Group E. *1.0 or less*

Trypsin hydrolysate of beef serum, pepsin hydrolysate of beef serum albumin, nicotinic acid, melanin, dioxyphenylalanine, pepsin hydrolysate of beef serum, serine, lysine, cystine, egg albumin, casein, pepsin, urea, peptic-tryptic digest of pancreas, pepsin hydrolysate of beef serum globulin, glycollic acid, aspartic acid, erepsin hydrolysate of beef serum albumin, creatinine, oxamide, choline, bovine alpha pseudoglobulin, hydroxylamine, peptone, erepsin hydrolysate of beef serum, tyrosine, citrulline, succinimide, gluconic acid, uric acid.

SECTION III.

PRIMARY PHENOLIC ANTIOXIDANT: NORDIHYDROGUAIARETIC ACID

Group A. *Synergists with protective indices from 4.0 to 3.1*

None.

Group B. *3.0 to 2.1*

Methionine.

Group C. *2.0 to 1.6*

Phenylalanine, leucine, tryptophane, alanine, norleucine, milk protein hydrolysate No. 2, norvaline, valine, ascorbic acid.

Group D. *1.5 to 1.1*

Milk protein hydrolysate No. 1, threonine, isoleucine, proline, alpha-amino-isobutyric acid, dioxyphenylalanine, ninhydrin, glutamic acid, propamidine, cystine, casein, milk protein hydrolysate No. 000319, cysteine, ascorbyl palmitate, arginine, histidine, choline, levulinic acid, bovine alpha pseudo-globulin, serine, asparagin, glycerophosphoric acid, hydroxyproline, diiodotyrosine, citruline, uric acid, urea, creatinine.

Group E. *1.0 or less*

Trypsin hydrolysate of beef serum, amidol, casein hydrolysate made with sulfuric acid, trypsin hydrolysate of beef serum albumin, peptone, tyrosine, erepsin hydrolysate of beef serum globulin, melanin, aspartic acid, egg albumin, pepsin, gluconic acid, peptic-tryptic digest of pancreas, peptic digest of pancreas protein, acid precipitate from alkali treated beef serum protein, trypsin, nicotinic acid, lysine, erepsin hydrolysate of beef serum albumin, succinimide, casein hydrolysate made with barium hydroxide, oxamide, barbituric acid, sulfuric acid hydrolysate of beef serum protein, glycollic acid, trypsin hydrolysate of beef serum globulin, alkali treated beef serum protein, sulfuric acid hydrolysate of pancreas protein, arsphenamine, casein hydrolysate made with hydrochloric acid, erepsin hydrolysate of beef serum, pepsin hydrolysate of beef serum albumin, pepsin hydrolysate of beef serum globulin, pepsin hydrolysate of beef serum, hydroxylamine.

Acknowledgments

The pancreas and beef serum protein hydrolysates manufactured by Sharp and Dohme, Inc., were generously provided by R. H. Barnes of that company. The milk protein hydrolysates which were manufactured by the W. F. Straub Company were generously provided by C. P. Barnum of the University of Minnesota. Acknowledgments are also due Fred Bock, Albert Milgrom, and Miss Babette Springer for their technical assistance.

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Antioxidant Properties of Carrot Oil[1]

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WHILE purified carotene is quite susceptible to oxidation, carotene in carrots is remarkably stable and quite unaffected by autoclaving and drying on air. Occurrence of fat-soluble antioxidants in carrots has been described by Bradway and Mattill (1), but they excluded the presence of tocopherols on the following grounds: 1) Bioassays of carrots gave low results. 2) Wheat germ antioxidants were preferably soluble in petroleum ether while carrot antioxidants were not. 3) The distillation ranges under reduced pressure of the two factors were different.

In the following paper evidence will be presented which indicates that tocopherols are chiefly responsible for the antioxidant properties of the fat-soluble portion of carrots. Molecular distillation was used for the purpose of concentrating and characterizing the antioxidants. This technique has frequently been successful in dealing with labile fat-soluble substances. Robeson and Baxter (13) for example identified the antioxidant of shark liver oil as α-tocopherol by molecular distillation. For a discussion of the "analytical" technique of molecular distillation used in this work the original articles of Hickman (3) and Embree (2) should be consulted.

