FORD, J. E. (1958). *J. gen. Microbiol.* **19**, 161-172

B₁₂-Vitamins and Growth of the Flagellate *Ochromonas malhamensis*

By J. E. FORD

*The National Institute for Research in Dairying, Shinfield,
Reading, Berkshire*

SUMMARY: The flagellate *Ochromonas malhamensis* requires vitamin B₁₂ and responds only to those natural forms of the vitamin which are active also for higher animals. The relation between the rate of growth and the concentration of cyanocobalamin is described by an equation of the form of an adsorption isotherm. The 'inactive' analogues, pseudovitamin B₁₂ and Factor A, were taken up by *O. malhamensis* to about the same degree as cyanocobalamin, and inhibited competitively the growth response to cyanocobalamin, apparently by blocking a cell mechanism for 'binding' the vitamin.

At least half the marine algal flagellates and diatoms which have so far been studied in pure culture require an exogenous supply of vitamin B₁₂ (Droop, 1957*a*), and in the natural orders Chrysomonadina and Euglenida the requirement is probably characteristic (cf. Hutner, in Ford & Hutner, 1955). That so many of these planktonic organisms require vitamin B₁₂ explains the present keen interest among marine biologists in the ecological role of the vitamin, particularly about the question whether the concentration of available vitamin B₁₂ in the seas can fall so low as to limit the development of plankton communities. Droop (1957*b*) calculated that, on present evidence, there must be at all times more than enough vitamin B₁₂ in the sea for the crops which are encountered. Daisley (1957) argued that this view is based on a questionable assumption, namely, that determinations of the potency of vitamin B₁₂ for a few species, made under the conditions of laboratory pure culture, can be applied in an assessment of the natural situation. Daisley urged the need for much more information to be collected before any conclusion is drawn about the status of vitamin B₁₂ in marine ecology. The present paper illustrates that the factors involved are indeed complex, and is concerned with the influence of vitamin B₁₂ and of certain of its natural analogues on the growth of the freshwater chrysomonad *Ochromonas malhamensis*.

Ochromonas malhamensis is facultatively photosynthetic, and when grown in the light has relatively simple nutritional needs (Hutner, Provasoli & Filfus, 1958). For optimal growth in darkness its requirements are more complex, and have been worked out in some detail (Ford, 1958). The organism has an absolute and specific need for vitamin B₁₂, and responds selectively to the natural forms of the vitamin which are active also for higher animals (Coates & Ford, 1955).

METHODS

Test media. Most of the experiments involved growing *Ochromonas malhamensis* in darkness in a medium of the composition shown in Table 1. This medium allowed rapid and abundant growth, and was complete in the sense that the response to limiting amounts of cyanocobalamin was not enhanced by the presence of a variety of crude natural extracts. For incubations in light the medium was modified by the omission of casein hydrolysate, tryptophane, methionine and cystine, and by the inclusion of 0.3 g. NH_4Cl /100 ml. medium.

Table 1. *Composition of culture medium*

	(g.)		(mg.)
Acid hydrolysed casein, 'vitamin free'*	5	DL-Methionine	200
Glucose	10	L-Cystine	100
Diammonium hydrogen citrate	0.8	m-Inositol	10
KH_2PO_4	0.3	Choline chloride	2
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2	Thiamine	2
CaCl_2	0.15	p-Aminobenzoic acid	1
'Metals' solution†	10 ml.	NaCN	2
	(mg.)		
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	50	Biotin	10 μg .
DL-Tryptophane	100	Tween 80	1 ml.

The pH value of the solution was adjusted to 5.5, the volume being made up with distilled water finally to 1000 ml.

* Allen and Hanburys Ltd.

† After Hutner (private communication). The solution had the following composition: ethylenediamine tetra-acetic acid, 5 g.; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 6.15 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 11 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g.; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.04 g.; H_3BO_3 , 0.06 g.; KI, 0.001 g.; water to 1000 ml.

