STUDIES ON γ-CAROTENE AND RELATED CAROTENOIDS

I. γ-CAROTENE

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III. GAZANIAXANTHIN

IV. MICRO-DETERMINATIONS OF THE ISOPROPYLIDENE GROUP IN CAROTENOIDS

Thesis by

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It is a pleasure to acknowledge my indebtedness to Prof. L. Zechmeister for his guidance through the course of this research. I am particularly happy to have been able to learn the fundamentals of the chromatographic method under his direction.

Abstract

A series of investigations have been carried out with γ-carotene and related carotenoids.

In "Monkey flowers" (Mimulus longiflorus) a carotenoid occurs which apparently differs only in melting point from γ-carotene described by Kuhn and Brockmann[7]. The available evidence indicates that this compound is indeed γ-carotene but the cause of the melting point difference remains anomalous.

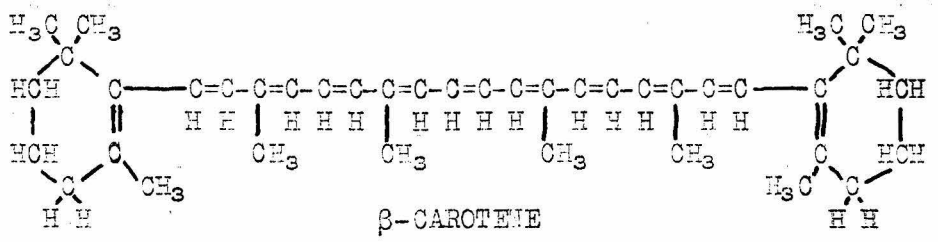
A new carotenoid, the second of its type, possessing four to five cis double bonds in the molecule has been isolated from the fruit of Butia capitata and Pyracantha angustifolia and its properties described. The compound, a hydrocarbon, has been termed pro-γ-carotene because of its relation to γ-carotene. Its most remarkable property is the almost instantaneous change in the spectrum upon treatment with iodine. The change is caused by the formation of a mixture of stereoisomers of the carotenoid, in which the all trans form predominates but in which the pro-carotenoid is absent. The stereoisomerization of pro-γ-carotene under the influence of several catalysts and experimental conditions has been studied chromatographically.

Experiments with gazaniaxanthin, discovered by Schon[16] in the flowers of Gazania rigens, have indicated that this carotenoid is dihydrorubixanthin. The stereoisomerization of the compound has been studied but the chromatographic separation of the stereoisomers is extremely difficult. The spectral curve of the substance exhibits the "cis peak" phenomenon[25] on isomerization.

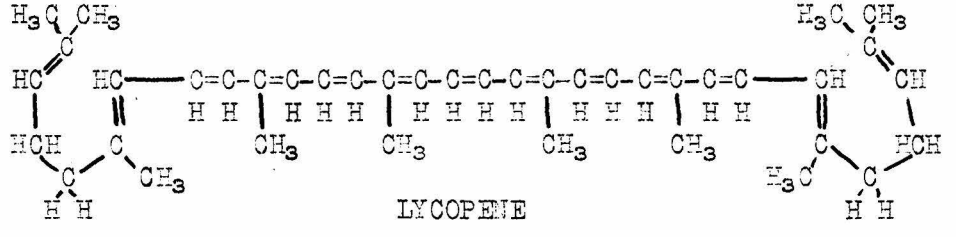
Isopropylidene determinations by ozonolysis have been carried out with a number of carotenoids. Gazaniaxanthin, which probably possesses an isopropyl group, behaves as if about 0.9 "isopropylidene" group were present while β-carotene seemingly possesses 0.3 "isopropylidene". These results have been correlated with the fact that the method is known to indicate the presence of some "isopropylidene" when an isopropyl group is present.

INTRODUCTION

The complete determination of the structures of β-carotene and lycopene[30] demonstrated that these carotenoids could be expressed by the following formulas:

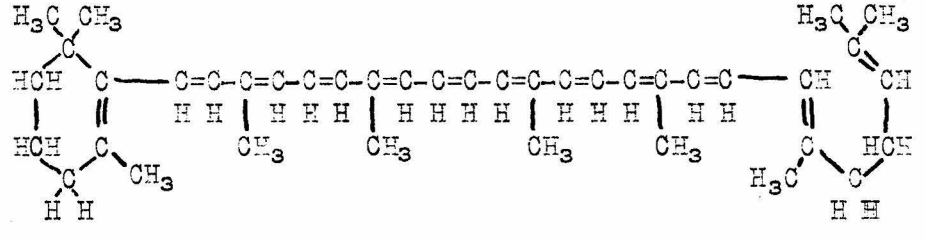


β-CAROTENE



LYCOPENE

Since β-carotene possesses two cyclic ends while lycopene is entirely acyclic, the occurrence of a carotenoid intermediate in structure, as expressed by this formula, was to be expected:



The first evidence that such a carotenoid does occur was found by Winterstein and Ehrenberg[21] in the fruit of the lily of the valley (Convallaria majalis) from which these workers were able to isolate only a few milligrams of crystals. The positions of the absorption maxima of these crystals corresponded to those expected for a substance intermediate

in structure between β-carotene and lycopene. Kuhn and Brockmann[7] then isolated this carotenoid, termed γ-carotene, in quantities of 0.1% from commercial carotene. The provitamin A activity, the presence of twelve double bonds and of one isopropylidene group, and the empirical formula, $C_{40}H_{56}$, were sufficient to prove that γ-carotene possessed one cyclic and one acyclic end. The position of the methyl side chains was postulated by analogy. Several authors[1,3,5,18,19,20] have isolated γ-carotene in small quantities from various sources but with the possible exception of marsh dodder investigated by Mackinney[12], no convenient source of γ-carotene was available.

The observation that chromatograms of extracts of orange "Monkey flowers" (Mimulus longiflorus) showed appreciable quantities of what seemingly was γ-carotene induced an investigation of these flowers. From this source, it was, indeed, possible to isolate a crystalline compound which possessed correct analytical data, spectra and adsorption behavior but the melting point of which differed markedly from that of γ-carotene. Since this carotenoid was obtained from flowers, while all previous isolations had been from other plant organs, there existed the possibility that the variation might arise from this source. Consequently, a considerable number of plant materials were investigated chromatographically in order to find possible sources of γ-carotene. Crystals were isolated from several sources but, as described in more detail in Section I, it was never possible to approach the highest recorded melting point of γ-carotene more closely than 28°.

While the search for sources of γ-carotene was in progress, prolycopene, $C_{40}H_{56}$, was detected and isolated[24,10]. All double bonds in lycopene probably possess an all-trans configuration, but according to Pauling[13],

seven of the thirteen double bonds may assume the cis-position. In prolycopene, it is postulated that five to six of these double bonds have changed to that configuration. This was the first instance in which a C_{40}-carotenoid with cis double bonds had been found to occur naturally. Prolycopene possessed a characteristic which set it apart from all known carotenoids. The visually observed spectral bands of the pure compound are very indistinct, but upon addition of a trace of iodine change with startling rapidity. A new band is formed at a higher wave length while the two original bands shift slightly and become distinct. This new spectrum approximates that of lycopene but is actually that of a complicated mixture of lycopene stereoisomers in which lycopene predominates but from which prolycopene has disappeared. Because this test was easily carried out in the spectroscope, all compounds separated chromatographically in the search for a new source of γ-carotene were thus examined.

Pro-γ-carotene[26] was found to occur in the fruit of the palm trees, Butia capitata and B. eriospatha. The quantities of pro-γ-carotene in these plants are so small that it was possible to isolate only 0.3 mg. per kilo, of fresh fruit. Further investigation disclosed that the berries of Pyracantha angustifolia[27] are a much better source of this pro-carotenoid. The properties of pro-γ-carotene were studied to the extent that the limited quantities available would permit.

Hydroxy derivatives of carotenoid hydrocarbons are not restricted to the more commonly occurring members of the series. γ-Carotene also forms monohydroxy compounds. Kuhn and Grundmann[8] isolated the first monohydroxy-γ-carotene, rubixanthin, the structure of which was elucidated save for the position of the hydroxyl on the ring. A second hydroxy-carotenoid related to γ-carotene, gazaniaxanthin, was isolated by Schön[16] who demonstrated the presence of a hydroxyl group and approximated the empirical formula. The spectrum clearly indicated a

γ-carotene chromophore. Because the plant material for the isolation of gazaniaxanthin was available, this carotenoid was investigated in order to elucidate further its structure as well as to study its stereoisomerization.

A considerable quantity of gazaniaxanthin had been isolated, some experiments had been conducted toward the determination of its structure and the preliminary study of the stereoisomerization had been completed when the author of this Thesis became engaged in other research. Therefore, it was necessary to leave the thorough investigation of this compound in an incomplete state.

A study of the stereoisomerization of γ-carotene had been intended but no work had been started upon this topic.

EXPERIMENTAL INTRODUCTION

In the experimental work described in this Thesis, certain materials, instruments and procedures were used many times. A repeated description of these is unnecessary. In this section, therefore, materials and instruments will be described and terms denoting the procedures defined. When the terms are used in later sections, they will have this definition unless otherwise indicated.

Materials and Instruments.--The greater portion of the chromatography was carried out on calcium hydroxide. The calcium hydroxide used is known commercially as Shell Brand lime, chemical hydrate, (98% through 325 mesh).

The most frequently used solvent was petroleum ether. This hydrocarbon mixture had a boiling range of 60-70°.

The visual spectra were obtained by means of an Evaluating Grating Spectroscope (Zeiss) using light filter BG-7 (Zeiss). All data refer to petroleum ether unless otherwise indicated.

The values of the spectral absorption curves were determined with a Beckman quartz photoelectric spectrophotometer at intervals of one mμ. near maxima and minima and five mμ. elsewhere. The solvent was Eastman "Practical" Hexane which had been treated with sulfuric acid and distilled.

Quantitative determination of carotenoids in solution was made with a Pulfrich Gradation Photometer. Light filter S 47 was employed with petroleum ether as the solvent.

Rotations were measured with a Schmidt and Haensch polarimeter. The reading errors were $\pm$ 0.01-0.02°.

All melting points are corrected. The melting point apparatus was an electrically heated Berl block, the temperature rise of which was controlled to 1.5-2.5° per minute. Melting points were taken in sealed capillaries filled with carbon dioxide. The capillary was introduced into the block 20° below the melting point.

Terms.--"Saponification" was carried out by adding 30% methanolic potassium hydroxide to the carotenoid solution in ether or petroleum ether. Because two liquid phases were present in most instances, the time of saponification was usually 16-20 hours. After saponification, the alkali and methanol were washed from the water-insoluble phase and the latter dried with sodium sulfate. If ether was the solvent, it was necessary to evaporate to dryness and then dissolve the residue in petroleum ether before chromatography, whereas petroleum ether solutions could be chromatographed immediately.

Solutions were "evaporated" in all glass apparatus under reduced pressure. Carbon dioxide was passed through the capillary into the system during the distillation and the water bath surrounding the distilling flask was maintained at 40°.

"Elution" was preceded by extrusion of the column from the chromatographic tube. After the separation of the layers by cutting, the elution itself was usually carried out with alcohol.

Before "rechromatographing" it was necessary to elute the zone and transfer to a suitable solvent, usually petroleum ether. After the solute had been transferred by the addition of water, the solvent washed free of alcohol, and dried with sodium sulfate, the zone was ready to rechromatograph.

When chromatograms are described, the figures to the left indicate the width of the zones in millimeters.

When acetone was used in the development of the column, the term "% acetone" denotes the percentage by volume of this solvent in ligroin.

After chromatography in a percolator, the chromatogram was removed by inverting and tapping on the glass.

I. γ-CAROTENE

I. γ-CAROTENE[28,17]

Introduction and Discussion

When chromatograms of extracts of orange "Monkey flowers" (Mimulus longiflorus, Grant, Scrophulariaceae) are examined, a substantial pink zone is present which possesses an absorption spectrum essentially identical with that of γ-carotene, $C_{40}H_{56}$. This carotene was detected by Kuhn and Brockmann[7] in commercial carotene where it occurs in quantities of about 0.1%. Neither this occurrence nor any natural source reported up to the present time[1,3,5,18,19,20], with the exception of the marsh dodder investigated by Mackinney[12], constitutes an easily available starting material for γ-carotene.

When the compound from Monkey flowers was obtained in crystalline form, all easily determined properties, save one, indicated that it was γ-carotene. However, the melting point, 133°, was markedly different from that reported for γ-carotene, 178°. This melting point was observed (within a degree or two) for all once recrystallized samples obtained in the various isolations. The analytical data, chromatograms of crystals, and microscopic investigation pointed to purity.

The many attempts to raise the melting point of the compound to 178° are recorded in Table II. Simple recrystallization from different solvents, exhaustive drying, evacuated capillaries during the melting point determination (as used by Kuhn and Brockmann), separate crystallization of the top and bottom halves of a zone, and treatment with iodine followed by chromatography and crystallization of the main zone were unavailing. In no case was the melting point essentially altered. However, when the

purification procedure prescribed by Kuhn and Brockmann was applied, that is, three adsorptions and four crystallizations, crystals were obtained which melted at 150°. This melting point was not changed by recrystallization from other solvents.

These facts made it desirable to obtain γ-carotene from other sources. It was possible to isolate crystals from commercial carotene, Cuscuta californica, Daucus carota and Gazania rigens. A very small quantity of crystals were also isolated after isomerization of pro-γ-carotene (Section II). With the exception of the material from Daucus carota, the crystals melted at approximately the same point as those isolated from Monkey flowers. No separation occurred in a mixed chromatogram between any two samples. When the crystals isolated from commercial carotene were carried through the procedure of Kuhn and Brockmann, the melting point rose to 151°. These data and the melting points of γ-carotene recorded in the literature are summarized in Table I.

Table I

Melting Points of γ-Carotene Samples Isolated from Various Sources

Source	Plant organ	M.p. °cor.	Authors
Commercial carotene (Hoffmann-La Roche, Basel)		178	Kuhn and Brockmann[7]
Commercial carotene (Barnett Labs., Long Beach)		128(151)	(This Thesis)
Rosa rugosa	Fruit	176.5	Willstaedt[18]
Gonocaryum pyriforme	Fruit	172 160-165	Winterstein[19,20]
Convallaria majalis	Fruit	170	Winterstein and Ehrenberg[21]
Cuscuta salina C. subinclusa	Stem	164-165	Mackinney[12]
Cuscuta californica	Stem	131	(This Thesis)
Daucus carota	Root	146	(This Thesis)
Gazania rigens	Petals	131-133	(This Thesis)
Mimulus longiflorus	Flowers	133(150)	(This Thesis)

The cause of the variations is not immediately apparent. Without doubt, Kuhn and Brockmann possessed the substance called γ-carotene, the structure of which is given in the Introduction to this Thesis. Other authors have had little evidence of the structure of the compound they called γ-carotene. One must assume, however, that the former experienced difficulty in attaining the melting point they recorded, because their procedure involved three adsorptions on alumina, followed by four recrystallizations, each preceded by boiling with methanol and washing with petroleum ether. Because this procedure was the only one able to change the melting point of the compound from Monkey flowers, it seems not unreasonable to assume that some alteration has taken place.

