The Precursors of the Xylene Ring in Riboflavine

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1. The nature of the precursors of the xylene ring in riboflavine was reinvestigated with growing as well as resting cells of *Eremothecium ashbyii*. 2. The incorporation of acetoin into riboflavine was very low; further, [2-¹⁴C]pyruvate and [1-¹⁴C]acetate were equally effective as precursors of lumichrome, and pyruvate was much more active as a precursor of acetoin. These results exclude acetoin as a direct precursor of riboflavine. 3. Addition of unlabelled glucose decreased the incorporation of [¹⁴C]acetate into riboflavine more than it decreased the conversion of acetate into carbon dioxide, indicating that acetate is not a direct riboflavine precursor. 4. The incorporation of various sugars and dilution experiments suggest that a derivative of the intermediates of the pentose phosphate cycle is the precursor of the xylene ring in riboflavine.

Masuda (1956) isolated a pteridine derivative, 6,7-dimethyl-8-ribityl-lumazine, from cultures of *Eremothecium ashbyii*, an organism active in the synthesis of riboflavine. On structural grounds and on the basis of chemical studies this author suggested that acetoin (3-hydroxy-2-butanone) was the precursor of the aromatic ring in riboflavine (ring A). This hypothesis was further strengthened by the finding of Goodwin & Treble (1958) that [1-¹⁴C]acetoin was incorporated into riboflavine better than [2-¹⁴C]acetate in whole cells of *E. ashbyii*. The more recent studies of Plaut (1963) show that a molecule of 6,7-dimethyl-8-ribityl-lumazine donates a 'four carbon unit' to another molecule leading to the formation of riboflavine and a derivative of 4,5-diaminouracil. This finding, however, does not exclude the possibility that acetoin is the ultimate precursor of the four carbon atoms in 6,7-dimethyl-8-ribityl-lumazine and hence of the aromatic ring of riboflavine. Kishi, Asai, Masuda & Kuwada (1959) presented evidence that cell-free preparations of *E. ashbyii* could bring about the condensation of acetoin with 4-aminoribotyl-5-aminouracil to form 6,7-dimethyl-8-ribityl-lumazine.

The earlier studies of Plaut (1954), however, show that both the carboxyl and methyl carbon atoms of acetate are incorporated into the aromatic ring of riboflavine. This is a strong evidence against acetoin being involved in this biosynthesis. Barring the direct reduction of acetate to acetaldehyde, a reaction known to occur in some anaerobic organisms only, the only known mechanisms of acetoin biosynthesis involve the loss of the carboxyl group of acetate. Klungsoyr (1954), working with non-

growing cells of *E. ashbyii*, showed very poor incorporation of carboxyl-labelled acetate into riboflavine. We have attempted to compare the incorporation of acetate, acetoin and pyruvate (a direct precursor of acetoin and acetate) into riboflavine in both growing and standing cultures of *E. ashbyii*. We also measured the effect of adding non-radioactive diluents.

Our results in general show poor incorporation of pyruvate, acetate and acetoin into riboflavine. This, taken together with the dilution experiments, suggests that none of these compounds is directly involved in the synthesis of riboflavine ring A. By using various labelled sugars we obtained results which lend support to the hypothesis that a derivative of the pentose phosphate-cycle intermediates may act as the ultimate precursor of the xylene ring in riboflavine.

MATERIALS AND METHODS

Biological material. *Eremothecium ashbyii* (American Type Culture Collection no. 6747) was maintained on agar plates of the medium used for growth and transferred periodically.

Chemicals. Radioactive chemicals were purchased from The Radiochemical Centre (Amersham, Bucks.). Other chemicals, unless otherwise indicated, were chemically pure or B.P. products from various commercial companies.

Growth conditions. Initially cells were grown with large inocula (5%) in an aqueous medium that contained (per litre): yeast extract (Difco), 2g.; Bacto peptone (Difco), 5g.; a centrifuged 25% (w/w) solution of corn-steep liquor (A. E. Staley Manufacturing Co., Decatur, Ill., U.S.A.), 20 ml.; glucose, 40g.

