# *In Vitro* Nitrosation of Methapyrilene

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Received October 5, 1978, from **Hoffmann-La Roche Inc., Nutley, NJ 07110*, and the ‡*Massachusetts Institute of Technology, Cambridge, MA 02139.* Accepted for publication December 18, 1978.

**Abstract** □ The reactions of sodium nitrite and methapyrilene were studied in aqueous solution at neutral pH and under simulated gastric fluid conditions. Reaction product formation was much more complex than nitrosation of the parent molecule dimethylamino moiety to form nitrosodimethylamine. Several new nitroso compounds were formed under the reaction conditions studied. The simultaneous incorporation of 2 moles of ascorbic acid/mole of nitrite ion prevented any destruction of methapyrilene under all conditions studied. The implications of these observations with respect to nitrosation theory, the general carcinogenicity of nitroso compounds, and methapyrilene dosage formulation are discussed.

**Keyphrases** □ Methapyrilene—nitrosation, *in vitro*, with sodium nitrite, nitrosodimethylamine formation, effect of pH, prevented by ascorbic acid □ Ascorbic acid—methapyrilene nitrosation, prevention of nitrosodimethylamine formation, *in vitro* □ Nitrosodimethylamine—formation from methapyrilene, prevention by ascorbic acid, *in vitro* □ Carcinogens—nitrosodimethylamine, formation from methapyrilene, prevention by ascorbic acid, *in vitro*

The formation of *N*-nitroso compounds by interaction of nitrite or other nitrosating agents such as gaseous nitrogen dioxide or dinitrogen trioxide with susceptible amines has received much attention over the past few years (1). A recent survey showed that of 100 nitrosamines studied, 80% were carcinogenic in animal studies (2).

## BACKGROUND

No animal species has proved immune so far to the carcinogenicity of *N*-nitroso compounds (3, 4). Dimethylnitrosamine, the simplest dialkyl nitrosamine, is carcinogenic in a wide variety of species including the mouse, hamster, mink, rat, guinea pig, rabbit, and rainbow trout. Dimethylnitrosamine is carcinogenic in the mink at the lowest dose tested in rodents, 50 μg/kg, given twice per week in the diet (5). In addition, the carcinogenicity of *N*-nitrosamines is organospecific. In the rat, for example, dimethylnitrosamine is a liver carcinogen regardless of the administration route (6).

These results are of particular concern to humans, because many of these suceptible amines are commonly ingested as pharmaceuticals, pesticide residues, and normal components of food. Additionally, nitrite is produced by microbiological nitrate reduction in the mouth and is ingested from exogenous sources such as cured meat products and many vegetables.

Ascorbic acid inhibits this reaction, as was first demonstrated in an *in vitro* study (7) in 1972. This protective effect of ascorbic acid was extended to *in vivo* studies (8), which demonstrated that aminopyrine–nitrite-induced hepatotoxicity in rats could be completely abolished by the concomitant feeding of sodium ascorbate.

The mechanism of this protective effect by ascorbic acid is currently believed to be due to the ability of ascorbic acid to compete successfully with susceptible amines for the available nitrite (nitrosating species) ion. This premise is based on detailed studies (9) of the anaerobic oxidation of ascorbic acid by nitrous acid. The initial attack by the nitrosating species on the 3-hydroxy group of ascorbic acid forms the nitrite ester, which subsequently decomposes to yield the semiquinone. Further reaction of the semiquinone with an additional mole of nitrosating species completes ascorbate oxidation to dehydroascorbic acid. This work was extended (10) to aerobic systems, and a kinetic model was developed that predicted the amount of ascorbate required to inhibit completely nitrosation of an amine in the presence of ascorbic acid.

Since that time, the conditions required for ascorbic acid to prevent nitrite-induced reactions involving many different amine substrates have needed evaluation. The subject of the present study is methapyrilene, a tertiary pharmaceutical amine found in many over-the-counter antihistamine and sleep aid products. A significant incidence of liver tumors and other liver lesions was reported in rats fed an aqueous solution of methapyrilene plus sodium nitrite (11). The authors presumed that this hepatotoxicity was due to dimethylnitrosamine formation in the stomach of the test animals. The present study was undertaken to investigate the methapyrilene reaction with nitrite and the potential utility of ascorbic acid in protection of methapyrilene from such reactions.

## EXPERIMENTAL

**Materials and Methods**—Methapyrilene hydrochloride[1] was commercial grade. Sodium nitrite[2] and isopentyl nitrite[3] were used as sources of nitrosating intermediates. Redistilled methylene chloride was used for all sample extractions and workups.

**Nitrosated Standards**—*Nitrosation of* N-(*2-Pyridyl*)-N′-*dimethylethylenediamine* (*VIII*)—The amine was purified by dissolving it in hydrochloric acid, extracting with methylene chloride, basifying the aqueous phase, and extracting the amine with ether.

The amine (165 mg, 1 mmole) was dissolved in 4 ml of ether and treated with 1 ml of isopentyl nitrite. After standing overnight, a gummy residue precipitated. The ether solution was decanted and heated to evaporate the solvent. Alcohol (7 ml) was added, and the resultant solution was evaporated by boiling and then under a nitrogen stream. The product (IX) was obtained as a reddish oil, pure by TLC.

*Nitrosation of 2-(2-Thienylmethylamino)pyridine* (*V*)—The recrystallized amine had a sharp melting point and dissolved cleanly in ether. One millimole (190 mg) was treated with 1.5 ml of isopentyl nitrite in 4 ml of ether for 2 days. The product (VI) was worked as for IX and yielded a reddish oil, pure by TLC. Both products were confirmed as nitrosamines by the use of UV photolysis and Griess reagent on their chromatograms.

