THE STEREOCHEMISTRY OF DEHYDRO-β-CAROTENE. STUDIES ON THE INTERACTION OF SOME CAROTENOIDS WITH BORON TRIFLUORIDE AND N-BROMOSUCCINIMIDE. PROVITAMIN A ACTIVITY OF A C_{42}-CAROTENOID.

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1953

The author wishes to express his deep appreciation to Professor Zechmeister for his interest and guidance in the problems connected with this research.

ACKNOWLEDGMENTS

I wish to thank the California Institute of Technology for generous financial assistance throughout the period of the author's graduate studies.

I am indebted to Professor H. J. Deuel, Jr. and Dr. J. Ganguly who carried out the bioassays for provitamin A activities and to Dr. A. Elek and Mr. G. Swinehart for the microanalytical determinations.

ABSTRACT

An extensive study of the stereoisomerization of dehydro-β-carotene is presented. Two of the _cis_ isomers have been isolated in crystalline form and described. The nature of "isocarotene" has been clarified.

Treatment of β-carotene with boron trifluoride under defined conditions leads chiefly either to its stereoisomerization or chemical conversion. Two of the several conversion products have been crystallized; a third, exhibiting strong fluorescence in ultraviolet light is also described.

Three new carotenoids, termed isocryptoxanthin, isocryptoxanthin methyl ether, and dimethoxy-β-carotene, formed by the addition of water or methanol to dehydro-β-carotene in the presence of boron trifluoride, are described. The structures of the first two compounds have been determined; the location of the substituent groups in the third is, at present, uncertain.

Dehydro-β-carotene, bisdehydro-β-carotene, and anhydroeschscholtzxanthin have been isolated from the end products of the reaction of β-carotene with N-bromosuccinimide. Bisdehydro-β-carotene is a new carotenoid whose probable structure is discussed.

The considerable provitamin A potency of 16,16'-homo-β-carotene is discussed from the standpoint of its novel structural features.

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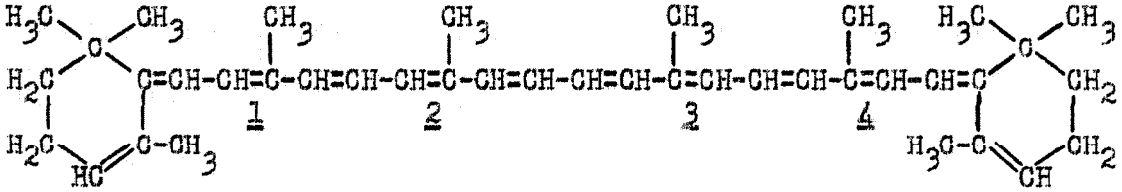
I. A STUDY OF STEREOISOMERIC DEHYDRO-β-CAROTENES.

A. THEORETICAL PART

1. Introduction.

In 1932, Kuhn and Lederer (21) had prepared a carotenoid, which they called "isocarotene", by a spontaneous conversion of β-carotene tetraiodide when standing in acetone solution. After a treatment with thiosulfate and, eventually, by repeated crystallizations from benzene-methanol these authors isolated a product which melted at 181° and showed spectral maxima in light petroleum (b.p. 70-80°) at 504, 475, and 447 mμ. By further crystallization of "isocarotene" from light petroleum Karrer, Schopp, and Morf (16) obtained glittering violet needles (m.p. 193°). They found that this compound had 12 conjugated double bonds and corresponded to the formula $C_{40}H_{54}$. In 1940 Karrer and Schwab (17) proposed the now generally accepted structure for the compound which they correctly termed "dehydro-β-carotene" (Formula I.).



Formula I. Dehydro-β-carotene. (The sterically unhindered double bonds are designated by <u>italicized</u> numbers.)

In this Thesis it will be shown that Kuhn's "isocarotene" preparation was a mixture of (mainly) three stereoisomeric dehydro-β-carotenes and unreacted β-carotene. Because all-*trans*-dehydro-β-carotene is very much less soluble than any of its *cis* isomers or β-carotene, it is possible, in principle, to concentrate these latter compounds in the mother liquors and thus to obtain a relatively pure crystalline all-*trans*-dehydro-β-carotene.

The melting point given by Karrer (16) corresponds to that of the all-*trans* form and indicates that he was probably able to eliminate all other components of Kuhn's "isocarotene" without applying chromatography. Since, as will be shown, the all-*trans* form constitutes but a small part of "isocarotene", the crystallization method of purification could not have been very efficient. It is interesting to note that neither of the authors mentioned reported the yields obtained after repeated crystallizations.

The application of chromatography as employed in this Thesis in addition to offering a simple means for the isolation of all-*trans*-dehydro-β-carotene also led to the detection and isolation in pure form of two crystalline *cis* isomers of this carotenoid. Neither of the previous authors reported any inhomogeneity in their final preparations, although Kuhn (21) had reported the presence of β-carotene in some crude preparations of "isocarotene" before he had developed the proper experimental conditions. Although many preparations of "isocarotene" were made following the directions of Kuhn and Lederer a β-carotene free sample was never obtained (cf. Table 1.).

2. Steric Possibilities in the Dehydro-β-carotene Set.

It is now well established that carotenoids and other compounds with long conjugated polyene systems are capable of undergoing reversible *cis-trans* isomerization. The most extensive work in this field has been done by Zechmeister and his collaborators, especially Escue, Lemmon, LeRosen, Pinckard, Polgár, and Schroeder (32).

The stereochemical study of dehydro-β-carotene is of special interest because, so far as is known, it is the first time that a C_{40}- carotenoid with a single bond in the central position has been investigated from this viewpoint, although Oroshnik et al. (25) have discussed certain stereochemical aspects of retro-vitamin A, a C_{20}- compound with one end grouping identical to that in dehydro-β-carotene.

Table 1.

Composition of Kuhn's "Isocarotene"

Component	Maxima in hexane (Beckman)	M.P.	Amount (in % of starting material)
All-trans-dehydro-β-carotene	502,471, 445 mµ.	193°	16
Neo-dehydro-β-carotene A*	497, 467, 442	161°	15
Neo-dehydro-β-carotene D*	494, 464, 440	178°	10
β-Carotene	479, 451	-	46
Unaccounted for	-	-	13

According to Pauling (26) only those double bonds which do not have a methyl group attached to the next adjacent carbon atom are capable of assuming cis configurations easily. The presence of such a methyl group would, because of a spatial conflict, diminish the probability of a cis configuration being formed if the usual methods are employed in the trans→cis isomerization experiments. However, in the synthesis of carotenoids as developed by Inhoffen, in Braunschweig, and Karrer, in Zurich,

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* This nomenclature is based on their respective positions in the chromatogram (on lime-celite) of the stereoisomeric mixture obtained by iodine catalysis of any steric form of dehydro-β-carotene.

certain double bonds are formed by the partial hydrogenation of triple bonds by means of specific catalysts. Such a conversion leads to the formation of a double bond with a _cis_ configuration (9).

Recently, by this method Garbers, Eugster, and Karrer (5) have synthesized a carotenoid-like molecule in which one or two "sterically hindered" double bonds in the conjugated system have a _cis_ configuration. These compounds show degraded spectra, i.e., no fine structure is present in their extinction curves; furthermore the extinction coefficients are very much lower than that of the all-_trans_ form, and the maxima are shifted to much lower wave lengths. When catalyzed with iodine, these "hindered" isomers disappear and the same stereoisomeric mixture is obtained as would also be produced by a similar catalytic treatment of the all-_trans_ form. The formation of a compound with a "hindered" _cis_ bond by the partial hydrogenation of an acetylenic bond is not surprising from the thermodynamic standpoint, for this step represents a decrease in the energy content of the molecule. In the case of the iodine catalyzed stereoisomerization of an all-_trans_ carotenoid, however, the energy requirements for the formation of a hindered _cis_ bond would be greater than for an unhindered _cis_ bond; therefore the probability of such a configuration being assumed would be small.*

Formula I shows the location of the four sterically unhindered double bonds in dehydro-β-carotene. Calculated on this basis, only 10 unhindered spatial forms are possible. In the course of the experiments to be

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* A brief discussion of a similar steric hindrance caused by certain hydrogen atoms in the benzene rings of the diphenyloctatetraene molecule has been given by Zechmeister and LeRosen (33). For the _trans_-_cis_ isomerization of diphenylbutadiene see Pinckard, Wille and Zechmeister (27).

described the ten isomers listed in Table 2 were observed.

Table 2.

Observed Stereoisomeric Dehydro-β-carotenes

(in order of descending chromatographic sequence on lime-celite)

Stereoisomer	Maxima in hexane solution (Beckman)
All-*trans*-dehydro-β-carotene	502, 471, 445 mμ.
Neo-dehydro-β-carotene A	497, 467, 442
Neo-dehydro-β-carotene B	494, 463, 439
Neo-dehydro-β-carotene C	491, 461, 435-6
Neo-dehydro-β-carotene D	494, 464, 439
Neo-dehydro-β-carotene E	487, 457, 432
Neo-dehydro-β-carotene F	489, 459, 433-4
Neo-dehydro-β-carotene G	482, 453, 430
Neo-dehydro-β-carotene H	479, 452, 429
Neo-dehydro-β-carotene J	(degraded, no fine structure)

The molecular extinction curves of these isomers are given in Figs. 1-7. The stereoisomers listed in Table 2, except for neo J, were obtained by iodine catalysis. The neo J isomer was isolated from melt isomerization products, and will be discussed further in Section 3d.

3. Stereoisomerization of Dehydro-β-carotene.

As mentioned earlier three easily crystallizable stereoisomeric dehydro-β-carotenes were found in "Isocarotene". Stereoisomerization experiments were performed with all three of these compounds.

It should be mentioned at this point that the cis isomers of most previously studied carotenoids which were adsorbed not much below their respective all-trans forms were crystallizable with difficulty, if at all.

a. Stereoisomerization by iodine catalysis.

In these experiments a solution of the carotenoid in hexane was treated with iodine (about 1% of the pigment weight) and illuminated under standardized conditions. Each of the three crystalline dehydro-β-carotenes gave stereoisomeric mixtures of nearly identical composition.

The percentage of all-trans-dehydro-β-carotene present in the stereoisomeric mixture was remarkably small. In analogous equilibria resulting from other C_{40}- carotenoids the all-trans form was the predominant component as shown by the following figures (32): lycopene, 57% all-trans; β-carotene, 48%; and α-carotene, 52%. In the dehydro-β-carotene series, as will be noted from Table 3, a cis form termed neo A is predominant (28-30%) while the all-trans compound constitutes only 17-18% of the stereoisomeric mixture.

b. Stereoisomerization by Refluxing Solutions.

Preliminary experiments showed that none of the three crystalline dehydro-β-carotenes was appreciably isomerized when allowed to remain in hexane solution at room temperatures for 24 hours. However, when such solutions were refluxed (in the absence of light) for 1 hour extensive stereoisomerization took place as shown in Table 4.

The stability of all-trans-dehydro-β-carotene toward thermal isomerization is not as great as that of α- or β-carotene, but is about the same as that shown by lycopene. In similar reflux experiments (32)

Table 3

Composition of the Stereoisomeric Equilibrium Mixtures Obtained by Iodine Catalysis in Light of Three Crystalline Dehydro-β-carotenes. (The figures designate percent of starting material as established photometrically. In numerous experiments these values did not vary more than 1-2%.)

	Steric form treated with iodine		
	All-trans-dehydro β-carotene	Neo-dehydro-β-carotene A	Neo-dehydro-β-carotene D
All-trans	17	18	17
Neo A	28	30	30
Neo B	4	5	3
Neo C	10	11	10
Neo D	12	15	15
Neo E	4	6	5
Neo F	6	6	3
Neo G	3	2	1
Neo H	1	1	1
Unaccounted for	15	7	16

the following percentages of the respective all-trans starting materials were recovered: α-carotene, 92%; β-carotene, 86%; lycopene, 45% (the last figure refers to a 30 minute reflux period).

As shown by Table 4, half of the all-trans and neo A forms of dehydro-β-carotene were present in their unchanged configurations after refluxing;

Table 4.

Composition of the Stereoisomeric Mixture Obtained By Refluxing Hexane Solutions of the Three Crystalline Dehydro-β-carotenes for 1 Hour.
(in % of starting material)

	Starting Material		
	All-trans-dehydro β-carotene	Neo-dehydro-β-carotene A	Neo-dehydro-β-carotene D
All-trans	53	5	2
Neo A	24	50	13
Neo B	-	4	-
Neo C	-	(*)	-
Neo D	4	16 *	70
Neo E	2	10	8
Unaccounted for	17	13	7

the still higher stability of the neo D isomer as compared with that of the all-trans form is an unusual feature. In most carotenoid sets those cis isomers which can be produced from the all-trans form by stereochemical conversions often show a degree of thermostability which may approach but never exceed that of the all-trans form.

c. Photochemical Stereoisomerization.

The hexane solutions were illuminated with two 40 watt fluorescent lamps at room temperature. Of all the isomerization methods employed, such

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* A small amount of neo C was present in this zone and estimated together with neo D.

illumination caused the least amount of steric rearrangement (Table 5).

Table 5.

Composition of the Stereoisomeric Mixtures Obtained by Illumination of Hexane Solutions of the Three Crystalline Dehydro-β-carotenes for 8 Hours. (in % of starting material)

	Starting Material		
	All-trans-dehydro-β-carotene	Neo-dehydro-β-carotene A	Neo-dehydro-β-carotene D
All-*trans*	84	5	1
Neo A	8	86	6
Neo D	2	3	91
Unaccounted for	6	6	2

Insolation (i.e., exposure to intense sunlight) of carotenoid solutions offers another means for causing their stereoisomerization. Because of inconstant atmospheric conditions (smog, rain, cloudiness) it was not possible to carry out an extensive study of the effect of insolation on dehydro-β-carotenes. During a three hour insolation period with the all-*trans* compound more than half of the pigment was converted into unidentified colorless products. As established chromatographically, the remaining pigment mixture showed the following composition: all-*trans*, 48%; neo A, 40%; and other *cis* isomers (mainly neo D), 12%.

d. Stereoisomerization by Melting Crystals.

The fusion of a sample of all-*trans*-dehydro-β-carotene (m.p. 193°) in an evacuated glass tube caused extensive stereoisomerization. As expected, a large fraction of the pigment was destroyed. The composition of the stereoisomerized mixture obtained from numerous experiments was quite uniform. Except for the absence of any neo H, all the steric forms of dehydro-β-carotene produced by iodine catalysis were also present here (Table 6). In the fusion experiments, however, an additional cis isomer was formed consistently and, since it constituted the lowest zone on the column, it was termed neo-dehydro-β-carotene J.

Table 6.

Composition of the Stereoisomeric Mixture Obtained by Fusing Crystals of All-trans-dehydro-β-carotene at 200° for $1\frac{1}{2}$ Minutes (in % of starting Material)

All-trans	8
Neo A	14
Neo B	2
Neo C	12
Neo D	12
Neo E	3
Neo F	1
Neo G	1
Neo J	1
Unaccounted for	46

The neo J isomer differed from all the others in that its extinction curve showed a degraded spectrum, i.e., a single broad band with almost no fine structure. Although in different experiments some minor variations at the top part of the curve were observed, the maximum was consistently at 446-448 mμ. in hexane (Fig. 5). These small spectral variations in the extinction curves were probably due to its rather rapid stereoisomerization in solutions. In one exceptional instance the maximum was located at 436 mμ. indicating that a different isomer was formed (Fig. 8).

When a dilute solution of the neo J isomer in hexane was treated with a trace of iodine and illuminated for one minute, a deepening of the color from a pale yellow to a bright yellow or orange was easily noticeable to the naked eye. When this change was followed with the visual spectroscope, the appearance of sharp bands of 496 and 467 mμ. could be observed. Chromatographic analysis of such solutions showed that the usual iodine catalyzed stereoisomeric mixture was present.

A number of *cis* carotenoids are known which also lack definite fine structure and which behave similarly when subjected to iodine catalysis, e.g., prolycopene and pro-γ-carotene (34), both naturally occurring pigments, but, so far as is known, the observations reported here represent the first instance of an *in vitro* conversion of an all-*trans* C_{40}- carotenoid into a *cis* isomer presenting the described spectral characteristics. Neo-dehydro-β-carotene J was also formed by melting crystals of the neo A and neo D isomers (Figs. 6 and 7).

Although the absence of fine structure in the extinction curve of the neo J isomer and the low value of its molecular extinction coefficient (about 60% of that of the all-*trans* form) are indicative of a poly*cis* structure, such a configuration can not be claimed with certainty for this compound, since, as mentioned earlier, Garbers, Eugster, and Karrer (5) have demonstrated that a carotenoid whose molecule contains a sterically hindered *cis* double bond shows very similar characteristics. Possibly, under the energetic conditions of the fusion experiments such a *cis* double bond could be formed. It is interesting to note that no neo J isomer appeared when neo-dehydro-β-carotene A was melted at 170°, although the yield of neo J amounted to 1% at 200°.

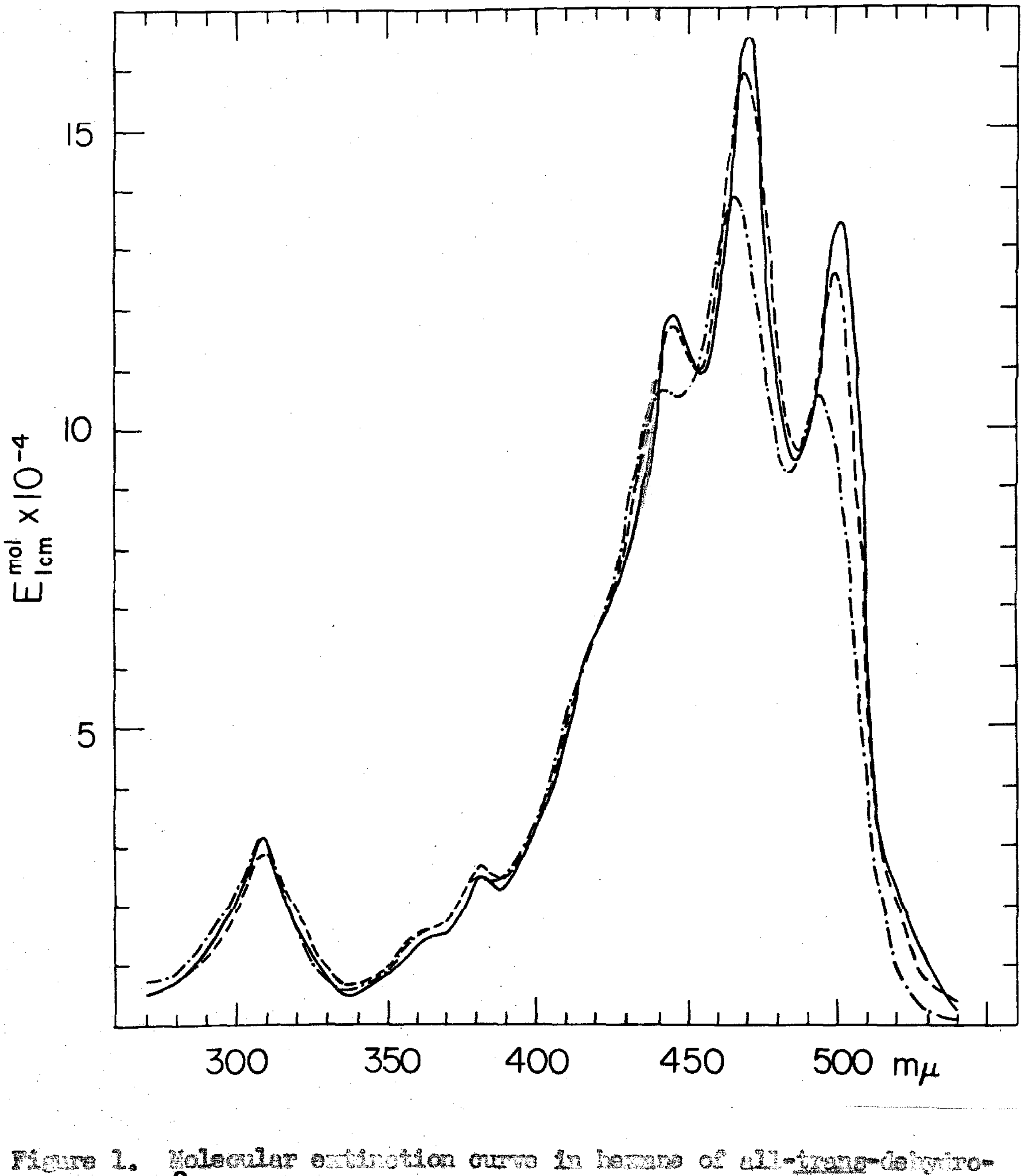


Figure 1. Molecular extinction curve in hexane of all-*trans*-dehydro-β-carotene: ——, fresh solution; – – –, after refluxing for 1 hour; –·–·–, after iodine catalysis in light.

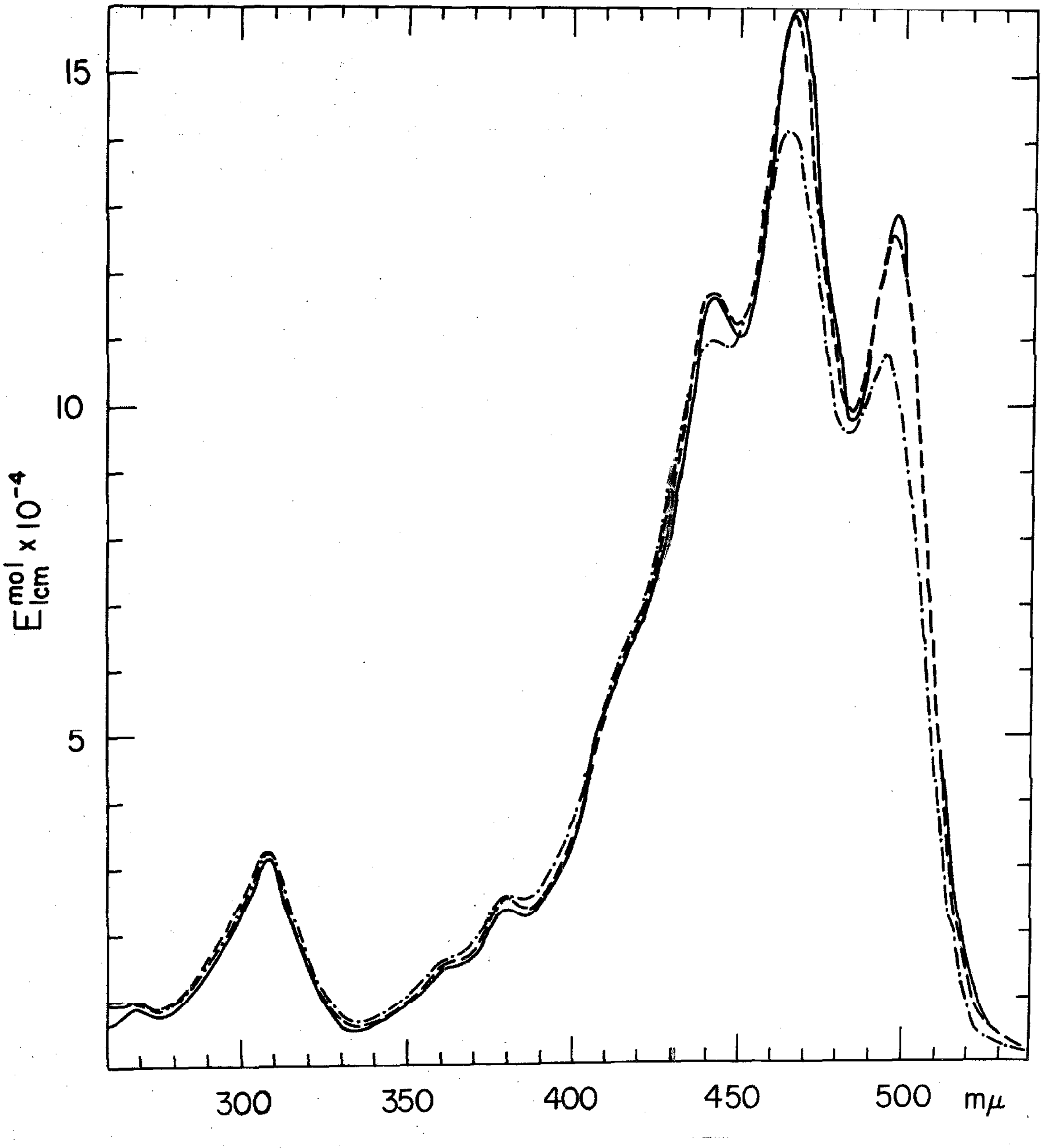


Figure 2. Molecular extinction curve in hexane of neo-dehydro-β-carotene A: ——, fresh solution; ----, after refluxing for 1 hour; -.-.-., after iodine catalysis in light.

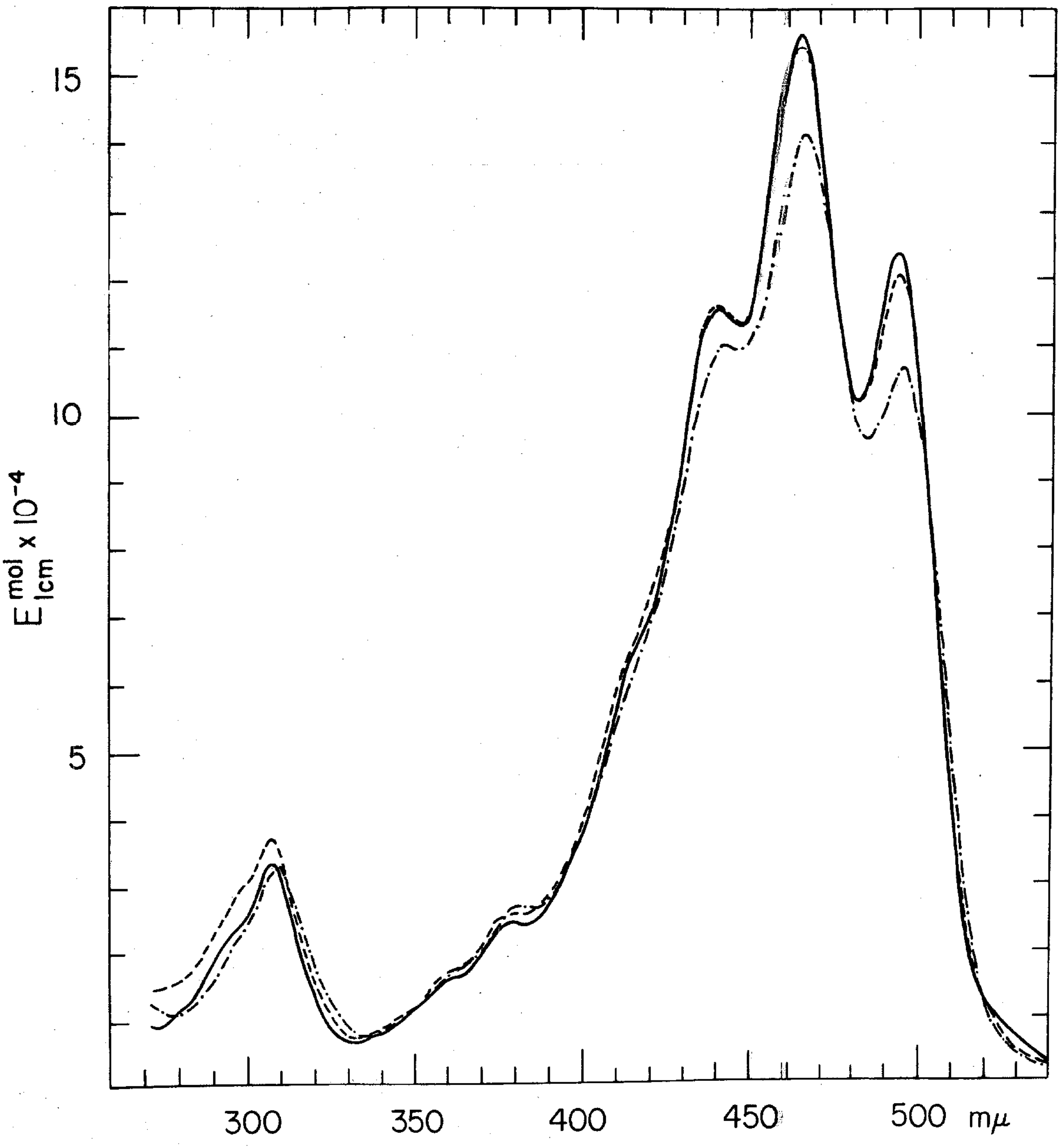


Figure 3. Molecular extinction curve in hexane of neo-dehydro-β-carotene D: ——, fresh solution; ----, after refluxing for 1 hour; -·-·-·, after iodine catalysis in light.

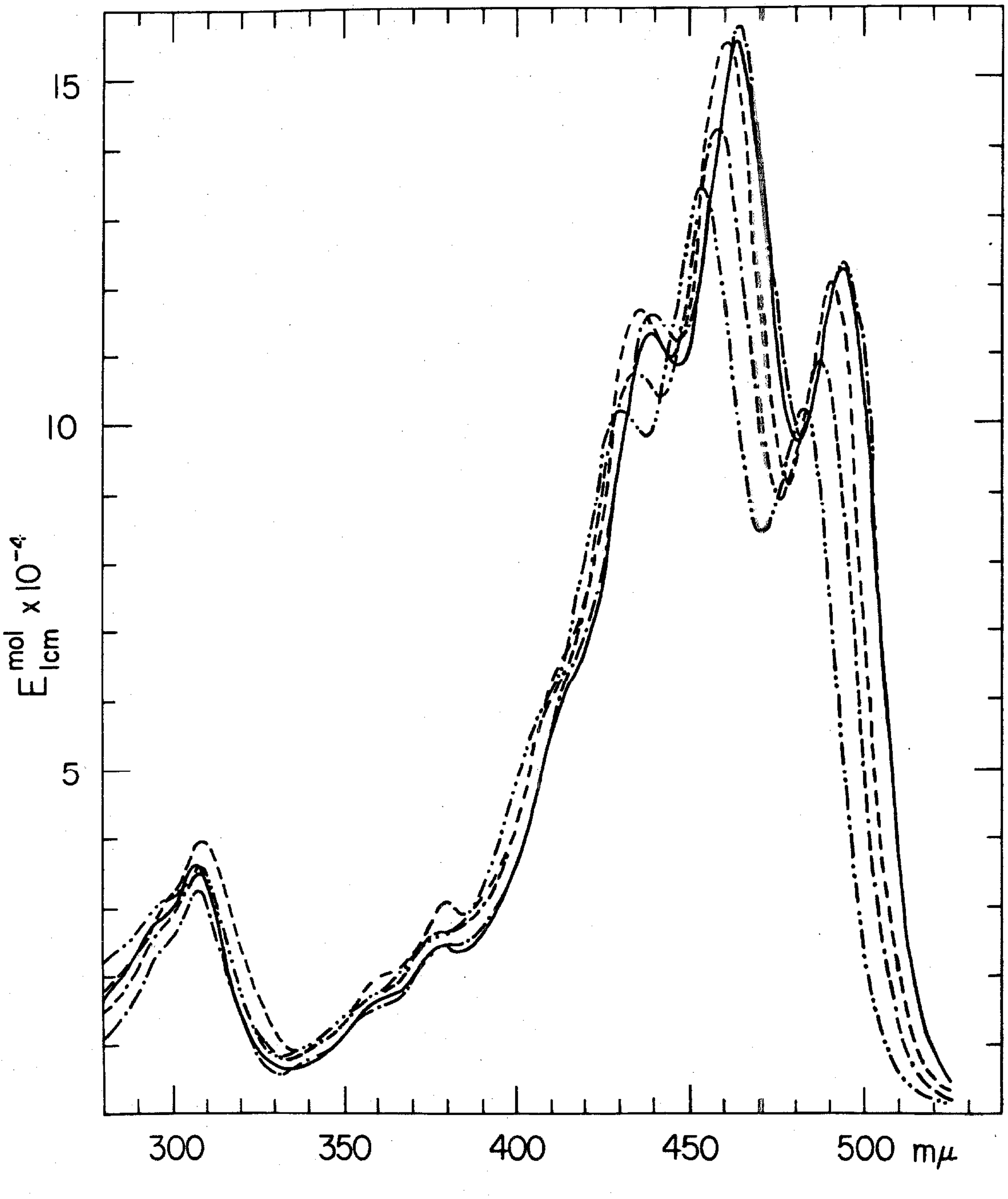


Figure 4. Molecular extinction curves in hexane of some *cis* dehydro-β-carotenes: ——, neo B; ----, neo C; -·-·-·, neo D; —·——, neo E; ··-··-, neo G.

