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Research Section

MUTAGENIC ACTIVITY OF PEPTIDES AND THE ARTIFICIAL SWEETENER ASPARTAME AFTER NITROSATION

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Abstract—Naturally occurring dipeptides, cholecystokinine (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 glycyl–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*-methylbenzylamine 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: volunteers 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). Nitrosation can also take place in stimulated macrophages and endothelial cells; here arginine serves as precursor 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 radiolabelled 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 two other peptides, Gly–Trp and cholecystokinine (CCK; a tetrapeptide hormone with *N*-terminal Trp). In preliminary tests, nitrosated aspartame was also mutagenic in TA100 (Shephard *et al.*, 1987).

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 endpoint; 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-H_3PO_4 (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–NH_2, 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

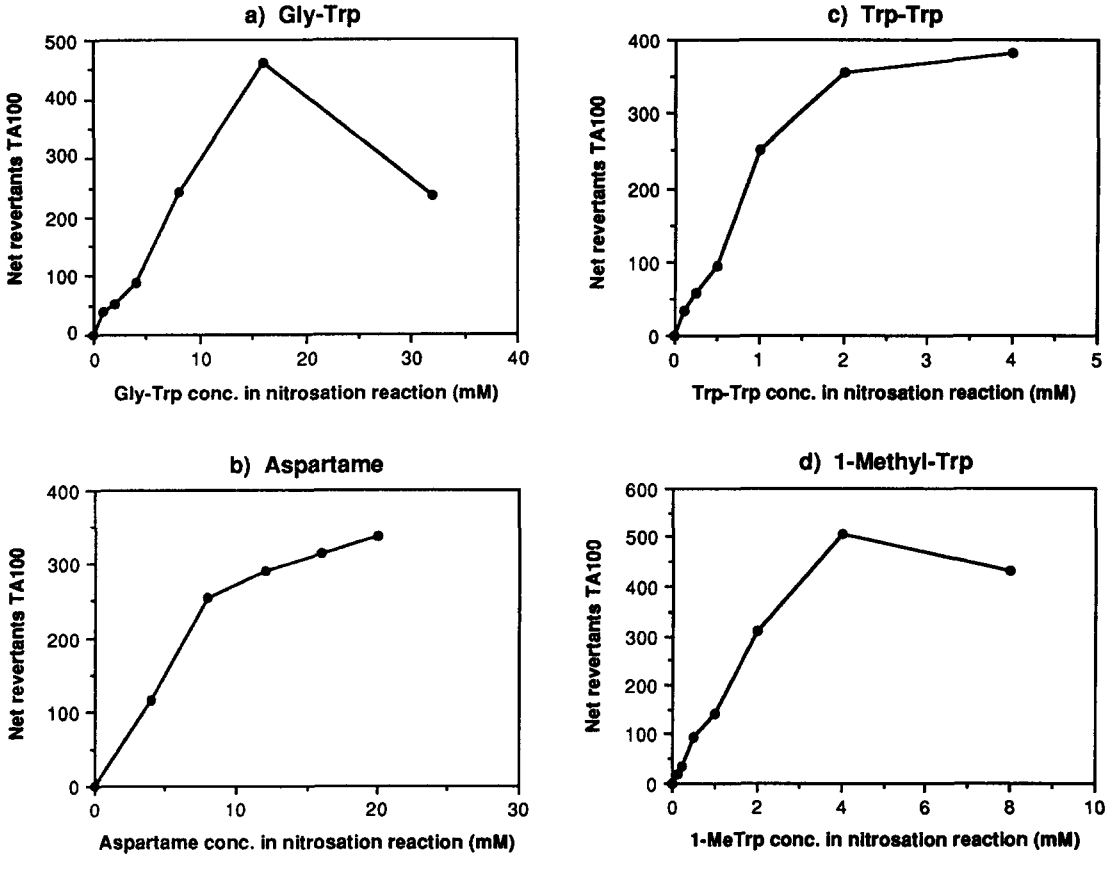

Fig. 1. Relationship between the concentration of precursor in the nitrosation reaction and the resultant mutagenic activity in *Salmonella typhimurium* TA100. The experimental conditions were: 30 min of nitrosation with 40 mM-nitrite at pH 3.5. The volume of nitrosation mixture per plate was: (a) Gly–Trp, 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)

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

*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

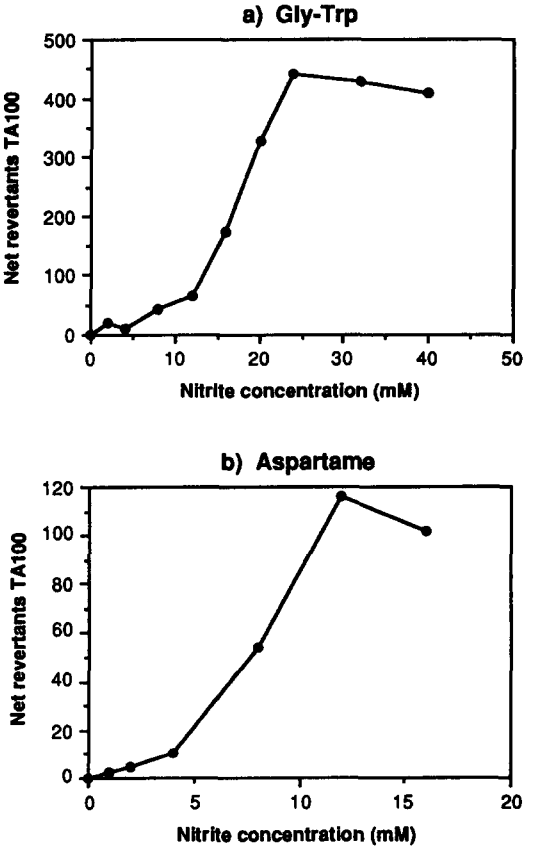

Fig. 2. Relationship between the concentration of nitrite in the nitrosation reaction and the resultant mutagenic activity in *Salmonella typhimurium* TA100. The experimental conditions were: (a) 8 mM-Gly–Trp, 10-min nitrosation and (b) 20 mM-aspartame, 5-min nitrosation. The pH of the reaction was 3.5. The numbers of revertants induced by 200 μl nitrosation mixture per plate are shown.

