Ribitol and Flavinogenesis in *Eremothecium ashbyii*

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1. Supplementation of cultures of *Eremothecium ashbyii* with ribitol leads to a twofold increase in riboflavin formation compared with unsupplemented cultures or those supplemented with ribose or ribulose phosphate. Addition of unlabelled ribitol decreases the incorporation of [$1\text{-}^{14}\text{C}$]ribose into riboflavin, indicating that free ribitol is preferred to ribose for incorporation into riboflavin. 2. The enzymes ribitol kinase, D-ribose reductase, D-ribose 5'-phosphatase and GMP nucleosidase were demonstrated in the cell-free extracts. Ribitol induces the formation of ribitol kinase. The enzyme is activated *in vitro* by the flavinogenic purines, guanine and xanthine. D-Ribose reductase shows a specific requirement for NADPH and forms free ribitol from ribose. 3. The activities of ribitol kinase, ribose 5'-phosphatase and GMP nucleosidase reach their maximal values before riboflavin formation reaches a maximum. 4. [$\text{U-}^{14}\text{C}$]GMP is taken up intact by the culture of *E. ashbyii* and is incorporated into riboflavin as well as into a blue fluorescent compound. The radioactivity from this compound is incorporated into riboflavin by the cell-free extract of *E. ashbyii*.

Micro-organisms have been used extensively to study the biosynthesis of riboflavin, since some of them produce this vitamin in large excess. Although considerable information is available on the biosynthesis of riboflavin, the mode of insertion of the ribitol moiety into the vitamin molecule still remains obscure (Bacher & Lingens, 1970; Baugh & Krumdieck, 1969; Goodwin, 1963; Goodwin & Jones, 1956; Harvey & Plaut, 1966; Maley & Plaut, 1959; McNutt, 1954; Plaut, 1961). The present investigation was undertaken to study the effect of availability of ribitol and guanine on enzymic constitution and flavinogenesis in *Eremothecium ashbyii*.

Materials and Methods

Organism

A flavinogenic strain of *Eremothecium ashbyii* (no. 0944), obtained from the Type Culture Collection, Tokyo, Japan, was maintained on Sabourauds' agar (Difco Laboratories, Detroit, Mich., U.S.A.) slant cultures. The yeast was cultivated in the liquid fermentation medium as described by Goodwin & Pendlington (1954), supplemented with 0.5% vitamin-free Casamino acids (Difco Laboratories). The inoculated flasks were incubated at $28 \pm 2^\circ\text{C}$ in a dark incubator; unless otherwise specified, the cultures were incubated for 4 days. The surface cultures grown in the above liquid medium ('normal medium') are termed 'normal cultures'; when ribose (5 or 10mg/50ml) or ribitol (5 or 10mg/50ml) or guanine (3mg/50ml) was included in this medium, the cul-

tures are referred to as 'ribose-supplemented', 'ribitol-supplemented' or 'guanine-supplemented' respectively.

Radioactivity experiments

To study the fate of [$1\text{-}^{14}\text{C}$]ribose during growth and flavinogenesis, the organisms were grown for 48h in the non-radioactive normal medium. Cells were collected by centrifugation and resuspended in the fermented broth to give a concentration of approx. 0.2mg dry wt./ml of suspension. Samples (5ml) from this suspension were distributed in 100ml sterile flasks and incubated at $28 \pm 2^\circ\text{C}$ in the dark with 5 μCi of carrier-free [$1\text{-}^{14}\text{C}$]ribose (specific radioactivity 7.5mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.), in the presence and absence of various amounts of cold ribitol. After 16h of incubation, cells were harvested by filtration and washed with 0.75% NaCl. Washed cells were extracted with hot 80% (v/v) ethanol two or three times by grinding in an unglazed mortar. Supernatant and washings after extraction were pooled (cell debris was saved), evaporated with an air jet at 37°C and redissolved in 80% ethanol. This concentrate was subjected to ascending paper chromatography on Whatman no. 3 paper sheets in 80% ethanol as the solvent system, to separate riboflavin from soluble nucleotides and sugars. The positions of the authentic standards were located by running a guide strip with each set of papers. The development was carried out for 16h. After air-drying, small strips of the chromatogram (0.5cm $\times$ 2.0cm) were cut out, put in 5ml of

toluene-based Bray's (1960) scintillation liquid and counted for radioactivity in a Packard Tri-Carb liquid-scintillation counter. The soluble nucleotides referred to in Table 6 and Fig. 1 are mainly GTP and GDP. The general term has been used because the conversion of GMP into adenine and xanthine nucleotides in these cultures was not analysed.

