Studies on the Biosynthesis of Riboflavin

2. FURTHER OBSERVATIONS ON NITROGEN METABOLISM AND FLAVINOGENESIS IN *EREMOTHECIUM ASHBYII**

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Purines are known to stimulate riboflavin synthesis in the fungus *Eremothecium ashbyii* (MacLaren, 1952; Goodwin & Pendlington, 1954*a, b*) and work with isotopes indicates that this is due to incorporation of the purine or a metabolite into the riboflavin molecule and not to an indirect effect (Plaut, 1953, 1954*a, b*; Plaut & Bromberg, 1954; Klungsoyr, 1954*a, b*; McNutt, 1954). McNutt showed that the riboflavin isolated after adding [8-¹⁴C]adenine to growing *E. ashbyii* was only slightly labelled, whilst that obtained after the addition of generally tagged adenine was strongly labelled. Plaut's work on *Ashbya gossypii* (an organism closely related to *E. ashbyii*) showed that the carbon of CO₂ and formate was incorporated into riboflavin almost exclusively in positions 2 and 4 respectively, and the methylene and carboxyl carbons of glycine into positions 4*a* and 9*a*, respectively. A similar labelling pattern also occurs in the purine molecule (see, for example, Buchanan & Sonne, 1946; Karlsson & Barker 1949).

Klungsoyr (1954*a, b*) also showed that formate carbon was incorporated into position 2.

The pyrimidines thymine, cytosine and uracil, and 4-amino-1:2-dimethyl-5-(*p*-1'-ribitylamino) benzene and 4:5-dimethylbenzimidazole were all incapable of stimulating flavin synthesis under conditions which allowed purine stimulation (Goodwin & Pendlington, 1954*b*); these observations also suggest that nitrogens 9 and 10 come from a purine-like precursor rather than an *o*-phenylenediamine derivative.

Goodwin & Pendlington (1954*a, b*) further observed that although most amino acids, when added to the basal medium containing minimal amounts of peptone, stimulate neither growth nor

flavinogenesis, glutamate and aspartate stimulate both, whilst serine, threonine and, to a much lesser extent, tyrosine stimulate riboflavin synthesis but have little or no effect on growth.

The present paper records further investigations into the role of purines and amino acids in flavinogenesis. A short account of some of this work has already appeared (Brown, Goodwin & Pendlington, 1954).

EXPERIMENTAL

Cultures. The strain of *Eremothecium ashbyii* used throughout was that obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Media. The basal medium was that described previously (Goodwin & Pendlington, 1954*b*). Appropriate amounts of bacteriological peptone (Evans) and the compounds under test were added to this medium. The pH was adjusted to 5.8 and, except where otherwise stated, the medium was sterilized by autoclaving at 15 lb./sq.in. for 15 min.

Materials. Amino acids, adenine, xanthine and alloxan were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. The methylpurines were the gift of Professor Sir Alexander Todd, F.R.S., and Dr A. H. Johnston; 2-amino- and 2-hydroxy-purine and the 4:5-diaminopyrimidines the gift of Professor A. Albert; 4-aminopterin, folic acid, leucovorin and azaxanthine the gift of the Lederle Laboratories (through the good offices of Dr J. R. Rueggsegger), and 4-amino-1:2-dimethyl-5-(*p*-1'-ribitylamino) benzene the gift of Dr E. S. Holdsworth.

Cultural conditions and analytical procedures. These have been previously described in detail, and the degree of reproducibility in the results has also been given (Goodwin & Pendlington, 1954*b*).

Washed cells were prepared by centrifuging as previously described (Goodwin & Pendlington, 1954*b*) decanting the medium and washing twice with sterile distilled water, the cells being centrifuged after each washing.

RESULTS

Effect of 4:5-diaminopyrimidines

As purines incorporated into riboflavin appear first to lose carbon 8 (McNutt, 1954), it was possible that 4:5-diaminopyrimidines would be intermediates. A number of these compounds have been tested at a level (8 mg. N/100 ml.) at which adenine and xanthine produce a two- to threefold increase

* Part 1: Goodwin & Pendlington (1954*b*).

