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ALKYLATING POTENCY OF NITROSATED AMINO ACIDS AND PEPTIDES

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The alkylating potency of unstable *N*-nitrosamino acids and *N*-nitrosopeptides was investigated *in vitro* using 4-(*para*-nitrobenzyl)pyridine (NBP) as nucleophile. Of the amino acids, Met and those with an aromatic side chain were the most potent. The relative overall alkylating potency was 23:10:5:4:2:1: for Trp, Met, His, Tyr, Phe and Gly, respectively. The homo-dipeptides were much more potent than the amino acids, with relative potencies of 400:110:100:8:3:1, for Trp–Trp, Tyr–Tyr, Met–Met, Asp–Asp, Phe–Phe and Gly, respectively. In the one-phase reaction system (in which NBP is already present during the nitrosation reaction at acidic pH), all amino acids tested showed a second-order reaction for nitrite. In the two-phase system (in which NBP is added only after bringing the nitrosation reaction mixture to neutrality), all amino acids tested except one again showed a second-order reaction for nitrite (Phe, His, Asp and the dipeptide artificial sweetener aspartame); only Met under these conditions had a reaction order of one for nitrite. This could mean that nitrosation of the side chain of Met produces a second *N*-nitroso product which is relatively stable in acid but reacts with NBP under neutral conditions. In the human stomach, this side-chain nitrosation might become more important than the reactions at the primary amino group, firstly because of the greater stability of the product(s) in acid and secondly because of the first-order reaction rate for nitrite. A decrease in nitrite concentration from the millimolar concentrations of the in-vitro assay to the micromolar concentrations in the stomach reduces the reaction rate by a factor of 1000 for the side-chain nitrosation, whereas a million-fold reduction will be observed for nitrosation of the amino group.

Although unstable primary *N*-nitroso compounds (NOC) formed endogenously in the gastric lumen are detoxified to a large extent by lumen nucleophiles, there is evidence that these labile NOC could in some cases be sufficiently stable that a fraction might diffuse through the stomach lining and cause local DNA damage (Huber & Lutz, 1984). Using in-vitro screening tests designed to mimic partially the situation in the stomach, a cross-section of dietary NOC precursers (including ureas, guanidines, primary amines, amino acids and dipeptides) was assayed for their activation-independent mutagenic activity and/or alkylating activity immediately following nitrosation (Shephard *et al.*, 1987). As some amino acids and dipeptides showed high reactivity as compared to other precursor classes, more comprehensive and detailed studies on the nitrosation and alkylating characteristics of amino acids and dipeptides have been undertaken.

Overall alkylating potency

The nitrosation and alkylation kinetics were measured by simple colorimetric assays using 4-(*para*-nitrobenzyl)pyridine (NBP) as nucleophile. The tests exploit the fact that alkylated NBP forms a blue chromophore at pH 10. In the so-called one-step NBP test, precursor, nitrite and NBP are present simultaneously at acidic pH. Electrophilic species generated by the breakdown of unstable NOC are trapped immediately by NBP. Experimental details have been described elsewhere (Shephard *et al.*, 1987).

The rates of NBP adduct formation by amino acid and dipeptide precursors in the one-step test are shown in Figure 1. Most striking was the enormous range of reaction rates, from Glu and Gln, which had no detectable activity (less than 1% of the rate of Gly), up to Trp–Trp which reacted 400 times faster than Gly — a span of over four orders of magnitude. Of the amino acids, Met and those with an aromatic side chain were the most reactive. The majority of the aliphatic amino acids were slightly less reactive than Gly. The homo-dipeptides tended to be more reactive than the corresponding amino acids. This was especially pronounced among the most potent amino acid–peptide pairs, where addition of a second residue increased the rate of adduct formation by a factor of 10–30 fold (Trp, 20-fold; Tyr, 29-fold; Met, 10-fold). Striking, too, was the increase in reactivity of aspartame (Asp–Phe-methylester, an artificial sweetener) over that of Asp-Asp (13-fold) and of Asp (180-fold). The reactivity of the peptides was governed mainly by the identity of the *N*-terminal amino acid (e.g., compare Ala–Gly and Gly–Ala, or Met–Gly and Gly–Met with the respective homodipeptides). As with the amino acids, peptides with an aromatic side chain had greater reactivity.

