Studies on the Biosynthesis of Riboflavin

NITROGEN METABOLISM AND FLAVINOGENESIS IN *EREMOTHECIUM ASHBYII*

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Many fungi are known to synthesize riboflavin in small amounts (e.g. Peltier & Borchers, 1947, list 240 flavin-producing species), but there are three which synthesize it and excrete it into the culture medium in very large amounts. These are *Candida* spp. (Burkholder, 1943; Tanner, Vojnovich & van Lanen, 1949), *Eremothecium ashbyii* (Guillermont, Fontaine & Raffi, 1935) and *Ashbya gossypii* (Wickerham, Flickinger & Johnston, 1946).

Although many patents have been filed covering the commercial production of riboflavin using these organisms (see a review by Pridham, 1952, for full details), the observations recorded have in general been only of limited value in furthering knowledge of the basic mechanisms concerned in this biosynthesis. The main reason for this is that the cheaply available materials found to stimulate riboflavin production are generally such biochemically complex products as molasses, fish meal, hide scraps, dried blood, etc.

A considerable amount of work has been carried out in an attempt to culture *E. ashbyii* on a fully defined medium. (Schopfer, 1944; Schopfer & Guilloud, 1945*a-c*; Dulaney & Grutter, 1950; Maclaren, 1952; Yaw, 1952; Hickey, 1953), but a survey of the literature makes it clear that the problem has not yet been completely solved. Our preliminary experiments confirmed this conclusion; it was found, however, that satisfactory and reproducible growth and riboflavin synthesis could be obtained using small amounts of peptone in an otherwise synthetic culture medium. Using this type of basal medium, an investigation has been carried out on the effect of various nitrogen-containing compounds on riboflavin synthesis by *E. ashbyii*. A preliminary report of some of these findings has already been made (Goodwin & Pendlington, 1954).

EXPERIMENTAL

Cultures. The culture used throughout this investigation was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland. It was maintained on malt agar slopes and subcultured every 7–10 days.

Media. The basal medium used contained per l.: 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.; KH_2PO_4 , 2.0 g.; biotin 0.04 mg.; thiamine, 0.1 mg.;

inositol, 0.2 g. To this was added varying amounts of bacteriological peptone (Evans) and the nitrogen compounds under test. The pH was adjusted to 5.8. Sterilization was carried out by autoclaving at 15 lb./sq.in. for 15 min. Although the peptone would probably contain sufficient quantities of inositol, biotin and thiamine, it was considered advisable to add always the optimum amounts advised by Schopfer (1944). This would eliminate possible variations in the vitamin content of different batches of peptone and also the difference in vitamin levels when various concns. of peptone were being compared.

All the amino acids used except tyrosine, phenylalanine and tryptophan and all the purines and pyrimidines were obtained from L. Light & Co. Ltd., Colnbrook, Bucks; tyrosine, phenylalanine and tryptophan were obtained from British Drug Houses Ltd., London; 1:2-dimethyl-4-amino-5-(*p*-1'-ribitylamino)benzene and 1:2-dimethyl-5-(*p*-1'-ribitylamino)benzene were the gift of Dr E. S. Holdsworth and 4:5-dimethylbenzimidazole the gift of Dr V. Petrow.

Cultural conditions. The media (15 ml.) were dispensed in Erlenmeyer flasks (50 ml.), inoculated and incubated in a water-jacketed incubator in the dark at 28° without shaking. The patent literature makes it clear that riboflavin synthesis is stimulated considerably in shake cultures. It was not possible to shake the cultures in the present investigation, but this is no drawback providing the conditions are always standardized. Each flask was inoculated with three drops of a spore suspension drawn from a 2-day-old culture grown on the basal medium plus 0.4% (w/v) peptone. This procedure was adopted following the observation of Tanner *et al.* (1949) that, with *Ashbya gossypii*, best yields of riboflavin were obtained when the inoculum vol. was about 0.5% of the total vol. of the medium and when it was obtained from young cultures. This point was not tested in *E. ashbyii*, but the procedure just described was found to be extremely satisfactory and to yield very reproducible results.

Analytical procedures. Three replicate flasks were examined in each experiment. The major part of the medium and mycelium was transferred to a thin, 15 ml. tared centrifuge tube and centrifuged in a small bench centrifuge for 5 min. A portion of the supernatant was taken for riboflavin determination and the remainder discarded; any mycelium remaining in the culture flask was transferred quantitatively to the centrifuge tube which was again spun for 5 min. The residual medium was decanted off and the mycelium twice washed thoroughly with distilled water, followed each time by centrifuging. The washed mycelium was then dried at 80° for 24 hr.

Riboflavin in the medium was determined spectrophotometrically by measuring the *E* value of the centrifuged medium at 445 m μ , the wavelength of max. absorption for

riboflavin in the visible region of the spectrum. By comparing this value with the $E_{1\%}^{1\text{cm}}$ (445 m μ .) value for pure riboflavin (301; Adamson, 1948), the amount of riboflavin in the medium could easily be calculated. This procedure was justified for a number of reasons: (a) the starting medium exhibited almost negligible absorption at 445 m μ ., (b) the absorption spectra of different specimens of riboflavin-containing media were not significantly different from that of pure riboflavin, and (c) paper chromatography of the medium according to the method of Crammer (1948) for riboflavin and its derivatives showed that the pigment was almost entirely riboflavin; apart from riboflavin there only existed minute traces of a spot with blue fluorescence in u.v. light with an R_f value very close to that given by Crammer (1948) for riboflavin phosphate. The riboflavin in the mycelium was extracted by adding 10 ml. of HCl to the washed residue in the centrifuge tube and autoclaving at 15 lb./sq.in. for 15 min. After cooling, the supernatant, was made up to an appropriate volume (usually 15 ml.) and the riboflavin determined spectrophotometrically. The residual mycelium was completely colourless.

