with corn silage the results were irregular. In one experiment more nitrogen dioxide was obtained, in another less, and in two others no effect was apparent. The carbon dioxide data for corn are equally erratic. The October 3 sample with added nitrate produced a large amount of both carbon dioxide and nitrogen dioxide, but in the other experiments there was no regular correlation between production of the two gases.

Addition of chloroform and toluene to the corn sample decreased carbon dioxide production slightly and nitrogen dioxide markedly. Sodium bisulfite reduced formation of both gases but particularly that of nitrogen dioxide if enough were used—1 gram per kg. seemed too little.

Small lots of other plant materials—cabbage, lettuce, and lawn clippings—were ensiled; the evolved gases, tested colorimetrically, all gave a positive test. With nothing added, from 0.4 to 0.8 ml. of nitrogen dioxide per kg. was released. With added nitrate the quantity of nitrogen dioxide from cabbage rose from 0.6 to 1.6 ml. per kg. Addition of 2.5% sodium chloride (as in the making of sauerkraut) reduced nitrogen dioxide production in one experiment but had no effect in another.

Summary

Five silos were tested for the production of nitrogen dioxide during the summer and fall of 1956. All gave positive tests. Three of the silos were equipped with drainage basins for collecting the expressed silage juice and in two cases the basins became filled with the brown fumes. A good colored movie film of the fumes was obtained. A sample of the gas in the basin analyzed about 10% nitrogen dioxide.

Gases from eight lots of experimental silage to which nitrogen-15–labeled nitrate or amino acids were added were analyzed by means of the mass spectrometer and found to contain from 3.4 to 14.8% nitric oxide and usually less than 0.5% nitrogen dioxide. The atom per cent excess nitrogen-15 was about the same in the nitric oxide as in the added nitrate, indicating that most of it came from the latter. As expected, the label in nitric oxide was about 10 times as high when $NaN^{15}O_3$ was added to the forage as when nitrogen-15–labeled amino acids were ensiled with the plant material. The percentage of $N^{14}N^{15}$ was about the same in the gases from the two silages, hence its formation is attributed to a Van Slyke reaction between nitrous acid and α-amino nitrogen.

Other lots of silage were made from several kinds of forage with various treatments. Added sodium nitrate usually increased production of nitrogen dioxide. Chloroform and toluene prevented formation of gas, and sodium bisulfite reduced the amount. Sterilization and reinoculation of the forage gave reduced but definite amounts of nitrogen dioxide. These data indicate that bacteria and not plant enzymes are responsible for the formation of the nitrogen oxides.

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Received for review March 27, 1957. Accepted October 7, 1957. Fermentation Subdivision, Division of Agricultural and Food Chemistry, 130th Meeting, ACS, Atlantic City, N. J., September 1956. Published with the approval of the Director, Wisconsin Agricultural Experiment Station. Investigation supported in part by funds from Red Star Yeast and Products Co., Milwaukee, Wis.

FEEDSTUFFS ANTIOXIDANTS

Stabilization of Alfalfa Carotenoids with *N,N'*-Diaryl-alpha,omega-diaminoalkanes

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Several *N,N'*-diaryl-α,ω-diaminoalkanes were synthesized and tested as antioxidants for the carotenoids of alfalfa. Increasing the length of the aliphatic portion of the molecule improved the antioxidant activity of the compounds, indicating lipide solubility to be an important factor. Compounds having methoxy groups in the para positions were more active than the corresponding methyl-substituted compounds, which in turn were more active than the unsubstituted substances.

THE ABILITY of various organic compounds to stabilize the carotene of dehydrated alfalfa meal has been reported (*2, 8, 11, 14*). The most promising appeared to be DPPD (*N,N'*-diphenyl-*p*-phenylenediamine) and Santoquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline). However, the Food and Drug Administration recently requested that addition of DPPD to feedstuffs be discontinued (*10*), because it caused toxic effects with certain test animals (*12*). Santoquin has been approved for use in poultry feeds only. Hence there still is need for an all-pur-

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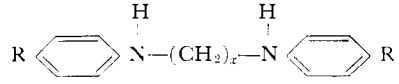
pose, nontoxic antioxidant for use with dehydrated alfalfa meal.

