Biosynthesis of Riboflavin and the Role of Acetoin (3-Hydroxybutan-2-one) in the Formation of the *o*-Xylene Ring

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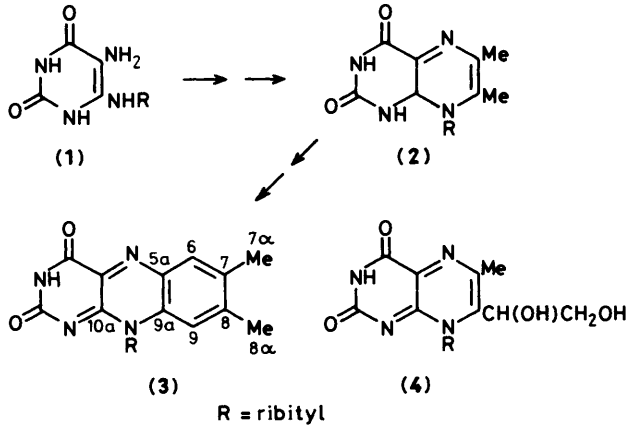
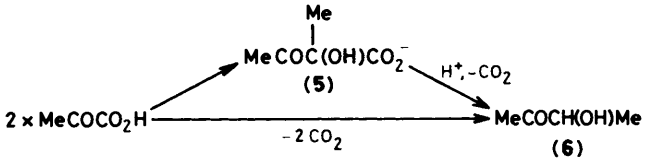
Incorporation experiments with [2-^{13}C]pyruvate and [4-^{14}C]acetoin ([4-^{14}C]-3-hydroxybutan-2-one), in *Eremothecium ashbyii* have shown that acetoin (**6**) is not a precursor of riboflavin. Growth studies with the same organism support this conclusion and indicate that the *o*-xylene ring and the ribityl side-chain of riboflavin differ appreciably in their biogenetic origins.

In the later stages of riboflavin (**3**) biosynthesis, 5-amino-2,4-dioxo-6-ribitylaminopyrimidine [ADRAP (**1**)] (or its 5'-phosphorylated derivative) is converted into 6,7-dimethyl-8-ribityllumazine [DMRL (**2**)], which is in turn converted into riboflavin (**3**). The additional C_4 unit required for the construction of the *o*-xylene ring is derived from the C_4 unit of a second molecule of DMRL (**2**) with release of a molecule of ADRAP (**1**) (Scheme 1); for recent reviews see references 1—4.



Scheme 1.

The origin of the C_4 units required for the synthesis of the *o*-xylene system of riboflavin (**3**) has been the subject of considerable speculation. A candidate earlier suggested for this role was acetoin (3-hydroxybutan-2-one) (**6**). Thus, various workers reported the conversion, by cell-free systems from *Eremothecium ashbyii* and *Aerobacter aerogenes*, of ADRAP (**1**) into DMRL (**2**) in the presence of acetoin (**6**).[5] Further, acetoin (**6**) was shown to be produced both by *E. ashbyii*[6] and by *A. aerogenes*.[7] Direct evidence was claimed by Goodwin and Treble[8] who reported that [1-^{14}C]acetoin was incorporated specifically into the *o*-xylene ring of riboflavin (**3**), with one half of the radioactivity located in the methyl groups, none in C-7 or C-8, and the remainder, by inference, in C-6 and C-9 [*cf.* (**3**), Scheme 1]. However, these results could not be reproduced by Ali and Al-Khalidi,[9] who found that incorporation of [1-^{14}C]acetoin into riboflavin in *E. ashbyii* was extremely low. Therefore they were not able to confirm the claim of specific incorporation of radioactivity into the *o*-xylene unit. Further, their experiments with other precursors (acetate, pyruvate) indicated, albeit indirectly, that acetoin (**6**) could not be a precursor of riboflavin (**3**). On the other hand, and more recently, mutants of *A. aerogenes* deficient in the ability to synthesize acetoin, were reported also to be limited



Scheme 2.

in their capacity to produce riboflavin (**3**), indicating a possible role for acetoin (**6**) in riboflavin biosynthesis.[10] In view of these contradictory results, which have not eliminated acetoin (**6**) from consideration as a precursor of riboflavin (**3**) in recent papers and review articles,[11] it seemed desirable to re-examine its role in riboflavin biosynthesis. Accordingly the experiments described below were undertaken in an effort to clarify this question.

