British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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Studies on the Biosynthesis of Riboflavin

4. PURINE METABOLISM AND RIBOFLAVIN SYNTHESIS IN *EREMOTHECIUM ASHBYII**

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(Received 15 May 1957)

It is now well established that purines stimulate the biosynthesis of riboflavin in the fungus *Eremothecium ashbyii* (MacLaren, 1952; Goodwin & Pendlington, 1954), and experiments with isotopically labelled adenine indicate that this is due to the direct incorporation of purine after loss of C₆ (McNutt, 1954).

Experiments with labelled glycine, formate and serine also indicate that rings B and C of riboflavin are built up in a very similar pattern to the purine rings (Plaut, 1953; 1954*a, b*; Goodwin & Jones, 1956). Naturally occurring pyrimidines do not stimulate flavinogenesis (Brown, Goodwin & Pendlington, 1955).

Goodwin & Pendlington (1954) found that xanthine and adenine both stimulated flavino-

genesis and that xanthine was the more efficient. As xanthine is structurally the purine most closely related to riboflavin, it is likely that other purines are active by virtue of their conversion into xanthine. One would expect that the first step in the conversion of adenine would be the formation of hypoxanthine, for an adenase (adenine deaminase) has been observed in a number of micro-organisms, especially fungi (Di Carlo, Schultz & McManus, 1951; Roush, 1954). The hypoxanthine produced would then be converted into xanthine by xanthine oxidase. Preliminary experiments showed, however, that hypoxanthine is much less active than adenine and thus would appear to be ruled out as an intermediate.

Because of these somewhat unexpected results it was decided (a) to extend the number of purines investigated to include hypoxanthine, uric acid and guanine, the ribosides adenosine and inosine, and the ribotide adenosine-3'-phosphate; (b) to follow the metabolism of these compounds during their incorporation into riboflavin, and (c) to examine the effect on riboflavin synthesis of certain purine antagonists and inhibitors of purine synthesis.

A brief report of some of these findings has already appeared (Brown, Goodwin & Jones, 1956).

* Part 3: Goodwin & Jones (1956).

EXPERIMENTAL

Cultures. The strongly flavinogenic strain of *Eremothecium ashbyii* used throughout the investigation was kindly supplied by Mrs J. Robson (Atomic Energy Research Establishment, Harwell). Apart from yielding more riboflavin it behaved towards purines in the same way as our original strain (Goodwin & Pendlington, 1954).

Media. The basal medium was that described previously (Goodwin & Pendlington, 1954). Appropriate amounts of bacteriological peptone (Evans) and the compounds under test were added to the medium, which was adjusted to pH 5.8 and generally sterilized by autoclaving at 15 lb./in.². When the compounds in use were known to be unstable to autoclaving, the media were sterilized by filtration.

Materials. The naturally occurring purines, their ribosides and ribotides were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. They were checked for purity by paper chromatography. The azapurines were a gift from Dr R. E. F. Matthews (Molteno Institute, Cambridge); azaserine and the triazoles were a gift from I.C.I. Pharmaceuticals Ltd. (Dr A. W. Crowther); the substituted pyrimidines and 6-mercaptapurine were a gift from the Wellcome Foundation (Dr L. G. Goodwin); the antibiotics were a gift from The Lederle Laboratories (Dr F. C. Ottati) and riboflavin and isoriboflavin were a gift from Roche Products Ltd. (Dr A. L. Morrison).

Cultural conditions. These have been previously described in detail (Goodwin & Pendlington, 1954).

Determination of dry weight and riboflavin. The methods employed have been described (Goodwin & Pendlington, 1954). Analyses were carried out in triplicate. The reproducibility of results with *E. ashbyii* was given in detail by Goodwin & Pendlington (1954) and Brown *et al.* (1955), and in order to conserve space the scatter of the results will not be given in the tables to follow.

Paper chromatography. The media to be examined were spotted directly on to Whatman no. 1 paper and chromatographed in a descending system, either propan-2-ol (170 ml.)—conc. HCl (sp.gr. 1.19; 41 ml.)—water (to 250 ml.) or butan-1-ol—acetic acid—water (4:1:5, by vol.) being used as solvent system. The former is recommended for separation

of purines, nucleosides and nucleotides (Wyatt, 1951) and the latter for riboflavin and its derivatives (Crammer, 1948). It was found that both solvent systems gave good separations of both groups of compounds; they could therefore be used for checking the identity of the various components of the media. The chromatograms were developed for about 20 hr. in a tank darkened to prevent photolysis of riboflavin and its derivatives. After removal from the tanks the sheets were dried in the dark in a current of warm air for 15 min. The purines and their ribosides or ribotides were located with ultraviolet light from a low-pressure Hg lamp emitting mainly the Hg line at 256 mμ. The compounds show up as dark spots on a blue-white background (Wyatt, 1955). Riboflavin and its derivatives were also visualized by ultraviolet light, but in this case a high-pressure Hg lamp (Hanovia) emitting mainly the Hg line at 365 mμ was used. Under this lamp the flavins fluoresce bright yellow or bright blue.

