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Diatoms as tools for assay of total B_{12} activity and cyanocobalamin activity in sea water

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ABSTRACT

The total cobamide concentration (vitamin B_{12} plus B_{12} analogs active for some organisms) can be determined in sea water by bioassay utilizing growth of the centric diatom *Bellerochea polymorpha* clone 675-d. Vitamin B_{12} activity can be determined by bioassay utilizing *Thalassiosira pseudonana* clone 3H; hence the activity of vitamin B_{12} analogs can be determined by difference. Useful range of the assay is 0.1 to 1 ng l^{-1}. Samples can be appropriately diluted with vitamin-free sea water.

The existence of several differentially active cobamides makes it desirable to assay water samples for these analogs of vitamin B_{12} as well as for cyanocobalamin. Organisms having the narrowest specificity (the mammalian type of response) in meeting a cobamide requirement utilize vitamin B_{12} itself $\alpha(5, 6$-dimethylbenzimidazolyl cyanocobalamin), its hydroxocobalamin form present under alkaline conditions, or derivatives with other substituted benzimidazole rings in the side chain. Some organisms such as the bacterium *Lactobacillus leichmannii* can use also the cobamides with adenine (pseudovitamin B_{12}) or guanine-like side chains. Certain other organisms, notably the bacterium *Escherichia coli,* can use in addition factor B, which lacks the nucleotide-like side chain altogether.

There are species of algae falling into each of these specificity patterns (Guillard, 1968; Provasoli and Carlucci, 1974), and some of these as well as other microorganisms have been used for bioassay of the vitamin in sea water (Table 1). The importance has been stressed of knowing the specificity of the dominant algae in a particular population and of assaying the water for the appropriate forms of cobamide (Provasoli, 1963; Provasoli and Carlucci, 1974).

Few studies have been made utilizing more than one assay organism in order to measure analog concentrations. Cowey (1956) found 6 ng l^{-1} vitamin B_{12} activity from *L. leichmannii* assay and 4 ng l^{-1} vitamin B_{12} activity from *O. malhamensis* assay of the same water sample from Aberdeen Bay. The major difference in re-

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Table 1. Response of vitamin assay organisms to vitamin B_{12} analogs, expressed as percentage of response observed with vitamin B_{12}.

Organism	B_{12}	Pseudo B_{12}	Factor B	Reference
Algae				
Amphidinium carteri	100	0	0	Provasoli & Carlucci, 1974
Euglena gracilis	100	100	0	Smith, 1965
Ochromonas malhamensis	100	0	0	Smith, 1965
Skeletonema costatum	100	5	5	Guillard, 1968
Thalassiosira pseudonana 3H	100	0	0	Guillard, 1968
Thalassiosira pseudonana 13-1	100	10-45	30	Guillard, 1968
Thalassiosira pseudonana 13-1	100	60	65	Carlucci & Silbernagel, 1966a
Bacteria				
Escherichia coli	100	100	100	Smith, 1965
Lactobacillus leichmannii	100	50-100	0	Smith, 1965; Cowey, 1956
Fungus				
Thraustochytrium globosum	100	0	0	Vishniac & Riley, 1961

sponse of these two organisms is utilization of pseudovitamin B_{12} by *L. leichmannii* at 50% effectiveness. Therefore the sea water analyzed can be estimated to contain about 4 ng l^{-1} each of vitamin B_{12} and pseudovitamin B_{12}, with factor B content not known. Assay of some other oceanic samples gave results for *L. leichmannii* assay similar to those for *O. malhamensis* assay. These analyses required extraction of the vitamin from a large volume of sea water because the organisms used require a fresh water medium. Preferred methods now use marine organisms for direct assay of sea water (Strickland & Parsons, 1972; Carlucci & Silbernagel, 1966a; Ryther & Guillard, 1962).

