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The Effects of Different Levels Of Octyl Hydroquinone On The Utilization Of Carotene By The Rat

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WOODS

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THE EFFECTS OF DIFFERENT LEVELS OF OCTYL HYDROQUINONE ON THE UTILIZATION OF CAROTENE BY THE RAT

By

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Biographical Sketch

Leon Alexander Woods Jr. was born May 14, 1925 in San Antonio, Texas. He attended both grade school and high school in San Antonio, Texas and was graduated from Phylis Wheatley Senior High School in May 1942. In September 1942 he entered Prairie View A. and M. College to pursue the B.S. degree, majoring in chemistry and minoring in biology. In December 1944 his college training was interrupted, being called into the Armed Services. In January 1947 he returned to school at Prairie View A. and M. College, after being honorably discharged from the Air Corps. In May 1948 he was graduated from Prairie View A. and M. College with the Degree, Bachelor of Science. His undergraduate school achievements included: Lieutenant Colonel in R.O.T.C.- 1942-43, tennis team- 1942-44, 1947-48, Science Editor of Panther- 1943-44 and Vice-president of Veteran's Club- 1947-48. In June 1949 he entered graduate school at Prairie View A. and M. College to pursue the M.S. degree in biochemistry. In September 1949 he received a fellow-ship from Prairie View A. and M. College to further his education. He is a member of Beta Kappa Chi Scientific Society and the American Chemical Society.

DEDICATION

Lovingly dedicated to my

fiancee', Doris Avonne Jackson

ACKNOWLEDGEMENTS

The writer's sincere thanks are given to the following: The entire library staff of Prairie View A. and M. College, for their able assistance in helping me to obtain reference materials; To Dr. E. G. High, for his inspiration, guidance and willingness to help me at any hour during the course of this research.

L. A. W.

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I.

INTRODUCTION

Substances which prevent polymerization or oxidative reactions from occuring are well known. Such substances have been termed "antioxidants" or "inhibitors".

A considerable amount of research has been devoted to the study of these "inhibitors" of reactions or "negative catalysts" during the past 30 years. It is pointed out that certain substances susceptible to polymerization or oxidation must be protected. Leather oils, rubber, fats, unsaturated hydrocarbons, aldehydes and some vitamins and their precursors are substances which are either easily oxidized or are subject to polymerization. Consequently, if their usefulness to man is to be retained they must be protected from oxidative deterioration or polymerization into unwanted substances.

Some aromatic amines are known to protect rubber and oils from aging. The polymerization of acrolein may be diminished by the addition of small quantities of resorcinol or other phenolic substances. Tetraethyl lead prevents the oxidation of benzaldehyde in motor fuels, and vitamin E is a well known antioxygenic substance for fats.

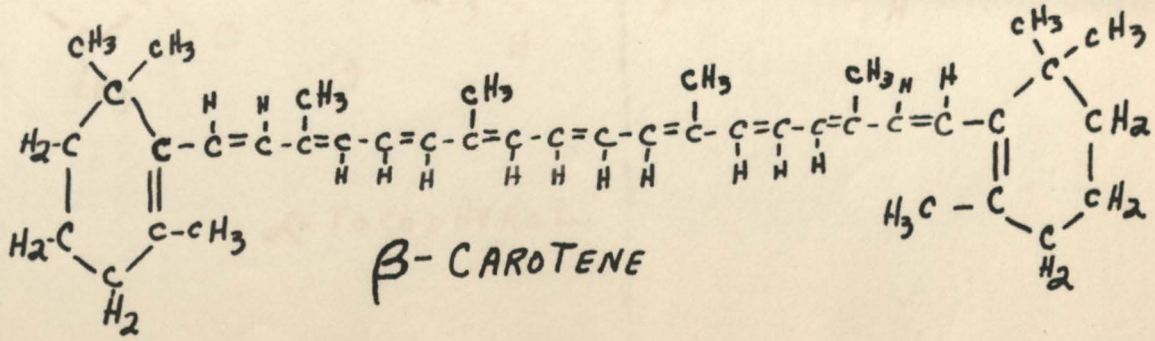
Much of the research done on antioxidants has been in the test tube with homogeneous systems. However, in biological systems one is not dealing with homogeneous conditions, thus the study of biological antioxidants becomes complicated. In addition, most metabolic processes cannot be controlled in living organisms as one may regulate the temperature of a test tube reaction, or control the concentration of the reacting substances therein.

It has been demonstrated in the test tube that antioxidants can function by breaking chain reactions involving free radicals and by being reversibly oxidized and reduced very easily. Some evidence seems to point to these phenomena taking place with biological antioxidants. The question of how biological antioxidants function in living organisms remains unanswered.

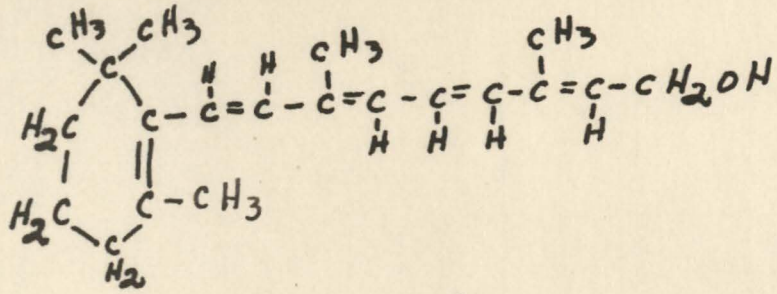
Vitamin A and its precursors are very important substances to the normal nutritional state of animals and are very susceptible to oxidative destruction. Consequently, the value of "biological antioxidants" to protect these substances becomes apparent.

This study reports on the effects of Octyl Hydroquinone as a biological antioxidant on the utilization of carotene by the rat. It was instigated with the hope of shedding some light on the mode of action of small amounts of vitamin E(a biological antioxidant) in sparing carotene and the mode of action of large amounts of vitamin E in becoming antagonistic to carotene utilization in the rat. From interpreted data, it is hoped to gain insight into the possible mechanism of the conversion of carotene to vitamin A.

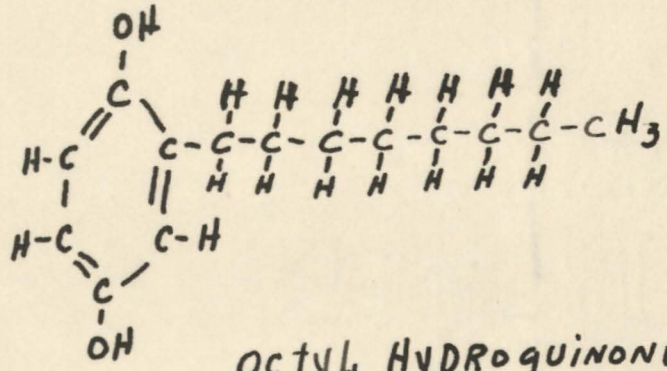
The following formulas are the important ones to be considered in this thesis:



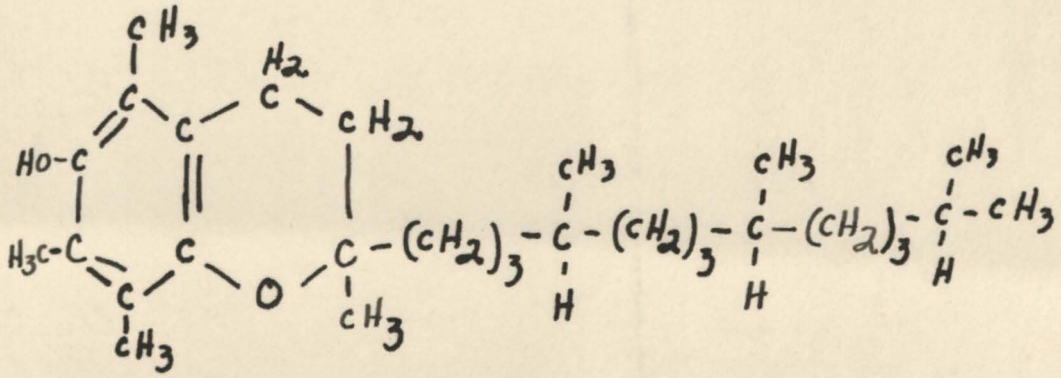
β- CAROTENE



VITAMIN A



OCTYL HYDROQUINONE



L-TOCOPHEROL

II.

HISTORICAL

The first carotenoid discovered was carotene by Wackenroder(1) in 1831, as ruby-red crystals from the root of the cultivated carrot and was given the name "Carotin". Due to the standard methods of nomenclature for organic compounds devised later, the name "Carotin" was changed to "Carotene". Since this discovery by Wackenroder the number of carotenoids identified and whose structure is known has increased to 30(2) and possibly more.

The association of vitamin A activity with the carotenoids was first observed by Steenbock(3) in 1919. von Euler, et al(4) and Moore(5) later confirmed Steenbock's observations. Of all the carotenoids having vitamin A activity, β -carotene is the most potent(6). Consequently, β -carotene has received the greatest attention as a provitamin A substance.

After the discovery of vitamin A by McCollum and Davis(7) in 1915, it has been shown that vitamin A is a very important substance to the normal nutritional state of animals. The need for this vitamin by animals, as well as for other vitamins, is recognized by its absence rather than by its presence. Cessation of growth, xerophthalmia, nyctalopia and lowered resistance to infection are manifestations of vitamin A deficiency. In addition, vitamin A plays an important role in the visual cycle. Wald(8) has been one of the outstanding workers on the visual cycle. The utilization of vitamin A by animals is therefore of great importance.

The oxidative destruction of carotene and vitamin A in solution and in the crystalline state is a well known phenomenon. Light, air and heat are capable of accelerating this destruction. Loss in color in the visible region of the spectrum may be considered as good evidence of destruction and decreasing provitamin A activity of carotene(9). In addition, isomerization of carotene and other carotenoids will cause a shift of absorption maximum from the visible region of the spectrum, while an increase is generally noticed in the ultraviolet region(10). This isomerization usually brings about a sharp reduction in provitamin A activity. Many workers have studied the isomerization of carotenoids and have reported their spectral characteristics and provitamin A activity(10,11,12,13). Gillam(14) in 1935, however, was the first to discover the isomerization phenomena in the field of C_{40}-carotenoids.

An antioxidant preventing the rampant destruction of carotene or vitamin A, while stored, or in the animal body prior to its utilization would be of economic and nutritional significance.

