I. SOME IRREVERSIBLE CONVERSIONS OF LUTEIN AT ELEVATED TEMPERATURES

II. INVESTIGATION OF THE EFFECT OF GLOBULIN DEPLETION ON ANTIBODY PRODUCTION IN RABBITS

III.

Thesis by

John William Sease

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

California Institute of Technology

Pasadena, California

1945

The author wishes to express his sincere appreciation to Professor Zechmeister, Professor Niemann, and Professor Campbell for their interest in the work reported in this Thesis and for their guidance throughout its course.

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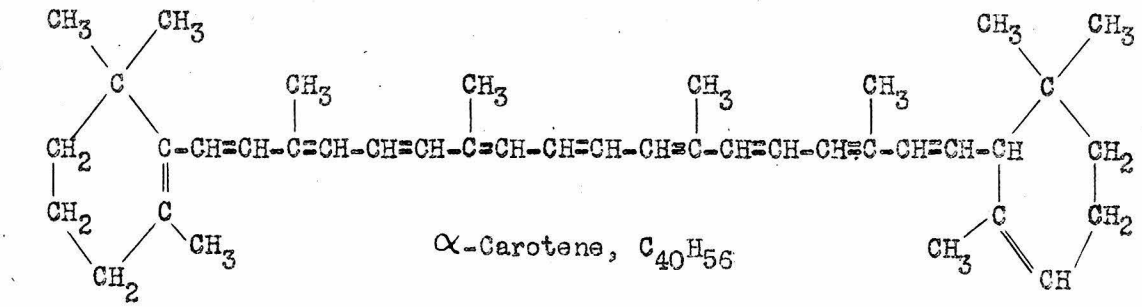
A. INTRODUCTION

A long chain of conjugated double bonds is one of the distinguishing characteristics of the group of naturally occurring polyene pigments which are designated as "carotenoids". Although the great majority of these compounds contains forty carbon atoms (Figure 1), a few pigments of lower molecular weight are known (Figure 2).

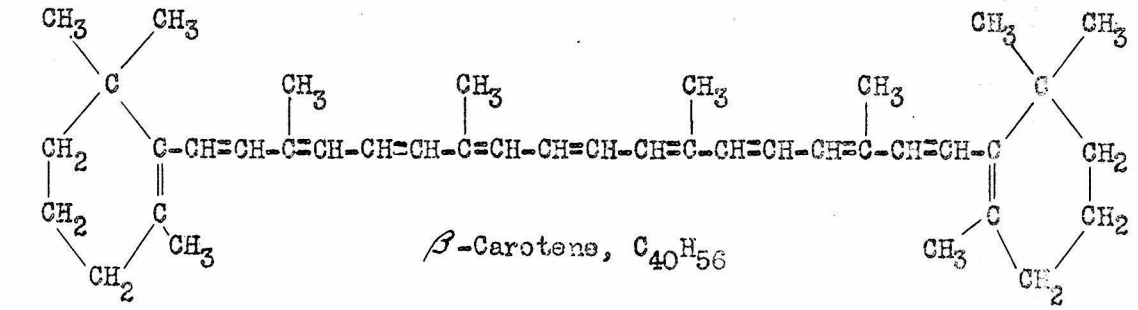
1. The Nomenclature of Dihydroxy Carotenoids

When Kuhn, Winterstein, and Lederer (41) showed that "xanthophyll", $C_{40}H_{56}O_2$, which had previously been considered to be homogeneous, actually was composed of two pigments, they proposed that the term "xanthophyll" be applied as a group name to those C_{40}-carotenoids which contain hydroxyl groups, and that the term "lutein" be reserved for the principal constituent of the xanthophyll mixture. "Lutein" had been used earlier by Willstatter and Escher (66) to describe the xanthophyll pigment isolated from the yolks of hen's eggs. Kuhn's nomenclature has been accepted by several other authors (67,62,48) and consequently will be used in this Thesis, although von Euler (4) prefers to refer to the egg yolk mixture as "lutein" and Karrer (22) uses the expression "phytoxanthine" as a collective term for all carotenoid alcohols.

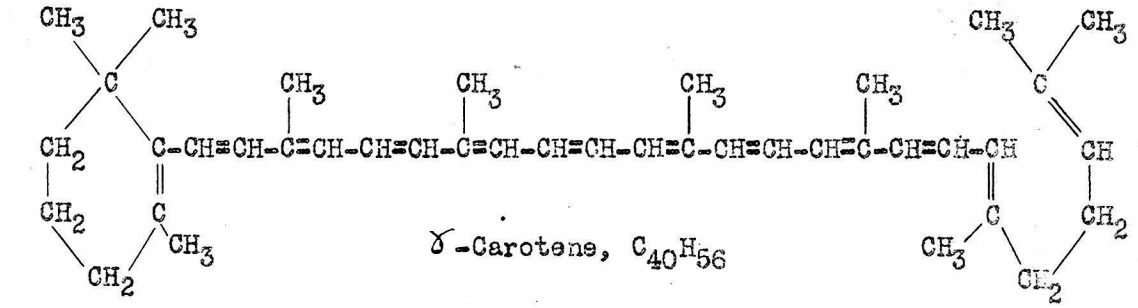
Soon after the two individual compounds had been isolated from xanthophyll their structures were clarified by Karrer and his colleagues (30,12), who showed that lutein was a dihydroxy-α-carotene and that the other pigment was a dihydroxy-β-carotene (Figure 1), which has been named "zeaxanthin".



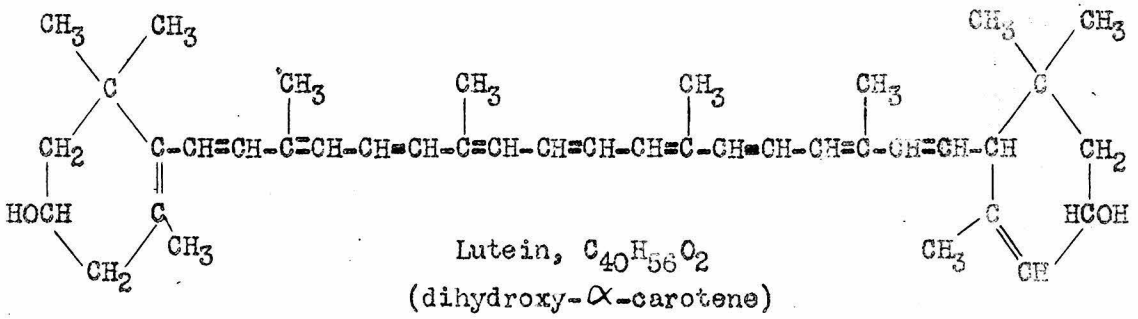
α-Carotene, $C_{40}H_{56}$



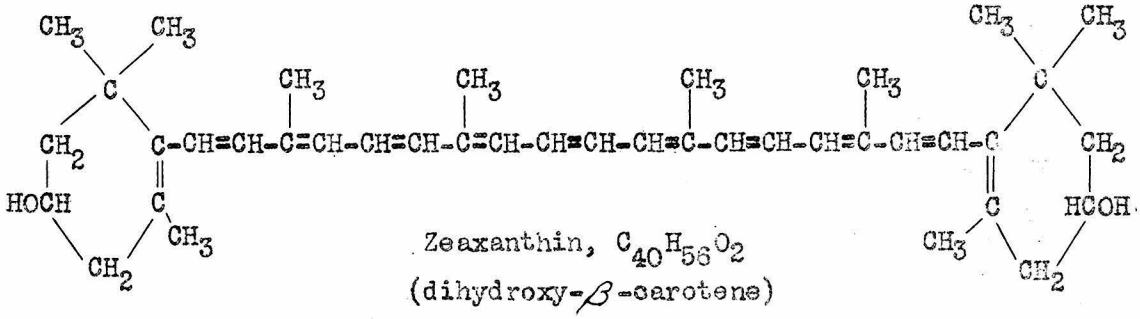
β-Carotene, $C_{40}H_{56}$



γ-Carotene, $C_{40}H_{56}$

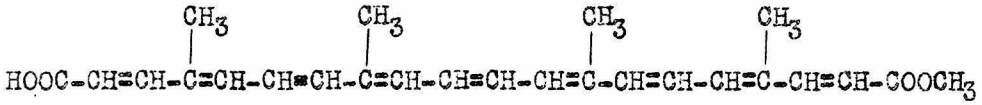


Lutein, $C_{40}H_{56}O_2$
(dihydroxy-α-carotene)

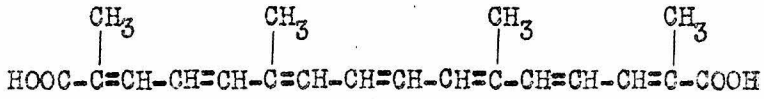


Zeaxanthin, $C_{40}H_{56}O_2$
(dihydroxy-β-carotene)

Figure 1. Some Carotenoids Which Contain Forty Carbon Atoms



Bixin, $C_{25}H_{30}O_4$



Crocetin, $C_{20}H_{24}O_4$

Figure 2. Some Carotenoids Which Contain Less Than Forty Carbon Atoms

2. A Brief History of Carotenoid Isomerization

Discovery of Carotenoid Isomers.- In view of the many examples of cis-trans isomerization in compounds containing double bonds (47), it seems surprising that the question of the existence of geometrical isomers of carotenoids did not arise as soon as the unsaturated nature of the chromophore was demonstrated (76,24,38). Yet six years elapsed between the discovery of two isomeric forms of bixin by Herzig and Faltis (10) in 1923 and the correct interpretation of this phenomenon as a cis-trans isomerization by Karrer and his associates (17).

In 1935 Gillam and El Ridi (5-7) were the first to observe that carotenoids containing 40 carbon atoms form isomers. These workers obtained two clearly defined zones when they chromatographed solutions of pure β-carotene repeatedly on alumina columns; the upper band showed the spectral maxima of β-carotene, while the lower pigment layer had the same maxima as α-carotene. Elution and readsorption of either pigment again gave rise to the same two layers, "the process thus being reversible and never complete". The lower zone, termed "pseudo-α-carotene", was not identical with α-carotene, since repeated chromatography of α-carotene on alumina yielded a new pigment, "neo-α-carotene", whose spectral maxima lie at shorter wave lengths than the starting material. A further proof that pseudo-α-carotene and α-carotene are not identical is that both β-carotene and pseudo-α-carotene are optically inactive, while α-carotene and neo-α-carotene are active.

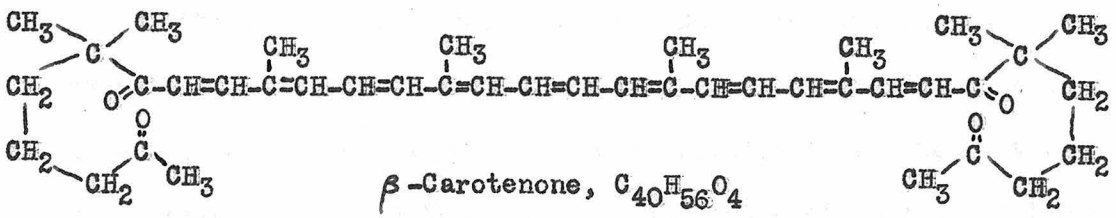
Gillam and his associates (5-7) attributed the transformation of β-carotene into pseudo-α-carotene and of α-carotene into neo-α-carotene to an action of the adsorbent employed and stated that the isomerization occurred only in the chromatographic column. This view soon became untenable, however.

Zechmeister and Cholnoky (73) observed a spontaneous, reversible isomerization in capsanthin solutions and Zechmeister and Tuzson (86,87) showed that solutions of chromatographically homogeneous lycopene, β-carotene, or cryptoxanthin, on standing at room temperature, undergo partial isomerizations which are spontaneous, reversible, and greatly accelerated by the action of heat. According to Zechmeister and Tuzson, "the progress of the isomerization is a function of time and it has nothing to do with a subsequent adsorption. The Tswett column only furnishes a suitable method for showing an effect already present in the solution. The isomerization may also be recognized by spectroscopic and colorimetric readings, independently of the chromatographic technique".

Interpretation of the Carotenoid Isomerization.- Gillam et al. (5-7) attributed the formation of pseudo-α-carotene and neo-α-carotene either to a double bond migration or to a trans-cis rearrangement. In the case of capsanthin, which contains a carbonyl group (67), Zechmeister and Cholnoky (73) at first also had to consider a keto-enol tautomerism. As more and more experimental data was obtained, however, it became increasingly apparent that the isomerizations involve trans-cis changes in double bonds instead of double bond migrations (74,81,80,88). The principal reasons which have led to this conclusion may be summarized as follows:

1) In the case of several carotenoids many more isomers are known than can be accounted for by double bond migrations (80,55,83);

2) β-Carotenone (Figure 3) isomerizes despite the fact that its chromophore is "blocked" at each end by a carbonyl group, so that a double bond could migrate only into a methyl sidechain, an improbable assumption (75);



β-Carotenone, $C_{40}H_{56}O_4$

Figure 3.

3) Lutein has never been observed to yield zeaxanthin during isomerization experiments, although the migration of but one double bond would be required to effect the transformation (80); also, α-carotene and β-carotene could not be interconverted (83,55);

4) The catalysts effective in carotenoid isomerizations are known to catalyze trans-cis rearrangements (80).

When the formation of a compound is irreversible, a structural alteration, rather than a steric change or a structural isomerization, should be considered the cause. Such is the case with the formation of the desoxyluteins described in this Thesis, the dihydrocarotenes of Polgár and Zechmeister (56), and probably many of the pigments of unclarified structure obtained by acid treatment of carotenoids (41,62,63,59) (see below).

Despite their altered structures both the desoxyluteins and the dihydrocarotenes are typical polyenes, which undergo cis-trans isomerization upon iodine catalysis.

3. Methods of Carotenoid Isomerization, with Special Reference to Lutein

Heat.- The action of heat in accelerating the spontaneous isomerization of lycopene, β-carotene, and cryptoxanthin solutions was reported by Zechmeister and Tuzson (87). They also showed that, when a benzene solution of lutein was

refluxed for thirty minutes, approximately 10% of the lutein was converted by a reversible process into another pigment, "neolutein A", which is identical with a pigment obtained by the iodine catalysis of lutein at room temperature (see below). A third pigment was detected in some of the experiments, but its relationship to lutein and to neolutein A has not yet been established. In independent experiments Strain (60-62) also isolated these two products from alcoholic solutions after heating. However, he did not demonstrate the reversibility of their formation, nor did he differentiate clearly between the effect of heat and the action of the adsorbent.

More recently it has been shown by Zechmeister in collaboration with Polgár (55,57,83) and with Lemmon (78) that carotenoids can be isomerized by melting the crystals in an oxygen-free atmosphere.

Acid.- Kuhn, Winterstein, and Lederer (41) refluxed solutions of lutein in methanol which was approximately 10^{-5} normal in oxalic or tartaric acid for one hour and isolated pigment crystals with increased optical rotation and decreased melting point. Strain (62) repeated this work and found by chromatographic analysis that the pigment mixture so obtained contained traces of strongly adsorbed material, which resembled the products formed by refluxing methanolic solutions of lutein, and much larger amounts of weakly adsorbed pigments, which passed through the column and exhibited the same partition behavior as cryptoxanthin, $C_{40}H_{56}O$, a monohydroxy-β-carotene. In a later paper (63) Strain reported that dilute acids had a similar effect on solutions of α-carotene, β-carotene, cryptoxanthin, and zeaxanthin. In independent experiments Quackenbush, Steenbock, and Peterson (59) treated both xanthophyll mixtures and pure lutein with dilute acetic, oxalic, sulfuric, and hydrochloric acids and succeeded in separating the lower, weakly-adsorbed layers into three components:

Color of Layer	Spectral Maxima in 65-75° Petroleum Ether (mμ.)
Dull red	476-448*
Yellow-orange	477, 447.5
Yellow-orange	477, 447

* Region of maximal spectral absorption

Unfortunately, the above experiments established neither the structure of any of these pigments formed on acid treatment of lutein nor the reversibility of the process. It is, therefore, impossible to state at the present time whether or not any of the observed products is a stereoisomer of lutein.

Iodine.- In 1929 Karrer, Helfenstein, Widmer, and van Itallie (17) found that bixin (Figure 2) is altered by treatment with iodine, which was known to be a catalyst for cis-trans conversions of unsaturated compounds (1,1b). They used iodine to convert this natural pigment into the so-called "β-bixin", an isomer detected earlier by Herzig and Faltis (10). The first application of this reagent to the isomerization of carotenoids of the C_{40}-series is due to Zechmeister and Tuzson (88), who succeeded in producing isomers of lycopene, β-carotene, cryptoxanthin, lutein, zeaxanthin, and taraxanthin by the action of small amounts of iodine (0.5-1% of the pigment) in petroleum ether or benzene solution. In the case of lutein, approximately 40% of the starting material was converted into two other pigments, designated as "neolutein A" and "neolutein B". These are adsorbed above lutein on the chromatographic column and show the following spectra:

	Spectral Maxima	
	In Benzene (mμ.)	In Light Petroleum Ether (mμ.)
Neolutein A	484, 452	471, 422
Neolutein B	484, 453	472, 443
Lutein	489, 458	477, 448

The reversibility of the process was demonstrated by treating a solution of each pigment with iodine; each of the equilibrium mixtures so obtained contained neolutein A, neolutein B, and lutein in about the same ratio as the mixture produced by a corresponding treatment of lutein itself.

Very recently it has been shown by Zechmeister and Polgár (83,57) that iodine has no catalytic effect in the dark, although exposure of the solutions to a light source for only a few seconds produces the iodine isomerization usually observed in diffuse laboratory light.

Light.- Within the last few years the sensitivity of carotenoids to light has been utilized by Zechmeister and collaborators (79) as a method for the preparation of stereoisomers; they showed that, for example, dilute, nearly colorless solutions of prolycopene turned intensely yellow upon exposure to sunshine ("insolation") for a few minutes. Soom afterward Zechmeister and Polgár (83) isomerized α-carotene and β-carotene by exposure to sunlight, light from a Mazda incandescent bulb, and light from a Hanovia ultraviolet lamp. The applicability of the method to some carotenoid alcohols was shown by Zechmeister and Lemmon (78), who obtained isomers of cryptoxanthin and zeaxanthin. It has not yet been applied to lutein solutions.

4. Stereochemical Considerations, with Special Reference to Lutein

Configuration of Naturally Occurring Carotenoids.- By far the largest part of the polyene pigments found in plants possess the "all-trans" configuration, i.e., all of the double bonds in the molecule exist in the trans form (68). This conclusion is based not only on the stability of these pigments and on chromatographic analyses of numerous plant extracts but also on the results of X-ray investigations of crystals (9,46) and on the interpretation of spectral data (49,51,54). Recently, however, two "pro-carotenoids", containing probably five or six cis double bonds, have been found in nature by Zechmeister in collaboration with LeRosen, Went, and Pauling (80), with LeRosen (45), with Schroeder (84,85), and with Escue (77). While these poly-cis compounds apparently are wide-spread in nature, their total quantity is extremely small in comparison with that of the all-trans pigments (68).

Stereochemical Possibilities for Lutein.- Not all the double bonds of the polyene chain are free to assume the cis configuration. The theory of resonance requires that for effective conjugation the single and double bonds of the chromophore must lie in the same plane (53). Pauling (54) showed that, under these conditions of coplanarity, steric hindrances prevent those double bonds which are adjacent to a C-CH_3 group (C=C-C(CH_3)) from assuming the cis configuration; however, the double bonds to which methyl side chains are directly attached (C=C(CH_3)) may exist in the cis form, since only slight steric hindrances exist. The central double bond of the C_{40}-carotenoid is, of course, free to assume either a cis or a trans configuration, since no methyl groups are attached either to the carbon atoms joined by the double bond or to the adjacent carbon atoms. Furthermore, any double bonds included in the cyclic end groups have their configurations fixed permanently and are not subject to stereoisomerization.