Determination of Antioxidant Effect

The antioxidant effect of different materials was compared by addition to a substrate of distilled methyl esters of olive oil fatty acids and determination of peroxide concentrations by a modification of Wheeler's method (14). Such a substrate is convenient for volumetric and colorimetric work, but quite unstable. It was freshly prepared before use and a sample of substrate was included in every analytical run to account for variations in the susceptibility to autoxidation between different batches of substrate. A typical substrate preparation is described below:

One hundred grams of olive oil were dehydrated by mixing with a small amount of benzene and distilling off the water-benzene azeotrope. The olive oil was refluxed with 300 ml. absolute methanol and 2.5 ml. concentrated sulfuric acid at the boiling point of methyl alcohol for about 16 hours. After cooling, fatty acids and glycerol were removed by adding ether and extracting with sodium carbonate solution. The product was dried overnight with drierite, the ether distilled off, and the crude methyl esters, weighing 91 gm., subjected to vacuum distillation in an asbestos-covered modified Claisen flask with electrically heated Vigreaux column. Most of the material (83 gm.) distilled between 190 and 200° at 10 mm. pressure. The saponification equivalent of this fraction was 293, the iodine value (Wijs' method) was 79.5.

For the estimation of the relative antioxidant effect the substrate and added test materials were incubated in glass-stoppered 125-ml. Erlenmeyer flasks in an ordinary water bath kept at 50 ± .25°C. Constant illumination from a 200-W. light bulb, about one foot above the surface of the water, was used to accelerate autoxidation. Every 24 hours the flasks were mixed by rotation, then a sample was withdrawn by pipette. After having once determined the weight of material delivered by this pipette at 50°, weighing of samples

[1] From a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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became unnecessary. Each sample was delivered into another 125-ml. Erlenmeyer flask fitted with a long glass tube in a rubber stopper. The tube acts as an air-cooled reflux condenser when the sample is heated with the glacial acetic acid-chloroform mixture and potassium iodide according to Wheeler's method. Thus samples are kept in an atmosphere of inert solvent vapors which displace the air and fill the flask, making the use of nitrogen unnecessary. Peroxide values were expressed as *milliequivalents* of $Na_2S_2O_3$ required by 1 kgm. of sample.

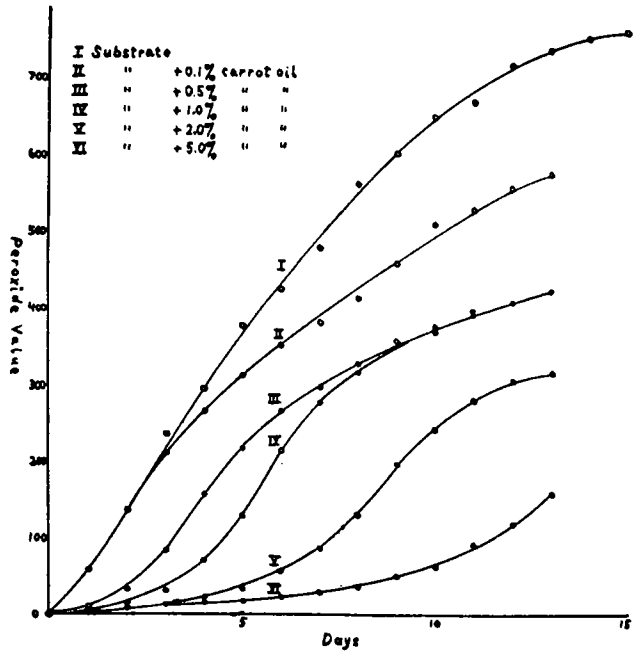

FIG. 1. Effect of various amounts of carrot oil on the autoxidation of the substrate.

Experiments with Carrot Oil

Thirty pounds of carrots were cleaned and grated, then finely ground with sand in a mortar to break up the cells. The brei was immediately transferred to acetone in a five-gallon bottle. The acetone inactivates bacteria and enzymes, keeps out air, dehydrates, and partially extracts the material. The bottle was stoppered and its contents mixed by laying it on its side and rolling it back and forth. The next day the acetone was separated from the solid matter. To accomplish this a stopper with two holes of different size was used. A piece of 120-mesh wire gauze was fitted into the larger hole and a suitably bent piece of glass tubing introduced into the smaller one in such a way that it would reach into the air pocket which forms when the bottle is laid sidewise or tipped over, mouth down. By applying pressure to this air pocket with a pressure bulb the liquid was forced out of the larger hole, while the solid was retained by the wire gauze. The acetone was changed three times after overnight contact, water was added to the combined acetone extracts and the fatty material extracted from this with petroleum ether. The pulp appeared quite dry and was extracted for two days with a 3:1 mixture of absolute methanol and ethyl ether. To this extract water was added and it was also extracted with petroleum ether. After drying the extracts over drierite, they were concentrated under reduced pressure at room temperature. For further extraction of the pulp three changes of chloroform were used. Finally, the pulp was extracted in a large Soxhlet apparatus with peroxide-free ether for 24 hours. From the combined extracts of 30 lb. of carrots 32 gm. of fatty material were obtained.