The cell debris, obtained after extraction with 80% ethanol, was resuspended in 80% ethanol and filtered through a membrane filter (pore size $0.5\mu\text{m}$: Selectron Filter, Carl Schleicher und Schüll, D 3354, Bassel, W. Germany). The membranes bearing the cell debris were dried at 60°C overnight, put into 5 ml of Bray's (1960) scintillation fluid and counted for radioactivity. The radioactive counts per unit weight of the cell debris were calculated to give the measure of the incorporation into nucleic acids, as no other constituent of cell debris was expected to incorporate radioactive GMP.

To study the incorporation of labelled GMP into riboflavin, a 5 ml sample of the culture (0.2 mg/ml of suspension, as described above) was incubated with $4\mu\text{Ci}$ of $[\text{U-}^{14}\text{C}]\text{GMP}$, diluted with 0.5 mg of unlabelled GMP/ $10\mu\text{Ci}$ of $[\text{U-}^{14}\text{C}]\text{GMP}$ (specific radioactivity 280–350 mCi/mmol), for various time-intervals. The extraction procedures were the same as described above. Guanine and its nucleotide derivatives were separated from riboflavin by the method of Lane (1963). Samples were applied to dried Whatman no. 3 paper sheets previously impregnated with ammonium sulphate [satd. $(\text{NH}_4)_2\text{SO}_4$ soln. – water (9:1, v/v)]. Development was carried out for 16 h by ascending chromatography in 80% ethanol as the solvent system. Authentic standards were run along with each set of papers and their positions located by cutting the paper into small strips ($0.5\text{cm} \times 2.0\text{cm}$), these being eluted in 0.5 ml of 0.1 M-HCl and the E_{249} values recorded. Radioactivity distribution in the samples was determined by counting the corresponding small strips in 5 ml of toluene-based Bray's (1960) scintillation fluid. The R_F values of GTP, GDP, GMP, guanosine, guanine, riboflavin and the blue fluorescent compound were found to be 0.05, 0.05, 0.19, 0.30, 0.58, 0.47 and 0.38 respectively. All radioactivity measurements were made in a Packard Tri-Carb liquid-scintillation counter.

Analytical methods

Riboflavin was determined spectrophotometrically at 445 nm with pure riboflavin as the reference compound (Goodwin & Pendlington, 1954). Ribose was determined by the orcinol method (Schneider, 1957). Ribitol was extracted from fresh mycelia by the method of Maruo *et al.* (1965) and was separated and isolated by paper chromatography with butan-1-ol–pyridine–water (6:4:3, by vol.) as the solvent system (Hanahan & Olley, 1958). The R_F values of glucose,

ribose, ribulose and ribitol were 0.36, 0.75, 0.23 and 0.56 respectively. Identification of the sugars on the guide strips was performed by spraying with periodate–benzidine–HCl reagent. Areas corresponding to ribitol were cut out and eluted at 30°C in water for 1 h. Ribitol was determined in the aqueous eluates by the colorimetric method of Hanahan & Olley (1958).

Guanine was estimated either by the spectrophotometric method (Vischer & Chargaff, 1948) or by the colorimetric method of Hitchings (1941).

Enzyme preparations and assays

The organisms were harvested by filtration at 5°C and frozen at -15°C . Frozen cells were ground manually at $0-5^\circ\text{C}$ with powdered glass (1:1, w/w) in 0.1 M-tris–HCl buffer, pH 7.5, until complete cell breakage had taken place. The disrupted suspension was centrifuged at 5000g for 15 min. The supernatant was centrifuged again at 7000g for 15 min at $0-5^\circ\text{C}$. The pale-yellow supernatant solution was used as the crude enzyme preparation. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. Enzyme activities were measured either immediately after preparation or the solution was kept overnight at -20°C and thawed only once. Controls without substrate or enzyme solution or with boiled enzyme were carried out, correction being made for any change observed in this incomplete test system. All the enzyme assays quoted exhibited a linear dependence of the extent of reaction with the time of incubation and of the rate with the concentration of enzyme protein.