It follows, therefore, from these considerations that lutein has only five double bonds available for trans-cis changes:

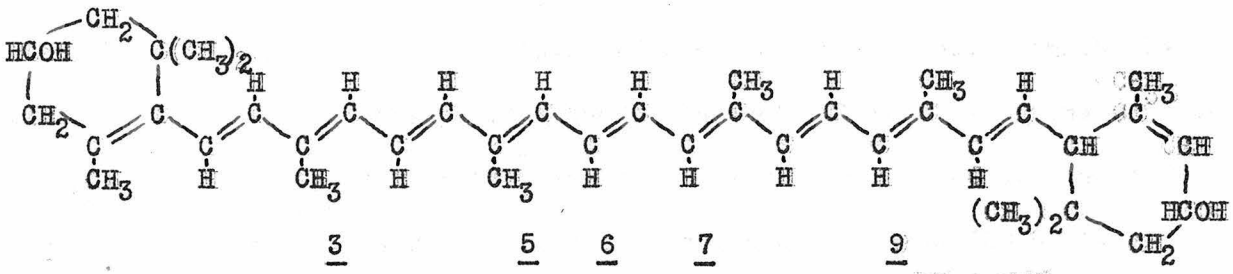


Figure 4. All-trans lutein

(The five stereochemically-effective double bonds are numbered)[1]

For an unsymmetrical molecule the number of stereoisomers is 2^n, where n is the number of stereochemically effective double bonds (80,54); this gives 32 as the theoretical number of members of the lutein stereoisomeric set[2].

Configuration of the Two Known Stereoisomers of Lutein.- A tentative assignment of configuration of neolutein A and neolutein B has been made by Zechmeister and Polgár (83), who inferred the presence of a single cis bond in each molecule from the fact that the spectral maxima of the neo-compounds lie at wave lengths 5-6 mμ. lower than those of the naturally occurring all-trans-lutein (79). The suggested locations of the cis double bond were based on the heights of the spectral maxima which lie at approximately 330 mμ. When an

[1] The following nomenclature, proposed by Zechmeister and Polgár (83), will be used in this Thesis: Each double bond of the conjugated system will be assigned an italicized number in order to avoid confusion with the numbering of carbon atoms: e.g., 3,6-di-cis-β-carotene. The lowest number will be given to the double bond in the β-ionone ring or, if the double bond of this ring is not part of the chromophore, to the conjugated double bond nearest the β-ionone ring. In the absence of such a system an α-ionone ring receives preference over an aliphatic terminal group.

[2] The term "stereoisomeric set" includes all cis-trans isomers of a given carotenoid (68).

all-*trans* carotenoid undergoes *trans-cis* isomerization, the extinction in the visual spectral region is markedly decreased, while a new maximum appears in the range 320-380 mμ. Zechmeister and Polgár, who discovered this effect (82), termed this maximum the "*cis*-peak" and the range of wave lengths in which it appears, the "*cis*-peak region". Theoretical considerations advanced by Pauling (54,79) show that the height of the *cis*-peak is determined by the degree of bending of the aliphatic chain (cf. also Mulliken (50,51)). A high *cis*-peak, such as that of neolutein A, corresponds to a *cis* double bond located in the middle of the conjugated chain, while a much lower *cis*-peak, as in the case of neolutein B, corresponds to a *cis* double bond near the end of the conjugated system. On this basis Zechmeister and Polgár concluded that neolutein A is probably *6*- or *5*-mono-*cis*-lutein and that neolutein B is probably *3*- or *9*-mono-*cis*-lutein.

B. DISCUSSION OF NEW EXPERIMENTS

1. Conversions of Lutein in Solution at Elevated Temperatures

Iodine Catalysis at Room Temperature.- Preliminary experiments confirmed the observations of Zechmeister and Tuzson (88) that iodine catalysis at room temperature yields neoluteins A and B, which may be separated from the unchanged all-trans-lutein on a calcium carbonate column. Both benzene-petroleum ether mixtures and solutions of acetone in petroleum ether were used as developers in an attempt to increase the sharpness of the separation and thus possibly to detect small amounts of isomers that had hitherto escaped discovery. These hopes were not realized, however, and, since neoluteins A and B have already been well-characterized by Zechmeister and Tuzson (88) and by Zechmeister and Polgár (83), work with iodine catalysis at room temperature was discontinued.

Fusion of Lutein Crystals.- Since prior experiments on the thermal isomerization of lutein (88,62) had not employed heating over 80°, it seemed desirable to investigate the changes occurring at higher temperatures. Accordingly, lutein crystals were melted for three minutes at 200° to 230°, lower temperatures being precluded by the melting point of lutein. Chromatographic analysis of the pigment mixture in the solidified melt showed an upper, heterogeneous layer which on rechromatography was found to contain both unchanged lutein and neolutein A. In addition to these two identified pigments and to some other colored products that did not possess the α-carotene or lutein chromophore, four layers were present whose spectral maxima, both before and after iodine catalysis, corresponded to those of mono-cis-luteins. More work will be necessary to establish whether these compounds are members of the lutein stereoisomeric set and whether one of them is identical with neolutein B.

The chromatogram of the melt also showed a heterogeneous yellow layer of much lower adsorbability than the lutein zones. Rechromatography of this mixture yielded several pigments having the spectral maxima either of all-trans-lutein or of neoluteins A and B; upon the addition of iodine to solutions of any of these pigments, the spectral maxima shifted to the equilibrium values for the lutein stereoisomeric set. The position of the layers on the Tswett column, their partition behavior, and their solubility in petroleum ether indicate that they are not related to either lutein or α-carotene (Figure 1), but rather that they constitute an entirely new group of carotenoids, which contain a single oxygen atom. Three of the pigments, termed the "desoxyluteins", have been crystallized and studied in some detail; the experimental data are discussed below in Section C.

The high temperatures employed in these melts caused considerable pigment losses, the total yield of pigment at the end of the melt varying from about 52% at 200° to about 8% at 230°. Therefore, it seemed desirable to search for a method which would permit heat treatment of lutein at temperatures between the boiling point of benzene and the temperature necessary to obtain homogeneous lutein melts.

Mixed Melts with Naphthalene.- It was found that homogeneous solutions are obtained when mixtures of lutein and naphthalene are fused at temperatures of 85° or higher. The presence of naphthalene in the melt mixture after cooling causes no difficulty, since naphthalene dissolves easily in benzene and is so weakly adsorbed on the chromatographic column that it passes into the filtrate before any appreciable movement of the pigment layers down the column has taken place. This new method, although it has been applied at the present time only to lutein, should prove more generally applicable and facilitate the study of thermal isomerization of carotenoids with high melting points.

Although the use of naphthalene permitted a great lowering of the melt temperatures, the total yields of pigment were not greatly increased, for example, a naphthalene melt for three minutes at 140° showed a 40% decrease in colorimetric value, while a three-minute fusion of lutein crystals at 200° caused only a 48% lessening of color intensity. Moreover, the products from three-minute lutein-naphthalene mixed melts at 85°, 110°, and 150° were found to be essentially the same as those from melts of lutein crystals at 200° or higher, namely, 1) an upper group of layers containing lutein, neolutein A, and possibly other stereoisomers of lutein and 2) a lower group of apparently monoxy compounds, among which were the desoxyluteins.

Naphthalene Melts in the Presence of Iodine.- When a combination of thermal and iodine catalysis was employed by carrying out mixed melts with naphthalene for three minutes at 85°, 110°, and 140° in the presence of 2 to 5 µg. of iodine per mg. of lutein, no unaltered lutein could be detected by chromatographic analysis. Good yields of the monoxy compounds previously observed, including the three desoxyluteins, were obtained (Table 1). The most striking feature of the chromatogram, however, was a series of pink and orange bands at the bottom of the column. These pigments, which are adsorbed weakly on calcium carbonate and exhibit an epiphasic behavior, apparently contain no hydroxyl groups. Their unique spectral characteristics mark them as a hitherto-unobserved group of carotenoids (see "Epiphasic Pigments" under Part D). Yields under the conditions studied are so low, 1-2%, that isolation and characterization of the individual pigments must await the development of improved preparative methods.

The use of iodine at elevated temperatures is a new feature and nothing is known about the behavior of carotenoids other than lutein under such conditions. An investigation with numerous polyene pigments, should be undertaken to determine the utility of the method both for the preparation of new

artifacts and, at somewhat lower temperatures, for the production of new stereoisomers.

Mixed Melts with Naphthalene and Orthoboric Acid, Tetraboric Acid, or Boric Oxide.- In order to investigate the combined effect of acid and heat, tetraboric acid was added to the lutein-naphthalene mixture before melting. Despite the insolubility of tetraboric acid in fused naphthalene, 50-60% yields of the previously-observed monoxy pigments were obtained at 140° in five minutes (Table 1). Only small amounts of dihydroxy pigments and traces of hydrocarbons were present; it is particularly interesting that no unaltered lutein could be detected. Similar pigments and yields were obtained with anhydrous orthoboric acid or boric oxide.

The use of these reagents increased the yield of the three desoxy-luteins to such an extent that large scale preparations, involving a total of 2 g. of lutein as starting material, could be undertaken profitably. Although application of the reaction to zeaxanthin (Figure 1) did not lead to any detectable quantity of alteration products, the method should nevertheless prove of importance.

Experiments in Benzene Solution with Boric Oxide.- A benzene solution of lutein was treated with boric oxide at room temperature for three days and then at 80° for two hours. The pigments produced were qualitatively the same as those found in mixed melts with naphthalene and either tetraboric acid or boric oxide at 110° or 140° in the course of five minutes.

Experiments in Pyridine Solution with Boron Trifluoride.- When lutein in pyridine solution was treated with boron trifluoride in ether for sixteen hours at room temperature, only traces of monoxy compounds were formed; however, approximately two-thirds of the lutein was converted into those

Table 1

Effect of Iodine, Orthoboric Acid, Tetraboric Acid, and Boric Oxide on Yields of Monohydroxy-compounds and Epiphasic Pigments Obtained from Lutein under Various Conditions

Components of Melt (in addition to lutein)	Time of Melt Min.	85°		110°		140°	
		Monohydroxy Compounds	Epiphasic Pigments	Monohydroxy Compounds	Epiphasic Pigments	Monohydroxy Compounds	Epiphasic Pigments
Naphthalene	3	none	none	trace	none	10%	none
Naphthalene and Iodine	3	37%	1.9%	22%	1.5%	--	--
Naphthalene and Orthoboric Acid	5	--	--	--	--	53%	none
Naphthalene and Tetraboric Acid	5	small	none	44%	trace	63%	trace
Naphthalene and Boric Oxide	1.5	--	--	--	--	56%	none
Naphthalene and Boric Oxide	5	--	--	--	--	68%	none
Naphthalene and Boric Oxide	10	--	--	--	--	61%	trace
Naphthalene, Tetraboric Acid, and Iodine	5	33%	0.7%	small	none	--	--

pink and yellow dihydroxy pigments which appear in very small amounts in boric acid-naphthalene melts (see above). These compounds are not members of the lutein stereoisomeric set (see "Dihydroxy Carotenoids" in Part D).

Mixed Melts with Naphthalene and Sodium Tetraborate.- When anhydrous sodium tetraborate was present, the reaction took an entirely different course, i.e., only dihydroxy pigments were obtained. Sufficient time was not available for a detailed investigation of the zones; however, neoluteins A and B appeared to be present above unchanged lutein. A lower layer, having its spectral absorption maxima at 462 and 434 mμ., showed a spectral behavior on treatment with iodine which was to be expected of a member of the stereoisomeric lutein set. Pending a final clarification of its structure this compound has been tentatively designated as "neolutein U" (see Section D).

2. A Survey of the Processes Occurring in Solutions of Lutein at Elevated Temperatures

On the basis of the postulated structures discussed below in Sections C and D for various artifacts obtained from lutein in these experiments certain remarks can be made about the processes occurring in solutions of lutein at elevated temperatures.

Formation of Lutein Stereoisomers.- The principal action of heat on lutein solutions is to produce trans-cis rotations. This is true not only for fused lutein crystals but also for solutions of lutein in naphthalene, benzene, or alcohol.

Formation of Monoxy Pigments.- Heat also produces small quantities of compounds containing but a single oxygen atom, among which are the desoxyluteins. By the addition of iodine to naphalalene melts at 80-110°

20-40% yields of the monoxy pigments can be obtained. A still further improvement in yield results when orthoboric acid, tetraboric acid, or boric oxide are added to the melt mixture. In this case no unaltered lutein can be detected among the few dihydroxy pigments observed on the chromatographic column, while the monoxy layers have 50-70% of the color intensity of the starting material.

The loss of an oxygen atom to give monoxy pigments could be explained by a simple dehydration with the formation of a new double bond. However, the present experimental results indicate that the desoxyluteins are formed without such an increase in the number of double bonds in the molecule (see page 24). If this is the case, the removal of an oxygen atom must be accompanied by a reductive process of some sort.

Although nothing can be said concerning the mechanism of desoxylutein I formation until its structure has been clarified, two possible mechanisms for the formation of desoxyluteins II and III suggest themselves. The process might well occur by a dehydration of the α-ionone endgroup of lutein to a cyclohexadiene derivative, followed by a subsequent hydrogenation to give desoxyluteins II and III, which are presumably cyclohexene derivatives (see Figure 5). Another possible mechanism is the formation of a tautomeric free radical which then would react in some unknown manner to form the desoxyluteins (see Figure 6).

The striking effect of the boron compounds is probably due to their strong electrophilic nature, which causes them to react with the unshared electron pairs of the hydroxyl groups in the lutein molecule and thereby greatly facilitates the removal of the hydroxyl group of the α-ionone ring.

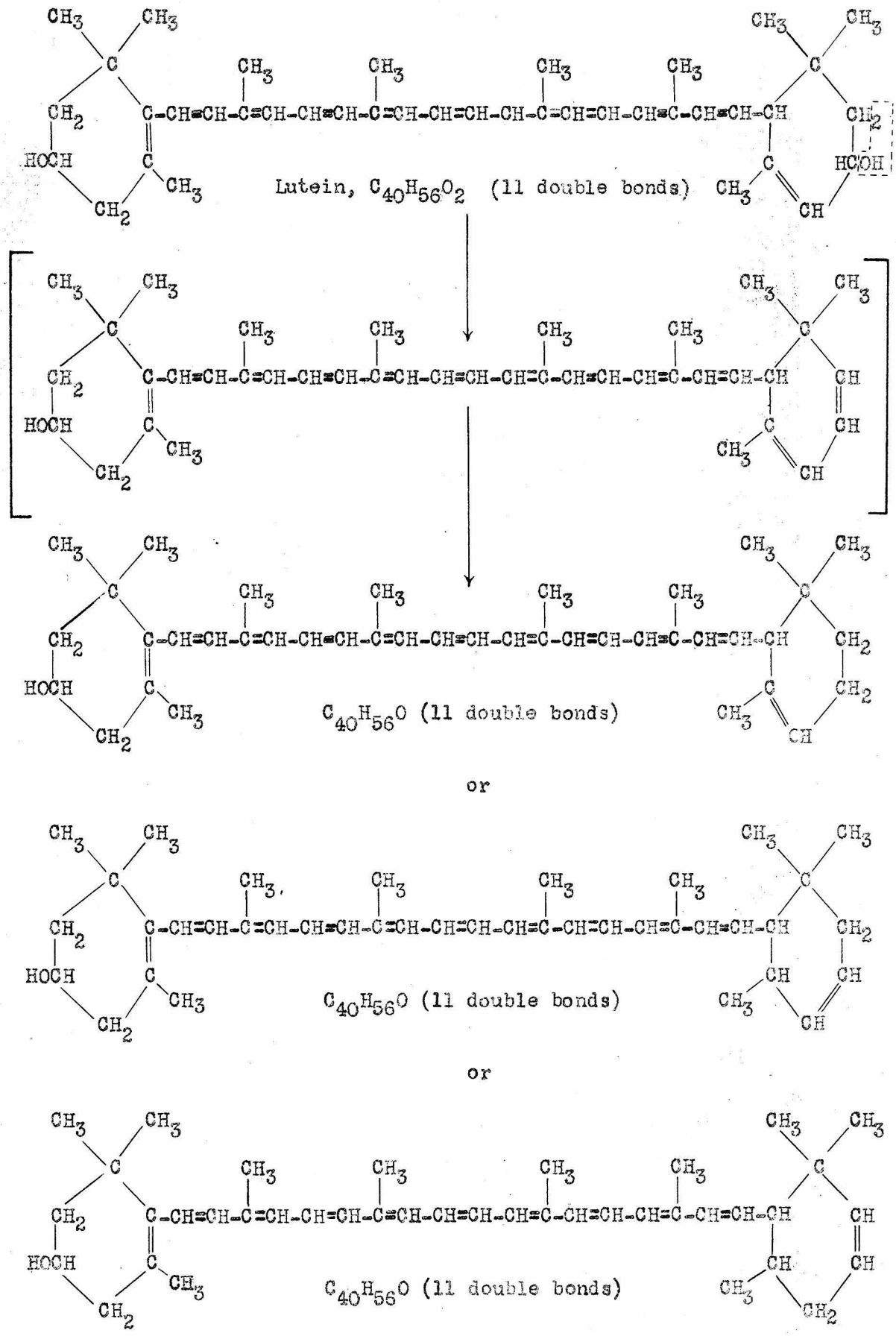


Figure 5. A Possible Mechanism for the Formation of Desoxyluteins II and III

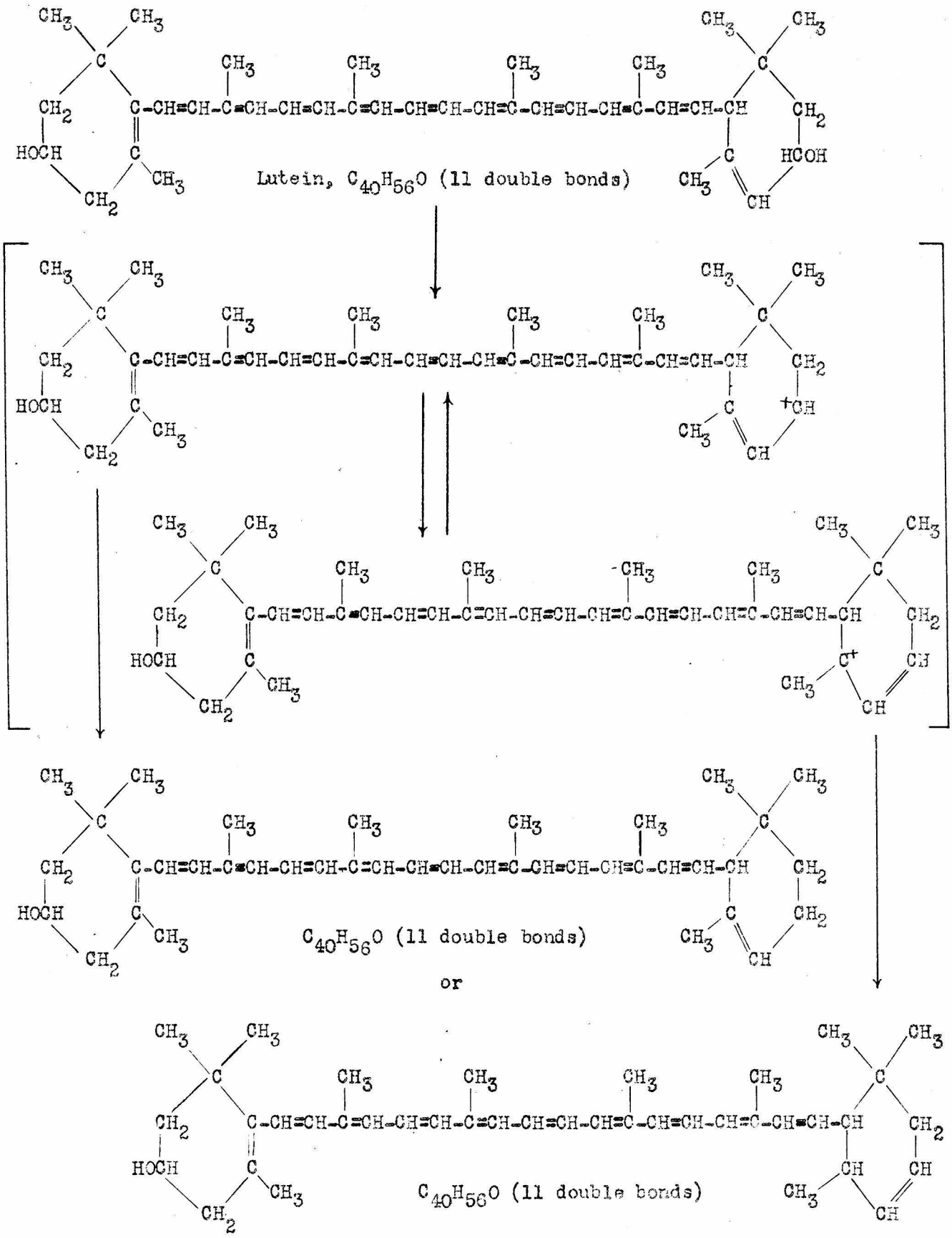


Figure 6. A Possible Mechanism for the Formation of Dosoxyluteins II and III

The formation of the desoxyluteins seems to be in agreement with the fact, observed by other investigators (41,59,63), that dilute solutions of xanthophylls or lutein on treatment with dilute acids yield pigments of apparently lower oxygen content. Moreover, Zechmeister and Lemmon (78) have isolated a compound of altered partition behavior from zeaxanthin melts. The exact relationship between these pigments and the desoxyluteins remains to be established, but it is undoubtedly a very close one.