**Analyses**—Methapyrilene determinations were performed by GLC[4] on an instrument equipped with a flame-ionization detector. The column, a 1.83-m × 0.635-cm o.d. 8% methyl silicone gum rubber[5] glass U-tube, was operated isothermally at 240° with a helium flow rate of 50 ml/min. Samples were injected as the free base after the reaction mixture had been rendered alkaline with sodium hydroxide and the compound had been extracted into distilled methylene chloride.

The reaction products of methapyrilene with sodium nitrite were extracted as outlined and analyzed by a combined GLC–mass spectrometer[6] system interfaced with a data dystem[7]. Methane was used as the GLC carrier gas and as the chemical-ionization reagent gas. A 1.02-m × 0.32-cm o.d. 3% methyl silicone gum rubber[5] column was used at an initial temperature of 140° and a programmed rate increase of 10°/min. The mass range of *m/e* 60–460 was scanned in 2 sec.

Electron-ionization mass spectrometry of the nitrosated standards of *N*-(2-pyridyl)-*N*′-dimethylethylenediamine and 2-(2-thienylmethylamino)pyridine were obtained by mass spectrometry[8] using a chamber voltage of 70 (standard ionizing voltage). Several columns were tried for GLC–electron-ionization mass spectrometry, with terephthalic acid-treated polyethylene glycol[9] proving the most useful. Injector temperatures of 150–200° and lower were compared.

TLC studies were performed using precoated 15-cm silica plates with and without the fluorescent additive for visualizing UV-absorbing compounds, as required. The solvent system was hexane–ether–meth-

[1] Lot 072/02470, J. B. Williams Co., Jersey City, N.J.
[2] J. T. Baker Chemical Co., Phillipsburg, N.J.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Model 220, Tracor Instruments, Austin, Tex.
[5] Se-30.
[6] Model 1015D, Finnigan Corp., Sunnyvale, Calif.
[7] Model 6000, Finnigan Corp., Sunnyvale, Calif.
[8] Hitachi-Perkin-Elmer RMU 6E mass spectrometer, Perkin-Elmer Corp., Norwalk, Conn.
[9] Carbowax 20M/TPA.

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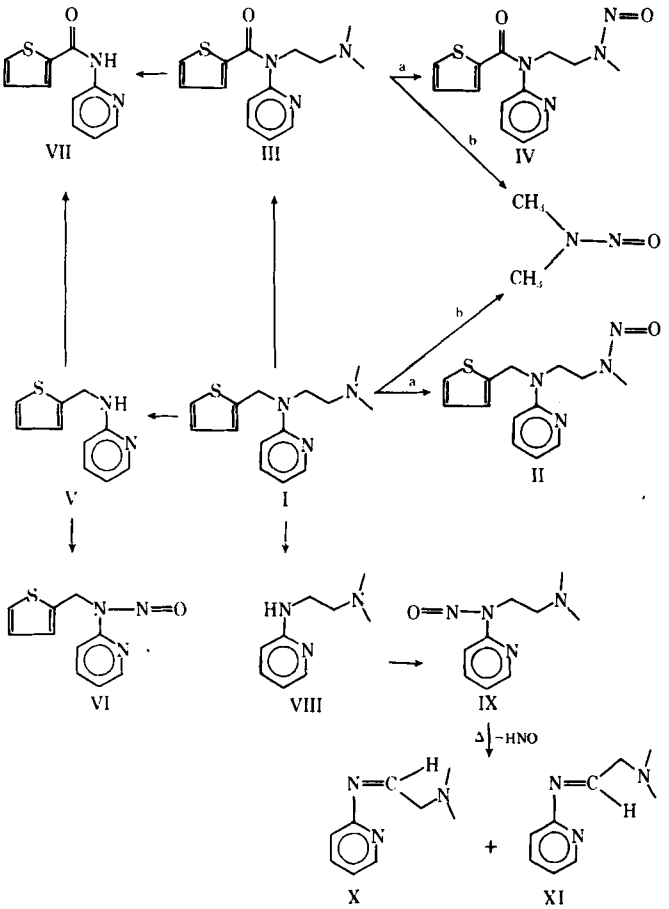
**Table I—TLC $R_f$ Values for Methapyrilene and Some Reaction Products on Silica Gel G**

| Compound | $R_f$ Value | Reaction to Spray Reagent | | |
|---|---|---|---|---|
| | | Iodoplatinate | Griess | Isatin |
| I | 0.5 | + | − | + |
| III | 0.4 | + | − | + |
| VIII | 0.25 | + | − | − |
| IX | 0.55 | + | + | − |

ylene chloride–methanol–concentrated ammonium hydroxide (40:15:20:10:0.25).

All compounds were visualized by UV on fluorescent plates. Nitrosamines were further identified by spraying the plate with Griess reagent followed by exposure to longwave UV light, which caused them to appear as pink spots. Basic nitrogen compounds were differentiated by spraying with iodoplatinate reagent, and thienyl compounds were detected by isatin reagent (12). The $R_f$ values were not highly reproducible (probably because some components of the solvent system are very volatile). The compounds, in order of increasing $R_f$ values, were VIII, I, IX, V, and VI (Scheme I and Table I).