The best criterion for determining the extent of carotene utilization by animals is growth increase of young animals and storage of vitamin A in the livers. Osborne(15) in 1918 stated that the liver is the chief site of storage of vitamin A. Sherman(16) and Moore(17) have shown that 90-95% of the vitamin A found in the rat's body was in the liver.

Vitamin E was first observed by Moore(18) in 1940 as having a synergistic effect on the utilization of vitamin A. Later, several workers furthered Moore's work, extending it to carotene(19). Hickman, et al(20) reported in 1944 that natural vitamin E(mixed tocopherols) enchanced the growth promoting power of vitamin A alcohol, vitamin A acetate and the U. S. P. reference oil. Harris, et al(21) in 1944 tested the sparing action of natural tocopherol concentrates on carotene and found that approximately 0.5mg. of natural mixed tocopherols was the optimum daily dose to demonstrate the sparing action of vitamin E on carotene, while larger quantities were neutral or antagonistic. Hickman, et al(22) reported again in 1944 on the sparing action of the tocopherols, testing the growth response of vitamin A and carotene when administered with the tocopherols, hydroquinone, lauryl hydroquinone and other substances. These workers concluded that the increased growth response to these substances was due to their repression of oxidation in and near the gastrointestinal tract. Hove and Hove(23), Lemeley, et al(24) and Kemmerer, et al(25) are some of the other investigators who have studied carotene destruction in vitro, vitamin A utilization and carotene utilization, respectively. The report by Davies and Moore(26) in 1941 in their description of vitamin A-E synergy put forth the suggestion that the increased storage of vitamin A in the livers of rats was due to repression of oxidation by the

tocopherol which behaved as an antioxidant and was undoubtedly the spark which started much work along this line.

At the present time some few substances classed as antioxidants are known which enhance growth of animals fed vitamin A or carotene, as: α , β , and γ -tocopherols, lauryl hydroquinone and catechol. And a host of substances have been found to be effective antioxidants in vitro, including; the tocopherols, hydroquinone, nordihydroguaiaretic acid, pyrogallol, flavonoids and possibly any ortho and para diphenolic substance. It appears that fat-soluble antioxidants are the most important ones to be considered in vivo. However, it has been observed by Halpern and Biely(27) in their report on the utilization of vitamin A in various carriers that vitamin A oils had a greater biological value in water emulsions than in vegetable oils, using depleted chicks as experimental animals. Water soluble antioxidants might therefore, be effective in protecting carotene and vitamin A in vivo in emulsions of water. It is very interesting to note that growth response has been the main criteria for evaluating antioxidants administered with carotene or vitamin A and not tissue deposition of vitamin A.

In addition to the previously mentioned importance of antioxidants in protecting carotene and vitamin A, antioxidants have been studied with the attempt to discover their mode of action. This might in turn uncover the

mechanism of carotene conversion to vitamin A in the animal body.

Due to the fact that vitamin A and carotene are fat-soluble these substances are usually found in oils, or are administered in some type of oily-media. It is a well known fact that fats and oils easily become rancidified and that the autoxidation of unsaturated fatty acids contained in both fats and oils may play the important role in this rancidification. The precursors in this autoxidation seem to be hydroperoxides which are formed at the double bonds of molecules(28). This period of peroxide formation may be considered the induction period, after which rapid oxidation takes place, though no conclusive evidence has been presented which indicates whether or not peroxides are formed during the oxidation of fats other than the formation of unsaturated hydroperoxides(29). Other mechanisms for fat oxidation, however, have been proposed.

Our lack of knowledge of fat metabolism has surely been an impediment to the advancement of knowledge on carotene and vitamin A metabolism and antioxidants related to carotene and vitamin A metabolism. The better understanding of any of these questions will undoubtedly serve as a valuable tool to clear-up confusion on any of the others.

It was previously mentioned that Harris, et al(21) suggested that larger quantities of mixed tocopherols(above the optimum level) when administered along with carotene,

may behave neutral or antagonistic to the provitamin from growth response data. Johnson and Baumann(30) employing tissue deposition as criterion for carotene utilization in the rat demonstrated that excess α -tocopherol decreased the utilization of carotene but it was neutral toward vitamin A utilization. High and Day(31) confirmed these findings, employing α -tocopheryl acetate, and reported further that the antagonistic effect of large amounts of α -tocopherol toward the utilization of carotene is not due to the hydroquinone or the phytyl moiety of the vitamin E molecule as such. However, hydroquinone is fat insoluble and this property alone is possibly responsible for its ineffectiveness in vivo. The in vivo antioxidant property of lauryl hydroquinone(22), a fat soluble hydroquinone, supports this hypothesis. As to whether the reversal effects of both small and large amounts of vitamin E on the utilization of carotene are due only to the antioxidant property of the substance is apparently unknown; however, this investigation was designed to increase our understanding on this subject.

III.

METHODS

A. Source and Preparation of Reagents

Chloroform- Both U. S. P. and recovered chloroform were dried over anhydrous reagent grade potassium carbonate and distilled from the same reagent. Chloroform thus prepared was kept in glass stoppered bottles containing a little anhydrous potassium carbonate.

Diethyl Ether(peroxide-free)- Commercial diethyl ether was mixed throughly with acidified ferrous sulfate solution, separated and the ether dried over anhydrous potassium carbonate then distilled from the same reagent. The peroxide-free ether thus prepared was kept in brown bottles in the dark.

Ethyl Alcohol- Undistilled 95% ethyl alcohol was used in preparing the alcoholic potassium hydroxide solution.

Activated Glycerol Dichlorohydrin- Glycerol dichlorohydrin, obtained from the Shell Chemical Corporation, Houston, Texas, was treated with antimony trichloride and distilled according to the method of Sobel and Werbin(32).

Hexane- C.P. n-hexane and recovered n-hexane(B.P. 60-71° C) were used as the solvent in all spectrophotometric work.

U. S. P. Vitamin A Reference Standard- U.S.P. Vitamin A Reference Standard was obtained from Pharmacopeia of the United States, Philadelphia, Pennsylvania (former address), 46 Park Avenue, New York 16, New York(present address). This Reference Standard was used in preparation of vita-

min A calibration curve, with glycerol dichlorohydrin as the chromogenic agent.

Vitamin E(Type IV-40 Concentrate of mixed tocopherols)- This substance was obtained from Distillation Products, Inc., Rochester, New York, being kindly supplied by Dr. Norris Embree. A predetermined amount of the mixed tocopherols was dissolved in olive oil to give the desired concentration. This solution was kept in ordinary Erlenmyer flasks in the refrigerator at 9°C.

Wesson Oil- Wesson Oil distributed by Wesson Oil and Snowdrift Sales Company, New Orleans, Louisiana, was obtained from a local grocery store.

Olive Oil- Olive oil was obtained from General Chemical Company, New York, New York.

B. Dietary Substances

Vitamin A Deficient Diet(Diet 301)

Composition	Parts	Source
Casein(Vitamin A-free)	18	Sheffield Farms Company, Inc., Norwich, N. Y.
Cane Sugar	58	———
Salts Mixture(12a)	4	———
Brewer's Yeast	8	Anheuser-Busch, Inc., St. Louis, Mo.
Crisco	10	Procter & Gamble, Cincinnati, Ohio

Vitamin A Deficient Diet(Diet 301) Cont'd

Composition	Parts	Source
Irradiated Yeast (Fleishmann's Hy-Dee)	2	Standard Brands, Inc., N. Y. C.

Salts Mixture(12a)

Constituents(All C.P.)	Parts
Sodium chloride(NaCl)	14.7
Potassium dihydrogen phosphate(KH_2PO_4)	40.9
Magnesium sulfate($MgSO_4 \cdot 7H_2O$)	8.7
Calcium carbonate($CaCO_3$)	40.0
Ferric citrate($Fe(C_6H_5O_7)$)	3.5
Potassium iodide(KI)	1.0
Manganous chloride($MnCl_2.4H_2O$)*	0.47
Zinc chloride($ZnCl_2$)	1.2
Copper sulfate($CuSO_4.5H_2O$)	0.5

* Both Manganous sulfate($MnSO_4.H_2O$) and Zinc sulfate ($ZnSO_4.7H_2O$) may be substituted for Maganous chloride and Zinc chloride, respectively.

The above salts mixture(12a) was prepared by first grinding each constituent to a fine powder in a mortar before being mixed throughly.

C. Preparation of Supplement Materials

Both the methods of preparing vitamin A-deficient animals and the method of supplementing were substantially

the same as that previously described by High(33).

β-carotene- The β-carotene used was obtained from General Biochemical, Inc., Chagrin Falls, Ohio, being 90% β and 10% α-carotene. The desired amounts of carotene commercial preparation were dissolved as previously described(33), in peroxide-free ether and then thoroughly mixed with a determined amount of Wesson Oil or olive oil ti give the desired concentration. The ether was removed under reduced pressure on a warm water bath to facilitate evaporation. The thus prepared carotene-Wesson Oil or carotene-olive oil solution was stored in low-actinic flasks and kept in a refrigerator at 9°C. These stock solutions of carotene were used as source of other solutions of carotene. The concentration of the stock solutions of carotene were 60 μgm./0.2 ml. of Wesson Oil or olive oil.

Octyl Hydroquinone- The Octyl hydroquinone smployed was obtained from the Tennessee Eastman Corporation, Kingsport, Tennessee, being kindly supplied by Dr. J. M. Knight. The desired amounts of octyl hydroquinone were dissolved in peroxide-free ether and then thoroughly mixed with a determined amount of Wesson Oil or olive oil to give the desired concentration. The ether was removed as described above in the preparation of the β-carotene solutions. Solutions thus prepared were stored in low-actinic flasks in the refrigerator at 9°C. The stock solutions of octyl hydroquinone were 10 mg./0.2 ml. of Wesson

Oil or olive oil.

D. Care of Animals

1. Method of Preparing Vitamin A Deficient Animals

All rats used in the experiments were albinos, either of Texas A. and M. College Strain or of Wistar Strain. To prepare vitamin A deficient animals for experimental purposes, weanling rats approximately three to four weeks old, were placed on vitamin A-deficient diet(diet 301), after being suitably marked for identification, and separated as to sex. The animals were placed in groups and kept in galvanized iron cages, 22 inches long, 20 inches wide and 15 inches high, as shown in Fig. 1, until vitamin A deficiency was shown by plateauing of weight or xerophthalmia. All rats were allowed to drink and eat "ad libitum" during this period. Approximately four weeks were required for the animals to develop vitamin A deficiency. As soon as vitamin A deficiency was established, the animals were divided into groups according to sex and growth record. Each rat was transferred for supplementation to an individual cage of galvanized iron, 9 3/4 inches long, 7 inches wide and 7 inches high. The individual cages were arranged in skyscraper fashion, as shown in Fig. 2. The room temperature varied somewhat where the animals were kept due to lack of controlled heating conditions, but the average temperature of the room was approximately 25°C.

