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THE AQUEOUS SULFITE-NITRITE SYSTEM: INVESTIGATIONS ON THE MUTAGENICITY OF SOME KNOWN PRODUCTS

W.H. Kalus*[1], R. Münzner[1] and W.G. Filby[2]

[1] Federal Research Center for Nutrition, Engesserstr. 20, D-7500 Karlsruhe

[2] Nuclear Research Center, D-7500 Karlsruhe

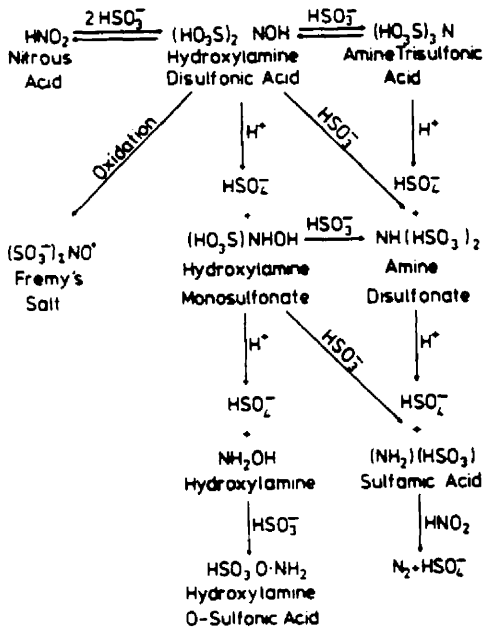
ABSTRACT
Some known reaction products of the two commonly used food additives, sulfite and nitrite, were examined for mutagenicity using the Salmonella/mammalian-microsome test. Potassium nitrosodisulfonate, potassium aminetrisulfonate and potassium hydroxylaminemonosulfonate were not mutagenic over a dose range of 0.01 - 10 mg/plate in the strains his G 46, TA 100 and TA 98. Potassium hydroxylaminedisulfonate showed a weak mutagenic activity in his G 46 and TA 100 with microsomal activation. Hydroxylamine-O-sulfonic acid was only weakly mutagenic in the excision-repair proficient strain his G 46 in the presence of S9.

INTRODUCTION

Chemicals have usually been tested individually for their biological effects. But by way of multiple transformations, for example in the biosphere or in the diet, new, potentially hazardous substances can be formed. Combination effects are therefore of particular practical interest. To date, investigations of this problem have been carried out to a limited extent only. As model substances we have chosen some of the reaction products of sulfite and nitrite. Both anions occur ubiquitously throughout the biosphere. Furthermore, they are widely used as food additives and may be produced in the organism from sulfur containing amino acids or nitrate, respectively.

The reduction of nitrous acid by sulfur dioxide has been a subject of research for many years. Whereas most of the early work was aimed at a detailed understanding of the mechanism of hydroxylamine synthesis more

recent interest in these reactions has been concerned with atmospheric aerosol formation and the chemical treatment of flue gases. A brief account of the reactions presently thought to occur in the oxidising sulfite-nitrite system is given in the simplified scheme (I) and discussed below.



Scheme I. Simplified scheme of the sulfite-nitrite oxidizing system

The definitive kinetic work on hydroxylamine synthesis was presented in a series of papers (1). Essentially the reaction to hydroxylaminedisulfonate was thought to involve the H+ catalysed decay of a primary addition product of sulfite on nitrite to the intermediate nitrosulfonic acid - an as yet still not isolated substance. Further addition of a sulfite ion to this lead to the observed product hydroxylamindisulfonate. The initial fairly simple reaction is complicated by secondary reactions involving, mainly depending on pH, reactant concentration and reaction duration, hydrolysis and/or further sulfonation, leading to highly sulfited products e.g. aminetrisulfonic acid, aminedisulfonate (scheme I). Recently,

Oblath et al.(2) presented data on the formation of hydroxylaminedisulfonic acid from sulfite and nitrite in weakly acidic solution. Although a unique mechanism could not be formulated three concurrent processes were proposed to explain the observed kinetics. The same authors later presented data on the reaction of nitrite with hydroxylaminemonosulfonate (3). A newer in-

vestigation of the system has cast further light on the matter. Littlejohn et al. (4) have employed the Laser Raman technique to follow the build up of various species with time using different sulfite/nitrite ratios. In this work it was possible, for the first time, to observe all stable species and their temporal dependence in the reaction system. The composition of mixtures after longer reaction times turned out to be very critically dependent on the initial concentration ratios and hence the probability of secondary hydrolytic or sulfonation reactions. Interestingly, the free sulfite ion does not appear to participate. In the presence of oxygen (5) or organic peroxides (6) the formation of the nitrososulfonate radical anion was observed.

