crystals or exposure of solutions to sunshine. Zeaxanthin is more photosensitive than cryptoxanthin. Extinction curves are given for fresh, refluxed, and iodine-catalyzed solutions. Cryptoxanthin and zeaxanthin develop *cis*-peaks at 348 mμ (in benzene); however, no peak was observed upon iodine catalysis in darkness. On the basis of optical data the most probable configurations are: neocryptoxanthin B = 6-mono-*cis*-cryptoxanthin, and neocryptoxanthin U = 3-mono-*cis*- or 9-mono-*cis*-cryptoxanthin; neo A is a di-*cis* compound; neozeaxanthin A = 6-mono-*cis*-zeaxanthin and neozeaxanthin B = 5-mono-*cis*-zeaxanthin; neozeaxanthin C is probably a di-*cis* isomer.

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[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, NO. 945]

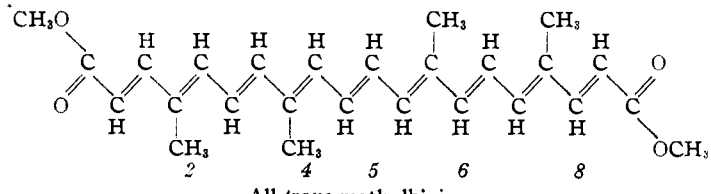
A Stereochemical Study of Methylbixin

BY L. ZECHMEISTER AND R. B. ESCUE

Bixin, $CH_3OOCC_{22}H_{26}COOH$, the main pigment in the seeds of the Annato tree (*Bixa orellana*, L.), was the first polyene for which stereoisomerism was demonstrated. Herzig and Faltis[1] in a single unreproducible experiment obtained from the seeds, instead of the well-known bixin, an isomer, termed "β-bixin," with higher melting point and greater stability than bixin, and with spectral maxima at longer wave lengths than those of bixin. Karrer and his associates[2] reported later that natural bixin can be converted into β-bixin by iodine, and they correctly interpreted this reaction as a *cis*→*trans* rearrangement. According to Kuhn and Winterstein[3] catalytic amounts of the halogen are sufficient to effect this transformation. Furthermore, they showed that both bixins yield the same dihydro compound, which is oxidized in air into β-bixin.

The following names have been used[4] for the two bixins and, in an analogous manner, for the two free carboxylic acids (norbixins) and the two dimethyl esters (methylbixins): ordinary bixin = natural bixin = *cis*-bixin = α-bixin = labile bixin = lower melting bixin = bixin II; isobixin = *trans*-bixin = β-bixin = stable bixin = higher melting bixin = bixin I.

Since no other isomer seems to have been described[5] and since the reversibility of the bixin isomerization by iodine catalysis so far as we know has not been claimed, we have re-investigated this field[6] by making use of some methods which were first applied to C_{40}-carotenoids.[7,8] Methylbixin was the most suitable starting material because of its greater solubility and markedly weaker adsorbability than that of bixin. Furthermore, because of its symmetrical molecule (see the formula) methylbixin can exist in only twenty stereoisomeric forms, whereas the corresponding number for bixin is thirty-two.[9,10]



All-*trans*-methylbixin
(The stereochemically effective double bonds are numbered.)

In the course of our experiments five members of the methylbixin set (and traces of other isomers) have been observed in chromatograms[11]; two new pigments, the neomethylbixins A and C, have been isolated as crystals. We designate

TABLE I

VISUALLY OBSERVED SPECTRAL MAXIMA OF SOME STEREOISOMERIC METHYLBIXINS LISTED IN THE SEQUENCE OF DECREASING ADSORPTION AFFINITIES

	In petroleum ether (b. p. 60–70°), mμ		In benzene, mμ	
Natural methylbixin	485	453.5	503	470
All-*trans*-methylbixin	490	457	508.5	475
Neomethylbixin A	485	454	502.5	469
Neomethylbixin B	471	444.5	491	458
Neomethylbixin C	479.5	449	496	463

(1) J. Herzig and F. Faltis, *Ann.*, **431**, 40 (1923).

(2) P. Karrer, A. Helfenstein, R. Widmer and Th. B. van Itallie, *Helv. Chim. Acta*, **12**, 741 (1929).

(3) R. Kuhn and A. Winterstein, *Ber.*, **65**, 646 (1932), and **66**, 209 (1933).

(4) *Cf.* L. Zechmeister, "Carotinoide," J. Springer, Berlin, 1934, pp. 239-251.

(5) A third bixin termed "isobixin" could not be reproduced; *cf.* J. F. B. van Hasselt, *Rec. trav. chim. Pays-bas*, **30**, 1 (1911), and **33**, 192 (1914); P. Karrer and T. Takahashi, *Helv. Chim. Acta*, **16**, 287 (1933).

(6) L. Zechmeister and R. B. Escue, *Science*, **96**, 229 (1942).

(7) *Cf. e. g.*, A. Polgár and L. Zechmeister, THIS JOURNAL, **64**, 1856 (1942); L. Zechmeister and A. Polgár, *ibid.*, **66**, 137 (1944).

(8) L. Zechmeister, A. L. LeRosen, W. A. Schroeder, A. Polgár and L. Pauling, *ibid.*, **65**, 1940 (1943).

(9) L. Pauling, *Fortschritte Chem. organ. Naturstoffe*, **3**, 203 (1939).

