Investigations into the *Euglena* method for the assay of the vitamin B₁₂ in serum

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SYNOPSIS Details are given of a modified *Euglena* method for the assay of B₁₂ in serum. Serum in very small quantities is usually inhibitory to the growth of the *Euglena*. In general, this inhibition is most marked when the organism is growing prolifically. For estimating serum B₁₂ values, a standard curve is used from B₁₂ standards containing 0.01 ml. of serum of low B₁₂ content, so that growth in test serum solutions is compared with growth in serum-containing standards. The recovery of B₁₂ added to serum, incomplete when estimated from aqueous standards, is now complete and the variation in results from batch to batch is markedly reduced.

Various factors—such as medium, light, and inoculum—can affect growth in aqueous and serum solutions differently and are an important cause of variations between batches and laboratories.

Optimal conditions were studied and they are essential for the most sensitive and accurate assay, but if the results are estimated from serum-containing standards accurate values can be obtained even under less than optimal conditions.

Subnormal serum B₁₂ concentrations can be detected visually as early as 24 hours after the start of assay. For diagnostic purposes the assay can be read at three days, provided that appropriate dilutions are used, conditions are optimal, and growth is measured in a 1 cm. cell.

Microbiological assay with *Euglena gracilis* has been widely used to measure the vitamin B₁₂ concentration of serum. Initially, *Euglena gracilis* var. *bacillaris* was used as the test organism (Ross, 1950, 1952) but subsequently Hutner, Bach, and Ross (1956) introduced the *z* strain. Using this strain and an improved medium, much more growth is obtained, the incubation period is shorter, and the assay is more sensitive.

The *Euglena* method has several advantages for assaying B₁₂ in serum. It has a high specificity and is very sensitive. Because whole serum is assayed, preliminary extraction is unnecessary and direct measurements can be made of both bound and free B₁₂. However, growth conditions need careful control and the results often vary widely in different laboratories (Table I). It is also general experience that considerable variation may occur between results in different assay batches. In addition, in my experience, it is rarely possible to obtain full recovery of B₁₂ added to serum.

In the assay the serum B₁₂ concentration is usually determined by comparing the growth of the organism in serum with its growth in aqueous

standards of B₁₂. A possible explanation for the shortcomings of the method is that the *Euglena* reacts differently in aqueous and serum solutions.

The work described in this paper was designed to investigate this point and to establish the optimal growth conditions necessary for the most sensitive assay.

MATERIALS AND METHODS

The method is basically that of Hutner, Bach, and Ross (1956) but modifications based on the work described in this paper are included.

Glassware used, and its rigid cleaning and preparation, was essentially that recommended by Hutner *et al.* (1956). A detergent, Diversey Pyroneg (Deosan Ltd.), however, was used for cleaning and was shown not to affect the assay. Glass-distilled water produced by the Loughborough all-glass still was used throughout. The assay medium was that recommended by Hutner *et al.* (1956).

THE ASSAY ORGANISM The *z* strain of *Euglena gracilis* was used¹. It was maintained by weekly subculture into the

¹The organism was obtained from the Culture Collection of Algae and Protozoa, Botany School, Downing Street, Cambridge.