Elution of the purine spots was carried out with 0.1 N-HCl by the method of Dent (1947); the flavin derivatives were similarly eluted with water. These eluted spots were then examined spectrophotometrically, the eluate from a blank piece of chromatography paper of the same area and *R_F* as the spot under consideration being used in the compensating cell.

Spectrophotometric determination of adenine and hypoxanthine in the presence of riboflavin

Kalckar (1947) showed that in a system in which adenine is being converted into hypoxanthine without any net change in total purine, the amount of hypoxanthine formed can be calculated from measurements of the changes in extinction (ΔE) at 265 and 240 mμ. The production of 1 μg. (0.0074 μmole) of hypoxanthine/ml. of solution is indicated by $\Delta E_{265 \text{ m}\mu}$ (1 cm.) - 0.059 and $\Delta E_{240 \text{ m}\mu}$ (1 cm.) + 0.032. This calculation cannot be directly applied when, as in the metabolism of adenine by *E. ashbyii*, (a) interference in the region 240–265 mμ is caused by another substance, and (b) the total purine concentration is falling. In the present case, chromatographic analysis and absorption-spectral measurements of media not containing purines showed that riboflavin was the only interfering substance. Allowance for

Table 1. *Spectrophotometric determination of adenine and hypoxanthine in the presence of riboflavin*

Initial adenine concn. 0.875 mM ($\equiv$ 0.875 μmole/ml.). See Experimental section for details of calculation.

Medium	10 ⁻¹ E values			
	240 mμ	251 mμ	265 mμ	445 mμ
1. Uninoculated	0.560	0.985	1.105	—
2. Culture (5 days)	0.642	0.984	0.981	0.0260
3. Culture (5 days), corrected for riboflavin	0.619	0.945	0.914	—
4. Values calc. for (3) assuming only adenine present	0.536	0.945	1.062	—
ΔE	0.083	—	- 0.148	—

Adenine metabolized to compounds other than hypoxanthine = $[(0.985 - 0.945)/0.985] \times 100 = 4.1\%$.

Amount of hypoxanthine produced (μmole/ml.) =

$$\left. \begin{aligned} (a) & (0.083 \times 10 \times 0.0074)/0.032^* = 0.191 \\ (b) & (-0.148 \times 10 \times 0.0074)/-0.059^* = 0.186 \end{aligned} \right\} 0.188.$$

Adenine converted into hypoxanthine = $(0.188/0.875) \times 100 = 21.5\%$.

Therefore adenine unchanged = 74.4%.

* Values given by Kalckar (1947).

riboflavin absorption in the 240–265 $m\mu$ region can be made by measuring $E_{445\ m\mu}$ (the maximum of riboflavin absorption in the visible region of the spectrum) and from the absorption spectrum of pure riboflavin, calculating its contribution to the observed $E_{240-265\ m\mu}$ values.

As hypoxanthine and adenine exhibit an isosbestic point at 251 $m\mu$ at the pH of the medium (5.8), any difference between $E_{251\ m\mu}$ corrected for riboflavin absorption and $E_{251\ m\mu}$ of the uninoculated medium represents the amount of adenine which has been metabolized but *not* to hypoxanthine. The $E_{240\ m\mu}$ and $E_{265\ m\mu}$ values corresponding to $E_{251\ m\mu}$ (corrected), if all the purine remaining were adenine, can be calculated, and consequently the amount of hypoxanthine ($\mu\text{g./ml.}$) formed is given by $E_{265\ m\mu}(\text{corr.}) - E_{265\ m\mu}(\text{calc.})/0.059$, or $E_{240\ m\mu}(\text{corr.}) - E_{240\ m\mu}(\text{calc.})/0.032$. An example (Table 1) will illustrate the steps in the calculation. A similar calculation can be made for the adenosine-inosine system. After making allowances for the various corrections to be applied, the method is probably accurate to $\pm 5\%$.

Spectrophotometric measurements were carried out with the Unicam SP. 500 and SP. 600 instruments.

RESULTS

Relative efficiency of purines and purine derivatives. Previously the flavinogenic activity of only xanthine and adenine had been reported (Goodwin & Pendlington, 1954). These purines have now been

compared with hypoxanthine, guanine and uric acid. Adenosine has been re-examined because the observation of McNutt (1954), that adenosine is less effective than adenine, was not in agreement with our previous results (Goodwin & Pendlington, 1954). Adenosine-3'-phosphate and inosine were also tested.