Nitrosamine analyses are most conveniently and quite specifically performed using a thermal energy analyzer (13). This detector can be coupled with either a gas or liquid chromatograph. The nitrogen–nitrogen bond of the nitrosamine (nitrosamide) is thermally split in a pyrolysis chamber. The resulting reaction products, parent amine (or amide) plus the liberated nitric oxide radical, and any other components that emerge from the pyrolysis oven are then swept through a cold trap. The nitric oxide radical, one of the few substances that will not be trapped, passes into the reaction chamber where it is oxidized with ozone to yield an excited nitrogen dioxide molecule. As this molecule relaxes to the ground




*Scheme I—Proposed reaction products of methapyrilene with sodium nitrite. The reaction products of I and III arise through nitrosative attack on the dimethylamino group. When the leaving group is $-CH_3$, the resulting product is the unsymmetrical dialkylnitrosamine (path a). When the leaving group is the substituted ethyl, both I and III yield nitrosodimethylamine (path b).*

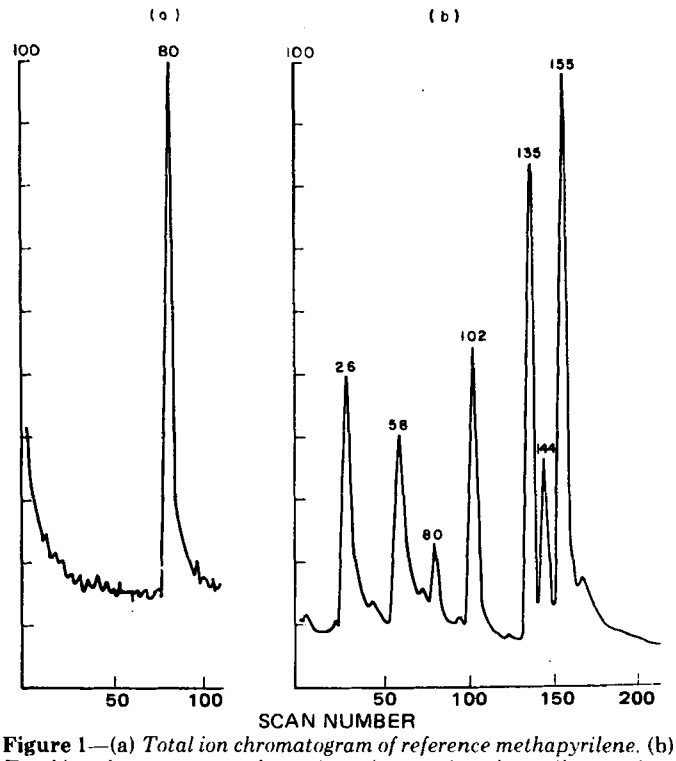



**Figure 1**—(a) *Total ion chromatogram of reference methapyrilene.* (b) *Total ion chromatogram of reaction mixture of methapyrilene and nitrite.*

state, it emits a characteristic energy (in the near IR), which is detected using a photomultiplier tube.

Nitrosodimethylamine analyses were performed by a GLC–thermal energy analyzer using a column of polyethylene glycol[10] on a potassium hydroxide-treated support. Methylene chloride solutions were generally analyzed isothermally at 145° and an attenuation of 0.1. Some analyses were performed using a temperature program between 100 and 180° at an attenuation of 0.1, when solvent tailing under isothermal conditions presented an analytical problem.

Compounds VI, IX, nitrosodimethylamine, and the reaction products of methapyrilene and nitrite at 37° were examined using a high-pressure liquid chromatography (HPLC)–thermal energy analyzer. These studies employed a prepacked column[11] and a mobile phase of 95% isooctane/5% acetone at a flow rate of 2 ml/min. The thermal energy analyzer detector was operated with an oven temperature of 450° and a reaction chamber pressure of 2 torr. The cold trap was held in a dry ice–isopropyl alcohol bath.

**Reactivity of Methapyrilene with Nitrite as a Function of Temperature**—Methapyrilene solutions (1 mg/ml) were prepared in pH 3.7 acetate buffer. Mole ratios of sodium nitrite to methapyrilene were adjusted by adding solid sodium nitrite to the solution to yield 8.6, 4.3, 1.0, and 0.5 mole ratios. Reactions were carried out by sealing 10-ml aliquots of the reaction mixtures in 20-ml amber all-glass ampuls. Reaction temperatures of 90, 70, 50, and 37° were maintained by immersion of the samples in constant-temperature water baths. Reaction time was 4 hr. Samples were stored under refrigeration pending analyses. In this particular study, nitrosodimethylamine determinations were performed by GLC using an electrolytic conductivity detector[12] operated in the reductive mode without a catalyst (14).

**Mild Reaction Conditions**—Studies were performed under simulated gastric fluid conditions at 37° and pH 3.7 for 4 hr in glass-stoppered vessels. The methapyrilene concentration was 1 mg/ml, and the sodium nitrite concentration was 2 mg/ml.

For a similar experiment involving the production of nitrosodimethylamine over 1–5 days under room temperature conditions at pH 6.2, the following sample workup was used. During storage, the samples were shielded from light. Afterwards, each sample was extracted with 3 × 100 ml of methylene chloride, dried over sodium sulfate, and con-

[10] Carbowax 20M.

[11] μPorisil, Waters Associates, Milford, Mass.

[12] Model 310, Hall electrolytic conductivity detector, Tracor Instruments, Austin, Tex.

