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Vitamin B_{12} binding by microalgal ectocrines: dissociation constant of the vitamin-binder complex determined using an ultrafiltration technique

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ABSTRACT: A simple, rapid ultrafiltration technique has been used to separate free vitamin B_{12} from that attached to the binding factor present in the media of cultures of the marine microalgae *Mantoniella squamata* and *Pavlova lutheri* (auxotrophic species), and *Dunaliella primolecta* and *Phaeodactylum tricornutum* (species with no B_{12} requirement). The dissociation constant (K_d) for the B_{12}-binder complex calculated from the results lies in the range 10^{-12} to 10^{-10} M indicating that the binder has a very high affinity for the vitamin. The ecological implications of the data are discussed in the light of our present understanding of B_{12} uptake by microalgae.

INTRODUCTION

Many microalgae release a substance which binds cyanocobalamin (vitamin B_{12}) (Provasoli and Carlucci, 1974; Pintner and Altmeyer, 1979). This binding factor is produced not only by algae auxotrophic for B_{12} but also by some which do not require the vitamin (Droop, 1968; Provasoli and Carlucci, 1974; Swift, 1980). Little is known about the properties of the algal B_{12}-binder other than that it is probably a protein since it is non-dialysable and is denatured by heat or proteolytic enzymes (Pintner and Altmeyer, 1979, and references therein). To date, the only algal B_{12}-binder that has in any way been characterised is a glycoprotein of MW ca. 200,000 produced by *Euglena gracilis* (Daisley, 1970).

An important parameter which has not been determined hitherto is the B_{12}-binder complex dissociation constant (K_d), which provides a measure of the affinity of the binding protein for the vitamin. We report here the measurement of the K_d value for the B_{12}-binder complex using the binding factor released by a number of marine microalgae and a simple, rapid ultrafiltration technique.

MATERIALS AND METHODS

Algal cultures. A list of the algae and culture media used is given in Table 1. All cultures were axenic. The media were sterilized by autoclaving at 121 °C for 15 min. Vitamin B_{12} (74 pM) was added only to the culture media for the stocks of auxotrophic algae. Cultures used for experiments were inoculated with 1 ml of stock and grown under the same environmental conditions (20 °C, 14 W m^{-2} warm white fluorescent light irradiance). B_{12} (18.4 pM) was again added only to media for the auxotrophic species. The chemostat culture of *Pavlova lutheri* (see Droop, 1968 for details) contained 7.4 pM B_{12} in the inflow. The age and the cell densities of the cultures when harvested are shown in Table 2. Cells were enumerated using a Coulter Counter Model B.

Preparation of culture medium containing B_{12}-binder. Cells and other particulate matter were removed by passing the cultures under slightly positive pressure first through a Whatman GF-F glass fibre filter and then through a Whatman WCN 0.2 μm pore cellulose nitrate membrane. The final filtrate was normally used within 1 h.

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Table 1. Algae and culture media

Algae	Strain No. SMBA[1]	Strain No. CCAP[2]	B_{12} requirement	Medium	Reference to Medium
Dunaliella primolecta Butcher	57	11/34	–	¾ ASP2[3]	Provasoli et al. (1957)
Mantoniella squamata (Manton & Parke) Desikachary	281	–	+	ASP2	Provasoli et al. (1957)
Pavlova (Monochrysis) lutheri (Droop) Green	261	–	+	S88	Droop (1968)
Phaeodactylum tricornutum Bohlin	14	1052/6	–	¾ ASP2[3]	Provasoli et al. (1957)

[1] Scottish Marine Biological Association strain number
[2] Equivalent Culture Centre for Algae & Protozoa (Cambridge) strain number
[3] 75% of the normal concentrations of NaCl, $MgSO_4.7H_2O$, KCl and $CaCl_2.2H_2O$. Concentrations of other constituents were as in Provasoli et al. (1957)

Separation of free and bound vitamin B_{12}. Free and bound vitamins were separated by ultrafiltration (Sophianopoulos et al., 1978; Whitlam and Brown, 1981). The apparatus used was the Micro-Partition System-1 (MPS) manufactured by Amicon Ltd (Fig. 1).

