sulfate and then saturated with gaseous hydrogen chloride. The hydrochloride which separated was filtered off, washed with benzene, dissolved in 95% ethanol and neutralized with dilute ammonium hydroxide. The addition of an excess of water caused an oil to separate. This oil was dissolved in hot dilute ethanol, and the resulting solution was cooled. The white crystals which separated melted at 101–103°, and did not depress the melting point of an authentic specimen of 4-(β-phenylethyl)-quinoline.[29]

**Acknowledgment.**—The authors are grateful to Howard A. Hartzfeld for assistance.

(29) B. Heymann and W. Koenigs, *Ber.*, **21**, 1424 (1888).

AMES, IOWA

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[CONTRIBUTION NO. 1868 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

# Stepwise Dehydrogenation of the Colorless Polyenes Phytoene and Phytofluene with N-Bromosuccinimide to Carotenoid Pigments

BY L. ZECHMEISTER AND B. KENNETH KOE

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Dehydrogenation of the colorless plant polyenes phytoene and phytofluene can be carried out with N-bromosuccinimide and some other reagents. This stepwise process takes place mainly at the ends of the conjugated system and leads through the following series: phytoene → phytofluene → ζ-carotene → neurosporene → lycopene. The artifacts were identified with the corresponding natural products. The exact mechanism of some of the steps mentioned is unknown.

The representatives of the naturally occurring $C_{40}$-polyenes with isoprenic structure belong either to the subclass of colorless, fluorescent compounds such as phytoene[1] and phytofluene[2] or to the carotenoid pigments proper containing longer conjugated systems that extend over 7 to 15 conjugated double bonds. As reported briefly,[3] a transition from the first to the second type can be achieved by dehydrogenation *in vitro*. So far as we know the most convenient agent for this purpose is N-bromosuccinimide (NBS); various other compounds such as N-bromoacetamide (NBA), *p*-benzoquinone, diphenoquinone, isatin and *o*-nitrosonitrobenzene, can also be applied in principle but the yields are very low in the latter three instances.

In the present study the experimental conditions of the following dehydrogenation steps are described: phytoene (3 conj. double bonds) → phytofluene (5) → ζ-carotene[4] (7) → neurosporene[5] (9) → lycopene, $C_{40}H_{56}$ (11). After having treated any of these polyenes with N-bromosuccinimide, a subsequent chromatographic resolution shows the presence of all those members of the above series that are more unsaturated than the compound treated. The main product is in each instance that polyene whose conjugated system contains two more double bonds than the starting material. However, neurosporene forms lycopene in very poor yields under the conditions applied. In contrast, according to Karrer and Rutschmann,[6] the further dehydrogenation of lycopene gives substantial amounts of dehydrolycopene ($C_{40}H_{52}$, 15 conj. double bonds), possibly because in that particular instance the two newly formed double bonds establish connection by conjugation of the main chromophore with the two formerly isolated double bonds located in the terminal isopropylidene groups of the lycopene molecule.

In the course of a rather extended study of the pertinent experimental conditions the following observations were made. Although the reaction involves liberation of hydrogen bromide, brominated compounds have not been encountered. Partial destruction of the conjugated system did take place in every instance but its extent could be limited by using not more than 1 mole reagent per mole polyene. It is advantageous to carry out such conversions in the presence of glacial acetic acid[7] whereby the dehydrogenation proceeds rapidly. Thus, upon treatment of practically non-fluorescent phytoene the strong fluorescence of newly formed phytofluene appeared within half a minute; and starting from phytofluene, the mixture turned a dark red in a minute or so. These two conversions require moderate heating, but the further ones some cooling. For yields *cf.* Table I. In the conversion of phytoene to phytofluene the yields were reduced by the presence of N-phenylmorpholine, sodium ace-