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/μmol, respectively). Mutagenicity was negligible in TA102. Nitrosated Gly–Trp was more active in TA100 than in TA98 (560 and 70 revertants/μmol, 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.

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

Bacterial strain		Aspartame (revertants/μmol precursor)	Gly–Trp (revertants/μmol precursor)
TA100	−S-9	300	560
	+S-9	75	350
TA98	−S-9	10	70
	+S-9	<4*	20
TA102	−S-9	<6*	nd
TA104	−S-9	100	nd

nd = not done

*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 descibed in the text.

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

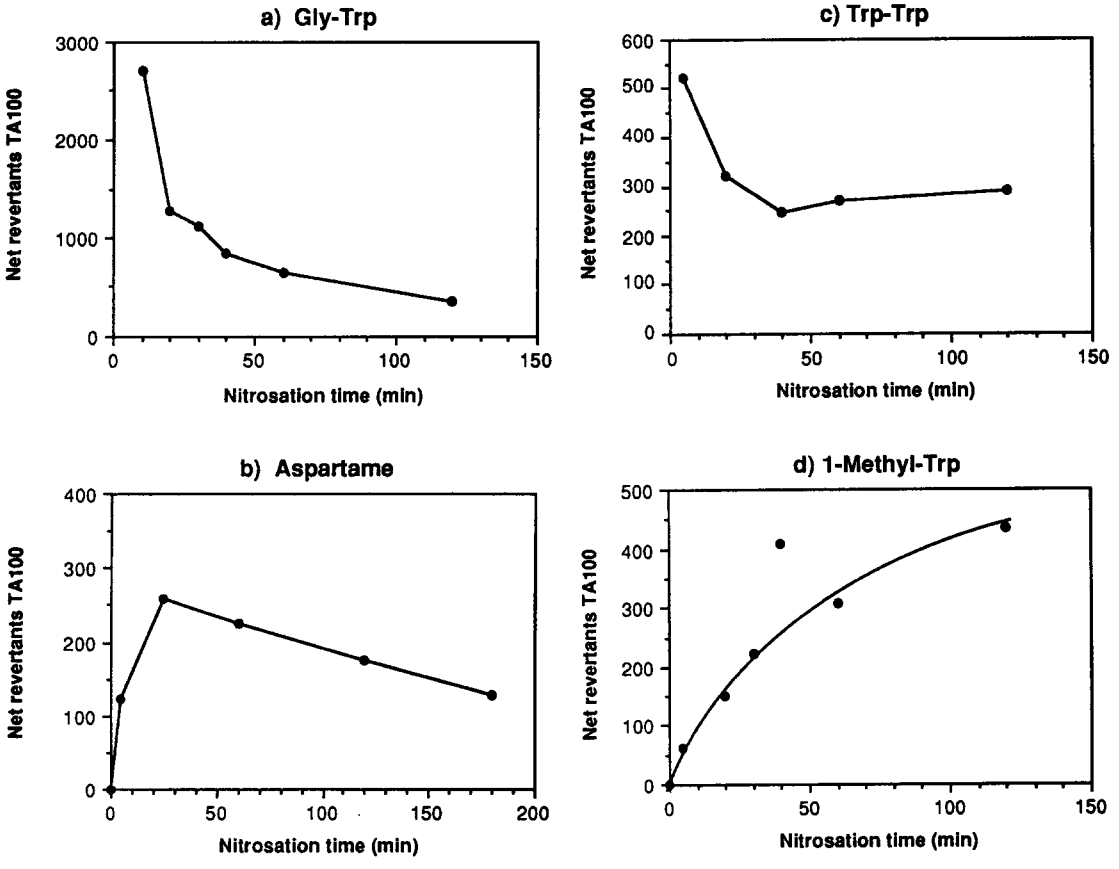

Fig. 3. Time-course study of the formation of products mutagenic to *Salmonella typhimurium* TA100 by the nitrosation of various dipeptides and 1-methyl-Trp. The conditions of the nitrosation reaction were: 40 mM-nitrite, pH 3.5 and (a) 8 mM-Gly–Trp, (b) 8 mM-aspartame, (c) 2 mM-Trp–Trp, or (d) 0.5 mM-1-methyl-Trp. The numbers of revertants induced by 200 μl nitrosation mixture per plate are shown.

Table 3. Estimated average daily intakes of selected nitrosatable peptides and aspartame in the Western diet

Nitrosatable precursor	Major dietary source	Estimated daily intake (g/person)	Reference
Gly peptides	Bound in protein	5–10*	Belitz and Schormüller, 1965
Trp peptides	Bound in protein	1–2*	
Aspartame	Artificially sweetened foods	Median = 0.02–0.07 Maximum ≃ 0.4	*Food and Chemical Toxicology*, 1991

*Based on a typical protein intake of 100 g/person/day (Schweiz. Bauernsekretariat, 1983). These estimates include oligopeptides of all sizes.

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 10^3 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.

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