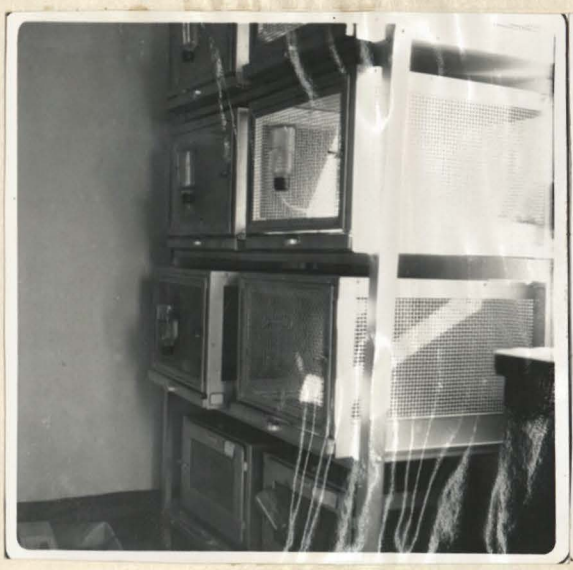


FIG. 1

FIG 2

2. Method of Supplementing

In all cases the period of supplementation lasted 20 days. Several methods of administering supplements were tried but it was found that the employment of calibrated droppers to administer the supplements was the best method. The supplements, either dissolved in Wesson Oil or olive oil, were administered by placing the calibrated number of drops of the desired substance on the bottom of a supplement cup. This material in each case was covered with a very thin layer of vitamin A deficient diet. It was observed that the vitamin A deficient animals usually had lost their appetites, which could be restored by placing one or two drops of the carotene solution supplement directly in their

mouths; the rest of the supplement being placed on the bottom of cup as previously described. Consequently, for the first day or two animals were supplemented in this manner. All other supplements were administered by placing supplement on the bottom of cup and covering it with a very thin layer of the vitamin A deficient diet. The vitamin A deficient diet was removed from the cages in the mornings and the supplements were given at noon. Following the consumption of all the supplement, the supplement cups were removed and replaced by the regular food cups containing the vitamin A deficient diet. The period of consumption of supplements varied from a few minutes to about thirty minutes. The animals were allowed to drink and eat "ad libitum" on vitamin A deficient diet until the next morning when the same procedure was repeated until the end of supplementation period. The weight of each rat was recorded at the beginning of the supplementation period and every two days until the termination of the experiment.

Approximately 16 hours after the administration of the last supplement each rat was sacrificed by employing ether, and liver and kidneys were promptly removed, weighed, and vitamin A content determined.

E. In Vitro Materials and Apparatus

The carotene and octyl hydroquinone solutions previously made up for supplementation were employed in the in vitro experiments. A Sargent constant temperature bath

apparatus with pipette leads for oxygen was used to study the rate of destruction of carotene, as shown in Fig. 3. The temperature of the water was thermostatically controlled to 60° C and 37.5° C throughout the experiments. Soft glass test tubes 19 X 150 mm. were used to hold the test solutions in the water bath in one set of experiments, while colorimeter tubes 15 X 125 mm. were employed in another set of experiments. The rate of destruction of carotene was speeded up by bubbling oxygen from a tank into the solutions while in the water bath. Although the total flow of oxygen in any given period of time was not measured, the amount passing into the tubes in a given experiment was maintained constant. All solutions used in each experiment were removed from the refrigerator and allowed to come to room temperature, then various amounts were extracted and pipetted into the tubes to give the desired concentration. The solutions contained in the colorimeter tubes were diluted solutions of both carotene and the antioxidant. This was done in order to obtain readings directly on the Klett-Summerson Colorimeter without making any further dilutions. The solutions in the test tubes were of the same concentrations fed to the animals. Dilutions were made from these solutions by pipetting out the desired amounts, diluting them with hexane and reading them on the Beckman Spectrophotometer(Model DU). The readings taken on the Spectrophotometer were at wave lengths of 450 and 436 millimicrons,

using hexane as solvent. The readings taken on colorimeter necessitated the use of a 420 millimicron filter.

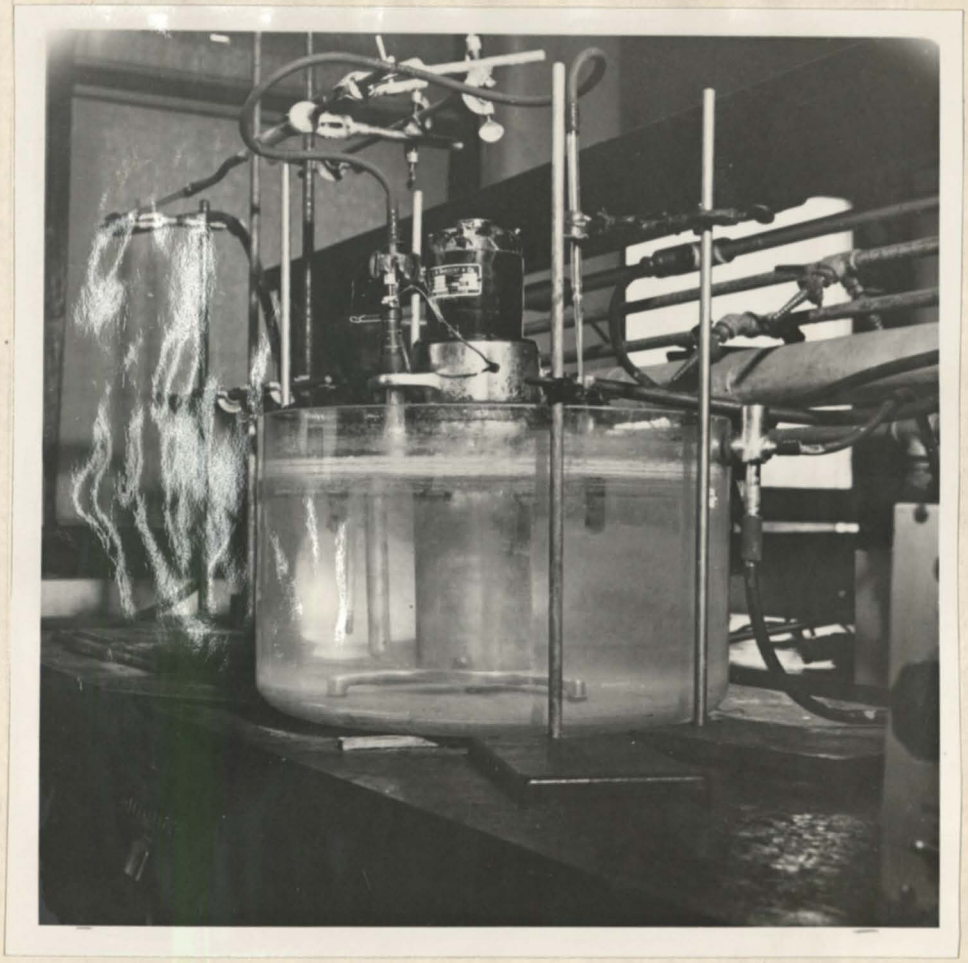


FIG. 3

F. Preparation of Calibration Curves

1. Vitamin A Calibration Curve

The vitamin A calibration curve was made by employing the U. S. P. Vitamin A Reference Standard. A

known quantity of the vitamin A standard was dissolved in dry chloroform to give a solution of known vitamin A concentration per milliliter. This was considered as the stock solution. From the original stock concentration of Vitamin A in chloroform, various dilutions were made with chloroform. 2 ml. aliquots of original solution and of the various dilutions were added to 4 ml. of activated glycerol dichlorohydrin reagent in colorimeter tubes. After mixing thoroughly and allowing 2 to 2½ minutes to elaspe for maximum color development, readings were made on a Klett-Summerson Colorimeter using a 540 millimicron filter. Calibration curve obtained from this data is shown in Fig. 4.

2. β -Carotene Calibration Curves

β -Carotene calibration curves were made using the 60 µgm. of Carotene/0.2 ml. of olive oil solution. These calibration curves were made employing the Klett-Summerson Colorimeter and the Beckman Spectrophotometer. One ml. of the above solution was accurately pipetted into a 100 ml. volumetric flask and diluted to the mark with hexane. This solution and various dilutions of it were read on the Beckman Spectrophotometer at 450 and 436 millimicrons. The calibration curves obtained from this data is shown in Fig.5. The carotene calibration curve made on the colorimeter employed the use of the 60 µgm carotene solution, also. Dilutions were made with olive oil, rather than hexane in this case.

A 420 millimicron filter was used for the colorimeter readings. The calibration curve thus prepared is shown in Fig. 6.

