IN-VITRO ASSAYS TO DETECT ALKYLATING AND MUTAGENIC ACTIVITIES OF DIETARY COMPONENTS NITROSATED *IN SITU*

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Nitrosation of dietary components has been combined with the 4-(*para*-nitrobenzyl)pyridine (NBP) colorimetric test for screening alkylating agents and with the Ames test for the detection of mutagenic activity. This allowed the investigation of short-lived nitrosation products of dietary components which generate electrophilic degradation products requiring no metabolic activation (natural amino acids and some derivatives, ureas, guanidines, primary alkyl and aryl amines). In a first system, precursor, nitrous acid and NBP were present simultaneously. All amino acids tested, except glutamic acid and glutamine, gave positive results. The reactivities spanned more than three orders of magnitude, with the aromatic amino acids and methionine the most active; two primary amines, tryptamine and histamine, were also strongly reactive. All guanidines tested, except the amino acid arginine, gave negative results. A second system consisted of two phases: NBP was added only after destruction of residual nitrite and adjustment of the pH to neutrality. This system was useful for the study of ureas, which are stable in acid but not in neutral media. The range of responses covered more than two orders of magnitude. Most amino acids and primary amines also gave positive results, but could be assessed only after analysing the kinetics of the competing reactions and choosing appropriate reaction times. In a third system, *Salmonella typhimurium* strain TA100 replaced NBP. Representatives of the class of amino acids, ureas, the primary amine tryptamine, and aniline became highly mutagenic upon nitrosation. Methylguanidine was only weakly mutagenic under the present assay conditions. The results indicate that further studies with unstable nitrosation products of dietary components are required to understand more thoroughly the role of endogenous nitrosation in gastric cancer.

In studies of compounds that could produce genotoxic carcinogens upon nitrosation, the precursors that lead to unstable *N*-nitroso compounds have been given relatively little attention. One reason for the lack of interest has been the problem of handling reactive compounds. A recent report on studies of precursors in the human diet pointed out the need for additional effort on ureas and amides, aryl amines, guanidines and amino acids (Shephard *et al.*, 1987 and this volume). Test systems have thus been developed in which labile nitrosation products formed in nitrosation assays can be analysed for alkylating or mutagenic activity *in situ* or immediately after their generation.

NBP was introduced by Epstein *et al.* (1955) as an analytical reagent for alkylating agents. Alkylation of the pyridine nitrogen followed by alkaline deprotonation of a benzylic hydrogen results in a dark blue-violet chromophore with an absorption maximum at 580 nm. The applications of this reagent to detect activation-independent carcinogens,

including *N*-nitroso compounds, have been compiled by Archer and Eng (1981) in the introduction to a paper describing the formation of alkylating intermediates from *N*-nitrosodiethylamine by a chemical oxidation system. The modifications described below include a system in which precursors are nitrosated in the presence of NBP in order also to trap highly labile alkylating reaction products.

Salmonella bacteria have for some time been used as indicator organisms to detect activation-independent mutagenic reaction products formed in a nitrosation assay, without extracting *N*-nitroso compounds from the incubation mixture. Positive results with defined precursors have been reported with guanidines (Endo *et al.*, 1974), aminoantipyrine and aniline (Boido *et al.*, 1980), ureas and carbamates (Takeda & Kanaya, 1982), tryptophan, tryptamine and 5-hydroxytryptamine (Gatehouse & Wedd, 1983), ranitidine (De Flora *et al.*, 1983) and diverse amine drugs (Andrews *et al.*, 1984), as well as with indole-3-acetonitrile from Chinese cabbage (Wakabayashi *et al.*, 1985a). We describe a modification of the system which allows bacteria to be present when the nitrosation reaction mixture is neutralized.

Alkylation of NBP *in situ*: NBP one-phase system

In our new system, the nitrosation and alkylation with NBP are allowed to proceed simultaneously. In order that all components can be brought into solution, the solvent system has to be a compromise between an acidic buffer (250 mM phosphate, pH 2.5) and an aprotic solvent mixture (ethylene glycol plus acetone). Under standard conditions at 37°C, a concentration of 40 mM is used both for the test compound and for nitrite; NBP is added at 47 mM. At intervals of 15 min to 1 h, aliquots of the reaction mixtures are made basic by the addition of triethylamine with vigorous mixing. Absorption at 580 nm is measured after exactly 30 and 60 sec, and the absorbance at t = 0 sec is calculated by extrapolation, assuming a first-order decay of the colour due to instability of the NBP-test compound adduct.