(10) L. Zechmeister, A. L. LeRosen, F. W. Went and L. Pauling, *Proc. Natl. Acad. Sci.*, **27**, 468 (1941).

(11) A. Winterstein mentioned in Klein's "Handbuch der Pflanzenanalyse," Vol. IV, p. 1403 (1933), that *cis*- and *trans*-bixin can be separated chromatographically; no experimental directions were given; *cf.* A. Winterstein and R. Stein, *Z. physiol. Chem.*, **220**, 247 (1933).

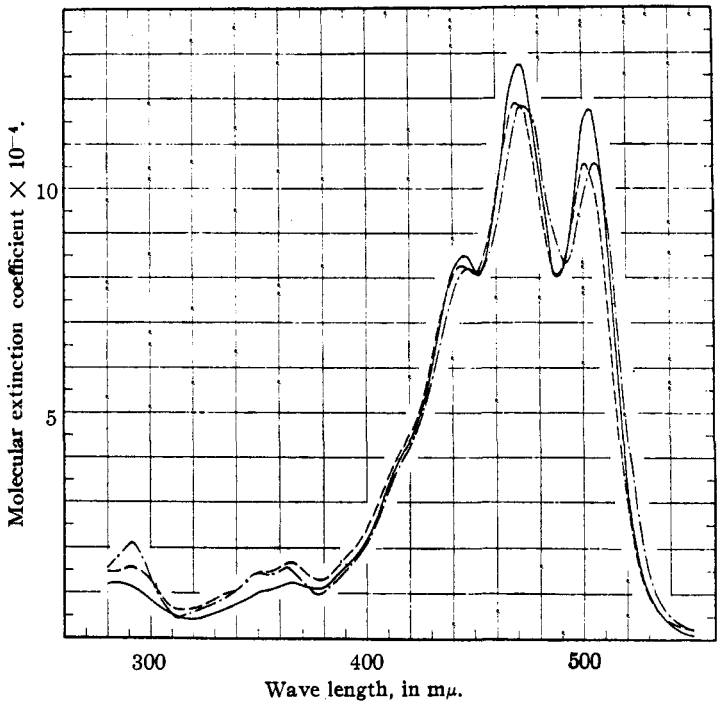


Fig. 1.—Molecular extinction curves of natural methylbixin in benzene: ——, fresh solution of natural methylbixin; - - -, mixture of stereoisomers after refluxing in darkness for forty-five minutes; and —·—·, after iodine catalysis at room temperature in light.

(Table I) that stereoisomer whose double bonds have the same configuration as those in naturally occurring bixin (=norbixin II-monomethyl ester) as "natural methylbixin"; the methyl ester of β-bixin (=norbixin I-monomethyl ester) is termed "all-*trans*-methylbixin."

The position of natural methylbixin in chromatograms strongly reminds us of neo-β-carotene U, whereas the neo-forms A, B and C are adsorbed below the all-*trans* member in both sets.

Extinction curves of the bixins and methylbixins have been reported in the literature.[12] Figures 1 to 4 show the alterations of the curves of the four main isomeric methylbixins under the influence of refluxing and iodine catalysis. The iodine equilibrium curves obtained from the isomers are identical within experimental error. However, the curves taken after refluxing are not identical because under the conditions applied true equilibria are not reached.

A characteristic "*cis*-peak" appears in benzene solutions in each case at 363–364 mμ. As in the corresponding lycopene curve, the *cis*-peak maximum of methylbixin shows a fine structure. Natural methylbixin possesses only a slight peak and neo C a moderately high one, whereas neo A is responsible for the main effect observed in the iodine equilibrium mixture (Fig. 8).

Light is needed for the development of the *cis*-peak in the presence of iodine.[13] The extinction of all-*trans*-methylbixin remained practically unaltered in the *cis*-peak region when the catalyzed solution was kept in darkness; however, a five-second illumination caused a major effect (Fig. 5).

In the absence of catalysts, a fifteen-minute exposure to sunshine did not essentially alter the extinction curve of all-*trans*-methylbixin; natural methylbixin proved also to be relatively photo-stable under similar conditions. However, the configurations of the neo forms A and C are much more photosensitive (Figs. 6 and 7). The alterations which are represented in these curves are nearly

(12) *Cf. e. g.*, A. Smakula, *Angew. Chem.*, **47**, 657 (1934); P. Karrer and E. Würgler, *Helv. Chim. Acta*, **26**, 116 (1943); the original of the latter paper was not available to us.

(13) *Cf.* A. Polgár and L. Zechmeister, THIS JOURNAL, **66**, 186 (1944).