**Table II—Reaction of Methapyrilene with Nitrite as a Function of Temperature and Mole Ratio [a]**

| Nitrite–Methapyrilene Mole Ratio | Methapyrilene Analysis, % retention | | | | Nitrosodimethylamine at 90°, % of theory |
|---|---|---|---|---|---|
| | 90° | 70° | 50° | 37° | |
| 8.6:1 | 2 | 50 | 97 | 99 | 12 |
| 4.3:1 | 7 | 59 | 96 | 99 | 8 |
| 1:1 | 47 | 67 | 98 | 99 | 0.1 |
| 0.5:1 | 75 | 82 | 98 | 100 | <0.04 |

[a] Reaction time was 4 hr using pH 3.7 acetate buffer. Methapyrilene = 1 mg/ml.

centrated to 10 ml in an apparatus[13]. Heptane, 1 ml, was added, and the sample was further concentrated to 1 ml with nitrogen. Samples were then quantitatively vacuum transferred to remove nonvolatile material and analyzed by the GLC–thermal energy analyzer on a polyethylene glycol[10]–potassium hydroxide-treated column at 100°.

**Ascorbic Acid Influence**—The influence of ascorbic acid on the nitrite–methapyrilene reaction was evaluated by incorporating 0–2 moles of ascorbic acid/mole of sodium nitrite present. In all cases, the reaction mixture pH was adjusted to compensate for the acid addition.

## RESULTS AND DISCUSSION

**Methapyrilene Reaction Products**—The methapyrilene molecule contains a dimethylamine moiety, which could be a potential source of nitrosodimethylamine. Tertiary amines have been reported to undergo nitrosative dealkylation to form a nitrosamine in the presence of a nitrosating intermediate (15, 16). The optimum condition for many aqueous nitrosations is usually about pH 3.4, the pKa for nitrous acid. This pH generally reflects the mutual optimization of the two reactive species: (*a*) the formation of the nitrosating intermediate, which increases with increasing acid concentration, and (*b*) the concentration of the unprotonated, more electrophilic form of the amine, which is, of course, dependent on the pKa of the specific base.

**Reaction Products of Methapyrilene and Nitrite**—Methapyrilene nitrosation at pH 3.7 was investigated as a function of temperature and mole ratio of nitrite (Table II). At 90° with an 8.6 mole excess of nitrite, the parent amine almost completely disappeared. As the temperature and mole ratios decreased, the stability of methapyrilene substantially increased.

**GLC-Chemical-Ionization Mass Spectrometric Analysis**—These analyses were performed to identify the reaction products at 90° and the highest mole ratio of nitrite. The total ion chromatograms for the reference standard (methapyrilene base) and a sample containing a mixture of the methylene chloride-extractable reaction products obtained under different reaction conditions are reproduced in Fig. 1. Proposed structures for the individual reaction products can be found in Scheme I and Table III. No definite structure could be made for scan 144. The presence of a carbonyl function in III, IV, and VII was confirmed by GLC coupled to an IR detection system[14].

At 90°, the major reaction product appeared to be III, in which the methylene bridge between the ethylenediamine and thienyl moieties had been oxidized to a keto group. One possible mechanism for the formation of such an amide structure would involve attack of the nitrosyl ion on this group to form a C-nitroso compound, which could rearrange to the corresponding oxime. Hydrolysis of the oxime would subsequently yield the amide plus hydroxylamine.

One interesting aspect of this experiment can be seen in Table II. At 90°, a definite break in both methapyrilene retention and nitrosodimethylamine formation occurred at nitrite–methapyrilene mole ratios at and below 1:1 compared to the higher ratios. Not only was the retention of the parent compound improved, but the conversion of methapyrilene to nitrosodimethylamine was almost negligible. One interpretation of this observation would indicate that the parent amine consumes 1 mole of nitrite prior to attack of the dimethylamino function. This idea is supported by the fact that III was the principal oxidation product under these conditions. Moreover, the dealkylation products observed at 90° involved cleavage of the parent molecule in the ethylenediamine portion of the molecule and not in the thienyl moiety (*viz.*, V and VII).

**Table III—Molecular Weights of the Products of the Reaction of Methapyrilene (I) and Sodium Nitrite**

| Peak, scan number | Molecular Weight | Possible Structure |
|---|---|---|
| 26 | 190 | V |
| 58 | 204 | VII |
| 80 | 261 | I |
| 102 | 275 | III |
| 135 | 276 | II |
| 155 | 290 | IV |

[13] Kuderna-Danish, SGA Scientific Inc., Bloomfield, N.J.
[14] RS1, Norcon Inc., Norwalk, Conn.

**Room Temperature Storage**—Based on these findings, the system was reexamined using considerably less stringent reaction conditions to identify the initial reaction products. Solutions of 1 mg of methapyrilene/ml of distilled water were stored at room temperature in the presence of 2 mg of sodium nitrite/ml. The pH of this mixture was 6.6. Under these conditions, substantial conversion (~1%/day) of the parent compound into *N*-(2-pyridyl)-*N'*-dimethylethylenediamine (VIII) was observed (Table IV). In the absence of nitrite, on the other hand, no deterioration of the parent compound was observed under identical storage periods. The appearance of this particular secondary amine is not unusual because its synthesis involves a cleavage of the activated methapyrilene methylene bond. One can only speculate as to why it was not observed at 90°.

Compound VIII was isolated by TLC at $R_f$ 0.25 (Table I), and its identity was confirmed by GLC–mass spectrometric analysis of both the free amine and its trimethylsilyl derivative. Furthermore, it behaved identically to a known standard in all tests performed. The nitrosation of VIII was studied in simulated gastric fluid (pH 1.3) and acetate buffer (pH 3.7) using the same amine and nitrite-ion concentrations described for methapyrilene. After 4 hr of reaction, both samples were more than 90% converted to the corresponding nitrosamine. After 24 hr, the reaction was essentially complete in both cases as estimated by TLC.