TABLE I

YIELDS, ESTABLISHED PHOTOMETRICALLY, IN THE STEPWISE DEHYDROGENATION OF SOME POLYENES

| Dehydrogenation step | Reagent | Yield based on starting mater., % | Yield based on converted starting mater., % |
|---|---|---|---|
| Phytoene → phytofluene | NBS | 26 | 40 |
| Phytoene → phytofluene | NBA | 16 | 31 |
| Phytoene → phytofluene | Quinone | 9 | 35 |
| Phytofluene → ζ-carotene | NBS | 28 | 40 |
| ζ-Carotene → neurosporene | NBS | 19 | 27 |
| Neurosporene → lycopene | NBS | 4 | 7 |

(1) J. W. Porter and F. P. Zscheile, *Arch. Biochem.*, **10**, 547 (1946); J. W. Porter and R. E. Lincoln, *ibid.*, **27**, 390 (1950); W. J. Rabourn and F. W. Quackenbush, *ibid.*, **44**, 159 (1953).

(2) L. Zechmeister and A. Polgár, *Science*, **100**, 317 (1944); L. Zechmeister and A. Sandoval, *Arch. Biochem.*, **8**, 425 (1945); THIS JOURNAL, **68**, 197 (1946); V. Wallace and J. W. Porter, *Arch. Biochem. Biophys.*, **36**, 468 (1952); *cf.* H. H. Strain, *J. Biol. Chem.*, **127**, 191 (1939); "Leaf Xanthophylls," Carnegie Inst. of Washington, 1938.

(3) B. K. Koe and L. Zechmeister, *Arch. Biochem. Biophys.*, **41**, 236 (1952). Previously, squalene, $C_{30}H_{50}$, had been dehydrogenated to polyene pigments in our laboratory by J. Dale, *ibid.*, **41**, 475 (1952).

(4) H. A. Nash, F. W. Quackenbush and J. W. Porter, THIS JOURNAL, **70**, 3613 (1948); H. A. Nash and F. P. Zscheile, *Arch. Biochem.*, **7**, 305 (1945); *cf.* also H. H. Strain, ref. 2.

(5) F. T. Haxo, *Arch. Biochem.*, **20**, 400 (1949).

(6) P. Karrer and J. Rutschmann, *Helv. Chim. Acta*, **28**, 793 (1945).

(7) L. Bateman, J. I. Cuneen and H. P. Koch, *J. Chem. Soc.*, 3045 (1950); G. Dupont, R. Dulou and N. Defay, *Bull. soc. chim. France*, 310 (1949); R. Tschesche and F. Korte, *Ber.*, **84**, 77 (1951).

tate or barium carbonate while benzoyl peroxide and trichloroacetic acid were without marked effect.

The oily phytofluene formed was identified by spectral and analytical data, including catalytic hydrogenation as well as by comparison with carrot oil phytofluene. The likewise non-crystallizable ζ-carotene was compared with a corresponding pigment fraction *ex* carrot oil; and the crystalline dehydrogenation product neurosporene with crystals originating from *Neurospora crassa*[5] for which we are greatly indebted to Dr. F. T. Haxo of the Scripps Institution of Oceanography, La Jolla, California. Our end product, crystalline lycopene, could not be differentiated from the main pigment of ripe tomatoes. A minor, still further dehydrogenated compound, probably containing 13 conjugated double bonds and adsorbed above lycopene on the chromatographic column, has not yet been studied in detail but it is spectroscopically quite different from Karrer's dehydrolycopene.

The exact mechanism of some of the conversions discussed above cannot be given at the present time since the structures of phytofluene and phytoene have not yet been established with certainty.

It is possible, although still under discussion,[8] that the (enzymatic) dehydrogenation of colorless polyenes does play a part in the formation of plant carotenoids. We may mention in this connection that the final stage of the biosynthesis of some carotenoid pigments, among others lycopene, requires the presence of oxygen.[9]

