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III. THE STEREOCHEMISTRY OF METHYLBIXIN

Thesis by

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Acknowledgment

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Abstract

A chromatographic investigation of the carotenoid pigments of whole wheat flour has been made to establish its provitamin A activity. While xanthophyll (lutein) was found to predominate, 0.02 to 0.06 milligrams of β-carotene was detected in a kilogram of flour and its identity was established by the mixed chromatogram method. Whole wheat bread was found to be even poorer in carotene. Investigations of rye flour and bread gave essentially the same qualitative and quantitative results.

From one kilogram of the ripe seeds of Evonymus fortunei Rehd., 11 milligrams of prolycopene and 0.5 milligrams of pro-γ-carotene have been isolated.

Two new stereoisomers of methylbixin have been obtained in crystalline form and a third has been observed in solution. Various isomerization procedures have been tried on the four crystalline materials and the interconversion of these isomers has been demonstrated. A consideration of the absorption data has been used to assign tentative configurations to the four principal members isolated from the methylbixin set of stereoisomers.

I. THE PROVITAMIN A CONTENT OF AMERICAN WHOLE WHEAT FLOUR AND WHOLE WHEAT BREAD

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Introduction and Discussion

Before the advent of the roller mill, all flour was milled by grinding the grain between flat stones. This procedure led to an inclusion of considerable quantities of bran in the final, inferior portion of the flour thus imparting a darker color than that of fine grade flour. As a consequence, color in flour became closely associated in the mind of the public with low grade flour. While the introduction of the roller mill allowed the miller to produce very fine grade flours with only a trace of a pale cream color, the public continued to demand whiter flours and bread. Since it had been known for sometime that the color of flour bleached upon storage, it became a common practice for millers to store their products several months in order to produce the desired whiteness. By the middle of the nineteenth century there were attempts made to accelerate this bleaching by the use of sulfur dioxide, ozone, and other oxidizing agents. However, it was not until the turn of the twentieth century that nitrogen peroxide, chlorine, benzoyl peroxide, and some other bleaching agents used at the present were introduced in the milling industry.

As early as 1909, Wesner and Teller (1) reported that the yellow color removed from flour by aging or by oxidizing agents was not due to the inclusion of bran in the flour but was a natural pigment closely related to other vegetable colors. Two years later they stated (2) that they had satisfied themselves

that this material was of the same nature if not identical with carotene. The claim that β-carotene is the main fat-soluble pigment extractable from unbleached flour was more or less supported by numerous investigators (3). As progress was made in this field over a number of years, there was a definite decrease in the amount of "carotene" reported. This trend was noted by Widmark and Neymark (4) as well as Jörgenson (5) in their reviews of this subject.

Not until 1935 was there any reference in the literature asserting that the entire lipoid-soluble pigment of the whole wheat was other than provitamin A. Markley and Bailey (6) were the first to state that only a fraction of the wheat carotenoids can consist of carotene. However, their estimate of one-third to one-seventh of the total pigment as β-carotene was still much too high. Malmberg and von Euler (7) carried out a chromatographic analysis of an extract from 100 grams of wheat and were unable to detect any β-carotene or any other carotenoid possessing provitamin A activity. According to their conclusion, the main carotenoid was xanthophyll (lutein). It is interesting to note that they report a minor red zone in the lower region of their chromatograms which was epi-phasic when partitioned between 90% methanol and ligroin. However, the solutions were so weak that it was impossible to obtain an absorption spectrum of this fraction. In view of the work described in the experimental section, it is most probable that this zone was due to the presence of β-carotene traces. The presence of xanthophyll reported in these investigations is in accord with the previous work

of Bowen and Moore (8) who established the presence of this pigment in wheat germ oil. Scheunert and Schieblich (9) ran biological tests with vitamin A-deficient rats and were unable to detect any activity in whole wheat flour or bread.

Despite this experimental evidence to the contrary, the alleged rôle of flour carotene in nutrition was still accepted in authoritative treatises as late as 1939. Drummond and Wilbraham (10) report the carotene content of stone-ground white flour to be 0.2 milligrams per 100 grams (11) and deplore that "the latest perversion of scientific effort is the selective breeding experiments being carried out in Canada with the object of producing a pigment-free, i.e. vitamin A-free, wheat" (12). Copping (13) postulated that in a diet deficient in green vegetables and fats, bread may be an important source of vitamin A. The Council on Foods of the American Medical Association (14) reviewed his article and calculated that a pound loaf of bread made from unbleached flour would furnish approximately 0.6 to 1.2 milligrams of carotene.

The next year a paper dealing with the carotenoids of Hungarian wheat flour was published by Zechmeister and Cholnoky (15). These investigators were unable to detect any β-carotene but did isolate crystals of xanthophyll (lutein) in a sufficient quantity to complete characterization. According to their claims, unbleached Hungarian flours are practically or absolutely free of carotene, the content being estimated to be less than 0.01 milligrams per kilogram of flour. The isolation of crystalline xanthophyll confirm-

ed the opinion of Malmberg and von Euler (7) that this pigment constitutes the main portion of the wheat carotenoids. Binnington, Hutchinson, and Ferrari (16) have lately reached the same conclusion and state that "recent unpublished studies . . . indicate that the major carotenoid pigment is xanthophyll or its ester, rather than carotene itself". In an editorial of the Journal of the American Medical Association (17) reviewing the conclusions of Zechmeister and Cholnoky, it was suggested that the same technic should be used with other milling products and with grain from different localities. That the carotenoid content of certain plants may differ qualitatively with location has been reported, e.g., by Zechmeister and Schroeder (18).

Such analyses were carried out in this investigation (19) with three varieties of freshly milled, unbleached American whole wheat flour. The spectra of even crude total extracts indicate that the main constituent of the lipoid soluble pigment is xanthophyll (lutein), the maxima being, e.g., at 477.5 and 448.5 mμ in petroleum ether or at 506 and 473 mμ in carbon disulphide. The pigment content of the saponified extracts varied between 1.5 and 2.0 mg. per kilo flour. The dye can be roughly divided in the Tswett column into a main xanthophyll fraction and a more weakly adsorbed smaller portion. The latter does not exceed 1-2% of the total pigment and shows the spectrum of β-carotene, of which it mainly consists. In exceptional cases the chromatographic filtrate was also pale yellowish but only because of unsatisfactory saponification of some xanthophyll-esters; it was free of carotene.

On the basis of the present estimations the total amount

of provitamin A in the flour is 0.02-0.04 mg. per kilo (Table 1). Cryptoxanthin is not present.

Considering the length of the procedure, the use of strong alkali, the sensitivity of polyenes, and the presence of comparatively huge amounts of colorless accompanying material, it was necessary to ascertain that no substantial losses had occurred during the analysis. Therefore, 0.1 mg. of crystalline β-carotene was dissolved in the total extract freshly obtained from 1 kilo of flour and all usual procedures (including saponification) were carried out. Simultaneously a blank experiment was run with another kilo of the same flour. The "by-layers" of the two columns differed visibly; half of the added carotene was found, recovered and estimated. When, however, in two analogous experiments only 0.01 mg. of carotene was added, 0 and 25% of the latter was recovered. Obviously the limits of reliability of the method have been reached here.

Thus it is probable that the true carotene content of whole wheat flour is considerably less than 0.1 mg. per kilo, but it cannot be decided if it is nearer 0.02 or 0.06 mg. The A-requirements of an adult person would be covered by 50-100 kilos of flour daily.

Since in some older papers the point seems to have been missed that not flour- but bread-carotene should be considered from the nutritional viewpoint, 100% whole wheat bread was also investigated. If no pigment losses occurred during the baking process, the carotenoid content of the loaf would be near 60% that of the flour, i.e., 0.9-1.2 mg. total pigment and 0.01-0.04 mg. carotene per kilo. On the basis of Table 2, however,

which presents some data for dried crust and crumb separately, the corresponding true figures appear as follows: 0.26 mg. total pigment and 0.005 mg. of β-carotene per kilo of fresh bread or roughly the half of this amount in a commercial pound loaf. Experiments with rye flour revealed the presence of 2-3 mg. of total carotenoids and of 0.03-0.06 mg. of β-carotene per kilo (Table 3); a sample of pumpernickel bread contained 1/15 of these amounts.

Experimental

Small scale experiments with flour.--One kilo of flour was percolated with peroxide-free ether for about three hours. The extracts were bright yellow and showed a greyish blue fluorescence in ultraviolet light. The crude extract (1 liter) was saponified overnight with methanolic KOH. Half a liter of water was cautiously added, the alkaline solution re-extracted with ether and the combined ether solution washed alkali free, dried with sodium sulfate and evaporated in vacuo, under CO_2. This evaporation was repeated twice after the addition of small amounts of petroleum ether (b.p. 60-70°). The oily, darkish residue was dissolved in 25 cc. of the solvent mentioned, and chromatographed on a calcium hydroxide column (Shell) (18 x 3.5 cm.). After developing the column, a 30-40 mm. broad yellow zone (xanthophylls) was visible near the top and about 70 mm. deeper a carotene containing pale double line was noticed (0.5 and 2 mm. thick).

The xanthophylls were eluted with ether which was then evaporated. It is advisable to eliminate the last traces of this solvent by adding some petroleum ether and distilling it off in vacuo. The residue is dissolved in petroleum ether. The carotene-layers were eluted with an alcohol-petroleum ether mixture 1:2, washed free of alcohol and dried. All photometric values were taken in the Pulfrich gradation photometer (Zeiss, light filter S-47, photometric values as found by Cholnoky (in press)). The spectra were determined in an Evaluating grating spectroscope as devised by Loewe and Schumm (Zeiss; Jena light filters BG-7). (Table 1). The identity of carotene was repeated-

Table 1

Whole Wheat Flour Carotenoids

(The spectra refer to petroleum ether solutions in which pure lutein shows maxima at 477.5, 448 mμ, pure β-carotene at 484.5, 452.5 mμ.)

No.	Type of wheat	Commercial designation of the flour	Pigment in the total extract			Pigment in the Xanthophyll fraction			Pigment in the carotene containing fraction		
			mg./kg.	Spectrum (mμ)		mg./kg. (minimum figures)	Spectrum (mμ)		mg./kg.	Spectrum (mμ)	
1	California White Wheat	Local Entire	2.0	477.5	447.5	1.4	478	448	0.04	484	452.5
2	"	" "	1.9	477	447	1.3	477	448	0.04	484	452.5
3	Dark Northern Spring Wheat	Montana Entire	1.6	478.5	448.5	1.0	478	448	0.02	485	454.5
4	"	"	1.5	478	448.5	1.1	478.5	449	0.01	485	453.5
5	Dark Hard Winter Wheat	Eastern Entire	1.9	477	447	1.4	477.5	447	0.01	485	452.5

ly established by the fact that the main portion of the by-layers did not separate from added β-carotene in the mixed-chromatogram.

Large scale experiments with flour.--A series of such experiments carried out with 25-40 kilos followed in principle the method described above. Five-kilo portions of the flour were extracted in percolators, the weaker solutions being used for the next portion. About 1/2 liter of ether per kilo material was sufficient. Since the saponified solutions as used in this work were generally much more concentrated than those in the analytical experiments, large amounts of sterols and other colorless contaminants had to be eliminated. The latter were partially fluorescent and hence observable on the column, in ultraviolet light. Near 0° highly concentrated pigment solutions yielded abundant quantities of white crystals which were repeatedly centrifuged off in the cold room and washed with ice cold petroleum ether. The crystals can also be precipitated from light petroleum by alcohol at 0°. In this way it is easy to isolate 1/2 gram or more of long colorless crystals per kilo flour. Without such purifications the high viscosity of some xanthophyll containing solutions may delay the passing through the column and even obstruct it.

All layers were re-chromatographed several times on lime, the xanthophylls finally on calcium carbonate. In the course of these operations spontaneous pigment crystallization from petroleum ether repeatedly occurred but the crystals could in each case be identified as lutein. The carotene-containing layers were submitted to mixed chromatography with β-carotene

originating from another source; the larger by-layer showed identity. The upper, smaller layer showed absorption maxima at 480 and 450 mμ in petroleum ether. Subsequent investigations by Polgar and Zechmeister (20) on the isomerization of β-carotene allow this zone to be identified definitely as neo-β-carotene U. This is the only β-carotene isomer which is adsorbed above the all-trans form and possesses the proper absorption spectra (e.g., 481, 450 mμ). Since this isomer is easily produced from the all-trans-β-carotene, it is more probable that it was produced by isomerization than that it was present as such in the flour.

The details of the lengthy chromatographic fractionations of the xanthophylls can be omitted here. All total-xanthophyll layers were heterogeneous but it was impossible to separate any well-defined carotenoid reliably different from lutein. Some layers having lower spectra than lutein (e.g., 472.5, 442.5 mμ in petroleum ether) were partially reconverted into the latter by heating and must thus be qualified as neo-luteins formed during the operations (21). Other fractions showed approximately the maxima of lutein (e.g., 478.5, 450 mμ or 476.5, 446 mμ). Only in few cases was a higher spectrum found (481, 449 mμ), but the respective zones were very poor in dye. If the technic of iodine catalysis for the establishment of stereoisomeric identity had been available at the time of this investigation, a more satisfactory examination of the neo-luteins could have been made. This procedure would have allowed a simple qualitative check on whether or not a given pigment belonged to the lutein set. As applied in our laboratory at

Table 2

Carotenoids in 100% Whole Wheat Bread

(The weight ratio crust:crumb was 1:3 in the fresh bread.

Dry weights:crust 75%, crumb 65%.)

No.	Material (dried)	Pigment in the total extract			Pigment in the Xanthophyll fraction			Pigment in the carotene containing fraction		
		mg./kg.	Spectrum (mμ)		mg./kg. (minimum figures)	Spectrum (mμ)		mg./kg.	Spectrum (mμ)	
1a	Crust	0.28	478	447.5	0.18	477	446.5	0.005	485	453
21	Crust	0.32	477.5	446.5	0.20	476	446.5	0.005	484.5	463.5
1b	Crumb	0.39	476.5	447	0.29	477	447	0.005	484	451.5
2b	Crust	0.38	477.5	447.5	0.32	477	446.5	0.01	483	451.5

Table 3

Rye Flour Carotenoids

No.	Commercial designation of the flour	Pigment in the total extract			Pigment in the Xanthophyll fraction			Pigment in the carotene containing fraction		
		mg./kg.	Spectrum (mμ)		mg./kg. (minimum figures)	Spectrum (mμ)		mg./kg.	Spectrum (mμ)	
1	Oregon #1	2.1	478.5	447	1.6	477	447	0.03	485	453
2	Oregon #1	2.3	478	447	1.7	477	447.5	0.03	484.5	453
3	Pillsbury	2.7	479	448.5	1.8	478	448.5	0.05	484	452
4	Pillsbury	2.8	479	449	1.8	477.5	448.5	0.06	482	452

the present, it is only necessary to add a trace of iodine to a solution and observe the spectral shift. Within a minute the solution of any isomer attains a characteristic equilibrium value that approximates the absorption spectrum of the all-trans member of the set. Once this equilibrium spectrum has been determined for one member of a stereoisomeric set, it can be used in the identification of any other.