TABLE I
SERUM VITAMIN B₁₂ CONCENTRATIONS IN NORMAL SUBJECTS USING *EUGLENA GRACILIS*
AS TEST ORGANISM

Author	Year	Strain Used	No. of Subjects	Serum B ₁₂ Concentration (μg./ml.)		Type of Subject
				Range	Mean	
Mollin and Ross	1952	<i>Bacillaris</i>	65	100-720	358	Normal ¹
Killander	1953	<i>Bacillaris</i>	56	100-720	360	Normal
Heinrich and Lahann	1954	<i>Bacillaris</i>	—	62-460	238	—
Lear <i>et al.</i>	1954	<i>Bacillaris</i>	20	292-856	532	Normal
Pitney and Beard	1954	<i>Bacillaris</i>	56	86-460	212	Normal
Mollin and Ross	1957	<i>Bacillaris</i>	223	100-900	356	Normal and control ²
Dixit <i>et al.</i>	1956	<i>Bacillaris</i>	37	92-800	259	Indian normals
Dixit <i>et al.</i>	1956	<i>Bacillaris</i>	78	72-2160	592	Indian soldiers
Killander	1957b	<i>Bacillaris</i>	156	130-1200	501	Normal and control
Raccuglia and Sacks	1957	<i>Bacillaris</i>	180	144-1080	387	Normal
Miller	1958	<i>Bacillaris</i>	22	270-1270	640	Normal and control
Nicholas and Pitney	1958	<i>Bacillaris</i> and <i>z</i>	218	190-875 ³	456	Normal
Shinton	1959	<i>z</i>	120	130-750	390	Normal
Cooper	1959	<i>Bacillaris</i>	32	—	499	Control
Cooper	1959	<i>z</i>	27	—	305	Control
Banerjee <i>et al.</i>	1960	<i>Bacillaris</i>	51	98-940	282	Normal
Davis and Kelly	1962	<i>z</i>	100	160-875	398	Normal
Anderson and Pope	1962	<i>z</i>	149	163-925	472	Normal

¹Normal = healthy hospital staff or blood donors.

²Control = patients convalescing from minor disorders.

³These were the average of the values obtained by the *bacillaris* and *z* methods.

liquid stock culture medium recommended by Hutner *et al.* (1956).

VITAMIN B₁₂ STANDARDS Cytamen cyanocobalamin (Glaxo Laboratories Ltd.) of a concentration of 50 μg./ml. was used for the B₁₂ standards. This was diluted to 40 μg./ml., from which the following range of standards was usually set up:—0, 0.5, 1, 2, 4, 6, 8, 10, and 12 μg./ml. final concentration. In some experiments 15 and 19 μg./ml. standards were included. Three to six tubes were set up for each concentration.

Aqueous vitamin B₁₂ standards These contained the amounts of the standard concentrations of B₁₂ made up to 2 ml. with distilled water together with 2 ml. of double-strength medium.

Serum-containing vitamin B₁₂ standards These contained in addition small amounts of serum of B₁₂ concentration less than 30 μg./ml. The amounts added varied in some experiments, but routinely 0.1 ml. of a 1 in 10 dilution of serum in water was included in the B₁₂ standards. The B₁₂ content of the added serum was negligible.

COLLECTION OF BLOOD New and sterile glassware and syringes and needles washed, prepared, and sterilized, and known to be B₁₂ free, were used. Latterly, disposable syringes and needles were used and are recommended.

PREPARATION OF SERUM FOR ASSAY All sera were assayed in two or three assay batches. Routinely, unless the serum B₁₂ concentration was expected to be abnormally high or low, each serum was assayed initially at a 1 in 80 dilution. If the growth proved to be too high or too low in the first assay, a more suitable dilution was used in a subsequent

assay chosen from the following range: 1 in 20, 1 in 40, 1 in 80, 1 in 200, 1 in 400, or higher if needed. Dilutions less than 1 in 20 are inadvisable due to the precipitation of proteins interfering with the assay. The dilution used was selected to give a reading equivalent to between 2 and 10 μg./ml. on the standard curve.

Three tubes were set up for each serum and contained the appropriate amount of serum made up with distilled water to 2 ml. together with 2 ml. of double-strength medium.

CONTROL SERA Control sera of known B₁₂ concentrations, with and without added B₁₂, were included in each batch at three different dilutions for each serum. The dilution agreement and completeness of recovery of added B₁₂ served as checks on the accuracy of the assay. The sera used were as follows:—

1(a) = Pooled serum with a B₁₂ concentration less than 60 μg./ml.; 1(b) = serum 1(a) with the equivalent of 200 μg./ml. of added B₁₂; 2(a) = pooled normal serum with a B₁₂ concentration between 400 and 500 μg./ml.; 2(b) = serum 2(a) with the equivalent of 2,000 μg./ml. of added B₁₂.

To prepare these, 200 ml. of suitable sera was pooled. To 100 ml. was added sufficient B₁₂ to give the required concentration of added B₁₂. The sera, with and without added B₁₂, were divided into aliquots of about 2 ml., sufficient for assay in two batches, and were stored at -12 to -17°C.