This one-phase system seems to be highly versatile, because it makes possible the instantaneous trapping of electrophiles formed with nitrous acid. Results are shown in Table 1. Rates of reaction (A_{580}/min) were expressed in relation to glycine (set at 100%). All α-amino acids except glutamic acid and glutamine produced a positive result. The reactivities spanned more than three orders of magnitude, with the aromatic amino acids and methionine the most active. Acetylation of the amino group resulted in complete loss of alkylating activity; modification of the carboxylic acid function, however, gave rise to an increased response, indicated by the reaction rates of an ester and of a dipeptide of glycine. The dipeptide sweetening agent, aspartame (aspartylphenylalanine methyl ester), was the most potent alkylating agent under the present assay conditions. Ureas were not investigated in this system because *N*-methyl-*N*-nitrosourea was too stable in acid to react with NBP. All guanidines tested also gave negative results. Some primary amines containing an aromatic moiety were more effective alkylating agents than glycine, whereas methylamine ranked at 40% and propylamine reactivity was below the limit of detection. Data obtained with the aryl amine aniline are as yet inconclusive because the red colour observed must first be assigned to either side reactions or formation of other NBP-adduct chromophores.

NBP two-phase system

In the stomach, nitroso compounds formed under the acidic conditions of the lumen can diffuse into the lining cells, where they encounter neutral pH. In order to simulate this type

Table 1. Alkylating potential (measured by the NBP reaction) and mutagenicity in the Ames test of dietary components incubated with nitrous acid

Compound	NBP one-phase rate of reaction (% glycine)	NBP two-phase overall reactivity (% methylurea)	Ames test, two-phases (revertants/μmol)[a]
Amino acids			
Tryptophan	2300	ND[b]	2000[c]
Methionine	1100	20	90
Tyrosine	400	ND	450[c]
Glycine	*100*	16	260
Arginine	54	<2	24
Lysine	30	ND	<1
Glutamic acid	<1	ND	<1
Amino acid derivatives and dipeptides			
N-Acetylglycine	<0.1	<1	60[d]
Glycine ethyl ester	150	10 000	2000[c]
Glycylglycine	300	500	ND
Aspartame	8000	4200	200
Ureas			
Methyl urea	<0.1	*100*	170[c,d]
Carbamoyl β-alanine	ND	120	5900
Citrulline	7[e]	6	20[f]
Methylhydantoin	ND	<0.3	ND
Guanidines			
Methylguanidine	<0.1	<2	23[d]
Creatin(in)e	<0.1	<2	<1
Primary amines			
Tryptamine	900	8	(pH 3.4) 7800
Histamine	450	pink colour	18
Methylamine	40	ND	<1
Propylamine	<1	ND	ND
Aryl amine			
Aniline	red colour	red colour	(pH 3.4) 2400[c,d]

[a]Based on amount of precursor incubated

[b]ND, no data

[c]Derived from 0.08 or 0.8 mM initial concentration; high toxicity to bacteria at higher levels

[d]Overproportional mutagenic activity at higher concentrations tested

[e]Positive response probably due to amino acid function

[f]pH 3.4

of two-stage process, a protocol involving two phases was worked out. First, test compound and nitrite were incubated at pH 2.5 for up to 60 min. Residual nitrite was destroyed by addition of amidosulfonic acid for 5 min, and NBP was added in a slightly alkaline amphiphilic solvent mixture to raise the pH to neutrality. Aliquots were taken at various times up to 90 min, triethylamine was added and the absorbance at 580 nm was measured immediately.