Table 2 gives the results of a typical series of experiments. The important points in regard to flavinogenic activity are (a) guanine is better than xanthine, (b) hypoxanthine, inosine and uric acid are much poorer than adenine, and (c) adenosine and adenosine 3'-phosphate are very similar to adenine. Furthermore, none of the compounds tested stimulates growth. The weak action of hypoxanthine and uric acid tend to support the results of MacLaren (1952), but the observations with guanine, which were repeated many times, are unexpected but confirm a single observation of McNutt (1954). As *E. ashbyii* stores riboflavin in its mycelium as well as excreting it into the medium, a possible explanation of the quantitative differences between the purines was the differential accumulation of riboflavin in the mycelium in the presence of certain members. Table 3 shows that the riboflavin present in the mycelium remains constant over the

Table 2. *Effect of various purines, nucleosides and nucleotides on flavinogenesis by E. ashbyii*

Basal medium + 4 mg. of peptone N/100 ml. and 0.37 mM-purine.* 5-day cultures, static; temp. 28°; triplicate determinations: amounts were produced in 15 ml. of medium in 50 ml. conical flasks.

Purine added	Mycelium dry wt. (mg.)	Riboflavin	
		Total ($\mu\text{g.}$)	Amount (g./100 g. of dry mycelium)
None (control)	12.0	583	4.86
Adenine	12.5	920	7.35
Hypoxanthine	12.5	742	5.93
Xanthine	11.8	930	7.87
Guanine	12.2	1010	8.27
Uric acid	12.8	715	5.58
Adenosine	13.0	907	6.97
Inosine	12.9	723	5.32
Adenosine-3'-phosphate	13.0	870	6.69

* This value was chosen because it represents about the maximal solubility of guanine in the basal medium.

Table 3. *Riboflavin distribution in mycelium and medium in cultures of E. ashbyii grown in the presence of various purines, nucleosides and nucleotides*

Experimental details were as given in Table 2.

Purine added	Riboflavin ($\mu\text{g.}$)			
	In medium	In mycelium	Total	% of total in mycelium
None (control)	340	104	444	23
Adenine	575	147	722	20
Hypoxanthine	458	147	605	24
Xanthine	578	146	724	20
Guanine	652	139	791	18
Uric acid	450	130	580	22
Adenosine	572	135	707	19
Inosine	451	124	575	22
Adenosine-3'-phosphate	533	122	655	19

range 18–24% of the total, irrespective of the purine present. This confirms the observations of Goodwin & Pendlington (1954) made with other media, that the percentage of riboflavin in the mycelium is reasonably constant, although with their original strain of *E. ashbyii* it tended to be somewhat higher than that observed with the present strain. Dry weights could not be recorded in this experiment because the mycelia are destroyed by hydrolysis with *N*-HCl to liberate the bound riboflavin. Table 3 also confirms the results given in Table 2 concerning the relative efficiencies of the various purines.

*Metabolism of adenine and adenosine
by E. ashbyii*

Cultures of *E. ashbyii* metabolizing adenine and adenosine were examined chromatographically on paper and, apart from the original compounds, spots corresponding to hypoxanthine and inosine respectively were detected. The identity of these two compounds was confirmed by mixed chromatography with authentic specimens in two solvent systems (see Experimental) and by their absorption spectra after elution from the paper. Apart from large amounts of riboflavin, traces of lumichrome and flavinadenine dinucleotide, no other fluorescing or ultraviolet light-absorbing spots were detected. Specially significant was the absence of any trace of xanthine or xanthosine.

The spectrophotometric method for determining adenine and hypoxanthine in the presence of riboflavin was applied in a series of experiments involving media containing varying amounts of adenine. The results of a typical experiment given in Table 4 show that the percentage of 'adenine metabolized to compounds other than hypoxanthine' (ANH) is high at low initial concentrations and gradually decreases with increasing adenine concentrations; simultaneously, with the exception of a drop at 0.3 mM, the percentage of adenine not attacked also increases with increasing adenine concentrations. The percentage of adenine converted into hypoxanthine reaches a maximum at an adenine level of about 0.3 mM. This tendency, in association with a low residual adenine level, was observed in all similar experiments but the actual adenine level at which the peak was observed varied from 0.3 to 1 mM. When the amounts of adenine

metabolized (Fig. 1) are plotted it is clear that (a) the amount of hypoxanthine formed and the ANH fraction both tend to become constant in the region 0.3–0.6 mM-adenine, (b) the amount of unchanged adenine increases regularly with increasing adenine concentration, (c) the curve for riboflavin production tends to follow the curves for hypoxanthine and ANH, but above 0.6 mM-adenine it corresponds more closely to the ANH curve. The correspondence between the riboflavin and ANH curves is much more marked with adenosine (see later). It is specially interesting that riboflavin stimulation and ANH both tend to level out in the region 0.3 mM-adenine. This corresponds to maximal relative formation of hypoxanthine. In retrospect, this tendency for riboflavin stimulation to slow down in the region corresponding to 0.3 mM-adenine can be seen in our original results (Goodwin & Pendlington, 1954).

