Chlorophyll Formation in *Euglena gracilis* var. *bacillaris*: Interference by Vitamin Analogues

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SUMMARY

The formation of chlorophyll in non-proliferating etiolated cells of *Euglena gracilis* var. *bacillaris* is inhibited by some antimetabolite analogues of vitamins. The cell size and cell mass of light-grown euglenas is considerably increased in vitamin B₁₂ deficiency. The inhibitory effect of 2,6-diaminopurine on chlorophyll formation in non-proliferating euglenas was not annulled by vitamin B₁₂; 6-mercaptopurine, sulphanilamide and benzimidazole were without effect. Isoniazide inhibition is not reversed by niacin; surprisingly, the vitamin itself is markedly inhibitory. The inhibitory effect of niacin, however, is prevented by pyridine-3-sulphonate. Niacin and its analogues were more inhibitory to growth in the dark than in the light. The inhibition of growth by niacin in the light is annulled appreciably by either glucose or pyruvate, or an overwhelming concentration of vitamin B₁₂. Aminopterin, desoxypyridoxine and 2-chloro-*p*-aminobenzoic acid do not have any effect on chlorophyll synthesis in non-proliferating euglenas; the last mentioned inhibits the growth of the alga more in the dark than in the light. Thiamine deficiency inhibits growth; such sub-optimally grown euglenas also synthesize less chlorophyll per cell on subsequent illumination under non-proliferation conditions.

The negative growth response of light-grown *Euglena gracilis* var. *bacillaris* to niacin suggests a microbiological method of estimating this vitamin in biological materials and pharmaceutical preparations up to concentrations of 70 µg./ml. in the growth medium. The 50% inhibition level of niacin in the light is 46 µg./ml.

INTRODUCTION

Exposure of dark-grown euglenas to light in a non-proliferation medium brings about the synthesis of chlorophyll with several associated changes. The formation of chlorophyll is inhibited by antimetabolite analogues of purines, pyrimidines and amino acids (Dubash & Rege, 1967*a*). No direct evidence of the involvement of vitamins during the pigment formation in dark-grown non-proliferating euglenas is available; such an apparent independence may mean synthesis of these during growth irrespective of illumination. The effect of antimetabolite analogues of vitamins was therefore studied with a view to explore the possible association of vitamins with the synthesis of chlorophyll.

METHODS

Euglena gracilis var. *bacillaris* was grown, harvested and illuminated under non-proliferating conditions as described earlier (Dubash & Rege, 1967*a, b*). Measurement

of growth, chlorophyll and cell numbers and all other experimental details have already been described. Dry weights of euglenas were determined by drying samples of the washed cell suspensions at 50–60° for 12–14 hr.

RESULTS

Requirement of vitamin B 12 and the effect of its antimetabolite analogues on growth and chlorophyll synthesis

Euglena gracilis var. *bacillaris* exhibits a unique requirement of an exogenous supply of vitamin B 12 (Hutner & Provasoli, 1964); it was of interest to check whether this vitamin is required for chlorophyll synthesis or for other metabolic purposes.

Organisms grown under conditions varied with respect to vitamin B 12 showed variations in their chlorophyll content; sub-optimally grown organisms had a lower chlorophyll index per mg. dry wt. However, when the chlorophyll index was measured per organism, a slight enhancement was observed in the case of sub-optimally grown

Table 1. *Effect of vitamin B 12 deficiency on the synthesis of chlorophyll in Euglena gracilis* var. *bacillaris* grown in the light

Vitamin B 12 in medium ($\mu\text{g.}/\text{ml.}$)	Dry wt cell suspension ($\text{mg.}/\text{ml.}$)	Cell count of sus- pension ($\times 10^6/\text{ml.}$)	O.D. at 660 $m\mu$ of 10 ml. of methanol extract		
			Per ml. of suspension	Per mg. cells	Per 10^6 cells
50 (Optimal)	4.36	4.42	0.298	0.068	0.067
5 (Sub-optimal)	3.23	1.25	0.096	0.029	0.077

Table 2. *Comparison of dry weights of Euglena gracilis* var. *bacillaris* grown under different conditions of stress

Stress condition	Dry weight of 10^6 cells (mg.)	Dry wt of treated cells Dry wt of control cells = N	
Control*	0.99		1
Vitamin B 12 deficiency*	2.58		2.61
Control†	1.54		1
Grown at 13.3–17 °C.†	3.78		2.46

* *Euglena gracilis* var. *bacillaris* grown in light in the glutamate-malate medium of Hutner, Provasoli, Schatz & Haskins (1950).

