The B₁₂ Vitamins and Growth of the Flagellate *Prymnesium parvum*

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SUMMARY

Vitamin B₁₂ is an essential nutrient for the flagellate *Prymnesium parvum* when growing in axenic culture and apparently can be replaced only by a few related 'incomplete' analogues, i.e. only those devoid of one or both benzimidazole-methyl groups; and, of the substituted analogues, some (e.g. the mono-acid, the dicarboxylic acid and the ethylamide of B₁₂) can replace vitamin B₁₂, but some others of this group, e.g. pseudovitamin B₁₂, 2-methylmercaptoadenine cobamide, the ethylamide mono-acid, methylamide and anilide of the vitamin B₁₂, act as competitive inhibitors of the growth of *P. parvum*.

INTRODUCTION

Micro-organisms which respond to vitamin B₁₂ as a nutrient may be divided into three groups in accordance with their ability to accept some other metabolite in place of vitamin B₁₂ (Lascelles & Cross, 1955). In the present paper vitamin B₁₂ means α -(5,6-dimethyl benzimidazolyl)-cobamide cyanide. The first group comprises a mutant of *Escherichia coli* for which methionine may replace vitamin B₁₂ (Davis & Mingioli, 1950). The second group, comprising several lactobacilli, can have vitamin B₁₂ replaced by certain deoxyribosides and purines (Kitay, McNutt & Snell, 1950). To the third group belong micro-organisms such as *Ochromonas malhamensis*, whose vitamin B₁₂ requirement can only be satisfied by the vitamin itself or by some of its analogues (Ford, 1958). These micro-organisms may alternatively be divided into three groups, each with its own specific requirements in relation to the vitamin B₁₂ group (Kon & Pawelkiewicz, 1958). To the first group belongs *Escherichia coli* 113-3, which can satisfy its vitamin B₁₂ requirements not only by the benzimidazole and purine analogues but also by 'incomplete' analogues devoid of the nucleotide. The second group is represented by *Lactobacillus leichmanii* and *Euglena gracilis*; members of this group utilize vitamin B₁₂ and its benzimidazole or purine analogues. The third group includes *Ochromonas malhamensis*, which can utilize only the benzimidazole analogues.

Prymnesium parvum (Chrysomonadina) has been shown to require vitamin B₁₂ for its growth in axenic culture (Droop, 1954*a*; Rotberg, 1958); McLaughlin (1958) tested the specificity of its requirement for some of the B₁₂ analogues. In the present paper, an attempt has been made to determine the position of *P. parvum* within the aforementioned scheme by studying its specific vitamin B₁₂ requirements.

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Some of the B₁₂ analogues are known to inhibit the growth of various micro-organisms (Ford, 1958, 1959; Baker, Frank, Pasher, Hutner & Sobotka, 1960); these analogues were also tested for their effect on the growth of *P. parvum*.

METHODS

Of the two strains of *Prymnesium parvum* isolated, respectively, by Droop (1954*b*) and by Reich & Kahn (1954), the latter (Reich) strain has been used in this study. Cultures of *P. parvum* were grown in a simplified version of a culture medium for marine algae (Provasoli, McLaughlin & Droop, 1957), the composition of which is shown in Table 1.

Stock cultures of *Prymnesium parvum* were maintained on basal medium fortified

Table 1. *Composition of basal culture medium*

Glass-distilled water was used. The medium was adjusted to pH 8.2 and sterilized by autoclaving at 120° for 15 min.

NaCl	10.00 mg./ml.	FeCl ₃ .6H ₂ O	1 µg./ml.
MgSO ₄ .7H ₂ O	3.00 mg./ml.	NaMoO ₄ .2H ₂ O	1 µg./ml.
KCl	0.80 mg./ml.	ZnSO ₄ .7H ₂ O	0.15 µg./ml.
CaCl ₂ .2H ₂ O	0.10 mg./ml.	CoCl ₂ .6H ₂ O	0.003 µg./ml.
H ₃ BO ₃	0.01 mg./ml.	Casamino acids*	1 mg./ml.
NaNO ₃	0.2 mg./ml.	Tris† buffer	1 mg./ml.
Na ₂ HPO ₄	0.05 mg./ml.	Thiamine hydrochloride	10 mµg./ml.
MnCl ₂ .4H ₂ O	5 µg./ml.		