The efficiency of the conversion of adenine into riboflavin, as measured by the percentage of the

Fig. 1. Effect of concentration on the metabolism of adenine by *E. ashbyii*. Experimental conditions were as described in Table 2. O, Riboflavin in medium; x, ANH; ●, hypoxanthine produced; ▲, residual adenine.

Table 4. Influence of initial adenine concentration on adenine metabolism by *E. ashbyii*

Experimental conditions were as given in Table 2.

Initial concn. of adenine (mM) ...	Percentage converted				
	0.15	0.30	0.45	0.60	1.2
Conversion into hypoxanthine	28	64	43	31	43
ANH	57	34	28	29	19
Unchanged	15	2	29	40	38

ANH fraction which can be accounted for as riboflavin, is given in Table 5. On the assumption that the ANH fraction is incorporated into all the riboflavin produced (McNutt, 1954), the efficiency drops from essentially 100% at the lowest adenine level to 55% at the 0.6 mM level; the apparent increased efficiency at the 1.2 mM level reflects the increase in riboflavin synthesis after the plateau region is passed. If ANH is assumed to contribute only to that riboflavin produced above the basal level, then apart from one anomalous result at the lowest adenine concentration, the incorporation varies between 11 and 37% over the concentration range studied.

When the metabolism of adenosine is compared with that of adenine on an equimolar basis over a range of concentrations it will be seen (Table 6; Fig. 2) that the riboside is always completely metabolized, little or no residual adenosine ever being detected; this is due to the greater production of inosine. The great similarity in both cases of the amounts not converted into hypoxanthine or inosine accounts for the equal flavinogenic activity of equimolar amounts of the free base and of its riboside. The good correspondence between the fraction not metabolized and riboflavin production is also well demonstrated in Fig. 2. The reason for the greater conversion of adenosine into inosine compared with the formation of hypoxanthine from adenine is not yet obvious.

Table 5. *Efficiency of incorporation of adenine into riboflavin by E. ashbyii*

Calculated from data in Table 4 and Fig. 1 and based on total riboflavin in medium and mycelium, that in the mycelium being assumed to be 20% of that in the medium (Table 3).

Initial concn. of adenine (mM)	Incorporation of ANH into riboflavin (%)	
	Assuming incorporation into all riboflavin produced	Assuming incorporation only into riboflavin produced in excess of that found in normal cultures
0.15	118	28
0.30	105	29
0.45	76	14
0.60	55	11
1.20	70	37

Effect of acetate on adenine metabolism

The presence of acetate in the basal medium is known to inhibit flavinogenesis by *E. ashbyii* (Brown *et al.* 1955). An investigation into the effect of the presence of acetate on adenine metabolism showed that, compared with the normal medium, the situation is markedly changed (Table 7). The relative amounts of unchanged adenine and ANH are considerably increased and decreased respectively, whilst the percentage converted into hypoxanthine is greatly increased. The actual amounts are given in Fig. 3 (cf. Fig. 1) from which it can be seen that only a trace is not accounted for as either unchanged adenine or hypoxanthine. The failure of the purine to stimulate flavinogenesis in the presence of acetate is also clearly demonstrated.

Fig. 2. Effect of concentration on the metabolism of adenosine by *E. ashbyii*. Experimental conditions were as described in Table 2. O, Riboflavin in medium; ×, adenosine metabolized but not to inosine; ▲, inosine produced. No significant amounts of residual adenosine were noted at any concentration.

Table 6. *Influence of initial adenosine concentration on adenosine metabolism by E. ashbyii*

Experimental conditions were as given in Table 2.

Initial concn. of adenosine (mM) ...	Percentage converted				
	0.15	0.30	0.45	0.60	1.2
Adenosine converted into inosine	28	69	69	79	97
Adenosine metabolized but not to inosine	63	34	35	26	10
Unchanged adenosine	9	0*	0*	0*	0*

* In all these cases the sum of the first two fractions slightly exceeded 100.

Metabolism of other purines in relation to flavinogenesis in E. ashbyii

No purine metabolites were detected by paper chromatography of cultures metabolizing hypoxanthine, xanthine and guanine. Spectroscopic measurements indicated that these purines gradually disappear from the culture medium, the last to

the greatest extent (Table 8). When the amounts of the purines metabolized are taken into account, their flavinogenic activities are very similar. It also appears that, at low concentrations, McNutt's view that exogenous purines are incorporated directly into riboflavin may not be correct, for the experiments recorded in Table 8 indicate 100 % incorporation into riboflavin, although no account has been taken of the riboflavin remaining in the mycelium (generally about 20 %, see Table 3).