Bread.--The crust and crumb samples were dried overnight at 45°, milled, extracted in 1-2.5 kilo portions with ether and worked up as described above for small scale experiments (Table 2).

Rye flour and bread.--These samples were treated in the same manner as the small scale experiments with whole wheat flour and bread. All chromatograms were analogous to those described and the presence of β-carotene was confirmed by mixed chromatography (Table 3).

Summary

The carotene content of some freshly milled, unbleached American whole wheat flours is, as estimated by means of chromatography, of the order of magnitude of 0.02 to 0.06 mg. β-carotene per kilo. Whole wheat bread is even much poorer in carotene. Thus wheat flour and bread are of no importance as provitamin A sources in human nutrition.

The results seem to be in accord with those reported by Pulkki and Puutula (22). An abstract of this work became available after this paper was in print.

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II. ISOLATION OF PROLYCOPENE AND PRO-γ-CAROTENE FROM EVONYMUS FORTUNEI

II. ISOLATION OF PROLYCOPENE AND PRO-γ-CAROTENE FROM EVONYMUS FORTUNEI

Introduction

It has been reported (1) that the ripe seeds of Evonymus europaeus L. (Celastraceae) contain unesterified zeaxanthin, $C_{40}H_{56}O_2$, as the main polyene pigment. The same statement is valid for Evonymus fortunei, var. color., Rehd., commonly termed "winter-creeper euonymus," from 1 kilo of which 1300 mg. of zeaxanthin were isolated. Upon evaporation of its saponified ether extract and addition of petroleum ether, abundant quantities of zeaxanthin crystallize. This section describes an investigation of some of the pigments (about 200 mg. per kilo, of which one-fourth is β-carotene) which remain in the mother liquor. The mixture can be resolved by chromatographic analysis. In addition to some twenty less interesting pigments, two representatives of a stereochemically new class of natural carotenoids (2) were separated; viz., prolycopene, $C_{40}H_{56}$, and pro-γ-carotene, $C_{40}H_{54}$. The yields of pure crystals were 11 mg. and 0.5 mg. per kilo of seeds respectively. Hence, Evonymus fortunei may serve as a source of prolycopene while it does not offer any larger yield of pro-γ-carotene than does the fruit of Butia capitata (3). According to Zechmeister and Schroeder (4) Pyracantha angustifolia is the best starting material for the isolation of pro-γ-carotene at the present time.

Interest in the pro-carotenoids was aroused by the presence of a largely cis-configuration in these pigments. Of the

seven sterically available double bonds in prolycopene, at least five or six must be cis to explain the large deviation of the spectral maxima from all-trans-lycopene. Likewise pro-γ-carotene must possess four or five of its six possible bonds in cis position. When a trace of iodine is added to a petroleum ether (b.p. 60-70°)solution of these pigments, an immediate shift of about 33 mμ toward longer wave-length in the position of the absorption maxima is noted. The new spectrum is that of a complex equilibrium mixture of stereoisomers in which the respective all-trans pigments are prevalent.

Previous to the isolation of prolycopene from the tangerine tomato (a variety of Lycopersicum esculentum), no C_{40}-carotenoid had been observed in nature which possessed a partially cis configuration. Investigations of many plant materials soon led to the discovery of pro-γ-carotene in the fruit of Butia eriospatha and Butia capitata (Becc.). The prefix "pro" was used for these new pigments to differentiate them from the artificially produced "neo"-carotenoids. Both prolycopene and pro-γ-carotene crystallize easily and are fairly heat resistant in this form. Their solutions undergo isomerization when heated or exposed to brilliant sunlight as well as when catalyzed by iodine. Compared to their all-trans forms, these isomers are more soluble and possess lower melting points.

The large number of cis double bonds present in these new pigments may be contrasted to the configurations of bixin and crocetin. Each of these carotenoid carboxylic acids was isolated in two crystalline forms termed "stable" or "trans"

and "labile" or "cis". However, the small spectral differences between the two types indicate that only one double bond has the cis configuration in these cases. A re-investigation of the stereochemistry of methylbixin is reported in the third section of this thesis.

Experimental

The material was collected in Denton, Texas. The intensely orange-red hulls of 1 kilo of seeds were scraped off by rubbing between two layers of wire gauze in a mortar. Small particles of hull remained on the stones and were neglected. The pigment- and lipid-rich hulls were ground with sand and extracted with peroxide-free ether by repeated shaking. The dark extract (2 liters) was saponified over concentrated methanolic potassium hydroxide for 20 hours, then washed alkali-free, dried with sodium sulfate, and evaporated *in vacuo* at 40° as far as possible. To the dark, partially crystalline residue, petroleum ether was repeatedly added and evaporated. Finally, the oily residue was dissolved in the minimum volume of chloroform. On addition of 3 volumes of petroleum ether the main bulk of zeaxanthin crystallized out.

The mother liquor was poured on calcium hydroxide (Shell brand lime, chemical hydrate, 98 per cent passing through a 325 mesh screen) in a percolator (45 x 20 x 8 cm.). After washing the chloroform from the adsorbent with petroleum ether, the chromatogram was developed in the course of 3 hours with petroleum ether containing 2 per cent and later 3 per cent acetone. The light orange filtrate was discarded; it contained among other polyenes a portion of the β-carotene.

The cone was cut into three parts. The upper section (160 mm. from the top) was composed of an orange-brown (20 mm.) and a red (140 mm.) part, both of which were heterogeneous. Then followed an orange section (100 mm.) containing the two "pro" compounds and some minor pigments. In the

lowest section β-carotene predominated (60 mg.). The middle section was eluted with alcohol, transferred to petroleum ether, and developed on a calcium hydroxide column (28 x 7 cm.) with 1 liter of petroleum ether containing 2.5 per cent acetone and then with 0.5 liter containing 4 per cent. The following chromatogram appeared (on the left side the height of the zones is given).

55	mm.,	pink
40	"	orange, contained prolycopene
4	"	greenish yellow
3	"	light orange
5	"	yellow
20	"	orange, contained pro-γ-carotene
7	"	greenish yellow
15	"	faint orange
0.5	"	green line
15	"	colorless
7	"	pink, β-carotene

Prolycopene.--This zone was cut out, eluted with ether, dried, and evaporated in vacuo. The residue was dissolved in the minimum amount of benzene and crystallized in a centrifuge tube by cautious addition of several volumes of methanol. The microscope showed typical prolycopene crystals intermixed with much colorless crystalline material. The latter could not be removed by recystallization from benzene and methanol and only partially by treatment with methanol at 40°. It was almost completely removed by short centrifuging at slow speed. The heavy pigment crystals settled and the suspended colorless com-

pound was decanted. Minor amounts of prolycopene in the decanted liquid were recovered by repeating the process. The last trace of the contaminant was removed by a short treatment with methanol at 40° and rapid centrifuging. The yield was 11 mg.; m.p., 109-110° (corrected; in a sealed tube filled with CO_2). For the purpose of analysis the sample was dried at about 45° in a high vacuum for 45 minutes; it was free of ash.

Analysis--$C_{40}H_{56}$. Calculated. C 89.48, H 10.52

Found. " 89.00, " 10.72

Mol. wt., calculated, 537; found, 529 (in exaltone)

In a mixed chromatogram the pigment did not separate from prolycopene obtained from tangerine tomatoes (2). The spectral maxima of fresh solutions were: in carbon disulfide 500.5, 469.5 mμ (after the addition of iodine, 540.5, 500.5, 466 mμ); and in petroleum ether 470, 442 mμ (with iodine, 501, 469, 441 mμ).

Pro-γ-carotene.--This zone of the above chromatogram was eluted with alcohol, transferred into 20 cc. of petroleum ether, and rechromatographed on a calcium hydroxide column (27 x 5 cm.). Minor layers located both above and below the main orange pigment were discarded and the latter was rechromatographed on a smaller column (20 x 3 cm.) of the same adsorbent. This showed only traces of other pigments, much above pro-γ-carotene. The latter was eluted with ether and the evaporation residue was crystallized from benzene and methanol as described for prolycopene. No colorless contaminant was present. The yield was 0.5 mg.; the crystal form was typical for pro-γ-carotene. In a mixed chromatogram no separation took place

from a sample isolated from Pyracantha angustifolia (4). Spectral maxima in carbon disulfide were 492.5, 459 mμ (with iodine, 527.5, 490 mμ) and in petroleum ether, 461.5, 431.5 mμ (with iodine, 490, 457.5 mμ).

Summary

From 1 kilo of the ripe seeds of Evonymus fortunei Rehd., 11 mg. of prolycopene, $C_{40}H_{56}$, and 0.5 mg. of pro-γ-carotene, $C_{40}H_{56}$, have been obtained in crystalline form.

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III. THE STEREOCHEMISTRY OF METHYLBIXIN

III. THE STEREOCHEMISTRY OF METHYLBIXIN

Introduction

Bixin, the natural pigment extractable from the seeds of the Annato tree (Bixia orellana, L.), has found limited industrial application as a food pigment and as a dye for silk and cotton. Investigations concerning its chemical constitution extend from the early work of Etti (1) in 1874. The empirical formula, $C_{25}H_{30}O_4$, was established by Karrer, Helfenstein, Widmer, and van Itallie (2) as well as Kuhn and Ehmann (3) in 1929 and the true structural formula, by Kuhn and Winterstein (4). Without a doubt the most outstanding pioneer investigation of bixin was carried out by van Hasselt (5). He established that this pigment is the methyl derivative of a compound which he termed "norbixin". Both norbixin and bixin may be methylated with dimethyl sulphate to yield the same product, methylbixin. This clearly indicated the presence of two esterifiable groups in norbixin. Van Hasselt prepared methylethylnorbixin in two manners by ethylating bixin and by methylating ethylnorbixin and reported that the two products were not identical. This led him to state that the two esterifiable positions were not identical in norbixin. He also reported that a new isomer, "isobixin", could be obtained by the partial hydrolysis of methylbixin. This experiment could not be duplicated by Kuhn and Winterstein (6) or Karrer and Takahashi (7) so that the existence of this compound is problematical.

In 1923, Herzig and Faltis (8) observed that the esterification of norbixin with methanol in the presence of hydrogen chloride led to a methylbixin that differed from the usual

methylation product. This "β-methylbixin" was characterized by a higher melting point, longer wave-length absorption spectrum, and decreased solubility. Upon saponification, the corresponding β-bixin and β-norbixin showed analogous differences from the "natural" compounds. On one occasion Herzig and Faltis were able to extract a small quantity of β-bixin from annato seeds. Karrer, Helfenstein, Widmer, and van Itallie (2) interpreted this correctly as a case of geometrical isomerism and demonstrated that iodine was capable of converting the natural product into its isomeric form. According to Kuhn and Winterstein (4), catalytic amounts of iodine are sufficient in this isomerization. They also established the stereochemical nature of the change by preparing the same dihydrobixin from the two forms. Piperidine solutions of either derivative were easily oxidized by atmospheric oxygen to β-bixin. In view of the separations described in the experimental section of this thesis, it is interesting to note that Winterstein (9) reported without experimental data that the two bixin isomers were chromatographically separable.

Based upon the following data, Kuhn and Winterstein (10) assigned an asymmetric structural formula (Fig. 1). to bixin in 1928.

1. Bixin is the mono-methylester of a dicarboxylic acid (8).

2. The existence of two methylethylnorbixins indicates a non-symmetric molecule (5) (8).

3. Upon catalytic hydrogenation, nine double bonds are found (8).

4. Ozonolysis of methylbixin yields methylglyoxal and β-acetyl-acrylic acid-methylester (11). These indicate the

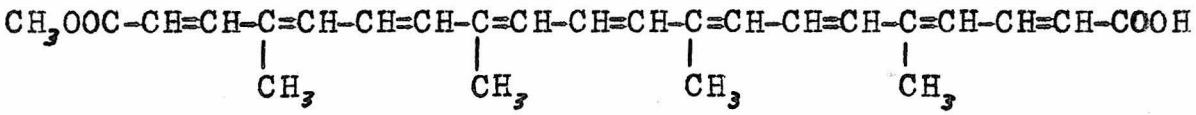


Figure 1. Asymmetric bixin formula.

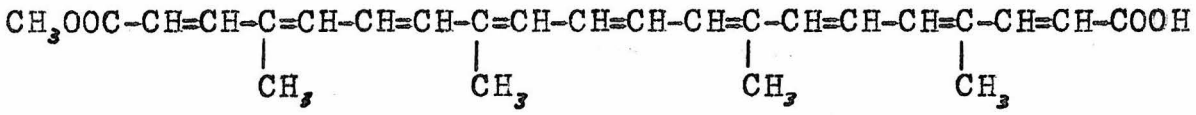


Figure 2. Symmetric bixin formula.

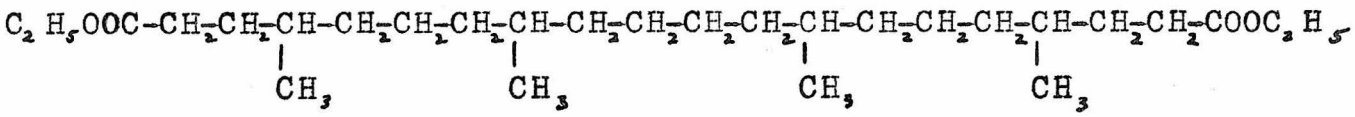
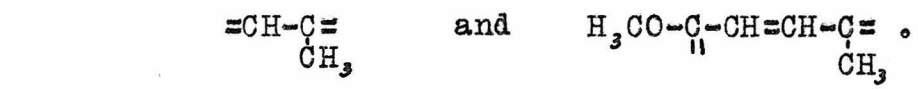
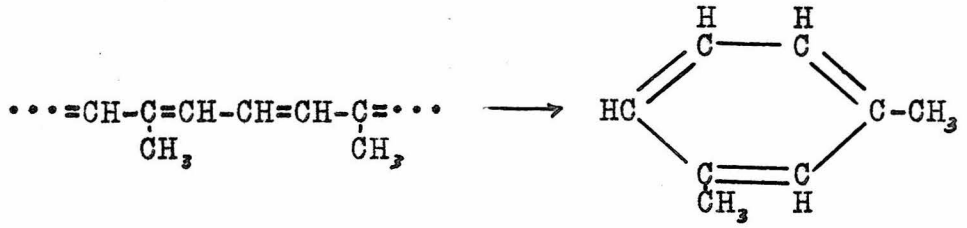


Figure 3. Perhydro-norbixin-diethylester.

presence of the following structures:



5. Thermal decomposition leads to the production of m-xylene (5).



6. The similarity of absorption spectra with that of β-carotene indicates an aliphatic structure (12) with conjugated double bonds.