Other studies suggest that vitamin B_{12} analogs are significant in the environment. Burkholder and Burkholder (1956) analyzed mud samples and suspended matter (mainly phytoplankton and detritus) from Bahia Fosforescente, Puerto Rico, and Long Island Sound, using *E. coli* and *O. malhamensis*. Activity from *E. coli* assay was 4-15 times that from *O. malhamensis* assay. Starr, Jones and Martinez (1957) tested isolates of a number of marine bacteria for vitamin B_{12} production. Seventy percent of the clones produced B_{12} activity according to *E. coli* assay and about half of those clones indicated vitamin B_{12} production with *E. gracilis* assay. In the clone with greatest vitamin production the activity for *E. coli* was almost four times the activity for *E. gracilis*. In two-thirds of those cultures producing vitamin B_{12}, excreted vitamin was detected in the culture medium; in the rest, vitamin B_{12} was detected only in the cell residue. Some experimental difficulties occur which can affect the values of ratios of B_{12} activity as assayed with two organisms. First, the sample

treatment procedure may be more tolerable to one organism; second, the response of *E. coli* to methionine, for the synthesis of which vitamin B_{12} is required, may result in overestimation of concentration of vitamin B_{12} + analogs despite efforts to correct for this.

Haines and Guillard (1974) tested the capacity of each of 14 different hetero-trophic marine bacteria to grow mutually with each of two *Thalassiosira pseudonana* auxotrophs (clones 3H and 13-1, see Table 1) in sea water medium initially free of vitamins and organic carbon sources. They applied yield values from standard re-sponse curves (Guillard and Cassie, 1963) to the algal cell number to estimate vitamin production by each bacterium. The range of values for the ratio of vitamin activity for (13-1) to vitamin activity for (3H) was 0.3 to 8, with a mean value greater than 2. These experiments are not comparable to standard vitamin assay, however. Excretory products of the two algal clones might be different in suitability for growth of a given bacterium, so that there is departure from bioassay procedure in which all nutrients except the one for analysis are known to be present in excess.

The procedure described here estimates cobamides of the vitamin B_{12}, pseudo-vitamin B_{12} and Factor B types by assay using two organisms, one with coliform (widest) and one with mammalian (narrowest) specificity. For diatoms, the three cobamides mentioned are representative of the variety of forms that exist (Guillard, 1968).

The small centric diatom designated clone 657-d (Guillard and Cassie, 1963) now described as *Bellerochea polymorpha* (Hargraves and Guillard, 1974) has coli-form specificity (Guillard, 1968) and requires thiamin (Hargraves and Guillard, 1974). The cell crop produced with Factor B or pseudovitamin B_{12} is greater than or equal to that produced with vitamin B_{12}, when all are tested at the 4 ng l^{-1} level (Guillard, 1968). *T. pseudonana* clone 3H has mammalian specificity and deter-mines vitamin B_{12} alone with good precision (Ryther and Guillard, 1962; Guillard, 1968). The concentration of analogs can be calculated by subtraction, or the ratio total cobalamins: vitamin B_{12} can be used.

1. Methods

Stock cultures. Stock cultures of *B. polymorpha* 675-d and *T. pseudonana* 3H were maintained in tubes or flasks of f/2 medium (Guillard and Ryther, 1962; Guillard, 1975) prepared in vitamin-free sea water with the standard vitamin mixture re-placed by 5 ng l^{-1} vitamin B_{12} and thiamin added for *B. polymorpha* only, 0.5 μg l^{-1} or more. Nitrate, phosphate, and silicate stock solutions were sterile and handled aseptically to avoid possible cobamide production by bacteria. Glassware was cleaned as described previously (Ryther and Guillard, 1962). Cultures were grown at 14, 18 or 20°C in continuous light or on a light-dark cycle. They were trans-ferred at fortnightly intervals. An inoculum for assay was prepared by transfer into

medium containing 0 or 1 ng l^{-1} vitamin B_{12}. After growth occurred, a dilution was made in vitamin-free sea water such that initial density in the assay medium was about 300 cells ml^{-1} (675-d) or 200 cells ml^{-1} (3H) using an inoculum volume of 0.1 or 0.2 milliliter.

Vitamin standards. Crystalline cyanocobalamin was purchased from Schwarz/Mann Div. of Becton-Dickinson. Primary stock solutions of 250 mg l^{-1} were made, with allowance for water of hydration (11%, Smith, 1965). They were sterilized by passage through a 0.22 μm membrane filter (GS, Millipore Corp.) and stored frozen in sterile tubes. Aseptic dilutions were made before use by pipetting into sterile vitamin-free water in tubes. Similar procedures were used for the other vitamins.