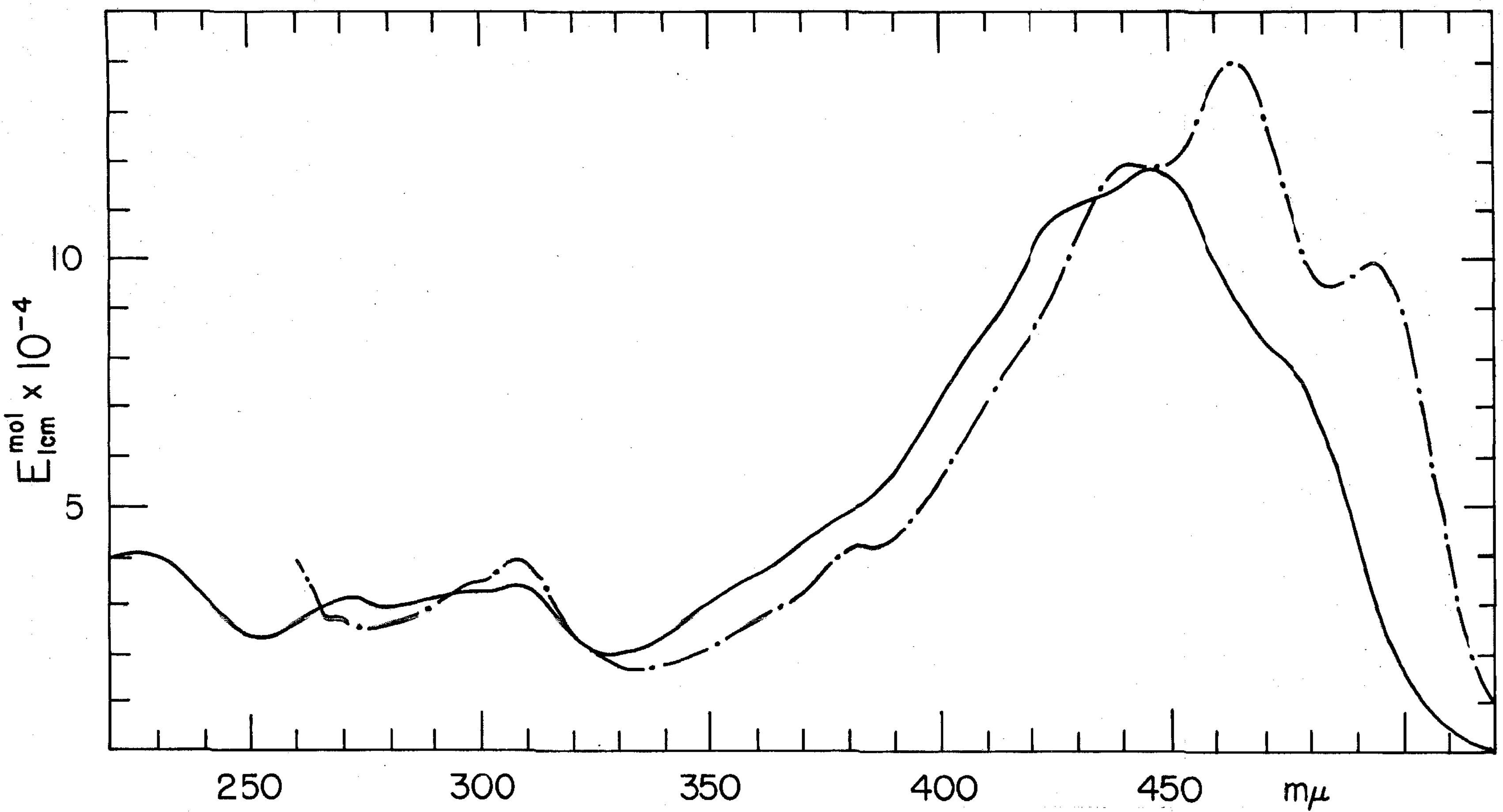


Figure 5. Molecular extinction curve in hexane of neo-dehydro-β-carotene J (from fusion of all-*trans*); ——, fresh solution; -·-·-, after iodine catalysis in light.

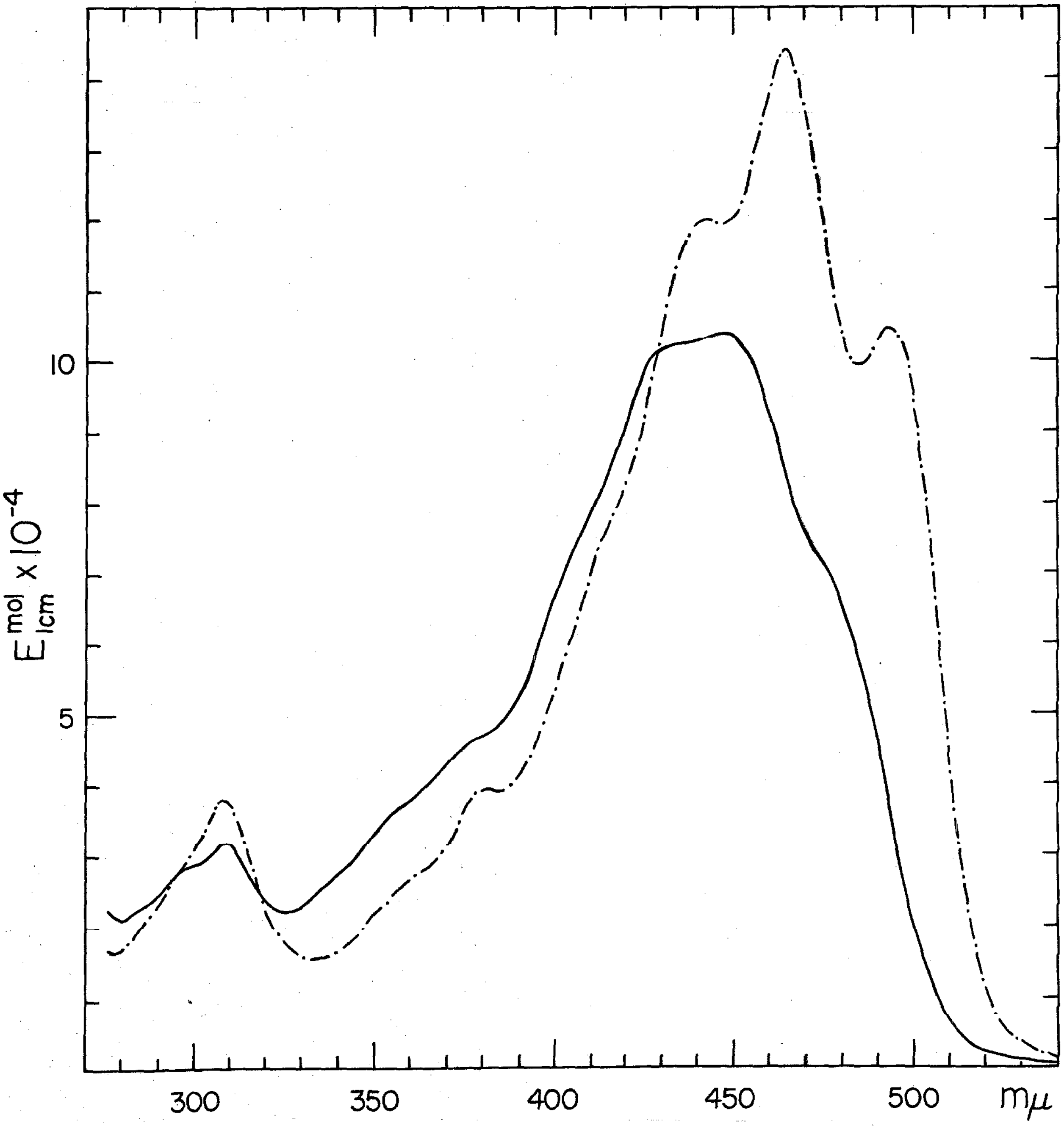


Figure 6. Molecular extinction curve in hexane of neo-dehydro-β-carotene J (from fusion of neo A isomer): ——, fresh solution; -·-·-·, after iodine catalysis in light.

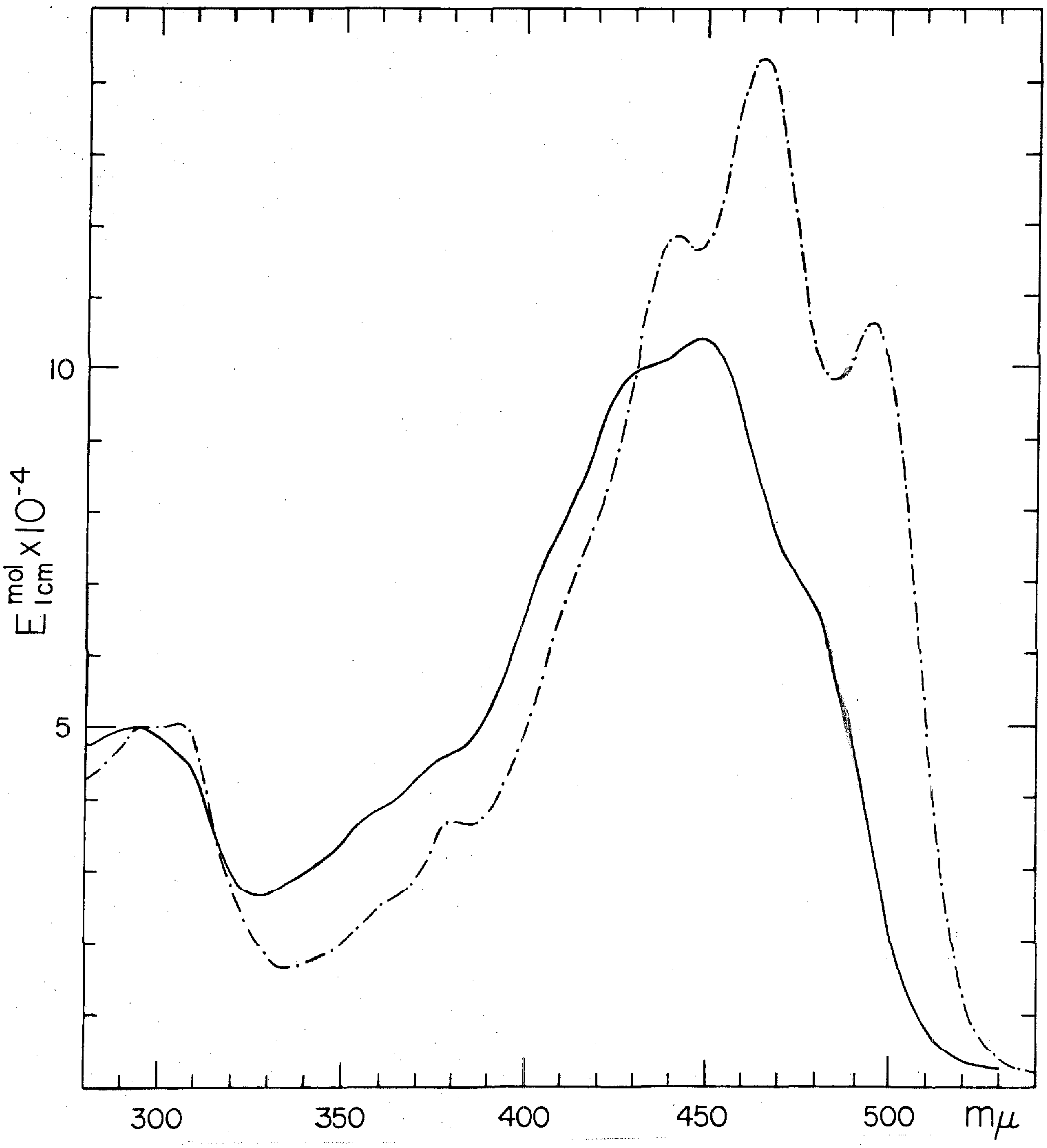


Figure 7. Molecular extinction curve in hexane of neo-dehydro-β-carotene J (from fusion of neo D isomer): ——, fresh solution; -·-·-·, after iodine catalysis in light.

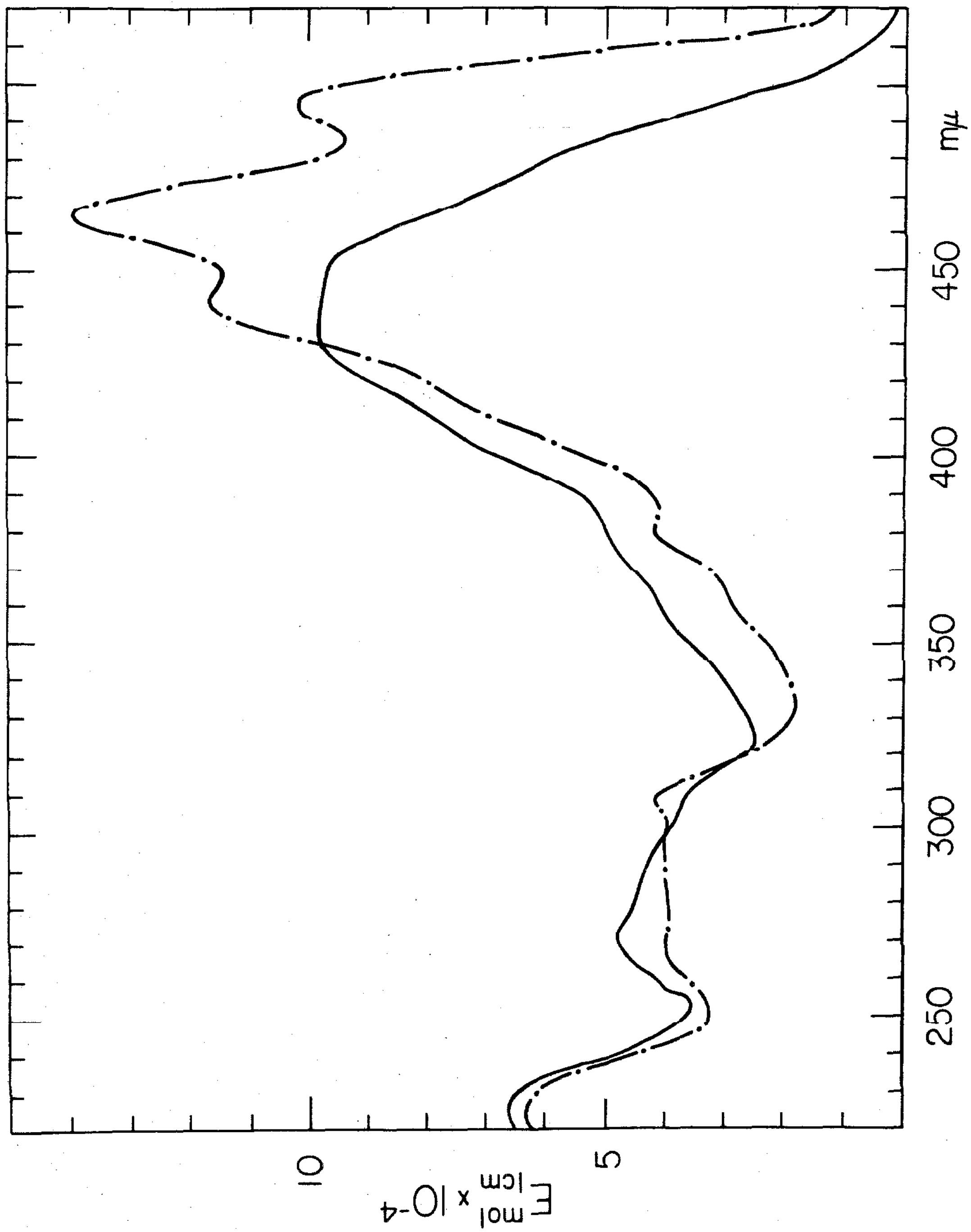


Figure 8. Molecular extinction curve in hexane of neo isomer of dehydro-β-carotene formed by fusion of the all-*trans* form: ——, fresh solution -·-·-·, after iodine catalysis in light.

B. EXPERIMENTAL PART

1. Materials and Methods.

In all of the experimental work reported in this Thesis, certain standard techniques, equipment, and reagents were used. In order to eliminate undue repetition a description of these is given in this section which applies to all the work herein, unless stated otherwise.

a. Adsorbents and Solvents.

The brand of calcium hydroxide used was Sierra Hydrated Lime, Superfine, United States Lime Products Corp., Los Angeles. In order to obtain an increased flow rate in chromatograms this material was mixed with Celite, No. 545, Johns-Manville Co. The term lime-celite refers to a 2:1 mixture (by weight). The alumina used was Activated Alumina, Grade F, 80-200 mesh, Aluminum Ore Co., East St. Louis, Ill., reground to 200 mesh. When the flow rate of some reground batches was very low, the alumina was mixed with celite (4:1 by weight). In general, the developed column was removed by extrusion with a wooden pestle.

For chromatographic work hexane (commercial grade), acetone (C.P.-USP), and benzene (Reagent Grade) were used. Acetone-hexane or benzene-hexane mixtures were often used as developers. The figures given in these cases (e.g., "5% acetone in hexane") refer to volume percent. Acetone was used for all elutions; in some instances acetone-methanol 5:1 was employed in order to elute strongly adsorbed pigments. For crystallizations only reagent grade solvents were used (except for hexane, which was commercial grade). For spectroscopic work in the ultraviolet region a purified hexane was prepared. Phillips' commercial grade was treated with fuming sulfuric acid until its optical density (after washing and a final distillation) was close to that of distilled water in the region 200-300 mμ.

b. General Notes.

Evaporations and concentrations were performed in <u>vacuo</u> (water aspirator) while a stream of pure dry nitrogen was bubbled through the solution. Sintered glass funnels were used for elutions, and where possible, all-glass apparatus was used for evaporations, washings, etc.

Visual spectroscopic readings were made with an Evaluating Grating Spectroscope, Zeiss (Loewe-Schumm modification) with light filter BG-7. Extinction curves were taken with the Beckman photoelectric spectrophotometer, Model DU. The quantitative extinction curves are based on two or more independent weighings.

All melting points were taken on an electrically heated Berl block in evacuated capillaries and are corrected.

2. <u>Preparation of Dehydro-β-carotene.</u>

a. β-carotene

The β-carotene used was obtained from a crude crystalline carotene preparation available in 1 gm. ampules from Barnett Laboratories, Long Beach, Calif. Each gram of this material was chromatographed on lime-celite from a hexane solution, in a large conic percolator, (48 x 21 x 8 cm.) or, alternatively, on six, 30 x 8 cm. lime-celite columns. Except for minor zones near the top only β-carotene (upper main zone) and α-carotene were observed in these chromatograms (ratio, 3:1). After development with hexane was complete, the percolator was inverted and the adsorbent cake released by gentle tapping of the glass walls. The β-carotene zone was cut out and eluted. The pigment was then transferred to hexane by the addition of water and the solution thoroughly washed in the LeRosen apparatus (23). After drying over anhydrous sodium sulfate, the solution was evaporated to dryness and the residue crystallized from the minimum amount of hot benzene or chloroform by the addition of methanol (4 to 5 volumes). The crystals were centrifuged, washed once in the centrifuge tube with hot methanol and then dried in the Abderhalden apparatus at 55° and 0.1 mm. pressure for 1 hour. Yield, 350-400 mg.

In some other experiments the commercial crude carotene mixture was twice recrystallized from benzene-methanol (or chloroform-methanol) without a preliminary chromatographic isolation of pure β-carotene. Such samples were found to contain 70% β-carotene and were used directly for the preparation of dehydro-β-carotene since the α-carotene content did not affect the next step. One gram of crude carotene crystals gave about 650 mg. of twice crystallized product.

b. Formation of Kuhn's "Isocarotene" from β-carotene.

The method described here is a modification of that given by Kuhn and Lederer (21). Although a crystalline preparation containing 70% β-carotene (see above) was used in later experiments, only those experiments which were based on the use of a pure crystalline β-carotene for the preparation of dehydro-β-carotene will be described in detail. The use of the 70% β-carotene mixture merely involved reducing the amounts of all necessary solvents and reagents by 1/3 for a given amount of starting material.

To 500 ml. of a solution of 800 mg. of iodine in hexane, contained in a 3 l. round bottom flask and cooled to -10°, was added, with good mechanical stirring, a solution of 800 mg. of β-carotene in 1 liter of hexane, also cooled to -10°. The β-carotene was added over a one minute period and stirring was continued for 2 more minutes. The black precipitate of β-carotene iodide was then filtered off without suction on a large fluted filter paper (diam., 40 cm.) and washed into $1\frac{1}{2}$ l. of acetone with $\frac{1}{2}$ l. of the same solvent. Occasional swirling was necessary to bring all the iodide into solution. The dark brown solution was allowed to stand at room temperature for 20-30 min. The solution was then transferred to a large separatory funnel and vigorously shaken with a solution of 5 gm. of sodium thiosulfate in 250 ml. of water. This last procedure caused the solution to turn red. Four hundred milliliters of hexane was then added followed by sufficient water to transfer nearly all of the pigment to the epiphase. The aqueous-acetone hypophase, which remained somewhat yellow was discarded. The hexane layer was washed in the continuous apparatus and dried. After evaporating to dryness, the bright red, powdery residue was transferred to a centrifuge tube with the minimum amount of hot benzene (50°) and crystallized out by the dropwise addition of warm methanol. Yield, 250-300 mg. of the pigments constituting the "isocarotene" described by Kuhn and Lederer.

c. Chromatographic analysis of Kuhn's "Isocarotene".

Eighty-two milligrams of recrystallized "isocarotene" (m.p. 172°) was dissolved in 150 ml. of hexane and developed on a 30 x 8 cm. lime-celite column with 8% acetone in hexane. A description of the chromatogram appears below (figures to left indicate width of zones in mm.):

4	reddish-brown	
45	interzone	
70	orange-red - - - - -	Zone I
2	interzone	
30	reddish-orange - - -	Zone II
2	interzone	
35	reddish-orange - - -	Zone III
15	interzone	
5	pale yellow - - - -	Zone IV
	Filtrate (colored)	

Each of the numbered zones was cut out, eluted, and transferred with water to hexane. The colored filtrate, which amounted to several hundred milliliters, was washed free of acetone, then, after drying, concentrated to about 50 ml. and developed with hexane on a 30 x 8 cm. lime-celite column.

1	brownish	
60	several yellowish zones (traces)	
20	yellow - - - - - - -	Zone V
3	interzone	
40	orange-brown - - - -	Zone VI
6	interzone	
10	orange-yellow - - -	Zone VII
5	interzone (slightly colored)	
10	yellow - - - - - - -	Zone VIII

The numbered zones from the above chromatogram were also cut out, eluted and transferred to hexane. The amount of pigment in each zone was estimated photometrically on the basis of previously determined molecular extinction coefficients (see the section below for the characterization of individual dehydro-β-carotene isomers). The composition of the "isocarotene" produced in this typical experiment was as follows:

Fraction I: All-*trans*-dehydro-β-carotene: 16% of "isocarotene" mixture.

Fraction II: Neo-dehydro-β-carotene A: 15%

Fraction III: Neo-dehydro-β-carotene D: 10%

Fraction IV: A minor stereoisomer of dehydro-β-carotene: 0.4%

Fractions V-VII: Unreacted β-carotene and some of its stereoisomers: 46%

Starting material unaccounted for: 13%

A chromatographic resolution of "isocarotene" without preliminary recrystallization showed approximately the same composition:

All-*trans*-dehydro-β-carotene:	11%
Neo-dehydro-β-carotene A:	12%
Neo-dehydro-β-carotene D:	8%
Other stereoisomeric dehydro-β-carotenes:	1%
Unreacted-β-carotene (including stereoisomers):	50%
Unaccounted for:	18%

d. Isolation and characterization of all-*trans*-dehydro-β-carotene and two crystalline stereoisomers (neo A and D).

For the purpose of isolating pure all-*trans*-dehydro-β-carotene and some stereoisomers, the hexane solution, obtained from 800 mg. of β-carotene by its reaction with iodine and subsequent treatment with thiosulfate and acetone etc. as described in section b., was concentrated to 300 ml. and developed directly on lime-celite with 6% acetone in hexane. Two 30 x 8 cm. columns were required, the corresponding zones of which were combined.

6	brownish-red	
4	interzone	
4	yellow	
15	interzone	
60	orange-red - - - - - -	Zone I
2	interzone	
65	reddish-orange - - - -	Zone II
2	interzone	
50	orange - - - - - - - -	Zone III
12	orange-yellow	
4	yellow	
3	interzone	
5	purple	
5	interzone	
10	pale yellow	

	Filtrate	

Each of the zones I-III was cut out, eluted and transferred to hexane. The three fractions were crystallized as described below for the case of the all-<u>trans</u>-dehydro-β-carotene fraction (from Zone I).

The solution was evaporated to dryness and the dark red, crystalline residue transferred to a 15 ml. centrifuge tube with a few milliliters of warm chloroform. (Fractions II and III which are more easily soluble than the <u>trans</u> form may be transferred with warm benzene. The centrifuge tube was placed in a bath at 50° and the chloroform completely evaporated by directing a gentle stream of dry nitrogen at its surface through a capillary tube. The residue was then dissolved in the minimum amount of benzene (about 2 ml.) at 50° and crystallized with constant stirring by slow dropwise addition of methanol down the walls of the centrifuge tube. Thus, the methanol was warmed to about the bath temperature before reaching the benzene solution. Dark purple-red, glittering crystals of dehydro-β-carotene appeared after a few drops of methanol had been introduced, but the addition was continued until the total volume amounted to 4-5 milliliters. (Fractions II and III crystallized less easily and required somewhat larger amounts of methanol.) The tube was removed from the bath and allowed to stand at room temperature for 1 hr. The crystals were then sharply centrifuged and dried in an Abderhalden apparatus at the temperature of refluxing acetone and 0.1 mm. pressure for 1 hr.

Yields: 28 mg. of the all-trans form (plus 2 mg. in mother liquor); 48 mg. of neo A form (plus 15 mg. in mother liquor); 18 mg. of neo-dehydro-β-carotene D (plus 15 mg. in mother liquor). All the unreacted β-carotene (about 300 mg.) was collected in the filtrate of the chromatograms. Thus about 25% of the 800 mg. of β-carotene which had reacted had been converted to dehydro-β-carotenes; the 400 mg. of β-carotene which was unaccounted for had evidently been converted to colorless destruction products and, to a lesser degree, to unidentified pigments.

Characterization of the crystalline products.

1.) All-trans-dehydro-β-carotene.

The compound crystallized from benzene-methanol in long red plates with characteristically cut ends (Fig. 9). Macroscopically, the glittering crystals were purplish red in color.

Melting point: 192-193° (corr.).

Solubility: Sparingly soluble in hexane, more easily in benzene or (especially) chloroform. Insoluble in methanol.

Partition test: On partitioning between hexane and 95% methanol the compound is found entirely in the epiphase.

Analysis: Calculated for $C_{40}H_{54}$: C, 89.83; H, 10.17. Found: C, 89.80, 89.91; H, 9.64, 9.95.

Spectral maxima, in hexane: 504, 474 mμ. (visual); 502, 471, 445 mμ. (Beckman).

Molecular extinction coefficient (at λ max): $E^{\text{mol.}}_{\text{1cm.}} = 16.6 \times 10^4$

(in hexane).

Chromatographic behavior: On lime-celite the compound requires 6-8% acetone in hexane for development. Its zone was located well above that of β-carotene but below lycopene on this adsorbent.

2.) Neo-dehydro-β-carotene A.

This isomer crystallized from benzene-methanol in red diamond shaped plates (Fig. 10). Macroscopically, the crystals had a glittery, dark red appearance.

Melting point: 161-163° (corr.).

Solubility: More soluble in hexane, benzene, and chloroform than the all-trans form but less so than β-carotene. Practically insoluble in methanol.

Partition test: Completely epiphasic.

Analysis: Calculated for $C_{40}H_{54}$: C, 89.83; H, 10.17. Found: C, 89.67, 89.70; H, 10.31, 10.22.

Spectral maxima, in hexane: 500, 470 mμ. (visual); 497, 467, 442 mμ. (Beckman).

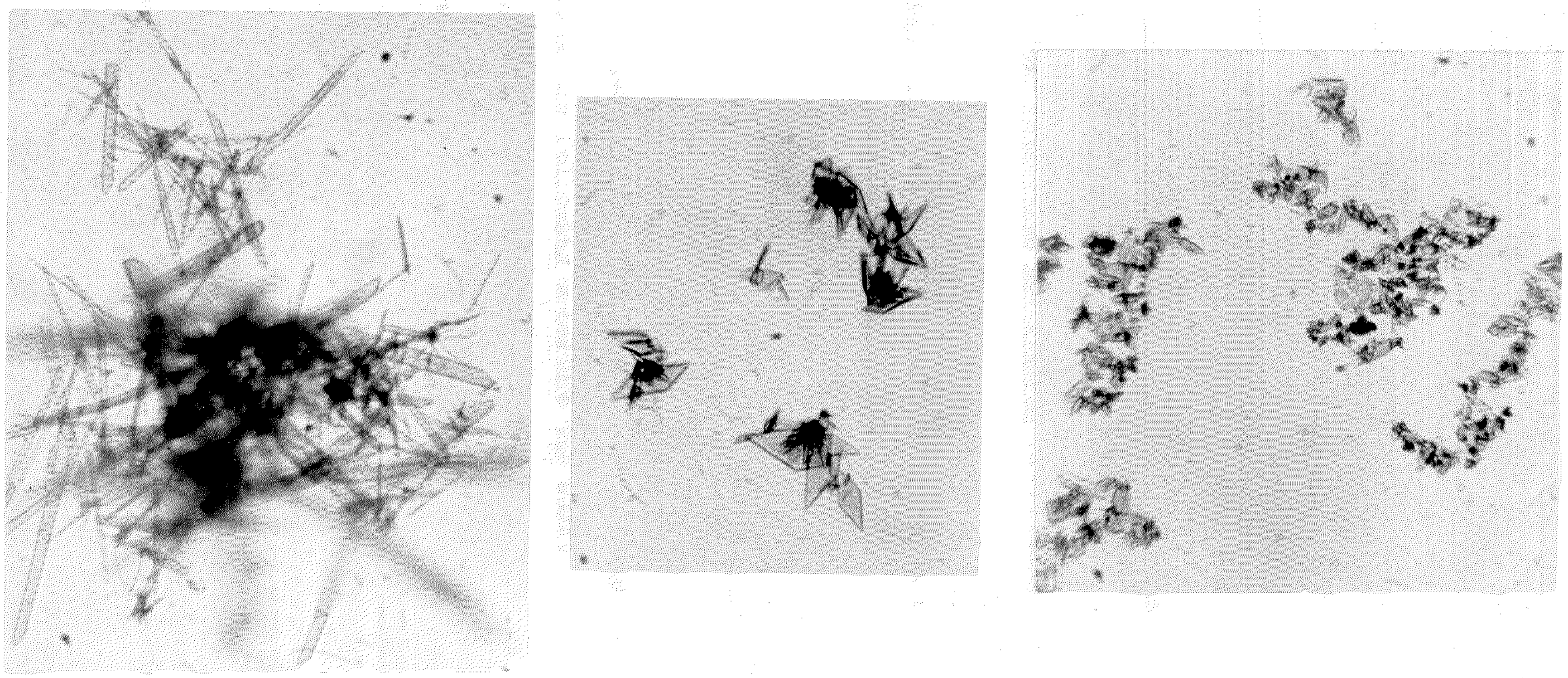
Molecular extinction coefficient (at $\lambda_{max.}$): $E^{mol.}_{1cm.} = 16.0 \times 10^4$ (hexane).