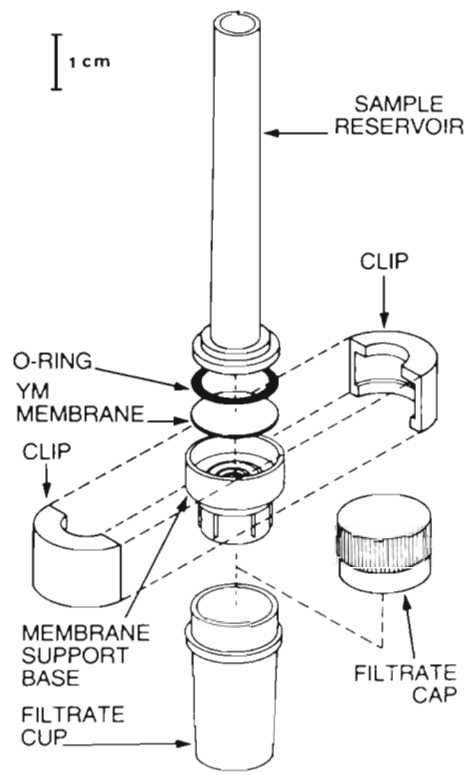

Fig. 1. Micro Partition System (MPS-1) used to separate free and bound vitamin B_{12}. Diagram reproduced by permission of Amicon Ltd

The units were fitted with 14 mm diameter Amicon ultrafiltration membranes, type YMT, which have low non-specific binding characteristics (typically < 5 %) and retain 99 % protein. Because the B_{12}-binder solutions were so dilute, it was found that slightly positive pressure could be used as the driving force instead of centrifugation.

Pressure was applied by a manually operated 5 ml plastic syringe connected with silicone rubber tubing to the top of the MPS reservoir. During filtration, the unit was clamped at an angle of 45°. Small amounts of ultrafiltrate which remained on the underside of the apparatus were transferred to the collecting cup with a micropipette.

Evaluation of the MPS apparatus. (a) *Non-specific adsorption.* The possibility of losses caused by non-specific adsorption of B_{12} by the apparatus was investigated by adding ^{57}Co-B_{12} to 0.2 µm membrane-filtered uninoculated medium to give concentrations of 33.5 and 100 pM and passing the solutions through the MPS membrane. (b) *Variation of B_{12} concentration in the ultrafiltrate.* The theory of ultrafiltration (Whitlam and Brown, 1981) predicts that, although the concentration of the solutes retained by the membrane increases as filtration proceeds, re-equilibration between bound and free ligand – in this case B_{12} – should maintain the unbound ligand, i.e. that passing through the membrane, at the same concentration. To verify this, filtrate from a *Phaeodactylum* culture was equilibrated with several concentrations of B_{12} (see Fig. 2 for details). One ml of solution was then transferred to the MPS and about 100 µl ultrafiltrate collected in the receiver cup, the precise volume being determined by weighing, and its radioactivity measured as described below. The cup was then returned to the MPS system and another fraction collected, and so on until about 70 % of the original solution had been ultrafiltered.

Stability of the B_{12}-binder. The stability of the binder when heated was tested by autoclaving samples of the culture filtrates for 10 min at 121 °C. Samples of culture filtrates were also treated with 'pronase' (50 µg ml^{-1}), a non-specific proteolytic enzyme (method as described in Pintner and Altmeyer, 1979). ^{57}Co-B_{12} was then

added to samples from each treatment to give a concentration of 100 pM, incubated for 1 h and finally ultrafiltered as described above.

