Studies on the Biosynthesis of Riboflavin

3. THE UTILIZATION OF ^{14}C -LABELLED SERINE FOR RIBOFLAVIN BIOSYNTHESIS BY *EREMOTHECIUM ASHBYII**

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(Received 12 January 1956)

Considerable evidence now exists indicating that the biosynthetic routes of rings B and C of riboflavin in the moulds *Eremothecium ashbyii* and *Ashbya gossypii* are closely related to that of the purines. For example, the pattern of labelling of the carbon atoms of rings B and C and of the purine molecule is identical after the addition of $\text{H}^{14}\text{CO}_2\text{H}$, $\text{NaH}^{14}\text{CO}_3$ and [*carboxy*- ^{14}C]glycine and [α - ^{14}C]glycine (Plaut, 1954). Furthermore, the addition of purines to the culture medium of *E. ashbyii* stimulates flavinogenesis very considerably (Maclaren, 1952; Goodwin & Pendlington, 1954) and this is due to its incorporation, after the removal of $\text{C}_{(8)}$, into the riboflavin molecule (McNutt, 1954).

Although glycine is incorporated into riboflavin (Plaut, 1954), it does not specifically stimulate flavin synthesis in *E. ashbyii* under conditions where serine is active (Goodwin & Pendlington, 1954). The only other amino acid to behave in the same manner as serine is threonine; tyrosine, but not phenylalanine, is very slightly active. As serine cannot be replaced by glycine plus formate (Brown *et al.* 1955) and, as the stimulatory effects of serine and purines were cumulative, it was suggested that serine might provide carbon units for synthesis of the aromatic ring A (Goodwin & Pendlington, 1954).

The present paper reports an investigation designed to test this suggestion; it is concerned with the incorporation of carbon from uniformly carbon-labelled serine and from DL- $[\beta$ - $^{14}\text{C}]$ serine into riboflavin by *E. ashbyii*.

EXPERIMENTAL

Cultures and cultural conditions. The strain of *E. ashbyii* used was kindly provided by Mrs J. Robson (A.E.R.E., Harwell, Berks). It produced more riboflavin than our original strain (Goodwin & Pendlington, 1954; Brown *et al.* 1955); otherwise it behaves similarly in that flavinogenesis is stimulated by serine and by adenine.

The medium used was that of Goodwin & Pendlington (1954), 1 l. containing: glucose, 10 g.; CaCl_2 , 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g.; NaCl, 0.2 g.; bacteriological peptone, 0.4 g.

Cultures were grown at 28° in Roux bottles, generally four for each experiment, each containing 100 ml. of medium. They were connected together in series so that CO_2 -free air could be drawn through continually, and the metabolic CO_2 was removed and trapped in KOH. The labelled serine was added, without dilution, in aqueous solution 48 hr. after inoculation, and the mould harvested 48 hr. later. After centrifuging off the cells and removing the riboflavin-containing medium, the residual riboflavin in the cells [30–40% of the total under our conditions (Goodwin & Pendlington, 1954)] was quantitatively recovered by adding a small volume of 2N-HCl and autoclaving at 15 lb./in.² for 10 min. The two riboflavin extracts were combined and assayed spectrophotometrically (Goodwin & Pendlington, 1954). The greater part was then reduced to dryness under vacuum and the residue dissolved in 0.5N-NaOH (40 ml.). This solution was placed in a shallow glass dish so that the depth of liquid was about 1.5 cm., and the riboflavin converted into lumiflavin by irradiation with an unfiltered mercury-arc source (Hanovia, 350 W) placed at a distance of 15 cm. Conversion was generally complete within 8 hr., and this could be checked by taking a small sample, acidifying with 2N-HCl, and shaking with an equal volume of CHCl_3 ; as lumiflavin is transferred quantitatively to the CHCl_3 phase, the reaction is judged complete when the water phase is colourless.

