The B₁₂ Vitamins and Methionine in the Metabolism of *Prymnesium parvum*

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SUMMARY

Methionine or ethionine, although capable of serving as sole nitrogen source for *Prymnesium parvum*, cannot replace or spare the vitamin B₁₂ nutrient requirement for the growth of this flagellate. In the presence of vitamin B₁₂ methionine counteracted the inhibition of growth exerted by some vitamin B₁₂ analogues which are substituted at the benzimidazole part of the molecule. No such effect was observed against the inhibition by certain other analogues. Analogue FIII (α -(5-hydroxybenzimidazolyl)-cobamide cyanide) replaced vitamin B₁₂ in the presence of methionine, and to a lesser degree also in the presence of other methyl donors such as methylmethionine sulphonium iodide, dimethylpropiothetin, choline chloride and betaine. The conclusion drawn is that for *P. parvum* analogue FIII is capable of replacing vitamin B₁₂ in all the metabolic pathways other than methyl-group synthesis. For the latter process, the benzimidazole methyl groups present in vitamin B₁₂ and analogue FIII_m (α -(5-methoxybenzimidazolyl)-cobamide cyanide) are indispensable.

INTRODUCTION

Vitamin B₁₂ participates in many biochemical reactions (see Heinrich, 1957, 1962). Davis & Mingioli (1950) showed that vitamin B₁₂ is essential for the growth of a mutant of *Escherichia coli* and that methionine can replace the vitamin for this mutant. Helleiner, Kisluk & Woods (1957) and Guest, Helleiner, Cross & Woods (1960) showed that vitamin B₁₂ is necessary for methionine synthesis by cell-free extracts of *E. coli*. A dependence on vitamin B₁₂ is also known for *Ochromonas malhamensis*, an allied flagellate of *Prymnesium parvum*, the growth of which is enhanced by methionine in the presence of vitamin B₁₂ (Hutner, Provasoli & Filfus, 1953; Johnson, Holdsworth, Porter & Kon, 1957; Ford, 1958). The growth of *O. malhamensis* is inhibited by ethionine, but this inhibition can be annulled by methionine. High concentrations of methionine, however, are also inhibitory (Johnson *et al.* 1957).

Vitamin B₁₂ is an essential nutrient for *Prymnesium parvum* (Droop, 1954; Rotberg, 1958), and may be replaced by some of its analogues, while other analogues inhibit the growth of *P. parvum* even in the presence of vitamin B₁₂ (Rahat & Reich, 1962). To date, no compound has been found which can replace vitamin B₁₂ for *P. parvum*, except the few related compounds just mentioned. Though some amino acids may serve as sole nitrogen source for *P. parvum*, vitamin B₁₂ is still an

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essential nutrient (Rahat & Reich, 1963). It is the purpose of the present paper to discuss the function of vitamin B₁₂ and some of its analogues in the metabolism of *P. parvum*, and the possible role of this vitamin in the synthesis of methionine and free methyl groups by this flagellate.

METHODS

The Reich strain of *Prymnesium parvum* was used in the present work. Data on medium composition and methods of culture are given elsewhere (Rahat & Reich, 1963). The amount of growth of *P. parvum* was determined at the end of 12 days by measuring the optical density of a culture with a Junior Coleman Spectrophotometer at 480 m μ . A list of vitamin B₁₂ analogues used in the present work is given in Table 1.

Table 1. *Compounds of the vitamin B₁₂ group used in the present work*

In this paper vitamin B₁₂ means α -(5, 6-dimethylbenzimidazolyl) cobamide cyanide. Other analogues are presented by name and code.

Abbreviation used	The analogues
1. B ₁₂	α -(5,6-dimethylbenzimidazolyl) cobamide cyanide
2. FIII	α -(5-hydroxybenzimidazolyl) cobamide cyanide
3. FIII _m	α -(5-methoxybenzimidazolyl) cobamide cyanide
4. PVB ₁₂	α -(adenyl) cobamide cyanide
5. FA	α -(2-methyladenyl) cobamide cyanide
6. 2MMA Cob	α -(2-methylmercaptoadenyl) cobamide cyanide
7. EAM	B ₁₂ ethylamidmonoacid
8. MA	B ₁₂ methylamide
9. An	B ₁₂ anilide

Vitamin B₁₂ commercial, Nutritional Biochemical Co., U.S.A.; analogues 2–6 received from K. Bernhauer, Technische Hochschule, Stuttgart, Germany; analogues 7–9 received from E. Lester Smith, Glaxo Laboratories, England.