in flavin synthesis, and the results (Table 1) show that: (a) the compound analogous to adenine (4:5:6-triaminopyrimidine) inhibits both growth and riboflavin synthesis considerably (about 60 %); (b) 4:5:6-triamino-2-hydroxypyrimidine (corresponding to 6-amino-2-hydroxypurine, which has not yet been found in nature) has little effect on growth but markedly inhibits riboflavin production; (c) 4:5-diamino-2:6-dihydroxypyrimidine (analogous to xanthine) and the remaining compounds have little effect. The important conclusion from these experiments is that the stimulating effects of adenine and xanthine are not reproduced by their 4:5-diaminopyrimidine analogues. Because of their instability, the pyrimidines were sterilized by filtration; 4:5-diamino-2:6-dihydroxypyrimidine is particularly unstable and on standing for 5 days at 28° in uninoculated media its absorption spectrum changes from one with a single sharp peak at 273 m μ . to one with two rather shallow peaks near 340 and 410 m μ . (Fig. 1). A similar change also occurs in inoculated media as can be seen when the absorption due to riboflavin (the riboflavin curve is that of a control experiment run at the same time) is subtracted from the gross curve of the inoculated medium. The resulting curve almost exactly coincides with that of the uninoculated

Fig. 1. The absorption spectra of: (a) Basal medium + 4:5-diamino-2:6-dihydroxypyrimidine after incubating alone for 5 days at 28° (—●—). (b) Same medium incubated after inoculation with *E. ashbyii*, for 5 days at 28° (—). (c) The contribution of riboflavin to curve (b) (—●—). (d) The difference spectrum of curves (b) and (c) (—).

Table 1. The effect of some 4:5-diaminopyrimidines on flavinogenesis by *Eremothecium ashbyii*

Basal medium plus 4 mg. peptone N/100 ml. and 8 mg. N/100 ml. of the pyrimidines; 5-day cultures; temp. 28°; triplicate determinations; pyrimidines sterilized by filtration.

Pyrimidine added	No. of experiments	Dry wt. (% of control)*		Riboflavin (% of control)*	
		Mean	Range	Mean	Range
4:5:6-Triamino	5	50.0	30.5-64.1	38.8	22.4-55.6
4:5:6-Triamino-2-hydroxy	3	90.2	67.5-109.0	35.9	9.5-58.6
4:5-Diamino-2:6-dihydroxy	3	104.6	94.4-112.0	95.0	87.0-102.3
4:5-Diamino-2-hydroxy	2	107.0	104.0-110.0	104.6	79.3-130.0
2:4:5-Triamino-6-hydroxy	1	104.0	—	102.0	—

* As experiments were carried out at different times, the results are expressed as percentages of the appropriate controls.

Table 2. The combined effect of certain pyrimidines and purines on riboflavin synthesis by *Eremothecium ashbyii*

Amounts produced in 15 ml. basal medium + 4 mg. peptone N/100 ml. with the addition of purines and pyrimidines at a level of 8 mg. N/100 ml.; 5-day cultures; temp. 28°; triplicate analyses.

Compound added	Dry wt. (mg.)		Riboflavin		
			(μg./15 ml. medium)		(g./100 g. dry mycelium)
	Mean	Range	Mean	Range	Mean
None (control)	13.1	12.6-13.5	84	81-90	0.64
4:5:6-Triamino-2-hydroxypyrimidine	14.3	13.6-15.3	8	6-13	0.1
4:5:6-Triaminopyrimidine	8.4	8.1-8.8	39	37-43	0.47
Adenine	17.3	17.2-17.4	241	239-244	1.39
Xanthine	15.0	14.3-15.9	253	205-283	1.69
4:5:6-Triamino-2-hydroxypyrimidine + adenine	17.3	14.1-20.3	103	99-111	0.61
4:5:6-Triamino-2-hydroxypyrimidine + xanthine	20.2	19.2-21.2	111	93-123	0.55
4:5:6-Triaminopyrimidine + adenine	16.9	16.2-17.6	267	260-275	1.58
4:5:6-Triaminopyrimidine + xanthine	14.8	14.2-15.5	106	96-124	0.71

medium. This also indicates that the pyrimidine is not utilized to any appreciable extent. The formation of the artifact in the medium may account for the lack of stimulation of riboflavin synthesis.

Examining the two inhibitory pyrimidines in conjunction with adenine and xanthine, it was repeatedly found (see Table 2 for the results of a typical experiment) that with the xanthine analogue both adenine and xanthine overcame the inhibition of riboflavin production but did not stimulate production above normal as they do in its absence. With the adenine analogue both purines overcome the growth inhibition but, whilst xanthine brings the riboflavin synthesis back to normal, adenine increases it above normal to the same extent as in the absence of the pyrimidine.

4:5:6-Triaminopyrimidine is the only member of the series which suppresses the stimulation of riboflavin production by threonine. In two experiments the mean stimulations in the presence of threonine and this pyrimidine were only 13.6 and 6.9 % of the values observed in the basal medium with threonine alone.