Determination of the dissociation constant, K_d. Prior to the determination of K_d, a preliminary trial was made to ensure that the binding capacity of the filtrate was approaching saturation at the highest concentrations of B_{12} used in each experiment. Using a micropipette, various volumes of ^{57}Co-cyanocobalamin (Amersham International plc, specific activity 11.04 GBq μmol^{-1}), were placed in a series of vials to give the required range of final concentrations (normally from 14 to 200 pM) and 2.5 ml of the culture filtrate was then added to each vial and gently mixed. The vials were incubated at 20°C for 1 h. A 0.2 ml sample was then transferred to a plastic 'minivial', its precise volume being found by weighing. The radioactivity in this aliquot was measured to enable the concentration of B_{12} in the incubation mixture to be calculated. A further 1 ml of filtrate from each vial was transferred to MPS units for ultrafiltration as described above. Radioactivity in the 'minivials' and MPS units was measured using a sodium iodide well crystal/photomultiplier counting head coupled to a 'Panax Reigate Series' scaler, type RAD 54. Counting efficiency in the 'minivials' and MPS cups was identical.

Calculation of K_d. Assuming the system was at equilibrium:

$$\text{Free } B_{12} + \text{Binder} \rightleftharpoons B_{12} - \text{Binder complex}$$

$$\text{Concentrations} \quad F \quad B_{max}-B \quad B$$

$$\text{the dissociation constant } K_d = F(B_{max}-B)/B \qquad (1)$$

where F = concentration of free B_{12} (in the ultrafiltrate); B = concentration of bound B_{12} = total B_{12} concentration – F; B_{max} = maximal B_{12} binding capacity of the culture medium (expressed as moles B_{12} bound l^{-1}).

Equation (1) transforms to

$$B = \frac{B_{max}F}{K_d + F} \qquad (2)$$

K_d is thus also the half-saturation constant for B_{12} binding (equal to the concentration of free vitamin present when half of the binding sites are occupied). Values of B_{max} and K_d were obtained by fitting the experimental data to this expression using the method of Droop et al. (1982; Appendix 2). Some examples of these fits are given in Fig. 3.

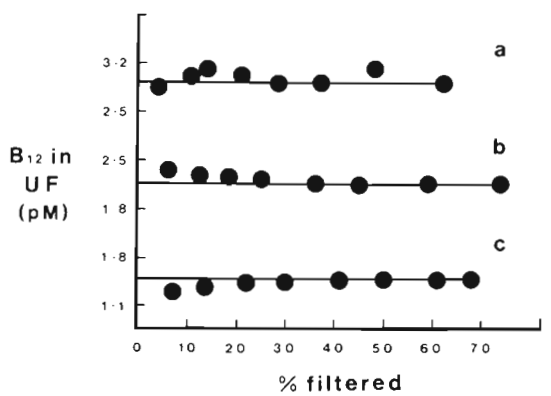

Fig. 2. Variations of the concentration of vitamin B_{12} in the ultrafiltrate (UF) with percentage volume filtered for *Phaeodactylum tricornutum* binding factor. UF B_{12} concentrations: (a) pM; (b) pM × 10^{-1}; (c) pM × 10^{-2}. Initial B_{12} concentrations before ultrafiltration were (pM): (a) 20.1; (b) 67.0; (c) 201.0. The % B_{12} bound was: (a) 85; (b) 66; (c) 29

RESULTS AND DISCUSSION

Evaluation of the MPS Apparatus

Non-specific adsorption

This was not significant. The decrease in the concentration of B_{12} in the ultrafiltrate of binder-free solutions was less than 5 %.

Variation of B_{12} concentration in the ultrafiltrate

The concentration of B_{12} in the ultrafiltrate did not vary significantly after passage of 200 µl (Fig. 2), indicating that the behaviour of the algal B_{12}-binder system conforms to theory (Whitlam and Brown, 1981). In experiments to determine K_d, at least 200 µl of ultrafiltrate was therefore collected. Apparent variations at lower concentrations may be attributed mainly to the greater degree of uncertainty in radioactivity counting statistics at lower concentrations of ^{57}Co-B_{12}. The MPS therefore provides a rapid and convenient method for separating free and bound B_{12} when the binder is present at the low concentrations typical of algal culture media (see Pintner and Altmeyer, 1979).

Stability of the B_{12} binder

Exposure to heat or treatment with a proteolytic enzyme destroyed the ability of the culture filtrates to bind B_{12}. More than 95 % of the ^{57}Co-B_{12} added to the denatured binder was recovered in the ultrafiltrate as opposed to ca 10 % in the controls. Denaturation of the binders by heat and by a protease implies that the binders are most probably proteins. This is consistent with the observations of Daisley (1970) and Pintner and Altmeyer (1979).

