NITRATE REDUCTION AND CAROTENE STABILITY

Effect of Nitrate and Some of Its Reduction Products on Carotene Stability

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Studies were made to determine whether nitrate or certain of its reduction products cause β-carotene destruction, as measured by reduction in optical density of solutions or extracts at 440 mμ. Under a variety of conditions, nitrate did not. Nitrite in neutral or alkaline medium caused little or no destruction, but addition of copper increased its effect. In an acid medium, nitrite additions caused rapid β-carotene disappearance, even at pH levels often found in biological materials. Nitric oxide and nitrogen tetroxide were also found to destroy β-carotene rapidly, and the effect of nitrite in acid medium may have been the result of the formation of gaseous nitrogen oxides. Results of studies with corn silage, rumen juice, and abomasum juice point to the possible significance of these findings in animal nutrition and in certain experimental work.

MANY OF OUR COMMON FORAGES accumulate high levels of nitrate nitrogen under certain conditions (16). When these forages are ingested by ruminants, the nitrate is reduced to nitrite which may cause death as a result of methemoglobinemia (2, 3). Ensiling may result in the formation of various nitrogen oxides in gaseous form (11). Some work has suggested that nitrates in feeds may be involved in vitamin A deficiencies in livestock (6, 7, 9, 12). While it cannot be concluded from the literature that carotene or vitamin A destruction by nitrate or its reduction products is a contributing factor in these deficiencies, this possibility should not be overlooked.

The studies described here were designed to determine whether nitrate or its reduction products can be expected to destroy carotene during storage or following the ingestion of feed.

Materials and Methods

A number of different experiments were made during the course of this study. For purposes of brevity, and because similar experiments gave similar results, only certain of them are discussed in detail here.

Experiment 1. This experiment dealt with the effects of nitrate, nitrite, acid, base, and copper on carotene stability in alcoholic solutions. To three series of duplicate Evelyn tubes were added 1.0 ml. of water or a solution of ammonium hydroxide (2.5M), acetic acid (2.5M), hydrochloric acid (2.5M), cupric chloride (25 p.p.m. of Cu), or of

both the hydrochloric acid and cupric chloride. To one of these series, 1.0 ml. of 0.8M sodium nitrate solution was added, to another series 1.0 ml. of 0.8M sodium nitrite solution. These, and the third series of tubes (control) were then made up to 5.0 ml. with water. Next, 20 ml. of an alcoholic solution of carotene (19.9 ml. of ethanol + 0.1 ml. of vegetable oil containing 28 μg. of β-carotene) was added to each. As soon as the ethanolic carotene solution was added to a tube, it was stoppered, mixed, and read (at about 1 minute) at 440 mμ on an Evelyn colorimeter previously calibrated for β-carotene determinations. The tubes were then placed in the dark at room temperature and read again at 10, 24, and 48 hours.

Experiment 2. To determine what effect pH might have on the destruction of carotene in the presence of nitrite, aliquots of a 0.1M sodium acetate solution were adjusted with glacial acetic acid to about the following pH levels: 6.0, 5.5, 5.0, 4.5, and 4.0. Twenty milliliters of each of these and of the unadjusted sodium acetate solution was measured into three 25 × 150 mm. test tubes. Two tubes at each pH were then treated, one tube at a time, as follows. Three milliliters of a solution of β-carotene in oil and ethanol (0.03 ml. of vegetable oil containing 8.4 μg. of β-carotene per 3.0 ml. of ethanol) was added. Then 1.0 ml. of 0.8M sodium nitrite solution was added, the tube was immediately stoppered and shaken vigorously for 10 seconds, 15.0 ml. of n-hexane was added, and shaking was resumed for 15 seconds. The hexane layer was withdrawn and color measured photometrically at 440 mμ. The third tube for each pH level served as a control and was treated in the same

manner except that 1.0 ml. of water was added instead of the sodium nitrite solution.

Experiment 3. To determine what effect solutions of nitrate or nitrite might have on the carotene in alfalfa, freshly cut leaves were air dried, finely ground (1-mm. screen), and further dried overnight without heat in a vacuum. After mixing, four 5.0-gram samples were weighed into beakers, and 8.0 ml. of water, 0.18M sodium nitrate, 0.09M sodium nitrite, or 0.72M sodium nitrite solution was added. After mixing to wet the alfalfa and distribute the solution, the samples were transferred to test tubes and packed. The tubes were then stoppered and placed in the dark at 4° C. After 4 days storage, the contents of each were mixed, and 3.0-gram portions were analyzed for β-carotene by the AOAC method (1).