Chromatographic behavior: Separates from all-trans form on lime-celite upon developing with 6-8% acetone in hexane and occupies a lower position than the trans isomer.

3.) Neo-dehydro-β-carotene D.

This isomer crystallized from benzene-methanol in almond shaped crystals (Fig. 11); the individual crystals were smaller than the corresponding units of the trans or neo A forms.

Melting point: 177-178° (corr.)



Figures 9-11 (left to right): All-<u>trans</u>-dehydro-β-carotene, neo-dehydro-β-carotene A; and neo-dehydro-β-carotene D crystallized from benzene-methanol. Magnification 430 x.

Solubility: Easily soluble in hexane, benzene, and chloroform. Almost insoluble in methanol.

Partition test: When partitioned between hexane and 95% methanol only a trace of coloration is imparted to the hypophase.

Analysis: Calculated for $C_{40}H_{54}$: C, 89.83; H, 10.17. Found: C, 89.02, 89.66; H, 10.21, 10.07.

Spectral maxima, in hexane: 497, 467 mμ (visual); 494, 464, 440 mμ. (Beckman).

Molecular extinction coefficient (at λ max.): $E^{mol.}_{1cm.} = 15.6 \times 10^4$ (hexane).

Chromatographic behavior: This isomer separates from, and is located below, the neo A form when developed on lime-celite with 6-8% acetone in hexane.

3. Stereoisomerization of dehydro-β-carotene.

a. Iodine catalyzed stereoisomerization.

In all quantitative studies of the *cis-trans* isomerization of dehydro-β-carotene the following procedure was used:

A weighed sample of the pigment was dissolved in hexane to give a concentration of 10-15 mg./100 ml. A few drops of a solution of iodine in hexane, calculated to contain about 1% of the pigment weight, was then added and thoroughly mixed with the solution in a suitably sized Pyrex volumetric flask. The flask was then placed at a distance of 60 cm. from a pair of 3500° Mazda white fluorescent lamps (40 watts; 120 cm. long) and illuminated for 30 minutes. After illumination the mixture of isomers formed was resolved chromatographically and the amount of the individual components determined photometrically. Since the molecular extinction coefficients of only the three crystalline steric forms of dehydro-

β-carotene were determined quantitatively, those of the other stereoisomers had to be determined indirectly on the basis of the known extinction value for the iodine catalyzed stereoisomeric mixture ($E^{mol.}_{1cm.} = 14.0 \times 10^4$ at $\lambda_{max.}$, in hexane). Just before taking the absorption spectra of the various *cis*-*trans* isomers they were rechromatographed and any minor zones found on the column were eliminated. All the neo isomers except A and D were found to be very susceptible to thermal isomerization, even at room temperature.

Iodine catalysis of all-trans-dehydro-β-carotene. Typical experiment: A solution of 11.7 mg. of the pigment in hexane was isomerized and then developed on a 28 x 5.8 cm. lime-celite column with 8% acetone in hexane. In the description of the chromatogram which follows the figures to the right indicate the quantities of the individual isomers expressed in % of the starting material. The spectral data refer to the positions of the main maxima in hexane solution as determined with the Beckman spectrophotometer.

1	yellow-brown			
45	interzone			
28	orange-red	(all-*trans*)	502, 471 mμ.	17%
4	interzone			
30	reddish-orange	(neo A)	497, 467	28%
2	yellow	(neo B)	494, 463	4%
2	interzone			
20	yellow-orange	(neo C)	491, 461	10%
10	orange to pink	(neo D)	494, 464	12%
4	interzone			
8	yellow	(neo E)	487, 457	4%
1	interzone			
6	orange	(neo F)	489, 459	6%
3	interzone			
7	pale yellow	(neo G)	482, 453	3%
3	interzone			
6	very pale orange	(neo H)	479, 452	1%

15% of the starting material could not be accounted for.

Each of the pigments was shown to be a dehydro-β-carotene and not a structurally altered product by treating the hexane solution with a trace of iodine and recovering (chromatographically) the all-<u>trans</u> form of dehydro-β-carotene. The latter was identified by its spectrum and in the mixed chromatogram test.

The isomers termed neo C and neo D could not be separated well enough on lime-celite to permit an accurate determination of their amounts. It was found, however, that complete separation could be achieved on alumina when developed with a benzene-hexane mixture, 3:1. It is interesting that in the latter chromatogram the relative positions of the two isomers were inverted.

The neo A and neo B forms do not separate well either, even on alumina columns. In this case, since the amount of the neo B isomer formed was small, any errors made in its quantitative estimation could not have significantly affected the accuracy of the overall analysis of the stereoisomeric mixture. For purposes of spectral readings and extinction estimations the top part of the neo B zone was neglected when it was cut out of the column; thus the possibility of its contamination by the neo A isomer was minimized.

<u>Iodine catalysis of neo-dehydro-β-carotene A.</u> Typical experiment: 8.7 mg. of the carotenoid in hexane was isomerized and then developed on a 28 x 5.8 cm. lime-celite column with 8% acetone in hexane.

1	yellow-brown		
35	interzone		
20	orange-red	(all-*trans*)	18%
2	interzone		
28	reddish-orange	(neo A)	30%
2	yellow	(neo B)	5%
3	interzone		
20	yellow	(neo C)	11%
8	orange (with pinkish leading edge)	(neo D)	15%
2	interzone		
6	pale yellow	(neo E)	6%
3	interzone		
6	pale orange	(neo F)	6%
5	interzone		
10	very pale yellow	(neo G)	2%
6	interzone		
10	very pale buff	(neo H)	1%

Starting material unaccounted for, 7%.

Iodine catalysis of neo-dehydro-β-carotene D. Typical experiment: 13.9 mg. of the carotenoid in hexane solution was isomerized and then chromatographed as stated for the neo A form.

1	yellow-brown		
40	interzone		
25	orange-red	(all-*trans*)	17%
3	interzone		
25	reddish-orange	(neo A)	30%
3	yellow	(neo B)	3%
2	interzone		
15	yellow	(neo C)	10%
10	orange (with pinkish leading edge)	(neo D)	15%
3	interzone		
8	yellow	(neo E)	5%
2	interzone		
5	orange	(neo F)	3%
5	interzone		
8	very pale yellow	(neo G)	1%
5	interzone		
10	very pale buff	(neo H)	1%

Unaccounted for: 16%

b. Stereoisomerization by fusion.

A sample of the carotenoid (15-17 mg.) was sealed, *in vacuo*, in a glass tube (diam., 7 mm.) and kept in an oil bath at 200° for 1½ minutes.

(A longer period of immersion led to excessive destruction.) After cooling in ice water the tube was opened; the contents were dissolved in 2-3 ml. of benzene and diluted to 50 ml. with hexane. This solution was developed on a lime-celite column with 6% acetone in hexane. The composition of the mixture was determined in the same way as stated above for the isomerization by iodine catalysis.

Isomerization of all-*trans*-dehydro-β-carotene. Typical experiment: The hexane solution obtained from the fusion of 15 mg. of compound was developed on a 24 x 4.8 cm. lime-celite column with 6% acetone in hexane. Figures to the right show percentage composition.

1	yellow		
4	brownish-red		
20	interzone		
18	orange-red	(all-*trans*)	8%
2	interzone		
15	reddish-orange	(neo A)	14%
1	yellow	(neo B)	2%
3	interzone		
12	yellow-orange	(neo C)	12%
4	orange-pink	(neo D)	12%
10	yellow	(neo E)	3%
1	interzone		
5	pale orange	(neo F)	1%
4	interzone		
8	very pale yellow	(neo G)	1%
5	interzone		
8	very pale buff	(neo J, possibly a poly-*cis* isomer)	1%
	Filtrate	pale yellow-green	

All the fractions, except the all-*trans* and neo A forms, had to be rechromatographed before the spectra were taken. After elution and transfer into hexane, some contaminants absorbing in the ultraviolet regions were eliminated by repeatedly washing with 95% methanol.

Some larger scale experiments were undertaken to obtain the "poly-*cis*" isomer in amounts sufficient for crystallization; however, this substance could not be crystallized since all attempts led to almost complete stereoisomerization.

The pigment collected in the filtrate did not contain any dehydro-β-carotene isomers and was, undoubtedly, a decomposition product of the starting material. In all, 46% of the all-*trans*-dehydro-β-carotene was unaccounted for.

Stereoisomerization of neo-dehydro-β-carotene A. It was found that a temperature of 170° was not sufficiently high to cause the formation of any "poly-*cis*" isomer, whereas at a temperature of 200° approximately 1% of the starting material appeared in the "poly-*cis*" form. A detailed chromatographic analysis of the isomeric mixture, beyond the estimation of "poly-*cis*" compound, was not undertaken. Qualitatively, the chromatogram was very similar to that shown in the case of the all-*trans* melt.

As was demonstrated in the theoretical section of this Part, the "poly-*cis*"-dehydro-β-carotene has a degraded spectrum but develops the usual maxima upon iodine catalysis.

Stereoisomerization of neo-dehydro-β-carotene D. A melt isomerization of this isomer was undertaken with 17 mg. of substance to ascertain whether a "poly-*cis*" isomer was formed. This was found to be the case; the yield amounts to approximately 1% of the starting material upon fusion at 200° for 1½ minutes.

Isomerization of a stereoisomeric mixture of dehydro-β-carotenes. A "poly-*cis*" form was obtained also by fusion of a sample of Kuhn's "isocarotene", from which all β-carotene had been removed chromatographically. Again, only about 1% of this form was isolated.

c. Stereoisomerization by refluxing solutions.

The general procedure was as follows:

A dilute solution of the carotenoid in hexane was refluxed in an all glass apparatus for 1 hour. The heating source was a Glas-Col heating mantle and the voltage was adjusted so that the hexane vapors rose about 1 inch in the condenser. The flask and condenser were covered with a black cloth to exclude light. The isomerized solution was resolved chromatographically and its composition determined spectrophotometrically.

Stereoisomerization of all-*trans*-dehydro-β-carotene. The refluxed solution of 8.5 mg. of substance in 200 ml. of hexane was developed on a 28 x 5.8 cm. lime-celite column with 6% acetone in hexane.

2	brownish		
45	interzone		
45	orange-red	(all-*trans*)	53%
2	interzone		
25	pale orange	(neo A)	24%
2	interzone		
10	pale yellow	(neo D)	4%
2	interzone		
4	very pale yellow	(neo E)	2%

Seventeen percent of the starting material used was not recovered.

Stereoisomerization of neo-dehydro-β-carotene A. The starting material amounted to 4.2 mg. and was dissolved in 100 ml. of hexane. The isomerized solution was developed on a 24 x 4.8 cm. lime-celite column as stated above:

1	orange-brown		
25	interzone		
6	orange-red	(all-*trans*)	5%
1	interzone		
40	reddish-orange	(neo A)	50%
2	yellow	(neo B)	4%
2	interzone		
8	pale orange	(neos C and D)	16%
8	interzone		
15	yellow-brown	(neo E)	10%
2	interzone		
8	very pale orange	(neo F)	2%

Thirteen percent of the starting material could not be accounted for.

Stereoisomerization of neo-dehydro-β-carotene D. 8.0 mg. of substance was dissolved in 200 ml. of hexane. The refluxed solution was developed on a 28 x 5.8 cm. lime-celite column as stated above.

1	yellowish		
30	interzone		
10	pale orange	(all-*trans*)	2%
4	interzone		
20	pale orange	(neo A)	13%
5	interzone		
45	orange	(neo D)	70%
1	interzone		
10	yellow	(neo E)	8%

Only 7% of the starting material could not be accounted for.

d. Photochemical stereoisomerization.

In each of the experiments a solution of 2 mg. of pigment in 25 ml. of hexane, contained in a Pyrex volumetric flask, was illuminated for 8 hours at a distance of 30 cm. from the light source described in the section on iodine catalyzed stereoisomerization. After illumination the solutions were developed on 20 x 3.8 cm. lime-celite columns with 6% acetone in hexane.

Stereoisomerization of all-*trans*-dehydro-β-carotene.

1	pale yellow		
15	interzone		
55	orange-red	(all-*trans*)	84%
1	interzone		
8	pale reddish-orange	(neo A)	8%
2	interzone		
5	pale yellow-orange	(neo D)	2%

Six percent of the starting material was unaccounted for.

Stereoisomerization of neo-dehydro-β-carotene A.

1	pale yellow-brown		
20	interzone		
8	pale orange	(all-*trans*)	5%
1	interzone		
50	reddish-orange	(neo A)	86%
2	interzone		
6	pale yellow-orange	(neo D)	3%

Six percent of the starting material was unaccounted for.

Stereoisomerization of neo-dehydro-β-carotene D.

1	pale yellow		
20	interzone		
5	very pale pink	(all-*trans*)	1%
2	interzone		
6	pale orange-red	(neo A)	6%
3	interzone		
35	orange	(neo D)	91%

Two percent of the starting material was unaccounted for.

II. A STUDY OF THE INTERACTION OF BORON TRIFLUORIDE WITH β-CAROTENE.

A. THEORETICAL PART

1. Introduction.

Twelve years ago H. H. Strain (30) reported that some carotenoids in carbon tetrachloride solutions were converted by boron trifluoride into "unstable blue pigments" which, by treatment with alcohol, were reconverted to yellow pigments that "did not exhibit definite absorption maxima". As far as is known, there is no further literature on this subject.

It will be shown that a brief treatment of β-carotene in hexane solution with boron trifluoride leads principally to its cis-trans isomers*, while a more prolonged treatment with this reagent completely converts the starting material to other products, some colorless. Two of the pigments present in the complex reaction mixture obtained from the latter experiment have been isolated in crystalline form.

2. Stereoisomerization of β-Carotene by the Action of Boron Trifluoride.

When a hexane solution of β-carotene was shaken with boron trifluoride etherate for 1 to 5 minutes the reagent (hypo-) phase turned dark blue. Upon mixing the liquid with 95% methanol, the original orange-red color of the β-carotene solution reappeared. After transferring the pigments to hexane, chromatographic analysis showed that the β-carotene was extensively stereoisomerized. The results of a 1 minute and a 5 minute experiment are compared in Table 7. The extent of stereoisomerization within a given time was essentially constant, whether the experiments were performed in total darkness or in normal indoor lighting. The boron

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* Some preliminary observations were made in this laboratory by J. Leemann.

trifluoride catalyzed *cis-trans* isomerization of β-carotene, therefore, must proceed by means of a mechanism different from that of the light dependent iodine catalyzed isomerization, and is possibly related to acid catalyzed stereoisomerizations (28).

Table 7.

Pigment Mixture Obtained from β-Carotene by a Brief Treatment with Boron Trifluoride at Room Temperature.

(The figures refer to % of starting material as estimated spectrophotometrically.)

Component of the Mixture	Duration of Treatment	
	1 Min.	5 Min.
Neo-β-carotene U	19	17
All-*trans*-β-carotene	36	35
Neo-β-carotene A	6	6
Neo-β-carotene B	14	12
Other neo isomers of β-carotene	5	3
Pigments not belonging to the β-carotene set (estimated as "β-carotene")	1	2
Unaccounted for (including some fluorescent destruction products)	19	25

3. Some New Compounds Obtained from β-Carotene by Prolonged Treatment with Boron Trifluoride.

As mentioned above, in the boron trifluoride stereoisomerization experiments some pigments not belonging to the β-carotene set appeared in small quantities on the column; furthermore, a colorless product which showed blue fluorescence in ultra-violet light was collected in the chromatographic filtrate. Since the 5 minute treatment produced more of these new substances than did shorter experiments, the shaking time was extended for the purpose of further increasing the yields. It was found that upon shaking the hexane solution of β-carotene with the reagent for a full hour, the hexane phase became almost colorless and, after the addition of methanol, the recovered pigment mixture did not include either β-carotene or any of its steric forms. Subsequent chromatograms showed the presence of at least five new pigment zones. The main zone was much more weakly adsorbed than β-carotene and was collected in the filtrate together with the fluorescent substance previously mentioned. More strongly adsorbed were three pale, greenish-yellow zones, the upper two of which were located above β-carotene in the mixed chromatogram test (Table 8).

Pigment IV did not form a homogeneous zone and, except for demonstrating its non-identity with β-carotene in a mixed chromatogram test, was not investigated further.

Pigment I crystallized easily from benzene-methanol and melted sharply at 214° (corr.); its spectral curve is given in Fig. 12 .

Pigment II (Fig. 13) was established to be a <u>cis</u> form of I; because it was formed only in very small amounts no attempt was made to crystallize it.

Pigment III crystallized with some difficulty from benzene-methanol; it melted at 157-158° (corr.) (spectral curve, Fig. 14).

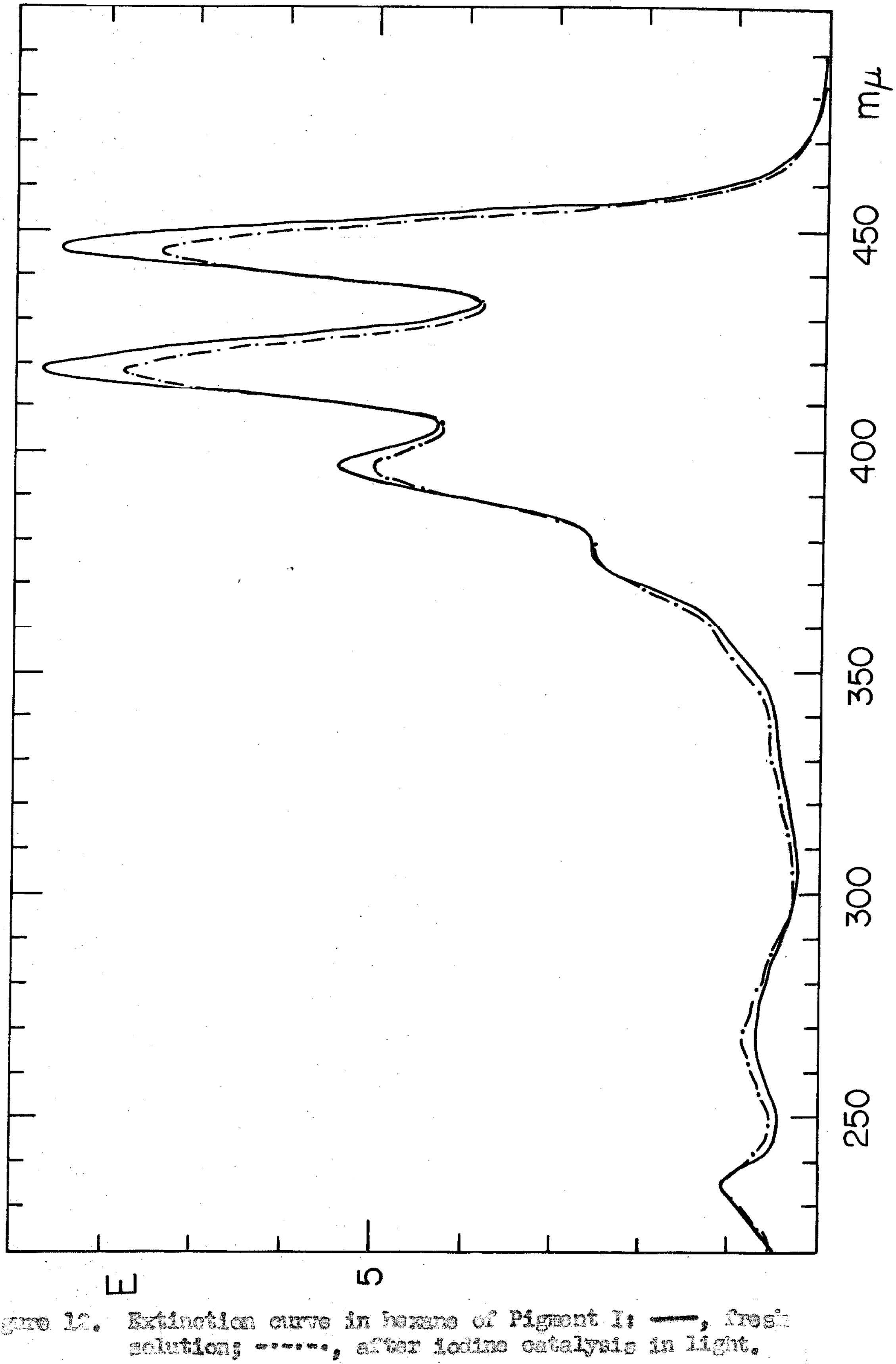


Figure 12. Extinction curve in hexane of Pigment I: ——, fresh solution; -·-·-·, after iodine catalysis in light.

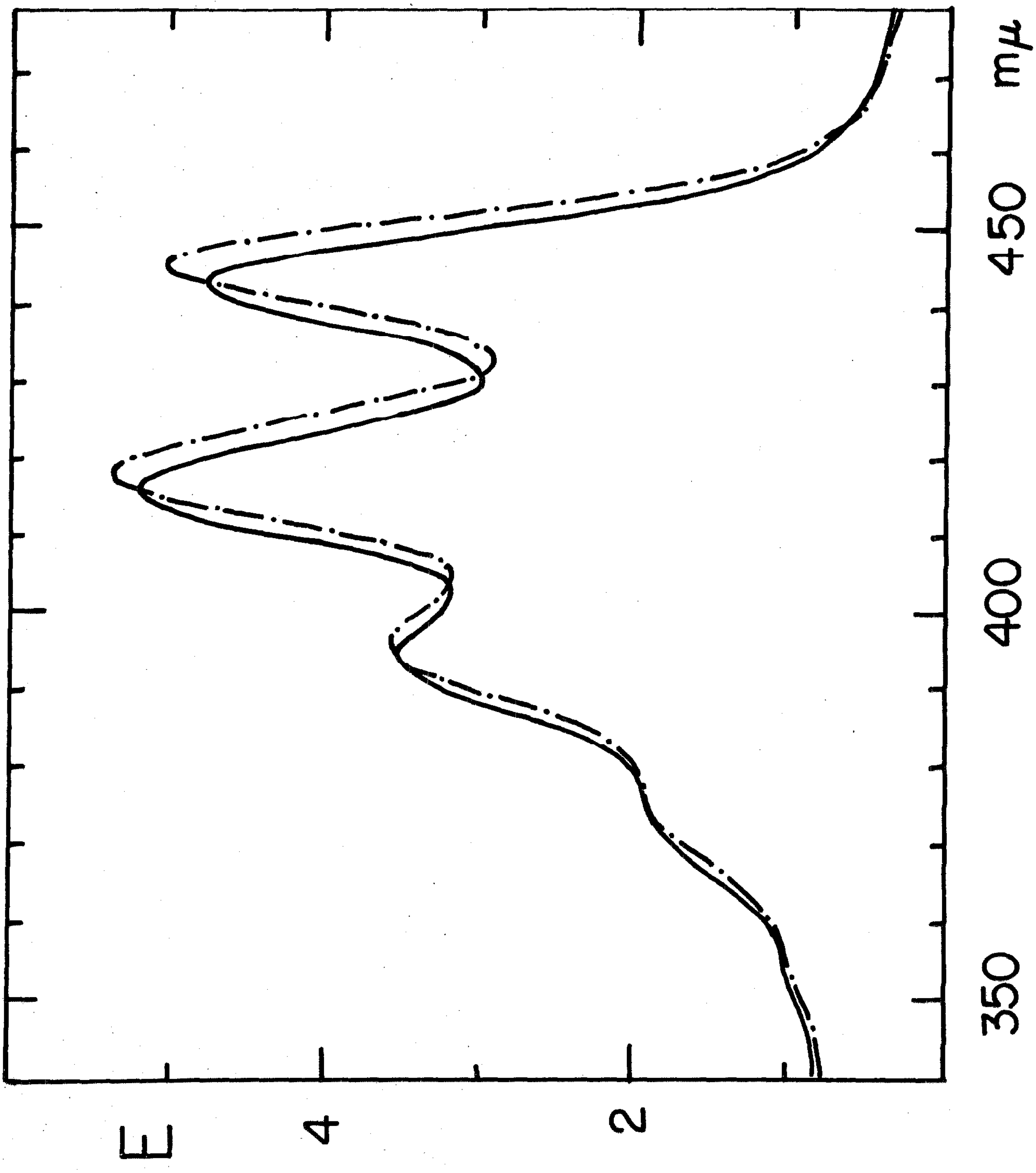


Figure 13. Extinction curve in hexane of Pigment II: ——, fresh solution; —·—·—, after iodine catalysis in light.

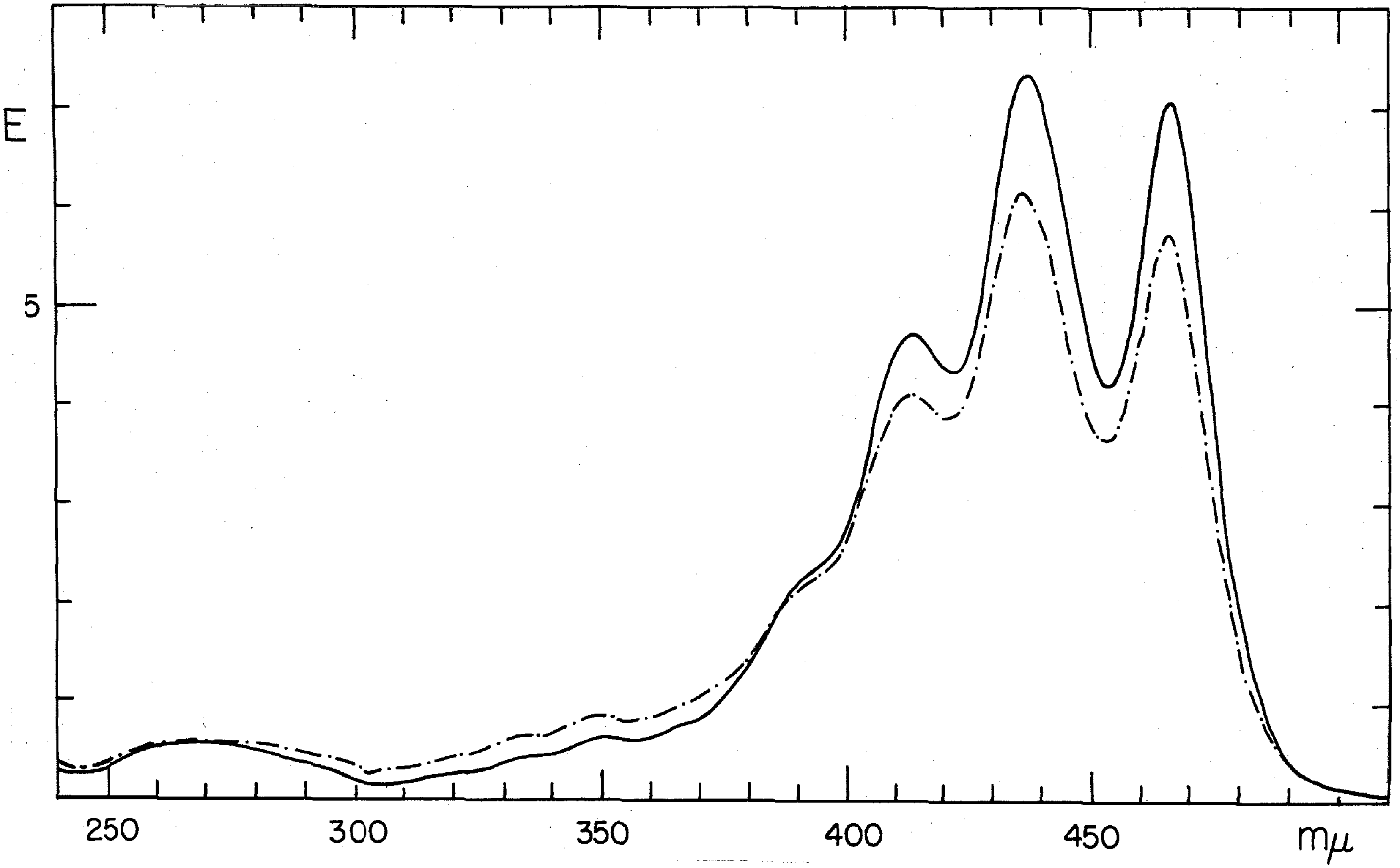


Figure 14. Extinction curve in hexane of Pigment III: ——, fresh solution; –·–·–, after iodine catalysis in light.

Table 8.

Visually Observed Spectral Maxima in Hexane of Some New Pigments Obtained from β-Carotene by Treatment with Boron Trifluoride and Subsequently with 95% Methanol.

(In descending order of adsorption on lime-celite)

Pigment I	448, 422 mμ. (very sharp maxima)
Pigment II	444, 419 mμ. (very sharp maxima)
Pigment III	468, 441 mμ. (very sharp maxima)
Pigment IV	483, 455, 428 mμ. (somewhat blurred maxima)

Analysis showed that Pigment I was not a hydrocarbon but had the approximate composition $C_{40}H_{56}O$. Pigment III, on the other hand, was a hydrocarbon whose analysis was indicative of the formula $C_{40}H_{56}$ or $C_{40}H_{54}$. Since, however, the positions of the spectral maxima of Pigments I and III indicate the presence of, respectively, 8 and 9 conjugated double bonds further experiments would be required for a structural clarification.

The colorless fluorescent zone mentioned was separated from some pigmented compounds by adsorption on alumina. The absorption curves as obtained from two different experiments are shown in Fig. 15. It may be noted that maxima at 228 mμ. are characteristic for a number of naphthalene derivatives (cf. the pertinent curves in (4)). The conversion of carotenoids to naphthalene derivatives is not unknown, Kuhn and Winterstein (22) having isolated 2,6-dimethylnaphthalene, among other compounds, in trace amounts by the thermal decomposition of these pigments.