RESULTS

Reproducibility of results

Table 1 shows twelve triplicate values obtained for riboflavin synthesis in the basal medium containing 5 mg. peptone N/100 ml. These results were obtained over a period of 18 months and show that whilst reproducibility between flasks in any one experiment is usually excellent, the amounts of riboflavin produced under apparently identical cultural conditions at different times can vary considerably. The reason for these variations is not known, but they emphasize the need for adequate controls. The 'experimental' flasks showed the same range of variations. Occasionally (about 1 in 200 flasks), an aberrant flask is obtained, in which riboflavin synthesis is very much greater than expected, but growth is very much reduced.

Although most of the stimulatory effects dealt with in this paper are large, generally at least 50% above normal, it is obvious from Table 1 that under optimum conditions it would be possible to demonstrate significant stimulations of the order of 10–20% above normal.

The effect of age on growth and riboflavin synthesis

A typical time curve for growth and flavinogenesis by *E. ashbyii* cultured on the basal medium containing 0.24% (w/v) peptone (Fig. 1), shows that growth was complete after 5 days but that riboflavin production, increasing at almost the same rate as growth during the first 5 days after inoculation, continued until about the seventh or eighth day. Thereafter, net production of riboflavin was almost nil. Following these observations, it was decided that in subsequent experiments only 5- and/or 9- to 10-day cultures would be examined.

Fig. 1. Dry wt. and riboflavin production by *E. ashbyii* cultured on basal medium containing 0.24% (w/v) peptone. Temp. 28°. Amounts per 15 ml. medium in 50 ml. conical flasks. ●—●, dry wt.; ○—○, riboflavin.

Table 1. Reproducibility of growth and riboflavin production by *E. ashbyii*

Triplicate analyses made during different experiments; production in 15 ml. medium in 50 ml. conical flasks. Incubation time 5 days; temp. 25°. Basal medium + 5 mg. peptone N/100 ml.

Expt. no.	Riboflavin (μg./15 ml.)				Dry wt. of mycelium (mg.)			
	Individual values			Mean	Individual values			Mean
1	145	159	152	152	9.8	9.7	9.7	9.7
2	122	177	170	156	11.4	12.5	11.1	11.7
3	185	175	158	173	10.5	13.3	11.7	11.8
4	185	214	178	192	8.2	6.9	7.0	7.4
5	250	305	249	268	13.1	11.3	11.3	11.9
6	180	188	188	185	13.6	12.4	14.5	13.5
7	145	150	140	145	10.7	10.0	8.4	9.7
8	106	102	111	106	12.8	12.1	12.3	12.4
9	167	176	193	175	—	10.7	11.7	11.2
10	146	130	136	137	8.6	10.5	6.2	8.4
11	182	208	178	189	11.8	11.0	8.4	10.4
12	254	246	238	246	7.1	7.0	7.0	7.0

*The effect of the concentration
of peptone*

The effect of varying the concentration of peptone was examined in the presence and absence of 0.1% (w/v) glycine. Table 2 shows that the presence of glycine had no effect on the action of peptone on riboflavin production; there does appear to have been in this case a slight stimulatory effect on growth, although this was not confirmed in other experiments (see, for example, Table 4).

Comparing Fig. 1 and Table 2, it will be seen that

the observations recorded in Fig. 1, that growth but not flavinogenesis is complete after 5 days' incubation, have been confirmed.

Table 1 also indicates that the optimum concentration of peptone for riboflavin synthesis is about 0.24%, if the amount per unit weight of mycelium but not the total yield is the criterion; this is most marked in 10-day cultures. Further experiments (Table 3) showed, however, that the spread is rather wide, and that the highest concentrations of riboflavin are produced over a peptone concentration range of 0.16–0.40% (w/v).

Table 2. *Growth and riboflavin synthesis by E. ashbyii in presence and absence of glycine and in presence of varying amounts of peptone*

Dry wt. and riboflavin per 15 ml. medium in 50 ml. conical flasks; basal medium described in Experimental section; temp. 28°.

Concn. of peptone* % (w/v)	Five-day cultures			Ten-day cultures		
	Dry wt. (mg.)	Riboflavin		Dry wt. (mg.)	Riboflavin	
		Total amount (μ g.)	Amount (g./100 g. dry mycelium)		Total amount (μ g.)	Amount (g./100 g. dry mycelium)
		Medium without glycine				
1.92	32.8	904	2.76	42.6	1499	3.52
0.96	33.4	768	2.32	33.7	2052	6.08
0.48	30.2	715	2.41	27.9	1856	6.65
0.24	21.1	442	2.25	21.0	1644	7.82
0.12	16.4	465	2.82	13.0	1071	8.22
0.06	11.0	281	2.99	11.8	717	6.07
0.03	8.5	166	1.95	9.8	405	4.03
Medium containing glycine 1.0% (w/v)						
1.92	41.4	947	2.34	54.7	1614	2.95
0.96	40.1	799	2.00	38.4	1884	4.90
0.48	29.0	723	2.49	26.4	1640	6.22
0.24	26.1	656	2.51	20.7	1385	7.99
0.12	20.8	491	2.39	18.5	1021	5.55
0.06	16.0	282	1.84	16.1	692	4.31
0.03	9.1	124	1.36	11.3	276	2.41

* Peptone contains 12.5% N.