Formation of Epiphasic Pigments.- The formation of epiphasic pigments in small amounts when lutein solutions are treated with iodine at 80-110° apparently involves both dehydrating the α-ionone and the β-ionone end-groups of lutein, and moving the isolated double bonds of the α-ionone group into conjugation with the main chromophore. It is not clear, however, why these epiphasic pigments should be detected after iodine treatment but not after mixed melts with the boric acids, which are much more efficient than iodine in facilitating the removal of a hydroxyl group from the α-ionone group of lutein.

C. STRUCTURES OF THE DESOXYLUTEINS

As mentioned above, three compounds of this class have been isolated in the crystalline state from pigment mixtures produced in boric acid-naphthalene melts. Analyses showed the formula $C_{40}H_{56}O(\pm H_2)$, and the names "desoxylutein I", "desoxylutein II", and "desoxylutein III" were accordingly assigned to the pigments, pending a final clarification of their structures.

All three desoxyluteins showed a partition behavior intermediate between that of dihydroxy carotenoids and that of hydrocarbons, a behavior to be expected of monoxy compounds which contain either a hydroxyl or a keto group (67,42,43,8,64,65). Furthermore, the following adsorption sequence[1] shows that the desoxyluteins are, in general, above hydrocarbons and below dihydroxy carotenoids on the chromatographic column:

Adsorbed Most Strongly	Zeaxanthin	
	Lutein	
	Lycopene	
	Desoxylutein I	
	Cryptoxanthin	
	Desoxylutein II	
	γ-Carotene	Can be separated only with the greatest difficulty
	Desoxylutein III	
	β-Carotene	
Adsorbed Most Weakly	α-Carotene	

[1] The adsorption sequence was determined on 1:1 calcium carbonate-calcium hydroxide mixture, using 1-10% acetone in petroleum ether as developer.

Any formulas proposed for the desoxyluteins must take into account the following experimental facts:

1) The partition behavior of all three compounds is intermediate between that of lutein (a dihydroxy-α-carotene) and α-carotene (a hydrocarbon).

2) Acetates of desoxyluteins I and II have been prepared.

3) Upon catalytic hydrogenation at room temperature each of the three compounds adds eleven moles of hydrogen.

4) All three compounds have typical polyene absorption spectra (Figures 7-9), which shift to lower wave lengths and develop cis-peaks (82) upon iodine catalysis. The visual absorption maxima of desoxyluteins II and III have the same positions as those of all-trans-α-carotene or all-trans-lutein, and they shift in a similar manner when catalyzed with iodine. The spectrum of desoxylutein I is unlike that of most carotenoids, since it possesses no fine structure; also the absorption maxima lie at wave lengths about 17 mμ. longer than lutein without any corresponding increase in the manner of carbon-carbon double bonds.

5) In biological assays for vitamin A activity in the rat all three compounds gave negative results when 10 μg. were fed daily per rat; this precludes the presence of β-ionone rings as end groups (67).

1. Structures of Desoxylutein II and Desoxylutein III

While the above evidence is not sufficient to determine the structures of desoxyluteins II and III completely, some progress toward that goal is nevertheless possible. Since zeaxanthin fails to react in the boric acid-naphthalene melt, it seems reasonable to assume that the α-ionone ring and hydroxyl group of lutein are involved in the reaction rather than the

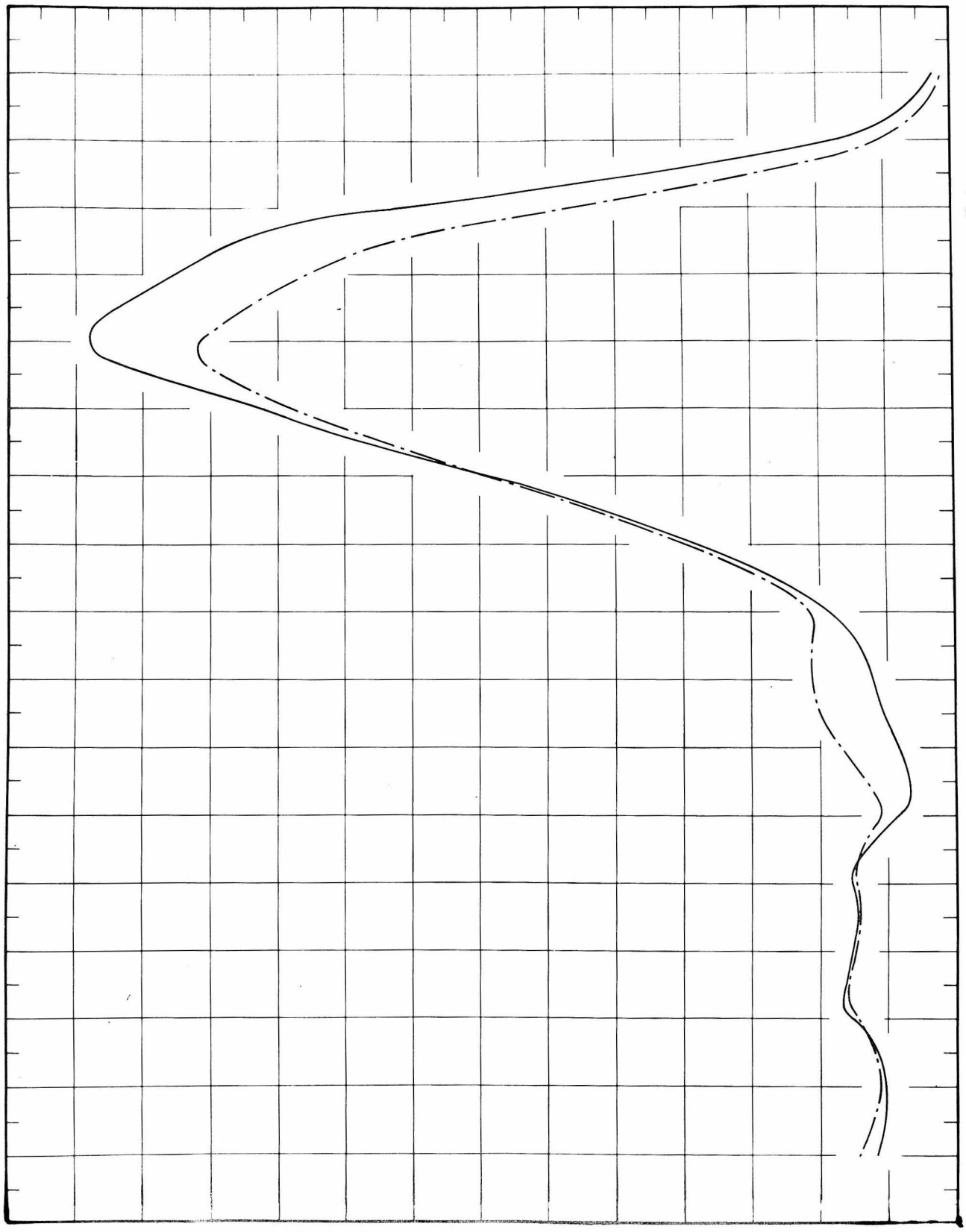


Figure 7. Molecular extinction curves of desoxylutein I in hexane: ——, fresh solution of the all-trans compound; —·—·—, on iodine catalysis at 25°.

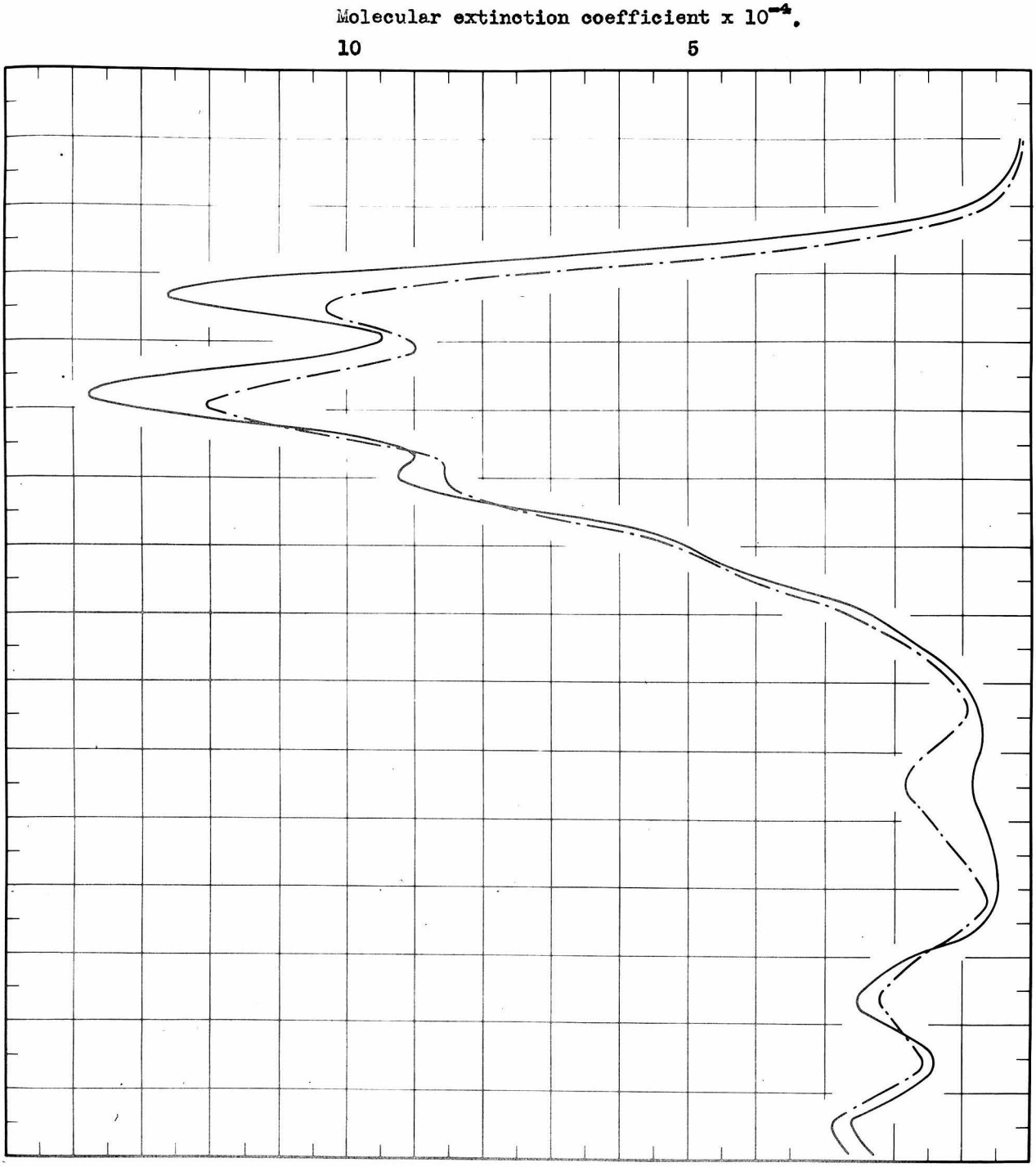


Figure 8. Molecular extinction curves of desoxylutein II in hexane; ——, fresh solution of the all-trans compound; —·—; on iodine catalysis at 25°.

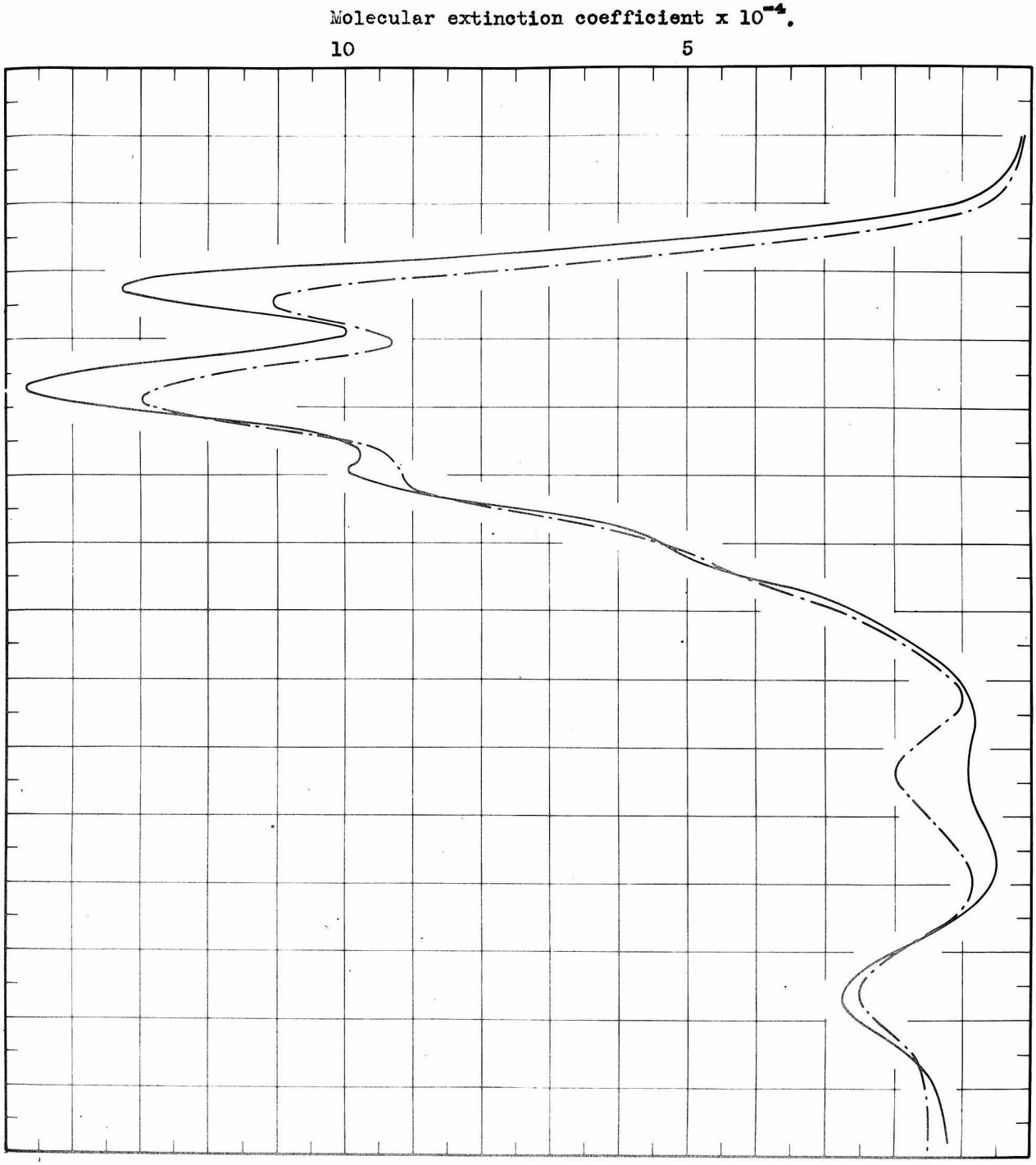


Figure 9. Molecular extinction curves of desoxylutein III in hexane: ———, fresh solution of the all-trans compound; —·—·—·, on iodine catalysis at 25°.

β-ionone ring and its hydroxyl group; this assumption seems to be in agreement with the observation of Strain (63) that zeaxanthin is more resistant than lutein to the action of acids. The absorption spectra show that ten of the double bonds are involved in the chromophore. Therefore, both desoxylutein II and desoxylutein III may be written as in Figure 10.

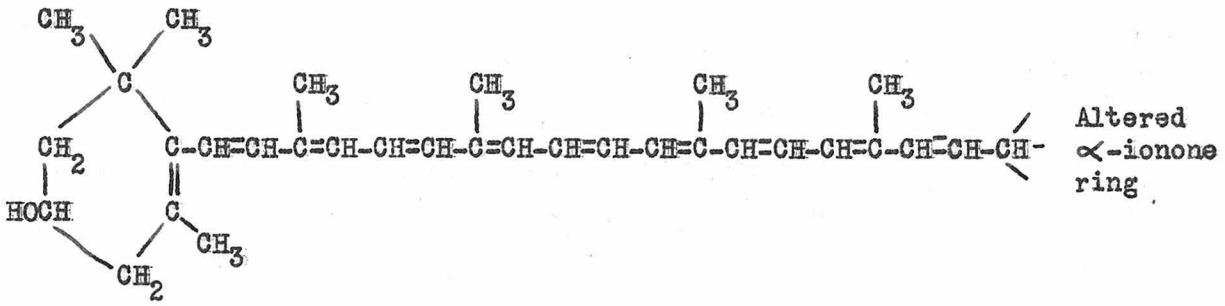


Figure 10. Desoxyluteins II and III

In the remaining unclarified portion of the molecule each of the desoxyluteins contains one isolated double bond.