The nitrosamine was separated from the reaction mix with methylene chloride and isolated by solvent evaporation. IR spectrophotometry confirmed the presence of a nitroso group and indicated a structure compatible with IX. The fact that VIII reacted more readily with nitrite than methapyrilene is understandable since it is a secondary amine. Secondary amines classically are the most readily nitrosated amines, with the exception of the tertiary amine aminopyrine (17).

Further confirmation of the structure of the nitroso derivative of VIII by direct mass spectrometry was attempted. The compound yielded a fairly clean spectrum in which the highest *m/e* was 163, corresponding to M − 31 for the *N*-nitroso derivative of VIII (inlet temperature of 160°). When the inlet temperature was lowered to 65°, a similar spectrum was obtained, but with additional peaks at *m/e* 164 and 179, presumably M − 30 and M − 15, respectively. In neither case was the molecular ion observed, so the resulting spectra, while consistent with the structure of IX, cannot be considered firm evidence that the desired product was obtained. GLC–mass spectrometry on terephthalic acid-treated polyethylene glycol[9] yielded complex chromatograms with injector temperatures at 150–200°. Lower injector temperatures gave simpler chromatograms, but this result may have been due either to less decomposition or simply to fewer components being flash evaporated.

Useful mass spectra were obtained for only two of these components. Both gave an apparent molecular ion at *m/e* 163 and probably correspond to the isomeric imines formed by HNO elimination of the parent nitrosamine during GLC (X and XI, Scheme I).

Similarly prepared samples were also analyzed for nitrosodimethyl-

**Table IV—Oxidation of Methapyrilene (1 mg/ml) in Water at pH 6.6 in the Presence of Nitrite (2 mg/ml)**

| Hours at Room Temperature | Percent VIII[a] |
|---|---|
| 24 | 0.5 |
| 48 | 1.8 |
| 72 | 2.5 |
| 98 | 4 |
| 168 | 7.5 |

[a] Estimations by TLC compared to an authentic standard.

**Table V—Effect of Ascorbic Acid on the Methapyrilene–Nitrite Reactions**

| Ascorbic Acid–Nitrite Mole Ratio | Concentrations[a] | Reaction Conditions[b] | pH | Percent Present | | | |
|---|---|---|---|---|---|---|---|
| | | | | Methapyrilene[c] | III[d] | VII[d] | Nitrosamine[d] |
| 0:1 | A | 1 | 3.7 | 91 | + | + | — |
| 0.5:1 | A | 1 | 3.7 | 94 | + | + | — |
| 1:1 | A | 1 | 3.7 | 98 | + | + | — |
| 2:1 | A | 1 | 3.7 | 100 | ND | ND | — |
| 0:1 | B | 1 | 3.7 | 89 | + | + | — |
| 0.5:1 | B | 1 | 3.7 | 99 | + | + | — |
| 1:1 | B | 1 | 3.7 | 100 | + | + | — |
| 2:1 | B | 1 | 3.7 | 100 | ND | ND | — |
| 0:1 | A | 2 | 3.7 | — | — | 10 | + |
| 0.5:1 | A | 2 | 3.7 | — | — | 5 | + |
| 1:1 | A | 2 | 3.7 | — | — | 1–2 | + |
| 2:1 | A | 2 | 3.7 | — | — | ND | ND |
| 0:1 | B | 2 | 3.7 | — | — | 10 | + |
| 0.5:1 | B | 2 | 3.7 | — | — | 4 | + |
| 1:1 | B | 2 | 3.7 | — | — | 2 | + |
| 2:1 | B | 2 | 3.7 | — | — | ND | — |
| 0:1 | A | 3 | 6.6 | — | ND | 1 | — |
| 2:1 | A | 3 | 6.6 | — | ND | ND | — |
| 0:1 | A | 4 | 6.6 | — | ND | 4 | — |
| 2:1 | A | 4 | 6.6 | — | ND | ND | — |
| 0:1 | A | 5 | 1.0 | — | ND | 2 | — |
| 2:1 | A | 5 | 1.0 | — | ND | ND | — |
| 0:1 | A | 5 | 2.0 | — | ND | <0.2 | — |
| 2:1 | A | 5 | 2.0 | — | ND | ND | — |
| 0:1 | A | 5 | 3.0 | — | ND | <0.2 | — |
| 2:1 | A | 5 | 3.0 | — | ND | ND | — |
| 0:1 | A | 5 | 4.0 | — | ND | <0.2 | — |
| 2:1 | A | 5 | 4.0 | — | ND | ND | — |
| 0:1 | A | 5 | 5.0 | — | ND | 0.2 | — |
| 2:1 | A | 5 | 5.0 | — | ND | ND | — |

[a] A, sodium nitrite = 2 mg/ml and methapyrilene = 1 mg/ml; and B, sodium nitrite = 10 mg/ml and methapyrilene = 5 mg/ml. [b] 1 = acetate buffer, 37°, 36 hr; 2 = acetate buffer, room temperature, 10 days; 3 = distilled water, room temperature, 21 hr; 4 = distilled water, room temperature, 4 days; and 5 = simulated gastric fluid, 37°, 4 hr. [c] Analysis by GLC. [d] Estimation by TLC. The + indicates that the compound was present but not estimated, and ND indicates that none was detected. Estimation of VII was based on a methapyrilene standard.

amine with the GLC–thermal energy analyzer after 1- and 5-day periods. The 1-day sample contained 0.33 ppm and the 5-day contained 0.104 ppm of nitrosodimethylamine. These values correspond to 16.5 and 5.2 μg of nitrosodimethylamine/50 mg of methapyrilene. The nitrosodimethylamine loss between Days 1 and 5 was due to chemical–physical factors that caused aqueous solutions of this nitrosamine to be unstable over long periods. The fact remains, however, that some production of nitrosodimethylamine can take place even under such mild conditions.

