Creatinine Nitrosation To Yield 5-Oxocreatinine 5-Oxime and 1-Methyl-5-oxohydantoin 5-Oxime: Reaction Rates, Identification of *syn* and *anti* Oxime Isomers, and Their Interconversion by Nitrite†

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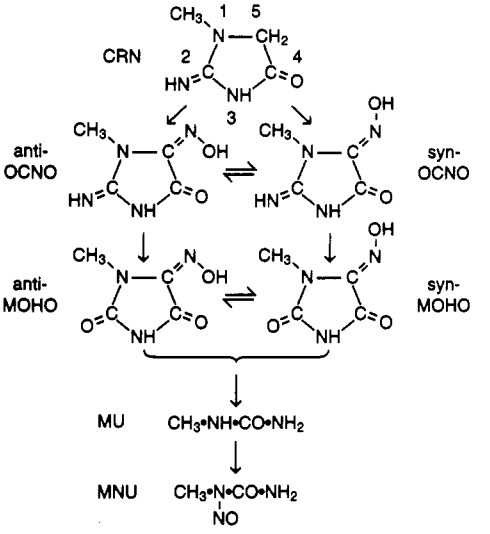
Aqueous nitrosation of creatinine (CRN) yields 5-oxocreatinine 5-oxime (OCNO) and 1-methyl-5-oxohydantoin 5-oxime (MOHO). Nitrosation of OCNO yields MOHO. Because further nitrosation of MOHO yields methylurea, which can be nitrosated to give the carcinogen methylnitrosourea, the CRN → OCNO → MOHO reactions were reinvestigated. On treatment of CRN with six batches of nitrite at pH 1, MOHO yield but not OCNO yield dropped sharply as the nitrite level was reduced. MOHO yield was 0.8% from CRN and 11.2% from OCNO under similar conditions. For CRN and OCNO nitrosation by single batches of nitrite, the pH maximum was 2.7–3.0 and third-order kinetics were followed, with reaction rates proportional to [nitrite].[2] OCNO showed pK_a values of 2.7 and 8.9. For OCNO nitrosation, the stoichiometric and real rate constants were, respectively, 17 and 0.4 times those for CRN nitrosation. Four products of a OCNO nitrosation experiment were separated by HPLC and provisionally identified as *syn*- and *anti*-OCNO and *syn*- and *anti*-MOHO, with a predominance of the *anti* isomers. Nitrite catalyzed the isomerization of all four oximes (a novel effect of nitrite). Nitrosation of CRN to give methylnitrosourea may be too slow and complex to present a hazard.

INTRODUCTION

Large amounts of creatinine (CRN) occur in some foods; e.g., dried fish contains 4.1 g of CRN/kg and fried bacon 3.3 g of CRN/kg (Mirvish *et al.*, 1981). CRN is produced by the dehydration of creatine when these foods are dried or fried (Mirvish *et al.*, 1982). Hence, even a tiny conversion of CRN to carcinogens could present a hazard. Fried beef contains the mutagen and carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), which is formed from creatine (or, presumably, CRN), phenylalanine, and glucose (Skog and Jägerstad, 1991). Also, CRN is nitrosated to give 5-oxo-CRN 5-oxime (OCNO) and 1-methyl-5-oxohydantoin 5-oxime (MOHO; Scheme I) (Schmidt, 1912; Greenwald and Levy, 1948; Archer *et al.*, 1971). (These oximes were incorrectly named without the 5-oxo prefix in all previous reports.) Nitrosation of CRN at C-5 produces OCNO, which is deaminated by nitrosation of the primary amino group at C-2 to give MOHO. Dried fish and fried bacon yielded methylurea (MU) when they were treated with nitrite and then denitrosated (Mirvish *et al.*, 1980). The MU precursor in dried fish was identified as CRN (Mirvish *et al.*, 1982). MU is readily nitrosated to give methylnitrosourea (MNU), a potent carcinogen (Mirvish, 1975). Hence, MNU could arise from CRN via the sequence CRN → OCNO → MOHO → MU → MNU (Scheme I).

