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Biochem. J. (1960) **75**, 53

Studies in Flavinogenesis

6. THE ROLE OF THREONINE IN RIBOFLAVIN BIOSYNTHESIS
IN *EREMOTHECIUM ASHBYII**

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Goodwin & Pendlington (1954) showed that only two amino acids, L-serine and L-threonine, specifically stimulated the production of riboflavin in the

riboflavin (I) in *Ashbya gossypii* arise from glycine and that C-2 is derived from formate, and Goodwin & Jones demonstrated that C-1 and C-2 of serine were found in C-4a and 9a of riboflavin and C-3 of serine in C-2 of riboflavin. Thus serine was incorporated into the riboflavin molecule after being split into a C₁ unit (formate) and glycine.

The mechanism of threonine incorporation has now been studied with L-[¹⁴C]threonine. Part of this work has already been briefly reported (Goodwin & Horton, 1959).

EXPERIMENTAL

Organism. Our normal strain of *Eremothecium ashbyii* (Brown, Goodwin & Jones, 1958) was used throughout the investigation.

Medium. The medium used was the basal medium described by Goodwin & Pendlington (1954) with bacteriological peptone added to a concentration of 0.04%. After adjusting to pH 5.8, the medium was autoclaved at 15 lb./in.² for 15 min.

(I)

mould *Eremothecium ashbyii*. The mechanism of stimulation by L-serine was elucidated by Goodwin & Jones (1956), using [¹⁴C]serine. It had already been shown by Plaut (1954) that C-4a and C-9a of

* Part 5. Goodwin & McEvoy (1959).

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Cultural conditions. The organism was grown at 28° in continual-shake culture in 250 ml. Erlenmeyer flasks, each containing 50 ml. of medium. The flasks were inoculated with 2 ml. of a 48 hr. culture of *E. ashbyii* (Goodwin & Pendlington, 1954).

Isolation of riboflavin. The cells from the culture flasks were combined and filtered. The residual yellow mycelium was boiled with water for about 1 min. and again filtered; the residue was white. The combined filtrates were evaporated to small volume under reduced pressure and absolute ethanol (five times the volume of the concentrate) was added. The resulting precipitate, containing much protein and a trace of flavinadenine dinucleotide, was filtered off and the filtrate evaporated to dryness *in vacuo*. The residue was dissolved in a minimal volume of water and the pH adjusted when necessary with dilute hydrochloric acid to between 5.0 and 5.5. A freshly prepared saturated solution of sodium dithionite was added dropwise until all the riboflavin was reduced, as indicated by the sudden disappearance of the characteristic greenish fluorescence in u.v. light. The air in the reaction flask was immediately replaced by nitrogen and the flask stoppered and kept overnight at 4°. Next day the precipitated leucoriboflavin was centrifuged off and washed three times with dilute sodium dithionite solution and then twice with ice-cold water. The leucoriboflavin was dissolved in warm water and oxidized by blowing air through the solution, which was subsequently filtered. The filtrate was then decreased in volume by vacuum distillation, until riboflavin just began to crystallize out. The solution was immediately filtered and

left at 0–4° to crystallize. The crystals were centrifuged and washed with small volumes of ice-cold water. The purity of the riboflavin was checked by spectroscopic measurement at 266 and 445 m μ (Adamson, 1948), and by paper chromatography in butanol-acetic acid-water (4:1:5, by vol.) once in each phase (i.e. one chromatogram was run successively in each phase, the direction of solvent flow being the same in each case).

Degradation of riboflavin

Conversion into lumiflavin and lumichrome. Riboflavin (32.5 mg.) was dissolved in 200 ml. of 0.5 N-sodium hydroxide and the solution placed in a crystallizing dish (16 cm. diam.) which gave a depth of liquid of 1 cm. The dish was placed in a copper container through which cold water was continuously circulated so that the temperature of the riboflavin solution never rose above 15°. The solution was agitated with a magnetic stirrer and irradiated for 8 hr. with a glass-envelope mercury-arc lamp (Phillips 120 w at a distance of 15 cm.). This source of u.v. radiation gave better results than an unfiltered mercury arc in a quartz envelope (Hanovia) previously used in this Laboratory (Goodwin & Jones, 1956). The solution was then adjusted to pH 5.0 with acetic acid and the resulting lumiflavin and lumichrome were extracted repeatedly with chloroform. The combined chloroform extracts were evaporated to small volume and chromatographed on paper with the butanol-acetic acid-water system (organic phase). This separated lumiflavin and lumichrome well. The lumiflavin was eluted with warm water and crystallized

Fig. 1. Degradation of riboflavin.

from methanol. Its purity was checked spectrophotometrically by the use of the data of McNutt (1956), and by rechromatographing the sample in the aqueous phase of the butanol-acetic acid-water system, when it was found that its specific activity remained constant.