Effect of 8-azapurines on growth and flavinogenesis of E. ashbyii

The action of a number of 8-azapurines has been examined in order to see if some light would be thrown on the metabolism of purines in relation to flavinogenesis. To conserve space the results are combined and values recorded as percentages of the basal values obtaining in each particular experiment (Brown *et al.* 1955). Fig. 4 shows that (a) 8-aza-adenine tends to inhibit flavinogenesis specifically (e.g. at 0.125 mg./100 ml. growth is only slightly inhibited whilst riboflavin production is inhibited to the extent of 75 %) and (b) 8-azaguanine and 8-azahypoxanthine inhibit both growth and flavinogenesis equally, although the 8-azahypoxanthine effect is very much less marked and even at a concentration of 40 mg./100 ml. it exerts only a 40 % inhibition. The 8-aza-adenine effect is similar to that previously recorded for 8-azaxanthine (Brown *et al.* 1955), although the latter is much less toxic. A concentration of nearly 70 mg./100 ml. of 8-azaxanthine is required before slight inhibition of growth is observed. Up to its maximal solubility

Fig. 3. Effect of acetate on the metabolism of adenine by *E. ashbyii*. Experimental details were as described in Table 7, with acetate added at a level of 0.1 M. O, Riboflavin levels; ×, ANH; ●, hypoxanthine produced; ▲, residual adenine.

Table 7. *Effect of acetate on purine metabolism in E. ashbyii*

Experimental conditions were as given in Table 2, except that 0.8 % (w/v) sodium acetate (anhydrous) was added to the basal medium.

Initial concn. of adenine (mM) ...	Percentage conversion			
	0.112	0.195	0.353	0.60
Conversion into hypoxanthine	57.6	42.1	32.2	22.1
ANH	14.6	4.9	3.8	2.6
Unchanged	27.8	53.0	64.0	75.3

Table 8. *Metabolism of xanthine, hypoxanthine and guanine by E. ashbyii*

Experimental conditions were as given in Table 2; purines were added to medium at a level of 0.37 mM.

	Purine added		
	Hypoxanthine	Xanthine	Guanine
Riboflavin content* (μg./15 ml.)	510	580	665
Purine remaining (%)	66	47	30
Purine utilized incorporated into total riboflavin (%)	107	87	96
Purine utilized incorporated into riboflavin produced above basal level (%)	21	20	25

* Basal level 315 μg./15 ml.

(0.5 mg./100 ml.) 8-azaisoguanine had no inhibitory effect on either growth or flavinogenesis.

With levels of 8-aza-adenine (0.125 mg./100 ml.) and 8-azaguanine (1 mg./100 ml.) which almost completely inhibit growth and flavinogenesis, the presence of the corresponding purine reverses both effects to a considerable extent (Tables 9 and 10). The inhibitory effect of 8-aza-adenine is also reversed by xanthine and, perhaps somewhat unexpectedly, by hypoxanthine. Although in the experiment recorded in Table 9 the purines were used at a level of 20 mg./100 ml., it has been found that concentrations as low as 5 mg./100 ml. are equally effective; this level of guanine is also effective against 8-azaguanine (Table 10). In none of the experiments discussed in this section was there any detectable metabolism of the 8-azapurines.

Action of 6-mercaptopurine

6-Mercaptopurine, a purine inhibitor in *Lactobacillus casei* (Elion, Hitchings & Vanderwerff, 1951), when tested up to the level of maximum solubility (2 mg./100 ml.) had no effect on growth of *E. ashbyii* but stimulated riboflavin synthesis (Table 11). The organism can presumably remove the sulphhydryl group from this compound.

Fig. 4. Effect of certain 8-azapurines on growth and riboflavin synthesis in *E. ashbyii*. Experimental conditions were as described in Table 2; the values are recorded as the percentages of the appropriate control values. ●, Riboflavin; ○, dry weight; A, 8-aza-adenine; B, 8-azaguanine; C, 8-azahypoxanthine.

Table 9. Effect of 8-aza-adenine on riboflavin production in *E. ashbyii* in the presence of excess of purines

Experimental details were as given in Table 2; 8-aza-adenine was added at a level of 0.125 mg./100 ml., the purines at a level of 20 mg./100 ml.

Purine added to medium	Mycelium dry wt. (mg.)	Riboflavin (μg./15 ml.)
None (control)	16.2	271
8-Aza-adenine	7.3	99
Adenine	16.3	550
Xanthine	16.9	555
Hypoxanthine	15.3	475
8-Aza-adenine + adenine	16.9	434
8-Aza-adenine + xanthine	14.6	506
8-Aza-adenine + hypoxanthine	15.6	464

Table 10. Effect of 8-azaguanine in the presence and absence of guanine on growth and flavinogenesis by *E. ashbyii*

Experimental conditions were as described in Table 2. Guanine was added at a level of 5 mg./100 ml., 8-azaguanine at a level of 1 mg./100 ml.