The final pH of the medium was 5.6. Cells were grown

with continuous shaking in flasks filled to 10–20% of their capacity. The temperature was maintained at $29 \pm 1^\circ$.

Collection of mycelia. Twenty-four-hours-old cultures were filtered through muslin under suction and washed with cold saline. The resulting mycelial pad could then be peeled off the cloth and weighed directly. The cells were suspended in Krebs–Ringer phosphate buffer containing 10 mg. of guanine/100 ml. for experiments with non-growing cells. The manner of incubation with radioactive compounds is described in the Results section.

Extraction of riboflavine. The incubation was stopped by acidifying to pH 1 with conc. HCl. The suspension was then autoclaved for 20 min. at 15 lb./in². To the resulting hydrolysate enough Na₂SO₄ was added to saturate the solution. Riboflavine was extracted three times with 1 vol. of benzyl alcohol. At this stage benzyl alcohol forms an emulsion with the aqueous hydrolysate. The emulsion was easily broken by centrifugation and the upper benzyl alcohol layer pipetted out. The benzyl alcohol extracts were pooled, diluted with 4 vol. of ether and the riboflavine was twice extracted with 5 ml. of water.

Column chromatography of riboflavine. Florisil (60–100 mesh) (Floridin Co., Warren, Pa., U.S.A.) was suspended in 2% (v/v) acetic acid. When the earth settled, the slightly turbid supernatant fluid was decanted. The earth was resuspended in the 2% acetic acid and packed as a slurry in columns 1.5 cm. diam. and 10 cm. high. The aqueous extract of riboflavine was placed directly on the Florisil column. The column was washed with 500 ml. of 2% (v/v) acetic acid and the yellow band was eluted with 20% (v/v) pyridine.

Estimation of riboflavine. Riboflavine was measured by the intensity of its yellow colour at 445 m μ by using the millimolar extinction coefficient of 11.3. The extinction coefficient of riboflavine was checked with an authentic sample of riboflavine (Eastman Kodak Co.).

Crystallization of riboflavine to constant specific activity. After assaying the initial amount of riboflavine eluted from the columns, 25 mg. of non-labelled riboflavine was added and the solution evaporated to dryness. The riboflavine was crystallized to constant counts/min./ μ mole of the riboflavine, from the following solvents: 10% (w/v) acetic acid, 70% (v/v) propanol and conc. HCl diluted with water (4–5 vol.). One crystallization from each solvent was always enough to achieve constant specific activity.

Preparation of lumichrome. Lumichrome was prepared by the procedure of Karrer, Salomon, Schöpp, Schlitter & Fritzsche (1934). A dilute solution of purified riboflavine containing 20 mg./100 ml. was prepared in 50% (v/v) methanol. The solution was placed in Erlenmeyer flasks of 250 ml. capacity and irradiated (three fluorescent lamps, 15 w each) for 72 hr. During this period the intense yellow colour of the solution turned pale green. The methanolic solution was evaporated to dryness *in vacuo* and the yellow material was taken up in two 3 ml. portions of 0.1 N-NaOH. The alkaline solution was shaken once with 50 ml. of chloroform and the chloroform discarded. The alkaline liquid was brought to pH 2 with conc. HCl and the lumichrome extracted five times with 50 ml. of chloroform.

Chromatographic purification of lumichrome. A portion (10 g.) of Celite 545 (L. Light and Co. Ltd., Colnbrook, Bucks.) was triturated in a mortar with 4 ml. of water and packed tightly into a column (1.5 cm. diam.). The combined chloroform extract was passed through the column and

washed with an additional 50 ml. of chloroform saturated with 0.1 N-HCl. The lumichrome passed through the column, leaving a band of yellow impurities on top of the column.

To test the purity of lumichrome, it was chromatographed on Whatman no. 1 filter paper in the different solvent developed by Whitby (1952). In all cases it gave a single spot which corresponded in R_f to the five-times-crystallized lumichrome prepared on a large scale by the procedure of Karrer *et al.* (1934).

