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A Screening Procedure for the Formation of Nitroso Derivatives and Mutagens by Drug-Nitrite Interaction

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A general procedure for screening for the formation of nitroso derivatives and/or mutagens by drug-nitrite interaction was established. A test drug (50 mM) was reacted with nitrite (500 mM) at pH 3.0—3.5 and 37°C for 4 h. The residual nitrite in the reaction mixture was decomposed with ammonium sulfamate. The nitrite-free reaction mixture was assayed for both nitroso derivatives and mutagens by colorimetry of Griess reagent-positive substances formed by treatment with hydrogen bromide and by mutagenesis assay using *Salmonella typhimurium* TA98 and TA100 as tester strains, respectively.

Keywords——nitroso derivative; mutagen; nitrosation; drug-nitrite interaction; aminopyrine; dimethylamine; morpholine; *N*-methylurea; ethyl *N*-methylcarbamate

Nitroso compounds, which are among the most potent carcinogens known, are formed *in vivo* by the reaction of nitrogen-containing compounds with nitrite.[1-3] A number of nitrosatable precursors are present in the environment, and possible human risk arising from the nitrosation products of these precursors has been discussed.[4,5]

Drugs are among the nitrosatable precursors to which humans are exposed, and the formation of hazardous substances by drug-nitrite interaction is an important problem in the evaluation of the safety of drugs.[6] Chemical and biological studies on the nitrosation products of some drugs have been reported,[2,7] but most drugs have been used in medical treatment without any information on the possible human risk from drug-nitrite interaction. To establish the safety of drugs, it is necessary to screen out the drugs which give hazardous products on interaction with nitrite, but no procedure suitable for this purpose has been reported so far. We report here on a procedure suitable for screening out the drugs which form nitroso derivatives and/or mutagens by drug-nitrite interaction.

Materials and Methods

Chemicals——*N*-Nitrosomorpholine was synthesized by the reaction of morpholine with sodium nitrite in dilute hydrochloric acid and purified by distillation (bp_{12-13} 103°C). Other chemicals were of reagent grade and were obtained from commercial sources. They were used without further purification.

General Procedure for the Screening of Drug-Nitrite Interaction——Nitrosation: To 0.1 mmol of test material in a tube fitted with a glass stopper, 1.5 ml of dilute hydrochloric acid, which contained sufficient acid to acidify the reaction mixture to pH 3.0—3.5, and 0.5 ml of 2 M sodium nitrite were added. The stoppered tube was shaken at 37°C for 4 h. The final concentration of the test material was 50 mM and that of nitrite was 500 mM.

Removal of Residual Nitrite: The tube was immersed in an ice bath, then 0.5 ml of 2 M ammonium sulfamate was added to the reaction mixture, and the whole was allowed to stand for 5—10 min in an ice bath with gentle shaking.

Colorimetric Determination of Nitroso Compound: A modification of the Eisenbrand and Preussmann procedure[8] was used to determine the nitroso derivatives formed. To 20 μl of the nitrite-free reaction mixture, 2 ml of 1.5% hydrogen bromide in glacial acetic acid was added. The mixture was stirred and left to stand for 15 min at room temperature, then 4 ml of freshly prepared Griess reagent (0.5% sulfanilic acid–0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 30% aq. acetic acid) was added. The whole was mixed and allowed to stand for 15 min at room temperature, then the absorbance was determined at 550 nm. The results were converted to NDMA[9] equivalents according to the results obtained from a concurrent run

using the standard solution of NDMA, and the yield of nitroso derivatives was expressed in mole percent based on the amount of material used for the reaction.

Determination of Mutagenic Activity: A modification, *i.e.*, preincubation (for 20 min at 37°C),[10] of the procedure according to Ames *et al.*[11] was used to determine the mutagenicity of the reaction product, using *S. typhimurium* TA98 and TA100 as tester strains. Up to 200 μl of the nitrite-free reaction mixture per plate was used for the assay. S-9 mix contained 2 μmol of NADPH, 150 μl of S-9 fraction prepared from the liver of rats treated with polychlorinated biphenyls (500 mg/kg), 2.5 μmol of glucose 6-phosphate, 0.25 unit of glucose 6-phosphate dehydrogenase, 4 μmol of magnesium chloride, 16.5 μmol of potassium chloride and 50 μmol of sodium phosphate buffer (pH 7.4) in a total volume of 0.5 ml. The mean number of spontaneous revertants per plate for TA98 without S-9 mix, TA98 with S-9 mix, TA100 without S-9 mix and TA100 with S-9 mix were 25, 36, 148 and 141, respectively. Mutagenicity of a sample was evaluated from the slope of the linear portion of the dose-response curve and is presented in terms of the number of revertants per μmol of parent compound.

