Factors Affecting the Bacteriostatic Action of Sodium Nitrite[1]

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Sodium nitrite is known to be responsible for the production of the desirable heat-stable pink color in cured meats. It has also been used as an inhibitor of bacterial spoilage of fish (Tarr, 1941a, b). However, in cured meats it is not known to have any practical preservative action relative to those organisms not inhibited by the high sodium chloride content. Many varieties of staphylococci and various lactic acid bacteria grow quite readily on cured meats, but it has been observed in this laboratory that staphylococci rarely, if ever, are found in large numbers in the interior of cured meat items. In view of this observation, it was decided to investigate the effect of various other factors on the inhibition of staphylococci by sodium nitrite.

Materials and Methods

Analytical. Nitrite added to bacteriological media was determined colorimetrically using the Griess reagent (A. O. A. C., 1945). Deproteinization was found to be unnecessary, and the color density was determined in a Coleman spectrophotometer, at a wavelength of 522 mμ.

Sulfhydryl groups were determined by the method of Shinowara (1935, 1935–36) using the phospho-18-tungstic acid reagent. Cysteine was determined directly; the cystine in the same sample was reduced to cysteine and the concentration of cystine was then calculated by difference. Color density was determined spectrophotometrically at 750 mμ.

The pH was determined using a glass electrode Beckman Model H pH meter.

Bacteriological. For most of the experiments a complex medium was employed which contained 1.0 per cent tryptone, 1.0 per cent yeast extract, 0.5 per cent K_2HPO_4 and 1.0 per cent glucose. The medium was prepared double strength, sterilized by autoclaving at 121 C for 20 minutes, sterile solutions of the variable components added, and made up to single strength with sterile distilled water. In a number of experiments, as indicated, the glucose was omitted from the basal medium, sterilized separately, and added aseptically. The sodium nitrite was sterilized by Seitz filtration of a 10 per cent stock solution.

The cultures used for most of this work were strains 161 and 196 of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*). These were typical coagulase-positive strains (Evans and Niven, 1950) and were isolated from outbreaks of food poisoning by Dr. G. M. Dack of the University of Chicago.

The inoculum consisted of one drop of a 10^{-2} dilution of a 24-hour broth culture.

For aerobic growth, 18-mm culture tubes containing 10 ml of medium were placed in a nearly horizontal position in the incubator to give a large surface area. For anaerobic growth the medium was covered with a vaspar seal. In all of the experiments reported here maximum incubation was for 48 hours at 30 C since the nitrite may disappear at a rapid rate, even in the uninoculated media under certain conditions.

Results

Effect of pH. Tarr (1941a, b) reported that the pH of the medium greatly influences the bacteriostatic power of nitrite. Preliminary experiments, typical of that presented in table 1, confirmed Tarr's observations. In these experiments, *S. aureus*, strain 196, was cultured aerobically in the complex medium at varying pH levels.

Using a pKa value of 3.4 for nitrous acid the minimum bacteriostatic concentration of this hypothetical undissociated acid was calculated for the various pH levels employed. It will be noted that the concentration of nitrous acid for bacteriostasis remains relatively constant throughout the pH range tested, thus relating the bacteriostatic action of nitrite to the acid. In other words, as the pH of the medium is lowered by one unit the bacteriostatic effect of added nitrite is increased approximately 10-fold.

In these experiments in which the organism was cultured aerobically, the glucose was autoclaved in the double strength medium and the nitrite added aseptically. When sterile glucose was added aseptically to the medium after autoclaving there was no significant change in the results.

Anaerobic tolerance and the effect of manner of glucose sterilization. When similar studies were conducted anaerobically it was noted that sterilization of glucose

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TABLE 1. *Effect of pH on the aerobic nitrite tolerance of Staphylococcus aureus, strain 196, when glucose is autoclaved in a complex medium*

pH*	Nitrite Concentration		Calculated Undissociated HNO_2†	
	Growth‡	No growth	Growth	No growth
	ppm	*ppm*	*ppm*	*ppm*
6.90	3,500	4,000	1.12	1.28
6.52	1,800	2,000	1.37	1.52
6.03	600	700	1.38	1.61
5.80	300	400	1.20	1.60
5.68	250	400	1.32	2.12
5.45	140	180	1.25	1.50
5.20	80	150	1.12	2.10
5.05	40	80	0.92	1.84

* The pH values were determined on duplicate tubes of medium at the time of inoculation.