Once the existence of stereoisomeric forms of bixin was recognized, it was no longer necessary to postulate differences in the structure of the two halves of the molecule. Kuhn and Winterstein (6) then proposed the accepted symmetric formula (Fig. 2). which agrees with the synthesis (13) of perhydro-norbixin-diethylester (Fig. 3). The synthetic product was found to be identical with that produced by the catalytic hydrogenation of diethylnorbixin. Additional evidence was supplied by the oxidation of lycopene to bixin-dialdehyde which was converted into trans-norbixin (14). The thermal decomposition products of bixin and lycopene (6) also supported this view. Thus, fifty-eight years after the first investigation of bixin, its structural formula was secured.

Previous stereochemical investigations of bixin.--Karrer and Solmssen (15) made the first attempt to determine the actual stereo-configuration of bixin. They carried out a controlled oxidation of bixin by shaking a benzene solution of the pigment

with an aqueous solution of potassium permanganate and sodium bicarbonate. The mixture of oxidized products was separated by chromatographic adsorption. Apo-1-norbixinal-methylester (Fig. 4) and apo-3-norbixinal-methylester (Fig. 6) were obtained in a yield of 1 to 7% of the starting material while only traces of apo-2-norbixinal-methylester (Fig. 5) were obtained. With natural (labile) bixin and trans (stable) bixin, the following conclusion was drawn:

> "Aus der Tatsache, dass die Apo-1-norbixinal-methylester und Apo-2-norbixinal-methylester aus labilem und stabilem Bixin verschieden, die Apo-3-norbixinal-methylester aber identisch sind, geht hervor, dass die Isomerie der beiden Bixine höchst wahrscheinlich auf verschiedener konfigurativer Ausbildung an derjenigen Doppelbindung beruht, die, von der unveresterten Carboxylgruppe des Bixins abgerechnet, die dritte in der Kette ist" (Fig. 7).

Both "labile and stable" apo-1-norbixinal-methylester as well as apo-3-norbixinal-methylester were obtained in sufficient quantity to be completely characterized by melting points, absorption spectra, and analysis of the oximes. It was found possible to convert the labile form into the stable form by the addition of iodine in a manner completely analogous to the conversion of the respective bixins. The corresponding apo-2-norbixinal-methylesters were never obtained in crystalline form but were characterized only by their spectra. It was reported that the addition of iodine to a solution of apo-2-norbixinal-methylester from natural bixin caused its absorption maxima to shift to those characteristic of the product obtained from trans-bixin.

The steric configuration proposed by Karrer and Solmssen is incompatible with the view of Pauling (16) that such a bond

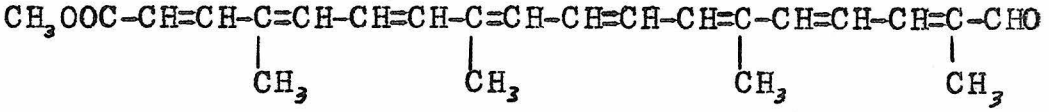


Figure 4. Apo-1-norbixinal-methylester.

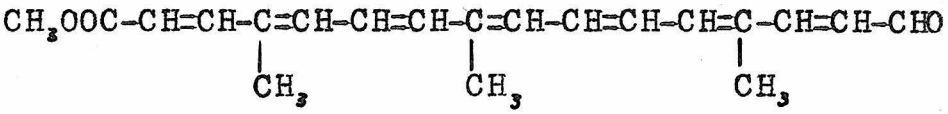


Figure 5. Apo-2-norbixinal-methylester.

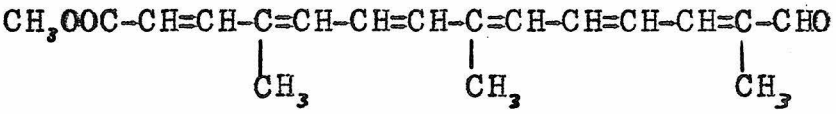


Figure 6. Apo-3-norbixinal-methylester.

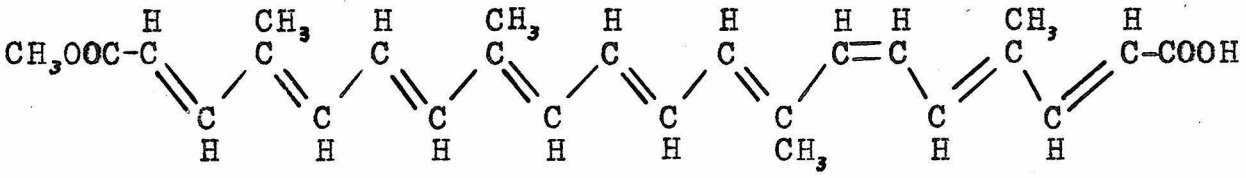


Figure 7. Labile bixin (according to Karrer and Solmssen).

can not assume cis configuration (17). Since resonance of the conjugated double bonds require that the system be coplanar, steric interaction of the hydrogen and methyl groups prevent a cis configuration of any double bond adjacent to a C-CH_3 group. The exclusion of such types leaves only five stereochemically effective double bonds in bixin. Hence, the total number of cis-trans isomers should be thirty-two for the unsymmetric bixin but only twenty for the symmetric methylbixin.

Stereochemical Investigation of Methylbixin.--Recent developments in the field of carotenoid isomerization have made a stereochemical re-investigation of bixin desirable. In the experimental section of this thesis, methylbixin has been used to avoid certain disadvantages of the natural product. Bixin is comparatively difficultly soluble in organic solvents and its free carboxyl group does not allow convenient chromatographic separation. The symmetrical nature of methylbixin makes it a simpler stereochemical investigation.

Even qualitative experiments indicated that the effect of iodine catalysis upon methylbixin was not a simple conversion to its all-trans form. Chromatographic adsorption revealed the presence of at least four stereoisomers. When each of these isomers was separately catalyzed with iodine, all gave the same qualitative and quantitative chromatograms. This was confirmed by the isolation of all-trans-methylbixin from each iodine catalyzed pigment.

Two new stereoisomers of methylbixin have been crystallized and a third has been observed in solution. Table 1 lists all of the members of this set which have been detected at the present

Table 1

Physical Characteristics of the Observed Stereoisomeric Methylbixins

	Melting Point	Absorption Spectra Petroleum Ether (mμ)		Benzene (mμ)	
Natural Methylbixin	161°	485	453.5	503	470
All-trans-methylbixin	198°	490	457	508.5	475
Neomethylbixin A	190°	485	454	502.5	469
Neomethylbixin B	...	471	444.5	491	458
Neomethylbixin C	150°	479.5	449	496	463

Table 2

Molecular Extinction Coefficients of Stereoisomeric Methylbixins in Benzene at Positions of Maxima and Minima Values

Natural Methylbixin

(mμ)	$E^{mol}_{cm} \times 10^{-4}$
284	1.2
320	0.4
366	1.2
378	1.1
445	8.5
452	8.1
471	12.8
488	8.0
503	11.7

All-trans-methylbixin

(mμ)	$E^{mol}_{cm} \times 10^{-4}$
297	2.5
316	0.3
363	0.8
374	0.7
448	8.7
454	8.6
475	13.0
493	8.5
508	12.2

Neomethylbixin A

(mμ)	$E^{mol}_{cm} \times 10^{-4}$
293	1.8
308	0.9
363	3.5
380	1.3
444-8	7.4
470	10.7
489	7.4
502	9.1

Neomethylbixin C

(mμ)	$E^{mol}_{cm} \times 10^{-4}$
297	2.0
314	0.9
363	2.2
378	1.5
438	7.0
446	6.8
465	9.8
482	6.6
495	8.2

with their melting points and visual spectra. Molecular extinction curves for the compounds and the mixtures of stereoisomers resulting from refluxing their solutions or iodine catalysis are given in Figures 8 to 12. Values characteristic of the molecular extinction coefficients at maxima and minima are listed in Table 2.

A great deal of preliminary investigation of adsorbents was necessary before a satisfactory material was found which allowed the separation of the stereoisomeric methylbixins. Initially, calcium hydroxide was used with petroleum ether-acetone development. While this system had been found applicable to a wide variety of other carotenoids, methylbixin gave poorly developed chromatograms. Benzene allowed a somewhat better development. However, it was found necessary to abandon calcium hydroxide when experiments revealed that the methylbixin adsorbates were incompletely eluted by acetone, alcohol, methanol, or ether. A number of calcium carbonate preparations were investigated before one (Merck, Heavy Powder) was found which showed promising differentiating power. Even in the best cases, only a few millimeters of separation were attained between zones of stereoisomers. Fortunately, adjacent zones of the chromatogram differed sufficiently in their color to make a clean separation of the individual adsorbates possible.

At times a tendency of a few zones to undergo further differentiation was noted. However, the pigments isolated from such sections showed no differences in melting points or spectra and there seems to be no justification for considering them

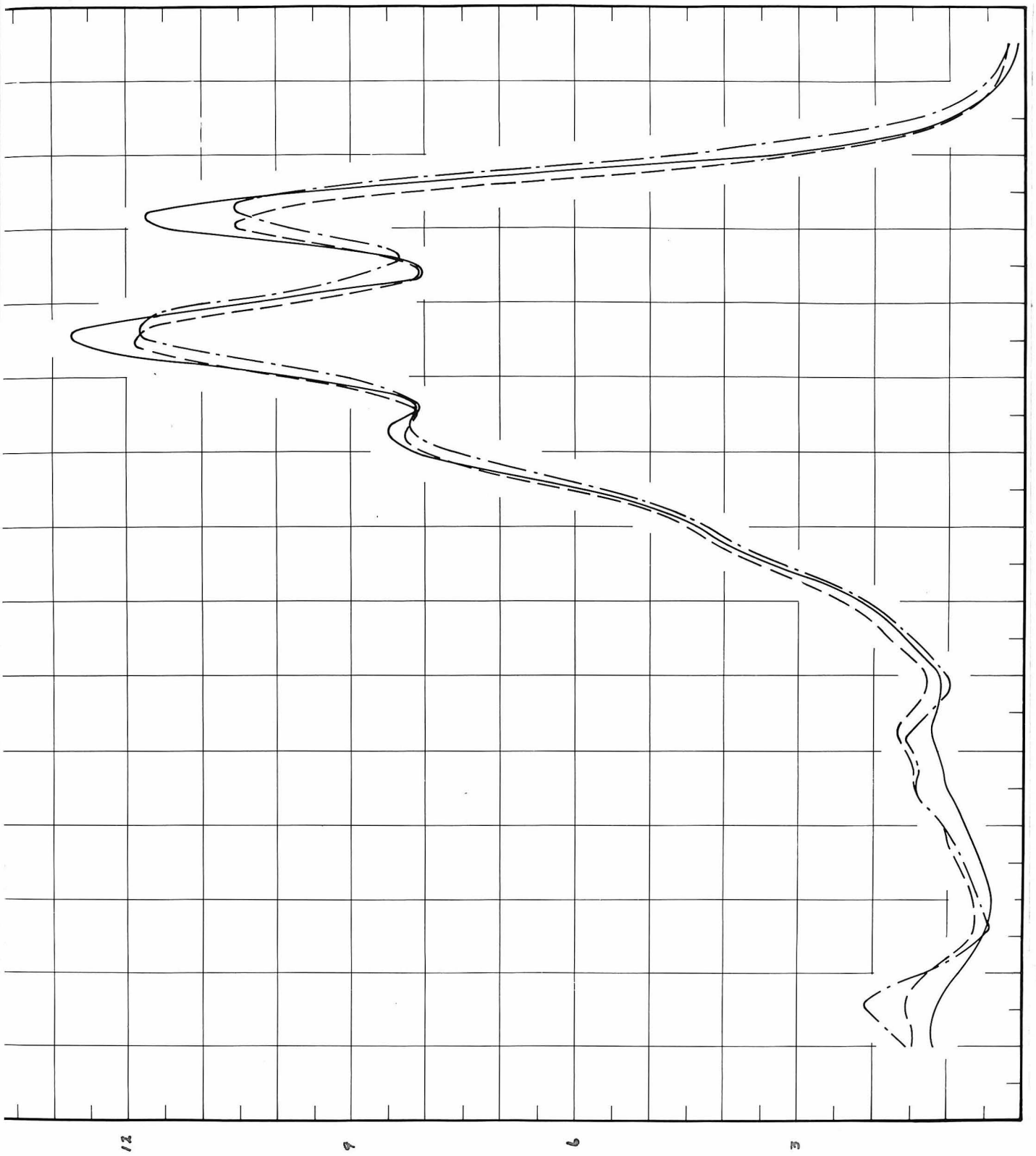


Figure 8. Molecular extinction curves of natural methylbixin in benzene: ——— fresh solution of crystals, - - - mixture of stereo-isomers after refluxing, and -·-·-· after iodine catalysis.

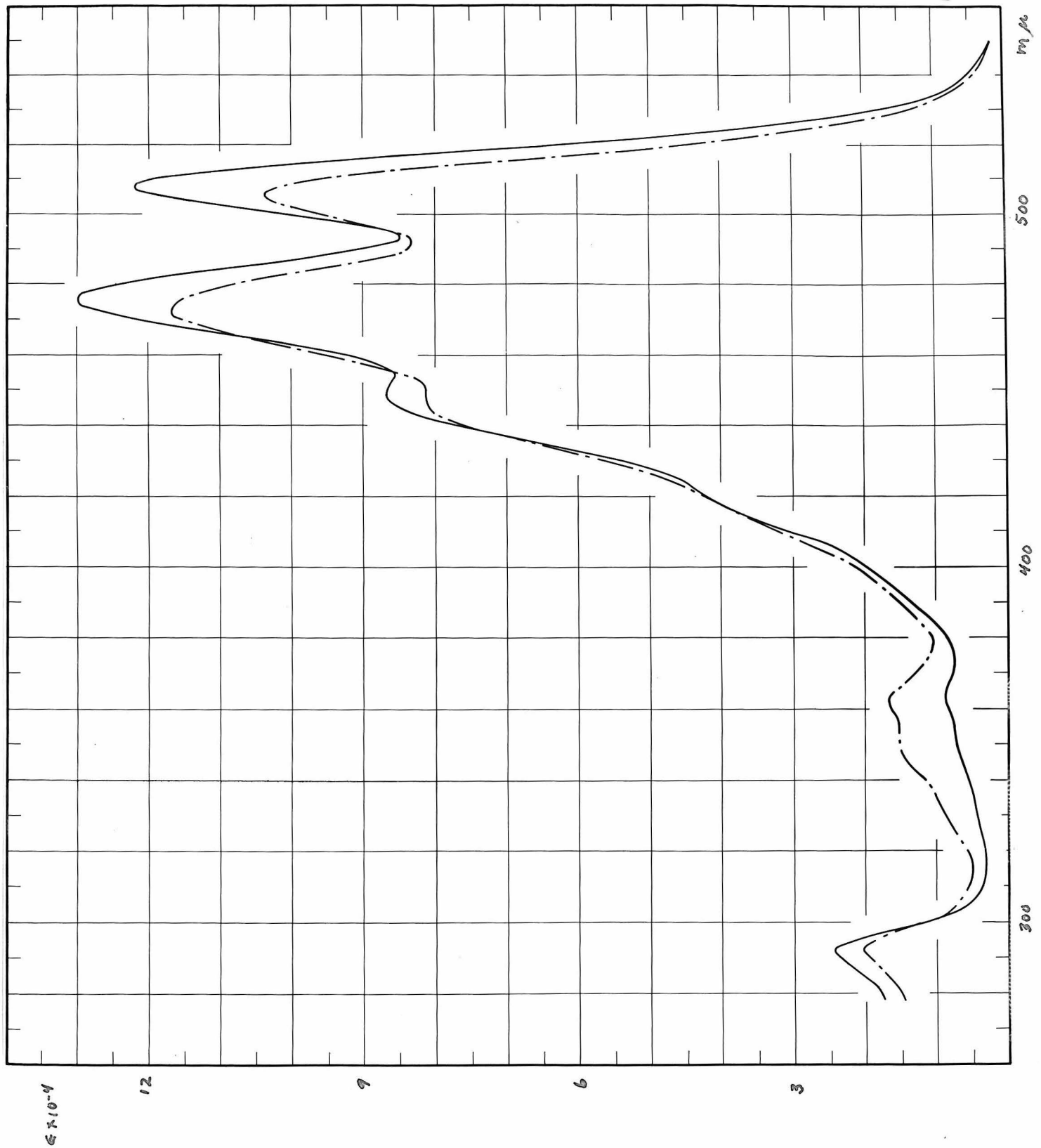


Figure 9. Molecular extinction curves of all-trans-methylbixin in benzene: ——— fresh solution of crystals, ·-·-·- mixture of stereoisomers after refluxing or iodine catalysis.

to be different stereoisomers. Prolonged development reunited many of these zones into chromatographically homogeneous samples. The cause of this phenomenon is unknown.