RESULTS

Experiment showed that methionine and ethionine could be used by *Prymnesium parvum* as sole nitrogen sources. No growth inhibition occurred with either of these amino acids even at high concentrations (Fig. 1). In a second series of experiments, almost identical growth curves were obtained when the above-mentioned amino acids were added at concentrations of 0.25 mg./ml. (Fig. 2) and 2.0 mg./ml. Neither methionine nor ethionine show such sparing of vitamin B₁₂ for *P. parvum* as is known for *Escherichia coli* (Davis & Mingioli, 1950) and *Ochromonas malhamensis* (Johnson *et al.* 1957). Even though no sparing of vitamin B₁₂ was detected, nevertheless methionine, when added to media containing vitamin B₁₂ and growth-inhibitory analogues, was found to counteract the inhibition caused by some of these analogues (Fig. 4). This annulment of inhibition by methionine was clearly evident when the compounds FIII, PVB₁₂, 2MMA Cob and FA were the inhibitors; these are substituted in the nucleotide part of the molecule. However, with inhibitory analogues which were substituted in the propionamide of the corrinoid part of the molecule (e.g. compounds MA, EAM, An), only a slight annulment of inhibition was observed, which may have been due merely to the addition of another organic metabolite.

Table 2 shows the growth of *Prymnesium parvum* in media devoid of vitamin B₁₂, in the presence of the inhibitory analogues and with or without methionine. It can be seen at once that of all the analogues tested only FIII replaced vitamin B₁₂ in the presence of methionine. As pointed out earlier only analogues substituted in the

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Growth of *Prymnesium parvum* in inorganic medium with methionine or ethionine as sole nitrogen source. In these experiments Casamino acids were omitted from the basal medium (see Rahat & Reich, 1962). —, Methionine; ---, ethionine.

Fig. 2. Growth of *Prymnesium parvum* at various suboptimal concentrations of vitamin B₁₂ in the presence of methionine or ethionine (0.25 mg./ml.). ---, Vitamin B₁₂; —, vitamin B₁₂ + methionine;, vitamin B₁₂ + ethionine.

Fig. 3. Growth of *Prymnesium parvum* at various concentrations of methionine, in the presence of compound FIII (10 mμg./ml.) with vitamin B₁₂ omitted.

Analogue added (see Table 1)

Fig. 4. Effect of methionine or ethionine (4 mg./ml.) on the growth of *Prymnesium parvum* in the presence of vitamin B₁₂ and growth inhibitory analogues. Vitamin B₁₂ was added at 100 μg./ml. and the respective analogues at 100 mμg./ml. Broad open column = with methionine; right half (solid black) = with ethionine; left half (vertical rules) = no addition.

nucleotide part of the molecule had their growth-inhibitory effect annulled by methionine. Of these analogues, only FIII has the benzimidazole group unchanged, whereas in compounds FA, PVB₁₂ and 2MMA Cob (see Table 1), this group is replaced by adenine or its derivative.

In the absence of vitamin B₁₂ but with 10 μ g. FIII/ml. + 8 mg. methionine/ml., growth of *Prymnesium parvum* was equal to that obtained with vitamin B₁₂ alone (Fig. 3). Higher concentrations of compound FIII gave no additional growth. Noteworthy, however, is the fact that, whereas growth of *Ochromonas malhamensis* is inhibited by 0.8 mg. methionine/ml., for *P. parvum* maximal growth was attained in the presence of 8 mg. methionine/ml. An attempt was made to replace methionine, in the presence of compound FIII, by other potential methyl donors with homocysteine added as a methyl acceptor. As can be seen from Table 3, these methyl donors exerted an effect qualitatively similar to that of methionine.

Table 2. *Growth of Prymnesium parvum in the absence of vitamin B₁₂ but $\pm$ methionine and various analogues*

Analogues (at 2 μ g./ml.)	Optical density reading (O.D. $\times$ 1000)	
	With methionine (4 mg./ml.)	Without methionine
FA	13	8
FIII	268	50
2MMA Cob	18	13
PVB ₁₂	13	8
EAM	22	18
MA	31	22
An	22	18

Table 3. *Growth of Prymnesium parvum in the presence of compound FIII (α -[5-hydroxybenzimidazolyl]-cobamide cyanide; 10 μ g./ml.) and potential methyl-donors with homocysteine as methyl acceptor*

Methyl-donor added	Optical density reading (O.D. $\times$ 1000)
MMT	65
MMT + HCSTE	114
Thet	60
Thet + HCSTE	108
Ch	91
Ch + HCSTE	114
Bt	75
Bt + HCSTE	119
HCSTE	65
No addition	46

MMT = Methylmethionine sulphonium iodide (100 μ g./ml.); Thet = Dimethyl propiothetin HCl (100 μ g./ml.); Ch = Choline chloride (1.0 mg./ml.); Bt = Betaine (1.0 mg./ml.); HCSTE = Homocysteine (100 μ g./ml.).

It is known that vitamin B₁₂ participates in nucleic acid metabolism (Dinning & Young, 1960; Manson, 1960; Downing & Schweigert, 1956*a, b*). On the assumption that compound FIII in presence of methionine might be replaced by a nucleotide, various nucleotides were added to basal media free from any members of the vitamin B₁₂ group. The nucleotides tested, at 0.1 mg./ml., were: adenine, adenosine, deoxyadenosine, adenylic acid, cytidine, cytosine, cytidylic acid, guanosine, thymine, thymidine, uridine, RNA, DNA. No growth occurred in any culture tested.