Various amounts of carrot oil were added to the substrate and peroxide values were determined every 24 hours over a period of 13 days. Figure 1 shows the action of carrot oil on the rate of peroxide formation in the substrate. The antioxidant effect is greater with higher carrot oil concentrations and prolonged even with very low concentrations.

In order to determine whether the inhibitors could be concentrated in the non-saponifiable fraction of carrot oil the following experiment was carried out. About one gram of carrot oil was dissolved in 25 ml. chloroform. Ten ml. absolute methanol, containing 1.5 gm. KOH previously dissolved by refluxing, were added and the mixture kept at 50° for half an hour under a continuous stream of nitrogen with periodic mixing. The mixture was added to 30 ml. freshly boiled water and shaken with peroxide-free ether in a separatory funnel. The ether extracts, containing the non-saponifiable material, were combined and evaporated after drying. The alkali-soluble matter was liberated from the aqueous solution by acidification with HCl and extracted with ether. By evaporating the ether from this and from a sample of the original ether extract three specimens were obtained: carrot oil, its non-saponifiable, and its alkali-soluble fraction. Enough substrate was added to make each specimen weigh 2% of the weight of substrate and the samples were incubated at 50° for 10 days (Figure 2). The

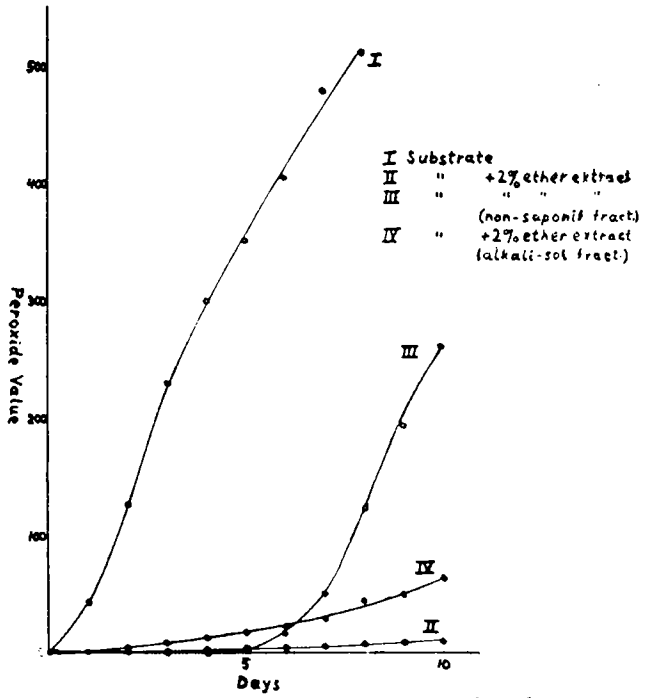

FIG. 2. Distribution of antioxidant properties of carrot extract between the non-saponifiable and alkali-soluble fraction.

alkali-soluble fraction did not have as great an inhibitory effect on the oxidation of the substrate as the untreated oil. The peroxide value of the substrate containing the non-saponifiable matter was zero until the end of the fourth day, i.e., it afforded greater reduction in the rate of oxidation than the original carrot oil, but after the fifth day the values sharply rose so that they soon exceeded even those of the alkali-soluble fraction.

The effect of acetylation on the antioxidant activity of carrot oil was investigated. About 2 gm. of carrot oil were dissolved in approximately 50 ml. chloroform in a 300-ml. Erlenmeyer flask fitted with a separatory funnel. Nitrogen was bubbled through the liquid. After the flask had been well flushed, about 10 ml. acetyl chloride were slowly dropped in through the

funnel with constant mixing by agitation. After 15 minutes 100 ml. distilled water were added. The mixture was shaken and centrifuged. The chloroform layer was washed repeatedly with water in a separatory funnel, then dried over drierite.

At the same time it was attempted to get an insight into the role of carotene in the antioxidant properties of carrot oil by inactivating carotene by hydrogenation. The hydrogenation of carrot oil was carried out according to the low pressure procedure proposed by Quaife and Harris (11). The hydrogenated carrot oil was divided into two portions. To one portion enough carotene, recrystallized as described later, was added to bring up the content of unchanged carotene in the hydrogenated oil to that originally present, i.e., to about 2%.

