Research Section

MUTAGENIC ACTIVITY OF PEPTIDES AND
THE ARTIFICIAL SWEETENER ASPARTAME AFTER
NITROSATION

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(Accepted 26 January 1993)

Abstract—Naturally occurring dipeptides, cholecystokinin (CKK, a tetrapeptide hormone) and the
artificial sweetener aspartame were nitrosated for 10–30 min with 40 mm-nitrite (pH 3.5, 37°C), and the
resultant products examined for mutagenicity in Salmonella typhimurium TA100. Specific mutagenicities
(net revertants per μmol precursor) spanned four orders of magnitude, with CCK being the most potent
precursor (4700 revertants/μmol) followed by tryptophyl-tryptophan (Trp-Trp; 1000 revertants/μmol).
Aspartame and glycylyl-Trp (Gly–Trp) had intermediate activity (300 revertants/μmol), while Gly–Gly and
methionyl-methionine were only weakly mutagenic (20 and 12 revertants/μmol, respectively).
The dipeptides of aspartic acid, phenylalanine and tyrosine had no detectable mutagenicity (limits of
detection 0.5, 40 and 5 revertants/μmol, respectively). Kinetic studies with aspartame and Gly–Trp
suggested that the mutagenic products arose primarily from nitrosation of the primary amine rather than
the amide or indole group. The mutagenicities of nitrosated aspartame and Gly–Trp were higher in TA100
than in TA98, and higher without than with enzymatic activation (S-9 mix) in both strains. The time-
course study of Trp–Trp nitrosation showed the production of at least two mutagens: a potent but unstable
mutagenicity was seen at very short nitrosation times and a more stable but weaker effect was obtained
after more than 60 min of nitrosation. Not only the absolute specific mutagenicity but also the nitrite
dependence of the nitrosation reaction and the stability of the nitroso product must be taken into account
in determining the risk posed by endogenous nitrosation of foods in the human stomach. Under stomach
conditions, nitrosation of the side-chains of certain Trp peptides would be expected to contribute more
to the endogenous burden of nitrosated products than nitrosation of aspartame or Gly peptides.

INTRODUCTION

The normal human diet contains many constituents
that can be nitrosated in the gastro-intestinal tract
(particularly in the stomach) to form potentially
carcinogenic N-nitroso compounds (NOCs). Tumour
formation as a result of endogenous nitrosation has
been demonstrated in rats given N-methylbenzyl-
amine in the diet and nitrite in the drinking water for
2 yr (Sander, 1971; Tahira et al., 1988). Endogenous
nitrosation is also known to occur in humans: volun-
teers given a large dose of proline or thioproline and
nitrate (the latter is converted to nitrite by oral cavity
bacteria) excrete increased amounts of N-nitroso
products in the urine (Bartsch et al., 1989). Nitrosa-
tion can also take place in stimulated macrophages and
endothelial cells; here arginine serves as precurs-
or for the nitrosating agent (Leaf et al., 1989). The
magnitude of the health risk posed by endogenous
nitrosation of dietary components in the normal
acidic stomach, in comparison with other sites of
endogenous nitrosation and with exogenous sources
of NOCs, is still controversial.

The contribution of nitrosation of primary amino
groups to the endogenous NOC burden received
little attention until recently, because nitrosated
primary amines were considered to be too unstable
to be genotoxic (Ridd, 1961). However, recent in vitro
kinetic studies with aspartic acid (Asp), the dipeptide
artificial sweetener aspartame (aspartylphenylalanyl-
methylester) and glycine (Gly) ethylester, all three
compounds bearing a primary amino group, showed
that the alkylating activity of the nitrosated products
was surprisingly stable (half-lives of 500, 200 and
30 min, respectively, in acidic, and 60, 15 and 2 min,
respectively, in neutral aqueous buffers). In each case,
the amount of alkylating agent increased with the
square of the nitrite concentration in the nitrosation
reaction (Meier et al., 1990).

Two in vitro assays using 4-(p-nitrobenzyl)-pyridine
(NBP) as model nucleophile were recently developed
to measure the alkylating potential of unstable NOCs
following nitrosation (Shephard et al., 1987). With
these tests, a cross-section of dietary precursors,
including ureas, guanidines, amino acids, peptides

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and primary amines, were compared for their overall nitrosation speed/alkylating activity. The results showed that the primary amine of some dipeptides, especially those with N-terminal tryptophan (Trp) or N-terminal Gly, and aspartame ranked very high as compared with other precursor classes. However, detailed studies on the stability of nitrosated Trp peptides could not be carried out with this system because coloured nitrosation products interfered with the colorimetric measurement of the NBP adducts. For the same reason, the indole precursor class could not be included in this comparison.