† The amount of undissociated nitrous acid was calculated from the dissociation curve employing a pKa of 3.4.

‡ Growth was determined after 48 hours at 30 C.

TABLE 2. *Effect of the manner of adding glucose on the nitrite tolerance of Staphylococcus aureus, strain 196, at pH 6.55 in a complex medium*

Incubation Conditions	Nitrite Concentration			
	Glucose sterilized in the medium		Glucose added aseptically	
	Growth	No growth	Growth	No growth
	ppm	*ppm*	*ppm*	*ppm*
Aerobic	1,800	3,000	1,600	1,800
Anaerobic	20	40	800	1,000

in the medium greatly enhanced the bacteriostatic effect of nitrite. Calculations of the amount of undissociated nitrous acid again indicated that this was the substance associated with bacteriostasis anaerobically, as it was aerobically.

Results presented in table 2 demonstrate that when the inhibitory level of nitrite was determined at a constant pH, glucose autoclaved in the medium had no effect on the aerobic tolerance, but decreased the anaerobic tolerance about 30-fold. It should also be noted that when glucose was added aseptically the aerobic tolerance was about twice the anaerobic tolerance.

When sucrose was used rather than glucose, autoclaving in the medium had no effect on the anaerobic tolerance and all results were equivalent to those obtained when glucose was added aseptically. Also, when no fermentable carbohydrate was added, the anaerobic nitrite tolerance was of the same order of magnitude as that found when glucose was added aseptically.

Nitrite tolerance of other microorganisms. Three additional strains of coagulase-positive staphylococci were tested and gave results similar to those with strain 196. As indicated in table 3, a number of other bacterial species were tested for their tolerance to nitrite. Considerable variation was noted among these bacteria. In general, aerobic tolerance to nitrite was higher than anaerobic tolerance among these microorganisms, but for the majority of the strains tested the differences were not as marked as with *S. aureus*.

The *Lactobacillus*, *Pediococcus*, *Streptococcus lactis*, enterococcus, and gram negative species tested were relatively tolerant to nitrite. On the other hand, the *Streptococcus pyogenes* strain tested was extremely sensitive. Quite unexpectedly, *Streptococcus salivarius* and *Streptococcus mitis* were relatively sensitive under anaerobic conditions (glucose autoclaved in the medium) but were resistant under aerobic conditions. These two species, in contrast to *S. aureus*, are generally considered to be "indifferent" to oxygen with respect to their growth requirements and do not possess the heme-containing respiratory enzyme systems for obtaining energy aerobically.

Reversal of anaerobic nitrite inhibition. Screening of a number of amino acids and reducing substances revealed that sulfhydryl compounds (sodium thioglycollate, cysteine, or glutathione) would reverse the nitrite inhibition observed when glucose was autoclaved in the medium. Ascorbic acid and other reducing substances that do not contain a sulfhydryl group were ineffective.

Representative data obtained with glutathione for *S. aureus*, strain 196, are presented in table 4. In all experiments the sulfhydryl-containing substances were

TABLE 3. *Minimum inhibitory concentrations of nitrite for a variety of microorganisms at pH 6.6 to 6.7*

Species	Glucose Sterilized in Medium		Glucose Added Aseptically	
	An-aerobic	Aerobic	An-aerobic	Aerobic
	ppm	*ppm*	*ppm*	*ppm*
Streptococcus salivarius (13 strains)	80	4,000	3,000	6,000
Streptococcus mitis	40	4,000	800	2,000
Streptococcus lactis	6,000	10,000	4,000	6,000
Streptococcus liquefaciens (4 strains)	800	6,000	4,000	4,000
Streptococcus faecalis (16 strains)	4,000	6,000	4,000	5,000
Streptococcus pyogenes	2	20		
Lactobacillus casei, strain 7469	4,000	8,000		
Lactobacillus arabinosus, strain 17–5	8,000	25,000		
Pediococcus cerevisiae, strain 8081	8,000	25,000		
Bacillus megatherium	80	4,000		
Escherichia coli	2,000	4,000	4,000	4,000
Aerobacter aerogenes	2,000	4,000		
Proteus vulgaris	400	4,000		
Salmonella typhosa	800	2,000		
Salmonella aertrycke (*typhimurium*)	2,000	4,000		
Shigella flexneri (*paradysenteriae*)	100	2,000		