To help establish the significance of these reactions, we now report studies on the rates and kinetics of OCNO and MOHO formation from CRN and of MOHO formation from OCNO. We wished to determine whether these reactions are likely to occur in food products or under the acidic conditions (from pH 0.9 for fasting gastric juice to pH 5.5 just after a meal) of the human stomach. We also describe the provisional identification of *syn* and *anti* forms of OCNO and MOHO and their isomerization by nitrite.

Scheme I. Structure (with Numbering) of CRN and Its Conversion by Nitrite to *syn*- and *anti*-OCNO and -MOHO and to MU and MNU



METHODS

1. Synthesis and UV Spectra of OCNO and MOHO. Solutions of 6.1 g of CRN in 10% HCl were added dropwise to aqueous solutions of $NaNO_2$ (analytical reagent grade, Mallinckrodt Inc., Paris, KY). The mixtures were reacted without cooling to prepare OCNO or at 0 °C to prepare MOHO (Archer *et al.*, 1971). The crude precipitates were recrystallized from hot water. The products of six syntheses of OCNO, each from 6.1 g of CRN, were combined to give 9.66 g (21%) of OCNO, mp (dec) 250 °C. MOHO yield from 6.1 g of CRN was 1.62 g (21%), mp (dec) 192–194 °C (lit. mp 255 °C for OCNO and 180–191 °C for MOHO). UV maxima in water: 232 nm (ε, 6300) for CRN, 250 (11 400) and 290 (5400) nm for OCNO, and 225 (7500) and 282 (6900) nm for

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† Dedicated to Dr. Rolf Preussmann, Deutsches Krebsforschungszentrum, Heidelberg, Germany, on the occasion of his 65th birthday.

0021-8561/93/1441-2051$04.00/0

MOHO. UV spectra of OCNO were determined at intervals of ≤1 pH unit from pH 1 to pH 12 using citrate, phosphate, and borax buffers. UV maxima for OCNO were at 231 (9900) and 285 (9700) nm at pH 1, 251 (15 000) and 296 (5200) nm at pH 4, 252 (14 700) and 296 (5100) nm at pH 7, and 269 (10 200) and 312 (10 600) nm at pH 12.

2. Extent and Kinetics of CRN and OCNO Nitrosation. (*a*) *Method A.* $NaNO_2$ in 30 mL of water was added in six equal portions over 2–3 min every 30 min (total reaction time, 3 h) to solutions of CRN, OCNO, or MOHO (1 mmol) in 145 mL of water in a thermostated water bath at 25 °C. The pH was adjusted with HCl to 1.0 after each nitrite addition. Solutions were stirred only during nitrite additions. Volumes were then brought to 250 mL with water, and pH was readjusted to 1.0.

(*b*) *Method B.* A single batch of $NaNO_2$ was added to CRN or OCNO solutions, which were adjusted to the desired pH with HCl and reacted for 1–3 h in a water bath at 25 °C. Reactions were otherwise performed as in method A, except that OCNO was nitrosated at 10% of the usual scale.

(*c*) *Steps Common to Both Methods.* For reactions with >100 mM nitrite, a 5-mL sample was rotary-evaporated for 10 min at 20–25 Torr and 22–25 °C to remove most nitrogen oxides. In all reactions, ammonium sulfamate (10% solution, 0.7 g/g of $NaNO_2$) was added dropwise to destroy the remaining nitrite. Ten milliliters of 0.02 M sodium phosphate buffer at pH 6.0 (buffer A) and NaOH or HCl were added to bring the volume to 10 or 20 mL and the pH to 6.0. Solutions were stored overnight at 6 °C.

(*d*) *HPLC.* Samples of solution (20 or 100 μL) were subjected to HPLC on a 250 × 4.6 mm column of 5-μm C_{18} Ultrasphere ODS (Alltech, Arlington Heights, IL), which was developed with buffer A–methanol (19:1) at 0.8 mL/min and usually monitored at 267 nm. CRN, X, OCNO, Y, and MOHO were typically eluted at 6.0, 8.3, 9.2, 10.4, and 14.2 min, respectively (CRN was detected at 230 nm). Yields were calculated by comparing peak areas with those of standard solutions of OCNO (for OCNO and X) or MOHO (for MOHO and Y). UV in buffer A at 267 nm: ε, 0 (CRN), 7700 (OCNO) and 5200 (MOHO). The detection limit of OCNO or MOHO was 50 ng. MOHO yields from OCNO were corrected for 2.0% MOHO present in the OCNO sample.