Ribitol kinase was assayed by measuring the residual ribitol after incubation by the colorimetric method of Hanahan & Olley (1958), after the removal of proteins and phosphorylated compounds from the incubation mixture with the $\text{Ba}(\text{OH})_2$ – ZnSO_4 reagent of Somogyi (1945). The standard assay mixture contained: tris–HCl buffer (pH 7.2), $100\mu\text{mol}$; MgCl_2 , $20\mu\text{mol}$; ATP, $20\mu\text{mol}$; ribitol, $25\mu\text{mol}$; enzyme solution containing 200–500 μg of protein. The total volume was 3.0 ml. The mixtures were incubated for 30 min at 30°C .

D-Ribose reductase was assayed at 30°C by measuring the rate of formation of NADP^+ from NADPH in the presence of ribose. The standard assay mixture contained: tris–HCl buffer (pH 7.5), $100\mu\text{mol}$; NADPH, $0.25\mu\text{mol}$; D(–)-ribose, $10\mu\text{mol}$; enzyme solution containing 100–200 μg of protein. The total volume was 2.0 ml. Controls without ribose were run to make corrections for any endogenous oxidation. The formation of NADP^+ was measured at 340 nm in a Beckmann DU-2 spectrophotometer. For product identification, D-ribose in the above assay mixture was replaced by $0.01\mu\text{Ci}$ of $[\text{1-}^{14}\text{C}]\text{-ribose}$ (specific radioactivity 7.5 mCi/mmol) and the

total volume of the assay mixture was 0.5ml. After incubation for 30min at 30°C, these samples were subjected to paper chromatography as described above. Areas corresponding to ribose and ribitol were cut out, put into 5ml of Bray's (1960) scintillation fluid and counted for radioactivity.

Ribose 5'-phosphatase was measured at 30°C by measuring the release of P_i from ribose 5'-phosphate. The standard assay mixture contained: tris-HCl buffer (pH 7.5), 200 μ mol; $MgCl_2$, 5 μ mol; D-ribose 5'-phosphate, 5 μ mol; enzyme solution containing 100–200 μ g of protein. The total volume was 2.0ml. Incubation was carried out at 30°C for 1 h. The P_i released was measured in 5% (v/v) trichloroacetic acid supernatants by the method of Fiske & Subba-Row (1925).

GMP nucleosidase was assayed by the method of Wang (1955), free ribose liberated by the enzymic reaction being measured by the method of Nelson (1944). The standard assay mixture contained: sodium acetate buffer (pH 6.0), 200 μ mol; GMP, 3 μ mol; enzyme solution containing 250–500 μ g of protein. The final volume was 2.0ml. Incubation was carried out at 30°C for 2 h. The activity of a non-specific phosphatase was closely associated with GMP nucleosidase, and therefore the product estimated was free ribose, instead of ribose 5'-phosphate. A similar case was reported by Hurwitz *et al.* (1957) with AMP nucleosidase from *Azotobacter vinelandii*. The total reducing sugar estimated by Nelson's (1944) method was therefore essentially free ribose. Errors were eliminated by running a number of appropriate controls.

Enzyme units. One unit of ribitol kinase is that amount of the enzyme which phosphorylates 1 μ mol of ribitol/30min under the defined experimental conditions.

One unit of GMP nucleosidase is that amount of the enzyme which liberates 1 μ mol of free ribose/2h under the defined experimental conditions.

One unit of ribose reductase is that amount of the enzyme which causes a change of 0.01 E_{340} unit/2min under the defined experimental conditions.

One unit of ribose 5'-phosphatase is that amount of the enzyme which liberates 1 μ mol of P_i /h under the defined experimental conditions.