Carrot oil and the three samples of modified carrot oil were mixed with the substrate in 2% concentration. Figure 3 shows that carrot oil lost more of its

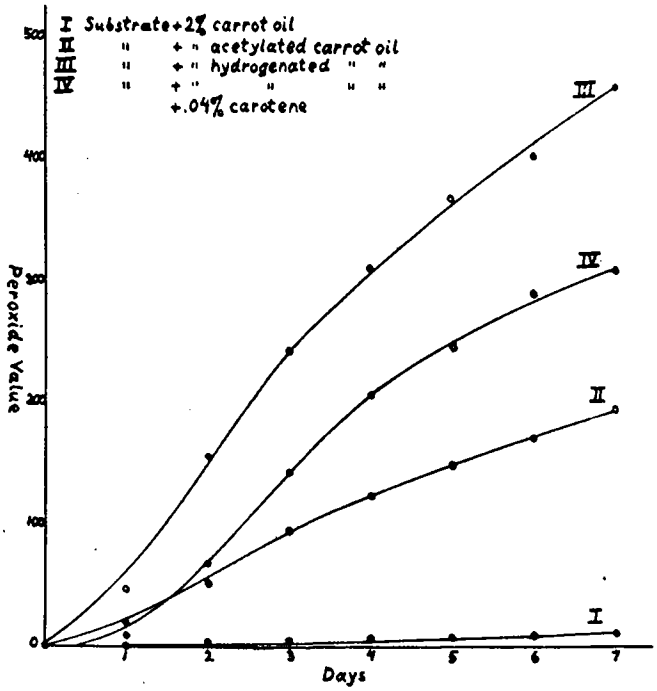

FIG. 3. Effect of acetylation and hydrogenation on the antioxidant properties of carrot oil. Effect of restoring the carotene to hydrogenated carrot oil.

antioxidant effect by hydrogenation than by acetylation, and that restoration of carotene to hydrogenated carrot oil also restored some, but by no means all of it.

Molecular Distillation of Carrot Oil

Over 200 gm. Constant Yield Oil [3] (C.Y.O.) were melted, and, to get a good dispersion, an amount of chloroform solution of carrot oil calculated to produce a 2.5% solution of carrot oil in C.Y.O. was added. The chloroform was taken off under vacuum. A small amount of the 2.5% carrot oil solution was set aside for testing of the original distilland. A 500-cc. cyclic falling-film molecular still (3) was charged with 200 gm. of the solution. After a period of degassing, a distillation was carried out, beginning with a distillation temperature of 110° and allowing 10 minutes for the collection of each fraction. The column heat was raised 10° for each succeeding fraction, but the preheat was kept at 110°. Thus, 12 fractions, ranging in distillation temperature from 110° to 220°, were obtained.

The fractions were analyzed for carotene and tocopherol. The carotene content of the original distilland was 1.08 mg./gm. and increased with distillation temperature from .015 mg./gm. for fraction 110° to 3.5 mg./gm. for the distillation residue. No tocopherol could be detected in any of the fractions, but the original carrot oil was found to contain 0.5% tocopherol. The presence of γ- (and δ-) tocopherol was excluded by a special test (10). A modification of the Emmerie-Engel method (11) was used to eliminate the interference from carotene. The original distilland used for molecular distillation, calculated on the basis of this analysis, contained 12.5 mg.% tocopherol, an amount which might have escaped detection.

Five grams of each fraction were added to 25 gm. substrate. Thus, the resulting solutions contained 16.67% of each fraction. The results of a 12-day peroxide assay are given in Figure 4. Peroxide values of the unprotected substrate and the substrate to which 16.67% C.Y.O. had been added were 463 and 553 respectively at the end of 12 days, as compared to 6.0 for the substrate protected by 16.67% of the fraction withdrawn at 190°. There the effect reached a maximum and then decreased. Tocopherols have an elimination maximum around 190°, but the temperature of the elimination maximum depends on the construction of the still and the mode of distillation. Besides, the fraction containing the highest concentration of tocopherol does not necessarily have the