Effect of aminopterin

Aminopterin was examined for its effect on riboflavin synthesis because of its known inhibitory effect on the biosynthesis of purines (Woolley & Pringle, 1950; Buchanan & Wilson, 1953). Fig. 2 shows that with increasing concentration aminopterin increasingly inhibits growth. Up to a concentration of 2 µg./100 ml., on the other hand, riboflavin synthesis is considerably stimulated; above this level synthesis is reduced, but to the same extent as growth, so that the concentration (amount excreted/g. dry mycelium) remains at its elevated level.

When threonine was tested in the presence of aminopterin at levels of 20 and 200 µg./100 ml., it did not overcome growth inhibition in either case (Table 3); with the lower concentration it was, however, still able to exert its flavinogenic action, but this was eliminated at the higher concentration of aminopterin. Adenine examined under the same conditions partly overcame the growth inhibition of aminopterin whilst its stimulation of riboflavin synthesis was only slightly reduced. The growth

Fig. 2. The effect of aminopterin on growth and riboflavin synthesis in *E. ashbyii*. Amounts produced in 15 ml. basal medium containing 4 mg./100 ml. peptone N. (5-day cultures; temp. 28°). ●, Dry wt.; ○, riboflavin/15 ml. medium; ×, riboflavin (g./100 g. dry mycelium).

Table 3. The effect of aminopterin on purine and threonine stimulation of riboflavin synthesis by *Eremothecium ashbyii*

Amounts produced in 15 ml. basal medium containing 4 mg./100 ml. peptone N plus adenine or DL-threonine with aminopterin; temp. 28°; 5-day cultures; triplicate determinations.

Compound added	Concn. (mg./100 ml.)	Dry wt. (mg.)		Riboflavin		
		Mean	Range	(µg./15 ml.)		(g./100 g. dry mycelium)
				Mean	Range	Mean
None (control)	—	11.1	10.5–11.5	154	152–158	1.40
Aminopterin	0.02	5.2	4.0–5.8	251	211–283	5.06
Aminopterin	0.20	2.1	1.7–4.2	121	118–127	5.92
DL-Threonine	15.0*	8.5	7.0–10.3	366	255–460	4.55
Adenine	7.7†	12.0	11.1–13.2	359	296–359	2.72
Aminopterin + DL-threonine	0.02 15.0*	3.0	2.6–3.5	376	343–399	13.65
Aminopterin + DL-threonine	0.20 15.0*	3.6	3.4–3.7	158	147–169	4.43
Aminopterin + adenine	0.02 7.70†	7.8	6.6–9.9	407	384–428	5.36
Aminopterin + adenine	0.20 7.70†	5.9	4.7–7.9	243	236–256	4.26

* ≡ 2 mg. N of L-isomer. † ≡ 4 mg. N.

Table 4. *The effect of aminopterin on riboflavin synthesis in Eremothecium ashbyii in the presence of folic acid and leucovorin*

Amounts produced in 15 ml. basal medium + 4 mg. peptone N/100 ml., to which was added aminopterin, alone or with folic acid or leucovorin; triplicate analyses; temp. 28°; 5-day cultures.

Compounds added to basal medium	Amount (µg./100 ml.)	Dry wt. (mg.)		Riboflavin		
		Mean	Range	(µg./15 ml.)		(g./100 g. dry mycelium)
				Mean	Range	
None (control)	—	10.2	9.4–11.2	70	69–70	0.68
4-Aminopterin	4.0	4.6	4.2–5.0	153	143–167	3.33
4-Aminopterin + leucovorin	4.0	4.6	4.2–5.4	123	114–131	2.71
	4.0					
4-Aminopterin + leucovorin	4.0	4.9	4.6–5.2	130	127–145	2.69
	40.0					
4-Aminopterin + leucovorin	4.0	10.1	9.3–11.7	109	97–123	1.07
	400.0					
4-Aminopterin + folic acid	4.0	5.9	5.2–7.4	129	124–136	2.22
	4.0					
4-Aminopterin + folic acid	4.0	5.8	5.1–6.5	128	117–138	2.23
	40.0					
4-Aminopterin + folic acid	4.0	6.4	6.8–8.0	116	108–128	1.81
	400.0

Fig. 3. The effect of increasing concentrations of peptone on the action of aminopterin on growth and flavinogenesis in *E. ashbyii*. Amounts produced in 15 ml. basal medium containing varying amounts of peptone; concn. of aminopterin 4 µg./100 ml. 5 day cultures; temp. 28°. ●, Dry wt.; ○, riboflavin/15 ml. medium; ×, riboflavin (g./100 g. dry mycelium).

inhibition of aminopterin is overcome by 100 times the concentration of citrovorum factor (leucovorin) which does not decrease riboflavin synthesis to the control level, although there is some slight reduction; folic acid at the same concentration reduces riboflavin synthesis to the same degree but does not stimulate growth to the same extent as

does leucovorin. Increasing concentrations of peptone also overcome the growth inhibition of aminopterin without decreasing to any marked extent the riboflavin levels (Fig. 3).