MEASUREMENT OF TOTAL VITAMIN B₁₂ IN SERUM The tubes containing standards and solutions for assay were heated in a boiling water bath for 15 minutes. This released the B₁₂ bound to serum protein and also served to sterilize the assay tubes adequately.

MEASUREMENT OF UNCOMBINED VITAMIN B₁₂ IN SERUM
The solutions of serum for assay were either not heated or heated at 50°C. for half an hour which sterilized the assay tubes but freed very little B₁₂ from the protein.

INOCULUM A seven-day-old culture was used. The inoculum was washed and prepared as recommended by Hutner *et al.* (1956) and was finally suspended in 20 ml. of sterile single-strength medium. The preparation of the inoculum was varied where stated.

INOCULATION After heating, the tubes were distributed at random in the perspex racks, in which they were inoculated and incubated. Three drops of inoculum were delivered into the stock culture tubes and thereafter one drop was added to each assay tube using a sterile Pasteur pipette calibrated to deliver 50 drops per millilitre.

WATER BATH FOR INCUBATION Figure 1 is the diagram of the water bath, a modification of that used by Ross (1952), and was designed and assembled by the Postgraduate Medical School. It consists of a perspex tank mounted on an aluminium cabinet. The two rows of five perspex racks holding 54 tubes each are supported 2.7 inches above the bottom of the tank on ledges along the sides and down the centre of the tank. The metal cabinet houses lights, motors, and a fan and has removable panels on the sides and perforated ends to allow a free flow of air.

The light and temperature requirements of the *Euglena* are supplied and controlled in the following way:

Light There are four 3 ft. 30 watt 'warm white' fluorescent strip lights in the cabinet beneath the tank. The light is diffused by means of one or two layers of Cinemoid filter placed between sheets of opal glass just beneath the tank. The lights are controlled by a Variac transformer and it is possible to vary the amount of light over a range of 25 to 250 foot-candles, measured with a Weston Master III Universal exposure meter held at the level of the top of the tank.

A black cloth is placed over the perspex tank to cut out all outside light, so that the assay tubes receive light only from the strip lights below. There is slightly less light at the edges but this only affects very large batches of up to 540 tubes. With smaller batches the periphery of the bath is left free.

Temperature There is no heating element. Thermostats regulating the heating element occasionally fail and as a rise of 2 or 3°C. is critical, large batches may be ruined.

The temperature is maintained by the heat given out by the lights enclosed in the cabinet. It is also dependent on the temperature of the room and the amount of covering on top of the bath. In order to prevent the temperature from rising above that required, a thermostat in the tank controls a fan in one end of the cabinet. This causes air to pass through the cabinet beneath the tank, and the water can be maintained at the temperature required provided that temperature changes in the room are not too great.

The mixing of the water by a stirrer at one end of the tank is facilitated by the fact that there is 2.7 inches of water in the tank beneath the racks, as well as the water surrounding the assay tubes. This ensures that the temperature of the water is uniform in all parts of the tank.

INCUBATION Each assay batch was incubated in the water bath for four and three-quarter days, but a shorter incubation period is recommended. The optimal amount of light, measured as previously mentioned, was 50 foot-candles. The optimal temperature was 28.5°C. These conditions were varied where stated in some of the experiments.

MEASUREMENT OF GROWTH A homogeneous suspension of *Euglena* was obtained by shaking and growth was measured in a Unicam colorimeter SP 300 using a red filter (Ilford 204). When using the range of B₁₂ standards 0 to 12 µg./ml. a more accurate curve is obtained by

FIG. 1. Water bath for the incubation of the *Euglena* assay.

measuring growth in a 0.5 cm. cell, but for the sake of uniformity in this paper all growth was measured in a 0.25 cm. cell unless otherwise stated. Occasionally corrections had to be made for turbidity of the supernatant due to slight protein precipitation. In this case the optical density (O.D.) of the supernatant after centrifugation was measured and subtracted from the reading of the whole suspension.