B_{12} binding constants

K_d, the dissociation constant for B_{12} binding, is both a measure of the affinity of the binder for the vitamin (the smaller the value, the greater the affinity) and also equivalent to the half-saturation constant for binding (see Equations 1 and 2). Table 2 gives the dissociation constants and the vitamin binding capacities of the algal binders. The K_d values demonstrate that (a) B_{12} is tightly complexed by the binders and (b) since K_d is also the half-saturation constant, that these substances are very efficient at binding low concentrations of vitamin. The relevance of this will be discussed later.

Highley et al. (1967) found that the 1 : 1 complex formed between B_{12} and hog intrinsic factor gradually dimerized over a period of 7 h, and Pintner and Altmeyer (1979) thought that, in their experiments, more than 1 mole of B_{12} was attached per mole of algal binder. There was, however, no evidence from the plots of our data (Fig. 3) that either of these effects had occurred during the 1 h incubation employed in this study.

Table 3 summarises the association constants ($K_a = K_d^{-1}$) for B_{12} binders from a variety of sources. K_a values for algal binders lie in the range 10^{10} to 10^{12} M and are similar to those of mammalian origin but significantly greater than the K_a for the binding protein from the bacterium *Escherichia coli*.

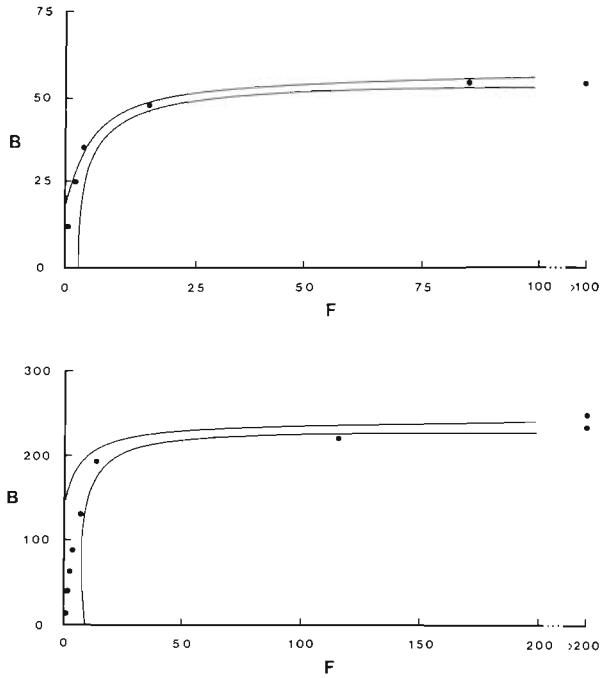

Fig. 3. Computer iterative fit of data summarised in Table 2. B = bound B_{12} (pM), F = free B_{12} (pM). Data points are shown within 95 % fiducial limits, except for points on the truncated part of the abscissa. (A) *Dunaliella binder* (expt. 1) K_d = 3.35 (pM). Value of truncated point = 145.5 pM; (B) *Pavlova* binder (expt. 1) K_d = 2.97 (pM). Values of truncated points: upper 238.8 pM, lower 309.9 pM

Table 2. Microalgal ectocrines: dissociation constants of B_{12} complexes and capacities for B_{12} binding