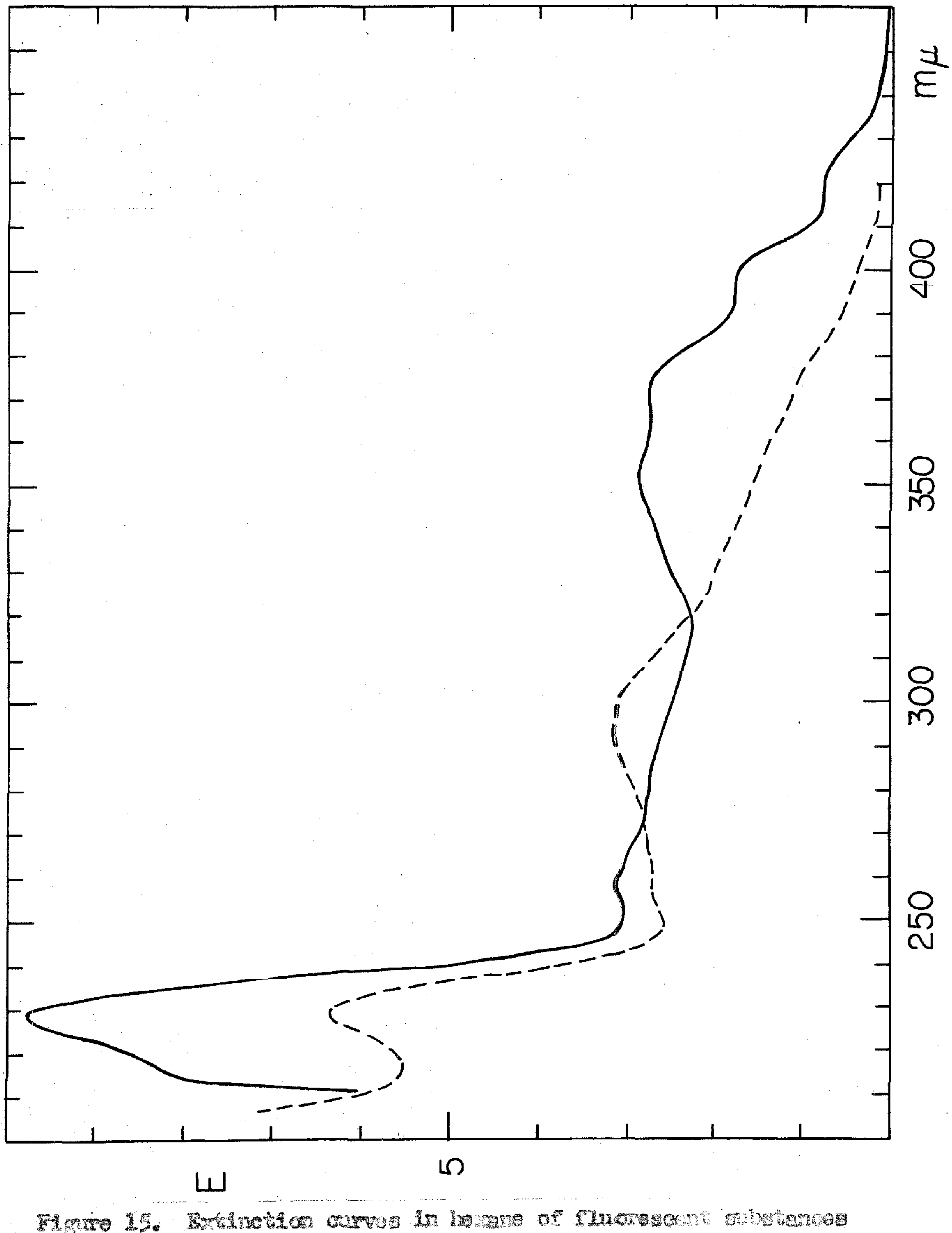


Figure 15. Extinction curves in hexane of fluorescent substances obtained in seperate experiments by the action of boron trifluoride on β-carotene.

B. EXPERIMENTAL PART

1. *Materials.*

The boron trifluoride etherate used was either the Eastman Kodak product, redistilled, or prepared by passing the gas into ethyl ether (Reagent Grade) as described in Part III. β-Carotene was prepared from a commercial carotene sample (Barnett Labs., Long Beach, Calif.) by crystallization of the chromatographically isolated product.

2. *Stereoisomerization Experiments.*

One minute experiment: A solution of 20 mg. of β-carotene in 25 ml. of hexane contained in a 50 ml. volumetric flask was shaken (by hand) with 2 ml. of the etherate for 1 minute. The reagent phase turned dark blue immediately. The contents were then added to 100-200 ml. of 95% methanol in an Erlenmeyer flask and swirled for a few seconds until a bright orange-red color appeared. The pigment mixture was transferred to the hexane phase by the addition of water and the colorless aqueous-methanol hypophase discarded. The solution was washed in the LeRosen apparatus for 10 minutes, dried over sodium sulfate, and developed with hexane on a 28 x 5.8 cm. lime-celite column (Figures to right indicate spectral maxima in hexane (Beckman)).

20	colorless	
3	orange	unidentified; 478, 451 mµ.
35	interzone	
50	yellow	neo-β-carotene U; 475, 447
45	orange	all-*trans*-β-carotene; 480, 452
15	pale yellow	neo-β-carotene A; 467, 440
25	orange-yellow	neo-β-carotene B; 470, 444
5	interzone	
5	orange	a *cis*-β-carotene; 468, 442
2	yellow	a *cis*-β-carotene; - , 442

	Filtrate	slightly fluorescent in ultraviolet light

Each zone was cut out, eluted, and transferred to hexane solution. The pigments obtained from the two bottom zones could not be identified with certainty but were definitely members of the β-carotene set as shown by visual observation of the spectral shift upon addition of iodine to their hexane solutions. The pigment of the top zone could not be identified either but all others were well-known members of the β-carotene set. The composition of the stereoisomerized mixture was determined spectrophotometrically; for this purpose the unidentified pigment adsorbed at the top of the column was assumed to have the β-carotene extinction coefficient at $\lambda_{max.}$. The following results were obtained (in % of the starting material): β-carotene, 36%; neo-β-carotene U, 19%; neo A, 6%; neo B, 14%, other cis-β-carotenes, 5%; unidentified pigment, 1%; unaccounted for, 19%.

Five minute experiment: A solution of 10 mg. of β-carotene in 25 ml. of hexane contained in a 50 ml. volumetric flask was vigorously shaken with 1 ml. of boron trifluoride etherate for 5 minutes. The liquid was then treated as described for the 1 minute experiment; the resulting hexane solution was developed with hexane on a 20 x 3.8 cm. lime-celite column. (See description of chromatogram on following page.)

Except for the appearance of another unidentified pigment zone, the chromatogram was similar to that obtained from the 1 minute experiment. It was noted, however, that the filtrate was more strongly fluorescent. The isomerized mixture showed the following composition (in % of the starting material): β-carotene, 35%; neo-β-carotene U, 17%; neo A, 6%; neo B, 12%; other cis-β-carotenes, 3%; unidentified pigments, 2%; unaccounted for, 25%.

20	colorless	
3	orange	unidentified; 478, 451 mμ.
15	interzone	
6	pale yellow	unidentified; 452
15	interzone	
40	yellow	neo-β-carotene U
2	interzone	
40	orange	all-trans-β-carotene
2	interzone	
10	pale yellow	neo-β-carotene A
15	orange-yellow	neo-β-carotene B
2	interzone	
5	pale orange	a cis β-carotene
2	pale yellow	a cis β-carotene
	Filtrate	fluorescent in ultraviolet light

3. Formation of Some Conversion Products of β-Carotene.

Each of six, 30 mg. samples of β-carotene, dissolved in 60 ml. of hexane in a 125 ml. glass stoppered Erlenmeyer flask was shaken mechanically with 3 ml. of boron trifluoride etherate for 1 hour. At the end of this period the hexane epiphase was almost decolorized while the etherate layer was dark blue. The contents of each flask were added to 200 ml. of 95% methanol and the pigment mixture transferred to the hexane phase by the addition of water. The six hexane solutions were then combined, washed, dried, and concentrated to 100 ml. This solution was then developed on two, 30 x 8 cm. lime-celite columns with hexane. (See description of chromatogram on following page.)

The corresponding zones from the two columns were combined, eluted and transferred to hexane. The two filtrates were also combined (cf. Section b).

2	reddish-brown	
30	interzone	
3	pale red	
10	yellowish-green	Pigment I
5	interzone	
10	very pale yellowish-green	Pigment II
35	interzone	
45	pale greenish-yellow	Pigment III
2	orange	
10	interzone	
75	pale yellow to pale yellow-orange	
60	several very pale blurred zones	

Filtrate, yellowish; strongly fluorescent in ultraviolet light

----

a. Treatment of the pigment zones.

1.) Pigment I. After evaporation of its hexane solution, the residue was dissolved in the minimum amount of hot benzene (1-2 ml.) and crystallized in a 5 ml. centrifuge tube by slow, dropwise addition of methanol at room temperature. Yield, 5 mg.

Melting point: 214° (corr.)

Solubility: Sparingly soluble in hexane; soluble in benzene but distinctly less so than β-carotene. Insoluble in methanol.

Partition test: When partitioned between hexane and 95% methanol the pigment was found completely in the epiphase.

Analysis: C, 86.90; H, 10.33.

Chromatographic behavior: Moved slowly on lime-celite with hexane; adsorbed well above β-carotene but below dehydro-β-carotene.

Crystal form: Pigment I crystallized easily from benzene-methanol in yellow rectangular plates (Fig. 16). Macroscopically, the crystals appeared dull orange in color.

2.) Pigment II had maxima in hexane at 444, and 419 mμ. (visual). After the addition of a trace of iodine the maxima shifted to 446, and 421 mμ. Chromatographic resolution of the isomerized solution showed the presence of a pigment that was chromatographically and spectroscopically indistinguishable from Pigment I. Hence, Pigment II was a cis form of I.

3.) Pigment III initially failed to crystallize from benzene-methanol. After it had been rechromatographed on alumina, it was possible to crystallize it by rapid addition of methanol to its warm benzene solution. At first, only a cloudiness appeared but crystals were formed after allowing the liquid to stand at 4° for a few hours. Yield, 5 mg.

Melting point: 158-159° (corr.)

Solubility: Very soluble in hexane and benzene; insoluble in methanol.

Partition test: Completely epiphasic in the hexane-95% methanol system.

Analysis: C, 89.33; H, 10.17

Chromatographic behavior: Adsorbed below but not easily separable from β-carotene.

Spectrum, in hexane: 468, 441 mμ. (visual); 467, 437, 414 mμ. (Beckman).

Crystal form: The crystals from benzene-methanol were light yellowish-orange in color, very small, and of rather irregular shape. They tended to form clumped aggregates (Fig. 17).

Chromatographic analysis of the iodine-treated hexane solution of Pigment III showed that the pigment had remained mainly unaltered. The

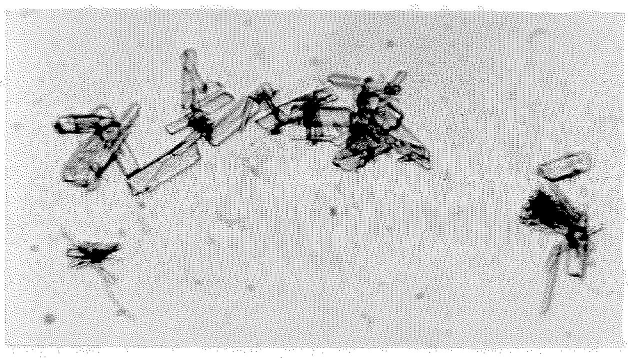


Figure 16 (top). Pigment I from benzene-methanol (430 x).

Figure 17 (bottom). Pigment III from benzene-methanol (430 x).

only other zone on the column had maxima in hexane at some 4 mμ. lower wave length than those of the starting material (Fig. 18). The location of the spectral maxima of this new pigment (462, 433, 410 mμ.) was unaltered by treatment with a trace of iodine; only a slight decrease in its extinction values could be observed.

4.) Pigment IV did not form a homogeneous zone on lime-celite but discrete zones could not be obtained. The visually observed maxima (blurred) in hexane were located at 483, 455, and 428 mμ. In the mixed chromatogram test this pigment was adsorbed well below β-carotene.

b. Treatment of the fluorescent filtrates.

The combined chromatographic filtrates (Sec. 3) were concentrated to 50 ml. After washing and drying, the yellowish-orange solution was developed with hexane on a 28 x 5.8 cm. alumina column. Under these conditions a weakly adsorbed colorless zone (detected by a dull blue fluorescence in ultraviolet light) was easily separated from the pigments, all of which were strongly adsorbed on the column. Only one fluorescent zone was observed. The latter was cut out, eluted, transferred to hexane and completely evaporated; all attempts to crystallize the very pale yellow, oily residue failed. In hexane solution the oil, which had an intense, blue fluorescence in ultraviolet light, showed a single distinct maximum at 228 mμ.

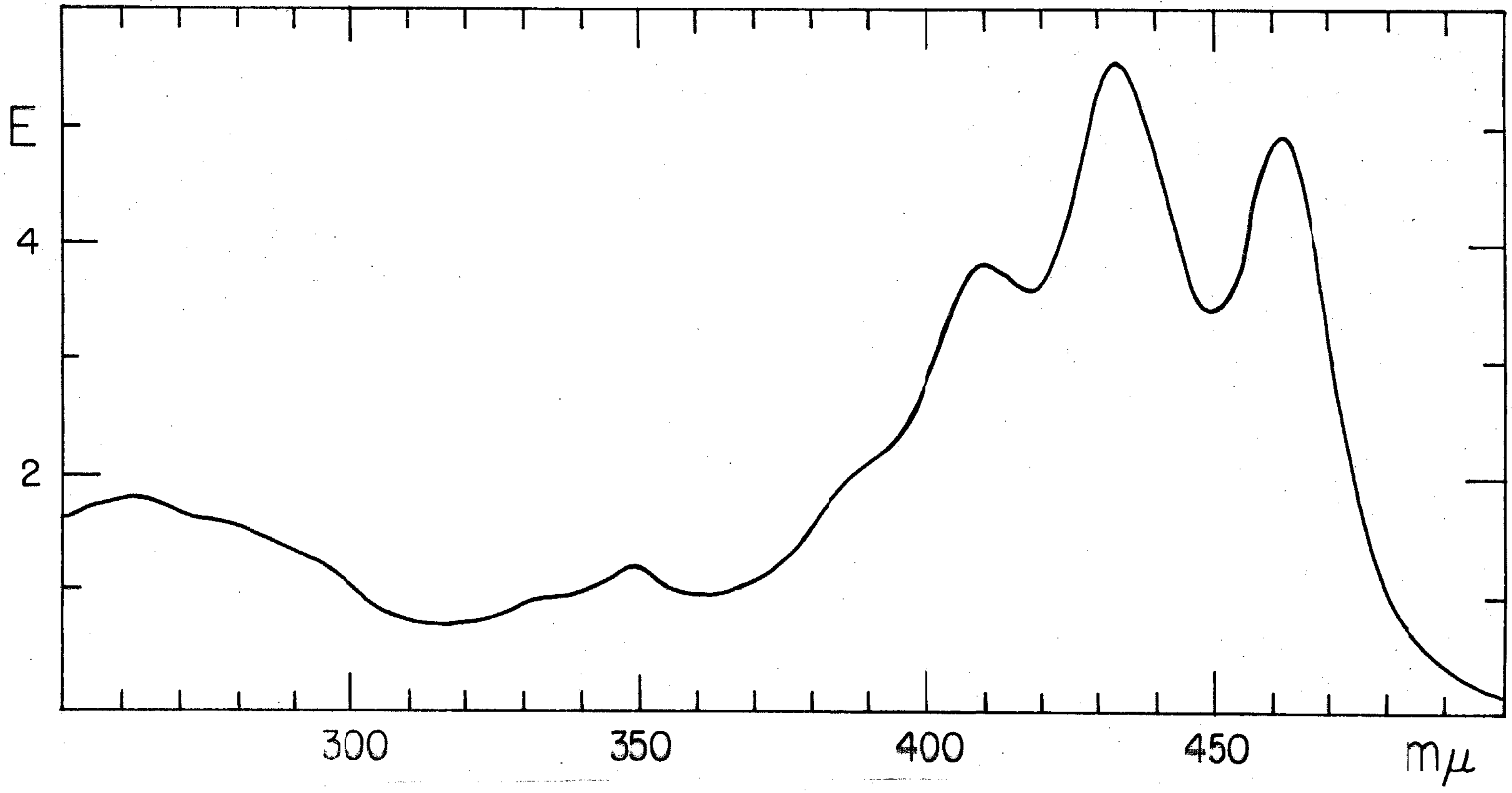


Figure 18. Extinction curve in hexane of substance formed irreversibly from Pigment III by iodine catalysis in light.

III. SOME NEW CAROTENOIDS PRODUCED BY THE ACTION OF WATER OR METHANOL ON THE BORON TRIFLUORIDE-DEHYDRO-β-CAROTENE COMPLEX.

A. THEORETICAL PART

1. Introduction.

When boron trifluoride etherate is added to a hexane solution of dehydro-β-carotene, $C_{40}H_{54}$, a dark blue or greenish-blue color appears in both phases owing to the formation of a complex between the two compounds. Complexes are also formed between boron trifluoride and other carotenoids, e.g., lycopene or β-carotene. Carotenoids, in general, also form blue-colored complexes with some other strongly electrophilic compounds such as antimony or arsenic trichloride (in chloroform solution) and with concentrated sulfuric acid. Furthermore, carotenoid epoxides (5) and carotenoid aldehydes, as well as polyhydroxylated carotenoids, form blue-colored complexes with concentrated HCl, also. The nature of many of these complexes is unknown; however, the β-carotene complex with antimony trichloride has been discussed by Meunier (24). In general, these complexes are broken by water or methanol.

As was shown in Part II, a few minutes treatment of β-carotene solutions with boron trifluoride etherate and subsequently with methanol led chiefly to stereoisomerization. It was found, however, that a similar brief treatment of dehydro-β-carotene did not yield stereoisomers of the starting material. When methanol was added a complicated mixture was obtained, one third of which consisted of colorless breakdown products not investigated in this Thesis. The colored components of the end product were found, by chromatographic analysis of the hexane solution, to consist of three main pigments (and their stereoisomers). These were β-carotene (18% of the starting material), a mono-methoxy-β-carotene $C_{40}H_{55}OCH_3$

(40%, half of it in the all-trans form and half in cis forms), and a dimethoxy-β-carotene, $C_{40}H_{54}(OCH_3)_2$ (6%). The latter two compounds were previously unknown.

When the dehydro-β-carotene-boron trifluoride complex was decomposed with water instead of methanol, a different mixture was obtained whose hexane solution was resolved chromatographically. The total quantity of pigment components in this instance amounted to about 45% of the starting material. The product contained β-carotene (12%, including stereoisomers), but the main pigment zone in the chromatogram was a mono-hydroxylated β-carotene, $C_{40}H_{55}OH$ (33%, about a third in cis forms).

The product obtained as mentioned was termed isocryptoxanthin, and those obtained from the methanol reaction, isocryptoxanthin methyl ether and dimethoxy-β-carotene, respectively. None of these compounds had been mentioned earlier in the literature.

2. Isocryptoxanthin (4-hydroxy-β-carotene).

Isocryptoxanthin (and its neo A isomer) were adsorbed well above all-trans-β-carotene on the lime-celite column. It crystallized easily from benzene-methanol. Its absorption spectrum was very similar to that of β-carotene (Fig. 19), which indicated identical chromophores. When partitioned between hexane and 95% methanol isocryptoxanthin was found to be about equally distributed between the two phases. Such distribution is a characteristic feature of mono-hydroxylated carotenoids. Analysis showed the composition, $C_{40}H_{56}O$. The compound was less soluble in hexane than β-carotene. As further confirmation of the hydroxylic nature of isocryptoxanthin, a well-crystallized acetyl derivative was prepared. As would be expected, this derivative was completely epiphasic and, in contrast

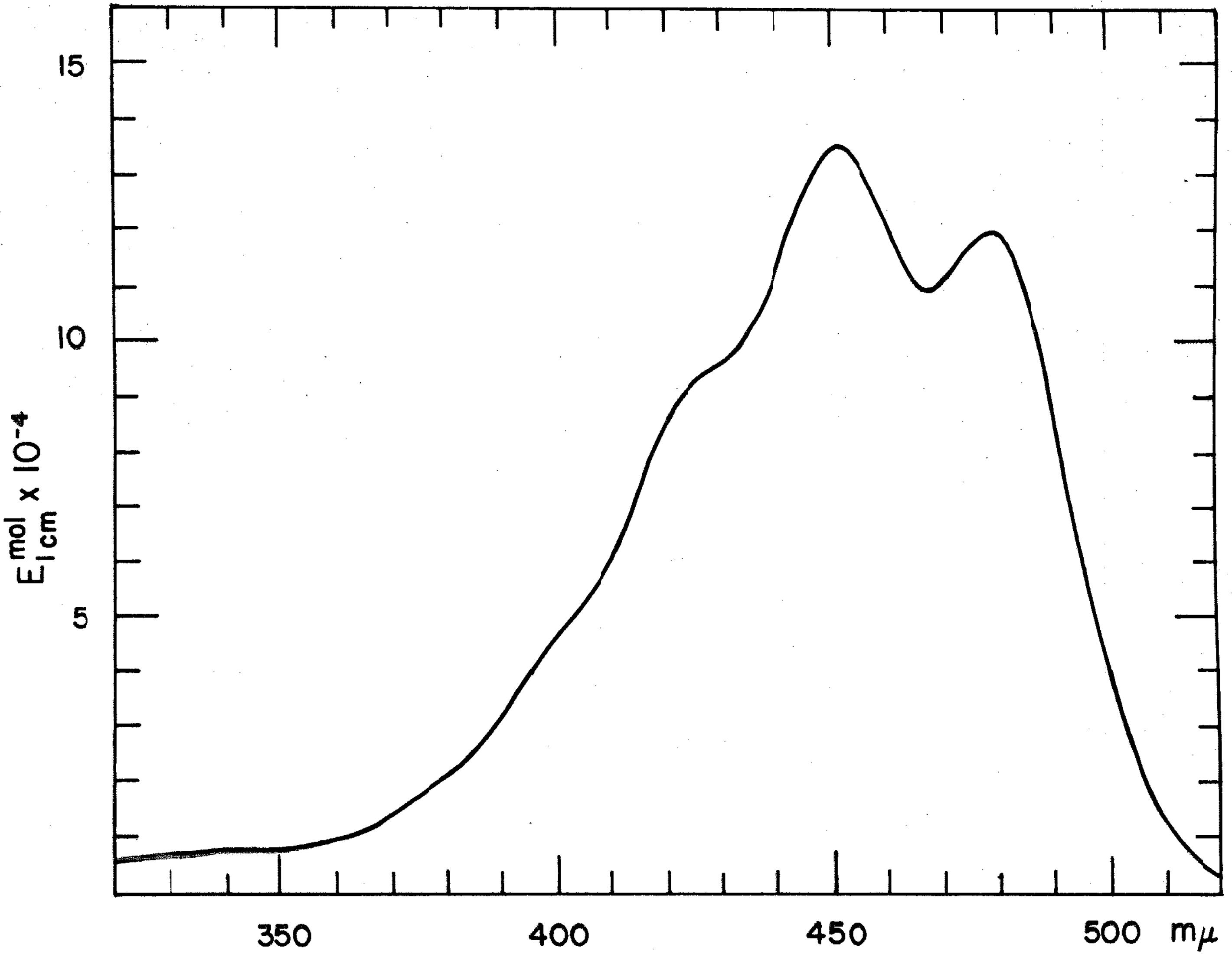
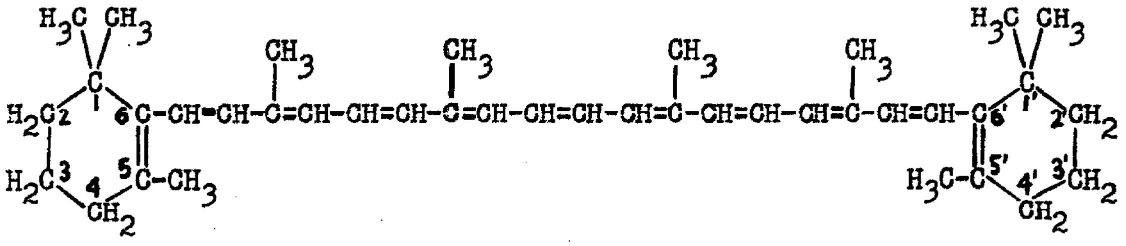


Figure 10. Molecular extinction curve in hexane of isocryptoxanthin (4-hydroxy-β-carotene).

to isocryptoxanthin, could be developed easily on lime-celite with 1 or 2% acetone in hexane. Furthermore, when the acetate was saponified with 15% methanolic KOH, isocryptoxanthin was recovered. Isocryptoxanthin was thus established to be a mono-hydroxy-β-carotene. One such compound was already well known, namely the naturally occurring cryptoxanthin.

In order to compare isocryptoxanthin with cryptoxanthin, 30 mg. of a pure crystalline preparation of the latter was isolated from 4.5 Kgs. of ripe persimmons (Diospyros kaki L.) according to Chatterjee and Zechmeister (1). The two compounds were extraordinarily similar. They melted only 1° apart (isocryptoxanthin, 164°; cryptoxanthin, 165°) and could not be distinguished spectroscopically or in the partition test. It was possible, however, to separate a mixture of the two compounds chromatographically on the lime-celite column, but only after prolonged development. Although two distinct zones were observed, no sharp colorless interzone appeared, for once separated the two zones moved down the column at very similar rates. In order to determine the adsorption sequence, unequal amounts of cryptoxanthin and isocryptoxanthin were used in this mixed chromatogram test. When isocryptoxanthin was the major component, the uppermost zone was the larger one. Isocryptoxanthin, therefore, is adsorbed above cryptoxanthin in the system used.

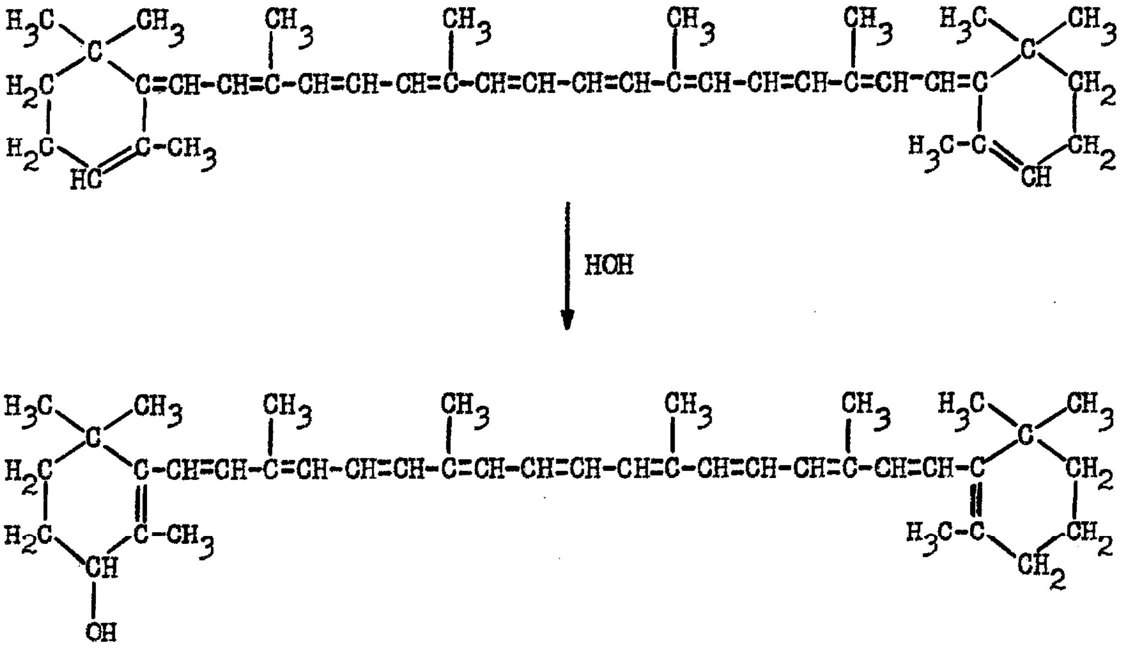
The next step was to determine the location of the hydroxyl group in the isocryptoxanthin molecule. Since this compound resembled cryptoxanthin so closely, it was reasonable to assume that the group was located in a ring. (No carotenoids are known which have substituent groups in the polyene chain linking the two β-ionone rings or in any of the various methyl groups.) In the β-carotene molecule there are only three positions available in either ring for substituent groups, namely, 2, 3 and 4. (see Formula II).



Formula II. β-Carotene, showing the numbering of the β-ionone rings.

It had been proposed by Kuhn and Grundmann (20), and generally accepted, that the hydroxyl group in cryptoxanthin occupies position 3. Recently it has been suggested by Goodwin and Taha (6) that cryptoxanthin is not 3-, but 4-hydroxy-β-carotene; however, Karrer (11), reviewing the complete evidence available concerning the location of the hydroxyl group showed conclusively that there was no valid basis for Goodwin's suggestion. As will be shown here isocryptoxanthin, not cryptoxanthin, has its hydroxyl group located in the 4 position.

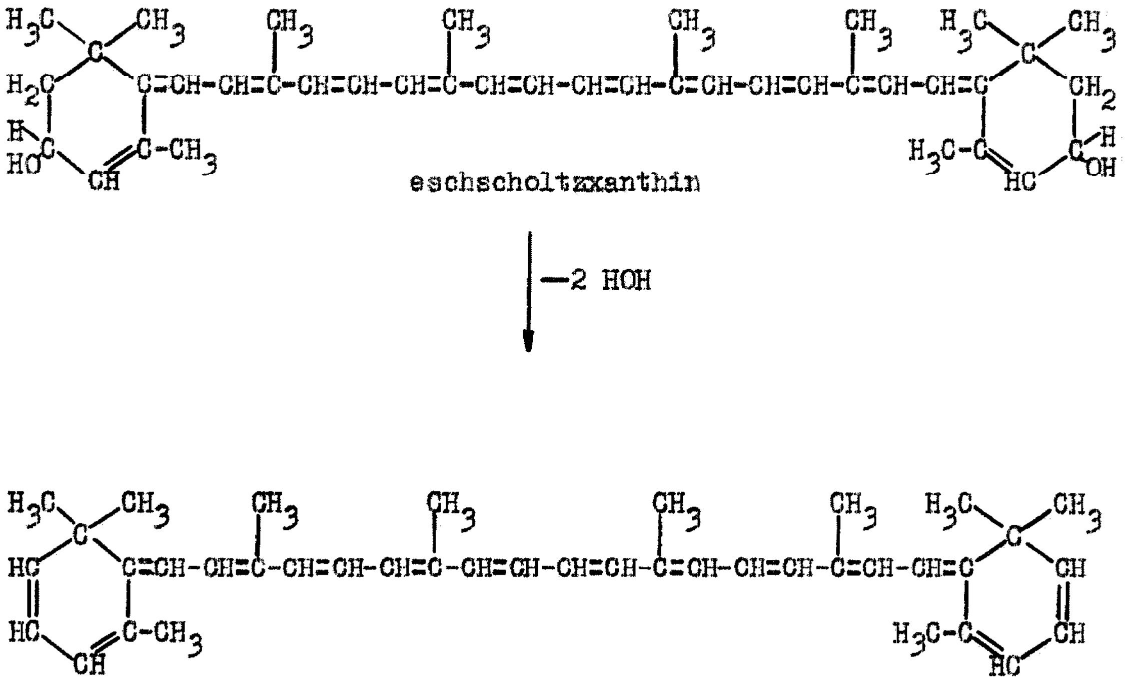
It would be difficult, indeed, to justify assigning the hydroxyl group in isocryptoxanthin to position 2, for no satisfactory explanation of its formation from dehydro-β-carotene could then be offered. On the other hand, if isocryptoxanthin were 4-hydroxy-β-carotene, its formation could be accounted for by a Thiele addition of HOH to the ends of the conjugated system in dehydro-β-carotene under the conditions described for the boron trifluoride experiment (Scheme I). Although boron trifluoride is an effective catalyst for many reactions (e.g., polymerizations and esterifications) this is the first instance in which the application of this reagent has caused the addition of the elements of water to the ends of a conjugated polyene system.