Degradation to *N*-methyl-1:2-diamino-4:5-dimethylbenzene. Lumiflavin (30 mg.) was dissolved in 5*N*-sodium hydroxide (16 ml.) and the solution heated at 150° for 10 hr. in a stainless-steel bomb. The cooled reaction mixture was extracted five times with 50 ml. portions of diethyl ether. The combined extracts were evaporated to dryness *in vacuo* and the residue was dissolved in 50 ml. of dry ether. The solution was allowed to stand over pellets of potassium hydroxide for 4–5 hr.; the ether was then removed *in vacuo* and the residue dried overnight in a vacuum desiccator (over sulphuric acid). The dry residue was dissolved in dry peroxide-free ether and transferred to a 15 ml. centrifuge tube. The dihydrochloride of *N*-methyl-1:2-diamino-4:5-dimethylbenzene was precipitated by passing dry hydrogen chloride gas through the solution. The hydrochloride was centrifuged down, washed with dry ether and stored in a desiccator before determination of its radioactivity.

Isolation of C-6 and C-7 and the C-methyl groups. Lumichrome was oxidized to acetic acid by the conventional procedure of Kuhn & Roth (1933). The acetic acid was collected by steam distillation and converted into its sodium salt, which was counted. By this means C-6 + C-7 + C-methyl groups were isolated.

Isolation of C-2 and C-4. Lumiflavin (30 mg.) was heated with 2*N*-sodium hydroxide at 100° for 30 min. and the resulting urea (C-2) was isolated as dioxanthyl urea (Goodwin & Jones, 1956). The keto acid formed at the same time was isolated and sublimed at 215° in an atmosphere of nitrogen; C-4 was liberated as carbon dioxide, which was collected in barium hydroxide (Goodwin & Jones, 1956).

Isolation of C-4a + C-9a. The lactam obtained after decarboxylating the keto acid (see previous paragraph) was isolated as previously described (Goodwin & Jones, 1956). The activity in C-4a + C-9a is obtained by subtracting the activity of *N*-methyl-1:2-diamino-4:5-dimethylbenzene from that of the lactam.

An outline of the degradations used is given in Fig. 1.

Preparation of crude homogenates. Cells of *E. ashbyii* were harvested after incubation for 48 hr., washed twice with 0.15*M*-phosphate buffer, pH 7.6, suspended in the minimum volume of the same buffer and disintegrated at 0° in a small Waring Blender.

Determination of threonine aldolase. The microdiffusion method of Karasek & Greenberg (1957) was employed, conventional Conway units being used.

Labelled materials. L-[¹⁴C]Threonine was purchased from A.E.R.E., Harwell, Bucks.

Measurements of radioactivity. All activities were measured on a windowless gas-flow counter (Nuclear Measurements Corp. Inc., Chicago, Ill., U.S.A.) as previously described (Goodwin, 1958).

RESULTS

Incorporation of L-[¹⁴C]threonine into riboflavin by Ererothecium ashbyii

L-[¹⁴C]Threonine is actively incorporated into riboflavin under our experimental conditions, in which

the labelled material is added to the cultures 48 hr. after inoculation and the riboflavin is extracted after a further 48 hr. Percentage incorporations up to 4.5 have been obtained and these figures are similar to those obtained with generally labelled L-[¹⁴C]serine, where the maximum uptake recorded was in the region of 7% (Goodwin & Jones, 1956). Degradation of the resulting labelled riboflavin as described in the Experimental section indicated that the activity was entirely confined to C-4a and C-9a (Table 1). These are the carbon atoms which normally arise from glycine (Plaut, 1954) and thus threonine must be metabolized to glycine before incorporation into riboflavin.