Action of azaxanthine

Increasing concentrations of azaxanthine have comparatively little effect on growth of *E. ashbyii*, but a pronounced effect on riboflavin synthesis, so that the concentration of the vitamin drops considerably (Fig. 4). When tested at a concentration of 4 mg. N/100 ml., azaxanthine inhibits the stimulation due to adenine by 55 % but that due to xanthin to an extent (10.9 %) which is probably not significant (Table 5).

Effect of alloxan and

4-amino-1:2-dimethyl-5-(D-1'-ribitylamino)benzene

Previously, alloxan and 4-amino-1:2-dimethyl-5-(D-1'-ribitylamino)benzene (I) had only been examined separately for stimulation of flavinogenesis (Goodwin & Pendlington, 1954b) and in neither case was any stimulation observed. As alloxan was examined at a level (20 mg./100 ml.) which appeared slightly toxic to the organism, it has now been examined over a wide range of concentrations, and it will be seen from Fig. 5 that no stimulation can be observed. At a concentration of 4 mg./100 ml., riboflavin synthesis is strongly inhibited and to a greater extent than growth, whilst, at 128 mg./100 ml., alloxan completely inhibits growth.

Alloxan and (I) were examined together, because Smith & Emmart (1949) claimed that they

Table 5. *The effect of azaxanthine and purines on stimulation of riboflavin production in Ererothecium ashbyii*

Azaxanthine and xanthine added at a level of 4 mg. N/100 ml. to basal medium containing 4 mg. peptone N/100 ml.; 15 ml. medium; temp. 28°; 5-day cultures. Triplicate determinations.

Material added to basal medium	Dry wt. (mg.)		Riboflavin		
	Mean	Range	($\mu\text{g./15 ml.}$)		(g./100 g. dry mycelium)
			Mean	Range	Mean
None (control)	14.2	13.4-14.3	72	69-77	0.51
Azaxanthine	13.8	13.2-14.9	63	60-65	0.45
Adenine	17.1	15.4-18.9	161	152-166	0.95
Xanthine	15.8	14.3-16.7	200	185-208	1.26
Azaxanthine + adenine	17.2	17.2-17.3	103	103-105	0.60
Azaxanthine + xanthine	15.9	15.4-16.6	177	165-191	1.11

Fig. 4. The effect of azaxanthine on growth and riboflavin synthesis in *E. ashbyii*. (Amounts produced in 15 ml. basal medium containing 4 mg. peptone N/100 ml.; 5-day cultures; temp. 28°.) ●, Dry wt.; ○, riboflavin/15 ml. medium; ×, riboflavin (g./100 g. dry mycelium).

stimulated riboflavin synthesis in the human tubercle bacillus, and because Banerjee, Dittmer & du Vigneaud (1945) stated that these two substances reacted chemically to form riboflavin. We have found that a mixture of these two substances react spontaneously on incubating at 28° for 5 days, producing very small traces of riboflavin but mainly a compound with an absorption spectrum quite different from that of riboflavin (Fig. 6). Like riboflavin, it possesses an intense green fluorescence which is alkali-labile; it is also reduced by dithionite and reoxidized by shaking in air, both reactions being less rapid than with riboflavin. It can be separated from riboflavin by chromatography on paper. Using the solvent system of Crammer (1948) it runs just ahead of riboflavin. This material appears almost certainly to be the anil

Fig. 5. The effect of alloxan on growth and riboflavin synthesis in *E. ashbyii*. Amounts produced in 15 ml. basal medium containing 4 mg. peptone N/100 ml.; 5-day cultures; temp. 28°. ●, Dry wt.; ○, riboflavin.

(II), for Tischler, Wellman & Ledenberg (1945) have shown that very little riboflavin but a large amount of the anil is synthesized when alloxan and (I) react; unfortunately Tischler *et al.* did not report the absorption spectrum of the anil.