However, the recrystallized material prepared by the method of Karrer *et al.* (1934) was not pure and on chromatography on paper it gave two spots. Spectral analysis of these spots showed that lumichrome was contaminated with traces of riboflavine. Therefore the whole material was purified by passing it through Celite columns and recrystallizing once more from acetic acid.

Estimation of lumichrome. The chromatographically pure material was used as a standard. Complete spectra were taken in 0.5 N-NaOH and in 0.1 N-HCl. The shape of these spectral curves agreed with those reported by McNutt (1954). The molar extinction coefficients were calculated at different wavelengths on this sample on the basis of the nitrogen content of the material, as determined by a micro-Kjeldahl method. These values are reported in Table 1. For routine analysis lumichrome was estimated from the extinction at 340 and 425 m μ of its solutions in 0.5 N-NaOH.

Isolation of acetoin from mycelial cultures. Whenever incorporation of radioactivity into acetoin was to be determined, the following procedure was followed. To a suitable portion of the cell suspension 50 mg. of unlabelled acetoin was added and the final volume made up to 50 ml. with water. The mixture was distilled *in vacuo* almost to dryness. The clear distillate contained the acetoin.

Conversion of acetoin into 2,4-dinitrophenylosazone. 2,4-Dinitrophenylhydrazine (1.5 g. dissolved in 50 ml. of 30% w/v, perchloric acid) was mixed with the distillate containing the acetoin and the mixture refluxed for 6 hr. on a water bath. The voluminous precipitate of the osazone was filtered off on a Buchner funnel, washed with 10 ml. of water and dried at 50°.

Crystallization of osazone to constant specific activity. The osazone was crystallized alternately from aniline and nitrobenzene until the radioactivity per milligram of osazone became constant. In one case, after the osazone had been crystallized several times from the above solvents, it was recrystallized from several litres of acetic acid. The recrystallized material showed no change in specific radioactivity.

Organic synthesis of 3-hydroxy[1-¹⁴C]butan-2-one (acetoin). The procedure of Brady, Rabinowitz, van Baalen & Gurin (1951) was used to synthesize acetoin except that the synthesis was scaled down to 10 m-moles of lactonitrile.

Table 1. Molar extinction coefficient of lumichrome

	In 0.1 N-HCl		In 0.5 N-NaOH		
	262	350	265	340	425
Maxima (m μ)	33.3	8.81	40.3	6.50	6.95
10 ⁻³ $E_{\max}$ (cm. ² mole ⁻¹)					

Radioactive purity of acetoin. For the determination of the radioactive purity of acetoin as well as for further purification, the acetoin was chromatographed on a Celite column. A similar column was used by Neish (1950) to separate acetoin, diacetyl and butane-2,3-diol.

A portion (45 g.) of Celite 545 was wetted with 20 ml. of water and triturated thoroughly in a mortar. It was packed in a column of 2 cm. diam. The radioactive acetoin (0.4 g. of total synthesized product) was taken up in 2 ml. of water triturated with 5 g. of Celite and packed on the top of the column. Acetoin was eluted with ethyl acetate saturated with water and 10 ml. fractions were collected. Acetoin was determined colorimetrically and the radioactivity was estimated on the same sample. The profile obtained shows that acetoin comes out in the first few fractions of eluate and the curve of radioactivity corresponds to the curve of acetoin concentration.

The acetoin was recovered from ethyl acetate by diluting it with 2 vol. of normal pentane, and re-extracting with water. The aqueous extract was shaken again with normal pentane to extract the residual ethyl acetate. The aqueous acetoin was preserved at -12° .

Colorimetric estimation of acetoin. For rapid determination of acetoin a modification of the procedure of Happold & Spencer (1952) was used. A sample containing 20–100 μ g. of acetoin was mixed with 5 ml. of 0.1% (w/v) 2,4-dinitrophenylhydrazine dissolved in 5% (w/v) perchloric acid. It was placed in a tube tightly closed with a rubber stopper and boiled for 1 hr. in a water bath. After cooling, a pinch of kaolin was added to the tube, which was centrifuged. The supernatant was discarded and the precipitate was washed once with dilute HCl and once with water. The precipitate was extracted twice with 5 ml. of sodium ethylate. The extinction of the violet colour was read in a Klett photometer with a 540 $m\mu$ filter. The value was compared against a standard containing 40 μ g. of acetoin. The colour intensity is linear within the range 20–100 μ g. of acetoin. (Sodium ethylate is prepared by dissolving 3 g. of clean sodium in 1000 ml. of absolute ethanol.)