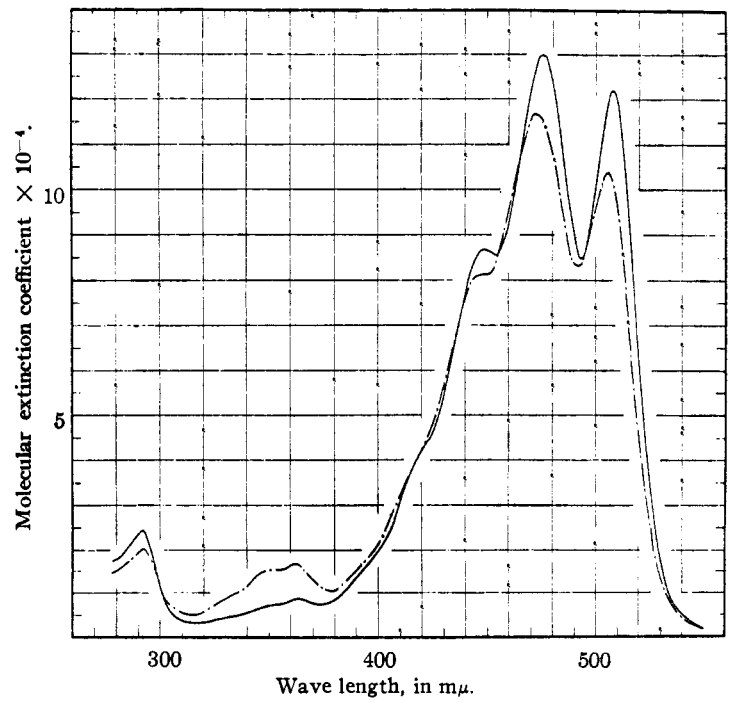


Fig. 2.—Molecular extinction curves of all-*trans*-methylbixin in benzene: ——, fresh solution of the all-*trans* compound; and —·—·, after iodine catalysis at room temperature in light. (The latter curve is practically identical with that obtained after refluxing forty-five minutes in darkness.)

exclusively caused by stereoisomerization. Photometric measurements after iodine catalysis of the insolated solutions proved that practically no destruction of pigment had taken place.

Interrelationship and Configuration of Some Stereoisomers.—It has been pointed out earlier[8] that there is a high probability for the formation of substantial amounts of mono-*cis* isomers from an all-*trans* carotenoid by isomerization. Furthermore, it has been stated that a difference of about 5 mμ from the visually established longest wave length maximum of the all-*trans* member of the set corresponds to one *trans*→*cis* rotation.[7,8] On the basis of the spectra listed in Table I we assume that natural methylbixin and neomethylbixin A are mono-*cis* compounds, neo C is a di-*cis* and neo B probably a tri-*cis* (or tetra-*cis*) methylbixin.

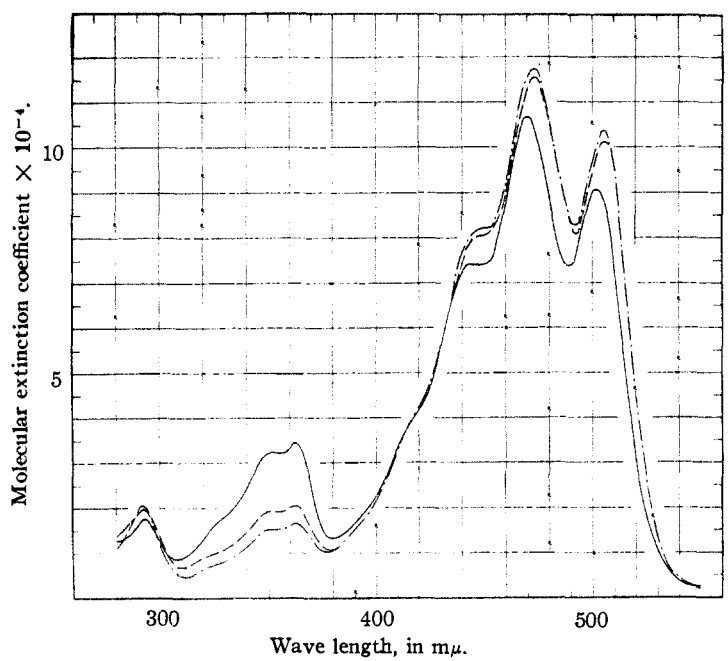
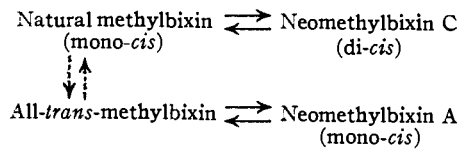


Fig. 3.—Molecular extinction curves of neomethylbixin A in benzene: ——, fresh solution of neomethylbixin A; - - -, mixture of stereoisomers after refluxing in darkness for forty-five minutes; and — · — ·, after iodine catalysis at room temperature in light.

These conclusions which are based on optical data are in accordance with the following observations of another type. It is a characteristic feature of the methylbixin set that no all-*trans* pigment (except traces) is formed, in the absence of catalysts, from natural methylbixin upon refluxing and *vice versa*. The thermostability and photostability of the *cis* double bond in natural methylbixin are so great that the molecule undergoes a second *trans*→*cis* rotation and gives substantial amounts of neomethylbixin C instead of yielding any marked amounts of the all-*trans* pigment. On the other hand, the neomethylbixin A is easily formed when all-*trans* methylbixin is refluxed and *vice versa;* no natural methylbixin appeared in the mixtures except perhaps traces.

If full arrows indicate easy interconversion and dotted arrows hindered interconversion, then the following scheme is valid.



The exposure of the respective isomers to sunshine and subsequent chromatography confirmed the above interrelationship. The results of this method are especially conclusive because of the simple character of the chromatograms (see Experimental Part).

The *cis* double bonds in the two mono-*cis* compounds are evidently of different character. Furthermore, it is reasonable to say that one of the two *cis* double bonds in neo C is identical in position with that in natural methylbixin.