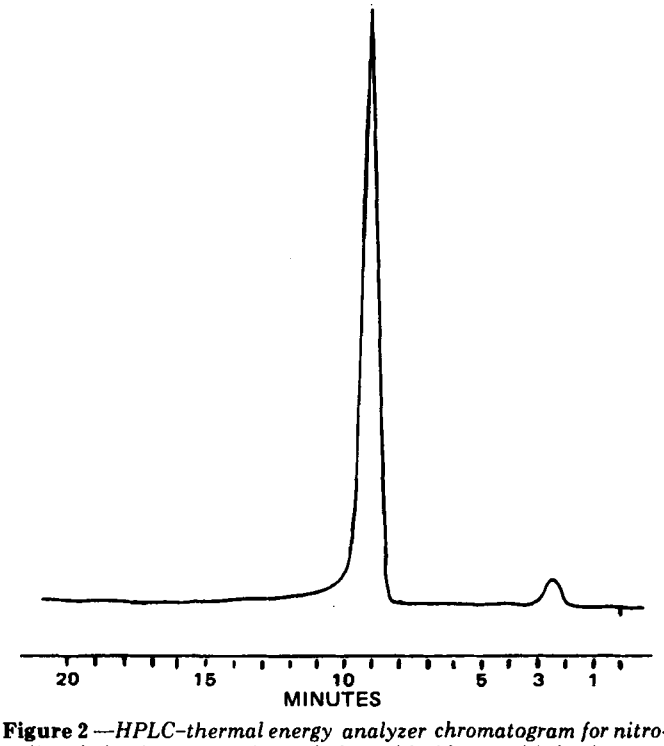



**Figure 2**—*HPLC–thermal energy analyzer chromatogram for nitrosodimethylamine, 5 ppm in methylene chloride, 10-μl injection, and attenuation 128×.*

**Gastric Fluid Conditions**—The potential for nitrosamine formation between an amine and nitrite is greatly enhanced under gastric fluid conditions as compared to the neutral solution. This situation directly relates to the hydrogen-ion influence on the nitrosating intermediate formation through the nitrite–nitrous acid equilibria (Schemes II and III):

$$NO_2^- + H^+ \rightleftharpoons HONO$$

*Scheme II*

$$2HONO \rightleftharpoons N_2O_3 + H_2O$$

*Scheme III*

The studies performed here for the methapyrilene system after 4 hr under simulated conditions yielded the following results. Conversion of methapyrilene to nitrosodimethylamine was 150 ng/ml from a maximum theoretical yield of 250 μg/ml, which represents a 0.06% conversion by GLC–thermal energy analyzer analysis. In addition to nitrosodimethylamine, several other peaks appeared in the HPLC–thermal energy analyzer chromatogram. The first peak in all HPLC–thermal energy analyzer chromatograms, appearing at 3 min after injection, was a solvent front response; it did not correspond to an *N*-nitroso compound, and its magnitude was unimportant. The remaining peaks corresponded to the same peaks derived from the nitroso derivative of V and the nitroso derivative of VIII standards, although not all peaks could be accounted for in this manner. Nitroso V represented yet another nitrosamine derivative, which could be formed from methapyrilene (Figs. 2–4).

These observations have important implications with respect to the experimental handling of methapyrilene–nitrite solutions, especially in animal feeding experiments such as those described previously (11). The present results indicate that the experimental protocol of mixing methapyrilene and sodium nitrite in drinking water prior to ingestion by test animals could lead to preformed nitrosodimethylamine in quantities (0.1–0.3 ppm in drinking water) comparable to those formed by incubation under simulated gastric fluid conditions for 4 hr. In addition, the substantial conversion of the parent amine to VIII complicates the scheme by introducing a secondary amine that has been shown to nitrosate much more rapidly than the parent amine under simulated gastric fluid conditions at 37°. For this and other reasons, caution should

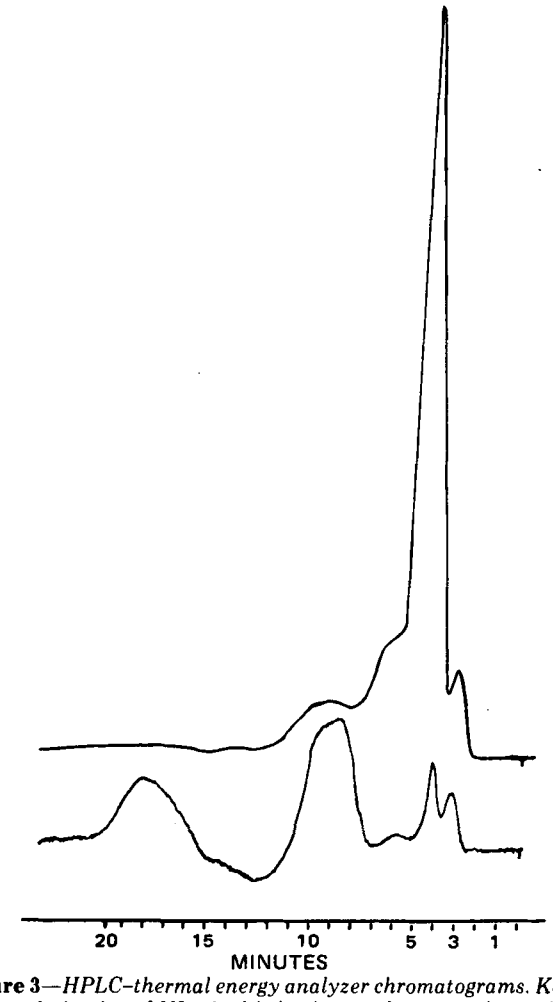



**Figure 3**—*HPLC–thermal energy analyzer chromatograms. Key: top, nitroso derivative of VI, 10-μl injection, and attenuation 128×; and bottom, nitroso derivative of IX, 10-μl injection, and attenuation 32×.*

