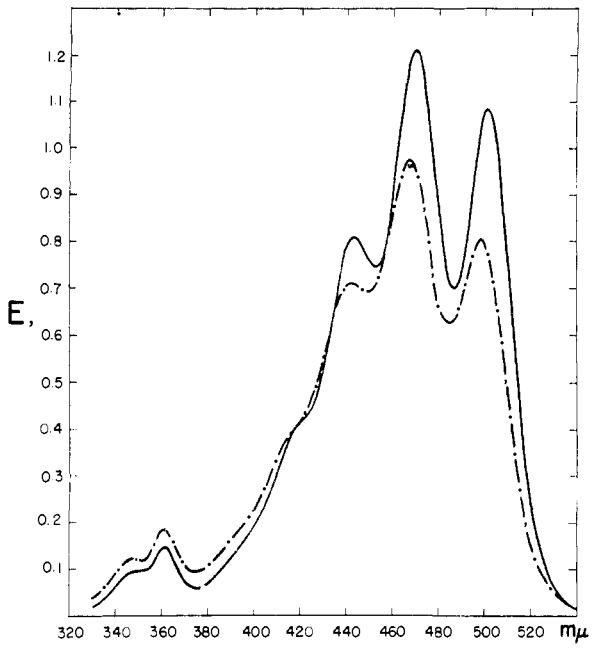

Fig. 3.—Extinction curves (in hexane) of lycopene obtained by dehydrogenation of neurosporene: ——, before, and -·-·-, after iodine catalysis (in light).

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
1 colorless interzone } (8 mg.)
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).

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PASADENA, CALIFORNIA

[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

RECEIVED FEBRUARY 3, 1954

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 3326 C from the National Institutes of Health, Public Health Service.

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of KOH were added. A solution of 0.675 g. of $KMnO_4$ in 12 ml. of water was added gradually with mechanical stirring, then the reaction mixture was heated in a water-bath at 50° for 5 minutes. The mixture was cooled and the excess $KMnO_4$ decomposed by the dropwise addition of 1 ml. of 3% H_2O_2. The MnO_2 was removed and washed thrice with water by centrifugation. The combined supernatant and washings were filtered, and 5.32 ml. of 0.085 *N* H_2SO_4 (0.45 meq.) was added slowly with stirring to neutralize a portion of the KOH. The solution was concentrated *in vacuo* at room temperature to about 3 ml. and transferred to a 13-ml. screw cap tube with washings to bring the volume to 5.3 ml. Ammonium sulfate, 0.676 g., and 2.6 ml. of NH_4OH (sp. gr., 0.90) were added. The tube, equipped with a sealed-in thermometer, was pressure sealed and heated in a water-bath at 70° for 40 minutes with agitation every 10 minutes. The mixture was cooled and transferred to a 100-ml. round-bottom flask and the NH_3 removed by distillation *in vacuo* at room temperature. The mixture was placed in a covered petri dish and allowed to evaporate to dryness slowly *in vacuo* over H_2SO_4. The dry residue was pulverized, transferred to a 50-ml. round-bottom flask and extracted 4 times with 15-ml. portions of *n*-butyl alcohol at 70°, stirring each for 30 minutes. The *n*-butyl alcohol extracts were evaporated to dryness *in vacuo* at room temperature to yield 0.257 g. which gave an assay for 94% urea by the urease method (80% of theoretical).[11] The total activity was 0.0404 μc. (86% of theoretical). Following this procedure 0.231 g. of urea with a specific activity of 1.27 mc. per millimole (21.2 μc. per mg.) was prepared.