3. Isolation of *syn*- and *anti*-OCNO and *syn*- and *anti*-MOHO. Two batches of 14.2 mg of OCNO were each reacted for 3 h at 25 °C with 200 mg of $NaNO_2$ in 20 mL of water adjusted with HCl to pH 2.7. The combined product was worked up as in section 2c to give 70 mL of solution, and a sample was analyzed by HPLC (section 2d). Seven 10-mL samples of the product were subjected to preparative HPLC on a 50 × 2.2 cm column of 10 μm P_{10} ODS 3/M20-50 (Whatman Inc., Clifton, NJ), eluted at 9 mL/min, and monitored at 267 nm. X, OCNO, Y, and MOHO were eluted at 17, 20.2, 26.1, and 40.5 min, respectively. Corresponding fractions from each HPLC were combined, evaporated at <3 Torr and 35 °C until salts began to precipitate, stored at 6 °C over $CHCl_3$, and filtered.

To desalt the filtrates, 10-mL lots of each fraction were subjected to a second preparative HPLC on the same column as before, with elution by double-distilled water. Retention times were similar to but more variable than those found previously. Each fraction was combined from several HPLC runs and evaporated as before to give the pure oximes, which were dissolved in water (100 mL for OCNO and MOHO, 10 mL for X and Y) and stored at 6 °C over $CHCl_3$. Samples of each solution were (a) subjected to analytical HPLC; (b) evaporated and analyzed on a modified AEI-MS-902 mass spectrometer by the electron impact method using a probe; (c) evaporated, suspended in ice-cold D_2O, dissolved by adding DCl dropwise, and immediately subjected to ^1H NMR analysis on a Varian XL-300 (300 MHz) instrument; and (d) treated with nitrite (see Results).

RESULTS

Mutagenicity Test of OCNO and MOHO. Solutions of 0.25, 0.5, and 1.0 mg of OCNO or MOHO/100 μL of dimethyl sulfoxide per plate were not mutagenic to strains TA-1535 and TA-98 of *Salmonella typhimurium* in the Ames test (Maron and Ames, 1983), performed using 100 μL of solution/plate with and without Aroclor-induced rat liver microsomes. These strains are sensitive to point and frame-shift mutations, respectively. Positive and negative controls gave satisfactory results.

Table I. Yields of OCNO and MOHO after Treatment of 1 mmol of CRN, OCNO, or MOHO with 6 Batches of Nitrite Added over 3 h at pH 1 and 25 °C[a]

compd treated	total $NaNO_2$, g	no. of expts	yield or recovery, % (mean ± SD) OCNO	MOHO
CRN	31	3	4.0 ± 0.6	39 ± 13
	15.5	3	6.5 ± 1.6	19 ± 12
	7.8	3	6.7 ± 0.4	13 ± 4
	3.9	6	6.3 ± 3.5	3.8 ± 2.8
	2.0	6	5.0 ± 1.4	0.6 ± 0.3
	1.0	6	1.6 ± 0.4	0
OCNO	2.0	3	81 ± 9	11.2 ± 0.6
	1.0	2	83 ± 1	4.6 ± 0.5
	0.5	4	10.8 ± 18	1.8 ± 0.4
	0.25	2	12.6 ± 2	1.2 ± 0.5
	0.125	2	95 ± 2	0.3 ± 0.1
	0.063	4	102 ± 8	0.2 ± 0.1
	0.032	4	99 ± 3	0
MOHO	31	5		60 ± 16

[a] Nitrosations were carried out by method A.