Reaction order for nitrite

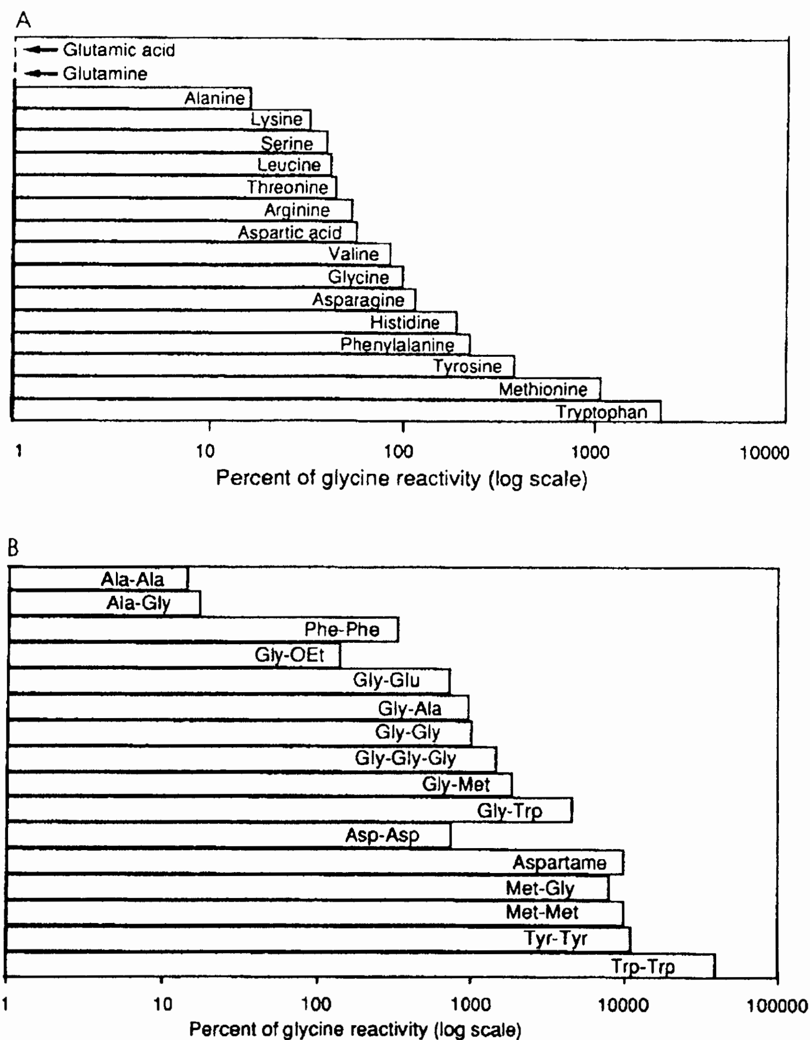
Preliminary work showed that the reactivity of both Gly and Gly–Gly increased proportionally to the square of the nitrite concentration, thus following amine- rather than amide-type kinetics (see Mirvish, 1975). Furthermore, *N*-acetylglycine had no reactivity (Shephard *et al.*, 1987). Thus, the alkylating activity being measured appeared to result from nitrosation of the primary amine; however, among the most potent amino acids, additional reactions on the side chains are also possible (Bonnett & Nicolaidou, 1977). The reaction order for nitrite was investigated for these special amino acids.

Using the one-step NBP test, the reaction rate was measured at three or more different nitrite concentrations in the range 10–75 mM (typically, 30, 40, 50 mM). When log(reaction rate) was plotted against log(nitrite concentration), the slope of the resulting line gave the reaction order for nitrite. With each amino acid tested, the production of alkylated NBP was, within experimental error, dependent upon the square of the nitrite concentration (data not shown). This suggested either that the side-chain reactions (i) had the same kinetic order as the reaction at the primary amine and thus were indistinguishable from it or (ii) occurred much more slowly than nitrosation of the primary amine, or that (iii) the product of side-chain nitrosation was stable at acidic pH and therefore undetectable.