The analytical data, the partition behavior, and the chromatographic position on the column of the compound from Monkey flowers prove conclusively that the substance is a hydrocarbon. Because the positions of the visible absorption maxima are identical with those of γ-carotene, it is probable that the difference between the two is slight. The conjugated double bond system must be identical. On the other hand, the isolated double bond might form an isobutylidene instead of an isopropylidene group without altering the visible spectrum for in neither case would it be in conjugation. A micro-isopropylidene determination according to the method of Kuhn and Roth[9] gave very nearly the theoretical quantity of acetone calculated for one isopropylidene group. From this result it may be concluded that the substance possesses such a group.

Further evidence that the compound is indeed γ-carotene may be adduced from the catalytic hydrogenation. This determination indicated the presence of twelve double bonds exactly as Kuhn and Brockmann[7] have found.

The cause of the melting point variation cannot be given with certainty, therefore. If it does not result from a purely crystallographic phenomenon, the presence of small amounts of a compound with very similar adsorption affinity and solubility should be taken into consideration. The latter possibility is strengthened by the fact that the γ-carotene zone of some plant extracts (see Table V) seemed to consist of two components on the Tswett column. This was most frequently observed on the first chromatogram of the crude extract but the heterogeneity usually did not appear upon rechromatographing. It was observed in Mimulus extracts in 1941 but not in 1942. It may be suggested that dihydro-γ-carotene would probably have the properties outlined. The pigment of the Mimulus flowers, which grow wild in Southern California, shows considerable variation in its composition. While one of the materials contained 60 mg. of lycopene, $C_{40}H_{56}$, per kilo of dry flowers and a nearly equal amount of zeaxanthin, $C_{40}H_{56}O_2$, another was free of zeaxanthin but contained cryptoxanthin, $C_{40}H_{56}O$, and an increased quantity of lycopene (95 mg.). The photometrically estimated γ-carotene contents were 45 mg. and 75 mg. per kilo respectively. In the best experiment 45.5 mg. of crystals per kilo were isolated, i.e., about 60% of the γ-carotene content. The total amount available was 280 mg. of crystals.

Flowers, therefore, which develop under natural conditions in the field contain no pro-carotenoids which possess several cis-double bonds and which are discussed in Section II. If, however, Monkey flower stems with buds were placed in water for several days and exposed only to diffuse light in the laboratory at room temperature, the flowers were noticeably different in tint and paler in color than flowers which developed on the

intact plant in the open. Parallel chromatograms of extracts of the two materials established the fact that under these two sets of conditions, the polyene mixtures differed both qualitatively and quantitatively with respect to the components found. The paler flowers contained a greater number of lycopene stereoisomers than the controls. The chromatogram of the paler flowers included considerable quantities of prolycopene, $C_{40}H_{56}$, and pro-γ-carotene, $C_{40}H_{56}$. The spectral maxima of these fractions were 467, 440 mμ. and 461, 431 mμ., respectively. Upon addition of iodine to the solutions, the bands showed the characteristic shift to 500.5, 469.5, 440 mμ. and 494, 461 mμ. Both pro-carotenoids have been identified by mixed chromatograms with samples from other sources.

In the light of the above observation, it is possible that prolycopene and pro-γ-carotene are precursors of lycopene and γ-carotene in the biosynthesis of the Mimulus pigment.

Experimental

The flowers were collected in Southern California during June and dried at 45-50° for 24 hours. (The dry weight was 23-24%.) The material was then kept under carbon dioxide in the dark and worked up within two weeks. A total of about 10 kilos of dry material was available, corresponding to 400,000-500,000 flowers.

Composition of the Pigment.--For the quantitative determination of the individual carotenoids, the procedure previous to chromatography was that described below for large scale experiments. Twenty grams of dried and milled flowers were used. The following chromatogram was obtained on a column (20 x 3 cm.):

11 yellow and red minor top zones
1 colorless interzone
12 red, lycopene (506, 474.5, 446 mμ.)
13 orange, neolycopene (497.5, 465.5, 436.5 mμ.)
3 colorless interzone
20 orange, cryptoxanthin (484.5, 453.5 mμ.)
7 yellow, unidentified (471.5, 442.5 mμ.)
10 colorless interzone
45 pink, γ-carotene (495, 462, 433 mμ.)
27 pale orange, partially neo-γ-carotene
7 colorless interzone
25 to the bottom and filtrate, β-carotene (485, 452.5 mμ.)

The photometrically estimated content of the γ-carotene zone was 75 mg. per kilo of dry flowers and was less than the lycopene content (95 mg.)

(1942). In flowers collected in 1941, however, only 45 mg. of γ-carotene per kilo was found while the lycopene value was 60 mg.

Isolation of γ-Carotene.--3.3 kilos of dried and milled Monkey flowers were extracted with petroleum ether in a large percolator (45 x 20 x 8 cm.). The total volume of the extract was 5 liters, the last portion of which was only faintly colored. These operations required 7 hours. After saponification, the dark wash water did not contain carotenoids. The deep red petroleum ether solution was halved and chromatographed simultaneously in two percolators (35 x 15 x 8 cm.). Each chromatogram was developed with one liter of 2.5% acetone and then with 2 liters of 5% acetone. In the course of the development β-carotene and substances which fluoresced in ultra-violet light were washed into the filtrate. The colored layers which appeared were the same as those indicated above. The irregularly shaped γ-carotene zone was cut out as well as possible and eluted with alcohol. The γ-carotene was rechromatographed on two cylindrical columns (28 x 7 cm.) with 5% acetone. After separating from the minor zones above and below it, the carotenoid was eluted with ether.

The solution was evaporated to dryness. The dark red, glassy residue crystallized on cooling. It was dissolved in the minimum amount of benzene and transferred into a 50 ml. centrifuge tube with a dropper. Upon careful addition of excess methanol while stirring, γ-carotene crystallized out.* After standing for 15 minutes, the crystals were centrifuged, washed with methanol, partially dried with a stream of carbon dioxide, and recrystallized

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*The suspension of crystals should not be cooled because this causes a precipitation of colorless material. If such a complication should occur, the contaminant can be removed from the γ-carotene by centrifuging, adding methanol and warming in a water bath until the methanol boils for several minutes. After rapidly centrifuging, the hot methanol is decanted from the γ-carotene crystals.

from benzene-methanol as above. (Petroleum ether + methanol or carbon disulfide + abs. ethanol are also suitable crystallizing mixtures.) The suspension of γ-carotene was kept in ice water for an hour. After filtering and drying in vacuo, the yield was 150 mg. or 45.5 mg. per kilo of dry flowers. Experiments starting with 1 kilo and 2 kilos yielded 18 mg. and 30 mg. of crystalline γ-carotene per kilo respectively. The combined mother liquors from all three experiments gave 9 mg. The total quantity of γ-carotene available was 280 mg. The crystals were chromatographically homogeneous when a solution was washed to the bottom of a very long tube (47 x 1.3 cm.).

Analytical Data for γ-Carotene.--For analytical purposes the samples were dried in high vacuum at 56° for 45-60 minutes. They were free of ash.

Carbon and hydrogen

Calcd. for $C_{40}H_{56}$:	C, 89.48;	H, 10.52
Found:	C, 89.36;	H, 10.78
	89.91	10.53

Molecular weight in exaltone

Calcd. for $C_{40}H_{56}$:	M. W., 537
Found:	M. W., 492

Isopropylidene*

Calcd:	$(CH_3)_2C=$ groups per mole, 1.0
Found:	$(CH_3)_2C=$ groups per mole, 0.95, 0.95

Number of double bonds

5.943 mg. with 3.0 mg. PtO_2 added 3.26 ml. of hydrogen (22°, 741 mm.)
10.14 mg. with 5.5 mg. PtO_2 added 5.44 ml. of hydrogen (24°, 741 mm.)

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*For complete data of these analyses and control determinations carried out simultaneously, see Table VIII.

Calcd. for $C_{40}H_{56}$: Double bonds, 12.0

Found: Double bonds, 11.9, 11.5

Properties of γ-Carotene.--(a) Melting point. The melting poimt of γ-carotene after various treatments is recorded in Table II.

(b) Crystal form. Macroscopically the crystals appeared almost purple. The microscope showed very small clustered crystals. They tended to be rhombic in shape. Each rhombus was orange brown but regions where two were superimposed appeared intensely orange or reddish orange.

(c) Absorption Maxima in Various Solvents. The absorption maxima of γ-carotene were as follows: in carbon disulfide, 532.5, 494.5, 461 mμ.; in benzene, 509.5, 476, 447 mμ.; and in petroleum ether, 495, 462, 433 mμ. Upon addition of iodine the maxima shifted to 529.5, 491.5, 458 mμ.; 505, 472.5 mμ.; and 491.5, 459 mμ. respectively.

The spectral curve of γ-carotene is shown in Fig. 1. The carotenoid exhibits the "cis peak" phenomenon[25].

(d) Partition Behavior. The compound was epiphasic when partitioned between petroleum ether and 90-95% methanol.

(e) Adsorption Characteristics. γ-Carotene is readily adsorbed by calcium hydroxide and alumina but, as is to be expected, is not retained by calcium carbonate. When developing the carotenoid on calcium hydroxide, it is convenient to employ 5-10% acetone. Ether or alcohol are suitable eluents.

Other Carotenoids in the Flowers of Mimulus.--In one experiment 20 mg. each of zeaxanthin and lycopene per kilo of dry material were isolated in crystalline form. These carotenoids, as well as cryptoxanthin and β-carotene (which were not crystallized) were identified by mixed chromatograms, by spectra and by chromatographic positions. The absorption

spectra were: zeaxanthin, 484.5, 453.5 mμ.; lycopene, 506, 474.5, 446 mμ.; cryptoxanthin, 484.5, 453.5 mμ.; and β-carotene, 485, 452.5 mμ.

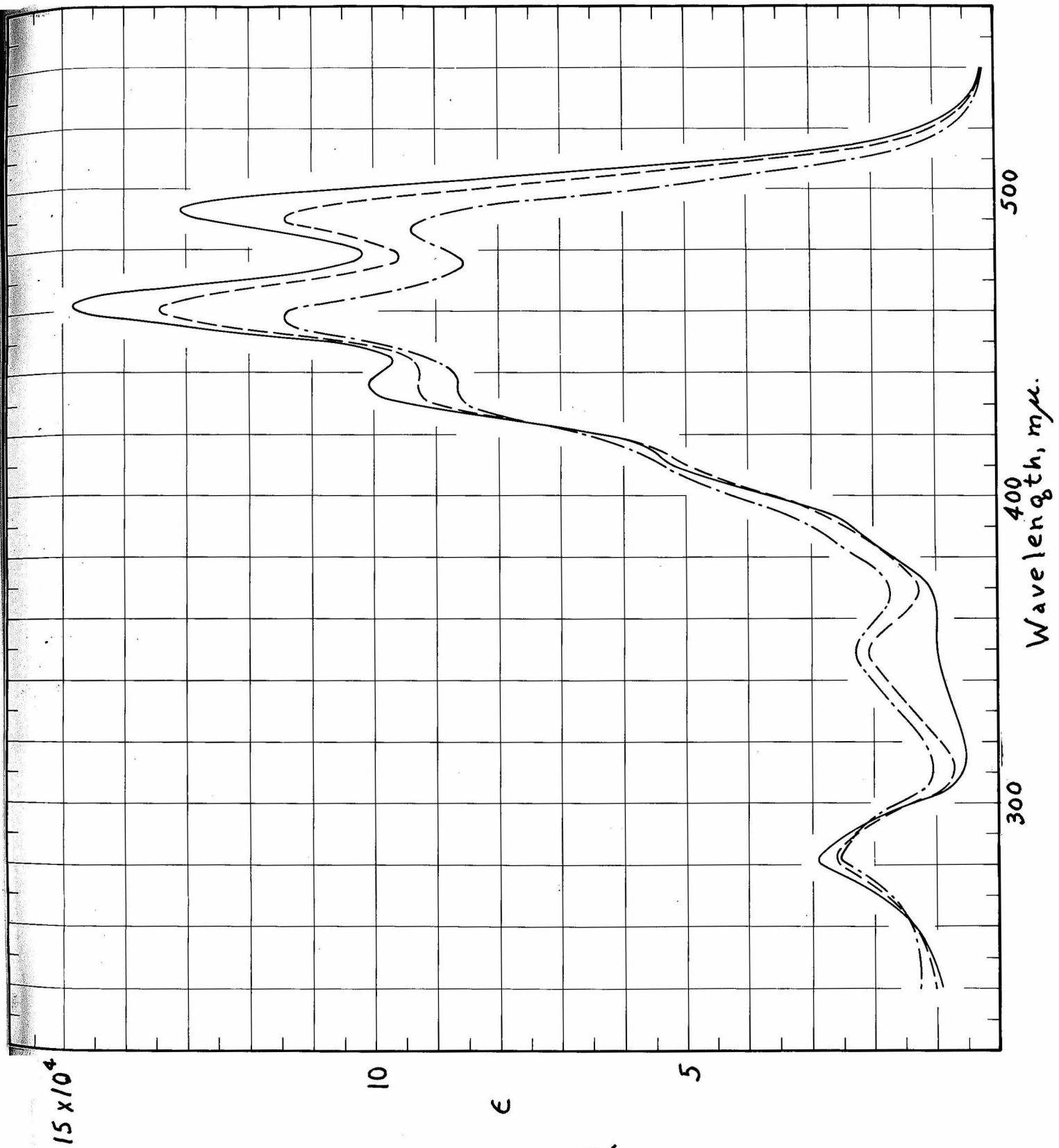


Fig. 1. Molecular extinction curves of γ-carotene in hexane: ___ fresh solution of the all-trans compound; - - - after refluxing; .-.- after iodine catalysis.