Each of the four crystalline stereoisomers was subjected to the four isomerization procedures described in detail in the experimental section. Both natural and all-trans-methylbixins were found to be very stable when exposed to sunshine, "insolation", while neomethylbixins A and C undergo considerable isomerization. Only a single isomer was produced in appreciable amounts from any given pigment. When benzene solutions of the pigments were refluxed, only one-third of the natural or all-trans-methylbixin isomerized while two-thirds of the neomethylbixins A and C underwent stereochemical changes.

Melting the crystals of the stereoisomeric methylbixins caused extensive destruction. When kept at its melting point for fifteen minutes, 95% of natural methylbixin was destroyed. It was found possible to decrease these losses to 25% by shortening the duration of the melt to one minute. Chromatograms of melt isomerizations always contained zones of irreversibly formed pigments that did not belong to the methylbixin set. One of these zones was adsorbed within the neomethylbixin B region and could not be separated from it conveniently. For this reason, the colorimetric values for neo B are high in such experiments. Only all-trans-methylbixin showed appreciable thermal stability under these conditions.

All members of the methylbixin set gave the same iodine equilibrium within the limits of experimental error. This allows its molar extinction curve to be used for the determina-

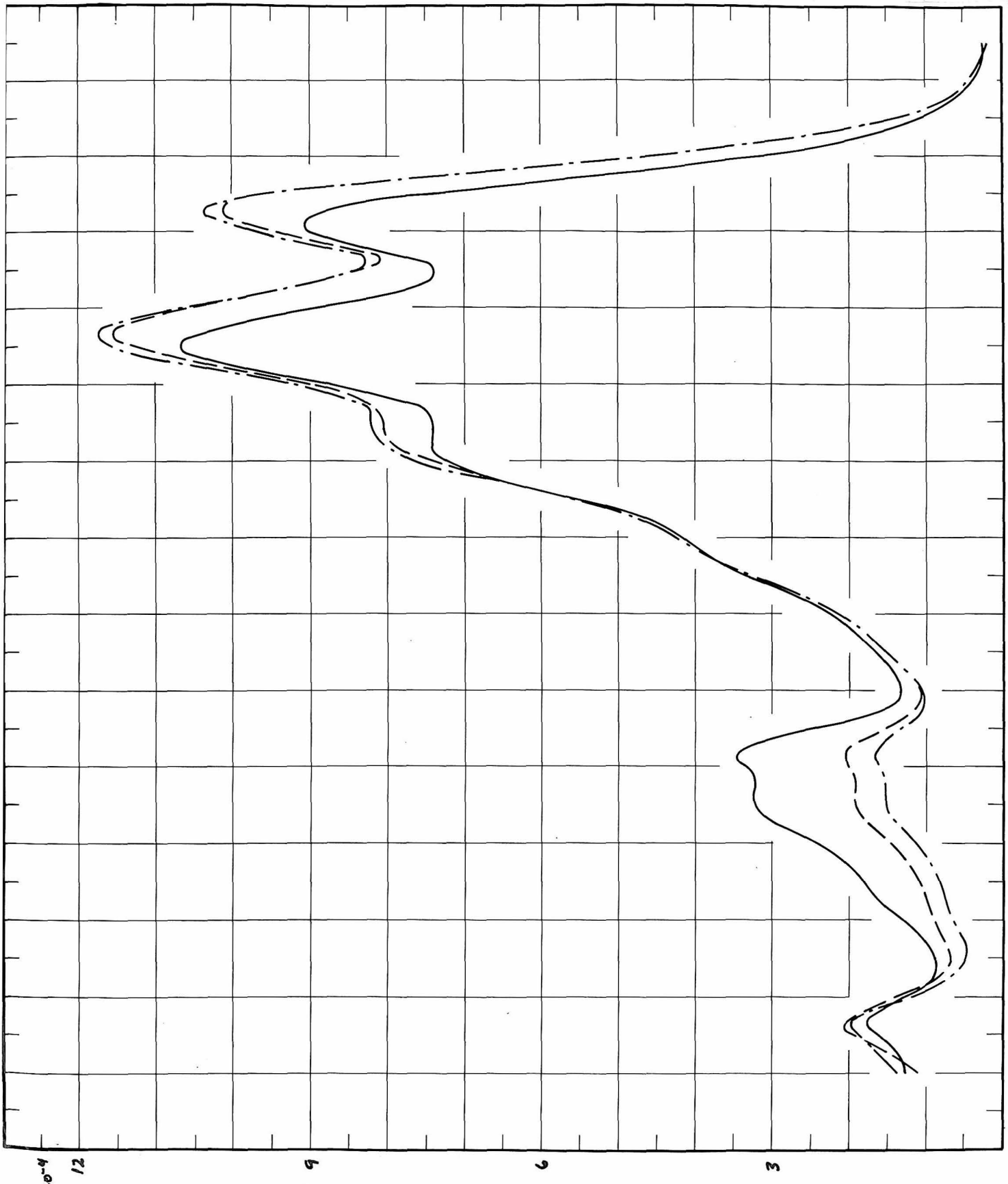


Figure 10. Molecular extinction curves of neomethylbixin A in benzene: ——— fresh solution of crystals, - - - mixture of stereoisomers after refluxing, and -.-.- after iodine catalysis.

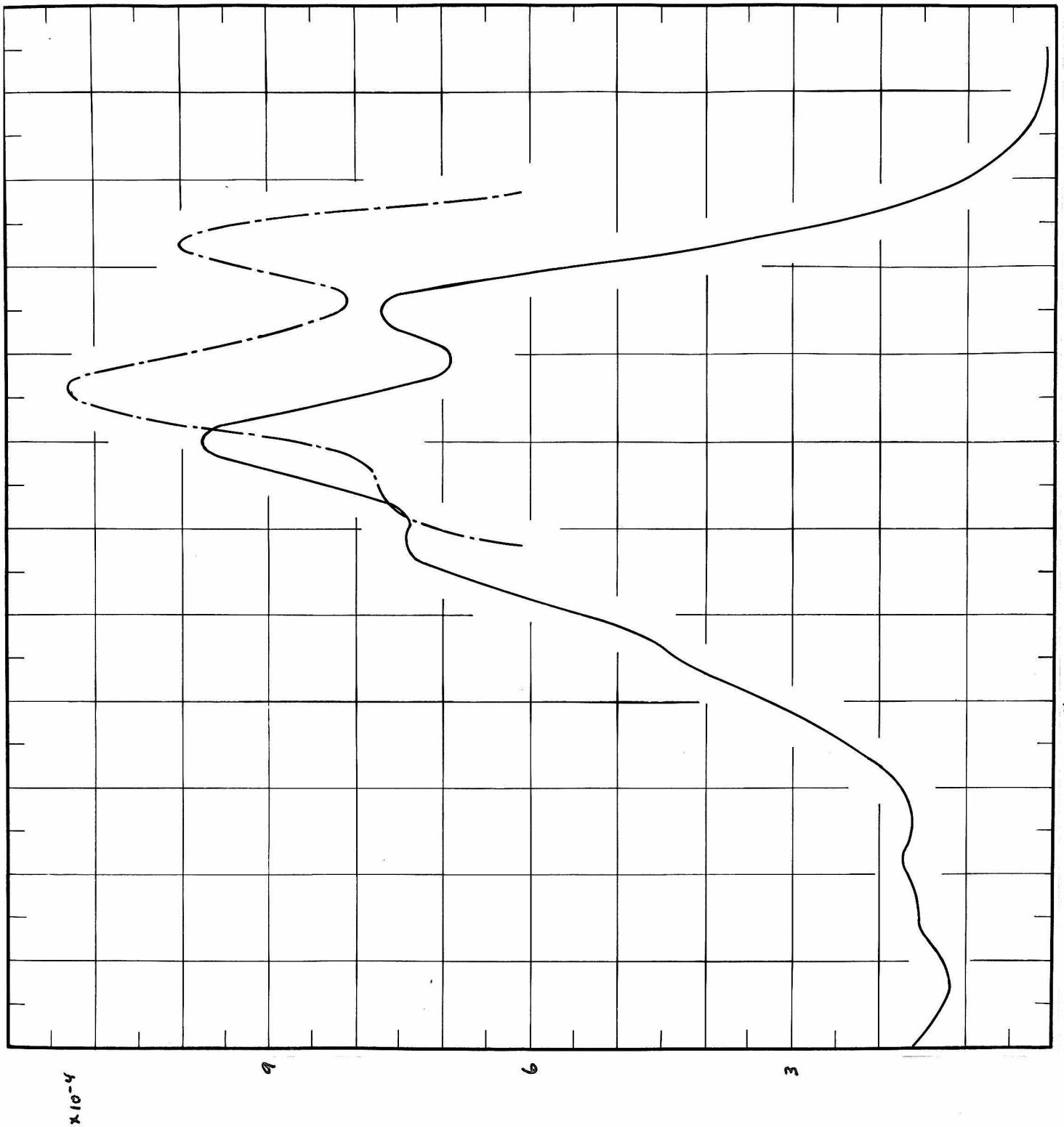
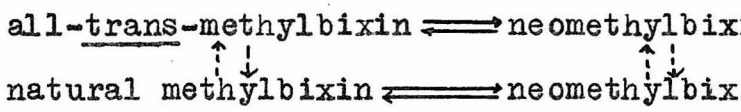


Figure 11. Molecular extinction curve of neomethylbixin B in benzene: —— fresh solution, and -•-•- after iodine catalysis.

tion of pigment concentrations. Illumination has a great effect upon the rate of isomerization in iodine catalyzed solutions (Fig. 14). It is to be noted that natural methylbixin does not appear in this equilibrium.

If the iodine equilibrium is excluded from consideration, the following remarkable relationship is noted between the members of the set. The isomerization of natural methylbixin leads to the production of neomethylbixin C as the principal product and vice versa. This same type of easy interconversion is noted with all-trans-methylbixin and neomethylbixin A. These results are illustrated in the following diagram with full arrows indicating the most probable isomerization product.



Neomethylbixin B occurs to such a minor extent in these isomerization mixtures that its relationship to these two systems can not be decided.

Tentative Assignment of Configurations.--When a double bond assumes a cis configuration, the hydrogen atoms of the adjacent C-H groups overlap their normal van der Waals radii. This leads to a repulsion that tends to push the molecule out of complete coplanarity and results in a shift of the absorption maxima toward shorter wave-lengths. Since many neo-carotenoids differ in absorption spectra from their all-trans form by about 5 mμ, this shift has been interpreted as being due to the cis rotation of one trans bond. (16) (17). An examination of the spectral differences of the stereoisomeric methylbixins indicates that this figure is also valid in this set. Thus,

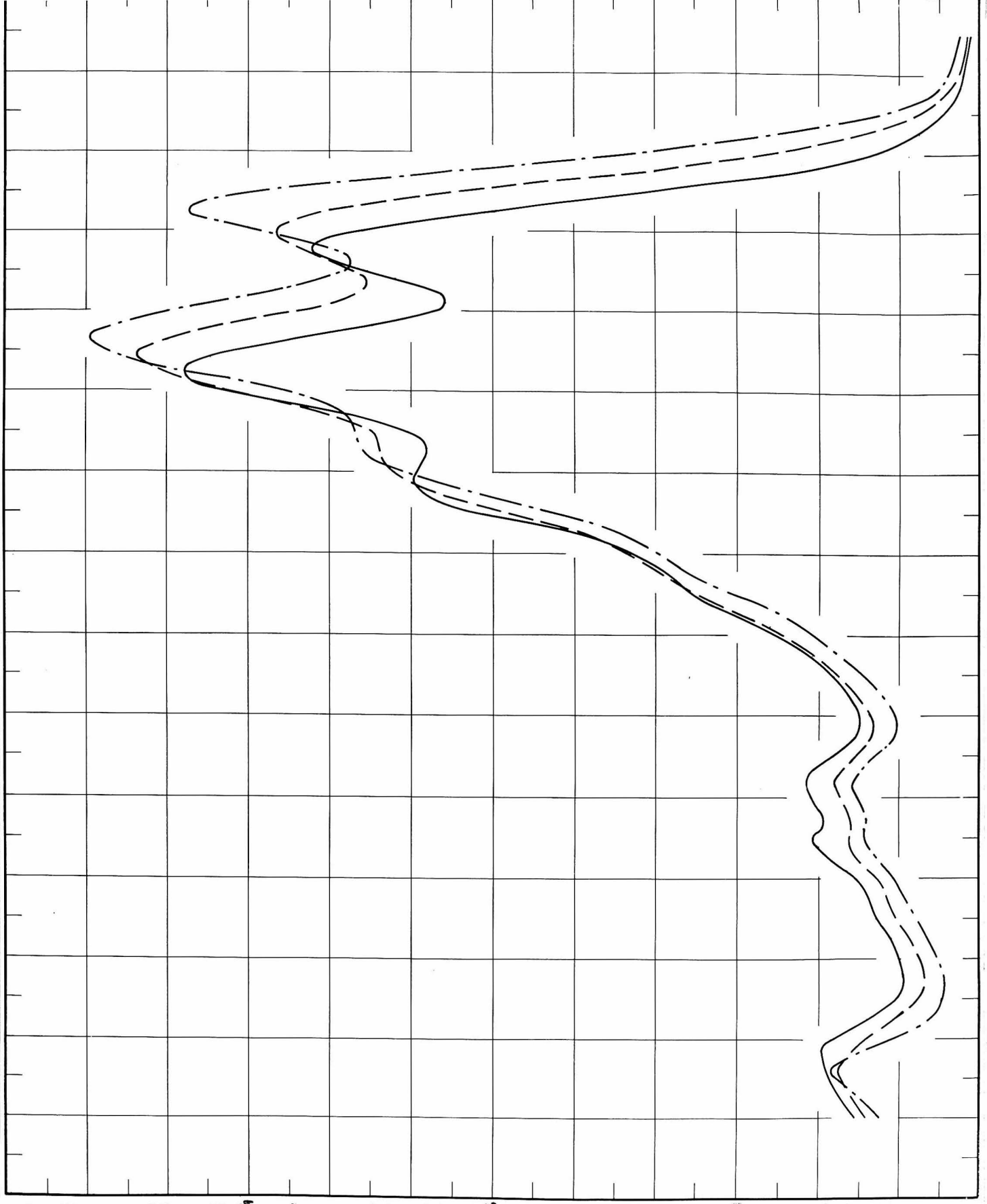


Figure 12. Molecular extinction curves of neomethylbixin C in benzene: ——— fresh solution of crystals, - - - mixture of stereoisomers after refluxing, and -.-.- after iodine catalysis.

