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The Influence of Certain Derivatives of Vitamin B₁₂ upon the Growth of Micro-Organisms

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SUMMARY: Seven analogues of vitamin B₁₂ were examined for their influence upon the growth of *Ochromonas malhamensis*, *Euglena gracilis*, *Lactobacillus leichmannii* ATCC 4797 and *Escherichia coli* 113-3. The analogues were active in promoting the growth of *E. gracilis* and *L. leichmannii*, but were essentially inactive for *O. malhamensis*. For *E. coli* three of the analogues were active and four inactive. In *O. malhamensis* and in *E. coli*, the 'inactive' analogues antagonized the growth-promoting action of cyanocobalamin, apparently by saturating the cells' mechanism for 'binding' the vitamin and so preventing its uptake.

Tests were also carried out with *Ochromonas malhamensis* and *Escherichia coli* on twelve derivatives of benziminazole. None of these proved inhibitory towards *E. coli*, at least up to 100 µg./ml. culture medium. Eight of the compounds were inhibitory towards *O. malhamensis*; the effects were relatively small, however, and were possibly unrelated to the metabolism of vitamin B₁₂.

At least fourteen analogues of vitamin B₁₂ occur in nature. They are all closely related chemically but differ in their growth-promoting activities for different micro-organisms. Of the analogues so far tested, only those few that contain a benziminazole moiety in their nucleotide have proved active in promoting the growth of higher animals. The remainder are inactive, and even in some instances have been shown to antagonize the action of vitamin B₁₂ (Coates *et al.* 1956). Certain micro-organisms, among them the protozoan *Ochromonas malhamensis*, share this animal pattern of response to the B₁₂-vitamins. Other micro-organisms, including the familiar assay organisms *Lactobacillus leichmannii*, *Euglena gracilis* and *Escherichia coli* 113-3, are less exacting and can make equivalent use of several of the B₁₂-group vitamins (cf. Coates & Ford, 1955).

Tests with *Ochromonas malhamensis* (Ford, 1958) and with a group of soil bacteria (Ford & Hutner, 1957), for which the analogues, pseudovitamin B₁₂ and Factor A, are essentially inactive, revealed a form of metabolite-anti-metabolite relationship between cyanocobalamin and each of these 'inactive' analogues. The analogues inhibited growth apparently by saturating the cells' mechanism for trapping and storing cyanocobalamin. The process of absorption required the presence of an intact nucleotide in the molecule of the B₁₂-vitamin. Factor B, which constitutes the non-nucleotide portion of all the B₁₂-vitamins that have so far been characterized, was not absorbed and was neither active nor antagonistic.

The present paper reports an extension of these earlier studies. Through the kindness—deeply appreciated—of Professor K. Bernhauer (Biochemisches Laboratorium der Aschaffenburg Zellstoffwerke, A.G., Stokstadt-am-Main) and Dr E. L. Smith, F.R.S. (Glaxo Laboratories Ltd., Greenford,

Middlesex) I have been able to examine in a number of micro-organisms the effects on growth and cyanocobalamin uptake of several derivatives of cyanocobalamin, prepared by chemical modifications of the structures of naturally occurring forms of the vitamin. A more limited study has also been made of the effects of various benziminazole derivatives on the growth of *Ochromonas malhamensis* and of *Escherichia coli*. It was prompted by reports (cf. Funk & Nathan, 1958) that various substituted benziminazoles inhibit the growth of bacteria and flagellates that require vitamin B₁₂.

MATERIALS

Analogues of vitamin B₁₂

Pseudovitamin B₁₂, vitamin B₁₂ monocarboxylic acids, vitamin B₁₂ methylamide, vitamin B₁₂ ethylamide, vitamin B₁₂ anilide (all made by Dr E. Lester Smith; described by Smith, Parker & Gant, 1956), 'benzyl B_{12III}' (an analogue of cyanocobalamin, containing in its nucleotide the benzyl ether of 5-hydroxybenziminazole, made by Professor K. Bernhauer; described by Gross, Friedrich & Bernhauer, 1957) and 'MMHP' (an analogue of pseudovitamin B₁₂, containing 2-methyl-mercapto-6-hydroxypurine in its nucleotide, made by Professor K. Bernhauer; described by Friedrich & Bernhauer, 1957) were studied.