Endogenous nitrosation studies have been carried out in rats with several primary amine precursors. Evidence of genotoxicity was found with three compounds: oral gavage of radioactive methylamine, tyramine or Trp followed by sodium nitrite resulted in the formation of radio-labelled DNA adducts specifically in the stomach. Borderline results were obtained when rats were treated with nitrite and the precursors Gly-ethylester and Asp (Lutz et al., 1990; Meier et al., 1990).

In the context of in vitro nitrosation studies on indole derivatives, Ochiai et al. (1986) found that two tryptophan dipeptides, Trp–Trp and Trp–Gly, were mutagenic in Salmonella typhimurium strain TA100 following nitrite treatment; however, no mutagenic effect was observed with other two peptides, Gly–Trp and cholecystokinin (CCK; a tetrapeptide hormone [CCK]; a tetrapeptide hormone (with N-terminal Trp). In preliminary tests, nitrosated peptides could not be carried out with this system because coloured nitrosation products interfered with the coolorimetric measurement of the NBP adducts. For the same reason, the indole precursor class could not be included in this comparison.

The purpose of the present study was three-fold: first, to examine whether the alkylating potential of nitrosated peptides observed in the chemical model system was significant in a system with a biological end point; secondly, to gather more information on nitrosated peptides bearing the indole side-chain and thirdly, to compare the possible contributions of peptides and indoles to the daily burden of mutagenic products arising from endogenous nitrosation.

MATERIALS AND METHODS

Materials

Dipeptides [Trp–Trp, tyrosyl–tyrosine (Tyr–Tyr), methionyl–methionine (Met–Met), Gly–Trp, Asp–Asp, phenylalanyl–phenylalanine (Phe–Phe)] were from Sigma Chemical Co. (St Louis, MO, USA) except for Gly–Gly and aspartame, which were purchased from Wako Pure Chemical Industries (Osaka, Japan). CCK was from the Peptide Institute, Inc. (Minoh-shi, Osaka, Japan). Methylurea was from Wako Pure Chemical Industries. Harmaline·HCl and 1-methyl-Trp were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Bacterial strains

S. typhimurium strains TA100, TA98, TA102 and TA104 were generously provided by Professor B. Ames (University of California, Berkeley, CA, USA). Liver microsomal (S-9) fractions were obtained from the livers of rats treated with polychlorinated biphenyls (Kanechlor KC 500; Kanegafuchi Chemical Co., Tokyo, Japan).

Standard method for nitrosation and Ames test

The precursor (final concentration 1–40 mM, 114 mM for Asp–Asp) and nitrite (final concentration 40 mM) were combined in sterile citrate (80 mM)–phosphate buffer (pH 3.0) to give a final pH of 3.5 in a total volume of 2 ml. The concentrations in the Results refer to the nitrosation reaction. The reaction was carried out in a shaking water-bath (37°C) for 10 or 30 min. Unreacted nitrite was destroyed by adding an excess of ammonium sulphamate (10% molar) and vortexing briefly. Duplicate aliquots of 25, 50, 100 and 200 μl nitrosation mixture were immediately pipetted into sterile test-tubes and subjected to the mutation assay.

Neutral phosphate buffer (0.5 ml, 100 mM-phosphate, pH 7.4) and 0.1 ml of an overnight culture of S. typhimurium TA100 were immediately added, and the mixtures were preincubated at 37°C for 30 min. The time between stopping the nitrosation reaction and adding the bacterial suspension was not longer than 2 min. After plating out and 2 days of incubation at 37°C, the revertant colonies were counted. Control TA100 plates had typically 100–110 spontaneous revertants. The specific mutagenicity was calculated from the linear portion of the dose–response curve and expressed as net revertants/μmol precursor/plate. In some (negative) cases, the highest precursor concentration tested was less than 40 mM because of insolubility. The specific mutagenicity of each precursor was determined in two to four independent experiments. Variation between individual experiments averaged 10–15%.

Nitrosation reaction kinetics

Precursor concentration dependence. Gly–Trp and aspartame concentrations were varied over the range 1 to 32 mM, and those of Trp–Trp and 1-methyl-Trp over the range 125 μM to 8 mM. Nitrite concentration was kept constant at 40 mM. Nitrosation was carried out for 30 min. At each concentration, eight appropriate aliquots of nitrosation mixture were tested with S. typhimurium TA100 as described under Standard method for nitrosation and Ames test.

