Vol. 95, No. 2, 1980
July 31, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 835-841

DESMUTAGENIC ACTIONS OF ASCORBIC ACID AND CYSTEINE ON A NEW PYRROLE MUTAGEN FORMED BY THE REACTION BETWEEN FOOD ADDITIVES; SORBIC ACID AND SODIUM NITRITE

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Received June 4,1980

SUMMARY; Mutagenicity of 1,4-dinitro-2-methyl pyrrole, a new mutagen isolated from the reaction mixture of sorbic acid and sodium nitrite, was found to be destroyed by treatment with ascorbic acid or cysteine. Chemical studies revealed that the loss of mutagenicity was due to reduction from the C-nitro to C-amino group.

INTRODUCTION

It has been reported by a number of investigators that ascorbic acid (Vitamin C) reacts with nitrite and prevents formation of N-nitrosamines *in vitro* as well as *in vivo* system (1, 2). Ascorbic acid has also been shown to inhibit mutations induced by N-nitroso compounds such as N-methyl-N-nitrosoguanidine and dimethylnitrosamine (3). It was also reported that other antioxidants such as tert-butyl-4-hydroxyanisole (BHA), 3-tert-butyl-4-hydroxytoluene (BHT), α-tochopherol and selenium were able to inhibit bacterial mutations induced by malonaldehyde or β-propiolactone (4). Recently, inactivation of the mutagenicity of amino acid and protein pyrolysates with vegetable factors and peroxidases has been reported (5-7).

In these years, we have extensively studied mutagenic principles formed by the reaction of sorbic acid with sodium nitrite. Finally, we concluded that the main mutagenic product is 1,4-dinitro-2-methyl pyrrole (DNMP) (8-10).

The following abbreviations are used in this text.
1,4-dinitro-2-methyl pyrrole; DNMP, 1-nitro-2-methyl-4-amino pyrrole; NMAP, 5-nitro-2-furaldehyde semicarbazone; nitrofurazone, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; AF-2, high pressure liquid chromatography; HPLC, ultra violet; UV, infrared; IR and nuclear magnetic resonance; n.m.r.

0006-291X/80/140835-07$01.00/0

Sorbic acid and sodium nitrite are used as food additives in all over the world; both of them are considered relatively safe because of their natural existency.

This paper reports that the new pyrrole mutagen isolated as a reaction product between sorbic acid and sodium nitrite was destroyed chemically with ascorbic acid or cysteine.

MATERIALS AND METHODS

CHEMICALS

Ascorbic acid and cysteine were purchased from Wako Chemical co. Ltd. and DNMP was isolated and purified from the reaction mixture of sorbic acid with sodium nitrite by the method described previously (8).

INSTRUMENTAL ANALYSIS

1. Quantification of DNMP by HPLC
   The amount of DNMP was quantified using HPLC (column; μ-Bondapak C-18, solvent system; H_2O:CH_3OH=85:15, UV detector; 254 nm, elution speed; 1.0 ml/min. and pressure; 1500 psi.
2. Insruments for structural elucidation
   UV; Hitachi 200-10 spectrometer, IR; Jasco A-3, n.m.r.; Jeol JNM-FX-100, mass spectrum; Jeol JNM-D-100, high-resolution mass spectrum; Jeol JMS-OISG and polarography; Fuso Denki Model 312.

TEST OF MUTAGENICITY

Mutagenicity was tested by the Ames test using Salmonella typhimurium TA 100 and 98 strains. Various amounts of test compounds were diluted with dimethylsulfoxide. S-9 mix was prepared from liver homogenate of rats given polychlorinated biphenyls (500 mg/kg) on five days before sacrifice (9).

INCUBATION OF DNMP WITH ASCORBIC ACID OR CYSTEINE

DNMP (1 mM) and ascorbic acid or cysteine (8 mM) were dissolved in buffer solutions of different pHs; pH 1.5 and 3.5 (0.2 M sodium citrate-HCl), pH 5.0 (0.2 M sodium acetate-acetic acid) and pH 6.8 (0.2 M KH_2PO_4-Na_2HPO_4). The mixed solutions were incubated at 37°C for one hour. After the incubation, 10 μl of the reaction mixture was employed in the HPLC analysis for quantification, then the remaining solution was adjusted to pH 4.2 and extracted three times with ethyl acetate and concentrated in vacuo. Extracts were dissolved in dimethylsulfoxide and used for measurement of the mutagenicity.

LARGE SCALE ISOLATION OF NMAP

Large scale reaction of DNMP (10 mM) with ascorbic acid (80 mM) has been carried out in 0.2 M phosphate buffer (pH 6.8) at 37°C for one hour, then the reaction mixture was extracted three times with n-butanol. After concentration, the condensate was extracted again with methanol and then purified by preparative HPLC (column; Bondapak C-18 porasil B and solvent system; H_2O:CH_3OH=9:1) and finally pure NMAP was isolated (yield; 32%).