Barbituric-2-C^{14} Acid.—Urea-C^{14}, 0.231 g., was converted to barbituric-C^{14} acid by an adaptation of the method of Slimmer and Stieglitz.[12] Freshly cut sodium, 0.20 g., was dissolved in 4.0 ml. of absolute ethanol. Two ml. of this solution was added to the urea in the reaction tube. The urea was dissolved with heating and to the hot solution was added a solution of 0.62 ml. of diethyl malonate in 1.0 ml. of absolute ethanol. The contents were rapidly mixed and the tube (1.5 × 13 cm.) was sealed and placed in an oven at 108° for 7 hours. The reaction mixture was dissolved in 17 ml. of hot water, then acidified with 1.0 ml. of concentrated HCl and placed in the refrigerator. Two crops of crystals were filtered and dried, yielding 0.374 g. (82% of theoretical) with a specific activity of 1.38 mc. per millimole (10.8 μc. per mg. (84% of theoretical)).

1-(D-Ribitylamino)-2-*p*-tolylazo-4,5-dimethylbenzene.—3,4-Dimethylaniline[13,14] was condensed with D-ribose to yield 3,4-dimethylaniline-N-D-ribopyranoside,[15] which on hydrogenation over Pd on $CaCO_3$ at 60 p.s.i. for three hours at 70° yielded N-D-ribityl-3,4-dimethylaniline. This material was converted to 1-(D-ribitylamino)-2-*p*-tolylazo-4,5-dimethylbenzene.[16]

D-Riboflavin-2-C^{14}.—The 0.374 g. of barbituric-2-C^{14} acid was condensed with an equimolar quantity (1.09 g.) of 1-(D-ribitylamino)-2-*p*-tolylazo-4,5-dimethylbenzene following the method of Tishler, *et al.*[16,17] The crude D-riboflavin-2-C^{14} was recrystallized from hot water to yield 0.504 g. (46% of theoretical) of orange crystals. The specific activity was 1.26 mc. per millimole (3.4 μc. per mg.; 41% of theoretical). Identity of the D-riboflavin-2-C^{14} was established by the usual physical properties as well as paper chromatography and microbiological assay using *Lactobacillus casei*.

3,4-Dihydro-3-keto-4-(D-1′-ribityl)-6,7-dimethylquinoxaline-2-carboxyureide.—1-(D-Ribitylamino)-2-phenylazo-4,5-dimethylbenzene[18] was catalytically reduced and the resulting phenylenediamine condensed with alloxan.[19,20]

3-Hydroxy-6,7-dimethylquinoxaline-2-carboxyureide.—4,5-Dimethyl-2-nitroaniline was catalytically reduced and the resulting phenylenediamine condensed with alloxan to yield a yellow solid which was recrystallized from 50% acetic acid; m.p. 274–275° dec.

Anal. Calcd. for $C_{12}H_{12}O_3N_4$: C, 55.4; H, 4.7; N, 21.5. Found: C, 55.2; H, 5.3; N, 21.9.

3,4-Dihydro-3-keto-4-methyl-6,7-dimethylquinoxaline-2-carboxyureide.—3,4-Dihydro-3-keto-4-methyl-6,7-dimethylquinoxaline-2-carboxyureide was prepared from 4,5-dimethyl-2-nitroaniline using the method of Kuhn.[21]

Metabolism of D-Riboflavin-2-C^{14} by *Lactobacillus casei*.—Forty-day incubations were carried out in 8-ounce amber bottles. The basal medium was the usual one except that it contained twice the amount of peptone. Each bottle contained 50 ml. of basal medium solution and 50 ml. of radioactive riboflavin solution containing 182 μg. (0.619 μc.). The bottles were inoculated with *L. casei* and incubated at 37°. After the incubation period five bottles were joined in series and aerated for one hour. Carbon dioxide was collected in saturated $Ba(OH)_2$. Barium carbonate plates were prepared following the method of Dauben[22] and their activities measured by a mica end-window Geiger–Müller counter.

Microbiological Assay.—Solutions for microbiological assay of the radioactive riboflavin in the cells and medium were prepared by the method of Rahn.[23] The cells were washed with isotonic saline and destroyed by sonic oscillation.