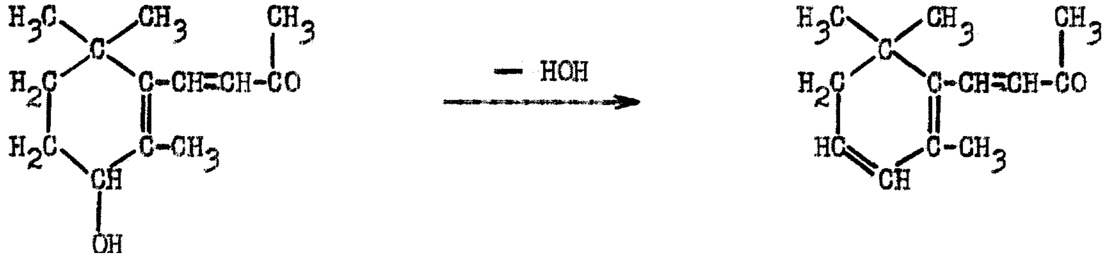
Scheme I. Formation of isocryptoxanthin from dehydro-β-carotene.

It was emphasized by Karrer (11) that compounds containing a hydroxyl group adjacent to a conjugated system (i.e., an allylic hydroxyl group) undergo dehydration with extraordinary ease under the influence of acid, with the formation of an additional double bond. He reported (14) that eschscholtzxanthin, $C_{40}H_{52}(OH)_2$, a pigment detected in the blossoms of Eschscholtzia californica by Strain (31), was dehydrated to a hydrocarbon with 14 double bonds merely by acidifying a chloroform solution with a trace of HCl (Scheme II, below). Karrer also found (12) that 4-hydroxy-β-ionone was dehydrated under similar conditions (Scheme III, below.) In his discussion of the position of the hydroxyl group in cryptoxanthin (11) this author pointed out that since cryptoxanthin did not show this property its hydroxyl group was very probably not in the 4 position.

Isocryptoxanthin, however, did show this property; the dehydration was accomplished by acidifying a chloroform solution of the pigment and allowing it to stand for a few minutes at room temperature. The HCl was introduced by adding a drop or two of HCl saturated chloroform to the solution. A stereoisomeric mixture of dehydro-β-carotenes was formed in 70% yield together with about 5% of β-carotene, as determined by chromatographic and spectrophotometric methods. Except for some minor brownish destruction products which remained at the top of the column during development, no pigments other than those mentioned were found.



Scheme II. Dehydration of eschscholtzxanthin.



Scheme III. Dehydration of 4-hydroxy-β-ionone.

This "acid chloroform test" offers the only rapid means for differentiating isocryptoxanthin from cryptoxanthin. One or two milliliters of a dilute isocryptoxanthin solution (only a few micrograms are needed) change in color from yellow to orange-red within 30 seconds upon the addition of one drop of chloroform (C.P.) saturated with HCl. The color change is a result of the formation of a system with 12 conjugated double bonds from one composed of 11 conjugated double bonds. Cryptoxanthin undergoes no visible alteration when subjected to this test. In addition to the all-<u>trans</u> form of isocryptoxanthin, neo-isocryptoxanthin, as well as isocryptoxanthin acetate, also give the same color reaction.

All the evidence presented is consistent with the proposed 4-hydroxy-β-carotene structure for isocryptoxanthin. The contention of Goodwin (6) that cryptoxanthin is the 4-hydroxy compound is thus disproved on the basis of new observations.

3. Isocryptoxanthin Methyl Ether (4-Methoxy-β-carotene) and Dimethoxy-β-carotene.

a. Isocryptoxanthin methyl ether.

The main pigment isolated from the interaction of the dehydro-β-carotene-boron trifluoride complex with methanol was crystallized from benzene-methanol. It had an absorption spectrum (Fig. 20) which was nearly indistinguishable from that of β-carotene. Although this new compound was adsorbed above, and easily separated from β-carotene on lime celite, it was possible to cause it to migrate with pure hexane. This behavior served to differentiate it from isocryptoxanthin. Analysis of the compound showed that it had one oxygen atom. Since it was much more easily developed on lime-celite than isocryptoxanthin it did not seem probable that the new pigment had a free hydroxyl group. This was confirmed by partitioning the compound between hexane and 95% methanol; it showed much more pronounced epiphasic character than would be expected from a hydroxylated carotenoid. The possibility of its being an epoxide of dehydro-β-carotene was eliminated when it was found that its ether solution did not color the acid phase when shaken with concentrated HCl. It had been found by Karrer (10)that all known carotenoid epoxides impart a blue coloration to the acid in this test. Finally, a Zeisel determination showed the presence of one methoxyl group.

When a chloroform solution of the methoxy-β-carotene was acidified with a trace of acid chloroform, according to the method described above for the dehydration of isocryptoxanthin, a stereoisomeric mixture of dehydro-β-carotenes was formed in 73% yield, together with 9% of β-carotene. Thus it was concluded that the methoxyl group was located in 4 position. For confirmation, some isocryptoxanthin was methylated directly (based on the method described by Karrer (19) for the methylation of zeaxanthin, a naturally occurring dihydroxy-β-carotene).

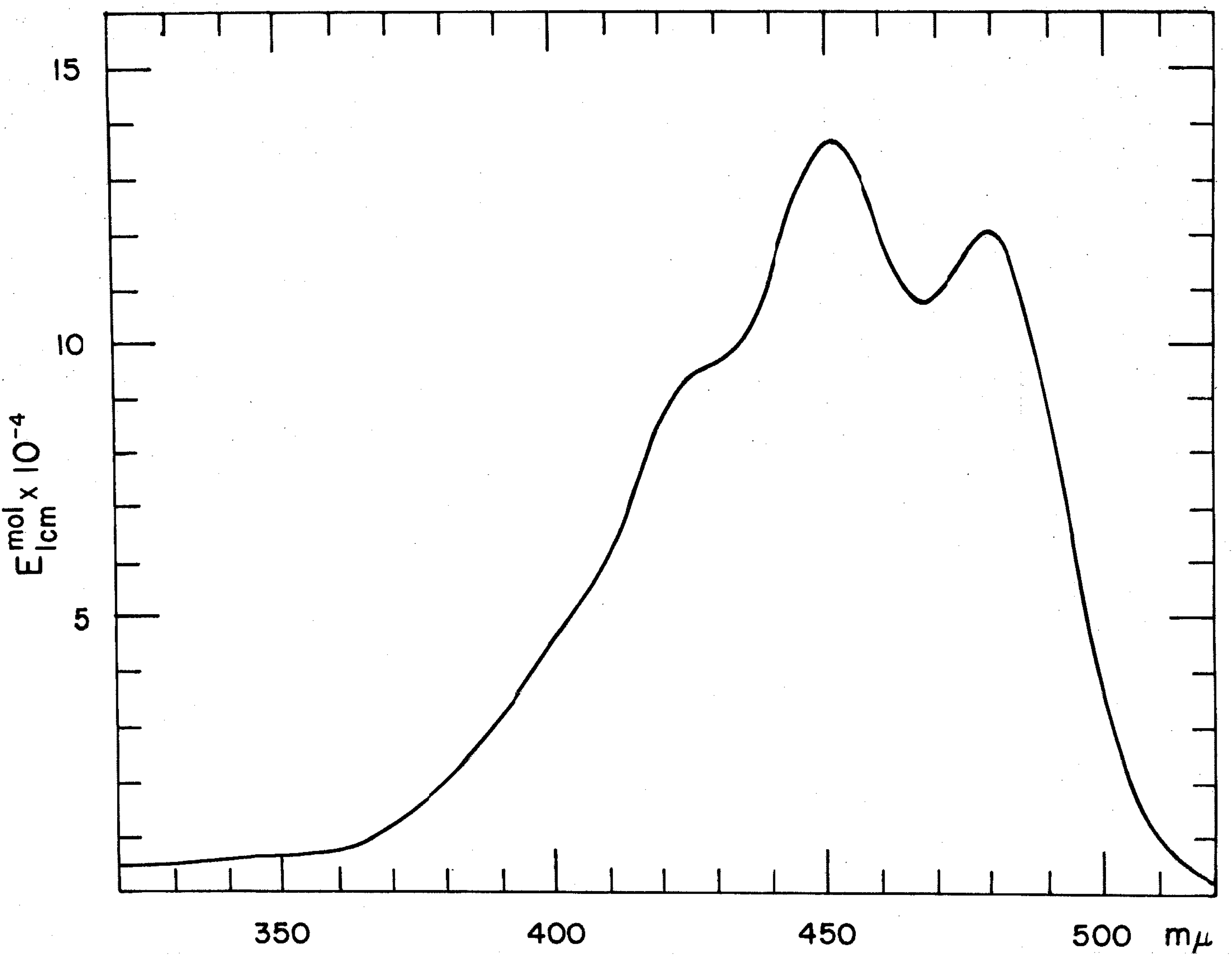


Figure 20. Molecular extinction curve in hexane of isocryptoxanthin methyl ether (4-methoxy-β-carotene).

This isocryptoxanthin methyl ether was found to be identical with 4-methoxy-β-carotene, obtained via the BF_3 complex, both spectroscopically, in melting point (135°), in the mixed chromatogram test, and the "acid chloroform test". In addition, some cryptoxanthin methyl ether (so far not described in the literature) was prepared from naturally occurring cryptoxanthin in order to compare it with 4-methoxy-β-carotene. Although the two compounds dould not be differentiated either spectroscopically or in the mixed chromatogram test, cryptoxanthin methyl ether, in contrast to the 4-methoxy compound did not undergo any color change in the "acid chloroform test". In this respect, the two methylated derivatives behaved in an analogous manner to the respective unmethylated compounds.

b. Dimethoxy-β-carotene

Adsorbed above isocryptoxanthin methyl ether, on the chromatogram of the reaction mixture obtained by treating the boron trifluoride-dehydro-β-carotene complex with methanol, was a second new carotenoid showing a β-carotene spectrum (Fig. 21). In the mixed chromatogram test the new compound was located below isocryptoxanthin and easily separated from it. Analysis and a Zeisel estimation indicated the presence of two oxygen atoms and the formula, $C_{40}H_{54}(OCH_3)_2$. Although somewhat more hypophasic than isocryptoxanthin methyl ether, the compound was considerably less hypophasic than isocryptoxanthin when partioned between hexane and 95% methanol. Thus, all the evidence was consistent with the formulation of the new compound as a dimethoxy-β-carotene.

The position of the two methoxy groups could not be determined with as much certainty as were the positions of the single substituent groups in isocryptoxanthin and its methyl ether, for in the present case a simple, if unusual, type of Thiele addition of methanol to the conjugated system of

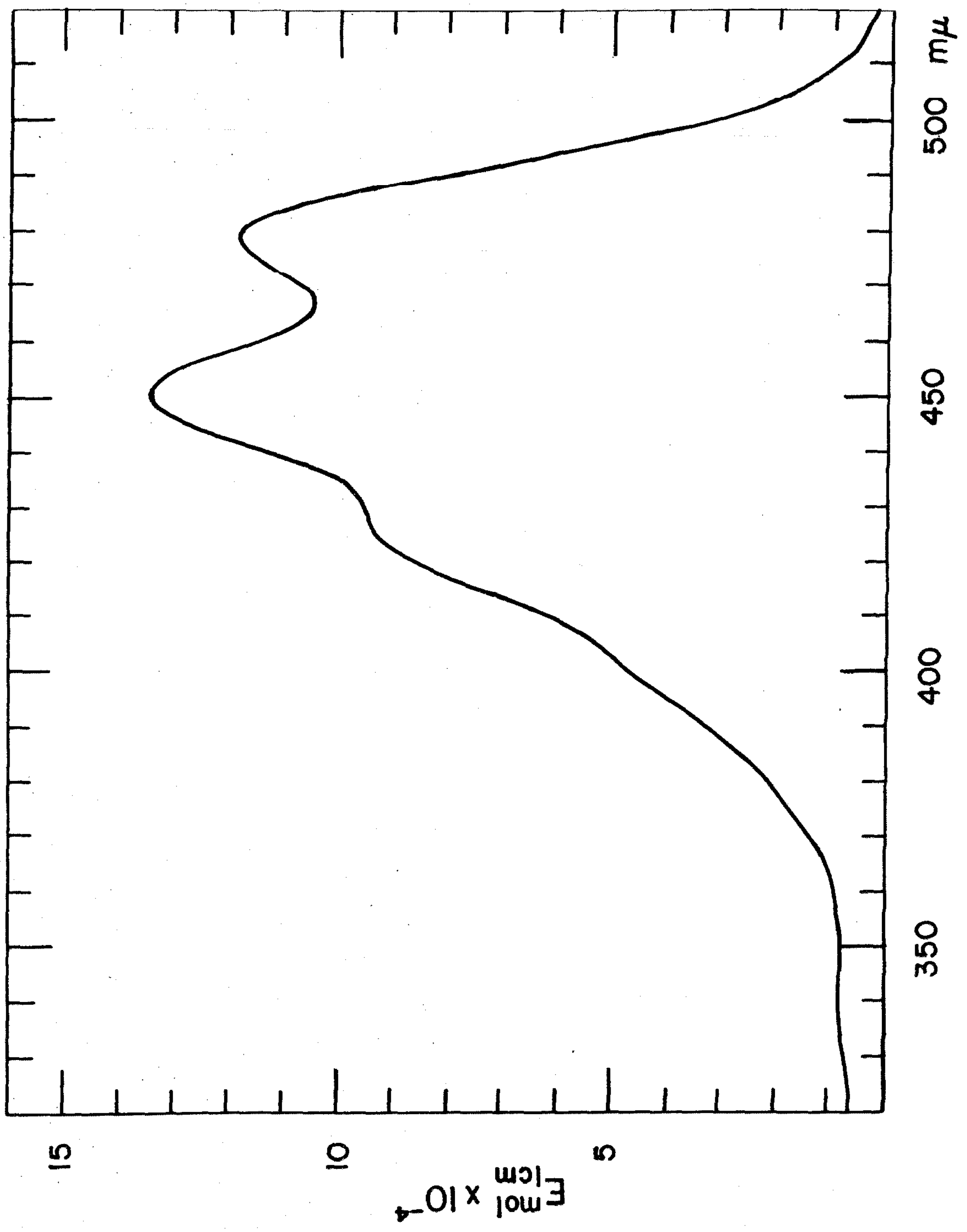


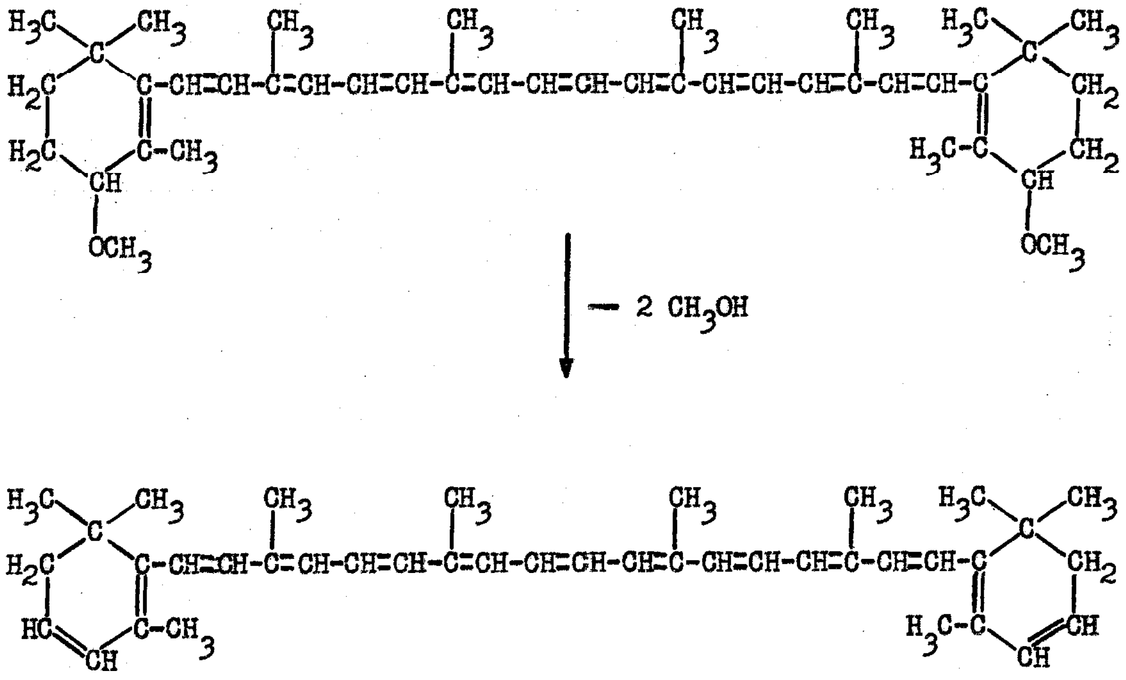
Figure 21. Molecular extinction curve in hexane of dimethoxy-β-carotene.

dehydro-β-carotene would not explain the formation of a compound with two methoxyl groups and 11 conjugated double bonds.

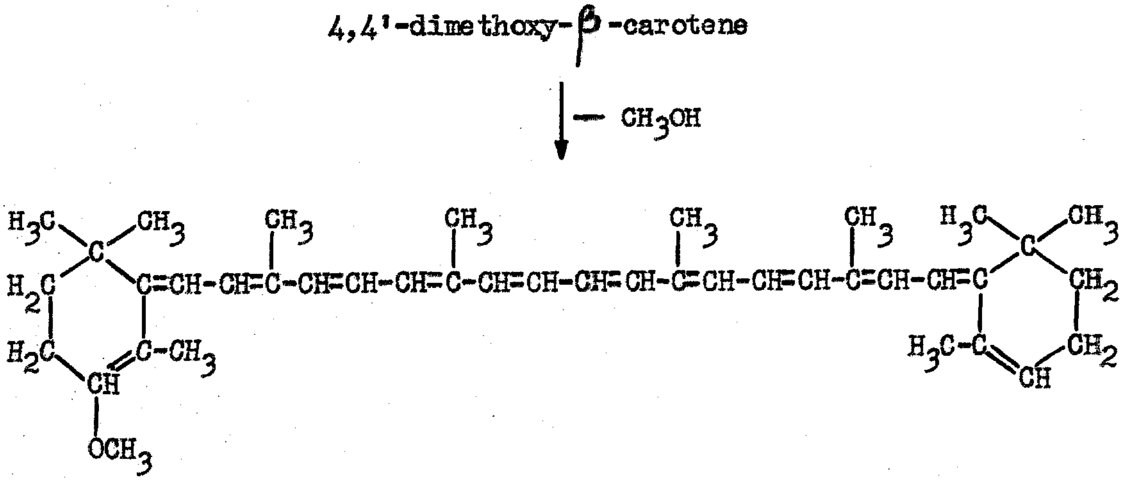
A dilute, yellow-colored chloroform solution of dimethoxy-β-carotene turned red when a trace of HCl was introduced. After transfer into hexane, chromatographic analysis showed that some of the dimethoxy-β-carotene had remained unchanged, whereas, under the same conditions isocryptoxanthin or isocryptoxanthin methyl ether were completely converted to other products, mainly dehydro-β-carotenes. Among the conversion products isolated from the chromatogram just mentioned, two could be identified by spectroscopic and mixed chromatogram tests, namely, dehydro-β-carotene and bisdehydro-β-carotene. In addition, a carotenoid which was spectroscopically identical to dehydro-β-carotene but adsorbed above it was obtained. Because of the small yield it was not possible to characterize this compound further. It is believed, however, that it represents a methoxylated dehydro-β-carotene.

The available evidence at the present time concerning the location of the methoxyl groups in dimethoxy-β-carotene is inconclusive, and further experiments will be required before its structure can be clarified. The formation of some bisdehydro-β-carotene (a carotenoid with 13 conjugated double bonds; see Scheme IV, below) would indicate that these groups are in the 4 and 4' positions, by analogy with the dehydration of eschscholtzxanthin, mentioned above.

The reaction product mentioned which appeared above the dehydro-β-carotene zone in the chromatogram could possibly be interpreted as having resulted from the elimination of the elements of <u>one</u> molecule of methanol (Scheme V, below).



Scheme IV. Formation of bisdehydro-β-carotene from 4,4'-dimethoxy-β-carotene.



Scheme V. Formation of 4-methoxy-dehydro-β-carotene from 4,4'-dimethoxy-β-carotene.

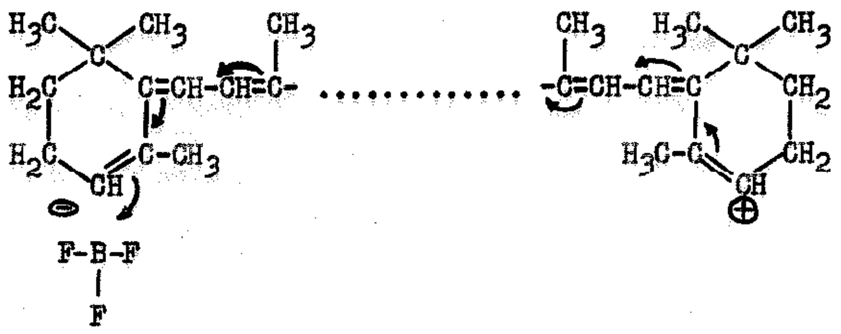
Some bioassays have shown that dimethoxy-β-carotene has about 1/6 the provitamin A activity of β-carotene in the rat (see Section 4). On the other hand, zeaxanthin (3,3'-dihydroxy-β-carotene) and, in general, all carotenoids which do not possess at least one unsubstituted β-ionone ring show no provitamin A effect. This would indicate that both methoxyl groups were in the same ring, were it not for two additional facts: 1. Almost all carotenoids, in the all-*trans* configuration, which do have one unsubstituted β-ionone group display about half the provitamin A activity of β-carotene. 2. Exceptional cases in which carotenoids with substituents in both β-ionone rings show some provitamin A effect are known, e.g., β-carotene diepoxide which according to Karrer, *et al* (13), has a potency equal to about 1/7 that of β-carotene.

4. Two Proposed Mechanisms for the Formation of Isocryptoxanthin and its Methyl Ether from Dehydro-β-carotene.

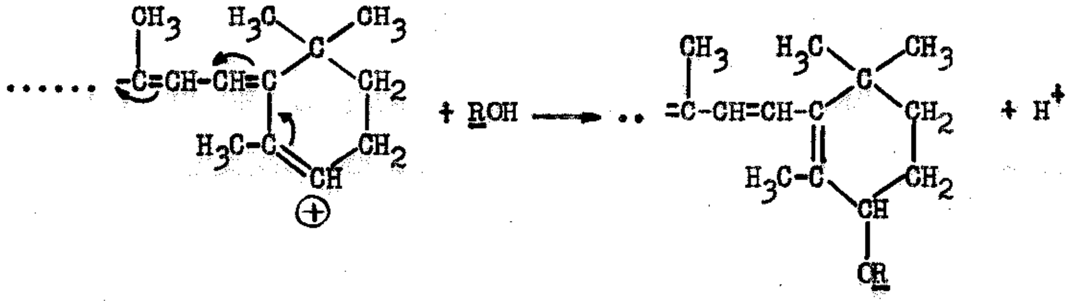
In the following discussion two mechanisms, *a* and *b*, are proposed, but the possibility of both occurring simultaneously is not excluded.

a.) In the mechanism proposed here the water or methanol reacts with the complex of boron trifluoride and dehydro-β-carotene.

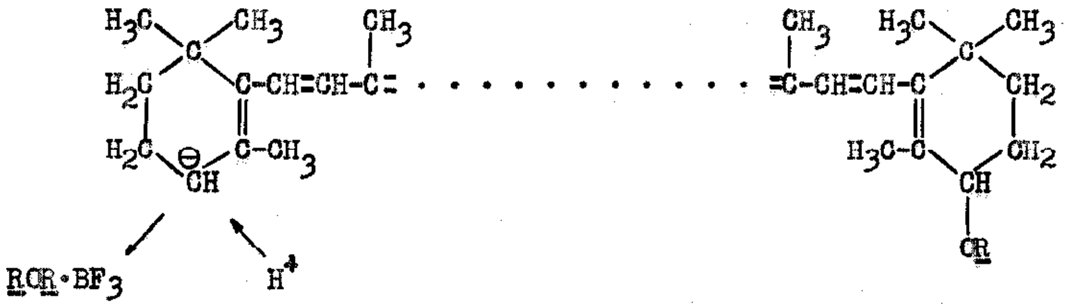
Step 1. Boron trifluoride is attracted to one end of the chromophoric system in dehydro-β-carotene. Additional electrons are drawn from the adjacent carbon atom to this site which has now become momentarily electron deficient because of the proximity of the boron trifluoride. Thus a localized dipole is formed. However, because of the extraordinary mobility of the electrons in a long conjugated polyene system, polarization of the whole molecule takes place:



Step 2. The positive end of the molecule is then subject to nucleophilic substitution by water or methanol (R = H or CH_3).



Step 3. One of the hydrogen ions, abundantly present in the acid medium because of the hydrolysis (or methanolysis) of boron trifluoride, displaces BF_3 which is removed, primarily, as a complex with ether, water or methanol:

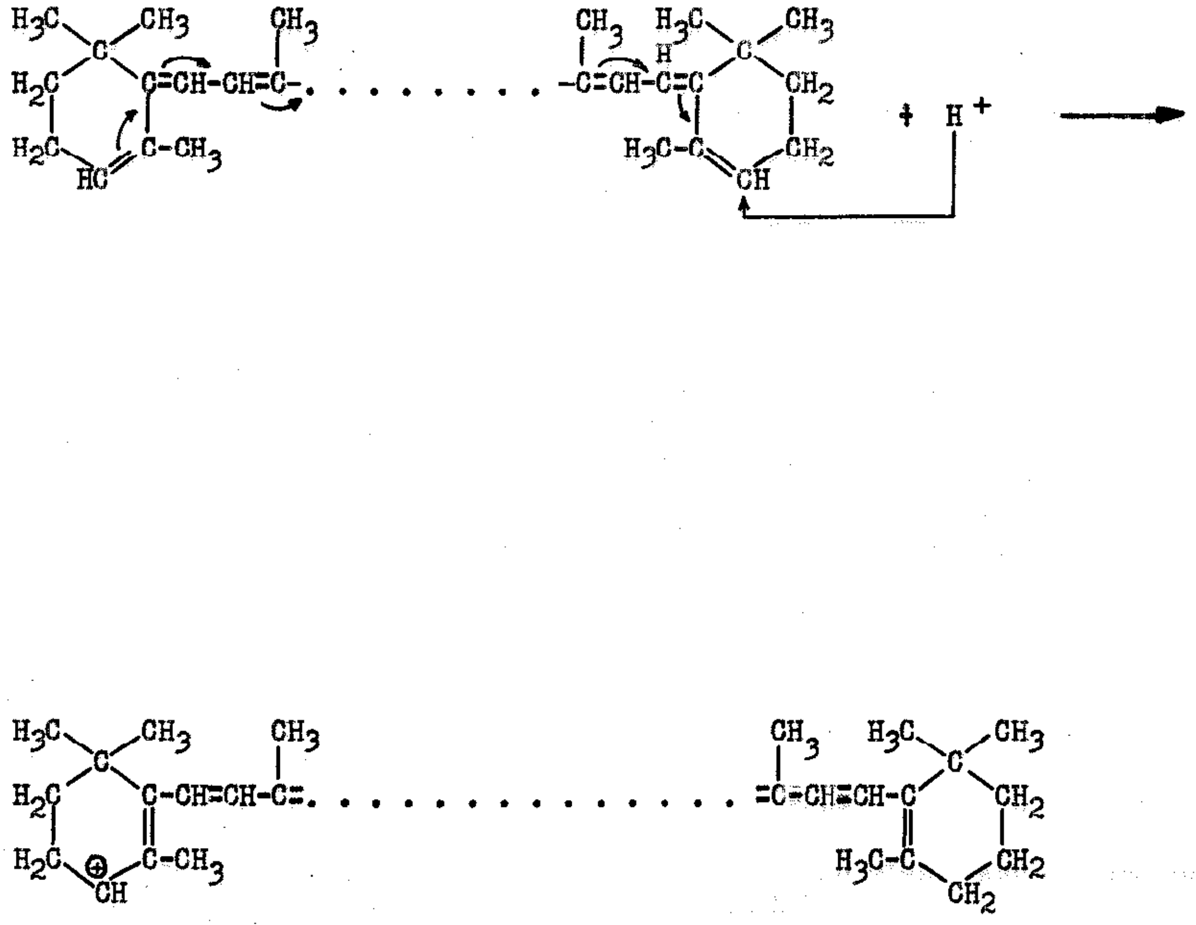


b.) The second possible mechanism proposed is an extension of that generally accepted for the acid catalyzed hydration of the olefinic bond.

