D.) % and of HAA recovered were 107 ± 3 (S.D.) % of those calculated to be originally present in the pellets. At the time of death of the animals, most of the 14C was still present in the pellets. The pellets recovered from the mice surviving the longest time after surgery retained less 14C than did the pellets recovered from mice surviving shorter periods of time. The elution rate of HKYN and HAA was slower in these mice, which were placed in a restricted environment for several days, than was found to
The urinary CO₂ contained the largest quantity of the \(^{14}\text{C}\) eluted from the pellets. Though with time a variable quantity of compound was eluted from the pellets, averages of 58.6 ± 11.7 (S.D.) % of total HKYN-\(^{14}\text{C}\) and of 55.3 ± 7.7 (S.D.) % of total HAA-\(^{14}\text{C}\) that disappeared from the pellets were metabolized to and exhaled as respiratory \(^{14}\text{CO}_2\). The cumulative respiratory \(^{14}\text{CO}_2\) exhaled by all animals during the periods of collection is presented for HKYN in Chart 1 and for HAA in Chart 2. The urine and feces contained the 2nd largest quantity of eluted \(^{14}\text{C}\). Of the \(^{14}\text{C}\) eluted from the pellet, the mean % in the urine was 14.1 ± 6.9 (S.D.) for HKYN and 18.4 ± 10.7 (S.D.) for HAA. The mean % of eluted \(^{14}\text{C}\) excreted in the feces was 20.5 ± 3.4 (S.D.) for HKYN and 14.6 ± 8.4 (S.D.) for HAA.

Low levels of radioactivity were found in the tissues and carcasses of the animals. Since a small, fairly constant fraction of the \(^{14}\text{C}\) eluted from the pellets was found in the washed tissues, the mean cpm/mg (wet weight) of tissue were computed to provide an estimate of the comparative radioactivity present (Chart 3). The bladder was found to contain a much higher specific activity after exposure to

be the case (8) in mice allowed more room for activity and exercise.

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At the end of 24 hr, 64.4 ± 12.9 (S.D.) % was present as injection of HKYN, 53.7 ± 14.0 (S.D.) % of the re the bladder contained lesser quantities of '4C. With i.p. tamed 48.5 ±9.0 (S.D.) ¶@, and the bladder contents and of the '4C recovered was found in respiratory CO2. At the these compounds.

3-hydroxy-L-kynurenicine (HKYN) and 3-hydroxyanthranilic acid covered was found in resj)iratory CO2 after 1 hr (Chart 5). HKYN or HAA than did the kidneys, liver, heart and lungs, or carcass (P<0.01).

Introduction of 14C-labeled HKYN or HAA i.u. and i.p.— 14CO2 was detected in the breath 1 hr after the i.u. instillation of HKYN (Chart 4). At the end of 6 hr, 31.8 ± 13.5 (S.D.) %, and at the end of 24 hr, 39.7 ± 10.0 (S.D.) % of the 14C recovered was found in respiratory CO2. At the completion of the study period, the carcass and feces contained 48.5 ± 9.0 (S.D.) %, and the bladder contents and the bladder contained lesser quantities of 14C. With i.p. injection of HKYN, 53.7 ± 14.0 (S.D.) % of the 14C recovered was found in respiratory CO2 after 1 hr (Chart 5). At the end of 24 hr, 64.4 ± 12.9 (S.D.) % was present as 14CO2, and the carcass and feces contained 19.0 ± 8.3 (S.D.) %. The urine and bladder contained lesser quantities of 14C at the end of 24 hr. A significantly greater quantity of 14CO2 was obtained following i.p. injection than after i.u. instillation of HKYN (0.05>P>0.02). Conversely, a highly significantly greater quantity of 14C was present in the carcass and feces following i.u. instillation than after i.p. injection of HKYN (P<0.01).

