Anal. Calcd. for $C_{10}H_6N_2O_5$: C, 51.28; H, 2.56; N, 11.97. Found: C, 51.22; H, 2.51; N, 11.6.

In an attempt to apply Witt's[7] method for the production of diazo compounds phenyl-β-naphtholaminomethane was treated with ten times its weight of concentrated nitric acid. After standing for one hour it was poured into water and the precipitated material was converted into the potassium salt of 1,6-dinitro-2-naphthol as previously described.

Benzoic acid was also formed in this reaction. It was isolated by acidifying the alkaline mother liquor and recrystallizing the precipitated material from hot water.

Acetyl Derivative of 1,3-Diphenyl-4,2-β-naptho-iso-oxazine.—This was obtained by warming 2.0 g. of the condensation product, II, with 10.0 cc. of acetic anhydride on the water-bath for two hours; yield, 1.6 g.; m. p. 170°.

Anal. Calcd. for $C_{26}H_{21}NO_2$: C, 82.3; H, 5.55; N, 3.69. Found: C, 82.0; H, 5.7; N, 4.1.

(7) Witt, *Ber.*, **42**, 2953 (1909).

Acetyl Derivative of Phenyl-β-naphtholaminomethane.—This was obtained by the hydrolysis of the above-mentioned compound with concentrated hydrochloric acid for six hours in the cold; m. p. 225°.

N-Acetyl-phenyl-α-(β-methoxynaphthyl)-aminomethane was obtained when the acetyl derivative of phenyl-β-naphtholaminomethane was methylated as previously described for the urethan. It melted at 186° and was identical with the compound prepared by Ray and Moomaw.[3] This shows that the acetyl group in the oxazine was attached to nitrogen as Ray and Moomaw prepared their acetyl compound from phenyl-α-(β-methoxy-naphthyl)-aminomethane.

Summary

The reaction between nitrous acid and phenyl-β-naphtholaminomethane leads to the formation of N-nitroso-1,3-diphenyl-4,2-β-naphtho-iso-oxazine and 1,6-dinitro-2-naphthol.

CINCINNATI, OHIO RECEIVED JUNE 27, 1934

[CONTRIBUTION FROM THE BIOCHEMICAL LABORATORY, STATE UNIVERSITY OF IOWA]

The Association of Fat-Soluble Vitamins and Antioxidants in Some Plant Tissues[1]

BY ELIZABETH M. BRADWAY AND H. A. MATTILL

In earlier papers[2] the evidence was reviewed that vitamin A or carotene, and especially vitamin E as it exists in foods, are easily destroyed by oxidation in the presence of autoxidizable substances, and that their survival in such association depends, in part at least, upon the protective action of naturally occurring or added inhibitors. The stability of carotene in different solvents under various conditions[3] and the vulnerability of vitamin E to catalyzed autoxidation of fats[4] have been the subjects of more recent studies. The proved presence of antioxidants in some vegetable oils and the isolation of an antioxidant from lettuce[5] which was separate and distinct from the vitamins and sterols, prompted the suggestion that such inhibitors might be the protective agencies in all plant tissues, whereby the labile fat-soluble vitamins are preserved in an environment otherwise favorable to oxidation.

If the presence of natural antioxidants is related to the abundance and stability of carotene or vitamin E in vegetable foods, it should be possible to demonstrate the existence of inhibitors especially in such vegetables as are rich sources of these vitamins or whose vitamin content is little diminished by drying or by exposure to other conditions favoring oxidation. Attempts to isolate naturally occurring antioxidants might also yield information on their chemical behavior perhaps on the particular form in which they exist in the plant, as well as on other constituents of plant oils. Carrots, the earliest known source of carotene, suffer no appreciable loss of their vitamin A value by autoclaving at 15 pounds pressure and subsequent drying at room temperature.[6] The tomato, whose vitamin A is especially stable,[7,8] seems also to contain vitamin E. These two vegetables were chosen for this study along with wheat germ oil whose content in vitamin E is the highest of any of the vegetable oils. The methods for separating the unsaponifiable constituents were essentially those employed in the study of lettuce[9] with appropriate modifications where necessary.

(1) Presented at the Philadelphia meeting of the American Society of Biological Chemists, April 29, 1932.

(2) (a) Cummings and Mattill, *J. Nutrition*, **3**, 421 (1931); (b) Olcovich and Mattill, *J. Biol. Chem.*, **91**, 105 (1931).