The O.D. readings were averaged and the standard curve was plotted on linear graph paper as O.D. $\times$ 100 against the final B₁₂ concentration in $\mu\mu\text{g./ml.}$

The growth curves of the aqueous and serum-containing B₁₂ standards have been designated 'aqueous curve' and 'serum curve' respectively.

ESTIMATION OF SERUM B₁₂ CONCENTRATION The three O.D. readings for each serum were averaged and the B₁₂ concentration was estimated from the standard curve allowing for the dilution at which it was assayed. In this paper comparisons have been made between the serum B₁₂ concentrations estimated from the aqueous curve and the serum curve. The present results suggest that estimations from the serum curve represent the true value.

RESULTS

RECOVERY OF VITAMIN B₁₂ ADDED TO SERUM Recovery experiments were carried out in which amounts of 150, 200, and 2,000 $\mu\mu\text{g./ml.}$ of B₁₂ (cyanocobalamin) respectively were added to three different sera. The sera, with and without added B₁₂, were assayed in repeated and consecutive batches and the results were estimated from aqueous B₁₂ standards as in the method of Hutner *et al.* (1956). The recovery often varied in individual assay batches and was rarely complete (Table II). The mean recovery for the different sera ranged from 82 to 87%, but in individual batches the recovery varied from 65 to 107%.

EFFECT OF KCN ON RECOVERY It has been shown that the addition of cyanide considerably increases the yield of B₁₂ in the supernatant when serum proteins are precipitated by heat (Spray, 1955; Killander, 1957a; Girdwood, 1960; Matthews, 1962).

Using the *Euglena* method, where whole serum is assayed, the recovery of cyanocobalamin added to serum was usually very slightly higher than that of hydroxocobalamin, when 200 and 2,000 $\mu\mu\text{g./ml.}$ of

either was added to each of two sera. The addition of KCN (5 $\mu\text{g.}$ per assay tube) to the medium did not improve the recovery of cyanocobalamin, whereas it did seem to improve slightly the recovery of hydroxocobalamin. The recovery of both forms of B₁₂ when KCN was added was therefore similar, but far from complete, and in this particular experiment was only 68%.

EFFECT OF HEAT ON RECOVERY It is possible that heating at 100°C. for 15 minutes does not completely liberate all the B₁₂ from the serum protein or that heating destroys some B₁₂. The effect of heating was therefore investigated.

Aqueous B₁₂ standards, and control sera, with and without KCN (5 $\mu\text{g.}$ per assay tube), were heated at 100°C. for three, 10, 15, or 30 minutes and a further set autoclaved at 118°C. for five minutes. Recovery was incomplete and similar for each procedure.

EFFECT OF ADDITION OF SERUM TO VITAMIN B₁₂ STANDARDS ON GROWTH AND RECOVERY The effect of adding serum to the B₁₂ standards was studied in order to determine whether the inadequate recovery of B₁₂ added to serum was due to a depressing effect of serum on the growth.

Effect of addition of small amounts of serum on growth The effect on growth of varying amounts of serum, ranging from trace amounts to the amounts usually assayed, was studied by adding 0.00001, 0.0001, 0.0002, 0.001, 0.01, 0.05, 0.1, and 0.2 ml. of pooled serum (B₁₂ concentration 18 $\mu\mu\text{g./ml.}$) to eight different sets of B₁₂ standards respectively. The growth at each concentration of B₁₂ was compared to that in standards to which no serum had been added (Fig. 2). When more than 0.01 ml. of serum had been added corrections were made to the O.D. readings for the B₁₂ content of the added serum and for turbidity of the supernatant (see Materials and Methods)².

At B₁₂ concentrations of more than 1.0 $\mu\mu\text{g./ml.}$ growth was less in all the serum-containing standards.

²The true concentrations of the standards containing added serum were considered to be the sum of the crystalline B₁₂ in the standards and the assayed concentration of B₁₂ in the added serum.