When alloxan and (I) were tested together for flavinogenic capacity in *E. ashbyii*, no stimulation could be observed when the riboflavin was measured spectrophotometrically using the incubated, uninoculated medium in the compensating cell. Using water in the compensator, the absorption

spectrum of the uninoculated medium (Fig. 6) indicates anil synthesis (the reason for the slight flattening of the 410 $m\mu$. maximum is not known) and that of the inoculated medium a summation of the spectrum of riboflavin normally formed in the basal medium and that of the anil. When this riboflavin absorption is subtracted from that of

the inoculated medium, the spectrum obtained is almost exactly that of the uninoculated medium. This indicates no significant utilization of the anil for flavinogenesis.

Effect of some uncommon purines

The availability of small amounts of 2-methylhypoxanthine and 2-methyladenine, which have recently been shown to be components of some forms of vitamin B₁₂ (Brown & Smith, 1954), and 2-amino- and 2-hydroxy-purines (not known naturally) allowed us to test them in two experiments for their flavinogenic power. The limited results (Table 6) are somewhat variable, but suggest that the methyl derivatives are probably more effective flavinogenic agents than the 2-hydroxy- and 2-amino-purines. More interesting is the observation that the stimulations of threonine and methyl purines appear to cancel each other out whilst threonine in the presence of 2-hydroxy- and 2-amino-purine behaves normally. It has not been possible to examine this anomalous behaviour further.

Effect of possible metabolites of serine and threonine and related compounds

DL-alloThreonine. This compound has the same stimulatory effect on riboflavin synthesis as has DL-threonine; in one experiment the mean stimulation in the presence of DL-allothreonine was 79.6% of that in the presence of the same amount of DL-threonine.

Possible metabolites of serine and threonine. It has already been shown that serine but not glycine

Fig. 6. The absorption spectra of: (a) The crystalline compound produced from alloxan and 4-amino-1:2-dimethyl-5-(D-1'-ribitylamino)benzene (—). (b) The basal medium of alloxan plus 4 mg. peptone N/100 ml., alloxan (14.2 mg./100 ml.) and 4-amino-1:2-dimethyl-5-(D-1'-ribitylamino)benzene (I) (7.2 mg./100 ml.), uninoculated but incubated at 28° for 5 days. (—●—). (c) Same as (b) but inoculated with *E. ashbyii* at 28° for 5 days (—). (d) The basal medium without alloxan and I, inoculated and incubated for 5 days at 28° at same time as (a) and (b) (---). (e) The difference spectrum of (c) and (d). (—●—).

Table 6. *The effect of some uncommon purines either alone or in combination with DL-threonine on growth and flavinogenesis in Eremothecium ashbyii*

Amounts produced in 15 ml. basal medium plus 4 mg. peptone N/100 ml. and a purine at a level of 4 mg. N/100 ml. DL-Threonine added at a level of 2 mg. N/100 ml. 5-day cultures; temp. 28°; 2 experiments, each value is the mean of those with triplicate flasks.

Purine added	Dry wt. (% of control)				Riboflavin (% of control)			
	A		B		A		B	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
In absence of threonine								
2-Aminopurine	119	117-121	110	108-118	129	128-130	97	95-100
2-Hydroxypurine	122	117-126	106	95-113	151	146-158	99	91-119
2-Methylhypoxanthine	93	72-113	—	—	191	182-199	133	121-145
2-Methyladenine	—	—	—	—	—	—	142*	—
Adenine	127	117-139	119	114-124	209	190-230	194	168-209
In presence of threonine								
2-Aminopurine	127	123-135	—	—	208	174-226	—	—
2-Hydroxypurine	140	131-150	—	—	175	158-192	—	—
2-Methylhypoxanthine	93	—	—	—	116	111-121	84	69-108
2-Methyladenine	—	—	—	—	—	—	96	88-108
Adenine	100	95-106	—	—	—	—	249	232-266
Threonine alone	95	95-96	—	—	146	130-162	145	140-154

* Only one determination.

— Signifies value not determined.

stimulates flavinogenesis in *E. ashbyii* (Goodwin & Pendlington, 1954*b*); the possibility that glycine + formate might also be active has now been ruled out; ethanolamine, another compound related to serine, is, however, stimulatory, although to a lesser degree than serine (Table 7).

Of possible threonine metabolites and related compounds, lactate, α -oxobutyrate, α -hydroxybutyrate, $\alpha\beta$ -dihydroxybutyrate, α -aminobutyrate and *n*-butyrate, when tested at a level equivalent to 3 mg. threonine N/100 ml., were non-stimulatory; pyruvate, however, produced a definite stimulation, although it was less than that obtained using an equivalent amount of threonine. The stimulatory effect of pyruvate was also observed in the

basal medium, growth is reduced to the same level as that obtained in the absence of both glucose and acetate, indicating that acetate is probably not utilized; riboflavin synthesis is also reduced and to a much greater extent than is growth (Table 10). The mycelia produced under these conditions are almost completely white.