† Readings of Buetow (1962): *Euglena gracilis* var. *bacillaris* (SM-L1), a streptomycin bleached mutant, grown in the dark with acetate as the sole carbon source in the medium of Cramer & Myers (1952).

euglenas (Table 1). This indicated an increase in size and cellular mass in vitamin B 12 deficiency. Buetow (1962) observed a similar decrease in the growth rate and increase in cellular mass of euglenas at lower incubation temperatures. Table 2 shows a comparison of the growth characteristics of the euglenas grown under different conditions of stress.

Although vitamin B 12 deficiency distorts cell metabolism and brings about these characteristic biochemical lesions during growth, results in Table 3 show that the

vitamin plays no significant role during chlorophyll synthesis in non-proliferating organisms. The 2,6-diaminopurine inhibition of chlorophyll synthesis was not annulled to any extent by the presence of vitamin B₁₂. 6-Mercaptopurine, sulphanilamide and benzimidazole do not inhibit pigment synthesis.

Table 3. *Effect of anti-metabolite analogues of vitamin B₁₂ on chlorophyll synthesis in non-proliferating etiolated organisms of Euglena gracilis var. bacillaris*

Analogue	Chlorophyll synthesized/cell/ 72 hr (%)	
	Control	+ 1 m μ g./ml. B ₁₂
None	100	100
2,6-Diaminopurine	63.9	65.0
6-Mercaptopurine	114.0	102.8
Sulphanilamide	102.8	95.2
Benzimidazole	97.2	80.5

All analogues were added at a concentration of 1×10^{-3} M in the non-proliferation medium.

Table 4. *Effect of antimetabolite analogues of vitamins on chlorophyll synthesis in non-proliferating etiolated organisms of Euglena gracilis var. bacillaris*

Analogue	Concentration in the non- proliferation medium (μ g./ml.)	Inhibition of greening (%)
None	.	0
Isoniazide	2000	65.5
3-Acetylpyridine	200	-11.5
Pyridine-3-sulphonic acid	200	-17.1
Aminopterin	20	10.7
2-Chloro- <i>p</i> -amino benzoic acid	25	0
Desoxypyridoxine	100	4.9

Table 5. *Effect of anti-metabolite analogues of vitamins on the growth of Euglena gracilis var. bacillaris*

Analogue	Concentration in the growth medium (μ g./ml.)	Inhibition of growth (%)	
		Light	Dark
None	.	0	0
Niacinamide	50	14.0	68.5
Isoniazide	50	-6.0	14.6
3-Acetylpyridine	50	44.5	76.0
Pyridine-3-sulphonic acid	50	0	22.4
Niacin	50	62.0	75.4
Aminopterin	20	3.1	1.5
2-Chloro- <i>p</i> -amino benzoic acid	25	54.0	63.8

Effect of antimetabolite analogues of vitamins on chlorophyll synthesis and growth of Euglena gracilis var. bacillaris

Several other antimetabolite analogues of vitamins were tested for their effect on chlorophyll synthesis (Table 4) and growth (Table 5) of *Euglena gracilis* var. *bacillaris*. Aminopterin and desoxypyridoxine had little effect on chlorophyll synthesis in non-

proliferating euglenas. Among the niacin derivatives tried, only isoniazide was inhibitory, whereas 3-acetylpyridine and pyridine-3-sulphonate were, in fact, stimulatory. However, these had varying effects on the growth of the euglenas. 2-Chloro-*p*-aminobenzoic acid and 3-acetyl pyridine were quite inhibitory to growth (Table 5) and more so in the dark, whereas pyridine-3-sulphonate and isoniazide were only inhibitory in the dark; aminopterin was ineffective. 2-Chloro-*p*-aminobenzoic acid and sulphanilamide had no effect on chlorophyll synthesis in non-proliferating euglenas although both inhibited growth.