be used in the interpretation of carcinogenicity experiments as solely the result of *in vivo* nitrosation of the parent amine.

**Ascorbic Acid Effect**—Ascorbic acid was applied successfully as a nitrosamine inhibitor in a number of *in vivo* (8, 18) and *in vitro* (7) systems involving drug nitrosation. Table V summarizes the results for the application of various mole ratios of ascorbic acid to nitrite ion for two different concentrations of nitrite and methapyrilene under different reaction conditions. As predicted (10), the presence of 2 moles of ascorbic acid/mole of nitrite was effective in preventing oxidative and nitrosative damage to the methapyrilene molecule. This result was true for all reaction conditions (Table V). Results not reported in Table V indicate that ascorbic acid is just as effective a protective agent at 90°. Although TLC results can be considered encouraging, this procedure lacks the sensitivity and specificity afforded by the thermal energy analyzer.

Analyses of methapyrilene–nitrite mixtures containing 2 moles of ascorbic acid/mole of nitrite were performed with the thermal energy analyzer. The reaction conditions were the same as those described under *Experimental* for 5 days at room temperature and 4 hr in simulated gastric fluid at 37°. No thermal energy analyzer responsive peaks were detected in either sample (Fig. 5). Based on the lowest detectable limit for nitrosodimethylamine under the conditions of analysis, a maximum of 0.0001% of nitrosodimethylamine could be present in the ascorbic acid-containing samples in contrast to 0.06 and 0.03% of this nitrosamine found in the gastric fluid and 1-day room temperature samples, respectively. These results indicate that ascorbic acid is an extremely effective agent in protecting against methapyrilene nitrosation. Hence, it follows that susceptible pharmaceutical amines could be safeguarded against *in vivo* nitrosation if they were compounded with ascorbic acid.

This suggestion has already been made (7). Technologically, however, other considerations come into play. If the nitrosation rate of the amine is sufficiently slower than the ascorbate and nitrite reaction, one can safely conclude that *in vivo* nitrosation of the amine will be halted successfully. Aside from kinetic considerations, this would presume that the ascorbic acid solution rate is comparable to or better than that for the susceptible amine. One formulation approach to assure this could be a T-T tablet with ascorbic acid comprising the outer tablet.

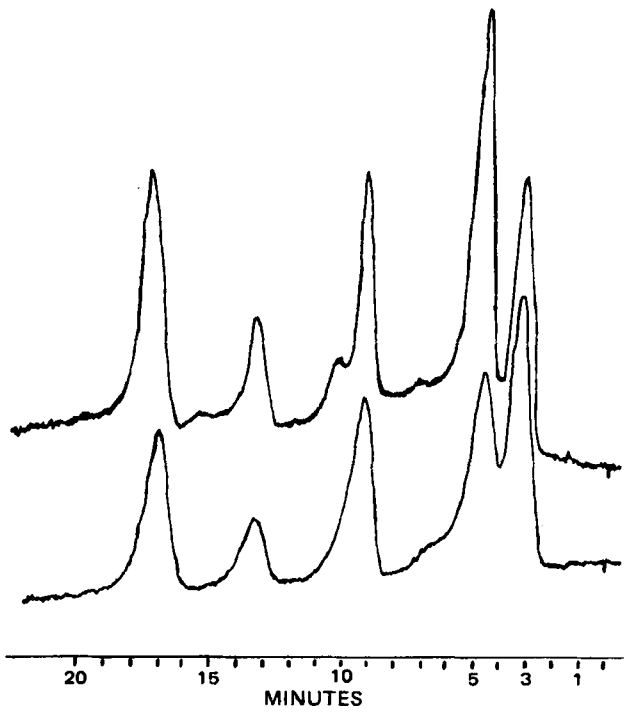



**Figure 4**—*HPLC–thermal energy analyzer chromatograms. Key: top, methapyrilene dissolved in sterile distilled water and immediately reacted with nitrite for 4 hr at 37°, 10-μl injection of methylene chloride extract, and attenuation 16×; and bottom, methapyrilene incubated at room temperature in sterile distilled water for 7 days and then reacted with nitrite for 4 hr at 37°, 10-μl injection of methylene chloride extract, and attenuation 32×.*

Recent experimental work has shown that yet another precaution must be taken. Aminopyrine is a popular headache remedy on the European market. It is also a tertiary amine, which nitrosates extremely rapidly in