It has been pointed out[8] that in long conjugated systems a central mono-*cis* isomer is expected to show the highest *cis*-peak. As a rough approximation, the *cis*-peak effect of each member of the set can be taken proportional to the square of the distance between the center of the chromophore and the mid-point of the straight line between its ends. The values for the molecular extinction coefficients × 10^{-4} at the *cis*-peak wave length minus the corresponding value for the all-*trans* compound are

Natural methylbixin : 0.4	Neomethylbixin A : 2.8
All-*trans*-methylbixin : 0	Neomethylbixin C : 1.4

Figure 8 shows that neomethylbixin A must be interpreted as the 5-mono-*cis* and natural methylbixin as the 2-mono-*cis* isomer. The considerable peak of neo C indicates the presence of a central double bond and we suggest that this isomer is 2,5-di-*cis*-methylbixin. The curve of neo B is flat in the *cis*-peak region but because its lability and the difficulties of separation, no exact data can be given. The skeleton models of the four main stereoisomeric methylbixins are represented in Fig. 9.

The intensity of the principal absorption peak has already been used by Pauling[9,10] in the assignment with confidence of the all-*trans* structure to the natural C_{40}-carotenoids. Pauling pointed out, using the example of lycopene and neolycopene A for the discussion, that the integrated absorption coefficients should be approximately proportional

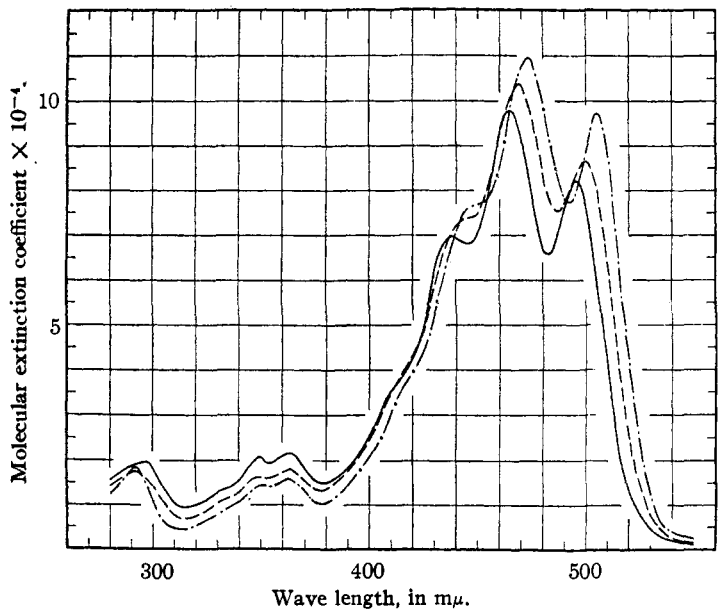


Fig. 4.—Molecular extinction curves of neomethylbixin C in benzene: ——, fresh solution of neomethylbixin C; – – –, mixture of stereoisomers after refluxing in darkness for forty-five minutes; and —·—·, after iodine catalysis at room temperature in light.

to the squares of the actual distances between the ends of the system. For the four members of the methylbixin set, we found experimentally that the maximum intensity ratios are, all-*trans*:natural: neo A:neo C = 1:0.93:0.80:0.82 while the ratios calculated on the basis of the models (with the single bond-double bond angles taken as 125°16′) are 1:0.87:0.82:0.79; the agreement is satisfactory, considering the experimental error and the approximate nature of the theory.

The configuration proposed for natural methylbixin is in contradiction with some results published (with reservation) by Karrer and Solmssen.[14] In a remarkable paper, these authors concluded, on the basis of the stereochemical relationship of some partial oxidation products, that natural bixin (or methylbixin) contains a *cis*-double bond in the 7-position. This double bond, however, belongs to the sterically hindered type.[9,10]

Some other data of the older literature are in very good accordance with the content of the present paper. Van Hasselt[5] found that two different methyl ethyl esters of "natural" norbixin can be prepared. Although he attributed this observation to a structural difference in the two molecule halves, it was shown by Karrer, *et al.*,[15] that the cause for the lack of symmetry is stereochemical and that a symmetrical structural formula must be given to norbixin. The existence of the two methyl ethyl esters shows independently of our configurational assignments that natural methylbixin cannot contain a centrally located *cis* double bond.

(14) P. Karrer and U. Solmssen, *Helv. Chim. Acta*, **20**, 1396 (1937).

(15) P. Karrer, P. Benz, R. Morf, H. Raudnitz, M. Stoll and T. Takahashi, *Helv. Chim. Acta*, **15**, 1218 and 1399 (1932), *cf.* footnote 3.

We believe that the experiments described below are in accordance with previous observations concerning the C_{40}-carotenoids and that they provide us with an enlarged basis for further conclusions. The experimental data for methylbixin indicate that, as was found also for the lycopene set,[8] the *trans* configuration around any double bond investigated is thermodynamically more stable than the *cis*.

Acknowledgment.—We wish to thank Professor Linus Pauling for valuable advice, furthermore Professor A. J. Haagen-Smit and Dr. G. Oppenheimer for microanalyses.

Experimental Part

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 does not completely elute these bixin adsorbates. For the separation of the methylbixin stereoisomers, 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.