Table 3. *Growth and riboflavin synthesis by E. ashbyii in presence of varying peptone concentrations*

Dry wt. and riboflavin production per 15 ml. medium in 50 ml. conical flasks; basal medium supplemented with varying amounts of peptone.

Concn. of peptone* % (w/v)	Five-day cultures			Nine-day cultures		
	Dry wt. (mg.)	Riboflavin		Dry wt. (mg.)	Riboflavin	
		Total amount (μ g.)	Amount (g./100 g. dry mycelium)		Total amount (μ g.)	Amount (g./100 g. dry mycelium)
0.040	19.0	185	1.01	10.8	345	3.19
0.080	21.2	340	1.45	15.6	684	4.38
0.160	25.0	610	2.42	19.5	1052	5.35
0.240	27.2	686	2.52	25.5	1333	5.23
0.320	29.5	725	2.45	29.9	1574	5.26
0.400	30.6	717	2.35	30.3	1581	5.22
0.480	31.3	725	2.32	32.8	1563	4.76

* The peptone used contained 12.5% N.

Effect of various amino acids on flavinogenesis

Two control media were used: *A* consisted of the basal medium plus peptone ($\cong 0.005\%$, w/v, N); which gave a reasonable growth (about 10 mg. dry wt. per 15 ml.) but only a comparatively small production of riboflavin. Medium *B* consisted of the basal medium plus 0.025% (w/v) peptone N. This gave about twice the dry weight produced by *A* and more than twice the amount of riboflavin. By adding various N sources to medium *A* in amounts calculated to bring the total N content of the medium to that of *B*, the ability of the N sources to stimulate the synthesis of riboflavin could be assessed and compared with that of peptone. Table 4 gives the results obtained when most of the known amino acids except tyrosine and tryptophan (see later) were examined in this way. It will be seen that most of these amino acids are incapable of stimulating either growth or flavinogenesis, whilst L-cysteine inhibited growth completely. Two amino acids, however, DL-serine and DL-threonine, specifically stimulated riboflavin production. L-Asparagine, L-aspartic acid and L-glutamic acid stimulated both growth and riboflavin production equally, as evidenced by the same concentrations of riboflavin in these cultures as in the *B* controls. They could not,

therefore, be considered to have a specific effect on riboflavin. A few amino acids (e.g. L-cystine) tended to inhibit growth slightly without affecting riboflavin synthesis. This resulted in a somewhat higher riboflavin concentration than in the control medium, but as the effect was slight by comparison with that of DL-threonine and DL-serine and, more important, because the amount of riboflavin was never greater than that in the controls, these amino acids were not examined further.

Of the two specifically stimulating amino acids, threonine was examined in more detail and it was shown (Fig. 2) that maximal stimulation was achieved with 0.5 mg. L-threonine N/100 ml. Further experiments showed that D-threonine was inactive but in no way inhibitory, whilst, as might have been expected, DL-threonine was (below the plateau level) one half as active as the L isomer. In the presence of 25 mg. peptone N/100 ml., the effect of L-threonine is almost obliterated, presumably because sufficient is present in the extra peptone.

The two common amino acids not included in Table 4 are L-tyrosine and L-tryptophan. They were not sufficiently soluble at the pH of the medium to be examined at a level of 20 mg. N/100 ml. They were, therefore, examined at lower concentrations

Table 4. *Growth and riboflavin synthesis by E. ashbyii in presence of various amino acids*

Dry wt. and riboflavin production per 15 ml. medium in 50 ml. conical flasks; temp. 28° ; media *A* and *B* contain the basal medium + 5 mg./100 ml. and 25 mg./100 ml. of peptone N respectively: other media contain 5 mg. peptone N/100 ml. plus an amino acid at a concentration equivalent to 20 mg. N/100 ml.

Medium	Five-day cultures			Ten-day cultures		
	Dry wt. (mg.)	Riboflavin		Dry wt. (mg.)	Riboflavin	
		Total amount (μ g.)	Amount (g./100 g. dry mycelium)		Total amount (μ g.)	Amount (g./100 g. dry mycelium)
Control <i>A</i>	13.7	230	1.68	14.0	395	2.82
L-Alanine	7.9	143	1.82	9.2	365	3.38
DL- α -Amino butyric acid	9.6	118	1.51	7.5	333	4.44
L-Arginine	13.6	176	1.29	16.4	443	2.71
L-Asparagine	27.2	506	1.95	24.5	915	3.73
L-Aspartic acid	20.5	593	2.90	26.3	1025	3.90
DL-Citrulline	14.5	253	1.75	13.0	540	4.15
L-Cysteine		No growth			No growth	
L-Cystine	11.1	283	2.58	9.7	348	3.27
L-Glutamic acid	17.5	547	3.12	19.0	842	4.43
Glycine	13.7	141	1.07	17.7	360	2.05
L-Hydroxyproline	13.4	213	1.59	9.0	337	3.74
DL-Histidine	16.9	179	1.06	—	—	—
DL-Isoleucine	15.6	166	1.06	16.7	235	1.45
L-Leucine	15.2	190	1.26	18.3	425	2.33
L-Lysine	13.1	192	1.43	13.6	368	2.71
DL-Methionine	14.8	135	0.91	16.2	194	1.19
DL-Ornithine	15.8	152	0.96	—	—	—
L-Phenylalanine	10.9	114	1.30	13.4	288	2.15
L-Proline	16.4	191	1.16	20.7	328	1.59
DL-Serine	14.5	343	2.32	12.3	973	7.80
DL-Threonine	10.9	428	3.93	13.9	865	6.23
L-Valine	18.4	176	0.95	19.5	359	1.84
Control <i>B</i>	22.6	473	2.11	24.3	985	4.06

Table 5. *Growth and riboflavin synthesis by E. ashbyii in the presence of small amounts of L-tyrosine*

Media A and B are the basal medium plus 5 and 13 mg. peptone N/100 ml., respectively; dry wt. and riboflavin production per 15 ml. medium in 50 ml. conical flasks; temp. 28°.

