SOME NUTRITIONAL REQUIREMENTS FOR BIOSYNTHESIS OF RIBOFLAVIN BY EREMOTHECIUM ASHBYII

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Eremothecium ashbyii Guilliermond is a remarkable organism because of its capability of synthesizing relatively large amounts of riboflavin under appropriate conditions. This vitamin is synthesized to a limited extent by a rather wide variety of microorganisms (Pridham, 1952), but those known particularly for their riboflavin producing abilities along with *E. ashbyii* are *Ashbya gossypii* (Wickerham *et al.*, 1946; Tanner *et al.*, 1948), some yeasts of the *Candida* genus (Tanner *et al.*, 1945), and certain clostridia (Meade *et al.*, 1947).

Most microorganisms which require riboflavin for growth are satisfied with less than 1 μ g per ml. Some strains of *E. ashbyii* are capable of producing in excess of 1,000 μ g per ml under proper conditions, and yields of this order are being obtained currently in industry.

E. ashbyii, a cotton parasite, was described first by Guilliermond (1935) and by Guilliermond, Fontaine, and Raffy (1935) who observed the presence of a yellow pigment which sometimes crystallized in the vacuoles of the hyphae.

Data regarding the nutritional requirements of *E. ashbyii* have not been entirely in agreement. Schopfer (1944) reported biotin to be essential for growth while thiamin and *i*-inositol were indicated as supplementary factors. In contrast, Dulaney and Grutter (1950) found that if certain amino acids were present in concentrations above 0.1 per cent, then only *i*-inositol was essential. Yaw (1952) has supported Dulaney and Grutter (1950) on the essentiality of inositol. Phytin can replace inositol under certain conditions (Minoura, 1950a). The role of a number of amino acids was studied also by the above investigators and by Schopfer and Guilloud (1945a). It does not appear that stimulation of growth of the organism and riboflavin formation are parallel (Minoura, 1950b).

Unidentified nutritional factors evidently exist for *E. ashbyii* (Schopfer, 1944; Schopfer and

Guilloud, 1945a). A heat-labile factor, present in milk products, was reported by Desieve (1947). The fact that lipids are significant in the metabolism of *E. ashbyii* has been reported (Rudert, 1945; Phelps, 1949b; Hickey, 1952); however, Yaw (1952) found no advantage for oleic acid under the conditions employed. MacLaren (1952) studied the effects of purines and pyrimidines on growth and riboflavin formation and found that xanthine particularly promoted the formation of riboflavin in contrast to Yaw's (1952) findings, while uracil was suppressive.

Cultural variations of *E. ashbyii* have been described in some detail by Schopfer and Guilloud (1945b). These consisted mainly of the yellow, flavin producing variants, a poorly sporulating, cream-colored form which produced relatively little flavin, and white forms which produced only traces.

It is the purpose of this paper to present data on certain of the nutritional requirements of *E. ashbyii* other than for biotin, thiamin, and *i*-inositol as they affect riboflavin biosynthesis. The data concern particularly conditions which result in riboflavin yields of about 800 μ g per ml and higher, and are supplementary to data supplied recently (Hickey, 1952).

EXPERIMENTAL METHODS

Analytical method. A 10 to 12 ml sample of medium was acidified with about 0.2 g of NaH_2PO_4 in a test tube. This was mixed, covered, and steamed for about 20 minutes. After steaming, the sample was centrifuged and the supernatant liquid was assayed fluorophotometrically after appropriate dilution.

Cultural methods. *E. ashbyii* can be carried on agar slopes, the composition of which is 1.5 per cent agar, 0.8 per cent maltose, 1.2 per cent glucose, 0.1 per cent yeast extract (Difco), and 1.5 per cent trypticase,² pH 6.0. These are auto-

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claved for 20 to 30 minutes at 15 pounds per square inch. Incubation is at 28 C for 5 to 7 days after which the culture is refrigerated. It may remain viable for 2 or 3 months, but transfers should be made monthly. Lyophilization of the culture has not been successful.