## Experimental

**Materials and Methods.**—Adsorbents: (a) a 3:1:1 mixture of alumina (Aloreo, Grade F, Aluminum Ore Co., East St. Louis, reground to −200 mesh), lime (Sierra Hydrated Lime, Superfine, U. S. Lime Products Corp., Los Angeles) and Celite No. 545 (Johns-Manville Co.); (b) a 85:15 mixture of lime and Hyflo-Super Cel (Johns-Manville Co.); (c) a 3:1 mixture of magnesia ("Seasorb" 43; Food, Mach. and Chem. Corp., San José, Calif.) and Celite. The N-bromosuccinimide and N-bromoacetamide were manufactured, respectively, by Arapahoe Chemicals, Inc., Boulder, Colo., and Matheson, Coleman and Bell, Inc., East Rutherford, N. J. Chromatographic zones were cut out and eluted with acetone. Sharp light was avoided throughout the work. Evaporations were carried out *in vacuo* while nitrogen bubbled through. Solutions were washed in a continuous apparatus[10] and dried with sodium sulfate. Spectral data refer to the Beckman instrument.

**Preparation of Phytoene and Phytofluene.**—According to Rabourn and Quackenbush[1] carrot oil (Nutrit. Research Assoc., South Whitley, Ind.) is a suitable starting material for this purpose. Considering the low pigment content of the oil, we have adopted it as a starting material but used a procedure different from that of the authors mentioned. A solution of 120 g. of oil in 250 ml. of hexane was adsorbed on alumina–lime–Celite contained in a percolator (48 × 21 × 8 cm.) and developed with benzene–hexane 1:3 until the strongly greenish fluorescent phytofluene had almost reached the base of the "cone." The receiver containing the phytoene fraction (about 600 mg.) was changed at this point and by further washing a crude phytofluene fraction was obtained, also containing stereoisomers and sizable amounts of pigment, mainly α-carotene.

(a) **Phytoene.**—After concentration, this fraction was developed on two 27 × 5.5 cm. alumina–Hyflo Super-Cel columns with 3 l. of hexane per column. The phytoene zone was then located by streaking the column along its main axis with 2% permanganate solution. Where the streak crossed the zone (about half-way down the column) the reagent turned brown within 1–2 min. (Considerably later the whole streak would react.) The phytoene zone was eluted, transferred with water into hexane, washed acetone-free, shaken 3 times with 95% methanol in order to eliminate some impurities, washed again and dried; photochemically established yield about 400 mg.

(b) **Phytofluene.**—This fraction containing approximately 300 mg. of phytofluene was, after concentration, developed with hexane + 5% acetone on two 30 × 8 cm. magnesia–Celite columns until the phytofluene zone had reached the bottom. After cutting and elution, this fraction was treated as described for phytoene; yield 150 mg.

**Conversion of Phytoene into Phytofluene (a).**—The evaporation residue of a colorless hexane solution containing 105 mg. of phytoene was dissolved in 7.5 ml. of carbon tetrachloride. While stirring mechanically in a bath of 40°, a solution of 33.8 mg. of N-bromosuccinimide in 2.5 ml. of glacial acetic acid (40°) was poured in. After 6 more min. stirring the liquid had turned orange in color and was rinsed by means of 40 ml. of hexane into a separatory funnel containing a 5% sodium bicarbonate solution that was covered with 30 ml. of hexane. The liquid was swirled with water and the epiphase (about 75 ml.) washed acid-free, dried, and developed with hexane on a 30 × 8 cm. lime–Celite column (the figures on left designate thickness of zones, in mm.; the spectral maxima refer to hexane solutions; fl. = fluorescent or fluorescence)

| | | |
|---|---|---|
| 20 | red and orange-yellow pigments and blue fl. zones | |
| 15 | pale yellow, no fl. | |
| 30 | greenish fl.: phytofluene-like substances (2 mg.) | 367–368, 348, 332 mμ |
| 25 | empty interzone | |
| 125 | strong greenish fl.: phytofluene (23 mg.) | 367, 348, 332 mμ |
| 13 | greenish-gray fl.: a *cis*-phytofluene (1 mg.) | 366, 347, 331 mμ |
| 18 | weaker greenish-gray fl.: a *cis*-phytofluene (0.5 mg.) | 366, 347, 331 mμ |
| 54 | empty interzone | |
| Filtrate: 38 mg. of unreacted phytoene and unidentified greenish fl. substances | | |

The 125-min. zone was eluted and, after transfer into hexane, was combined with corresponding fractions obtained in two parallel experiments (total, 72 mg. of phytofluene). After rechromatography on two 30 × 8 cm. alumina–lime–Celite columns (developer, benzene–hexane 1:3), the combined eluate of the two respective main zones was washed and treated with methanol as described. For analysis a suitable fraction was evaporated completely, dried in high vacuum, in darkness, in an Abderhalden apparatus, over $P_2O_5$ and paraffin for 6 hours.