Purine added	Mycelium dry wt. (mg.)	Riboflavin (μg./15 ml.)
None (control)	65.5	230
Guanine	60.1	435
8-Azaguanine	2.8	62
8-Azaguanine + guanine	—	305

Table 11. Effect of 6-mercaptopurine on flavinogenesis in *E. ashbyii*

Experimental details were as given in Table 2.

Concn. of 6-mercaptopurine (mg./100 ml.)	Dry wt. (mg.)	Riboflavin (μg./15 ml. of medium)
0	17.1	195
0.10	17.5	185
0.25	16.9	239
0.50	16.9	236
1.0	17.5	255
2.0	16.8	282

Action of azaserine

Azaserine, which is reported to function by preventing the incorporation of formate into purines (Skipper, Bennett & Shabel, 1954), was found to inhibit growth and flavinogenesis equally, inhibition being complete at a level of 0.1 mg./100 ml. This inhibition is not overcome by DL-serine even at a level of 80 mg./100 ml. (Table 12).

Adenine, however, at a level of 20 mg./100 ml. does stimulate growth slightly and, more significantly, exerts its usual strong flavinogenic action. This action of adenine does not allow serine to

exert its usual strongly stimulatory effect (Goodwin & Pendlington, 1954) because in the presence of azaserine a mixture of adenine and serine does not stimulate riboflavin to any greater extent than does adenine alone.

Effect of certain substituted pyrimidines

As riboflavin may be synthesized from purines via a 4:5-diaminopyrimidine after the elimination of $C_{(8)}$, it was considered that pyrimidines substituted with other groupings in the 4 and 5 positions might have an inhibitory effect on flavinogenesis. A number of such compounds, which have anti-malarial activity, were examined. To conserve space, only the effects of the highest concentrations (20 $\mu\text{g./100 ml.}$; maximal solubility of most of the compounds examined) are recorded in Table 13. As the experiments were carried out at different times, the results are recorded as a percentage of the appropriate control values. It will be seen that in all cases except one, riboflavin production was inhibited to a greater extent than growth and that this was most marked in the chlorophenyl derivatives. Experiments with 2:6-diamino-4-ethyl-5-*p*-chloro-

phenylpyrimidine in the presence of xanthine, showed no significant inhibition of xanthine stimulation of flavinogenesis.

Effect of certain 1:2-dihydro-s-triazenes

These were examined after a report (Foley, Modest, Farber & Haley, 1955) that certain triazenes inhibited pteroylglutamic acid systems in a number of micro-organisms, and that there was a weak riboflavin inhibition (Foley & Haley, 1955) in *L. casei*. The basic structure of the compounds is

and the compounds examined had the following substituents: (a) R_1 , phenyl; R_2 , phenyl; R_3 , H; (b) R_1 , 4'-chlorophenyl, R_2 and R_3 methyl; (c) R_1 , 3':4'-dichlorophenyl, R_2 and R_3 methyl; (d) R_1 , 2'-chlorophenyl, R_2 and R_3 methyl; (e) R_1 , phenyl, $R_2 + R_3$, methyl. In no case, at the highest concentration examined, did any of these compounds show any significant growth or riboflavin inhibitory action. (We are grateful to Mr D. Treble for help with some of these experiments.)

Table 12. Effect of azaserine, alone and in the presence of adenine and DL-serine on flavinogenesis in *E. ashbyii*

Experimental conditions were as described in Table 2. Azaserine was used at a level of 0.05 mg./100 ml., DL-serine at 80 mg./100 ml. and adenine at 20 mg./100 ml.

Compounds added to basal medium	Dry wt. (mg.)	Riboflavin ($\mu\text{g./15 ml.}$)
1. None (control)	11.4	403
2. Azaserine	0.9*	69
3. Serine	11.1	574
4. Adenine	10.4	552
5. Serine + adenine	11.8	740
6. Azaserine + serine	3.0	56
7. Azaserine + adenine	5.7	317
8. Azaserine + serine + adenine	5.3	308

* Too small for accurate assessment.

Table 13. Effect of certain 4:5-substituted pyrimidines on growth and flavinogenesis by *E. ashbyii*

Experimental details were as given in Table 2; results are expressed as percentage of appropriate control; concn. of added pyrimidine, 20 $\mu\text{g./100 ml.}$

Substituents in 2:6-diamino-4- R_2 -5- R_1 -pyrimidine		Dry wt. (% of control)	Riboflavin (% of control)
R_1	R_2		
3:4-Dichlorophenyl	Ethyl	84	56
4-Chlorophenyl	Ethyl	89	37
4-Chlorophenoxy	Methyl	44	43
Phenoxy	Hydrogen	87	68
4-Chlorophenyl	Methyl	110	69
4-Nitrobenzyl	Methyl	78	67

isoRiboflavin

isoRiboflavin [5:6-dimethyl-9-(D-1'-ribityl)-iso-alloxazine] restricts growth of riboflavin-deficient rats more than does a simple deficiency of riboflavin itself (Emerson & Tishler, 1944), but shows no signs of inhibiting the utilization of riboflavin by *L. casei* (Foster, 1944). In the present investigation no inhibition of growth or riboflavin synthesis in *E. ashbyii* by isoriboflavin was observed.