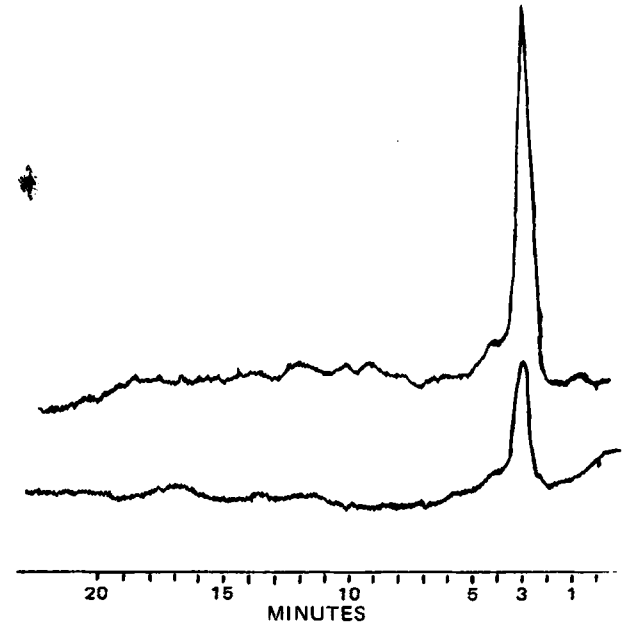



**Figure 5**—*HPLC–thermal energy analyzer chromatograms. Key: top, same as Fig. 4 (top) with twice molar ascorbic acid added with nitrite, 10-μl injection of methylene chloride extract, and attenuation 16×; and bottom, same as Fig. 4 (bottom) with twice molar ascorbic acid, 10-μl injection of methylene chloride extract, and attenuation 16×.*

the presence of nitrite to yield nitrosodimethylamine (20). Eisenbrand *et al.* (21) showed that tablets containing 300 mg of aminopyrine and protected with 160 mg of ascorbic acid contained 88, 89, and 57 μg/kg of nitrosodimethylamine as purchased. These same tablets, when exposed for about 12 hr to a continuous air flow containing 1 ppm of nitrogen dioxide, comparable to many urban atmospheres (chamber volume of 50 $m^3$, air flow of 50 $m^3$/hr), were found to contain 5090 μg/kg of nitrosodimethylamine. Obviously, the heterogeneous admixture of aminopyrine and ascorbic acid in a tablet of this nature is less than effective in protecting the aminopyrine crystals from attack by nitrogen dioxide molecules.

Aminopyrine is unusual in being a tertiary amine that is more rapidly nitrosated than most secondary amines. This fact is at least justified under aqueous nitrosation conditions if one considers that its pKa is 5.04. Whether or not the same aberrant behavior extends to its ability to be directly nitrosated by gaseous nitrogen dioxide as compared to other amines is not known.

Successful tablet formulations containing ascorbic acid to protect against the nitrosation of a susceptible amine should preclude the possibility of direct gas phase exposure of the amine to nitrogen dioxide or contain suitable experimental assurances that this nitrosation route is not possible. Protection of this type of susceptible amine would probably involve encapsulation of the amine crystals or possibly the entire tablet with a suitable barrier.

## SUMMARY AND CONCLUSIONS

Methapyrilene contains a dimethylamine moiety, which, on the basis of previous studies (8, 15), was suspected of reacting with a nitrosating intermediate to form nitrosodimethylamine, a potent animal carcinogen. The present studies demonstrated that very small yields of nitrosodimethylamine are obtained in neutral solutions at room temperature after 1 day and also under conditions simulating gastric digestion (*i.e.*, 4 hr at 37° at pH 3.7). Severe reaction conditions (4 hr at 90° at pH 3.7) are required to obtain high yields (over 20%) in the reaction of methapyrilene with nitrite.

In near neutral solution at room temperature and under conditions simulating the environment of the stomach, the major pathway for the reaction of methapyrilene and nitrite is completely different than that leading to nitrosodimethylamine formation. One observed reaction is the formation of the secondary amine *N*-(2-pyridyl)-*N'*-dimethylethylenediamine (VIII) through nitrite oxidation of the thiophene-activated methylene bridge of the parent amine. A recent report (22) indicating that methapyrilene is metabolized *in vivo* to nonsulfur-containing metabolites tends to support this finding. Another secondary amine can be formed by removal of the side chain on the central nitrogen in the molecule leading to 2-(2-thienylmethylamino)pyridine. Evidence suggests that the formation of these secondary amines could lead to the generation of the corresponding nitrosamines under the acid conditions of the stomach.

Ascorbic acid, when present in a 2:1 molar ratio to nitrite ion, was completely effective in preventing the oxidative and nitrosative damage of the methapyrilene molecule in all systems studied.

The production of small, but toxicologically significant, amounts of nitrosodimethylamine and ~1%/day of VIII from methapyrilene under neutral aqueous conditions in the presence of nitrite indicates that caution should be exercised in any experimental protocol involving solution storage for the following reasons. Animal feeding studies cannot preclude the presence of preformed nitrosamines (*e.g.*, nitrosodimethylamine). In addition, ~1%/day of VIII, a secondary amine that has been shown to nitrosate much more rapidly than methapyrilene under simulated gastric fluid conditions, is formed.

The concept of protecting susceptible pharmaceutical amines from nitrosation by using ascorbic acid, either as its acid or salt form, is probably a viable goal. However, consideration must be given not only to aqueous sources of nitrosating intermediates but also to gaseous ones.

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## ACKNOWLEDGMENTS


The technical assistance of Mr. J. P. Keating and Mr. M. P. Lanyi of Hoffmann-La Roche Inc. and Dr. J. S. Wishnok and Mr. W. Bishop of the Massachusetts Institute of Technology is gratefully acknowledged. The *N*-(2-pyridyl)-*N'*-dimethylethylenediamine standard was graciously supplied by Dr. W. Schroeder of Burdick and Jackson Laboratories.