Maintenance of organism and preparation of inocula. The stock cultures were maintained in the basal medium (Table 1), supplemented with 0.1 μg . cyanocobalamin/ml. and autoclaved at 115° for 15 min. in 10 ml. portions held in 50 ml. conical flasks. They were incubated at about 27° under constant illumination at 240 foot-candles, and subcultured from 0.5 ml. inoculum at 4-day intervals. Grown under these conditions the cultures reached about 5,000,000 organisms/ml. at 4 days, and were used at this stage as inocula for the growth tests to be described. Some of the experiments required relatively large amounts of culture. These were grown as described above, but were grown in larger flasks ('penicillin bottles') each containing 400 ml. of medium, and from proportionately larger inocula.

Growth-rate tests. Most of the growth-rate tests were incubated in the dark. They were set up in optically matched Pyrex test tubes (19 × 150 mm.) each containing 5 ml. test medium. The tubes were plugged with cotton wool and autoclaved for 10 min. at 115°. After cooling to room temperature each tube was inoculated with one drop of undiluted 4-day culture and placed in a shaking machine in an air incubator operating at 29°. After the chosen time intervals the cultures were removed from the incubator and their relative optical densities (at 580 $m\mu$) determined in a Lumetron model 400 A photometer.

All the cultures were examined microscopically, and in some the mean diameters of the organisms were determined and counts made.

Tests of growth in the light were done in the modified medium (see above), and were set up in 50 ml. flasks and incubated at 27° in a thermostatically-controlled cabinet, uniformly illuminated at 180 foot-candles. Apart from that, they were performed as described above for the tests incubated in darkness.

Sources of B₁₂-vitamins. Cyanocobalamin was kindly provided by Glaxo Laboratories (Greenford) Ltd; vitamin B_{12 III} was kindly given by Dr K. Bernhauer (Biochemisches Laboratorium der Aschaffenburg Zellstoffwerke, A.G., Stockstadt-am-Main), and pseudovitamin B₁₂, Factor A, Factor B, Factor C and Factor D by Drs E. S. Holdsworth and J. W. G. Porter. All these analogues of vitamin B₁₂ occur naturally. They are red, cobalt-containing substances and are closely related chemically to cyanocobalamin (for review see Kon, 1955). The differences in chemical structure, where these are known, are confined to the nucleotide portion of the molecule. Thus, cyanocobalamin nucleotide is 5:6-dimethylbenzimidazole- α -D-ribofuranose phosphate, and vitamin B_{12 III} nucleotide is the 5-hydroxybenzimidazole ribotide. In pseudovitamin B₁₂ the nucleotide is based on adenine, and in Factor A on 2-methyladenine. Factor B has no nucleotide, and constitutes the non-nucleotide portion of the molecule in the above three analogues. The structures of Factors C and D are not yet known.

RESULTS

Vitamin B₁₂ and the growth of Ochromonas malhamensis

Fig. 1 shows the progression of growth in the dark (plotted as log₁₀ optical density readings) with time of incubation at different concentrations of cyanocobalamin. Growth with no added cyanocobalamin was very slight, and can be attributed to carry-over in the inoculum, and in part also to some vitamin contained in the 'vitamin-free' casein hydrolysate used in the medium.

In actively growing cultures the organisms were highly motile and ranged in diameter from c. 5 to 15 μ , averaging about 10 μ . Older cultures tended increasingly to contain cell debris and large agglomerates of non-motile small organisms, and accurate counting was not possible. In young cultures the counts were roughly proportional to the optical density readings, but the latter were preferred as a measure of total growth product. Measurements of the optical densities of serial dilutions of cultures of *Ochromonas malhamensis* at different stages of growth showed that they obeyed Beer's law within the limits of accuracy of the photometer.

No abnormalities in morphology were noticed as being characteristic of vitamin B₁₂ depletion. Even after incubation for 138 hr. the organisms grown with no added vitamin appeared quite normal, and were all highly motile. These cultures were remarkable only for their very slow rate of growth. In contrast, cultures grown at high concentrations of cyanocobalamin grew very rapidly to the stage of 'ageing'. At this stage, numbers increased faster than the rate of production of new cell material (as measured by optical density),

and the organisms diminished in size from a volume of *c.* $650\mu^3$ to *c.* $200\mu^3$. These aged organisms were non-motile and characteristically acorn-like in appearance; their contents were granular and confined at one end. On further incubation the organisms autolysed completely, and the cultures became quite clear. But this process of ageing in the cultures is only indirectly relevant to the metabolism of vitamin B₁₂, and can probably be attributed to the exhaustion of other nutrients in the culture medium.

