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A SUGGESTED METHOD FOR THE ASSAY OF VITAMIN B_{12} IN SEA WATER

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(Text-figs. 1–3)

One of the difficulties encountered in the use of fresh water organisms for the assay of vitamin B_{12} of marine origin is the elimination of salt from the extracts. This has suggested that a marine organism might be used with advantage. Indeed on several occasions marine or supra-littoral species have been proposed as possible assay subjects, but acceptable techniques await development (Lewin, 1954; Droop, 1954; Sweeney, 1954). *Monochrysis lutheri*, a supra-littoral euryhaline species, has advantages over others in being small, robust and giving heavy yields in synthetic media. The method employed in some *Monochrysis* assays carried out in the early spring of 1954 will be described.

Principle

If a sample of sea water to be assayed, containing Z mμg B_{12}/l., is divided into portions A and B and A is diluted r times with a B_{12}-less synthetic sea-water substitute, the difference in B_{12} content of the two preparations then is

$$Z\frac{r-1}{r}. \tag{1}$$

If, now, each of these preparations is given the same graded doses of B_{12} the regressions of the responses so obtained can be compared and the value of Z ascertained. In Fig. 1

$$Z=\frac{rM}{r-1}. \tag{2}$$

For a parallel line method, such as this, the doses require to lie on that region of the response curve where linear increase of dose causes linear increase of yield measurements. When the optical measurement used is per cent absorption, this region is between doses of 3 and 18 mμg B_{12}/l. for *Monochrysis*.

The choice of three dose levels, say 3, 6, 9 mμg B_{12}/l., with replications, gives a symmetrical six-point layout which can be treated statistically in the manner described by Finney (1952) for parallel line assays. That is, tests can be made for 'regression', 'difference between preparations', 'linearity' and

'divergence from parallelism', and fiducal limits of the estimate can be calculated.

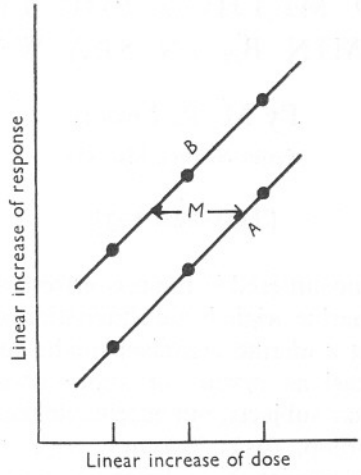

Fig. 1. Diagram of linear parallel line assay. Linear increase of response (vertical) against dose (horizontal) for preparations *A* and *B*. *M* (measured horizontally), difference between regressions in terms of dose.

Preliminary Assay

When the expected value of Z is entirely unknown it is necessary to perform a preliminary assay in order to ensure a convenient value for M. (M can be reduced by diluting the whole sample.) A very rough idea of Z can be obtained by adding to a pilot sample the ingredients (less the water) of the nutrient solution given on p. 437, and comparing the growth obtained with typical *Monochrysis* dose response curves (Fig. 3; Droop, 1954). For expected values of Z up to 10 mμg/l. no dilution of the original sample is necessary other than the twofold one performed by the addition of the nutrient solution (see below). But for larger expected values of Z a proportional further dilution of the original sample should be made.

A value of r of less than 3 is too small for convenience and one much greater tends to cause a lack of parallelism between the two regressions. This is occasioned by the fact that autoclaved enriched natural sea water is not as favourable a medium as the enriched synthetic sea-water.

Experimental

For the detailed assay a sample of 200 ml. is large enough. The predetermined initial dilution is made if necessary and the sample is divided in the

ratio 3:1. The smaller portion is then diluted threefold. Dilutions are effected with the following freshly prepared artificial sea water:

NaCl	30 g
$MgCl_26H_2O$	5 g
KCl	0·75 g
$CaSO_4$	1 g
H_2O (glass distilled) to	1 l.

The two portions are then mixed with their own volumes of the following freshly prepared nutrient solution and the pH adjusted to 7·5:

KNO_3	200 mg
K_2HPO_4	20 mg
Thiamin	1 mg
'T.M.2'[1]	20 ml.
'S.W.2'[1]	10 ml.