In some experiments a small proportion of the medium was reduced to about one-sixth of its volume, the pH adjusted to 5.0 and $\text{Na}_2\text{S}_2\text{O}_4$ added until the E_h (redox potential) of the medium was reduced to +0.05 v (Hines, 1945; McMillan, 1945). After standing overnight the precipitated reduced riboflavin was centrifuged off and extracted with warm (60–90°) 75% (v/v) aqueous propan-2-ol; during this extraction the riboflavin was oxidized to its original state. After concentration the riboflavin was chromatographed twice on paper with butanol–water–acetic acid (4:5:1, by vol.) as solvent system. Its activity was measured after each chromatography and gave concordant results; the crude riboflavin precipitated by $\text{Na}_2\text{S}_2\text{O}_4$ always gave much higher values.

The amount of lumiflavin produced from the main portion of riboflavin was assayed spectrophotometrically (Goodwin & Pendlington, 1954), the CHCl_3 removed and a known weight of unlabelled lumiflavin added, the amount being usually about five times greater than the amount of labelled material so that a final weight of 10–15 mg. was obtained. Weighed amounts of this were taken for ^{14}C assay. The homogeneity of the lumiflavin was checked by paper chromatography with the butanol–water–acetic acid solvent system.

* Part 2: Brown, Goodwin & Pendlington (1955).

One portion of the lumiflavin was converted into the keto acid 1:2-dihydro-1:6:7-trimethyl-2-oxo-3-quinoxalinecarboxylic acid by dissolving in 2*N*-NaOH and heating for 30 min. at 100° (Kuhn & Rudy, 1934; Plaut, 1954) and the urea so formed was precipitated as dioxanthylurea (Fosse, 1914). The crystalline dioxanthylurea was removed by centrifuging, washed repeatedly with absolute ethanol and then water, and dried and weighed. Weighed portions were then taken for ¹⁴C assay. The residual solution containing the keto acid was acidified with glacial acetic acid and the keto acid extracted with CHCl₃. The solvent was removed and the acid sublimed in a microsublimation tube in a stream of N₂; the CO₂ produced was thus swept out of the apparatus, trapped in Ba(OH)₂ (Plaut, 1954) and counted as BaCO₃.

The second portion of lumiflavin was converted into *N*-methyl-4:5-dimethyl-*o*-phenylenediamine by the method developed by Plaut (1954). Lumiflavin (5 mg.) was dissolved in 5*N*-NaOH (5 ml.) and heated in a stainless-steel bomb for 10 hr. at 150°. After cooling to room temp., the contents of the bomb were extracted with ether, the extract was dried over KOH for 3–4 hr. and the ether removed *in vacuo*. The residue was then dried *in vacuo* at 40° for 2 hr. and then dissolved in a small volume (2–4 ml.) of anhydrous ether. The diamine dihydrochloride was precipitated in a centrifuge tube (15 ml.) by passing hydrogen chloride through the solution; after centrifuging, the supernatant was removed and the precipitate washed twice with dry ether and then placed immediately in a desiccator containing soda lime and dried at 50° for 6–8 hr. (Plaut, 1954). (The dihydrochloride is an extremely deliquescent substance.) It was then weighed and counted. The complete degradation is summarized in Fig. 1.

Measurement of radioactivity. Samples were plated on stainless-steel planchets (area 2.8 cm.²) and assayed with a Geiger-Müller end-window counter, working at a geometry of approximately 5%. Counts were carried to a standard error of ± 5% and were corrected to zero thickness from a standard graph. In each experiment all the results are referred to the counts in the total riboflavin produced.

Radioactive materials. Uniformly labelled L-[¹⁴C]serine (specific activity 5.3 × 10⁶ counts/min./mg.) was obtained from The Radiochemical Centre, Amersham. A small amount of DL-[β-¹⁴C]serine (specific activity 0.5 × 10⁶ counts/min./mg.) was most generously given to us by Dr E. A. Adelberg, Department of Bacteriology, University of California, Berkeley, California, U.S.A. Serine was added to the medium from an aqueous solution without dilution with unlabelled material.

RESULTS

Incorporation of labelled serine into riboflavin

Rings A, B and C. The results of two experiments, one with uniformly labelled L-[¹⁴C]serine and the other with DL-[β-¹⁴C]serine are recorded in Table 1.

