

Antibacterial Effect of Cysteine-Nitrosothiol and Possible Precursors Thereof

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The postulated intermediate of nitrite-myoglobin reaction, cysteine-nitrosothiol, was prepared and its antibacterial effect was tested on *Salmonella* strains, *Streptococcus faecium*, and spores and vegetative cells of *Clostridium sporogenes*. Cysteine-nitrosothiol showed a higher inhibitory effect than nitrite. Preliminary results on the effect of simultaneous use of nitrite and cysteine on *Clostridium sporogenes* spores were also presented.

Since nitrosamines were identified in foods (3), interest has been focused on precursors of nitrosamines as meat additives, initially nitrate and nitrite. Due to a lack of sufficient information, entirely different and extreme views evolved (19). The prohibition of nitrate and nitrite would lead, as it is well known, not only to the disappearance of cured meat products because of taste and color problems (although results of MacNeil and Mast [12] showed good flavor formation with special seasoning without nitrite), but also to the decrease of microbiological safety. This health risk, the danger of *Clostridium botulinum*, is considered as the only acceptable argument by health authorities in favor of the continued use of nitrate and nitrite.

Since a definite trend towards prohibition of nitrate and nitrite can be noticed on one hand, and since, on the other, these compounds evidently cannot be omitted from antibacterial use, it is clear that food chemists and microbiologists must preserve nitrite usage in meat industry even if at lower concentrations.

We might consider the general view against nitrate, as a precursor to qualitatively and quantitatively uncontrollable derivatives. At the same time, there is much information on the nitrite concentrations required to control clostridial growth. On the basis of works of Greenberg (6), Leistner et al. (11), and Farkas et al. (4) on the sporostatic effect in food medium, an initial concentration of 150 to 250 μg of nitrate per g of NaNO_2 is generally needed, supposing normal conditions (F_0 below 0.5, spore count below $10^5/\text{g}$). Because of the danger of botulism, this level cannot be decreased.

Nevertheless, there are data that suggest

some kind of revision of these abovementioned concentrations. As has been known for many years, the concentration of nitrite necessary for sporostatic effect depends highly on initial count and the heat treatment (17). On the other hand there is information available which suggests that the microbiological activity of nitrite can be increased by the help of reducing agents and heat treatment (1, 9, 16; W. R. Schack and R. E. Taylor, Canadian patent 674, 171), in which case authors suggest formation of an active intermediate of nitrite.

Mirna and Hofmann (13) describe the identification of the nitrosothiols that are formed from the reaction of nitrite and $-\text{SH}$ compounds. They consider these substances as an active intermediate (activated nitrite) of the reaction of nitrite + myoglobin \rightarrow nitrosomyoglobin.

Taking into consideration the analogies of both fields, one automatically assumes that, by the help of an additional compound or treatment properly chosen, the effectiveness of nitrite from the viewpoint of both color formation and bacteriostatic (sporostatic) action can be increased.

This fact has two consequences. (i) To acquire the desired effect, nitrite may be in lower concentration than needed without these additives. (ii) On the other hand it may be that the same intermediate compounds bring about the increase of both the bacteriostatic and the color-forming effects.

On the basis of the remarkable increase in the microbial activity of nitrite caused by cysteine, which was published by Johnston and Loynes (8) and whose result was confirmed by our investigations, we came to the assumption that per-

haps nitrosothiols formed from -SH compounds and nitrite may also have a microbiological effect similar to their role in cure-pigment formation, as reported by Mirna and Hofmann (13). Our experiments were therefore aimed at studying the effect of cysteine-nitrite combination, formation of nitrosothiol from cysteine and nitrite, and the effect of nitrosothiol.

Although emphasis is generally on inhibition of spores after a heat treatment of above 100 C, in practice temperatures below 100 C are also very important with meat products. Therefore, the reaction of vegetative bacteria, spores, and vegetative cells of sporeformers onto nitrite or nitrosothiol addition were investigated at temperatures at and below 100 C.

MATERIALS AND METHODS

Spore suspension. Spores from *Clostridium sporogenes* (from the collection of Central Food Research Institute, Budapest, Hungary) were prepared, harvested, and maintained according to Riemann (18). Inoculation, imitating practice, was carried out prior to a heat treatment of 100 C for 10 min (F_0 0.1).

Medium. Beef bouillon (one part beef, three parts water) was supplemented with 0.5% peptone and 2% NaCl and sterilized at 121 C for 20 min. This medium (pH 6.2), deaerated before inoculation by boiling, was distributed in vials, and anaerobic conditions were assured by height of liquid only. This medium contained no added Eh-lowering substances to avoid disturbance of experiments on the effect of reducing additives.