Acetoin may be formed in micro-organisms from pyruvate, either by condensation to α-acetolactate (2-hydroxy-2-methyl-3-oxobutanoate) (**5**) followed by decarboxylation under the sequential action of α-acetohydroxy acid synthetase[12] and α-acetolactate decarboxylase,[13] or directly, under the action of pyruvate decarboxylase[14] (Scheme 2). Incorporation of [2-^{13}C]pyruvate into riboflavin (**3**) *via* acetoin (**6**) would therefore be expected to lead to riboflavin (**3**) enriched in ^{13}C at C-5a, -9a, -7, and -8.

In three separate experiments, [2-^{13}C]pyruvate was administered to *E. ashbyii*, together, in two of the experiments, with [2-^{14}C]pyruvate. Riboflavin (**3**) was isolated and purified as the tetra-*O*-acetate. Incorporation of pyruvate was very low (0.02, 0.016% from ^{14}C activity measurements in the two experiments with radiolabelled pyruvate). Enrichment factors were measured relative to signals due to the acetyl groups of non-biosynthetic origin. These are shown in columns 1—3 of Table 1, and their sum is given in column 4. (A sum rather than an average is shown because incorporations varied between experiments and average enrichment factors would be meaningless.)

The values from Table 1, column 4 show that significant incorporations were observed only for the carbon atoms of the ribityl side chain and for C-6, -9, -7α, and -8α. A lower, but probably significant, enrichment was found for C-5a and C-10a. Essentially zero enrichment factors were found for C-9a, -7, and -8. This pattern of incorporation is almost precisely the opposite of what would be expected if pyruvate were incorporated into riboflavin *via* acetoin (**6**) (*cf.* Schemes 1 and 2).

[4-^{14}C]Acetoin ([4-^{14}C]-3-hydroxybutan-2-one), synthesized from 2-methylpropenal and [^{14}C]iodomethane,[15] was incorporated into riboflavin (**3**) in *E. ashbyii* (incorporation 0.022, 0.012% in duplicate experiments). The riboflavin (**3**) was

Table 1. Enrichment factors in the carbon atoms of riboflavin (**3**) following incorporation of [2-^{13}C]pyruvate[a] in *E. ashbyii*

Carbon atom	Expt. no. 1	2	3	Sum (1 + 2 + 3)
4	−0.06	−0.03	0.1	0.01
2	−0.03	−0.18	0.1	−0.11
10a	0.03	0.49	0.18	0.7
4a	0.01	−0.19	0.36	0.18
9a	0.11	−0.08	0.2	0.23
5a	−0.11	0.21	0.65	0.75
8	0.01	−0.04	−0.07	−0.1
7	0.05	−0.02	0.12	0.15
9	0.30	0.60	0.5	1.4
6	0.17	0.55	0.44	1.16
8α	0.08	0.38	0.50	0.96
7α	0.14	0.46	0.52	1.12
1′[b]	0.97	0.79	0.59	2.35
2′	0.21	0.78	0.30	1.29
3′	0.24	0.67	0.50	1.41
4′	0.15	0.49	0.49	1.13
5′	0.25	0.59	0.27	1.11

[a] Amount administered: 50 mg (Expt. 1), 100 mg (Expts. 2, 3); [b] C-1′ to C-5′ are the carbon atoms of the ribityl side-chain.