TABLE 1
Synthetic medium A

AGENT	AMOUNT PER LITER
Glucose.....	5 g
K ₂ HPO ₄	1 g
KH ₂ PO ₄	1 g
MgSO ₄ ·7H ₂ O.....	0.7 g
NaCl.....	1 g
<i>i</i> -Inositol.....	6.7 mg
Thiamin.....	0.5 mg
Biotin.....	0.34 µg
Distilled water.....	to 1 liter
pH = 6.5	

TABLE 2
Riboflavin production in medium A containing single amino acids or their amides

AMINO ACID 0.2 g PER 100 ML	6 DAYS	
	pH	Riboflavin µg/ml
L-Leucine.....	6.0	29
L-Arginine.....	5.8	13
L-Asparagine.....	7.7	87
DL-Norleucine.....	6.5	11
L-Glutamic acid.....	7.2	86
L-Glutamine (natural).....	5.1	51
L-Aspartic acid.....	7.7	80
None.....	6.5	4

A loop transfer of the organism to a liquid medium composed of 1 per cent peptone, 1 per cent glucose, 0.1 per cent yeast extract, and 0.2 per cent beef extract, pH 6.0 to 6.2, shaken on a reciprocal shaker at 28 C allows good growth in 24 to 48 hours. The shake flask is a 300 ml Erlenmeyer containing 80 ml of medium. This culture may be used as inoculum, or additional generations may be made.

Specific strains have not proven particularly stable in character, and strain selection by well known methods is advantageous.

Fermentation studies. In order to select amino

acids which were capable of supporting growth of and riboflavin production by *E. ashbyii*, synthetic basal medium A, shown in table 1, was employed.

The amino acids were studied at a concentration of 2 g per liter. This low level was maintained in order to suppress the effects of impurities and the potential toxicities of high levels of some single amino acids.

After 6 days of reciprocating shaking of 150 ml of media in 1 liter Erlenmeyer flasks at 28 to

TABLE 3
Synthetic medium B

AGENT	AMOUNT PER LITER
Maltose.....	10 g
Glucose.....	15 g
K ₂ HPO ₄	1 g
KH ₂ PO ₄	1 g
NaCl.....	1 g
MgSO ₄ ·7H ₂ O.....	0.7 g
FeSO ₄ ·7H ₂ O.....	5 mg
<i>i</i> -Inositol.....	30 mg
Thiamin.....	1.5 mg
Biotin.....	0.7 µg
Distilled water.....	to 1 liter
pH = 6.2	

30 C, the following amino acids allowed the formation of less than 10 µg per ml of riboflavin: glycine, α-alanine, β-alanine, L-tryptophan, L-tyrosine, L-histidine, L-proline, L-lysine, L-cystine, L-hydroxyproline, DL-valine, DL-serine, DL-isoleucine, DL-β-phenylalanine, taurine, and DL-threonine. The growth of the organism in these media was also very sparse.

Some amino acids and their amides supported better growth than others, and higher riboflavin yields also were obtained. Data on these are given in table 2.

Increasing the glutamate concentration to 1 per cent raised the riboflavin yield to about 100 µg per ml, while the use of 0.8 per cent glutamate supplemented with 0.07 per cent leucine, 0.07 per cent arginine, and 0.07 per cent asparagine raised the riboflavin yield at 7 days to 202 µg per ml. Supplementing 0.8 per cent glutamate with 0.2 per cent casamino acids (Difco, vitamin-free) resulted in 205 µg per ml. Studies with other amino acid mixtures in medium A showed no

particular improvement, nor did a number of ash residues such as corn steep ash. Investigations of organic growth factors therefore were undertaken.

Certain advantages have been observed (Larson, 1952; Hickey, 1952) in the combined use of glucose and maltose over glucose alone in the nutrition of *E. ashbyii* for riboflavin formation. Maltose as a sole sugar, incidentally, supports

Since considerable improvement both in growth of organism and in riboflavin yield was obtained with the addition of as little as 0.01 per cent yeast extract, the presence of one or more organic growth factors is indicated. The factor also was present in gliadin and in liver extract (Lilly); it was not demonstrated in zein.