The present study was undertaken to assess the possible mutagenic effect of some of the available stable reaction products of sulfite-nitrite interactions.

EXPERIMENTAL

Chemicals:

Commercially available materials were used without further purification. Nitrosodisulfonate radical anion (Fremy's salt) was obtained from Sigma Chemical Co. (Deisenhofen FRG). Sodium nitrite and sodium sulfite analytical grade were purchased from Merck (Darmstadt. FRG). Hydroxylamine-O-sulfonic acid (HOMS) was supplied by Fluka AG (Neu Ulm, FRG). Potassium hydroxylaminedisulfonate (HADS) was synthesized from nitrite-ion and bisulfite by the method of Rollefson et al. (7), and potassium hydroxylaminemonosulfonate (HAMS) by the technique of Degener et al. (1). Aminetrisulfonate (ATS) was synthesized by the procedure given by Sisler et al. (8). The prepared compounds were stored by a vacuum desiccator under P_2O_5 until use.

The interaction of Fremy's salt with some components of the sulphite-nitrite system

In order to check the possible formation of further secondary products formed during the course of the mutagenicity tests the decomposition kinetics of the somewhat unstable Fremy's salt in the presence of some model compounds was investigated at pH 7.2 (Ames-buffer) by ESR spectroscopy. The reactions were generally sufficiently slow to be followed by conventional mixing outside the cavity and subsequently drawing the mixture into the cell by suction. The initial concentration of Fremy's salt was 5×10^{-4} M

and the substrates were maintained at tenfold excess in order to approximate pseudo-first order conditions. Whereas mixtures of sulfite, HADS, HAMS and HADS/HAMS (equimolar) had no discernible influence on the spontaneous decay of Fremy's salt (< 2 x 10^{-7} mole $l^{-1}s^{-1}$) HOMS increased it considerably to ca. 2.3 x 10^{-6} mole l^{-1} s^{-1} corresponding to a half life of about 15 mins under our conditions. Sulfamic acid, also a product of the sulfite-nitrite interaction is known to inhibit the chain decomposition of Fremy's salt by scavenging the proposed chain carrying intermediate HNO_2.

Mutagenicity Testing

The standard plate-incorporation test was carried out according to the procedure of Ames et al. (9). The order of mixing the test components was soft agar (2.0 ml), bacteria (0.1 ml), either S9-mix or phosphate buffer (pH 7.4) (0.5 ml), and finally the test substance, freshly dissolved in ice-cold aqua dest. (0.1 ml). In the experiments with strain his G 46 0.25 ml of bacteria culture were employed. The mixture was poured onto a minimal agar plate. Preliminary experiments showed that the use of ice-cold water was essential in order to achieve reproducible results, especially in the case of the nitrosodisulfonate radical anion.

Salmonella typhimurium strains his G 46, TA 98 and TA 100 were used. For information concerning the genotype of these strains see Ames et al. (9). The liver homogenate 9000 x g supernatant fraction was prepared from three pooled livers of male Sprague-Dawley rats treated with Aroclor 1254 (i.p. 500 mg/kg body weight) as described (9). The S9-mix contained 20% S9-fraction.

RESULTS AND DISCUSSION

Mutagenicity Testing

Table I and II show the results of the mutagenicity testing of five reaction products of sulfite and nitrite. The substances were tested over a three-log dose range in the presence and absence of S9-mix. The results of the concentration range showing a weak mutagenic effect were confirmed by using a narrower range of doses. The test substances were considered mutagenic if the response was twice the background mutation rate. As only with microsomal activation a mutagenic response was seen assaying potassium hydroxylaminedisulfonate and hydroxylamine-O-sulfonic acid the data without S9-mix are not shown. Also the results obtained with TA 98 were omitted

because the five tested compunds failed to show a mutagenic response in this strain.