To distinguish between these possibilities, more detailed experiments were carried out using the two-step NBP test, which is a closer approximation of the situation *in vivo*. In this system, the nitrosation and alkylation reactions are separated spatially and temporally:

nitrosation is first carried out at acidic pH in the absence of NBP, followed by alkylation of NBP at neutral pH. The experimental details have been described previously (Shephard *et al.*, 1987). The reaction order for nitrite was determined again for Met, His and Phe. Asp and aspartame were also tested, since the latter had shown high reactivity in the one-step test. Because of interfering colour reactions, Trp and Tyr could not be tested in the two-step system. Very short nitrosation times (20, 40 and 60 sec) were used in order to obtain the initial nitrosation reaction rate, before significant breakdown of NOC could occur.

Fig. 1. Overview of 4-(*para*-nitrobenzyl)pyridine (NBP) alkylating activity of A, amino acids and B, peptides following nitrosation in the NBP one-step test



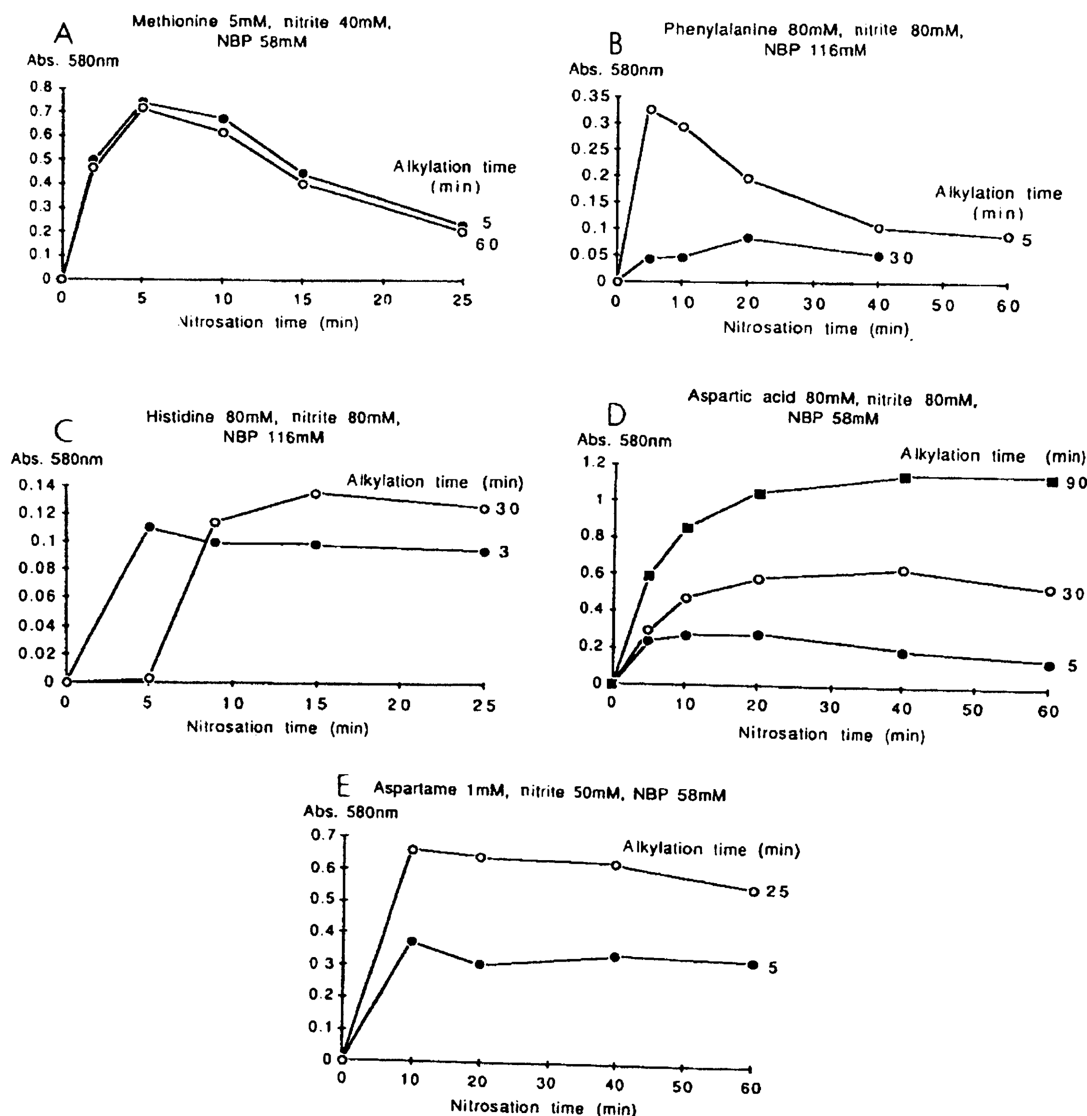
Under these conditions, Phe, His, Asp and aspartame all again showed the expected second-order reaction for nitrite (data not shown). Nitrosation of Met, however, showed a *linear* dependence on nitrite concentration. The difference in the behaviour of Met in the two test systems could be due to the production of a nitrosated product at the side chain. In the human stomach, this side-chain nitrosation might become more important than the reactions at the primary amino group, firstly because of the greater stability of the