Rudert (1945) has shown that certain lipids could be used to some degree as energy sources for

TABLE 4
Evidence of factors for Eremothecium ashbyii in yeast extract and in gliadin

MEDIUM	3 DAYS		5 DAYS		GROWTH
	pH	Riboflavin	pH	Riboflavin	
		$\mu\text{g/ml}$		$\mu\text{g/ml}$	
Vitamin-free basal*	6.0	54	6.1	51	+
Normal basal†	6.2	94	6.8	157	+
Normal basal† + 0.01% yeast extract	6.5	320	6.9	650	++++
Normal basal† + 0.1% yeast extract	7.0	460	7.3	600	++++
Normal basal† + 0.1% casamino acids‡	6.2	96	6.8	155	+
Normal basal† + 0.1% casein§	6.2	155	6.9	245	+
Normal basal† + 0.3% tryptic digest of gliadin...	6.1	285	5.8	405	++++

* Normal basal B† without added biotin, thiamin, and inositol.

† Synthetic basal B plus 0.95% monosodium glutamate, 0.6% leucine, and 0.5% asparagine.

‡ Difco, vitamin-free.

§ Labco, vitamin-free.

the growth of *E. ashbyii* very poorly (Schopfer, 1944). A reevaluation of vitamin and mineral requirements in a basal medium containing maltose resulted in synthetic medium B described in table 3.

When synthetic medium B was supplemented with 0.95 per cent monosodium glutamate, 0.6 per cent leucine, and 0.5 per cent asparagine, the following vitamins and growth factors showed no improvement over the controls: niacin, niacinamide, pantothenic acid, pyridoxine, *p*-aminobenzoic acid, folic acid, thymine, adenine, xanthine, guanine, uracil, and B₁₂ concentrate ("reticulogen"). These observations on growth factors are in general agreement with reported data (Dulaney and Grutter, 1950; Yaw, 1952). The absence of activity in crystalline insulin and in trypsin eliminated streptogenin. Considerable growth stimulation and improvement in riboflavin yield were found in yeast extract and in a tryptic digest of gliadin as indicated in table 4.

Where the yield of riboflavin was relatively high, the growth had been heavy in contrast to the sparse growth in the lower yielding media.

the metabolism of *E. ashbyii* in place of carbohydrates. The significance of lipids as nutritional supplements promoting riboflavin formation by *E. ashbyii* was reported by Phelps (1949b). When a modified basal B containing glutamate was supplemented with oils such as corn, cottonseed, poppyseed, wheat germ, peanut, and others, very pronounced improvements in growth and production of riboflavin were observed. This effect did not occur when glycerol was the supplement but sodium oleate showed the effect quite strongly. Table 5 contains data showing the effects of yeast extract and sodium oleate on riboflavin formation by *E. ashbyii*.

The use of 1 per cent asparagine plus 1 per cent glutamic acid in place of 2 per cent glutamic acid, and the inclusion of both yeast extract and sodium oleate at 0.1 per cent levels resulted in no improvement in yield. Sodium oleate evidently can replace yeast extract under the preceding conditions. This does not prove that oleate is the active factor of yeast extract. Experiments showed that the optimum concentration of sodium oleate for riboflavin formation by *E. ashbyii*

under the preceding conditions was about 0.05 to 0.07 per cent.

Using a basal medium now also modified by the inclusion of 0.06 per cent sodium oleate, U.S.P., further studies were made to detect additional factors. The glutamate and oleate containing basal was not improved by supplementary vitamin-free casamino acids or by a number of indi-

is peculiar that the effect was less pronounced with other casein preparations, but relatively low yields were obtained with four other caseins at 0.1 per cent concentration levels. The apparent retarding action of the corn prolamin, zein, is in considerable contrast to the enhancing action of the wheat prolamin, gliadin. Antagonism may be indicated by gelatin and zein.

TABLE 5
Replacement of yeast extract by oleate in Eremothecium ashbyii nutrition

MEDIUM	3 DAYS		5 DAYS	
	pH	Riboflavin $\mu\text{g/ml}$	pH	Riboflavin $\mu\text{g/ml}$
Basal B* + 2% glutamic acid.....	5.8	67	6.4	180
Basal B* + 2% glutamic acid + 0.1% yeast extract.....	6.9	495	7.9	620
Basal B* + 2% glutamic acid + 0.1% Na oleate.....	6.9	460	7.6	590

* Supplemented with 2 mg % H_3BO_3 , 2 mg % $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, and 50 mg % CaCl_2 .