Table II

Melting Point of γ-Carotene after Various Treatments

	Treatment	Method of Drying	M.p.[a] (°)
1.	Twice crystallized from petroleum ether-methanol	Vacuum desiccator	130
2.	Same as 1	25°, 0.15 mm., 50 min.	130
3.	Same as 1	50°, 0.04 mm., 45 min.	135
4.	Crystallized from benzene-methanol	No drying	127
5.	Same as 4	Vacuum desiccator	123
6.	Same as 4	50°, 0.05 mm., 15 min.	131
7.	Sample from another isolation	Vacuum desiccator	138.5
8.	Sample from a third isolation	56°, 0.1 mm., 30 min.	132-3
9.	Same as 8 but melting point was taken in an evacuated capillary		133-4
10.	Partial Kuhn and Brockmann procedure	56°, 0.1 mm., 45 min.	144-5
11.	Same as 10 but melting point was taken in an evacuated capillary		144-5
12.	Same as 10 but recrystallized from petroleum ether-methanol	56°, 0.1 mm., 45 min.	145-6
13.	Complete Kuhn and Brockmann procedure	56°, 0.1 mm., 45 min.	149.5-150
14.	Same as 13 but recrystallized from carbon disulfide-ethanol	56°, 0.1 mm., 45 min.	146.5-7
15.	Sample treated with iodine, chromatographed, crystallized	56°, 0.1 mm., 45 min.	131.5-2
16.	Upper half of a zone after crystallizing	56°, 0.1 mm., 45 min.	126-7
17.	Lower half of a zone after crystallizing	56°, 0.1 mm., 45 min.	125.5-6

a) The melting points of crystals from the first six treatments were taken rapidly to see if any major change in melting point had taken place. They were not taken accurately.

Summary of Section I

1. A carotenoid has been isolated from Monkey flowers (Mimulus longiflorus, Grant, Scrophulariaceae) in yields of 45.5 mg. per kilo of dry flowers.

2. This carotenoid is probably γ-carotene, although the melting point, 133°, is much below that reported in the literature, 178°.

3. When grown in the field, Monkey flowers contain no pro-carotenoids but under certain conditions it is possible to bring about their formation in the flowers.

II. PRO-γ-CAROTENE

II. PRO-γ-CAROTENE[26,27]

Introduction and Discussion

A remarkable class of naturally occurring polyenes is composed of those C_{40}-carotenoids which contain several cis-double bonds in their chromophores. Such a configuration manifests itself in a lower melting point, higher solubility and especially by a spectrum in which the bands have been shifted to much shorter wave lengths than those present in the spectrum of the corresponding all-trans-carotenoid. On addition of iodine to solutions of these compounds, the corresponding all-trans isomer is formed in each case together with minor stereoisomers. This becomes evident by an instantaneous shift in the spectra. After catalysis, the first maxima are located at approximately thirty mμ. longer wavelength than those of the original solutions.

These new compounds crystallize well and are then fairly heat resistant in this form. Their solutions, however, undergo isomerization, slowly when heated and very rapidly when treated with iodine or other suitable catalysts[29,23,2] e.g., hydrochloric acid. Melting of crystals also causes isomerization. By these methods a complicated mixture of stereoisomers is formed in which large amounts of the corresponding ordinary carotenoid usually occur. The latter forms the top zone in a chromatogram of the mixture while the unchanged fraction of the starting material forms the lowest zone (or one near the lowest).

It has been suggested[24] that the prefix "pro" be attached to the current name of the all-trans carotenoid in order to designate the compounds belonging to the class under consideration. Such a nomenclature does not point to any essential difference in structure from the so-called "neo"-carotenoids which have been detected by isomerization of the all-trans

compound. The absorption maxima of the neo-isomers are only moderately lower than those of the starting carotenoid, the spectrum of which differs greatly from that of the pro-carotenoid.

A final nomenclature would designate the spatial arrangement of each double bond, e.g., "3,5-cis-γ-carotene." It can, however, be introduced only after a determination of the configuration. Tentative names and prefixes, used in the literature at the present time, will then have to be discarded ("labile, stable, neo, pro," etc.). The simple prefixes cis and trans should be reserved for those cases in which the indicated configuration is valid for all conjugated double bonds or for all those which in principle can assume that configuration.

At present the most important difference between pro-carotenoids and neo-carotenoids lies in the fact that it has not yet been possible to obtain the former from ordinary carotenoids, which, when kept at room temperature, heated, or catalyzed, yield considerable amounts of their neo-forms.

The first representative of the pro-series, prolycopene, $C_{40}H_{56}$, was observed in Tangerine tomato (Lycopersicum esculentum) extracts[24] and later crystallized.[10]

In this Section a description is given of the isolation and properties of pro-γ-carotene, $C_{40}H_{56}$. The following absorption maxima (in petroleum ether) characterize the compounds in question.

Lycopene	504.5,	473.5,	445.5 mμ.
Neo-lycopene A	500.5,	470,	441
Prolycopene		470.5,	445
γ-Carotene	495	463	mμ.
Neo-γ-carotene A	489.5,	458.5	
Pro-γ-carotene		464	(435)

Pro-γ-carotene occurs in the ripe fruit of the palm trees, Butia capitata and B. eriospatha Becc. In these plants a complicated polyene mixture is present which includes γ-carotene, lycopene, β-carotene and sometimes prolycopenes. The pro-γ-carotene content of the material used for the isolation is 40 to 50 times less than the prolycopene content of the Tangerine tomato. Approximately 0.3 mg. of pure crystals can be isolated per kilo of B. capitata fruit.

In order to find a practical source of pro-γ-carotene, a great variety of plant material was tested for "pro"-carotenoids by means of extraction, chromatography, and iodine catalysis.

As a result of these analyses, it has been found that the ripe fruit of Pyracantha (Cotoneaster) angustifolia Schneid. (Pomoideae) constitutes the only practical source of pro-γ-carotene at the present time; 27.7 mg. were obtained in crystalline form from 1 kilo of air-dried berries (about 3 kilos of fresh material). The same quantity also yielded 28.4 mg. of prolycopene. Furthermore, a second member of the stereoisomeric series, lycopene-prolycopene, was isolated (7.3 mg. of crystals) but it may not be a natural product.

Because pro-γ-carotene and prolycopene are hydrocarbons, the question arises whether the occurrence of "pro" compounds in the vegetable kingdom is restricted to this type. A minor constituent in the Pyracantha berries gave information on this point. Since its spectrum is identical with that of pro-γ-carotene before and after the addition of iodine, both must possess a similar chromophore. On the other hand, the behavior of the compound in the partition test and especially its increased adsorption affinity as compared with pro-γ-carotene (and even γ-carotene) prove the presence of a hydroxyl group. Because of the small quantities available, this

monohydroxy-pro-γ-carotene has not yet been prepared in crystalline form. It should be noted that all "pro" compounds known at the present time possess at least one aliphatic end-group in their molecules.

The isomerization of pro-γ-carotene caused by melting the crystals, by heating in solution and by iodine or hydrochloric acid catalysis is described in the experimental part. Each minor layer (except some, formed by hydrochloric acid catalysis) of the chromatogram of such an isomerization mixture gives a similar mixture of stereoisomers when cut out, eluted and catalyzed.

Of the 64 theoretically possible stereoisomers[10,13] of γ-carotene, 10 have been observed in this investigation. Of a total of eleven conjugated double bonds six may assume the cis-configuration. In pro-γ-carotene, however, one or possibly two such double bonds remain in the trans-configuration. This is confirmed by the formation of small amounts of a stereoisomer, the absorption maxima of which are of 4-6 mμ. shorter wave length than those of pro-γ-carotene. It seems, therefore, that the conjugated double bond system of the latter contains 6 or 7 trans- and 5 or 4 cis-double bonds.

Two of the compounds formed by hydrochloric acid catalysis do not belong to the stereochemical series of pro-γ-carotene. These substances have spectra which approximate that of α-carotene, and which decrease the wave-length upon addition of iodine. These facts would indicate that not only has pro-γ-carotene, assumed the all-trans configuration but also that the acyclic end has cyclized.

It was possible to isolate a small quantity of crystals of the all-trans form of pro-γ-carotene from the isomerization experiments with the latter. These crystals melted at 130°. From the results described in Section I, it would seem that these crystals were γ-carotene.

Experimental

Isolation of Pro-γ-carotene

Isolation from the Fruit of Butia capitata.--Twenty kilos of the ripe, bright yellow fruit of Butia capitata (2-3 cm. in diameter; collected in Southern California in November) were mashed in a mortar, freed from the seeds, and kept in methanol overnight. Contact with the solvent longer than one to two days should be avoided; if the material cannot be worked up promptly, the whole fruit may be preserved for some time by placing in large jars containing a 2-3 cm. layer of methanol.

The material was pressed out in a fruit press, the liquid discarded and the wet fibrous cake was passed twice through an electric grinder. One liter of methanol and one liter of petroleum ether were added to four portions, each corresponding to 5 kilos of fresh fruit. These solvents form a two-phase system. The mixture of material and solvents was shaken mechanically for fifteen minutes and permitted to stand some time. The upper layer was intensely orange; the lower only slightly colored. This extraction was repeated twice, the volume ratio of methanol and petroleum ether added being now 1:10. A final treatment with pure petroleum ether is advantageous. The solid residue was pressed out and passed through an electric grinder between extractions. A total volume of 37.5 liters of petroleum ether and 6.5 liters of methanol was employed to extract the 20 kilos of fresh fruit. A large volume of water was added slowly to the two phase extract. The upper phase of petroleum ether was washed three times, dried quickly with sodium sulfate, and concentrated in vacuo at 40° to about 4 liters. The liquid was diluted with half a volume of ether and saponified. While washing free of alkali, a semi-solid material appeared at the interface and was discarded. After

drying, the solution was concentrated in vacuo to 100 ml. and the evaporation repeated after 200 ml. of petroleum ether had been added. The dark red, viscous liquid was diluted with petroleum ether to one liter.

After filtration to remove colorless material, the solution was chromatographed in a conical percolator (32 x 15 x 8 cm.), in order to obtain a rough separation of the carotenoids. In the course of developing with 5 liters of petroleum ether numerous layers appeared. The upper section consisted of minor zones and a main red lycopene zone accompanied by some of its neoforms. The middle section had a red zone of γ-carotene at the top and a brilliant orange zone of pro-γ-carotene at the bottom. Finally, the lower section contained β-carotene which constitutes about one-half of the polyene content of the fruit. Each of the sections was cut out.

The pro-γ-carotene solution was rechromatographed twice on a smaller column (25 x 4 cm.) which was developed first with petroleum ether and then with 2% acetone. The orange zone (75 mm. wide) was eluted with ether. After evaporation of the solvent, the dry residue was dissolved in the minimum amount (0.5 ml) of petroleum ether in a centrifuge tube. On cautious addition of a few milliliters of methanol, with stirring, red crystals appeared immediately but were mixed with some colorless particles of microscopic size.

The sample was centrifuged and washed with methanol in the centrifuge tube in which it was recrystallized from benzene on addition of methanol. The crystals were treated three times with methanol at 40° and recrystallized from benzene and methanol. These latter operations were repeated. The yield was 5.9 mg. or approximately 0.3 mg. per kilo of fresh fruit. In the mother liquor only small quantities of pro-γ-carotene were present while a new compound (Table V) having a one-banded spectrum (432.5 mμ) was accumulated.

A total of 14 mg. of pro-γ-carotene was isolated from several experiments of this scale.

Isolation from the Fruit of Pyracantha angustifolia.--The Pyracantha berries were picked in November and December in Southern California and dried in air at room temperature. On prolonged standing the yields diminished rapidly. One kilo of the air-dried material was coarsely ground in a mill, kept under ether for 3 hours, then filtered on a Buchner funnel, washed with ether, and treated once more in the same manner. The extract (4.5 liters) was saponified. After saponification, the dark wash water did not contain carotenoids.

The solution of the dark red, viscous residue in 1 liter of petroleum ether was chromatographed in a large percolator (45 x 20 x 8 cm.) on calcium hydroxide. The chromatogram was developed with 5 liters of petroleum ether and then with 1% acetone. The complicated chromatogram was composed of three sections: (a) a strongly adsorbed, poorly differentiated top section (7 cm. wide), (b) a main section (6 cm.) containing several orange and yellow zones including prolycopene and pro-γ-carotene, and (c) the lowest section of the cone, occupied by large amounts of β-carotene preceded and followed by some of its stereoisomers. This last section and the yellow, fluorescing filtrate were discarded. The three sections were separated by cutting.

Fractionation of Main Section--After elution with alcohol the carotenoids were rechromatographed (column 28 x 7 cm.) with 2% acetone.

The chromatogram had the following appearance:

80 several minor layers near top

35 bright orange, prolycopene (470.5, 441 mμ)

5 orange, traces

12 yellow, unidentified carotenoid 1 (464, 438 mμ)

10 several minor layers

50 orange, pro-γ-carotene (462, 432.5 mμ.)

10 yellow, unidentified carotenoid 2 (457.5, 430.5 mμ.)

20 several minor layers

The presence of pro-γ-carotene and of prolycopene was detected by the addition of iodine to the respective solutions in a spectroscopic cell. By this catalysis intense new spectra appeared almost instantaneously (493, 460 mμ. and 501.5, 470 mμ.).

Pro-γ-carotene--This zone was rechromatographed and developed with 2% acetone; the main component was eluted with ether. Upon evaporation of the ether *in vacuo* a dark red, crystalline residue remained. The latter was dissolved in the minimum amount of benzene at 20° and transferred into a 15 ml. centrifuge tube with a dropper. About 10 ml. of methanol were then added with stirring, first drop by drop until red crystals appeared, and later more rapidly. After standing in ice water for 1/2 hour, the crystals were centrifuged and washed with methanol in the same tube. After recrystallization from benzene and methanol, the yield was 25.1 mg. The mother liquor gave 2.6 mg. The total yield corresponds to 45 per cent of the pro-γ-carotene content of *Pyracantha* determined photometrically. A mixed chromatogram with pro-γ-carotene from *Butia capitata* established the identity of the two samples.

Analytical Data for Pro-γ-carotene.--For the purpose of analysis the samples from each source were dried in high vacuum for 45 minutes. The sample from *Butia* (*B.c.*) was dried at room temperature while that from *Pyracantha* was dried at 50°.

Carbon and hydrogen

Calcd. for $C_{40}H_{56}$: C, 89.48; H, 10.52

Found: C, 88.99; H, 10.65 (B.c.)
89.79 10.55 (P.a.)
89.94 10.52 (P.a.)

The values obtained with the sample from Butia are calculated for the ash free substance. The other contained no ash.

