Transport of the Renal Carcinogen 3-Hydroxymethyl-1-[[3-(5-nitro-2-furyl)allylidene]amino] hydantoin by Renal Cortex and Cooxidative Metabolism by Prostaglandin Endoperoxide Synthetase

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ABSTRACT

Transport of the renal carcinogen 3-hydroxymethyl-1-[[3-(5-nitro-2-furyl)allylidene]amino] hydantoin (HMN) by the renal cortex and metabolism by the kidney was evaluated. Organic acid and base transport by renal cortical slices was determined using [131I]Hippuran and [14C]tetraethyl-ammonium, respectively. HMN caused a dose-dependent reversible inhibition of [131I]Hippuran accumulation but did not alter [14C]tetraethyl-ammonium uptake. By contrast, benzidine inhibited organic base but not acid transport. The decrease in absorbance at 405 nm was used as an index of microsomal metabolism of HMN. Reduced nicotinamide adenine dinucleotide phosphate-dependent metabolism of HMN was not observed with either cortical or medullary microsomes. However, there was prostaglandin endoperoxide synthetase-mediated metabolism of HMN. Specific substrate, cofactor, and inhibitor studies suggest that metabolism occurs by the prostaglandin hydroperoxidase activity of prostaglandin endoperoxide synthetase. At least one product of HMN metabolism was characterized and shown to be different from HMN by its high-pressure liquid chromatographic and ultraviolet spectral properties. The renal mixed-function oxidase system, lipid peroxidation, nitroreduction, and lipooxygenase did not seem to be involved in HMN metabolism. These results are consistent with the hypothesis that the kidney is a site for cooxidative metabolism of chemicals which elicit carcinogenic and nephrotoxic effects in the kidney. Facilitated transport of HMN into renal tissue by the organic acid transport system may explain the greater potential for HMN to elicit renal carcinogenesis compared to other tissues.

INTRODUCTION

Diets containing 0.23% HMN (29) cause renal tumors and severe nephrotoxicity in rats. Neoplastic lesions were the result of malignant transformation of the tubular epithelium. Nephropathy was characterized by necrosis of tubular epithelium and resulted in death of the animal. Information about the metabolic disposition of HMN and about the mechanism of HMN-induced nephropathy has not been reported.

The entry of certain compounds into the kidney is facilitated by special systems for handling these compounds. Organic acid and base transport systems have been well characterized in the renal cortex (7, 21). Some organic acids transported by the former system include penicillin (5), aspirin (9), and phenacetin (13). The organic acid transport system is an important contributor to the nephrotoxic effects exhibited by some compounds. Both the renal tubular secretion and nephrotoxic effects of certain organic acids are prevented by the administration of probenecid, an inhibitor of organic acid transport (5, 22).

Renal oxidative metabolism of drugs has been shown to occur by at least 2 separate independent oxidative pathways (35). Mixed-function oxidase-mediated metabolism was demonstrated. This metabolism is dependent upon NADPH and is prevented by metyrapone and carbon monoxide. The second pathway, cooxidative metabolism, is initiated by fatty acid substrates specific for prostaglandin endoperoxide synthetase and blocked by specific inhibitors of that enzyme. The mixed-function oxidase pathway predominated in the renal cortex while cooxidation predominated in the inner medulla. The 2 pathways exhibited similar activities in the renal outer medulla. These pathways have been consistently demonstrated using enzymatic assays (34, 36), optical spectra (3, 33, 34), electron paramagnetic spectra, and sodium dodecyl sulfate: gel electrophoresis (3). The mixed-function oxidase system has received considerable investigative attention. However, little attention has been given to cooxidative metabolism by prostaglandin endoperoxide synthetase.