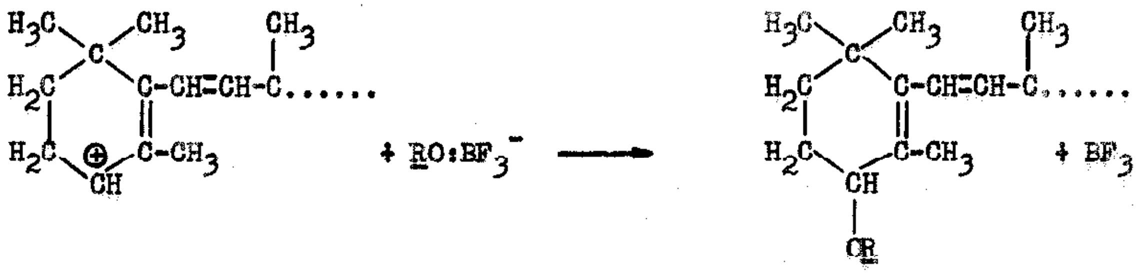
Step 1. Hydrogen ion is made available as the result of complex ion formation between boron trifluoride and hydroxyl or methoxyl ion:

$$BF_3 + \underline{R}OH \longrightarrow \underline{R}O{:}BF_3^- + H^+$$

Step 2. A hydrogen ion attacks the conjugated system in dehydro-β-carotene with the resulting formation of a carbonium ion. The positive charge appears at the other end of the system because of induced electron shifts throughout the chromophore:

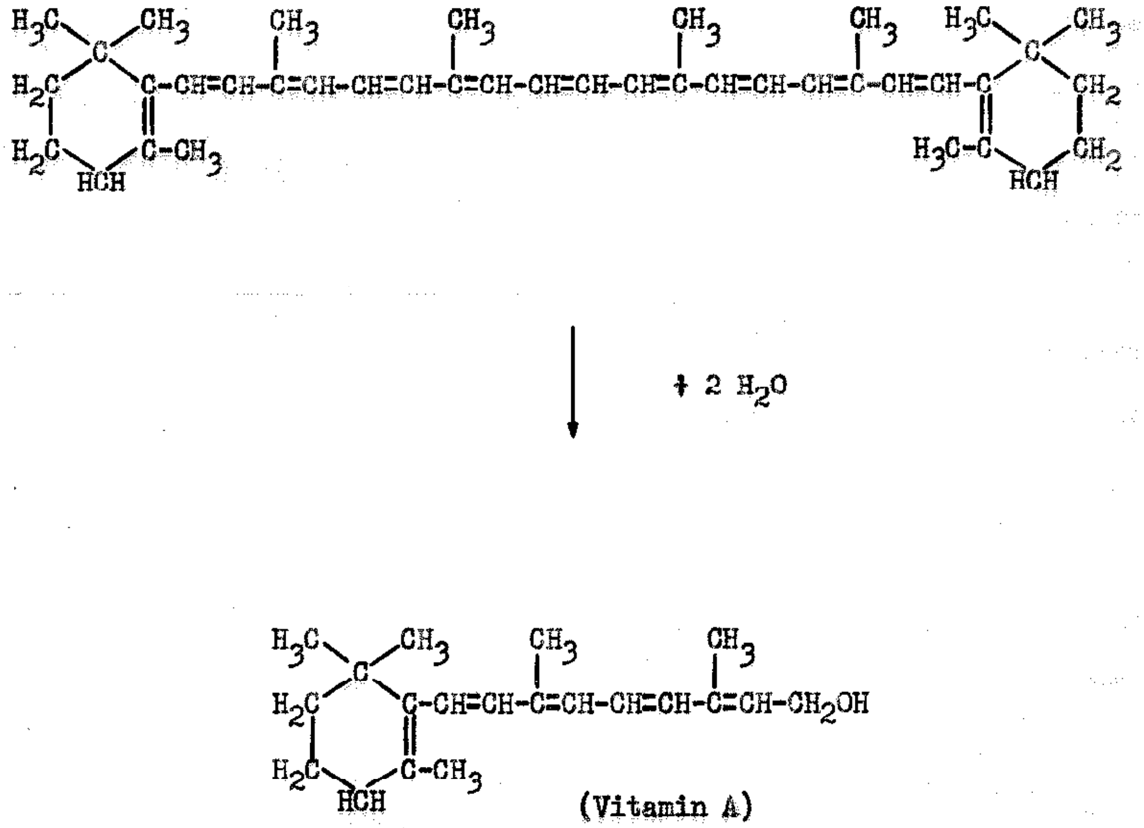


Step 3. The nucleophilic ion, $\mathbf{R}O:BF_3^-$, then attacks the molecule at the point of minimum electron density, whereby BF_3 is regenerated:



5. Provitamin A Activities of Isocryptoxanthin, Isocryptoxanthin Methyl Ether, and Dimethoxy-β-carotene.

As is well known, β-carotene has strong provitamin A activity by virtue of the fact that the animal organism is able to split this molecule enzymatically at its central double bond and thus produces two molecules of vitamin A. This reaction is represented schematically, below.



(Vitamin A)

According to some authors, an oxidative cleavage takes place in the center of the β-carotene molecule and the resulting aldehyde group is reduced in a second step.

Evidently, no other carotenoid can show as strong a provitamin A effect as β-carotene. Those carotenoids tested, half of whose molecules are structurally identical with one half of the β-carotene molecule, also show strong provitamin A activity, but only about 50% of that exhibited by the latter, while if no unsubstituted β-ionone ring is present the compound is inactive. Cryptoxanthin, as would be expected, exhibits strong provitamin A activity (57%); therefore it was of interest to determine the provitamin A activity of the isomeric isocryptoxanthin. Isocryptoxanthin methyl ether and dimethoxy-β-carotene were also tested; these were of special interest since the provitamin A activities of methoxylated β-carotenes have not been reported heretofore. The rat bioassays, were kindly carried out at the University of Southern California by Drs. H. J. Deuel, Jr. and J. Ganguly.

It was found that isocryptoxanthin had 48% of the vitamin A potency of β-carotene, and the methyl ether 52%. Both results are in complete agreement with the structures assigned to these compounds in this Thesis. Dimethoxy-β-carotene also showed provitamin A activity, namely, 16% that of β-carotene. This result can not readily be interpreted at the present time, for if the two methoxyl groups were located in the same ring the bioactivity should amount to about 50% (β-carotene = 100%), while if each ring had one methoxyl no provitamin A potency should be observed. Although the well crystallized sample showed chromatographic homogeneity, the possibility that it is a mixture of two (or more) dimethoxy derivatives of the types mentioned can not be excluded.

B. EXPERIMENTAL PART

1. Materials.

The dehydro-β-carotene used was of high purity, being chromatographically homogeneous and free from colorless impurities as shown by microscopic examination of the crystals. Its preparation from β-carotene is described in Part I.

Boron trifluoride etherate was prepared from the gas available in small cylinders from the Ohio Chemical & Manufacturing Co., Cleveland, and Reagent Grade diethyl ether. Except for the use of commercially available boron trifluoride gas, the directions given by Hennion (7) were followed. The etherate darkened on long standing, but this was found to have little or no effect on its potency.

2. 4-Methoxy-β-carotene and Dimethoxy-β-carotene.

a. Formation

To a solution of 60 mg. of all-*trans*-dehydro-β-carotene in 250 ml. of hexane, contained in a 750 ml. Erlenmeyer flask, was added 5 ml. of boron trifluoride etherate. The flask was vigorously swirled by hand for 60 seconds (the etherate is not appreciably soluble in hexane) and then 300 ml. of 95% methanol was added; the dark greenish-blue color of the carotenoid-boron trifluoride complex immediately changed to dark red. The liquid was transferred to a separatory funnel and water was added to transfer all pigments to the upper phase. The aqueous-methanol hypophase was discarded and the epiphase was washed in a continuous apparatus. After drying with anhydrous sodium sulfate, the hexane solution was adsorbed at the top of a 30 x 8 cm. lime-celite column and developed with 2-3% acetone in hexane.

Description of the chromatogram (the figures designate thickness in mm.):

1	yellow	
1	purple	
10	orange	
3	purple	
10	interzone	
5	pale pink	
20	interzone	
20	orange	Zone I (dimethoxy-β-carotene)
15	interzone	
25	yellow	Zone II
5	interzone	
60	orange	Zone III (4-methoxy-β-carotene)
2	interzone	
25	yellow	Zone IV
2	interzone	
15	orange	Zone V (β-carotene)
2	interzone	
10	pale yellow	Zone VI
10	interzone	
10	pale yellow	Zone VII

Each of the zones I-VII was cut out and eluted with acetone. After transferring to hexane, each fraction was rechromatographed on a smaller column. Repeated rechromatography of fraction III was sometimes found to be necessary before it crystallized easily. No attempts were made to crystallize fractions II, IV, VI, and VII as these were found to be stereoisomers of the others (cf. section c., below).

Fractions I, III, and V were crystallized according to the procedure described below for 4-methoxy-β-carotene.

The hexane solution (about 50 ml.) was evaporated to dryness <u>in vacuo</u>. (The temperature of the bath should not exceed 45° since excessive stereoisomerization of carotenoids would increase crystallization difficulties.) The dry, powdery residue was dissolved in 2-3 ml. of cold benzene and transferred to a 5 ml. centrifuge tube.* The tube was placed in a bath of water kept at about 40° and the solvent evaporated by directing, through a capillary, a gentle stream of pure dry nitrogen at the surface. The residue was then dissolved in 10 drops of benzene, and methanol was slowly added from a dropper by allowing it to run down the side of the tube. Thus, the methanol was warmed approximately to the bath temperature before reaching the benzene solution. During these operations the solution was stirred constantly with a thin pointed glass rod. In all, 4 to 5 volumes of methanol were added, and, in favorable cases, glittering red crystals appeared before this addition was complete. The tube was then allowed to stand at room temperature for 30 minutes. (Stronger cooling was not found to be advisable, as at times, a colorless or pale yellow crystalline material precipitated which was difficult to remove by recrystallization.) The red crystals were centrifuged down and the mother liquor decanted. The pigment was washed twice, in the centrifuge tube, with methanol at room temperature, and then dried in an Abderhalden apparatus, in the presence of P_2O_5, in good vacuum (0.1 mm.), at 55° (refluxing acetone), for 1 hour. Yield: 8-9 mg. of 4-methoxy-β-carotene, 2-3 mg. of dimethoxy-β-carotene, and 5-6 mg. of β-carotene, in all, 27-28% of the starting material. The mother liquors were found to contain 1-3 mg. of additional pigments.

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* If, after the hexane has been evaporated, the residue is of oily or partially oily nature, difficulties in crystallization will be encountered later. In such a case the residue should be dissolved in hexane and rechromatographed on lime-celite.

b. Characterization

1.) Dimethoxy-β-carotene

The crystals from benzene-methanol formed very small irregular plates. Under the microscope (magnification 430 x), single crystals appeared light orange, while clumped groups were dark red. Macroscopically, the dried crystals were dark red.

Melting point: 152° (Berl block; evac. capillary; corr.)

Solubility: Soluble in hexane, more easily in benzene or chloroform. Almost insoluble in methanol.

Partition test: When partitioned between hexane and 95% methanol, the compound was mainly epiphasic. However it was more hypophasic than β-carotene.

Analysis: Calculated for $C_{40}H_{54}(OCH_3)_2$: C, 84.50; H, 10.13. Found: C, 84.33; H, 10.02. C, 84.43; H, 10.84. (Second result corrected for 1.5% ash).

Methoxyl determination (Zeisel): Calculated: 10.37%. Found: 9.45%.

Spectrum: Maxima in hexane: 484, 453 mμ. (visual); 479, 450-451 mμ. (Beckman).

Molecular extinction coefficient: $E^{mol.}_{1cm.} = 13.4 \times 10^4$ at $\lambda_{max.}$

Chromatographic behavior: Easily developed on lime-celite with 2-3% acetone in hexane. Adsorbed well above β-carotene in the mixed chromatogram test.

2.) 4-Methoxy-β-carotene

This compound crystallized from benzene-methanol in oval shaped clusters (Fig.22). Macroscopically, the dry crystals appeared dark red.

Melting point: 133-135°

Solubility: Soluble in hexane; very soluble in benzene or chloroform; almost insoluble in methanol.

Partition test: The compound showed an essentially, but not fully epiphasic behavior. In this respect it stands between β-carotene and dimethoxy-β-carotene.

Analysis: Calculated for $C_{40}H_{55}OCH_3$: C, 86.86; H, 10.31. Found: C, 86.03, 86.11; H, 10.60, 10.09.

Methoxyl determination: Calculated: 5.46%. Found: 5.05%

Spectrum: Maxima in hexane, 484, 453 mμ. (visual); 479-480, 451 mμ. (Beckman).

Molecular extinction coefficient: $E^{mol.}_{1cm.} = 13.5 \times 10^4$ at $\lambda_{max.}$

Chromatographic behavior: On lime-celite the zone of this compound appears between those of dimethoxy-β-carotene and β-carotene.

3.) β-carotene.

The compound crystallized very easily from benzene-methanol in red hexagonal plates characteristic of β-carotene. The crystals were dark red in color.

Solubility: Soluble in hexane, but somewhat less so than the two methoxy derivatives described above.

Partition: Only a trace of hypophasic behavior was observed. Identical in this respect with authentic β-carotene.

Analysis: Calculated for $C_{40}H_{56}$: C, 89.48; H, 10.37. Found: C, 89.35; H, 10.37.

Spectrum: The absorption spectrum in hexane was identical with that of an authentic sample of β-carotene.

Chromatographic behavior: Not separable from authentic β-carotene in the mixed chromatogram test.

c. Identification of Fractions II, IV, VI, and VII.

1.) Fraction II.

This fraction was separable from 4-methoxy-β-carotene only with some difficulty. It had maxima in hexane at 473, 449 mμ. (Beckman), and showed a small cis-peak at 340 mμ. These values corresponded closely with those observed for neo-β-carotene U (28). Upon isomerizing this fraction with iodine, all-trans-4-methoxy-β-carotene was formed and isolated chromatographically. It was identified by specroscopic readings and in the mixed chromatogram test. Consequently, Fraction II was termed neo-4-methoxy-β-carotene-U.

2.) Fraction IV.

This fraction showed maxima at 470 and 445 mμ. (Beckman, in hexane) and a strong cis-peak at 338 mμ. Upon iodine catalysis the main maximum shifted to 446-447 mμ. and the height of the cis-peak decreased. From a chromatogram of the isomerized mixture 4-methoxy-β-carotene and β-carotene were isolated (ratio 2:1). Fraction IV was thus identified as a mixture of neo-4-methoxy-β-carotene and neo-β-carotene U.

3.) Fractions VI and VII.

Tests similar to those described above showed these fractions to be neo isomers of β-carotene.

3. Isocryptoxanthin

a. Formation.

To a solution of 35 mg. of all-trans-dehydro-β-carotene in 100 ml. of hexane, contained in a 300 ml. Erlenmeyer flask, was added 3 ml. of boron trifluoride etherate. The mixture was vigorously swirled by hand for 60 seconds and then transferred to a 1-liter separatory funnel containing 400 ml. of acetone. Water was added slowly until two phases separated and the upper (hexane) phase contained nearly the total pigment. The aqueous-acetone hypophase was discarded, and the hexane layer washed in the continuous washing apparatus until the yellowish-brown color changed to a clear dark red. This usually took about 15 minutes.

After drying, the hexane solution was filtered from a reddish-brown, gummy material which precipitated during the washing process. The filtrate (about 100 ml.) was developed on a 27 x 5.8 cm. lime-celite column with 6-8% acetone in hexane.

1	brown	
12	brownish-red	
15	interzone	
4	purple	
5	interzone	
1	yellow	
3	interzone	
1	purple	
3	interzone	
1	yellow	
10	interzone	
40	reddish-orange	Zone I (isocryptoxanthin)
2	interzone	
12	yellow	Zone II (neo-isocryptoxanthin)
30	interzone	
8	very pale yellow	
35	interzone	
8	very pale yellow	
25	interzone	
8	very pale yellow	
35	interzone	
15	yellow	Zone III (β-carotene)

Zones I-III were cut, eluted, and, after transfer to hexane, each was rechromatographed on lime-celite. Fractions I and II required 8%

acetone for development, and fraction III, pure hexane. After crystallization of I and III from benzene-methanol (according to the method described above for 4-methoxy-β-carotene), the samples were dried in the Abderhalden apparatus at 80° (refluxing benzene) for an hour. Yield, 5 mg. of isocryptoxanthin and 2 mg. of β-carotene. Photometric measurements showed an additional 9 mg. in the mother liquors and as *cis* isomers (not crystallized).

b. Characterization of Isocryptoxanthin and β-Carotene

1.) Isocryptoxanthin

The crystals obtained from benzene-methanol are shown in Fig. 23. Under the microscope (430 x), single crystals appeared orange-brown, but where they crossed a light to dark red color was observed depending on the thickness of the conglomerate. The general shape was that of oval plates with blunted ends. Macroscopically, the somewhat glittery crystals appeared dark red.

Melting point: 162-164°

Solubility: Rather sparingly soluble in hexane; more easily in benzene or chloroform. Slightly soluble in hot methanol.

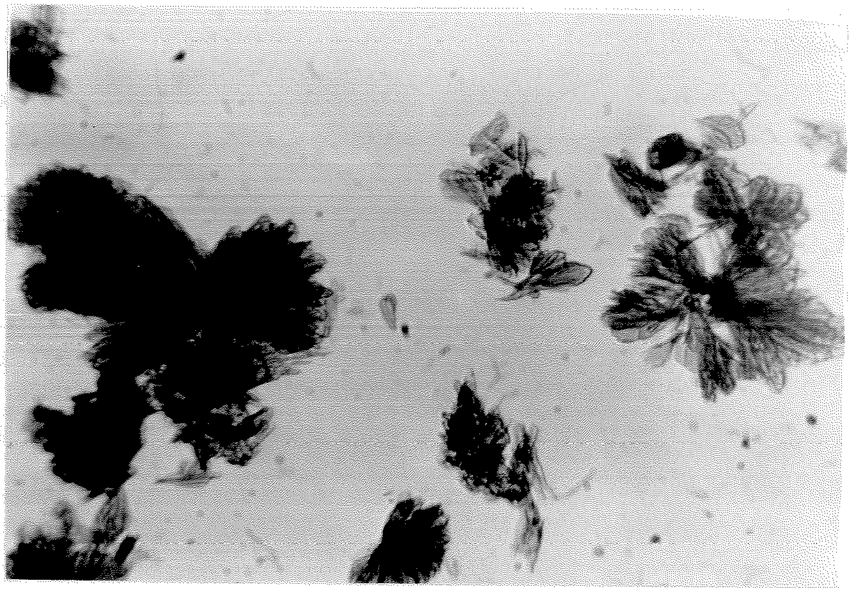
Partition test: Strongly, but not completely, hypophasic.

Analysis: Calculated for $C_{40}H_{55}OH$: C, 86.87; H, 10.22. Found: C, 86.97, 86.52; H, 10.54, 10.24.

Spectrum: Maxima in hexane, 484, 453 mμ. (visual); 479, 451 mμ. (Beckman).

Molecular extinction coefficient: $E^{mol.}_{1cm.} = 13.5 \times 10^4$ at $\lambda_{max.}$

Chromatographic behavior: Strongly adsorbed on lime-celite.



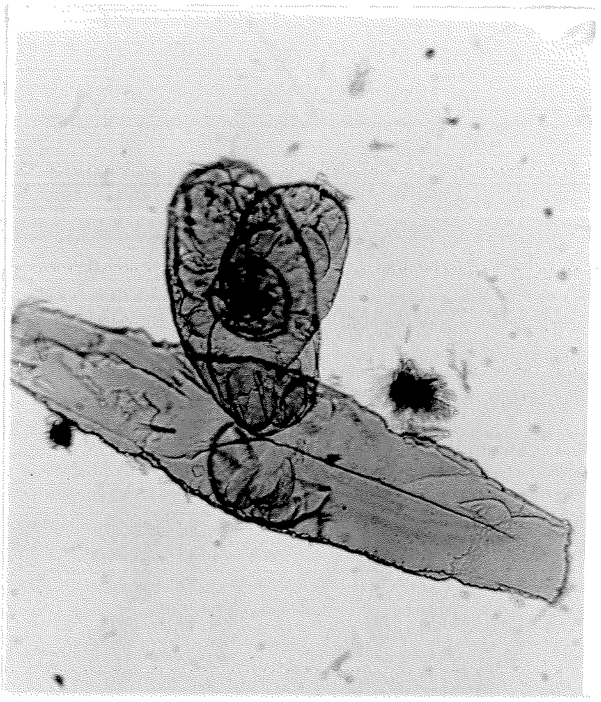


Figure 22 (above): Isocryptoxanthin methyl ether from benzene-methanol (430 x).

Figure 23 (below): Isocryptoxanthin from benzene-methanol (430 x).

Separated with difficulty from cryptoxanthin in the mixed chromatogram test; adsorbed above the latter. Prolonged development with 8% acetone in hexane was required.

2.) β-carotene.

The crystals from benzene-methanol were cleanly formed hexagonal plates. The compound was in all respects identical with authentic β-carotene.

Analysis: Calculated for $C_{40}H_{56}$: C, 89.48; H, 10.52. Found: C, 89.32; H, 10.76.

c. Identification of Fraction II.

It is claimed that this fraction was a neo isomer of isocryptoxanthin for the following reasons: The maxima in hexane were located at 477 and 446 mμ. (visual), i.e., at about 6 mμ. shorter wave lengths than the corresponding maxima of all-trans-isocryptoxanthin. Upon isomerizing this fraction with a trace of iodine (in light), the main isomer which was formed, and separated chromatographically, was found to be all-trans-isocryptoxanthin as shown by spectral observation and in the mixed chromatogram test.

d. Acetate and Methyl Ether of Isocryptoxanthin.

1.) Isocryptoxanthin acetate.

The method used was based on that of Kuhn and Grundmann (20) for the preparation of cryptoxanthin acetate, with some minor modifications. Thirty milligrams of isocryptoxanthin were dissolved in 2.5 ml. of dry pyridine (c.p.), in a 15 ml. centrifuge tube. One ml. of acetic anhydride (c.p.) was added and the mixture heated at 70° for 45 minutes. The solution was then transferred to a small separatory funnel, diluted with 50 ml. of hexane, and washed free of pyridine and acetic anhydride (about 10 min. washing time was required). The dried hexane solution was developed on a 27 x 5.8 cm. lime-celite column with 2% acetone in hexane. The acetyl derivative formed an orange-brown zone which moved down the column at a moderate rate together with some pale orange and yellow minor stereoisomers. In contrast, unreacted isocryptoxanthin remained near the top during development. The acetate zone was cut out, eluted, and transferred with water to hexane. The dried hexane solution was completely evaporated. The residue was crystallized in a small centrifuge tube from benzene-methanol (1:4) and dried for 1 hour at 55° in the Abderhalden apparatus. Yield: 4 mg.

Melting point: 108°

Partition test: Entirely epiphasic.

Chromatographic behavior: Easily developed on lime-celite with 2% acetone in hexane. Adsorbed above β-carotene but well below isocryptoxanthin.

Spectrum: Identical to that of isocryptoxanthin. (Maxima at 479 and 451 mμ. in Beckman.)

Saponification: About half of the sample underwent saponification when 10 ml. of a dilute hexane solution was shaken with 10 ml. of 15% methanolic KOH for 10 min. at room temperature. This estimate was made photometrically after chromatographic resolution of the reaction product.

2.) Isocryptoxanthin methyl ether.

The method used was based on the reported by Karrer and Takahashi (19) for the methylation of zeaxanthin, with a number of modifications in the procedure.

To 8.6 mg. of isocryptoxanthin contained in a 5 ml. centrifuge tube was added 0.5 ml. of tert.amyl alcohol (dried by distilling from metallic potassium). The mixture was heated to 90° and toluene (dried over P_2O_5) was added dropwise, with constant stirring, until all the isocryptoxanthin dissolved. Two pieces of potassium metal, each about the size of a grain of rice, were introduced and stirred about until dissolved. The solution was then allowed to cool to 35°, and 3 ml. of methyl iodide (free from iodine) were added. The corked centrifuge tube was kept at 35° for 30 minutes. The liquid was transferred to a small separatory funnel and diluted with 25 ml. of hexane. After washing and drying, the hexane solution was developed on a 24 x 4.8 cm. lime-celite column with 2% acetone in hexane. The isocryptoxanthin methyl ether appeared as a yellow-brown zone which moved down the column quite readily. The major zone which was unreacted isocryptoxanthin, remained near the top. No stereoisomers of either of these two substances were observed, presumably because the methylation had been carried out in a basic medium. Yield, about 0.5 mg.

Melting point: 133-137°

Partition test: Slightly hypophasic.

Spectrum: In hexane, 479, 450-451 mµ. (Beckman).

Chromatographic behavior: The compound did not separate from an authentic sample of 4-methoxy-β-carotene or from cryptoxanthin methyl ether in the mixed chromatogram test on lime-celite.

4. Action of Acid Chloroform

To a solution of 2-4 mg. of the compound (isocryptoxanthin, 4-methoxy-β-carotene or dimethoxy-β-carotene) in 10 ml. of chloroform (c.p.), 4 drops of chloroform saturated with HCl were added. The solution was then kept at room temperature for 25 minutes, and shaken with 10 ml. of a 5% NaHCO$_3$ solution. After drying over sodium sulfate, the chloroform was evaporated, the dry residue dissolved in 10-20 ml. of hexane and developed on a 20 x 3.8 cm. lime-celite column with 6% acetone in hexane.

Description of the chromatogram obtained from isocryptoxanthin as starting material:

1	brownish-yellow		
25	interzone		
15	pale red	Zone I	
3	interzone		
25	red	Zone IIa)	cut out together
2	yellow	Zone IIb)	
8	interzone		
20	orange-red	Zone III (contains two stereoisomers)	
6	interzone		
8	orange-yellow	Zone IV	
60	interzone (incl. a few very pale zones)		
5	yellow	Zone V	

The chromatogram obtained from 4-methoxy-β-carotene in the acid chloroform reaction was qualitatively identical to that described above, there being an exact correspondence zone for zone. Zone I was all-*trans*-dehydro-β-carotene, and Zones II-IV were stereoisomers of this compound. Zone V was β-carotene (including stereoisomers). The identity of the respective fractions was established by spectra and mixed chromatogram tests. Estimates of the amount of substance in the various fractions were made photometrically (Tables 9 and 10).

Table 9.

Conversion Products Obtained from Isocryptoxanthin by Treatment with Acid Chloroform

(% of Starting Material)

All-*trans*-dehydro-β-carotene	16
Neo A + B isomers	30
Neo C + D isomers	18
Other isomers	5
β-Carotene (including isomers)	5
Unaccounted for	26

Table 10.

Conversion Products Obtained from 4-Methoxy-β-carotene by Treatment with Acid Chloroform

(% of Starting Material)

All-*trans*-dehydro-β-carotene	19
Neo A and neo B isomers	30
Neo C and neo D isomers	19
Other steric forms	6
β-Carotene (including isomers)	4
Unaccounted for	22

The interaction of dimethoxy-β-carotene with acid chloroform was more complex as shown by the following chromatogram:

1	yellow-brown	
3	interzone	
3	pale red	Zone I
2	pale red	Zone II
3	interzone	
3	pale orange-red	Zone III
10	pale red with yellow leading edge	Zone IV
3	interzone	
5	very pale yellow	
3	interzone	
5	very pale pink	
3	interzone	
10	red-purple	Zone V
2	interzone	
10	several very pale zones	
6	interzone	
15	yellow	
4	interzone	
15	orange-brown with pink trailing edge	Zone VI
6	pale yellow	

All numbered zones were cut out, eluted and transferred to hexane. The mixture of Fractions I and II was rechromatographed to obtain a more satisfactory separation. Fraction V was also rechromatographed. The following spectral maxima were observed (visual, in hexane):

Fraction I	503, 473 mμ.	unidentified; possibly a methoxy-dehydro-β-carotene
Fraction II	498, 469 mμ.	unidentified; possibly a neo isomer of above
Fraction III	504, 474 mμ.	all-*trans*-dehydro-β-carotene
Fraction IV	497, 467 mμ.	mixture of neo-dehydro-β-carotenes
Fraction V	519, 487, 460 mμ.	bisdehydro-β-carotene
Fraction VI	483, 453 mμ.	unchanged dimethoxy-β-carotene

Fraction V did not separate in the mixed chromatogram test from an authentic sample of bisdehydro-β-carotene (a carotenoid with 13 conjugated double bonds, cf. Part IV), prepared from β-carotene by dehydrogenation with N-bromosuccinimide.

No quantitative estimate of the conversion products could be made because of the complexity of the product and small amounts of its components. It was evident, however, that the conversion was less extensive, if more complex, than in the case of isocryptoxanthin or its methyl ether.

5. Preparation of Isocryptoxanthin, Isocryptoxanthin Methyl Ether, and Dimethoxy-β-carotene from a Stereoisomeric Mixture of Dehydro-β-carotenes.

In the experiments previously described, a pure crystalline all-*trans*-dehydro-β-carotene was employed as starting material for the preparation of isocryptoxanthin, isocryptoxanthin methyl ether, and dimethoxy-β-carotene. It was found that a stereoisomeric mixture of dehydro-β-carotenes could also be used for the preparation of these compounds. Although its use resulted in chromatograms of increased complexity, the over all yields, based on β-carotene, were better owing to the greatly increased amount of starting material made available. (As was shown in Part I, the all-*trans* form constituted but a relatively small fraction of the stereoisomeric dehydro-β-carotenes produced from β-carotene iodide by the action of acetone.)

Preparation and treatment of a stereoisomeric mixture of dehydro-β-carotenes.

The three principal steric forms of dehydro-β-carotene (i.e., all-*trans*, neo A, and neo D) produced from β-carotene as described in Part I were separated from extraneous products by adsorption on lime-celite. About 400 mg. of these pigments were ultimately obtained in hexane solution (photometric estimate) and, after evaporation to dryness, 150-200 mg. could be crystallized from benzene-methanol. These crystals were treated as follows:

To 30-40 mg. of the crystalline mixture dissolved in 100 ml. of hexane were added 5 ml. of boron trifluoride etherate. The flask was vigorously swirled for 2 minutes and then treated with water or methanol to form isocryptoxanthin, or its methyl ether and dimethoxy-β-carotene, exactly as prescribed for the pure all-<u>trans</u> compound.

The mother liquor remaining from the crystallization of the dehydro-β-carotene mixture was also utilized for the preparation of the three new substituted β-carotenes in the following manner:

After dilution to 100 ml. with hexane, the mother liquor was shaken with four, 100 ml. portions of 95% methanol, discarding the methanol phase after each washing. The hexane phase was then washed with water, dried, and diluted to 500 ml. with additional solvent. Each 100 ml. portion of this solution was treated with 5 ml. of boron trifluoride etherate in the same manner as described above for the crystalline starting material.

IV. ON THE INTERACTION OF β-CAROTENE AND N-BROMOSUCCINIMIDE

A. THEORETICAL PART.

1. Introduction and General Remarks.

The formation of dehydro-lycopene, $C_{40}H_{52}$, an entirely aliphatic carotenoid with 15 conjugated double bonds, by the action of N-bromosuccinimide on lycopene, $C_{40}H_{56}$, was observed by Karrer and Rutschmann (15) eight years ago. Up to the present time this seems to be the only instance reported in which a C_{40}- carotenoid has been dehydrogenated by this method.

The corresponding reaction of β-carotene (only partially aliphatic) with N-bromosuccinimide described in this Thesis was carried out by refluxing in carbon tetrachloride. The resulting mixture was resolved by means of chromatographic analysis and the individual pigments estimated spectrophotometrically. A typical experiment of this type led to a complicated mixture, the composition of which appears in Table 11.

About one half of each of the three dehydrogenated β-carotenes was isolated in pure, crystalline all-<u>trans</u> form, showing the following characteristics: Dehydro-β-carotene, m.p. 193°, maxima in hexane (Beckman): 502, 471, 445 mμ. Bisdehydro-β-carotene, m.p. 204°, maxima: 519, 487, 461. Anhydro-eschscholtzxanthin, m.p. 193°, maxima: 531, 498, 472.

The ease with which Karrer and Rutschmann dehydrogenated lycopene compared with the difficulty involved in dehydrogenating β-carotene may be attributed to the following factors: a) The bromination of lycopene

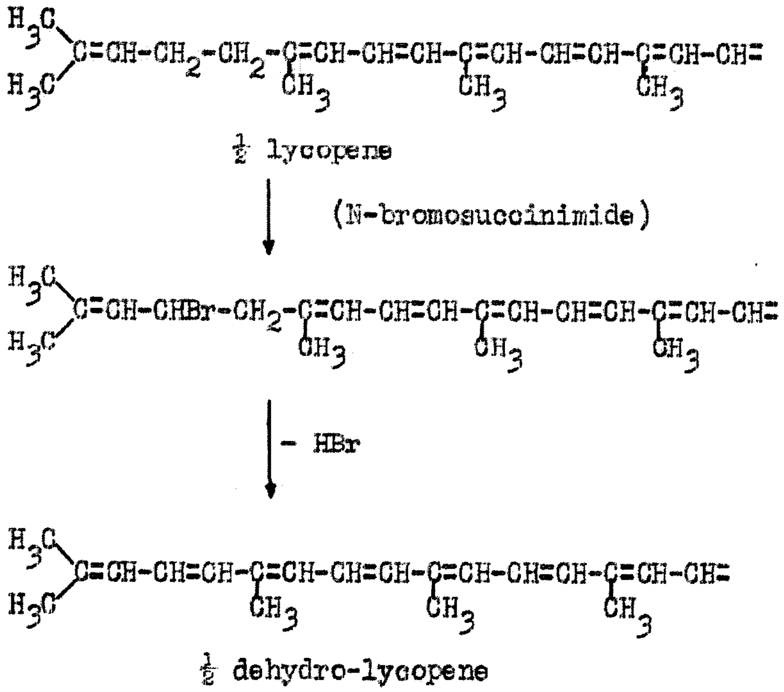
Table 11.