It seems probable that the carbon skeleton undergoes no alteration during the removal of the hydroxyl group from the α-ionone ring. If this is true, desoxyluteins II and III are cyclohexene derivatives whose structural formulas can be found in Figure 5; each of the three formulas shown is in agreement with all the experimental evidence cited above. The possibility cannot be excluded, however, that a carbon-carbon bond is broken in the melt to give a γ-carotene carbon skeleton (Figure 1); the molecule would then contain either an additional double bond (Figure 11) or two additional hydrogen atoms (Figure 12). The structures of Figure 11, each of which contains twelve double bonds, can be definitely ruled out by the hydrogenation data, but the hydrogenated monocyclic pigments of Figure 12 have the same number of double bonds as the bicyclic pigments of Figure 5 and differ from them only in having two more hydrogen atoms. The uncertainty of the carbon-

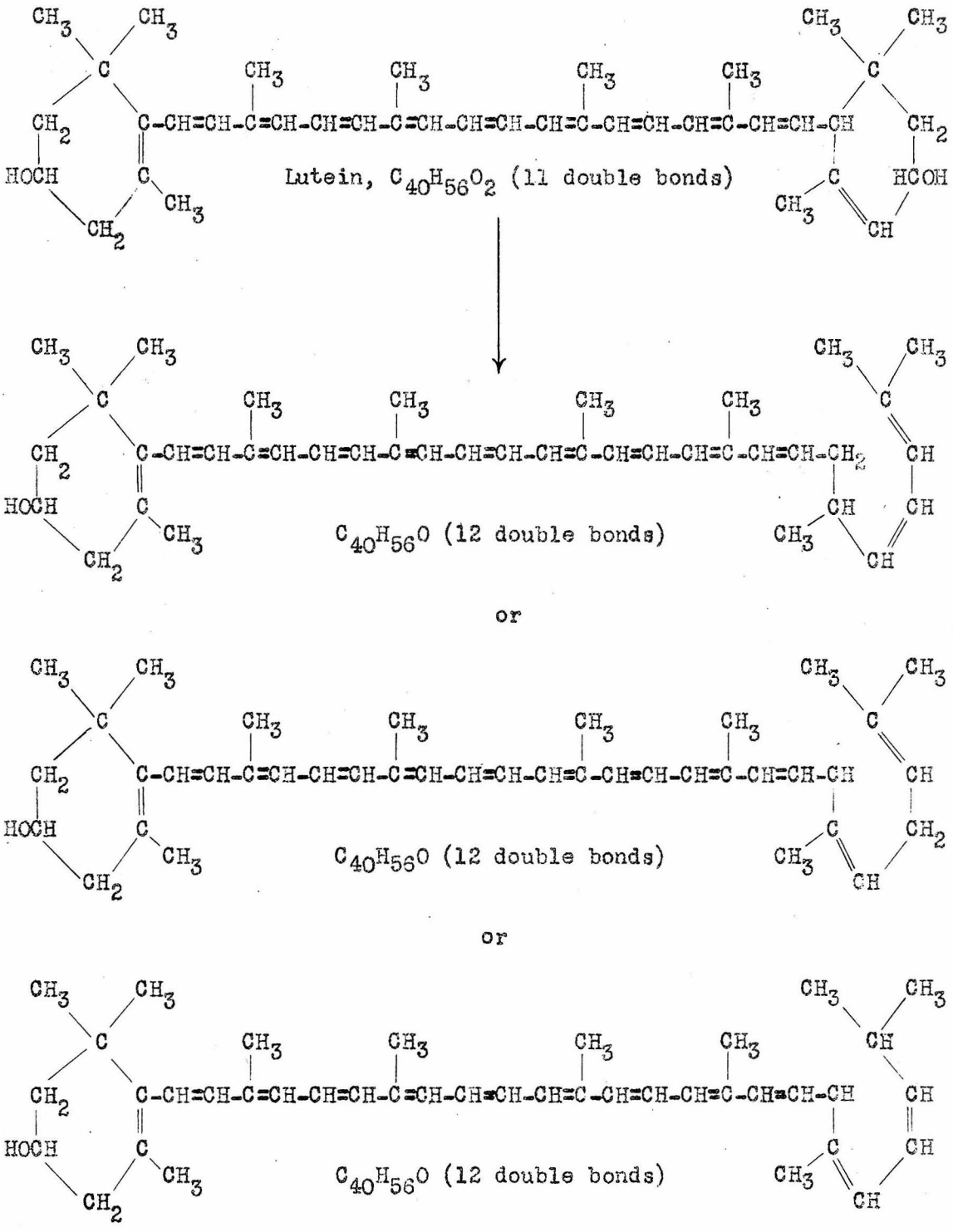


Figure 11. Improbable Structures for Desoxyluteins II and III

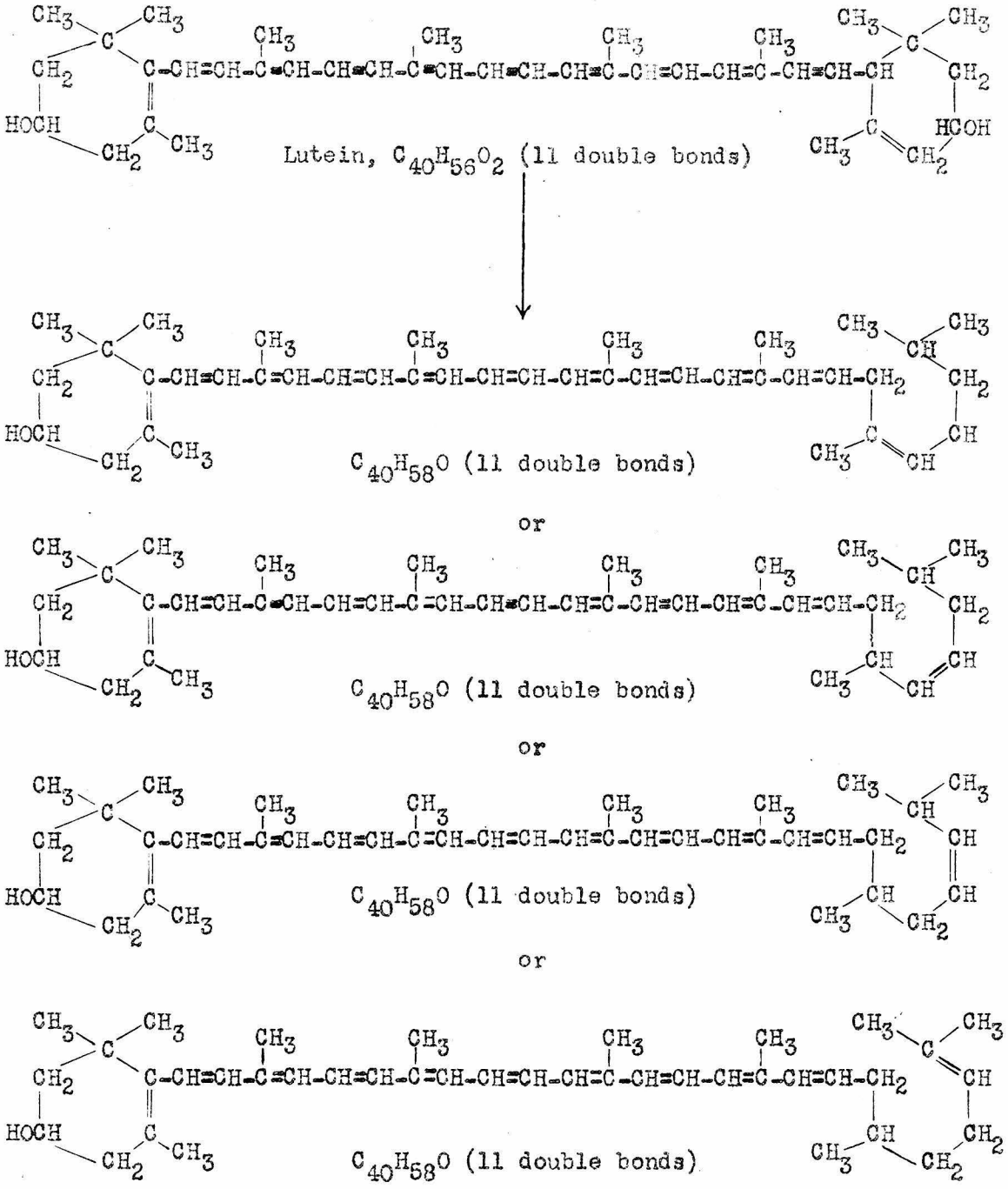


Figure 12. Possible Structures for Desoxyluteins II and III

hydrogen determinations (±0.3% for carbon and ±0.5% for hydrogen) is so great compared to the differences between the percentages calculated for $C_{40}H_{56}O$ and for $C_{40}H_{58}O$ (Table 2) that it is impossible to decide between the two formulas. Consequently, desoxyluteins II and III may be either bicyclic carotenoids, as suggested in Figure 5, or monocyclic carotenoids, as suggested in Figure 12.

Table 2

Calculated for $C_{40}H_{56}O$	C, 86.89; H, 10.22
Calculated for $C_{40}H_{58}O$	C, 86.58; H, 10.53

In the final establishment of the structures of desoxyluteins II and III oxidative degradations should be of great value. With permanganate, chromic acid, or ozone under the proper conditions (20,21,14,36,18,11,31), it should be possible to isolate characteristic fragments from the α-ionone end of the molecule; typical fragments to be expected are shown in Figure 13. The other, β-ionone end group will yield α,α-dimethylsuccinic acid and α,α-dimethylmalonic acid with permanganate or chromic acid, but no α,α-dimethylglutaric acid (29,16,15,52). Under milder conditions which permit the isolation of larger-sized degradation products, derivatives such as apo-2-α-carotenal (3), apo-2-zeaxanthinal (β-citraurin), and apo-3-zeaxanthinal (27,28,23) might be obtained (Figure 14).

Zerevitinoff determinations for active hydrogen (39,40,69,70) as well as additional attempts at esterification must be carried out with desoxylutein III to confirm its present classification as a monohydroxy carotenoid. If the oxygen is present instead as a ketone group, which may or may not be enolizable, treatment with hydroxylamine will yield an oxime under all but the most adverse conditions, i.e., when the carbonyl group lies at the end of

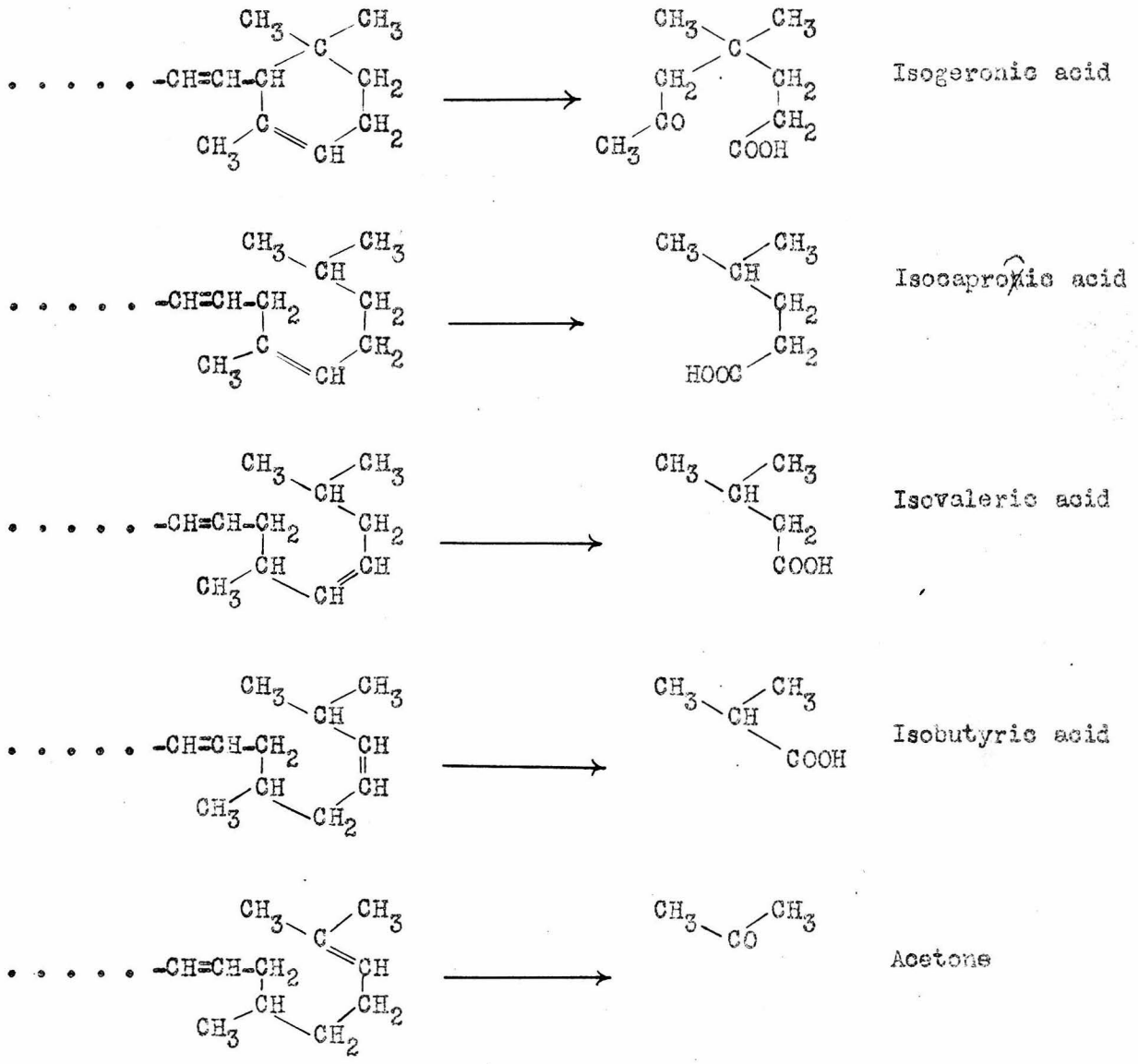
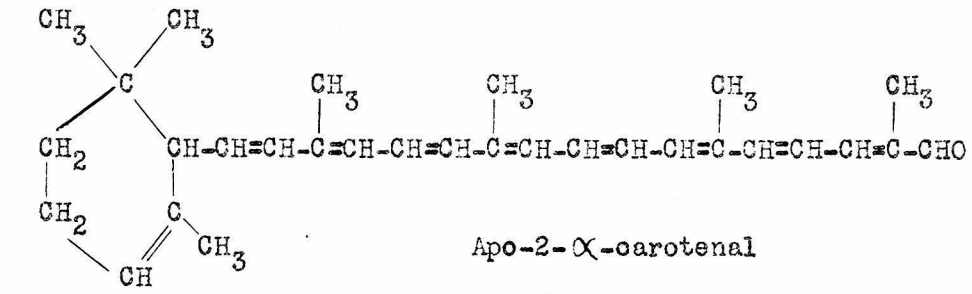
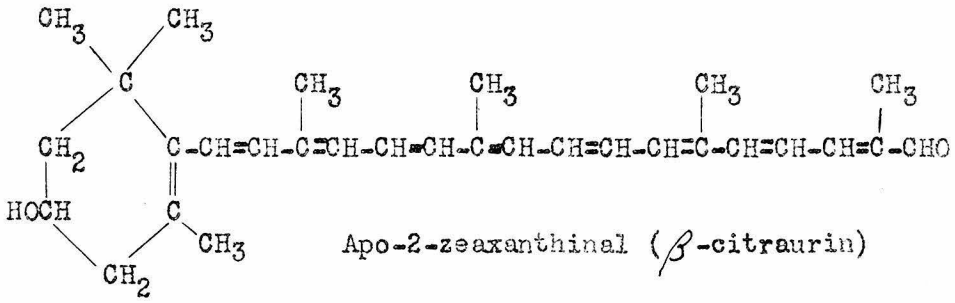


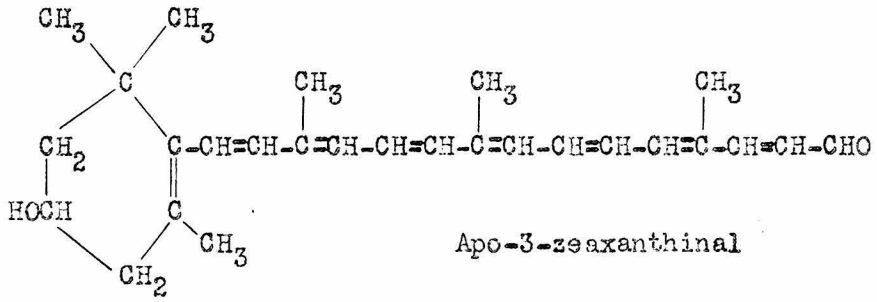
Figure 13. Degradation Products to Be Expected on the Basis of Various Structures Suggested for Desoxylutein II and III



Apo-2-α-carotenal



Apo-2-zeaxanthinal (β-citraurin)



Apo-3-zeaxanthinal

Figure 14. Degradation Products to Be Expected on the Basis of Various Structures Suggested for Desoxyluteins II and III

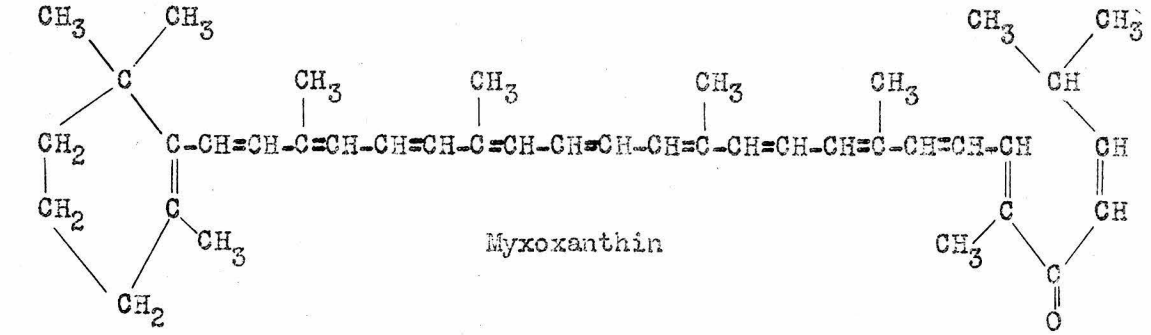
the conjugated polyene chain (31,33). This case can be excluded, however, since the α-carotene chromophore with its ten double bonds does not contain a conjugated carbonyl group and, moreover, the solutions of desoxylutein III in methanol and petroleum ether do not exhibit different colors, as do carresponding solutions of carotenoids which contain a carbonyl group in conjugation with the main chromophore (67).

2. Structure of Desoxylutein I

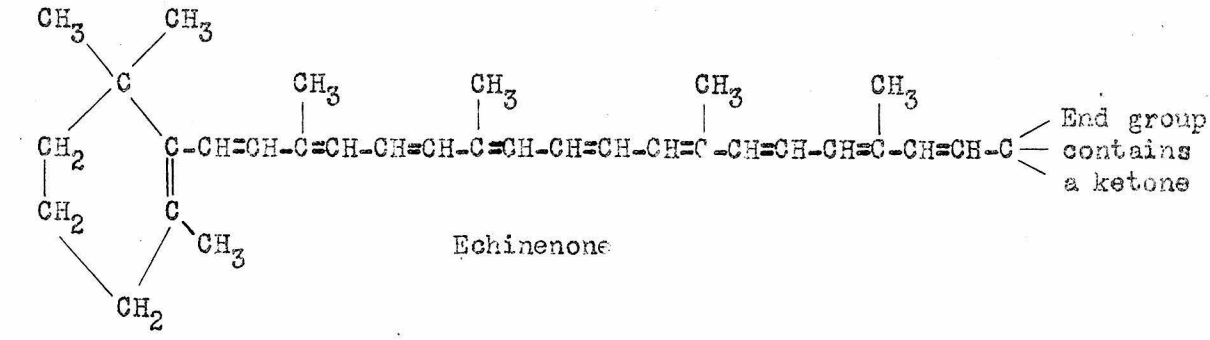
Even a partial determination of the structure of desoxylutein I is not possible at the present time, since its blurred absorption spectrum cannot be interpreted in terms of any known chromophore. Only a few other carotenoids are known which have similar spectra (Figure 15). Their visually observed absorption maxima are given in Table 3, along with those of desoxylutein I. Heilbron and Lythgoe (8) ascribe the blurred spectrum of myxoxanthin to the

Table 3

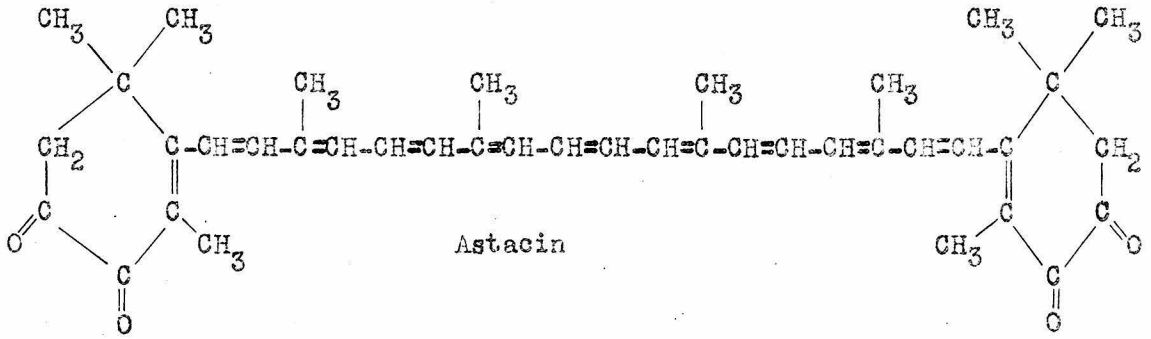
	Absorption Maxima in Carbon Disulfide (mμ.)	Absorption Maxima in Petroleum Ether (mμ.)
Myxoxanthin (8)	488	465
Echinenone (42,43)	(520), 488, (450)	-
Astacin (37)	505-515	-
Capsanthin (71,72)	543, 504	506, 475
Methyl azafrin (34)	476, 446, 419	447, 423
Desoxylutein I	(537), 493, 457	(493.5), 459