Gas Chromatographic Determination of Nitrosamines——The nitrite-free reaction mixture was extracted with an equal volume of ethyl acetate. An equal volume of 3 mM NDIBA in ethyl acetate was then added to the ethyl acetate extract as an internal standard. Analysis was run on a Shimadzu GC-4CMPF gas chromatograph fitted with a hydrogen flame ionization detector and a glass column (3 m × 4 mmϕ) packed with 20% polyethyleneglycol 20 M on Chromosorb W. The temperatures of the column oven and detector were set at 160 and 210°C, respectively. Nitrogen was used as a carrier gas (60 ml/min). The retention times of NDMA, NMOR and NDIBA (internal standard) were 4.2, 28.7 and 10.1 min, respectively. The quantity of nitrosamine was determined from the ratio of the peak area of the nitrosamine to that of the internal standard.

Results

Effect of the Concentration of Nitrite on Nitrosation

Morpholine, dimethylamine and piperidine (50 mM) were individually reacted with nitrite (50—750 mM) at pH 3.4 and 37°C for 4 h, and the formation of nitroso derivatives was determined colorimetrically. As shown in Table I, 100 mM nitrite was required for the quantitative nitrosation of morpholine, and 400 mM nitrite for dimethylamine. With 500 mM nitrite, piperidine was nitrosated in 70% yield.

Table I. Effect of Concentration of Nitrite on the Nitrosation of Secondary Amines

Concentration of nitrite (mM)	Yield of nitroso derivative (mol%)		
	Morpholine	Dimethylamine	Piperidine
50	74	7	2
100	103	20	6
200	103	57	22
400	101	99	57
500	102	106	69
750	102	106	89

Amines (50 mM) were reacted with nitrite at pH 3.4 and 37°C for 4 h.

Effect of Temperature on the Nitrosation of Dimethylamine

At 0, 25 and 37°C, 50 mM dimethylamine was reacted with 500 mM nitrite at pH 3.4, and the time-courses of nitrosation were determined colorimetrically (Table II). At 37°C, quantitative nitrosation was observed within 2 h. The rate of nitrosation was lower either at 25°C or at 0°C than at 37°C.

Decomposition of Nitrite by Ammonium Sulfamate

To 2 ml of 500 mM sodium nitrite, whose pH had been adjusted to 3.4—5.0 by prior addition of a suitable amount of hydrochloric acid, 0.5 ml of 2 M ammonium sulfamate was added at 0—4°C (in an ice bath). The amount of nitrite that remained in the reaction mixture

Table II. Effect of Temperature on the Nitrosation of Dimethylamine

Time of reaction (h)	Yield of nitroso derivative (mol%)		
Reaction at:	0°C	25°C	37°C
0.25	5	24	48
0.5	8	40	73
1.0	14	61	91
2.0	23	76	98
4.0	33	76	107

Dimethylamine (50 mM) was reacted with nitrite (500 mM) at pH 3.4.

Table III. Decomposition of Nitrite by Ammonium Sulfamate

Time of reaction (min)	Nitrite remaining in the reaction mixture (%)			
Reaction at:	pH 3.4	pH 3.7	pH 4.0	pH 5.0
0.5	36	—a)	—	—
1.0	0	78	97	99
2.5	0	0	98	—
5.0	0	0	49	99
10.0	0	0	0	98

Nitrite (400 mM) was reacted with ammonium sulfamate (400 mM) at 0—4°C.
a) Not determined.