DISCUSSION

Methionine and ethionine can both be used by *Prymnesium parvum* as sole nitrogen source. Even at high concentrations, neither of these two amino acids exerts on *P. parvum* the growth inhibitory effect they can exert on *Ochromonas malhamensis* (Johnson *et al.* 1957). This may possibly be attributable to a low uptake by *P. parvum* of methionine and ethionine into sites where they are required in unchanged form, whereas at sites where they function as catabolic substrates these amino acids may be utilized even at high concentrations. Since with *P. parvum* there is no antagonistic interaction between methionine and ethionine, it must be assumed that the metabolic system of this organism is either so specific as to prevent ethionine from interfering with the action of methionine, or is such as to utilize both amino acids equally well, enabling ethionine to be incorporated into cell protein, as is the case with *Tetrahymena* (Gross & Tarver, 1955).

The addition of methionine, but not of ethionine, to media containing vitamin B₁₂ and one of the growth-inhibitory benzimidazole-substituted analogues annulled the inhibitory effect of the latter. There was no such annulment in cultures in which the inhibitory analogues were ones substituted in the corrinoid part of the molecule. This shows that the various inhibitory analogues exert their inhibitory effects at different sites. The benzimidazole-substituted analogues apparently inhibit methionine or methyl-group synthesis, and therefore their inhibition is annulled when methionine is added. The propionamide-substituted analogues seem to inhibit some other biosynthetic pathway. To obtain the above-mentioned annulment, a relatively high concentration of methionine was required. This also might be explained by assuming a low uptake of methionine as such, at the sites where it is needed unchanged. Perhaps it is at these sites that methionine is synthesized when vitamin B₁₂ is not subject to competitive inhibition of an inhibitory analogue. Methionine, while obviously synthesized only in the presence of vitamin B₁₂, shows no sparing effect for this vitamin. It seems likely therefore that only a small part of the vitamin B₁₂ is utilized in methyl metabolism, the rest participating in other metabolic processes.

As mentioned before, the growth of *Prymnesium parvum* is inhibited by benzimidazole-substituted analogues (e.g. compounds FA, PVB₁₂ and 2MMA Cob, Table 1) even in the presence of vitamin B₁₂; however, the addition of methionine enables growth. This suggests that, of the functions of vitamin B₁₂ in the organism, only that of methionine synthesis is inhibited by these analogues. Hence the addition of methionine enables growth of *P. parvum* even in the presence of such inhibitory analogues. For *Ochromonas malhamensis* the inhibition was shown to be exerted on the uptake of the vitamin (Ford, 1958).

Such a mechanism of action of B₁₂ antagonists cannot be excluded in *P. parvum*. A final conclusion whether *Ochromonas* and *Prymnesium* are different in this respect will have to await further experiments on both organisms.

Compound FIII_m (-5-methoxy) replaces vitamin B₁₂ for *Prymnesium parvum* while compound FIII (-5-hydroxy) inhibits growth, even in the presence of vitamin B₁₂ (Rahat & Reich, 1963). However, compound FIII in the presence of methionine replaces vitamin B₁₂ for *P. parvum*. No precursors of methionine or allied amino acids tested had such effect with compound FIII. This emphasizes the importance

of the methyl groups in methionine, compound FIII_m and vitamin B₁₂. There is, however, the possibility that compound FIII in the presence of labile methyl groups of methionine may be converted to FIII_m or even to vitamin B₁₂ proper. If this be so, then the reverse transfer of methyl groups should be possible, i.e. transformation of a methionine precursor into methionine as a result of transmethylation of the vitamin B₁₂ benzimidazole methyl groups. In *P. parvum* cultures containing compound FIII instead of vitamin B₁₂, the addition of various methyl donors (methyl-methionine sulphonium iodide, dimethylpropiothetin, choline, betaine) in the presence or absence of homocysteine as methyl acceptor gave enhancement of growth, qualitatively like that attained with vitamin B₁₂. This again emphasizes the importance of free methyl groups for the growth of *P. parvum* in the absence of vitamin B₁₂, which obviously participates in the synthesis of these groups. Compound FIII has a benzimidazole moiety in its molecule, and can replace vitamin B₁₂ in the presence of methionine. In the other nucleotide-substituted analogues tested the benzimidazole moiety was replaced by a purine group. It would seem, therefore, that compound FIII can assume all the functions of vitamin B₁₂ except that of methionine or methyl group synthesis, for which the methyl groups of the benzimidazole moiety are indispensable. The latter syntheses are actually inhibited by the presence of hydroxybenzimidazole moiety in the molecule of FIII. Thus, the provision of methionine can relieve vitamin B₁₂ from one of its functions, methionine synthesis, and compound FIII can assume the remaining functions of the vitamin. Attempts to find a compound that can replace FIII in the presence of methionine have thus far been unsuccessful.

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