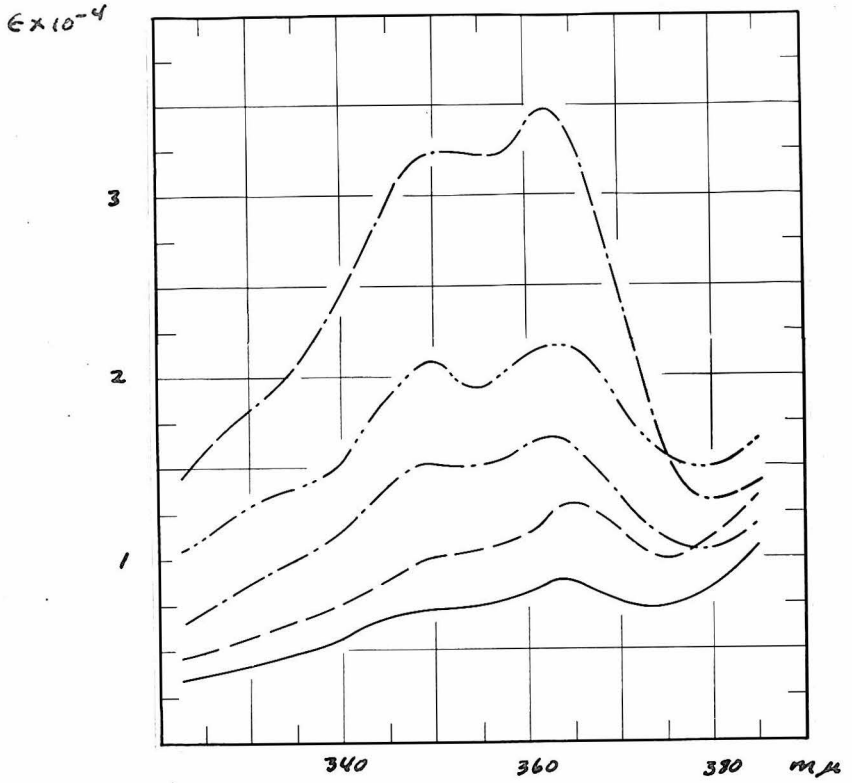


Figure 13. Molecular extinction curves of methylbixin stereoisomers in the cis-peak region (in benzene): ——— all-trans, - - - natural, -•-•-•- neo C, - -•- -• neo A, and -•-•- iodine equilibrium.

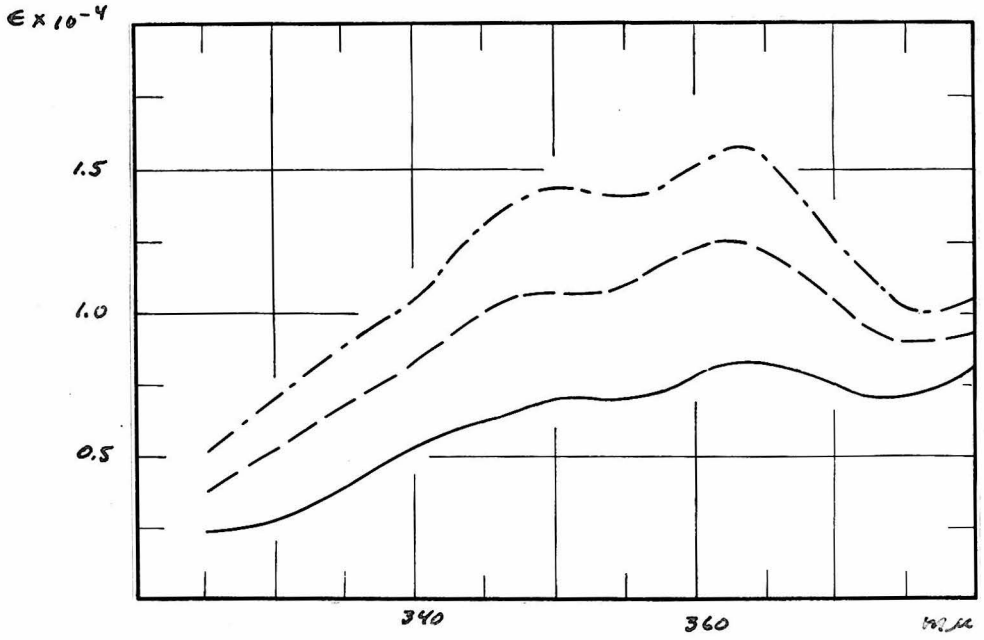


Figure 14. Effect of irradiation of the iodine catalyzed equilibrium: ——— molecular extinction curve of all-trans methylbixin after standing 30 minutes in darkness with iodine (in benzene), - - - after 5 seconds of irradiation, and -•-•- after 30 seconds or longer irradiation.

natural methylbixin and neomethylbixin A differ by 5 mμ in petroleum ether from the all-trans form and should be classified as mono-cis-methylbixins. The 10.5 mμ difference noted for neomethylbixin C suggests a di-cis-methylbixin; the 19 mμ difference of neomethylbixin B would be consistent with a tetra-cis-methylbixin.

Zechmeister and Polgár (18) noted that when iodine was added to an all-trans carotenoid, certain changes occurred in the molecular extinction curves. In general, the height of the visual maxima decreased and shifted toward shorter wavelength values while a distinct increase in absorption was observed in a certain region of the ultra-violet. This behavior has been interpreted as being due to the formation of isomers having appreciable absorption maxima in this region from the the all-trans pigment. The development of this "cis-peak" in iodine catalyzed solutions of all-trans-methylbixin and its dependence upon light are illustrated in Figure 14.

Theoretical considerations of this effect by Zechmeister, LeRosen, Schroeder, Polgár, and Pauling are now in press. It has been shown that the cis-peak offers certain measure of the deviation of the molecule from a linear shape. Thus, all-trans pigments and pro-carotenoids show little absorption in this region. A maximum effect is to be expected with the central, mono-cis-isomer. If one cis double bond is located near the end of the conjugated system, little effect will be noted.

Figure 13 shows extinction curves in the cis-peak region for the four crystalline methylbixins and their iodine equilibrium. Natural methylbixin is seen to possess a cis-peak only

slightly greater than the all-trans form. Since the centrally located double bond, 5, is excluded by the existence of two methylethylnorbixins, a choice must be made between the 2 and 4 stereochemically effective double bonds. The small cis-peak observed would not be expected for 4-cis-methylbixin so the configuration of 2-cis-methylbixin has been assigned to natural methylbixin (Fig. 15, II).

Neomethylbixin C is a di-cis isomer with a distinct cis-peak. The interconversions of neomethylbixin C and natural methylbixin suggest that both isomers possess a 2-cis double bond and that a rotation about a central position produces the other isomer. A centrally located double bond must be cis in neomethylbixin C to explain the height of its cis-peak. While the structures 2,4- or 2,6-di-cis-methylbixin can not be excluded, the most probable structure is 2,5-di-cis-methylbixin (Fig. 15, IV).

Neomethylbixin A has been assigned the structure of 5-cis-methylbixin (Fig. 15, III) in view of its maximum cis-peak effect. The isomerization of this pigment in the melt to yield moderate amounts of neomethylbixin C supports the assignment of 2,5- rather than 2,4- or 2,6-di-cis-methylbixin for neo C.

Since all of the possible tetra-cis isomers are expected to be more or less linear, a measurement of the cis-peak of neomethylbixin B will not allow preference to be given to any one of the three possible configurations. The cis-peak observed for neo B has not been included in Figure 13 since it is identical with the iodine equilibrium values which are given.

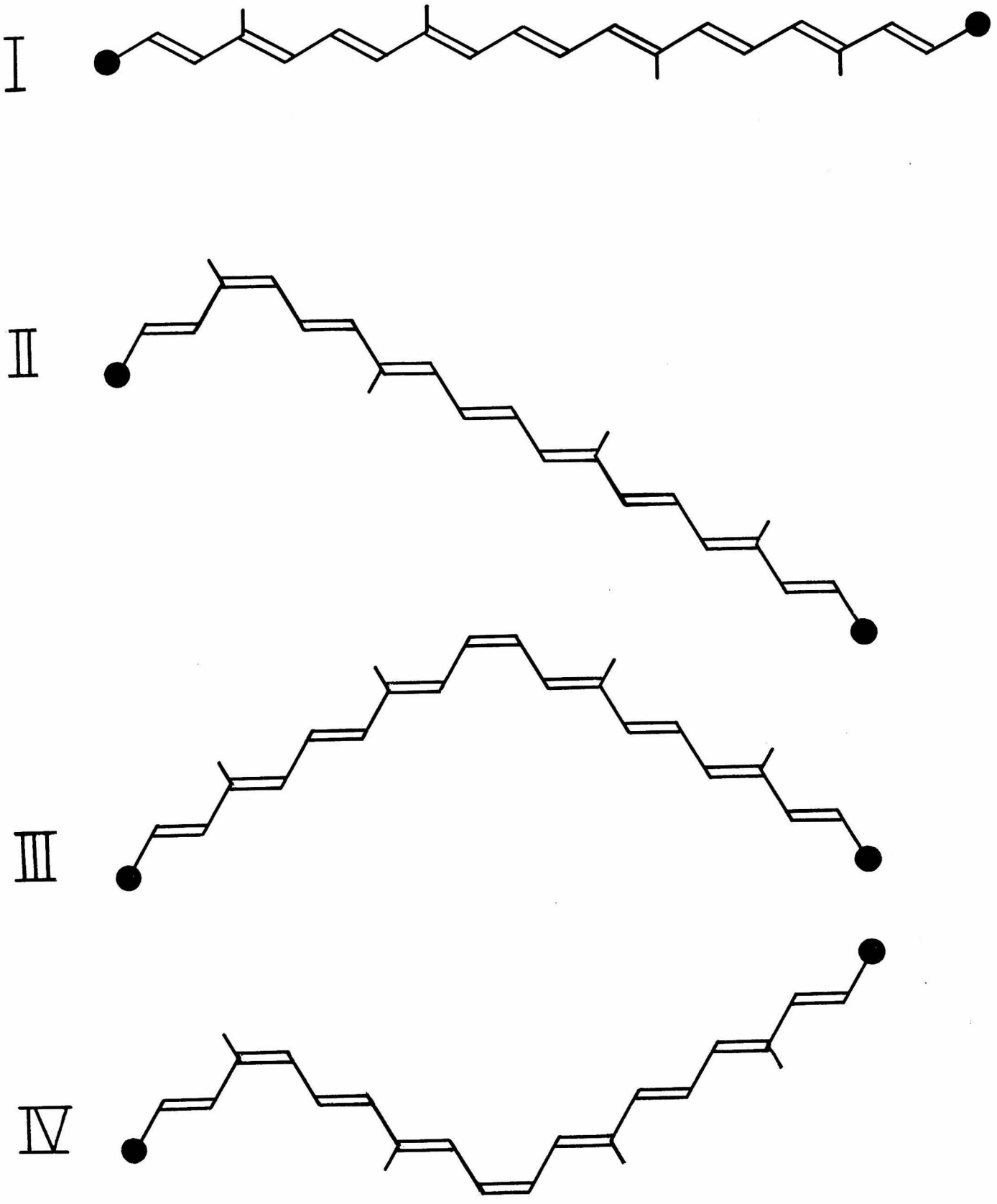


Figure 15. Probable configurations of the four crystalline methylbixins. I. All-trans-methylbixin. II. Natural methylbixin. III. Neomethylbixin A. IV. Neomethylbixin C.

Experimental

Materials and Methods.--The designation "petroleum ether" refers to Skellysolve B (b.p. 60-70°). Calcium carbonate (Merck, Heavy Powder) was used as the adsorbent. Bixin is so strongly adsorbed on this material even from benzene solutions that a separation from methylbixin is easily accomplished. Ether or methanol do not completely elute these bixin adsorbates. For the separation of the methylbixin stereosiomers, development with a benzene-petroleum ether mixture (1:3) was used unless otherwise specified. These pigments can be conveniently chromatographed when dissolved in any mixture of benzene and petroleum ether if the former does not exceed 25%; a lower benzene content is distinctly advantageous since it secures a narrow pigment zone for the subsequent development. The best available eluent is acetone. Spontaneous crystallization may occur from petroleum ether solutions.

For melting point determinations, the pigments were sealed in tubes filled with carbon dioxide. The samples were introduced into an electrically heated Berl-block 20° below the melting point and the temperature was increased 2 to 3° per minute.