(3) Baumann and Steenbock, *ibid.*, **101**, 561 (1933); Turner, *ibid.*, **105**, 443 (1934).

(4) Waddell and Steenbock, *J. Nutrition*, **4**, 79 (1931).

(5) Olcott and Mattill, *J. Biol. Chem.*, **93**, 65 (1931).

(6) Steenbock and Boutwell, *J. Biol. Chem.*, **41**, 163 (1920).

(7) Sherman, Quinn, Day and Miller, *ibid.*, **78**, 293 (1928).

(8) Steenbock and Schrader, *J. Nutrition*, **4**, 267 (1931).

(9) Olcott and Mattill, *J. Biol. Chem.*, **93**, 59 (1931).

Tomatoes.—Canned tomatoes, completely dried at 100° in a vacuum oven (four to six hours), were treated with hot 90% methyl alcohol in a continuous extractor until the solvent was no longer colored (twenty hours). After the usual process of alcoholic saponification (at 45–50° for twelve hours) dilution, and extraction with peroxide-free ether, this solution of the unsaponifiable constituents was washed free of soaps, dehydrated with anhydrous sodium sulfate and the residue, after removal of the ether, was dissolved in warm petroleum ether. On standing at −5° under an inert gas this solution deposited sterols, which have not been examined further. The solvent in the filtrate was replaced by hot methyl alcohol and from this solution, on standing at −5°, lycopene and carotene separated. The lycopene, freed from carotene by washing with acetone, was examined as described below. The methyl alcohol filtrate was diluted with water to 92% alcohol (by volume) and was repeatedly extracted with petroleum ether; the residues after removal of the solvents were tested for vitamin E and antioxidant by the methods described elsewhere.[10] The results of the tests on these several fractions are given in Table I. These results are similar to those obtained from lettuce with

TABLE I

VITAMIN E AND ANTIOXIDANT IN TOMATO

	Vitamin E, minimum dosage[a]	Antioxygenic index[b]
Whole dried	5 g. (7) (11) (5) (2)[c]	
Unsaponifiable	50 mg. (3) (1) (0) (2)	3+
Petroleum ether fraction	25 mg. (2) (0) (1)	1
Methyl alcohol fraction[d]		
T_{13}	75 mg. (0) (0) (0)	5.5
T_{14}		40
T_{15}		89–92

[a] The amount required to cause a successful gestation in rats on a sterility producing diet. Only the tests with minimum dosage are reported. [b] The antioxygenic index is the ratio of the induction period of the autoxidizable fat containing the inhibiting substance in a concentration of 0.1% to the induction period of the same fat without addition of the inhibitor. [c] The figures in parentheses are the number of young in the litter. [d] Three different runs. No assays were attempted on Fractions T_{14} and T_{15}.

respect to the separations accomplished by different organic solvents and it is at once evident that diphasic separation of the methyl alcohol soluble material as between 92% methyl alcohol and petroleum ether rather completely segregated vitamin E from the antioxygenic material. To obtain still more active concentrates the small amounts of material available were fractionally distilled *in vacuo*, with the results given in Table II.

Not all of the fractions were tested and while no greater concentration was secured, probably because of destruction during distillation, it may nevertheless be said that the most active vitamin E fraction was that which distilled above 200°. This temperature is in fair agreement with that of the active distillates from lettuce and from

(10) (a) Mattill, *J. Biol. Chem.*, **90**, 141 (1931); (b) Olcott and Mattill, *ibid.*, **104**, 423 (1934).

TABLE II

CONCENTRATES OF VITAMIN E AND ANTIOXIDANT FROM TOMATO

Petroleum Ether Soluble Fraction Distillates (0.1–0.2 mm.)

	Temp. range, °C.	Vitamin E, minimum dosage
I	90–115	100 mg. (0) (0)
II	185–200	50 mg. (0) (0)
III	210–225	25 mg. (1) (0)

Methyl Alcohol Soluble Fraction Distillates (0.1–0.2 mm.)

		Antioxidant index
I	90–115	82–90
II	135–150	18–23
III	190–200	2+
IV	Residue	1.2

wheat germ oil[10b] and of Evans and Burr's preparation[11] from wheat germ oil. In the absence of further information one may conclude tentatively that the vitamin E obtained from these three sources is the same substance.