* Casamino acids = Bacto vitamin-free casamino acids (Difco).

† Tris = 2-amino-2-hydroxy methyl propane-1,3-diol.

Table 2. *Compounds of the vitamin B₁₂ group used in the present work*

In this paper 'vitamin B₁₂' stands for α-(5,6-dimethylbenzimidazolyl) cobamide cyanide. Other analogues are presented by specific name.

Abbreviations used	The analogues
1. B ₁₂	α-(5,6-dimethylbenzimidazolyl) cobamide cyanide
2. 5MBIA Cob	α-(5-methylbenzimidazolyl) cobamide cyanide
3. BIA Cob	α-(benzimidazolyl) cobamide cyanide
4. FIII _m	α-(5-methoxybenzimidazolyl) cobamide cyanide
5. FIII	α-(5-hydroxybenzimidazolyl) cobamide cyanide
6. PVB ₁₂	α-(adenyl) cobamide cyanide
7. FA	α-(2-methyladenyl) cobamide cyanide
8. 2MMA Cob	α-(2-methylmercaptoadenyl) cobamide cyanide
9. FB	cobinamide cyanide
10. FIB	cobinamide phosphoribose
11. DMBIA	5,6-dimethyl benzimidazole
12. MO	B ₁₂ monoacid
13. DA	B ₁₂ dicarboxylic acid
14. EA	B ₁₂ ethylamide
15. EAM	B ₁₂ ethylamidemono acid
16. MA	B ₁₂ methylamide
17. An	B ₁₂ anilide

Vitamin B₁₂ commercial, Nutritional Biochemicals Co., U.S.A. Analogues 2–10 received from K. Bernhauer, Technische Hochschule, Stuttgart, Germany. Analogue 11 received from Merck, Sharp and Dohme, U.S.A. Analogues 12–17 received from E. Lester Smith, Glaxo Laboratories, England.

with suboptimal quantities (10 $\mu\text{g.}/\text{ml.}$) of vitamin B₁₂. In some of the experimental cultures, however, vitamin B₁₂ was intentionally omitted. All cultures were grown in 16 $\times$ 160 mm. test tubes fitted with aluminium caps to prevent possible organic contamination. Inoculation was accomplished by transferring 0.1 ml. of a 10- to 12-day culture into 5 ml. of basal medium containing the substances to be tested. The cultures were kept in a thermostat under constant illumination at $20 \pm 2^\circ$. Growth measured as optical density (O.D.) was determined after incubation for 12 days with a Junior Coleman Spectrophotometer at 480 m μ . Control cultures of *P. parvum*, grown in basal medium + 100 $\mu\text{g.}$ vitamin B₁₂/ml., attained a count of about 5×10^6 organisms/ml.

The various compounds of the vitamin B₁₂ group to be tested (Table 2) were kept sterile in solution in sealed glass ampoules, and were added to the medium before autoclaving.

RESULTS

The nutrient requirement of *Prymnesium parvum* satisfied by vitamin B₁₂ could not be replaced by various amounts of DNA, RNA, thymidine, thymine, deoxyadenosine, adenosine, cytidine, guanosine or uridine. Attempts to stimulate the growth of *Prymnesium parvum* in the absence of vitamin B₁₂ by addition of various amino acids, such as alanine, glycine, homocysteine, homocystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan or valine, were unsuccessful; negative results were obtained also with betaine or

Table 3. *Growth of Prymnesium parvum in the presence of incomplete vitamin B₁₂ analogues*