The pseudovitamin B_{12} and Factor B analog were originally purified in the laboratories of Dr. W. M. Scott of the College of Medicine, Wayne State University, Detroit, and Dr. K. Bernhauer of the Lehrstuhl für Biochemie, Technische Hochschule, Stuttgart. Solutions were diluted quantitatively and then resterilized by filtration. Factor B stock solution contained 1 mg l^{-1}; pseudovitamin B_{12} contained 0.25 mg l^{-1}. Thiamin hydrochloride (Nutritional Biochemicals Corp.) stock solution was prepared at 1 g l^{-1}.

Treatment of water samples. Water samples were filtered through washed 0.45 μ membrane filters (HA, Millipore Corp.) in glass apparatus and stored frozen in clean polyethylene bottles until assayed. Thawing was begun at 2°C and completed in lukewarm water. The thawed sample was diluted immediately to 90% strength with fresh glass-distilled water, filtered again, and promptly prepared for assay.

Some of the sea water from the samples was made vitamin-free by treatment with washed activated charcoal ("Norit-A", Fisher Scientific Co.) in a procedure modified (Swift, 1973) from earlier work (Ryther and Guillard, 1962). About ten grams of charcoal were put into a 0.5 l glass graduate cylinder. Fresh distilled water, from glass apparatus, was added to fill the cylinder and the suspension was shaken several times. After settling, the volume of charcoal was noted in order to convert dry weight to wet volume. The wash water was then pumped or siphoned away. The washing was repeated five times more. After the final decanting to a level above the settled charcoal, the mass of charcoal remaining after washing was calculated. A portion of the slurry containing one gram of charcoal was then pipetted into each liter of filtered sea water being treated. Additional distilled water was added to dilute the sea water to 90% strength. The slurry was stirred periodically or continuously for at least an hour and then filtered to remove the charcoal.

2. Bioassay procedures

Nutrient addition. The sea water sample and vitamin-free sea water were enriched with nutrients to the strength of medium f/2 with vitamins omitted. Nitrate, phosphate, silicate and thiamin solutions were diluted from sterile stock solutions, and

chelated trace metals from refrigerated stock, so that a small volume sample could be prepared accurately. Thiamin was added to medium for *T. polymorpha* 675-d at 0.5 μg l^{-1} to 2.5 μg l^{-1}; below 0.1 μg l^{-1}, thiamin limitation interferes with vitamin B_{12} assay (Swift, 1973).

Sterilization. Autoclaving was used to sterilize the samples. Autoclaving retains about 85% of vitamin activity (thiamin, biotin, or vitamin B_{12}) and is satisfactory if standards and samples are treated alike (Swift, 1973). Trial of an alternate method of sterilization was made: sea water was sterile-filtered and nutrient solutions sterilized by autoclaving were added aseptically. This method used with bioassay algae gave erratic results; often less growth occurred with increased vitamin concentrations. We conclude that the f/2 medium used here is more favorable for growth when ingredients are autoclaved together.

Autoclaving also destroys proteins which bind vitamin B_{12}. Such proteins have been observed in culture medium and may be present in the environment (Droop, 1968). However the vitamin bound to proteins is potentially available. Laboratory studies show binding proteins can be degraded by bacteria or denatured under sterile conditions at a rate dependent on temperature (Pintner and Altmyer, 1973). Skeggs (1966) recommends autoclaving or heating samples to destroy vitamin B_{12} binding proteins in order to avoid erratic assay results from nonuniform utilization of vitamin B_{12} initially present in bound form.

Medium for assay utilizing *B. polymorpha* was autoclaved 10 minutes in culture tubes. Medium for assay utilizing *T. pseudonana,* in flasks, was autoclaved 15 minutes. Return to atmospheric pressure required 15-30 minutes.

B. polymorpha bioassay. Assays utilizing *B. polymorpha* were performed in 20 × 150 mm culture tubes with teflon-lined screw caps using 10 ml volume. Flasks were not used because the different dilutions and the standards necessary would have required excessive sample volume and incubator space. Cultures were incubated at 21 C ± 2 C above cool white fluorescent lights at intensity of 0.028 ly min^{-1}, measured with a Weston foot-candle meter, on 14:10 LD cycle. Tubes were stirred with a vortex mixer once a day. About 14 days incubation was required to reach maximum density.

Samples were assayed in triplicate at two dilution levels, 50% and 30%, with the vitamin-free medium used as dilutent. (Assay of the first few samples from the region being studied was made in duplicate at 20, 40, 60, 80 and 100% strengths to determine the dilutions most suitable.)