Results and Discussion

Effects of ribose, ribitol and ribulose phosphate on flavinogenesis

Addition of ribose or ribitol to growing cultures of *E. ashbyii* results in the stimulation of growth in the early phase of its cultivation. The presence of ribitol rather than ribose or ribulose phosphate results, in addition, in a considerable increase in flavinogenesis

(Table 1). We also observed that on the seventh day of cultivation 60% of the added ribitol had been utilized in the ribitol-supplemented cultures, resulting in about a twofold increase in riboflavin production; this suggests that ribitol may be utilized for flavinogenesis. It was surmised that free ribitol may be a preferable substrate to ribose or ribulose phosphate. Experiments with $[1-^{14}C]$ ribose supported this view. The results listed in Table 2 indicate that although about 65–70% of added $[1-^{14}C]$ ribose is taken up, the incorporation of $[1-^{14}C]$ ribose into riboflavin is low (approx. 1.5% in 16h of incubation of cells with 5 μ Ci of $[1-^{14}C]$ ribose); the incorporation of radioactivity from $[1-^{14}C]$ ribose into riboflavin is diluted in the presence of exogenous unlabelled ribitol, though the uptake of $[1-^{14}C]$ ribose and the intracellular concentration of $[1-^{14}C]$ ribose in these systems remain practically the same. Under these conditions $[1-^{14}C]$ ribose incorporation is channelled to soluble nucleotides and nucleic acids (Fig. 1), and the label in riboflavin decreases considerably. These results suggest that once ribitol is available exogenously to the cultures for flavinogenesis the organism uses ribose for nucleotide synthesis. During the competition in the utilization of ribose and ribitol for riboflavin synthesis, the organism prefers ribitol to ribose. This supports the view (Goodwin & Pendlington, 1954; McNutt, 1954; Forrest & McNutt, 1958) that the ribitol moiety of riboflavin may not arise from the ribose residue of guanosine and that ribitol is separately attached to some intermediate in the biogenic pathway.

Table 1. *Comparison of the flavinogenic nature of ribose, ribitol and ribulose phosphate in E. ashbyii*

The culture was grown in 10ml of the normal medium on a rotary shaker. Each compound (1.0mg) was added aseptically to the growing culture 48h after inoculation. Mycelia were harvested 4 days after the addition of the named compounds and the fermented broth was analysed for riboflavin production. Results are the means of three sets of experiments.

Additions to the normal medium	Riboflavin produced (μ g/10ml of broth)
Nil	38
Ribose	43
Ribulose phosphate	50
Ribitol	90
Guanine	100
Guanine+ribose	109
Guanine+ribitol	136
Guanine+ribulose phosphate	93

Table 2. Incorporation of $[1-^{14}\text{C}]$ ribose into riboflavin by *E. ashbyii*

The control system contained $5\mu\text{Ci}$ of $[1-^{14}\text{C}]$ ribose. Details of the experiment are given in the Materials and Methods section.				
System	$10^6 \times$ Uptake of $[1-^{14}\text{C}]$ ribose (c.p.m./5 ml of culture)	Intracellular concn. of $[1-^{14}\text{C}]$ ribose (c.p.m./mg dry wt. of cells)	$10^4 \times$ Incorporation into riboflavin (c.p.m.)	Total riboflavin produced ($\mu\text{g}/5\text{ ml}$)
Control	5.44	986	7.6	48.4
Control + $200\mu\text{g}$ of ribitol	5.48	1001	3.76	70.0
Control + $500\mu\text{g}$ of ribitol	5.85	931	3.2	85.0
Control + 1.0 mg of ribitol	5.84	941	3.0	95.0
				Sp. radioactivity of riboflavin (c.p.m./ μg of riboflavin)
				1.57×10^3
				4.8×10^2
				3.7×10^2
				3.4×10^2

Fig. 1. Effect of unlabelled ribitol on the incorporation of $[1-^{14}\text{C}]$ ribose

Incorporation into riboflavin (●), nucleic acids (▲) and soluble nucleotides (○) by *E. ashbyii* is shown.