Experiment 4. To determine the effect of added nitrates on the stability of carotene in corn silage, green corn plants at a soft dough stage were chopped into about 1/4-inch lengths and mixed. Three equal portions were weighed and spread out in a large pan. One portion was sprayed, with mixing, with 50 ml. of water per 800 grams of green forage. The second portion of 800 grams was sprayed with 50 ml. of 4% potassium nitrate solution, and the third with 50 ml. of 20% potassium nitrate solution. Immediately after spraying, 250-ml. bottles were tightly packed full with forage and were sealed. The bottles were placed in an incubator at 34°C. Samples of the unsprayed green forage were immediately analyzed for moisture by drying in the forced draft oven at 75° C. for 48 hours, and for β-carotene by the AOAC method. At 7 and 15 days, the contents from one bottle for each

Table I. β-Carotene Destruction in Solutions with Added Nitrate and Nitrite (Experiment 1)

| | Per Cent β-Carotene Destroyed | | | | | | | | | | | |
| | Control | | | | Nitrate Present (0.032M)[a] | | | | Nitrite Present (0.032M)[a] | | | |
Solution	1 Min.	10 Hr.	24 Hr.	48 Hr.	1 Min.	10 Hr.	24 Hr.	48 Hr.	1 Min.	10 Hr.	24 Hr.	48 Hr.
Mixture only[b]	0	0	0	0	0	0	0	0	0	0	1	3
With NH_4OH (0.1M)	0	0	0	0	0	0	0	0	0	0	0	0
With CH_3COOH (0.1M)	0	0	0	0	0	0	2	4	100	100	100	100
With HCl (0.1M)	1	8	19	38	1	10	17	34	100	100	100	100
With $CuCl_2$ (1.0 p.p.m. Cu)	0	1	1	4	0	0	1	3	0	37	70	78
With HCl (0.1M) and $CuCl_2$ (1.0 p.p.m. Cu)	6	47	64	81	8	47	63	82	100	100	100	100

[a] Concentration in alcoholic solution.

[b] Mixture containing 5 ml. of water, 19.9 ml. of ethanol, and 0.1 ml. of β-carotene (28 μg.) in oil solution.

treatment were removed, chopped quickly with a scissors, mixed, and analyzed in duplicate for moisture and β-carotene.

Experiment 5. For use in an artificial rumen technique, 40 ml. of strained rumen juice from a steer on a low nitrate hay ration was introduced into each of eight bottles. To each, 1.0 gram of finely ground alfalfa was added. Then 1.0 ml. of water was added to four of the flasks (control), 1.0 ml. of 8% sodium nitrate solution to the remainder. One flask from each treatment was immediately analyzed for nitrites and for β-carotene. For nitrites, 0.5 ml. of liquid was withdrawn, treated with lead acetate, and analyzed for nitrites by an adaptation of the α-naphthylamine method as described by Whitehead (15). β-Carotene was determined by treating the entire contents of a bottle with 0.5 gram of magnesium carbonate and then grinding the mixture in a Waring Blendor for 1 minute with 60 ml. of ethanol. After adding 40 ml. of n-hexane, the grinding was continued for 2 minutes. The mixture was filtered with suction through a layer of Filter Cel and the residue washed with 20 ml. of ethanol. The residue, Filter Cel, and filter paper were washed back into the grinder with 80 ml. of ethanol and ground for 2 minutes, filtered, and washed as before. The grinding and filtration were then repeated, using n-hexane instead of ethanol. The combined filtrates were then freed of alcohol and analyzed according to the AOAC method.

The remaining flasks were incubated at 37° C. in an atmosphere of carbon dioxide. As required. pH was adjusted to 7.0 with saturated sodium carbonate solution. At no time did the pH in any bottle fall below 6.5. Samples of 0.5-ml. volume were removed from each bottle for nitrite determination at 4, 8, 12. and 20 hours. At 20 and at 48 hours, one bottle from each treatment was removed for carotene analysis.