Extent and Kinetics of CRN and OCNO Nitrosation. OCNO and MOHO yields were determined by HPLC after nitrosation of CRN and OCNO by six portions of $NaNO_2$, added over 3 h at pH 1 (Table I). As the total amount of $NaNO_2$ was reduced from 31 to 1.95 g, OCNO yield from CRN remained relatively constant at 4.0–6.7% and was still 1.6% with 1.0 g of $NaNO_2$, but MOHO yield from CRN dropped sharply, with no MOHO detected when 1.0 g of nitrite was used. This was consistent with the sequence of reactions CRN → OCNO → MOHO. The yield of MOHO from OCNO was much greater than that from CRN; e.g., with 2 g of $NaNO_2$, the MOHO yield was 0.6% from CRN and 11.2% from OCNO (Table I). MOHO was detected when OCNO was treated with only 63 mg of $NaNO_2$. When MOHO was treated with the highest amount (31 g) of $NaNO_2$, 40% was lost (Table I, last row). In the absence of nitrite, aqueous solutions of CRN, OCNO, and MOHO were stable for 3 h at pH 1 and 25 °C.

Tables II and III show OCNO and MOHO yields from CRN and MOHO yields from OCNO when single batches of nitrite were added. OCNO yield from CRN was relatively constant at 1.4–5.7%, except at the lowest nitrite level of 36 mM. The pH maximum was 3.0 for CRN and 2.7–3.0 for OCNO nitrosation. These tables include values of the rate constants k_1 (eq 1) and k_2 (eq 2) for OCNO plus MOHO formation from CRN and for MOHO formation from OCNO.

$$\text{rate} = k_1[\text{amine}][\text{nitrite}]^2 \quad (1)$$

$$\text{rate} = k_2[R_2NH][HNO_2]^2 \quad (2)$$

$$\text{rate} = k_3[R_2NH][H^+][HNO_2] \quad (3)$$

In the absence of catalysis, *N*-nitrosation usually follows eqs 1 and 2 or eq 3 (Mirvish, 1975; Mirvish *et al.*, 1991). Equation 1 involves the stoichiometric concentrations (irrespective of state of ionization) of the reactants. Equations 2 and 3 involve concentrations of the reactive species, HNO_2 and the unprotonated amines. Table IV gives the mean values for k_1 and k_2. The mean k_1 values are derived only from the k_1 values at pH 3 (CRN

Table II. OCNO and MOHO Yields on Nitrosation of CRN with a Single Batch of Nitrite at 25 °C[a] and Rate Constants k_1 (eq 1) and k_2 (eq 2)[a]

pH	nitrite, mM	CRN, mM	reaction time, h	no. of expts	% yield (mean ± SD) OCNO	% yield (mean ± SD) MOHO	10^2k_1, M^{-2} min^{-1}	k_2, M^{-2} min^{-1}
1.0	320	5	3	5	5.7 ± 0.7	1.0 ± 0.2	0.039	25
2.0	290	5	3	3	3.3 ± 0.2	6.5 ± 1.2	0.71	4.9
2.7	290	5	1	2	3.4 ± 0.7	13 ± 2	3.5	6.6
3.0	290	5	1	4	3.7 ± 0.6	18 ± 1	4.9	6.5
3.3	290	5	1	2	3.2 ± 0.1	16 ± 1	4.3	4.9
3.6	290	5	1	3	3.5 ± 0.7	14 ± 3	3.8	4.8
4.0	290	5	3	3	2.8 ± 0.5	12 ± 4	1.1	2.0
3.0	36	5	1	2	0.5 ± 0	0.1 ± 0	9.0	11.9
3.0	72	5	1	2	1.4 ± 0.2	0.6 ± 0.1	7.0	9.2
3.0	145	5	1	2	3.2 ± 0.2	3.8 ± 0.6	6.0	7.9
3.0	290	2.5	1	3	3.8 ± 0.2	16 ± 1	4.5	6.0
3.0	290	1.25	1	3	4.6 ± 0.2	17 ± 2	4.9	6.5

[a] Nitrosation was performed by method B. Values of k_1 were obtained from the integral form of the third-order rate equation derived from eq 1 (first order for base, second order for nitrite) (Frost and Pearson, 1961), taking the product as the sum of OCNO and MOHO yields. Values of k_2 (eq 2) were derived from k_1 using eq 4.

$$k_2 = k_1[1 + \text{antilog}(pK_a - pH)][1 + \text{antilog}(pH - 3.36)]^2 \quad (4)$$

This was obtained by combining eqs 1 and 2 and applying the Henderson–Hasselbach equation, in which the acids are HNO_2 and $R_2NH_2^+$, the bases are NO_2^- and R_2NH, the 3.36 term is the pK_a of HNO_2 (Turney and Wright, 1959), and the pK_a term is 4.8 [the pK_2 of CRN (Perrin, 1965)].