*Anal.* Calcd. for $C_{40}H_{68}$: C, 87.51; H, 12.49. Found: C, 88.05; H, 12.02 (Br absent).

Catalytic hydrogenation: 20.102 mg. of subst. adsorbed in the presence of 28 mg. of $PtO_2$, 5.84 ml. of hydrogen (0°, 760 mm.); calcd. 7.0 and found 7.1 double bonds.

This preparation showed exactly the extinction curve of natural phytofluene.[11] In the mixed chromatogram test it did not separate either from phytofluene *ex* carrot oil or from a sample obtained by dehydrogenation of phytoene with *p*-benzoquinone (see below), following the elimination from each test substance of a *cis* form by chromatographic resolution on a very long column.[11]

(b).—Dehydrogenation of 105 mg. of phytoene with 26 mg. of N-bromoacetamide under the conditions outlined resulted in the formation of 17 mg. of phytofluene while 52 mg. of the starting material remained unchanged.

(c).—The dehydrogenation of phytoene with *p*-benzoquinone requires higher temperatures and is best carried

(8) L. Zechmeister and A. Sandoval, ref. 2; J. W. Porter and R. E. Lincoln, ref. 1; J. Bonner, A. Sandoval, Y. W. Tang and L. Zechmeister, *Arch. Biochem.*, **10**, 113 (1946); G. Mackinney, C. O. Chichester and P. S. Wong, THIS JOURNAL, **75**, 5428 (1953). General Survey: T. W. Goodwin, "The Comparative Biochemistry of Carotenoids," Chapman & Hall, Ltd., London, 1952.

(9) V. N. Lubimenko, *Mém. Acad. Sci. Pétrograd*, VIII, p. 33, Nr. 12 (1916); B. M. Duggar, *Washington Univ. Stud.*, **1**, 22 (1913); R. Kuhn and W. Wiegand, *Helv. Chim. Acta*, **12**, 499 (1929); L. Zechmeister and L. v. Cholnoky, *Z. physiol. Chem.*, **199**, 22 (1930); *Ber.*, **67**, 170 (1934).

(10) A. L. LeRosen, *Ind. Eng. Chem., Anal. Ed.*, **15**, 105 (1943).

(11) B. K. Koe and L. Zechmeister, *Arch. Biochem. Biophys.*, **46**, 100 (1953); F. J. Petracek and L. Zechmeister, THIS JOURNAL, **74**, 181 (1952).

out in the absence of solvents. Thus, a mixture of 33.5 mg. of phytoene and 9.3 mg. of the quinone (resublimed) was sealed in an evacuated tube and immersed in a bath of refluxing xylene for 3.5 hours. The product (a bright red mass mixed with large, colorless crystals of hydroquinone) was dissolved in hexane–acetone, the latter was eliminated by washing, and the orange hexane solution chromatographed on a 20 × 3.5 cm. column as described above.

85 empty (except for minor pigment and weakly fl. zones at top)
62 strong green fl.
22 grayish-green fl.
19 grayish-green fl.
} 3 mg. phytofluene, including *cis* forms 366–367, 348, 331 mμ
21 empty section
Filtrate: 23 mg. of unreacted phytoene and some bluish-green fl. substances

**Conversion of Phytofluene into ζ-Carotene.**—A total of 1.3 g. of phytofluene was dehydrogenated in 13 portions, each containing 100 mg. of substance in 7.5 ml. of carbon tetrachloride (40°) to which, while stirring, a solution of 33 mg. of N-bromosuccinimide in 2.5 ml. of glacial acetic acid was added (40°). Four minutes later the darkened solution was rinsed over (using 30 ml. of hexane) into a separatory funnel containing some 5% sodium bicarbonate solution covered with hexane. After washing and drying, the total solution (*ex* 1.3 g. of phytofluene) was developed with hexane–acetone 9:1 on six 30 × 8 cm. lime–Celite columns. Each chromatogram showed the following sequence.