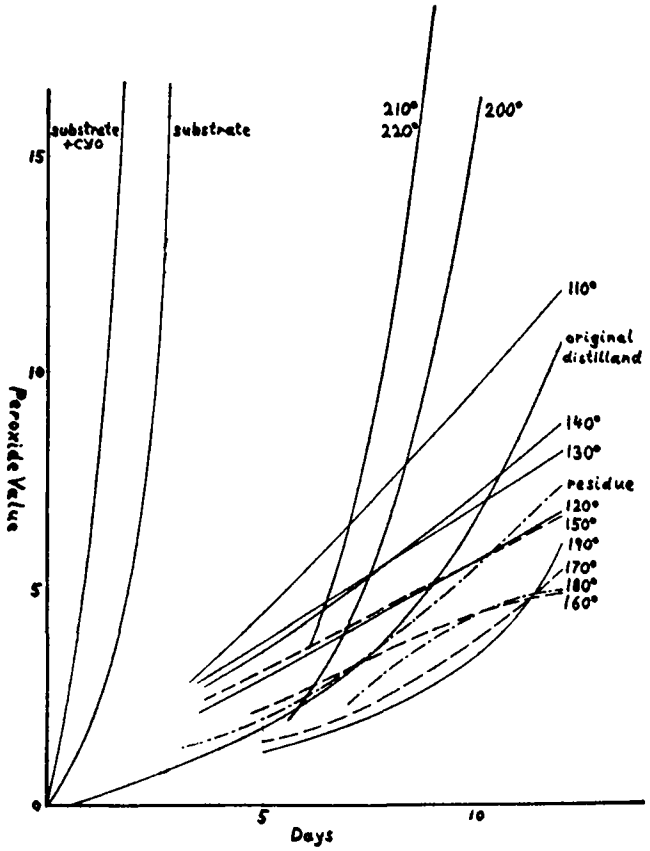

FIG. 4. Effect of fractions from the molecular distillation of carrot oil on the autoxidation of the substrate.

[3] Distillation Products, Inc.

greatest antioxidant effect, as shown later. The situation is complicated by the fact that one is dealing with a complicated mixture of substances, several of which may have an effect on autoxidation. In order to arrive at a definite conclusion it is necessary to subject pure tocopherol plus any substance or substances known to occur in carrot oil and to be of influence to a molecular distillation under the same conditions, to test the resulting fractions in a comparable manner, and to compare both elimination maxima.

Experiments with Tocopherol and Carotene

The effect of pure α-tocopherol and of pure carotene on the autoxidation of the substrate was investigated. Pure natural d α-tocopherol [4] was dissolved in the substrate in concentrations ranging from 1 to 100 mg.%. The effect of various concentrations of tocopherol on the autoxidation of the substrate at 50° is shown in Figure 5. In 5 mg.% and higher concentrations no induction period was noticeable within the experimental period of 14 days. Instead, oxidation proceeded at a slow, even rate. This rate was faster, the higher the tocopherol concentration. Samples having tocopherol concentrations of 25 mg.% or more consequently showed greater peroxide content than the original substrate during the period in which it itself oxidized rather slowly.

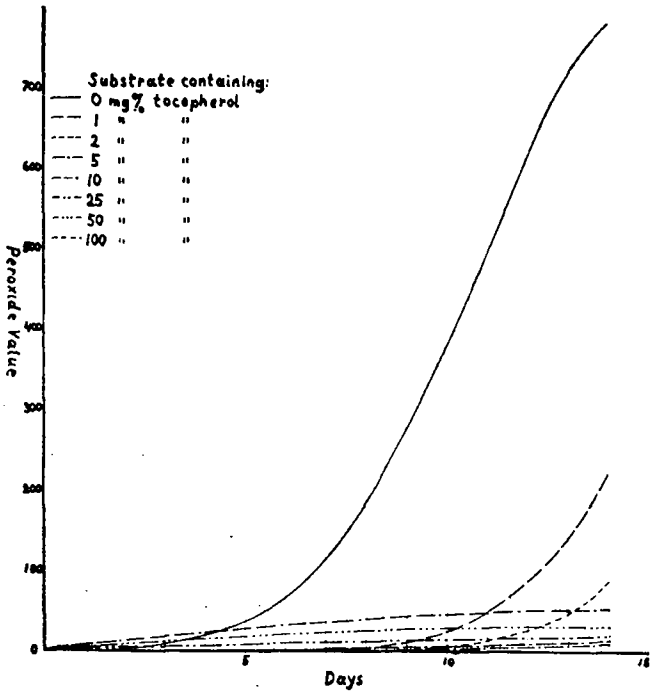

FIG. 5. Effect of various concentrations of pure α-tocopherol on the autoxidation of the substrate.

Carotene from carrot oil [5] was recrystallized by dissolving in chloroform and precipitating with absolute methanol. The precipitate was allowed to age for about an hour, then filtered off with a fritted glass filter and washed on the filter with methanol. This procedure was repeated and the product was dried in a vacuum desiccator overnight. The effect of carotene in concentrations from 0.2 to 30 mg.% on the autoxidation of the substrate is shown in Figure 6. The sample containing 0.2 mg.% carotene had peroxide values similar to those of the original substrate up to the fourth day. From then on they were appreciably lower. The peroxide values at the 1 mg.% level were consistently lower. When the carotene content was increased above 4 mg.%, the peroxide values were higher than those of the untreated substrate until about the fifth day, but lower afterwards. The peroxide values were higher the higher the carotene concentration. All samples containing carotene eventually assumed similar rates and peroxide values, far below those of the substrate. Carotene was completely bleached in all samples by the end of the third day.