Fig. 1

Fig. 2

Fig. 1. Influence of the cyanocobalamin concentration on the growth of cultures of *Ochromonas malhamensis*. A, no added cyanocobalamin; B, 0.025 mμg./ml.; C, 0.05 mμg./ml.; D, 0.1 mμg./ml.; E, 0.5 mμg./ml.; F, 1.0 mμg./ml.

Fig. 2. Influence of cyanocobalamin concentration on the rate of growth of *Ochromonas malhamensis* during the first 42 hr..

It seemed probable, from Fig. 1, that growth of the cultures was exponential only during the early hours of incubation. Plotting the relative growth rates shown during the first 42 hr. against the concentrations of cyanocobalamin gives a conventional rate/concentration curve (Fig. 2) which, according to Hinshelwood (1946), follows an equation of the form of an adsorption isotherm. It is described by the equation $K/K_{\max} = C/C_1 + C$, where K is the relative growth rate and $K_{\max}$ the rate when $C = \infty$; C is the concentration of cyanocobalamin, and C_1 is that value of C at which the value of K lies half-way between zero and $K_{\max}$. $1/K$ plotted against $1/C$ gives a straight line in this equation (Fig. 3). From this $K_{\max}$ can be read off as 3 divisions/24 hr., and C_1 (the concentration of cyanocobalamin which allows half maximal growth-rate) as 0.000013 μg./ml. Under the conditions used, the rate/concentration relation appeared to be logarithmic. However, Hinshelwood (1946) showed that at very low concentrations, the growth rate (K) would become proportional to the concentration of cyanocobalamin (C).

Droop (1957*b*) pointed out that the requirements of several flagellates for vitamin B₁₂ are very low, and are quantitatively similar. *Monochrysis lutheri*, *Euglena gracilis* and a *Stichococcus* sp. each required about 3 molecules of cyanocobalamin/ μ^3 of living alga. From the present experiments the minimal requirement for *Ochromonas malhamensis* is found to be of the same order. When grown in the light, the organisms required about 1 molecule of cyanocobalamin/ μ^3 ; grown in darkness they required about 3 molecules/ μ^3 . But

Fig. 3

Fig. 3. Influence of cyanocobalamin concentration on the rate of growth of *Ochromonas malhamensis*: $1/K$ against $1/C$.

Fig. 4

Fig. 4. Uptake of cyanocobalamin added in graded amounts to portions of a culture of *Ochromonas malhamensis*.

whether the similarity of all these estimates is more than fortuitous is open to question. In *O. malhamensis* at least, the requirement varies with the conditions of culture. For example, Hutner, Sanders, McLaughlin & Scher (1957) showed that over the temperature range 30–37° the requirement for cyanocobalamin increased 500-fold; and even 30° is much higher than the temperatures prevailing in Malham Tarn, whence *O. malhamensis* was first isolated. However, taking Droop's assumption that requirements as low as 3 molecules of cyanocobalamin/ μ^3 are fairly typical (and in the natural environment one can conceive the possibility of even lower requirements), his estimate that shortage of vitamin B₁₂ does not limit the size of marine plankton crops seems to ignore what is clearly an important consideration, namely, that of the influence of the concentration of vitamin B₁₂ upon the rate of growth.

The uptake of B₁₂-vitamins by Ochromonas malhamensis

Uptake of cyanocobalamin. Cyanocobalamin was added in graded amounts to 40 ml. portions of a culture of *Ochromonas malhamensis*, grown with limiting amounts of cyanocobalamin, as described on page 162. The portions were shaken for 2 hr. at 30° and centrifuged; organisms and supernatant fluids were then extracted and assayed separately for cyanocobalamin, with *Lacto*-

bacillus leichmannii as assay organism (cf. Coates *et al.* 1958). The distribution of the added vitamin between organisms and culture medium is shown in Fig. 4.