Algae	Experiment No.	Age of culture (d)	Cell density $N \times 10^{-3}$ (10^6 l^{-1})	Dissociation constant[1] K_d (pM)	Binding factor released per 10^6 cells (in B_{12} equivalents)[1] B_{max}/N (fmole)	Number of data points	Cell volume[2] (μm^3) and assumed shape	Reference for cell dimensions
Dunaliella primolecta	1	15	1.27	3.35 (0.36)	44.5 (0.35)	6	32–45 ovoid	Butcher (1959)
	2[3]	–	–	2.76 (7.78)	23.4 (2.1)	6		
	3	6	1.38[4]	7.68 (1.34)	49.6 (1.4)	7		
Mantoniella squamata	1	14	6.35	1.24 (1.92)	5.21 (0.18)	7	14–48 sphere	Manton and Parke (1960)
	2	7	2.86	0.94 (1.11)	5.91 (0.10)	7		
Pavlova lutheri	1	22	3.81	2.97 (0.76)	61.9 (0.62)	9	36–150 square plate	Droop (1953)
	2	7	3.56	3.41 (0.23)	36.0 (0.36)	6		
	3	Chemostat[5]	1.19	0.94 (0.52)	125 (1.3)	9		
	4	Chemostat[6]	1.06	1.02 (1.48)	22.5 (0.48)	7		
Phaeodactylum tricornutum	1	22	3.71	45.3 (5.96)	7.76 (0.27)	8	–	–
	2	26	5.91	18.8 (1.91)	16.8 (0.60)	6		
	3	35	8.80	28.2 (7.46)	6.90 (0.60)	6		

[1] Standard errors in parentheses
[2] Volume range based on minimal and maximal dimensions given in reference
[3] Medium from the previous experiment held at 20 °C for 5 h then diluted threefold with B_{12}-free medium
[4] The higher cell population with a shorter culture time was due to the use of a denser inoculum
[5] Filtered S88 medium from a chemostat culture (dilution rate 0.1 d^{-1})
[6] Filtered medium from *Pavlova lutheri* cells which had been taken from a chemostat (3), harvested by centrifuging ($10^4 \times g$ for 1 min) and re-suspended in fresh B_{12}-free medium for 3 h at 20 °C

Table 3. Comparison of values of the association constants for B_{12} binders from various sources

Source of binder	Association constant-K_a (M^{-1})	Reference
Algae		
Dunaliella primolecta	2.2×10^{11} *	This study
Mantoniella squamata	5.8×10^{11} *	This study
Pavlova lutheri	3.2×10^{11} *	This study
Phaeodactylum tricornutum	3.3×10^{10} *	This study
Bacteria		
Escherichia coli	2×10^{8}	Taylor et al. (1972)
Mammals		
Pig gastric intrinsic factor	4×10^{9}	Francis et al. (1977)
Human gastric intrinsic factor	3×10^{9}	McGuigan (1967)
Human gastric intrinsic factor	6×10^{9}	Hippe and Olesen (1971)
Human gastric intrinsic factor	1.5×10^{10}	Allen and Mehlman (1973)
Transcobalamin I and II	3×10^{11}	Hippe and Olesen (1971)

* Reciprocal of mean value of K_d in Table 2

The binder from *Phaeodactylum tricornutum* (a diatom) showed a lower affinity for B_{12} than that from the other algae. It is difficult to argue that this is a reflection of the lack of a B_{12} requirement by this alga since binder from *Dunaliella primolecta* (a chlorophyte), also a non-requirer, has a K_d similar to that of the two B_{12} auxotrophs. The lower affinity may be related to the *specificity* of the binder, i.e. its ability to bind analogues of B_{12}. Many diatoms are able to utilize a range of B_{12} analogues for growth whereas chlorophytes are more specific (Provasoli and Carlucci, 1974; their Table 27.2). However, this question can only be resolved when more data are available for K_d and binding specificity for a wide range of algae.

Binding capacity

In Table 2 we list the binding capacities of the culture filtrates used in our experiments. Messina and Baker (1982) estimated that, on a cellular basis, *Gonyaulax tamarensis* released 120 times more binder than *Cyclotella cryptica* but the same amount per unit volume of cell material. Our data suggest that binder production by *Mantoniella squamata* was lower than that of the other flagellates studied even if differences in cell size are taken into account (Table 2).

Role of algal B_{12} binders

As far as we are aware, this is the first report of dissociation constants for algal B_{12} binders. Previous attempts to determine K_d using equilibrium dialysis were unsuccessful (Droop, pers. comm.). These constants, together with other data obtained in our experiments, make it possible to reinterpret some of the published data concerning algal B_{12} binders and to discuss their likely role and effects in cultures and in natural waters.