TABLE 6
Flavogenetic activity of various agents in oleate containing medium

MEDIUM	3 DAYS		5 DAYS	
	pH	Riboflavin $\mu\text{g/ml}$	pH	Riboflavin $\mu\text{g/ml}$
Basal*	6.7	235	7.8	280
Basal* + 0.1% gelatin.....	6.8	60	8.2	80
Basal* + 0.1% egg albumin.....	7.1	295	8.1	495
Basal* + 0.1% peptone (Bacto).....	6.8	120	8.2	180
Basal* + 0.1% blood serum, dry basis.....	7.2	195	8.1	270
Basal* + 0.1% trypticase.....	7.9	320	8.4	370
Basal* + 0.1% gliadin.....	7.3	580	8.3	800
Basal* + 0.1% zein.....	7.5	60	7.4	85
Basal* + 0.1% trypsin.....	7.1	310	7.9	310
Basal* + 0.1% Labco vitamin-free casein.....	7.6	560	8.5	850
Basal* + 0.1% GBI vitamin-free casein.....	7.6	230	8.5	400
Basal* + 0.1% New Process† casein.....	7.6	185	8.6	285

* Basal B + 1.9% monosodium glutamate + 0.06% Na oleate + 2 mg % H_3BO_3 + 2 mg % $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ + 50 mg % CaCl_2 , pH 6.0.

† Sheffield Farms Company, Inc., New York, New York.

vidual amino acids. The effectiveness of supplementation of 1.9 per cent glutamate medium with 0.1 per cent of various complex nitrogenous agents is shown in table 6.

As can be seen, a wide range of assays can be obtained using various supplements. Data of particular interest are the high riboflavin yields resulting when 0.1 per cent supplements of gliadin or Labco vitamin-free casein were employed. It

Incremental replacement of monosodium glutamate with Labco vitamin-free casein resulted in riboflavin assays in excess of 1,000 μg per ml. Data are given in table 7. Significant improvement of assays occurred when casein replaced the glutamate through a concentration of about 0.3 per cent, after which changes were relatively small. Assays from 1,000 to 1,500 μg per ml have been obtained in other similar experiments when

Labco casein replacement concentration ranged from about 0.3 to 2.0 per cent. Other casein preparations when used in a 2 per cent concentration in place of glutamate resulted in assays of 1,000 μg per ml or better as with Labco vita-

larly; the other is designated as factor B which has been found particularly in Labco vitamin-free casein and in gliadin.

Since sodium oleate or oleic acid was not established definitely as the active agent supplying

TABLE 7
Replacement of glutamate with casein and the effect on riboflavin formation

MEDIUM	3 DAYS		5 DAYS	
	pH	Riboflavin	pH	Riboflavin
		$\mu\text{g/ml}$		$\mu\text{g/ml}$
Basal* + 2% monosodium glutamate.....	6.8	250	7.8	300
Basal* + 1.9% monosodium glutamate + 0.1% casein†.....	7.5	615	8.0	750
Basal* + 1.8% monosodium glutamate + 0.2% casein.....	7.6	795	8.0	980
Basal* + 1.7% monosodium glutamate + 0.3% casein.....	7.6	850	8.0	1,030
Basal* + 1.5% monosodium glutamate + 0.5% casein.....	7.2	920	8.0	1,110
Basal* + 1.0% monosodium glutamate + 1.0% casein.....	7.2	1,040	8.1	1,180
Basal* + 0% monosodium glutamate + 2.0% casein.....	7.0	930	7.9	1,360

* Basal B + 0.06% Na oleate + 2 mg % H_3BO_3 + 2 mg % $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ + 50 mg % CaCl_2 , pH 6.4.

† Labco vitamin-free casein.