Chromatograms.—The chromatograms were developed in the *n*-butyl alcohol, water, acetic acid—4:5:1 system (hereafter referred to as solvent system number 1) and examined under ultraviolet light, the fluorescent spots outlined and radioautographs made on E. K. Blue Brand X-ray film. Flavins in the medium and the destroyed cells were extracted with liquid phenol by the method of Yagi.[24]

Detection of Spots.—The non-fluorescent substances were detected as follows.

Oxaluric Acid[25] and Lactic Acid.—Formation of the ammonium salts and treatment with Nessler reagent.[26]

Uracil and Barbituric Acid.—Formation of the silver salts and treatment with 0.1% *sym*-diphenylcarbazone.[27]

Urea.—The paper was sprayed with a 0.15% ethanol solution of brom thymol blue. When dry it was sprayed with 1% urease solution.

Alloxan.—The paper was sprayed with a 4.5% solution of boric acid in 5% acetic acid. When dry it was sprayed with a 0.2% solution of 4-methyl-1,2-diaminobenzene in glacial acetic acid. The alloxazine is formed immediately at room temperature.

Results

A representative sample of the cultures was assayed microbiologically for the riboflavin in the cell suspension, the medium and the cells. The results are given in Table I.

During incubation 29 μg. per 100 ml. or 16% of the riboflavin was destroyed. It has been shown previously[23] that riboflavin in sterile medium under these experimental conditions undergoes no deterioration.

The activity found in the $BaCO_3$ samples shows that some of the riboflavin-C^{14} is metabolized to

(11) In four preparations of urea using non-radioactive KCN, assays of urea were 97, 98, 95 and 97% and the yields were 79, 83, 79 and 84%, respectively.

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TABLE I

DESTRUCTION OF D-RIBOFLAVIN-2-C^{14} BY *L. casei*

	Concn., μg. per 100 ml.	Recovd. ribo-flavin, %
Culture medium before incubation	182	..
Cell suspension	153	84
Medium	145	80
Cells	4.4	2

$C^{14}O_2$. The specific activity of the $BaC^{14}O_3$ remained constant after two reprecipitations. The $BaC^{14}O_3$ was decomposed in one side arm of an H-shaped closed vessel and the liberated $C^{14}O_2$ passed by diffusion through the cross-arm packed with glass wool into the other side of the vessel which contained saturated $Ba(OH)_2$ solution. The radioactivity values were corrected for self-absorption by the method of Yankwich, *et al.*[28] The specific activity of the $BaC^{14}O_3$ before purification was 1.21 c.p.m. per mg. After each reprecipitation the value was 0.67 c.p.m. per mg. Since 0.253 g. of barium carbonate was collected from the aeration of five culture bottles, the total activity in the barium carbonate of 168 c.p.m. is equivalent to 0.0051 μc. This indicates that 0.17% of the radioactive riboflavin was metabolized to CO_2. These results are similar to those from experiments in which thiamine-C^{14} was injected into rats. There too, 0.2% of the administered radioactivity appeared as CO_2.[4]

Paper chromatography of the phenol extract of the cells in solvent system No. 1 produced fluorescent spots with R_f values of 0.04 and 0.09. These were the same R_f values shown by FAD and FMN from tissue extracts which were run simultaneously. The radioautographs of the chromatograms showed, in addition to exposed areas for FAD and FMN, exposed areas corresponding to an R_f value of 0.78. The corresponding spots on the paper did not fluoresce. Chromatography of the cell extract in the 5% Na_2HPO_4–isoamyl alcohol system (hereafter referred to as system number 2) again showed the presence of FAD and FMN. Radioautographs gave exposed areas on the film corresponding to FAD, FMN and the unknown substance with an R_f value of 0.12.