Concn. of tyrosine added (mg. N/100 ml.)	Five-day cultures			Ten-day cultures		
	Dry wt. (mg.)	Riboflavin		Dry wt. (mg.)	Riboflavin	
		Total amount (μg.)	Amount (g./100 g. dry mycelium)		Total amount (μg.)	Amount (g./100 g. dry mycelium)
0 (control A)	10.8	285	2.64	8.7	547	6.29
0.5	8.3	262	3.16	9.3	492	5.32
1.0	10.7	282	2.64	8.5	529	6.24
2.0	9.7	370	3.81	9.0	539	6.00
4.0	10.4	393	3.78	9.0	624	6.99
8.0	7.2	465	6.42	12.8	741	5.67
0 (control B)	15.1	469	3.10	15.1	1036	6.90

Fig. 2. The effect of the addition of varying amounts of L-threonine to the basal medium plus 0.005% (w/v) peptone N, on dry wt. and riboflavin production. Cultural conditions are the same as described for Fig. 1. ●—●, dry wt.; ○—○, riboflavin.

Table 6. *Growth and riboflavin synthesis by E. ashbyii in presence of small amounts of DL-phenylalanine*

Media A and B are the basal medium plus 5 and 13 mg. peptone N/100 ml., respectively. Dry wt. and riboflavin production per 15 ml. medium in 50 ml. conical flasks; 5 day cultures; temp. 28°.

Concn. of phenylalanine added (mg. N/100 ml.)	Dry wt. (mg.)	Riboflavin	
		Total amount (μg.)	Amount (g./100 g. dry mycelium)
0 (control A)	11.1	347	3.19
0.5	8.9	327	3.71
1.0	8.7	289	3.32
2.0	9.2	278	3.01
4.0	9.8	320	3.28
8.0	7.4	271	3.66
0 (control B)	18.1	574	3.35

(0.5–8.0 mg. N/100 ml.) and it was found that DL-tryptophan was without effect on flavinogenesis, although growth was inhibited slightly; at the highest concentration flavinogenesis as well as growth appeared to be inhibited. L-Tyrosine, on the other hand, stimulated riboflavin synthesis, but was much less effective than threonine (Table 5). The first unequivocal response was obtained with a level of 4.0 mg. N/100 ml., whilst with threonine maximal response was obtained with 0.5 mg. N/100 ml. (Fig. 2).

The possibility existed that the weak effect of tyrosine was due to an impurity in the sample used. Paper chromatography revealed only one spot (tyrosine) and no other amino acid was detected. The presence of a trace impurity in tyrosine appears to be unlikely, for such an impurity might be expected to occur also in other amino acids, e.g. L-phenylalanine which is probably isolated from a similar source. DL-Phenylalanine was examined in more detail but with entirely negative results (Table 6).

Paper chromatography (Crammer, 1948) of the media in which riboflavin synthesis had been stimulated showed that in all cases the flavin produced was chromatographically indistinguishable from riboflavin.

Effect of purines and pyrimidines

Following the observations of Maclaren (1952) that certain purines stimulated flavinogenesis by *E. ashbyii*, a number of purines and pyrimidines and their derivatives were tested under the same conditions as were the amino acids. Table 7 shows that xanthine and adenine (as well as adenosine) specifically stimulated riboflavin considerably whilst the pyrimidines and their derivatives had no such effect. There is some inhibition of both growth

and riboflavin synthesis with thiouracil but no specific effect on riboflavin; alloxan is very much more inhibitory than thiouracil.

More detailed examination of adenine and xanthine (Tables 8 and 9) show that xanthine tends to be somewhat more effective than adenine but that, in contrast to the observations with the flavinogenic amino acids, the stimulation continues to increase up to the highest levels examined. It will be noticed that with adenine there was no stimulation of growth even with the highest concentrations, but that with xanthine there appeared to be some growth stimulation at high concentrations.

Table 7. *The effect of purines and pyrimidines on riboflavin production by E. ashbyii*

Controls *A* and *B* contain the basal medium +5 and 25 mg. peptone N/100 ml. respectively. The various purines and pyrimidines were added to *A* in amounts equivalent to 20 mg. N/100 ml. Six-day cultures; temp. 28°. Amounts are those produced by 15 ml. medium in 50 ml. flasks.

Culture medium	Dry wt. (mg.)	Riboflavin	
		Total amount (μg.)	Amount (g./100 g. dry mycelium)
Control <i>A</i>	13.0	290	2.23
<i>A</i> + adenine	11.4	572	5.02
<i>A</i> + adenosine	12.7	644	5.06
<i>A</i> + xanthine	13.7	788	5.76
<i>A</i> + thymine	10.8	187	1.73
<i>A</i> + cytosine	11.5	301	2.63
<i>A</i> + uracil	12.1	228	1.88
<i>A</i> + thiouracil	7.0	169	2.46
<i>A</i> + alloxan	1.4	32	2.27
Control <i>B</i>	22.8	685	3.00

Table 8. *The effect of varying concentrations of adenine on riboflavin synthesis by E. ashbyii*

Media *A* and *B* contain the basal medium +5 and 45 mg. peptone N/100 ml. respectively; 5-day culture; temp. 28°. Amounts produced in 15 ml. medium in 50 ml. conical flasks.