From this and similar experiments it was evident that, at 'physiological' concentrations, the uptake of cyanocobalamin was proportional to the concentrations added. At higher concentrations the uptake was somewhat more

Fig. 5. Uptake of different B₁₂-vitamins, added in graded amounts to equal portions of a culture of *Ochromonas malhamensis*.

efficient, and was again proportional to the amounts added. A transition from 'inefficient' to 'efficient' uptake is reflected in an abrupt increase in the slope of the uptake curve, and is probably associated with the property of the culture fluid of 'binding' vitamin B₁₂ (see p. 167).

Uptake of vitamin B₁₂ analogues. Of the naturally occurring analogues of vitamin B₁₂, *Ochromonas malhamensis* responds only to vitamin B₁₂ III. The other natural analogues have little or no activity, and are represented in this work by Factor A, Factor B and pseudovitamin B₁₂ (for reviews of literature on B₁₂ vitamins, see Kon, 1955; Ford & Hutner, 1955). To determine whether these biological properties are related to the efficiencies with which the

different analogues are taken up by the organisms the following experiment was made.

To 40 ml. portions of *Ochromonas malhamensis* culture were added cyanocobalamin, vitamin B_{12 III}, Factor A, Factor B or pseudovitamin B₁₂, each in amounts of 0, 0.01, 0.05, 0.2, 1.0, and 5.0 μ g. The portions of culture were shaken gently for 1 hr. at 29° and then centrifuged. The organisms were extracted and assayed for the different B₁₂-vitamins by using *Lactobacillus leichmannii* and *Escherichia coli* 118-3 as assay organisms (Ford, 1958). The results are shown in Fig. 5.

Because of technical limitations in the assay, the values given for Factor B and pseudovitamin B₁₂ are only approximate. It was quite clear, however, that whereas very little, if any, of Factor B was taken up, the remaining compounds were taken up, and in about the same amount. It would seem then that the inactivity of Factor A and pseudovitamin B₁₂ as growth promoters cannot be attributed to inefficient uptake; the compounds are inherently inactive. Factor B was quite inactive for *Ochromonas malhamensis*, even when it was given together with the 'missing' nucleotide moiety of the cyanocobalamin molecule.

Mechanism of uptake of B₁₂-vitamins

Although several of the B₁₂-vitamins, active and inactive, are taken up by *Ochromonas malhamensis*, it can be shown that cyanocobalamin is taken up preferentially from mixtures containing the inactive analogues. The process seems to be associated with the presence in the organisms of a specific vitamin B₁₂-binding component. Extracts of disrupted *O. malhamensis* have the property of binding cyanocobalamin, vitamin B_{12 III}, Factor A and pseudovitamin B₁₂ in roughly equivalent amount, in the sense of preventing their passage through a cellophan membrane. With these cell extracts, as with intact organisms, cyanocobalamin itself is preferentially bound. Gregory & Holdsworth (1953) reported very similar behaviour of cyanocobalamin in sow's milk and in preparations of 'intrinsic factor'. The property of binding vitamin B₁₂ is typically associated with the gastric secretion in man and in certain higher animals, and is believed to be concerned with uptake of the vitamin. From present indications it may well prove that the mechanism for the uptake of vitamin B₁₂ is essentially the same in *O. malhamensis* as in the higher animals. Certainly *O. malhamensis*, with its animal-like specificity for the vitamin, should prove a most useful model for studies on vitamin B₁₂ metabolism. These observations just outlined about the mechanism of vitamin B₁₂-uptake will be further discussed in a later paper. They are quoted here to help the understanding of experiments (p. 168) on competitive relationships between the B₁₂-vitamins.

Binding of vitamin B₁₂ in culture fluids. In cultures of *Ochromonas malhamensis* the property of binding vitamin B₁₂ is not confined to the organisms; it appears also in the culture fluid during growth. In proportion to the vitamin B₁₂ requirement of the organisms, this extracellular binding capacity is very large.