Effect of niacin derivatives

In an attempt to study the possible annulment by the vitamin of the inhibition of chlorophyll synthesis by the antimetabolite analogues of niacin it was observed that 200 $\mu\text{g./ml.}$ niacin was itself inhibitory to the synthesis of chlorophyll in non-proliferating euglenas. The effects of the various antimetabolite analogues of niacin along with this vitamin were therefore studied (Table 6). Figure 1 shows the negative growth-response relationship between niacin concentration and growth of *Euglena gracilis* var. *bacillaris*.

Table 6. *Effect of niacin and its anti-metabolite analogues on chlorophyll synthesis in non-proliferating etiolated organisms of Euglena gracilis var. bacillaris*

Analogue	Concentration in the non- proliferation medium ($\mu\text{g./ml.}$)	Inhibition of greening (%)
None	.	0
Niacin	200	95.6
Niacinamide	2000	100
Isoniazide	2000	65.6
3-Acetylpyridine	200	-11.5
Pyridine-3-sulphonic acid	200	-17.1
Niacin + 3-acetylpyridine	.	100
Niacin + pyridine-3-sulphonic acid	.	-54.1

Both niacin and its amide were inhibitory to growth as well as chlorophyll synthesis (Tables 5, 6). The growth inhibition with niacin was more marked in the dark than in the light as with other antimetabolite analogues of this vitamin. The inhibition of chlorophyll synthesis by niacin was completely overcome by pyridine-3-sulphonate (Table 6). Attempts to reverse this anomalous inhibition of growth by niacin have been met with only partial success. Table 7 shows a 50 % annulment of the inhibition in the light by glucose or by a hundredfold increase in vitamin B₁₂ in the growth medium. There was, however, no annulment by these supplements in the dark.

Among the several Krebs-cycle intermediates tested for their effect on the niacin inhibition of growth, only pyruvate annulled the inhibition completely in light and partially in the dark. β -Glycerolphosphate and α -ketoglutarate, however, could partially annul the niacin inhibition in the light but not in the dark. Succinate, citrate, malate and fumarate all aggravated the inhibition, particularly in the light. These results are given in Table 8.

Effect of thiamine

The effect of thiamine deficiency on growth and chlorophyll synthesis in *Euglena gracilis* var. *bacillaris* is shown in Table 9. Thiamine-deficient organisms had a 15 % lower potential for chlorophyll synthesis than normal organisms when they were illuminated under non-proliferating conditions.

Fig. 1. Effect of niacin on the growth of *Euglena gracilis* var. *bacillaris* in the light (—) and in the dark (----).

Table 7. *Annulment of niacin growth inhibition of Euglena gracilis* var. *bacillaris* with glucose and vitamin B 12

Vitamin B 12 in medium (μg./ml.)	Niacin in medium (46 μg./ml.)	Glucose 1 %	Growth in		Inhibition of growth (%)	
			Light (Klett)	Dark	Light	Dark
50 (normal)	—	—	179	.	0	.
	—	+	182	240	0	0
	+	—	90	.	50	.
	+	+	133	17	26	93
5000 (100-fold)	—	—	186	.	0	.
	+	—	135	.	27	.
	—	+	.	276	.	0
	+	+	.	14	.	98

Assay of niacin with Euglena gracilis var. *bacillaris*

The *Euglena gracilis* assay of vitamin B 12 (Hutner, Bach & Ross, 1956) owes its world-wide acceptance to its extreme sensitivity and its specificity for vitamin B 12 (Hutner, 1961; Hutner & Provasoli, 1964). The inhibitory effect of niacin on the photosynthetic growth of the euglenas was striking and offered an elegant method for

assaying this vitamin microbiologically, taking advantage of the typical negative-growth response curve which is shown in Fig. 1. A linearity of response was shown for niacin concentrations up to 70 $\mu\text{g./ml.}$ in the basal medium with a 50% inhibition level of the vitamin corresponding to 46 $\mu\text{g./ml.}$