Derivatives of benziminazole

Benziminazole, 5-methylbenziminazole, 5-aminobenziminazole, 5-nitrobenziminazole, 2-(*o*-hydroxyphenyl)benziminazole, 5:6-dimethylbenziminazole, 5:6-dichlorobenziminazole, 2:5-dimethylbenziminazole, 3:4-dimethyl-6-D-ribitylaminobenzene, 1-amino-3:4-dimethyl-6-D-ribitylaminobenzene, 5:6-dimethylbenziminazole- α -D-ribofuranose phosphate (α -ribazole), 5:6-dimethylbenziminazole- β -D-ribofuranose phosphate (β -ribazole) were studied.

METHODS

Microbiological methods

For the tests with *Euglena gracilis* the 'Z' strain of this organism was used in the assay procedure recommended by Hutner, Bach & Ross (1956).

The tests with *Lactobacillus leichmannii* ATCC 4797 were carried out by the method of Skeggs, Nepple, Valentik, Huff & Wright (1950), modified as described by Coates, Ford, Harrison, Kon & Porter (1953). With *Escherichia coli* 113-3 a tube assay technique was used essentially as described by Burkholder (1951). The basal medium was modified by the inclusion of sodium cyanide (2 mg./l. final strength medium) and the substitution of thiomalic acid for thioglycollic acid. For comparing the effects of the vitamin B₁₂ analogues on the growth rate, a heavy inoculum of exponentially growing cells was given, and the period of incubation was reduced to 10 hr. Cyanobalamin was included in the basal medium at a growth-limiting level, and the various analogues were tested over a wide range of concentrations.

Assays with *Ochromonas malhamensis* were done as described by Ford (1958). An examination of the influence on growth rate exerted by the different

analogues of vitamin B₁₂, and by the various derivatives of benziminazole, was carried out as described by Ford (1958). So also were the studies on vitamin B₁₂ uptake by resting cells, except that in the experiments now described ⁵⁸Co-labelled cyanocobalamin was employed, and measured with a scintillation counter containing a thallium-activated sodium iodide crystal.

An extract of *Ochromonas malhamensis* cells was prepared in the following manner. Two litres of 4-day culture grown with 0.0001 µg. cyanocobalamin/ml. were centrifuged, and the supernatant fluid was discarded. The residue was reconstituted to a thick brei by the addition of 25 ml. of 0.3 M-sucrose solution and transferred to a 150 ml. conical flask, together with 30 ml. of 3 mm. glass beads. The flask was stoppered tightly and shaken vigorously for 20 min. in a Towers-Gilson vibratory shaker (Towers Ltd., Widnes, England). The contents of the flask were then filtered through a plug of glass wool in the stem of a small funnel, and centrifuged at 10,000 g for 20 min. The supernatant liquor was carefully decanted for use in the experiment described on p. 698.

The technique of ultrafiltration through cellulose sausage skin has been described by Gregory (1954).

RESULTS

Measurements of the growth-promoting activities of the vitamin B₁₂ analogues

The relative growth-promoting activities of the vitamin B₁₂-analogues for the four different test micro-organisms are shown in Table 1. None of the seven compounds was active for *Ochromonas malhamensis*; three were active for *Escherichia coli*, and all were active for *Lactobacillus leichmannii* and *Euglena gracilis*.

Table 1. *Relative growth-promoting activities of different analogues of vitamin B₁₂, measured with different micro-organisms. The activity of cyanocobalamin is taken as 100*

	<i>Ochromonas malhamensis</i>	<i>Escherichia coli</i>	<i>Lactobacillus leichmannii</i>	<i>Euglena gracilis</i>
Vitamin B ₁₂ methylamide	0	0	14	26
Vitamin B ₁₂ ethylamide	0	0	12	13
Vitamin B ₁₂ anilide	0	0	48	7
Benzyl-vitamin B ₁₂ III	0	15-45	36	30
Vitamin B ₁₂ monoacids	0	0	23	184
Pseudovitamin B ₁₂	0	8	62	70
'MMHP'	0	43	25	Not tested

Influence of the analogues upon the growth-rate of Ochromonas malhamensis and Escherichia coli

Six of the vitamin B₁₂-analogues were tested for their effects upon the growth responses of *Ochromonas malhamensis* and *Escherichia coli* to cyanocobalamin. The results of these tests are illustrated in Figs. 1 and 2, which show approximately the rates at which cultures of the organisms grew in presence of a constant level of cyanocobalamin and with graded concentrations of the various analogues. Under the conditions of test employed, growth rates were nearly proportional to the logarithms of the final optical densities of the cultures. The

ratios, concentration of vitamin B₁₂ analogue: concentration of cyanocobalamin, at which the rates of growth were reduced to one half that of the culture grown with cyanocobalamin alone, were taken as an index of the antagonistic action of the analogues. These ratios are given in Table 2.

