Formation of Nitrosoproline in Rats (37160)

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Since the observation by Magee and Barnes in 1956 (1) that nitrosodimethylamine will induce hepatic cancer in the rat, increasing attention has been given nitrosamines as potential carcinogenic agents. It has been found that the nitrosamines are, in general, potent carcinogens (2–4). Nitrosamines will form *in vitro* through the nitrosolation of secondary amines under conditions of pH, etc., similar to those of the stomach (5), and certain secondary amines are nitrosolated in the stomach of rats (6, 7). The reaction also occurs *in vitro* in aspirated gastric juice (8, 9). Although nitrosamines have been detected in a number of natural products, with a single exception (10, 11) the levels have been found to be low (12–14). The significance of exposure to these levels of nitrosamines has not been determined (15–18).

L-Proline and hydroxy-L-proline are the secondary amines to which the population is exposed to the greatest extent. *N*-Nitroso-L-proline (NSoP) and its decarboxylation product, the carcinogen nitrosopyrrolidine, might be expected to present the greatest endogenous hazard of the nitrosamines.

Since nitrite ion is absorbed in the stomach and foregut, and the imino groups of proline and hydroxyproline are not available for nitrosolation until the imino-peptide bond is hydrolyzed by the specific intestinal peptidase, prolidase, normally ingested nitrite ion and free imino acids are not likely to be found together in the gastrointestinal tract. However, current trends in the use of protein hydrolysates as flavoring agents, either in conjunction with or in place of monosodium glutamate, greatly increase the potential for free imino acids and nitrite ion being present together under chemical conditions which are very favorable to nitrosolation (19). Therefore, we investigated the possibility of *in vivo* formation of NSoP from L-proline and sodium nitrite in rats.

During these studies we attempted to prepare NSoP by several methods in which sodium nitrite is added to an acidic solution of the imino acid (20–22); however, we were unable to obtain yields in the quantity desired. The method of Heyns and Königsdorf (23) was modified to overcome this difficulty; yields were generally good and the nitrosolated compound was free of inorganic salts as by-products.

Methods and Materials. L-proline was obtained from the Mann Research Company and L-[^{14}C]-proline (uniformly labeled, 214 mCi/mmole) was obtained from the New England Nuclear Corporation. Radioactivity measurements were made with a Packard Model 3375 liquid scintillation spectrometer, using a scintillation phosphor prepared by dissolving 4.0 g of 2,5-diphenyloxazole (PPO, Packard Instrument Company) and 0.100 g of 1,4-bis-2-(4-methyl-5-phenyloxazoyl) benzene (DMPOPOP) (Packard Instrument Company) in a mixture of 700 ml of toluene and 300 ml of methanol. An Atomic Accessory Model SA-160 strip scanner was used to locate radioactive spots on paper chromatograms.

Preparation of N-nitroso-L-proline. Standard NSoP was prepared by using a modification of the method of Heyns and Königsdorf (23) in which an excess of nitrogen trioxide dissolved in 1.2 *N* HCl was added to a solution of non-radioactive proline and [^{14}C]-proline and the mixture was allowed to stand at 8° for 4–7 days.

The reaction mixture was chromatographed on a 15 × 370 mm Dowex-50 column and

eluted with 0.2 *M* citrate buffer, pH 3.25; NSoP was found in the 50–70 ml fraction. Of the radioactivity added to the reaction mixture, 87% appeared in this fraction and the remainder was recovered as L-proline.

After acid hydrolysis, the product yielded nitrite, as shown by a positive Greiss test, and proline, as shown by chromatography. The purity of the product was authenticated by mass spectroscopy.[1]

Table I gives the R_f values for NSoP and proline in several solvent systems after ascending paper chromatography on Whatman No. 1 paper. NSoP in quantities greater than 100 μg can be located on paper as a yellow spot after uv irradiation.

Procedure. Young adult Osborne–Mendel rats were fasted for 24 hr before the experiments. The test doses of L-proline, which were prepared to contain 25 μCi of [^{14}C]-proline (0.014 mg) and dissolved in 1 ml of water, were given by stomach tube and were followed by the test doses of sodium nitrite, also dissolved in 1 ml of water. After 15 min, the rats were killed by decapitation, the proximal and distal ends of the stomach were ligated, and the stomach was removed. The contents were collected and the stomach was quickly washed out with about 15 ml of cold saline. The washing was added to the stomach contents and 1 ml of saturated ammonium sulfamate was added to remove any excess nitrite from the mixture. A portion was taken for column chromatography and kept frozen until analysis.

The remainder of the stomach contents was acidified to pH 1 and the NSoP was extracted into ethyl ether. The ethyl ether extraction in control experiments with stomach contents adjusted to pH 1.0 was found to be 80% efficient. An aliquot of the ether extract was counted and the amount of NSoP was calculated from the specific activity of the administered proline. The quantities of NSoP which are reported in Table II are based upon actual counts obtained and are not adjusted for efficiency of extraction. The remainder of the ether extract was used for paper chromatography to confirm the identity of the labeled compounds.