Prostaglandin endoperoxide synthetase has been shown to cooxidatively metabolize N-[4-(5-nitro-2-furyl)-2-thiazoyl]formamide, a 5-nitrofuran compound structurally related to HMN (38). Purified prostaglandin endoperoxide synthetase has been shown to consist of 2 separate activities, fatty acid cyclooxygenase and prostaglandin hydroperoxidase (20). Cooxidative metabolism was shown to involve the hydroperoxidase activity (37). Subsequent studies have shown that cooxidative metabolism generates reactive intermediate(s) by a free radical pathway (32). [14C]Benzidine cooxidation by renal medullary slices and microsomes results in the covalent binding of radioactive material to proteins and nucleic acids (23, 32, 36). Covalent binding to protein has also been observed following cooxidative metabolism of [14C]diethylstilbestrol (8). Immunohistofluorescence techniques have localized prostaglandin synthesis to the renal tubular epithelium (28). This is also the site of HMN-induced carcinogenic lesions (29). Studies of subcellular localization of prostaglandin endoperoxide synthetase have demonstrated that the enzyme system is associated with the endoplasmic reticulum and nuclear membrane (24). This subcellular localization would make both the cytoplasm and nucleus
of cells susceptible to cooxidative damage. Cooxidative metabolism of 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene by prostaglandin endoperoxide synthetase has been shown to result in the formation of a mutagen(s) (15). In view of the fact that HMN is a specific renal carcinogen and nephrotoxin, the present study was designed to elucidate possible mechanisms of those effects by examining the renal transport and the oxidative metabolism of HMN.

**MATERIALS AND METHODS**

**Materials.** [131I]Hippuran (0.2 mCi/mg) was purchased from Mallinckrodt, Inc., St. Louis, Mo. [1-14C]Tetraethylammonium bromide (4.4 mCi/mmol) was purchased from New England Nuclear, Boston, Mass. Scintillation fluid (ACS) and Nuclear-Chicago solubilizer were purchased from Amersham Searle Corp., Arlington Heights, Il. Gas mixtures were obtained from Liquid Carbonic Corp., Chicago, Ill. Indomethacin, aspirin (acetylsalicylic acid), methemoglobin (type I, bovine), butylated hydroxytoluene, NADPH, vitamin E, Tween 20, benzidine dichlorohemazine, and 9,12,15-octadecatrienioic acid were purchased from Sigma Chemical Co., St. Louis, Mo. Cumene hydroperoxide was purchased from Fluka A. G. Chemische Fabrik, Buchs, Germany. 5,8,11,14-Eicosatetraenoic acid and 8,11,14-eicosatrienoic acid were purchased from Nu Check Prep, Inc., Elysian, Minn. Protoporphyrin IX and manganese heme were purchased from Porphyrin Products, Logan, Utah. 15-HPETE was synthetized as described previously (37). HMN was the generous gift of Clin Midy, Montpellier, France; ethoxyquin was the gift of Monsanto Chemical Co., St. Louis, Mo.; SKF-525A was the gift of Smith, Kline & French Laboratories, Philadelphia, Pa.; and prostaglandin E₂ was the gift of The Upjohn Co., Kalamazoo, Mich. The purity of HMN was greater than 99.5% as judged by high-pressure liquid, thin-layer, and gas chromatography. All other chemicals were purchased in the highest possible grade from standard sources. Male New Zealand rabbits weighing 1.5 to 2.0 kg were obtained from Eldridge Laboratory Animals, Barnhart, Mo.

**Preparation of Microsomes.** Rabbits were anesthetized with i.v. sodium thiopental (20 mg/kg). Kidneys were quickly removed and placed in ice-cold 0.9% NaCl solution, and the cortex and inner medulla were separated by careful dissection. Ram seminal vesicles were obtained fresh from a local packing plant and dissected free of adjacent tissue. Microsomes from each tissue were prepared by methods described previously (34). Prostaglandin endoperoxide synthetase was solubilized from ram seminal vesicles with 1.5% Tween 20 using procedures published previously (17). Aliquots were stored frozen at −40°. Protein content was determined in microsomes by the method of Lowry et al. (14) and in solubilized preparations by the method of Bensadoun and Weinstein (4) using bovine serum albumin as the standard.