Table 2. Production of riboflavin (**3**) and acetoin (**6**) by washed mycelium incubated with single carbon sources

Carbon source (wt/mg/15 ml)	Acetoin (μg ml^{-1})	Riboflavin (μg ml^{-1})	Tissue wt. (mg, 15 ml sample)
Glucose	17.6	11.9	7.6
Sucrose	14.2	8.5	6.9
Fructose	9.0	15.3	8.1
Ribose	0.1	3.1	4.8
Glycerol	0.3	6.7	5.6
Glycerol	0.2	5.8	3.5
Sodium pyruvate	7.35	0.5	2.7
Sodium 2-oxobutanoate[a]	1.5	0	1.9
Control[b]			6.3

[a] The acetoin response in the Westerfeld test may be attributable to 2-hydroxypentan-3-one. [b] The control samples were autoclaved without further incubation to determine the amounts of tissue added to each sample before the second incubation period

purified as the tetra-*O*-acetate, re-hydrolysed to riboflavin (**3**) and subjected to Kuhn-Roth oxidation. The barium acetate obtained (C-7, -8, -7α, and -8α) contained only 16.8% of the total activity of the riboflavin (**3**), which contrasts with the value of 50% expected if acetoin (**6**) were a specific precursor, and a value of 23.5% for random distribution of activity throughout the riboflavin. (The figure of 50% is based on the assumption that if acetoin *were* a specific precursor, the radioactivity derived from it would be equally divided between the two biogenetic C_4 units of the *o*-xylene ring.) In order to confirm that the low incorporation of acetoin (**6**) was not attributable to its failure to penetrate the cells, the lipid fraction from the mycelium was isolated in one experiment, saponified, and the radioactivity of the liberated fatty acids was measured (1.2% incorporation).

The biogenesis of the *o*-xylene ring is closely related to the biogenesis of the 5,6-dimethylbenzimidazole (DBI) unit of coenzyme B_{12}, of which riboflavin is a precursor.[16–22] Information on the origin of the DBI unit of coenzyme B_{12} is thus directly relevant to riboflavin biogenesis. Of particular interest is the high incorporation by *Propionibacterium shermanii* of radioactivity from [1-^{14}C]ribose into the carbon atoms of DBI that are derived from the methyl groups of the lumazine (**2**).[17,22]

The precursor role of an intermediate of the pentose phosphate pathway in riboflavin biosynthesis was first proposed by Al-Khalidi.[9] Although this suggestion was later withdrawn,[23] recent results with the yeast *Pichia guilliermondii* have confirmed the significant incorporation of label from D-[1-^{14}C]-ribose and D-[1-^{14}C]ribitol into the *o*-xylene unit,[11a] and more pertinently, label from D-[1-^{13}C]ribose was found to label the methyl carbon atom specifically.[24] These results have prompted reconsideration of the possible role of a five-carbon sugar of the pentose pathway of metabolism as a precursor of the *o*-xylene ring of riboflavin. However, the incorporation results obtained to date, with four significant exceptions (see below), have been derived solely from experiments with ribose or ribitol labelled in C-1. Other evidence has been indirect and conflicting. Thus, Logvinenko *et al.* found that in extracts of riboflavin-dependent mutants of *Pichia guilliermondii*, riboflavin was formed in the presence of 2,5-diamino-6-hydroxy-4-ribosylaminopyrimidine-5′-phosphate, NADPH, and ribose-5-phosphate.[25] The requirement for a monosaccharide was slightly satisfied by glucose-6-phosphate and fructose-5-phosphate but not by ribose, ribitol, fructose, glucose, or pyruvate. On the other hand, in extracts of *Escherichia coli*, 6,7-dimethyl-8-ribityl-lumazine (**2**) was synthesized from ADRAP (**1**) in the presence of NAD, but in the absence of other carbon sources.[26] Also, Mailänder and Bacher found that in a *Salmonella typhimurium* mutant that was incapable of converting guanosine into free guanine, activity from guanosine labelled uniformly with ^{14}C in the ribose moiety was converted into riboflavin without dilution of the label.[27] Moreover, the riboflavin was labelled exclusively in the ribityl side chain.

The marked difference between these results and others obtained using [1-^{14}C]-labelled ribose or ribitol is attributable to the inability of the *S. typhimurium* mutant to release from guanosine free ribose that might enter the general metabolic pool.

Thus, although these results do not disprove hypotheses concerning the derivation of the *o*-xylene unit of riboflavin from pentose precursors, they do strongly indicate that the ribose side-chain of guanosine and the ribityl components of later intermediates are not the source of the *o*-xylene unit. In this connection it should be noted that in our experiments with [2-^{13}C]pyruvate, label was incorporated more efficiently into the ribityl side chain of riboflavin than into the *o*-xylene ring, suggesting that the pathways to these two components diverge at an early stage.