Vegetative bacteria. *Streptococcus faecium* (our isolation), *Salmonella enteritidis*, *S. anatum*, *S. choleraesuis*, var. *kunzendorf* (origin, Hungarian Meat Inspection Service), and vegetative forms of *C. sporogenes* were used in tests. Inoculation was carried out prior to heat treatment at 60 C for 10 min. Anaerobic conditions were provided for clostridia only.

Preparation of nitrosothiol. Red crystalline cysteine-nitrosothiol was prepared by the method of Mirna and Hofmann (13), and the crystals were refrigerated until use. Concentrations of nitrosothiol are given as NaNO_2 equivalents throughout this article.

Investigations with cysteine. These tests were carried out in raw diluted meat suspension. To one part beef three parts water was added, and the mixture was macerated for 15 min at 40 C and coarsely filtered. This liquid, which also contained meat particles in suspension, contained 2% NaCl. In these latter experiments only spores of *C. sporogenes* were tested. Heat treatment was at $F_0 = 0.4, 0.8, 1.6$; the temperature was maintained at 100 C.

Application of additives. All additives (nitrite, cysteine-nitrosothiol, and cysteine) were added to the media as a filter-sterilized solution before inoculation and heat treatment. A 0.2-ml additive solution was introduced into 10 ml of medium.

Detection of bacterial growth. All samples were incubated after treatments at 30 C for 2 weeks. Putrid odor and turbidity of samples with clostridia and turbidity with all other organisms were observed.

RESULTS

The effect of nitrosothiol on various bacteria in comparison with nitrite is shown in Tables 1 to 4. On the basis of the data, it appears that cysteine-nitrosothiol, as a suggested intermediate of color formation in cured meats, exhibits an inhibitory effect (at least in medium) on those bacteria against which nitrite is also effective. It should also be mentioned that nitrosothiol has the same inhibitory effect in a lower concentration as nitrite does in a higher one. In agreement with the results of Leistner et al. (11), the nonsporeformers we tested show rather different tolerance against nitrite. For example, whereas growth of *Streptococcus faecium* was not at all inhibited by nitrite concentrations used in meat industry nor by

TABLE 1. Effect of nitrite and nitrosothiol on vegetative cells of *Clostridium sporogenes*^a

Nitrite concn (added $\mu\text{g/g}$)	Spoiled/total	Nitrosothiol concn as NaNO_2 equivalent ($\mu\text{g/g}$)	Spoiled/total
0	15/15	0	15/15
50	15/15	30	9/15
100	10/15	60	1/15
150	0/15	120	0/15

^a Initial count $10^6/\text{ml}$. Broth samples containing 2% NaCl, pH 6.2, with nitrite or nitrosothiol additive were inoculated before heat treatment at 60 C for 10 min. Spoilage values were recorded after 2 weeks of incubation at 30 C.

TABLE 2. Effect of nitrite and nitrosothiol on spores of *Clostridium sporogenes*^a

Nitrite concn (added $\mu\text{g/g}$)	Spoiled/total	Nitrosothiol concn as NaNO_2 equivalent ($\mu\text{g/g}$)	Spoiled/total
0	10/10	0	10/10
50	8/10	30	5/10
100	0/10	60	0/10
150	0/10	120	0/10

^a Initial count $10^6/\text{ml}$. Broth samples containing 2% NaCl, pH 6.2, with nitrite or nitrosothiol additive were inoculated before heat treatment at 100 C for 10 min. Spoilage values were recorded after 2 weeks of incubation at 30 C.

TABLE 3. Effect of nitrite and nitrosothiol on *Streptococcus faecium*^a

Nitrite concn (added $\mu\text{g/g}$)	Turbid/total	Nitrosothiol concn as NaNO_2 equivalent ($\mu\text{g/g}$)	Turbid/total
0	15/15	0	15/15
50	15/15	30	15/15
100	15/15	60	15/15
150	15/15	120	15/15

^a Initial count $4.3 \times 10^6/\text{ml}$. Broth samples containing 2% NaCl, pH 6.2, with nitrite or nitrosothiol additive were inoculated before heat treatment at 60 C for 10 min. Turbidity was recorded after 2 weeks of incubation at 30 C.

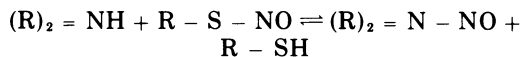
nitrosothiol, this latter compound proved inhibitory to *Salmonella* strains. (It should be mentioned that Leistner and his co-workers [11] found inhibitory activity of the regular concentration of nitrite with salmonellae, but failed in finding any such effect with staphylococci.)

DISCUSSION

Cysteine-nitrosothiol, which was identified by Mirna and Hofmann (13) and whose color-forming ability was also proved by us, has a definite microbiological inhibitory effect. This effect is comparable to that of nitrite; in medium it is even more effective. Accepting and enlarging the assumption of Johnston and Loynes (8) and Mirna and Hofmann (13), it seems probable that nitrosothiols, among them cysteine-nitrosothiol, may be considered as intermediates which could transfer the nitroso group directly to components of bacterial cell or

to meat pigment.