The uniformly labelled serine gives a labelling pattern consistent with it being converted into glycine and a one-carbon fragment before incorporation into the riboflavin molecule. Considerable activity is found in C₍₃₎ which arises from formate (Plaut, 1954) and in C_(4a) plus C_(9a) which arise from glycine (it was not possible to separate C_(4a) and

Fig. 1. Degradation of riboflavin.

Table 1. *Distribution of activity in riboflavin produced by Eremothecium ashbyii grown in the presence of either uniformly labelled [¹⁴C]serine or [β -¹⁴C]serine*

Labelled serine was added to 48 hr. cultures on basal medium of Goodwin & Pendlington (1954); cultures were harvested 48 hr. later; 100 ml. of medium was used in each Roux bottle; temp., 28°.

Compound isolated	Uniformly labelled serine		[β - ¹⁴ C]Serine	
	Total activity (counts/min.)	Percentage of total activity incorporated in lumiflavin	Total activity (counts/min.)	Percentage of total activity incorporated in lumiflavin
Total counts added	2 090 000	—	105 000*	—
Lumiflavin	115 000	—	3 850	—
Dixanthylurea (C ₍₃₎)	29 600	25.7	3 000	78
CO ₂ (C ₍₄₎)	0†	0	0	0
'Oxo-acid' (C _(4a) + C _(9a))	77 000	66.9	250	6.5
N-Methyl-4:5-dimethyl-o-phenylenediamine (Ring A + C ₍₁₎)	0	0	0	0
Percentage of total activity found in lumiflavin	5.5	—	3.7	—

* Twice the number of counts were added as DL-serine.

† 0 indicates that the counts were not significantly different from the background.

C_(9a)). From a consideration of the relative activities of C₍₃₎ and C_(9a) plus C_(4a), it appears that both units derived from serine are incorporated to approximately the same extent.

The greater part of the activity of [2-¹⁴C]serine is found in C₍₃₎, indicating that the β -carbon atom of serine is a good source of 'formate'. The small but significant activity found in C_(4a) plus C_(9a) indicates that the one carbon unit arising from [2-¹⁴C]serine can to some extent be incorporated into glycine.

In neither case is there any detectable activity in the aromatic ring A, and thus the suggestion that serine stimulates flavinogenesis by providing a carbon precursor for this aromatic ring must be discarded.

The ribitol side chain. A number of experiments (Table 2) have shown that after addition of uniformly labelled serine, the activity of the resulting riboflavin and lumiflavin are not significantly different. Thus it appears that serine contributes no carbon atoms to positions 2', 3', 4' and 5' of the ribityl residue. As the inactivity of N-methyl-5:6-dimethyl-o-phenylenediamine (Table 1) shows that serine is also not incorporated in C₍₁₎, it can be concluded that serine metabolites play no part in ribitol formation.

It is noteworthy that the incorporation of serine (4–7%) into riboflavin is much greater than that previously recorded (0.01%) for acetate (Klungsoyr, 1954). Plaut (1954) did not record the percentage incorporation of the various labelled compounds used in his experiments.

Dilution out of serine by adenine. Our first experiments with labelled serine were carried out in media containing adenine to increase the yield of riboflavin. It was soon apparent that under these conditions adenine was almost completely diluting out the serine. Table 3 demonstrates the very

Table 2. *Incorporation of uniformly labelled serine into riboflavin and lumiflavin by E. ashbyii*

Riboflavin was isolated 48 hr. after addition of labelled serine to 48 hr. cultures; 100 ml. of basal medium was used in each Roux bottle; temp., 28°. Determinations on two portions of the same riboflavin sample were made in each experiment.

Total activity added	Total activity in riboflavin (counts/min.)	Total activity in lumiflavin (counts/min.)	Incorporation into riboflavin (%)
308 000	20 900	19 850	6.8
	21 200	20 500	6.9
2 080 000	96 600	112 000	4.7
	113 000	118 000	5.5

marked reduction in incorporation of uniformly labelled serine into riboflavin in the presence of adenine. Similar results were obtained with [β -¹⁴C]serine; because of the low initial activity of the added serine, the counts in the isolated riboflavin were not significantly different from the background counts.