Ochromonas malhamensis and *Escherichia coli* were alike in that, for both organisms, vitamin B₁₂ methylamide and vitamin B₁₂ ethylamide were potent antagonists, and the 'anilide' markedly less antagonistic. The monoacids were strongly active against *E. coli* and only weakly inhibitory towards *O. malhamensis*. Benzyl B₁₂III and MMHP, on the other hand, promoted the growth of *E. coli* but inhibited the growth of *O. malhamensis*. In this behaviour

Fig. 1

Fig. 2

Fig. 1. Influence of vitamin B₁₂ analogues on the growth of *Ochromonas malhamensis* in medium containing 0.15 mμg. cyanocobalamin/ml.

Fig. 2. Influence of vitamin B₁₂ analogues on the growth of *Escherichia coli* 113-3 in medium containing 0.2 mμg. cyanocobalamin/ml.

—, vitamin B₁₂ methylamide; ·····, vitamin B₁₂ ethylamide; ---, vitamin B₁₂ anilide; — · — ·, vitamin B₁₂ monoacids; — — —, Benzyl vitamin B₁₂III; — · · —, 'MMHP'.

In both figures, optical densities are plotted on a logarithmic scale, so that the curves indicate approximately the rates of cell division at the different concentrations of the analogues. The line $kc/2$ shows the concentrations of the different analogues at which the growth-rate was about half that in the control cultures.

Table 2. Concentrations of vitamin B₁₂-analogues, relative to cyanocobalamin, at which growth-rates were reduced to half those of control cultures grown with cyanocobalamin alone.

	Relative concentration of analogue required to reduce growth rate by 50 %	
	<i>Ochromonas malhamensis</i> test	<i>Escherichia coli</i> test
Vitamin B ₁₂ methylamide	240	126
Vitamin B ₁₂ ethylamide	580	83
Vitamin B ₁₂ anilide	2,800	6,400
Benzyl-vitamin B ₁₂ III	4,500	Not antagonistic
Vitamin B ₁₂ monoacids	26,000	330
'MMHP'	12,000	Not antagonistic

Escherichia coli was grown in basal medium containing 0.2 mμg. cyanocobalamin/ml. and *Ochromonas malhamensis* in medium containing 0.15 mμg. cyanocobalamin/ml.

they resembled the naturally occurring analogues pseudovitamin B₁₂ and Factor A (Ford, 1958).

Influence of benziminazole derivatives upon the growth-rate of Ochromonas malhamensis and Escherichia coli

The analogues listed on p. 694 were tested at concentrations of 0.01, 0.10, 1.0, 10 and 100 µg./ml. for their effects upon the rate of growth of *Ochromonas malhamensis* and *Escherichia coli* in culture media containing 0.1 mµg. cyanocobalamin/ml. None of the compounds depressed the growth of *E. coli*. At 100 µg./ml., 5-methylbenziminazole and 2:5-dimethylbenziminazole were slightly stimulatory.

Benziminazole, 5-methylbenziminazole, 5-nitrobenziminazole, 5:6-dimethylbenziminazole, 5:6-dichlorobenziminazole and 2:5-dimethylbenziminazole were slightly inhibitory towards *Ochromonas malhamensis* at 10 µg./ml. and 2-(*o*-hydroxyphenyl) benziminazole caused a marked depression of growth. At 100 µg./ml. the effects of the different analogues on relative growth rate were more pronounced, as follows: growth-rate in absence of benziminazole analogue, 100; with 5-aminobenziminazole, 100; with α -ribazole, 98; with 3:4-dimethyl-6-D-ribityl-aminobenzene, 95; with β -ribazole, 93; with 1-amino-3:4-dimethyl-6-D-ribityl-aminobenzene, 91; with 2:5-dimethylbenziminazole, 88; with benziminazole, 75; with 6-nitrobenziminazole, 73; with 5-methylbenziminazole, 64; with 5:6-dimethylbenziminazole, 27; with 5:6-dichlorobenziminazole, 16, and with 2-(*o*-hydroxyphenyl)benziminazole, 8.