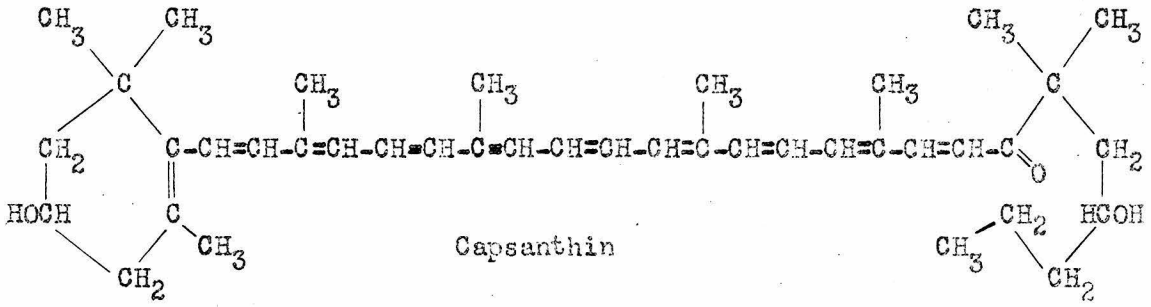
Myxoxanthin



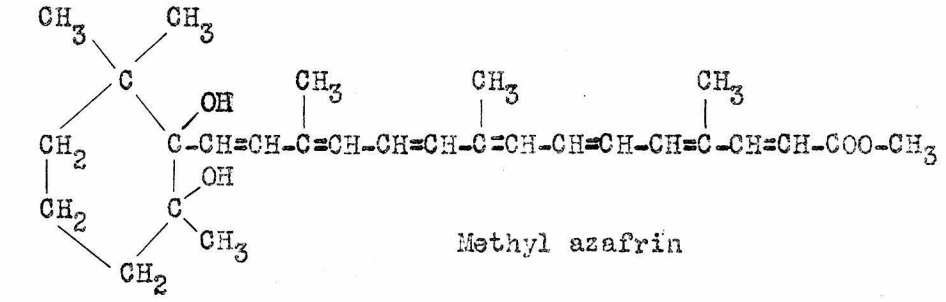
Echinenone



Astacin



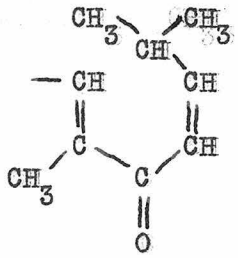
Capsanthin



Methyl azafrin

Figure 15. Some Carotenoids Having Blurred Absorption Spectra

presence of the end group,



which contains a keto group in conjugation with eleven double bonds on one side and a single double bond on the other, although they give no theoretical justification for such a belief. However, in the case of capsanthin (71,72) and methyl azafrin (34) such a grouping is not present. As a result the only certainty at the present time seems to be that a lack of fine structure in the visible region requires at least one carbonyl group conjugated with the main chromophore. While a necessary condition, this is, however, not a sufficient one, since numerous compounds with conjugated ketone groups show a fine structure: for example, semi-β-carotenone and β-carotenone (67) (Figure 3). The spectral evidence therefore indicates that desoxylutein I contains a conjugated ketone group. This conclusion is not, however, in agreement with the other experimental evidence. Such a conjugated carbonyl group could not undergo esterification without first being enolized and this, in turn, should be manifest in an altered spectral curve for the ester, a shift which was not observed experimentally. Furthermore, no significant color difference exists between solutions of desoxylutein I in methanol and in petroleum ether; this dependence of color on solvent is characteristic for carotenoids containing a conjugated ketone group (67).

Another ambiguity prevails with respect to the results of the catalytic hydrogenation, since ketone groups may or may not be reduced under the conditions employed (32,8,11). It is impossible to say at the present time whether

the hydrogenation data indicate ten carbon-carbon double bonds and a reducible carbonyl group, or eleven carbon-carbon double bonds and a non-reducible oxygen function.

D. Nature of Some Other Compounds Formed from Lutein

Of the great number of unidentified pigments observed in the reported experiments a few compounds for which some experimental data is available to permit partial characterization will be mentioned briefly here. For convenience the pigments have been divided into two classes: 1) dihydroxy carotenoids, and 2) epiphasic pigments.

1. Dihydroxy Carotenoids

"Neolutein U".- The name "neolutein U" has been tentatively assigned to a pigment, obtained from sodium tetraborate-naphthalene melts, which has visual absorption maxima at 462.5 and 434.5 mμ. Upon the addition of iodine the bands blur for a few seconds and then reappear at 474 and 443 mμ., which are the iodine equilibrium values for the α-carotene and lutein stereoisomeric sets. This spectral difference of about 14 mμ. from the all-trans-form, together with the adsorption affinity and hypophasic partition behavior of the pigment, seems to point to a di- or tri-cis-lutein (79).

After three days' standing at 5° a petroleum ether solution of the pigment was chromatographed and found to contain three layers whose visual spectral maxima and chromatographic adsorption behavior corresponded to those expected for neolutein A, all-trans-lutein, and the unchanged pigment. Iodine catalysis of neolutein U yielded only two pigments, presumably lutein and neolutein A. If the compound in question belongs to the lutein stereoisomeric set, the absence of neolutein B from the equilibrium mixtures is surprising. Further work must be done to clarify the status of this compound; large amounts of starting material will be required since the yields are only about 1%, estimated colorimetrically as lutein.

Pigments Which Are Not Stereoisomers of Lutein.- The pink and yellow pigments, designated in the experimental section as "X" and "Y", are formed

in small amounts by the action of boric acid or boric oxide, either in naphthalene melts or in refluxed benzene solution. Yields of 20-30% were obtained by treating a pyridine solution of lutein with boron trifluoride in ether. Since both pigments lie above lutein in the Tswett column and are hypophasic, they undoubtedly contain two free hydroxyl groups.

The upper pigment, "X", has visual absorption maxima in petroleum ether at 482 and 451 mμ., which go to 478 and 448 mμ. upon addition of iodine. On the basis of the close spectral resemblance to the β-carotene chromophore the compound could be all-trans-zeaxanthin. Under the energetic experimental conditions employed the migration of a double bond into conjugation would not be surprising and could furnish a simple explanation for the change in the spectrum. The identity or non-identity of this pigment with zeaxanthin can be easily established when work is resumed.

The lower of the two pigments, "Y", possesses the same absorption spectrum in petroleum ether as neolutein A or neolutein B, namely, 471 and 442 mμ., shifting to 474 and 444 mμ. on the addition of iodine. Chromatographic analysis of this iodine equilibrium mixture showed neither neolutein A, neolutein B, nor lutein, so that the compound apparently does not belong to the lutein stereoisomeric set.

2. Epiphasic Pigments

These compounds are formed in small amounts by the action of iodine at temperatures of 80°, or higher. The total yield, which was only about 1%, was divided among numerous stereoisomers, so that crystallizable amounts of any single pigment were not obtained. In the absence of analytical data the adsorption behavior and the epiphasic partition behavior of these compounds apparently justify their characterization as hydrocarbons.

All of the compounds have typical polyene chromophores with three sharply defined maxima and a behavior on iodine isomerization which indicates that they belong to the same stereoisomeric set. Unfortunately, the chromophore, which has absorption maxima at 520, 488, and 457 mμ., is an unknown one; of the known carotenoids the closest spectroscopically is rhodoxanthin, whose chromophore contains eleven carbon-carbon double bonds with a conjugated carbonyl group at

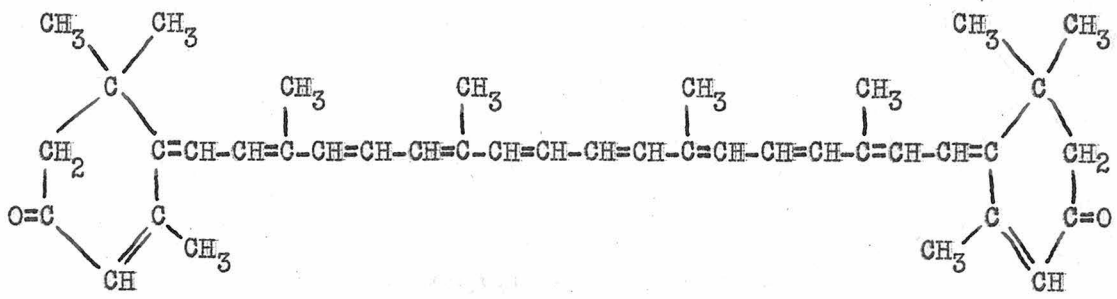
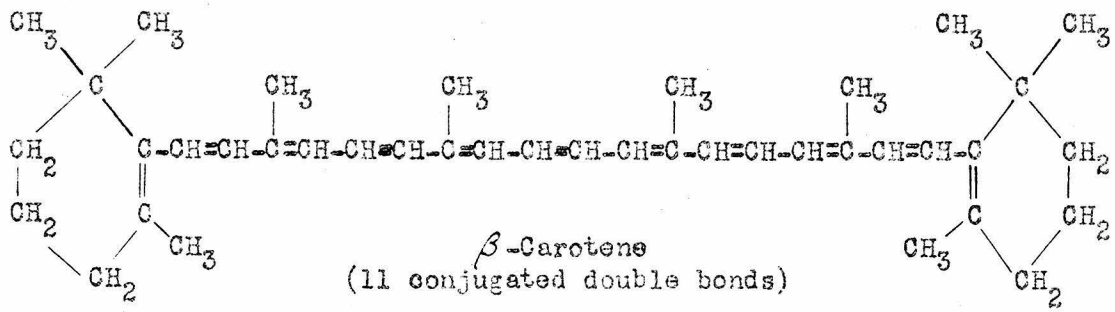


Figure 16. Rhodoxanthin

Spectral Maxima in Petroleum Ether
524, 489, 458 mμ.

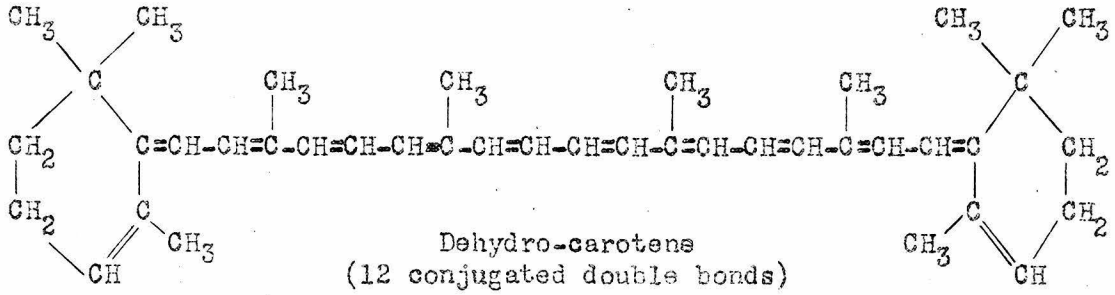
each end (32). Such a diketone is ruled out by the epiphasic partition behavior but a replacement of the two conjugated ketone groups by a single carbon-carbon double bond should give a chromophore with its first absorption maximum at approximately the same wave length, namely 17-20 mμ.[1] greater than that of dehydro-carotene (35,25,26) or 34-40 mμ. greater than that of β-carotene (Figure 17). Hydrocarbons containing other than two alicyclic rings are apparently ruled out on the basis of spectral data (Figure 18).

[1] The increase in the wave length of the first absorption maximum caused by the addition of a single carbon-carbon double bond to the chromophore is 17 mμ. in going from α-carotene to γ-carotene or 20 mμ. in going from β-carotene to dehydro-carotene (Figure 17).



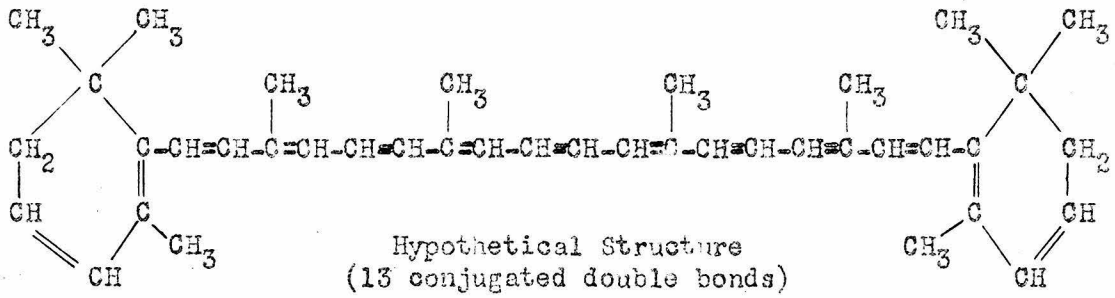
β-Carotene
(11 conjugated double bonds)

Spectral Maxima in Petroleum Ether
484, 452 mμ.



Dehydro-carotene
(12 conjugated double bonds)

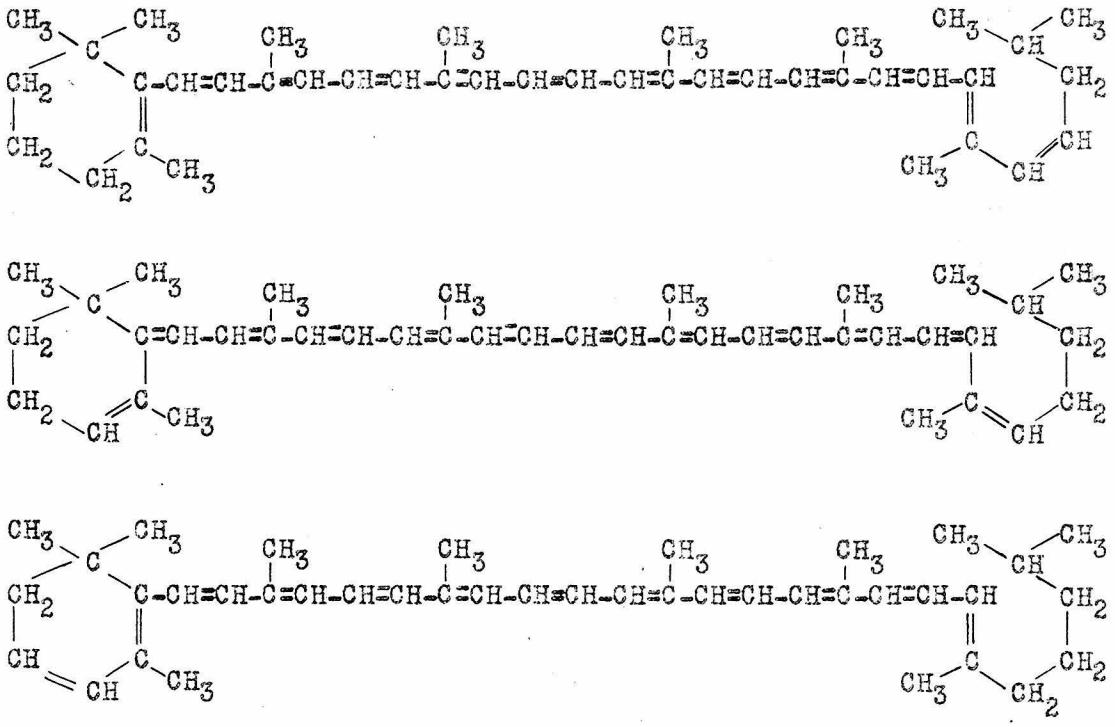
Spectral Maxima in Petroleum Ether
504, 475, 447 mμ.



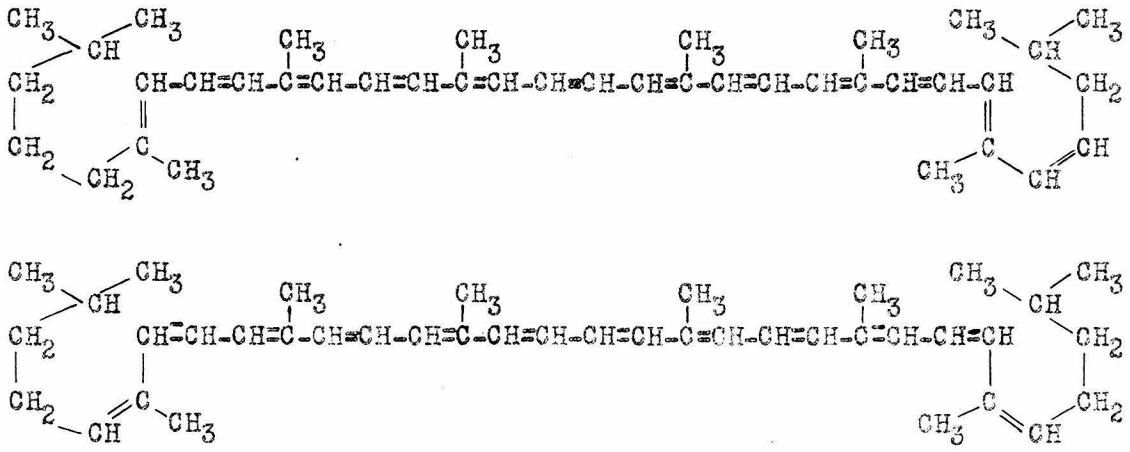
Hypothetical Structure
(13 conjugated double bonds)

Expected First Absorption Maxima in Petroleum Ether
484 + (34-40) = 518-524 mμ. or 504 + (17-20) = 521-524 mμ.

Figure 17. Relation Between Chromophore and Wave Length of First Absorption Maximum



Expected First Absorption Maximum in Petroleum Ether
495 + (17-20) = 512-515 mμ.



Expected First Absorption Maximum in Petroleum Ether
507 + (17-20) = 524-527 mμ.

Figure 18. Improbable Structures for Epiphasic Pigments

E. EXPERIMENTAL DETAILS

1. Introduction

Certain reagents, techniques, and analytical methods, which were used repeatedly in the experimental work, will be described in the following section to avoid repetition.

Reagents.- Tetraboric acid[1] was prepared from Merck Reagent orthoboric acid, either anhydrous or with water of crystallization, by heating in a porcelain evaporating dish at approximately 250° until foaming ceased; usually 20-30 minutes were required. The melt was allowed to cool to room temperature, ground to a fine powder, and stored in a tightly-stoppered bottle.

Boric oxide was prepared from Merck Reagent boric acid, either anhydrous or with water of crystallization, by heating in a porcelain evaporating dish, cautiously at first until foaming had ceased and then at red heat for forty-five minutes. The melt was allowed to cool to room temperature, ground to a fine powder, and stored in a tightly-stoppered bottle.

The petroleum ether used was Skellysolve B, b.p. 60-70°. Chromatographic developers were prepared by diluting this petroleum ether with 1, 2.5, 5, or 10 volume percent of acetone. Also used as developers were 3:1, 1:1, 1:3, and 1:4 petroleum ether-benzene mixtures.

The adsorbents were Merck Heavy Powder calcium carbonate, USP, and Shell Brand calcium hydroxide, chemical hydrate, 98% through 325 mesh. A 1:1 mixture of these adsorbents, prepared by mechanical shaking for at least 45 minutes, was also employed. The adsorbents were packed in suitable columns of the following sizes (Scientific Glass Apparatus Co., Bloomfield, N. J.):

[1] In addition to tetraboric acid some metaboric acid may be present in the product (48a).

Column Size	Inner Diameter (mm.)	Length (mm.)
No. 1	11	130
No. 2	19	200
No. 3	38	230
No. 4	48	270
No. 5	58	300
No. 6	80	330

Techniques.- The pigment zones were "eluted" by washing on a sintered glass filter with 96% ethanol until the pigment had passed completely into the filtrate; in some cases petroleum ether or ether were added to the ethanol. This solution was then placed in a continuous washer (44) and ether, petroleum ether, or benzene was added. Upon addition of water the pigment migrated into the upper phase and the solution was washed with running water for 5-10 minutes to remove all traces of alcohol. Finally, the organic solvent was dried over anhydrous sodium sulfate for 5-10 minutes.

The partition behavior was determined by shaking a benzene or petroleum ether solution of pigment with 85% methanol and estimating the relative intensity of color in the two phases. The experiment was then repeated with 95% methanol. A final test was carried out by mixing a sample of the original solution with an equal volume of absolute methanol and adding the minimum amount of water which would give a separation into two phases. Pigments which are found in the upper phase are said to be "epiphasic", while those pigments which are found in the aqueous phase, even with 85% methanol, are termed "hypophasic".

Characterization and Analytical Data.- Visual absorption maxima were read in a Loewe-Schumm Evaluating Grating Spectroscope, Zeiss, using a 2 mm. blue Zeiss BG-7 filter. When maxima for a pure substance had been read, 5-7 drops of solvent containing 30-60 µg. of iodine per ml. were added to the spectroscopic cell and the maxima were recorded again.