Determination of radioactivity. All samples were plated on preweighed glass planchets and counted on a gas-flow counter (Nuclear-Chicago Corp.) in which 1 μ C gives 600 000 counts/min. The planchets were reweighed and whenever applicable corrections for self-absorption were applied.

The samples were counted for 10 min. If activities of less than 10 counts/min. were obtained the counting was continued for an additional 10 min. The background count was about 18 counts/min.

RESULTS

Incorporation of [1- 14 C]acetate and [2- 14 C]pyruvate into acetoin in growing cells. Pyruvate and acetaldehyde are the only known metabolic precursors of acetoin. The label from carboxyl-labelled acetate metabolized through the tricarboxylic acid cycle will appear in pyruvic acid in the carboxyl group only, and therefore will not label acetoin. The direct reduction of acetate to acetaldehyde is known to occur in some anaerobic organisms only. The only other known route through which carboxy-labelled acetate can label acetoin would involve its incor-

poration into glucose, via carboxyl-labelled pyruvic acid, the randomization of the isotope in the glucose through the hexose monophosphate shunt, the subsequent production of pyruvate and its conversion into acetoin. Such a route would involve great dilutions. As carboxyl-labelled acetate is known to be incorporated into riboflavin, it was necessary to find the relative magnitude of the incorporation of acetate and pyruvate into acetoin in *E. ashbyii*.

Two flasks each containing 400 ml. of a 24 hr.-old culture were incubated with 1 μ mole containing 10^6 counts/min. of either [1- 14 C]acetate or [2- 14 C]pyruvate (the direct precursor of [1- 14 C]acetate). After 12 hr. the incubation was stopped by adding 5 ml. of conc. hydrochloric acid. Acetoin was isolated as the 2,4-dinitrophenyl osazone and recrystallized four times alternately from aniline and nitrobenzene. Constant specific activity was obtained after the first crystallization. The results showed specific activities 40 and 255 counts/min./ μ mole for the acetoin produced from [1- 14 C]acetate and [2- 14 C]pyruvate respectively.

Incorporation of acetate and pyruvate into riboflavin and acetoin in standing cultures. To obtain a correlation between the labelling of riboflavin and acetoin by acetate and pyruvate the incorporation of these substrates into both acetoin and riboflavin was studied simultaneously in standing cultures. Cell suspensions of *E. ashbyii* in Krebs-Ringer phosphate buffer containing guanine were incubated with [2- 14 C]pyruvate, [1- 14 C]acetate and [2- 14 C]acetate with or without non-radioactive diluents. At the end of 12 hr. the reaction was stopped and the incubation medium was divided into two equal portions: one portion was used to isolate riboflavin; to the other portion 50 mg. of unlabelled acetoin was added and the osazone prepared and crystallized to constant specific activity (Table 2). Though [2- 14 C]pyruvate was incorporated about 60 times as effectively as [1- 14 C]acetate into acetoin its incorporation into riboflavin was less than one and a half times as effective as the incorporation of [1- 14 C]acetate. Comparing [2- 14 C]pyruvate with [2- 14 C]acetate, the incorporation of [2- 14 C]acetate was about three times that of the pyruvate into riboflavin whereas it labelled acetoin less than one-third as effectively as pyruvate.

The addition of 15 molar equiv. of unlabelled acetate to incubations with labelled pyruvate and vice versa showed very little effect.