Analogue* added		Optical density reading (O.D.) $\times$ 1000	
		In presence of 100 $\mu\text{g.}/\text{ml.}$ vitamin B ₁₂	In absence of vitamin B ₁₂
FB	1 $\mu\text{g.}/\text{ml.}$	337	27
FIb	1 $\mu\text{g.}/\text{ml.}$	367	22
DMBIA	1 $\mu\text{g.}/\text{ml.}$	409	27
FB + DMBIA	1 $\mu\text{g.}/\text{ml.}$ each	—	27
FIb + DMBIA	1 $\mu\text{g.}/\text{ml.}$ each	—	18
BIA Cob	100 $\mu\text{g.}/\text{ml.}$	—	208
5MBIA Cob	100 $\mu\text{g.}/\text{ml.}$	—	229
B ₁₂	100 $\mu\text{g.}/\text{ml.}$	—	328

* For code, see Table 2.

choline. These findings can be compared to results with *Ochromonas malhamensis* (another chrysomonad) where methionine can spare vitamin B₁₂ and enable some growth even in the absence of the latter (Hutner, Provasoli & Filfus, 1953; Johnson, Holdsworth, Porter & Kon, 1957). Table 3 shows growth of *P. parvum* in media containing 'incomplete' analogues of vitamin B₁₂ in which some part of the molecule is missing.

As seen from Table 4, the remaining vitamin B₁₂ analogues fell into two groups with respect to their influence on the growth of *P. parvum*. Members of the one

Fig. 1. Growth of *Prymnesium parvum* in media containing growth-enhancing analogues of vitamin B₁₂. —··—, Vitamin B₁₂; —●—●—, FIII_m*; ---, MO; —, EA; ····, DA.

* For code, see Table 2.

Table 4. Growth of *Prymnesium parvum* in media containing substituted vitamin B₁₂ analogues; in the presence or absence of vitamin B₁₂

Analogue added* (1 μg./ml.)	Optical density reading (o.d.) × 1000	
	In presence of vitamin B ₁₂	In absence of vitamin B ₁₂
	100 μg./ml.	
FA	22	9
PVB ₁₂	22	9
2MMA Cob	22	18
FIII	22	9
FIII _m	328	444
MO	523	337
DA	456	244
EA	509	237
EAM	31	22
MA	22	22
An	36	18
No analogue added	469	20

* For code, see Table 2.

group, namely FIII_m, MO, DA and EA, replaced the vitamin quantitatively; however, each of these analogues elicited a different growth response (Fig. 1).

The second group of analogues included FA, PVB₁₂, 2MMA Cob, FIII, EAM, MA and An; these had an inhibitory effect on the growth of *Prymnesium parvum* even in the presence of vitamin B₁₂. Since it had been claimed by Droop, McLaughlin, Pintner & Provasoli (1959) that FIII replaces vitamin B₁₂ for *P. parvum*, an attempt was made to compare the growth of the 'Droop' and the 'Reich' strains in the presence of this analogue. Such comparison showed that both strains reacted in similar fashion to compound FIII, the growth of both being inhibited by this

Fig. 2. Growth of *Prymnesium parvum* in media containing the inhibitory analogues and vitamin B₁₂ at various ratios. —, Vitamin B₁₂ constant (100 μg./ml.), analogues according to ratio. - - -, Analogues constant (1 μg./ml. (except PVB₁₂ and 2MMA Cob, 50 mμg./ml.)), vitamin B₁₂ according to ratio.

analogue. The source and amount of compound FIII used in the work of Droop *et al.* was not indicated by these authors. This apparent discrepancy of results remains.

The inhibitory analogues were further tested quantitatively for their inhibitory effect. For this, two parallel series of media were used: the first series contained 100 μg. vitamin B₁₂/ml., while the amount of the analogue tested varied successively from a ratio of analogue/B₁₂ of 1:1 to 10,000:1 (w/w), except for PVB₁₂ and 2MMA Cob, for which the ratios were from 31.25:1 to 500:1 (unbroken line in Fig. 2). Media of the second series contained 1 μg. analogue/ml., with the exception of PVB₁₂ and 2MMA Cob, of which 50 mμg./ml. were given, while the amount of vitamin B₁₂ varied according to the desired ratio (broken line in Fig. 2).