All extinction curves were taken on a Beckman Spectrophotometer (2) with petroleum ether solutions freshly prepared from crystals; a few drops of benzene were used to aid in dissolving the desoxylutein crystals.

All optical rotations were measured with a Schmidt and Haensch polarimeter with a cadmium lamp as light source; chloroform was used as solvent.

Photometric estimations were carried out by reading the extinctions in either petroleum ether or benzene with a Pulfrich Gradation Photometer, Zeiss, and blue (S47 or S49) light filters. The colorimetric extinctions were converted into concentrations of lutein or lycopene by means of the curves in use in this laboratory.

Melting points were taken in an electrically-heated Berl block (1) with the rate of heating adjusted to give a temperature rise of less than 2° per minute near the melting point. All samples were sealed under carbon dioxide.

Carbon-hydrogen analyses were carried out by the standard Pregl microcombustion method.

Molecular weights were determined by the Rast freezing point lowering method, using either exaltone (cyclopentadecanone) or camphor as solvent.

Catalytic hydrogenations were carried out in the apparatus devised by Prater and Haagen-Smit (58) with platinum oxide catalyst and a mixture of methylcyclohexane and glacial acetic acid as solvent.

The biological assays for vitamin A activity were carried out in the standard manner by placing young rats on a vitamin A-free diet which contained all the other factors essential for normal growth. This diet was supplemented by a daily dose per rat of 10 μg. of the test carotenoid in 0.1 ml. of Wesson oil. Lack of vitamin A activity was evidenced by cessation of growth, ophthalmia, and finally death. Dr. C. E. P. Jeffreys kindly supervised these tests.

Acknowledgment.- The author is indebted to Dr. C. E. P. Jeffreys for rat tests, to Mr. R. M. Lemmon and Mr. H. Pinckard for assistance in some preparative experiments, and to Professor A. J. Haagen-Smit as well as Dr. G. Oppenheimer and Mr. G. Swinehart for microestimations.

2. Preparation of Lutein

The isolation of lutein from Tagetes patula extracts was described by Kuhn, Winterstein, and Lederer (41). A modified procedure making use of chromatography was used in the following preparation.

One kg. of air-dried petals of Tagetes erecta (grown in Southern California) was ground in a Wiley mill and percolated with 5 liters of ether. Thirty percent methanolic potassium hydroxide was added to the ether extract until a second phase separated and the liquid was allowed to stand overnight at room temperature; the saponified extract was then washed free of alkali and methanol, dried over anhydrous sodium sulfate, and evaporated in vacuo at 30-40°. The residue was dissolved in 450 ml. of benzene at 40° and the pigment crystallized by the addition of three volumes of petroleum ether and cooling to 5°. After recrystallization from chloroform-petroleum ether the crude lutein crystals weighed 10 g.

The purification of this material was carried out with 500 mg. portions. The solution of each portion in 100 ml. of benzene was developed with benzene on a No. 6 calcium carbonate column. The main zone was eluted with an ether-ethanol mixture, washed alcohol free, dried over sodium sulfate, and evaporated in vacuo at 30-40°. The residue was crystallized from 25 to 30 ml. of chloroform by the addition of four volumes of petroleum ether and gave on the average about 165 mg. of lutein. These glittering crystals were chromatographically homogeneous and in a mixed chromatogram they did not separate from lutein obtained from another source. The analytical and optical data were correct.

3. Preliminary Experiments

Fusion of Lutein Alone.- Sixty mg. of lutein were sealed under carbon dioxide in a Pyrex tube and heated in a dibutylphthalate bath at 200-205° for three minutes; the tube was then cooled rapidly in ice water. The pigment was dissolved in a few milliliters of benzene, diluted with three volumes of petroleum ether, and poured on a No. 4 calcium carbonate column. Development with 1:4 benzene-petroleum ether yielded the following chromatogram:

Layer Thickness in mm.	Color	Spectral Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
50	Yellow (Complex mixture; "Zone A")		
20	Colorless		
3	Yellow	476.5, 445	477, 447.5
30	Colorless		
30	Yellow (Complex mixture; "Zone B")		

Zone "A" was eluted, transferred into benzene, and diluted with three

volumes of petroleum ether before rechromatography on a No. 3 calcium carbonate column. Development was with 1:3 benzene-petroleum ether, followed by 1:1 and 3:1 benzene-petroleum ether and finally by pure benzene. The following zones were observed:

Color	Spectral Maxima in Benzene (mμ.) Original	After Iodine Catalysis
Yellow	485.5, 455	487.5, 456.5
Pink	488.5, 456.5, (429)	490.5, 457.5
Yellow (Neolutein A)	483.5, 452.5, (425)	486, 454.5, (426.5)
Pink	489, 456.5	490.5, 458.5
Yellow	485, 453.5	487.5, 456
Yellow	484.5, 453.5	486.5, 456
Yellow	481.5, 450.5	487, 455.5
Yellow (Lutein)	487, 455[illegible].5	487, 454.5

The neolutein A and lutein layers were identified by means of mixed chromatograms.

A petroleum ether solution of "Zone B" was chromatographed on a No. 3 column packed with 1:1 calcium carbonate-calcium hydroxide. Development with 1% and then with 2.5% acetone in petroleum ether gave the following layers:

Color	Spectral Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
Pink	(484), 455.5, 427	456.5
Pink	480.5, 448.5	477, 446.5
Orange	475.5, 445, (418)	474, 443
Yellow	469, 438	474.5, 444
Orange	476.5, 444.5	476, 444
Yellow	479.5, 438.5	475.5, 444.5
Yellow	474	475

Pigment Losses in the Lutein Melt.- Three to six mg. of lutein were sealed in a small Pyrex tube under carbon dioxide and melted for three minutes. After rapid cooling the pigment was dissolved in benzene and estimated photometrically. The following values were obtained (calculated as "lutein"):

Temperature of Melt	Percent Recovery of Pigment
(Blank)	94.5%
200°	50.5%
210°	31.2%
220°	21.2%
230°	7.6%

Lutein-Naphthalene Melts.- Sixty mg. of lutein and 330 mg. of naphthalene were mixed together and sealed under carbon dioxide in two Pyrex tubes (8 mm. outside diameter). The mixture was melted by immersing the tubes in a dibutylphthalate bath at 150° for three minutes. After rapid cooling the solidified melts were dissolved in 8 ml. of benzene and then 32 ml. of petroleum ether was added. The solution was poured on a No. 4 calcium carbonate column and developed with benzene. The following picture resulted:

Layer Thickness in mm.	Color	Spectral Maxima in Benzene (mμ.) Original	After Iodine Catalysis
5	Yellow		
5	Colorless		
25	Yellow		
5	Colorless ("Zone A")		
10	Yellow		
10	Colorless		
30	Yellow		
15	Colorless		
30	Yellow (Lutein)	490.5, 458.5	488, 455.5
4	Colorless		
10	Orange ("Zone B")		

The top layers, "Zone A", were combined, eluted, transferred into benzene, and four volumes of petroleum ether were added. The solution was poured on a No. 5 calcium carbonate column, developed with 1:3, 1:1, and 3:1 benzene-petroleum ether mixtures and, finally, with pure benzene. The following chromatogram was obtained:

Layer Thickness in mm.	Color	Spectral Maxima in Benzene (mμ.) Original	After Iodine Catalysis
10	Yellow	488.5, 457.5	488.5, 457.5
5	Colorless		
5	Orange	489.5, 457.5	490.5, 457.5
5	Colorless		
20	Yellow (Neolutein A)	484.5, 453.5	487.5, 456.5
2	Colorless		
2	Orange	487.5, 455.5	489.5, 457
5	Colorless		
10	Yellow {"Zone C"; not neolutein B)	485.5, 454.5	487.5, 456.5
5	Colorless		
40	Yellow	488.5, 456.5	488.5, 456.5

Neolutein A was identified by a mixed chromatogram with neolutein A freshly prepared by the action of iodine on lutein; "Zone C", which showed approximately the same spectrum as neolutein B, was tested in the same way with neolutein B but two pigment zones were obtained.

An attempt to crystallize "Zone B" was unsuccessful. Accordingly, the solvent was evaporated, and the residue was dissolved in petroleum ether with the aid of a few drops of benzene. 0.5 ml. of petroleum ether containing approximately 15 μg. of iodine were added and the solution was allowed to stand for thirty minutes at room temperature before being chromatographed on a No. 3 column packed with 1:1 calcium carbonate-calcium hydroxide mixture. Upon development with 1, 2.5, and 5% acetone in petroleum ether the following chromatogram appeared:

Layer Thickness in mm.	Color	Spectral Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
15	Yellow (faint)	477.5, 447.5	477.5
15	Colorless		
5	Pink (Desoxylutein I)	492.5 (very blurred)	wave length decreases; too blurred to be read
10	Colorless		
18	Yellow-orange (Desoxylutein II)	477, 447.5	475.5, 445
2	Colorless		
4	Yellow	470.5, 441	475, 444.5
12	Colorless		
10	Yellow-orange (Desoxylutein III)	477.5, 446	476, 445
5	Colorless		
10	Yellow	473.5, 442.5	474.5, 443.5

The top and bottom layers of this chromatogram were found to be epiphasic when partitioned between 85% methanol and petroleum ether, but with 95% methanol a portion of the pigment, estimated as approximately one-fifth, migrated into the aqueous phase.

Lutein-naphthalene melts at 85° and 110° gave essentially the same bands above the layer of unchanged lutein. However, at 85° no lower layer of mono-hydroxy-compounds was observed and at 110° only a trace.

Yields Obtained in Lutein-Naphthalene Mixed Melts.- Twelve mg. of lutein were mixed with naphthalene and melted for three minutes at 140°. Photometric estimation of the three main zones gave the following yeilds (calculated as

"lutein"):

Neolutein A, 13%; Lutein, 27%; Monohydroxy-compounds, 10%.

Mixed Melts with Naphthalene and Iodine.- Twenty-three mg. of lutein and 100 mg. of naphthalene were mixed together in a small Pyrex tube (8 mm. outside diameter) and a tiny crystal of iodine (about 50 µg.[1]) was added. The tube was filled with carbon dioxide, sealed, and heated to 140° for three minutes. The solidified melt was dissolved in the minimum amount of benzene and three volumes of petroleum ether were added. The solution was chromatographed on a No. 3 calcium carbonate column and developed with 1:1 benzene-petroleum ether:

Layer Thickness in mm.	Color	Absorption Maxima in Benzene (mµ.) Original	After Iodine Catalysis
3	Yellow	490.5, 458	492, 460
3	Colorless		
5	Yellow (heterogeneous)	488, 456.5	489.5, 457
7	Colorless		
2	Orange-yellow	495.5, 461.5	492, 459.5
50	Faint yellow and pink bands		
20	Yellow		
2	Pink		
2	Colorless		
3	Yellow (Middle layers; "Zone A")		
20	Colorless		
25	Pink and orange bands (Lower layers; "Zone B")		

[1] In a preliminary experiment in which 1-2 mg. of iodine was added almost all the pigment was bleached during the melt.

"Zone B" was adsorbed only weakly and no satisfactory separation could be achieved before its zones reached the bottom of the column.

The layers of "Zone A" were combined for elution and transferred to petroleum ether. When developed on a No. 3 calcium hydroxide column with 1, 2.5, and 5% acetone in petroleum ether the following chromatogram resulted:

Layer Thickness in mm.	Color	Absorption Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
20	Faint yellow bands		
5	Pink (Desoxylutein I)	492.5, 458	492, 459
10	Yellow } (Desoxylutein II	477, 447	475.5, 445.5
4	Orange } plus unidentified pigment)		
2	Colorless		
6	Yellow	472, 442	475.5, 445
10	Colorless		
12	Yellow (Desoxylutein III)	479, 449.5	476, 445.5
3	Colorless		
15	Yellow	470, 441	476.5, 445

The top and bottom layers were epiphasic when partitioned between 85% methanol and petroleum ether, but divided between the two phases when 95% methanol was employed.

The layers of "Zone B" were transferred into petroleum ether and developed on a No. 3 calcium hydroxide column with petroleum ether and 1% acetone in petroleum ether:

Layer Thickness in mm.	Color	Absorption Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
4	Faint pink bands	No readable spectrum	
4	Pink ("Zone C")	519.5, 487.5, 457.5	518.5, 486.5, 456.5
12	Faint pink bands	(516-8), 485, 445.5	(517-8), 487, 456.5
7	Orange-pink ("Zone D")	484.5, 454	483.5, 453
5	Yellow	478, 449.5	483, 452
3	Pink	478, (449)	484, 452.5
13	Orange-pink	477.5, 446	483, 451.6
6 3	Pink-violet Violet	481	482
5	Pink and violet bands	No readable spectrum	

Pigments "C" and "D" were epiphasic. The lower of these two zones was found in mixed chromatograms to be adsorbed on calcium hydroxide below kryptoxanthin and γ-carotene but above β-carotene.

Similar experiments, carried out at 85° and at 110°, gave middle layers which on rechromatographing were found to be essentially the same as those produced at 140°. Only traces of the lower epiphasic layers were found in the 85° melt, but the 110° melt contained numerous bands, four of which had spectra going to 517, 484.5, 455 mμ. upon the addition of iodine. The petroleum ether solutions of these four bands were combined, concentrated to 20 ml. in vacuo, and treated with iodine in petroleum ether for 20 minutes at room temperature. The solution was then chromatographed on calcium hydroxide and developed with 2.5%, 5%, and 10% acetone in petroleum ether:

Layer Thickness in mm.	Color	Spectral Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
2	Pink		
18	Colorless		
2	Pink	519, 487.5, 456, (431.5)	516.5, 485, 455.5
2	Colorless		
7	Pink	520, 487.5, 457, (432)	517, 484.5, 454.5
2	Colorless		
10	Pink Bands	516, 485, 455, (430)	517, 484.5, 455.5
3	Colorless		
5	Pink	514, 483, 453.5, (428)	516.5, 484.5, 454.5
10	Colorless		
5	Pink	510.5, 479.5, 451	516.5, 484.5, 454.5
5	Colorless		
5	Pink	512, 481, 451	516.5, 485, 454.5

Reflux with Iodine in Benzene.- Fifteen mg. of lutein were dissolved in 10 ml. of benzene and 1 ml. of benzene containing 0.2 mg. of iodine was added. The solution was refluxed for thirty minutes, additional 0.5 ml. portions of iodine solution being added at the end of ten and twenty minutes. Two volumes of petroleum ether were added and the solution was poured on a No. 3 calcium carbonate column. Development with 1:1 benzene-petroleum ether gave a chromatogram which closely resembled that obtained in the 110° melt with naphthalene and iodine.

Fusion of Lutein with Naphthalene and Tetraboric Acid.- One hundred mg. of lutein was mixed with 1.5 times its volume of naphthalene plus its own

volume of tetraboric acid, and sealed in two Pyrex tubes (8 mm. outside diameter) under carbon dioxide. The tubes were kept at 140° for five minutes and then cooled rapidly. The benzene solution (15 ml.) was diluted to 125 ml. with petroleum ether, and developed with 5 and 10% acetone in petroleum ether on a No. 6 column packed with 1:1 calcium carbonate-calcium hydroxide mixture:

Layer Thickness in mm.	Color	Spectral Maxima in Benzene (mμ.) Original	After Iodine Catalysis	Pigment Yield[1] (estimated as "lutein")
20	Orange	489.5, 457	489.5, 456.5	
5	Pink	492.5, 459.5	490.5, 458	19%
12	Yellow	486.5, 454.5	489, 456	
7	Orange	489.5, 457.5	487.5, 456	
15	Colorless			
30	Pink (Desoxylutein I)			6%
10	Colorless			
40	Yellow-orange (Desoxylutein II)			16%
25	Yellow			9%
10	Yellow-orange (Desoxylutein III)			7%
15	Yellow			7%

The petroleum ether solutions of the five lower layers were evaporated <u>in vacuo</u> at room temperature; the residues were transferred to 15 ml. centrifuge tubes with minimum quantities of ether, and evaporated to dryness in a stream of carbon dioxide. Crystallization from benzene-methanol yielded 3.5 mg. of

[1] Starting material = 100%.

desoxylutein I, 10.3 mg. of desoxylutein II, and 2 mg. of desoxylutein III; all attempts to crystallize the other layers yielded only yellow or orange oils.

Reflux of Lutein in Benzene Solution with Boric Oxide.- Thirty mg. of boric oxide were added to a solution of 15.5 mg. of lutein in 10 ml. of benzene and allowed to stand overnight at room temperature; the solution was then refluxed for two hours and kept for two days at room temperature. Four volumes of petroleum ether were added and the solution was chromatographed on a No. 4 column packed with calcium hydroxide, 5 and 7.5% acetone in petroleum ether being used as developer. The following layers, each separated from its neighbors by colorless interzones, were obtained:

Color	Spectral Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
Yellow	490.5, 456.5[1]	488.5, 455.5[1]
Mixture, pink above, yellow below ("Zone A")		
Pink (Desoxylutein I)	About 492 (very blurred)	
Yellow (Desoxylutein II)	476.5, 447.5	473.5, 445
Yellow (Desoxylutein III)	478, 447	476, 445.5

After elution "Zone A" was transferred to benzene and six volumes of petroleum ether were added. The solution was poured on a No. 3 column containing a 1:1 mixture of calcium carbonate and calcium hydroxide and the chromatogram was developed with 5, 10, 15, and 20% acetone in petroleum ether. Three well-separated layers appeared:

[1] In Benzene.

Color	Spectral Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
Pink ("Pigment X")	481, 451	478, 448
Yellow ("Pigment Y")	471.5, 442	474, 443.5
Orange (Lutein)	476.5, 447.5	473, 444.5

"Pigment X" was hypophasic when partitioned between 85% methanol and petroleum ether; the other two pigments were essentially hypophasic with 85% methanol and completely so with 95% methanol. On treatment with iodine neither the red nor the yellow layers gave rise to lutein isomers.

Treatment of Lutein with Boron Trifluoride in Pyridine.- Four mg. of lutein were dissolved in 10 ml. of pyridine and 5 ml. of 45% boron trifluoride in ether (Eastman, practical grade) was added slowly with cooling to keep the temperature below 30°. The solution was allowed to stand overnight and then washed free of pyridine. After drying over sodium sulfate the solution was evaporated in vacuo at room temperature. The residue was taken up in a few milliliters of benzene. Several volumes of petroleum ether were added and the solution was developed on calcium hydroxide (No. 3 tube) with 2.5, 5, and 10% acetone in petroleum ether:

Color	Spectral Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
Pink ("Pigment X")	482, 450.5	478.5, 448.5
Yellow ("Pigment Y")	470.5, 441.5	473.5, 443.5
Orange (Lutein)	476, 446.5	473.5, 444
Yellow (trace at bottom of column)	476, 446	474, 443.5

The three principal layers were all essentially hypophasic when partitioned

between 85% methanol and petroleum ether and entirely hypophasic with 95% methanol; the trace of yellow pigment found at the bottom of the column was epiphasic under all conditions. The lutein was identified by a mixed chromatogram test.

Melt of Lutein with Anhydrous Sodium Tetraborate in Naphthalene.- Eight mg. of lutein, 51.8 mg. of naphthalene, and 6.0 mg. of anhydrous sodium tetraborate were sealed in a Pyrex tube (about 12 mm. outside diameter) under carbon dioxide; the tube was kept at 140°(±5°) for five minutes, and cooled rapidly. The solid was dissolved in a few milliliters of benzene, and six to eight volumes of petroleum ether were added before pouring on a No. 3 column packed with calcium carbonate. 1, 2.5, and 5% acetone in petroleum ether were used as developers:

Layer Thickness in mm.	Color	Spectral Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
4	Colorless		
2	Yellow	478, 447	481, 448.5
2	Colorless		
8	Yellow	471, 441.5	474.5, 445
2	Colorless		
3	Yellow	472, 442	475, 444
50	Colorless		
30	Yellow	478, 447	474.5, 444
4	Colorless		
10	Yellow	477.5, 447	475.5, 445
2	Colorless		
5	Yellow (Neolutein U)	462.5, 434.5	475.5, 443.5 Blurs on addition of iodine, then gives good spectrum

All zones were hypophasic with 85% methanol.