Table III. MOHO Yield from OCNO after Nitrosation with a Single Batch of Nitrite and Rate Constants k_1 (eq 1) and k_2 (eq 2)[a]

pH	nitrite, mM	OCNO, mM	reaction time, h	no. of expts	MOHO yield, % (mean ± SD)	k_1,[b] M^{-2} min^{-1}	k_2,[c] M^{-2} min^{-1}
1.0	83	5.7	3	2	4.5 ± 2.1	0.043	2.2
2.0	72	5.7	3	3	17.7 ± 5.1	0.24	1.6
2.4	72	1.0	3	3	34 ± 6	0.46	1.7
2.7	72	1.0	3	6	46 ± 5	0.68	2.0
3.0	72	1.0	3	4	45 ± 4	0.66	2.0
3.3	72	1.0	3	2	39 ± 1	0.55	2.4
4.0	83	5.7	3	3	4.0 ± 1.0	0.037	1.1
2.7	18	1.0	1.5	3	5.3 ± 0.4	1.98	5.9
2.7	36	1.0	1.5	3	12.2 ± 1.8	1.15	3.4
2.7	72	1.0	1.5	3	31.7 ± 4.1	0.83	2.5
2.7	72	0.5	1.5	3	31.5 ± 4.2	0.82	2.4
2.7	72	0.25	1.5	3	33.8 ± 0.5	0.89	2.6

[a] Nitrosations were performed by method B. [b] The k_1 values were calculated as in Table II and are based on MOHO yield. [c] The k_2 values were derived from k_1 as in Table II but taking pK_a as 2.7, the lower pK_a of OCNO.

Table IV. Means of k_1 and k_2 Values in Tables II and III

reaction	values as M^{-2} min^{-1}, mean ± SD (no. of results) k_1	k_2
CRN → OCNO + MOHO	0.061 ± 0.017 (6)[a]	5.6 ± 1.7 (9)[b]
OCNO → MOHO	1.06 ± 0.48 (6)[c]	2.2 ± 0.6 (11)[d]

[a] For all results at pH 3. [b] For all results except those at pH 1 or with ≤72 mM nitrite. [c] For all results at pH 2.7. [d] For all results except that with 18 mM nitrite.

nitrosation) or pH 2.7 (OCNO nitrosation), because k_1 varied (and is expected to vary) with pH.

The pK_a values used to derive k_2 from k_1 (Table II) included a value of 2.7 for OCNO. This pK_a was calculated from the sharp shift of the lower of two UV maxima of OCNO from 231 nm at pH 1 to 251 nm at pH 4–7 (see Methods). A second pK_a of 8.9 for OCNO was derived from the sharp shift of its upper UV maximum from 295 nm at pH 8 to 310 nm at pH 10. According to its UV spectrum, OCNO was stable in 0.1 N HCl or 0.1 N NaOH for 1 h at 21–23 °C. We attribute the pK_a of 2.7 to protonation of the guanidine group (probably at N-2) by analogy to CRN. We attribute the pK_a of 8.9 to deprotonation of the oxime group, because the pK_a for oximes with α-keto groups (resembling the 4-carboxyl group of OCNO) is 8.4–9.5; e.g., it is 9.3 for biacetylmonoxime (Green and Saville, 1956). The UV spectrum of MOHO did not change sharply with pH.

Values for k_3 (eq 3) varied much more than those for k_2; e.g., for CRN nitrosation, k_3 for row 4 was 180 times that for row 1 of Table II. For OCNO nitrosation, k_3 for row 4 was 31 times that for row 5 of Table III. Hence, both nitrosations mainly followed eqs 1 and 2. Nevertheless, some reaction of CRN by eq 3 was suggested by the high k_2 at pH 1 and with 36 and 72 mM nitrite at pH 3. Reaction by eq 3 is favored at low pH and low nitrite concentration because its rate is proportional to $[H^+]$ and $[HNO_2]$ (and not to $[HNO_2]^2$). The extensive decomposition of HNO_2 at pH 1 (Mirvish *et al.*, 1975) would also raise the apparent k_2.