B_{12} binder as an ectocrine

The physiological role of the B_{12} binding protein released by microalgae remains an enigma. Droop (1968) suggested that the binder is an *ectocrine*, i.e. a substance secreted by phytoplankton which may have growth-promoting or growth-inhibiting properties and, as a result, may influence species succession (Smayda, 1980). Certainly the K_d values (equivalent to the half-saturation constant for binding) of the algal binders indicate that they would be very efficient at sequestering the low concentrations of vitamin B_{12} present in natural waters. Messina and Baker (1982) found that the growth response of *Skeletonema costatum* to vitamin B_{12} decreased in the presence of exudate from *Gonyaulax tamarensis* and *Cyclotella cryptica* at vitamin concentrations and cell densities possible in the environment. This led them to argue that, under natural conditions, the growth of phytoplankton having little or no B_{12} requirement would be favoured because the binder would suppress the growth of auxotrophic species. Our data suggest, however, that this would be true only in unusual circumstances. The steady state concentration of B_{12} binder in a chemostat culture (in B_{12} equivalents l^{-1}) is given by $\tau N/\mu$ where τ is the specific rate of release of the inhibitor in B_{12} equivalents (fmoles 10^6 cells^{-1} d^{-1}), N is the number of cells l^{-1} (in millions) and μ is the specific growth rate (equal to the dilution rate) (Droop,

1968). Using the data from Experiment 3 with *Pavlova lutheri* (Table 2), the value of τ may be calculated to be 12.5 fmoles 10^6 cells^{-1} d^{-1} If this value is typical, it would clearly take several months for a population of even 10^6 cells l^{-1} to release enough binder to inactivate the picomolar concentration of B_{12} reported to be present in coastal waters (Swift, 1980).

Furthermore, using values for the B_{12}-binding capacity of the ectocrine released by phytoplankton and the dissociation constant for binding representative of those in Table 2, *viz.* 40 fmoles per 10^6 cells and 2 pM, and assuming that sea water contains B_{12} in solution (bound plus free) at a concentration of 1 pM, and that the binder concentration is proportional to the cell population, it may be calculated that, whereas the ectocrine released by a population of 10^9 cells l^{-1} would bind 95 % of the vitamin, the value would fall to 63 % for a cell density of 10^8 l^{-1} and to only 12 % for one of 10^7 l^{-1}. Even in the experiments of Messina and Baker (1982), the final population of *Skeletonema costatum* growing in B_{12}-limited cultures (0.37 pM = 0.5 pg B_{12} ml^{-1}) was reduced only by about 37 % by the exudate from (effectively) 8.8×10^6 cells l^{-1} of *Cyclotella cryptica*. We therefore concur with Droop (1968) that severe inhibition of the growth of B_{12} requiring species would be expected only after the development of very high cell densities, such as occur in exceptional blooms, in supra-littoral pools and, of course, in cultures.

B_{12} binder and uptake of the vitamin

An alternative to the 'ectocrine hypothesis' is the idea that the algal B_{12}-binder is somehow involved in the uptake of the vitamin by the cells. It may be significant, in this context, that the association constants for algal B_{12}-binders are very similar to those for Transcobalamin I and II (Table 3) which facilitate transport of B_{12} in mammals (Ellenbogen 1975). However, whereas in mammalian systems uptake of B_{12} by cells or tissues normally takes place only if it is bound to proteins such as transcobalamin or 'intrinsic factor' (see DiGirolamo and Huennekens, 1975; Ellenbogen, 1975), the presence of B_{12}-binder in algal cultures actually obstructs uptake of the vitamin by cells (Droop, 1968). Provasoli and Carlucci (1974) suggested that the B_{12}-binding protein is involved in transport of B_{12} into cells and that, when excess is produced, it is excreted into the medium. The similarity of the half-saturation constants for B_{12} binding by the *Pavlova lutheri* ectocrine (mean K_d values 3.1 pM, see Table 2) and those for surface adsorption by the same species – 0.88 pM for exponentially growing cells and 2.55 pM for stationary phase cells (Droop, 1968) lends credence to this view. The observations that, although both *Phaeodactylum tricornutum* and *Dunaliella* spp. do not require B_{12} but nevertheless retain a high affinity uptake system for the vitamin (Droop, 1968; Scott, 1981) and also produce a high affinity binder, provide additional support for the transport hypothesis.