Incorporation of acetate, pyruvate and acetoin into riboflavin in standing cultures. In this experiment 5% cell suspensions of *E. ashbyii* in Krebs-Ringer phosphate buffer containing guanine were incubated with [1- 14 C]acetate, [2- 14 C]acetate, [2- 14 C]pyruvate and 3-hydroxy[1- 14 C]butan-2-one (radioactive acetoin). Because of the larger endogenous dilution expected with pyruvate and acetate, the

Table 2. *Incorporation of acetate and pyruvate into riboflavine and acetoin*

Each flask contained 17 μ moles of the radioactive substrate (10×10^6 counts/min.), 50 ml. of a 5% (w/v) suspension of washed *E. ashbyii* cells in Krebs-Ringer phosphate buffer, 5 mg. of guanine and, whenever indicated, 250 μ moles of unlabelled substrate. The flasks were incubated for 12 hr. at 30° on a shaker incubator (100 strokes/min.). The reaction was stopped by adding 1.5 ml. of conc. HCl.

Labelled substrate	Unlabelled substrate	Riboflavine		Acetoin	
		μ moles formed	Counts/min./ μ mole	μ moles formed	Counts/min./ μ mole
[1- ¹⁴ C]Acetate	—	2.0	375	560	38
[1- ¹⁴ C]Acetate	Pyruvate	2.1	280	720	31
[2- ¹⁴ C]Acetate	—	2.6	1450	570	600
[2- ¹⁴ C]Acetate	Pyruvate	2.5	1500	575	630
[2- ¹⁴ C]Pyruvate	—	2.8	560	560	2130
[2- ¹⁴ C]Pyruvate	Acetate	2.3	413	570	2700

Table 3. *Incorporation of acetoin, acetate and pyruvate into lumichrome and carbon dioxide*

Results presented are the averages of quadruplicate flasks. Each flask was provided with a suspended well containing 1 ml. of N-NaOH and contained 15 ml. of 5% (w/v) cell suspension in Krebs-Ringer phosphate buffer with 10 mg. of guanine/100 ml. Flasks were incubated at 30° for 12 hr. The reaction was stopped by the addition of 0.3 ml. of conc. HCl. The incubation was continued for an additional 1 hr. to trap the liberated CO₂. The riboflavine was isolated from the incubation and degraded as described in the Materials and Methods section.

Labelled substrate	Riboflavine		Percentage converted into CO ₂
	μ mole formed	Counts/min./ μ mole of lumichrome	
[1- ¹⁴ C]Acetoin	0.50	19	0.24
[2- ¹⁴ C]Pyruvate	0.53	192	4.4
[2- ¹⁴ C]Acetate	0.24	510	6.11
[1- ¹⁴ C]Acetate	0.83	125	28.0

specific activity of the added acetate and pyruvate (6.67×10^4 counts/min./ μ mole) was made higher than the specific activity of the added acetoin (1.25×10^4 counts/min./ μ mole). Additional flasks were also incubated with radioactive substrate combined with non-labelled diluent.

The liberated carbon dioxide was trapped in 1N-sodium hydroxide placed in a well suspended from the stopper of the flask. Riboflavine was isolated and crystallized to constant specific activity. The purified riboflavine was converted into lumichrome. Lumichrome was purified and assayed for its radioactivity. The average results of this experiment are in Table 3. The replicates in this experiment agreed within 30% of the mean.

These results indicate that acetoin was poorly metabolized and poorly incorporated into ribo-

flavine. The 19 counts/min./ μ mole are of marginal significance and can possibly be due to counting errors as the amount counted in all cases was about 10% of the total riboflavine formed.

The addition of unlabelled diluents (250 μ moles) of acetate or pyruvate to the flasks containing radioactive acetoin did not significantly change the incorporation. Similarly the addition of unlabelled acetoin or acetate to the flasks containing the labelled pyruvate and the addition of unlabelled pyruvate and acetoin to the flasks containing the radioactive acetate did not alter the incorporation drastically.

Effect of glucose on the incorporation of acetate into riboflavine and carbon dioxide. During the course of experiments with growing cells it was observed that when sufficient glucose was present in the culture medium the incorporation of acetate or pyruvate into riboflavine was exceedingly poor. Therefore the effect of glucose was examined in two experiments.