Table 8. *Effect of Krebs cycle intermediates on the niacin inhibition of growth of Euglena gracilis var. bacillaris*

Krebs' cycle acid added* (1%)	Inhibition of growth with 50 $\mu\text{g./ml.}$ niacin (%)	
	Light	Dark
None	65.0	83.5
Glucose	33.0	96.8
Pyruvate	0	45.8
Lactate	59.0	87.0
Succinate	84.0	84.0
Malate	78.0	89.5
α -Ketoglutarate	43.5	92.6
β -Glycerophosphate	37.5	87.0
Fumarate	78.8	92.3
Citrate	82.2	83.1

* Additions of Krebs' intermediates were to the basal medium containing glutamate (1%) and malate (0.2%) but without glucose.

Table 9. *Thiamine requirement for growth and greening of Euglena gracilis var. bacillaris*

Thiamine in growth medium ($\mu\text{g./ml.}$)	Inhibition of growth (%)		Inhibition of chloro- phyll synthesis in non-proliferating cells (%)
	Light	Dark	
0	46.0	16.0	15.0
1000 (basal)	0	0	0

DISCUSSION

Although Venkataraman, Netrawali & Sreenivasan (1965) suggested on the basis of incorporation of labelled ^{14}C -formate that in DNA synthesis in *Euglena gracilis* var. *bacillaris* vitamin B 12 has a greater role in the reduction of ribotides to deoxyribotides than in the reduction of formate to thymine methyl and that the vitamin B 12-dependent conversion of glutamate to β -methyl aspartate also contributed to thymine synthesis, its exact function in this organism still remains obscure. The role of vitamin B 12 in chlorophyll synthesis in euglena has not been explored. The dry weight of euglenas grown under conditions of vitamin B 12 deficiency (Table 2) increased nearly 2½-fold as was the case of cultures grown at low temperatures (Buetow, 1962). Gigantism was also apparent from results given in Table 1 on the basis of the chlorophyll index of the euglenas grown under sub-optimal vitamin B 12 conditions. Both higher optimal temperatures and adequate vitamin B 12 favour cell multiplication with a constant fragmentation of cellular material which prevents cell gigantism due to 'stagnation'. Cell enlargement has also been observed in the z strain of *Euglena gracilis* (Epstein, Weiss, Causeley & Bush, 1962) grown under sub-optimal vitamin B 12 conditions. The occurrence of a wide variation in cell size in response to anti-metabolites or metabolite

deficiency has been reported by several workers (Beck, Hurlock & Levin, 1960; Dubash & Rege, 1967*b*; Epstein & Weiss, 1960*a*; Ford & Goulden, 1959; Rueckert & Mueller, 1960). Such varied size differentiation, along with the cellular accumulation of materials which render the organisms more opaque, must throw doubt on turbidimetric growth measurements. The vitamin B 12 assay with *Euglena* (Hutner *et al.* 1956) would, in the light of these experiments, be much more reliable if chlorophyll were taken as the index of growth rather than turbidity. The chlorophyll content of vitamin B 12-deficient organisms did not show such a wide variation (Table 1). The strong inhibition by acetate and niacin (see Coelho, Dubash & Rege, 1964) both of common occurrence in biological materials and pharmaceutical preparations and of growth stimulation by various Krebs's intermediates (Coelho *et al.* 1964) suggests need for caution when assaying vitamin B 12 or niacin with this alga. The graded negative growth response of photosynthetically growing *Euglena gracilis* var. *bacillaris* to the presence of niacin may prove useful for the assay of this vitamin in biological materials and pharmaceutical preparations.