Refluxing experiments were carried out in darkness while a slow stream of carbon dioxide was introduced into the all-glass apparatus. In isomerization experiments by melting crystals, the pigments were sealed under carbon dioxide, kept fused in a dibutylphthalate bath, and then cooled rapidly in ice water. The iodine catalyzed solutions (contained in 25 ml. glass volumetric flasks) were exposed to diffuse daylight for thirty minutes or illuminated by a 3500° white fluorescent Mazda lamp

(tube length, 120 cm.) at 60 cm. distance for fifteen minutes. This same procedure was used in the experiment illustrated by Figure 14. Transparent quartz test tubes (22 mm. diameter) from which the air had been displaced by carbon dioxide were used in the insolation experiments. Concentrations were determined upon iodine catalysis in a Pulfrich Gradation Photometer (light filter S47) on the basis of the following values:

k	0.0	0.2	0.4	0.6	0.8	1.0
mg. pigment in 100 ml. benzene	0.00	0.065	0.145	0.215	0.315	0.390

Visual spectra were taken with an Evaluating Grating Spectroscope (Zeiss; light filter BG-7, 2 mm. thick). The spectral data refer to petroleum ether solution unless otherwise indicated. For this purpose, other solvents were displaced from the column by washing with petroleum ether immediately before extrusion. Each pigment listed below in the chromatograms as a member of the methylbixin set shifted its maxima to 488, 455 mμ ($\pm$0.5 mμ) when catalyzed with iodine. The extinction curves were determined in a Beckman Photoelectric Spectrophotometer (19). All crystalline samples were dried in high vacuum. The concentration of solutions was established in many cases from the molar extinction curve of the iodine equilibrium with an accuracy of $\pm$2.6%. When it was desired to obtain molar extinction curves of chromatographic eluates, the following technic was used. After cutting, the adsorbates were washed with petroleum ether (b.p. 28-38°) and sucked dry with a vacuum pump. Then the pigment was eluted with ice cold acetone and transferred into benzene.

1. Natural Methylbixin

Isolation.--The usual procedures of preparation were modified in order to avoid higher temperatures as well as the isolation of bixin as an intermediate. The final product was chemically and stereochemically identical with samples obtained by the methylation of bixin with diazomethane or dimethyl sulfate.

The Bixia seeds (250 g.) were shaken mechanically for about 1/4 hour with 1.5 g. of potassium hydroxide in 400 ml. of absolute methanol until the pigmented coating was removed from the stones. The dark red solution was filtered and diluted with 200 ml. of amhydrous methyl acetate. Ten milliliters of dimethyl sulfate was added and the liquid was allowed to stand overnight. Upon longer standing, oily material may appear. Purple crystals which were contaminated with bixin separated and were re-methylated. The crystals (2.5 to 3 g.) were extracted by alternative shakings with equal volumes of chloroform and benzene. The combined and filtered solution (100 ml.) was drawn into a column (23 x 4.8 cm.); the methylbixin was washed into the filtrate with pure benzene. After concentration to 25 ml. and addition of 90 ml. of methanol, pure methylbixin crystallized (Fig. 16).

The solution was cooled to -10° and the crystals were filtered, washed with ice cold methanol, and dried in high vacuum. M.p. 161-161.5° (cor.). The yield was 1.4 g. but it is dependent upon the quality of the seeds.

Analysis--Calculated for $C_{24}H_{24}O_2(OCH_3)_2$: C, 76.44; H, 7.90; OCH_3, 15.19.

Found: C, 76.45, 76.33; H, 7.93, 7.98; OCH_3, 14.98, 15.08.

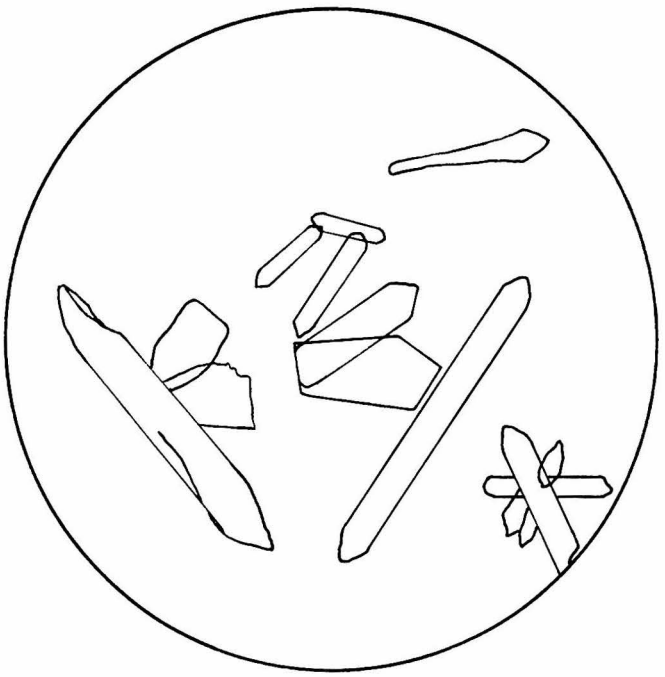


Figure 16. Natural methylbixin crystallized from benzene and methanol (100x).

(a) Cis-trans Isomerization of Natural Methylbixin upon Standing and Refluxing.--The extent of spontaneous isomerization in benzene-petroleum ether solution at 25° is about 3% in 24 hours. It must be remarked that even fresh solutions occasionally show a separation into blurred regions when chromatographed. The fractions could not be differentiated by any means.

A solution of 5 mg. of methylbixin in 20 ml. of benzene was refluxed for one hour, concentrated to 2 ml., diluted with 15 ml. of petroleum ether, and chromatographed (18 x 1.9 cm.). Figures to the left of the description always designate the zone width in millimeters.

The colorimetric ratio of unchanged starting material: stereoisomers (essentially neo C) was 74:26.

25 colorless
34 orange-red, unchanged methylbixin: 486, 454 mμ
1 colorless
1 pink, all-trans: 488.5, 456 mμ
1 orange-red, neo A: 485, 453 mμ
1 orange, neo B (?)
1 colorless
40 yellow-orange, neo C: 479, 449 mμ

(b) Cis-trans Isomerization upon Melting Crystals of Natural Methylbixin.--When the pigment was kept fused for fifteen minutes, 95% of the initial color intensity disappeared. However, fusion for one minute was found to be satisfactory. Eight milligrams was kept at 165°, then dissolved in 5 ml. of benzene, diluted with 3 volumes of petroleum ether, and developed on a column (18 x 2.5 cm.).

15 yellow, irreversible
3 colorless
5 pink, traces
50 red-orange, unchanged starting material: 485, 454 mμ
10 pink, all-trans: 489, 456.5 mμ
10 orange, neo A: 485, 453 mμ
9 yellow, mainly neo B: 474, 444.5 mμ
2 yellow-orange, (see below)
32 orange, neo C: 479, 448.5 mμ
Filtrate: yellow, irreversible

Those zones designated as being irreversible do not belong to the methylbixin set. The colorimetric ratio of unchanged natural methylbixin: all-trans: neo A: neo B: neo C was 28: 17: 16: 8: 31.

The by-product contained in the 2 mm. zone was obtained in larger quantity by melting 100 mg. of methylbixin. It is not a member of the methylbixin set but shows a characteristic spectral curve (Fig. 22).

(c) Cis-trans Isomerization of Natural Methylbixin by Iodine Catalysis at Room Temperature.--Two milligrams of pigment in 1 ml. of benzene was diluted with 10 ml. petroleum ether and

catalyzed with 20 μg. of iodine. The solution was kept for fifteen minutes in diffuse daylight and chromatographed (18 x 1.9 cm.).

25	colorless
39	red-orange, mainly all-trans: 489, 457 mμ
10	orange, neo A: 484, 453 mμ
1	yellow, neo B traces: 473, 444.5 mμ
21	orange, neo C: 479, 448.5 mμ

(The uppermost fraction of the 39 mm. zone showed a 2 mμ shorter wave-length maxima than indicated.)

The colorimetric ratio of all-trans: neo A: neo C was 72: 19: 9.

(d) Photochemical Cis-trans Isomerization of Natural Methylbixin.--Two milligrams of pigment dissolved in 3 ml. of benzene and diluted with 10 ml. of petroleum ether was insolated for fifteen minutes (final temperature, 30°), and developed on a column (18 x 1.9 cm.).

32	colorless
68	orange-red, unchanged natural methylbixin: 485, 453 mμ
1	almost colorless
1	pink } very little
1	orange } pigment
1	yellow }
30	orange, neo C: 478.5, 449 mμ

The colorimetric ratio of unchanged natural methylbixin: minor isomers: neo C was 90: 2: 8. See Figure 17 for molar extinction curves.

2. All-trans-Methylbixin

Isolation.--This pigment was prepared according to the method of Kuhn and Winterstein (4) by catalyzing 500 mg. of natural methylbixin in 20 ml. of ethyl acetate with 30 mg. of iodine. Upon standing at 0°, crystals appeared which were recrystallized from benzene-methanol to give 200 mg. of chromatographi-

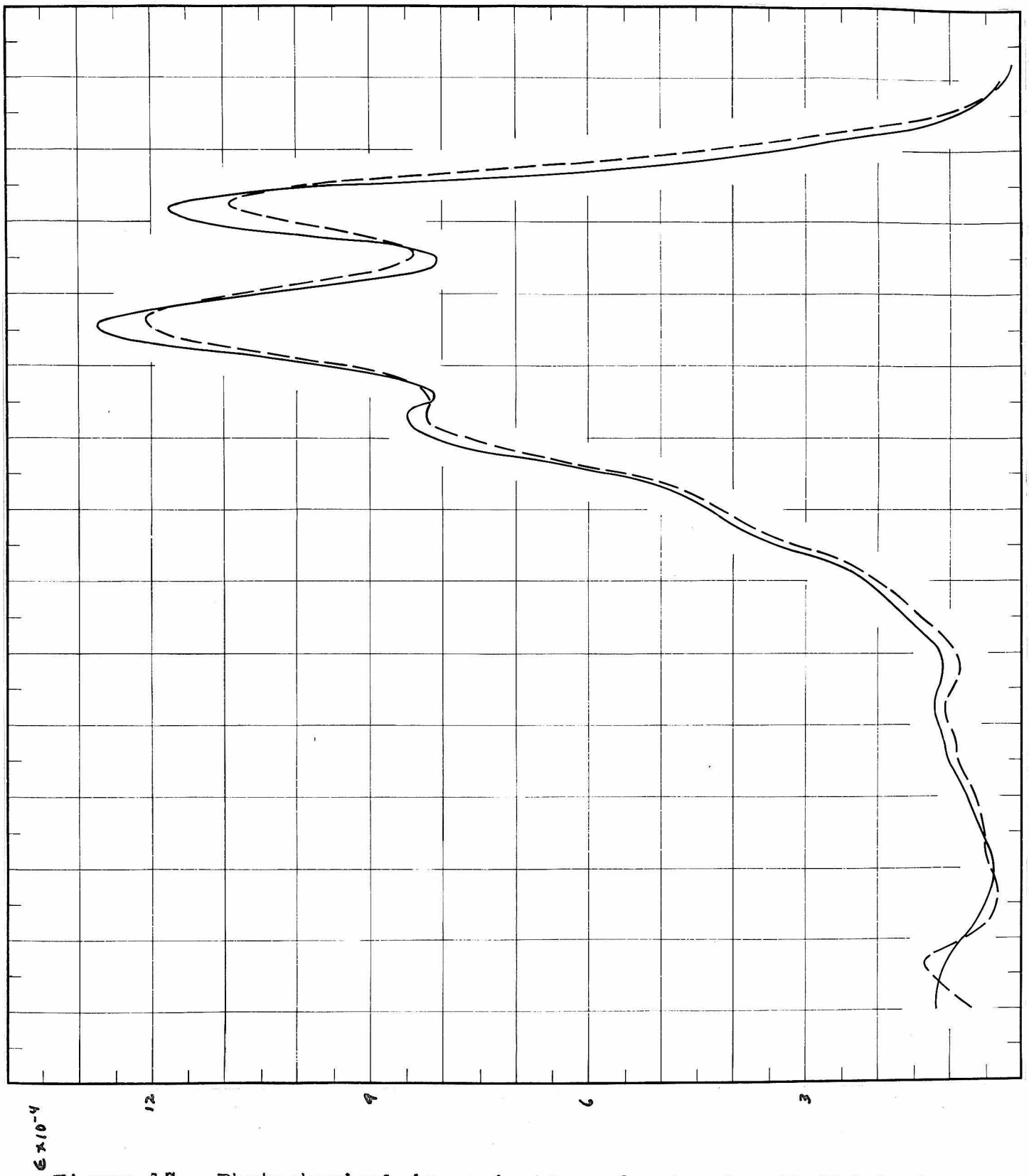


Figure 17. Photochemical isomerization of natural methylbixin in benzene: ——— molecular extinction curve of original solution, and - - - sample after 15 minutes insolation.

cally homogeneous crystals (Fig. 18); m.p. 198° (cor.). The influence of polar solvents on the spectral curve is illustrated by Figure 19.

Analysis--Calculated: C=76.44; H=7.90; Molecular weight=408.5

Found: C=75.90; H=7.72; Molecular weight=388.

(a) Cis-trans Isomerization of all-trans-Methylbixin upon Refluxing.--Three milligrams of pigment in 20 ml. of benzene was refluxed for an hour. The solution was concentrated to 3 ml., diluted with 5 volumes of petroleum ether, and chromatographed (18 x 1.9 cm.).

20	colorless	
30	orange-red, unchanged all-trans: 490, 458 mμ	
9	orange, neo A: 485, 453 mμ	
0.5	yellow	minor isomers: 480.5, 449 mμ
1.5	colorless	
2	orange	
2	colorless	
2	orange	

The colorimetric ratio of unchanged all-trans: neo A: minor isomers was 63: 35: 2.

(b) Cis-trans Isomerization upon Melting Crystals of all-trans-Methylbixin.--Eight milligrams of pigment was kept fused at 200° for one minute, dissolved in 5 ml. of benzene, diluted with 20 ml. of petroleum ether, and chromatographed (18 x 2.5 cm.).

24	yellow, irreversible
45	colorless
25	orange-red, unchanged all-trans: 489, 456 mμ
8	orange, neo A: 485, 453 mμ
3	almost colorless
15	lemon yellow, mainly irreversible
20	orange, neo C: 479, 449 mμ
2	almost colorless
2	faint orange (traces)

The colorimetric ratio of unchanged all-trans: neo A: neo C was 57: 19: 24.

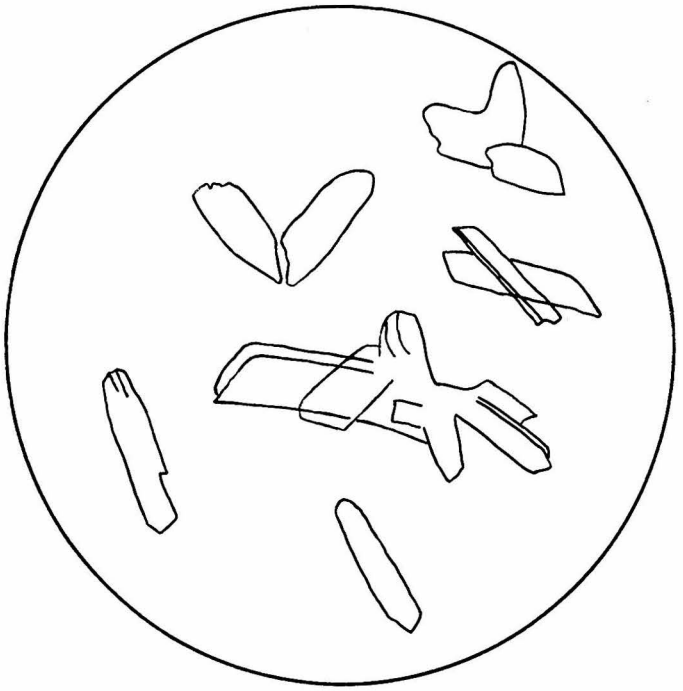


Figure 18. All-trans-methylbixin crystallized from benzene-methanol (430x).