Antibiotics

A number of antibiotics were examined in the search for a specific inhibitor of riboflavin synthesis. None of the following had such an effect (the figures in parentheses indicate the concentration range at which the antibiotic began significantly to inhibit growth): chloramphenicol (100 mg./100 ml.), puromycin (5–10 mg./100 ml.), achromycin (50–100 mg./100 ml.) and aureomycin (0.2–0.5 mg./100 ml.). Penicillin had no effect on growth even at a level of 200 mg./100 ml.

DISCUSSION

There is now no doubt that adenine, guanine and xanthine, but not hypoxanthine or uric acid, greatly stimulate flavinogenesis in *E. ashbyii* (Tables 2 and 3) and that this stimulation is due to direct incorporation of the purines after the removal

at some stage of $C_{(8)}$ (McNutt, 1954). Two questions arise from the present investigation: (a) Is the large amount of riboflavin produced by *E. ashbyii* on media not supplemented with purines due to excessive production of endogenous purines? (b) What are the steps involved in the conversion of exogenous purines into riboflavin?

With regard to (a), if it is assumed that adenine and guanine are produced in excess of normal growth requirements and that this excess is funnelled into riboflavin synthesis, then most of the recorded observations can be accounted for. For example: (i) the incorporation of ^{14}C -labelled serine into riboflavin is almost completely diluted out by adenine (Goodwin & Jones, 1956); (ii) acetate, which inhibits flavinogenesis both on non-purine and purine-containing media (Brown *et al.* 1955), also strongly inhibits the metabolism of exogenous adenine (Fig. 3 and Table 7); (iii) azaserine, which probably inhibits the synthesis of purines at the stage just before the cyclization of the glycol ribotide (Baddiley, 1955), has a general inhibitory effect both on growth and flavinogenesis with no specific action on the latter (Table 12); (iv) aza-adenine and azaguanine both inhibit riboflavin synthesis to a greater extent than growth (Fig. 4). Azahypoxanthine, on the other hand, has only very slight effect on either growth or flavinogenesis, presumably because hypoxanthine is not concerned to any significant extent in the metabolism of this organism; furthermore, exogenous hypoxanthine is in fact only weakly flavinogenic. The preferential inhibition of flavinogenesis by azaxanthine (Brown *et al.* 1956) would suggest that adenine and guanine are converted into riboflavin via xanthine, which is indeed the purine most closely related to rings B and C of riboflavin.

With regard to the mechanism of conversion of exogenous purines into riboflavin, the following possibilities exist for adenine, assuming that it is deaminated at some stage before the loss of $C_{(8)}$.

1. $\rightarrow$ hypoxanthine $\rightarrow$ xanthine	}	$\rightarrow$ 4:5-diaminouracil
2. $\rightarrow$ isoguanine $\rightarrow$ xanthine		
3. $\rightarrow$ 2:8-dihydroxyadenine $\rightarrow$ uric acid		

If it is assumed that the glyoxaline ring can be opened before deamination but that deamination occurs before the tricyclic ring system is formed, then the further possibilities arise:

2a. $\rightarrow$ isoguanine $\longrightarrow$	$\rightarrow$ 4:5:6-triamino-2-hydroxypyrimidine
3a. $\rightarrow$ 2:8-dihydroxyadenine $\longrightarrow$	$\uparrow$
4. $\rightarrow$ 4:5:6-triaminopyrimidine $\longrightarrow$	$\downarrow$
	$\rightarrow$ 4:5-diaminouracil

It is also possible that 4:5:6-triamino-2-hydroxypyrimidine might be incorporated into the riboflavin nucleus before deamination.