13 several brown zones
4 pink
2 colorless interzone
10 orange-red: lycopene, etc., 495–496, 465, 438 mμ
50 colorless interzone
60 orange-yellow: neurosporene (7 mg.), 465, 436, 413–414 mμ
60 colorless interzone
5 pale orange
70 greenish-yellow: ζ-carotene (62.5 mg.), 422, 397, 376 mμ
7 colorless interzone
19 greenish-gray fl.: unreacted phytofluene (66 mg.) contaminated by some minor pigment zones
Filtrate: yellow, greenish fl.

The combined eluate of the six ζ-carotene zones was transferred into hexane and rechromatographed on four 30 × 8 cm. magnesia–Celite columns (developer, hexane–acetone 9:1). The greenish-lemon-yellow ζ-carotene zone separated easily from some α-carotene and phytofluene. For analysis the solution of a 30-mg. sample was twice rechromatographed on alumina–lime–Celite (developer, benzene–hexane 2:3), then extracted with methanol, washed methanol-free, dried and evaporated. The light orange-yellow oil was dried in high vauum.

*Anal.* Calcd. for $C_{40}H_{64}$: C, 88.15; H, 11.85. Found: C, 87.96, 88.09; H, 12.05, 12.00.

The substance did not separate in the mixed chromatogram test, on magnesia–Celite, from a ζ-carotene sample obtained from carrot oil. On lime–Celite it could be resolved to give an all-*trans* zone (top; maxima in hexane, 425, 400, 379, 360–1 mμ, *cf.* Fig. 1) and two *cis* forms (423, 398, 378 mμ and 421, 396, 375–6 mμ).

**Conversion of ζ-Carotene into Neurosporene.**—A solution of 115 mg. of substance in 8.6 ml. of carbon tetrachloride (0°) reacted with a likewise precooled glacial acetic acid solution (2.9 ml.) of 37.5 mg. of N-bromosuccinimide for 4 min. while stirring in an ice-bath and then treated as described in the foregoing section. The resulting dark red hexane solution from two parallel experiments was adsorbed on lime–Celite (30 × 8 cm.) and developed with hexane–acetone 9:1

15 yellow and violet minor zones
5 almost colorless interzone
14 two red zones: lycopene, etc. (0.9 mg.)
55 colorless interzone
100 yellow orange: neurosporene (47 mg.)
15 colorless interzone
70 greenish yellow: unchanged ζ-carotene (86 mg.)
26 colorless section

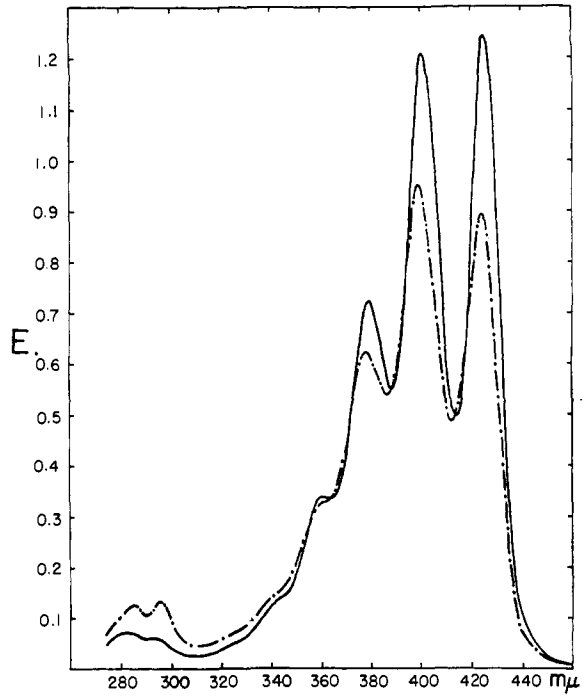


Fig. 1.—Extinction curves (in hexane) of ζ-carotene obtained by dehydrogenation of phytofluene: ——, before, and - - - -, after iodine catalysis (in light).