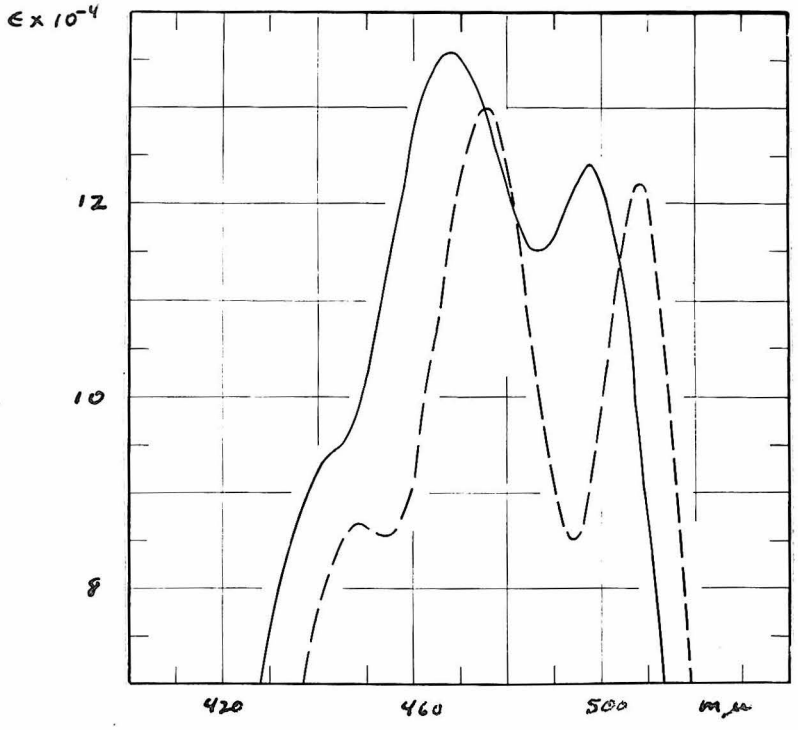


Figure 19. Effect of polar solvents upon the visual spectrum of all-trans-methylbixin: - - - benzene solution and ——— ethyl alcohol solution.

(c) Cis-trans Isomerization of all-trans-Methylbixin by Iodine Catalysis at Room Temperature.--A solution of 2 mg. of pigment in 2 ml. of benzene was chromatographed (18 x 1.9 cm.) after standing for thirty minutes with iodine and diluting with 15 ml. of petroleum ether.

50	colorless
30	orange-red, unchanged all-trans: 489, 456 mμ
5	orange, neo A: 484, 452 mμ
5	almost colorless
25	light orange, neo C: 479, 448 mμ

The colorimetric ratio of unchanged all-trans: neo A: neo C was 72: 19: 9.

(d) Photochemical Cis-trans Isomerization of all-trans-Methylbixin.--Two milligrams of pigment in 3 ml. of benzene was insolated for fifteen minutes (final temperature, 31°) and chromatographed (18 x 1.9 cm.) after dilution with 10 ml. of petroleum ether.

25	colorless
25	orange-red, unchanged all-trans: 489, 458 mμ
2	orange, neo A: 485.5, 453 mμ

The colorimetric ratio of unchanged all-trans: neo A was 94: 6.

3. Neomethylbixin A

Isolation.--A solution of 100 mg. of natural methylbixin in 10 ml. of benzene was catalyzed with 0.5 mg. of iodine and kept in daylight for thirty minutes at 25°. After dilution with 3 volumes of petroleum ether, it was chromatographed (27 x 5.8 cm.). The broad orange layer located directly below the all-trans zone was eluted with acetone and rechromatographed immediately on a smaller column (24 x 4.8 cm.). This chromatogram showed only traces of other isomers. However, since the uppermost section of the neo A zone usually contains consider-

able amounts of the all-trans isomer, about 1/5 of the main zone was rejected. A third chromatogram showed perfect homogeneity. The pigment was transferred into benzene, evaporated to 2 ml., and crystallized by the addition of excess methanol (Fig. 20). Yield, 20 mg.; m.p. 190-191° (cor.).

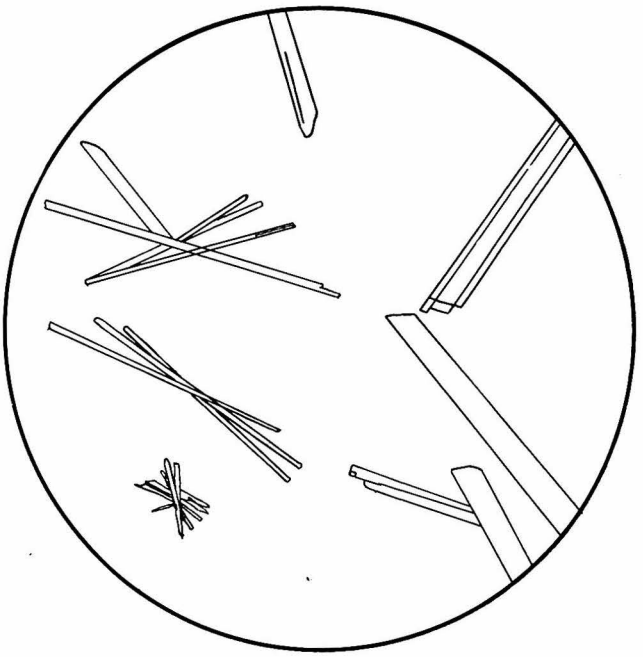


Figure 20. Neomethylbixin A crystallized from benzene-methanol (430x).

The sample was recrystallized from benzene-methanol. This isomer is much more soluble and less stable than natural or all-trans-methylbixin. Fresh solutions of even the purest crystals showed a slight contamination (about 3%, exceptionally 6%) with all-trans-methylbixin.

(a) Cis-trans Isomerization of Neomethylbixin A upon Refluxing.--The solution of 3 mg. pigment in 20 ml. of benzene was refluxed

for an hour, concentrated to 3 ml., diluted with three volumes of petroleum ether, and chromatographed (18 x 1.9 cm.).

22 colorless
50 pink, all-trans: 490, 458 mμ
22 orange, unchanged neo A: 485, 453 mμ
2 yellow, neo B: 475, 447 mμ
9 light orange, neo C: 477.5, 447.5 mμ

The colorimetric ratio of all-trans: unchanged neo A: neo B: neo C was 63: 32: 2: 3.

(b) Cis-trans Isomerization upon Melting Crystals of Neomethylbixin A.--Five milligrams of pigment was kept at 195° for one minute, dissolved in 5 ml. of benzene, diluted with 15 ml. of petroleum ether, and chromatographed (18 x 2.5 cm).

20 yellow, irreversible
20 colorless
30 pink, all-trans: 488.5, 455.5 mμ
10 orange, unchanged neo A: 484.5, 453 mμ
15 greenish yellow, neo B (+ irreversible): 474, 447 mμ
22 yellow-orange, neo C: 479, 449 mμ

Irreversibly formed pigments were also noted in the chromatographic filtrate. The neo C zone showed some tendency to separate into three sections with blurred borders; however, an examination did not reveal any spectral differences.

The colorimetric ratio of all-trans: unchanged neo A: neo B: neo C was 53: 25: 6: 16.

(c) Cis-trans Isomerization of Neomethylbixin A by Iodine Catalysis at Room Temperature.--Three milligrams of pigment in 5 ml. of benzene was catalyzed and chromatographed (18 x 1.9 cm.) after standing half an hour.

40 colorless
44 pink, all-trans: 490, 456.6 mμ
16 orange, unchanged neo A: 484.5, 453.5 mμ
2 yellow, neo B: 474, 446.5 mμ
28 yellow-orange, neo C: 478, 448.5 mμ

The colorimetric ratio of all-trans: unchanged neo A: neo B:

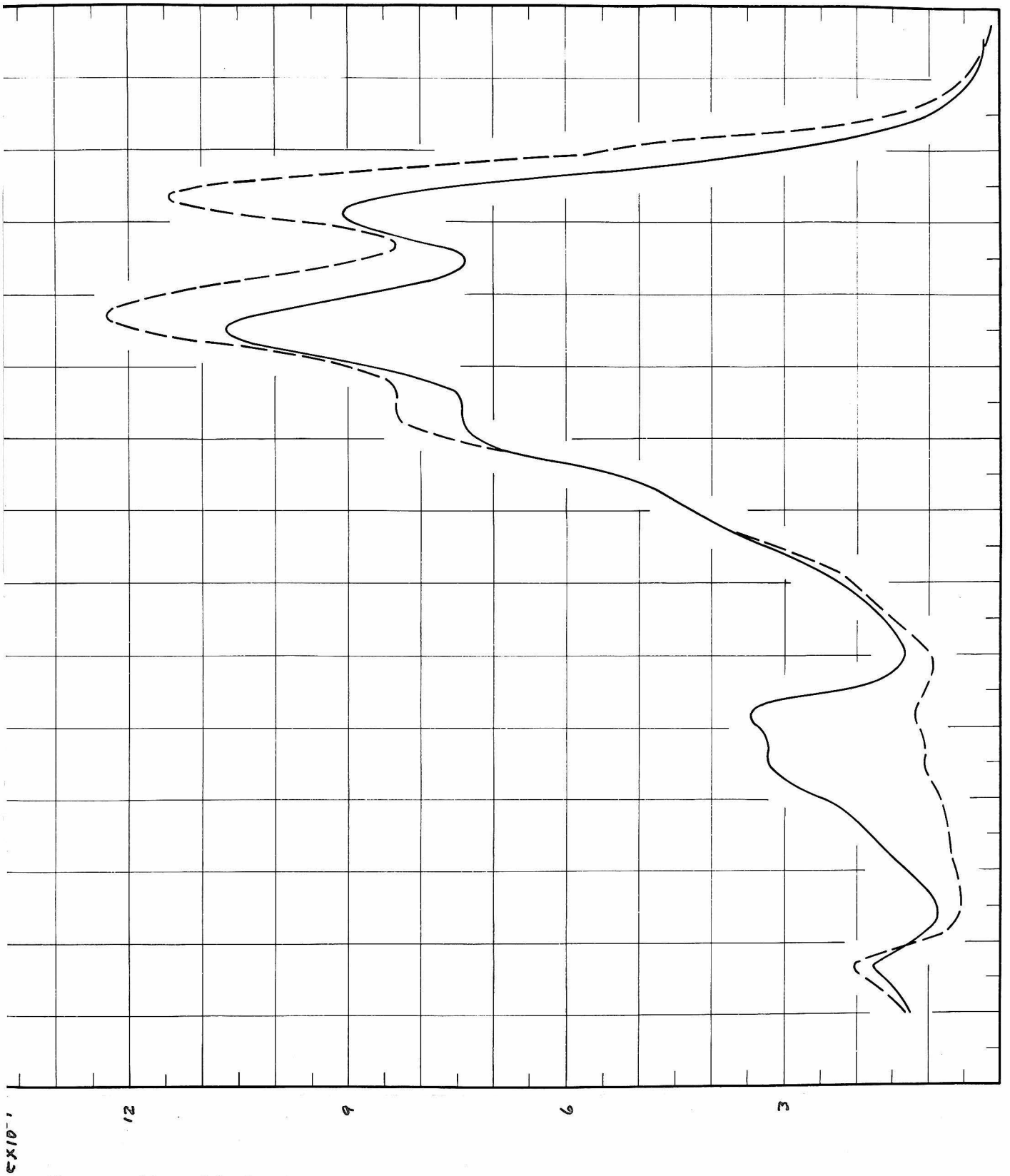


Figure 21. Photochemical isomerization of neomethylbixin A in benzene: ——— molecular extinction curve of original solution, and - - - sample after 15 minutes insolation.

neo C was 64: 25: 2: 9.

(d) Photochemical Cis-trans Isomerization of Neomethylbixin A.--After fifteen minutes insolation, the benzene solution of 3 mg. of pigment was diluted with five volumes of petroleum ether and chromatographed (18 x 1.9 cm.).

60	colorless
33	pink, mainly all-trans: 490, 458 mμ
25	pinkish orange, mainly neo A: 484.5, 453 mμ
1	yellow (traces)

Unsatisfactory separation of the zones allows only an approximation to the colorimetric ratio of all-trans: unchanged neo A as being 45: 55. The effect of insolation of the molar extinction curve is illustrated in Figure 21.

4. Neomethylbixin B

Several attempts were made to obtain sufficient amounts of this isomer to enable a more thorough investigation. The molar extinction curve of Figure 11 is thought to be reasonably correct. This sample was isolated from an iodine catalyzed solution of 100 mg. of natural methylbixin. The pigment from the eluate of the neomethylbixin B zone was transferred into benzene and its absorption spectra taken in the Beckman Photoelectric Spectrophotometer. Iodine was added and the pigment concentration was established from the molar extinction values of the iodine equilibrium.

