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A METHOD FOR THE MEASUREMENT OF VITAMIN B_{12} CONCENTRATION IN SEA WATER

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(Text-fig. 1)

The vitamin B_{12} concentration in sea water is so very low that only the most sensitive microbiological assay techniques are likely to be capable of measuring it, but the salt concentration is so high as to be inhibitory to most of the organisms conventionally used for the microbiological assay of the vitamin. Nevertheless, the consideration that vitamin B_{12} concentration may have an influence on the amounts and types of life present in sea areas has persuaded several investigators to attempt its measurement.

The methods so far employed have each their own advantages and disadvantages (Droop, 1954, 1955; Lewin, 1954; Sweeney, 1954; Cowey, 1956; Adair & Vishniac, 1958). The method to be described here offers advantages in sensitivity, permitting direct measurement of the vitamin in small quantities of oceanic waters after further dilution; samples to be measured pass through comparatively few different containers and thus there is less risk of their accumulating adventitious vitamin; simple and easily cleaned apparatus is used, and much less bench-work is involved than when methods other than dilution are used for lowering the salt concentration.

I am deeply indebted to Dr L. R. Fisher and Dr S. K. Kon who introduced me to the subject of vitamins in the sea and their possible significance, and to Dr J. E. Ford for his advice on microbiological techniques.

Thanks are also due to Dr G. I. M. Ross, then at the Vincent Square Laboratories, Westminster Hospital, who provided the culture of *Euglena gracilis* strain *z* with details of the growth medium before their publication; and to Dr R. Johnston of the Scottish Home Department Marine Laboratory, Aberdeen, for providing sea-water samples.

MATERIAL AND METHODS

Principle

The organism used is the freshwater flagellate *Euglena gracilis* strain *z*, grown in the medium of Hutner, Bach & Ross (1956). Carsted (cited by

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Hutner, Bach & Ross, 1956) found that this organism shows a growth response not only to cyanocobalamin (the form of vitamin B_{12} active for man and other higher animals), but also to two of its analogues, pseudovitamin B_{12} and Factor A. In this it resembles *Monochrysis lutheri* (Droop, 1955) but differs from some other marine algae (Droop, 1957). In his 1957 paper, Droop mentions that in the sea the analogues of vitamin B_{12} may have an importance equal to that of the vitamin.

The assay is carried out in a manner rather different from that described by Hutner *et al.* (1956); instead of measuring the growth of the *Euglena* population turbidimetrically, the chlorophyll from the cells is extracted and measured colorimetrically by the procedure of Heinrich & Lahann (1952). Various chlorophyll-measuring techniques resembling this procedure have of course long been familiar to marine biologists, who use them to obtain estimates of the phytoplankton standing crop in the sea (e.g. Harvey, 1934; Atkins & Parke, 1951).

Chlorophyll production was found to be proportional to the amount of vitamin B_{12} supplied to the *Euglena* population during its growth; which provides an exceptionally sensitive index of the vitamin concentration.

The sea-water sample has to be diluted with distilled water before use in order to reduce its salt concentration to a level which will not interfere with the growth of *Euglena*.

Several different dilutions of the sea-water sample, and at least two flasks of each dilution, are assayed together. Other flasks containing known amounts of vitamin B_{12} are necessary in each assay to provide the calibration.

Cleaning of glassware

Careful cleaning of the glassware for use in the assay is essential, as otherwise it might contribute significant amounts of vitamin B_{12}; perhaps even more than would be contained in the sample itself. The extent to which the glassware can be freed of vitamin B_{12} may well be the factor which imposes a practical limit on the accuracy and sensitivity of the assay.

Conical flasks of 50 and 100 ml. capacities are used. Before being brought into use for sea-water assays, flasks are filled with an approximately 10% (w/v) solution of sodium hydroxide and then steamed. They are then washed in turn with distilled water, dilute hydrochloric acid and several changes of distilled water.

With flasks used for sea-water assays and no other purpose, it has been found sufficient to wash them in a dilute detergent (Lissapol; I.C.I.) followed by distilled water acidulated with hydrochloric acid; then to fill them with distilled water and autoclave them, repeating this two or three times. The clean flasks are left in a drying oven until they are needed.

Preparation of medium

The medium recommended and described by Hutner *et al.* (1956) is prepared in solution at five times single strength, and stored in Polythene bottles at $-20°$ C.