The results of the present investigation allow the elimination of some of these possibilities. The first possibility can be discarded because on a molar basis hypoxanthine is very much less flavinogenic

than adenine (Table 2), and in any case a considerable amount of adenine is converted into hypoxanthine (Table 4). Possible permeability effects cannot account for this difference because under comparable conditions about the same proportion of added hypoxanthine and adenine are metabolized. A small fraction of the hypoxanthine produced from adenine must obviously be converted into riboflavin, but the capacity of this route can only be very limited and most of the hypoxanthine is excreted. The second possibility could not be tested because isoguanine was not available. If, however, this pathway did take place one would expect azaisoguanine to inhibit flavinogenesis; unfortunately this is a very insoluble compound, but at the level of maximal solubility (0.5 mg./100 ml.) it had no obvious effect. It should be noted that this is a concentration above that (0.125 mg./100 ml.) at which 8-aza-adenine produces specific inhibition of flavinogenesis (Fig. 4). The preliminary conversion of adenine into 2:8-dihydroxyadenine (route 3), which occurs in animal tissues (Bendich, Brown, Phillips & Thiersch, 1950), followed by deamination to uric acid, can probably be ruled out because of the poor flavinogenic activity of uric acid (Table 2). This failure of uric acid to stimulate riboflavin synthesis cannot be due to permeability effects. That some does get into the cells is demonstrated by its slight but definite action; that being so, if it were the true precursor sufficient should have penetrated over a period of 5-10 days to produce a much greater stimulation of riboflavin synthesis. The explanation for the poor activity of uric acid is much more likely to be the same as that observed with hypoxanthine, i.e. the capacity of the route uric acid $\rightarrow$ riboflavin must be very limited. Routes 2a, 3a and 4 may be ruled out as 4:5:6-triamino-2-hydroxypyrimidine and 4:5:6-triaminopyrimidine strongly inhibit flavinogenesis (Brown *et al.* 1955). Thus adenine probably does not lose $C_{(8)}$ before its amino group but, if it is deaminated before it loses $C_{(8)}$,

hypoxanthine cannot be an intermediate. Because of its structure and its strong flavinogenic activity xanthine is the most likely intermediate; it may be produced via route 2 but there is no direct evidence for this. No xanthine has ever been detected either spectroscopically or chromatographically when *E. ashbyii* is metabolizing adenine. This is, however,

no conclusive argument against its role as an intermediate. Once it is produced in the cell it may exist only for a very short time as such and be rapidly converted into riboflavin. On losing C₈, xanthine would be converted into 4:5-diaminouracil or a related compound before conversion into riboflavin. This is such a reactive and unstable substance that its stimulatory activity could not be assessed (Brown *et al.* 1955). It can, however, be easily converted chemically into riboflavin analogues (Birch & Moye, 1957), and recently it has been shown to be a normal metabolite of *E. ashbyii* (Goodwin & Treble, 1957). However, the problem of whether xanthine and 4:5-diaminouracil are intermediates in the conversion of adenine into riboflavin can only be finally settled with isotopically labelled adenine; this is now under investigation.

In the above discussion no consideration has been given to the question of whether purines are incorporated into riboflavin as free bases or as ribosides or ribotides. Adenosine, inosine and adenylic acid exert flavinogenic effects similar to that of adenine (Tables 2 and 3). Whatever the state of the purines when being incorporated into riboflavin, *E. ashbyii* must possess a very active system for interconverting free purines and their ribosides and ribotides.

Finally, the position of guanine is interesting because on a molar basis it is more effective than the postulated obligatory intermediate xanthine. However, calculated on the amount of purine disappearing from the medium, xanthine and guanine are equally active (Table 8), and the question which arises is why they, and hypoxanthine as well, are metabolized to different degrees. As stated previously the block in hypoxanthine metabolism is probably due to its slow rate of conversion into xanthine. A possible explanation of the guanine effect is that it is deaminated to xanthine in a region of the cell which allows faster conversion of xanthine into riboflavin than do other regions reached by exogenous xanthine.

SUMMARY

1. As stimulators of riboflavin synthesis by *Ermothecium ashbyii*, purines fall into this order of decreasing effectiveness: guanine, xanthine, adenine, hypoxanthine, uric acid. Adenosine, adenosine-3'-phosphate and inosine have the same activity as their parent compounds.

2. A spectrophotometric method has been devised to determine the amount of hypoxanthine formed from adenine in a system in which the total purine concentration is changing.

3. By this method the metabolism of adenine and adenosine by *E. ashbyii* has been studied. Adenine is converted partly into hypoxanthine, partly into

riboflavin and possibly other compounds, and part remains unchanged. No xanthine could be detected. The quantitative aspects of these reactions have been studied in detail. Qualitatively adenosine behaves similarly to adenine. Quantitatively the amount converted into riboflavin is the same, but all the residue is converted into inosine; there is no unchanged adenosine remaining.

4. Acetate inhibits the metabolism of adenine.

5. Hypoxanthine, guanine and xanthine slowly disappear from the culture medium of *E. ashbyii* but no purine metabolites could be detected.

6. The effect of flavinogenesis of a number of 8-azapurines, 4:5-disubstituted pyrimidines, azaserine, 1:2-dihydro-*s*-triazenes and a number of antibiotics, has been examined.

7. A possible mechanism of conversion of purine into riboflavin has been discussed.

We are extremely grateful to the Agricultural Research Council for financial assistance, and to Miss B. M. Eales for skilled technical assistance.

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