VITAMIN "A" CALIBRATION CURVE

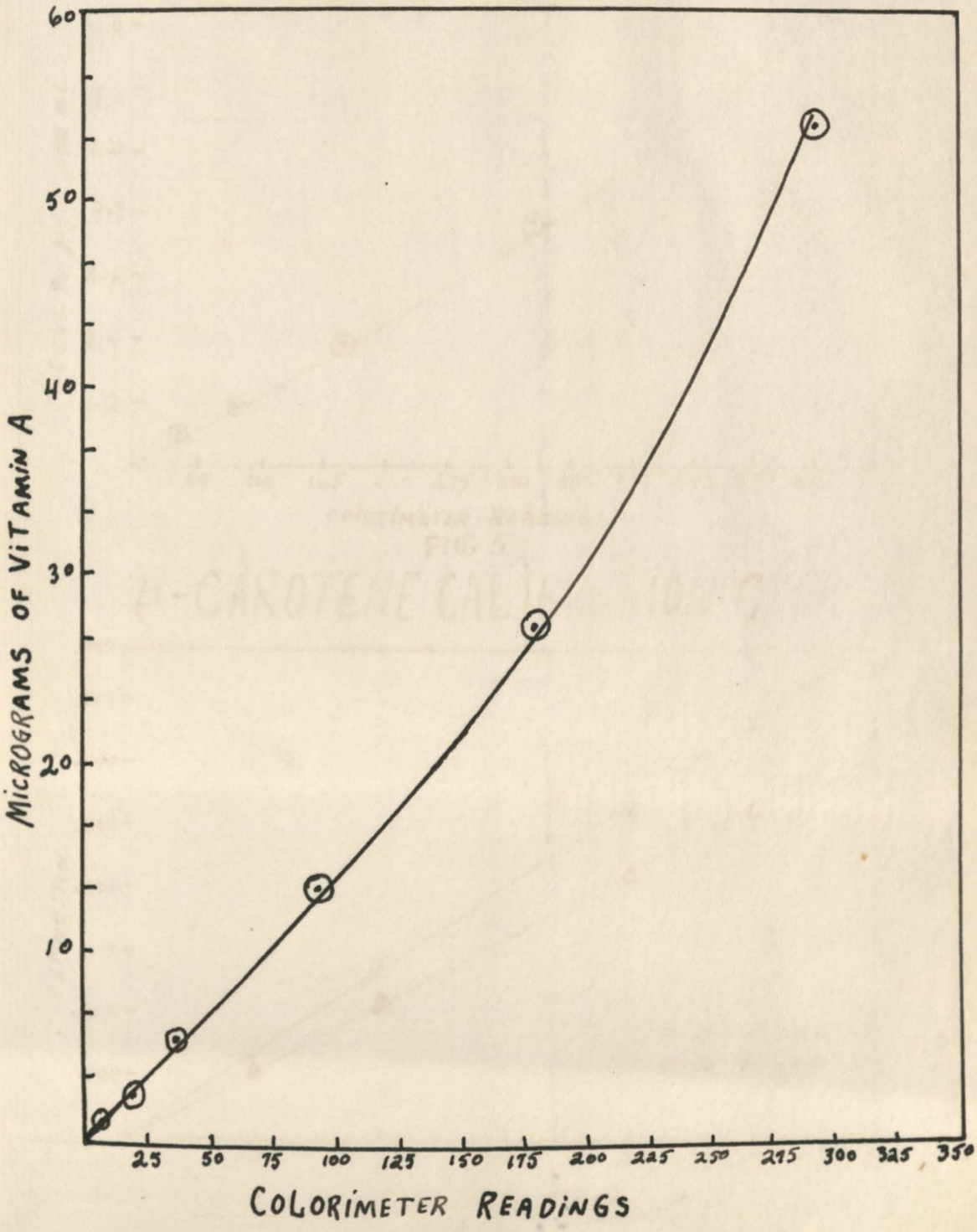


FIG. 4

β-CAROTENE CALIBRATION CURVE

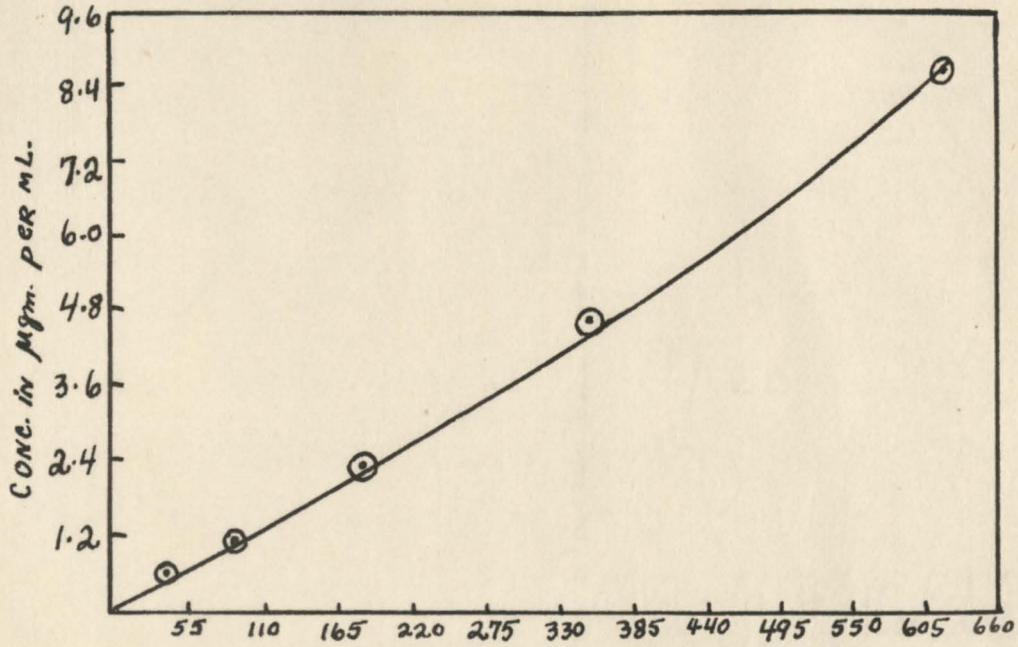


FIG. 5

β-CAROTENE CALIBRATION CURVE

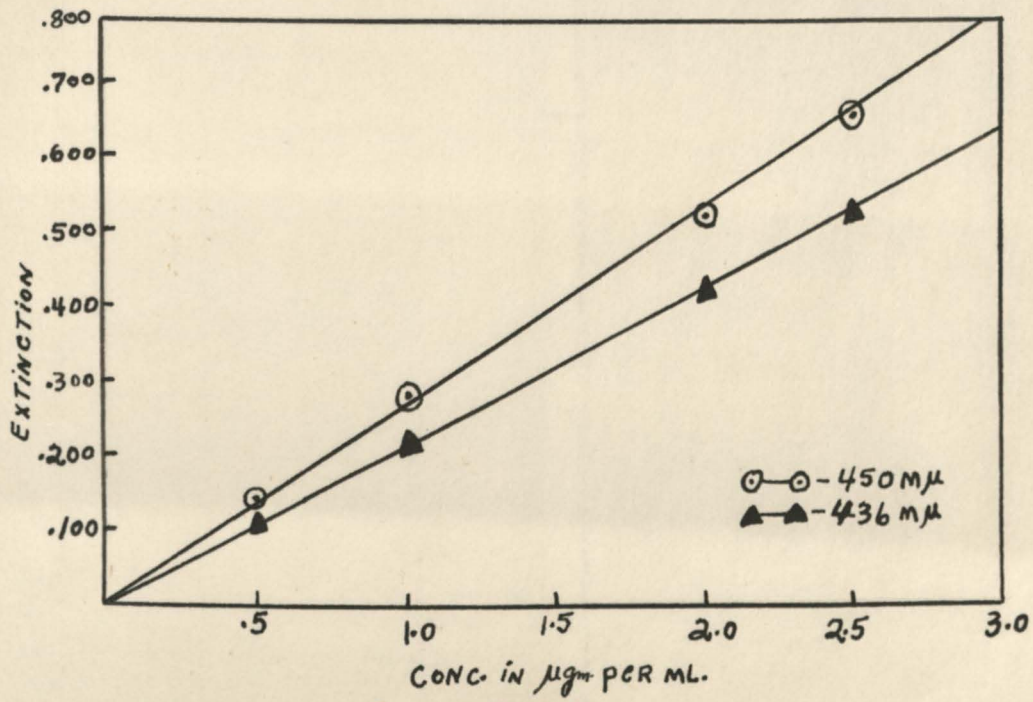


FIG. 6

The procedure of analysis for vitamin A in the tissues was the same as previously described by High(33).

G. Procedure of Analysis for Vitamin A in The Tissues

Approximately 16 hours after administration of the last supplement, each rat was sacrificed by employing ether. The livers and kidneys were removed promptly, and each organ was added to a previously weighed 250 ml. Erlenmyer flask containing approximately 50 ml. of water. Following the weighing of each flask plus tissues to ascertain the weight of the tissue, each organ was homoganized in a waring blendor. The homogenates were saponified with 25 ml. of 15% ethanolic potassium hydroxide at 50° C for approximately one-half hour. Following the cooling of the saponified tissues, they were extracted in 500 ml. separatory funnels with four 50 ml. portions of peroxide free ether; followed by a 50 ml. water wash, a 50 ml. 10% potassium hydroxide wash, and three 50 ml. water washes or until wash water gave no color with phenolphthalein indicator solution.

The ether extracts prepared above were dried over anhydrous potassium carbonate, one-half to one hour, filtered and the potassium carbonate was washed with two 25 ml. portions of ether, in order to remove adhering vitamin A, and the wash solutions were combined with the ether extracts which were evaporated to dryness in a 250 ml. suction flask under reduced pressure. In order to facilitate evaporation,

each flask was heated gently on a warm water bath during this operation. The dried residues were dissolved in 10 to 20 ml. of chloroform, the quantity depended upon the concentration of vitamin A, and 2 ml. aliquots of these solutions were added to 4 ml. of activated glycerol dichlorohydrin in a colorimeter tube. After mixing the solutions throughly and allowing them to stand for 2 to 2½ minutes for maximum color development, the intensity of colors developed were ascertained with a Klett-Summerson colorimeter employing a 540 millimicron filter. The values obtained were converted into micrograms of vitamin A by employing a calibration curve for the reagent used and the aliquot of solution employed.

H. Procedure of Analysis of β-Carotene Solutions

In the experiments employing the use of the colorimeter tubes, dilute solutions of the carotene stock solution and of the octyl hydroquinone stock solution were used. However, the concentrations of these solutions were proportional to the amount fed to the animals. Readings were taken on the Klett-Summerson colorimeter at various time intervals. The readings were made by taking the colorimeter tubes from the water bath and reading them directly on the colorimeter employing a 420 millimicron filter. This was done as quickly as possible. By employing the β-carotene calibration curve, previously made, as shown in Fig. 5, the colorimeter readings

were converted to µgm. per ml. of carotene, and thus gave the amount of carotene destroyed up to each time interval. The temperature of the bath was 60° C during all such analysis.

The solutions contained in the test tubes were of the same concentration administered to the animals, except in the experiment run at 37.5° C. At various time intervals, one ml. of the solutions was accurately pipetted out, diluted with hexane in a 25 ml. volumetric flask and readings were made on the Beckman Spectrophotometer at wave lengths of 450 and 436 millimicrons.

The values obtained were converted into micrograms of carotene present by employing a calibration curve (Fig. 6.) obtained with known quantities of carotene measured with a Beckman Spectrophotometer at the two wave lengths.

IV.

EXPERIMENTAL

A. Results and Discussion on In Vivo Data

1. Carotene and Octyl Hydroquinone Data

Several series of experiments were conducted during the summer of 1950, but due to adverse experimental conditions, poor results were obtained, consequently, no report will be made of this data.