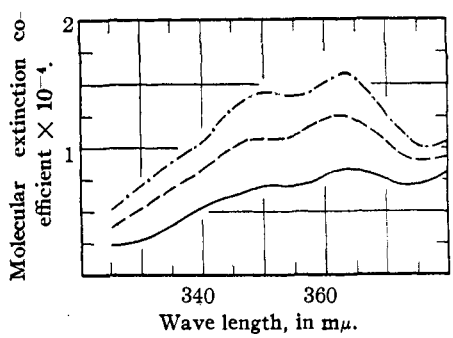


Fig. 5.—Influence of illumination on the development of the *cis*-peak effect in an iodine catalyzed solution of all-*trans*-methylbixin in benzene: ——, molecular extinction curve after standing for thirty minutes in darkness with iodine; – – –, after five seconds illumination; and —·—·, after thirty seconds illumination.

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. The melting point values may be influenced by isomerization.

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 dibutyl phthalate bath, and then

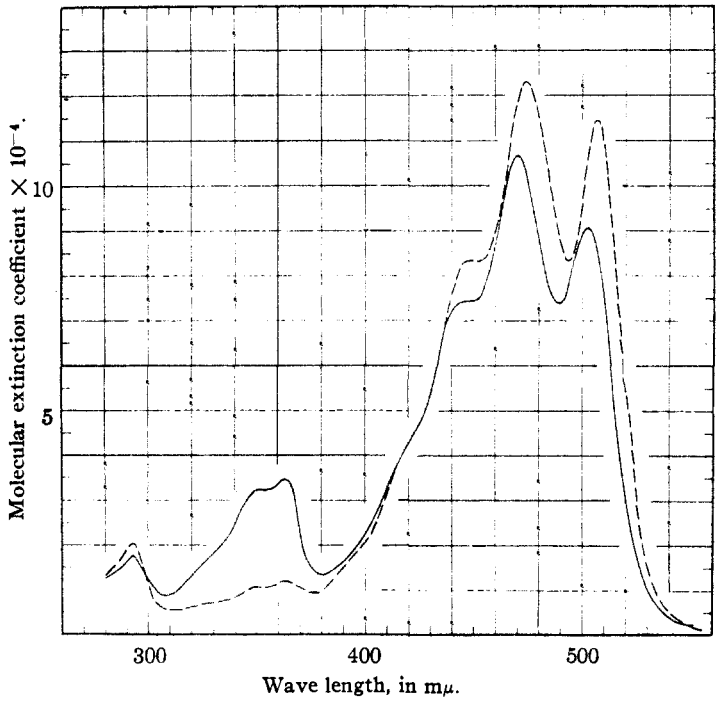


Fig. 6.—Photochemical isomerization of neomethylbixin A in benzene: ——, fresh solution of neomethylbixin A; and - - -, mixture of stereoisomers after fifteen minutes insolation.

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 Fig. 5. 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 S 47) on the basis of the following values.

k	0.2	0.4	0.6	0.8	1.0
Pigment in 100 ml. benzene, mg.	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 solutions 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 visually observed 488, 455 mμ (±0.5 mμ) when catalyzed with iodine. The extinction curves were determined in a Beckman Photoelectric Spectrophotometer.[16]

All crystalline samples were dried in high vacuum. The concentration of solutions was established in many cases from the equilibrium curve (Table II) upon iodine catalysis with an accuracy of ±2.5%. Since the columns were developed with benzene–petroleum ether mixtures but the concentrations had to be established in pure benzene, the adsorbates were washed after cutting with petroleum ether (b. p. 28–38°). The latter was eliminated by means of a vacuum pump and the pigment was eluted with ice cold acetone and transferred into benzene.

(16) H. H. Cary and A. O. Beckman, *J. Optical Soc. Am.*, **31**, 682 (1941).

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 *Bixa* seeds (250 g.) were shaken mechanically for about one-fourth 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 anhydrous 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 remethylated. The crystals (2.5 to 3 g.) were extracted by alternate shakings with equal volumes of chloroform and benzene. The combined and filtered solution (100 ml.) was drawn into a column (23 × 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 glittering, pure methylbixin crystallized (Fig. 10). After cooling to −10° 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 seeds.

Anal. Calcd. for $C_{24}H_{26}O_2(OCH_3)_2$: 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.

TABLE II

MOLECULAR EXTINCTION COEFFICIENTS OF SOME MEMBERS OF THE STEREOISOMERIC METHYLBIXIN SET AND OF THE IODINE EQUILIBRIUM MIXTURE AT THE MAXIMA (*italicized*) AND MINIMA IN BENZENE

Wave length, mμ		Wave length, mμ	
Natural methylbixin		Neomethylbixin A	
284	1.20	*293*	1.76
320	0.4(0)	308	0.8(8)
366	1.21	*362*	3.5
378	1.10	380	1.31
445	8.5	*444–8*	7.4
452	8.1	*470*	10.7
471	12.8	489	7.4
488	8.0	*502*	9.1
503	11.7		
		Neomethylbixin C	
All-*trans*-methylbixin		*297*	1.98
292	2.45	314	0.9(4)
316	0.3(0)	*363*	2.17
363	0.8(0)	378	1.50
374	0.7(3)	*438*	7.0
448	8.7	446	6.8
454	8.6	*465*	9.8
475	13.0	482	6.6
493	8.5	*495*	8.2
508	12.2		
Iodine equilibrium mixture			
363	1.6		
450	8.1		
473	11.5		
505	10.3		