Tris-(hydroxymethyl)-aminomethane 1 g.
Glass distilled water to 1 l.

There are now two solutions, one (*A*) containing one-third as much of the original sample as the other (*B*). These solutions are then decanted into test-tubes in 6 ml. aliquots and vitamin B_{12} added as follows:

18 $\mu\mu$g	to each of four tubes containing solution				*A*
18 $\mu\mu$g	,,	,,	,,	,,	*B*
36 $\mu\mu$g	,,	,,	,,	,,	*A*
36 $\mu\mu$g	,,	,,	,,	,,	*B*
54 $\mu\mu$g	,,	,,	,,	,,	*A*
54 $\mu\mu$g	,,	,,	,,	,,	*B*

The tubes are plugged and autoclaved for a few moments at 15 lb. and inoculated with 0·02 ml. of a stock culture of *M. lutheri* and incubated at room temperature (16–22° C.) in continuous 'warm-white' fluorescent light of 100 ft. candles until growth is completed.

Example Assay

All assays of samples of surface water taken in February and March 1954 from Keppel Pier, Millport, on incoming tides, gave results lying between 5 and 10 mμg B_{12}/l. A single example will, therefore, be sufficient to illustrate the method.

Preliminary experiments had suggested that the water was unlikely to yield more than 10 mμg B_{12}/l., so preliminary overall dilutions were not necessary.

The sample, taken from Keppel on 5 April 1954, was assayed immediately.

[1] For compositions of these metal solutions see Droop (1955).

The results are shown in Table I and Fig. 2. Inspection suggests that there is a difference of $M=2{\cdot}4$ mμg/l. between the two regressions.

TABLE 1. ASSAY OF VITAMIN B_{12} IN SEA WATER

The sample is attenuated by a factor of 6 in preparation *A* and one of 2 in preparation *B*.

	Dose 3 mμg/l. B_{12}				Dose 6 mμg/l. B_{12}				Dose 9 mμg/l. B_{12}			
A	30	33	26	36	43	48	42	36	63	54	50	57
B	42	44	34	41	59	54	48	52	70	61	62	67

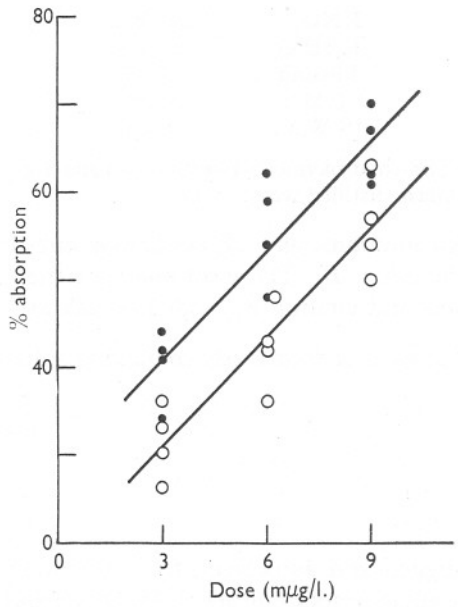

Fig. 2. Assay of Keppel Water, 5 April 1954. Dose of vitamin B_{12} in mμg/l.

$$Z=\frac{rM}{r-1}d, \tag{3}$$

where d represents total overall dilutions, including that effected on addition of the nutrients. Substituting,

$$Z=\frac{3\times 2{\cdot}4}{3-1}\times 2$$

$$=7{\cdot}2.$$

The calculated value of M is obtained from the equation

$$M=\frac{\bar{y}_B-\bar{y}_A}{b}-(\bar{x}_B-\bar{x}_A),$$

where $b=\frac{\Sigma S_{xy}}{\Sigma S_{xx}}$ (Finney, 1952), which when evaluated from the results set out in Table I, gives $M=2{\cdot}344$.

In the symmetrical assay both the calculation of M and the arithmetic for the analysis of variance are much simplified by making use of orthogonal contrasts afforded by the layout as described by Finney (1952).