Concn. of adenine added (mg. N/100 ml.)	Dry wt. (mg.)	Riboflavin	
		Total amount (μg.)	Amount (g./100 g. dry mycelium)
0 (control <i>A</i>)	11.3	215	1.90
0.25	10.4	223	2.14
0.50	11.3	304	2.69
1.0	10.6	344	3.25
2.0	11.8	362	3.07
4.0	11.4	422	3.70
8.0	10.6	442	4.17
16.0	12.0	495	4.13
20.0	12.6	562	4.46
30.0	12.8	597	4.66
40.0	11.3	720	6.37
0 (control <i>B</i>)	21.5	576	2.68

It was also found that even in the presence of 25 mg. peptone N/100 ml. small amounts of the purines stimulated riboflavin synthesis. For example, in one experiment the addition of 8 mg. adenine N/100 ml. increased flavinogenesis by 36 %.

Table 9. *The effect of varying concentrations of xanthine on riboflavin synthesis by E. ashbyii*

Experimental conditions as outlined in Table 8.

Concn. of xanthine added (mg. N/100 ml.)	Dry wt. (mg.)	Riboflavin	
		Total amount (μg.)	Amount (g./100 g. dry mycelium)
0 (control <i>A</i>)	15.9	366	2.29
0.25	13.7	401	2.92
0.50	13.6	437	3.22
1.0	15.0	512	3.40
2.0	15.9	708	4.42
4.0	14.4	699	4.86
8.0	18.1	722	3.98
16.0	17.8	962	5.38
20.0	21.1	995	4.84
30.0	18.9	1137	6.04
40.0	(Not examined—xanthine not sufficiently soluble)		
0 (control <i>B</i>)	30.6	770	2.52

Table 10. *The effect of uracil on growth and riboflavin synthesis by E. ashbyii*

The control is the basal medium +0.005 g. peptone N/100 ml. 5-day cultures; amounts produced in 15 ml. medium in 50 ml. conical flasks; temp. 28°.

Amount of uracil added to medium (mg. N/100 ml.)	Dry wt. (mg.)	Riboflavin	
		Total amount (μg.)	Amount (g./100 g. dry mycelium)
0 (control)	10.8	262	2.43
50	8.0	92	1.03
150	10.2	130	1.28
200	11.6	121	1.04
250	9.5	161	1.69

Table 11. *The effect of uracil on the adenine-stimulated synthesis of riboflavin by E. ashbyii*

Control medium is basal medium +0.005% (w/v) peptone N; 5-day cultures; amounts produced by 15 ml. medium in 50 ml. conical flasks; temp. 28°.

Amounts (mg. N/100 ml.) added to basal medium		Dry wt. (mg.)	Riboflavin	
Adenine	Uracil		Total amount (μg.)	Amount (g./100 g. dry mycelium)
0	0	9.7	145	1.50
4	0	9.6	252	2.71
4	2	12.6	280	2.2
4	4	10.8	254	2.4
4	8	11.7	229	2.0
4	16	11.2	210	1.9

The results given in Table 7 indicated that uracil had only a doubtful inhibitory effect on riboflavin synthesis when tested at a low concentration compared with that used by Maclaren (1952), who obtained marked inhibition. Uracil was accordingly tested under our conditions at the levels employed by Maclaren. It will be seen (Table 10) that at concentrations of 50 mg. N/100 ml. and above very little effect on growth was observed but that flavinogenesis was inhibited to the extent of about 50%. If, as Maclaren suggested, uracil exerts its inhibitory action by blocking the incorporation of a purine into the riboflavin molecule, then this effect should be most marked under conditions where purines would normally be stimulating riboflavin synthesis. Table 11 shows that under these conditions using adenine as the purine, there was only very slight inhibition even when the uracil concentration was 4 times that of adenine.

*Effect of derivatives of
4:5-dimethyl-o-phenylenediamine*

Other nitrogenous compounds which might be precursors of riboflavin are 5:6-dimethylbenzimidazole and 1:2-dimethyl-4-amino-5-(p-1'-ribitylamino)benzene. These, together with 1:2-dimethyl-5-(p-1'-ribitylamino)benzene, have been examined in the same way as the previous compounds at a level equivalent to the level of adenine (4 mg. N/100 ml.) used in previous experiments. The results of one of

these experiments are recorded in Table 12. No stimulation was observed and in fact there was in two cases slight but definite inhibition. This inhibition has been confirmed in other experiments (see, for example, Table 14).

Miscellaneous nitrogen compounds

A number of other nitrogenous compounds have also been examined and the results of one such experiment are recorded in Table 13. It will be seen that glucosamine considerably inhibits both growth and riboflavin synthesis equally, whilst $(\text{NH}_4)_2\text{SO}_4$ is without obvious effect. Urea is interesting in that in all experiments at the higher level it has proved non-stimulatory for riboflavin; at the lower level, on the other hand, it is occasionally slightly stimulatory, as in the experiment recorded in Table 13. The reason for this spasmodic stimulation is not known.