B_{12} incorporation by microalgae, in common with bacterial (Bradbeer, 1979) and mammalian cells (DiGirolamo and Huennekens, 1975), is biphasic: *Ochromonas* (Reeves and Fay, 1966; Bradbeer, 1971), *Euglena* (Sarhan et al., 1980); *Pavlova* (Droop, 1968) and *Brachiomonas* and *Dunaliella* (Scott, 1981). The primary phase is very rapid (completed within 1 min), insensitive to metabolic inhibitors and is thought to involve adsorption of B_{12} to cell surface receptors or carrier sites, while the secondary phase, which is slower (lasting up to at least 1 h), energy-dependent and affected by metabolic inhibitors and temperature, is presumed to represent active transport of the vitamin into the cell (Bradbeer, 1971; Sarhan et al., 1980).

Thus, in order for microalgae to incorporate B_{12} from the culture medium, the primary surface uptake sites have to compete with the binding factor for the vitamin. Assuming that the rate of dissociation of the B_{12}-binder complex is rapid enough to maintain equilibrium, the distribution of B_{12} between the uptake sites and the binder would be a function of the relative affinities of the two for the vitamin and also their relative concentrations:

By analogy with Equation (1), vitamin binding to the cell sites can be described by the equation

$$K_c = F(C_{max}-C)\,N/CN = F(C_{max}-C)/C \qquad (3)$$

where K_c = the dissociation constant for cell surface B_{12} uptake sites; C = amount of B_{12} attached to 10^6 cells; N = number of cells (in millions) l^{-1}; C_{max} = the maximal binding capacity (in B_{12} equivalents) of 10^6 cells. The form of Equation (3) not containing N may be rearranged to

$$C = \frac{C_{max}F}{K_c + F} \qquad (4)$$

which is the form of the Langmuir adsorption isotherm. Thus, K_c is identical to the half-saturation constant for B_{12} binding by the cell surface.

Thus the ratio

$$\frac{B_{12}\text{ bound to cell surface l}^{-1}}{B_{12}\text{ attached to binding factor l}^{-1}} = \frac{CN}{B} = \frac{C_{max}N}{B_{max}} \quad \frac{K_d+F}{K_c+F}$$

$$(\text{if } K_d \triangleq K_c, \text{ as discussed earlier}) \triangleq \frac{C_{max}N}{B_{max}}$$

$$= \frac{\left\{\begin{array}{c}\text{Maximal } B_{12}\text{ surface binding}\\ \text{capacity of } 10^6 \text{ cells}\end{array}\right\}}{\left\{\begin{array}{c}\text{Binding factor in } B_{12}\text{ equivalents}\\ \text{released by } 10^6 \text{ cells}\end{array}\right\}}$$

Droop (1968) found that the cell surface B_{12}-binding capacity of *Pavlova lutheri* was 20.5 fmoles per 10^6 cells for exponentially growing cultures but only 2.04 fmoles per 10^6 cells for stationary phase cells. If the data in Table 2 for 7 and 22 d old batch cultures are applied respectively to these 2 phases of growth, it may be calculated that, whereas in logarithmic phase cultures about 36% of the vitamin would be bound to the cell surfaces, the figure would be only about 3% for stationary phase cultures. Distribution of the smaller percentage of vitamin on the cells amongst the greater population present in the stationary phase would mean that the cell surface B_{12} concentration would be considerably lower than in the logarithmic phase. It is interesting to note that the decrease in the proportion on the cells would be due mainly to the lower cell surface binding capacity of the cells rather than to an increase in the amount of binder released in the stationary phase.

While this alone provides an adequate explanation for the auto-inhibitive effect of the production of B_{12} binder by auxotrophic phytoplankton, the possibility that the rate of dissociation of the B_{12}-binder complex might be too slow to maintain the supply of B_{12} to newly produced cellular binding sites and thus also retard growth should not be overlooked.

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