In Expt. 1 cells were grown as described previously and samples of the culture were withdrawn aseptically at intervals for glucose analysis. After 48 hr. incubation the concentration of glucose fell below 1.0 mg./100 ml. At that point 100 ml. portions of the culture were transferred to two sterile flasks. One flask was incubated with 20 μ moles of [2-¹⁴C]-acetate (1.5×10^6 counts/min.) alone, and the other with the same [2-¹⁴C]acetate with the addition of 2 g. of unlabelled glucose. During incubation the flasks were aerated with carbon dioxide-free air and the evolved carbon dioxide was trapped in 1N-sodium hydroxide. The incubation was stopped after 12 hr. Riboflavine was isolated, crystallized to constant specific activity and counted. It was further degraded to lumichrome, which was purified and counted. The results are shown in Table 4, Expt. 1.

In Expt. 2 cells were grown for 24 hr., harvested

and resuspended with 19 vol. of Krebs–Ringer phosphate buffer containing guanine. Portions (100 ml.) of the suspension were incubated with $10 \mu\text{moles}$ of the labelled substrate containing 2×10^6 counts/min. In certain flasks (as indicated) $2500 \mu\text{moles}$ of glucose or $5000 \mu\text{moles}$ of sodium pyruvate were added; the incubation time was 6 hr., after which the radioactivity of carbon dioxide, riboflavin and lumichrome were determined as described in the Materials and Methods section. The results of this experiment are shown in Table 4, Expt. 2.

It is clear from these experiments that the presence of glucose inhibited the incorporation of acetate into the lumichrome to values below 10% of the control value. This inhibition seems to be of

the same magnitude in growing and resting cells. The conversion of acetate into carbon dioxide was also inhibited but to a much smaller degree. The addition of amounts of pyruvate equivalent to the glucose had much less effect on the incorporation of acetate into lumichrome. It is also noted that $[1-^{14}\text{C}]\text{glucose}$ and $[6-^{14}\text{C}]\text{glucose}$ are better incorporated into lumichrome than acetate.

A calculation of the ratio of the isotope in lumichrome to the isotope in riboflavin shows that the lumichrome part of the riboflavin molecule contained 50–56% of the isotope when $[2-^{14}\text{C}]\text{acetate}$ was used, under vastly divergent conditions.

Incorporation of monosaccharides into riboflavin. In the previous experiment the addition of glucose was shown to dilute the incorporation of acetate

Table 4. *Incorporation of acetate and glucose into riboflavin and carbon dioxide*

See text for experimental details

	Labelled substrate	Unlabelled substrate	μmoles formed	Riboflavin		Percentage converted into CO_2
				Counts/min./ μmole of riboflavin	Counts/min./ μmole of lumichrome	
Expt. 1 (growing cells)	$[2-^{14}\text{C}]\text{Acetate}$	—	13.5	900	483	34
	$[2-^{14}\text{C}]\text{Acetate}$	Glucose	16.1	59	30	20
Expt. 2 (non-growing cells)	$[2-^{14}\text{C}]\text{Acetate}^*$	—	2.74	735	368	59
	$[2-^{14}\text{C}]\text{Acetate}$	Glucose	2.12	59	33	16
	$[2-^{14}\text{C}]\text{Acetate}^*$	Pyruvate	1.19	522	289	60
	$[6-^{14}\text{C}]\text{Glucose}^*$	—	3.02	1412	522	24
	$[\text{U}-^{14}\text{C}]\text{Glucose}^*$	—	2.60	1307	503	44

* Average of duplicate experiments, which agreed in each case to better than 30% of the mean.

Table 5. *Incorporation of various sugars into riboflavin and carbon dioxide*

See text for experimental details.