When this experiment was repeated with 2.2 mg. of lutein and the neo-layer estimated colorimetrically as "lutein" in the Pulfrich photometer, the yield was found to be about 1%.

When iodine was added to a petroleum ether solution of "Neolutein U" and the solution after a few minutes standing was developed on a No. 2 calcium carbonate column with 2.5% acetone in petroleum ether, two yellow layers resulted; the spectral absorption maxima of the upper, minor zone were at 470.5 and 441 mμ., while the first band of the lower, main layer was at 475.5 mμ.; upon the addition of iodine both gave maxima at 473 and 443 mμ.

A solution of the neolutein which had stood at 5° for 3 days was chromatographed on a No. 2 calcium carbonate column, using 2.5% acetone in petroleum ether as developer. Three yellow zones appeared:

Position on the Column	Spectral Maxima in Petroleum Ether (mμ.) Original	After Iodine Catalysis
Top	469, 441.5	472.5
Middle	477	473
Lower	460, 433	472.5, 443.5

Fusion of Zeaxanthin with Tetraboric Acid or Boric Oxide in Naphthalene.- The zeaxanthin used for these experiments was furnished by Professor Zechmeister. In a test of its purity 1-2 mg. were dissolved in a few drops of benzene and the solution was diluted to 10 ml. with petroleum ether. This solution was then poured on a No. 2 column packed with a 1:1 mixture of calcium carbonate and calcium hydroxide and developed with 5, 10, 15, and finally 20% acetone in petroleum ether. The chromatogram was homogeneous except for a small layer, estimated to be 5% or less of the total pigment, which lay below the zeaxanthin zone.

Seven mg. of the zeaxanthin were mixed in a small glass tube (about 12 mm. outside diameter) with 55 mg. of naphthalene and 47 mg. of tetraboric acid, sealed in carbon dioxide, heated for five minutes with constant shaking in a dibutylphthalate bath at 140° (±5°), and cooled in an ice bath. The solution in 1-2 ml. of benzene and 15 ml. of petroleum ether was developed with 5, 10, 15, and 20% acetone in petroleum ether on a No. 3 column containing a 1:1 mixture of calcium carbonate and calcium hydroxide:

Layer Thickness in mm.	Color	Spectral Maxima in Benzene (mμ.) Original	After Iodine Catalysis
20	Colorless		
15	Yellow	491, 458.5	493, 458.5
5	Colorless		
25	Orange-yellow (Zeaxanthin)	496.5, 462.5	493.5, 460
2	Colorless		
5	Yellow	489.5, 456	493, 459
5	Colorless		
10	Orange (Impurity)	495, 460	492.5, 459
Diffuse	Orange	491.5, 457.5	492.5, 459

The experiment was repeated with a mixture of 4.5 mg. of zeaxanthin, 38 mg. of naphthalene, and 88 mg. of boric oxide. On development with 5, 10, 15, and 20% acetone in petroleum ether, the resulting chromatogram was similar to that of the metaboric acid melt:

Color	Spectral Maxima in Benzene (mμ.) Original	After Iodine Catalysis
Yellow	490.5, 458	493.5, 459
Orange-yellow	493, 460	491, 459.5
Orange-yellow (Zeaxanthin)	494, 461.5	492.5, 460.5
Yellow	484.5, 452.5	493, 460
Orange (Impurity)	493, 460	493, 459
Yellow	489.5, 456	491, 459

Yields of Monohydroxy and Epiphasic Compounds Obtained by the Fusion of Lutein under Various Conditions.- In order to choose the best conditions for a large-scale preparation of the desoxyluteins, numerous small-scale experiments with 4-10 mg. of pigment were carried out. The weight of naphthalene employed was six to eight times that of the lutein. Iodine was added in the form of a tiny crystal of approximately 50 μg., while the weight of the boron compound was six to nine times that of the lutein. The melt was dissolved in a minimum amount of benzene, diluted with three volumes of petroleum ether and developed on a No. 3 calcium carbonate column with 1:1 benzene-petroleum ether.

The monohydroxy layers were transferred to petroleum ether and rechromatographed on calcium carbonate-hydroxide mixture. When significant amounts of the desoxyluteins appeared, the layers were combined, eluted, and estimated photometrically as "lutein". The epiphasic layers were also transferred to petroleum ether, and catalyzed with iodine. After standing for thirty minutes the solution was developed on calcium hydroxide with 5% acetone in petroleum ether; if pink layers appeared, they were combined, eluted, and estimated as

"lycopene". The results of this series of experiments are given in Table 1 (See p. 17).

4. Large Scale Preparation of Desoxyluteins I, II, and III

One gram of purified lutein was divided into forty 25-mg. portions. Each was mixed with 140 mg. of naphthalene and 60 mg. of finely-powdered tetraboric acid and sealed under carbon dioxide in a small glass tube (about 12 mm. outside diameter). Each tube was agitated in a dibutylphthalate bath at 140° (±5°) for five minutes and then rapidly cooled. The tubes were crushed in a large mortar and their combined contents were dissolved in 200 ml. of cold benzene. Four volumes of petroleum ether were then added and the solution was divided into eight parts, each of which was chromatographed on a No. 6 column packed with 1:1 calcium carbonate-calcium hydroxide mixture. Upon development with 5% acetone in petroleum ether the following chromatogram appeared:

Layer Thickness in mm.	Color
15	Pink, orange, and yellow (Minor zones of unidentified pigments)
15	Colorless
50	Dark pink (Desoxylutein I)
40	Yellowish-orange (Desoxylutein II)
4	Dark pink
6	Brownish-orange
40	Yellow with orange tint (Desoxylutein III)
20	Yellow

The chromatographic filtrate was yellow and contained at least two additional pigments which gave a weakly adsorbed heterogeneous column section when rechromatographed on calcium hydroxide with petroleum ether developer. The upper portion of the band was orange with a spectrum too blurred to be read, while the lower portion was yellow and showed spectral maxima at 469.5 and 442.5 mμ. in petroleum ether; these values decreased about 1.5 mμ. upon iodine catalysis.

The desoxylutein I zones from each of the eight columns were combined; a similar pooling was carried out for the various zones of desoxylutein II and for those of desoxylutein III. After elution each of the three pigments was transferred into petroleum ether, washed alcohol-free, dried, and developed with 5% acetone in petroleum ether on No. 6 columns containing a 1:1 mixture of calcium carbonate and calcium hydroxide. Two columns each were needed for the rechromatography of desoxyluteins I and III and four columns for desoxylutein II. Desoxylutein I formed a 110 mm. pink zone, with a minor yellowish-orange layer immediately below it. The chromatograms of desoxylutein II showed a 30 mm. layer of desoxylutein I above the main (70 mm.) yellowish-orange zone; several zones lay below it. The chromatogram of desoxylutein III was practically homogeneous and consisted of a 110 mm. yellowish-orange layer.

After elution with ethanol each of the three compounds was transferred into ether, washed free of alcohol, dried, and evaporated *in vacuo* at room temperature. Each residue was dissolved in the minimum amount of ether, transferred to a 15-ml. centrifuge tube, and evaporated to dryness with a stream of carbon dioxide. After dissolving rapidly in 0.5-1.0 ml. of benzene at 40-50°, 4-5 volumes of methanol were added with stirring at room temperature. Pigment

crystals appeared immediately. The mixture was kept overnight at 5° and then centrifuged. The crystals were washed with 1 ml. of methanol, centrifuged, and recrystallized as before. When the methanol was added gradually over a period of several hours at room temperature, larger, glittering crystal units appeared.

While the isolation of desoxyluteins I and II never afforded any difficulties, it was not easy to obtain a reliable separation of desoxylutein III from the bottom zone of the chromatogram. Impure solutions yielded an oil and had to be rechromatographed before crystals could be obtained.

The yields of crystals obtained in several experiments with tetraboric acid and boric oxide were as follows:

Starting Material	Percent Yield[1]		
Lutein	Desoxylutein I	Desoxylutein II	Desoxylutein III
1.0 g.	3.8%	10.0%	4.3%
1.0 g.	3.0%	12.7%	3.2%
0.1 g.	3.5% (6%)	10.3% (16%)	2% (7%)

Figures in parenthesis are colorimetric estimations in the Pulfrich photometer, calculated as "lutein".

5. Characterization of the Desoxyluteins

Desoxylutein I

Description and Analytical Data.- This compound, when crystallized from benzene-methanol, forms long prisms with peaked ends, partially grouped

[1] Starting material = 100%.

in aggregates (Figure 19). Their color under the microscope is a rich reddish-orange which changes to brownish-red at crossings (Desoxyluteins II and III appear yellow or brownish by comparison). The crystals melted at 149° (cor.) and gave the following analytical data:

Carbon-Hydrogen:-

Calculated for $C_{40}H_{56}O$:	C, 86.89	H, 10.22
Found:	C, 87.09	H, 10.33
	C, 86.29	H, 9.76
	C, 86.59	H, 9.57

These data were corrected for 1.6, 1.5, and 0.5% ash respectively.

Molecular Weight:-

Calculated for $C_{40}H_{56}O$:	553
Found (in camphor):	524

Catalytic Hydrogenation:- 9.730 mg. of substance in methylcyclohexane and glacial acetic acid, with 9.73 mg. of PtO_2 catalyst, added 4.70 ml. of hydrogen (over mercury, 21.5°, 743.5 mm.): 4.729 mg., with 2.23 mg. of catalyst, added 2.33 ml. (22.5°, 739.5 mm.). Found: 10.8 and 10.9 double bonds.

Solubility:- The compound is easily soluble in cold benzene, ether, and chloroform, but much less so in petroleum ether. The solubility in methanol is very slight. On partition between petroleum ether and 85% methanol the pigment showed a purely epiphasic behavior, while a portion estimated at approximately 20%, migrated into the lower phase when 95% methanol was employed.

Visually Observed Spectrum.- As would be expected from an examination of the extinction curve (Figure 7) the visually observed spectrum of desoxylutein I has an essentially different character from those of desoxyluteins

Figure 19

Desoxylutein I, crystallized from benzene-methanol.
Approximately 250 diameters

II and III or from that of lutein itself. The limits of the two bands are indistinct and the interspace between them is never clear. In petroleum ether, at very low concentrations, a main blurred band can be observed at 458-459 mμ. and a much weaker one at approximately 494 mμ. Even a small increase in concentration, however, produces an extended shadowed area, extending from about 450 to 550 mμ., within which no maxima can be seen (traces of desoxylutein II as impurity have the same effect as increasing the concentration). In benzene the bands are even more blurred and unreadable. In carbon disulfide the main maxima were found near 537 and 492 mμ. Upon the addition of iodine the spectrum blurs and migrates toward shorter wave lengths. The maxima of the equilibrium mixture in petroleum ether are located at about 491.5 and 459.5 mμ.

Adsorption Behavior.- On a calcium carbonate-calcium hydroxide mixture, 1:1, with 5-10% acetone in petroleum ether as developer, desoxylutein I is adsorbed below gazaniaxanthin (a hydroxy-γ-carotene), lutein, and lycopene but above cryptoxanthin, desoxyluteins II and III, γ-carotene, and β-carotene.

Isomerization by Iodine Catalysis.- A solution of 1 mg. of chromatographically homogeneous desoxylutein I in a few drops of benzene was diluted to 15 ml. with petroleum ether and 15 μg. of iodine in 0.5 ml. of petroleum ether were added. After standing for twenty minutes the solution was chromatographed on a No. 2 column containing calcium carbonate-calcium hydroxide mixture. On development with petroleum ether containing 5% acetone a new zone appeared below the unchanged portion of the all-trans form. The neocompound showed blurred maxima at 486.5 and 452 mμ. in petroleum ether, which changed to 489.5 and 456.5 mμ. upon the addition of iodine. The equilibrium mixtures formed by the iodine treatment of desoxylutein I and neodesoxylutein

I are chromatographically identical.

Acetate of Desoxylutein I.- Five mg. of desoxylutein I in 1.5 ml. of anhydrous pyridine were treated at room temperature with 0.5 ml. of acetic anhydride for twenty-four hours. The pigment was then transferred with water into petroleum ether and washed free of pyridine. After drying over anhydrous sodium sulfate, the solution was chromatographed on a No. 3 column containing calcium carbonate-calcium hydroxide mixture. Development with 5% acetone in petroleum ether gave two zones, the lower one of which was the larger and contained the acetylated pigment. The eluate of the latter was transferred into ether, and evaporated in vacuo; the residue was crystallized from benzene-methanol as small plates; m.p. 139° (cor.). Its spectrum did not differ from that of the unesterified compound; the partition behavior, however, was purely epiphasic. Saponification of the acetate in petroleum ether with cold 30% methanolic potassium hydroxide yielded a homogeneous pigment which did not separate from desoxylutein I in the mixed-chromatogram test.

Desoxylutein II

Description and Analytical Data.- The characteristic crystal forms of this compound are represented in Figure 20. The color of the crystals resembles that of lutein, both macro-and microscopically. The crystals melted at 156.5-158° (cor.) and yielded the following analytical data:

Carbon-Hydrogen:-

Calculated for $C_{40}H_{56}O$:	C, 86.89	H, 10.22
Found:	C, 86.73	H, 9.93
	C, 86.78	H, 10.12
	C, 86.47	H, 9.82

The data are corrected for 0.5, 0.5, and 0.4% ash respectively.

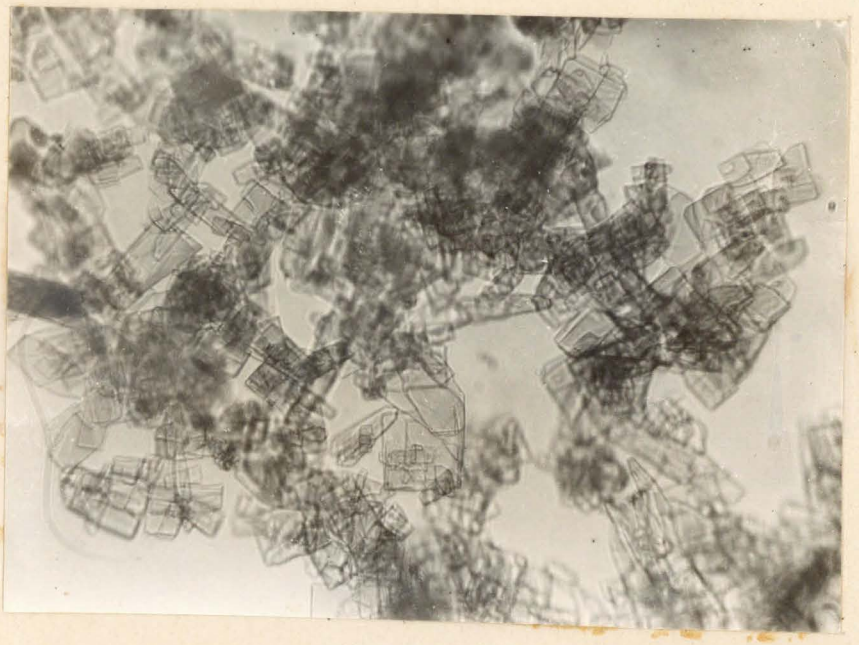


Figure 20

Desoxylutein II, crystallized from benzene-methanol.
Approximately 250 diameters

Molecular Weight:-

Calculated for $C_{40}H_{56}O$: 553

Found (in exaltone): 569 and 577.

Catalytic Hydrogenation:- 10.284 mg. of substance, with 5.31 mg. of PtO_2, added 5.09 ml. of hydrogen (22.5°, 742.0 mm.): 6.369 mg. with 2.41 mg. of catalyst, added 3.05 ml. (22.5°, 741.5 mm.). Found: 11.0 and 10.7 double bonds.

Solubility.- The solubility and partition behavior are the same as in the case of desoxylutein I.

Visual Spectrum.- The following maxima were observed:

Solvent	Spectral Maxima (mμ.) Original	After Iodine Catalysis
Carbon Disulfide	508.5, 473.5, 444	505.5, 471.5
Benzene	490.5, 458, 432	488, 456
Petroleum Ether	477, 446.5	475, 444
Hexane	476.5, 446	473.5, 443
Ethanol	480, 449	

Optical Rotation.- The rotation observed for a 1 dm. thickness of a chloroform solution containing 13.83 mg. per 10 ml. was less than the uncertainty of the measurements; therefore $[\alpha]_{Cd} = 0° (\pm 10°)$.

Adsorption Behavior.- When developed with petroleum ether containing 5% or 10% acetone on a calcium carbonate-calcium hydroxide column (1:1), the compound is adsorbed below lutein, lycopene, desoxylutein I, and cryptoxanthin, but above γ-carotene, desoxylutein III, and β-carotene.

Stereoisomerization by Iodine Catalysis.- In an experiment similar to

that carried out with desoxylutein I two stereoisomers were observed below the unchanged portion of the all-trans-desoxylutein II. The spectral maxima were located at 468.5 and 439.5 mμ. for the upper stereoisomer and at 467 and 438 mμ. for the lower one. Petroleum ether solutions of both isomers, as well as the all-trans-pigment, shifted their maxima to 473.5 and 443.5 mμ. upon iodine catalysis. The three mixtures of stereoisomers obtained in this manner were chromatographically identical.

Acetate of Desoxylutein II.- This ester, which was prepared as described above for desoxylutein I, forms plates, most of which have curved sides. The crystals melted at 141° (cor.), after softening at about 139°. The acetate showed a purely epiphasic behavior. After saponification the pigment was found in a mixed chromatogram to be identical with desoxylutein II. The positions of the visually observed spectral maxima were not changed either by the acetylation or by the saponification of the ester.

Desoxylutein III

Description and Analytical Data.- The crystals viewed under the microscope show a typical barrel-like or boat-like shape (Figure 21); their color is similar to that of desoxylutein II. The crystals melted at 162° (cor.), after softening. The following analytical data were obtained:

Carbon-Hydrogen:-

Calculated for $C_{40}H_{56}O$:	C, 86.89	H, 10.22
Found:	C, 86.92	H, 9.74
	C, 87.19	H, 10.13

The samples were ash-free. In unfavorable cases the carbon values were markedly different (87.75 and 85.96).