Time course. 8 mM-Gly–Trp, 8 mM-aspartame, 2 mM-Trp–Trp and 0.5 mM-1-methyl–Trp (optimal concentrations, as determined above) were nitrosated with 40 mM-nitrite for various periods. After stopping the reaction with ammonium sulphamate, eight aliquots of nitrosation mixture were immediately tested with S. typhimurium TA100 without S-9 as described above.

Nitrite concentration dependence. 8 mM-Gly–Trp and 8 mM-aspartame were nitrosated with various concentrations of nitrite (1–40 mM) for 10 min and
5 min, respectively. To compensate for the various amounts of nitrite, the pH was quickly readjusted with an appropriate volume of 250 mM-H₃PO₄ (determined beforehand). The reaction was stopped with an excess of ammonium sulphamate. Duplicate aliquots of nitrosation mix (200 µl) were tested with *S. typhimurium* TA100 as described above. Two independent experiments were carried out, and the variation between them averaged 10–15%.

**pH dependence.** Phosphate buffers with a pH between 1.5 and 4.0 (intervals of 0.5 pH units) were prepared, and aspartame nitrosation was carried out at the different pHs for 30 min using 8 mM-aspartame and 40 mM-nitrite. Following nitrosation, 1 M-NaOH was added to the preincubation mixture (where necessary) to bring the solution to neutrality, and mutagenicity was tested with *S. typhimurium* TA100 as described above.

**Strain dependence**

Aspartame (20 mM) was nitrosated under standard conditions. Mutagenicity was examined with *S. typhimurium* TA100, TA98, TA102 and TA104 in parallel. Gly–Trp (8 mM) was similarly nitrosated and tested with *S. typhimurium* TA100 and TA98. Spontaneous revertants in strains TA98, TA102 and TA104 were 9, 225 and 140 colonies per plate, respectively.

**S-9 mix dependence**

The effect of substituting 500 µl S-9 mix (10% S-9) for the pH 7.4 phosphate buffer was examined with Gly–Trp and aspartame (both 8 mM) in TA100 and TA98. The samples were nitrosated with 40 mM-nitrite for 10 and 30 min, respectively.

**RESULTS**

**Specific mutagenicity**

Several of the peptide precursors showed a definite, dose-dependent mutagenicity in *S. typhimurium* TA100 following nitrosation. The specific mutagenicities of the precursors following nitrite treatment spanned four orders of magnitude (Table 1). The hormone tetrapeptide CCK (Trp–Met–Asp–Phe–NH₂, where Trp is the N-terminal amino acid and the carboxyl group of Phe is present as the amide derivative), with almost 5000 revertants/µmol after

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**Fig. 1. Relationship between the concentration of precursor in the nitrosation reaction and the resultant mutagenic activity in *Salmonella typhimurium* TA100. The experimental conditions were: 30 min of nitrosation with 40 mM-nitrite at pH 3.5. The volume of nitrosation mixture per plate was: (a) Gly–Trp, 200 µl; (b) aspartame, 100 µl; (c) Trp–Trp, 200 µl and (d) 1-methyl-Trp, 25 µl.
Table 1. Specific mutagenicity in *Salmonella typhimurium* TA100 of various peptides incubated in the absence of rat liver S-9 fractions after nitrosation with 40 mM-nitrate at pH 3.5 for 30 min (except where otherwise indicated)

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Concentration in nitrosation reaction (mM)</th>
<th>Mutagenicity (revertants/μmol precursor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCK tetrapeptide</td>
<td>1.25</td>
<td>4700*</td>
</tr>
<tr>
<td>Trp-Trp</td>
<td>1.0</td>
<td>1000</td>
</tr>
<tr>
<td>Aspartame</td>
<td>8</td>
<td>300</td>
</tr>
<tr>
<td>Gly–Trp</td>
<td>8</td>
<td>300</td>
</tr>
<tr>
<td>Gly–Gly</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Met–Met</td>
<td>20</td>
<td>12*</td>
</tr>
<tr>
<td>Phe–Phe</td>
<td>5</td>
<td>&lt;40†</td>
</tr>
<tr>
<td>Tyr–Tyr</td>
<td>20</td>
<td>&lt;5†</td>
</tr>
<tr>
<td>Asp–Asp</td>
<td>114</td>
<td>&lt;0.5†</td>
</tr>
<tr>
<td>1-Methyl-Trp</td>
<td>0.25</td>
<td>8000</td>
</tr>
<tr>
<td>Harmaline</td>
<td>1</td>
<td>4000</td>
</tr>
<tr>
<td>Methylurea</td>
<td>4</td>
<td>12,500</td>
</tr>
</tbody>
</table>

*Nitrosation time = 10 min.
†Values given are limits of detection, which were dependent on solubility.