Recent work by Floss and his co-workers has revealed a complex pattern of incorporation of glucose and glycerol into riboflavin in *Ashbya gossypii*.[28] These results are clearly highly significant but as yet not readily interpretable in terms of later intermediates in the pathway.

Some further results obtained in the present studies are relevant to riboflavin biosynthesis.

Thus, when washed mycelium was incubated with a single carbon source, the results in Table 2 were obtained. It is noteworthy that of the carbon sources tested, only glucose, sucrose, fructose, ribose, and glycerol were able to stimulate significant flavinogenesis, with fructose being the most effective. The determination of acetoin and riboflavin production showed parallel levels with glucose, sucrose, and fructose but not with ribose and glycerol. Significantly, pyruvate supported acetoin production but not flavinogenesis, providing further evidence against the precursor role of acetoin in riboflavin biosynthesis. Other compounds tested (inositol, glyceric acid, glycerophosphate, glyceraldehyde, amino acids, and nucleotides) were unable to support growth or flavinogenesis, and extensive autolysis of the culture occurred. When various precursors were added to cultures in full medium at the start of flavinogenesis, the results shown in Table 3 were obtained. Thus glyceric acid, glycerophosphate, and gluconate were markedly stimulatory

Table 3. Effect of supplements on the production of riboflavin (3) and acetoin (6) on addition to media B or C at the start of flavinogenesis

Supplement (mg ml^{-1})	Percent of control values for		
	Acetoin	Riboflavin	Tissue wt.
Glycerol (5)	—	96	104
Glyceraldehyde (1)	—[a]	82	98
Glyceric acid (0.5)	92		81
Sodium β-glycerophosphate (0.5)	90	303	102
Calcium α/β-glycerophosphate (0.5)	72	211	63
Calcium gluconate (0.25)	88	179	112

[a] Glyceraldehyde interferes with the Westerfeld acetoin assay.

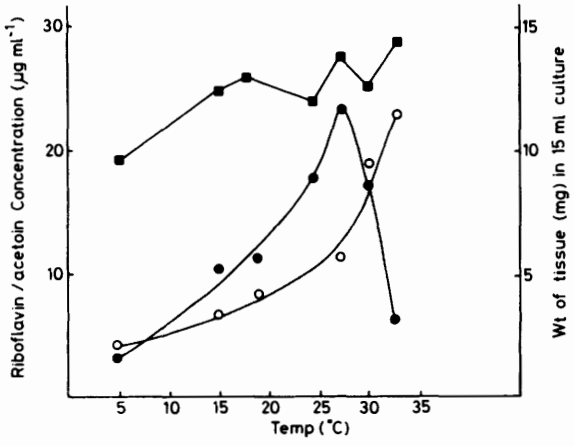


Figure. Effect of temperature on mycelial growth (■—■), and on riboflavin (3) (●—●) and acetoin (6) (○—○) production in *Eremothecium ashbyii*. The organism was grown in medium B for 42 h at 27 °C and thereafter at the indicated temperature for a further 80 h

relative to glycerol or glyceraldehyde. Addition of acetoin in amounts ranging from 20 μg ml^{-1} to 1 mg ml^{-1} to cultures at any time during the first three days of growth caused only a marginal (6—14%) reduction in riboflavin production.

An interesting relationship between riboflavin production, acetoin production, and temperature was noted (Figure). Thus maximum riboflavin production occurred at 27 °C, with a sharp reduction above 30 °C, whereas the rate of acetoin production increased steadily over the temperature range investigated.

All of our experimental results thus point consistently to the rejection of acetoin as a precursor of the *o*-xylene unit of riboflavin and by implication of the 1,2-dimethylbenzimidazole unit of coenzyme B_{12}. Further, although demonstrating the ability of various carbohydrate sources to support flavinogenesis, they do not point specifically to any particular compound as a significantly better precursor than others. The problems encountered in studies of riboflavin production are typical of those encountered in studies of metabolites derived almost directly from primary metabolites. In such cases the results of incorporation studies in particular are less easily interpretable than in studies of secondary metabolism when specialised pathways of varying length lead from the primary metabolic pool to the secondary product. In such cases the application of different techniques is strongly indicated.