An external standard series for *B. polymorpha* used the charcoal-treated vitamin-free sea water. For each of three forms, vitamin B_{12}, pseudovitamin B_{12}, and Factor B, tubes were prepared with concentrations of 0, 0.2, 0.4, 0.6, 1.0 1.8, and 3.0 ng l^{-1}. Internal standards were not used because total vitamin concentrations would have been beyond the useful range of the assay.
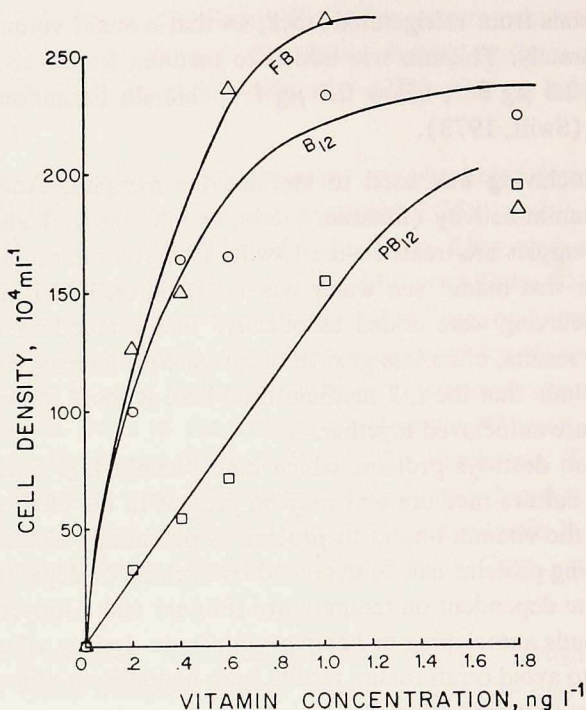
Figure 1. Cell crop produced by *B. polymorpha* (clone 675-d) in response to vitamin B_{12} and two of its analogs. Circles: vitamin B_{12} (curve B_{12}). Triangles: Factor B (curve FB). Squares: pseudovitamin B_{12} (curve PB_{12}).

T. pseudonana bioassay. Assays utilizing *T. pseudonana* 3H were performed using 50 ml of the prepared sample in a 125 ml erlenmyer flask, capped by a glass 50 ml beaker. These were incubated under 0.048 ly min^{-1} illumination for six days at 21 C (14:10 LD cycle) or nine days at 16 C (12:12 LD cycle). Cultures were swirled once a day. External cyanocobalamin standards were made, usually 10 flasks in the range 0-4 ng l^{-1}. The low linear portion of the response ends around 2 ng l^{-1}, but higher concentrations still provide usable standards (Ryther and Guillard, 1962; Swift and Taylor, 1974; Swift, 1973).

Determination of yield. The external standard flasks or tubes containing 1 ng l^{-1} vitamin B_{12} were counted daily until the density remained essentially the same for two days. At that time all of the cultures were counted.

Cell counts using a hemocytometer were made in duplicate on each tube or flask, counting at least 200 cells each time. Four counts were made on the standards, counting 300 cells in each.

Many cells are enlarged when their growth is halted by vitamin B_{12} depletion.
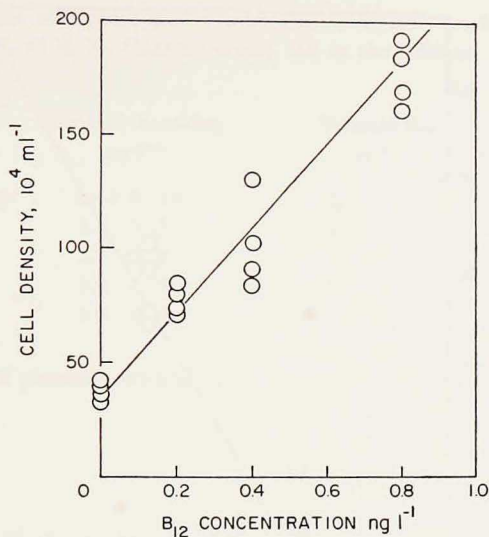
Figure 2. Standard curve for total cobamide assay using *B. polymorpha* 675-d. Slope, determined by least squares fit, is 187.5×10^4 cells pg^{-1}. Nonzero intercept occurs when inoculum is taken from early log phase cultures.