The animals in series I received 30 micrograms of carotene as the control group, and 30 micrograms of carotene plus 10 mg. of octyl hydroquinone as the experimental group. In this series Wesson Oil was used as the solvent for both the carotene and octyl hydroquinone solutions. The period of supplementation lasted 20 days. The average weight gain by the control group was 37 grams, as compared to 40 grams average weight gain by the group receiving carotene plus the 10 mg. level of octyl hydroquinone. No significant difference in weight gain was observed between each group. The average amounts of vitamin A found in the livers and kidneys of the control group were 30.0 and 17.0 micrograms, respectively; while the average amounts of vitamin A found in the livers and kidneys of the group receiving carotene plus 10 mg. of octyl hydroquinone were 16.0 and 6.7 micrograms, respectively. Statistical analysis revealed a significant difference between these groups. The data presented in Table I, Series I, summarizes the results of this experiment. Due to the fact that Wesson Oil contains about

0.04% tocopherol(20), it was decided to repeat this experiment, using olive oil as the solvent for carotene and octyl hydroquinone. Olive oil does not contain any tocopherol as demonstrated by Lemley, et al(34). In this repeat experiment the carotene and octyl hydroquinone were administered at the same levels as that administered in Series I. The period of supplementation was 20 days. The results of this experiment are summarized in Table II, Series II. The average weight gain of the control group in this series was slightly higher than the experimental group, in contrast to that shown in Series I, however, again no significant difference was found. However, from tissue deposition of vitamin A from carotene fed, the same trend was observed as in Series I. The control rats in this series deposited an average of 45.6 and 6.3 micrograms of vitamin A in their livers and kidneys, respectively. The group receiving the 10 mg. level of octyl hydroquinone deposited an average of 33.6 and 4.9 micrograms of vitamin A in their livers and kidneys, respectively. Statistical analysis revealed a significant difference between the control group and the experimental group.

Table I (Series I) *

Group	Number of Rats and Sex	Weight Gain (GRAMS)	Micrograms of Vitamin A		
			Kidneys	Livers	Total
I (Control)	1 ♂	54	17.0	45.0	62.0
	1 ♀	52	15.0	22.0	37.0
	1 ♀	37	22.0	34.0	56.0
	1 ♀	34	15.0	17.0	32.0
	Average	37	17.0	30.0	47.0 ± 7.3
II (Expt'l)	1 ♂	53	5.0	17.0	22.0
	1 ♀	45	8.5	16.0	24.5
	1 ♀	37	8.0	15.0	23.0
	1 ♀	37	0 .0	15.0	15.0
	1 ♀	26	12.0	17.0	29.0
	Average	40	6.7	16.0	22.7 ± 2.4

The key to Tables I thru III is found on page 31.

Table II (Series II) **

Group	Number of Rats and Sex	Weight Gain (GRAMS)	Micrograms of Vitamin A		
			Kidneys	Livers	Total
I (Control)	1 ♀	41	10.5	47.5	58.0
	1 ♀	37	8.5	50.0	58.5
	1 ♀	31	3.5	49.5	53.0
	1 ♀	26	2.5	34.5	37.0
	Average	34	6.3	45.6	51.6 ± 5.1
II (Expt'l)	1 ♀	56	7.0	40.0	47.0
	1 ♀	35	2.5	35.0	37.5
	1 ♀	21	2.5	22.0	24.5
	1 ♀	11	7.5	37.5	45.0
	Average	31	4.9	33.6	38.5 ± 5.1

Although the growth shown by the control groups in Series I and II, in comparison with the groups receiving 10 mg. of octyl hydroquinone in these same series cannot be considered as showing a significant difference, a definite trend can be seen from tissue deposition of vitamin A. In each series the control groups deposited a significantly higher amount of vitamin A in their livers and kidneys than did the groups receiving 10 mg. of octyl hydroquinone. This seems to indicate that octyl hydroquinone acts antagonistic to vitamin A deposition when administered at the 10 mg. level.

Table III, Series III, shows the results of administering octyl hydroquinone at the 0.5 mg. level. Since the 10 mg. level acted antagonistic to vitamin A deposition, the 0.5 mg. level was tested to see if a sparing action would exist at this level, resulting in an increased deposition of vitamin A. Carotene was administered at the same levels in this series as in Series I and II. Olive oil was used as the solvent for the carotene and the octyl hydroquinone. The period of supplementation was 20 days. It should be pointed out that at the 0.5 mg. level a sparing or synergistic effect on carotene utilization seems to exist. Statistical analysis revealed that no significant difference existed between the control and experimental groups of this series. However, the trend seemed to be in favor of

0.5 mg. level of octyl hydroquinone increasing the tissue deposition of vitamin A. Therefore, the control rats of Series II and III were combined. In the later instance, a definite significant difference was observed, showing that the 0.5 mg. level of octyl hydroquinone increased the tissue deposition of vitamin A. Whether or not the 0.5 mg. level of octyl hydroquinone is the optimum level to demonstrate synergism, was not determined, therefore other levels slightly below and above the 0.5 mg. level should be studied to find the optimum level.

Table III (Series III) **

Group	Number of Rats and Sex	Weight Gain (GRAMS)	Micrograms of Vitamin A		
			Kidneys	Livers	Total
I (Control)	1 ♀	64	2.5	58.5	61.0
	1 ♀	53	8.0	64.5	72.5
	1 ♀	47	5.0	48.0	53.0
	1 ♀	47	3.0	64.0	67.0
	1 ♀	46	3.5	57.5	61.0
	1 ♀	28	5.0	64.0	69.4
	Average	48	4.5±.8	59.4±2.6	64.0± 2.8
II (Expt'l)	1 ♀	71	7.5	45.5	52.5
	1 ♀	59	7.5	48.0	55.5
	1 ♀	58	7.5	81.0	88.5
	1 ♀	57	7.5	56.5	64.0
	1 ♀	56	3.0	75.5	78.5
	1 ♀	49	8.0	82.0	90.0
	Average	58	6.8±.7	65.0±6.8	71.5± 6.7

* Texas A. & M. College Strain Rats

** Wistar Strain Rats

The Standard error of mean was calculated from the formula: $S.E.M. = \sqrt{\frac{\Sigma (x-x^1)^2}{n(n-1)}}$, where S.E.M. =

standard error of mean, x = individual values, x^1 = the group mean, n = number of individuals.

B. Results and Discussion on In Vitro Data

1. Carotene and Octyl Hydroquinone Data

Preliminary experiments were conducted to determine under what conditions the carotene solutions could be destroyed that would be most applicable to study. One of the preliminary experiments conducted was the thermal decomposition of the carotene solution in an oven at 68.8° C. Ultraviolet radiation and the employment of hot water baths were used in other experiments. The data on the later two experimental methods will not be reported here. The typical thermal decomposition curve obtained from the carotene destruction experiment employing the oven is shown in Fig. 7, Series A. The carotene solution minus the octyl hydroquinone showed rapid decomposition, while the carotene plus the 10 mg. level of octyl hydroquinone solution showed only negligible decomposition up to 184 hours.

From the above mentioned preliminary experiments it was decided to study the rate of destruction of carotene at 60° C, employing a constant temperature bath with oxygen bubbling into the solutions, in the presence of diffused laboratory light. The first set of experiments conducted under these conditions compared the

relative destruction of carotene alone with carotene plus 10 mg. and 0.5 mg. of octyl hydroquinone. These solutions and all subsequent solutions used in the in vitro experiments were made employing olive oil as solvent. Fig. 8, Series I, shows the results of the first set of experiments. The carotene solution was completely destroyed between 33 and 40 hours, while the carotene plus the 10 mg. and 0.5 mg. levels of octyl hydroquinone showed only slight decomposition between 33 and 40 hours. This data indicates that the octyl hydroquinone protected the carotene from destruction, however 10 mg. of octyl hydroquinone seemed to offer more protection then did the 0.5 mg. level of octyl hydroquinone. Analysis of the solutions in this series were made on the Klett-Summerson colorimeter, employing a 420 millimicron filter. A check was made on the results of Series I, employing the Beckman Spectrophotometer; this was Series II. The results of Series II may be seen in Fig. 9, Series II. The same relative results were obtained in this series as was in Series I, however the carotene was almost completely destroyed in this instance at 16 hours, as compared to negligible destruction of the carotene plus the 0.5 mg. level and 10 mg. level solutions of octyl hydroquinone. Although more concentrated solutions of carotene were used in Series II, the larger tubes employed and the amount of

oxygen bubbling into the solutions may account for the more rapid destruction of carotene.

To ascertain if the 10 mg. level of octyl hydroquinone offered more protection than did the 0.5 mg. level, a long term experiment was conducted to conclusively determine this. Fig. 10, Series III, shows that up to 80 hours the 10 mg. level of octyl hydroquinone offered greater protection than did the 0.5 mg. level for the carotene solution.

To be sure that the octyl hydroquinone was not forming colored oxidation products that had absorption at the wave lengths of the carotene analysis, viz., 436 millimicrons and 450 millimicrons, a series of experiments were conducted to determine this. The results of this experiment are shown in Fig. 11, Series IV. It was observed from this experiment that only a negligible increase in color due to oxidation of the octyl hydroquinone could be detected at 436 millimicrons and 450 millimicrons. These results lend weight to the hypothesis that the maintanence of color by the solutions containing the octyl hydroquinone was due to the antioxidant effect of octyl hydroquinone and not to colored oxidation products of octyl hydroquinone. The possibility remains, however that octyl hydroquinone may have reacted with the carotene in solution to give colored products. However, this possibility would be

rather difficult to investigate in vitro. In vivo experiments could be conducted by administering the oxidized solutions of both carotene and carotene plus octyl hydroquinone to vitamin A-deficient rate thus illustrating the relative biological potency of these two solutions. However, this method would be open to criticism.

Fig. 12, Series V, shows the results of observing the destruction of carotene at 37.5° C. In this series all experimental conditions were the same as previously described, except for the temperature change. This experiment was conducted at body temperature to be sure that the same sparing effects of octyl hydroquinone demonstrated at 60° C, could be shown at 37.5° C. The results obtained at 37.5° C show that the same sparing effect by octyl hysroquinone could be deomonstrated at this temperature. It is therefore believed that a correlation can be made between the in vivo results with that of the in vitro results.

2. Carotene and Vitamin E Data

All of the previously described in vitro experiments were conducted in part to substantiate the octyl hydroquinone is capable of acting as a biological antioxidant. To add more evidence to the belief that octyl hydroquinone is a biological antioxidant, a well known biological antioxidant, vitamin E, was tested under the

same experimental conditions, as was octyl hydroquinone. The results of this experiment are shown in Fig. 13, Series VI. The same protecting effect exhibited by octyl hydroquinone was demonstrated by vitamin E.