Experimental

L-Threonine, myoinositol, DL-glyceric acid, sodium β-glycerophosphate, calcium α/β-glycerophosphate, pyruvic acid, peptone (from soybean, type III), and yeast extract were obtained from the Sigma Chemical Company Ltd. D-Ribose, DL-glyceraldehyde, riboflavin, methacrolein (2-methylprop-2-enal), and acetoin dimer were obtained from the Aldrich Chemical Company Ltd. D-Glucose, sucrose, glycerol, D-fructose, L-valine, L-leucine, L-isoleucine, glycine, L-serine, and agar powder were obtained from B.D.H. Chemicals Ltd. Calcium gluconate was obtained from Hopkin and Williams Ltd. Radiochemicals were obtained from Amersham International plc. and sodium [2-^{13}C]pyruvate from MSD Isotopes (Merck Frosst Canada Inc.). *Eremothecium ashbyii* (IMI 14783) was obtained from the Commonwealth Mycological Institute, Kew, Surrey. Radioactivity measurements were determined using an LKB Rackbeta scintillation counter and ^{13}C n.m.r. spectra were determined on a Bruker WH-400 n.m.r. spectrometer through the courtesy of Dr. O. W. Howarth and the S.E.R.C. High Field n.m.r. service, University of Warwick. Light petroleum refers to the fraction of b.p. 60—80 °C.

Growth of Eremothecium ashbyii.—Most media and additives were sterilised by autoclaving for 20 min at 115 °C (10 p.s.i.). Reducing sugars were sterilised separately but sucrose was sterilised in the salt–yeast extract solution. Heat-labile compounds and isotope solutions were sterilised by filtration through Sartorius Minisart filters (0.22μ). The organism was maintained on 1.4% agar slopes of a medium containing Peptone (1%), yeast extract (0.1%), sucrose (2%), and mineral salts as in medium B (see below). The mycelium was subcultured onto fresh slopes every 14 days. Slopes were removed from the incubator (27 °C) after 7 days and kept at 5 °C, when they were found to retain their viability for at least 10 weeks. For experiments in liquid culture, colonies were transferred to 10 ml volumes of the selected medium which was shaken briefly and the suspension was incubated for 18 h at 27 °C. Small aliquots of the inoculum were then added aseptically to 100 times their volume of culture medium, and incubation was continued. Acetoin was determined by the Westerfeld method.[29] Riboflavin was determined by measurement of its absorbance at 445 nm at pH 4—5 with correction for absorbance by the medium used. Riboflavin tetra-acetate was determined by measurement of its absorbance at 450 nm in chloroform or ethyl acetate. The same method was used to estimate riboflavin and riboflavin tetra-acetate for radioactivity measurements. Tissue weight for tissue grown during preparative experiments was determined by collecting the mycelium by filtration through gauze, washing with water and air drying to constant weight. For routine determinations during experiments to investigate the effect of supplements it was found more convenient to use a chromic acid oxidation procedure. For this, the mycelium was collected by centrifugation (5 min at 2 000 g), washed four times with cold water, and suspended in water (0.5 ml). Chromic acid reagent [chromium trioxide 5% w/v in sulphuric acid (6M, 2 ml)] was added and the suspension was heated at 80 °C for 2 h. The digests were cooled, aliquots (1 ml) were diluted to 5 ml with water and the absorbance of the resulting solution was measured at 585 nm. Calibration against known weights of tissue showed that the relationship between absorbance and tissue weight was linear over a range of 0—22 mg mycelium.

Growth Media.—Medium A contained (g l^{-1}) ammonium sulphate (0.72) and ammonium hydrogen phosphate (0.26) (pH 5.5). Medium B contained ammonium sulphate (3.0), magnesium sulphate heptahydrate (0.7), calcium nitrate (0.4), sodium chloride (0.5), potassium dihydrogen phosphate (1.0), and dipotassium hydrogen phosphate (0.1) (pH 4.4). Medium C contained ammonium sulphate (2.16), ammonium hydrogen phosphate (0.78), magnesium sulphate heptahydrate (0.7), calcium nitrate (0.4), and sodium chloride (0.5) (pH 5.5).