ACETYLATION OF NMAP

Acetylation of NMAP (20 mg) was carried out by treating with acetic anhydride/pyridine (0.5 ml, each) at room temperature. A monoacetate derivative of NMAP was purified by preparative HPLC (column; Bondapak C-18 porasil B and solvent system; H_2O:CH_3OH=85:15) and 14 mg of monoacetate derivative was obtained.

Table 1. Inactivation of the mutagenicity of DNMP by treatment with ascorbic acid or cysteine.

pH	Mutagenicity (% of control)		
	None	Ascorbic acid	Cysteine
1.5	100	94	99
3.5	100	104	104
5.0	100	90	82
6.8	100	0	2

Incubation was carried out with or without ascorbic acid or cysteine in 10 ml buffer solution of 1 mM DNMP and other conditions were described in the text. Ethyl acetate extracts were dissolved in 1 ml dimethylsulfoxide and 50 µl of the solution were used in the Ames test. The strain TA 100 of Salmonella typhimurium was used for the mutagenicity assay. Spontaneous revertants were subtracted and the number of revertants without ascorbic acid or cysteine were taken as 100%.

RESULTS

EFFECTS OF ASCORBIC ACID OR CYSTEINE ON MUTAGENICITY OF DNMP

As shown in Table 1, the mutagenic activity of DNMP was completely abolished by incubation with ascorbic acid or cysteine at pH 6.8, while it was decreased slightly at pH 5.0 but unaltered at pH 3.5 or below.

HPLC ANALYSIS OF THE ASCORBIC ACID OR CYSTEINE EFFECTS

Results of the quantitative HPLC measurements of DNMP after treatment with ascorbic acid or cysteine are shown in Fig. 1. It is clearly indicated

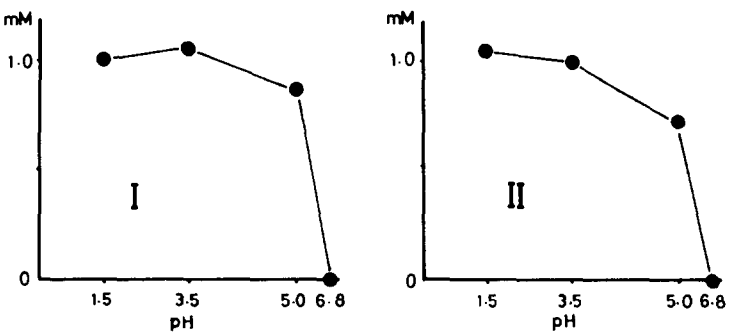


Fig. 1. Degradation of DNMP (1 mM) by ascorbic acid or cysteine (8 mM) at various pHs.

The amount of DNMP was determined by HPLC.
Column; µ-Bondapack C-18
Solvent system; H_2O-methanol (85:15)
I; DNMP + ascorbic acid
II; DNMP + cysteine

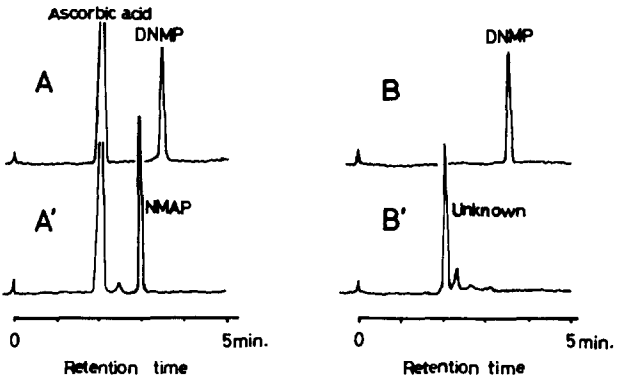


Fig. 2. HPLC chromatograms of the reaction mixture of DNMP (1 mM) with ascorbic acid or cysteine (8 mM) at pH 3.5 and 6.8.

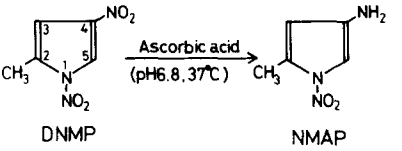
A ; DNMP + ascorbic acid, pH 3.5.
A'; DNMP + ascorbic acid, pH 6.8.
B ; DNMP + cysteine, pH 3.5.
B'; DNMP + cysteine, pH 6.8.

that degradation of DNMP is remarkably dependent upon the pH of the treatment and this pH effect was well correlated with that observed in the mutagenicity test. It was thus clarified that the loss of the mutagenicity is due to degradation of DNMP as a result of the reaction with ascorbic acid or cysteine. However, detailed examinations on the retention time of the reaction products in HPLC chromatograms shown in Fig. 2 indicate that the degradation process of DNMP by ascorbic acid is apparently different from that by cysteine.

STRUCTURAL ANALYSIS OF THE REACTION PRODUCT

In order to confirm the degradation process on the chemical and biological bases, we isolated NMAP from a large scale reaction mixture of DNMP and ascorbic acid.