Composition of the Reaction Product of
β-Carotene and N-Bromosuccinimide
(the figures indicate % of starting material)

Unchanged β-carotene	25
Unidentified products (pigments showing extraordinarily strong adsorption affinities and colorless, non-fluorescent compounds; both types probably including halogenated substances)	60
Dehydro-β-carotene	9
Bisdehydro-β-carotene	4
Anhydro-eschscholtzxanthin	2

probably occurs at the carbon atoms within the chain which are next adjacent to the isolated double bonds; it is well known that allylic brominations by N-bromosuccinimide are difficult when the double bonds are part of a conjugated system. b) The necessary dehydrobromination is facilitated by the presence of double bonds on both sides of each of the brominated carbon atoms which then fall into conjugation with the newly formed double bonds (Scheme VI). c) Since dehydro-lycopene is insoluble in carbon tetrachloride it is continuously removed from the reaction mixture as it formed. None of these factors is operative in the case of the β-carotene dehydrogenation.

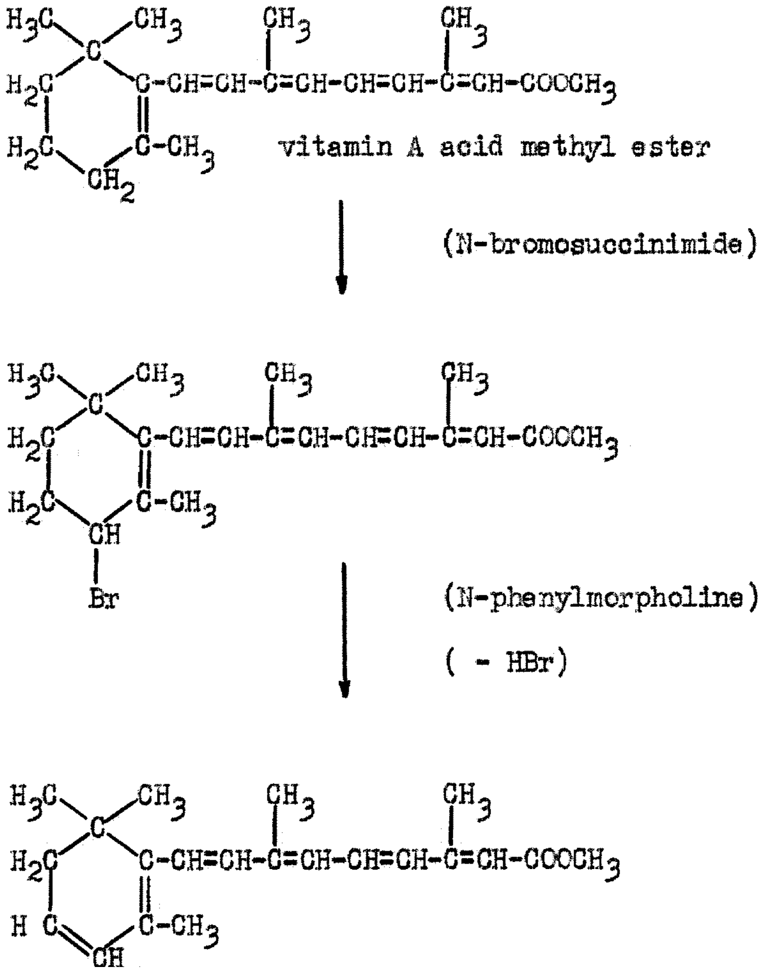
The use of N-phenylmorpholine to facilite the elimination of HBr has been described by Farrar et al. (3) for the dehydrogenation of Vitamin A acid methyl ester (Scheme VII.). A tentative application of this method to β-carotene actually decreased the yield somewhat; at the



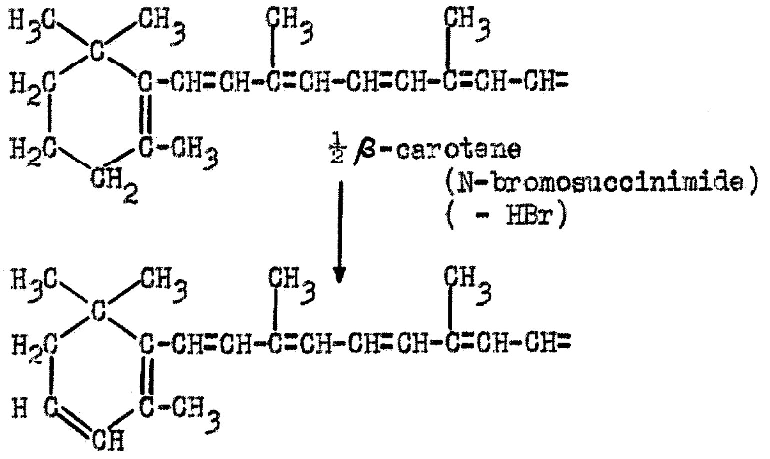
Scheme VI.

present time no explanation of this phenomenon can be offered since, in principle, the two reactions should be very similar (Scheme VIII).

Since N-bromosuccinimide is thought to react <u>via</u> a free radical mechanism, many workers have used light or peroxides to facilitate free radical formation. However, neither illumination of the refluxing reaction mixture nor the use of benzoyl peroxide affected the yields in the β-carotene dehydrogenation. It was found that the addition of $NaHCO_3$ or $BaCO_3$ did increase the yields slightly. A refluxing period of 6 hours and 2:1 mole ratio of N-bromosuccinimide:β-carotene gave the best yields.



Scheme VII



Scheme VIII

2. Structure of Bisdehydro-β-carotene.

Bisdehydro-β-carotene is thought to have the structure represented by either Formula III or IV on the basis of the following considerations:

a) Analysis showed that the compound is a hydrocarbon.

b) Catalytic hydrogenation proved the presence of 13 double bonds.

c) The location of the spectral maxima in hexane, viz. 519, 487, 461 mμ. (Fig. 24), indicates that the double bonds are conjugated. Dehydro-β-carotene having 12 conjugated double bonds has its maxima located about 17 mμ. lower than the corresponding maxima of the bisdehydro compound.

d) Bisdehydro-β-carotene is less strongly adsorbed than lycopene (which has only 11 conjugated double bonds but a completely aliphatic structure) thus indicating that neither of the ring systems of the β-carotene molecule had been opened during the conversion.

At the present time it is not possible to decide between the two proposed structures (Formulas III and IV). The marked similarity in the shapes of the bisdehydro- and dehydro-β-carotene extinction curves and the absence of distinctive <u>cis</u> peaks in the corresponding curves of their neo isomers is most plausibly explained on the basis of the unsymmetrical structure (Formula IV), since then the arrangement of the double bonds in the two compounds would be identical. If bisdehydro-β-carotene had the symmetrical structure represented by Formula III, it would bear the same relationship to vitamin A_2 (3) as β-carotene does to vitamin A_1 (cf. Scheme IX). Bisdehydro-β-carotene could then be expected to show considerable growth promoting properties in the rat.

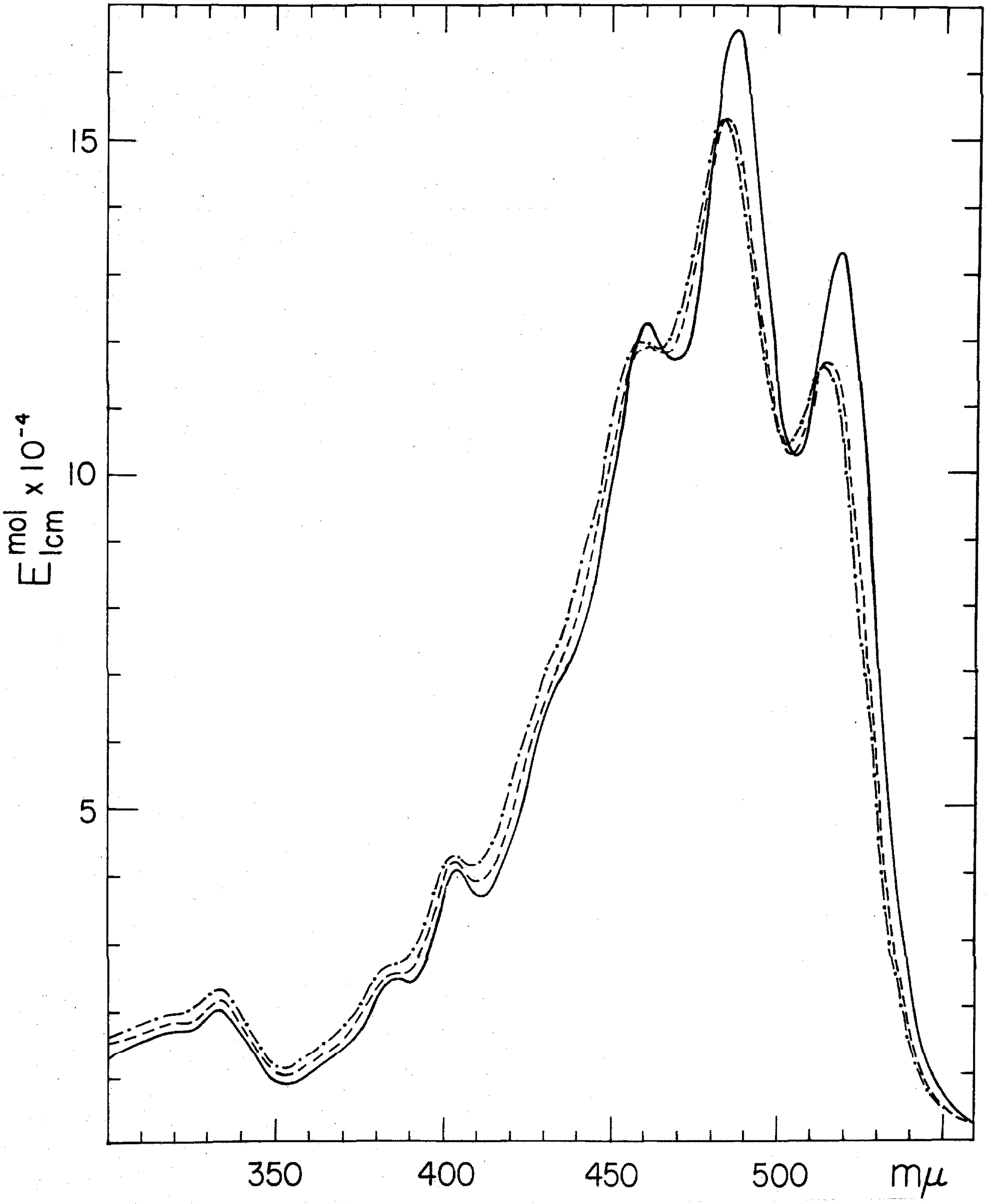


Figure 24. Molecular extinction curve in hexane of all-*trans*-bisdehydro-β-carotene: ——, fresh solution; - - - -, after refluxing for one hour; -·-·-·-, after iodine catalysis in light.

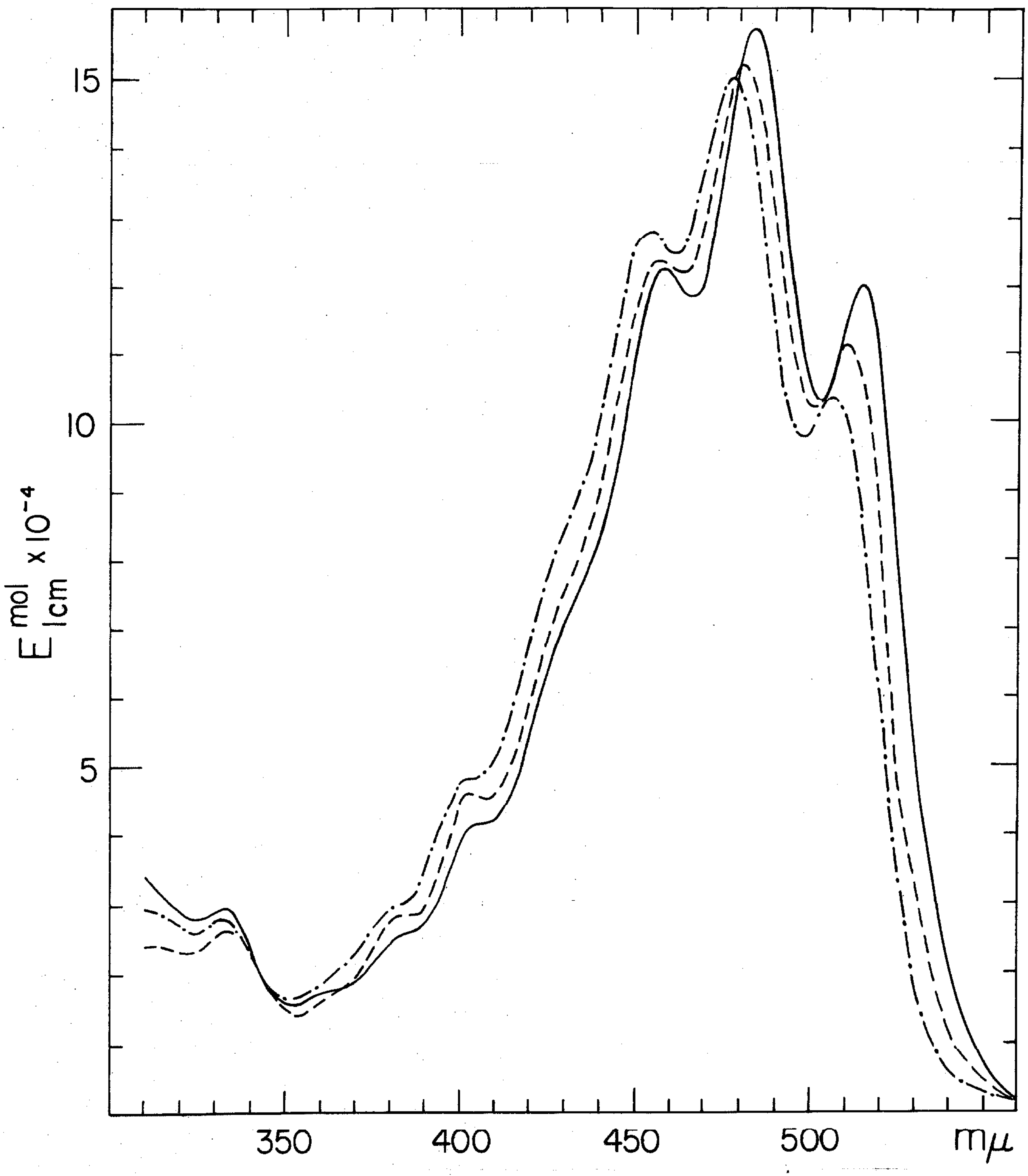


Figure 25. Molecular extinction curves in hexane of some cis bisdehydro-β-carotenes: ——, neo A; – – –, neo B; –·–·–·, neo C.

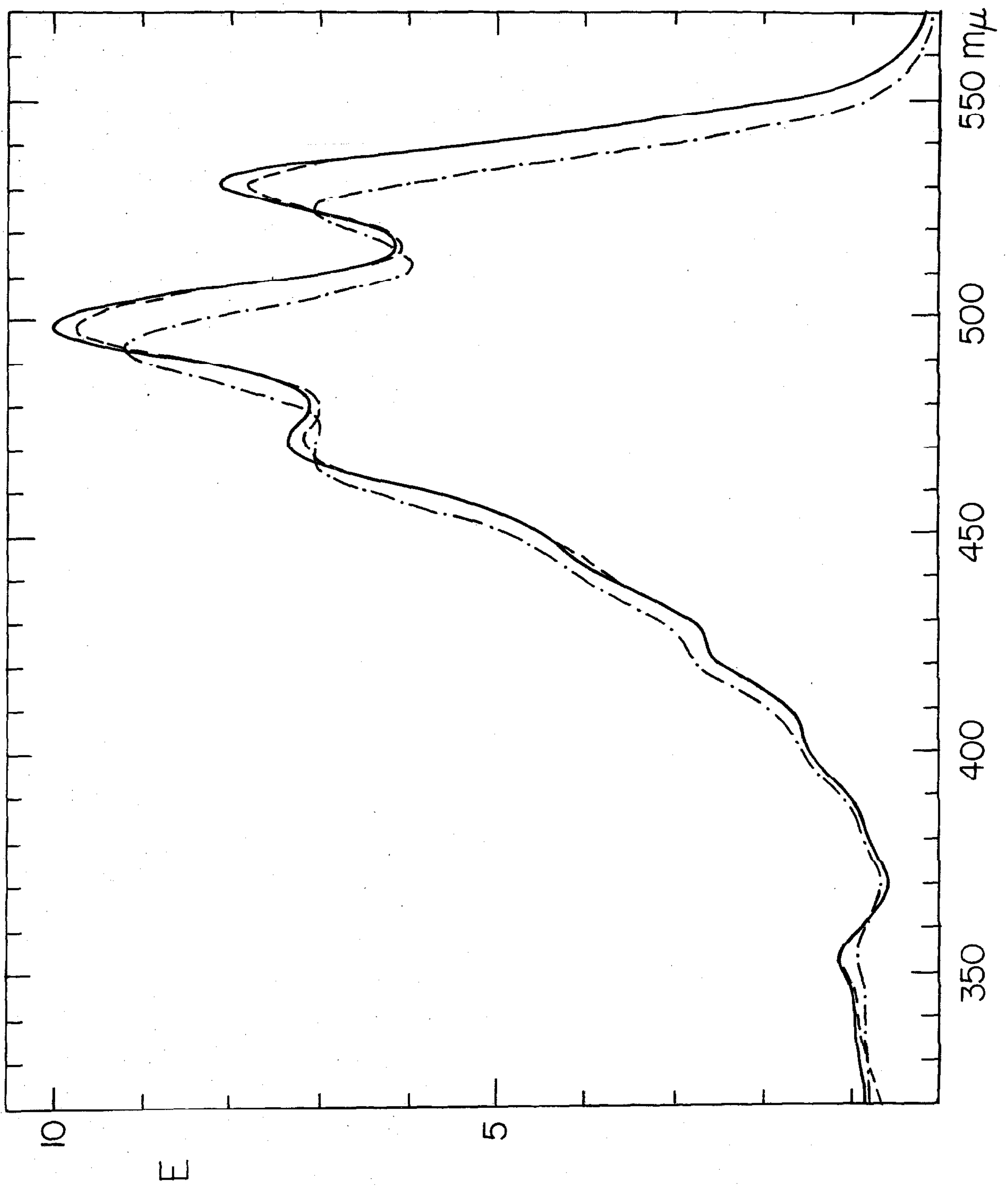
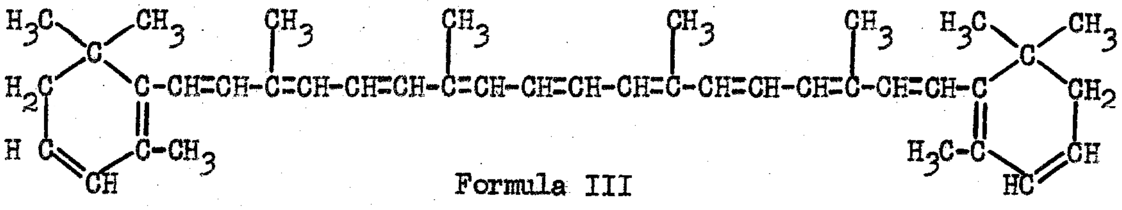
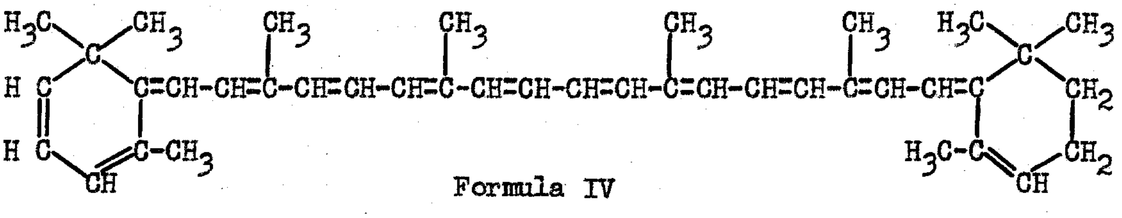


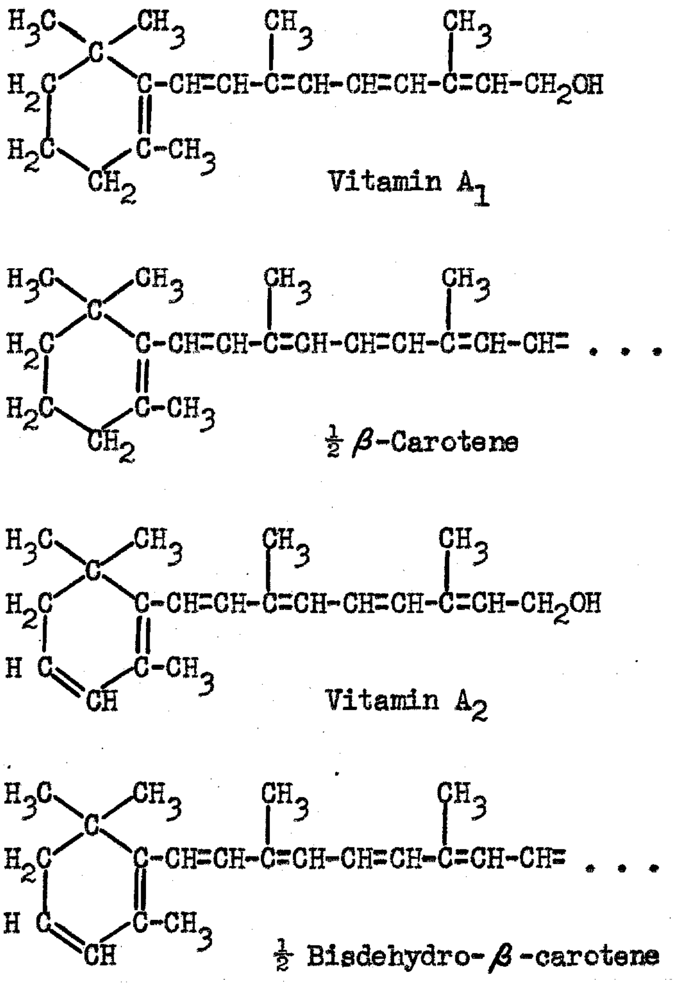
Figure 26. Extinction curves in hexane of anhydro-eschscholtzxanthin: ——, all-trans; ----, "neo A"; -·-·-, "neo B".



Formula III



Formula IV



Vitamin A_1

$\frac{1}{2}$ β-Carotene

Vitamin A_2

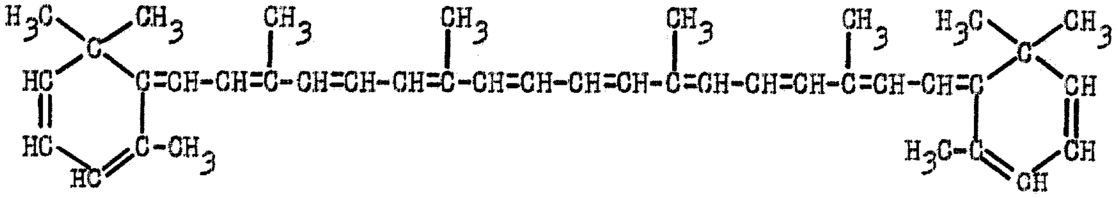
$\frac{1}{2}$ Bisdehydro-β-carotene (see text)

Scheme IX

Should this be the case, then its formulation as a symmetrical molecule would be established. A negative result, however, would not be indicative of any definite structure. Bioassays of this nature are now being carried out at the University of Southern California and will be reported later.

3. Anhydro-eschscholtzxanthin.

By the dehydration of a naturally occurring hydroxylated carotenoid, viz. eschscholtzxanthin, Karrer and Leumann (14) obtained a compound, $C_{40}H_{50}$, termed "anhydro-eschscholtzxanthin" (Formula V). The method of dehydration used by these authors was mentioned in Part III of this Thesis in connection with the dehydration of isocryptoxanthin.



Formula V Anhydro-eschscholtzxanthin

It is believed that one of the products obtained from β-carotene by the action of N-bromosuccinimide is identical with Karrer and Leumann's anhydro-eschscholtzxanthin on the basis of the comparative data presented in Table 12 .

As can be seen from Formula V , only a single arrangement of the 14 double bonds is possible unless the N-bromosuccinimide reaction had opened one or both of the rings in the β-carotene molecule. However this

Table 12.

Comparison of Some Characteristics of Anhydro-eschscholtzxanthin Preparations

	Sample of Karrer and Leumann	Sample obtained in the present study
Spectral maxima in hexane (visual)	531, 500 mμ.	534, 499 mμ.
Melting point	196°	193°
Analysis (Calcd. for $C_{40}H_{50}$: C, 90.50 H, 9.50)	C, 89.69; H, 9.63	C, 89.89; H, 10.10

possibility may be excluded on the basis of the markedly weaker adsorption affinity of the compound in comparison with lycopene.

B. EXPERIMENTAL PART

1. Materials.

The N-bromosuccinimide was obtained from Arapahoe Chemicals, Inc., Boulder, Colo. Recrystallization of this product from water offered no advantages.

2. Preparation of Bisdehydro-β-carotene and Anhydroeschscholtzxanthin.

To a solution of 200 mg. of β-carotene in 60 ml. of carbon tetrachloride were added 132 mg. of N-bromosuccinimide and 300 mg. of sodium bicarbonate. This liquid, contained in a 100 ml. round bottom flask, was refluxed for 6 hours using a 200 watt incandescent lamp as a source of heat. After refluxing, the solution, then somewhat darker than before the reaction, was cooled to room temperature under the tap and filtered. The filtrate was concentrated, in vacuo, to about 20 ml., diluted with 80 ml. of hexane and developed on a 30 x 8 cm. lime-celite column with 3% acetone in hexane:

15	brownish-red	
10	interzone	
75	several red and orange zones	Section I
40	interzone	
45	yellowish-orange	Section II

Sections I and II were cut out separately, eluted and transferred to hexane solution. Section II consisted of unreacted β-carotene of which, according to a spectrophotometric estimation, 52 mg. were recovered.

The hexane solution of Section I was evaporated to dryness, the crystalline residue transferred with the minimum amount of hot benzene, to a 15 ml. centrifuge tube and crystallized from the hot solution by dropwise addition of methanol with constant stirring. Yield, 28 mg. These crystals were dissolved in 150 ml. of hexane and developed on a 30 x 8 cm. lime-celite column with benzene-hexane (1:3). The following rather blurred chromatogram was obtained (figures to the right indicate the visually observed spectral maxima in hexane):

3	brownish-red		
20	interzone		
10	pale red		
15	interzone		
25	orange-red	Zone I	504, 474 mμ.
50	reddish-purple	Zone II	522, 490
25	purple	Zone III	534, 499
15	orange	Zone IV	500, 469
10	orange-red	Zone V	518, 489
5	pale orange	Zone VI	494, 461
3	interzone		
10	pale orange	Zone VII	515, 484
5	yellowish-orange	Zone VIII	491, 460
5	interzone		
10	pale orange	Zone IX	510, 481

Each numbered zone was cut out, eluted and transferred to hexane The pigments contained in the various fractions are listed below.

Fraction I	all-<u>trans</u>-dehydro-β-carotene
Fraction II	all-<u>trans</u>-bisdehydro-β-carotene
Fraction III	anhydro-eschscholtzxanthin
Fractions IV, VI and VIII	<u>cis</u> isomers of dehydro-β-carotene
Fractions V, VII and IX	<u>cis</u> isomers of bisdehydro-β-carotene

The dehydro-β-carotenes, which constituted the bulk of the dehydrogenated pigments formed, were well known on the basis of observations reported in Part I of this Thesis. The crystallization and identification of bisdehydro-β-carotene and anhydro-eschscholtzxanthin are described below in Sections a. and b.

a. Bisdehydro-β-carotene.

The benzene-hexane solution of this compound (Fraction II) was completely evaporated in <u>vacuo</u> and the reddish-purple crystalline residue transferred to a 3 ml. centrifuge tube by means of 1-2 ml. of warm benzene. The tube was then kept in a bath at about 40° and the benzene evaporated by directing a gentle stream of nitrogen at its surface until about $\frac{1}{2}$ ml. of solution remained. The carotenoid was then crystallized from the warm solution by dropwise addition of methanol down the sides of the centrifuge tube. After 3-4 volumes of methanol had been added the tube was removed from the bath and kept at room temperature for an hour. The crystals were centrifuged and, after decanting the mother liquor, washed with methanol, centrifuged again and dried in <u>vacuo</u> (0.2 mm.) over P_2O_5 at the temperature of refluxing acetone for one hour. Yield, 2-2.5 mg. A total of 35 mg. of bisdehydro-β-carotene was isolated from numerous similar experiments.

<u>Characterization.</u>

<u>Crystal form</u>: Crystallized from benzene-methanol in oblong, pale purple plates (Fig. 27). Macroscopically, the crystals appeared dark purple.

<u>Melting point</u>: After sintering at 190° the crystals melted sharply at 204°.

<u>Solubility</u>: Sparingly soluble in hexane, more easily in benzene. Insoluble in methanol.

<u>Partition test</u>: When partitioned between hexane and 95% methanol the compound was found entirely in the epiphase.

Analysis: Calculated for $C_{40}H_{52}$: C, 90.16; H, 9.84. Found: C, 89.40; H, 10.37.

Spectral maxima, in hexane: 522, 490 mµ (visual); 519, 487, 461 mµ (Beckman).

Molecular extinction coefficient at $\lambda_{max.}$: $E^{mol.}_{1cm.} = 16.6 \times 10^4$ (in hexane).

Hydrogenation: 5.269 mg. of substance (with 11.53 mg. of PtO_2 catalyst) added 2.76 ml. of hydrogen (0°, 760 mm.). Calculated for $C_{40}H_{52}$: 13 double bonds. Found: 12.5 double bonds. As a control, a parallel hydrogenation was run with 5.056 mg. of β-carotene and 11.09 mg. of the catalyst. Calculated for $C_{40}H_{56}$: 11 double bonds. Found: 10.6 double bonds.

Chromatographic behavior: Adsorbed below dehydro-β-carotene and above β-carotene on lime-celite, when developed with 3% acetone in hexane or a 1:3 benzene-hexane mixture.

b. Anhydro-eschscholtzxanthin.