**Microsomal Incubation Conditions and Analysis of HMN Metabolism.** The rate of oxygenation of HMN was determined by the rate of decrease in absorbance at 405 nm and at 25°. An extinction coefficient of 22.9 mM/cm for HMN was used. Kinetic measurements were made using a Beckman Acta VI recording spectrophotometer with a scale expander and a temperature control device. Reaction mixtures contained the following components: 0.1 M phosphate buffer (pH 7.8); microsomal protein; 0.025 mM HMN; 0.0012 mM methemoglobin; and various test substances in a final volume of 0.8 ml. Reactions were started by the addition of fatty acid. Following addition of arachidonoyl acid, the reaction was linear with respect to protein concentration and time. Inhibitors were added to both the sample and reference cuvets.

Inhibitors were preincubated with microsomes for 2 min at room temperature prior to addition of methemoglobin and fatty acid. All inhibitors and initiators of cooxidation were found to elicit their respective effects in a dose-dependent manner (38).

**Incubation Conditions and Analysis of HMN Transport by Renal Cortex.** The organic acid transport system was evaluated by the technique of Cross and Taggart (7) as described previously (13). Rabbit cortical slices were prepared with a Stadie-Riggs microtome. Slices were added to flasks containing 2 ml Krebs-Ringer acetate, [131I]Hippuran (2 m), and test substances as indicated in the results. The flasks were gassed with 95% O₂:5% CO₂ and incubated for 60 min at 37°. Slices were then removed and blotted on filter paper, and their radioactivity was determined. An aliquot of the medium was also measured for radioactivity. From these 2 values, the S:M was calculated.

The organic base transport system was evaluated using techniques described previously (12). Rabbit cortical slices were incubated in 2 ml of Krebs-Ringer bicarbonate buffer containing 1 mg/ml glucose. Before the addition of slices, vials containing media were equilibrated with a gas phase of 95% O₂:5% CO₂. Slices were incubated for 60 min at 37° in a shaking water bath. All vials contained 4 μM [14C]tetraethylammonium and the indicated test agents. Slices were solubilized in 1 ml of Nuclear-Chicago solubilizer following an overnight incubation at 47°. Mixtures were cooled and neutralized, and ACS was added. β-Radiation measurements were performed with a Searle ISOCAP/300 liquid scintillation system equipped with a photon monitor which prevents errors due to chemical quenching. Quench corrections were made for all samples. The S:M for [14C]tetraethylammonium was calculated as described above for [131I]Hippuran.

**High-Pressure Liquid Chromatography.** One ml of reaction mixture was extracted with 4 ml of ethyl acetate. The organic layer was washed with water, dilute bicarbonate, and again with water; dried with sodium sulfate; evaporated; and chromatographed on a Zorbax-SIL column (0.6 x 25 cm). An Instrumentation Specialties Company Model 1440 high-pressure liquid chromatograph was used. The column was eluted isocratically with methanol:hexane:methylene chloride (15:50:35) at a flow rate of 1.1 ml/min. Fractions were collected and used for spectral analyses. Results represent the mean ± S.E. of at least 3 separate experiments.

**RESULTS**

The effect of HMN on organic acid and base transport systems was evaluated (Table 1). The effects of HMN were compared to the aromatic amine carcinogen and nephrotoxin benzidine. Both organic acid and base transport systems are energy dependent. Therefore, the validity of each test system was evaluated by incubating cortical slices in the absence of oxygen. Slices incubated with 95% N₂:5% CO₂ exhibit dramatically lower organic acid and base S:M values compared to the corresponding control values. The diluent for HMN, DMSO, was also examined for its effect on transport. DMSO (final
The metabolism of HMN by cortical and inner medullary microsomes was evaluated (Chart 2). Incubation of microsomes with 0.025 mM HMN in the presence or absence of 1 mM NADPH did not result in detectable metabolism. However, following addition of 0.062 mM arachidonic acid, cortical and inner medullary metabolism of HMN was 2.2 ± 0.1 and 9.5 ± 0.7 nmol per mg protein per min, respectively. This metabolism was completely blocked by addition of 0.05 mM indomethacin. 15-HPETE elicited a dose-dependent increase in inner medullary HMN metabolism. The rate of HMN metabolism with 0.016 and 0.032 mM 15-HPETE was 4.4 ± 0.2 and 8.9 ± 0.4 nmol per mg protein per min, respectively. This metabolism was consistent with metabolism of HMN by the prostaglandin hydroperoxidase activity of prostaglandin endoperoxide synthetase.