Ribitol kinase

Ribitol kinase, an enzyme similar to ribulokinase (Simpson & Wood, 1968), which phosphorylates ribitol has been detected in this organism. The enzyme reaches its maximal activity on the fourth day of cultivation during active riboflavin synthesis (Fig. 2). The enzyme shows optimum activity at pH 7.0 and can also phosphorylate xylitol. The cell-free extracts of ribitol-supplemented cultures show a threefold increase in the activity of this enzyme over the normal and ribose-supplemented cultures (Table 3), demonstrating its inducible nature. The flavinogenic purines guanine and xanthine were found to activate this enzyme, guanine being a more effective stimulator than xanthine (Table 4). Also, the activation by guanine was more pronounced for the enzyme extracted from ribitol-supplemented cultures. Cell-free extracts from guanine-supplemented cultures, assayed in the absence of guanine, show about a 2.4-fold increase in ribitol kinase activity, thereby indicating an influence of this purine in the physiological regulation of this enzyme.

Aldose reductase, ribose 5'-phosphatase and GMP nucleosidase

The organism possesses an NADPH-dependent aldose reductase, which reduces ribose to ribitol; free ribose is a better substrate for this enzyme than is D-ribose 5'-phosphate or ribulose 5'-phosphate.

Table 3. Activities of ribitol kinase, ribose reductase and nucleosidase in *E. ashbyii*

For experimental details see the text. n.d., Not determined.

Growth condition of the culture	Enzyme activity (units/mg of protein)		
	Ribitol kinase	D-Ribose reductase	GMP nucleosidase
Normal culture	0.41	3.8	6.06
Ribose-supplemented culture	0.47	4.22	3.2
Ribitol-supplemented culture	1.20	2.2	9.8
Guanine-supplemented culture	0.99	n.d.	8.04

Table 4. Ribitol kinase and its activation by flavinogenic purines in *E. ashbyii*

The assay mixture was as described in the Materials and Methods section.

Additions to the assay mixture	Enzyme activity (units/mg of protein)	
	Normal cultures	Ribitol-supplemented cultures
Nil	0.42	1.24
Guanine (3.3mm)	1.60	11.2
Xanthine (3.3mm)	1.04	5.0

Fig. 2. Enzyme activities and flavinogenesis during cultivation of *E. ashbyii*

Ribitol kinase (○), ribose 5-phosphatase (●) and GMP nucleosidase (△) activities and flavinogenesis (▲) on different days of cultivation in *E. ashbyii* are shown.

The enzyme from the 4-day-old mycelia showed K_m values 1.2 and 2.0mm with D(–)-ribose and ribulose 5'-phosphate respectively, indicating an increased

affinity of the enzyme for ribose in comparison with ribulose phosphate. The K_m value for the reverse reaction:

was 7 mm, thereby suggesting that conversion of ribose into ribitol is more favourable. Free ribitol, the product of ribose reductase, formed over a reaction period of 30min was detected by paper chromatography as described in the Materials and Methods section. Further confirmation of the nature of the enzymic reaction was obtained by using [1-¹⁴C]ribose as the substrate: 4.9×10^4 c.p.m. of [1-¹⁴C]ribose was converted into 4.82×10^4 c.p.m. of [1-¹⁴C]ribitol within 30 min at 30°C, which amounts to 95% conversion. Reports of aldose reductase from other sources have appeared (Chiang & Knight, 1959; Hayman & Kinoshita, 1965; Scher & Horecker, 1966; Watson *et al.*, 1969). It is noteworthy that ribitol-supplemented cultures show less enzyme activity than do normal and ribose-supplemented cultures, which may be due to the presence of exogenous ribitol in the medium (Table 3). It appears from these results that in this organism free ribose can be converted into free ribitol, which is subsequently phosphorylated by ribitol kinase. It was also observed that free ribose could be made available by the presence of a non-specific phosphatase (Fig. 2), which was detected and showed optimum activity at pH7.5. K_m values for D-ribose 5'-phosphate and D-glucose 6-phosphate at pH7.5 were 2.7 and 5.0mm respectively.

The presence of a non-specific nucleosidase was

Table 5. *Substrate specificity of GMP nucleosidase of E. ashbyii*

Cell-free extracts from 4-day-old normal cultures were prepared as described in the text. The activity of nucleosidase in these extracts was determined with the named nucleotides. The assay system was the same as described in the text.