The results presented in Table I and II indicate that potassium nitrosodisulfonate, potassium aminetrisulfonate and potassium hydroxylaminemonosulfonate were not mutagenic in the strains his G 46 ad TA 100. Potassium hydroxylaminedisulfonate showed in the narrow dose range of 2 - 10 mg/plate a weak, but reproducible mutagenic activity in his G 46 and TA 100. Hydroxylamine-O-sulfonic acid was only mutagenic in the excision-repair proficient strain his G 46, but failed to induce mutations in the excision-repair deficient strain TA 100. At a concentration of 5 mg/plate hydroxylamine-O-sulfonic acid inhibited growth of his G 46 and TA 100.

Table I: Mutagenicity testing of some reaction products of sulfite and nitrite in strain his G 46 with metabolic activation

Conc. mg/plate	I	II	III	IV	V
0	33[a]	35	33	35	27
0.01	40	37	31	41	23
0.1	49	38	40	68	26
1.0	59	38	58	98	25
2.0	57	35	69	51	30
5.0	54	31	71	toxic	22
10.0	41	24	79	toxic	21

[a] Number of His^+ revertants per ml distributed on 4 plates

I	Potassium nitrosodisulfonate (Fremy's salt)	$(SO_3K)_2NO$
II	Potassium aminetrisulfonate (ATS)	$(KSO_3)_3N$
III	Potassium hydroxylaminedisulfonate (HADS)	$(KSO_3)_2N\text{-}OH$
IV	Hydroxylamine-O-sulfonic acid (HOMS)	NH_2OSO_3H
V	Potassium hydroxylaminemonosulfonate (HAMS)	$KSO_3NH\text{-}OH$

In earlier studies on the mutagenicity of sulfite-treated fruit and vegetable juices it was observed that mutagenic activity was only evident in

the excision-repair proficient strain his G 46 but not in the excision-repair deficient strains TA 1535 and TA 100 of Salmonella typhimurium (10). It was suggested that excision-repair dependent mechanisms are responsible for this greater sensitivity of strain his G 46 toward the sulfite-treated

Table II: Mutagenicity testing of some reaction products of sulfite and nitrite in strain TA 100 with metabolic activation

Conc. mg/plate	I	II	III	IV	V
0	125[a]	124	114	126	129
0.01	123	123	122	128	144
0.1	115	121	111	133	145
1.0	111	112	135	125	145
2.0	119	105	185	62	127
5.0	112	129	245	toxic	125
10.0	118	121	144	toxic	142

[a] Number of His^+ revertants per plate

I	Potassium nitrosodisulfonate (Fremy's salt)	$(SO_3K)_2NO$
II	Potassium aminetrisulfonate (ATS)	$(KSO_3)_3N$
III	Potassium hydroxylaminedisulfonate (HADS)	$(KSO_3)_2N\text{-}OH$
IV	Hydroxylamine-O-sulfonic acid (HOMS)	NH_2OSO_3H
V	Potassium hydroxylaminemonosulfonate (HAMS)	$KSO_3NH\text{-}OH$

juices. Indeed, later investigations with several strains of E. coli of varying DNA-repair capacities indicated that excision-repair is necessary for a co-mutagenic effect by bisulfite (11).

The observation that mutagenicity of hydroxylamine-O-sulfonic acid was only demonstrable with strain his G 46 but not with TA 100 is important for practical mutagenesis testing in that sulfonic acids may be detected with greater sensitivity in the excision-repair proficient strains than in repair-deficient strains.

In as far as the binding of the potentially hazardous nitrosating agent nitrite by sulfite results in the fairly innocuous substances investigated here, this process may be looked upon as a blocking mechanism against nitrosamine formation.

Similarly, the possibility of formation of genotoxic substances arising via secondary reactions of the nitrosodisulfonate radical anion in real situations seems to be negligible on account of their, in general, very low rates of reaction.

An important aspect of this study was to examine to what extent the cooperative mutagenic effects of bisulfite in combination with nitrite observed by Khoudokormoff et al. (12) and Hayatsu et al. (13) could be accounted for by the specific compounds studied here. According to our results it can be said that the effects may be semi-quantitatively accounted for by the two compounds of low mutagenicity III and IV studied in this work. We recommend, however, that the search be continued, perhaps with the emphasis on testing for the non-isolable intermediates (short lived ions or other free radicals) generated under controlled conditions.

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