Table V. Mass Spectra of OCNO, X, MOHO, and Y

ions[a]	m/z	"OCNO" (*anti*-OCNO), %[b]	"X" (*syn*-OCNO), %	m/z	"MOHO" (*anti*-MOHO), %	"Y" (*syn*-MOHO), %
M	142	71	79	143	47	54
M − OH	125	100	100	126	100	100
M − NO	112	26	18	113	10	10
M − HNCO	99	12	14			
$CH_3NC{\cdot}NOH$	72	33	24	72	45	69
$OC{\cdot}NCO$				70	10	11
$OC{\cdot}NH{\cdot}CN$	69	21	31			
$CH_3{\cdot}NH{\cdot}CN$	56	68	27	56	7	10
C_2H_2NO				56	7	9
$CH_3{\cdot}NCN$	55	66	32	55	7	11

[a] Accurate masses of all peaks agreed with the assigned formulas to within 4 millimass units. [b] Percent relative intensity.

Isolation and Provisional Identification of *syn*- and *anti*-OCNO and *syn*- and *anti*-MOHO. Our OCNO sample contained 10% of peak X, eluted on HPLC just before OCNO. Our MOHO sample contained 5% of peak Y, eluted between OCNO and MOHO. The four compounds were isolated from a mixture which was obtained by nitrosating 28.4 mg of OCNO and contained X, OCNO, Y, and MOHO in the ratio 21:33:5:41, respectively. The four compounds were separated by preparative HPLC. Inorganic salts were removed by a second HPLC of each fraction, with elution by water. Analytical HPLC of the individual fractions showed only single peaks. We obtained 150, 4800, 670, and 8000 μg of X, OCNO, Y, and MOHO, respectively. All four compounds were stable for 24 h at 6 °C in distilled water and for 2 h at pH 1 and 25 °C, as indicated by HPLC.

The mass spectra of OCNO and MOHO (Table V) were similar to those reported by Archer *et al.* (1971). The mass spectra of OCNO and X were similar except for more prominent peaks at *m/z* 56 and 55 for OCNO than for X, and those of MOHO and Y were similar. This indicated that X was an isomer of OCNO and Y was an isomer of MOHO. In particular, the molecular (M), M − OH, and M − NO ions at *m/z* 142, 125, and 112 were similar for OCNO and X; and the M, M − OH, and M − NO peaks at *m/z* 143, 126, and 113 were similar for MOHO and Y. The apparent ketocyanamide peak (*m/z* 69, C_2HN_2O, ? $OC{\cdot}NHCN$) in the spectra of OCNO and X probably arose from C-2 to C-4. The apparent methylcyanamide peak (*m/z* 56, $C_2H_4N_2$, ? $CH_3{\cdot}NH{\cdot}CN$) occurred mainly in the

Table VI. Products Obtained after Nitrite Treatment of *syn* and *anti* % Isomers of OCNO and MOHO[a]

reactant		nitrite,	no. of	products, % initial reactant (mean ± SD)				
name	amount, μg	mM	expts	*anti*-OCNO	*syn*-OCNO	*anti*-MOHO	*syn*-MOHO	total[b]
anti-OCNO	100	145	3	61 ± 4	21 ± 1	29 ± 12	1 ± 1	113
anti-OCNO	100	14.5	3	97 ± 13	17 ± 2	0	0	113
anti-OCNO	100	1.45	3	123 ± 13	2 ± 1	0	0	125
anti-OCNO	100	0[c]	1	99	0	0	0	99
syn-OCNO	75	145	3	18 ± 1	39 ± 12	16 ± 6	8 ± 2	81
anti-MOHO	335	145	3	0	0	89 ± 9	5 ± 0	94
anti-MOHO	335	14.5	3	0	0	94 ± 5	4 ± 1	98
anti-MOHO	335	1.45	1	0	0	92	0	92
anti-MOHO	335	0[c]	1	0	0	98	0	98
syn-MOHO	200	145	3	0	0	39 ± 2	57 ± 3	96