Table IV gives a complete summary of all in vitro experiments, including Series A, I, II, III, IV, V and VI. The curves shown in Fig.'s 7 thru 11 represent carotene destruction data obtained by using the 450 millimicron β-carotene calibration curve, since the analysis for carotene at 436 millimicrons were equivalent to those at 450 millimicrons.

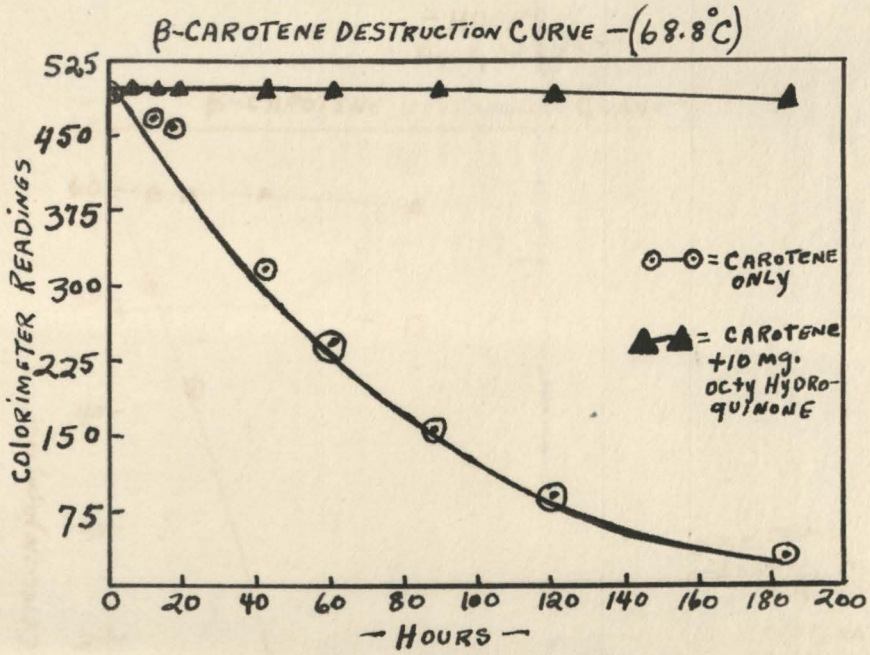


FIG. 7, SERIES A

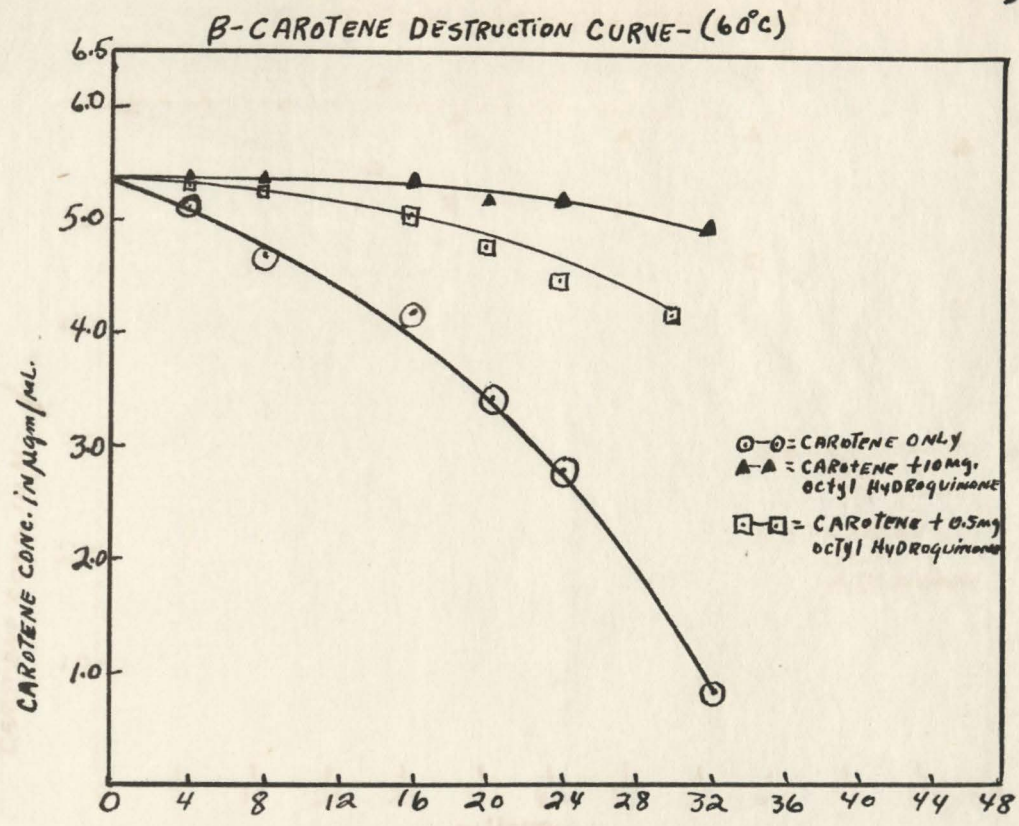


FIG. 8, SERIES I

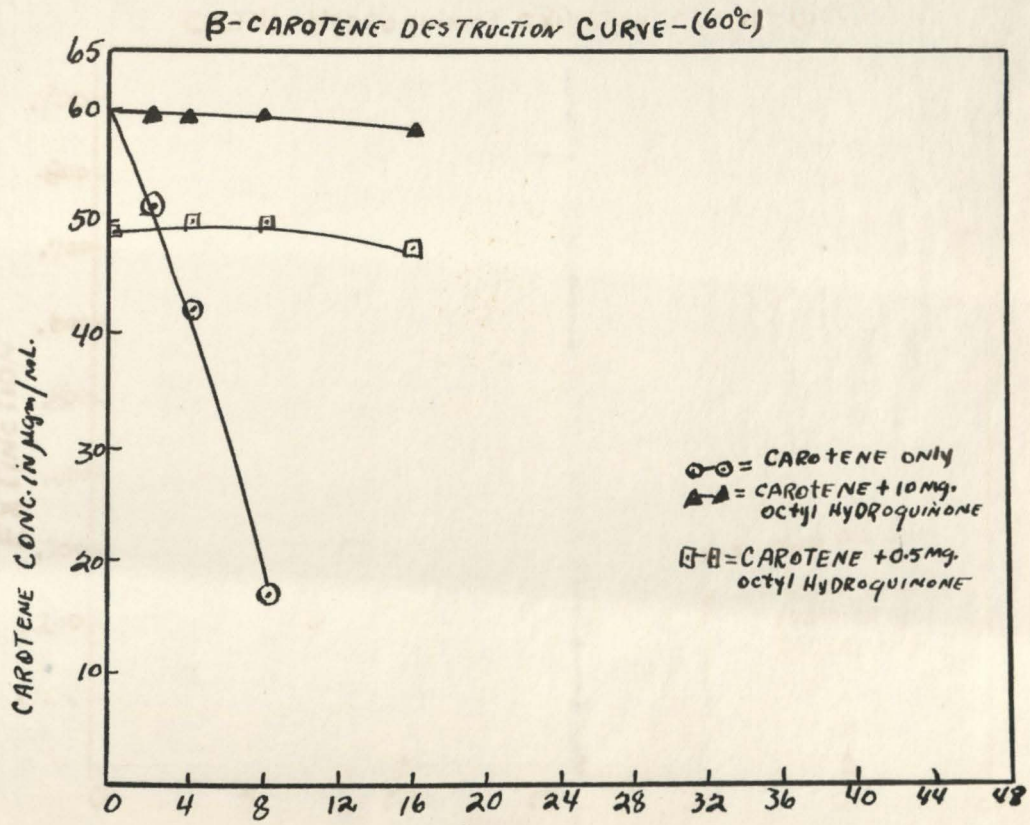


FIG. 9, SERIES II

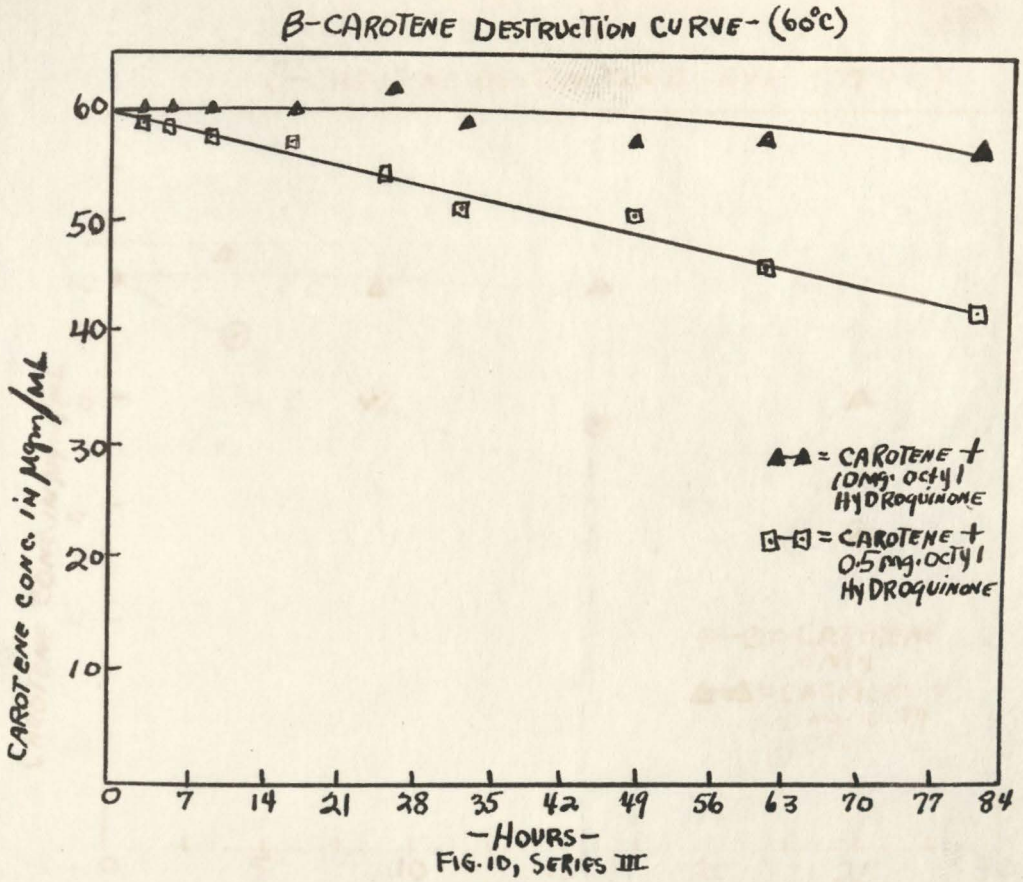


FIG. 10, SERIES III

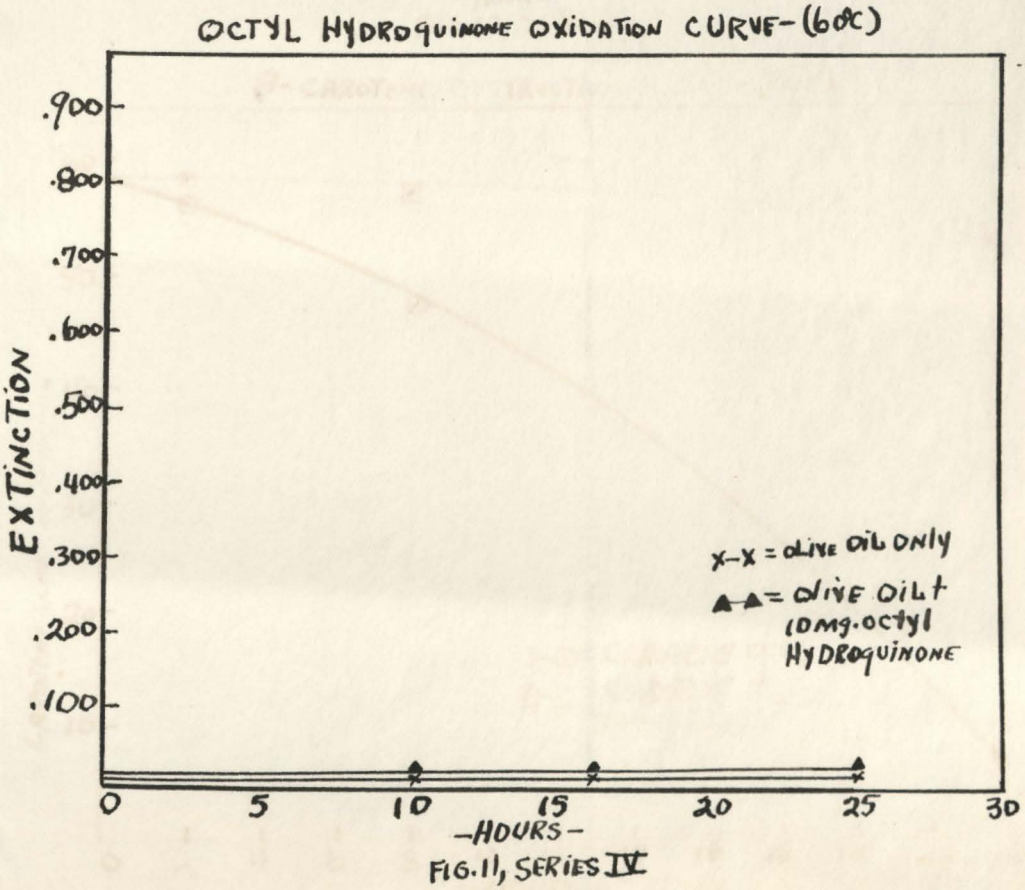


FIG. 11, SERIES IV

β-CAROTENE DESTRUCTION CURVE-(37.5°C)

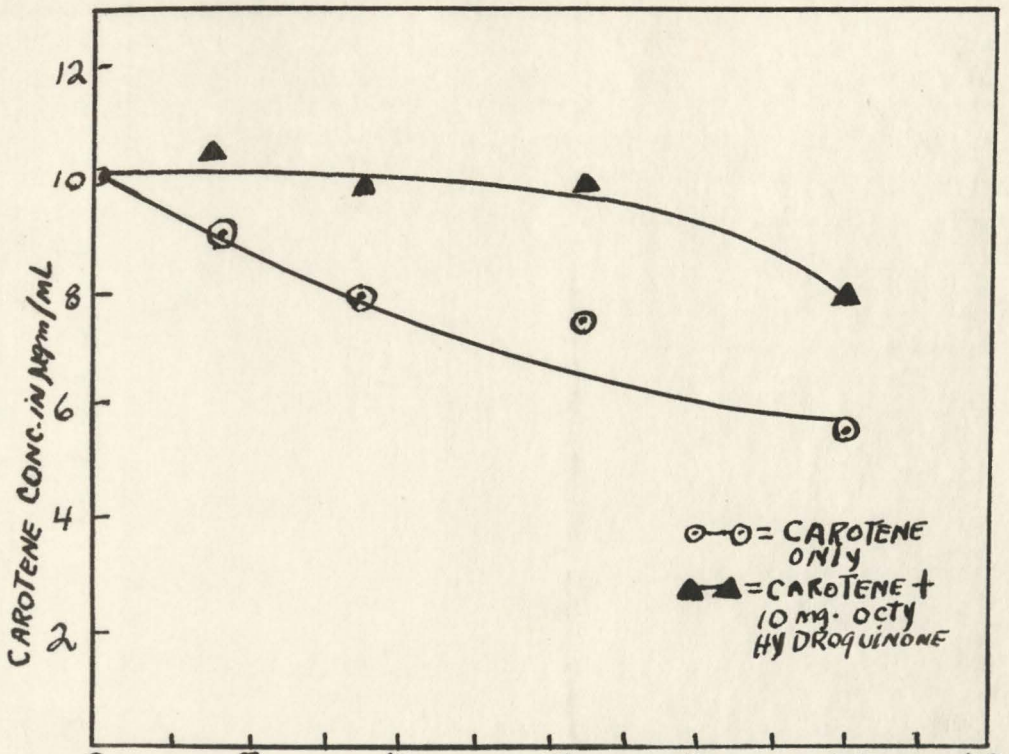


FIG. 12, SERIES V

β-CAROTENE DESTRUCTION CURVE-(60°C)

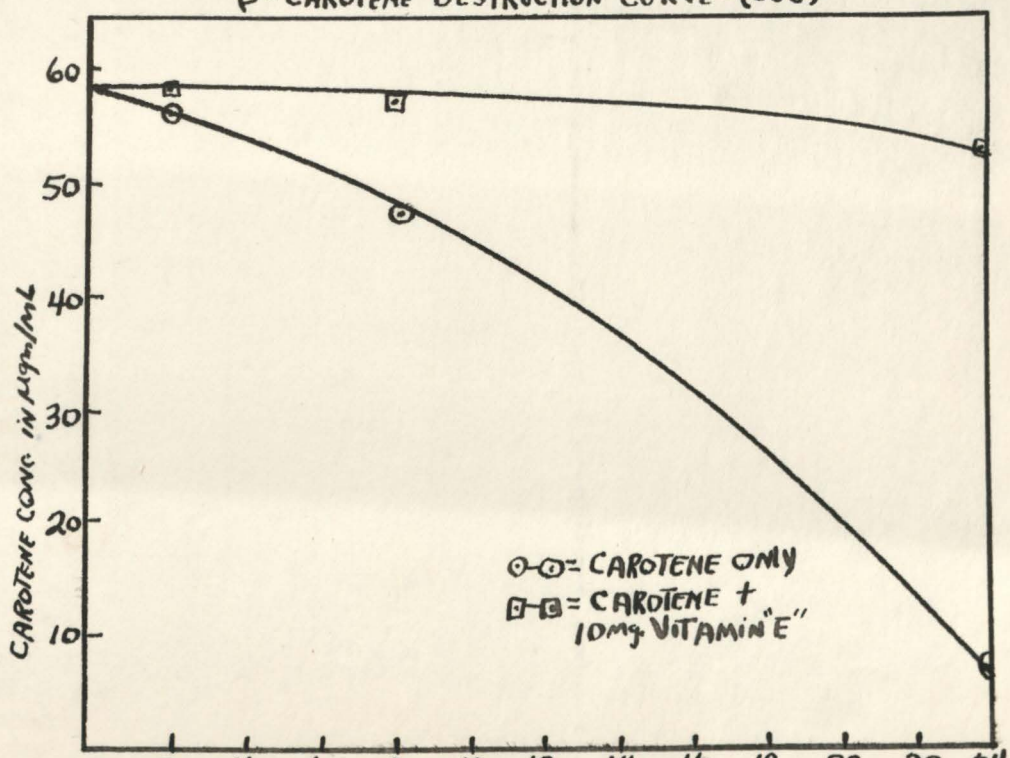


FIG. 13, SERIES VI

TABLE IV

SERIES	CAROTENE CONC. IN μgm./ML.								
	HOURS								
A (60°C)	0	2	4	16	48	60	120	136	184
C	6.8	6.8	6.7	6.2	3.8	3.1	1.2	.90	.33
C ≠ 10 (e)	6.9	6.9	6.9	6.9	6.9	---	6.8	6.7	6.6
I (60°C)	0	2	4	8	16	20	24	32	48