The cooxidative metabolism of HMN was further evaluated using solubilized microsomal preparations as a source of prostaglandin endoperoxide synthetase. The substrate requirements for cooxidation of HMN by prostaglandin endoperoxide synthetase were examined (Table 2). Arachidonic acid elicited a dose-dependent increase in HMN metabolism. 8,11,14-Eicosatrienoic acid also elicited cooxidation. Prostaglandin E2 and 9,12,15-octadecatrienoic acid were not effective. Cumene hydroperoxide elicited a dose-dependent increase in cooxidation. Like the medullary preparations, 15-HPETE elicited cooxidation with the solubilized microsomal preparations.

Various agents known to preferentially alter the fatty acid cyclooxygenase or hydroperoxidase activities of prostaglandin endoperoxide synthetase were examined for their effects on HMN cooxidation (Table 3). Arachidonic acid-mediated metabolism was completely blocked by 0.025 mM indomethacin, 0.1 mM ethoxyquin, and 0.1 mM butylated hydroxytoluene. The mixed-function oxidase inhibitor SKF-525A (0.4 mM) did not

Table 1

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Organic acid (S:M)</th>
<th>Organic base (S:M)</th>
</tr>
</thead>
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<tr>
<td>95% N2:5% CO2</td>
<td>2.9 ± 0.4</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>Control</td>
<td>10.4 ± 1.2</td>
<td>12.3 ± 0.3</td>
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<tr>
<td>Diluent (0.13% DMSO)</td>
<td>11.7 ± 1.2</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td>HMN (0.5 mM)</td>
<td>4.9 ± 0.2</td>
<td>13.1 ± 0.5</td>
</tr>
<tr>
<td>Benzidine (0.5 mM)</td>
<td>9.8 ± 0.9</td>
<td>8.9 ± 0.4</td>
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</tbody>
</table>

* Mean ± S.E.
* p < 0.01 compared to corresponding control value.

Chart 1. Concentration-dependent inhibition of cortical [31]Hippuran accumulation by HMN (n = 4 or more). Bars, S.E.

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alter HMN cooxidation. By contrast, cumene hydroperoxide-mediated cooxidation was not inhibited by indomethacin. Ethoxyquin inhibited cumene hydroperoxide-mediated cooxidation, but SKF-525A was not effective. Dose-response effects of each inhibitor were observed.

The heme and heme protein requirements for HMN cooxidation were examined (Table 4). In the absence of methemoglobin, solubilized microsomes did not metabolize HMN even in the presence of arachidonic acid or cumene hydroperoxide. The methemoglobin (heme protein) requirement could not be replaced by addition of protoporphyrin IX, Mn²⁺-heme, or FeCl₃. However, HMN metabolism was not observed when methemoglobin and arachidonic acid were incubated with either heated microsomes (3 min at 100°C) or in the absence of microsomes.

High-pressure liquid chromatographic analysis was used to characterize products of cooxidative metabolism. Samples were prepared using inner medullary microsomes, extracted, and subjected to high-pressure liquid chromatographic analysis. Chart 3A is the elution profile of a control incubation containing complete reaction mixture without arachidonic acid.