[a] Aqueous solutions (5 mL) of reactant and $NaNO_2$ were adjusted to pH 2.7 with HCl, diluted to 10 mL, and kept for 3 h at 25 °C. Reactions were stopped and analyzed by HPLC as in sections 2c and 2d under Methods. [b] Values >100% are attributed to experimental error. [c] In runs without nitrite, reactant solutions were incubated for 3 h at pH 1.

spectra of CRNO and X, where it probably arose mostly from N-1 to C-2, and also occurred as a minor peak in the spectra of MOHO and Y, where it may have arisen from N-1 to N-3. MOHO and Y also showed a peak at *m/z* 70 (C_2NO_2, ? OC·NCO), which was probably derived from C-2 to C-4.

The ^1H NMR spectra in D_2O·DCl showed methyl peaks at 3.35, 2.94, 2.92, and 2.63 ppm for OCNO, X, MOHO, and Y, respectively. In the *syn* isomers (with the hydroxy *syn* to the *N*-methyl group), the hydroxy groups are expected to deshield and cause an upfield shift of the methyl protons more than the hydroxy groups of the *anti* isomers, as occurs with the *syn–anti* isomers of nitrosamines (Karabatsos and Taller, 1964). On this basis and because the major products are most likely to be the less hindered *anti* isomers, we provisionally identify X, OCNO, Y, and MOHO as, respectively, *syn*- and *anti*-OCNO and *syn*- and *anti*-MOHO (Scheme I).

Treatment of Individual Isomers of OCNO and MOHO with Nitrite. Samples of each of the four purified isomers were treated with nitrite for 3 h at pH 2.7 and 25 °C. The products were analyzed by HPLC (Table VI). Nitrosation of *syn*- and *anti*-OCNO by 145 mM nitrite produced about 50% yields of the two isomers of MOHO. *anti*-OCNO produced almost entirely *anti*-MOHO, whereas *syn*-OCNO yielded more *anti*- than *syn*-MOHO. Treatment of *anti*- and *syn*-OCNO with 145 mM nitrite also isomerized 26 and 32%, respectively, of the recovered OCNO. The same treatment isomerized 5% of *anti*-MOHO and 39% of *syn*-MOHO. Whereas the OCNO → MOHO reaction required 145 mM nitrite, isomerization of *anti*-OCNO and *anti*-MOHO was detected with only 1.45 and 14.5 mM nitrite, respectively. The oximes did not isomerize at pH 1 in the absence of nitrite (Table VI). Hence, (a) isomerization of OCNO and MOHO was effected by lower nitrite concentrations than those needed to convert OCNO to MOHO, and (b) the *syn*-OCNO → *anti*-MOHO and *syn*-MOHO → *anti*-MOHO reactions were much more extensive than the corresponding *anti* → *syn* reactions, as expected if *anti*-MOHO was the less hindered isomer. There was little evidence that *anti*-OCNO was more favored than *syn*-OCNO.

DISCUSSION

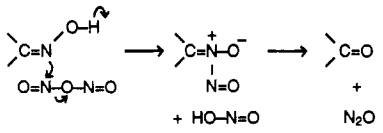
CRN was initially nitrosated with six additions of nitrite at pH 1 (Table I) because these conditions had been used to study MU formation from certain foods and from CRN (Mirvish *et al.*, 1980, 1982). Nitrite was added in six lots to maintain [HNO_2] above 50 mM, because high concentrations of HNO_2 rapidly decompose to NO and HNO_3 (Turney and Wright, 1959; Mirvish *et al.*, 1975). When 31 and 1.0 g (2.6 and 0.08 M) of $NaNO_2$ were used to nitrosate CRN, MOHO yields were 39 and 0%, respectively (Table I). When CRN was treated with 2.1 and 0.1 M nitrite at pH 1 and was then treated with acid to convert MNU to MU, the MU yields were 2.7 and 0.02%, respectively (Mirvish *et al.*, 1982). When CRN, OCNO, and MOHO were treated with 0.1 M nitrite under similar conditions, MU yields were 0.02, 0.02, and 7.0%, respectively. The finding that MU yield from CRN paralleled that of OCNO and the relatively high MU yield from MOHO indicate that MU arose from CRN via its conversion to MOHO. Our results are somewhat higher than those of Sen and Seaman (1988) and N. P. Sen (personal communication), who found that reaction of CRN with 0.4 M nitrite for 1 h at pH 1 gave only a 0.0003% yield of MNU as determined by HPLC–thermal energy analysis.