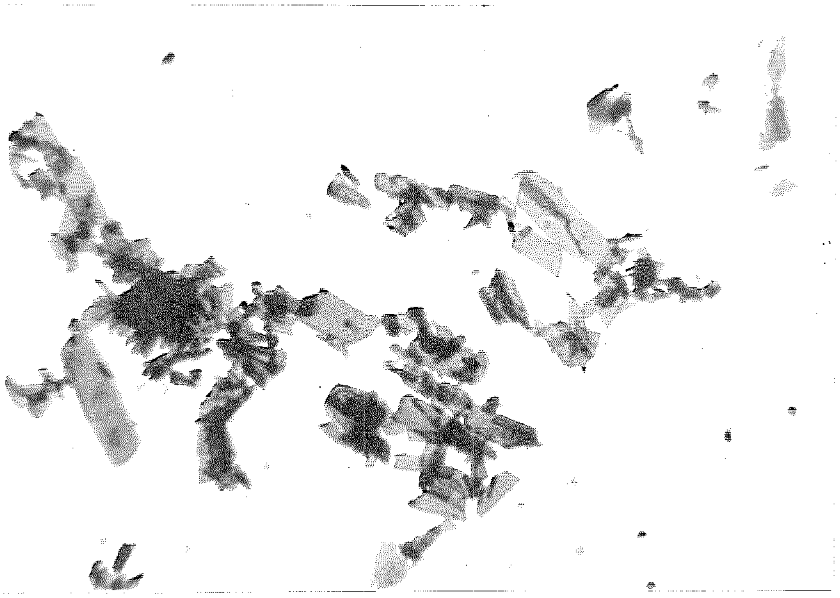
This compound was crystallized from benzene-methanol according to the procedure described above for bisdehydro-β-carotene. Yield, about 1 mg. A total of 14 mg. was isolated from numerous similar experiments.

Characterization.

Crystal form: Individual crystals from benzene-methanol were brownish-purple; overlapping crystals appeared almost black. In general, the crystals formed bulky conglomerates but occasionally parallel twinning and fan-like groupings were found, (Fig. 28). Macroscopically, the crystals appeared very dark purple.

Melting point: 192-193°

Solubility: More soluble in hexane and benzene than bisdehydro-



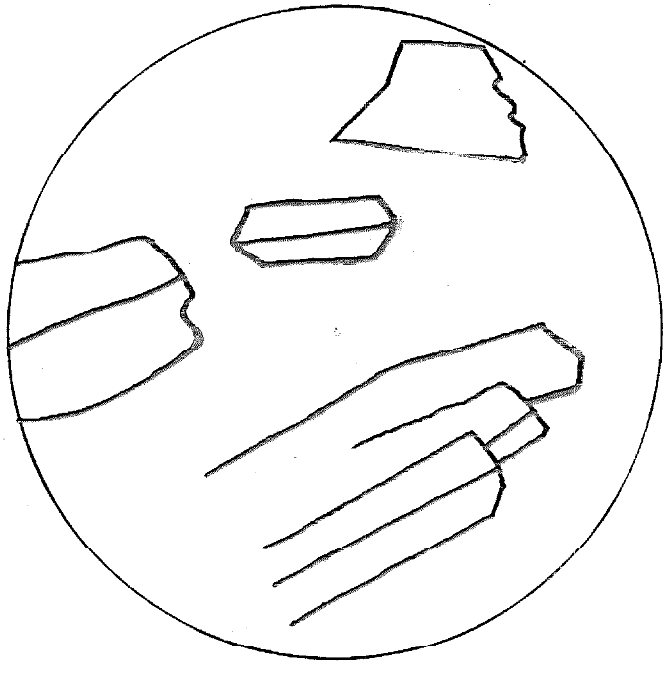


Figure 27 (above): Bisdehydro-β-carotene from benzene-methanol (430 x).

Figure 28 (below): Anhydro-eschscholtzxanthin from benzene methanol.
Sketch of typical crystal forms.

β-carotene; insoluble in methanol.

Partition test: Entirely epiphasic in the hexane-95% methanol system.

Analysis: Calculated for $C_{40}H_{50}$: C, 90.50; H, 9.50. Found: C, 89.89; H, 10.10

Spectral maxima, in hexane: 534, 499 mµ. (visual); 531, 499, 472 mµ. (Beckman).

Molecular extinction coefficient, at $\lambda_{\text{max.}}$: $E^{\text{mol.}}_{\text{1cm.}} = 16.0 \times 10^4$.

Chromatographic behavior: Adsorbed below bisdehydro-β-carotene but above β-carotene on lime-celite, when developed with 3% acetone in hexane.

3. Some Stereoisomers of Bisdehydro-β-carotene.

A solution of 3 mg. of bisdehydro-β-carotene in 100 ml. of hexane containing about 1% of the pigment weight in iodine was illuminated under the same conditions as prescribed for the iodine catalysis of dehydro-β-carotene (Part I, Section B., 3a.). The isomerized solution was developed on a 28 x 5 cm. lime-celite column with benzene-hexane (1:3):

5	very pale purple		
10	interzone		
5	very pale pink		
15	red	all-*trans*	521, 490, 457 mµ.
10	orange-red	neo A	518, 485, 456 (Fig. 25)
20	interzone		
15	pale orange-red	neo B	514, 483 (Fig. 25)
20	interzone		
20	pale orange	neo C	510, 481 (Fig. 25)

Before extrusion, the column was freed from benzene by washing with hexane. Each zone was cut out, eluted, and transferred to hexane. Estimates of the amount of pigment in each fraction were made spectrophotometrically. The composition of the stereoisomeric mixture (in % of starting material) is given below:

All-_trans_-bisdehydro-β-carotene	24%
Neo-bisdehydro-β-carotene A	25
Neo B	13
Neo C	6
Unaccounted for	32

The abnormally large percentage of destroyed pigment is indicative of the sensitivity of this compound.

4. Some Stereoisomers of Anhydro-eschscholtzxanthin.

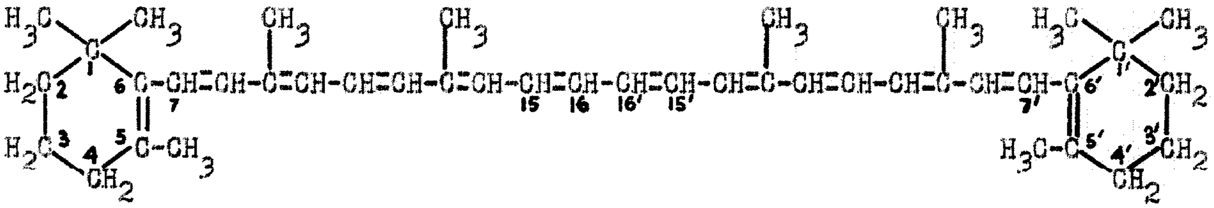
Systematic stereoisomerization experiments were not carried out with this compound. However, chromatographic resolution of a hexane solution which had been kept at 4° for about 3 days showed the presence of small amounts of two new substances both adsorbed below anhydro-eschscholtzxanthin. They were _cis_ isomers of the latter since chromatographic resolution of their iodine catalyzed hexane solutions showed the presence of a pigment that was chromatographically and spectroscopically identical with anhydro-eschscholtzxanthin. The more strongly adsorbed of the _cis_ isomers had spectral maxima identical to that of the all-_trans_ compound (cf. Fig. 26).

APPENDIX

A. PROVITAMIN A POTENCY OF A β-CAROTENE HOMOLOGUE.

1. Introduction and General Remarks.

Recently, a homologue of β-carotene was obtained by total synthesis by Inhoffen, Bohlmann, Aldag, Bork, and Leibner (8). This new compound, termed 16,16'-homo-β-carotene (Formula VI) differs from β-carotene in the presence of two extra CH groups in the central part of the chromophore and is the first representative of a carotenoid of this type. Although the last step in this synthesis (partial saturation of acetylenic bonds by means of a poisoned catalyst) led to the formation of a molecule with two centrally located cis bonds, these authors were also able to obtain the all-trans form by iodine catalysis (Fig. 29).



Formula VI 16,16'-homo-β-carotene.
(The numbering system follows the nomenclature of Karrer.)

Bioassays of the all-trans and di-cis spatial forms were carried out with rats. In accordance with the usual procedure, the supplements were administered in Wesson oil solutions*. As shown in Table 13., both

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* This work has been published in collaboration with H. J. Deuel, Jr., H. H. Inhoffen, J. Ganguly and L. Zechmeister (2). Determinations of the spectral characteristics and the degree of stability, under standardized conditions, of the compounds in Wesson oil were carried out in this laboratory, while the bioassays were performed at the University of Southern California. I wish to thank Prof. H. H. Inhoffen, Braunschweig, Germany for the two synthetic samples.

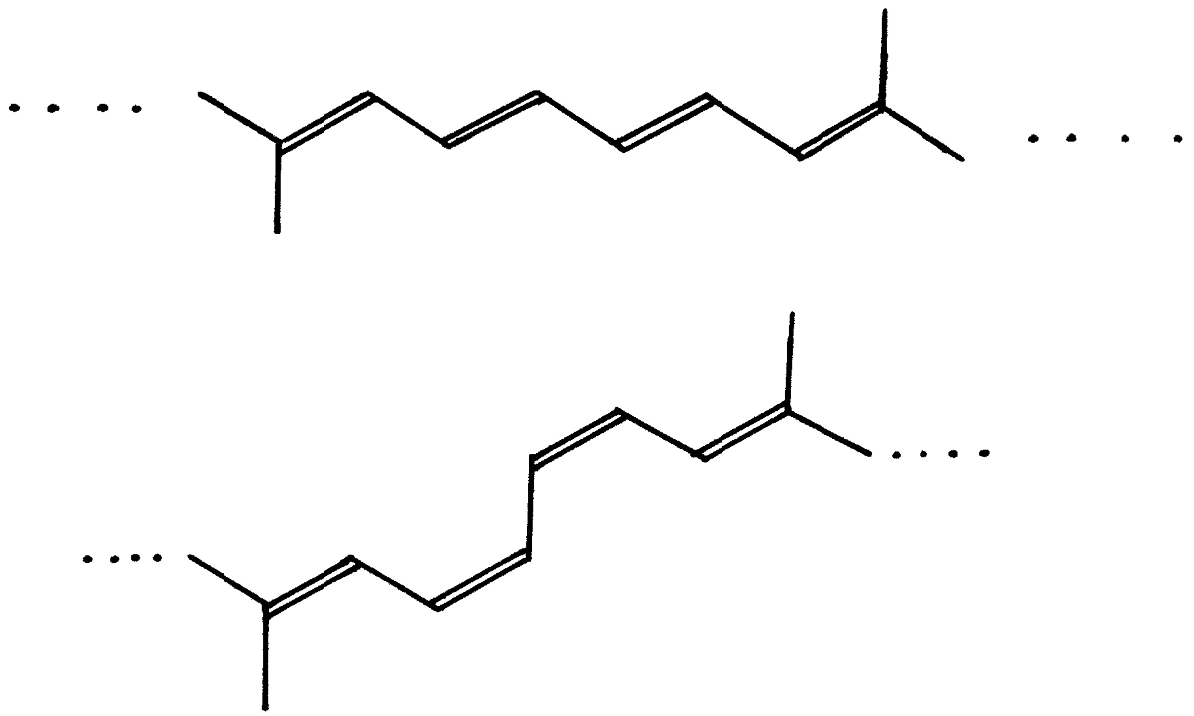


Figure 29. All-<u>trans</u>-16,16'-homo-β-carotene (above), and its central di-<u>cis</u> isomer (below) (skeleton models).

spatial forms were active.

Table 13.

Relative Provitamin A Activities in the Rat

Compound	Relative potency (%)
All-*trans*-β-carotene	100
All-*trans*-16,16'-homo-β-carotene	22
15,16,15',16'-di-*cis*-homo-β-carotene	20

On the basis of the considerable provitamin A potency of homo-β-carotene the following conclusions may be drawn:

a) The conversion of a carotenoid to vitamin A takes place even when the number of carbon atoms in the C-C skeleton of the provitamin molecule exceeds forty. (It had been found earlier by Karrer and Solmssen (18) that β-apo-2-carotenal, a carotenoid with thirty carbon atoms showed strong provitamin A potency.)

b) The absence of an isoprenic structure in the central portion of the chromophore does not prevent enzymatic attack in that region.

c) It is not essential, for a successful enzymatic attack, that the provitamin A have a double bond at the center of the molecule.

The practically equal biopotencies of the all-*trans* and di-*cis* forms of homo-β-carotene can be explained, tentatively, by the fact that both have straight molecular shapes.

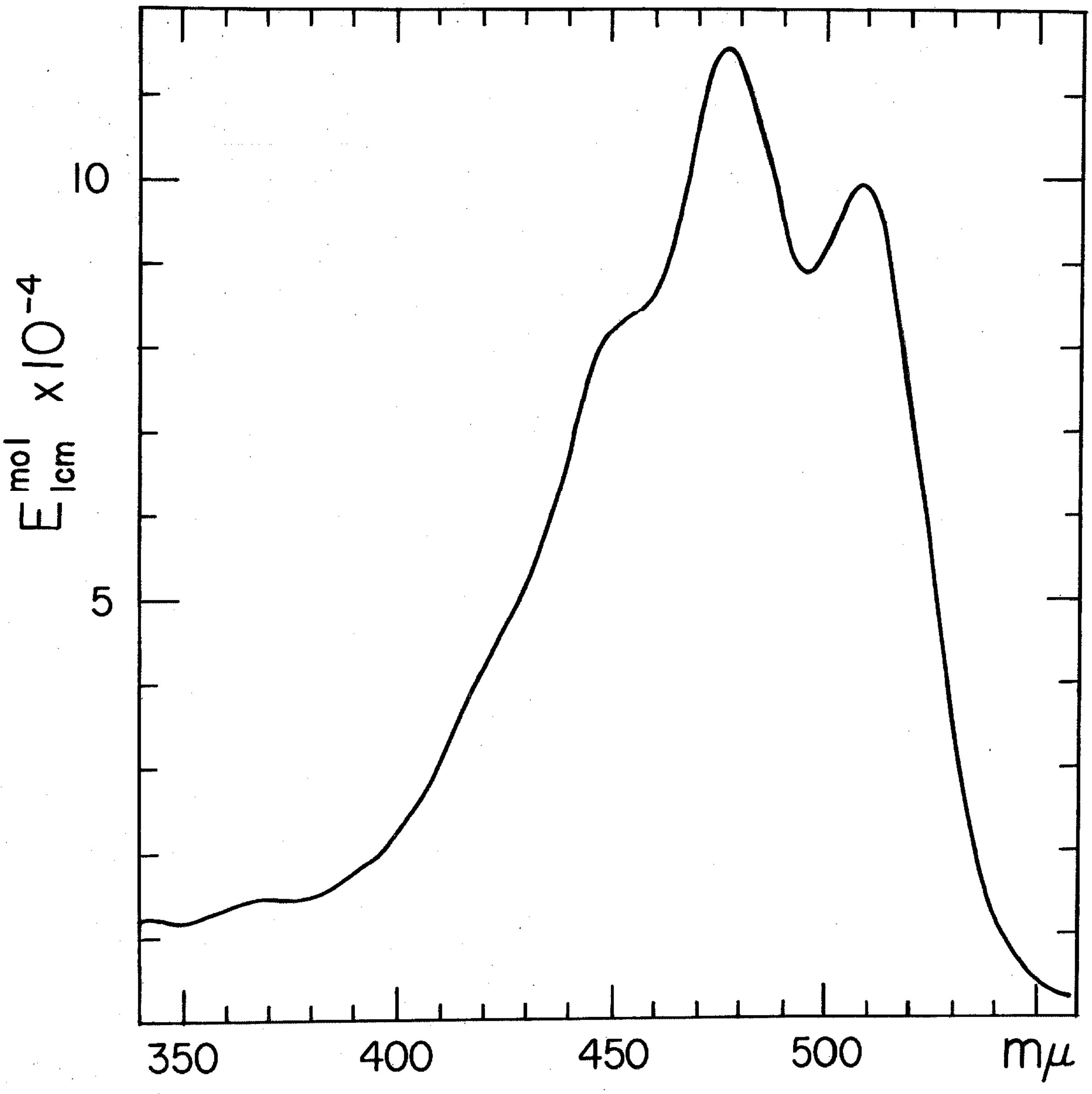


Figure 30. Molecular extinction curve in Wesson oil of all-*trans*-16, 16'-homo-β-carotene.

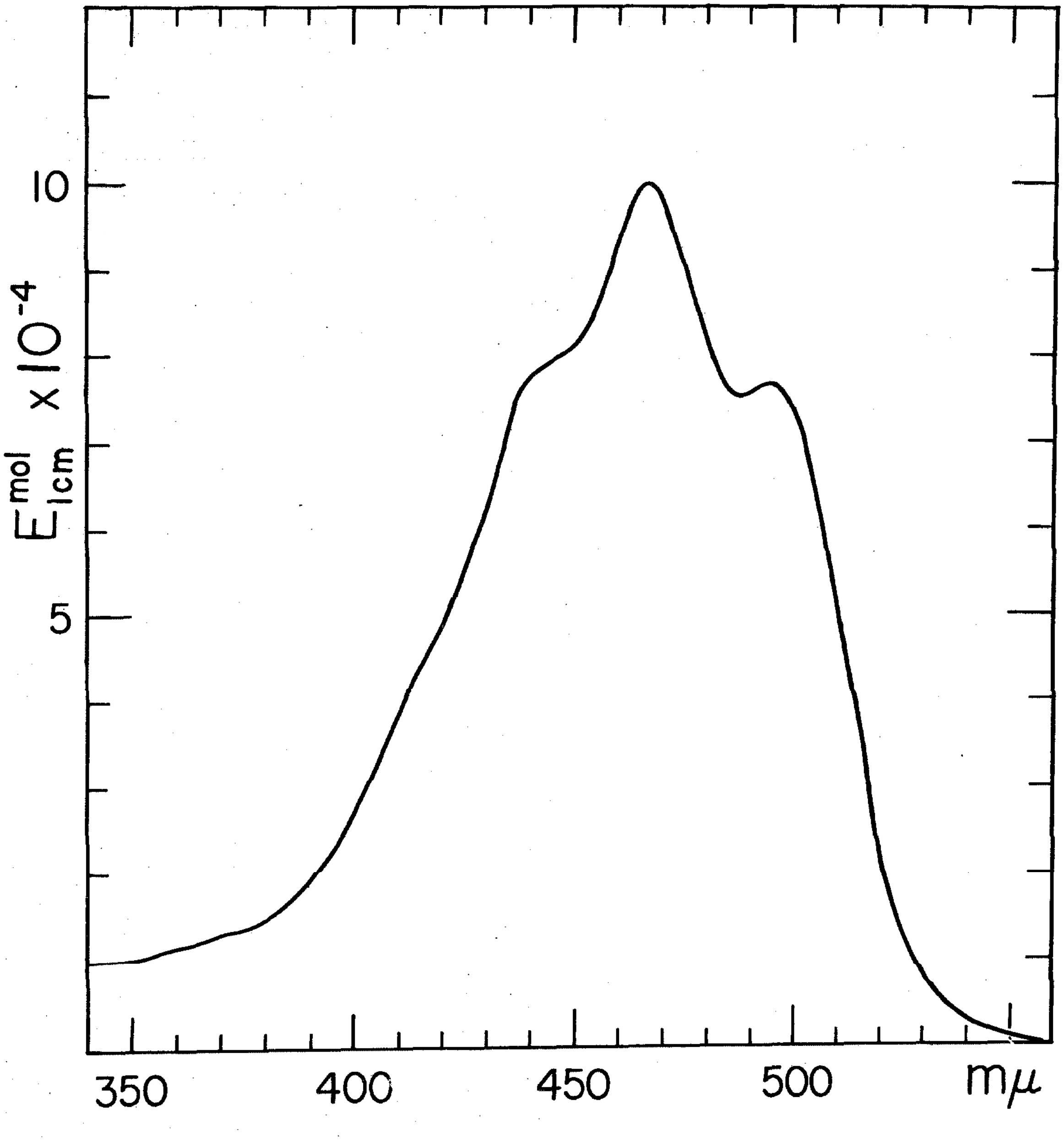


Figure 31. Molecular extinction curve in Nesson oil of di-*cis*-16,16'-homo-β-carotene.

2. Experimental.

In order to prepare the supplements, the carotenoids were dissolved in peroxide free diethyl ether and transferred to flasks having ground-glass stoppers; the ether was then removed under gentle suction, leaving the pigment as a thin film on the walls of the flask. About 90-100 ml. of Wesson oil (containing 0.5 mg. of α-tocopherol per 0.1 ml. of solution) was immediately introduced, the air displaced by nitrogen, and the flasks stoppered. They were allowed to stand for 3-4 days in the dark at room temperature, and were shaken occasionally. The oily solutions were then passed through sintered-glass funnels of medium porosity and the concentration of the pigment in each filtrate determined spectrophotometrically. They were then diluted to give concentrations of 1, 2, or 4 µg./ 0.2 ml. of solution in the case of the synthetic C_{42} compounds, and 0.5 and 1.0 µg. in the case of all-trans-β-carotene. All solutions were stored under nitrogen in 10 ml. brown bottles at 5°. New samples were used twice weekly during the assay period.

Spectral readings were made at $\lambda_{max.}$ with a Beckman spectrophotometer, Model DU, before and after the bioassay. No significant changes could be noted, demonstrating that both the di-cis and all-trans configurations of homo-β-carotene show remarkable resistance toward thermal stereoisomerizations in Wesson oil solutions.

The values for the molecular extinction coefficients in Wesson oil are: All-trans-16,16'-homo-β-carotene, $E^{mol.}_{1cm.} = 11.5 \times 10^4$ (at 477 mµ.); di-cis isomer, $E^{mol.}_{1cm.} = 10.0 \times 10^4$ (at 466-467 mµ.). For the molecular extinction curves, cf. Figs. 30 and 31 .

REFERENCES

1. Chatterjee, A. and Zechmeister, L: J. Am. Chem. Soc. 72, 254 (1950).

2. Deuel, H. J. Jr., Inhoffen, H. H., Ganguly, J., Wallcave, L. and Zechmeister, L.: Arch. Biochem. and Biophys. 40, 352 (1952).

3. Farrar, K., Hamlet, J. C., Henbest, H. B. and Jones, E. R. H.: J. Chem. Soc. (Lond.) 1952, 2657.

4. Friedel, R. A. and Orchin, M.: Ultraviolet Spectra of Organic Compounds, John Wiley & Sons, New York, 1951.

5. Garbers, G. F., Eugster, C. H. and Karrer, P.: Helv. chim. Acta 35, 1850 (1952).

6. Goodwin, T. W. and Taha, M. M.: Biochem. J. 48, 513 (1951).

7. Hennion, G. F. Hinton, H. D. and Nieuland, J. A.: J. Am. Chem. Soc. 55, 2858 (1933).

8. Inhoffen, H. H., Bohlmann, F., Aldag, H. J., Bork, S. and Leibner, G.: Ann. 573, 1 (1951).

9. Inhoffen, H. H. and Siemer, H.: Fortschr. Chem. org. Naturstoffe 9, 1 (1952).

10. Karrer, P.: Fortschr. Chem. org. Naturstoffe 5, 1 (1948).

11. Karrer, P.: Helv. chim. Acta 34, 2160 (1951).

12. Karrer, P. and Eugster, C. H.: Helv. chim. Acta 34, 1400 (1951).

13. Karrer, P., Jucker, E., Rutschmann, J. and Steinlein, K.: Helv. chim. Acta 28, 1150 (1945).

14. Karrer, P. and Leumann, E.: Helv. chim. Acta 34, 445 (1951).

15. Karrer, P. and Rutschmann, J.: Helv. chim. Acta 28, 793 (1945).

16. Karrer, P., Schöpp, K. and Morf, R.: Helv. chim. Acta 15, 1158 (1932).

17. Karrer, P. and Schwab, G.: Helv. chim. Acta 23, 578 (1940).

18. Karrer, P. and Solmssen, U.: Helv. chim. Acta 20, 682 (1937)

19. Karrer, P. and Takahashi, T.: Helv. chim. Acta 16, 1163 (1933).

20. Kuhn, R. and Grundmann, C.: Ber. 66, 1746 (1933).

21. Kuhn, R. and Lederer, E.: Ber. 65, 637 (1932).

22. Kuhn, R. and Winterstein, A.: Ber. 66, 1733 (1933).

23. LeRosen, A. L.: Ind. and Eng. Chem. (Anal. Ed.) 14, 165 (1942).

24. Meunier, P. and Vinet, P.: Chromatographie et Mesomerie, Masson & Cie, Paris, 1947.

25. Oroshnik, W., Karmas, G. and Mebane, A. D.: J. Am. Chem. Soc. 74, 295 (1952).

26. Pauling, L.: Fortschr. Chem. org. Naturstoffe 3, 203 (1939).

27. Pinckard, J. H., Wille, B. and Zechmeister, L.: J. Am. Chem. Soc. 70, 1938 (1948).

28. Polgar, A. and Zechmeister, L.: J. Am. Chem. Soc. 64, 1856 (1942).

29. Polgar, A. and Zechmeister, L.: J. Am. Chem. Soc. 65, 1528 (1943).

30. Strain, H. H.: J. Am. Chem. Soc. 63, 3448 (1941).

31. Strain, H. H.: J. Biol. Chem. 123, 425 (1938).

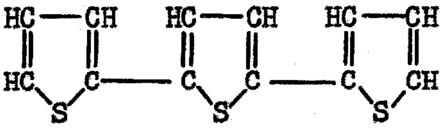
32. Zechmeister, L.: Chem. Rev. 34, 267 (1943).

33. Zechmeister, L. and LeRosen, A. L.: J. Am. Chem. Soc. 64, 2755 (1942).

34. Zechmeister, L., LeRosen, A. L., Schroeder, W. A., Polgar, A. and Pauling, L.: J. Am. Chem. Soc. 65, 1940 (1943).

Propositions submitted by L. Wallcave

1. Within recent years certain organic compounds in crystalline form or in solutions have been used, in conjunction with photomultipliers, as scintillation counters for γ-radiation or energetic particles. So far, only aromatic or partially aromatic hydrocarbons such as stilbene, terphenyl, naphthalene, and anthracene have been investigated. (Cf. R. Hofstadter: Nucleonics 6, (No. 5), 70 (1950)). I propose these investigations be extended to include α-dithienyl and α-terthienyl, compounds containing sulfur and exhibiting some aromatic character. Terthienyl is strongly fluorescent in ultraviolet light.



α - TERTHIENYL

2. When a solution of phosphotungstic acid, $H_3PW_{12}O_{40}$, was photochemically reduced in the presence of isopropyl alcohol the colorless solution turned black. (L. Chalkley: J. Chem. Phys. 56, 1084 (1952)). Upon oxidation with silver nitrate the original acid was again formed. The composition of the black acid could not be determined precisely, but Chalkley suggested the formulas, $H_4PW_{12}O_{40}$ or $H_2PW_{12}O_{38}$. I propose that the reduced acid, as indicated by its color, contains tungsten in a mixture of two or more oxidation states.

3. A white solid showing the empirical composition AlH_3 can be isolated from the reaction mixture formed by the interaction of aluminum chloride and lithium hydride. (A. E. Finholt, A. C. Bond, Jr.

and H. I. Schlesinger: J. Am. Chem. Soc. 69, 1199 (1947)). I propose that X-ray diffraction studies be made with this substance to supplement available information on the nature of the covalent aluminum bonds in such compounds as Al_2Cl_6 and $Al_2(CH_3)_6$.

4. Myxoxanthin, $C_{40}H_{54}O$, is a keto-carotenoid of undetermined constitution but which may have the γ-carotene carbon skeleton. (Cf. P. Karrer and E. Jucker: Carotenoids, Elsevier, New York, 1950, p. 225). I propose that the carbonyl group in myxoxanthin be reduced by means of the Wolff-Kishner method and the resultant hydrocarbon compared with authentic γ-carotene.

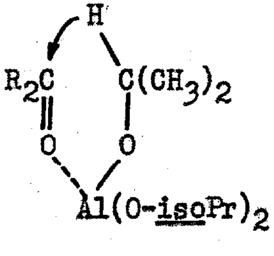
5. The Thiele addition of water or methanol to the conjugated system in dehydro-β-carotene in the presence of boron trifluoride (reported in this Thesis) should also occur with other electrophilic reagents such as aluminum chloride or antimony trichloride. Furthermore, the use of boron trifluoride as a catalyst for the addition of water or alcohols to conjugated dienes or polyenes should be investigated.

6. The adsorption affinities on lime of dehydro-β-carotene ($C_{40}H_{54}$; 12 conjugated double bonds), bisdehydro-β-carotene ($C_{40}H_{52}$; 13 conjugated double bonds), and anhydro-eschscholtzxanthin ($C_{40}H_{50}$; 14 conjugated double bonds) decrease in that order (reported in this Thesis). Since these compounds all have the same carbon skeleton, I propose that this unexpected sequence is associated with the steric effects of the methyl groups located near the ends of the conjugated system.

7. T. W. Goodwin has reported (Biochem. J. 51, 458 (1952)) the presence of a colorless, fluorescent polyene with a phytofluene like

absorption spectrum in the berries of <u>Lonicera japonica</u>. Since it was absorbed less strongly than phytofluene, which is dodecahydro-lycopene ($C_{40}H_{68}$), Goodwin suggested that the new compound is dodecahydro-β-carotene. I suggest that the new compound may well be the <u>cis</u> phytofluene reported by F. J. Petracek and L. Zechmeister (J. Am. Chem. Soc. <u>74</u>, 184 (1952)). I also suggest that dodecahydro-β-carotene, should it exist, would be found in carrots, in which β-carotene is the main pigment and where lycopene is found only occasionally, in small amounts. Therefore, I propose that the phytofluene from carrots be compared with that obtained from tomatoes, in which lycopene is predominant. The situation in the case of <u>Lonicera japonica</u> is unclear because considerable amounts of both β-carotene and lycopene are found in these berries and thus might be expected to contain both phytofluene (dodecahydro-lycopene) and dodecahydro-β -carotene.

8. According to M. J. S. Dewar (<u>Electronic Theory of Organic Chemistry</u>, Oxford Univ. Press, London, 1949, p. 136) the Meerwein-Ponndorf reduction using aluminum isopropoxide may proceed by means of a hydride ion transfer within a cyclic intermediate, as follows:



I propose the use of boron isopropoxide in the reaction to test this hypothesis considering the fact that boron alkoxides form stronger "acceptor" bonds with carbonyl groups than do the corresponding aluminum compounds. (Cf. N. V. Sidgwick: The Chemical Elements and Their Compounds, Vol. 1, Oxford Univ. Press, London, 1950, p. 421).

9. In the metalation of triphenylamine with butyl lithium the metal atom substitutes a ring in the *meta* position. In corresponding reactions with aniline, diphenylamine, and N-phenylcarbazole the substitution occurs in the *ortho* position. (H. Gilman and S. M. Spatz: J. Org. Chem. **17**, 860 (1952)). These authors suggest that the exception to the "*ortho* rule" in the case of triphenylamine results from "the steric hindrance of the free rotating phenyl groups". I propose that this explanation is at best only partly correct since the metalation should then occur at the *para* position. I further propose that the metal atom is directed into the *meta* position by the presence of a considerable positive charge on the nitrogen atom owing to resonance effects; thus this process would be independent of any steric factors.

10. A serious problem in the analysis of many organic compounds is the preparation of ash-free samples. I propose the use, for this purpose, of ethylenediamine N-tetra-acetic acid, $(HOOCCH_2)_2NCH_2$-$CH_2N(CH_2COOH)_2$, sold under the trade name "Sequestrene".