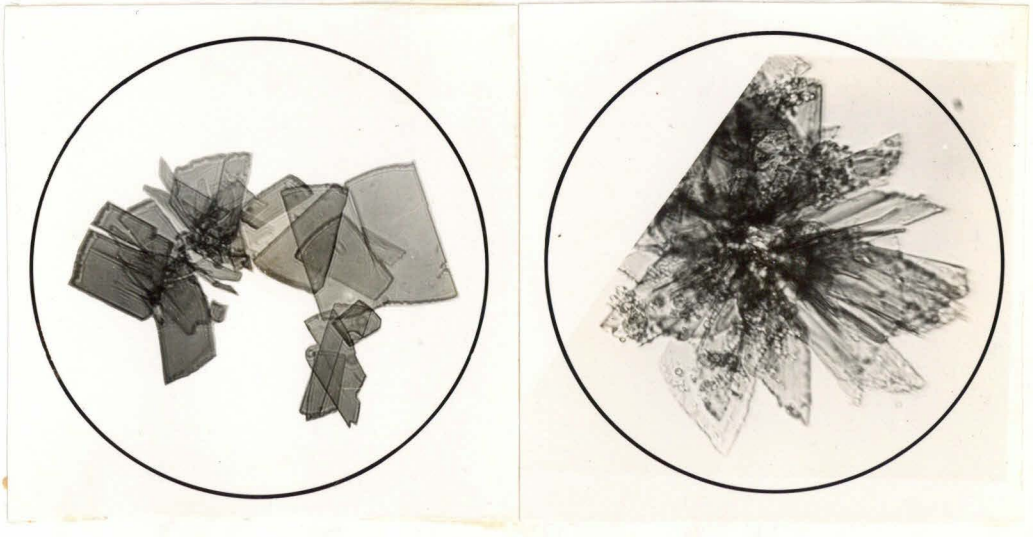
Molecular weight

Calcd. for $C_{40}H_{56}$: M. W., 537

Found: M. W., 515 (B.c.), 558 (P.a.)

The molecular weight determinations were carried out in exaltone (cyclopentadecanone, m.p. 63°) which has a molar freezing point depression constant of 21.3. The samples were altered only slightly during the determination as proved by a chromatogram of a petroleum ether solution of the melt.

Properties of Pro-γ-carotene.--(a). Melting point. Pro-γ-carotene isolated from Butia melted at 118-9° after previous shrinkage; the crystals obtained from Pyracantha melted at 121-2° after softening near 119°. The melting point is lower than that of γ-carotene but higher than that of prolycopene (111°). (b). Crystal form. Macroscopically, pro-γ-carotene forms brick-red, glittering plates. Under the microscope individual crystals are dull brownish-yellow with intensely orange crossings. Curved crystal edges are typical (Fig. 2).



(a) (b)

Fig. 2. -- Pro-γ-carotene, crystallized from benzene and methanol (430 x): (a) slow crystallization; (b) rapid crystallization.

(c). Absorption Maxima in Various Solvents. The absorption maxima of pro-γ-carotene were determined visually in a number of solvents both before and after the addition of iodine. The data obtained with the crystals from Butia are presented in Table III. With the sample obtained from Pyracantha,

Table III

Spectra of Pro-γ-carotene in Various Solvents

($m\mu$.)[a)]

Solvent	Before addition of iodine		After addition of iodine		
Carbon disulfide	493.5	460.5	528	490.5	(433.5)
Acetone	(493.5)	463	493	462	(434)
Pyridine	481	450.5	481.5	452.5	
Benzene	477	477.5	507.5	474.5	(444)
Carbon tetrachloride	475.5	445.5	506	473	443
Chloroform	473	(444)	505	470.5	(441.5)
Dioxane	470	442	500	469.5	(441.5)
Ethanol	(465)	(437)	(491.5)	466	(436.5)
Ethyl acetate	464	(436)	492.5	463.5	(436)
Petroleum ether	464	(435)	493.5	461	(433.5)
Ether	462	(435)	494	461	(433)
Methanol	462	(434)	(490)	460	

a)Parentheses indicate that the band is very indistinct. In some solvents pro-γ-carotene shows a faint shadow at higher wave lengths. These solvents with the approximate position of the shadow are: carbon disulfide, 527; chloroform, 502; dioxane, 499; petroleum ether, 492; ethyl acetate, 493; ether, 491; and methanol, 491 $m\mu$.

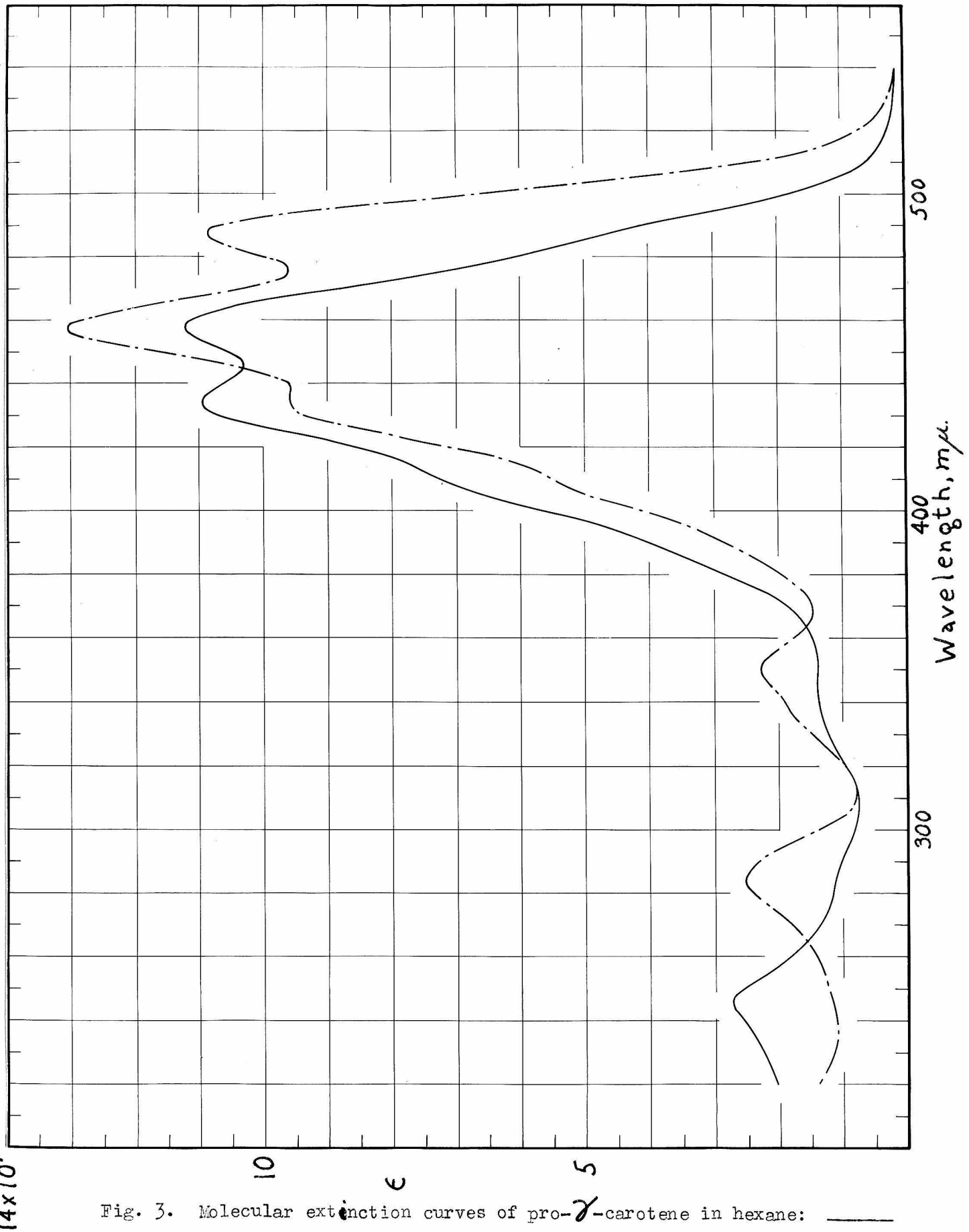


Fig. 3. Molecular extinction curves of pro-γ-carotene in hexane: ——— fresh solution; .-.- after iodine catalysis.

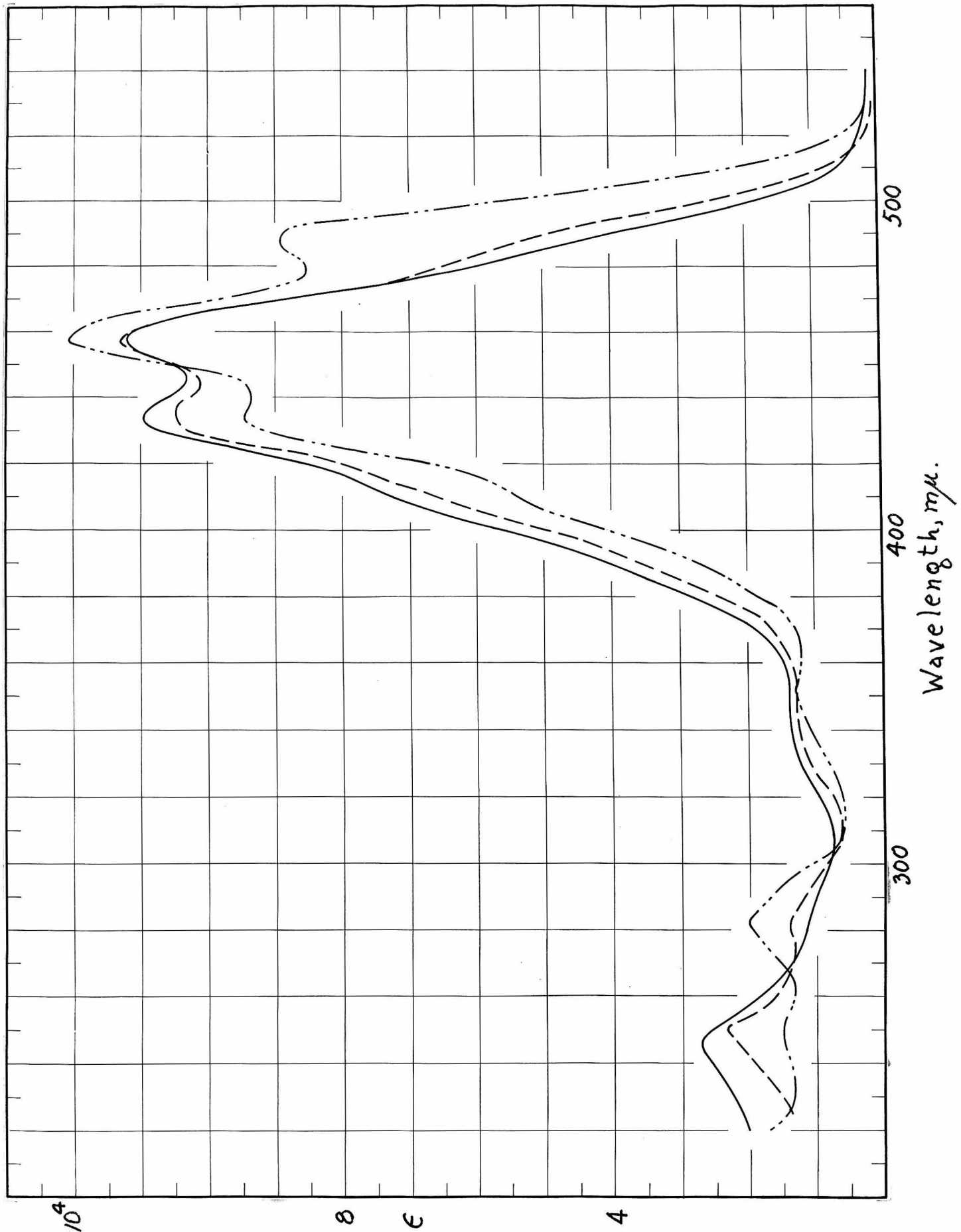


Fig. 4. Effect of solar radiation on pro-γ-carotene: _____ fresh solution; - - - 5 min. irradiation; ..-..- 30 min. irradiation.

(f). Photometric Determination. The concentration of pro-γ-carotene in solution may be determined by means of the Pulfrich Gradation Photometer using light filter S 47 (k = extinction coefficient; c = mg. substance per 100 ml. of petroleum ether solution)

k	0.3	0.5	0.7	0.9
c	0.20	0.33	0.47	0.61

Stereoisomerization of Pro-γ-carotene under Various Conditions.

(a). Isomerization with Iodine at Room Temperature. 1.4 mg. of pro-γ-carotene crystals in 100 ml. of petroleum ether was treated with a solution of 35 μg. of iodine in 0.3 ml. After standing one and one-half minutes at room temperature, the solution was poured on a column (20 x 3 cm.) and was completely adsorbed five minutes later. When developed with 2% acetone, a chromatogram was obtained in which the layers were separated by colorless interzones (Table IV).

Table IV

Stereoisomers of Pro-γ-carotene Obtained By Iodine Isomerization[a)]

Color of adsorbate	Spectrum (mμ) in petroleum ether: Before addition of iodine			After addition of iodine	
Red (γ-carotene)	495	463	(435)	492.5	460
Orange	489.5	458.5		492	460.5
Orange	489	458		492.5	460.5
Pink	488.5	457.5		492.5	460.5
Orange	488.5	457		492.5	460.5
Orange	489	457		492	460
Orange (pro-γ-carotene)	463	(434		492.5	460.5

[a)] In the order of decreasing adsorption affinity on calcium hydroxide.

(b). Isomerization by Heating in Solution. The petroleum ether solution of about 1 mg. of pro-γ-carotene was refluxed for thirty minutes, and developed on a column with 1% acetone. Most of the starting material remained unchanged while some γ-carotene appeared at the top; two minor zones were observed below pro-γ-carotene, the one immediately below having the spectrum 458.5, (432.5) mμ.; on addition of iodine the spectrum changed to 493, 461 mμ. The lowest zone was present only in traces.

(c). Isomerization by Melting of Crystals. A small quantity of pro-γ-carotene was melted and kept between 130 and 135° for twenty minutes, in a sealed tube filled with carbon dioxide, and then plunged into ice water. The chromatogram of the petroleum ether solution, after developing with 3% acetone, consisted of seven zones: γ-carotene, three neo-isomers (about 488, 457 mμ.), pro-γ-carotene and finally two pigments below, having absorption maxima at 489.5, 458 mμ.and 457.5 mμ., respectively. The weak adsorbability of the zone immediately below pro-γ-carotene is remarkable; on the basis of its spectrum one would rather expect a position much above pro-γ-carotene. After addition of iodine the spectrum of each stereoisomer was converted within a few seconds into that of the same equilibrium mixture (492.5, 460 mμ).