Specific mutagenicity was calculated from at least two independent experiments consisting of eight plates each, as described in the text.

10 min nitrosation, was the most potent precursor followed by Trp–Trp with 1000 revertants/μmol. Aspartame and Gly–Trp, with 300 revertants/μmol, had intermediate activity, while Gly–Gly and Met–Met were only weakly mutagenic. Asp–Asp, Phe–Phe and Tyr–Tyr had no detectable mutagenicity (limits of detection 0.5, 40 and 5 revertants/μmol, respectively) after nitrosation even at high levels of precursor and nitrite. For comparison, two Trp analogues (1-methyl-Trp and harmaline) and methylurea (included as calibration standard to allow comparison with other precursor classes reported previously; Shephard et al., 1987) were nitrosated and tested for mutagenicity under the same conditions.

Nitrosation reaction kinetics

The unstable mutagenic nitrosation product(s) of some of the more potent precursors, particularly Gly–Trp and aspartame, were characterized in greater detail with respect to their nitrosation kinetic parameters and the specificity of the mutagenic response.

Precursor concentration dependence. The relationship between the concentration of the precursor in the nitrosation reaction and the resultant mutagenicity was examined over a large concentration range with Gly–Trp, aspartame, Trp–Trp and 1-methyl-Trp (Fig. 1). In each case, a linear dose response was observed with bactericidal effects at high doses, although mutagenicities produced by Gly–Trp and Trp–Trp with nitrite decreased during the 30-min nitrosation reaction as described below.

Nitrite concentration dependence. Experiments were carried out with various nitrite concentrations in the nitrosation reaction while the peptide concentration was kept constant. With Gly–Trp, which can produce revertants without appreciable cytotoxicity after nitrosation, a quadratic relationship between nitrite concentration and induced revertants was seen (Fig. 2a). This result implies that the amount of the mutagen ("NO-Gly–Trp") formed was proportional to the square of the nitrite concentration. With aspartame, the results were less clear-cut. The products of aspartame nitrosation appeared to be quite cytotoxic, giving only a relatively narrow span of interpretable results between background and maximum levels of revertants, thus making a non-linear relationship more difficult to detect. However, after 5 min of nitrosation, a reproducible tendency towards a quadratic relationship between nitrite concentration and induced revertants was observed at the sensitivity limits of this biological system (Fig. 2b).

Time course. The curves of mutagenic activity plotted against nitrosation time (Fig. 3) are very interesting. They show the formation of different kinds of mutagens from a single amine precursor. The mutagenic activity produced by nitrosation of 1-methyl-Trp increased with time up to 120 min, suggesting that the major mutagenic product(s) are stable (Fig. 3d). In contrast, with Gly–Trp and Trp–Trp the maximum mutagenic activity was
detected less than 5 min after addition of nitrite (Fig. 3a,c). The mutagens produced by nitrosation of Trp–Trp seemed to be composed of both unstable and stable compounds, and those of Gly–Trp of a very rapidly and a relatively slowly degrading compound(s). With aspartame, the situation was different again: the mutagenicity increased to a peak value at 25 min of nitrosation and decreased slowly thereafter (Fig. 3b).

pH dependence. Over the pH range 1.5 to 4.0, the highest number of revertants was observed when aspartame was nitrosated at pH 3.5 for 30 min (data not shown).

Bacterial strain dependence and effect of S-9 mix

The mutagenicity of nitrosated aspartame and Gly–Trp in various bacterial strains and the effect of added S-9 mix are shown in Table 2. Nitrosated aspartame was most mutagenic in S. typhimurium TA100, followed by TA104 and TA98 (300, 100 and 10 revertants/#mol, respectively). Mutagenicity was negligible in TA102. Nitrosated Gly–Trp was more active in TA100 than in TA98 (560 and 70 revertants/#mol, respectively). Addition of S-9 mix reduced effectively the mutagenicity of both nitrosated dipeptides in TA100 and TA98; the effect was more pronounced with aspartame than with Gly–Trp.

**DISCUSSION**

The data from the kinetic studies with Gly–Trp and aspartame suggest that mutagenicity results from nitrosation of the amine, rather than amide bond: quadratic dependence on nitrite concentration (Fig. 2) is expected with the nitrosation of a primary amine group; in contrast, the nitrosation of an amide bond appears to be first-order in nitrite. For Gly–Trp, the observed dependence is close to quadratic, with a calculated exponent of 1.75. The mutagens produced by nitrosation of Trp–Trp seemed to be composed of both unstable and stable compounds, and those of Gly–Trp of a very rapidly and a relatively slowly degrading compound(s). With aspartame, the situation was different again: the mutagenicity increased to a peak value at 25 min of nitrosation and decreased slowly thereafter (Fig. 3b).