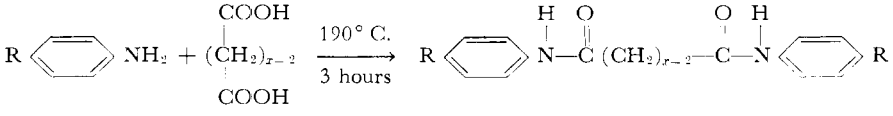
Mitchell, Beauchene, and Silker (*11*) found that monomethylaniline possessed greater antioxidant activity than aniline when applied to dehydrated alfalfa, and that the presence of a hexamethylene bridge between the nitrogen atoms of two aniline molecules gave still greater activity. A number of compounds of the latter type have been employed as stabilizers for poly(vinyl acetal) resins (*4*) and as antioxidants for rubber (*3*, *7*, *15*). In this investigation, certain compounds having this general structure were synthesized by introducing methyl and methoxy substituents into the para positions of the rings and varying the chain length from one to 10 carbons:



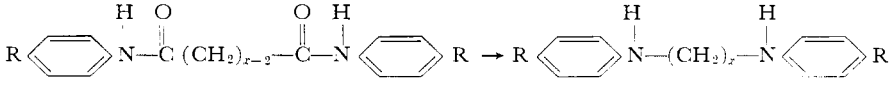
R = H, CH_3, OCH_3
X = 1 to 10

These compounds were tested for their ability to inhibit carotene and xanthophyll destruction during storage of alfalfa meal.

Synthesis of Compounds. Most of the compounds were prepared by condensing the appropriate aromatic amine and dibasic acid, and reducing the resulting diamide. The amine and dibasic acid were heated at 190° C. for 3 hours:



The diamides so obtained were purified by washing successively with 5N hydrochloric acid, hot water, and diethyl ether. They then were reduced at room temperature with lithium aluminum hydride, with tetrahydrofuran as a solvent:



Certain compounds were not prepared by these general reactions. Efforts to reduce oxanilide to N,N'-diphenylethylenediamine with lithium aluminum hydride were unsuccessful. However, the compound was obtained by reaction of aniline with ethylene chlorohydrin in the presence of sulfuric acid. N,N'-Di-(*p*-methylphenyl)methylenediamine and N,N'-di-(*p*-methoxyphenyl)methylenediamine were prepared by reaction of 10 grams of *p*-toluidine and *p*-anisidine, respectively, with 5 ml. of 40% formaldehyde in 1.5 liters of water at 0° C. according to the method of Eibner (*5*). Details of these syntheses will be reported elsewhere.

Determination of Xanthophyll. Attempts to determine xanthophylls showed that the method of Bickoff and coworkers (*1*) frequently gave erroneous results because of the presence of a varying amount of chlorophyll in the final xanthophyll solution. The following method eliminates this difficulty.

One gram of alfalfa meal in a 125-ml. Erlenmeyer flask was hydrated by adding 3 ml. of water, 60 ml. of acetone were added, and the flask was swirled gently to mix the contents. After soaking for 16 hours in the dark, the liquid was filtered through cotton and the green extract was collected in a 500-ml. separatory funnel. The flask and the meal were washed with acetone until the washings were colorless.

To the extract and washings were added 20 ml. of a 20% solution of potassium hydroxide in absolute ethyl alcohol. The separatory funnel was stoppered and shaken intermittently for 5 minutes to saponify the chlorophylls. To the extract were added 150 ml. of Skellysolve B and 250 ml. of distilled water, and again the separatory funnel was shaken vigorously. The aqueous phase was drawn off and extracted with 100 ml. of Skellysolve B to ensure complete removal of the carotenoids. The Skellysolve B extracts were combined and washed twice with 150 ml. of distilled water.

The Skellysolve B solution was dried over anhydrous sodium sulfate, after which it was chromatographed on a 2.5 × 7 cm. column of hydrated lime and Super-Cel (3 to 1 by weight). Skellysolve B was passed through the column until the carotene was eluted completely. The xanthophylls were eluted by washing the column with a solution consisting of 5% absolute ethyl alcohol in Skellysolve B. The eluate was diluted to 250 ml. and its absorbance was measured at 444 mμ by means of a Beckman DU spectrophotometer. An absorptivity of 225 was used for calculating the amount of xanthophyll per gram of meal.