The main zone was eluted, transferred into hexane, rechromatographed (lime–Celite; hexane + 5% acetone), evaporated to dryness, dissolved in a small amount of chloroform (50°), crystallized by cautious addition of abs. ethanol until cloudiness appeared and then kept at 20°; yield 10 mg. of slender, orange-yellow needles grouped in sheaf, fan or star forms.

After recrystallization from benzene–methanol the sample melted at 110–112° (*in vacuo*); m.p. of similarly recrystallized neurosporene *ex* Neurospora 112–115°, and of the mix-

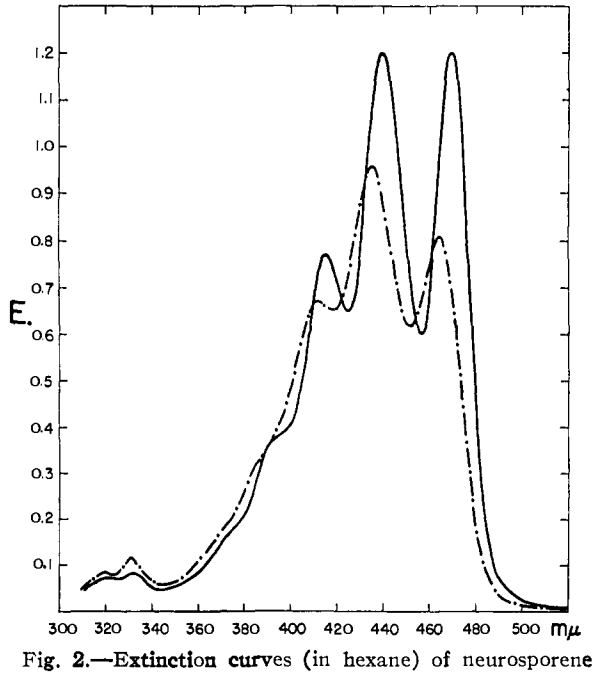


Fig. 2.—Extinction curves (in hexane) of neurosporene obtained by dehydrogenation of ζ-carotene; ——, before, and - - - -, after iodine catalysis (in light).

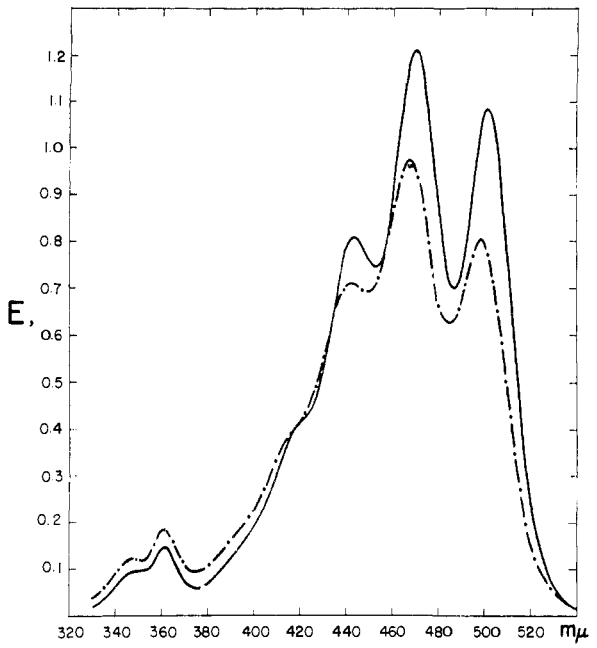


Fig. 3.—Extinction curves (in hexane) of lycopene obtained by dehydrogenation of neurosporene: ——, before, and -·-·-, after iodine catalysis (in light).

ture 111–115°. The spectral curve is given in Fig. 2. Our neurosporene preparation did not separate in the mixed chromatogram test from Haxo's neurosporene sample.

*Anal.* Calcd. for $C_{40}H_{60}$: C, 88.81; H, 11.19. Found: C, 89.29, 89.18; H, 10.66, 10.94.