From Fig. 2 it is clear that, in a medium containing both vitamin B₁₂ and the inhibitory analogues, the growth of *P. parvum* was dependent on the ratio of analogue to vitamin B₁₂ and not on the absolute amount of inhibitor. From the above figures, the inhibition indices of the various analogues may be roughly estimated; their approximate values are given in Table 5.

Table 5. *Inhibition indices of various vitamin B₁₂ analogues in growth of Prymnesium parvum*

Analogue*	Inhibition index
FIII	500
MA	500
PVB ₁₂	175
2MMA Cob	100
An	80
EAM	80
FA	10

* For code, see Table 2.

DISCUSSION

The nutrient requirement of *Prymnesium parvum* for vitamin B₁₂ is very exacting. Vitamin B₁₂ was not replaced by any of the various unrelated metabolites tested, nor could the 'incomplete' analogues substitute for it. These facts at once place *P. parvum* among the most exacting of the micro-organisms which have a nutrient requirement satisfied by vitamin B₁₂. Furthermore, *P. parvum* is apparently incapable of synthesizing vitamin B₁₂ even when some of the preformed parts of this vitamin are supplied. On the other hand, analogues which lack one or both of the benzimidazole methyl groups can substitute for vitamin B₁₂. This could be explained by assuming either that *P. parvum* is capable of adding the missing methyl groups to analogues such as benzimidazolyl cobamide cyanide, or that the function of vitamin B₁₂ is not affected by the absence of these groups. The former assumption seems the more plausible, since compound FIII_m, which has a methoxybenzimidazole group, replaced vitamin B₁₂, whereas compound FIII, with an hydroxybenzimidazole group at the same site, inhibited growth. Analogues in which a purine replaces the benzimidazole of the vitamin B₁₂ molecule, such as compounds FA, 2MMA Cob and PVB₁₂, all inhibited growth. This indicates that the benzimidazole part of the molecule is essential for the normal function of vitamin B₁₂ in *P. parvum*.

There are conflicting reports in the literature about the influence on the growth of micro-organisms of analogues substituted in the propionamide groups of the corrinoid part of the vitamin B₁₂ molecule. Thus, analogue MO (B₁₂ monoacid), which can replace vitamin B₁₂ for *Prymnesium parvum*, was reported by Baker *et al.* (1960) to do the same for *Ochromonas malhamensis*. Ford (1959), however, found it inhibitory for the growth of *O. malhamensis*. Such discrepancy in the data for *O. malhamensis* is found also with regard to analogue MA (vitamin B₁₂ methylamide), which inhibited the growth of *P. parvum*. Ford (1959) found that *O. malhamensis* adapts to the utilization of the inhibitory MA, or that vigorously growing cultures can probably transform this analogue to vitamin B₁₂. Analogues

DA (B₁₂ dicarboxylic acid) and EA (B₁₂ ethylamide), which are inhibitory for *O. malhamensis* (Ford, 1959; Baker *et al.* 1960), were in the present study able to substitute for vitamin B₁₂. Such contradictory results may be attributable either to differences in the experimental procedures used and their effect on the analogues, or to differences in ability of the given micro-organism to take up the analogues and change them to the functional forms. As the coenzyme forms of B₁₂ are known to contain different analogues (Barker, Weissbach & Smyth, 1958; Barker *et al.* 1960*a*; Barker *et al.* 1960*b*), it follows that a micro-organism's requirements for a given coenzyme form can determine the respective influence of the analogues on its growth. Further work on the particular function of each analogue is therefore desirable.

The inhibitory effect of analogues of vitamin B₁₂ on the growth of *Prymnesium parvum* has, in the present work, been shown to depend on the ratio of analogue to vitamin B₁₂ in the culture medium and not on the absolute amount of analogue. This indicates a form of competitive inhibition, with analogue and vitamin competing for a particular enzyme site in *P. parvum*. However, as pointed out by Woolley (1952), 'the inhibition index as found in a living organism may represent the resultant of several forces rather than the relative affinity of the two compounds for a specific protein'.

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