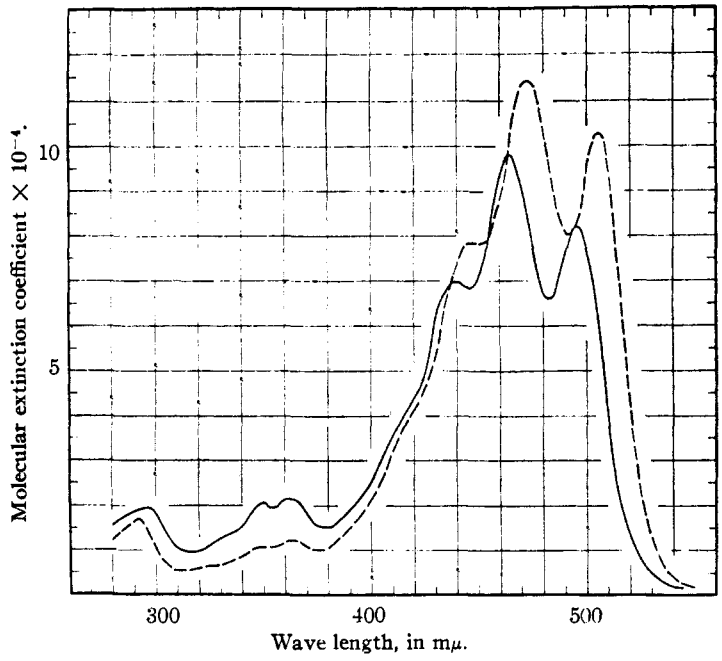


Fig. 7.—Photochemical isomerization of neomethylbixin C in benzene: ——, fresh solution of neomethylbixin C; and – – –, mixture of stereoisomers after fifteen minutes insolation.

The molecular extinction coefficients are compared with those of some other members of the set in Table II.

(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 twenty-four 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, and extended development may cause them to recombine.

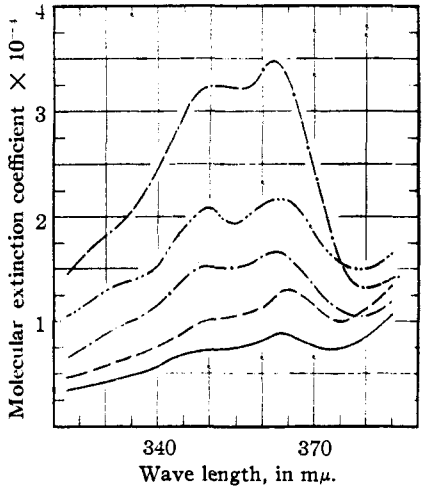


Fig. 8.—Molecular extinction curves of some members of the stereoisomeric methylbixin set in the *cis*-peak region in benzene: ——, all-*trans*-methylbixin; – – –, natural methylbixin; — · — ·, iodine equilibrium mixture; — · · · —, neomethylbixin C; and · — · — ·, neomethylbixin A.

A solution of 5 mg. of methylbixin in 20 ml. of benzene was refluxed for an hour, concentrated to 2 ml., diluted with 15 ml. of petroleum ether, and chromatographed (size of column 18 × 1.9 cm.). (The figures on the left designate zone width in millimeters.)

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

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

(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 and, after dilution with 3 vol. of petroleum ether, developed on a column (18 × 2.5 cm.) with benzene–petroleum ether 1:5 and later with the usual 1:3 mixture.

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 darker orange (see below)
32 orange, neo C: 479, 448.5 mμ
Filtrate: yellow, irreversible pigment

The colorimetric ratio was, unchanged natural methyl bixin: all-*trans:* neo A:neo B:neo C = 28:17:16:8:31.

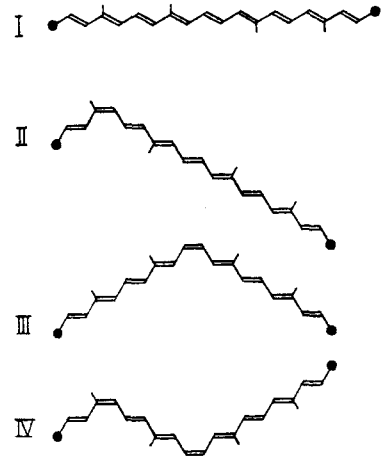


Fig. 9.—Suggested configurations of the four crystallized members of the methylbixin set: I, all-*trans*-methylbixin; II, natural methylbixin; III, neomethylbixin A; and IV, neomethylbixin C. (The carboxyl groups are represented by black circles.)

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 and shows a characteristic spectral curve. The first two of its four

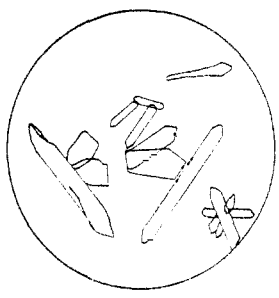


Fig. 10.—Natural methylbixin, crystallized from benzene and methanol (100×).