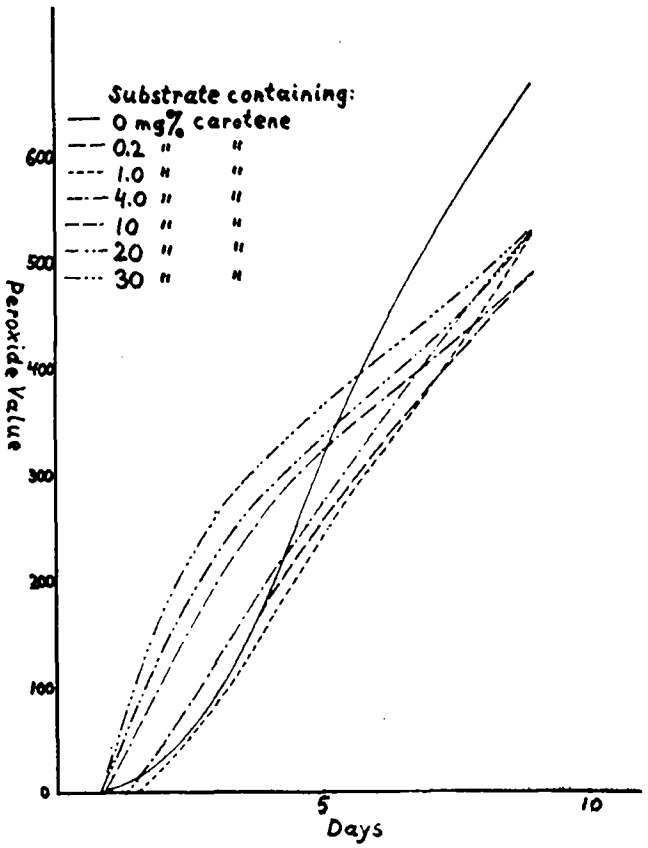

FIG. 6. Effect of various concentrations of pure carotene on the autoxidation of the substrate.

A mixture of α-tocopherol and carotene with C.Y.O. was distilled in the same manner as the carrot oil. The concentration of tocopherol was 12.5 mg.% and the concentration of carotene 107.5 mg.%, just as in the carrot oil distillation. Both substances, identical with those used in the preceding experiments, were dissolved in the molten C.Y.O. by adding them in chloroform solution and taking off the solvent under vacuum. Tocopherol values were obtained, but they were so inconsistent that they were considered unreliable. Carotene concentrations ranged from 0 for fraction 110° to 3.7 mg./gm. for the residue. The fractions were assayed in the same manner as those obtained from carrot oil (Figure 7). As in the distillation of carrot oil, the antioxidant effect increased roughly with the distillation temperature until a maximum activity was reached around 190°. Fractions 110°, 210°, and 220° were nearly devoid of antioxidant activity, the residue even accelerated the oxidation of the substrate.

[4] Distillation Products, Inc.

[5] Nutritional Research Associates, Inc.

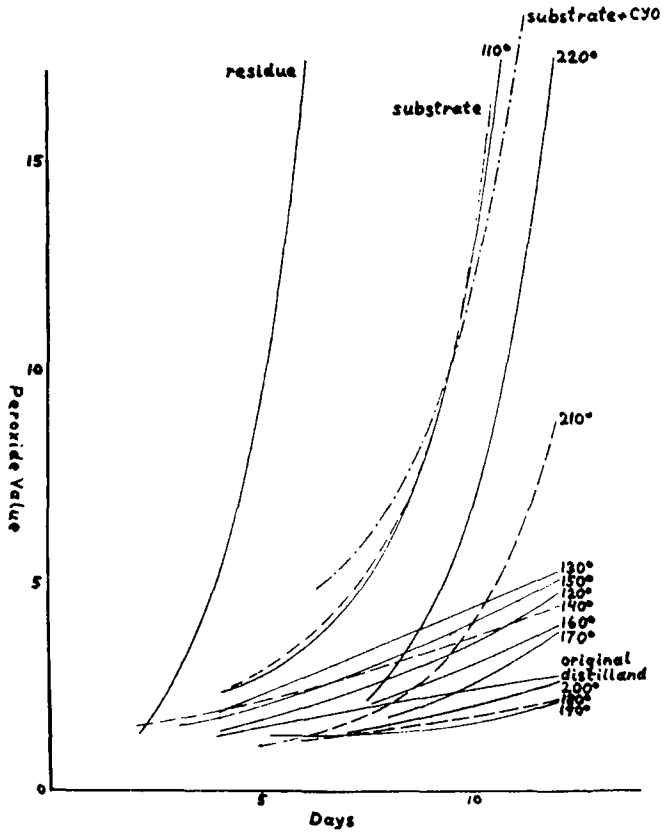

FIG. 7. Effect of fractions from the molecular distillation of a tocopherol-carotene mixture on the autoxidation of the substrate.