In this system, the overall response is dependent on a number of reaction rates: that of the formation and decay of *N*-nitroso compounds in acid, that of the *N*-nitroso compounds with NBP and other nucleophiles under neutral conditions, and the stability of the NBP adduct in neutral solution. The colour observed is therefore highly dependent on the reaction periods chosen. This complicates the experiment, but it allows analysis of reaction kinetics, so that the experimental findings can be extrapolated to the situation in humans.

The results derived from a 10-min/10-min incubation are shown in Table 1. Methylurea was used as the standard precursor and set at 100 percent, because the nitrosoureas are stable in acid and develop an alkylating potential only in neutral or alkaline solution. The optimal conditions for amino acids and primary amines have not yet been established. It is noteworthy, however, that the amino acid derivative *N*-acetylglycine gave negative results at a limit of detection of 1% methylurea, whereas the formation of esters or peptides resulted in a markedly increased response. As with the one-phase system, all guanidines gave negative results.

Nitrosation assay procedure followed immediately by the Ames test

The alkylating potential does not indicate whether a reactive product can also penetrate biological membranes and react with DNA or whether the resulting adduct is biologically relevant. In order to investigate these questions, *S. typhimurium* TA100 was used as an indicator organism for genotoxicity. Because low pH is toxic to the bacteria and because nitrite is mutagenic, the nitrosation reaction could not be performed in the presence of the bacteria. A one-phase system was therefore impossible, but the conditions used in the two-phase system could be adapted.

In our improved protocol, test compound (normally at 8 and 80 mM) was incubated with nitrite (80 mM) for 30 min at 37°C in a 20 mM phosphate buffer pH 2.4 (amino acids and derivatives, ureas and guanidines) or pH 3.4 (primary amines, aniline, and citrulline). Residual nitrite was destroyed with amidosulfonic acid (1.1 molar equivalents; 5 min, including a sterile filtration). The pH was brought to near neutrality by adding the reaction mixture in 300 mM phosphate buffer pH 8, to which the bacteria had been added 1 min before. This procedure ensured the presence of the bacteria as soon as the pH was neutral. Incubation with the bacteria was continued for 30 min to increase the sensitivity of the Ames test. Then, the bacteria were plated to score for revertants, and an aliquot was assayed for survival. This control was essential for correct interpretation of the results, because it was found that strong mutagenicity was almost always paralleled by potent cytotoxicity, so that only data in which survival was good could be evaluated quantitatively. As a control for mutagenicity of the test compound without nitrosation, each test compound was also incubated with nitrate instead of nitrite; negative results were obtained in all cases.

Some results are shown in Table 1. For a quantitative comparison with the mutagenic potency of other chemicals, the data are expressed as induced revertants per μmol precursor. Since, for instance, β-propiolactone gives rise to 4000 revertants per μmol, the mutagenic potency of many dietary precursors incubated with nitrite is quite remarkable.

With increasing concentrations of the precursors marked with footnote[d] in Table 1, an overproportional increase in the mutagenic response was found. Such nonlinearity was never observed with NBP alkylation; thus, it is probably related to the specific indicator system, i.e., the use of bacteria. It remains to be elucidated whether processes other than alkylation are responsible for this additional mutagenic effect. If similar reactions are possible in stomach lining cells, this would add a new dimension to nitrite risk evaluation.

Conclusions

The data presented clearly show that a great number of dietary components acquire electrophilic and mutagenic potential upon incubation with nitrous acid. For many classes of precursors, the response was much higher than anticipated.

The NBP one-phase system makes possible an overview of the various dietary precursor classes. Such information might facilitate the choice of compounds for supplementary in-vivo tests. The tests could include investigations of DNA binding in the stomach after treatment of animals with precursor and nitrite. Preliminary attempts have been made in this laboratory with a primary amine, methylamine, and with the urea compound dicarbamoylputrescine. Oral administration of ^{14}C-methylamine to rats, followed immediately by gavage with nitrite, resulted in the formation of 7-methylguanine in DNA isolated from the stomach (Huber & Lutz, 1984).

The two-phase systems are necessary for the biological interpretation of the data because they allow analysis of the kinetics of the various reactions. Such information is basic to the understanding of the underlying chemical processes and a prerequisite for extrapolating from the conditions used *in vitro* to the situation prevailing in the human stomach *in vivo*.