For column chromatography, the samples of stomach contents were centrifuged and the supernatant fractions were adjusted to pH 3.25 with citrate buffer. Five-milliliter portions were placed on a Dowex-50 column

[1] The mass spectrum of the product was the same as that of an authentic sample of NSoP prepared by Dr. W. Lijinsky, and a mixed melting point was not decreased.

TABLE I. R_f Values of Proline, Nitrosoproline, and Ethyl Ether Extracts of the Stomach Contents of Rats Given Proline and Sodium Nitrite.[a]

Solvent system	R_f value		
	Proline	Nitrosoproline	Ether extracts of stomach contents
Phenol:ethanol: NH_4OH (7.5 *N*) (15:4:0.5)	0.74	0.52	0.50
n-Butanol:methyl ethyl ketone:water: dicyclohexylamine (10:10:5:2)	0.13	0.62	0.62
n-Butanol:propionic acid:water (4:1:1)	0.27	0.71	0.71
Pyridine:*tert*-amyl alcohol:water:diethylamine (10:10:7:0.3)	0.43	0.58	0.56
n-Butanol:acetic acid: water (4:1:5)	0.28	0.80	0.78

[a] Ascending chromatography on Whatman No. 1 paper.

TABLE II. Total Amount of Nitrosoproline (μg) Recovered from Stomach Contents of Rats Given L-Proline and Sodium Nitrite.[a]

Proline administered (mg)[b]	$NaNO_2$ administered (mg)					
	6.9		0.69		0.069	
	Av.	Range	Av.	Range	Av.	Range
5.8	24 (5)	11–39	3.4 (3)	2.5–5.0	Trace[c] (4)	—
0.59	2.7 (3)	2.2–3.7	0.25 (3)	0.19–0.36	Trace[c] (3)	—
0.072	0.14 (3)	0.13–0.17	0.12 (4)	0.013–0.27	Trace[c] (2)	—
0.014	0.037 (3)	0.020–0.069	0.011 (3)	0.007–0.013	Trace[c] (3)	—

[a] Numbers of animals are given in parentheses.

[b] Includes 25 μCi (0.014 mg) of uniformly labeled [^{14}C]-proline.

[c] The quantity was not large enough to confirm identity by chromatography.

and the compounds were eluted with the citrate buffer. Fractions of 3 ml were collected and 0.2 ml of each was counted for ^{14}C radioactivity.

Results and Discussion. Table II shows the amounts of NSoP in the ether extracts of gastric contents from rats given the various doses of L-proline and sodium nitrite. Only one labeled compound was found after column and paper chromatography of the ether extracts; its R_f in the various solvent systems used for paper chromatography was the same as that of NSoP (Table I).

L-Proline was the major peak found after column chromatography of the unextracted stomach contents. Minor peaks, as yet unidentified, were found in addition to the NSoP peak.

The data given in Table II indicate that the amount of NSoP formed under these experimental conditions is related to the quantity of each reactant administered. Therefore it might be assumed that proline and sodium nitrite at even lower concentrations could react to form NSoP under proper conditions. Some additional experiments were performed in which 0.014 mg of [^{14}C]-proline was administered alone, without sodium nitrite, to rats. The ether extracts of the stomach contents of these rats contained minute amounts of ^{14}C-labeled products; the radioactivity was even less than that found after administration of 0.069 mg of sodium nitrite (Table II). These labeled compounds could be [^{14}C]-NSoP which resulted from a reaction between [^{14}C]-proline and endogenous nitrite or an ethyl ether-soluble contaminant of the labeled proline standard.

To obtain further evidence concerning the identity of the *in vivo* reaction product, the stomach contents of several rats given the highest levels of proline and nitrite were pooled and extracted with ethyl ether. The material, although not pure, was found by chromatography to contain only one ^{14}C-labeled compound, *i.e.*, NSoP. The mass spectrum of the extracted material indicated that NSoP was a constituent of the slightly contaminated sample.

The results were obtained under fasting conditions and it is difficult to extrapolate the data to usual feeding conditions where many other molecules capable of reacting with either nitrite or proline might be present and competing. Furthermore, the environmental consequences of the findings reported here are difficult to evaluate in the absence of chronic feeding studies with NSoP. The methods used might well serve as a model system to determine the possibility of *in vivo* formation of known carcinogenic nitrosamines from the parent amines and sodium nitrite.

Summary. Fasting rats were given L-proline and sodium nitrite by stomach tube. *N*-Nitroso-L-proline was found in the stomach contents after 15 min; the amounts were related to the quantity of reactants administered. The results show that nitrosamines can be formed in the stomach of rats under the experimental conditions described here.

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