Stability of Carotene in Autoxidizing Substrate

It was possible to get some information regarding the stability of carotene in the autoxidizing substrate by comparing the color of the incubated solutions during the experiments on peroxide development in a photoelectric colorimeter. The colorimeter readings are not proportional to carotene concentrations, but afford a ready comparison of relative values. By plotting time against colorimeter readings the relative stability of carotene in the fractions from the molecular distillation of carrot oil in the substrate is represented in Figure 8. The carotene content rose with the distillation temperature. Colorimeter readings for fractions up to 180° were strikingly constant. In the higher fractions destruction of carotene started sooner, the higher the distillation temperature. The fractions from molecular distillation of the tocopherol-carotene mixture behaved in a very similar manner.

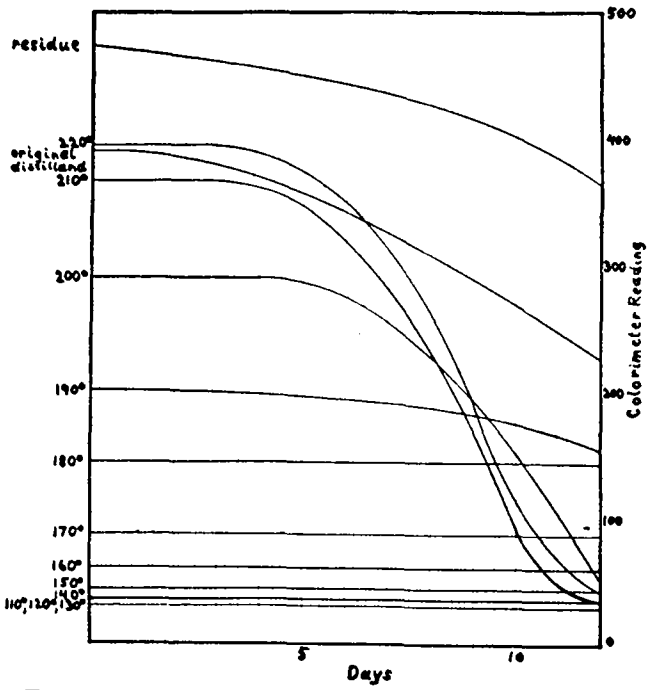

FIG. 8. Change of carotene content with time in the fractions from molecular distillation of carrot oil during the autoxidation of the substrate.

Discussion

Tocopherol was found to be present in carrot oil to the extent of approximately 0.5%. In 1946 Quaife and Harris (12) published the tocopherol concentration in carrot oil of unspecified origin as 1.620 mg./gm. During the course of this investigation the author was informed that H. A. Mattill had carried out a bioassay for vitamin E on some carrot oil produced by Nutritional Research Associates inc., in 1940. With Dr. Mattill's kind permission his unpublished data of that bioassay are quoted below:

"Amount fed	Number of young born
1 gm	3
500 mg	7
500 mg	7
250 mg	Resorption
250 mg	Resorption
250 mg	10 (9 dead)
250 mg	2 (1 ptly. consumed)"

This indicates an α-tocopherol concentration of approximately 1%. Much has been learned about bioassays for vitamin E since 1940, and it might be possible to obtain a more accurate figure, but it would have to be based on the assumption that all of the tocopherol is present in the α-form. But, since α-tocopherol has twice the biological activity of β-tocopherol, the result may still be in error. While it could not be decided whether tocopherol is present in the α- or β-form, or both, γ- and δ-tocopherol have been shown to be absent by chemical means. It was possible to show that tocopherol is not present to any extent in the esterified form, as Olcott (7) assumed in order to explain the low vitamin E activity of carrots in Bradway's experiments (1), since the unsaponified carrot oil had appreciable antioxidant effect. The drop in antioxidant activity on acetylation (Figure 3) explains itself as due to the inactivity of the tocopherol acetate.