Combined effect of various nitrogen sources

The effect on riboflavin synthesis of the addition of other nitrogenous substances to a medium containing L-threonine at its optimum level has been examined. In the results set out in Fig. 3, riboflavin production is recorded as the percentage stimulation above the control level. It was not considered necessary to record the dry weights of the mycelia, although they were measured, because, as shown earlier, none of the substances examined

Table 12. *The effect of 4:5-dimethyl-o-phenylenediamine derivatives on riboflavin synthesis by E. ashbyii*

Control medium is basal medium + 0.005% (w/v) peptone N; 5-day cultures; amounts produced by 15 ml. medium in 50 ml. conical flasks; temp. 28°.

Compound tested	Amount added* (mg./100 ml.)	Dry wt. (mg.)	Riboflavin	
			Total amount ($\mu\text{g.}$)	Amount (g./100 g. dry mycelium)
0 (control)	0	10.4	175	1.68
5:6-Dimethylbenzimidazole	7.4	9.8	126	1.28
1:2-Dimethyl-4-amino-5-(p-1'-ribitylamino)benzene	3.9	10.1	148	1.46
1:2-Dimethyl-5-(p-1'-ribitylamino)benzene	4.1	10.2	178	1.74

* These amounts are the molecular equivalents of 7.7 mg. adenine, which contains 4.0 mg. N.

Table 13. *The effect of some miscellaneous nitrogenous compounds on riboflavin synthesis by E. ashbyii*

Control media A and B contain the basal constituents + 5 and 25 mg. peptone N/100 ml. respectively. Amounts produced by 15 ml. of medium in 50 ml. conical flasks; temp. 28°; 5-day cultures.

Constituent added to medium	Concentration of constituent (mg. N/100 ml.)	Dry wt. (mg.)	Riboflavin	
			Total amount ($\mu\text{g.}$)	Amount (g./100 g. dry mycelium)
0 (control A)	0	11.3	213	1.89
Glucosamine	20	4.8	70	1.52
Urea	5	10.0	297	2.97
Urea	20	11.9	241	2.03
$(\text{NH}_4)_2\text{SO}_4$	10	9.6	239	2.49
$(\text{NH}_4)_2\text{SO}_4$	20	9.9	228	2.31

has any obvious effect on the growth of the organism. Fig. 3 shows that the addition of the other stimulatory amino acids DL-serine and L-tyrosine did not enhance the effect due to L-threonine alone and that urea and $(\text{NH}_4)_2\text{SO}_4$ were also inactive. The addition of the purine adenine, however, enhanced the L-threonine effect and produced more riboflavin than could be expected from a simple summation of the increases obtained when these two substances were tested separately.

Similar experiments carried out using the basal medium containing 4 mg. adenine N/100 ml. and to which other nitrogen sources were added, again showed (Fig. 4) the extra stimulation in the presence of L-threonine and demonstrated that DL-serine was equally effective. Some stimulation was

also obtained with urea and to a slight extent with L-tyrosine, whilst $(\text{NH}_4)_2\text{SO}_4$ was completely inactive.

It was thought that the failure of 5:6-dimethylbenzimidazole and 1:2-dimethyl-4-amino-5-(D-1'-ribitylamino)benzene to stimulate riboflavin synthesis might be due to the absence of the appropriate coupling compound (e.g. alloxan). Consequently, they were tested in the presence of small non-inhibitory amounts of alloxan (4 mg. N/100 ml.) and adenine (4 mg. N/100 ml.) and DL-threonine (2 mg. N/100 ml.). In the first case there was no stimulation of riboflavin synthesis and in the latter there was no potentiation of the adenine and threonine effects.

Fig. 3. The combined effect of L-threonine and one other N source on riboflavin synthesis. Results are expressed as % increase over production on the basal medium plus 0.005 % (w/v) peptone N. Cultural conditions as described in Fig. 1. A, L-threonine alone (1 mg. N/100 ml.); B, DL-serine alone (10 mg. N/100 ml.); C, A + B; D, L-tyrosine alone (5 mg. N/100 ml.); E, A + D; F, A + urea* (5 mg. N/100 ml.); G, A + $(\text{NH}_4)_2\text{SO}_4$ * (10 mg. N/100 ml.); H, adenine alone (4 mg. N/100 ml.); I, A + H.

* $(\text{NH}_4)_2\text{SO}_4$ alone is without stimulatory action, whilst urea alone has a slight effect (Table 10).

Fig. 4. The combined effect of the addition of adenine and one other N source on riboflavin synthesis. Results expressed as described in Fig. 3 and experimental conditions as described in Fig. 1. A, adenine alone (8 mg. N/100 ml.); B, A + L-threonine* (1 mg. N/100 ml.); C, A + L-tyrosine* (5 mg. N/100 ml.); D, A + urea* (5 mg. N/100 ml.); E, A + $(\text{NH}_4)_2\text{SO}_4$ * (10 mg. N/100 ml.); F, A + DL-serine* (10 mg. N/100 ml.).

* For control values of the additional N source values see Fig 3 and Table 13.

Table 14. *The relative amounts of riboflavin in mycelium and medium*

Amounts produced in 15 ml. medium in 50 ml. conical flasks. Incubation time, 5 days; temp. 28°.

Medium	Riboflavin			
	In mycelium ($\mu\text{g.}$)	In medium ($\mu\text{g.}$)	Total ($\mu\text{g.}$)	% of total in mycelium
A* (basal medium + 5 mg. peptone N/100 ml.)	131	181	312	41.7
B* (basal medium + 5 mg. peptone N/100 ml.)	100	165	265	37.8
A + DL-threonine (2 mg. N/100 ml.)	168	263	431	39.0
A + adenine (4 mg. N/100 ml.)	153	297	450	34.0
B + glycine (20 mg. N/100 ml.)	59	115	174	34.0
B + 5:6-dimethylbenzimidazole	78	119	197	39.6
B + 1:2-dimethyl-5-(D-1'-ribitylamino)benzene	82	188	270	30.4
B + 1:2-dimethyl-4-amino-5-(D-1'-ribitylamino)benzene	87	137	224	26.9

* A and B are the same medium but the two series of experiments were carried out at different times.