Preparation of sea water

In the procedure to be described the assay is set up with four graded dilutions of the sample, each in triplicate. For such an assay 10 ml. of sea water are sufficient. Amounts for assays to be done with greater replication to provide higher precision can readily be calculated.

While awaiting assay, the sea water is stored cold with approximately 1% of the preservative of Hutner & Bjerknes (1948) (1 part by volume *o*-fluorotoluene, 1 part dichloroethane, 2 parts *n*-butyl chloride).

10 ml. of the sea water are placed in a 100 ml. conical flask, together with 0·5 ml. citrate-phosphate buffer of pH 4·6 (McIlvaine, 1921*) and 0·5 ml. of a freshly prepared 0·001% solution of NaCN. 60–80 ml. of glass-distilled water are then added, and the flasks heated in the autoclave in flowing steam for 15 min. This treatment eliminates any volatile preservative present; and any vitamin B_{12} not originally in the free cyano form is converted into it (Ford, 1952; Coates & Ford, 1955). In the free cyano form, the vitamin is relatively stable, and nutritionally available to the assay organism.

After the steaming, the flask is cooled and the contents made up to 100 ml. with distilled water, which represents a 1 in 10 dilution of the original sea water.

This degree of dilution has been found to be usually suitable for bringing samples within the sensitivity range of the assay. A lesser degree of dilution should if possible be avoided as higher salt concentrations interfere increasingly with the growth of *Euglena* (Hutner *et al.*, 1956).

Layout of assay.

This prepared sea water is now distributed into twelve flasks as follows:

2 ml., 4 ml., 8 ml., and 16 ml. portions respectively into each of three flasks

To each of these flasks are then added 4 ml. of the five-times strength medium and, where necessary, distilled water to make up the volume to 20 ml.

Parallel series of flasks are prepared containing graded amounts of cyanocobalamin within the concentration range of 0·025–0·20 $\mu\mu$g/ml. For this standard range a solution containing 0·25 $\mu\mu$g cyanocobalamin/ml. distilled

* McIlvaine's buffer is described in laboratory handbooks, e.g. Vogel, 1939.

water is prepared by serial dilutions with distilled water from a stock solution containing 5 μg cyanocobalamin/ml. 25% aqueous ethyl alcohol, and is distributed as follows:

2 ml., 4 ml., 8 ml. and 16 ml. portions respectively into each of three flasks

Medium and distilled water are then added as to the test extracts.

At least one other flask is made up to contain medium and water only, with no added vitamin B_{12}. This flask provides the experimental blank in the subsequent colorimetric measurement.

The flasks are closed with tight-fitting metal caps,* or are plugged with non-absorbent cotton-wool and covered with greaseproof paper caps. They are then autoclaved at 10 lb. pressure for 10 min.

Preparation of inoculum

For the *Euglena* inoculum 4–7-day-old cultures are used, grown at room temperature under approximately 200 f.c. illumination in 10 ml. portions of single-strength medium containing 80 $\mu\mu$g cyanocobalamin/ml. Such cultures contain about 10^4 cells/cu.mm.

Before use, the inoculum culture is washed so as to reduce carry-over of vitamin B_{12}, and to remove the inhibitory factor present in the culture liquors (Kristensen, 1955; cf. Ford, Gregory & Holdsworth, 1955).

This washing is performed by centrifuging down the cells and decanting the growth medium. The cells are re-suspended in sterile single-strength medium containing no vitamin B_{12}, and are again centrifuged and the medium is discarded. This procedure is repeated and the cells are finally suspended in 10 ml. of the single-strength medium containing no vitamin B_{12}.

The washing procedure is carried out under aseptic conditions to minimize risk of contamination of the *Euglena* culture.

Inoculation and Incubation.

The assay flasks, cooled to room temperature after the autoclaving, are each inoculated with one drop of the washed *Euglena* suspension. During this inoculation care must again be taken to prevent contamination by air-borne organisms.

The flasks are then incubated for about 10–12 days under 180 f.c. illumination and at approximately 28° C.

During the period of incubation the flasks are daily given a brief shaking to break any clumping of the *Euglena* cells. They are put back in random rearrangement.

* Moncrieff Ltd. of Perth supply flasks suitable for use with 'Oxoid' caps.

Reading of assay.

After incubation, the contents of each flask are poured into a centrifuge tube, the *Euglena* cells centrifuged down, and the medium decanted; 3 ml. acetone are added to each tube and the cells stirred up. Chlorophyll leaves the cells and goes into solution. The cell debris is again centrifuged down and is extracted with a further 1 ml. acetone; this extract is added to the first. The volume is finally made up to 4 ml. just before the colorimetric measurement to compensate for any evaporation of the acetone.