Braunshtein & Vilenkina (1949) reported the presence of an enzyme, threonine aldolase, which splits threonine into glycine and acetaldehyde, and this has been considerably purified from sheep liver by Karasek & Greenberg (1957); it is also present in much lower activity in yeast. The existence of this enzyme in *E. ashbyii* would account for the labelling pattern observed in riboflavin synthesized in the presence of L-[¹⁴C]-threonine (Table 1). Its presence has now been demonstrated in crude homogenates of *E. ashbyii* with the technique of Karasek & Greenberg (1957). Samples of homogenates were placed in the outer compartments of Conway units and a solution of semicarbazide was placed in the inner compartments. The production of semicarbazone was determined by measuring $E_{224\text{m}\mu}$ of the solution in the centre well. Table 2 shows that on incubating L-threonine with homogenates of *E. ashbyii*, acetaldehyde is produced in excess of that formed endogenously. The percentage conversion is, however, low (0.06%). As the endogenous acetaldehyde formation was always relatively high in

Table 1. *Incorporation of generally labelled L-[¹⁴C]threonine into riboflavin by Ererothecium ashbyii*

Compound isolated	Specific activity (counts/min./ μmole)
Riboflavin	4100
Lumiflavin	4300
Acetic acid (C-6, C-7 plus adjacent methyl groups)	0
Dioxanthylurea (C-2)	0
Carbon dioxide (C-4)	0
<i>N</i> -Methyl-1:2-diamino-4:5-dimethyl- benzene (aromatic ring + C-1 + side-chain methyl groups)	0
Lactam (carbon atoms in <i>N</i> -methyl-1:2- diamino-4:5-dimethylbenzene plus C-4a + C-9a)	4100

Table 2. *Formation of acetaldehyde from threonine by homogenates of Eremothecium ashbyii*

Homogenates were produced as described in the Experimental section; L-threonine (125 μ moles) was in the outer compartment of the Conway unit; temp. 27°; time 2 hr.

Material in outer compartment	Acetaldehyde produced (μ mole)	
Threonine alone	0.0349	0.0291
Boiled homogenate	0.0622	0.0627
Homogenate alone	0.0956	0.1020
Homogenate plus threonine	0.2045	0.2115
	0.2159	0.2203

Table 3. *Formation of [14 C]acetaldehyde from generally labelled L-[14 C]threonine by homogenates of Eremothecium ashbyii*

Experimental conditions were as described in Table 2, but with incubation time 24 hr.

	Counts/min.
Activity of L-[14 C]threonine added to each unit	0.5×10^6
Activity of acetaldehyde recovered from centre well of each unit	850, 590, 720, 740
Activity in centre well of unit with L-[14 C]threonine but no homogenate present in outer compartment	20, 38

spite of various attempts to decrease it, the reality of the enzymic reaction was confirmed by using L-[14 C]threonine as substrate. Table 3 demonstrates that significant activity is found in the centre well when the labelled L-threonine is incubated with homogenates of *E. ashbyii*. The amount of radioactivity recovered in the centre well represents a conversion of 0.14 %, which is very similar to that found in the series of experiments quoted in Table 2. In order finally to demonstrate that acetaldehyde was being formed, unlabelled acetaldehyde was added to the combined contents of the centre wells of the units used in the radioactive experiment, and the acetaldehyde semicarbazone was allowed to crystallize. The crystalline material was found to be radioactive and it retained its activity after further recrystallization.

DISCUSSION

The results obtained in this investigation make it clear that C-1 and C-2 of threonine are incorporated by *Eremothecium ashbyii* into C-4a and C-9a of riboflavin after cleavage of the threonine to glycine and acetaldehyde by threonine aldolase. As serine and threonine are the only specifically flavinogenic amino acids under our conditions (Goodwin & Pendlington, 1954), and as previous isotope work has indicated that serine provides both formate and

glycine for riboflavin synthesis (Goodwin & Jones, 1956), it follows that glycine must be the limiting factor in the biosynthetic pathway in *E. ashbyii*. As glycine itself fails to stimulate flavinogenesis (Goodwin & Pendlington, 1954), the results with serine and threonine suggest that they are stimulatory for one of two reasons: (a) serine and threonine aldolases liberate an activated form of glycine and the organism either lacks the ability to activate glycine or activates it so slowly compared with the rate of formation of endogenous 'active glycine' that no stimulation of flavinogenesis can be observed; (b) the cell wall of *E. ashbyii* is permeable to serine and threonine but not to glycine. The first possibility is probably correct, but as tracer amounts of [14 C]glycine are incorporated into riboflavin in the closely related *Ashbya gossypii* (Plaut, 1954), *E. ashbyii* can probably activate glycine, but only very slowly. The observation that *E. ashbyii* does not use glycine for growth (Dulaney & Grutter, 1950) cannot be taken as indicating that it is not metabolized to a small extent; indeed, neither threonine nor serine stimulates growth in *E. ashbyii* (Goodwin & Pendlington, 1954).