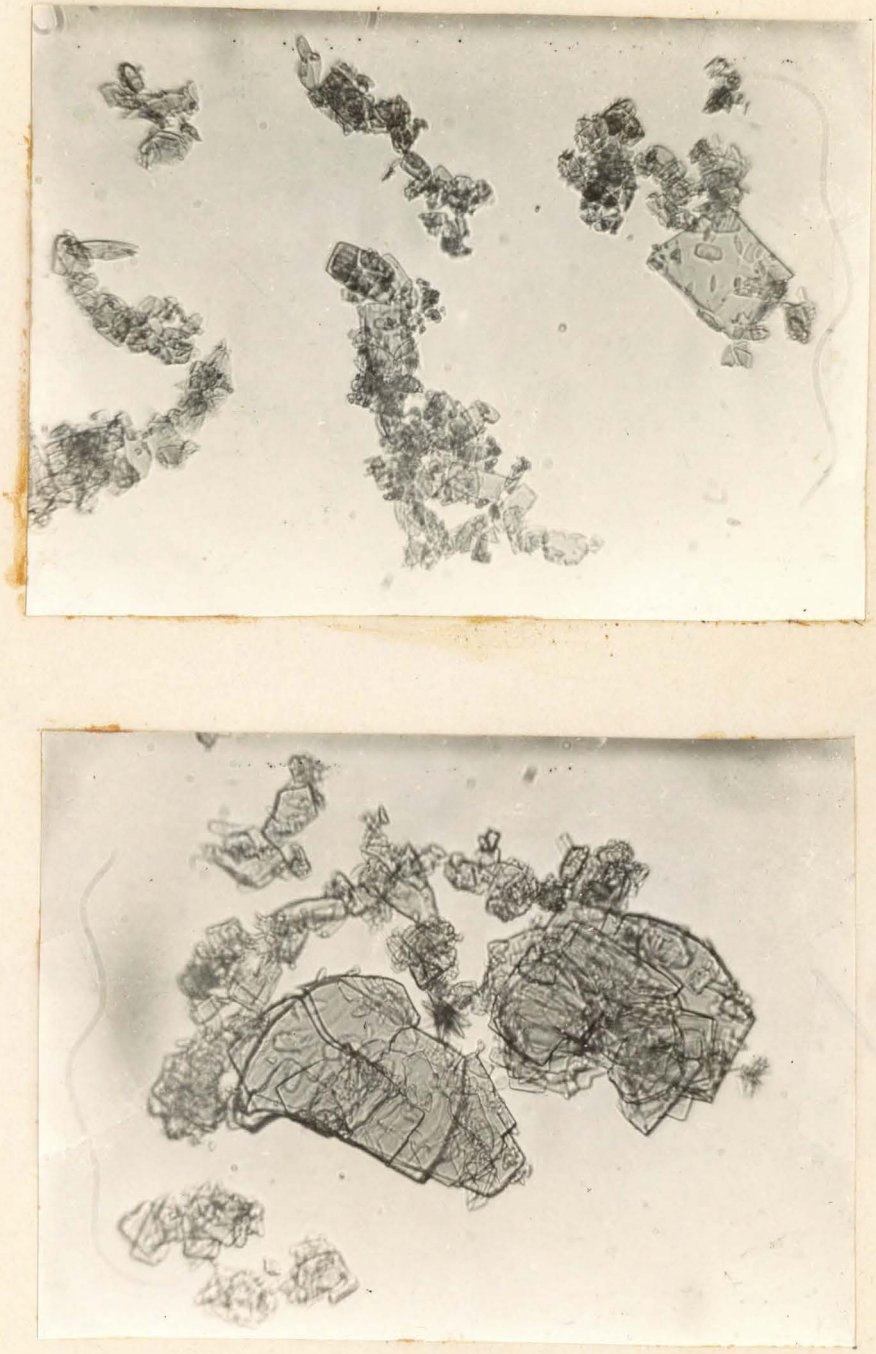


Figure 21

Desoxylutein III, crystallized from benzene-methanol.
Approximately 250 diameters

Molecular Weight:-

Calculated for $C_{40}H_{56}O$: 553

Found (in exaltone): 547

Catalytic Hydrogenation:- 9.300 mg. of desoxylutein III, with 4.49 mg. of PtO_2, added 4.61 ml. of hydrogen (22°, 740.5 mm.); 7.774 mg. with 3.49 mg. of catalyst, added 3.72 ml. (23°, 742.0 mm.). Found: 11.0 and 10.7 double bonds.

Solubility.- The solubility and partition behavior are the same as in the case of desoxylutein I.

Visual Spectrum.- The following data are characteristic:

Solvent	Spectral Maxima (mμ.) Original	After Iodine Catalysis
Carbon Disulfide	508.5, 474, 444.5	506, 472
Benzene	491, 458.5, 432	489.5, 456.5
Petroleum Ether	478, 448	476, 445
Hexane	477.5, 447	475.5, 445
Ethanol	481.5, 450.5	Blurs, shifts to shorter wave lengths

Adsorption Behavior.- Desoxylutein III is adsorbed slightly below γ-carotene and well below desoxylutein II, cryptoxanthin, desoxylutein I, lycopene, and lutein when developed on a 1:1 calcium carbonate-calcium hydroxide column with petroleum ether containing 2.5-5% acetone. Under similar conditions β-carotene occupied a place on the column considerably below that of desoxylutein III.

Stereoisomerization by Iodine Catalysis.- The chromatogram of the iodine-catalyzed solution showed, in addition to unchanged all-trans pigment and a

minor irreversible top layer, a neo-compound adsorbed below the main zone. Its spectral maxima in petroleum ether were at 468 and 439 mμ.; after the addition of iodine the equilibrium mixture showed maxima at 474.5 and 443.5 mμ. This mixture proved to be chromatographically identical with that produced by the iodine catalysis of desoxylutein III.

Acetate of Desoxylutein III.- A crystalline acetate of desoxylutein III could not be obtained.

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II. INVESTIGATION OF THE EFFECT OF GLOBULIN DEPLETION ON ANTIBODY PRODUCTION IN RABBITS

II. INVESTIGATION OF THE EFFECT OF GLOBULIN DEPLETION ON ANTIBODY PRODUCTION IN RABBITS

A. INTRODUCTION

It is now generally accepted that antibodies are associated with a specific protein fraction of the blood serum known as γ-globulin. Numerous explanations have been advanced to explain the appearance of these antibodies soon after the injection into the animal of the corresponding antigen.

According to the theory advanced by Pauling (1) the presence of an antigen at the site or sites of γ-globulin synthesis causes the long polypeptide chains to fold in such a manner that they assume configurations complementary to the antigen, thus acquiring the ability to combine with it. If the production of γ-globulin is a normal activity of the body, and not a specific response to the presence of antigen, the theory suggests that, in the presence of a certain amount of antigen, an increased rate of γ-globulin production should lead to a higher proportion of antibody molecules in the serum. This follows from the assumption that only a fraction of the circulating γ-globulin of the serum will be replaced during the effective presence of the antigen in the body, so that only a fraction of the globulin will be specific for that antigen.

The method we proposed to apply to stimulate an increased rate of γ-globulin formation was a severe depletion of the circulating globulins brought about by bleeding the animals at frequent intervals and reinjecting their erythrocytes suspended in an amount of isotonic homologous serum albumin sufficient to replace the volume of serum lost. A somewhat similar

experiment has been reported by Cannon and his co-workers (2) who brought about the desired depletion by prolonged feeding of the experimental animals with a protein-free diet. In his experiments no positive conclusions could be drawn concerning the effect of this treatment on the production of antibodies; immunization was attempted either during the starvation period or following it when the animal was being fed a diet rich in proteins, and hence was presumably synthesizing proteins rapidly.

The experiments described here are of a preliminary nature and failed to demonstrate whether it would actually be possible to observe the postulated effect. Several unexpected difficulties were encountered during the investigation and it now appears that a much more refined technique will be required to find the answer. The experimental section which follows describes what has been done, while some suggestions for further work are embodied in the discussion following.

B. EXPERIMENTAL

A. First Experiment

Depletion Technique.- Thirteen young rabbits, ranging in weight from 2.1 to 2.8 kg., were separated arbitrarily into a group of six controls and a group of seven test animals. Approximately 30 ml. of blood was taken from each test animal every 48 hours. The first two bleedings were from the marginal ear vein into paraffined 50 ml. centrifuge tubes, while the remainder were by heart puncture with a No. 20 gauge needle; in every case 2 ml. of 10% sodium citrate was present in the centrifuge tube or syringe to prevent clotting.

The cells from each rabbit were centrifuged down and washed three times with sterile 0.9% saline solution. The cells were then resuspended in sufficient 3% rabbit albumin solution, containing 0.9% sodium chloride, to bring the volume of the suspension up to that of the whole blood originally taken from the animal. The suspension was filtered through sterile gauze and reinjected by marginal ear vein into the same rabbit from which the cells came originally. The entire process of bleeding, washing, and reinjecting required 2 to 3 hours.

Analyses for Serum Albumin and Globulin.- To 1 ml. of citrated plasma was added 0.35 ml. of 2% calcium chloride solution; the serum was then diluted to 5 ml. with 0.9% sodium chloride solution and allowed to clot at 37°. The liquid was expressed from the clot and the fibrin was then removed and discarded. 4.6 ml. of saturated ammonium sulfate was then added and the solution was kept at room temperature for twenty to thirty minutes before centrifuging down the globulin. The globulin precipitate was dissolved

in saline and suitable aliquots of it and the albumin supernatant were taken for analysis by a modified Folin microcolorimetric method (3). Since no calibration factor for albumin was available, both albumin and globulin values are reported as rabbit serum globulin.

Immunological Techniques.- At the end of the depletion period (5 to 8 bleedings per rabbit) each rabbit was injected intraperitoneally with 100 mg. of ovalbumin in 5 ml. of isotonic saline. The immunizing injection was repeated after seven days. Ten days after the first injection preliminary ring tests with 1% ovalbumin and undiluted serum showed positive results in all but one of the four surviving rabbits and all but one of the six controls. Accordingly, three days later 15 ml. of blood was taken from the ear of each rabbit and the undiluted sera titrated, with the results in Table II. After eight days the rabbits were again bled and their sera titrated; the results are given in Table III.

B. Second Experiment

Preparation of 3% Albumin Solution.- Rabbit serum was diluted with an equal volume of saturated ammonium sulfate and the precipitate filtered off. The supernatant was dialyzed against running tap water for 2 to 3 days and then concentrated to the original volume of the serum by evaporation in the dialyzing membranes, which were suspended in a current of warm air. The solution was sterilized by passing through a Seitz filter and stored at 5°.

When an attempt was made to increase the severity of the depletion conditions by increasing the amount of each bleeding from 30 ml. to 50 ml. and by decreasing the interval between bleedings from 48 hours to 24 hours, seven of the ten test animals died within 24 hours. The difficulty was

traced to the albumin solution, which was found to be toxic when 30 ml. of the 3% solution was injected into each of three rabbits.

C. Third Experiment

More albumin was prepared as before, but the solution, instead of being stored after sterilization, was lyophilized. A sterile 3% solution was prepared in isotonic saline.

The test animals were bled 50 ml. each from the heart for four consecutive days. At the first bleeding 40 to 50 ml. of 3% albumin was injected into each rabbit immediately after bleeding. After the second bleeding 50 ml. of a suspension of erythrocytes, made up of two volumes of pooled, washed cells in three volumes of albumin solution, was injected, and after the third bleeding 40 ml. of albumin solution was administered. The hematocrit fell rapidly during the depletion experiments and was approximately 20% at the fourth day. It was intended to bleed only four times and inject the first immunizing dose of ovalbumin on the fifth day; however, only one rabbit survived the fourth bleeding and it died three days after the first ovalbumin injection.

Table I

Rabbit No.	Weight in kg. Bleeding 1st	8th	Albumin/Globulin Ratio in Serum* Bleeding 1st	2nd	3rd	4th	5th	6th	7th	8th
200	2.2	2.0	419/183	387/154	220/165	342/144	209/122	302/129	244/168	247/218
202	2.6	2.3	494/173	512/178	419/166	454/96	334/57	485/103	358/84	407/193
203	2.7	2.6	—	399**/162	430/116	478/108	272/70	485/122	202/129	411/84
205	2.4	2.1	476/132	426/94	403/139	354/129	147/96	298/130	195/151	258/141
207	2.8	2.6	—	—	**	446/56	379/64	383/144	399/36	391/71

* Numerator is µg. of albumin per ml. of serum, expressed as serum globulin. Denominator is µg. of globulin per ml. of serum.

** First bleeding for this rabbit.

Table II

Titration of Antiovalbumin Sera*

	Rabbit No.	Ovalbumin Dilution 1:5000	1:7500	1:11,250	1:16,875	1:25,313	1:39,970	Saline control
Depleted	202	+	+	+	+	++	−	−
	203	+	+	++	+	+	+	−
	205	−	−	−	−	−	−	−
	207	+	+	+	+	+	+	+
Control	204	−	−	−	−	−	−	−
	206	+	+	+	+	+	+	+
	213	−	−	−	−	−	−	−
	215	+	+	+	+	+	++	+
	216	+	+	+	+	+	++	+
	217	+	+	+	+	+	++	+

*Test was read visually after 20 to 30 minutes at 37°.

Table III

Titration of Antiovalbumin Sera*

	Rabbit No.	Antigen Dilution 1:20,000	1:30,000	1:45,000	1:67,500	1:101,250	1:151,875	1:227,813	Saline control
Depleted	202	400	460	650	max. 870	(1000)			133
	203	212	216	220	246	280	315	(max?)	186
	205	190	204	178	183	180	165		254
	207	246	310	270	325	---	max. 365	356	435
Control	206	202	165	169	244	max. ---	155	236	690
	213	248	345	270	315	---	400	475	115
	215	152	152	max. 160	128	148			166
	216	94	240	max? 320	max? 400	550	600		---
	217	165	280	520	max. 570	---	900	1300	445

* The figures are colorimetric readings representing relative amounts of precipitate. The indicated maxima were estimated visually one half hour after mixing.

C. DISCUSSION

In the first experiment we had hoped to be able to drop the globulin content of the blood of the rabbits employed to a low level during the course of a short series of bleedings, while reinjecting, in so far as possible, the other components of the blood in order to keep disturbing physiological complications to a minimum. Although the majority of the animals survived, Table I shows that no significant increase in the ratio of albumin to globulin can be noted in the bled animals during the course of the experiment, nor can any significant trend be noted in the absolute amounts of globulin in the serum.

Although no decrease in the amount of globulin in circulation had been shown by analysis of the fractionated sera it was felt that, since a large quantity of globulin had been removed in the course of two weeks, it was probable that the rabbits would be synthesizing globulins at an increased rate. Therefore we decided to proceed with the immunization in the hope of observing some significant difference between the response of the bled animals and the controls. Tables II and III show the results of experiments on the immune sera so obtained. On the basis of this evidence no conclusions concerning differences in the antibody titers of the two sets of rabbits can be drawn.

Due to this failure an attempt was made to use a much stronger program of depletion. It proved to be too strenuous for the animals to survive, so no tests could be run on immunization. In all probability it was the lack of a sufficient supply of red cells, combined with the general severe treat-

ment which caused the death of the rabbits.

At this point the experiments were dropped. In suggesting future paths which this research should take it appears to the authors that the whole problem of the depletion technique should be investigated more thoroughly and carefully. It will be impossible to draw any conclusions from the results of immunization until there is at hand a group of rabbits which has been depleted so thoroughly that a definite and easily demonstrable drop in the globulin content of the serum is obtained. This will probably require a very strenuous program of bleeding and reinjection, with special emphasis on the maintainance of the hematocrit at a point sufficient to sustain life.

Acknowledgement

This investigation was carried out in collaboration with Mr. A. E. Senear. The authors wish to thank Professor Dan H. Campbell, who suggested the problem and directed the work, Mr. George Feigen, who performed the heart punctures and gave many helpful suggestions, and Mary L. Sease, who assisted with the experimental work.

References

(1). L. Pauling, J. Am. Chem. Soc. 62, 2643 (1940).

(2). P. R. Cannon, W. E. Chase, and R. W. Wissler, J. Immun. 47, 133 (1943).

(3). D. Pressman, Ind. Eng. Chem., Anal. Ed. 15, 357 (1943).

III. Two copies of this section have been submitted to Professor Niemann.

SUMMARY

I. Lutein, a dihydroxy-α-carotene, is partially converted into stereoisomers by the action of heat; the transformations occur not only on the fusion of lutein crystals at 200° and in refluxing benzene solution, but also in mixed melts with naphthalene, a new method introduced to bridge the gap between 80° and 200°. Traces of compounds containing but a single oxygen atom are also formed under these conditions.

Greatly increased amounts of monoxy compounds, in yield sufficient for preparative purposes, can be obtained by adding to the naphthalene solution of lutein either orthoboric acid, tetraboric acid, or boric oxide, reagents which have not been used heretofore in the carotenoid field.

Three of the new monoxy conversion products have been crystallized. They have been tentatively termed "desoxyluteins I, II, and III". Their main characteristics are described and some structural features are discussed. Desoxylutein I is particularly interesting, since its spectral extinction band shows no fine structure.

When traces of iodine are added to lutein solutions at 80-150°, lutein stereoisomers and the previously observed monoxy derivatives are obtained; in addition, small quantities of compounds containing no hydroxyl groups are formed.

II. An attempt was made to determine the effect of globulin depletion on antibody production in rabbits. From one-sixth to one-third of the circulating blood volume was removed by heart puncture at 24 or 48 hour intervals; the erythrocytes were separated from the plasma, suspended in a volume of isotonic homologous serum albumin equal to that of the discarded plasma, and the suspension was reinjected. At the end of the depletion period the rabbits were immunized with hen ovalbumin and the titers of the antisera were compared with those of a normal, control group, similarly immunized.

Satisfactory conditions for the depletion of the animals were not found in these preliminary experiments. The depletion protocol proved either too mild, in which case no discernable difference in titer between the depleted and control groups could be noted, or too severe, in which case the rabbits did not survive.

III.

PROPOSITIONS

I. The methods developed by Kuhn and Winterstein for the synthesis of the diphenyl polyenes may be extended to the synthesis of polyene chains containing methyl substituents by using derivatives of crocetin or norbixin as starting materials; such an extension offers promising possibilities for the synthesis of certain naturally occurring carotenoids.

R. Kuhn and A. Winterstein, Helv. Chim. Acta 11, 87, 116, 123, 144, (1928)

II. The shift (approximately 11 mu.) of the absorption maxima of carotenoids toward shorter wavelengths when an acyclic end-group is cyclicized to a β-ionone ring is due principally to a steric interaction.

L. Pauling, Fortsch. Chem. organ. Naturstoffe 3, 203 (1939)

III. Pauling (1) has classified the double bonds of a conjugated polyene chain containing methyl substituents as "stereochemically effective" and "stereochemically ineffective", the latter being prevented from assuming the cis form by steric factors. These considerations may be tested experimentally by a study of the cis-trans isomerization of various synthetic polyenes which posess methyl groups attached to the carbon atoms of the chromophore (2).

1. L. Pauling, Fortsch. Chem. organ. Naturstoffe 3, 203 (1939)
2. R. Kuhn and M. Hoffer, Ber. 65, 656 (1932)
F. G. Fischer and K. Hultzsch, Ber. 68, 1726 (1935)
F. G. Fischer and H. Schulze, Ber. 75, 1467 (1942)

IV. The development of chromatograms of some colorless, non-fluorescing substances may be observed visually on fluorescing columns prepared from

an adsorbent containing small quantities of an inorganic fluorescent material, such as zinc sulfide.

V. The failure of attempts to resolve a racemic acid (or base) by converting to a pair of diastereoisomeric salts and chromatographing on an optically inactive adsorbent (1) should have been foreseen. Resolution on an optically inactive adsorbent may be achieved only in the case of diastereoisomeric molecules (2).

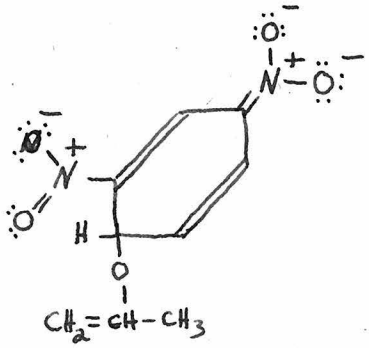
1. H. B. Hass, T. de Vries and H. H. Jaffé, J. Am. Chem. Soc. 65, 1486 (1943)
2. M. M. Jamieson and E. E. Turner, J. Chem. Soc. 1942, 611

VI. Amperometric titrations involving certain metal ions may be carried out using a cathode of the metal being titrated.

E. Salomon, Z. Electrochem. 4, 71 (1897)

VII. I propose that a study be made of the rate and extent of antibody formation in animals whose plasma contains a globulin concentration higher than normal; such a concentration can be maintained by the intravenous injection of globulin in isotonic saline.

VIII. The color formed when m-dinitrobenzene reacts in basic ethanol with an aliphatic aldehyde or ketone which contains an α-hydrogen atom is due to the formation of an ion of the type:



(In the case of acetone)

J. V. Janovsky and L. Erb, Ber. 19, 2158 (1886)
J. V. Janovsky, Ber. 24, 971 (1891)
B. v. Bitto, Liebigs Ann. 269, 377 (1892)
F. Reitzenstein and G. Stamm, J. prak. Chim. 81, 167 (1910)
J. Meisenheimer, Liebigs Ann. 323, 224 (1902)

IX. A thorough knowledge of First Aid for all types of chemical accidents should be required of each graduate student before he is permitted to begin research work.

Lange, Handbook of Chemistry, 5th Ed., Handbook Publishers, Inc., Sandusky, O. (1944)
Handbook of Chemistry and Physics, 28th Ed., Chemical Rubber Publishing Co., Cleveland (1944)
The Merck Index, 5th Ed., Merck and Co., Rahway, N. J. (1940)
The Merck Manual, 7th Ed., Merck and Co., Rahway, N. J. (1940)
Y. Henderson and H. W. Haggard, Noxious Gases and the Principles of Respiration Influencing Their Action, Reinhold Publishing Corp., New York (1943)
K. Egli and E. Rüst, Die Unfälle beim chemischen Arbeiten, Rascher et Cie., Zurich (1925)

X. This proposition is classified as confidential.