### Table 3

**Effect of various test agents on arachidonic acid and cumene hydroperoxide-mediated cooxidation of HMN by solubilized ram seminal vesicle microsomal prostaglandin endoperoxide synthetase**

Where indicated, the concentration of arachidonic acid and cumene hydroperoxide was 0.062 and 0.25 mM, respectively.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions (µM)</th>
<th>Specific activity (nmol/mg protein/min)</th>
</tr>
</thead>
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<tr>
<td>Experiment 1</td>
<td></td>
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<tr>
<td>Arachidonic acid</td>
<td>Indomethacin (0.025)</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>Ethoxyquin (0.100)</td>
<td>ND⁴</td>
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<tr>
<td>Arachidonic acid</td>
<td>SKF-525A (0.400)</td>
<td>4.6 ± 0.1</td>
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<tr>
<td>Cumene hydroperoxide</td>
<td>Indomethacin (0.025)</td>
<td>10.5 ± 0.3</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>Ethoxyquin (0.100)</td>
<td>9.6 ± 0.3</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>SKF-525A (0.400)</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>FeCl₃ (2.5)</td>
<td>ND</td>
</tr>
</tbody>
</table>

⁴ ND, not detected.

### Table 4

**Effects of heme and heme protein on arachidonic acid and cumene hydroperoxide-mediated cooxidation of HMN by solubilized ram seminal vesicle microsomal prostaglandin endoperoxide synthetase**

Where indicated, the concentration of arachidonic acid and cumene hydroperoxide was 0.062 and 0.25 mM, respectively.

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<td>Methemoglobin (2.5)</td>
<td>ND⁴</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>Protoporphyrin IX (2.5)</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>Mn²⁺-heme (2.5)</td>
<td>ND</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>FeCl₃ (2.5)</td>
<td>ND</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>Methemoglobin (2.5)</td>
<td>9.8 ± 1.1</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>Protoporphyrin IX (2.5)</td>
<td>ND</td>
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<tr>
<td>Cumene hydroperoxide</td>
<td>Mn²⁺-heme (2.5)</td>
<td>ND</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>FeCl₃ (2.5)</td>
<td>ND</td>
</tr>
</tbody>
</table>

⁴ ND, not detected.

Authentic HMN exhibits a similar elution profile. In the presence of 0.125 mM arachidonic acid (Chart 3B), 2 peaks were observed. Peak II corresponds to authentic HMN and Peak I represents a product of HMN metabolism. Samples incubated with 0.125 mM arachidonic acid and 0.025 mM indomethacin are illustrated in Chart 3C. The elution times for HMN, indomethacin, and HMN metabolite are 13.2, 10.9, and 8.6 min, respectively. UV absorption spectra of Peaks I and II material are shown in Chart 4. Peak II exhibited absorbance peaks in both the 305- and 405-nm regions. By contrast, Peak I showed absorbance only in the 325- to 280-nm range, characteristic of the hydantoin moiety.

### DISCUSSION

The results are consistent with HMN transport into renal tubular cells and subsequently undergoing renal cooxidative metabolism by prostaglandin endoperoxide synthetase. The dose-dependent inhibition of [³¹¹I]Hippuran by HMN suggests that there is competition between Hippuran and HMN for transport by the organic anion transport system. It is proposed that the 5-nitro group functions as a carboxylate anion. This point is supported by studies with nitrofurantoin, 1-{[5-nitro-furfurylidene]amino}hydantoin, a 5-nitrofuran with a structure similar to HMN. Nitrofurantoin behaves as a weak acid and is accumulated in renal slices by organic anion transport (1, 27). Alternatively, HMN could act as a more nonspecific transport inhibitor rather than as a specific competitive inhibitor. How-
ever, HMN had no effect upon organic cation transport by the renal cortex. The most likely explanation of these data, therefore, is HMN transport by the organic anion transport system.