The fact that up to 4 % of added [14 C]threonine is incorporated into riboflavin in intact cells of *E. ashbyii* compared with the conversion of only 0.14 % of the threonine into glycine and acetaldehyde by crude homogenates in 24 hr. suggests that threonine aldolase is quickly inactivated on breaking up the cells. This may be the explanation of the low activity of threonine aldolase observed by Karasek & Greenberg (1957) in yeast extracts compared with liver extracts.

The present experiments indicate that the failure of homoserine to stimulate flavinogenesis in *E. ashbyii* (Brown, 1956) is almost certainly due to the fact that an enzyme does not exist which will liberate 'active glycine' from homoserine. The observations of Braunshtein & Vilenkina (1949) and Karasek & Greenberg (1957), that threonine aldolase also acts effectively on L-allothreonine, indicate that L-allothreonine should also be strongly flavinogenic in *E. ashbyii*; Pendlington (1955) reported that this is indeed the case.

The failure of any activity to appear in the aromatic ring of riboflavin formed in the presence of generally-labelled threonine demonstrates that free acetaldehyde is not an intermediate in the formation of the aromatic ring, although we have found that homogenates of *E. ashbyii* rapidly decarboxylate pyruvate with the production of acetaldehyde and Goodwin & Treble (1958) have shown that the aromatic ring probably arises from pyruvate via a C₄ compound such as acetoin. The present results indicate that a C₄ compound of this type cannot be formed by the condensation of one molecule of pyruvate and one molecule of

acetaldehyde with the liberation of carbon dioxide, thus:

SUMMARY

1. *Eremothecium ashbyii* incorporates generally-labelled L-[¹⁴C]threonine into riboflavin to the extent of 4%.

2. C-1 and C-2 of threonine are incorporated as glycine into C-4a + C-9a of riboflavin. C-3 and C-4 of threonine are not incorporated into riboflavin.

3. The enzyme threonine aldolase, splitting threonine into glycine (C-1 + C-2) and acetaldehyde (C-3 + C-4), is present in *E. ashbyii*.

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Biochem. J. (1960) 75, 57

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The Lactic Dehydrogenase of *Propionibacterium pentosaceum*

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The occurrence in *Propionibacterium pentosaceum* of enzymes which are able to catalyse an oxidation of lactate in the presence of fumarate has been demonstrated by the work of Barker & Lipmann (1944). The evidence presented by these authors suggested the participation in the system of a type of lactic dehydrogenase different from that occurring in animal tissues.

They worked exclusively with dried cell preparations and their attempts to extract active fractions from such cells failed. A crude preparation, which carried out a fast lactate-fumarate dismutation, was obtained by Lara (1954) by grinding frozen cells of *P. pentosaceum* with alumina, essentially as described by McIlwain (1948).

The present paper describes the methods of preparation and some properties of a fraction capable of catalysing the oxidation of lactate in the presence of several redox-dyes, and also some inhibition studies.

MATERIALS AND METHODS

Organism and media. *P. pentosaceum* strain E. 2-1, obtained from Professor C. B. van Niel's collection, was cultured and harvested essentially as described by Barker & Lipmann (1944); tryptone, however, was omitted from the medium.

Methods. All reagent solutions were prepared with twice-distilled water, and the enzyme dilutions were made with 0.5% (w/v) bovine plasma-albumin solution (Armour). All operations with the enzyme were done in a cold room maintained at 0-4°, and the centrifugings were performed in a Servall centrifuge, model SS-1, at 25 000 g for 15 min. unless otherwise specified. Oxygen consumption was determined in a Warburg apparatus of conventional type, and the extinction determinations were made in 3 ml. cuvettes on a Beckman spectrophotometer model DU. Protein was determined by the method of Gornall, Bardawill & David (1949), with a coefficient of 0.095 (extinction at 540 mμ, 1 cm. light path, given by a solution of 1 mg. of protein/3 ml. of reaction mixture in the presence of 1.5 ml. of biuret reagent), and pyruvate was estimated by the