Vitamin B 12, however, does not seem to have any significant role in chlorophyll synthesis in non-proliferating euglenas as is shown by the inability of vitamin B 12 to annul the inhibition by 2,6-diaminopurine. Although in growing euglenas, 6-mercaptapurine competes with vitamin B 12 presumably by interfering with dimethylbenzimidazol function rather than inducing purine deficiency (Epstein & Timmis, 1963; Epstein & Weiss, 1960*a, b*), no inhibition of chlorophyll synthesis by this antimetabolite analogue was observed (Table 3). Similarly, benzimidazole (Epstein & Timmis, 1963) and sulphanimide (Coelho, 1966), reported to have an anti-B12 activity for growth of *Euglena*, had no appreciable effect on greening. Epstein & Timmis (1963) have shown both aminopterin and amethopterin to be inactive for *Euglena*. Aminopterin and desoxypyridoxine have also no effect on chlorophyll synthesis in non-proliferating etiolated euglenas on illumination (Tables 4, 5).

Both niacin and its amide are inhibitory for growth as well as chlorophyll synthesis in *Euglena* (Tables 5, 6) and these as well as the other anti-metabolite analogues of vitamins are more inhibitory in the dark than in the light. This indicates that these inhibit some system of general metabolic significance and do not obstruct some step in chlorophyll biosynthesis. The inhibition of chlorophyll synthesis by niacin is completely annulled by pyridine-3-sulphonate. It is clear from Tables 5 and 6 that other substitutions in the β -position of the pyridine ring lower the toxicity of the compound. A probable site of action of niacin and niacinamide would be for the TPN-requiring glyceraldehyde-3-phosphate dehydrogenase formed during chlorophyll synthesis (Fuller & Gibbs, 1959; Brawerman & Konigsberg, 1960). However, the niacin inhibition is exerted even in the dark when the euglenas are grown heterotrophically. Schopfer & Keller (1951) have reversed the growth inhibition of *Euglena gracilis* caused by vitamin K 3 with niacin or niacinamide and Frank *et al.* (1963) have overcome the thalidomide growth inhibition in this alga by niacin, niacinamide, NAD and vitamin K 1. Although the latter authors have used a level of 10 $\mu\text{g./ml.}$ niacin, they fail to report any inhibition of growth, whereas from Fig. 1 it is apparent that a 10% growth inhibition does occur at such a level. Frank *et al.* (1963), however, did find 6-amino-niacinamide to inhibit the growth of euglenas and reversed this effect with niacin. In the present case it is not only that a usual metabolite for other organisms is acting as an inhibitor, but that a normal antimetabolite ana-

logue, pyridine-3-sulphonate, annuls this inhibition. The reversal, though partial, of niacin toxicity to the photosynthetic growth of euglenas by overwhelming concentrations of vitamin B₁₂ may suggest that the former interferes in the utilization of the latter. It is also possible that vitamin B₁₂ is rendered unstable in the presence of niacin, particularly during sterilization of the medium at high temperatures; such examples of non-compatibility are known to occur in pharmaceutical preparations. The reversal by glucose of this niacin inhibition (Table 7) in the light may suggest that the inhibitor obstructs the photosynthetic pathway for eliciting energy; however, the more pronounced inhibition in the dark is inconsistent with this. The extremely high activity of pyruvate and α -ketoglutarate, particularly in the light to prevent niacin inhibition (Table 7), may suggest that niacin interferes in some manner with the pyridine nucleotide metabolism. Both these α -keto acids normally serve to produce the reduced NADH. It is quite possible that vitamin B₁₂ may act by achieving the same end-results, as it is known that this vitamin serves in maintaining sulphydryl groups in the reduced form (Dubnoff & Barton, 1956). A similar explanation had been given for the inhibitory effect of thalidomide (Frank *et al.* 1963), which is claimed to interfere at a point where niacin is converted to NAD or with the utilization of NAD and vitamin K in cellular oxidations. Mention must also be made at this point of another observation of Coelho (1966) that many glucose metabolites, particularly α -ketoglutarate, spare the requirement of vitamin B₁₂ in this alga.

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