Medium D contained magnesium sulphate heptahydrate (0.5), potassium dihydrogen orthophosphate (0.35), potassium chloride (0.5), and sodium nitrate (2.0) (pH 4.6). In addition, each medium contained yeast extract (1) and sucrose (20). Medium A was a modification of the medium described by Davis.[30] Medium B was a modification of Mardashev's medium for yeast culture.[31] Medium D is a modification of the basic Czapek Dox medium.[32] Medium C was a hybrid of media A and B. It was found that incorporation of peptone into the media offered no advantages for flavinogenesis, but addition of peptone (10 g l^{-1}) to medium B was found to be beneficial for the maintenance of the cultures on 1.4% agar slopes. Media with ammonium sulphate as the main nitrogen source (B and C) gave better yields of riboflavin (and relatively less acetoin) than the Czapek Dox variation (D) with nitrate as the main source.

Effect of Added Carbon Sources.—Addition of glucose (1%) to medium A brought about a reduction of 26% in the amount of riboflavin produced. Increasing the sucrose concentration in media B or C (to 3%) caused no significant changes in the levels of acetoin or riboflavin produced, and only small (*ca.* 12%) increases in tissue weight. The addition of ribose, fructose, inositol, or glycerol in amounts up to 5 mg ml^{-1} to media B or C at any time during the first three days of incubation produced no significant changes in the level of acetoin or riboflavin production or tissue growth except for a reduction in acetoin concentration of about 42% when high levels of fructose were added. When 48 h mycelium (grown in medium B) was collected by filtration, washed several times with sterile buffered salt solutions, resuspended in mineral salt solutions with selected carbon sources and incubated for three days, only sucrose, fructose, ribose, glucose, and glycerol were able to support significant flavinogenesis as indicated in Table 2.

Other materials tested (inositol, glyceric acid, glycerophosphate, glyceraldehyde, amino acids, and nucleotides) were unable to support growth or flavinogenesis, and extensive autolysis of the culture took place. The ability of glycerol to support flavinogenesis in washed mycelium contrasts with its apparent inability to stimulate flavinogenesis, as compared with relatively low concentrations of glycerophosphate or calcium gluconate (Table 3) when added to cultures in media B or C at the start of flavinogenesis (about 42 h).

[4-^{14}C]*Acetoin.*—This synthesis was performed on a vacuum line. [^{14}C]Iodomethane (500 μCi) was transferred into a reaction flask cooled in liquid nitrogen together with unlabelled material (702 mg, 4.98 mmol). Dry ether (10 ml) was added, followed by magnesium turnings (153 mg). The mixture was allowed to warm to room temperature whereupon formation of the Grignard reagent began. After 1.5 h, a solution of 2-ethylpropenal (360 mg) in dry ether (5 ml) was added slowly. The mixture was stirred for 3 h, the reaction flask was disconnected from the vacuum line, and its contents were poured onto a mixture of crushed ice and ammonium chloride solution. After 30 min, the ethereal layer was separated and the aqueous layer was extracted with ether (2 × 20 ml). The ethereal solutions were combined, dried (Na_2SO_4) and evaporated. The residual 3-methylbut-3-en-2-ol (2.78 × 10^8 d.p.m.) was dissolved in chloroform (5 ml). The solution was cooled in an ice–salt bath and ozone was passed through it for 30 min. Hydrogen peroxide was decomposed by the addition of catalase. The chloroform solution was extracted with water (3 × 10 ml) to give a solution containing acetoin (64 mg, 25 × 10^7 d.p.m.). This stock solution was distilled and the distillate was sterilised by filtration prior to use (63% recovery). In trial experiments this procedure was shown to give acetoin of >99% purity.