TABLE 4. *Effect of glutathione on the anaerobic nitrite tolerance of Staphylococcus aureus, strain 196, at pH 6.65 in a complex medium*

	Control Medium		Medium Plus Glutathione (1000 ppm)*	
	Nitrite added	Nitrite recovered†	Nitrite added	Nitrite recovered†
	ppm	*ppm*	*ppm*	*ppm*
Growth	50	27	2,000	1,770
No growth	100	68	4,000	3,450

* The glutathione was sterilized by Seitz filtration and added aseptically. Glucose was autoclaved in the medium.

† These figures represent the residual nitrite concentration in uninoculated tubes of medium after 48 hours incubation at 30 C.

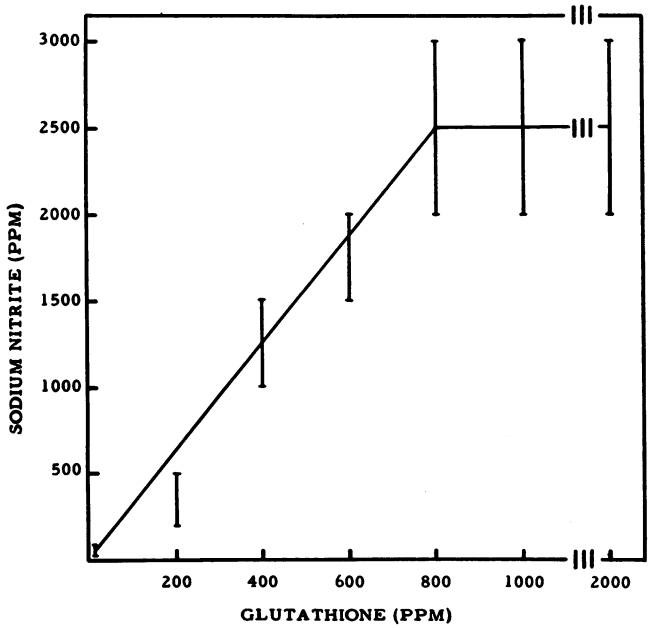

FIG. 1. The quantitative reversal of anaerobic nitrite inhibition by glutathione when glucose is autoclaved in a complex medium; pH 6.68. The vertical lines connect the tested maximum concentration tolerated, with the tested minimum inhibitory concentration for *Staphylococcus aureus*, strain 196.

added aseptically to the medium from a freshly prepared stock solution sterilized by filtration. Recovery values for nitrite indicated that glutathione did not destroy the added nitrite.

As demonstrated in figure 1, the reversal of anaerobic nitrite inhibition was directly proportional to the concentration of added glutathione up to a nitrite concentration of 2500 ppm. At levels of nitrite above this value there was no reversal.

Glutathione appeared to reverse the nitrite inhibition up to a level which approached the nitrite concentration tolerated under aerobic conditions. Glutathione did not affect the aerobic nitrite tolerance of *S. aureus*.

Similar experiments with *Streptococcus salivarius* and *Streptococcus mitis* revealed that the sulfhydryl compounds, especially cysteine, would also reverse the nitrite inhibition observed under anaerobic conditions. The results were not so dramatic, however. Cysteine at a concentration of 1000 ppm increased their anaerobic nitrite tolerance up to 10-fold. Higher concentrations of cysteine failed to increase the nitrite tolerance. These experiments indicate that other nutrilites may be important in overcoming the bacteriostatic effect of nitrite for these streptococci.