Nucleotide used as substrate (1.5 mM)	Enzyme activity (units/mg of protein)	Activity (% of that with GMP)
GMP	6.1	100
GDP	2.08	36
GTP	1.35	23
AMP	1.10	20
ADP	1.35	23
ATP	0.00	0
UMP	0.418	7
UDP	2.380	40
CMP	2.80	48
CTP	0.00	0
XMP	1.50	27

also detected (Table 3). The enzyme showed high activity when GMP was used as the substrate (Table 5). The other substrates tested showed no more than 50% of the activity observed with similar concentration of GMP. The enzyme showed optimum activity at pH 6.0 and retained about 50% of the activity when heated at 50°C for 2 h at this pH value. The K_m value with respect to GMP was 0.70 mM. The enzyme appears to be similar to the nucleosidase 'g' reported by Reese & Maguire (1968) in *Aspergillus foetidus*. Normal, ribitol-supplemented and guanine-supplemented cultures showed considerable activity of this enzyme; ribose-supplemented cultures, however, showed less enzyme activity (Table 3). Ribose is one of the products of this enzymic reaction and inhibits the enzyme *in vitro* at 2.5 mM. Decreased GMP nucleosidase activity (Table 3) in ribose-supplemented cultures could be due to the repression by exogenous ribose.

It was then decided to study the activities of ribitol kinase, GMP nucleosidase and D-ribose 5'-phosphatase at different phases of flavinogenesis. These results are presented in Fig. 2, which shows that the specific activity of each of these enzymes reaches a peak just before riboflavin synthesis reaches its maximal rate. These observations suggest the involvement of these enzymes in making available early precursors for flavinogenesis in *E. ashbyii*.

Purines rather than purine nucleotides stimulate flavinogenesis (Cheldelin & Baich, 1967; Goodwin, 1963). Guanine (or a close derivative) has been implicated as an obligatory precursor of riboflavin biosynthesis in *E. ashbyii* and other organisms (Baugh & Krumdieck, 1969). Generally cellular systems synthesize purines in the form of their nucleotides. It was therefore decided to investigate in this

Fig. 3. Distribution of $[U-^{14}C]GMP$ incorporated by the cells of *E. ashbyii*

Incorporation of $[U-^{14}C]GMP$ into (1) GTP, (2) GDP, (3) GMP, (4) guanosine, (5) guanine, (6) riboflavin (vitamin B_2) and (7) blue fluorescent compound is shown. ●, ^{14}C radioactivity; ○, E_{249} .

organism the availability of guanine and guanine precursors for flavinogenesis. With $[U-^{14}C]GMP$ (specific radioactivity 280–350 mCi/mmol) it was found that the nucleotide is taken up intact by the cells, and that GMP label is incorporated into riboflavin (Figs. 3 and 4). Incorporation into nucleic acids was considerably less than that into riboflavin,

indicating an involvement of GMP in riboflavin biosynthesis (Table 6). Within 1 h of adding radioactive GMP to the cell suspension a considerable amount of radioactive riboflavin is excreted into the medium. It was surprising that although an active GMP nucleosidase is present in this organism, no radioactivity was detected either in guanosine or guanine (Fig. 3). This may happen because either (a) guanine formed by the hydrolysis of GMP is channelled very quickly for incorporation into riboflavin or (b) the breakdown of the GMP molecule, loss of C-8 and/or the formation of the riboflavin precursor from guanine are simultaneous processes so that the formation of guanine in the free form is by-passed.

A blue fluorescent compound was found to be present in the mycelium of *E. ashbyii* during the early growth phase. This compound shows maximum extinction at 256nm and runs very close to guanosine