In the chromatography of the phenol extract of the culture medium in solvent system No. 1 the flavins were not completely separated from other fluorescent substances. The radioautographs of these chromatograms, however, showed exposed areas for FAD and riboflavin. In no case could the presence of FMN be demonstrated. Chromatography of the culture medium extract in solvent system No. 2 did not separate FAD from the large amount of riboflavin present.

The activities of the spots from the cell extract developed in solvent system No. 1 were measured and the results are shown in Table II.

The radioactive material in the phenol extract of the cells whose radioautographs indicated an R_f value of 0.78 in solvent system No. 1 and 0.12 in solvent system No. 2 was believed to be a metabolite resulting from the degradation of the riboflavin-

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TABLE II

RADIOACTIVITY OF CELL EXTRACT COMPONENTS

	c.p.m.	Disintegrations/min.	Distribution, %
C^{14}-Riboflavin std.	64.0	3820	..
FAD	10.7	640	87
FMN	0.7	42	6
Unknown	0.8	48	7

C^{14} molecule. This substance is not urea, oxaluric acid, lactic acid, uracil, alloxan, barbituric acid, lumichrome, lumiflavin, 3,4-dihydro-3-keto-4-(D-1'-ribityl)-6,7-dimethylquinoxaline-2-carboxyureide, 3-hydroxy-6,7-dimethylquinoxaline-2-carboxyureide nor 3,4-dihydro-3-keto-4-methyl-6,7-dimethylquinoxaline-2-carboxyureide, as indicated by their low R_f values in solvent system No. 1. These values, together with those of the culture medium in solvent system No. 1 and of the cell extract in systems No. 1 and No. 2, are as follows: Culture medium extract: riboflavin-C^{14}, 0.33; FAD, 0.04; cell extract: FAD, 0.04, 0.34; FMN, 0.09, 0.44; unknown, 0.78, 0.12. Urea, 0.41; oxaluric acid, 0.17; lactic acid, 0.65; uracil, 0.43; alloxan, 0.39; barbituric acid, 0.31; lumichrome, 0.63; lumiflavin, 0.41; 3,4-dihydro-3-keto-4-(D-1'-ribityl)-6,7-dimethylquinoxaline-2-carboxyureide, 0.40; 3-hydroxy-6,7-dimethylquinoxaline-2-carboxyureide, 0.63; 3,4-dihydro-3-keto-4-methyl-6,7-dimethylquinoxaline-2-carboxyureide, 0.68.

It was found further that lumichrome and the two latter substituted quinoxalines are not extracted by the phenol extraction procedure, and hence would not have appeared in the cell extract had either been the metabolite.

Discussion

D-Riboflavin-2-C^{14} provides a means by which the metabolism of riboflavin might be studied more extensively. As anticipated, *Lactobacillus casei* converts the assimilated riboflavin to flavin mononucleotide (FMN) and flavin–adenine dinucleotide (FAD). The relative distribution of the nucleotides found in the cells and the fact that no free riboflavin could be detected are in agreement with the results reported by Bessey, *et al.*,[29] for the levels of FAD, FMN and free riboflavin in rat tissue. If free riboflavin occurs in the *L. casei* cell in the same relative amount as it is found in the mammalian cell, its presence in the bacterial cell extract could not have been detected by the methods employed in this experiment.

It has been shown in studies by Rahn[23] that as the *L. casei* culture becomes older many of the cells die and the riboflavin content of the culture medium increases. It was anticipated, then, that at the end of the 40-day incubation period some of the riboflavin from the dead cells would have appeared again in the culture medium. The FAD found to be present in the culture medium after the cells had been removed must have been released by the dead cells during the incubation period. Apparently during the autolysis of the cell the FAD is not completely hydrolyzed.

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Since the riboflavin-C^{14} molecule is labeled in but one position, only the degradation of the urea-containing moiety can be followed. The metabolite found in this experiment, although as yet unidentified, can be characterized to some degree from the information obtained through the elimination of several ureides and the compounds resulting from the degradation of the ribityl group of riboflavin and from the cleavage of the pyrimidine ring. In further studies methods for obtaining larger quantities of the metabolite must be found to enable characterization by other means.