When an attempt was made to isolate this pigment from the melt of 100 mg. of natural methylbixin, the solution obtained showed the unusual type of extinction maxima illustrated in Figure 22. The first two maxima check with, and may be due to, those of neomethylbixin B. Since the iodine curve does not

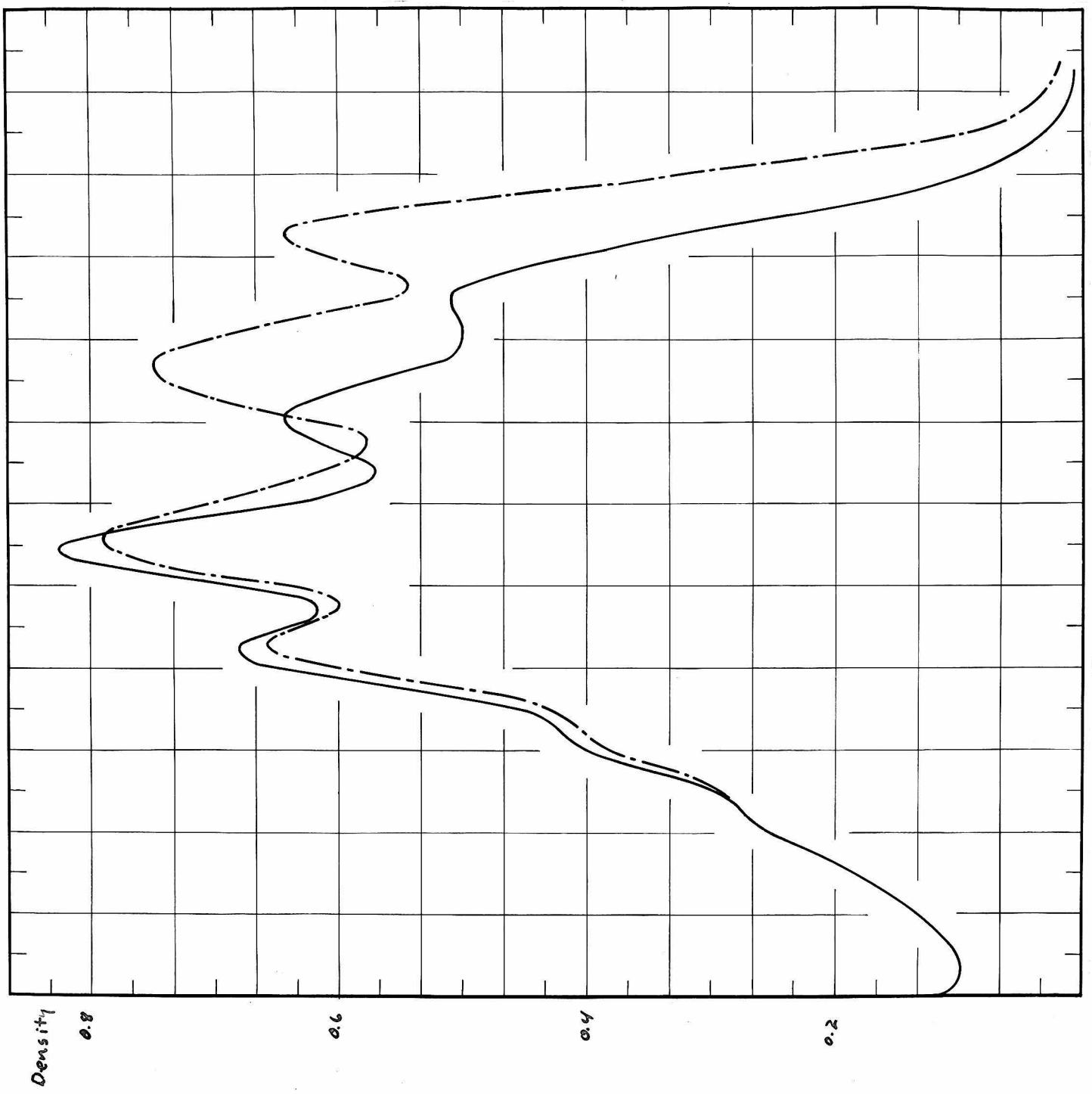


Figure 22. Extinction curve of eluate from neomethylbixin B zone of a melt isomerization of natural methylbixin:——— benzene solution of pigment, and ·-·-· after iodine addition.

conform to those of the methylbixin set, this material is certainly not a pure member of this group.

5. Neomethylbixin C

Isolation.--A solution of 300 mg. of natural methylbixin in 100 ml. of benzene-petroleum ether (1:1) was refluxed for an hour, diluted with petroleum ether to 250 ml., and developed on a column (30 x 8 cm.) with 2.5 liters of a 1:4 mixture. The development required three hours.

7	yellow (traces)
5	colorless
130	orange-red, unchanged natural methylbixin
1	colorless
2	pink
1	colorless
2	orange
1	yellow
60	bright orange, neomethylbixin C

In order to augment the yields, the zone of unchanged methylbixin was eluted with acetone, transferred into benzene, refluxed again, and chromatographed. The natural methylbixin zone of this second chromatogram was submitted to a third refluxing and adsorption analysis after the addition of a 50 mg. portion of starting material.

The combined acetone eluates of the three zones of neo C were transferred into petroleum ether solution and developed on a smaller column (27 x 5.8 cm.) with benzene-petroleum ether (1:5). Except for traces, the pigment was found to be homogeneous. It was transferred into petroleum ether and evaporated completely in vacuo. The red, oily residue was taken up in the minimum amount of benzene and transferred into a centrifuge tube. When methanol was added gradually with stirring, an oily suspension appeared. Crystallization may be effected by

scratching and cooling this suspension or by seeding with crystals. After cooling to -10°, the material was centrifuged and recrystallized (Fig. 23). The intensely colored mother liquors may be reworked.

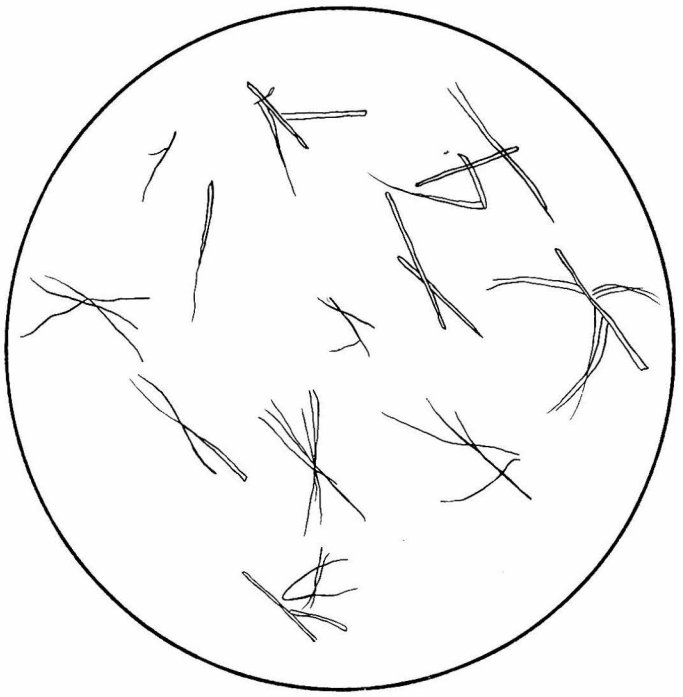


Figure 23. Neomethylbixin C recrystallized from benzene-methanol (430x).

The yield of crude neomethylbixin C crystals was 50 mg.; however, this quantity decreased to 25-30 mg. upon recrystallization. M.p. 150-151° (cor.).

(a) Cis-trans Isomerization of Neomethylbixin C upon Refluxing.--The benzene solution of 1 mg. pigment was refluxed for an hour, concentrated to 2 ml., diluted with 15 ml. of petroleum ether, and chromatographed (18 x 1.9 cm.).

15 colorless
5 red-orange, natural methylbixin: 485, 453.5 mμ
4 pink, all-trans: 490, 458 mμ
4 orange, neo A: 485, 453.5 mμ
3 yellow, neo B: 471.5, 444.5 mμ
36 yellow-orange, unchanged neo C: 479, 448.5 mμ

The colorimetric ratio of natural methylbixin: all-trans: neo A: neo B: unchanged neo C was 33: 12: 6: 3: 46.

(b) Cis-trans Isomerization upon Melting Crystals of Neomethylbixin C.--Five milligrams of pigment was kept molten at 155° for one minute, dissolved in 5 ml. of benzene, diluted with five volumes of petroleum ether, and chromatographed (18 x 2.5 cm.).

7 yellow, irreversible
40 colorless
37 orange-red, natural methylbixin: 485, 454 mμ
4 pink, all-trans: 489, 457.5 mμ
4 orange, neo A: 485.5, 452 mμ
10 yellow-orange, neo B: 471, 444 mμ
2 almost colorless
50 orange, unchanged neo C: 480, 448.5 mμ
Filtrate: yellow, irreversible

The colorimetric ratio of natural methylbixin: all-trans: neo A: neo B: unchanged neo C was 51: 4: 4: 5: 36.

(c) Cis-trans Isomerization of Neomethylbixin C by Iodine at Room Temperature.--Three milligrams of pigment were catalyzed in benzene with 20 μg. of iodine. After standing for thirty minutes, the solution was developed with benzene-petroleum ether (1:5) on a column (18 x 1.9 cm.).

20 colorless
49 orange-red, all-trans: 489, 456.5 mμ
20 orange, neo A: 484, 452.5 mμ
2 yellow
23 yellow-orange, unchanged neo C: 478, 448 mμ

The colorimetric ratio of all-trans: neo A: unchanged neo C was 63: 25: 12.

(d) Photochemical Cis-trans Isomerization of Neomethylbixin C.--

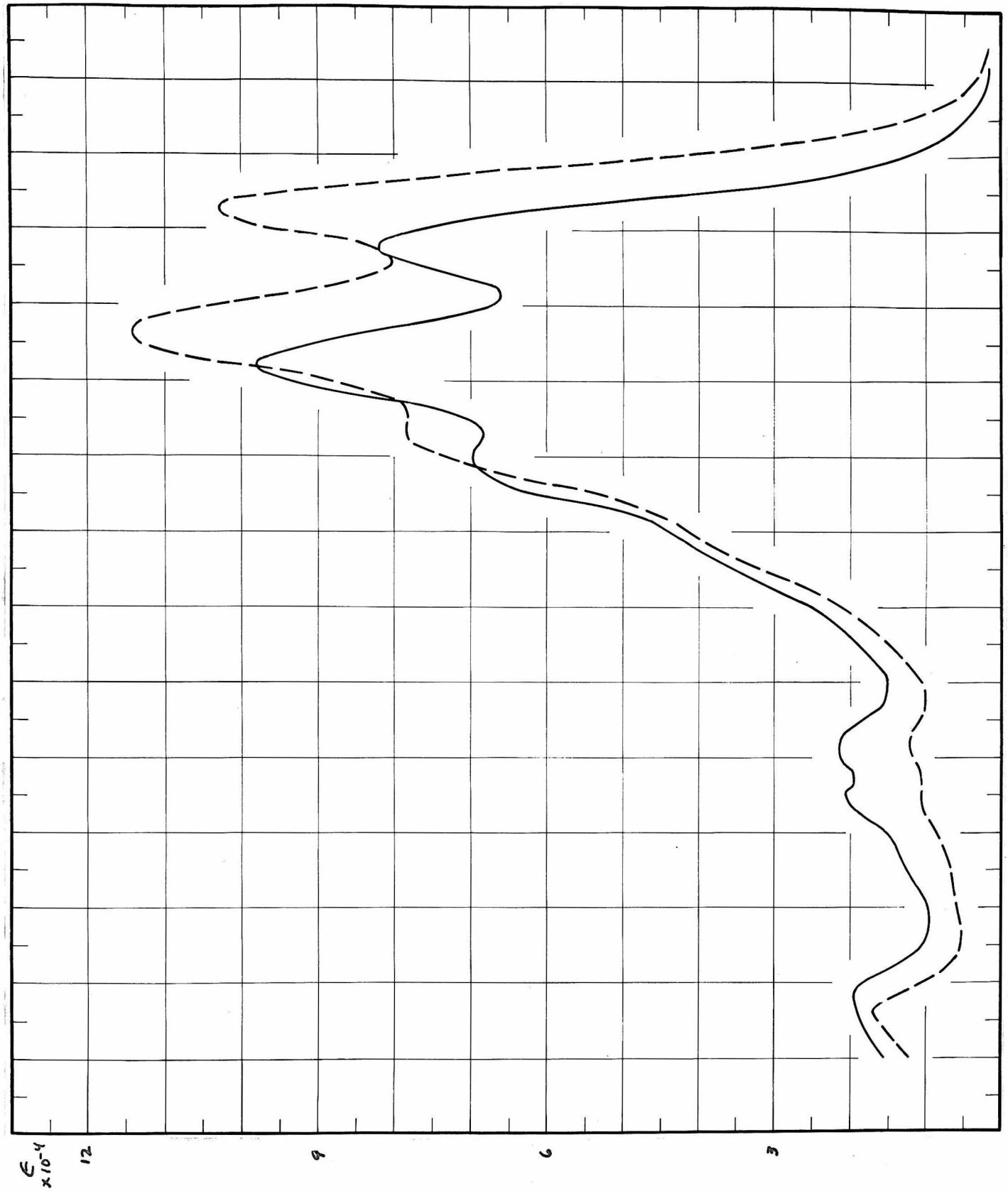


Figure 24. Photochemical isomerization of neomethylbixin C in benzene: ——— molecular extinction of original solution, and - - - sample after 15 minutes insolation.

A solution of 2 mg. of pigment in 3 ml. of benzene was insolated for fifteen minutes. After dilution with 10 ml. of petroleum ether, it was chromatographed (18 x 1.9 cm.).

23	colorless
15	orange-red, natural methylbixin: 485, 453.5 mμ
11	colorless
4	pink, neo A: 484.5, 453 mμ
3	colorless
65	yellow-orange, unchanged neo C: 478.5, 448 mμ

The colorimetric ratio of natural methylbixin: neo A: unchanged neo C was 21: 2: 77. Figure 24 illustrates the change of the molar extinction curve upon insolation.

Summary

Two new stereoisomers of methylbixin have been obtained in crystalline form and a third has been observed in solution. Various isomerization procedures have been tried on the four crystalline materials and the interconversion of these isomers has been demonstrated. A consideration of the absorption data has been used to assign tentative configurations to the four principal members isolated from the methylbixin set of stereoisomers.

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PROPOSITIONS

1. The examination of the stereoisomers of the two methylethylnorbixins reported by van Hasselt (1) would allow a definite determination of the steric configuration of neomethylbixin A.

(1) Rec. Trav. chim. Pays-Bas, 30, 1 (1911).

2. The steric configuration 1-cis-crocetin is proposed for "labile crocetin".

3. A polarographic investigation of bixin might extend our stereochemical knowledge of this subject.

4. G. E. Hilbert and E. F. Jansen (2) as well as E. S. Miller (3) have observed a shift of 150 to 190 Å in the absorption maxima of carotenoids toward longer wave-lengths at liquid air temperatures. This may be explained by the change of refractive index of the solvent under these conditions.

(2) J. Bio. Chem., 160, 97 (1934).

(3) Plant Physiol., 9, 179 (1934).

5. The cyclization of lycopene is feasible and should yield some amounts of the β-carotene isomer possessing α-ionone rings as terminal groups.

6. The ratio of molar extinction coefficient at the maximum absorption peak to that at the cis-peak is characteristic for a given stereoisomer. This ratio is independent of concentration and allows a quick identification of isomers possessing the same visual absorption maxima.

7. The failure of Kundt's law reported by W. R. Brode (4) should be qualified. It is proposed that an investigation of the infra-red absorption of such solutions will reveal considerable hydrogen bonding between the dye and alcohol.

(4) Chemical Spectroscopy, p. 187, John Wiley and Sons, London, 1943.

8. Application of the Refractivity Intercept of A. L. Ward and S. S. Kurtz (5) would allow a simple determination of the aromatic or aliphatic nature of hydrocarbons in a system of qualitative organic analysis.

(5) Ind. Eng. Chem. Anal. Ed., 10, 559 (1938).

9. Graduate students at the California Institute of Technology should make no attempt to begin their research during their first six months. This time could be profitably utilized in a course similar to Instrumental Analysis.

10. Exchange resins offer a convenient means of making sea water drinkable.