Experiment 6. In a study with the juice from the abomasum of a sheep, 0.5 gram of a corn oil solution of β-carotene (243 μg. of β-carotene) was ac-

curately weighed into each of two Potter-Elvehjem homogenizer tubes. As controls, 0.5 gram of corn oil was weighed into two additional tubes. Then 4.0 ml. of juice from the abomasum of a slaughtered sheep, strained through four layers of cheese cloth and having a pH of 3.0, was measured into each tube. After equilibrating for 10 minutes at 37° C. with grinding, 0.5 ml. of water was added to one tube containing vegetable oil and to one containing the vegetable oil–β-carotene mixture. At the same time, 0.5 ml. of 0.8M sodium nitrite solution was added to the remaining tubes. All tubes were incubated for 15 minutes with intermittent grinding. The contents of each were then washed into 100-ml. volumetric flasks with 25.0 ml. of water and with n-hexane to volume. The flasks were stoppered and vigorously shaken for about 1 minute. After standing a few minutes, a portion of the hexane was removed and the color was measured photometrically at 440 mμ.

Results and Discussion

Data for Experiment 1 with alcoholic solutions are presented in Table I. In the control series, only the hydrochloric acid and the acid plus copper had any appreciable effect. The very similar results in the series with added nitrate indicate that this ion does not destroy carotene. The copper-acid effect in the control and in the nitrate series appears to be more than an additive one, since the total loss here is considerably greater than the combined losses in the presence of either copper or hydrochloric acid alone. Nitrite itself had little or no apparent effect under neutral or alkaline conditions, but when acid was added with it the destruction was very rapid. The copper-nitrite mixture also destroyed carotene, but at a much slower rate when acid was not added than when it was.

The importance of pH in the destruction of carotene when nitrite is present is shown in greater detail by the results of Experiment 2 (Table II). At pH 6.25

Table II. Effect on β-Carotene Destruction of Adding Nitrite to Solutions of Various pH Values (Experiment 2)

pH of Buffer	pH of Mixture (Final)	β-Carotene Remaining, % of Control[a]
7.50	7.65	99
6.05	6.25	95
5.40	5.55	65
5.00	5.15	27
4.55	4.75	0
4.05	4.30	0

[a] Per cent of amount remaining in tube to which no nitrite was added.

and above, little effect was observed. As pH decreased, however. destruction increased. Apparently, only moderately acid systems are required to enhance the effect of nitrite on carotene stability.

Whether nitrite, as such, or nitrogen oxides formed from it under the conditions of the preceding experiments actually caused the destruction cannot be determined from the findings presented here. Nitrogen tetroxide is a well known bleaching agent which forms on contact of nitric oxide with oxygen. Nitric oxide is formed through the breakdown of the rather unstable nitrous acid. To study the effects of nitrogen tetroxide and nitric oxide on carotene, copper was reacted with a 1:1 water–concentrated nitric acid mixture, and the evolved gas was collected over 5% potassium hydroxide in a manner that would exclude oxygen. Introducing about 5 ml. of this colorless gas (nitric oxide) into 10 ml. of hexane or ethanol containing β-carotene, while at the same time passing through a rapid stream of nitrogen to exclude the oxygen of the air, caused almost instantaneous disappearance of the carotene. In addition, introducing a few milliliters of the gaseous nitric oxide into a test tube where it was allowed to oxidize to nitrogen tetroxide, and then introducing a solution of β-carotene in hexane or in ethanol, also resulted
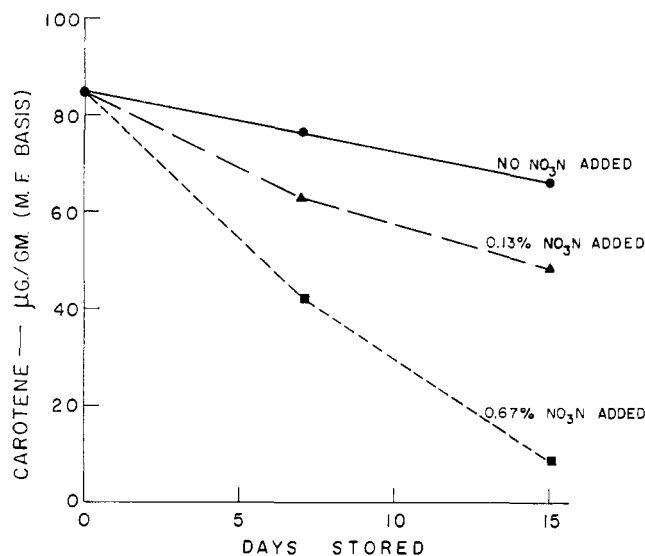
Figure 1. Effect of nitrate additions on carotene content of corn silage during storage
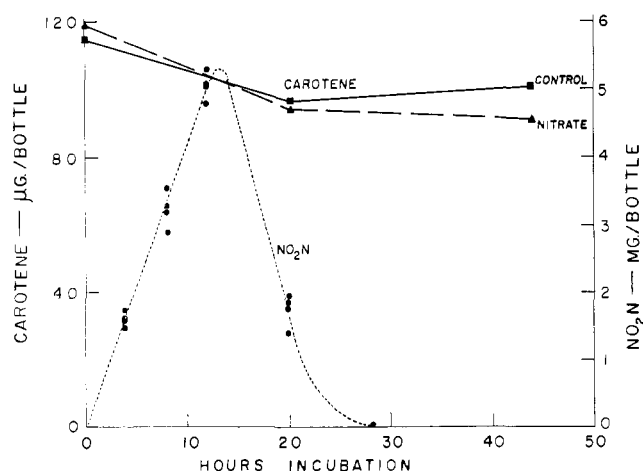