TABLE II

No. of Assays	B ₁₂ Added ($\mu\mu\text{g./ml. serum}$)	RECOVERY OF VITAMIN B ₁₂ ADDED TO SERUM					
		Estimated from Aqueous Curves			Estimated from Serum Curves		
		B ₁₂ Concentration of Serum ($\mu\mu\text{g./ml.}$)	% Recovery of Added B ₁₂		B ₁₂ Concentration of Serum ($\mu\mu\text{g./ml.}$)	% Recovery of Added B ₁₂	
			Range	Mean $\pm$ S.E.		Range	Mean $\pm$ S.E.
17	150	70	75-91	82 $\pm$ 1	84	95-117	104 $\pm$ 1
15	200	46	65-99	87 $\pm$ 3	48	90-104	98 $\pm$ 1
42	2000	387	71-107	85 $\pm$ 1	451	92-110	100 $\pm$ 1

FIG. 2. The effect on growth in vitamin B₁₂ standards of the addition of small amounts of pooled serum of pooled serum B₁₂ concentration 18 µg./ml. ○—○ aqueous standards ●—● serum-containing standards.

FIG. 3. Growth in aqueous vitamin B₁₂ standards ○—○ and in similar standards to which 0.01 ml. of serum of low B₁₂ content (less than 30 µg./ml.) has been added ●—●. Each curve is the mean of 40 consecutive assays.

The addition of 0.2 ml. of serum did not depress growth to any greater extent than did 0.001 ml., and there was even some depression of growth when as little as 0.00001 ml. of serum was added.

Effect of addition of serum on growth and recovery in repeated assays The effect of the addition of amounts of 0.01 ml. of serum of low B₁₂ content (less than 30 µg./ml.) to B₁₂ standards was studied in 40 consecutive assay batches. The difference in growth between aqueous and serum-containing standards often varied considerably from batch to

batch. The mean curve of growth of the serum-containing standards was significantly lower than that of the aqueous standards above 1.0 µg./ml. B₁₂ ($P < 0.001$) (Fig. 3). The recovery of 200 µg./ml. of B₁₂ added to serum of low B₁₂ content and 2,000 µg./ml. added to normal serum was incomplete and variable when estimated from the aqueous curves but complete when estimated from the serum curves. The mean recovery estimated from the aqueous curves was 86.1% at the 200 µg./ml. level and 85.4% at the 2,000 µg./ml. level and estimated from the

FIG. 4. The variation in growth in individual assays in aqueous ○—○ and serum-containing vitamin B₁₂ standards ●—●. Each curve is the mean of the 40 consecutive assays and the lines through the mean readings at each concentration of B₁₂ represent $\pm 2 \times S.D.$

TABLE III

VITAMIN B₁₂ CONCENTRATION OF 11 CONTROL SERA ASSAYED REPEATEDLY AND ESTIMATED FROM AQUEOUS AND SERUM-CONTAINING STANDARDS

Sera	No. of Assays	Estimated from Aqueous Curves				Estimated from Serum Curves			
		B ₁₂ Concentration (μg./ml.)			Coefficient of Variation (%)	B ₁₂ Concentration (μg./ml.)			Coefficient of Variation (%)
		Range	Mean	S.D.		Range	Mean	S.D.	
1	40	100-158	131	13.5	10.3	135-165	152	7.0	4.6
2	13	55-82	70	7.4	10.6	69-93	84	5.9	7.0
3 = 2 + 150 ¹	17	167-212	193	12.1	6.3	223-255	240	10.1	4.2
4	18	41-60	51	6.6	13.0	50-68	57	5.7	10.0
5 = 4 + 200	21	187-255	222	16.9	7.6	241-283	263	11.0	4.2
6	15	38-56	46	5.4	11.7	45-55	48	3.4	7.0
7 = 6 + 200	15	167-245	219	23.0	10.5	231-254	243	7.5	3.1
8	9	418-487	457	20.3	4.5	518-592	550	19.2	3.5
9 = 8 + 2,000	9	1,950-2,470	2,150	166	7.7	2,445-2,750	2,600	119	4.6
10	42	319-463	387	37.8	9.8	390-490	451	21.8	4.8
11	42	1,735-2,560	2,085	199	9.5	2,300-2,635	2,440	83.3	3.4

¹B₁₂ μg./ml.

serum curves was 102.2 and 99.6% respectively (also see Table II).