A culture (400 ml.) of *Ochromonas malhamensis*, grown as described earlier (p. 162) was centrifuged and the supernatant liquid decanted. To 9 ml. portions of this culture liquid were added 1 ml. portions of aqueous solutions containing 0, 0.01, 0.02, 0.05 or 0.1 $\mu\text{g.}$, respectively, of cyanocobalamin. Each sample was then ultrafiltered through Visking cellulose tubing, by the technique described by Gregory (1954), and the ultrafiltrates were tested for cyanocobalamin by means of *Lactobacillus leichmannii*. The results are shown in Table 2.

Table 2. *Binding of cyanocobalamin by Ochromonas malhamensis culture liquid*

Cyanocobalamin added ($\mu\text{g./ml.}$ culture liquid)	Cyanocobalamin in ultrafiltrate ($\mu\text{g./ml.}$)	Cyanocobalamin 'bound' ($\mu\text{g./ml.}$ culture liquid)
0	0	—
0.001	0	0.001
0.002	0	0.002
0.005	0.001	0.004
0.01	0.006	0.004

It is open to question whether the release of 'binding' material into the medium is connected with the mechanism for concentrating vitamin B_{12} from the environment. An analogy with the 'intrinsic factor' in the gastric secretion may be far-fetched, but does at least seem plausible. The degree of binding in the *Ochromonas malhamensis* culture liquid increased with the optical density of the culture, and was not particularly associated with old cultures. But, assuming that the secretion of a vitamin B_{12} -sequestering substance is a characteristic of actively growing organisms, it is difficult to conceive how, except under very restricted conditions of growth, such a substance might be of direct aid in trapping the vitamin. It seems much more likely that it actually inhibits growth. Thus, with *Euglena gracilis* Kristensen (1955) found that the culture liquid contained a thermolabile factor which severely inhibited the growth response to limiting concentrations of cyanocobalamin. And *E. gracilis* culture liquids, like those of *O. malhamensis*, bind vitamin B_{12} . When graded amounts of cyanocobalamin were added to a culture of *O. malhamensis* the efficiency of uptake by the organisms was greater at the higher concentrations. All this suggests that for *O. malhamensis* the cyanocobalamin complex is much less active or readily available than is the free vitamin.

Tests for synergism and antagonism

Effect of pseudovitamin B_{12} on the uptake of cyanocobalamin. Cyanocobalamin (0.02 $\mu\text{g.}$) was added with graded amounts of pseudovitamin B_{12} to 40 ml. portions of a culture of *Ochromonas malhamensis*. These samples were shaken for 2 hr. at 30° and centrifuged. The organisms and supernatant liquids were then separately extracted and assayed for cyanocobalamin by using *O. malhamensis* as assay organism (Ford, 1953). The influence of pseudovitamin B_{12} on the uptake of cyanocobalamin is shown in Fig. 6. At 0.25 $\mu\text{g.}$, pseudo-

vitamin B₁₂ increased the uptake of cyanocobalamin; above this quantity the uptake of cyanocobalamin was progressively inhibited. In other similar experiments, specimens of Factor A, Factor B and 5:6-dimethyl-benziminazole (DMB) were examined for their effects on cyanocobalamin uptake. Factor A behaved like pseudovitamin B₁₂. At low concentrations both compounds increased the uptake of cyanocobalamin, and in higher concentrations both were increasingly inhibitory. Factor A was, however, appreciably less inhibitory than pseudovitamin B₁₂. Factor B had no significant effect on cyano-

Fig. 6. Effect of pseudovitamin B₁₂ on the uptake of cyanocobalamin. Cyanocobalamin (0.02 μg.) was added to 40 ml. portions of a culture of *Ochromonas malhamensis*, together with graded amounts of pseudovitamin B₁₂. Figure shows distribution of the added cyanocobalamin between cells (●—●) and culture liquors (○—○), after incubation for 2 hr.

cobalamin uptake. DMB, even at 50 μg./ml., had no effect on uptake. DMB, has been shown to inhibit the uptake of cyanocobalamin by *Escherichia coli* (Oginsky & Smith, 1953), and to inhibit competitively the response of *Euglena gracilis* to the vitamin (Hendlin, 1953). DMB is present in the molecule of cyanocobalamin, but not in Factor B. And as Factor B is inactive for *Lactobacillus leichmannii* and very poorly taken up by *O. malhamensis*, it seems possible that the mechanism of uptake involves the linkage of the base (DMB) of the vitamin nucleotide with a receptor protein (or binding factor). The present finding indicates only that the free base does not hinder the uptake of cyanocobalamin by saturation of the binding mechanism.