(d). Isomerization with Conc. Hydrochloric Acid at Room Temperature. One mg. of pro-γ-carotene in 10 ml. of petroleum ether was mechanically shaken with 5 ml. of conc. hydrochloric acid for one hour. After the solution had been washed acid-free and dried, the chromatogram was developed with 2% acetone. Seven zones were formed: an unknown brownish zone fixed at the top, then γ-carotene, and three of its neoforms (spectra as in Expt. c); no unchanged pro-γ-carotene was present. The two lowest yellow

zones migrated much more rapidly than the other zones and showed spectra 477.5, 447.5 and 480, 450 mμ, respectively, which approximate that of α-carotene. On the addition of iodine the wave length of the maxima of the two lowest layers decreased a few millimicrons while each of the four γ-isomers gave the expected equilibrium spectrum (492.5 and 460 mμ.).

In the course of the above isomerizations some crystallized γ-carotene was isolated (494, 462, 434 mμ.). It did not separate in a mixed chromatogram from samples obtained from two different plants. These crystals of γ-carotene, in the same manner as other samples, melted at approximately 130°. So small a quantity (0.5 mg.) was available that no analysis or extended investigation was possible.

Other Carotenoids in Butia capitata.--The remaining sections of the initial chromatogram of the Butia extract were repeatedly chromatographed until the individual carotenoids were separated. A summary is presented in Table V. Very small quantities of crystals were isolated from the γ-carotene zones but in these cases also, the meager quantities of crystals made investigation impossible.

Other Carotenoids in Pyracantha angustifolia.--The carotenoids in the main section of the initial chromatogram of the Pyracantha extract were also investigated.

Prolycopene--This layer was cut out and rechromatographed. By developing with 5% acetone, minor layers were separated. The main zone was then eluted and isolated as described for pro-γ-carotene but a recrystallization was not carried out. The yield was 28.4 mg.; i.e., about 40 per cent of the quantity contained in the original extract as estimated photometrically. M.p., 110.5-111.5°. The shape of the crystals and their solubility corresponded

Table V

Carotenoids Present in the Ripe Fruit of *Butia capitata*[a]

Compound[b]	Spectrum (mμ.) in petroleum ether[c] Before addition of iodine			After addition of iodine		
Rubixanthin	496.5	464.5	(437.5)	494	461.5	
Lycopene	504.5	474	(445.5)	502	472	442
Neo-lycopene[d]	500.5	469	(440)	502	471	
Neo-lycopene[d]	500	469	(441)	502	471	(442)
A prolycopene		477	448	502.5	471	(443)
Unknown		472	444		469	442
γ-Carotene	497	465.5	(437)	495.5	463.5	
γ-Carotene	495	463		495.5	461.5	
A *prolycopene*		471.5	443.5	499.5	469.5	441.5
A *prolycopene*		466.5	439	500.5	469.5	422.5
A *prolycopene*	490.5	464	(437.5)	501	469	(440)
Neo-*γ*-carotene[d]	490	459.5	430	497	465.5	(436)
Pro-*γ*carotene		464	(435)	493.5	461	
Unknown			432.5			432.5
A *β-carotene isomer*	482	451.5		484.5	454	
β-Carotene	485.5	454		484.5	453.5	

[a] The compounds are listed in the order of decreasing adsorption affinity on calcium hydroxide. [b] Compounds italicized have been definitely identified, in most cases by mixed chromatogram. Other compounds have been tentatively identified by spectrum and column position. [c] Parentheses denote indistinct bands which are difficult to read. [d] The complete homogeneity of this zone is questionable.

with those of a sample from tangerine tomatoes. A mixed chromatogram with prolycopene from the latter source showed no separation. In the partition test (petroleum ether-95 per cent methanol) epiphasic behavior was observed. For the purpose of analysis the sample was dried in a high vacuum at room temperature for 45 minutes.

Analysis--for $C_{40}H_{56}$

Calcd. C 89.48, H 10.52

Found. 89.39, 10.63 (corrected for 0.7% ash)

Mol. wt. (in exaltone), calculated, 537; found, 575

The absorption maxima in carbon disulfide were 500, 468 mμ.(with iodine, 542.5, 502.5, 468.5 mμ.); in benzene, 482.5, 453 mμ.(with iodine, 518.5, 483, 452.5 mμ.); and in petroleum ether, 470.5, 441 mμ.(with iodine, 501.5, 469.5, 439.5 mμ.).

Minor Carotenoids--The unidentified carotenoid 1, after having been rechromatographed, showed absorption maxima at 464.5, 435.5 mμ.which were shifted on iodine catalysis to 469, 439 mμ. This equilibrium mixture when chromatographed and developed with 5% acetone separated into five layers; the spectrum of the main zone, adsorbed near the top, was 471, 442 mμ.

The unidentified carotenoid 2, after having been rechromatographed and treated with iodine, separated upon chromatographing into two isomers (458, 431 mμ.and 454, 426 mμ.respectively, from the top of the column) which gave identical spectra (457, 429 mμ.) on treatment with iodine.

One of the most interesting zones obtained from the Top Section of the initial Pyracantha chromatogram was that which possessed all the characteristics of a monohydroxy-pro-γ-carotene. The separation of this zone and investigation of the other carotenoids was carried out as follows.

Fractionation of the Top Section.--The carotenoids were eluted with alcohol, and rechromatographed on a smaller percolator (30 x 11 x 6 cm.) with 5% acetone. Five main fractions (Fractions I to V from top to bottom) appeared, each consisting of several components.

Fraction I (which among others contained some lutein) and Fraction III were of no particular interest.

Fraction II, when rechromatographed and developed with 10% acetone, separated into nine components; viz., lycopene, two neolycopenes, a monohydroxy-pro-γ-carotene, and five minor layers.

The monohydroxy-pro-γ-carotene zone was rechromatographed and was then homogeneous. The main absorption maxima were 461.5, 432.5 mμ. and on iodine catalysis, 492.5, 459 mμ. In contrast to pro-γ-carotene this compound was present in both phases if a drop of water was added to the solution in a petroleum ether-methanol mixture. When a petroleum ether solution was shaken with 90 per cent methanol, epiphasic behavior was observed. The adsorbability is also conclusive evidence for the presence of a hydroxyl group. On calcium hydroxide the compound is adsorbed below lycopene but much above pro-γ-carotene, as shown by mixed chromatograms. Furthermore, after the addition of iodine a main component is formed which does not separate on the column from a monohydroxy-γ-carotene (probably rubixanthin) obtained from another source. Finally, monohydroxy pro-γ-carotene is also adsorbed on calcium carbonate from petroleum ether, which, as is well known, does not occur with hydrocarbon carotenoids.

Fraction IV consisted mainly of an orange layer which showed maxima at 474, 442.5 mμ. (with iodine, 501.5, 469.5, 439 mμ.). In carbon disulfide the corresponding figures were 504, 471.5 mμ. and 543, 502.5, 468 mμ. This stereoisomer of lycopene was eluted with ether and crystallized as described

above for pro-γ-carotene. The yield was 7.3 mg.; m.p., 97-98°. The main stereoisomer formed by iodine catalysis did not separate from tomato lycopene in a mixed chromatogram.

Analysis--Calcd. for $C_{40}H_{56}$. Mol. wt. 537

Found. " " 563 (in exaltone)

Fraction V, when rechromatographed on calcium hydroxide with 25 per cent ligroin in benzene, separated into several minor and two main components, the latter possessing γ-carotene spectra (495, 462 mμ. and 494, 461 mμ.). On addition of iodine, maxima of shorter wave-length appeared.

Summary of Section II

1. A new carotenoid, pro-γ-carotene, has been isolated in quantities of 0.3 mg. per kilo of fresh fruit from Butia capitata, Becc. (Palmae) and of 27.7 mg. per kilo of dry berries from Pyracantha angustifolia, Schneid. (Pomoideae).

2. It is probable that four or five of the eleven conjugated double bonds in this molecule are in the cis-configuration.

3. Upon treatment of a solution of pro-γ-carotene with iodine, the spectrum changes with amazing rapidity to that of a mixture of stereoisomers which may be separated chromatographically.

4. Melting of crystals, refluxing of solutions and treatment of a cold solution with conc. hydrochloric acid also convert pro-γ-carotene into a mixture of stereoisomers. When hydrochloric acid is used, minor carotenoids are present which are not stereoisomers of pro-γ-carotene.

III. GAZANIAXANTHIN

III. GAZANIAXANTHIN

Introduction and Discussion

In the course of the investigations in the γ-carotene series, it has been desirable to study a monohydroxy carotenoid related to γ-carotene.

The first hydroxy-γ-carotene, rubixanthin, $C_{40}H_{55}OH$, was isolated from rose hips ("Hagebutten", Rosa canina, R. rubiginosa, etc.) by Kuhn and Grundmann[8]. They proved that the hydroxyl group is located on the β-ionone ring while the acyclic end terminates in an isopropylidene group. Because the plant material required for the isolation of rubixanthin was not available, the behavior of the related carotenoid, gazaniaxanthin, has been studied.

This carotenoid is the main component of the polyene pigment of the flowers of Gazania rigens, R. Br. (Compositae) in which it was detected by Schön[16]. The starting material, grown in Southern California, contained the same gazaniaxanthin but the pigment mixture was markedly different from that described by this investigator. The variation is understandable when the difference in geographical location is considered (Schön worked in Portugal). Table VI presents a comparison of the respective pigment mixtures.

Table VI

Main Components of the Carotenoid Pigment in Gazania rigens Flowers[a)]

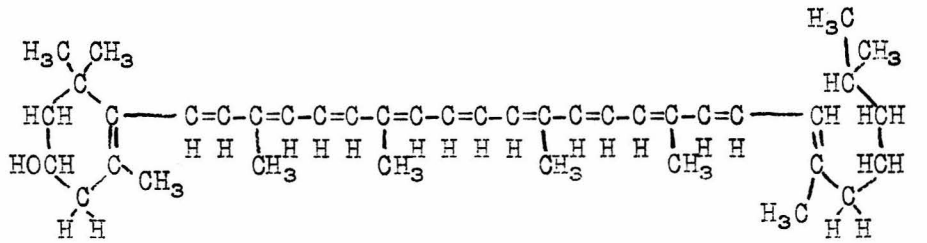
Grown in Portugal	Grown in Southern California
Lutein	Lutein
Rubixanthin	Gazaniaxanthin: 1400 mg./kilo
Gazaniaxanthin	Lycopene: 435 mg.
Unknown[b)]	Cryptoxanthin
γ-Carotene	γ-Carotene: 100 mg.
β-Carotene	β-Carotene: 60 mg.

a) The underlined compounds have been isolated as crystals. The figures refer to the content per kilo of dry flowers. The yields were: 30-45% of the gazaniaxanthin, 30% of the lycopene, and 13% of the γ-carotene content listed.

b) From Schön's description of this fraction it is not unreasonable to assume that this was a mixture of neo-gazaniaxanthins. The spectrum, identical with that of γ-carotene, may have been formed by re-isomerization.

In his paper, Schön not only described the isolation of gazaniaxanthin, but also suggested that it may be a monohydroxy-γ-carotene having the formula $C_{40}H_{54}O$ or $C_{40}H_{56}O$. He demonstrated the presence of a hydroxyl group by the synthesis of a crystalline acetate which has been confirmed by the determination of an active hydrogen according to the method of Zerewitinoff. Schön postulated that the hydroxyl group might be located "in the aliphatic side-chain, like in lycoxanthin and lycophyll"[22] but he rightly pointed out that such an assumption should be tested by a biological assay. Such an assay has now been carried out. Because gazaniaxanthin (like rubixanthin[8]) proved to be inactive as provitamin A in the rat, the hydroxyl group must be located on the β-ionone ring.

Analytical investigations to elucidate further details of the structure have led to contradictory results. A hydroxy-γ-carotene would possess twelve double bonds but catalytic hydrogenation has indicated the presence of only eleven in gazaniaxanthin. These must be conjugated in order to produce the spectrum observed. This result would indicate that the molecule does not contain an isolated double bond and consequently that the empirical formula is $C_{40}H_{58}O$. However, ozonolysis indicated the presence of one isopropylidene group[9]. Since compounds, such as thymol, give 0.3 "isopropylidene" group per mole, it may well be that carotenoids containing an isopropyl end-group would also yield acetone on ozonolysis. Therefore, gazaniaxanthin may be a dihydro-rubixanthin as indicated below in the tentative formula.



Gazaniaxanthin (?)

Fresh, dilute solutions of gazaniaxanthin are relatively stable at room temperature. In 24 hours only 4% of the pigment underwent trans-cis isomerization in petroleum ether, while upon refluxing in benzene for 15 minutes, an equilibrium was reached which contained about 30% neo-isomers. Within the same period of time, iodine (1-2% of the pigment) produced an equilibrium mixture containing 45% neo-compounds. Melting of the crystals also produced considerable quantities of stereoisomers.

From the standpoint of chromatographic technique, gazaniaxanthin and its isomers represent a difficult case. Even with the best absorbent and developer available, it was impossible to obtain colorless interzones between the isomers, all of which have less adsorption affinity than gazaniaxanthin. Although the outer appearance of the chromatogram was, at times, excellent, the differentiation inside the column usually did not justify the expectations. However, the stereoisomers of gazaniaxanthin obtained with iodine formed two distinct groups on the column, termed "neo-group I" and "II" (from top to bottom). The average spectra were nearly identical. Within group I, two stereoisomers, within II, four stereoisomers have been differentiated but for none is perfect homogeneity certain. The chromatograms of equilibrium mixtures obtained by refluxing solutions or melting crystals are similar.

As one proceeds down the column in the three cases, the wavelengths of the spectral maxima of the zones first decrease and then increase. The maxima of the middle zones are of 8-10 mμ. shorter wavelength than those of the all-trans compound while this difference is only 3-7 mμ. for the bottom zones (see Experimental Section).

The differences in the wavelengths of the absorption maxima of the neo-gazaniaxanthins are unusually small. The longest wavelength maxima of all but

one zone were located within the narrow range of 489.5 to 484 mμ. This behavior seems to be inherent in carotenoids related to γ-carotene. Thus, pro-γ-carotene forms five isomers which possess almost identical spectra (Section II of this Thesis) yet which may be separated chromatographically. On the other hand, the spectra of the twelve known neo-isomers of β-carotene[14] are different by 15 mμ. at the extremes and are so situated chromatographically that isomers with almost identical spectra cannot be confused. A similar situation obtains in the case of lycopene[10].

The spectral properties of natural (all-trans) gazaniaxanthin have been investigated from 540 to 230 mμ. in hexane (Fig. 5). If the solution is treated with iodine at room temperature or merely refluxed, the extinction coefficients decrease and the maxima shift toward the shorter wavelengths. Simultaneously, however, the very flat maximum at 349-350 mμ. is greatly increased and converted into a marked maximum. The latter is the so-called "cis-peak" defined by Zechmeister and Polgar[25]. That the equilibria between gazaniaxanthin and its stereoisomers formed by these treatments differ according to the method of isomerization, is evidenced by Fig. 5 as well as by the chromatograms described below.