14CO2 was present in the breath 1 hr after the i.u. introduction of HAA (Chart 6). At the end of 6 hr, 38.9 ± 10.3 (S.D.) % and at the end of 24 hr, 64.9 ± 6.4 (S.D.) % of the 14C recovered was present in respiratory CO2. At the end of the study period, the carcass and feces contained 22.5 ± 8.5 (S.D.) %, and the bladder contents and the bladder contained less 14C. Following i.p. introduction of HAA, 56.8 ± 3.1 (S.D.) % was present as 14CO2 after 1 hr (Chart 7). After 24 hr, 59.5 ± 2.2 (S.D.) % of recovered 14C was present as 14CO2. The carcass and feces, urine and bladder contained lesser quantities of 14C at the end of 24 hr. A statistical comparison of the % 14C recovered as 14CO2 in the urine and bladder contents, carcass and feces, and bladder at the end of 24 hr failed to detect any signif icant differences between i.u. and i.p. instillation of HAA.

**DISCUSSION**

Radiochemical technics have been used by other investiga tors (13, 16, 17, 19–21) in an effort to study the metabolism and distribution of bladder carcinogens, or to inves tigate the mode of interaction of the carcinogen with bladder epithelium. Henson et al. (16) and Somerville et al. (21) observed in the rat and the rabbit that the bladder and the red cells are capable of retaining systemically administered 2-naphthylamine-8-14C. They also (13) compared the relative absorption by the cat urinary bladder of 2-naphthylamine-8-14C and 2-amino-1-naphthyl-8-14C hydrogen sulfate instilled into the bladder and found that the former compound was absorbed into the blood to a much greater extent than was the latter. Radomski and Brill (19) administered oral doses of 2-naphthylamine-8-14C to dogs and found that the bladder mucosa contained only 0.01 % of the administered dose after 12 to 18 hr. Irving et al. (17) attempted to detect protein binding of 2-amino1-fluorenol-1-14C hydrochloride in the bladders of mice after surgical introduction of this compound suspended in paraffin. They concluded that the binding of 2-amino-1 fluoronol was negligible. However, this compound was also inactive as a bladder carcinogen when suspended in paraffin (2, 17) or cholesterol (2) and implanted into mouse bladders. Shimizu et al. (20) employed a radioautographic technic to demonstrate radioactivity in the bladder epithelium following i.p. administration of dL-tryptophan-3H to male mice. The grain counts observed were much less when bilateral ureteral ligation was done before dL-tryptophan-3H was injected, indicating that perhaps trypto phan or some of its metabolites were carried to the bladder by the urine. No identification of the substances adsorbed to the bladder was made (20).

In the present studies, during the period of acute, maximal exposure (8) of the mouse bladder to 14C-labeled HKYN or HAA suspended in cholesterol pellets and placed into the bladder under conditions identical with those employed to demonstrate the bladder carcinogenicity of HKYN and HAA (11), the major portion of radioactivity
that was eluted from the pellet was present in respiratory CO₂, feces, and the tissues and carcass of the mouse. These data suggest that in the case of these normally occurring, physiologic o-aminophenols, the urinary bladder may permit their passage to a considerable extent and that, contrary to previous suggestions (4—6, 12), the test substance might well be metabolized by the liver or other organs of the host with the attendant formation of large amounts of metabolites (Chart 8) of the test chemicals. The greater concentration of HKYN or HAA present in the bladder (Chart 3) during the period of maximal exposure of the bladder to these carcinogens may be related to the induction of the carcinomas that subsequently developed in this organ.

Less HKYN was metabolized to respiratory CO₂ after i.u. instillation than after i.p. injection, and more ¹⁴C was present in the carcass under the former conditions than the latter. The rate at which HAA was converted to respiratory ¹⁴CO₂ after i.u. instillation was slower than after i.p. injection. However, at the end of 24 hr, no significant differences were noted in the distribution of HAA after these 2 different modes of administration. The level of ¹⁴CO₂ excretion observed in these experiments compared favorably with that reported by Hankes et al. (15) after i.p. administration of HAA to mice.

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REFERENCES

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