C	5.3	5.1	5.1	4.7	4.2	3.4	2.8	.85	---
C ≠ .5 (e)	5.4	5.4	5.4	5.3	5.1	4.8	4.4	---	---
C ≠ 10 (e)	5.3	5.3	5.3	5.3	5.3	5.1	5.1	4.9	1.5
II (60°C)	0	2	4	8	16				
C	58.5	51.5	47.3	17.5	---				
C ≠ .5 (e)	45.6	46.5	41.5	30.0	---				
C ≠ 10 (e)	58.5	58.5	58.5	57.5	57.0				
III (60°C)	0	2	4	8	16	32	48	60	80
C ≠ .5 (e)	58.8	58.0	58.0	57.5	57.0	51.2	51.2	46.6	46.2
C ≠ 10 (e)	59.7	59.7	59.6	59.6	59.6	58.9	57.6	58.0	57.1
IV (60°C)	0	16	24						
Olive Oil	.015	.021	.007						
Olive Oil ≠ 10 (e)	.014	.027	.024						
V (37.5°C)	0	4	8	16					
C	10	9	8	7.4					
C ≠ 10 (e)	9.8	10.5	9.9	9.9					

TABLE IV (CONT'D)

SERIES	CAROTENE CONC. IN µgm/ML.				
	HOURS				
VI (60°C)	0	2	8	24	
C	58	57	47.5	7.5	
C + 10 (E)	58.5	58.6	57.5	54.5	

Key: C= Carotene dissolved in olive oil.
C + .5 (θ)= Carotene dissolved in olive oil plus .5 mg. Octyl hydroquinone dissolved in olive oil.
C + 10 (θ)= Carotene dissolved in olive oil plus 10 mg. Octyl hydroquinone dissolved in olive oil.
C + 10 (E)= Carotene dissolved in olive oil plus 10 mg. vitamin E dissolved in olive oil.