The kinetic results (Tables II–IV) demonstrate that the nitrosation of both CRN and OCNO mainly followed eqs 1 and 2, with a probable contribution under some conditions from eq 3. The low pK_a of 2.7 for OCNO compared to 4.8 for CRN is attributed to the electron-withdrawing effect of the oxime group in OCNO, which would hinder protonation, probably at N-2. Although CRN is nitrosated at a carbon atom (C-5), the kinetics are typical for *N*-nitrosation and show that only unprotonated CRN was nitrosated. Protonation of the guanidine group must impart sufficient positive charge to C-5 to prevent its nitrosation. According to the mean k_1 and k_2 values in Table IV, nitrosation of CRN proceeded 17 times more slowly than that of OCNO, whereas nitrosation of unprotonated CRN proceeded 2.5 times more readily than that of unprotonated OCNO. Hence, the slower nitrosation of CRN compared to CRNO is due to the higher pK_a of CRN, which results in only a small fraction of CRN being unprotonated and available for nitrosation at the optimum pH of 2.7–3.0.

The *syn* and *anti* forms of OCNO and MOHO did not isomerize readily, similar to aryl ketoximes and unlike most aliphatic ketoximes (Smith, 1966, p 32). However, these isomerizations were catalyzed by nitrite, with a tendency for the *anti* isomer to predominate in the case of MOHO (Table VI). This is apparently the first report of such an effect of nitrite. The mechanism of this reaction is unknown. *N*-Nitroso oximes are assumed to form when oximes are nitrosated, because N_2O and the parent carbonyl compounds are slowly produced (Scheme II) (Manning and Stansbury, 1959; Smith, 1966, p 66). However, these products are not expected to regenerate the isomerized oximes.

Our results suggest that CRN nitrosation to give MNU in food or in the stomach may not present a significant hazard because (a) MNU production from CRN involves many successive steps, of which at least three (CRN →

Scheme II. *N*-Nitrosation of Oximes and Generation of N_2O and the Carbonyl Compound



OCNO, OCNO → MOHO and MU → MNU) involve reaction with nitrite, and (b) the initial CRN → OCNO reaction is slow, with a k_1 just above that for the most slowly nitrosated amine in Table 2 of the review by Mirvish (1975). The corresponding k_2 value was about 1% of that for most amines, which demonstrates the slowness of *C*-compared to *N*-nitrosation. OCNO was not carcinogenic when injected into newborn mice (Wogan *et al.*, 1975), and OCNO and MOHO were not mutagenic in the Ames test (see Results). Nevertheless, dried salted fish contains 4.1 g/kg of CRN (Mirvish *et al.*, 1982), its use has been associated with stomach cancer, and nitrosamides related to MNU induce stomach cancer in rats (Mirvish, 1983). For these reasons and because MOHO yields MU much more readily than does CRN or OCNO (Mirvish *et al.*, 1982), it may be worthwhile to establish the facility of the MOHO → MU reaction and to determine if MOHO occurs in dried salted fish, even though neither OCNO nor MOHO was detected polarographically in nitrite-preserved meat (Davidek *et al.*, 1976).

ACKNOWLEDGMENT

We thank Dr. N. P. Sen for providing details of his experiments, Drs. B. Gold and L. Keefer for useful discussions, and J. Williamson for conducting the mutagenicity tests. This study was supported by NIH Grant R01-CA-30593 and Core Grant CA-36727 from the National Cancer Institute, Grant 89836 from the American Institute for Cancer Research, and Core Grant SIG-16 from the American Cancer Society.

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Received for review February 19, 1993. Revised manuscript received July 29, 1993. Accepted August 6, 1993.[⊛]

[⊛] Abstract published in *Advance ACS Abstracts,* October 1, 1993.