Effect of aging the complex medium. Double strength basal medium containing glucose was autoclaved and then aged for 7 days under the following conditions: 1) at room temperature in a slanting position, 2) in an anaerobic desiccator over pyrogallol and sodium carbonate, and 3) in a slanting position at 7 C. The volume was restored with sterile distilled water and nitrite tolerance under anaerobic conditions was determined in the usual manner using *S. aureus*, strain 161. The medium that was aged anaerobically and that aged in the refrigerator gave results comparable to freshly prepared medium. However, the medium that was aged aerobically at room temperature supported growth of the test organism in the presence of about 20 times as much nitrite as did the other media. This was in spite of the fact that the aged medium had a pH of 6.38 compared to a pH of 6.68 for the fresh medium. If the aged medium was steamed just prior to inoculation, the resulting nitrite tolerance was reduced to that found in freshly prepared medium.

Aeration in flasks on a mechanical shaker for 8 hours enhanced the anaerobic nitrite tolerance only 2-fold, indicating that simple saturation with air at room temperature would not explain the effect of aging 7 days at room temperature. Furthermore, the addition of hydrogen peroxide in varying concentrations up to .05 per cent did not enhance the tolerance to nitrite.

Studies using a synthetic medium. A synthetic medium was made up using 19 amino acids, adenine, guanine, uracil, xanthine, mineral salts, thiamine, nicotinic acid, K_2HPO_4, and glucose in amounts similar to those recommended by Gladstone (1937), and Porter and Pelczar (1941). For successful anaerobic growth it was necessary to add pyruvate aseptically at a concentration of 0.5 per cent (Fildes *et al.*, 1936). In this synthetic medium it was found (table 5) that autoclaving glucose with the other constituents decreased the apparent nitrite tolerance about 10-fold. Contrary to the findings in the complex medium, the addition of increased levels of cysteine did not reverse this added inhibition.

Studies with model systems. In an attempt to determine the reason for the lack of a cysteine effect in a synthetic medium, further experiments revealed that the addition of either 0.5 per cent pyruvate or fumarate to the complex medium also tended to nullify the cysteine effect. These results indicated that a chemical reaction takes place between cysteine and pyruvate or fumarate when these substances are mixed together.

TABLE 5. *Effect of cysteine concentration on the anaerobic nitrite tolerance of Staphylococcus aureus, strain 161, in a synthetic medium, pH 6.7*

Cysteine* Added	pH	Nitrite Concentration			
		Glucose sterilized in the medium		Glucose added aseptically	
		Growth	No growth	Growth	No growth
ppm		*ppm*	*ppm*	*ppm*	*ppm*
20	6.70	40	80		
25	6.60			400	800
100	6.60			400	800
400	6.65	40	80		
500	6.55			200	400
1000	6.55	20	40		

* The cysteine was sterilized by Seitz filtration and added aseptically to the sterile medium. The basal medium was devoid of cysteine or cystine and would not support anaerobic growth of the organism.

TABLE 6. *The apparent interaction of pyruvate and cysteine in a phosphate buffer at pH 6.7 under anaerobic conditions**

Time (Hours)	PPM Recovered as:			
	0.5 per cent pyruvate		No pyruvate	
	Cysteine	Cystine	Cysteine	Cystine
0	620	380	620	380
1	37	122	583	304
2	12	95	558	346

* The system contained 0.5 per cent K_2HPO_4 adjusted to pH 6.7 with HCl, 1000 ppm of cysteine, and pyruvate as indicated. Incubation was at 30 C.

Therefore, experiments were designed to demonstrate this chemical reaction in a model system.

Cysteine in a final concentration of 1000 ppm was added to a phosphate buffer, pH 6.7, containing 0.5 per cent pyruvate, and the mixture held at 30 C up to 2 hours under anaerobic conditions. The apparent cysteine-cystine content was determined in this mixture at the time intervals indicated in table 6. Under these conditions it was noted that pyruvate appears to destroy rapidly both cysteine and cystine in the system. The cysteine-cystine destruction was accompanied by a decrease in the pH of about 0.2 units. In the absence of pyruvate, cysteine was relatively stable in the phosphate buffer held under similar conditions.

Similar experiments indicated that fumarate also destroyed cysteine and cystine almost completely within 24 hours when held anaerobically in the phosphate buffer.