**Table 2. Mutagenicity of nitrosated aspartame and Gly–Trp in various *Salmonella typhimurium* strains with and without added rat liver S-9 fractions**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Aspartame (revertants/μmol precursor)</th>
<th>Gly–Trp (revertants/μmol precursor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA100 –S-9</td>
<td>300</td>
<td>560</td>
</tr>
<tr>
<td>+S-9</td>
<td>75</td>
<td>350</td>
</tr>
<tr>
<td>TA98 –S-9</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>+S-9</td>
<td>&lt;4*</td>
<td>20</td>
</tr>
<tr>
<td>TA102 –S-9</td>
<td>&lt;6*</td>
<td>nd</td>
</tr>
<tr>
<td>TA104 –S-9</td>
<td>100</td>
<td>nd</td>
</tr>
</tbody>
</table>

*Below detection limit.

Aspartame (20 mM) was nitrosated for 30 min, and Gly–Trp (8 mM) for 10 min with 40 mM-nitrite, pH 3.0. The specific mutagenicity was calculated by linear regression as described in the text.
has a linear nitrite dependence (Mirvish, 1975). In the NBP system, where no problem is caused by cytotoxicity, a clear second-order (quadratic) dependence on nitrite concentration was seen with the nitrosation of all peptides tested (Shephard, 1987; S. E. Shephard, unpublished results, 1989). Similarly, the observed maximum nitrosation rate at pH 3.5 is typical of the nitrosation of amines. Amide nitrosation, in contrast, has a linear dependence on proton concentration, and therefore would show no peak but would display a 10-fold increase in yield for every drop of 1 U in the pH (Mirvish, 1975).

The peptides showing the highest mutagenic activity in *S. typhimurium* TA100 were those bearing a Trp residue. With these compounds, both the amine and indole groups have been suggested to be the sites of nitrosation (Wakabayashi *et al.*, 1989). Previous studies have conclusively shown that the indole group is mutagenic on nitrosation, with mutagenic potency being very strongly influenced by the pattern of substitution; however, Trp derivatives require a free amine group to express mutagenicity (Ochiai *et al.*, 1986). In another report (Ohara *et al.*, 1988), nitrosated Trp gave at least eight different products. In the present study, the curves of mutagenic activity over nitrosation time suggest that both the indole and amine sites compete for the available nitrite, with the relative importance of each site varying considerably from derivative to derivative. With 1-methyl-Trp, for instance, indole nitrosation appears to be predominant, because the nitrosation time-course study showed the production of a stable mutagenic activity over 1 hr; this activity was very similar to that seen with harmaline, a /β/-carboline precursor bearing an indole but no amine function (data not shown). In contrast, with Gly–Trp (and perhaps with CCK) amine nitrosation may predominate: nitrite dependence was quadratic and the mutagenic activity, potent at very short nitrosation times, decreased extremely rapidly. This may explain why Ochiai *et al.* (1986), who used a 60-min nitrosation, could not detect any mutagenic activity with either Gly–Trp or CCK. Further, with Trp–Trp both types of nitrosatable sites may contribute to mutagenic activity: amine nitrosation would be predominant at short nitrosation times and indole nitrosation after longer times (Fig. 3). As demonstrated in these time-course studies, evaluation of the mutagenic potential of peptides as precursors of endogenous NOCs is highly dependent on the length of the nitrosation reaction chosen.

The mutagenic potentials of peptide precursors also depend strongly on the nitrite concentration used, since it has been shown that nitrosation of the primary amine of peptides has a quadratic nitrite dependence whereas indole nitrosation yield is proportional to nitrite concentration (Kurosky and Hofmann, 1972; Shephard *et al.*, 1990). Thus, if the millimolar nitrite concentrations used here were extrapolated to the micromolar concentrations found in the stomach, indole nitrosation yields would be expected to drop 10^4 times whereas amine nitrosation yields would decrease 10^6 times. The estimated average daily intakes of selected nitrosatable peptides and aspartame in the Western diet are given in Table 3. Under stomach conditions, precursors like the Trp derivatives that react preferentially at the indole sites would be expected to contribute more to the endogenous burden of nitrosated products than aspartame and the Gly peptides.

**Acknowledgements**—This study was supported by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan. S. E. Shephard was the recipient of a Foreign Research Fellowship from the Foundation for Promotion of Cancer Research of Japan.

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Mutagenicity of nitrosated peptides