The chlorophyll extract from the *Euglena* grown in one of the flasks containing no added vitamin B_{12} is used as the blank in the measurement. The intensity of the colour of each chlorophyll extract is read against this blank. In this laboratory a Beckman DU spectrophotometer with 1 cm Corex cells is used, the optical density reading, log I_0/I at 432 mμ, being taken.

Calculation of results

The optical density readings deriving from the standard series of cultures grown with known amounts of cyanocobalamin are used to construct a calibration curve (Fig. 1). The concentration of cyanocobalamin corresponding to the optical density readings obtained from each of the dilutions of the sea-water sample is then read from the curve, and an average value for the cyanocobalamin concentration in the sea water is calculated.

Internal standards

The extent of agreement between results calculated from the optical density measurements at different sample dilutions gives an indication of their validity. It can be further checked by the use of an 'internal standard'. If an internal standard is to be employed, the procedure for setting up the assay is modified as follows: before the final dilution step in the preparation of the sea-water sample, two equal portions are taken and to one is added a known amount of cyanocobalamin. Both portions are then made up to the same volume with distilled water to provide the solutions for two series of assay flasks. A comparison of these two series makes possible the calculation of the vitamin B_{12} concentration originally present in the sea water without reference to the 'external' (cyanocobalamin in distilled water) standard.

Agreement between the values calculated by means of such an internal standard and those obtained with reference to the external standard provides good evidence for the validity of the result.

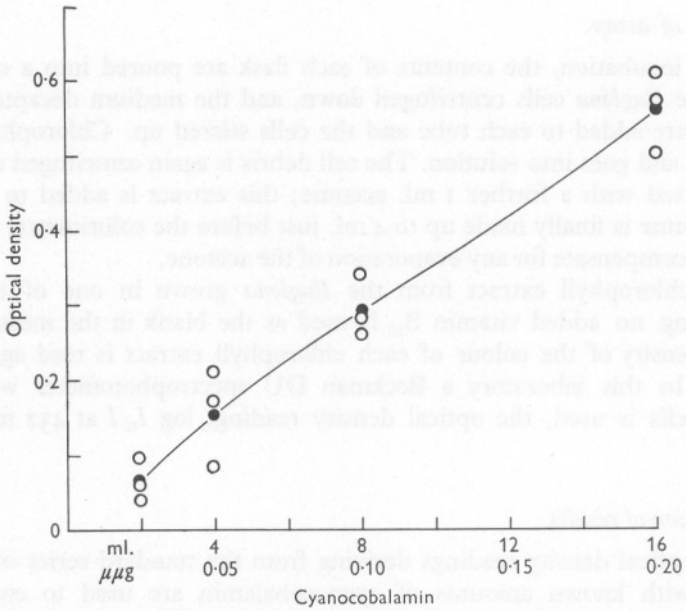

Fig. 1. Standard curve for assay of vitamin B_{12} by *Euglena gracilis* strain *z*. Standard cyanocobalamin 0·25 μμg/ml.; incubation for 12 days in this instance. ○ ○, values for individual flasks; ● ●, mean values. A smooth curve is drawn through the mean optical density values at the different levels of vitamin concentration. Slope and shape of the curve vary slightly from assay to assay. The horizontal ml. units refer to standard solution per flask, and the μμg units to cyanocobalamin per ml. The vertical units represent optical density of chlorophyll extract at 432 mμ.

TESTS OF THE *EUGLENA* ASSAY METHOD

Comparison with a previous method

Two 4 l. samples of surface sea water, collected 7 March 1956 at 58° 40′ N., 6° 10′ W. were supplied for assay by Dr R. Johnston of the Scottish Home Department Marine Laboratory, Aberdeen.

TABLE 1. COMPARISON OF METHODS OF MEASURING VITAMIN B_{12} IN SEA WATER

(Two surface samples taken 7 March 1956 at 58° 40′ N., 6° 10′ W.)