The stability of cysteine and nitrite was also determined in the phosphate buffer system without added pyruvate or fumarate. The results are presented in table 7. The results indicate that the nitrite was relatively stable, but the cysteine was partially destroyed in the presence of nitrite. No stoichiometric relationship between nitrite and cysteine could be demonstrated.

TABLE 7. *Stability of cysteine and nitrite in phosphate buffer at pH 6.7 under anaerobic conditions*

Additions	PPM Recovered After 24 Hours		
	Cysteine	Cystine	Nitrite
Cysteine, 1000 ppm*.....	484	283	—
Cysteine, 1000 ppm*, nitrite, 2000 ppm.....	186	46	1730
Nitrite, 2000 ppm.....	—	—	1790

* At zero time there were 620 ppm cysteine and 380 ppm cystine in the system.

Thus experiments in the synthetic medium were not successful in elucidating the exact quantitative relationships between nitrite and sulfhydryl substances as affecting the growth of *S. aureus*. Satisfactory anaerobic growth of the test organisms could not be achieved without the addition of pyruvate. This acid in turn destroyed the effectiveness of the sulfhydryl substances in overcoming the bacteriostatic properties of nitrite.

DISCUSSION

Several explanations have been proposed for the mechanism of nitrite bacteriostasis. The reaction of nitrous acid with alpha-amino groups is the basis for the well known Van Slyke reaction. The bacteriostatic properties of nitrite consequently have been attributed to interference with amino groups of the dehydrogenase systems of *Escherichia coli* (Quastel and Woolridge, 1927).

Philpot and Small (1938a, b) have indicated that nitrous acid can react with monophenols, such as tyrosine, and they attributed the inactivation of pepsin to this reaction. Also to be considered is the possibility that hydroxylamine may be produced from nitrite by a number of organisms (Lindsey and Rhines, 1932) and this substance in turn is responsible in some degree for nitrite bacteriostasis.

A very plausible explanation for the bacteriostatic action of nitrite lies in the ability of the decomposition product, nitric oxide, to react with heme pigments. The production of nitric oxide myoglobin is the basis for meat curing. Ingram (1939) reported that at pH 6 oxygen uptake by *Bacillus cereus* was strongly inhibited by nitrite, thus implying an interference with the cytochrome systems. On the other hand, Tarr (1941a) noted that among two *Achromobacter* strains and two micrococci (whose growth in broth was strongly retarded by nitrite) the aerobic respiration of only one *Achromobacter* strain was inhibited by nitrite at different pH levels. Consequently, he concluded that nitrite does not inhibit bacterial growth by sole virtue of its toxicity toward the aerobic respiratory catalysts.

Further evidence against this theory is given in the present study. Among the collection of bacteria studied, nitrite was strongly inhibitory toward some of the

streptococci even though these bacteria are devoid of the heme-containing respiratory catalysts. Also, nitrite was generally more bacteriostatic under anaerobic conditions where the cytochrome systems would not be important in the metabolism of the microorganisms.

The results presented in this study do not fit any simple theory as to the site of action of nitrite, especially for the staphylococci. Such a theory would have to explain 1) why nitrite is so effective anaerobically in a medium autoclaved with glucose, 2) why aerobic conditions, adding the glucose aseptically, or adding sulfhydryl substances decrease the effectiveness of nitrite, and 3) why aging the test medium containing autoclaved glucose decreases the anaerobic effectiveness of nitrite, and why steaming of this aged medium restores it equivalent to the freshly prepared medium.

Some of the data presented in this study might imply that nitrite inactivates certain bacterial enzyme systems that possess an active sulfhydryl group. Barron and Singer (1945) reported that among a large number of enzyme systems studied the pyruvate-metabolizing enzymes were the most sensitive to sulfhydryl inhibitors. Bernheim (1943), and Nord and Mull (1945) noted that nitrite inactivated the pyruvate-metabolizing enzymes of *Vibrio comma* and *Fusarium*, respectively.