product(s) in acid and secondly because of the first-order reaction rate for nitrite. A decrease in nitrite concentration from the millimolar concentrations of the in-vitro assay to the micromolar concentrations in the stomach reduces the reaction rate by a factor of 1000 for the side-chain nitrosation, whereas a million-fold reduction will be observed for the nitrosation of the amino group.

Nitrosation reaction profile

The time course of the nitrosation and alkylation reactions was investigated with the two-step test (Fig. 2). Met nitrosated very rapidly — the NOC concentration peaked after only 5 min of nitrosation — then broke down rapidly in acidic solution (half-life, 20 min at pH 2.5). The nitrosated product also reacted extremely rapidly with NBP: the reaction was finished by 5 min (no increase in product was seen after that time). Phe, in contrast,

Fig. 2. Time course of the nitrosation and alkylation reactions of selected amino acids and aspartame in the 4-(*para*-nitrobenzyl)pyridine) (NBP) two-step test



made two nitrosated products. One was nitrosated very rapidly (peak concentration after 5 min) and also alkylated NBP very rapidly; however, the NBP adduct was unstable at pH 7. At longer alkylation times, a second nitrosation peak emerged at 20 min of nitrosation, indicating a product that both nitrosated and alkylated more slowly. The former peak was probably due to the primary amine, the second, weaker reaction to the side chain. His similarly made two products, one with extremely rapid alkylation and a nitrosation peak at 5 min, and the second, again weaker, at longer nitrosation and alkylation times. Asp and aspartame showed yet another pattern of behaviour: they both made nitrosated products that were practically stable at acidic pH (half-life, > 200 min). Alkylation of NBP occurred rather more slowly but reached high levels after 90 min and longer. These nitrosation and alkylation profiles demonstrate the inhomogeneity of the amino acids with respect to nitrosating speed, reaction order for nitrite, alkylating potency and stability of nitroso product — factors that must be taken into account when evaluating the genotoxic risk posed by the in-vivo nitrosation of amino acids and peptides.

Although in-vitro screening tests clearly cannot replace in-vivo assays, they do offer valuable information for the ranking of chemical classes as well as of individual precursors with respect to the formation of alkylating NOC. On the basis of the results of these tests, in-vivo nitrosation experiments are now under way with important precursors.

References

Bonnett, R. & Nicolaidou, P. (1977) Nitrite and the environment. The nitrosation of alpha-amino acid derivatives. *Heterocycles*, 7, 637-659

Huber, K.W. & Lutz, W.K. (1984) Methylation of DNA in stomach and small intestine of rats after oral administration of methylamine and nitrite. *Carcinogenesis*, 5, 1729-1732

Mirvish, S.S. (1975) Formation of N-nitroso-compounds: chemistry, kinetics, and in vivo occurence. *Toxicol. Appl. Pharmacol.*, 31, 325-351

Shephard, S.E., Hegi, M. & Lutz, W.K. (1987) In-vitro assays to detect alkylating and mutagenic activities of dietary components nitrosated *in situ*. In: Bartsch, H., O'Neill, I.K. & Schulte-Hermann, R., eds, *The Relevance of* N*-Nitroso Compounds to Human Cancer: Exposures and Mechanisms* (IARC Scientific Publications No. 84), Lyon, IARC, pp. 232-236