This intermediate may have not only chemical and microbiological importance, but health aspects as well. It can be assumed that no direct HNO_2 formation occurs from nitrosothiol in meat or other foods, which would be a prerequisite for nitrosation of secondary amines, i.e., for formation of nitrosamine. The formation of nitrosamines from nitrosothiols could only occur if NO re-formed to NO_2 and through this to nitrous acid with the help of oxygen, or if the following reaction took place:



Because of the excess amount of sulfhydryl compounds, nitrosamine formation can only be a secondary possibility in both cases, a fact which substantially decreases the likelihood of nitrosamine formation in comparison with direct usage of nitrite.

Data (Table 5) support results of Johnston and Loynes (8) in proving the nitrite inhibition increasing property of cysteine. The ineffectiveness of nitrite without cysteine would perhaps need explanation mainly in comparison with its effect in medium. It should be kept in mind, however, that the medium in this experiment was meat suspension, and from results of Johnston et al. (9) we know that the presence of only 1% meat inhibits the Perigo effect (16); in other words it decreases the inhibitory action of nitrite. We might also mention that natural sulfhydryl groups of meat which were freed after the unfolding of protein during heating may also play a role in increasing the inhibitory effect of nitrite, and these groups are probably in concentrations diluted to ineffectiveness in our

TABLE 4. Effect of nitrite and nitrosothiol on *Salmonellae*^a

<i>Salmonella</i> sp.	Nitrite concn (added $\mu\text{g/g}$)	Turbid/total	Nitrosothiol concn as NaNO_2 equivalent ($\mu\text{g/g}$)	Turbid/total
<i>S. enteritidis</i> , $10^6/\text{ml}$ initial count	0	15/15	0	15/15
	50	15/15	30	9/15
	100	14/15	60	6/15
	150	15/15	120	3/15
<i>S. anatum</i> , $10^7/\text{ml}$ initial count	0	15/15	0	15/15
	50	15/15	30	10/15
	100	15/15	60	9/15
	150	15/15	120	3/15
<i>S. choleraesuis</i> , $10^6/\text{ml}$ initial count	0	14/15	0	15/15
	50	14/15	30	3/15
	100	15/15	60	0/15
	150	14/15	120	0/15

^a Broth samples containing 2% NaCl, pH 6.2, with nitrite or nitrosothiol additive were inoculated before heat treatment at 60 C for 10 min. Turbidity was recorded after 2 weeks of incubation at 30 C.

TABLE 5. Effect of nitrite, heat treatment, and cysteine-HCl (0.25%) on spores of *Clostridium sporogenes*^a

Cysteine	Heat treatment (F ₀)	No. spoiled/total				
		0 ^b	20	40	80	160
Absent	0.4		15/15	15/15	15/15	15/15
	0.8	15/15	15/15	15/15	15/15	15/15
	1.6	15/15	15/15	15/15	15/15	
Present	0.4		0/15	0/15	0/15	0/15
	0.8	0/15	0/15	0/15	0/15	0/15
	1.6	0/15	0/15	0/15	0/15	0/15

^a Initial count 10⁶/ml. Coarsely filtered meat extract containing 2% NaCl and various amounts of NaNO₂, with or without cysteine, was inoculated before heat treatment at 100 C. Spoilage values were recorded after 2 weeks of incubation at 30 C.

^b Added nitrite level (micrograms per milliliter).

meat suspension as compared to those in intact meat.

It has long been known that compounds containing sulfhydryl groups increase the effectiveness of nitrite from both chemical (i.e., color formation [10]) and microbiological (2, 8) points of view, and results of Mirna and Hofmann (13) indirectly emphasize the importance of these compounds. These data do not disagree with results that question the effect of SH-amino acids on nitrite (15), for there are other ways in which depletion of nitrite can occur.

On the basis of other data and our results there seem to be several possible alternatives for the food industry in its use of nitrite or its derivatives without harmful health consequences. Whether this will mean usage of reducing agents in higher concentrations (ascorbic acid, cysteine, etc., 1,000 to 2,000 µg/g) together with small concentrations (50 to 70 µg/g) of NaNO₂ (if nitrosothiols prove harmless) can be decided only after further investigation. Nevertheless, we may hope, knowing the abovementioned results, that there will be a method(s) for assuring proper microbiological safety even with nitrite concentrations previously considered sufficient for color formation only. Data should also be mentioned which suggest that some reducing agents (ascorbic acid and its compounds) are not only valuable from a bacteriostatic viewpoint but also are known as anti-nitrosamine formation agents (5, 7, 14).

The effect of sporostatic concentrations of cysteine-nitrite and ascorbic acid-nitrite in meat are under investigation in our laboratories. Results of these investigations and the effect of nitrosothiol in meat medium will be reported in the future.

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