Figure 2. Formation of nitrite from added nitrate and effect of nitrate addition on carotene in artificial rumen

(Nitrite values for control not plotted since none was detected at any time)

Table III. Effect of Added Nitrite on β-Carotene in Moistened Alfalfa Stored Cold (Experiment 3)

Treatment	β-Carotene Remaining After 4 Days Storage at 4° C., µG./G.[a]
5 Grams ground alfalfa + 8 ml. water	111
5 Grams ground alfalfa + 8 ml. 0.18M NaNO₃	117
5 Grams ground alfalfa + 8 ml. 0.09M NaNO₂	30
5 Grams ground alfalfa + 8 ml. 0.72M NaNO₂	15

[a] Dry basis. Initial value 143 µg. per gram.

in an almost immediate disappearance of the carotene. In view of these results, apparently under acid conditions the gaseous oxides of nitrogen rather than the nitrite cause the destruction of carotene.

In Experiment 3, alfalfa was used as the source of β-carotene. The data in Table III again indicate that the presence of nitrate does not cause carotene destruction while the presence of nitrite does. The effect of the nitrite appears to relate to its concentration. Here again, it cannot be assumed that the nitrite itself caused the destruction. However, the influence of copper in the presence of nitrite (Experiment 1) should not be overlooked. In a biological material, several substances which would catalyze a nitrite effect might possibly be present. Other experiments, not reported here, indicate that the rate of destruction of alfalfa β-carotene is relatively slow in the presence of nitrite, that the rate of destruction of β-carotene in neutral al-

coholic solutions containing copper and nitrite is also relatively slow at 4° C., and that oxygen and nitrite may interact to cause greater destruction of β-carotene than the sum of the effect of either one alone. Near neutrality, therefore, the mechanism of the effect of nitrite on β-carotene may not involve the gaseous oxides.

The data from an experiment with corn silage (Experiment 4) are shown in Figure 1. The corn used here was immature, was high in its content of carotene, and had a nitrate-nitrogen content of 0.03% on a moisture-free basis. The low level of added nitrate was equivalent to 0.13%, the high level to 0.67%, of nitrate nitrogen, again on a moisture-free basis. Under the conditions of this experiment, losses increased with increased nitrate additions and continued beyond the first week. For the low level of added nitrate, however, it appears that the effect of this addition expressed itself during the first week of storage, since the loss of carotene during the second week was about the same as for the control. Other experiments, not reported here, with corn silage made of more mature material confirmed the effect of added nitrate (0.5% nitrate nitrogen on a moisture-free basis) and indicated that carotene destruction resulting from the nitrate addition was confined to the first week of storage. Very probably, however, several factors not studied here are important in determining the extent and rate of carotene destruction resulting from the nitrate in ensiled forages.

Many workers have demonstrated the conversion of nitrate to nitrite in the rumen. When nitrate is incubated with rumen juice in vitro, nitrite appears quickly, reaches a high level in a few hours, and then drops off. The same

has been found true in in vivo studies (8, 13). The data obtained in Experiment 5 using an artificial rumen technique show that nitrite-nitrogen values were equivalent to between about 0.002 and 0.009M from about 4 to 25 hours of incubation (Figure 2). The highest level reached represented about 40% of the added nitrate nitrogen. By 26 hours, all nitrite nitrogen had disappeared. Small carotene losses were found in both the control and treated samples at 20 hours, none further occurring after that. Some indication of a slightly greater loss where nitrate had been added was found, but the effect was slight. In another experiment, not reported here, similar nitrite-nitrogen data were observed, but no loss of carotene was found in either the control or treated sample at 20 hours. This work, the data for Experiments 1 and 2, the report of Weichenthal et al. (14), and the findings that nitrate reduction in moistened plant material (10) or in incubated rumen juice (5) is negligible below pH 6 indicate no important role for nitrates in feeds in causing carotene destruction in the rumen. However, rumen fermentations vary greatly in their nature, and the limited work reported here does not allow for any definite conclusions in this matter. Case (4), for instance, observed the odor of nitrogen dioxide on opening the rumen of animals dead from nitrate poisoning, and under these conditions carotene destruction could be expected.