Influence of vitamin B₁₂ analogues and benziminazole derivatives upon the uptake by Ochromonas malhamensis of ⁵⁸Co-labelled cyanocobalamin

Forty-five conical flasks of 100 ml. capacity were arranged and numbered into nine series of five. To each flask was added 0.026 µg. (0.05 µc.) of ⁵⁸Co-labelled cyanocobalamin, together with 0, 0.05, 0.50, 5.0 or 50 µg. of one of nine compounds (seven vitamin B₁₂-analogues, 5:6-dimethylbenziminazole and 2-(*o*-hydroxyphenyl)benziminazole). Next was added 30 ml. of *Ochromonas malhamensis* culture, from 2 l. culture grown for 4 days in light with 0.1 mµg. cyanocobalamin/ml., as described by Ford (1958). The flasks and their contents were stood for 1 hr. at room temperature (c. 26°), being shaken at 10 min. intervals to prevent sedimentation of the organisms in the culture. The flask contents were then centrifuged, the supernatant liquors were decanted and the residua of compacted cells dissolved in 10 ml. portions of 4N-NaOH solution. The radioactivities of these solutions were then measured. The findings are shown in Fig. 3. The seven analogues of vitamin B₁₂ were alike in that at the higher test concentrations they prevented the uptake of cyanocobalamin, whereas at lower concentrations they facilitated uptake. The effects of the benziminazole derivatives were relatively small. The lowest test concentration of 2-(*o*-hydroxyphenyl)benziminazole enhanced slightly the uptake of cyanocobalamin; the highest concentration was slightly inhibitory. 5:6-Dimethylbenziminazole caused a small increase in cyanocobalamin uptake at all but the lowest test concentration.

Influence of vitamin B₁₂-methylamide upon the 'binding' of cyanocobalamin by an extract of Ochromonas malhamensis cells

To each of four test tubes was added 0.026 μg . (0.05 μc .) ^{58}Co -labelled cyanocobalamin, together with 0, 0.5, 5.0, or 50 μg . vitamin B₁₂-methylamide. Water was added to bring the content of each tube to 7 ml., followed by an extract of *Ochromonas malhamensis* cells to 10 ml. The whole was then ultrafiltered until about 3.5 ml. filtrate had collected. A 3 ml. portion of each filtrate was taken

Fig. 3

Fig. 3. Effects of vitamin B₁₂ analogues and of benzimidazole derivatives on the uptake of cyanocobalamin. ^{58}Co -labelled cyanocobalamin (0.026 μg .; 0.05 μc .) was added to 30 ml. portions of a culture of *Ochromonas malhamensis*, together with graded amounts of the test compound. After incubation for 1 hr., the cells were separated from culture fluid and dissolved in 4N-NaOH for scintillation counting. —, vitamin B₁₂ methylamide; — —, vitamin B₁₂ ethylamide; — — —, vitamin B₁₂ anilide; — — — —, vitamin B₁₂ monacids; — — — — —, benzyl vitamin B₁₂ III; — — — — —, 'MMHP'; — — — — —, Pseudovitamin B₁₂; — — — — —, 2-(*o*-hydroxyphenyl)benzimidazole; — — — — —, 5:6-dimethylbenzimidazole.

Fig. 4

Fig. 4. Influence of vitamin B₁₂-methylamide on the binding of cyanocobalamin. ^{58}Co -labelled cyanocobalamin (0.026 μg .; 0.05 μc .) was added to equal portions of an extract of *O. malhamensis* cells, together with graded amounts of vitamin B₁₂-methylamide. Each was then ultrafiltered, and the radioactivity of the filtrates measured.

and diluted to 10 ml. Finally, the radioactivities of these diluted filtrates were measured. The results are shown in Fig. 4. They indicate that the binding of added cyanocobalamin by an extract of *O. malhamensis* cells was progressively reduced by the simultaneous addition of progressively increasing amounts of vitamin B₁₂-methylamide.

Analysis of *Ochromonas malhamensis* cells grown with limiting cyanocobalamin showed that the soluble cell proteins contained only 10% of the cells' nitrogen, but nearly 30% of the cyanocobalamin, in a non-ultrafiltrable form. The experiment was therefore repeated, but substituting a particle-free cell preparation obtained by centrifuging a cell extract at 100,000 *g* for 20 min. The results showed that this preparation also had the property of binding added cyanocobalamin, and again this binding was prevented by the simultaneous addition of vitamin B₁₂-methylamide.