Under the conditions of the test the effectiveness of α-tocopherol in reducing the rate of peroxide formation in the substrate passed through a maximum at about 5 mg.% concentration (Figure 5). Oliver *et al.* (9) have shown that the rate of peroxide formation during the induction period of lard to which increasing amounts of tocopherol had been added similarly passes through a minimum at a tocopherol concentration of 7 mg.%.

Carotene is considered to be a pro-oxidant by some authors (1, 6, 15), but an antioxidant by others (4, 5). The reported antioxidant properties of carotene have been ascribed to the presence of antioxygenic material in the carotene used (8). This explanation seems plausible in the case of Newton's work (5) as inactivation of carotene by hydrogenation may have en-

hanced the antioxidant activity of his carotene-containing plant extracts, but the evidence presented by Monahagan (4) casts some doubt on the supposed prooxygenic effect of carotene. Under the conditions of the experiment in Figure 6 pure carotene inhibited the autoxidation of the substrate in concentrations below 3 mg.% but accelerated the initial rate of peroxidation in higher concentrations. All samples eventually contained oxidized carotene, but their final peroxide values were appreciably lower than those of the pure substrate.

Carrot oil lost most of its antioxidant activity on hydrogenation at 35 lb. with a catalyst of palladized $CaCO_3$ (Figure 3). Tocopherol is not likely to be affected by this treatment. The loss of carotene can hardly account for the loss in antioxidant activity on hydrogenation, although carotene showed a slight antioxidant effect when added to the hydrogenated carrot oil. It seems more likely that a trace of palladium chloride remaining with the carrot oil after hydrogenation acted catalytically on the oxidation.

The only evidence for the occurrence in carrot oil of other substances having an inhibitory effect on the autoxidation of the substrate was found in the study of saponified carrot oil (Figure 2). While the antioxidant activity of the alkali-soluble fraction was weak compared to that of the original carrot oil, it indicates the presence of some acidic inhibitors. The effect of the non-saponifiable fraction was stronger, but of shorter duration than that of the original carrot oil. This suggests that the concentration of tocopherol in the non-saponifiable fraction is greater, but that the synergistic action of the acidic substances is lacking.

The influence of fractions from the molecular distillation of the tocopherol-carotene mixture on the autoxidation of the substrate (Figure 7) is explainable on the basis of the behavior of the two components. The tocopherol concentration in the substrate sample containing 16.67% of the original distilland was about 2 mg.%, that of carotene about 18 mg.%. While the carotene concentration increased with the temperature of distillation, tocopherol apparently reached a maximum around 190° and then fell off again. Thus, each fraction contained these two substances in different proportions. Fraction 110°, containing almost none of either, had no influence on the oxidation of the substrate. Higher fractions had increasing antioxidant effect due to their increasing tocopherol content. Fractions 210°, 220°, and the residue contained small, decreasing amounts of tocopherol and large, increasing amounts of carotene, causing them to accelerate the reaction more and more. The fractions from the molecular distillation of carrot oil showed very similar behavior (Figure 4). Apparently, the elimination curve for tocopherol, if it had been obtained, would have the same maximum. The protective effect of carrot oil fractions on carotene oxidation was likewise quite similar to that of pure tocopherol. Comparison of the effects of the original distillands and the most potent fractions indicates that tocopherol was not too effectively concentrated.

Summary

A modified technique for the assay of antioxidants and a method for the preparation of carrot oil have been described. The antioxidant effect of carrot oil has been analyzed by molecular distillation and chemical treatment of the oil. Tocopherols appear to be chiefly responsible for the protection of the autoxidizable substrate and of carotene from oxidation. The effect of pure tocopherol and of pure carotene, and various mixtures of both, produced by molecular distillation, on the autoxidation of the substrate has been studied. The effectiveness of increasing tocopherol concentrations in reducing the initial rate of peroxidation of the substrate showed a maximum at 5 mg.% tocopherol concentration. Carotene was an inhibitor of this reaction in concentrations of less than 3 mg.%, but a pro-oxidant in higher concentrations. The antioxidant activity of the molecular distillate fractions of both the carrot oil and the mixture of pure tocopherol and carotene showed a maximum around a distillation temperature of 190° under the conditions of the experiment. The tocopherol content of carrot oil is considerable; it has been estimated at about 0.5 to 1%.

Acknowledgment

The author wishes to express his gratitude to H. A. Mattill for the use of his data, to P. L. Harris of Distillation Products inc., for carotene and tocopherol analyses, and to A. F. O. Germann, Nutritional Research Associates inc., for helpful information and a gift of carrot oil. The molecular still used has been kindly lent to us by Distillation Products inc.

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