The results for Experiment 6 on in vitro studies with juice from the abomasum of a sheep are given in Table IV. The addition of nitrite to the abomasum juice (pH 3.0) caused almost complete carotene destruction, even though the carotene was added in an oil which was not miscible with or completely emulsifiable

Table IV. Effect of Added Nitrite on β-Carotene Destruction in Sheep Abomasum Juice (Experiment 6)

Treatment	β-Carotene Found	
	μG.	% of theo-retical[a]
Abomasum juice + corn oil + water	1	..
Abomasum juice + corn oil + sodium nitrite	2	..
Abomasum juice + β-carotene in corn oil + water	219	90
Abomasum juice + β-carotene in corn oil + sodium nitrite	18	7

[a] 243 μg. added.

in the juice. In experiments involving the oral administration of nitrites and carotene (or possibly vitamin A) to monogastric animals, this effect cannot be neglected. Its importance in ruminants, however, is not clear. Wang et al. (13) report that nitrite nitrogen probably passes rapidly and directly from the rumen to the blood. Whether, however, nitrite might yet reach the abomasum in sufficient concentrations to cause significant carotene destruction needs study.

The studies discussed here indicate that β-carotene is not affected by the presence of nitrate nitrogen under a variety of conditions. Furthermore, the presence of nitrite nitrogen at about neutral or slightly alkaline pH levels apparently causes only very slow destruction of β-carotene. That provitamin A activity has not been affected cannot, however, be concluded. Copper enhances the rate of destruction when nitrite is present, and it is possible that other substances present in biological materials may do the same. As pH is decreased below 6, destruction in the presence of nitrite occurs rapidly and can result in serious losses of the provitamin. The nitrite effect in acid solutions appears to be the result of the decomposition of nitrous acid to yield gaseous oxides of nitrogen which cause rapid destruction of β-carotene.

Destruction of carotene in the rumen of animals fed forages containing nitrates does not appear to present a problem of practical consequence. On the other hand, high levels of nitrates in ensiled forages may enhance carotene losses considerably, and more study of this phase of the problem seems justified. Furthermore, in experimental work with monogastric animals, the rapid destruction of carotene in acid fluids to which nitrites are added must be considered. Since no biological tests for vitamin A activity were made in this work, it can only be assumed that the carotene destruction noted did not yield compounds with vitamin A activity. In view of the considerable amount of work done on this in the past, however, the assumption appears a valid one.

Acknowledgment

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STORAGE EFFECTS ON WINTER SQUASHES

Varietal Differences and Storage Changes in the Ascorbic Acid Content of Six Varieties of Winter Squashes

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TABLES OF FOOD VALUES usually consulted when estimating the nutrient intake of groups or individuals indicate that winter squash contains a relatively small amount of ascorbic acid. In such tables, winter squash is generally not identified by variety. The amount of data on specific varieties elsewhere in the literature is very limited.

Since increasing numbers of different varieties are becoming available to the consumer, this investigation was made to provide additional information on varietal differences and on the effect of storage on ascorbic acid content of six varieties of winter squashes.

Studies by Holmes and coworkers are of particular interest. Ascorbic acid determinations (fresh basis) on five varieties 6 weeks after harvest showed distinct varietal differences. Buttercup had a mean value of 39.6 mg. per 100 grams compared with 3.4 mg. per 100 grams for Des Moines (3). In addition, their findings suggest that changes in ascorbic acid content of winter squashes during storage are not necessarily the same for different varieties (4, 5).

Experimental

The varieties studied during 1957–58 were Baby Blue, Buttercup, Blue Hubbard, Silver Bell, and Sweet Meat of the Cucurbita maxima species and Butternut of the C. moschata species. The six squash varieties were grown in four replications on the University Horticultural Farm. The squashes were harvested between September 17 and 20, and placed in storage on slatted racks, keeping the fruit from the 24 plots separated.