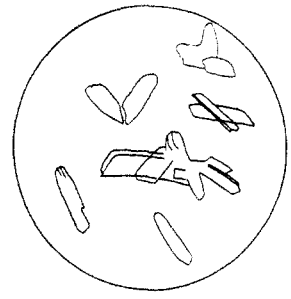


Fig. 11.—All-*trans*-methylbixin, crystallized from benzene and methanol (430×).

main maxima are identical in location with those of neomethylbixin B. The chromatographic separation of the two pigments is very difficult.

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

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

(The uppermost section of the 39-mm. zone showed 2 mμ shorter wave-length maxima than indicated.) The colorimetric ratio was, all-*trans:* neo A:neo C = 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 × 1.9 cm.) first with 20 ml. of benzene-petroleum ether 1:5 and then with 40 ml. of 1:3 mixture.

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

The colorimetric ratio was, unchanged natural methylbixin: minor isomers:neo C = 90:2:8.

2. All-*trans*-methylbixin

Isolation.—This pigment was prepared according to the method of Kuhn and Winterstein by catalyzing 500 mg. of natural methylbixin in 20 ml. of ethyl acetate with 30 mg. of iodine. On standing at 0°, crystals appeared which were recrystallized from benzene-methanol to give 200 mg. of chromatographically homogeneous crystals (Fig. 11); m. p. 198° (cor.). The influence of a polar solvent on the spectral curve is demonstrated by Fig. 12.

(a) *cis-trans* **Isomerization of All-*trans*-methylbixin upon Refluxing.**—Three milligrams of pigment in 20 ml. benzene was refluxed for an hour. The solution was concentrated to 3 ml., diluted with 5 vol. of petroleum ether and chromatographed (18 × 1.9 cm.).

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

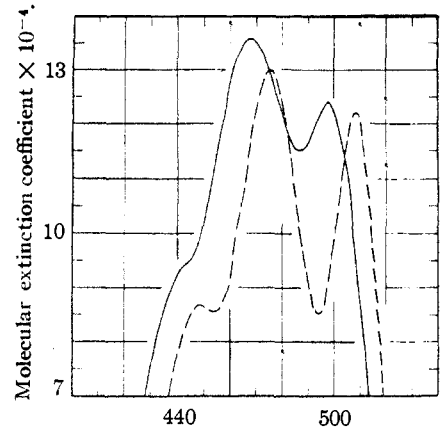


Fig. 12.—Molecular extinction curves of all-*trans*-methylbixin in the visible region: ———, in alcohol; - - - - -, in benzene.

The colorimetric ratio was, unchanged all-*trans:* neo A: minor isomers = 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 × 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 was, unchanged all-*trans:* neo A:neo C = 55:20:25.

(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, after standing for thirty minutes with iodine and diluting with 15 ml. of petroleum ether, was chromatographed (18 × 1.9 cm.).

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 was, unchanged all-*trans:* neo A: neo C(?) = 72:19:9.

(d) **Photochemical** *cis-trans* **Isomerization of All-*trans*-methylbixin.**—Two milligrams of pigment in 3 ml. of benzene, after dilution with 10 ml. of petroleum ether, was insolated for fifteen minutes (final temperature, 31°) and chromatographed (18 × 1.9 cm.).

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

The colorimetric ratio was, unchanged all-*trans:* neo A = 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 vol. of petroleum ether it was chromatographed (27 × 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 × 4.8 cm.). This chromatogram showed only traces of other isomers. However, since the uppermost section of the neo A zone usually contains considerable amounts of the all-*trans* isomer, about $^1/_6$ 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 addition of excess methanol (Fig. 13). The sample was recrystallized from benzene–methanol; yield, 20 mg.; m.p. 190–2° (cor.).

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 × 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 was, all-*trans:* unchanged neo A:neo B:neo C = 63:32:2:3.

(b) *cis-trans* **Isomerization upon Melting Crystals of Neomethylbixin A.**—Five milligrams of pigment was fused, kept at 195° for one minute, dissolved in 5 ml. of benzene, diluted with 15 ml. of petroleum ether and chromatographed (18 × 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 passed into 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 was, all-*trans:* unchanged neo A:neo B (+irreversible):neo C = 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 × 1.9 cm.) after standing half an hour.

40 colorless
44 pink, all-*trans:* 490, 456.5 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 was, all-*trans:* unchanged neo A:neo B:neo C = 64:25:2:9.

(d) **Photochemical** *cis-trans* **Isomerization of Neomethylbixin A.**—After a fifteen-minutes insolation of 3 mg. of pigment in benzene and dilution with 5 vol. of petroleum ether, the mixture was chromatographed (18 × 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)

Due to unsatisfactory separation, the following ratio is only approximately correct, all-*trans:* unchanged neo A = 45:55.

4. Neomethylbixin B

Small amounts of this isomer may be obtained by iodine catalysis or by refluxing neomethylbixin A solutions. Its extinction maxima were located at 490, 460 mμ, in benzene and shifted to 505, 472 mμ upon the addition of iodine. From one fusion experiment with natural methylbixin, a pigment of a different type was separated from the neo B zone. Its benzene solution showed four extinction maxima in the visual region (490, 461, 419 and 404 mμ), the two first being identical in position with those of neomethylbixin B. The addition of iodine did not produce the spectrum which is characteristic for the methylbixin set.