The most active antioxidant fraction was that which distilled between 90 and 115°, although a higher boiling fraction, even to 150°, still contained antioxygenic material; whether this was the same substance as in the lower boiling fraction or a different one could not be determined because of the small amounts available. The most active antioxidant distillates of lettuce and tomato are far apart on the temperature scale, lettuce 165–185°, as compared with tomato 90–115°. The antioxidant in tomato is evidently not the same substance as that found in lettuce.

The lycopene mentioned above was recrystallized four times from acetone by addition of methyl alcohol. The material thus purified had a melting point of 167–168°, which compares favorably with the commonly accepted figure (168–169°). It was tested for vitamin A and found to be inactive, as all others have found it. It also possessed no vitamin E activity. It was tested for antioxygenic properties and like carotene[2b] it was pro-oxygenic.[12] In a concentration of 0.04% an index of 0.5 was obtained, that is, the induction period of the autoxidizable fat was reduced one-half by the presence of lycopene. A sample of lycopene kindly sent us by Dr. Sando of the Bureau of Chemistry and Soils behaved similarly.

Wheat Germ Oil.—Wheat germ oil[13] was directly saponified, the sterols were removed from the unsaponifiable matter by crystallization first from petroleum ether and later from methyl alcohol. Diphasic separation of vitamin E and antioxygenic substance between 92% methyl alcohol and petroleum ether was not successful, as is shown by the data in Table III. The petroleum ether fraction was not only more potent as a source of vitamin E but was also more active in delaying oxidation, and the methyl alcohol portion contained little antioxidant and apparently no vitamin E in the doses administered.

Nor was it possible to separate the two by fractional distillation of the petroleum ether soluble material. As

(11) Evans and Burr, *Memoirs of the University of California*, Berkeley, **8**, 131 (1927).

(12) (a) Franke, *Z. physiol. Chem.*, **212**, 234 (1932); (b) Greenbank and Holm, *Ind. Eng. Chem.*, **26**, 243 (1934).

(13) The wheat germ oil was obtained through the courtesy of Mr. Neilsen of the Abbott Laboratories.

TABLE III

VITAMIN E AND ANTIOXIDANT IN WHEAT GERM OIL

	Vitamin E, minimum dose, mg.	Antioxygenic index
Unsaponifiable	250 (9) (6)	2.9–3.4
Petroleum ether fraction	75 (7) (4) (8) (11)	11–13
Methyl alcohol fraction	200 (0) (0) (0)	6–7

shown in Table IV the two fractions distilling between 180 and 225° were more active in both respects than any of the others. The temperature range for vitamin E confirms the figures quoted above; the temperature range for the antioxidant is a little higher than that of the substance distilled from lettuce and very much higher than that of the material occurring in tomato.

TABLE IV

PETROLEUM ETHER SOLUBLE FRACTION DISTILLATES (0.1–0.2 MM.) FROM WHEAT GERM OIL

		Vitamin E, minimum dose, mg.	Antioxygenic index
I	135–165°	110 (0) (0)	1.1
II	165–180°	75 (0) (0)	3.2
III	180–210°	25 (11) (7) (0)	12
IV	210–225°	25 (1) (0)	10
V	Residue		4.4

The difference in solubility behavior of the antioxidant in wheat germ oil as compared with that in lettuce and tomato may be due to the nature and amount of associated substances and their influence on its distribution between two immiscible solvents. More likely, however, the antioxygenic material from wheat germ oil is not the same substance as that from lettuce or tomato.

Carrots.—Canned carrots were dried by extraction with freshly distilled acetone according to the method of Holmes and Leicester[14] and were then extracted with hot methyl alcohol. Some sterols separated from the cold petroleum ether solution of the unsaponifiable matter. Following the successful diphasic separation of inhibitor from vitamin E it was necessary to remove further amounts of sterols and carotene before any antioxygenic activity could be demonstrated in the methyl alcohol soluble material. Antioxygenic indices of 3 to 5 were obtained from several of such preparations.

The association of carotene with an inhibitor in nature has been implied in a number of observations and the failure to appreciate the ready destruction of purified carotene by oxidation[15] contributed to the delay in recognizing carotene as a precursor of vitamin A. Attention has lately been drawn to the greater stability of crude carotene from carrots as compared with the purified material.[16] Crude carotene from lettuce[17] in 0.02% concentration in an autoxidizable animal fat mixture gave indices of 2.4 and 2.2; in 0.1% concentration, 1.4 and 1.6, whereas pure carotene, from whatever source, has never failed to behave as a pro-oxidant. The interesting observations of Monaghan and Schmitt[18] that carotene inhibits the oxygen uptake of rapidly oxidizing linoleic acid is not inconsistent with its pro-oxygenic capacity under other conditions and emphasizes the complexity of the processes of autoxidation as revealed by many recent papers.