**Conversion of Neurosporene into Lycopene.**—A solution of 75 mg. of neurosporene in 5.6 ml. of carbon tetrachloride reacted with 25 mg. of N-bromosuccinimide in 1.9 ml. of glacial acetic acid at 0° for 2 min. The dark red liquid was treated as described above and, finally, transferred into hexane. The combined reaction product of three similar experiments was developed with hexane–acetone 4:1 on lime–Celite (27 × 5.8 cm.)

| | | |
|---|---|---|
| 50 | several brown and two purple zones | |
| 25 | orange red | (8 mg.) |
| 1 | colorless interzone | |
| 4 | pink | |
| 3 | orange | |
| 10 | orange-red: lycopene | (9 mg.) |
| 16 | four orange zones (and interzones) | |
| 167 | empty section | |
| Filtrate: unreacted neurosporene (100 mg.) | | |

The 9-mg. fraction was rechromatographed, the main red zone eluted, transferred into hexane, evaporated and crystallized from chloroform–ethanol; long, quadrangular prisms typical for lycopene; yield 0.8 mg. When developed with benzene–hexane 3:2 on lime–Celite, this artifact did not separate from tomato lycopene. It showed the expected spectrum both before and after iodine catalysis (Fig. 3).

**Acknowledgment.**—We wish to thank Dr. A. Elek as well as Professor A. J. Haagen-Smit and Mr. G. Swinehart for analyses.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGY AND VITAL ECONOMICS, THE UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE AND DENTISTRY]

# Synthesis of D-Riboflavin-2-$C^{14}$ and its Metabolism by *Lactobacillus casei*[1]

BY EDWARD E. HALEY AND JOHN P. LAMBOOY

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The synthesis of D-riboflavin-2-$C^{14}$ with a specific activity of 3.4 μc. per mg. is described. Wöhler's method for the preparation of urea was modified to increase the yield to 79 to 84%, making it suitable for the convenient preparation of urea-$C^{14}$. D-Riboflavin-2-$C^{14}$ is metabolized by *Lactobacillus casei* to flavin–adenine dinucleotide (FAD), flavin mononucleotide (FMN), $CO_2$ and an unidentified compound.

Isotopic labels have been useful in elucidating the metabolic pathways of many of the B vitamins, particularly thiamine and nicotinamide.[2–7] Isotopically labeled D-riboflavin has not heretofore been prepared by chemical synthesis. Riboflavin-$C^{14}$ has been produced biosynthetically by a strain of *Ashbya gossypii*.[8] In the work to be reported, D-riboflavin was synthesized with $C^{14}$ incorporated into the 2-position of the molecule. Its specific activity, 3.4 μc. per mg., enables it to be useful in studies of riboflavin metabolism. D-Riboflavin-2-$C^{14}$ was provided as the riboflavin source in growing cultures of *Lactobacillus casei*. Metabolic products were extracted and identified by means of radioautographs of paper chromatograms.

## Experimental

**Urea-$C^{14}$.**—Wöhler's method was chosen for the preparation of urea-$C^{14}$, but was modified to give a marked increase in yield. $BaC^{14}O_3$ was converted to $KC^{14}N$ by the sodium azide method.[9a,9b] To a 50-ml. solution of 0.0076 g. of $KC^{14}N$ (0.0467 μc.) and 0.075 g. of KOH was added 0.275 g. of KOH to increase the concentration to 6.3 millimolar. This solution was concentrated to about 3 ml. by freeze-drying, with a loss of only 3% of the cyanide, then transferred with 2.5 ml. of water to a 50-ml. centrifuge tube for the next reaction. The quantitative conversion to $KC^{14}NO$ was carried out following a modification of the method of Gall and Lehman.[10] Carrier KCN, 0.321 g., and $Cu(OH)_2$ freshly prepared from 0.487 g. of $CuSO_4 \cdot 5H_2O$ and 0.30 g.

(1) This work was supported in part by Research Grant Number G 3320 C from the National Institutes of Health, Public Health Service.

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