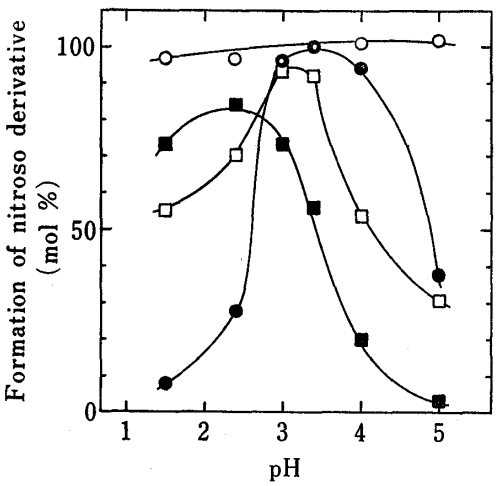

Fig. 1. pH-Profile of Nitrosation of *N*-Alkyl Compounds

N-Alkyl compound (50 mM) was reacted with nitrite (500 mM) at 37°C for 4 h.
○, morpholine; ●, dimethylamine;
□, *N*-methylurea; ■, ethyl *N*-methylcarbamate.

was determined by means of the Griess reagent at suitable intervals (Table III). The decomposition of nitrite was hardly observed at pH 5.0, whereas at lower pH, rapid decomposition was observed. In the pH region below 4.0, the nitrite disappeared within 10 min. The reaction of nitrite with sulfamate was accompanied by a decrease of pH of the reaction mixture from 3.0—3.5 to 1—2. The reaction mixture of 1 mmol of sodium nitrite and 1 mmol of ammonium sulfamate in 2.5 ml of water at pH 3.4 was neither mutagenic nor bacteriostatic to the two tester strains either in the absence or in the presence of S-9 mix in the dose range up to 200 μl/plate.

pH-Profiles of Nitrosation of *N*-Alkyl Compounds

Morpholine, dimethylamine, *N*-methylurea and ethyl *N*-methylcarbamate were reacted with nitrite at pH 1—5 and 37°C for 4 h. The concentration of chemicals was 50 mM and that of nitrite was 500 mM. Formation of nitroso derivatives was determined colorimetrically. As shown in Fig. 1, in the pH 3.0—3.5 region, most of the compounds used were nitrosated in more than 70% yield.

Effect of pH on the Nitrosation of *N*-Methylurea

N-Methylurea (50 mM) and nitrite (500 mM) were reacted at pH 1.5 or 3.5, at 37°C for 1 to 6 h, and the amount of *N*-nitroso-*N*-methylurea formed was determined spectrophotomet-

Table IV. Nitrosation of *N*-Methylurea

Time of reaction	Yield of *N*-nitroso-*N*-methylurea (mol%)	
	Reaction at: pH 1.5	pH 3.4
1.0 min	87	47
2.5 min	87	60
5.0 min	82	78
30.0 min	82	90
1.0 h	80	92
2.0 h	69	91
4.0 h	55	90
6.0 h	44	88

N-Methylurea (50 mM) was reacted with sodium nitrite (500 mM) at 37°C.

rically at 392 nm (ε_{392} 93). The time-course of the reaction is shown in Table IV. At pH 3.4, the yield was almost quantitative within 30 min and remained at a constant level up to 6 h. At pH 1.5, nitrosation was more rapid than at pH 3.4, but after reaching a maximum within 5 min, the yield decreased gradually.

Assay Results of Several Chemicals

Using the procedure described in Materials and Methods, dimethylamine, morpholine, *N*-methylurea, ethyl *N*-methylcarbamate and aminopyrine were assayed for the formation of nitroso derivatives and mutagens. The results are summarized in Table V. Dimethylamine and morpholine were nitrosated quantitatively, and the quantitative formation of the corresponding *N*-nitrosamine was also determined by gas chromatographic analysis. The mutagenicity of the reaction mixtures agreed closely with that of the corresponding *N*-nitrosamines (towards TA100 strain in the presence of S-9 mix: 30 His$^+$ revertants/μmol NDMA, 270 His$^+$ revertants/μmol NMOR). *N*-Methylurea was nitrosated quantitatively and the mutagenicity of the reaction mixture towards TA100 strain was in agreement with that of NMU (5200 or 5300 His$^+$ revertants/μmol, in the absence or in the presence of S-9 mix, respectively). Ethyl *N*-methylcarbamate and aminopyrine gave significant amounts of nitroso derivatives, and their reaction products were mutagenic.