DISCUSSION

All of the vitamin B₁₂-analogues tested were active for *Euglena gracilis* and *Lactobacillus leichmannii*. It remains to be investigated whether the compounds are intrinsically active, or whether these two micro-organisms can bring about their conversion to cyanocobalamin.

It has been shown (Ford, 1958) that pseudovitamin B₁₂ is taken up by *Ochromonas malhamensis* in about the same amount as cyanocobalamin. The inactivity of the analogue as a growth promoter cannot be attributed to inefficient uptake; the compound is inherently inactive. The process of vitamin B₁₂-uptake is associated with the presence in the organism of a specific vitamin B₁₂-binding component, which has the property of 'binding' cyanocobalamin and pseudovitamin B₁₂ in the sense of preventing their passage through a cellophane membrane. Cyanocobalamin is bound preferentially when it is added to a cell extract in admixture with pseudovitamin B₁₂. However, in relatively high concentration, pseudovitamin B₁₂ prevents the uptake of cyanocobalamin and so inhibits growth.

There remains the paradox that, at lower concentrations, pseudovitamin B₁₂ facilitates the uptake of cyanocobalamin and potentiates its growth-promoting activity (Ford, 1958). This is explained by the observation that, in cultures of *Ochromonas malhamensis*, the vitamin B₁₂-binding factor is not confined to the organisms: it appears also in the culture fluid during growth, and combines there with free vitamin B₁₂. In this complex-bound form the vitamin is far less readily absorbed than it is in its free form. One may suppose that, at relatively low concentration, pseudovitamin B₁₂ competes with cobalamin for the B₁₂-sequestering substance in the culture fluid, and so releases free cobalamin to the organisms. At higher concentrations the analogue tends increasingly to inhibit growth by blocking the intracellular binding mechanism.

It seems highly probable that all the B₁₂-analogues tested in the present study act on growth and vitamin B₁₂-uptake in *Ochromonas malhamensis* in this same fashion. Such quantitative differences as are apparent (Figs. 1, 3) can be

explained in terms of the relative affinities of analogue and vitamin for the binding substance.

The effects of the various B₁₂-analogues upon growth in *Ochromonas malhamensis* are shown most sensitively during the period of exponential growth, when the rate of cell division is governed by the concentration of available vitamin B₁₂ (Ford, 1958). Prolonging the period of incubation gives a different picture, that may be complicated by the effects of exhaustion of nutrients in the culture medium or by adaptation of the organism to the use of the analogue. Experiments now in progress suggest that *O. malhamensis* adapts fairly readily to the utilization of B₁₂-methanamide, or more probably that vigorously growing cultures can slowly transform this analogue to vitamin B₁₂. Thus the methanamide may be judged 'strongly inhibitory' at 48 hr., and yet appear at 96 hr. to potentiate the growth-promoting effect of vitamin B₁₂. Similarly, with mixtures of vitamin B₁₂ and pseudovitamin B₁₂, the amount of growth finally achieved is determined largely by the amount of vitamin B₁₂ present, and is little affected by the analogue. All this underlines the necessity, when reporting studies of this nature, to specify in some detail the conditions under which the tests were carried out.

None of the benzimidazole derivatives was strongly inhibitory. 2-(*o*-hydroxyphenyl)benzimidazole was the most active, and comparison of the response curves in Fig. 3 suggests that its mode of action might well be the same as that of the vitamin B₁₂-analogues. The effects were relatively slight, however, and were quite possibly non-specific.

In a recent publication, Baker, Pasher, Hutner, Herbert & Sobotka (1959) have reported that the methanamide, ethanamide and anilide of the monocarboxylic acid of vitamin B₁₂ were active in promoting the growth of *Euglena gracilis* and *Lactobacillus leichmannii*, and inactive for *Ochromonas malhamensis* and *Escherichia coli*. They pronounced that the three compounds were 'inactive as B₁₂-antagonists despite their previous designation as "antivitamin B₁₂" substances'. This conclusion should have been qualified by a statement that the concentrations of analogues used in the tests were relatively low, < 0.001 µg./ml. (S. H. Hutner, private communication). The authors had not enough of the analogues to enable them to try the higher concentrations tested in the present study. It can be seen from Fig. 1 that at the concentration of 0.001 µg./ml.—and indeed at 0.01 µg./ml.—no one of these three analogues inhibited the growth of *O. malhamensis*. Similarly, at 0.001 µg./ml. the analogues had no large effect upon the growth of *E. coli*.

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