A number of experiments have been carried out to see if these colourless mycelia produced in the absence of glucose would, as 'washed suspensions', synthesize riboflavin. Table 11 shows that riboflavin is synthesized (this is quite obvious even visually) in the presence of glucose and that synthesis appears to be slightly stimulated by the presence of adenine alone or adenine plus threo-

Table 7. *The effect of compounds related to serine on riboflavin synthesis in Eremothecium ashbyii*

Basal medium plus 4 mg. peptone N/100 ml. and equimolar amounts of possible serine precursors; 15 ml. medium; 5-day cultures; temp. 28°; triplicate determinations.

Precursor added	Amount (mg./100 ml.)	Dry wt. (mg.)		Riboflavin		
		Mean	Range	(μg./15 ml.)		(g./100 g. dry mycelium)
				Mean	Range	Mean
None (control)	—	9.2	7.4–10.8	52	40–60	0.57
Glycine	37.5	11.8	10.6–13.6	68	65–70	0.58
Sodium formate	25.0	8.0	7.6–10.6	50	45–55	0.62
Glycine + sodium formate	37.5 25.0	10.2	8.9–11.6	61	57–65	0.59
Ethanolamine hydrochloride	25.0	10.3	8.9–11.5	93	85–97	0.91
DL-Serine	25.0	7.6	6.6–8.4	173	160–175	2.25

Table 8. *The effect of pyruvate on riboflavin synthesis in Eremothecium ashbyii*

Basal medium plus 4 mg. peptone N and either 3.5 mg./100 ml. L-threonine N (added as twice the amount of DL-threonine), an equimolar amount of pyruvate or 10 mg./100 ml. adenine N, separately or in combination; 15 ml. medium; 5-day cultures; temp. 28°; triplicate analyses.

Compounds added to basal medium	Dry wt. of mycelium (mg.)		Riboflavin		
	Mean	Range	(μg./15 ml.)		(g./100 g. dry mycelium)
			Mean	Range	Mean
None (control)	13.2	12.2–14.1	134	130–142	1.02
Pyruvate	16.3	16.1–16.8	165	145–185	1.01
Adenine	15.7	14.5–17.3	269	237–325	1.71
DL-Threonine	18.5	17.2–19.8	230	205–245	1.25
Pyruvate + adenine	16.9	15.8–18.1	329	290–365	1.95
Pyruvate + DL-threonine	18.8	18.1–20.3	230	217–247	1.22

presence of adenine but not threonine (Table 8). Acetate, on the other hand, examined at the same level was always slightly inhibitory and this was also apparent in the presence of threonine and adenine (Table 9; the high dry weights in this experiment are unusual). The inhibitory effect of acetate has been followed at different concentration and Fig. 7 shows that riboflavin production is progressively decreased although growth is unaffected. The final pH of the medium is slightly increased in the presence of acetate. Furthermore, when acetate completely replaces glucose in the

nine, but not in the presence of threonine alone. The amounts of riboflavin synthesized are, however, so small compared with normal synthesis that this type of experiment appears at the moment to be of no great value.

DISCUSSION

Action of the 4:5-diaminopyrimidines

The incorporation of adenine into riboflavin in *E. ashbyii* which is accompanied by the loss of C₈ (McNutt, 1954) cannot be the simple incorporation

Table 9. *The effect of acetate on riboflavin synthesis in Eremothecium ashbyii*

Basal medium + 4 mg. peptone N/100 ml. and plus 3.5 mg./100 ml. L-threonine N (added as twice the amount of DL-threonine) or an equimolar amount of acetate or 10 mg./100 ml. adenine N. Amount produced in 15 ml. medium; 5-day cultures; temp. 28°; triplicate analyses.

Compounds added to basal medium	Dry wt. of mycelium (mg.)		Riboflavin		
	Mean	Range	(μg./15 ml.)		(g./100 g. dry mycelium) Mean
			Mean	Range	
None (control)	20.2	19.2-21.4	156	150-167	0.77
Acetate	25.7	20.6-29.3	96	82-105	0.38
Adenine	19.8	18.0-21.6	221	190-260	1.11
DL-Threonine	20.3	18.0-24.5	176	157-210	0.87
Acetate + adenine	31.1	28.5-34.0	183	150-230	0.58
Acetate + DL-threonine	32.7	27.2-36.6	169	132-185	0.51

Table 10. *Growth and riboflavin production in Eremothecium ashbyii in absence of glucose*

Basal medium + 4 mg. peptone N/100 ml. with glucose either removed or replaced by anhydrous Na acetate (1% w/v); 15 ml. medium; 5-day cultures; temp. 28°.