Production of carbon dioxide from serine. Table 4 demonstrates that, over 48 hr. after addition of uniformly labelled serine, 13% is metabolized to CO₂ and, after addition of [β -¹⁴C]serine, approximately 7% is recovered as CO₂. This latter value is calculated by assuming that D-serine is metabolically inert. This is based on the observation that although L-threonine stimulates flavinogenesis in a similar way to serine, D-threonine is inactive (Goodwin & Pendlington, 1954). If this assumption is accepted, the results obtained suggest that *E. ashbyii* converts only two serine carbon atoms into CO₂. It will also be seen that the production of CO₂ from serine is not affected by the presence of adenine.

Table 3. *Activity of riboflavin produced by E. ashbyii from labelled serine in the presence and absence of adenine*

Riboflavin was isolated 48 hr. after addition of labelled serine to 48 hr. cultures; 100 ml. of medium was used in each Roux bottle; temp., 28°.

	Total activity added (counts/min.)	Total activity in riboflavin (counts/min.)	Incorporation (%)
Basal medium	308 000	15 000	4.8
Basal medium + adenine (50 mg./100 ml.)	308 000	280	0.09

Table 4. *Activity of CO₂ produced by E. ashbyii from labelled serine in the presence and absence of adenine*

Riboflavin was isolated 48 hr. after addition of labelled serine to 48 hr. cultures; 100 ml. of medium was used in each Roux bottle; temp., 28°.

[¹⁴ C]Serine*	Total counts added (in 200 ml. of medium) (counts/min.)	Total activity in CO ₂ (counts/min.)	Percentage of total counts added in CO ₂
Basal medium	105 000	7 850	7.5
Basal medium + adenine (50 mg./100 ml.)	146 000	9 550	6.4
	76 000	6 040	7.8
Uniformly labelled serine			
Basal medium	2 080 000	274 000	13.2
Basal medium + adenine (50 mg./100 ml.)	817 000	103 000	12.6

* Twice as many counts added as DL-compound; it is assumed that D-serine is not converted into CO₂ (see text).

DISCUSSION

The reason why serine, but not glycine or glycine plus formate, is stimulatory appears from the present results to be its greater ease of conversion into active glycine or active formate. As both formate and glycine can be incorporated into riboflavin (Plaut, 1954), but as neither is stimulatory, either alone or in combination (Goodwin & Pendlington, 1954; Brown *et al.* 1955), and as the methylene carbon of glycine is not incorporated into C₂₀ of riboflavin, i.e. is not converted into formate (Plaut, 1954), it is possible that active formate is the limiting factor. Other situations are known in which serine is more effective than glycine; for example, (a) formate is essential for optimum conversion of pteroylglutamic acid into the citrovorum factor in *Streptococcus faecalis*; it can be replaced by serine but not by glycine (Broquist, Kohler, Hutchison & Burchenal, 1953), and (b) serine but not glycine causes a rapid disappearance of 5-amino-4-imidazole-carboxamide ribonucleotide (i.e. conversion into inosinic acid) in pigeon-liver homogenates (Buchanan, Levenberg, Flaks & Gladner, 1955).

The marked diluting out of chemically minute amounts of serine by adenine is explicable on the assumption of serine being incorporated into riboflavin via an adenine-like pool. The cumulative effect of adenine and serine when present in com-

parable amounts (Goodwin & Pendlington, 1954) can be due to the fact that formate, the limiting factor for purine-precursor synthesis in the basal medium, is supplied by serine whilst adenine provides extra purine precursor *in toto*. It must be assumed that units for synthesis of the aromatic ring and the ribityl side chain are not limiting.

SUMMARY

1. Uniformly labelled [¹⁴C]serine is incorporated into riboflavin by *Eremothecium ashbyii* to the extent of about 4–7%; incorporation is confined to positions 2 and 4a plus 9a; no activity was found in the aromatic ring or the ribityl side chain. About 13% of the label appears as carbon dioxide.