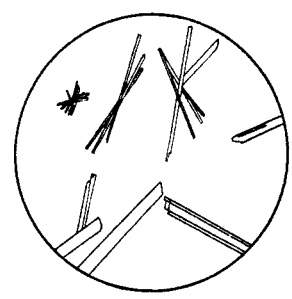


Fig. 13.—Neomethylbixin A, crystallized from benzene and methanol (430×).

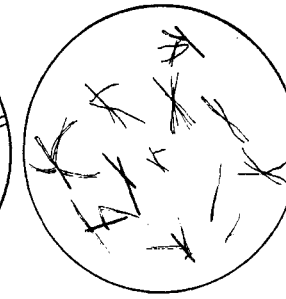


Fig. 14.—Neomethylbixin C, crystallized from benzene and methanol (430×).

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 (30 × 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, neo 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 developed with benzene–petroleum ether (1:5) on a smaller column (27 × 5.8 cm.). Except for traces, the pigment was found to be homogeneous. It was transferred into petroleum ether and completely evaporated *in vacuo*. The red, oily residue was taken up in the minimum amount of benzene and transferred to a centrifuge tube; the solvent was evaporated with a carbon dioxide stream at 25°. When methanol was added gradually with stirring, an oily suspension appeared which crystallized upon scratching and cooling. Upon seeding, the crystals appear directly. After cooling to −10°, they were centrifuged and recrystallized (Fig. 14). The intensely colored mother liquors may be reworked. The yield of crude neomethylbixin C crystals was 50 mg.; however, this quantity decreased to 25–30 mg. upon recrystallization; m. p. 150–1° (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 × 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 was, natural methylbixin: all-*trans*:neo A: neo B: unchanged neo C = 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 5 vol. of petroleum ether and chromatographed (18 × 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 was, natural: all-*trans*: neo A: neo B: unchanged neo C = 51:4:4:5:36.

(c) ***cis-trans* Isomerization of Neomethylbixin C by Iodine Catalysis at Room Temperature.**—Three milligrams of pigment was 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 × 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 was, all-*trans*: neo A: unchanged neo C = 63:25:12.

(d) **Photochemical *cis-trans* Isomerization of Neomethylbixin C.**—A solution of 2 mg. of pigment in 3 ml. of benzene was insolated for fifteen minutes and after dilution with 10 ml. of petroleum ether, chromatographed (18 × 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 was, natural: neo A: unchanged neo C = 21:2:77.

Summary

Besides "labile methylbixin," now named "natural methylbixin," and "stable" termed "all-*trans*-methylbixin," two other stereoisomers, neo A and C, have been isolated in crystals; several minor members of this set were observed in solution. The mutual conversion of the stereoisomers can be carried out by means of thermal methods, iodine catalysis or insolation. Natural and all-*trans*-methylbixin are practically not interconvertible by refluxing or exposure to sunshine. In contrast, the reversible formation of neo C from natural methylbixin and of neo A from the all-*trans* form takes place easily under similar conditions. On the basis of spectroscopic data, especially "*cis*-peak" measurements, configurations are suggested for the four main observed methylbixins.

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[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, NO. 947]

The Serological Properties of Simple Substances. VI. The Precipitation of a Mixture of Two Specific Antisera by a Dihaptenic Substance Containing the Two Corresponding Haptenic Groups; Evidence for the Framework Theory of Serological Precipitation

BY LINUS PAULING, DAVID PRESSMAN AND DAN H. CAMPBELL

The framework theory (lattice theory) of serological precipitation and agglutination, first proposed by Marrack,[1] was shown by Marrack and by Heidelberger and Kendall[2] to account for many experimental observations. Because of its simplicity and its compatibility with the available information about intermolecular forces, this theory was incorporated in his general theory of the structure and process of formation of antibodies by one of the present authors.[3]

Strong support of the framework theory has been provided during the past two years by the results of extensive studies of the reactions of antibodies and simple substances,[4] based upon the observations by Landsteiner and Van der Scheer[5] of the precipitation of antibody by certain simple substances containing two haptenic groups. It was found[4] from experiments with about fifty substances that all of those (about twenty) containing two or more haptenic groups (azophenylarsonic acid groups) per molecule gave precipitates with antiserum homologous to this haptenic group, and that none of the monohaptenic substances gave a precipitate. This fact is most readily accounted for by the framework theory.

The argument might be made, however, that no more than one of the haptenic groups of a molecule of a polyhaptenic substance is involved in interaction with antibody molecules, and that the difference in precipitability of polyhaptenic and monohaptenic substances with antiserum is due to some difference in properties of these two classes of substances, such as a tendency to asso-

(1) J. R. Marrack, "The Chemistry of Antigens and Antibodies," Report No. 194 of the Medical Research Council, His Majesty's Stationery Office, London, 1934; Second Edition, Report No. 230, 1938.

(2) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **61**, 559, 563; **62**, 467, 697 (1935); M. Heidelberger, *Chem. Rev.*, **24**, 323 (1939).

(3) Linus Pauling, THIS JOURNAL, **62**, 2643 (1940).

(4) Linus Pauling, David Pressman, Dan H. Campbell, and collaborators, THIS JOURNAL, **64**, 2994, 3003, 3010, 3015 (1942); **65**, 728 (1943).

(5) K. Landsteiner and J. Van der Scheer, *Proc. Soc. Exptl. Biol. Med.*, **29**, 747 (1932).