V.

GENERAL DISCUSSION AND CONCLUSIONS

To fully interpret the results of the in vivo data and the in vitro data, a number of factors must be considered. However, most of them are of a highly complexed nature and will only be mentioned briefly.

The conversion of β -carotene to vitamin A in the animal body is one of the factors, undoubtedly of a complexed order and as yet unsolved. However, one site of this conversion seems conclusively proven. Although it was previously thought that the liver was the site of carotene conversion to vitamin A, Ball, et al(35) postulated that the gut wall was one of the sites. Later Sexton, et al(36) and Glover, et al(37) in 1947, definitely proved that the conversion of carotene to vitamin A is capable of taking place in the gut wall of the small intestine. This is true, at least, in the rat and possibly in other species. It would therefore seem that an antioxidant must exert its influence on carotene either in the gastrointestinal tract or in the gut wall.

It was found in this investigation that a low level (0.5 mg.) of the octyl hydroquinone showed synergism toward vitamin A deposition from carotene fed to rats, while a high level(10 mg.) of the compound exerted an antagonism. On the assumption that carotene conversion to vitamin A is an oxidative cleavage, these findings support the hypothesis put forth by High(33) that any fat-soluble antioxidant at

a low level would enhance the deposition of vitamin A from carotene fed, while a high level would act antagonistic to vitamin A deposition from carotene fed, by interfering with the conversion of carotene to vitamin A. From the equation given by Taylor(38) that an inhibitor of any reaction would be proportional to its concentration, indicates that a high level or concentration of an antioxidant would have a greater inhibiting effect than a low level or concentration. This would lend further weight to the hypothesis that carotene conversion to vitamin A is an oxidative fission, probably at the 9:10 double bond. However, Swift(29) reported that large amounts of α -tocopherol accelerates the decomposition of methyl hydroperoxide oleate at 75° C. This latter report would indicate that large amounts of an antioxidant aids oxidation, rather than inhibiting it. To shed some light on the controversy just mentioned, both octyl hydroquinone and vitamin E were tested, the former at both low and high levels and the latter at a high level for their effect on the oxidation of carotene. The findings of these experiments indicate that an acceleration of carotene destruction by oxidation did not prevail at either the low or at the high level with either octyl hydroquinone or vitamin E. However, Swift's experiment was carried out at 75° C and the experiment reported here was carried out at 60° C. In addition, Swift's experiment dealt with preformed hydroperoxides, while the experiment reported here did not. Again, no conclusive evidence has been presented

indicating whether or not peroxides are formed during the oxidation of fats other than unsaturated hydroperoxides. Whether or not a parallel can be drawn between the oxidation of unsaturated fatty acids and fat oxidation in relation to the formation and decomposition of hydroperoxides is unknown.

The possibility that the effect of large amounts of vitamin E in exerting its antagonism was due either to the hydroquinone or the phytyl part of the molecule was tested by High and Day(31) and neither of these substances seemed directly responsible, thus indicating that the whole tocopherol molecule was involved, or at least not these fragments. Both hydroquinone and phytol were tested by these workers at a concentration stoichiometrically equivalent to their weight proportion in the tocopherol molecule. They also tested the effects of these substances at higher concentrations. Hydroquinone being fat-insoluble possibly would account for the fact that this substance exerts little if any effect on carotene in vivo. From the in vivo results reported here, that a high level (10 mg.) of octyl hydroquinone showed antagonism to vitamin A deposition from carotene fed to rats, while a low level (0.5 mg.) acted synergistic, would indicate that the former impairs the conversion of carotene to vitamin A, while the latter aids in this conversion. The in vitro results seem to substantiate such a hypothesis, since the 10 mg. level of octyl hydroquinone showed a greater ability

to protect carotene from oxidative destruction, while the o.5 mg. level did so to a lesser extent. In an attempt to parallel the in vitro results obtained employing octyl hydroquinone with vitamin E, this latter substance was tested in vitro under the same conditions as was octyl hydroquinone. The same relative results were obtained with vitamin E as were obtained with octyl hydroquinone, thus indicating that vitamin E and octyl hydroquinone possibly act similarly toward carotene metabolism.

In taking cognizance of every possible occurrence in the in vitro experiments, the isomerization of β-carotene in to neo-β-carotene was taken into account. As reported by Beadle and Zscheille(39) that when β-carotene is heated, neo-β-carotene(an isomer of β-carotene) is produced. This latter substance has a specific absorption coefficient at a wave length of 436 millimicrons of 196 which is coincident with the coefficient for β-carotene at this wave length. Therefore, it was decided in the in vitro analysis to measure carotene destruction at both 436 millimicrons and 450 millimicrons wave lengths of light, employing the Beckman Spectrophotometer. The results of the in vitro analysis revealed in several instances that a slight increase in absorption occurred at 436 and 450 millimicrons, up to 4 hours, only in the carotene plus octyl hydroquinone solutions analyzed. The control solutions containing only carotene showed a rapid decrease

in absorption at both 436 and 450 millimicrons. These findings further lend weight to the postulate that the octyl hydroquinone affords protection for carotene against oxidation. To have some evidence that the slight increased absorption and retention of color by the carotene plus octyl hydroquinone solutions was not due to colored oxidation products, the octyl hydroquinone solution in olive oil and the olive oil alone as the control were oxidized. A slight but insignificant absorption increase occurred at 436 and 450 millimicrons in tubs containing octyl hydroquinone in olive oil. These slight differences were due to experimental error. This in turn may account for the slight increased absorption of the carotene plus octyl hydroquinone solutions during the early stages of oxidation. However, the possibility remains that octyl hydroquinone may form colored oxidation products with carotene having absorption at 436 and 450 millimicrons. To investigate this latter possibility would be extremely difficult.

In an attempt to discover the manner in which carotene is converted to vitamin A in the animal body, a number of interesting theories have been produced by many workers, embracing the use of various methods. In vitro experiments have received the greatest attention by these workers. Olcott and McCann(40) suggested in 1931 that the conversion of carotene to vitamin A is via the carotenase

system. This led other workers to search for the enzyme capable of converting carotene to vitamin A. All such searches have been futile, although carotene destruction in vitro in the presence of certain fat enzymes has been shown(41,42). The vitamin A activity of β-carotene has generally been explained by symmetrical fission of the carotene molecule at its control double bond. Wendler, Rosenblum and Tishler(43) seem to be of the opinion that the central double bond might well be the most vulnerable position to reagent attack for reasons of its symmetrical and sterically least hindered disposition. It has been shown by these same workers(43) that oxidation of β-carotene with hydrogen peroxide-osmium tetroxide yielded vitamin A aldehyde, β-ionylideneacetaldehyde and 2, 7-dimethyl-actatriendial. The vitamin A aldehyde thus formed from β-carotene was found to have a biological potency approaching that of vitamin A. These findings by Wendler, Rosenblum and Tishler support the possibility that oxygen must play an important role in the conversion of carotene to vitamin A. Several other workers(44) in studying the conversion of vitamin A aldehyde(Retinene) to vitamin A in vivo, have suggested that the transformation of β-carotene into vitamin A, in vivo, is more likely achieved by oxidation of the former to retinene, which is then rapidly reduced to vitamin A, rather than by hydrolytic fission. Ball, et al(35) reported earlier along this same line in

1947, stating that quantitative experiments suggest that one molecule of vitamin A appears for each molecule of retinene absorbed, implying the existence of an efficient reductase. Here again the importance of available oxygen seems evident. Since the mild oxidation of unsaturated hydrocarbons may yield the aldehyde, it might well be that carotene is first oxidized to the aldehyde, which is then rapidly reduced to vitamin A alcohol. An available anti-oxidant in the gastrointestinal tract, such as vitamin E or octyl hydroquinone would control the oxygen supply necessary for carotene conversion or destruction. As pointed out by Michaelis(28) that the precursor of oxidation by O_2 is the formation of an "oxygenated complex" and that subsequent electron redistribution may lead to true oxidation. Such a phenomena as this is well known in biological systems. The autoxidation of unsaturated fatty acids is one reaction in which an oxygenated compound is the precursor of true oxidation. Michaelis(28) postulated that on exposure of the unsaturated fatty acid to oxygen, the well-known hydroperoxide is first formed. The thus formed hydroperoxide can oxidize a molecule such as hydroquinone or tocopherol to its semiquinone radical, the hydroperoxide itself being reduced to a compound of a lower level of oxidation. By electronic rearrangement this reduced hydroperoxide is converted into a compound which reduces the semiquinone back to the hydroquinone, itself

being oxidized to a compound which is, or will become the hydroperoxide again. This proposal on the mechanism of the action of an antioxidant by Michaelis seems an inviting possibility. Sufficient evidence has not been presented as yet to confirm this theory of Michaelis' and the work reported here can neither confirm or deny such a theory.

VI.

SUMMARY

A study has been made on the effects of both small and large amounts of octyl hydroquinone as a biological antioxidant on the metabolism of carotene. Vitamin A deposition in the livers and kidneys of rats from carotene fed was used as the main criterion for determining its effects. Also in vitro experiments were conducted in order to determine the antioxidant properties of both this substance and vitamin E for the purpose of interpreting the in vivo data.

1. Five-tenths mg. of octyl hydroquinone when fed along with carotene to vitamin A-depleted rats increased significantly the deposition of vitamin A from carotene fed as compared to rats receiving carotene only. On the other hand, 10 mg. of the compound decreased significantly the deposition of vitamin A from carotene fed as compared to rats that received carotene only as supplement.

2. Both 0.5 mg. of octyl hydroquinone and 10 mg. of the substance protected carotene from oxidative decomposition in vitro at a temperature of 60° C. The higher concentration of the substance (10 mg.) afforded the greater protection. Substantially the same results were obtained at a temperature of 37.5° C. Likewise, 10 mg. of vitamin E afforded a marked protection of carotene from oxidative decomposition at 60° C.

3. The significance of these observations in relation to the possible mode of action of both octyl hydroquinone

and vitamin E in small and large amounts on the utilization of carotene by the rat is discussed. Also graphic representation of some of the data is presented.

VII.

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