An amount of radioactive riboflavin in excess of that required for optimal growth was added to the culture medium in order to prolong the growth period and, hence, increase the total amount of riboflavin destroyed by the bacteria. As a result 290 μg. of riboflavin per liter was destroyed, whereas in experiments in which the culture medium contained 182 μg. per liter, 54 μg. per liter was destroyed during the same incubation period.[23]

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ROCHESTER, NEW YORK

[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

The Action of Acid on 2,7-Anhydro-β-D-*altro*-heptulopyranose[1]

BY L. P. ZILL AND N. E. TOLBERT

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The acid treatment of 2,7-anhydro-β-D-*altro*-heptulopyranose has been shown to give rise to two compounds other than D-*altro*-heptulose which have been tentatively identified as 2,7-anhydro-β-D-*altro*-heptulofuranose and 5-(1,2-dihydroxyethyl)-2-furfuraldehyde. A method has been developed for the isolation of small amounts of these two compounds by the use of thick paper chromatography. An alternative method of preparing 2,7-anhydro-β-D-*altro*-heptulofuranose is based on the ion-exchange chromatography of the borate complexes of the components formed by the acid treatment of the pyranose anhydride. The orcinol method has been applied to the quantitative determination of sedoheptulosan and the limitations of the method discussed. The compound responsible for the orcinol reaction has been tentatively identified as 5-(1,2-dihydroxyethyl)-2-furfuraldehyde.

Introduction

During the experimental development of a method for the separation of the borate complexes of D-*altro*-heptulose (compound IV) and 2,7-anhydro-β-D-*altro*-heptulopyranose (compound I) by ion-exchange chromatography[2] it was noted that a third orcinol-reacting component was formed by the acid treatment of I. It was further reported by Noggle[3] that chromatography of an acid-treated sample of I, using phenol–water as solvent, gave four spots which produced positive tests with the orcinol-TCA (trichloroacetic acid) spray test.[4,5]

The ordinary procedure for the isolation of IV from natural sources involves its acid conversion to the anhydride, I, which may be crystallized, a property not shared by the sugar itself.[6,7] The formation of compounds other than the free sugar provides a complicating factor and source of loss in such an isolation procedure. In addition, an inconvenience is introduced in that the acid-catalyzed reconversion to the free sugar takes place only to the extent of about 20% and requires an additional step to separate the sugar from residual I. These manipulations have been circumvented in recent work by the use of thick-paper chromatography for the isolation of radioactive IV extracted from plants.[8]

In view of these considerations and of the increasing importance of IV (and consequently of I) in biochemical studies, it was considered highly desirable to conduct a study of the acid treatment of I. This paper represents such a study. Two unknown compounds have been tentatively identified as 2,7-anhydro-β-D-*altro*-heptulofuranose (II) and 5-(1,2-dihydroxyethyl)-2-furfuraldehyde (VI). The proposed conversions which are completely analogous to similar reactions known to occur in the pentose and hexose sugars are given in the following scheme. Compounds I, II and VI are formed under the influence of acid and heat. In addition, the implication of these reactions in the application and limitations of the orcinol reaction for the quantitative determination of I are presented.

Experimental and Results

Crystalline I, often referred to as sedoheptulosan, was prepared for all experiments by the general procedure of La Forge and Hudson.[6,7] Absorption spectra were obtained with a model DU Beckman spectrophotometer. Colorimetric determinations were made with an Evelyn colorimeter equipped with filters giving the wave lengths indicated in the procedure. Because of the ease of removal, Dowex-50 was used instead of acid in several of the experiments.

Ion-exchange Chromatography of the Borate Complexes of Products Formed During the Acid Treatment of I.—A sample of I (420 mg.) in 20 ml. of distilled water containing

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