	Labelled substrate	Unlabelled substrate	μmoles formed	Riboflavin		Percentage converted into CO_2
				Counts/min./ μmole of riboflavin	Counts/min./ μmole of lumichrome	
First batch	$[1-^{14}\text{C}]\text{Glucose}^*$	—	1.27	1995	1194	33.5
	$[6-^{14}\text{C}]\text{Glucose}^*$	—	1.41	2320	843	26.4
	$[6-^{14}\text{C}]\text{Glucose}^*$	Ribose	1.54	1920	893	26.0
	$[1-^{14}\text{C}]\text{Ribose}$	—	1.65	3360	2300	7.2
	$[1-^{14}\text{C}]\text{Ribose}$	Glucose	1.38	725	486	
	$[\text{U}-^{14}\text{C}]\text{Erythritol}$	—	1.58	126	148	0.16
	$[\text{U}-^{14}\text{C}]\text{Erythritol}^*$	Glucose	1.18	62	118	0.04
Second batch	$[\text{U}-^{14}\text{C}]\text{Glucose}^*$	—	1.08	2526	1071	19.4
	$[\text{U}-^{14}\text{C}]\text{Glucose}^*$	Xylose	1.93	1900	584	20.3
	$[\text{U}-^{14}\text{C}]\text{Glucose}$	Glycerol	1.24	2730	800	25.3
	$[\text{U}-^{14}\text{C}]\text{Ribose}^*$	—	1.34	2154	989	3.6
	$[\text{U}-^{14}\text{C}]\text{Xylose}$	—	1.04	510	302	0.68
	$[\text{U}-^{14}\text{C}]\text{Xylose}$	Glucose	2.14	239	275	0.32
	$[\text{U}-^{14}\text{C}]\text{Glycerol}^*$	—	1.52	2434	1120	27.5

* Results are averages of duplicate flasks, which agree in all cases to better than 25% of the mean.

into the riboflavine more than the dilution by pyruvate. Glucose was also shown to be a more efficient precursor of the xylene ring in riboflavine.

The following experiment was designed to test the effectiveness of various monosaccharides and derivatives as precursors of the xylene ring of riboflavine. Because of the large number of flasks involved, the experiment was run in two separate batches.

E. ashbyii cells were grown for 24 hr. with aeration, harvested and resuspended in 19 vol. of Krebs-Ringer phosphate buffer containing 10 mg. of guanine/100 ml. Each flask contained 100 ml. of the cell suspension, 10 μ moles of radioactive substrate (2×10^6 counts/min.) and, whenever indicated, 2500 μ moles of non-radioactive diluent substrate (5000 μ moles for glycerol diluent). Incubation time was 6 hr. The isolation of carbon dioxide, riboflavine and lumichrome was as described in the Materials and Methods section.

The results of this experiment are presented in Table 5. Ribose was incorporated as efficiently as glucose, although its utilization (measured by conversion into carbon dioxide) is much poorer. The incorporation of xylose is poor but its conversion into carbon dioxide is very low whereas the incorporation of glycerol into carbon dioxide and riboflavine is similar to that of glucose.

The production of radioactive carbon dioxide from glucose 1-phosphate and glucose 6-phosphate is similar as is their incorporation into lumichrome.

DISCUSSION

Both acetate and acetoin have been suggested as the ultimate precursors of the xylene ring (ring A) in riboflavine. Plaut (1961), on the basis of the incorporation of [1- 14 C]acetate into riboflavine, favours the acetate hypothesis. However, Goodwin (1963), basing his arguments on structural grounds, on the incorporation of acetoin into riboflavine (Goodwin & Treble, 1958) and on the higher degree of randomization occurring in the incorporation of [2- 14 C]acetate compared with the incorporation of [1- 14 C]- or [6- 14 C]-glucose into the xylene ring of riboflavine (Plaut, 1954), advances the acetoin hypothesis.

We attempted to resolve this problem by studying the incorporation of radioactive substances in recrystallized riboflavine and in chromatographically pure lumichrome obtained therefrom. Our purification procedures led to a low recovery of lumichrome, which makes further degradation studies impractical. We assumed in all cases that the activity in lumichrome was essentially all in the xylene ring. This is justified since Plaut's (1954) results show very low incorporation of acetate and glucose in the pyrimidine ring of riboflavine (ring

c). Further, we ran all our incubations in the presence of large amounts of purines, which Goodwin & Jones (1956) have shown to decrease the incorporation of serine, a more direct precursor of the pyrimidine ring, to very low values.