EFFECT OF ADDITION OF SERUM TO AQUEOUS VITAMIN B₁₂ STANDARDS ON REPRODUCIBILITY OF RESULTS Figure 4 compares the variation in growth in standards containing 0.01 ml. of serum with that in the aqueous standards in the same 40 individual assay batches. The addition of serum had the effect of stabilizing growth, for there was less variation from batch to batch in the serum-containing standards.

The greater accuracy is reflected by the smaller variation in the mean recovery per batch estimated from the serum curves (95 to 107%, S.D. 3.8%) compared with recovery estimated from the aqueous curves (74 to 101% S.D. 6.9%).

The combined coefficient of variation for 11 control sera assayed at three dilutions per assay in replicate and consecutive assays was 5.1% when estimated from the serum curves, compared with 9.2% when estimated from the aqueous curves (Table III)³. In the triplicate assays on 769 test sera carried out in each instance in three different assay batches at one dilution per batch, the combined coefficient of variation was 5.3% for the serum curves compared with 8.0% for the aqueous curves.

The variation within a batch was small for either method of estimation. When the same serum was assayed at three dilutions, six or seven times within the same batch, the combined coefficient of variation at the various dilutions, assessed within six such batches, was 3.7% for either method.

There was fairly good agreement within a particular method of estimation between values of a serum

assayed at different dilutions. However, the agreement is probably better for the results calculated from the serum curves, for the combined coefficient of variation between the dilution values was greater (7.5% compared with 5.5%) for the values estimated from the aqueous curves assessed from the 11 control sera assayed at three dilutions in repeated batches.

EFFECT OF DIFFERENT CONDITIONS ON GROWTH AND RECOVERY IN AQUEOUS AND SERUM SOLUTIONS The foregoing results indicate, first, that the presence of traces of serum usually depresses the growth of the *Euglena* and, secondly, that there is less variation in the results from batch to batch when estimated from the serum curves. It seems that the growth of *Euglena* is affected differently in aqueous and serum solutions by small variations in growth conditions. The purpose of this section is to study the growth conditions and their effect on the growth of *Euglena* in aqueous and serum solutions.

TABLE IV

COMPARISON OF GROWTH AND RECOVERY USING TWO SELECTED DIFFERENT MEDIA IN SAME ASSAY BATCH

B ₁₂ (μg./ml.)	Medium A		Medium B	
	Aqueous	Serum-containing	Aqueous	Serum-containing
2.0	9.4 ¹	7.8	6.3	7.3
4.0	18.3	15.2	12.6	12.9
6.0	25.0	20.3	18.0	17.4
8.0	29.3	24.9	22.3	20.5
10.0	33.8	27.3	25.9	23.6
12.0	37.7	30.0	30.3	27.0
% Recovery of B ₁₂ added to serum	73	97	91	95

¹Growth measured as O.D. units × 100.

A = medium supporting good growth

B = medium supporting poor growth.

³All results reported in this paper were obtained using approximately optimal and constant conditions. In previous work, when conditions were not as well controlled, variation in results estimated from the aqueous curve was much greater.

FIG. 5. The effect of different intensities of light on the growth in serum-containing vitamin B_{12} standards. Curve 1 in either diagram is the mean growth in six assays at the optimal light intensity of 50 foot-candles. Curves 2, 3, 4, and 5 represent growth in the individual assays at light intensities of 250, 200, 100, and 75 foot-candles respectively, and curves 6 and 7 at light intensities of 37.5 and 25 foot-candles respectively.

Basal medium The effect on growth and recovery of five different lots of media was studied comparing aqueous and serum-containing standards in six to eight assay batches for each lot of medium. The same culture of *Euglena* was used and conditions were constant throughout. There was less variation in the mean growth in the serum-containing standards between the different lots of media. Mean recovery was constant and complete (99 to 103% mean 100.6%) when estimated from the serum curves, but was rarely complete (81 to 95% mean 85.8%) and the variation was greater when estimated from the aqueous curves. It is noteworthy that recovery in the latter was lowest with the medium that stimulated greatest growth. This was more obvious when growth and recovery were compared in the same assay batch using two selected media, one supporting good growth and the other poor growth (Table IV). Ammonium succinate was the only

ingredient which was different in the media and was obtained from different manufacturers.