Riboflavin in the mycelium

According to Deseive (1947) the mycelial riboflavin (which is mainly in the form of flavin-adenine dinucleotide (Forrest, private communication)) in *E. ashbyii* is negligible compared with that in the medium. On checking this under our conditions this was found not to be so. Table 14 shows that about 30–40% of the total riboflavin produced is in the mycelium. The possibility that the increased riboflavin observed in the medium in the presence of threonine, serine and purines might be due to the liberation of mycelial riboflavin into the medium is ruled out by the demonstration (Table 14) that in the presence of these stimulators the mycelial riboflavin is also increased, but not to the same extent as is the riboflavin in the medium. The results in Table 14 also show that the failure of glycine (which might be expected to be active because of its possible conversion into serine), 5:6-dimethylbenzimidazole and 1:2-dimethyl-4-amino-5-(p-1'-ribitylamino)benzene to stimulate riboflavin synthesis is not due to the failure to release riboflavin from the mycelium into the medium.

DISCUSSION

Comparison with earlier work on amino acid utilization

It is somewhat difficult to compare the results of the present amino acid experiments with those of other investigators (Schopfer & Guilloud, 1945*b*; Chin, 1947; Deseive, 1947; Hickey, 1953) because the experimental conditions were so different and, apart from Hickey, none of these investigators examined either serine or threonine. In Hickey's experiments they were examined in culture media in which they were the sole source of nitrogen, and, as might be expected from the present work, they did not support growth of the organism.

In agreement with Chin (1947) and Hickey (1953) it has been found that asparagine, aspartate and glutamate were good nitrogen sources for both growth and riboflavin synthesis, and it is difficult to explain the results of Schopfer & Guilloud (1945*b*) which indicated that no growth or riboflavin was produced on aspartate or glutamate.

The present work confirms the observations of Deseive (1947) that L-cysteine inhibits considerably growth and flavinogenesis.

It is interesting that in the present experiments glycine had little if any effect on growth of *E. ashbyii* and these results tend to confirm those of Dulaney & Grutter (1950), who concluded that glycine was not utilized by this organism. Schopfer (1944), on the other hand, found that glycine stimulated growth in the presence of some samples of peptone and not in others. Most of the amino acids fall into the same category as glycine, but it ought to be emphasized that it is not necessarily true that they are not metabolized by *E. ashbyii*. If, for example, growth alone had been the criterion, then L-threonine would have been considered inert.

Role of amino acids and purines in biosynthesis of riboflavin

The most likely explanation of the stimulation of riboflavin synthesis in *E. ashbyii* by certain amino acids and purines is that they are sources of specific building units which, under the prescribed experimental conditions, are limiting factors. It is possible, however, that the effect might be indirect and, although this appears unlikely, it remains for isotope studies, at present in progress, to decide this unequivocally. There is, however, some evidence that in the closely related organism *A. gosypii*, the biosynthesis of riboflavin follows a very similar course to that of the purines. Recent work by Plaut (1953*a*) with this organism has shown that ¹⁴C-labelled formate and CO₂ are incorporated almost entirely into C₂ and C₄ of riboflavin respectively. This agrees with the present views on the incorporation of these units into purines (see, for example, Brown, 1953). The position of glycine which is specifically incorporated into purines but which does not under our conditions stimulate riboflavin production appears to be somewhat anomalous. There is, however, no reason to believe that because it does not stimulate riboflavin synthesis it cannot be incorporated into the molecule, and very recently Plaut (1953*b*) has shown that incorporation can take place.

Assuming then the incorporation of the stimulatory amino acids and purines into riboflavin, many interesting possibilities arise. The fact that the amino acid stimulations are not cumulative suggests that they probably provide the same building unit. As the effect of amino acid plus purine is cumulative, the biosynthesis could be envisaged, using threonine and xanthine as examples, formally as follows:

On this scheme the amino acids function only by providing a carbon source, which is incorporated into the aromatic moiety of riboflavin. It is obvious that threonine and serine must provide the same or closely related units. It is not yet known whether the slight activity of tyrosine is possibly due to a limited ability of the fungus to split off serine from the molecule.

The observation that the addition of purine to a medium containing optimum amounts of L-threonine produces an effect greater than the sum of the two separate effects suggests that stimulation by L-threonine alone ceases at a low concentration because purines or their immediate precursors become the limiting factors. Addition of a purine results in the utilization of the remainder of the L-threonine and also in the stimulation of flavin biosynthesis due to the utilization of the purine itself; this is possible, for the conversion of purine into riboflavin is, even at low concentrations, only of the order of 20–30 %.

The fact that stimulation by purines alone does not stop at a comparatively low concentration but continues to increase even up to the highest concentration (a saturated solution) examined, would suggest that in the basal medium there is no shortage of 'aromatic precursors' such as may be provided by threonine. It could, however, also be ascribed to the fact that some of the purine is catabolized thereby yielding 'aromatic precursors'. It is, for example, possible from purely structural considerations, to envisage the release of serine.