Medium	Dry wt. (mg.)		Riboflavin		
	Mean	Range	(μg./15 ml.)		(g./100 g. dry mycelium) Mean
			Mean	Range	
Basal	13.9	12.6-14.9	77	67-85	0.56
Basal without glucose	4.9	3.2-7.2	9	7.5-10.0	0.19
Basal with acetate replacing glucose	2.4	1.0-3.4	7	5.0-8.5	0.26

Fig. 7. The effect of the addition of acetate to the basal medium on riboflavin synthesis and growth of *E. ashbyii*. Basal medium + 4 mg. peptone N/100 ml. 15 ml. medium, 5 day culture; temp. 28°. O, Dry wt.; ●, riboflavin; △, final pH; ×, riboflavin concn. (g./100 g. dry wt.).

of the resulting 4:5:6-triaminopyrimidine into riboflavin because this compound inhibits riboflavin synthesis. Two possible reasons for this are (a) adenine is first oxidized to xanthine, or (b) it is first converted into adenylic acid (or a related riboside

Table 11. *Riboflavin produced by washed cells of Eremothecium ashbyii*

Cells grown for 7 days on basal medium + 4 mg. peptone N/100 ml. with glucose (1% w/v) replaced by sodium acetate (1% w/v), washed and resuspended for 48 hr. in new media.

Transfer media	Riboflavin produced (μg./15 ml.)
Basal medium without glucose	None detectable
Basal medium	47, 40, 35, 35
Basal medium + adenine (15 mg. N/100 ml.)	52, 52, 60
Basal medium + DL-threonine (3 mg. N/100 ml.)	33, 40
Basal medium + adenine (15 mg. N/100 ml.) + DL-threonine (3 mg. N/100 ml.)	67, 67

or ribonucleotide) before either incorporation or oxidation to the xanthine derivative. As adenine but not xanthine overcomes the inhibition, it would seem that the reaction blocked was not adenine → xanthine, but a reaction common to both, perhaps the transfer of phosphoribose, with adenine but not xanthine being able to displace the inhibitor from the enzyme surface.

The failure of the xanthine analogue (4:5-diamino-2:6-dihydroxypyrimidine) to stimulate riboflavin synthesis would support the view that a ribityl or some similar derivative is concerned in flavin synthesis, although because of the instability of the analogue this conclusion cannot be

definite. Even taking all precautions probably little of the pyrimidine is actually presented to the organism.

The inhibition of riboflavin synthesis but not growth by 4:5:6-triamino-2-hydroxypyrimidine, compared with inhibition of both growth and riboflavin by 4:5:6-triaminopyrimidine, indicates a different site of action of the two inhibitors and suggests that although the biosynthetic pathways to purines and rings *B* and *C* of riboflavin are similar they must differ at one stage at least.

Action of aminopterin and azaxanthine

The inhibition of growth of *E. ashbyii* by aminopterin, which is counteracted by folinic acid, agrees with the established picture of the function of this inhibitor. Aminopterin appears to act in purine biosynthesis by inhibiting the incorporation of a one-carbon unit into C_2 , for Woolley & Pringle (1950) found that 4-amino-5-imidazolecarboxamide (4(5)-amino-5(4)-carbamoyl glyoxaline) (III) accumulated in aminopterin-inhibited cultures of *Escherichia coli*. No evidence has yet appeared that the probable acyclic precursor (IV), or its ribotide (Greenberg, 1953), of the imidazolecarboxamide also accumulates, although the insertion of C_8 to form the imidazole is also mediated through a one carbon fragment. This may, however, be due only to experimental difficulties. If increased riboflavin

synthesis in the presence of aminopterin is due to the shunt of a precursor (such as IV) common to both purines and riboflavin into the riboflavin channel, then it would appear (a) that insertion of C_8 in the purine was more sensitive to aminopterin than insertion of C_2 , (b) that perhaps IV combines with the *o*-xylene precursor of riboflavin to form V, and that the addition of C_2 to this compound is quite insensitive to aminopterin. Even if this explanation is not experimentally substantiated,

the effect of aminopterin again emphasizes that differences exist between the synthesis of purine and rings *B* and *C* of riboflavin.

Azaxanthine acts in the opposite sense to aminopterin in decreasing riboflavin synthesis but not growth; the separation of riboflavin from purine synthesis (growth) is again evident. The failure of azaxanthine to inhibit growth contrasts with its effect on *Lactobacillus casei* (Elion, Singer, Hitchings, Balis & Brown, 1953), but agrees with the report that it is of little value as a carcinostat (Hirschberg, Kream & Gellhorn, 1953). The observation that it inhibits adenine stimulation of riboflavin synthesis to a greater extent than that of xanthine is explicable on structural grounds, although Elion, Singer & Hitchings (1953) found adenine more effective than xanthine in reversing azaxanthine inhibition of the growth of *L. casei*.