The absorptivity of 225 was obtained by isolating the total xanthophylls of dehydrated alfalfa meal and obtaining the absorption spectrum of the mixture. Xanthophyll was isolated by extracting 1.5 kg. of meal with 10 liters of a solution of 33% acetone in Skellysolve B. The acetone was removed from the extract by washing with water, and the resulting Skellysolve B solution was drawn through a column containing a mixture of hydrated lime and Super-Cel (1 to 1 by weight) (*6*). The column was washed with Skellysolve B until the carotene was eluted completely. The xanthophylls next were eluted by washing the column with 5% acetone in Skellysolve B. The volume of the latter eluate was reduced to about 45 ml. by distillation at reduced pressure on a steam bath.

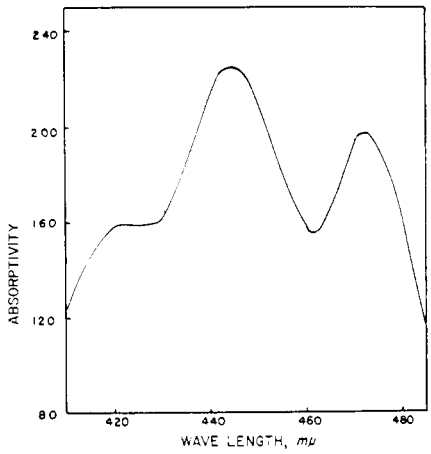


Figure 1. Absorption spectrum of total xanthophylls isolated from dehydrated alfalfa meal

The volume was adjusted to 300 ml. with acetone and the solution was held at −23° C. until the waxes separated out. The waxes were removed by cold filtration, the filtrate was concentrated to about 150 ml., and additional wax was removed by cooling. Alternate concentration and cooling were continued until no more wax was obtained. The wax fractions were combined and recrystallized from acetone to recover the small amount of xanthophyll retained by them. The final xanthophyll solution was concentrated to 20 ml., an equal volume of Skellysolve B was added, and the solution was allowed to stand at room temperature in the dark until crystallization occurred. The crystals were vacuum-dried at room temperature and used to prepare a Skellysolve B solution of known xanthophyll concentration. The absorption spectrum was obtained by means of the Beckman DU spectrophotometer. Absorption maxima occurred at 422, 444, and 472 mμ, and the calculated absorptivities at these wave lengths were 160, 225, and 199, respectively. The absorption curve is shown in Figure 1.

The absorptivity of 225 at 444 mμ obtained with the isolated total xanthophylls of dehydrated alfalfa is in good agreement with the estimated value of 231 at 445 mμ, reported by Bickoff and coworkers (*1*). Their absorptivity was calculated by separating each major component and using the absorptivities of the pure compounds for determining the concentration of each. The contribution of each component to the absorption at 445 mμ was determined and these were added to give the total absorptivity

Table I. Antioxidant Activity of Synthesized Compounds (0.05% Antioxidant)

Compound Added	% Retention after 6 Weeks at 37° C.	
	Carotene	Xanthophyll
None	32	60
N,N'-Diphenylethylenediamine	42	65
N,N'-Diphenyltetramethylenediamine	51	69
N,N'-Diphenyl-1,4-diamino-2-butene	35	62
N,N'-Diphenylhexamethylenediamine	57	73
N,N'-Diphenyloctamethylenediamine	60	74
N,N'-Diphenyldecamethylenediamine	48	70
N,N'-Di-(*p*-methylphenyl) methylenediamine	43	71
N,N'-Di-(*p*-methylphenyl) hexamethylenediamine	69	79
N,N'-Di-(*p*-methylphenyl) octamethylenediamine	69	79
N,N'-Di-(*p*-methylphenyl) decamethylenediamine	67	78
N,N'-Di-(*p*-methoxyphenyl) methylenediamine	48	76
N,N'-Di-(*p*-methoxyphenyl) hexamethylenediamine	67	89
N,N'-Di-(*p*-methoxyphenyl) octamethylenediamine	72	86
N,N'-Di-(*p*-methoxyphenyl) decamethylenediamine	69	84
Santoquin	74	87

of the mixture. They recognized that their absorptivity value did not include absorption due to the minor xanthophyll components, but believed the error due to this simplification to be small.