Effects of pseudovitamin B₁₂ on the rate of growth of Ochromonas malhamensis. Ford (1953) reported that under conditions specified for the assay of vitamin B₁₂ with *O. malhamensis*, pseudovitamin B₁₂ was for practical purposes inactive. Certainly the highest concentrations of pseudovitamin B₁₂ likely to be present in natural materials would have no appreciable influence on the degree of growth measured after incubation for 72 hr. Against this, it is reasonable to infer from the observations so far recorded in this paper that a form of metabolite-antimetabolite relationship should be demonstrable between cyanocobalamin and pseudovitamin B₁₂. Pseudovitamin B₁₂ was tested over a wide range of concentrations, and at three different concentrations of cyanocobalamin, for its effect on the rate of growth of *O. malhamensis*; the results are shown in Fig. 7.

It was found that the lower concentrations of pseudovitamin B₁₂ caused a slight but consistent increase in the growth rate. At higher concentrations of pseudovitamin B₁₂ growth was progressively inhibited and the inhibition was annulled by cyanocobalamin. Other analogues were also tested; Factor A behaved in the same manner as pseudovitamin B₁₂, while Factors B, C and D were quite inert and had no effect on the growth-rate.

Fig. 7. Influence of pseudovitamin B₁₂ on the rate of growth of *Ochromonas malhamensis* in media containing cyanocobalamin at: (1) 0.0002 μg./ml. (Δ—Δ); (2) 0.0005 μg./ml. (○—○); (3) 0.001 μg./ml. (●—●).

Stimulation of growth by the lower concentrations of pseudovitamin B₁₂ and Factor A can be interpreted as a further indication that bound cyanocobalamin is less readily absorbed than the free vitamin (see p. 168). It can be argued that by saturating the binding material in the culture liquid, these analogues would release free cyanocobalamin to the organisms. At higher concentrations the analogues would tend increasingly to inhibit growth by blocking the cell mechanisms for taking up and storing cyanocobalamin.

CONCLUSIONS

The pattern of response to the vitamin B₁₂ group of compounds shown by *Ochromonas malhamensis* seems to be broadly characteristic of a wide range of organisms, from soil bacteria (Ford & Hutner, 1957) to the chick (Coates *et al.* 1956). Many other organisms are less selective and can make use of one or more of the vitamin B₁₂-like compounds. A similar situation is probably found among those bacteria which synthesize vitamin B₁₂: some manufacture several of the B₁₂-vitamins, whereas others seem to produce only the classical vitamin B₁₂. No instance has yet been found of an organism for which any analogue of vitamin B₁₂ is active, and for which cyanocobalamin itself is inactive. Such an organism, if one exists, would be most useful for comparative studies. It seems that the B₁₂-requiring auxotrophs can be differentiated according to whether or not the analogues containing a purine nucleotide are active for them.

Competitive inhibition of cyanocobalamin by pseudovitamin B₁₂ has been reported in a number of soil bacteria (Ford & Hutner, 1957). Further study of one of these bacteria (isolate no. 56 of Lochhead & Burton, 1955) shows that it resembles *Ochromonas malhamensis* in the relation between its rate of growth and the concentration of cyanocobalamin.

There can be no doubt that in the soil (Lochhead & Thexton, 1951), in natural waters (Droop, 1957*a*) and in animals (Ford, Kon & Porter, 1952) the B₁₂-vitamins are of major ecological importance. As an aid to the understanding of the natural situation, studies of models with such organisms as *O. malhamensis* can be of great value.

In the matter of the importance of vitamin B₁₂ in marine productivity, the paramount need seems to be for more information. Little is known of the origins and distribution of marine vitamin B₁₂; even less is known of the relative abundance of the vitamin B₁₂ analogues, although Droop (1957*a*) concluded that these may have an importance equal to that of the vitamin. One can conceive that the evolution of a plankton community might be closely linked with the economy of the B₁₂-vitamins. But whether they are in fact of such over-riding importance in marine ecology is still open to question.