Incorporation of Pyruvate into Riboflavin.—The mycelium was grown in 12 bottles (120 ml) each containing medium B (20 ml). After 48 h incubation, the cultures were transferred to 12 500 ml bottles (to increase the degree of aeration) and to each bottle was added a solution of sodium hydrogen carbonate (2 ml, 12.5 mg), 2 ml of a solution of [2-^{13}C]sodium pyruvate (100 mg, 90 atom %; 2 ml), and sodium [2-^{14}C]pyruvate (25 μCi) in water (24.5 ml). Incubation was continued for a further 70 h. The bottles were sterilised in an autoclave and the mycelial suspension was filtered through gauze. The tissue residues were washed five times with water and dried (109 mg). The combined filtrate and washings (20 μg ml^{-1} riboflavin) was applied to a column of 12 g acid-washed Fuller's earth which was eluted successively with water (50 ml), acetic acid (4%, 50 ml), and water (50 ml). The riboflavin was then eluted with pyridine (25% aqueous solution, 200 ml) and the solution was evaporated under reduced pressure. The residue was treated with a mixture of acetic anhydride (4 ml), acetic acid (4 ml), and perchloric acid [1:1 mixture of perchloric acid (*d* 1.7) and water, 0.4 ml] at 0 °C. The mixture was allowed to warm to room temperature, stirred for 30 min and poured on to ice (25 g). The resulting mixture was extracted with chloroform (4 × 25 ml) and the combined chloroform solutions were concentrated and applied to a column of alumina (15 g, washed with ethyl acetate and activated by heating for 6 h at 300 °C). The column was washed with 5 bed volumes of chloroform, 3 bed volumes of ether, and 5 bed volumes of ethyl acetate, while being protected from light with aluminium foil. The riboflavin tetra-acetate was eluted with ethyl acetate–methanol (50:50), and the eluate was evaporated to give the crystalline riboflavin tetra-acetate. In three similar experiments the yields of riboflavin tetra-acetate were 19.4, 10.2, and 7.0 mg. In the two experiments in which sodium [2-^{14}C]pyruvate was administered, the incorporation of radioactivity was 0.02 and 0.036%. The three samples were examined by ^{13}C n.m.r. to give the results described in the text and in Table 1. The mycelial tissue residues (294 mg) from the experiments with radiolabelled pyruvate were ground up and extracted thrice with chloroform–methanol (2:1; 9 ml). The extracts were filtered through a glass fibre filter disc and evaporated. One tenth of the residue (total weight 25.3 mg) was heated at 100 °C for 2 h with sodium hydroxide solution (1M; 2.5 ml). The solution was diluted to 5.0 ml, extracted with ether (5 ml), acidified (HCl), and re-extracted with ether (3 × 5 ml). The radioactivity in the latter extracts, attributable to fatty acids, amounted to 3.7% and that in the non-saponifiable lipid fraction to 0.19% of the radioactivity administered.

Incorporation of [4-^{14}C]*Acetoin into Riboflavin* (3).—The mycelium was grown in 120 ml bottles containing medium B (18 ml) with 2% sucrose and 0.05% yeast extract. After 48 h growth, two cultures were added to two 500 ml bottles each containing guanosine 5′-monophosphate (4.8 mg), sodium hydrogen carbonate (18 mg), and [4-^{14}C]acetoin (7 ml of a filter-sterilised stock solution, 4 mg, 1.56 × 10^7 d.p.m.). Control flasks, used to monitor growth and flavinogenesis, contained similar solutions of inactive acetoin. After a further 144 h incubation the cultures were killed by the addition of formaldehyde solution (37—41%; 4 ml). The mycelium was removed by filtration through stainless steel gauze, washed with water, and dried (23.8 mg, total radioactivity: 3.88 × 10^5 d.p.m.). The culture medium filtrate was filtered through a glass fibre filter disc giving a combined volume of 102 ml containing riboflavin (23.2 μg ml^{-1}). Inactive riboflavin (10.6 mg) was added to the filtrate which was acidified to <pH 1 with sulphuric acid (1M), saturated with sodium sulphate, and extracted with benzyl alcohol (4 × 25 ml). The combined extracts were washed with sulphuric acid (0.05M, saturated with sodium sulphate), diluted with diethyl ether (200 ml) and light petroleum (200 ml), and extracted with aqueous sodium hydroxide (0.05M; 3 × 10 ml).