Antioxidant Activity. The compounds under investigation were applied to alfalfa meal at a rate of 1 pound per ton (0.05%) according to the method of Mitchell, Beauchene, and Silker (*11*). A solution of each compound was prepared by dissolving 0.266 gram in 25 ml. of acetone, and a 12.5-ml. aliquot of this solution was placed in a DeVilbiss No. 15 nasal atomizer. To this were added 10 ml. of a solution which consisted of 45.4 grams of Wesson oil and sufficient Skellysolve B to make a volume of 250 ml. One-half pound of alfalfa meal was placed in a small rotary mixer. The atomizer was connected to a source of air pressure by rubber tubing. The nozzle of the atomizer was inserted through a small hole in the center of the lid of the mixer, and the solution was sprayed on the meal while the mixer was being rotated at 37 r.p.m. The sprayed meal was transferred to a sheet of wrapping paper and placed in a darkened room for 2 hours to permit the solvent to evaporate. The meal was mixed and carotene was determined by the method of Silker, Schrenk, and King (*13*). Xanthophyll was determined by the method described above. The meals were stored at 37° C. for 6 weeks; then carotene and xanthophyll were determined again. The results are shown in Table I and portions are compared graphically in Figure 2.

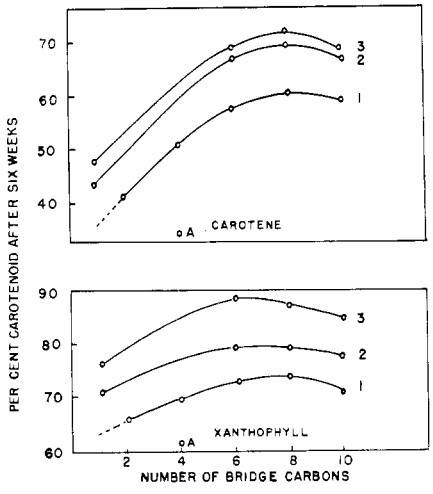


Figure 2. Relationship between chain length and antioxidant activity

1. No substituents
2. Methyl substituents in para positions
3. Methoxy substituents in para positions
A. *N,N'*-diphenyl-1,4-diamino-2-butene

Increasing the length of the carbon bridge up to six or eight methylene groups appreciably increased antioxidative activity. Methoxy groups substituted in the para positions gave greater activity than methyl groups, which in turn gave greater activity than the corresponding nonsubstituted compounds. The unsaturated compound, *N,N'*-diphenyl-1,4-diamino-2-butene (*A*, Figure 2), was much less active than its saturated counterpart, *N,N'*-diphenyltetramethylenediamine. The most active compound, *N,N'*-di-(*p*-methoxyphenyl) octamethylenediamine, was approximately equal to Santoquin, which was included in this study for comparative purposes.

Groups that tend to increase the electron density of the aromatic ring increase antioxidative activity. They may influence antioxidative activity by contributing to the resonance stabilization of intermediate free radicals. However, substituent criteria alone may not be sufficient for judging whether or not a certain compound will be a good antioxidant for the protection of the carotenoids in alfalfa or other forages. The compound must be capable of penetrating the plant tissue to the site of the carotenoids. As the carotenoids are dissolved in the lipide phase of the plant tissue, it would seem that the antioxidant should be lipide soluble also. Presumably the hydrocarbon nature of the polymethylene bridge of the compounds under investigation contributes to their penetration and solubility, which may account for the increase in activity with increasing chain length. The increase in activity within a given series of compounds would have been more pronounced than that shown in Figure 2, had the antioxidants been added at a constant molar level rather than at a constant weight. With increasing chain length, fewer active antioxidative sites were being supplied to the meal per gram of antioxidant, for the material was essentially being diluted with methylene groups.

In all instances the xanthophylls were more stable than carotene. Others have reported similar results (*9*). Carotene oxidation products may be formed which are retained on the adsorption column, but may possess a yellow color and be eluted with the xanthophylls.

These data indicate that more than one factor is involved in the ability of a chemical to function as an antioxidant for the carotenoids of alfalfa. They suggest that when a chemical grouping is found which imparts antioxidant activity to a compound, this activity may be enhanced by including other groups to increase the lipide solubility of the compound.

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Received for review June 15, 1957. Accepted August 1, 1957. Contribution 556, Department of Chemistry, Kansas State College. From a thesis submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in chemistry.