I would like to thank Dr M. R. Droop for his generous help in appraising some of the data presented in this paper. I am grateful also to Drs M. E. Gregory and S. K. Kon for helpful criticism and to Mr D. G. Scardifield for technical assistance.

REFERENCES

- COATES, M. E., DAVIES, M. K., DAWSON, R., HARRISON, G. F., HOLDSWORTH, E. S., KON, S. K. & PORTER, J. W. G. (1956). The activity for chicks of some vitamin B₁₂-like compounds. *Biochem. J.* **64**, 682.
- COATES, M. E. & FORD, J. E. (1955). Methods of measurement of vitamin B₁₂. *Biochem. Soc. Symp.* **13**, 86.
- COATES, M. E., FORD, J. E., HARRISON, G. F., KON, S. K. & PORTER, J. W. G. (1953). Vitamin B₁₂-like compounds. *Brit. J. Nutr.* **7**, 319.
- DAISLEY, K. W. (1957). Vitamin B₁₂ in marine ecology. *Nature, Lond.*, **180**, 1043.
- DROOP, M. R. (1957*a*). Auxotrophy and organic compounds in the nutrition of marine phytoplankton. *J. gen. Microbiol.* **16**, 286.

- DROOP, M. R. (1957*b*). Vitamin B₁₂ in marine ecology. *Nature, Lond.* **180**, 1041.
- FORD, J. E. (1953). The specificity of the requirement of *Ochromonas malhamensis* for cyanocobalamin. *Brit. J. Nutr.* **7**, 299.
- FORD, J. E. & HUTNER, S. H. (1955). Vitamin B₁₂ in the metabolism of micro-organisms. *Vitam. & Horm.* **13**, 101.
- FORD, J. E. & HUTNER, S. H. (1957). On the nature of the vitamin B₁₂ requirement in soil bacteria isolated by Lochhead and his co-workers. *Canad. J. Microbiol.* **3**, 319.
- FORD, J. E., KON, S. K. & PORTER, J. W. G. (1952). Cobalt in ruminant nutrition. *Chem. & Ind.* p. 495.
- GREGORY, M. E. (1954). The microbiological assay of vitamin B₁₂ in the milk of different animal species. *Brit. J. Nutr.* **8**, 340.
- GREGORY, M. E. & HOLDSWORTH, E. S. (1953). The combination of some vitamin B₁₂-like compounds with sow's milk whey and 'intrinsic factor' concentrates. *Biochem. J.* **55**, 830.
- HENDLIN, D. (1953). Discussion of papers on vitamin B₁₂. *Ann. N.Y. Acad. Sci.* **56**, 870.
- HINSHELWOOD, C. N. (1946). *The chemical kinetics of the bacterial cell*. Oxford: University Press.
- HUTNER, S. H., PROVASOLI, L. & FILFUS, J. (1953). Nutrition of some phagotrophic freshwater chrysomonads. *Ann. N.Y. Acad. Sci.* **56**, 852.
- HUTNER, S. H., SANDERS, M., McLAUGHLIN, J. J. A. & SCHER, S. (1957). Electrolyte requirements of protists and archaeometabolism. *Ann. N.Y. Acad. Sci.* **69**, 286.
- KON, S. K. (1955). Other factors related to vitamin B₁₂. *Biochem. Soc. Symp.* **13**, 17.
- KRISTENSEN, H. P. O. (1955). Investigations into the *Euglena gracilis* method for quantitative assay of vitamin B₁₂. *Acta physiol. scand.* **33**, 282.
- LOCHHEAD, A. G. & BURTON, M. O. (1955). Characteristics of vitamin B₁₂-requiring bacteria. *Canad. J. Microbiol.* **1**, 319.
- LOCHHEAD, A. G. & THEXTON, R. H. (1951). Vitamin B₁₂ as a growth factor for soil bacteria. *Nature, Lond.* **167**, 1084.
- OGINSKY, E. L. & SMITH, P. H. (1953). Effect of vitamin B₁₂ derivatives on oxidation and vitamin uptake by *Escherichia coli*. *J. Bact.* **65**, 183.

(Received 14 February 1958)