The analysis of variance of the example, set out in Table II, shows that the assay is satisfactory as far as 'difference between preparations', 'regression' and 'linearity' are concerned, but the variance due to 'deviations from parallelism' is too small.

TABLE II. ASSAY OF VITAMIN B_{12} IN SEA WATER. ANALYSIS OF VARIANCE.

Source	D.F.	Sums of squares	Mean square
Preparation	1	560·7	560·7
Regression	1	2450·3	2450·3
Parallelism	1	0·0	0·0
Quadratic	1	0·75	0·75
Difference of quadratics	1	5·3	5·3
Between doses	5	3017·1	603·4
Error	18	290·9	16·2
Total	23	3308	

The 95% fiducal limits to M are obtained by evaluating

$$\left[M-\bar{x}_A+\bar{x}_B\pm\frac{ts}{b}\left\{(1-g)\left(\frac{1}{N_A}+\frac{1}{N_B}\right)+\frac{(M-\bar{x}_A+\bar{x}_B)^2}{\Sigma S_{xx}}\right\}^{\frac{1}{2}}\right]\div(1-g),$$

where $g=\frac{t^2s^2}{b^2\Sigma S_{xx}}$ and the value of t for a 0·05 probability and 18 degrees of freedom is taken as 2·101 (Finney, 1952).

The limits to M are thus 3·357 and 1·471. Substituting in (3) we get for the B_{12} estimate of the sample: 7·03 mμg/l. with limits 10·07 and 4·41.

DISCUSSION

Monochrysis responds to pseudo-B_{12}, and factor A but not to factor B (Fig. 3) so that its specificity can be compared to that of *Lactobacillus leichmannii*.

The assay method described here has some advantages and some obvious disadvantages. It is reasonably sensitive and admits of statistical treatment, and its design is such as to eliminate all carry over effects, but its greatest advantage is that it is direct in the sense that it involves no extraction procedures which require much time and large samples and, moreover, are possibly subject to errors due to loss of the vitamin. Desalting methods (Provasoli & Pintner, 1953) are at present subject to similar errors. However, the advantages of the *Monochrysis* method are offset by the length of time taken for the assay to complete. *Monochrysis* is an obligate phototroph with a generation time of about 24 h, with the consequence that cultures take about 3 weeks to

mature. It is, therefore, certain that the *Monochrysis* method would never be used with advantage for routine purposes, though the possibility of its being useful in some instances is envisaged.

Monochrysis is one of many B_{12}-requiring marine phytoflagellates which can be handled in a similar manner. A number of such species with differing specificity patterns employed concurrently with the different forms of the vitamin could provisionally yield useful information of the B_{12} content of sea water. *Prymnesium parvum* or *Syracosphaera elongata*, for instance, are as specific as *Ochromonas* in their response.

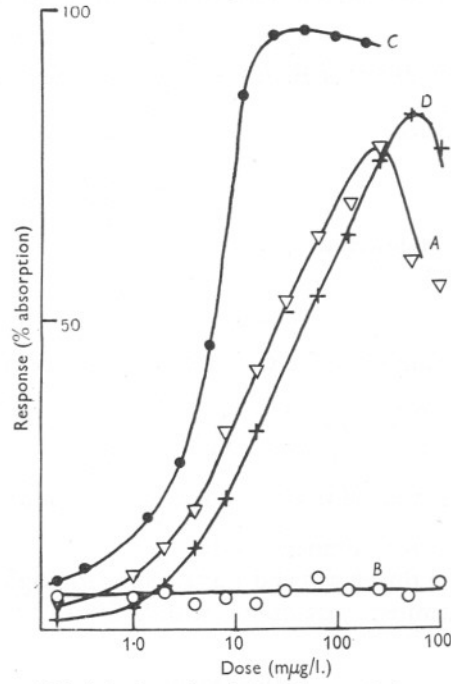

Fig. 3. Response of *M. lutheri* to vitamin B_{12} (curve *c*), factor *A*, pseudo-vitamin B_{12} (curve *D*), and factor *B* in presence of adenine.

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