A number of questions arise when the role of purines in flavinogenesis is further considered. They can only be solved by further work but may be briefly enumerated here: (a) although it is apparent that adenine can be deaminated, does this occur before or after incorporation into the riboflavin molecule? (b) is the ribose of adenosine removed or reduced to ribitol before incorporation of the purine? and (c) if 6:7-dimethylisalloxazine is first formed, is ribose attached and then reduced or is ribitol itself attached?

Role of o-phenylenediamine derivatives and pyrimidines in riboflavin biosynthesis

It is evident from this work that pyrimidines are not specifically incorporated into riboflavin, even in the presence of urea which, theoretically, could supply the unit for converting a pyrimidine into a purine. (The reason for the slight sporadic stimulation of urea alone is not yet obvious.) It has been found that in high concentration uracil actually inhibits riboflavin synthesis. This is in agreement with the original observations of Maclaren (1952), but we could not obtain as great an inhibition (50 % compared with 83 %). As it was found that the stimulatory power of adenine was reduced only

slightly, in the presence of a considerable excess of uracil, Maclaren's suggestion that uracil may act by competitively inhibiting the incorporation of purines into the riboflavin molecule may not be correct.

The failure of 1:2-dimethyl-4-amino-5-(D-1'-ribitylamino)benzene (I) to stimulate the synthesis of riboflavin alone or in the presence of non-toxic amounts of alloxan strongly suggests that a biosynthetic pathway similar to the chemical synthesis of Banerjee, Dittmer & du Vigneaud (1945) involving the condensation of (I) with alloxan, is not the major pathway in this organism, if it occurs at all. Smith & Emmart (1949), however, did observe a slight stimulation of riboflavin synthesis in *Mycobacterium tuberculosis* with both (I) and alloxan.

The absence of stimulation of riboflavin synthesis by 5:6-dimethylbenzimidazole (there is actually a slight inhibition) and (I) also makes it very unlikely that in *E. ashbyii*, at least, the pathway for riboflavin biosynthesis is similar to that for the cobalamins in other micro-organisms, e.g. *Escherichia coli*, where both 4:5-dimethyl-o-phenylenediamine and riboflavin itself stimulate the biosynthesis of cyanocobalamin in a vitamin B₁₂-requiring mutant (Ford & Holdsworth, 1954).

General aspects of amino acid metabolism

From the general point of view of amino acid metabolism, it is extremely interesting that serine is active in promoting flavinogenesis and glycine is not. Although glycine has been demonstrated to be the precursor of serine in many animal tissues and in some micro-organisms, e.g. *Torulopsis utilis* (Ehrensvärd, Sperber, Saluste, Reio & Stjernholm, 1947), it seems that *E. ashbyii* cannot bring about this conversion. From a similar point of view the activity of L-tyrosine and the inactivity of L-phenylalanine suggests that the latter cannot be converted into the former in *E. ashbyii*. This would appear to fit in with recent work on *Esch. coli* (Simmonds, Tatum & Fruton, 1947; Davis, 1950) which suggests that there are independent routes of synthesis of these two amino acids and that phenylalanine is not converted into tyrosine.

SUMMARY

1. *Eremothecium ashbyii* grown on a basal glucose-salts-vitamins medium supplemented with 0.24 % (w/v) peptone is fully grown after 5 days but continues to produce riboflavin up to 7–8 days. Maximal concentration of riboflavin is obtained over the range 0.16–0.40 % (w/v) peptone.

2. Using the basal medium supplemented with 0.005 % (w/v) peptone N, addition of L-threonine,

L-serine or L-tyrosine specifically stimulated riboflavin synthesis; L-tyrosine was the least effective. D-Threonine was inactive. L-Glutamate, L-aspartate and L-asparagine stimulated both growth and flavinogenesis, whilst L-cysteine inhibited both. Other amino acids tested were without obvious effect on riboflavin synthesis, but some inhibited growth slightly.

3. Under the same conditions the purines xanthine and adenine, and adenosine, stimulated flavinogenesis whilst most pyrimidines and their derivatives were without effect, although alloxan inhibited both growth and riboflavin synthesis. At very high concentrations, uracil inhibited riboflavin synthesis, although it did not inhibit the adenine-stimulated production of riboflavin.

4. Neither 5:6-dimethylbenzimidazole nor 1:2-dimethyl-4-amino-5-(D-1'-ribitylamino)benzene stimulated riboflavin production under the same conditions.

5. Of the other nitrogen-containing compounds tested, urea in low concentrations occasionally stimulated and $(\text{NH}_4)_2\text{SO}_4$ was without effect. D-Glucosamine was a powerful inhibitor of both growth and riboflavin.

6. The effects of the stimulatory amino acids are not cumulative, whilst those of a purine and an amino acid are.

7. The significance of these results in relation to the problem of the biosynthesis of riboflavin is discussed.

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On the Significance of γ -Glutamyl Transpeptidation in Peptide Biosynthesis

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Transpeptidation reactions have been receiving increasing emphasis in connexion with the syntheses of peptide and amide bonds and hence of proteins (Hanes, Hird & Isherwood, 1950, 1952; Hanes, Connell & Dixon, 1953; Waelsch, 1953; Fruton, Johnston & Fried, 1951; Fruton, 1950). Particular interest attaches to the γ -glutamyl transpeptidation

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reaction of Hanes *et al.* (1950, 1952) because the hypothesis has been advanced that glutamine and glutathione serve to direct the energy of the energy-rich bonds of adenosine triphosphate into the formation of peptide bonds by means of the transpeptidation and transamidation reactions (Waelsch, 1953). The hypothesis further assumes that the γ -glutamyl peptides formed by these reactions would either rearrange to α -peptides or by further exchanges