It is necessary to decide whether morphology is that of two cells, as yet unseparated, or one cell which has grown but is unable to divide further.

3. Results

Figure 1 shows standard curves for cell yield of *B. polymorpha* 675-d utilizing vitamin B_{12}, pseudovitamin B_{12} or factor B. The assay is very sensitive, particularly for factor B and vitamin B_{12}. Replicate experiments showed slight variation in the relative values of slopes of the three curves. The smaller yield with pseudovitamin B_{12} cannot be due to breakdown, because the predicted product is factor B, which is as potent as pseudovitamin B_{12}.

The standard curve for vitamin B_{12} was chosen to convert cell density into total equivalent concentration of vitamin B_{12} plus analogs. These values are subject to some underestimation because of the smaller yield of cells with pseudovitamin B_{12} as compared to the other forms. Figures 2 and 3 show standard curves for the two algae from one set of bioassays. Each concentration is calculated from the equation:

$$c = \frac{1}{0.9} \frac{n-a}{m} \frac{1}{D}$$

where c = concentration in sample
$\quad\quad n$ = cell density in assay culture
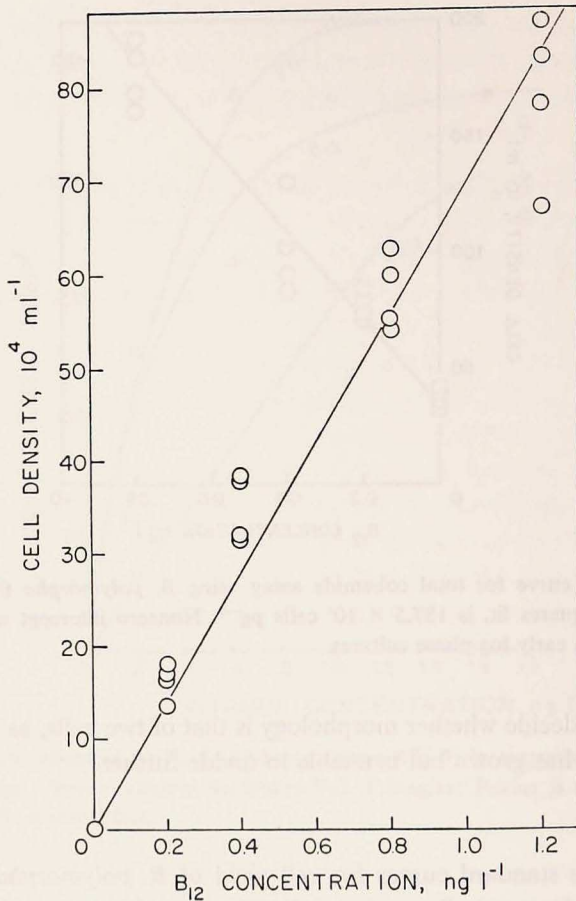$\quad\quad a$ = y-axis intercept of standard curve

Figure 3. Standard curve for vitamin B_{12} assay using *T. pseudonana* 3H. Slope, determined by least squares fit, is 69.7×10^4 cells pg^{-1}.

m = slope of standard curve

D = fraction of sample in tube

$\dfrac{1}{0.9}$ allows for initial dilution

Table 2 shows results for one station. Counts of replicate samples were as close as duplicate counts from the same tube. The precision of the assay for total coba-mides, average coefficient of variation about 30%, is not as good as that of the vitamin B_{12} assay using *T. pseudonana* 3H, which has precision of 10%. Cells of *B. polymorpha* 675-d are smaller than those of *T. pseudonana*, less refractile, and more fragile in senescence. Dividing cells are also more difficult to designate. Skeggs' review (1966) of vitamin B_{12} assay in biomedical applications cites 10% as expected

Table 2. Assay for total cobamides and vitamin B_{12} in water samples from station 2, 42°
38.4′ N, 69° 36.0′ W, of R. V. *Gosnold* cruise 168 in the offshore Gulf of Maine, August
13, 1970 (Swift, 1973).

Depth, m	Total Cobamides, ng l^{-1}	Vitamin B_{12}, ng l^{-1}	Ratio Cobamides: B_{12}
5	0.43 ± .15	0.30 ± .03	1.4
25	1.8 ± .5	0.54 ± .06	3.3
50	2.2 ± .8	0.9 ± .1	2.4
100	3.2 ± .5	1.25 ± .07	2.5
150	3.9 ± .8	1.7 ± .2	2.3

precision for assay of pharmaceutical preparations and 20% for assay of biological
samples.