The trans-cis isomerization of gazaniaxanthin induced by iodine catalysis also becomes manifest by the appearance of rather strong dextrorotation. The optical activity of the natural pigment in benzene or petroleum ether was too small to be observed in concentrations which permitted readings. In contrast, the iodine equilibrium mixture gave $[\alpha]_{Cd}^{25} = +160°$ (in petroleum ether) and a mixture of some neo-isomers, $[\alpha]_{Cd}^{25} = +220°$ which upon addition of iodine decreased to 160°.

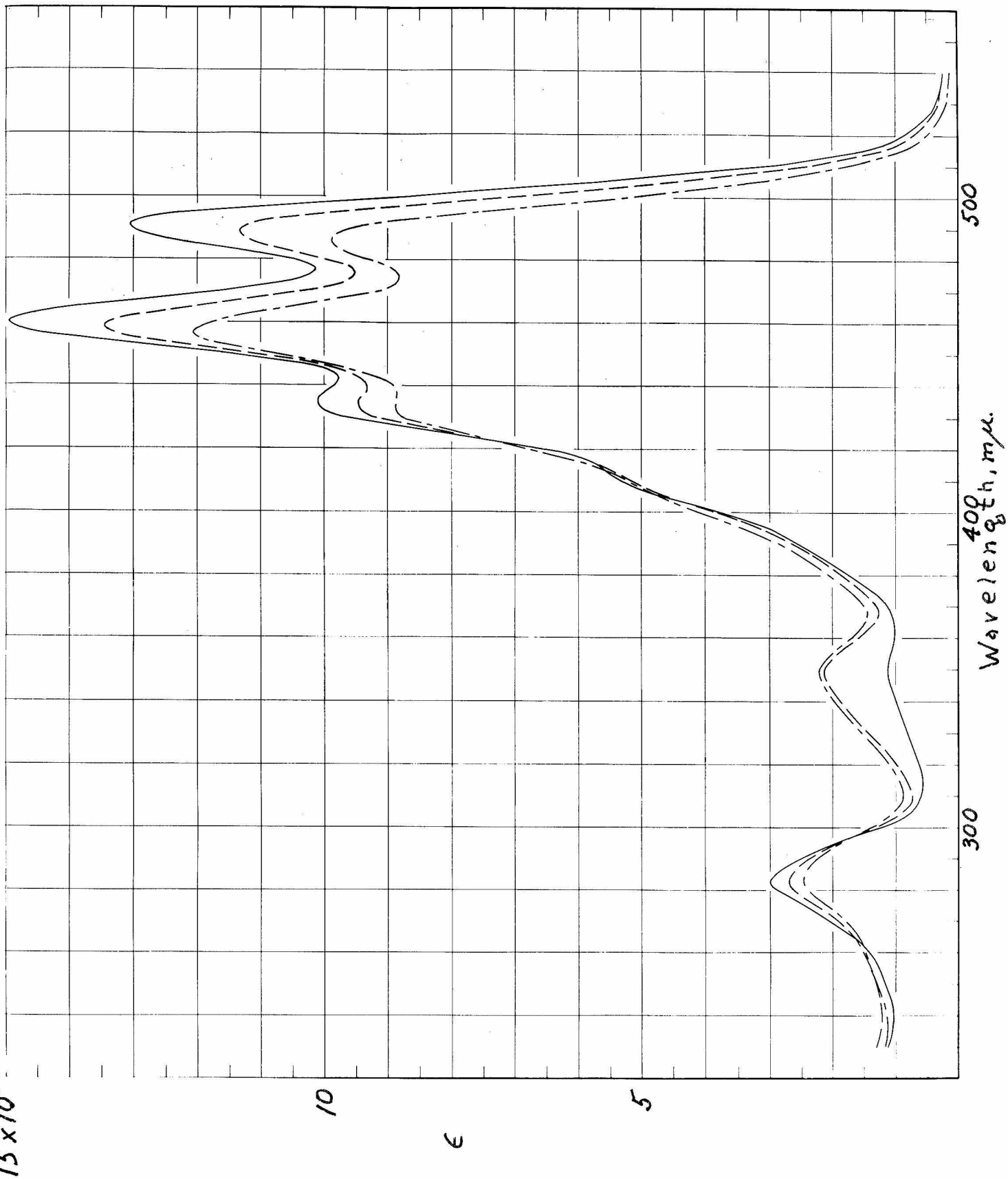


Fig. 5. Molecular extinction curves of gazaniaxanthin in hexane: _____ fresh solution of the all-trans compound; - - - after refluxing; -.-. after iodine catalysis.

Experimental

Isolation of Gazaniaxanthin from the Flowers of Gazania rigens.--One kilo of petals, collected within a week, was dried at 40-50° for 24 hours. The milled material was covered with petroleum ether for 2 hours in a percolator (35 x 15 x 8 cm.). Percolation was continued until 3.5 liters had passed through. The deep red solution was diluted to 5 liters and saponified. The solution was chromatographed* in two percolators (45 x 20 x 8 cm.), development requiring a total volume of 8 liters of petroleum ether and 10 liters of 10% acetone.

The two chromatograms showed minor top layers followed by a broad red zone of gazaniaxanthin and lycopene which did not separate with this development and were cut out together. Neolycopene and paler zones of cryptoxanthin and γ-carotene were immediately below. β-Carotene and minor pigments were washed into the chromatographic filtrate and discarded. The gazaniaxanthin-lycopene section was eluted with alcohol containing some petroleum ether and the combined cryptoxanthin-γcarotene zones with alcohol.

The petroleum ether solution of the gazaniaxanthin-lycopene section was rechromatographed in a percolator (35 x 15 x 8 cm.). The hydroxy-compound was retained on the column while the lycopene was washed into the filtrate by developing with 5% benzene in petroleum ether. To achieve this separation, Merck's calcium carbonate("Heavy powder") was employed. The choice of a carbonate which does not retain lycopene is important.

Gazaniaxanthin was eluted with ether and evaporated completely in vacuo.

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* If chromatography cannot be carried out immediately and the solution must stand in a cold room, it should be filtered before adsorption in order to remove gummy material which will clog the top of the column and may even cause it to split vertically.

The red, crystalline residue was dissolved in the minimum amount of cold benzene, transferred into two 50 ml. centrifuge tubes and crystallized at 25° by gradual addition of excess methanol with stirring. After standing at 5° overnight, the crystals were centrifuged, washed with methanol and dried with a stream of carbon dioxide in the centrifuge tube. Then they were dissolved in benzene at 45°. Methanol was added slowly until the first crystals appeared and then more rapidly. The suspension was kept at 25° for an hour and at 5° for two hours and then filtered. The yield was 560 mg. of pure gazaniaxanthin. After rechromatographing, the mother liquor yielded an additional 65 mg.*

Analytical data for Gazaniaxanthin

Carbon and hydrogen:

Calcd. for $C_{40}H_{54}O$:	C, 87.27;	H, 9.90.
for $C_{40}H_{56}O$:	86.89;	10.22.
for $C_{40}H_{58}O$:	86.58;	10.54.
Found:	C, 87.27;	H, 10.75.
	87.41	10.54.

Molecular weight:

0.297 mg. in 1.744 mg. of exaltone (k = 21.3): Δ = 7.2°.

Calcd. for $C_{40}H_{58}O$: mol. wt., 555.

Found: mol. wt., 504.

Number of double bonds:

10.17 mg. with 23.3 mg. PtO_2 added 5.12 ml. of hydrogen (24°, 743 mm.).
10.45 mg. with 32.5 mg. PtO_2 added 5.19 ml. of hydrogen (23°, 745 mm.).
7.52 mg. with 4.2 mg. PtO_2 added 3.65 ml. of hydrogen (20°, 746 mm.).

Calcd. for $C_{40}H_{58}O$: 11.0. double bonds.

Found: 11.2, 11.1 and 11.0 double bonds.

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* In two similar experiments with petals several weeks old, the yields were only 377 and 386 mg. per kilo of dry material.

Active hydrogen:

19.11 mg. gave 1.13 ml. of methane (30°, 742 mm.).

Calcd. for $C_{40}H_{57}OH$: OH. 1.0.

Found: OH. 1.3.

Isopropylidene groups:*

Calcd. for $C_{40}H_{58}O$: $(CH_3)_2C=$ groups per mole, 0.0.

Found: $(CH_3)_2C=$ groups per mole, 0.85, 0.85.

Properties of Gazaniaxanthin.--(a) Melting point. The melting point of gazaniaxanthin was 133-4°. This value, several degrees lower than that reported by Schön, was not altered by recrystallization.

(b) Crystal form. When suspended before filtration, the crystals had a golden glitter and a rather orange-brown appearance. Under the microscope they were lenslike in form or plates with rounded edges (Fig. 6). The color of the individual plates was brownish-orange; superimposed aggregates showed a purplish

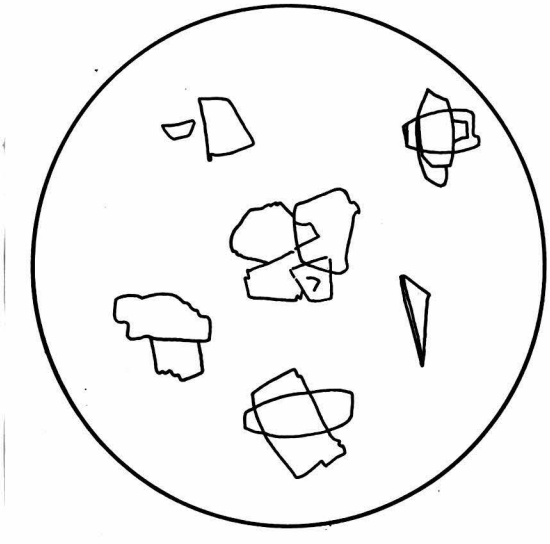

Fig. 6. Gazaniaxanthin from Benzene and Methanol.

* See Table VIII for data and simultaneous control determinations.

tint. At times needles were observed, grouped in rosettes similar to the photomicrographs of rubixanthin[8].

(c) Absorption Maxima in Various Solvents. The visually observed spectral maxima of gazaniaxanthin are listed in Table VII.

Table VII

Visually Observed Absorption Maxima of Gazaniaxanthin in Various Solvents

Solvent	Fresh solutions	After addition of iodine
Carbon disulfide	531 494.5 461	528.5 491 458.5
Benzene	509 476 447.5	505.5 473
Petroleum ether	494.5 462.5 434.5	491 459.5
Hexane	493.5 462 434	490.5 459
Absolute alcohol	494.5 462 434.5	No change

(d) Partition behavior. The partition behavior was identical with that described by Schön. The compound was epiphasic when partitioned between petroleum ether and 90% methanol but partially hypophasic when 95% methanol was employed.

(e) Adsorption characteristics. From petroleum ether solutions, gazaniaxanthin is more strongly adsorbed than lycopene, both on calcium hydroxide and on calcium carbonate. It is adsorbed much above cryptoxanthin.

(f) Photometric determination. The concentration of petroleum ether solutions may be determined with the Pulfrich photometer (light filter S 47); $\underline{k}$ = extinction coefficient, $\underline{c}$ = mg. of gazaniaxanthin in 100 ml. of solution, and $\underline{c}_I$ = mg. of iodine equilibrium mixture in 100 ml.

$\underline{k}$	0.3	0.5	0.7	0.9
$\underline{c}$	0.12(5)	0.21(5)	0.30(5)	0.39(5)
$\underline{c}_I$	0.15	0.25	0.36	0.47

(g) Polarimetry. With concentrations between 0.05 and 0.1 g. per 100 ml. using 1 dm. tubes, no rotations were observed in benzene or petroleum ether, while chloroform solutions showed a moderate rotation:

$$[\alpha]_{Cd}^{25} = +(100 \times 0.06°):(1 \times 0.092) = +65° \text{ (in } CHCl_3)$$

Upon the addition of a drop of concentrated iodine solution to the polarimeter tube the rotatory power increased within a few minutes:

$$[\alpha]_{Cd}^{25} = +(100 \times 0.14°):(1 \times 0.092) = +150° \text{ (in } CHCl_3)$$

On the other hand, if a petroleum ether solution of gazaniaxanthin was catalyzed with iodine and chromatographed, the heterogeneous neo-section located immediately below the zone of unchanged gazaniaxanthin gave:

$$[\alpha]_{Cd}^{25} = +(100 \times 0.10°):(1 \times 0.046) = +220° \text{ (in petroleum ether)}$$

A drop of iodine produced a decrease in the rotation:

$$[\alpha]_{Cd}^{25} = +(100 \times 0.07°):(1 \times 0.046) = +155° \text{ (in petroleum ether)}$$

Cis-trans Isomerization of Gazaniaxanthin. For the separation of gazaniaxanthin and its neo-forms the choice of the absorbent and developer is of more than usual importance because the zones tend to form an undifferentiated sequence on the column. The following adsorbents gave no separation: alumina (used by Schön with success in the isolation of gazaniaxanthin), zinc carbonate, calcium carbonate, calcium carbonate-hydroxide mixtures, magnesium carbonate, basic magnesium carbonate and magnesium oxide. It was only on calcium hydroxide that a separation of gazaniaxanthin from its stereoisomers and a further differentiation of the latter took place after prolonged development with first 5%, later 10% and finally 25% acetone. This procedure was used in the isomerization experiments described below. In the range of 5-25 mg. such a development required 1-3 hours and was usually continued until the lowest zone neared the bottom of the column. When the isomerization mixture obtained from 25 mg. of gazaniaxanthin was chromatographed on a 28 x 7 cm. column, 2-3 liters of the developer were required, of which 4/5 was 25% acetone.

(a) Isomerization of Gazaniaxanthin by Iodine Catalysis at Room Temperature.- A solution of 25 mg. of pigment in 3 ml. of cold benzene was diluted to 100 ml. with petroleum ether and treated with a solution containing 0.5 mg. of iodine. After standing an hour at 25° in diffuse light, the mixture was developed on a column (28 x 7 cm.) as described above.

110 colorless top section
60 pink, gazaniaxanthin, 494.5, 462.5, 434.5 mμ. (with iodine 490.5, 458.5 mμ.)
18 brownish orange, 487.5, 457 mμ. (491,459 mμ.)
15 lighter brownish orange, 487, 457 mμ. (491, 459 mμ.)
1-2 nearly colorless (between Groups I and II)
8 yellow, 484, 454 mμ. (491, 459 mμ.)
14 orange yellow, 485, 454.5 mμ. (491, 459 mμ.)
9 orange, 486, 456 mμ. (491, 459 mμ.)
5 pink, 487.5, 456.5 mμ. (491, 459 mμ.)