It would seem that the reason for anaerobic nitrite bacteriostasis for *S. aureus* and some of the streptococci in a medium containing autoclaved glucose could best be explained on a nutritional basis. The instability of the sulfhydryl amino acids when autoclaved in a medium containing a reducing sugar is well known. Riesen *et al.* (1947) made the interesting observation that the apparent degree of cystine destruction that occurred in a medium during autoclaving depended upon the test microorganisms employed for assay. In the present study, experiments with *S. aureus*, strain 161, indicated that as much as 90 per cent of the added cysteine content was destroyed when autoclaved with glucose in a synthetic medium for 20 minutes at 121 C. It should be emphasized, however, that in the complex medium employed throughout these studies the medium without added nitrite, even containing glucose during heat sterilization, supported maximum growth for all test microorganisms.

It is therefore logical to postulate that under conditions giving maximum sensitivity to nitrite (glucose autoclaved in the medium), a chemical change occurs, perhaps a condensation or addition, which involves the sulfhydryl constituents and another substance in the medium. The other substance might be a decomposition product of glucose. It would have to be assumed that this product is utilizable by the microorganism anaerobically except in the presence of nitrite. Minimum concentrations of nitrite would interfere with the anaerobic utilization of this complex, either at a physiological level, or else by reacting chemically with it. However, the product would be available under aerobic conditions or else the microorganism would be able to obtain sulfur from other medium constituents in the presence of oxygen.

To account for the reported aging effects it would have to be assumed that the complex is formed only under the influence of heat. Then upon exposure to oxygen at room temperature it would slowly dissociate, again making available sulfhydryl substances for the microorganism even in the presence of nitrite.

Under conditions giving minimum nitrite sensitivity (glucose not autoclaved in the medium), a second vital mechanism, perhaps not involving sulfur nutrition, would be affected by the nitrite to inhibit growth. Also, it might be postulated that those microorganisms which are relatively tolerant to nitrite under all conditions are able to utilize other sources of sulfur in their nutrition, thus eliminating the limited availability of a sulfur source in the presence of small concentrations of nitrite.

Evidence is presented in this study to indicate that a complex is formed between pyruvate (or fumarate) and sulfhydryl substances when added together at room temperature. Evidently, when nitrite is also added, this complex becomes unavailable for some microorganisms, especially *S. aureus*. That addition or condensation products are formed by the interaction of thiols with aldehydes, pyruvic acid or sugars seems to be well documented in the literature (Schubert, 1935, 1936, 1949; Cavallini, 1951). Such reactions between the sulfhydryl group of coenzyme A and substrate are now generally believed to be the basis for the biological activity of this coenzyme.

These studies indicate the need for further research concerning the availability of reaction products between thiols and carbonyl compounds in the nutrition of various microorganisms. Whether such studies will be useful in explaining the apparent inability of *S. aureus* to grow rapidly under anaerobic conditions in cured meats in the presence of nitrite remains to be seen. Further studies along these lines are now being conducted.

Summary

Tolerance toward nitrite varies widely among the different groups of bacteria tested.

A number of factors have been found to drastically affect the bacteriostatic action of sodium nitrite on food-poisoning staphylococci and some related organisms. The pH of the environment influenced the level of nitrite causing inhibition in a manner that tends to confirm the hypothesis that undissociated nitrous acid is the active form.

When glucose was autoclaved in the test medium the anaerobic growth of *Staphylococcus aureus* (*Micro-*

coccus pyogenes var. *aureus*), *Streptococcus salivarius*, and *Streptococcus mitis* was inhibited by much lower levels of nitrite than were required if the glucose was added to the medium after autoclaving. The aerobic tolerance to nitrite by these organisms was not greatly affected by the manner of adding the glucose.

The addition of sulfhydryl compounds such as cysteine or glutathione to a medium that had been autoclaved with glucose restored the nitrite tolerance to the same level as when the glucose was sterilized separately and added aseptically. Aging the medium for 7 days at 25 C after autoclaving also restored the nitrite tolerance to the higher level, but steaming this aged medium completely reversed this aging effect.

An explanation of these findings is offered based upon the assumption that the site of maximum sensitivity in these staphylococci is a system involving an essential sulfhydryl compound.

Evidence is presented to indicate that addition or condensation products are formed between pyruvate (or fumarate) and sulfhydryl substances when added together at room temperature. Nitrite appears to influence the availability of these products in the nutrition of some microorganisms.

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