The combined alkaline extracts were acidified with sulphuric acid (1M), extracted with diethyl ether to remove benzyl alcohol, and 'degassed' under reduced pressure to remove ether residues. The solution was applied to a column of Florisil (1.4 m × 10 cm), previously washed with 2% acetic acid. The column was eluted with 2% aqueous acetic acid (100 ml) and water (100 ml). The adsorbed riboflavin was eluted with 50% aqueous acetone containing pyridine (5%). The eluate was evaporated to give riboflavin (11.3 mg, 88% recovery, 1 009 d.p.m. mg^{-1}). The riboflavin was acetylated as previously described. The resulting tetra-acetate was applied to a column of alumina (15 g) which was eluted with chloroform (100 ml), ether (60 ml), and ethyl acetate (60 ml). The tetra-acetate was eluted with ethyl acetate–methanol (1 : 1) and the eluate was evaporated to give riboflavin tetra-acetate (8.0 mg, 369 d.p.m. mg^{-1}, 0.015% incorporation). Isolation of the fatty acid mixture from the mycelium as previously described gave material containing 259 × 10^3 d.p.m. mg^{-1} (0.83% of activity administered). A duplicate experiment in which two culture bottles were each incubated with [14-^{14}C]-acetoin 15.6 × 10^6 d.p.m., gave 1.43 mg, which, after dilution with inactive riboflavin (11.3 mg), purification and acetylation gave riboflavin tetra-acetate. After chromatographic purification as described above, there was obtained 8.3 mg (198 d.p.m. mg^{-1}, 0.12% incorporation). The residual mycelium (dry weight 29.6 mg) contained 1.98 × 10^5 d.p.m. The fatty acid fraction contained 379.9 × 10^3 d.p.m. (1.2% of the administered radioactivity). The riboflavin tetra-acetate from both experiments was shown to be radiochemically pure by reconversion into riboflavin for Kuhn-Roth oxidation as described below.

Kuhn-Roth Oxidation of Riboflavin Derived from [4-^{14}C]-*Acetoin.*—The combined riboflavin tetra-acetate from the duplicate incorporation experiments (10.97 mg) was diluted with inactive material to give 25.47 mg (107 d.p.m. mg^{-1}, 58.4 × 10^3 d.p.m. $mmol^{-1}$). This was heated for 1 h with concentrated hydrochloric acid (0.5 ml). The solution was diluted with water (4.5 ml), concentrated to 2 ml and cooled in ice–water to give crystalline riboflavin (12.8 mg, 155.8 d.p.m. mg^{-1}, 58.6 × 10^3 d.p.m. $mmol^{-1}$). The riboflavin was diluted with inactive material (95.3 mg) and oxidised by the Kuhn-Roth procedure.[33] The acetic acid produced was recrystallised as the hydrated barium salt (30.3 mg, 122 d.p.m. mg^{-1}, 1 100 d.p.m. $mmol^{-1}$), 16.8% of the total activity of the riboflavin. Calculated for random distribution of radioactivity: 1 540 d.p.m. $mmol^{-1}$ (23.5%).

Effect of Temperature on Riboflavin (**3**) *and Acetoin* (**6**) *Production by* E. ashbyii.—Optimal growth and riboflavin production were obtained at 26—27 °C. The Figure shows the effect of changing temperature on riboflavin and acetoin production. The mycelium was grown in medium B for 42 h at 27 °C. The temperature was then adjusted and analyses for riboflavin, acetoin, and tissue weight were carried out after a further 80 h at the new temperature. Similar effects were noted in medium C.

Effect of Acetoin (**6**) *on Riboflavin* (**3**) *Production.*—Addition of acetoin in amounts ranging from 20 μg ml^{-1} to 1 mg ml^{-1} to cultures in media B or C at any time during the first three days of growth caused no measureable change in mycelial growth and only a marginal (6—14%) reduction in riboflavin production. Acetoin added to cultures in medium B after 70 h growth was metabolised rapidly during the first 24 h, thereafter more slowly. Similar effects were noted with cultures in medium C.

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