Table V. Formation of Nitroso Compounds and Mutagens from Chemicals by Reaction with Nitrite

Chemicals	Formation of nitroso derivative[a] (mol%) Determined by		Mutagenicity of nitrosation product (Number of His$^+$ revertants/μmol parent compound)			
			TA 98		TA 100	
	Colorimetry	GLC	Without S-9 mix	With S-9 mix	Without S-9 mix	With S-9 mix
Dimethylamine	106	95[c]	—[b]	—	—	39
Morpholine	101	99[d]	—	—	—	390
N-Methylurea	100	N.D.[e]	—	—	5900	4500
Ethyl *N*-methylcarbamate	58	N.D.	—	—	46000	860
Aminopyrine	111	99[c]	73	26	60	150

Chemicals were treated as described in Materials and Methods.
a) Mean of two or three experiments.
b) No significant mutagenic activity was observed.
c) Yield of *N*-nitrosodimethylamine.
d) Yield of *N*-nitrosomorpholine.
e) Not determined.

Discussion

Dimethylamine, which is among the amines unreactive to nitrite,[2] was quantitatively nitrosated by the reaction of 50 mM amine with 500 mM nitrite at pH 3.0—3.5 and 37°C for 4 h. This set of reaction conditions was also suitable for nitrosation of several nitrosatable nitrogen-containing compounds including secondary amines and *N*-alkylamides (Fig. 1). Although the nitrosation of *N*-alkylamides has been reported to become progressively faster with increasing acidity, the yields of nitroso derivatives from *N*-methylurea and ethyl *N*-methylcarbamate after reaction for 4 h decreased with increasing acidity. In the case of nitrosation of *N*-methylurea, the decomposition of nitroso derivative formed by reaction with nitrite occurred at pH 1.5, but not at pH 3.4 (Table IV). *N*-Nitrosoalkylamides are unstable in acid, as well as in alkali.[12] Therefore, reaction at pH 3.0—3.5 seems to be appropriate for a general procedure applicable to the screening of a wide variety of chemicals and drugs.

Since nitrite interferes with the chemical and microbial assay of the reaction products, it is necessary to remove the residual nitrite in the reaction mixture. Removal of the residual nitrite without exposing the products to severely acidic conditions was accomplished by adding ammonium sulfamate, equimolar with nitrite used for the reaction, to the reaction mixture then allowing the whole to stand for several minutes in an ice bath.

As for the analytical method for the determination of nitroso derivatives, a nonspecific method seems to be suitable for screening, since the reaction product is unknown in most cases. For this purpose, colorimetric determination of the Griess reagent-positive substances formed from nitroso derivatives by treatment with hydrogen bromide according to Eisenbrand and Preussmann[8] was satisfactory.

Most carcinogenic nitroso compounds are mutagenic,[13] and the formation of a mutagen by drug-nitrite interaction has been reported recently.[14] In addition to chemical analysis, mutagenicity of the reaction product towards *S. typhimurium* TA98 and TA100 was assayed according to Ames *et al.*[11] The nitrite-free reaction mixture was usable in the mutagenesis assay without additional pretreatment.

On the basis of the results described above, the general procedure was established as described in Materials and Methods. The procedure provides a useful means of detecting the formation of nitroso derivatives and mutagens by drug-nitrite interaction. As shown in Table V, *N*-alkyl compounds of known reactivity to nitrite[2] gave reasonable results. On reaction with nitrite, aminopyrine, a drug which reacts readily with nitrite to yield NDMA and other products,[15] gave NDMA in quantitative yield. The formation of a mutagen other than NDMA is suggested, since mutagenicity of the reaction product(s) of aminopyrine and nitrite was found for both TA98 and TA100 strains both in the absence and in the presence of S-9 mix, whereas NDMA was mutagenic only towards TA100 strain in the presence of S-9 mix. 1-Diketobutyryl-1-phenyl-2-methyl-2-nitrosohydrazide hydrate,[16] a reaction product of aminopyrine with nitrite, has recently been shown to have mutagenic activity.

Although no information on the chemical structure or properties of the reaction product is available, the results of the screening procedure described above afford useful information as to whether or not a drug or chemical forms any hazardous products on reaction with nitrite. The versatility of the procedure has been shown in the screening of some nitrogen-containing drugs[17] as well as in that of pharmaceutical preparations of tranquilizers.[18]

The standardization of conditions for *in vitro* testing of drug-nitrite interaction has been recommended by the WHO Study Group on the Potential Carcinogenicity of Nitrosatable Drugs.[19] The recommendation does not seem to be applicable to the present screening purpose, since the reaction conditions recommended are milder than those of the procedure reported in this paper, and seem to be inadequate for efficient nitrosation. The recommended conditions might be useful for studies of drugs which have been proved to be readily nitrosatable.

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