2. [2-¹⁴C]Serine is incorporated almost exclusively into position 2; a small but definite labelling occurs in positions 4a plus 9a but none in the aromatic ring. About 7% of the label appears as carbon dioxide.

3. This isotope distribution is consistent with serine being converted into glycine and formate before incorporation into riboflavin.

4. Adenine dilutes out markedly the incorporation of labelled serine into riboflavin, but has no effect on the amount of serine metabolized to carbon dioxide.

We wish to thank the Agricultural Research Council for financial support.

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Biological Synthesis of Ascorbic Acid: the Conversion of Derivatives of D-Galacturonic Acid into L-Ascorbic Acid by Plant Extracts

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(Received 16 December 1955)

It was suggested by Isherwood, Chen & Mapson (1953, 1954) that the synthesis of L-ascorbic acid *in vivo* can be represented in outline as proceeding by one or both of the following routes:

In route (2), the third stage in the synthesis, namely the conversion of the lactone of L-galactonic acid into L-ascorbic acid, has been shown to occur *in vitro* in the presence of enzymes contained within mitochondria prepared from plant tissues (Mapson, Isherwood & Chen, 1954). The corresponding reaction in route (1), namely the conversion of L-gulonono- γ -lactone into L-ascorbic acid, was catalysed only to a negligible extent by the same extracts, and it is only recently that we have been able to prepare sufficiently active enzyme preparations for this reaction to be detected. The greater facility with which the last reaction in route (2) as compared with that in route (1) proceeded in the pea extracts led us to concentrate on the enzymic reaction involved in route (2) to the exclusion of (1).

In the present work, the second stage in the synthesis, namely the enzymic conversion *in vitro* of derivatives of D-galacturonic acid into derivatives of L-galactonic acid has been investigated and the enzyme mechanism associated with the reduction of the uronic acid characterized.

METHODS

Enzyme preparations

These were prepared from pea seeds which had been soaked for 24 hr. in water at 20° and germinated for a further 24 hr. at the same temperature. To reduce bacterial infection, the

dry pea seeds were immersed in 1% (w/v) HgCl₂ for 1 min. and then thoroughly rinsed in sterile water. The partly germinated peas (30 g.) were ground up with a mixture of sand and 40 ml. of a solution of sucrose (0.4M), phosphate 0.1M, pH 7.4, and 4 mM-Mg²⁺ at 0°. The brei was centrifuged at 500 g for 5 min. and the supernatant removed. This supernatant, designated as homogenate, was further separated by centrifuging at 20 000 g for 20 min. into mitochondria and soluble fraction, the latter containing the enzymes present in the soluble part of the cytoplasm.

In some experiments these preparations were fractionated further by precipitation with acetone at a low temperature, as described by Askonas (1951), or by fractionation with (NH₄)₂SO₄. The enzyme was precipitated by acetone between 30 and 70% (v/v), and by (NH₄)₂SO₄ in a concentration between 50 and 70% at neutral pH.

Chemicals

Methyl D-galacturonate (MDG) and the γ -lactones of L-galactonic, D-glucuronic and D-mannuronic acids were prepared as described by Isherwood *et al.* (1954), and the amide of galacturonic acid was prepared by the method of Isbell & Frush (1950). MDG labelled with ¹⁴C in the methyl group was prepared by a micro-adaptation of the usual method, with [¹⁴C]methanol. When this preparation was used with the pea enzyme, the derivatives formed were examined on a paper chromatogram by means of a Geiger counter.

n-Propyl D-galacturonate was prepared under conditions similar to those used for the methyl ester, substituting *n*-propanol for methanol. Owing to the relative insolubility of galacturonic acid in *n*-propanol the yields were small, and the crude material was not purified. Chromatographic examination of the syrup showed that it consisted, to the extent of at least 85%, of a fast-running compound, presumably the *n*-propyl ester, which on saponification with alkali gave only D-galacturonic acid.

The lactone of galactaric acid was prepared by dissolving 30 g. of the free acid in 2 l. of water, boiling for 20–30 min. until a clear solution resulted, and then evaporating the solution over a free flame until the volume was reduced to