TABLE 8
*Comparison of sodium oleate with yeast extract as sources of factor A for *Eremothecium ashbyii**

MEDIUM	2½ DAYS		4½ DAYS	
	pH	Riboflavin	pH	Riboflavin
		$\mu\text{g/ml}$		$\mu\text{g/ml}$
Basal*.....	6.5	85	6.5	135
Basal* + 0.01% Na oleate.....	6.5	25	6.4	30
Basal* + 0.1% Labco vitamin-free casein.....	6.4	100	6.4	145
Basal* + 0.1% Labco vitamin-free casein + 0.005% Na oleate..	6.8	300	6.7	360
Basal* + 0.1% Labco vitamin-free casein + 0.005% yeast extract†.....	6.7	455	7.0	610
Basal* + 0.1% Labco vitamin-free casein + 0.03% Na oleate..	7.2	480	7.9	630
Basal* + 0.1% Labco vitamin-free casein + 0.03% yeast extract†.....	7.3	670	7.9	895
Basal* + 0.1% Labco vitamin-free casein + 0.07% Na oleate..	7.1	420	7.9	575
Basal* + 0.1% Labco vitamin-free casein + 0.07% yeast extract†.....	7.5	735	8.2	905

* Basal B + 2 mg % H_3BO_3 + 2 mg % $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ + 50 mg % CaCl_2 + 1.9% monosodium glutamate, pH 6.3.

† Difco.

min-free casein. The difference in caseins was noted only at low casein levels as shown in table 6.

It seems evident, therefore, that two unidentified nutritional factors for *E. ashbyii* are indicated, in addition to the known factors, biotin, thiamin, and *i*-inositol. One is designated as factor A, which is supplied by sodium oleate and by certain oils, fats, and yeast extract particu-

larly; the other is designated as factor B which has been found particularly in Labco vitamin-free casein and in gliadin. Since sodium oleate or oleic acid was not established definitely as the active agent supplying factor A activity in yeast extract, an experiment was set up to clarify this question by parallel dilution of yeast extract and sodium oleate. The data are given in table 8. In addition, it was observed that activity was not extracted from aqueous yeast extract solution at pH 2.5 by benzene which should have extracted oleic acid.

From these data one must conclude that the

primary active agent in yeast extract is probably not oleate but something more potent since yeast extract with its salts, vitamins, and other agents is more effective than sodium oleate on an equal weight basis. Oleic acid exhibits both toxic and growth promoting properties to some organisms such as the lactic acid bacteria under certain conditions (Williams *et al.*, 1947). For *E. ashbyii*, however, under conditions thus far studied, sodium oleate has shown increasing activity through a concentration of 0.07 per cent, and no toxicity was demonstrated at 0.1 per cent at pH levels of 6 or higher. "Tween 80"^{*} and lecithin also showed activity while "Tween 40"^{*} was relatively inactive.

DISCUSSION

Some of the discrepancies in the literature regarding the nutrition of *E. ashbyii* might be related in part to strain differences and in part to difficulties in evaluation of the essentiality of a single factor if other deficiencies coexist. A number of nutritional studies have been reported in which riboflavin formation was in the 100 to 250 μg per ml range. Production figures of Moss and Klein (1947) and others (Phelps, 1949b; Hickey, 1952; Larson, 1952) have shown that in submerged culture *E. ashbyii* can produce riboflavin in the range of 1,000 to 2,480 μg per ml. These data suggest inadequacies in other, lower producing media.

The particular value of casein as a raw material for riboflavin production by *E. ashbyii* was indicated by Phelps (1949a,b). The differences in apparent flavogenetic properties of several caseins possibly reflect differences in manufacturing methods; however, finding a "vitamin-free" casein to have high activity was somewhat surprising since extraneous factors presumably were removed. Of interest is the fact that the animal nutritional quality of casein is reduced by ethanol extraction (Derse *et al.*, 1948).

The identity of factors A and B for *E. ashbyii* is not clear. Factor A type activity, as supplied by oleic acid and yeast extract, appears to be supplied by an agent much more potent than oleic acid and is not necessarily a fatty acid. Factor B activity is possibly a complex nitrogenous agent and evidently is not supplied by simple, well known amino acids. The presence of

both factors appears to be essential for maximum riboflavin formation.

SUMMARY

Conditions have been described which allow the formation of over 1,000 μg of riboflavin per ml by *Eremothecium ashbyii* in 4 to 5 days in submerged culture in fairly rigidly defined media.

Evidence is presented which indicates the existence of two unidentified nutritional factors for *E. ashbyii*. These are identified at present as factors A and B. Factor A activity is associated with oleic acid, some of its derivatives, and yeast extract. Factor B is associated with gliadin and some caseins including a "vitamin-free" casein.

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^{*} Atlas Powder Company. Tweens 40 and 80 are palmitic and oleic acid derivatives, respectively.

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