HMN was cooxidatively metabolized by prostaglandin endoperoxide synthetase. At least one product of HMN metabolism was characterized by high-pressure liquid chromatographic and UV spectral properties. In the synthesis of prostaglandins, fatty acid cyclooxygenase is responsible for the initial bis-dioxxygenation of unsaturated fatty acids (19, 20). The arachidonic acid product of the cyclooxygenase reaction, a 15-hydroperoxy prostaglandin, is subsequently reduced by prostaglandin hydroperoxidase. Arachidonic acid and 8,11,14-eicosatrienoic acid are both substrates for the cyclooxygenase enzyme, while 9,12,15-octadecatrienoic acid is not (2). Prostaglandin E₂ is the main product of rabbit renal medullary arachidonic acid metabolism (31). The present study and others (32, 33, 37, 38) have shown that prostaglandin E₂ does not initiate cooxidative metabolism. The cyclooxygenase enzyme demonstrates fatty acid specificity while the hydroperoxidase enzyme utilizes a broad range of substrates (20). This is consistent with both 15-HPETE- and cumene hydroperoxide-initiated cooxidation. Indomethacin is known to inhibit the cyclooxygenase but not the hydroperoxidase activities of prostaglandin endoperoxide synthetase (31). Therefore, arachidonic acid but not cumene hydroperoxide-mediated metabolism was inhibited by indomethacin. These results are all consistent with cooxidation being mediated by the hydroperoxidase activity of prostaglandin endoperoxidase synthetase. However, lipooxygenase products such as 15-HPETE can initiate cooxidation. Therefore, an increase in tissue fatty acid hydroperoxides as a result of tissue injury and/or inflammation could result in increased cooxidation by prostaglandin hydroperoxidase.

The heme and heme protein requirements for the cyclooxygenase and hydroperoxidase activities are different (19). Fatty acid cyclooxygenase can use either manganese heme or ferric heme while prostaglandin hydroperoxidase requires ferric heme. Utilizing the seminal vesicle preparation, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide cooxidative metabolism was demonstrated to require ferric heme (methemoglobin, hemin, or hematoporphyrin) (37). However, metabolism of [14C]arachidonic acid was similar in the presence of either manganese heme or ferric heme. Results in the present studies are consistent with a ferric heme requirement for HMN cooxidation.

The renal mixed-function oxidase system, lipid peroxidation, nitroreduction, and lipoxygenase activity did not seem to be involved in HMN metabolism. SKF-525A, a mixed-function oxidase inhibitor (26), did not inhibit HMN metabolism. In addition, NADPH, a necessary cofactor for mixed-function oxidases, was not required for cooxidation. The lack of cortical mixed-function oxidase-mediated metabolism of HMN is not related to the lack of mixed-function oxidase activity in the cortical fraction. Cortical microsomes exhibited characteristic cytochrome P-450 optical spectra, and the rate of NADPH-dependent metabolism of 1,3-diphenylisobenzofuran was similar to previous reports (33, 35). The lack of medullary mixed-function oxidase-mediated metabolism is consistent with undetectable inner medullary cytochrome 3-450 and mixed-function oxidase activities reported previously (3, 33, 35, 36). 9,12,15-Octadecatrienoic acid is susceptible to lipid peroxidation and is a substrate for lipoxygenase (11). However, this fatty acid did not initiate cooxidation. Nitroreductase activity is inhibited by oxygen (30) and has not been reported to be inhibited by indomethacin, ethoxyquin, or butylated hydroxytoluene. By contrast, prostaglandin synthesis requires oxygen (25) and is inhibited by these drugs (10).

An experimental model has been developed to describe the initial steps in HMN-induced renal cell carcinoma and nephrotoxicity. HMN enters the kidney by facilitated organic acid transport. Cells within the kidney then cooxidize HMN by the prostaglandin hydroperoxidase activity of prostaglandin endoperoxide synthetase. It is proposed that an activated cooxidative product(s) is formed which can covalently bind to macromolecules and initiate the carcinogenic process (16). Tubular epithelial cells are the site of HMN-induced lesions (29) and contain prostaglandin endoperoxide synthetase (28). Previous studies have shown that, during the cooxidative metabolism of [14C]benzidine by renal microsomes or by renal slices, 14C-labeled product(s) become covalently incorporated into proteins and nucleic acids (23, 32, 36). This model may explain the localization of their effects to the kidney and extra renal urinary system.

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