The petroleum ether soluble fraction from carrots contained no antioxidant and almost one gram was required to prevent resorptions in pregnant rats on the sterility producing diet, thus indicating that the amount of vitamin E in carrots is relatively small. This is borne out by the fact that 220 g. of canned carrots or 25 g. of dried (over calcium chloride) were the minimum amounts necessary to ensure the birth of litters.

The question naturally arises as to the chemical nature of these inhibitors and the form in which they exist in plant tissue; as yet a satisfactory answer cannot be given. It was work in this Laboratory which first demonstrated that the unsaponifiable portion of certain vegetable oils contained inhibitors for the autoxidation of animal fats.[19] That these are not the only types of inhibitors found in plants is evident from the antioxygenic effect of gossypol[10a] which has recently been confirmed.[20] Some glucosides, in particular the aglucone portion, may be inhibitors. At the suggestion of Dr. Sando, who kindly supplied the material, we examined a few glucosides, none of which was effective as an antioxidant; however, quercitin from quercitrin was a good antioxidant, as also has been demonstrated recently.[12b]

The mixtures of glucosides obtained from tomato and carrot pulp by the method of Schertz[21] were not antioxygenic. Portions of each were hydrolyzed with 10% potassium hydroxide or with 5% hydrochloric acid, and the hydrolyzate (made alkaline with sodium carbonate in the latter case) when extracted with peroxide-free ether, yielded an ether-soluble inhibitor from tomato (index 3 to 4) but none from carrot. Tomato juice was inactive both before and after hydrolysis. Similar tests with aqueous and acetone extracts of lettuce demonstrated no inhibitors either before or after hydrolysis.

It is unlikely that the inhibitors from the unsaponifiable portion of oils like wheat germ oil are glucosidic in origin unless hydrolysis of the glucoside takes place in the plant during the period of oil formation because the oil contains the active inhibitor before it is saponified. Nor is it probable that the types of inhibitors found in plants are confined either to the unsaponifiable fraction of the plant lipids or to the aglucone portion of the glucosides.

The authors are indebted to Dr. H. S. Olcott for his helpful suggestions.

Summary

1. The fat soluble vitamins in tomatoes, carrots and wheat germ oil are accompanied by inhibitors which prolong the induction period of autoxidizable fats.

2. After the carotenoid pigments and sterols are largely removed from the unsaponifiable matter, diphasic distribution between 92% methyl

(14) Holmes and Leicester, THIS JOURNAL, **54**, 716 (1932).

(15) Stephenson, *Biochem. J.*, **14**, 715 (1920); Drummond, Ahmad and Morton, *J. Soc. Chem. Ind.*, **49**, 291T (1930).

(16) Baumann and Steenbock, *Wis. Expt. Sta. Bull.*, **420**, 77 (1931).

(17) Gluesenkamp, unpublished observations.

(18) Monaghan and Schmitt, *J. Biol. Chem.*, **96**, 387 (1932).

(19) Mattill and Crawford, *Ind. Eng. Chem.*, **22**, 341 (1930).

(20) Royce, *Oil and Soap*, **10**, 123 (1933).

(21) Schertz, *Plant Physiol.*, **3**, 211 (1928).

alcohol and petroleum ether successfully separates the inhibitors from vitamin E in the case of tomato and carrot but from wheat germ oil the inhibitor as well as vitamin E are preferentially soluble in petroleum ether. From this fact and from the distillation ranges under diminished pressures it appears that the inhibitors in these three materials and in lettuce are probably all different substances. Similar evidence points to the identity of the vitamin E as obtained from different sources. Carrots contain only small amounts of vitamin E.

3. Lycopene, like carotene, is pro-oxygenic and shortens the induction period of autoxidizable fats. It is not active either as vitamin A or E. Crude carotene may be antioxygenic due to associated inhibitor.