	Assay method			
	Dilution; *E. gracilis z* Vitamin B_{12} in mμg/l.		Phenol passage; *Lb. leichmannii* Vitamin B_{12} in mμg/l.	
Sample	By external standard	By internal standard	By external standard	By internal standard
A	2·0	1·6	1·7	1·5
B	1·5	1·4	1·0	1·0

10 ml. portions of each were assayed as described; 3500 ml. portions of each were subjected to the techique used by Cowey (1956) of phenol passage and subsequent assay with *Lactobacillus leichmannii*. The results (Table 1) show that agreement between the results from the different methods was reasonably close. The accompanying paper (Daisley & Fisher, 1958) lists six more examples of sea water assayed by both the *Euglena* method and the method of Cowey (1956), as well as results for thirty-five other samples assayed by the *Euglena* method alone.

Variation between repeated assays of samples during storage

Three samples of sea water, also supplied by Dr Johnston, were stored for 9 months and assayed four times at intervals during that period. Each assay was carried out with the same degree of replication as that described in this paper, and results were calculated with reference to external standards. The results (Table 2) give no indication of a fall in the vitamin B_{12} concentration

TABLE 2. VARIATION BETWEEN REPEATED ASSAYS OF SAMPLES OF SEA WATER DURING STORAGE

(The figures refer to vitamin B_{12} concentrations as measured with *Euglena gracilis* strain *z*)

Sample collected:	Time	7 Mar. 1957	13 Mar. 1957	15 Mar. 1957
	Place	57° 02′ N. 1° 45′ W.	58° 30′ N. 0° 30′ E.	59° 25′ N. 4° 00′ E.
	Depth	10 m	10 m	10 m
		Vitamin B_{12} in mμg/l.		
Assay batch	25 Apr. 1957	1·6	1·4	0·9
	21 May 1957	1·2	1·4	0·8
	7 Aug. 1957	1·9	1·8	1·2
	9 Dec. 1957	1·4	1·1	1·0

during storage; they do, however, show some variation between results from different assay batches. The degree of variation is greater than is customarily obtained with other methods discussed by Coates & Ford (1955), but this may result from the higher sensitivity of the *Euglena* assay, which will make it more susceptible to the effects of adventitious vitamin B_{12} and to slight variations in the experimental procedure.

The precision of the assay method is, however, adequate for showing any considerable variation in vitamin B_{12} concentration between different samples, such as the several-fold differences between the concentrations in coastal and oceanic waters or between summer and winter water (Cowey, 1956), or water from different depths (Daisley & Fisher, 1958).

DISCUSSION

It is probable that assays with other chlorophyll- (or other pigment-) producing organisms, such as those mentioned by Droop (1954), could be increased in sensitivity by the procedure described: starting with a washed

depleted inoculum, growing a large volume of the organism, and then extracting its pigment into a small volume of solvent and measuring the colour intensity obtained.

If there were available a range of organisms each responsive specifically to a different member of the vitamin B_{12} family of compounds, it would be possible to make separate estimates of the concentrations of each such compound present in the sea; but it is an interesting fact that those organisms so far investigated which need members of the vitamin B_{12} family all respond to cyanocobalamin itself, in addition to any other analogue which they may utilize (see the lists given by Coates & Ford, 1955; Droop, 1957).

Some degree of differentiation would, however, be possible by comparison of results obtained with a cyanocobalamin-specific organism and an organism responding to several of the analogues, as was done by Cowey (1956) following the work of Ford (1953).

Not all the vitamin B_{12} which, in assay procedures such as that described in this paper, is made available to the assay organism is necessarily in a free and nutritionally active form in the original sea water. Some or even all may be bound in cells or cell fragments, or be combined with other substances. To differentiate between 'free' and 'bound' vitamin B_{12} in sea water, more complex procedures would have to be applied, as was found necessary with milk samples (Gregory, 1954); and if some proportion of the vitamin B_{12} in the sea was found to be in 'bound' forms, there would remain the problem that these might be differently available to different marine organisms.

Further work on these various aspects discussed is continuing.

Results obtained with the *Euglena* assay method with samples taken to study the vertical distribution of vitamin B_{12} in the sea are presented in a companion article (Daisley & Fisher, 1958).

SUMMARY

A sensitive method for the measurement of vitamin B_{12} concentration in sea water is described.

Small volumes, e.g. 10 ml., of sea water are diluted with distilled water and a microbiological assay using the fresh-water flagellate *Euglena gracilis* strain *z* is carried out. The diluted sea-water samples are enriched with a growth medium, washed inocula of the *Euglena* are added, and these cultures are grown for up to 2 weeks under constant conditions of temperature and illumination. The chlorophyll is then extracted from the cultures into small volumes of solvent and the optical densities measured. These optical density readings are related to the concentrations of vitamin B_{12} available to the *Euglena* populations during their growth.

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