on paper chromatography, but does not correspond to any of the guanine nucleotides (Fig. 3). On hydrolysis it liberates guanine, ribitol and P_i. It could be a nucleotide derivative resembling 9-phosphoribityl-purine, as suggested by Goodwin (1963). During our experiments with [U-¹⁴C]GMP we observed that some of the label is incorporated into this compound as well (Figs. 3 and 4), the incorporation increasing linearly with increase in incubation time. This radioactive blue fluorescent compound was isolated by paper chromatography. Cells were incubated for 8 h with 5 μCi of [U-¹⁴C]GMP (with 0.5mg of unlabelled GMP/10 μCi of [U-¹⁴C]GMP, specific radioactivity 280–350 mCi/mmol) as described above, and the final concentrate of the cell extract was subjected to ascending chromatography (Lane, 1963). The compound was located under u.v. light and also by detecting radioactivity in a guide strip. The area on the paper corresponding to this compound was eluted overnight at 30°C in 80% ethanol, which was evaporated at 37°C, and the residue was redissolved in 0.1 M-tris-HCl buffer, pH 7.2. This radioactive blue fluorescent compound (approx. 1000 c.p.m.) was added to a cell-free system containing: tris-HCl buffer (pH 7.2), 50 μmol; ATP, 5 μmol; crude enzyme solution containing 100–200 μg of protein. The total volume was 0.5 ml. The system was incubated in the dark for 1 h and deproteinized with trichloroacetic acid (5%, v/v). Pure riboflavin (50 μg) was added as a carrier. The supernatants were subjected to ascending chromatography in butanol-acetic acid-water (4:1:5, by vol.) as the solvent system. The chromatogram was developed for 16 h and, after air-drying, the areas corresponding to guanine, ribitol and riboflavin were cut out and counted for ¹⁴C as described in the Materials and Methods section. About 15% of the total radioactivity from the blue fluorescent compound is incorporated into riboflavin (Table 7), indicating that this compound might be a possible precursor of riboflavin. The low efficiency of this

Fig. 4. Time-course of incorporation of [U-¹⁴C]GMP into riboflavin and blue fluorescent compound by the cells of *E. ashbyii*

▲, Intracellular [U-¹⁴C]GMP; ●, riboflavin; ○, blue fluorescent compound. For details see the text.

Table 6. Incorporation of [U-¹⁴C]GMP into various components by *E. ashbyii*
For experimental details see the text.

Incubation time (h)	10 ⁷ × Uptake of [U- ¹⁴ C]GMP (c.p.m./5 ml)	Riboflavin (c.p.m.)	Incorporation of [¹⁴ C]GMP		
			Blue fluorescent compound (c.p.m./mg dry wt. of cells)	Soluble nucleotides (c.p.m./mg dry wt. of cells)	Nucleic acids (c.p.m./mg dry wt. of cells)
1	1.7	2.8 × 10 ⁵	90	0.79 × 10 ³	0.48 × 10 ³
3	3.5	2.5 × 10 ⁶	205	4.7 × 10 ³	1.6 × 10 ³
5	3.7	4.0 × 10 ⁶	300	11.2 × 10 ³	5.4 × 10 ³
8	3.8	4.7 × 10 ⁶	650	14.6 × 10 ³	44.2 × 10 ³
48	6.2	19.0 × 10 ⁶	5000	48.8 × 10 ³	7.8 × 10 ³

Table 7. Conversion of ^{14}C -labelled blue fluorescent compound into riboflavin by the cell-free extract of *E. ashbyii*

The reaction mixture contained: ^{14}C -labelled blue fluorescent compound (1000 c.p.m.); tris-HCl buffer (pH 7.2), 50 μmol ; ATP, 5 μmol ; enzyme solution containing 100–200 μg of protein. The total volume was 0.5 ml. Riboflavin (50 μg) was added as carrier after the system had been deprotenized with trichloroacetic acid (5%, v/v).

Substrate	Radioactivity (c.p.m./reaction mixture)	
	0 min	30 min
Blue fluorescent compound	1000	498
Riboflavin	0	152
Guanine	0	168
Ribitol	0	100

conversion could be due to (a) the absence of some of the limiting factors in the cell-free system, or (b) the unstable nature of the compound, as it was found to be degraded into guanine and ribitol during incubation (Table 7), or (c) some other unknown factors.

From the above results it appears that free ribitol is preferred to ribose for incorporation into the riboflavin molecule. Free ribose is converted into ribitol by D-ribose reductase and is subsequently phosphorylated by ribitol kinase. [^{14}C]GMP is incorporated into riboflavin as well as into the blue fluorescent compound. The conversion of the blue fluorescent compound into riboflavin *in vitro* suggests that this may also be a precursor of riboflavin.

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