4. Discussion

The useful range of the assay for total cobamides is 0.1 to 1 ng l^{-1}. Therefore
most samples must be diluted to some extent. In the case of work with Gulf of
Maine water, preliminary assays with *T. pseudonana* and *B. polymorpha* were per-
formed on selected samples. For *T. pseudonana* assay, concentrations were suitable
for assay without dilution. Results of preliminary assay with *B. polymorpha* at many
sample dilutions showed that cobamide concentrations were one to three times the
B_{12} concentrations. Thus medium containing 30% and 50% sample concentration
was selected for the subsequent assays. Similarly, oceanic waters such as the north
Pacific (Carlucci & Silbernagel, 1966b) contain vitamin B_{12} concentrations suitable
for *T. pseudonana* assay with slight or no dilution. Dilutions for the cobamide assay
should be selected using the assumption that cobamide concentrations will be three
times the B_{12} concentration. Water from nutrient-poor regions such as the Sargasso
Sea, where the maximum B_{12} concentration is 0.1 ng l^{-1} (Menzel and Spaeth, 1962)
should be assayed without much dilution for either organism. Any dilution would
be for salinity adjustment.

Water samples from estuaries or shallow coastal regions must be diluted for both
assays. Work by Cattell (1969) in the Strait of Georgia, and by Vishniac and Riley
(1962) in Long Island Sound (1961) has demonstrated annual variation between
four and 15 ng l^{-1} vitamin B_{12} in such regions.

No inhibition of growth was observed except when sterilization by means other
than autoclaving was used. Once prepared, the samples should be inoculated.
Vitamin B_{12} activity can decrease on exposure to light and to alkaline pH; inorganic
constituents in the medium can form precipitates and aggregates which could result
in nonuniform lag phase. It is preferable to continue storing the water samples
frozen, where they remain stable (Carlucci, Silbernagel and McNally, 1969), if
bioassay cultures cannot be inoculated within about a week after preparation.

The same type of assay could be performed utilizing *T. pseudonana* clone 13-1 in place of *B. polymorpha* 675-d. However, clone13-1 is less effective in utilizing analogs. Workers using different techniques to study specificity report different results (see Table 1), so further standardization of methods would be required.

The incubation could be shortened by employing radiocarbon uptake during the early stages of growth of the cultures as Gold (1964) and Carlucci and Silbernagel (1966a) describe.

The differentiation between pseudovitamin B_{12} and factor B in samples is dependent on having a clone responding to vitamin B_{12} and to pseudovitamin B_{12} only. Such a lactobacillus-like response is relatively rare. Among marine diatoms most clones capable of using pseudovitamin B_{12} can utilize factor B also. Of four clones (out of 23 studied) having the appropriate lactobacillus response, only one produced sufficient yield of cells with pseudovitamin B_{12} to be suitable for assay use (Guillard, 1968). This clone F-3, *Fragilaria* sp., was isolated from Great Pond, Falmouth, Mass. It was used in preliminary assay work before being lost by accident from the culture collection. Results with a water sample taken in Vineyard Sound, Massachusetts, in March 1968 indicate concentrations were: 1 ng l^{-1} B_{12}, 1.6 ng l^{-1} pseudovitamin B_{12} and 0.4 ng l^{-1} factor B. Clone F-3 was not suitable for assay because cells clumped together at the end of log phase growth. It would be useful to have another clone with B_{12} + pseudo B_{12} response available to use for assay. However, we do not know yet of any naturally abundant algae that can utilize pseudovitamin B_{12} and not factor B.

The different specificity of vitamin B_{12} response in marine algae (Guillard, 1968; Provasoli and Carlucci, 1974) suggests physiological differences which could be important in selection of the species or clones (physiological races) where low concentrations of vitamin B_{12} and its analogs occur. Our observation that concentrations of analogs exceed those of the vitamin indicate that the capacity to utilize analogs could be an advantage to a particular clone. High concentrations of analogs also suggest that the differential production of vitamin B_{12} (Starr, Jones and Martinez, 1957; Carlucci and Bowes, 1970a, 1970b; Droop, 1968) and its analogs by some microorganisms (presumably bacteria and algae) and the utilization of the vitamin and analogs by other microorganisms is a complicated process.

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