4. There are probably other types of inhibitors in plant tissues aside from those found in the unsaponifiable portion of the lipids and the glucosides.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF NEBRASKA]

Arsenicals Derived from 2-Amino-6-nitronaphthalene

BY LEON A. SWEET AND CLIFF S. HAMILTON

In 1915 Friedländer and Littner[1] stated that the products of nitration of β-acetnaphthalide were 2-amino-1-nitro-, 2-amino-8-nitro- and 2-amino-5-nitronaphthalene. Veselý and Jakeš[2] studied the reaction and were unable to find any trace of the 2,5-isomer, but obtained 2-amino-6-nitronaphthalene. Recently, Saunders[3] devised a method by which the three isomeric aminonitronaphthalenes were easily separated in two stages. His method was used in this work for the preparation of 2-amino-6-nitronaphthalene and although good yields were obtained the desired isomer comprised only 8% of the product. The nitration was studied for a range of temperatures but the ratio of yields was not altered. In view of the conflicting statements concerning the identity of this isomer, its structure was proved by reduction to 2,6-diaminonaphthalene, thus confirming the finding of Veselý and Jakeš.

2-Amino-6-nitronaphthalene was converted by the Bart[4] reaction into 2-arsono-6-nitronaphthalene. This was reduced with ferrous hydroxide to the corresponding amine.

Experimental

2-Amino-6-nitronaphthalene was prepared according to the method of Saunders.[3] It was reduced by means of tin and hydrochloric acid to give 2,6-diaminonaphthalene; m. p. 220° (Lange,[5] 216–218°). With benzoyl chloride in pyridine solution it formed **2-benzoylamino-6-nitronaphthalene,** which crystallized from ethanol in light yellow needles of m. p. 206°.

Anal. Calcd. for $C_{17}H_{12}N_2O_3$: C, 69.84; H, 4.14. Found: C, 69.67; H, 4.24.

2-Arsono-6-nitronaphthalene.—A solution of 2-amino-6-nitronaphthalene (9.4 g.) in 100 cc. of 6 *N* hydrochloric acid was stirred mechanically while cooled in a salt–ice mixture. A calculated volume of sodium nitrite solution (5 g. in 25 cc. of water) was added slowly during one hour. Meanwhile an arsenite solution was prepared by dissolving 20 g. of sodium metaarsenite and a few crystals of copper sulfate in a liter of water, and then made up to 1.5 liters by adding crushed ice. The arsenite solution was stirred mechanically while 85 cc. of 6 *N* sodium hydroxide was added slowly from one dropping funnel and the cold diazonium solution from another. The addition of alkali was begun, and after 5 cc. had been added both solutions were added simultaneously in such a ratio that the solution remained slightly alkaline. Stirring was continued for two hours, and then the temperature was raised to 60° for one hour. The solution was filtered and the arsonic acid precipitated by making acid to Congo red paper with hydrochloric acid; weight, 7.0 g. It was purified by dissolving in 0.5 *N* sodium carbonate solution and reprecipitating with acid, forming tiny glistening needles.

2-Arsono-6-aminonaphthalene was prepared from 2-arsono-6-nitronaphthalene by the method of Jacobs, Heidelberger and Rolf,[6] an alkaline ferrous hydroxide reduction. Reaction with acetic anhydride in glacial acetic acid yielded **2-arsono-6-acetylaminonaphthalene.** The amine in 0.5 *N* sodium carbonate solution condensed with

SUBSTITUTED 2-ARSONO-NAPHTHALENES

Substituents	Yield, %	Formula	Arsenic analyses, % Calcd.	Found	
6-Nitro-	47	$C_{10}H_8O_5NAs$	25.25	25.39	25.35
6-Amino-	87	$C_{10}H_{10}O_3NAs$	28.07	28.07	28.11
6-Acetylamino-	87	$C_{12}H_{12}O_4NAs$	24.25	24.28	24.14
6-Carbethoxyamino-	95	$C_{13}H_{14}O_5NAs$	22.12	22.27	22.32
6-β-Hydroxyethylamino-	90	$C_{12}H_{14}O_4NAs$	24.11	24.17	24.26

(1) Friedländer and Littner, *Ber.*, **48**, 330 (1915).
(2) Veselý and Jakeš, *Bull. soc. chim.*, [4] **33**, 942 (1923).
(3) Saunders, THIS JOURNAL, **54**, 636 (1932).
(4) Bart, *Ann.*, **429**, 55 (1922).
(5) Lange, *Chem. Zeit.*, **12**, 856 (1888).
(6) Jacobs, Heidelberger and Rolf, THIS JOURNAL, **40**, 1581 (1918).