In other experiments portions of the catalyzed solution were adsorbed on columns after 5, 15, and 30 minutes. Following elution with alcohol and transference into petroleum ether these colorimetric ratios were found:

Catalysis (min.)	0	5	15	30
Gazaniaxanthin: neosI :neos II	100:0:0	72:20:8	56:29:15	56:29:15

With neo-compounds as a starting material the equilibrium was also attained within 15 minutes.

The corresponding weight ratio at equilibrium was 50:30:20. Such figures can be obtained by the addition of iodine to each of the chromatographically separated pigment zones and the use of photometric values ("c_I") for the iodine equilibrium mixture.

(b) Isomerization by Refluxing.- For the best possible separation of isomers formed by this method, it is essential that the ratio of adsorbent to pigment be 2 to 3 times that used in experiments with iodine (see above). Fifteen mg. of gazaniaxanthin in 100 ml. of benzene were refluxed for an hour. After cooling rapidly to room temperature and diluting with 1 vol. of petroleum

ether, a chromatogram was obtained (28 x 7 cm.) from which all isomers and a small section of the unchanged gazaniaxanthin were cut out together. After elution with alcohol this mixture was rechromatographed (25 x 5 cm.).

90 colorless top section
50 pink, gazaniaxanthin, 494.5, 462.5, 434.5 mμ.
3 brownish pink, 488.5, 457.5 mμ. (with iodine: 491, 459 mμ.)
15 brownish pink, 487.5, 456.5 (490.5, 458.5 mμ.)
20 brownish orange, 486.5, 456 mμ. (490.5, 458.5 mμ.)
20 orange, 486, 455.5 mμ. (490.5, 459 mμ.)
20 orange brown, 487.5, 456.5 mμ. (490, 459 mμ.)
7 pink, 489.5, 458.5 mμ. (491, 459.5 mμ.)

The approximate rate of isomerization was established in a separate experiment:

Refluxing (min.)	0	15	30	60
Gazaniaxanthin: neos	100:0	71:29	70:30	68:32

(c) Isomerization of Solutions at Room Temperature.- Ten mg. of gazaniaxanthin in 25 ml. of petroleum ether was kept in the dark under carbon dioxide at 25-28°. Five ml. portions were chromatographed periodically (column 20 x 1.9 cm.). After cutting unchanged gazaniaxanthin from all its isomers, the relative color intensities of the two eluates were estimated in petroleum ether:

Time (days)	0	1	2	4	8
Gazaniaxanthin: neos	100:0	96:4	93:7	88:12	82:18

Neogazaniaxanthins are relatively stable under these conditions. For example, starting with a (chromatographically heterogeneous) neo-section which was located immediately below the all-trans zone, about 2 days were required to reach the equilibrium:

Time (days)	0	1	2	4
Gazaniaxanthin: neos	0:100	38:62	46:54	45:55

(d) Isomerization by Melting.- A sealed tube containing 20 mg. of the carotenoid was maintained at 140-5° for 5 min. After plunging into ice water,

the solidified mass was taken up in cold benzene and chromatographed (28 x 7 cm.) after dilution with petroleum ether.

100	colorless top section
60	pink, gazaniaxanthin, 494.5, 462.5, 434.5 mμ. (with iodine, 490.5, 458.5 mμ.)
5	brownish pink, 489, 457 mμ. (490.5, 459 mμ.)
20	orange brown, 488, 456.5 mμ. (491, 458.5 mμ.)
15	orange brown, 485, 454.5 mμ. (490.5, 458.5 mμ.)
15	yellow, 484, 453.5 mμ. (491.5, 459 mμ.)
15	yellow, 486, 454.5 mμ. (491, 458.5 mμ.)
12	pink, 489.5, 457.5 mμ. (492, 459 mμ.)
1	traces of color, 491, 460.5 mμ. (491, 459 mμ.)

Incomplete Experiments with Gazaniaxanthin.--Certain experiments with gazaniaxanthin were incomplete when the author of this Thesis became engaged in other research. They will now be discussed briefly.

Polarimetry.--It has been indicated that solutions of gazaniaxanthin in petroleum ether possess no rotation in concentrations which permit readings to be taken. However, when a petroleum ether solution of the gazaniaxanthin zone from an iodine isomerization mixture is inspected, a definite rotation is present. This was observed three times. In two instances the specific rotation was levo and, in the third, dextro, and was of the order of magnitude of 100-150°. The origin of the rotation in this case and the contradictory results have not been elucidated but it may be that the rotation originates in some change in the asymmetric carbon atom which presumably is present in gazaniaxanthin.

Irradiation.--The irradiation of carotenoids has been little studied. Yet it is a subject not devoid of interest when the simultaneous occurrence of carotenoids with chlorophyll and the probable, but unknown, function of the former in photosynthesis is considered.

Two samples of three mg. of gazaniaxanthin were each dissolved in twenty ml. of benzene; one solution contained 0.03 mg. of iodine. The solutions were sealed in thin-walled Pyrex bulbs under carbon dioxide and exposed to direct

solar radiation. After 34 hours of irradiation, the solution containing iodine had decreased in color intensity to a considerable extent, while the other had changed little, if at all, and was subjected to a total of sixty hours.

When these solutions were chromatographed with the usual development, isomers of gazaniaxanthin were present. The presence of these stereoisomers may perhaps be attributed as much to thermal isomerization as to any effect of radiation. The unique feature of the chromatograms consisted in the presence of yellow zones much less strongly adsorbed than gazaniaxanthin and its isomers. The chromatogram from the solution containing iodine possessed one such layer with the spectrum 476.5, 447.5 mμ. which was not altered upon treatment with iodine. The other chromatogram had two yellow zones, the upper with a spectrum, 478.5, 449.5 mμ. changing to 476, 447 mμ. with iodine while the lower had 478, 449.5 mμ. changing to 475.5, 446.5mμ. These spectra approximate those of α-carotene (or of a hydroxy-α-carotene). It may be postulated that the acyclic end of gazaniaxanthin has cyclized with the consequent loss of a double bond.

A similar experiment with a five-fold increase in concentration yielded negative results.

Crystallization of neogazaniaxanthin.--The instances in which neo-carotenoids have been isolated continue to multiply. Zeaxanthin[13], β-carotene[14] and methyl bixin[4] all yield crystalline isomers, while the pro-carotenoids are examples of more completely cis-carotenoids. It has also been possible to isolate a small quantity of a neo-gazaniaxanthin.

The neo-group I from an iodine isomerization was eluted with ether and evaporated to dryness. No crystals were obtained from benzene on the addition of methanol. After again evaporating to dryness the residue was dissolved in methanol and permitted to stand at 5° overnight. The crystals obtained formed

such extremely dense groups that individuals were distinguishable only at the edges. The 1.5 mg. isolated contained ten to fifteen per cent. gazaniaxanthin. The spectrum was 490, 458 mμ. and shifted slightly to higher wavelengths with iodine. The melting point was 121-2°.

Isolation of Other Carotenoids from Gazania rigens.--Although gazaniaxanthin is the main carotenoid in this material, certain other carotenoids were also isolated.

Isolation of Lycopene.--From the filtrate of the chromatogram of the gazaniaxanthin-lycopene section (see above), pure lycopene was isolated in a yield corresponding to 135 mg. per kilo of dry petals. The spectral maxima were: in carbon disulfide, 546, 507, 474.5 mμ. (with iodine, 543, 502, 469 mμ.); in benzene, 521, 486.5, 456 mμ. (517.5, 484, 453.5 mμ.); and in petroleum ether, 504.5, 473.5, 445 mμ. (501, 469.5, 441 mμ.).

Anal. Calcd. for $C_{40}H_{56}$: C, 89.48; H, 10.52

Found: 89.50 10.71

Isolation of γ-Carotene and Cryptoxanthin.--The petroleum ether solution of this section of the initial chromatogram was rechromatographed from this solvent in a percolator (35 x 15 x 8 cm.). The main zones, γ-carotene and cryptoxanthin, were eluted separately with ether and evaporated to dryness in vacuo.

a. γ-Carotene.- The residue was dissolved in the minimum amount of cold benzene, transferred into a centrifuge tube and methanol added cautiously with stirring. Upon standing at 5° a mixture of red and colorless crystals precipitated. After further addition of methanol, the suspension was kept at 5° for another day. It was then centrifuged and the solid repeatedly treated with boiling methanol for a minute or two. Each treatment was followed

by rapid centrifuging and decantation of the hot methanol which deposited white crystals upon cooling. These were filtered off. The filtrate, the mother liquor of the first centrifuging and the γ-carotene crystals were combined, rechromatographed and crystallized from benzene-methanol. After a single extraction with hot methanol and recrystallization, the microscope showed homogeneous red crystals of γ-carotene, the form of which was not unlike gazaniaxanthin described above; m.p. 131-3°*. The yield of pure γ-carotene was 13.5 mg. In the partition test epiphasic behavior was observed.

The spectral maxima were (in the visual spectroscope): in carbon disulfide, 533, 495.5, 462 mμ. (with iodine, 530, 492.5 459 mμ.); in benzene, 509.5, 477, 447.5 mμ. (506, 473.5 mμ.); and in petroleum ether, 495, 462.5, 434 mμ. (491.5, 459.5 mμ.).

Anal. Calcd. for $C_{40}H_{56}$: C, 89.48; H, 10.52

Found: 89.39 10.55

b. Cryptoxanthin.--When the dry residue (see above) was dissolved in a little benzene and diluted with methanol, only colorless material precipitated. After filtering, the pigment content of the filtrate was transferred into ether and saponified. The compound was chromatographed from petroleum ether, first on calcium carbonate and then on calcium hydroxide. Following elution with ether and evaporation, the dry residue was crystallized from benzene and methanol. The crystals were contaminated with colorless material, and were freed from the latter by recrystallization.

The yield was 1.3 mg., m.p. 163°. The behavior in the partition test corresponded to that of cryptoxanthin. In the mixed chromatogram test the

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* See Section I of this Thesis.

sample did not separate from cryptoxanthin obtained from another source.

The spectral maxima were: in carbon disulfide, 518, 482.5, 453.5 mμ. (with iodine, 513.5, 482 mμ.); in benzene, 497, 463.5 mμ. (495, 461.5 mμ.); and in petroleum ether, 483, 452.5 mμ. (481, 450.5 mμ.).

Anal. Calcd. for $C_{40}H_{56}O$: C, 86.89 H, 10.22.

Found: 87.26 10.46.

Summary of Section III

1. Catalytic hydrogenation indicates that gazaniaxanthin, first isolated by Schön from Gazania rigens, R. Br. (Compositae), is dihydro-rubixanthin.

2. The spectral curve of gazaniaxanthin exhibits the "cis-peak" phenomenon.

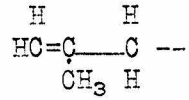
3. The stereoisomerization of gazaniaxanthin by iodine catalysis, by refluxing of solutions, and by melting of crystals has been investigated. The chromatographic separation of the stereoisomers is difficult.

4. The mixture of carotenoids in Gazania flowers grown in Southern California differs considerably from that of flowers grown in Portugal.

IV. MICRO-DETERMINATIONS OF THE ISOPROPYLIDENE GROUP IN CAROTENOIDS

IV. MICRO-DETERMINATIONS OF THE ISOPROPYLIDENE GROUP IN CAROTENOIDS

Introduction and Discussion

In the course of the investigations reported in this Thesis it became necessary to determine the presence or absence of an isopropylidene group in γ-carotene and gazaniaxanthin. Kuhn and Roth[9] have described a method for the micro-determination of isopropylidene groups which involves (1) ozonization of the compound in glacial acetic acid solution or suspension, (2) hydrolysis of the ozonide by means of potassium permanganate after partial neutralization of the acid with alkali, (3) distillation of the acetone formed, (4) conversion of the acetone into iodoform with iodine in alkaline solution, and (5) back-titration of the excess iodine with sodium thiosulfate after acidification. The method does not give quantitative results, for only 60-95% of the theoretical quantity of acetone is usually determined. This deficiency is ascribed to two factors. It is supposed that isopropylidene groups are in equilibrium with forms such as  because the deficit can be covered by formaldehyde or formic acid in many cases. It may also be connected with the known rearrangement of acetone peroxide to methyl acetate. As previously mentioned, compounds, such as thymol or isopropyl alcohol, which possess an isopropyl but no isopropylidene group, may yield acetone corresponding to approximately 0.3 isopropylidene group.

In the field of the carotenoids, the procedure has been employed by Kuhn in collaboration with Broekmann[7] and Gründmann[8] to determine the presence of an isopropylidene group in γ-carotene (from commercial carotene) and in rubixanthin (from rose hips). The results were 81 and 89% of the theoretical values for γ-carotene and 94% for rubixanthin. On a macro scale Karrer, Helfenstein,

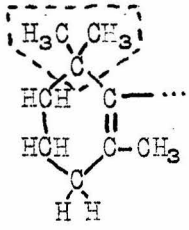
Pieper, and Wettstein[6] have obtained 80% of the calculated amount of acetone from lycopene.

In order to test the apparatus and become acquainted with the procedure, three determinations were carried out with lycopene which is the only easily available carotenoid containing an isopropylidene group. The percentages of the theoretical quantity of acetone obtained were 83, 84 and 83. In two determinations gazaniaxanthin yielded 94% of the theoretical for one isopropylidene group. These results made it desirable to test a substance of the same type which possessed no isopropylidene group. When β-carotene was ozonized, it yielded 32 and 35% of the theoretical amount of acetone for one isopropylidene group per molecule.

This result made a blank determination imperative. The blanks required 0.21 and 0.24 cc. of N/20 iodine solution. This cannot be expressed in terms of per cent. of the theoretical amount, however, because the effect of the correction depends on the size of the sample. The blank equals 5-30% of the iodine disappearing in the determination. When the correction is applied, the values are decreased, but in no case is an analysis rendered ambiguous.

When γ-carotene was ozonized, sufficient acetone was found to lead to the conclusion that this substance, as well as gazaniaxanthin, possessed an isopropylidene group. However, catalytic hydrogenation indicates the presence of only eleven double bonds in the latter and, therefore, makes it unlikely that an isolated double bond exists as discussed in Section III. The results of a direct method, such as hydrogenation, are to be more favorably regarded than those of a method, such as the isopropylidene determination, which is only semi-quantitative and which is known to yield acetone from isopropyl groups.