Possible metabolites of serine and threonine

The ability of ethanolamine to stimulate riboflavin synthesis indicates that it may be converted into the same riboflavin-stimulatory fragment as serine. Glycine + formate, however, cannot do this.

Of the metabolites of serine and threonine examined, only pyruvate stimulated flavinogenesis and its stimulation was less than that of equimolar amounts of the amino acids. As pyruvate was also effective in the presence of adenine but not threonine, these observations tend to confirm the suggestion that serine and threonine provide C units rather than C, N units for riboflavin synthesis (Goodwin & Pendlington, 1954b). It remains for isotope studies finally to decide this point.

Acetate is interesting in that although it is not apparently utilized for growth it does inhibit riboflavin synthesis. The reason for this is not apparent: it may be related to its slight buffering action on the medium so that the final pH is around 6.0 instead of 5.0. The studies of Hickey (1953) would suggest, however, that riboflavin synthesis is not particularly sensitive to pH changes. The present results with acetate agree with the isotope studies of Klungsøyr (1954a) in which only 0.01% of the added acetate was recovered as riboflavin. Plaut (1954b) does not record the efficiency of acetate incorporation into riboflavin in his experiments on the closely related *A. gossypii*.

SUMMARY

1. 4:5-Diamino-2:6-dihydroxy-, 4:5-diamino-2-hydroxy- and 4:5-diamino-2:6-dihydroxy-pyrimidine, at a level of 8 mg. N/100 ml., have little effect on either growth or flavinogenesis by *Eremothecium ashbyii*. 4:5:6-Triaminopyrimidine (I) inhibits growth and riboflavin synthesis equally (to about 50–60%), whilst 4:5:6-triamino-2-hydroxypyrimi-

dine (II) inhibits riboflavin synthesis by about 65 % but has little effect on growth. Compound (I) also inhibits riboflavin stimulation by adenine and xanthine and (II) that by xanthine only.

2. Concentrations of aminopterin up to 2 $\mu\text{g./100 ml.}$ inhibit growth but stimulate flavinogenesis considerably. Higher concentrations inhibit growth and flavinogenesis equally. Stimulation of flavinogenesis by adenine and threonine was not affected by 20 $\mu\text{g./100 ml.}$ aminopterin but is somewhat inhibited by a level of 200 $\mu\text{g./100 ml.}$ Folinic acid (leucovorin) is more effective than folic acid in overcoming the growth inhibition of aminopterin.

3. Azaxanthine inhibits riboflavin synthesis but not growth in *E. ashbyii*. Riboflavin stimulation by adenine is inhibited to a greater extent than that by xanthine.

4. Alloxan and 4-amino-1:2-dimethyl-5-(D-1'-ribitylamino)benzene together do not stimulate flavinogenesis, although they react spontaneously in the culture medium to form, not riboflavin, but what is probably the related anil.

5. 2-Amino- and 2-hydroxy-purine do not stimulate riboflavin synthesis markedly, whilst 2-methyladenine and 2-methylxanthine have a somewhat greater effect. None of these affects growth, but the first two inhibit the stimulation of flavinogenesis by threonine.

6. Pyruvate was the only non-nitrogenous compound related to serine or threonine which stimulated riboflavin synthesis. Ethanolamine, but not glycine + formate, was also active. *E. ashbyii* does not grow on acetate alone and, in the presence of glucose, acetate considerably inhibits riboflavin synthesis.

7. Washed colourless cells of *E. ashbyii* produced by culturing in the absence of glucose synthesize small amounts of riboflavin.

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Spectrophotometric Studies of the Cytochrome System of Heart Muscle

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Until quite recently, study of the cytochrome system has largely depended upon the use of the visual low-dispersion spectroscope. The classical observations of Keilin (1925) were made with this instrument, and many subsequent studies of respiratory enzyme systems have been based on its use (e.g. Ball, 1938; Keilin & Hartree, 1939, 1940; Slater, 1949, 1950; Tsou, 1951). Effects such as the

absence or presence of particular absorption bands under different conditions, or large differences in the kinetics of their appearance or disappearance may be satisfactorily investigated by this method. It has also been found possible to translate some of the effects observed into roughly quantitative terms by employing the double-wedge trough and optical system described by Keilin & Mann (1941).