Our results provide evidence against the involvement of either acetoin or acetate as the ultimate precursor of the xylene ring in riboflavine. The evidence against acetoin can be summarized as follows.

1. The very low incorporation of acetoin into riboflavine (Table 3). These results are contrary to those reported by Goodwin & Treble (1958). The reason for the difference is not clear as these authors have not yet reported the details of their experiments.

2. The incorporation of [2- 14 C]pyruvate into lumichrome was not better than the incorporation of its direct product [1- 14 C]acetate, whereas [2- 14 C]pyruvate was more effective than [1- 14 C]acetate as a precursor of acetoin, the ratio being 60:1 in standing cultures and 6:1 in growing cells (see Table 2).

3. [2- 14 C]Acetate was incorporated about one-fifth as effectively as [2- 14 C]pyruvate into acetoin, whereas its incorporation into lumichrome was 3 to 5 times as effective (Table 2).

4. The lower dilution of the incorporation of acetate into lumichrome by unlabelled pyruvate (a direct precursor of acetoin) compared with the dilution by unlabelled glucose (Table 4). It is not clear why pyruvate did not dilute the degradation of acetate into carbon dioxide; possibly it stimulates the tricarboxylic acid cycle to offset the dilution.

These last three points we take to show that the poor incorporation of acetoin is not due solely to a difficulty in its entry into the cells.

The evidence against acetate can be summarized as follows:

1. The higher degree of randomization of the isotope from acetate than the isotope from glucose in the xylene ring of riboflavine as shown by Plaut (1954) and pointed out by Goodwin (1963).

2. The very large dilution of the incorporation of acetate into lumichrome by non-radioactive glucose. This dilution is greater than the dilution of the degradation of acetate into carbon dioxide by glucose. It is also larger than the dilution of acetate incorporation into lumichrome by unlabelled pyruvate. These two facts show that the dilution of the incorporation of acetate into lumichrome is more than can be accounted for by the dilution of the specific activity of the radioactive acetate by acetate units formed from unlabelled glucose.

We would like to suggest as a tentative alternative hypothesis that a derivative of the intermediates of the pentose phosphate cycle acts as the

precursor of the xylene ring in riboflavine. The evidence favouring such a hypothesis can be summarized as follows.

1. The high amount of incorporation of ribose into riboflavine, especially if this is taken in conjunction with the poorer utilization of ribose than of glucose as measured by its conversion into carbon dioxide. The incorporation of xylose, though poorer than glucose, may be explained by its low degree of activation or entry into the cell. However, if the ratio of incorporation into lumichrome to oxidation to carbon dioxide is calculated (Table 5), ribose and xylose show almost equal values, which are approximately tenfold those obtained for glucose or glycerol.

2. The similar incorporation of isotope into the ribitol side chain and the lumichrome portion of the riboflavine molecules from various sources and under various conditions (Tables 3, 4 and 5), suggesting a common precursor of the xylene ring and the ribitol side chain which is presumably synthesized through a pentose (Plaut, 1961).

A similar isotope distribution has been observed by Plaut (1954) and Plaut & Broberg (1956). The evidence cited against acetate above and evidence 4 cited against acetoin can also be taken to favour this hypothesis.

The labelling pattern in riboflavine obtained from labelled acetate (Plaut, 1954) also fits this hypothesis if a four carbon atom sugar is incorporated into riboflavine, and if the major part of the pentose-cycle intermediates are produced through the transketolase, transaldolase reactions.

The observation that unlabelled glucose effectively dilutes the incorporation of pentoses into riboflavine while the reverse dilution is less pronounced may be a reflexion of the limited ability of the cells to absorb or activate pentoses. But it is not clear why xylose had a more marked effect than ribose on the incorporation of glucose into lumichrome.

As cells are not permeable to phosphate esters the direct testing of pentose-cycle intermediates as precursors of the xylene ring of riboflavine will have to await the preparation of cell-free extracts capable of affecting the synthesis of 6,7-dimethyl-8-ribotyl-lumazine.

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