NMAP is unstable upon heating and its mass spectrum showed only fragmentation peaks at m/e 125, 109 and 80, however, field desorption mass spectrum revealed a molecular ion peak at m/e 141. ^{13}C-n.m.r. spectrum in CD_3OD (internal standard; tetramethylsilane) showed a methyl carbon at σ21.3ppm (q), two olefinic carbons at σ102.0 (d) and σ117.8ppm (d) (3- and 5-carbons,



DNMP NMAP

Fig. 3. Transformation of DNMP to NMAP by ascorbic acid at pH 6.8 and 37°C.

respectively) and 2- and 4-carbons at σ159.6 (s) and σ160.4ppm (vice versa). ^{1}H-n.m.r. spectrum in CD_3OD showed a 3H singlet at σ2.36ppm and two olefinic protons at σ6.59 and σ7.86ppm (J=1.5 Hz). NMAP has absorption at ν_{max}^{KBr} 3500cm^{-1} (NH) in IR spectrum and λ_{max} (ethanol) 250nm (ε=2.3x10^4), 275nm (ε=7.9x10^3) and 315nm (ε=1.9x10^2) in UV spectrum.

Molecular formula of NMAP has been confirmed by high-resolution mass spectrum of the monoacetate derivative of NMAP and it gave a molecular ion peak at m/e 183.0638 ($C_7H_9O_3N_3$) and ^{1}H-n.m.r. spectrum in CD_3OD showed a 3H singlet due to N-acetyl group of amino group at σ2.44ppm. DNMP was polarographically reducible and it has a least negative half wave potential (-0.10 V. vs S.C.E.) for the conjugated C-nitro group (13), however, NMAP has no reduction wave and this result indicated that the conjugated C-nitro group was reduced (13, 14). From these instrumental analyses, the structure of NMAP has confirmed as shown in Fig. 3 and details of structural elucidation will be published elsewhere.

MUTAGENICITY OF DNMP AND NMAP

Mutagenicities of DNMP and NMAP were determined by the Ames test using Salmonella typhimurium TA 100 and 98 strains with or without S-9 mix. We found that DNMP was highly mutagenic in both strains (11), while NMAP gave no mutagenic activity at all (Fig.4). These results indicate that the loss of mutagenicity of DNMP is due to reduction of the conjugated C-nitro group to the amino group.

DISCUSSION

The loss of mutagenicity of DNMP by ascorbic acid or cysteine was confirmed by the Ames test in combination with the HPLC technique. The fact

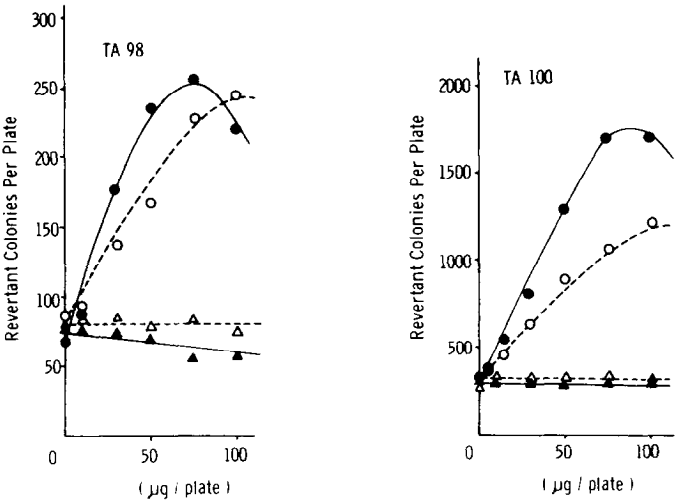


Fig. 4. Mutagenic activities of DNMP and NMAP in Salmonella typhimurium TA 100 and 98 strains with or without S-9 mix.

—●— ; DNMP without metabolic activation
---○--- ; DNMP + S-9 mix
—▲— ; NMAP without metabolic activation
---△--- ; NMAP + S-9 mix

that the mutagenic DNMP was converted to biologically inactive substances seems to be very important, especially, from the point of view of food safety since both of ascorbic acid and cysteine are popular as food constituents and that these reactions may occur during cooking, processing and digestion. Moreover, reduction of the conjugated C-nitro group of nitropyrrole to the C-amino group by ascorbic acid is also very interesting from viewpoint of a chemical reaction; this led us to examine the possibility of reduction of 2- or 5-nitro group in nitrofurazone and AF-2 (most of nitrofurans and nitropyrroles have nitro group at 2- or 5-position) (15, 16). However, these two compounds were not altered by treatment with ascorbic acid at all range of pH. Thus, the question whether ascorbic acid could reduce specifically the nitro group at 3- or 4-position in mutagenic nitropyrroles or nitrofurans is now under investigation. The reaction product of DNMP with cysteine was shown to be different from that of NMAP in the HPLC chromatograms. We are actually studying its biological and chemical properties.

The present experiments were done *in vitro* system but it is still necessary to determine whether this type of desmutagenic reaction(s) might take place during processing, cooking or digestion of foods in the human body.

ACKNOWLEDGEMENT

We wish to thank Dr. K. Tsuji of The Institute of Physical and Chemical Research for valuable discussion and suggestion. This work was supported by a research grant from Nissan Science foundation.

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