The source of the acetone (if it be acetone) from β-carotene and other carotenoids cyclised on both ends cannot be stated with certainty. It is evident from Table VIII that such compounds yield 24-30% of the acetone expected from one isopropylidene group. It seems unlikely that the acetone would arise from the middle section of the molecule. This would be expected to produce pyruvic (eventually acetic) and oxalic acids as the final products of a complete ozonolysis. One must, therefore, look to the cyclized end of the molecule for an explanation. While it is true that acetaldehyde or ethyl alcohol as well as methyl ketones will give the iodoform test, it is difficult



to imagine how acetaldehyde could survive the treatment with permanganate which precedes distillation in the experimental procedure. It is, likewise, difficult to imagine in what manner ethyl alcohol or any methyl ketone other than acetone could be formed. Under the drastic conditions of ozonization followed by oxidation, it seems probable that some acetone originates in that part of the cyclised end of the molecule within the dotted lines of the figure.

The procedure of Kuhn and Roth was not altered in any way in the present work. With one exception it was found sufficient to ozonize for two hours. The three lycopene samples were ozonized for 2, 2.5 and 3 hours but no difference can be attributed to this factor. Although carotenoids are not soluble in glacial acetic acid to the extent here required by the proportions of solute and solvent (0.2-0.6%), this offered no hindrance in the determination. With one exception all carotenoids had decolorized and gone into solution within an hour after ozonization had been started. Several samples required only 15-25 minutes. Celaxanthin ester proved refractory and had not completely decolorized after 3.5 hours ozonization. When ozonization was stopped at least 1/10

Table VIII

Micro-Isopropylidene Determinations with Carotenoids[a)]

Substance	mg. subs.	CC. N/20 I_2 used	CC. N/20 I_2 used (Corr.)	Moles C_3H_6= detn.	Moles C_3H_6= detn. (corr.)	Moles C_3H_6= calc.	% of theoretical
Blank		0.24	0.24				
Blank		0.21	0.21				
5,6-Dihydro-α-carotene	11.21	0.89	0.66	0.35	0.26	0.0	(26)
5,6-Dihydro-α-carotene	11.94	0.87	0.64	0.33	0.24	0.0	(24)
α-Carotene	15.18	1.05	0.82	0.31	0.24	0.0	(24)
5,6-Dihydro-β-carotene	8.92	0.76	0.53	0.38	0.27	0.0	(27)
Lutein	13.81	1.11	0.88	0.38	0.30	0.0	(30)
β-Carotene	15.19	1.09	0.86	0.32	0.25	0.0	(25)
β-Carotene	16.62	1.30	1.07	0.35	0.29	0.0	(29)
γ-Carotene	16.93	3.76	3.53	0.99	0.93	1.0	93
γ-Carotene	22.12	4.82	4.59	0.97	0.93	1.0	93
Gazaniaxanthin	13.50	2.75	2.52	0.94	0.86	0.0	(86)
Gazaniaxanthin	16.27	3.31	3.08	0.94	0.87	0.0	(87)
Celaxanthin ester	14.02	1.57	1.34	0.63	0.56	1.0	56
Lycopene	7.56	2.80	2.57	1.66	1.52	2.0	76
Lycopene	11.82	4.44	4.21	1.68	1.60	2.0	80
Lycopene	12.35	4.59	4.26	1.66	1.58	2.0	79
Prolycopene	7.93	3.15	2.92	1.78	1.64	2.0	82

[a)] Celaxanthin ester was isolated by Dr. A. L. LeRosen from Celastrus scandens[11]. 5,6-Dihydro-α-carotene and 5,6-dihydro-β-carotene were obtained by Dr. A. Polgar[15] by treating carotene with conc. hydriodic acid.

of the starting material was unchanged. Colorless solid particles, probably of ozonide, were also visible.

A summary of all determinations is presented in Table VIII.

Experimental

Determination of the Factors of Solutions.---The sodium thiosulfate solution was standardized against potassium iodate in an acid solution of potassium iodide with starch as indicator. The normality was 0.0517. A portion of the iodine solution was acidified, diluted and then titrated with the sodium thiosulfate solution. The normality was 0.0369.

The Course of the Analysis*.---The substance is placed at the bottom of the flask ..... (As much of the material to be analyzed as will form 1.5-2.5 mg. of acetone is weighed, from a long handled weighing tube into the clean dry flask K_1) and dissolved in 3 cc. of 99-100% acetic acid. It is important that it should be completely dissolved; the solution may be warmed for this. Substances insoluble in acetic acid are very finely ground in an agate mortar and ozonised finely dispersed in the acetic acid.

The ozone apparatus is first set in action and the speed of the oxygen current is regulated at 20 cc. per minute. The Siemens and Halske A.-G. ozonisator, Model Oz, gives under these conditions oxygen containing 3.2% of ozone** The standard joint S of the flask K_1 (Fig. 7) is secured with a steel spring, after previously moistening the inner part with metaphosphoric acid. In the second flask, K_2, 3 cc. of water are placed, the inner part inserted as above, the inlet tube is connected with the delivery tube of the

---

*This procedure is reproduced from F. Pregl and H. Roth, Quantitative Organic Microanalysis, 3rd Eng. Ed., Philadelphia: P. Blakiston's Son and Co. (1937).

**In this case an ozonisator built by Mr. Weiss was used.

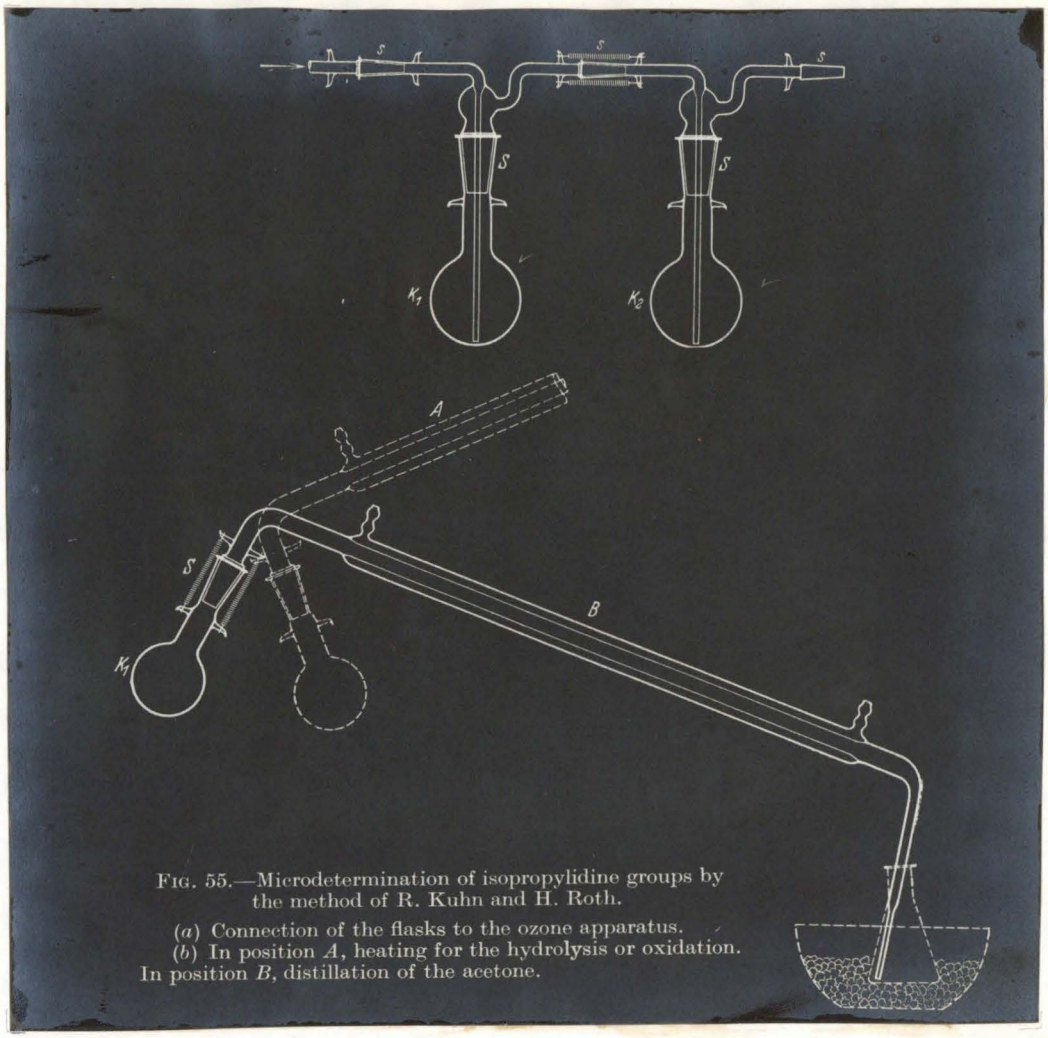


FIG. 55.—Microdetermination of isopropylidine groups by the method of R. Kuhn and H. Roth.

(*a*) Connection of the flasks to the ozone apparatus.
(*b*) In position *A*, heating for the hydrolysis or oxidation. In position *B*, distillation of the acetone.

Fig. 7

first flask, and the connections are secured with steel springs. The flask is then connected at s with the ozone apparatus and the second flask K_2 is cooled with melting ice. As a rule, the time required for ozonisation is from two to three hours.

The ozone apparatus is then disconnected and removed. After disconnecting the joints at S, the inlet tubes in both flasks are rinsed with about 10 cc. of water and removed. The contents of K_2 are then rinsed with about 10 cc. of water into the acetic acid solution of K_1, the acid is neutralized with 16 cc.

of 2 N sodium hydroxide, and 5 cc. of N potassium permanganate solution, with a few pieces of pumice, are placed in the flask. The condenser, the joint of which has been moistened with metaphosphoric acid, is inserted in the flask, the joint S secured, and the condenser is placed in position A and clamped. The solution is now boiled for ten minutes on the wire gauze, to decompose the ozonide and to oxidise the decomposition products which would affect the titration. Great care must be taken that the water is running freely in the condenser, during this. After removing the flame, one ascertains whether some permanganate is still present. If all should have been used up, 5 cc. more must be added after cooling and the heating repeated.

Distillation. The condenser and flask are then clamped in position B, without disconnecting. During the oxidation, 10 cc. of water have been cooled in a stoppered 100 cc. Erlenmeyer flask in melting ice, as in the diagram. This flask is now placed under the condenser so that the adapter is not at first immersed in the receiver, because then the water present would rise in the condenser during the cooling of the distillation flask. Only when the acetone begins to distill is the adapter dipped into the receiver. A Babo funnel is used for the distillation. After 20 cc. of distillate have been collected, the receiver is lowered, the adapter rinsed with 2-3 cc. of water, and the Erlenmeyer flask closed.

Titration. The acetic acid content of the distillate corresponds to a maximum of 30 cc. of N/10 sodium hydroxide. This distillate is immediately made alkaline with 5 cc. of 2 N sodium hydroxide for the iodoform reaction and mixed with 10 cc. of N/20 iodine solution from the microburet. The iodine is added rapidly drop by drop. Because, in the cold, the formation of iodoform is slow, the closed flask is allowed to stand for fifteen minutes at

room temperature whilst shaking frequently. To complete the determination one acidifies with 10 cc. of 2N sulfuric acid and titrates the unused iodine after two minutes with N/20 sodium thiosulfate solution, using starch as indicator.

Calculation. Because one molecule of acetone requires six atoms of iodine, one ml. of N/20 iodine solution corresponds to 0.484 mg. of acetone or 0.3505 mg. of $(CH_3)_2C=$.

$$\log 0.3505 = 9.54459 - 10$$

log (percentage of C_3H_6) = log (no. of ml. N/20 iodine used) + log (factor) + 2 - log (mg. of substance).

Example:

γ-Carotene, $C_{40}H_{56}$

Theoretical, 7.84%, C_3H_6, one C_3H_6 group.

mg. of substance, 16.93

No. of ml. of N/20 iodine used (corr.), 3.53

% of C_3H_6 found, 7.31 or 0.93 C_3H_6 group.

Summary of Section IV

1. Isopropylidene determinations have been carried out with a number of carotenoids.

2. Evidence indicates that an isopropyl group in a carotenoid molecule may act as an isopropylidene group.

3. Some "isopropylidene" is also found with carotenoids cyclized on both ends.

V. Two copies of this section were submitted to Professor Linus Pauling on May 3, 1943.

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PROPOSITIONS

1. Under some conditions pro-carotenoids can be caused to form in a plant which does not normally contain these compounds.[a] I propose that this plant be used in an investigation of the conditions for the formation of pro-carotenoids and for carotenoid biosynthesis in general.

[a] W. A Schroeder, J. Am. Chem. Soc., 64, 2510 (1942).

2. A nomenclature for carotenoid stereoisomers is proposed.

3. α-Carotene and 5,6-dihydro-β-carotene are indistinguishable chromatographically.[b] Dihydro-γ-carotene and γ-carotene probably would behave similarly. A study of the possibility of mixed crystals of the first pair would help to clarify the ambiguities connected with γ-carotene as reported in this Thesis.

[b] A. Polgar and L. Zechmeister, J. Am. Chem. Soc. 65 (1943)(in print).

4. A preliminary experiment indicates that irradiation may cause structural changes in the carotenoid molecule. A study of the action of irradiation might lead to information concerning the role of carotenoids in photosynthesis.

5. Chemicals used as adsorbents may vary exceedingly from lot to lot. A correlation of their properties with the studies of LeRosen[c] should lead to a rapid method of determining the suitability of a particular adsorbent or lot for chromatographic purposes.

[c] A. L. LeRosen, J. Am. Chem. Soc. 64, 1905 (1942).

6. The structure of "irone" has not yet been fully determined.[d] It seems probable that a chromatographic study of the phenylhydrazone (or related derivative) of the substance would aid the structural study.

[d] L. Ruzicka, C. Seidel, H. Schinz, and M. Pfeiffer, Helv. chim. Acta, 25, 188 (1942) and preceding papers.

7. The use of Karl Fischer's reagent[e] would greatly facilitate the determination of the water content of plants and other materials.[f]

[e] K. Fischer, Angew. Chem. 48, 394 (1935) and D. M. Smith, W. M. Bryant, and J. Mitchell, J. Am. Chem. Soc. 61, 2409 (1939).

[f] J. Mitchell, Ind. Eng. Chem., Anal. Ed., 12, 390 (1941).

8. Alcohols may be determined quantitatively by esterifying and then titrating the water formed with Karl Fischer's reagent.[g] The quantitative results are attributed to a shifting of the equilibrium by boron trifluoride. This explanation is incorrect.

[g] W. M. Bryant, J. Mitchell, and D. M. Smith, J. Am. Chem. Soc. 62, 1 (1940).

9. The teaching of organic chemistry at Caltech could be considerably promoted by the use of two-dimensional blackboard formulas and the frequent use of three-dimensional models.