It is probable that unexplained differences in batches of other chemicals may also produce this effect.

Effect of light The optimal light intensity for growth of the *Euglena* was first determined approximately by comparing growth in standards at light intensities from 25 to 250 foot-candles and was found to be about 50 foot-candles. The effect of different intensities of light was then studied more precisely in six assays by comparing the growth in aqueous and serum-containing standards at intensities of 250, 200, 100, 75, 37.5, and 25 foot-candles respectively with the growth at 50 foot-candles in each case. The six assays were carried out in two baths with all conditions identical except the light intensity. The same lot of medium and size and age of inoculum was used throughout the series and the maximum variation in

FIG. 6. The effect of optimal and excessive amounts of light on the growth in the aqueous $\circ$ — $\circ$ and serum-containing $\bullet$ — $\bullet$ vitamin B_{12} standards. The upper pair of curves in A, B, and C are the mean of six assays at the optimal light intensity of 50 foot-candles. The lower pairs of curves in A, B, and C are each the mean of two assays at light intensities of 250, 200, and 150 foot-candles respectively.

temperature from one bath to another or from one assay batch to another was 1°C. and was usually less than 0.5°C. Figure 5 compares the growth in the serum-containing standards at the different light intensities.⁴

The most satisfactory growth curve was again obtained at a light intensity of 50 foot-candles. When the light intensity was either decreased or increased there was less growth, the only exception being a slight increase in growth from 0 to 2 µg./ml. B₁₂ when the light was decreased. In the aqueous standards growth was usually considerably higher than in the serum-containing standards, the exception being at 150 foot-candles. There was an increase in growth in the aqueous standards at the higher concentrations of B₁₂ at light intensities of 200 and 250 foot-candles (Fig. 6)⁵, and for this reason the recovery of B₁₂ added to serum and estimated from the upper portion of the aqueous curve at a light intensity of 250 foot-candles was 15% lower than at 50 foot-candles, whereas estimated from the serum curve was approximately 100% at either light intensity.

⁴There were small variations between the six sets of control curves at 50 foot-candles and therefore for a true comparison small corrections had to be made to the readings of the other curves. To do this the respective 50 foot-candle curves were compared with the average of the six sets of curves and the readings at the 250, 200, 100, 75, 37.5, and 25 foot-candles were corrected accordingly.

⁵Small corrections were made to the readings of the growth curves at 250, 200, and 150 foot-candles as in Figure 5.

FIG. 7. The effect of temperature on the growth in the serum-containing vitamin B₁₂ standards.

The curve at the optimal temperature of 28.5°C. is the mean of four assays. The curves —●— and —○— represent growth at temperatures lower and higher than the optimal temperature respectively.

Effect of temperature Small changes in temperature had considerable effect on the growth of the *Euglena*. Preliminary experiments suggested that the optimal temperature for growth was 28.5°C. To determine this more precisely the growth in standards at temperatures of 23.5°C., 26°C., 31°C., and 31° to 33°C. respectively, was compared with the growth at 28.5°C. in four assays. These four assays were carried out in two baths with all conditions similar except for temperature. The temperatures were the average for the four and three-quarter days' incubation and were recorded twice daily using a maximum and minimum thermometer. The variation was not greater than ±0.5°C. except when the temperature range 31°C. – 33°C. was used. Figure 7 compares the serum curves at the different temperatures⁶.

As the temperature fell below 28.5°C. the growth progressively decreased at all concentrations. At 31°C. growth at lower concentrations of B₁₂ was much the same as at 28.5°C. but was markedly depressed at higher concentrations. When the temperature rose to 33°C. the depression of growth started at an even lower concentration of B₁₂ and was greater. Where growth was profuse there was interference with chlorophyll production and the

TABLE V
COMPARISON OF GROWTH AND RECOVERY USING
WASHED AND UNWASHED INOCULA

B ₁₂ (µg./ml.)	Inoculum A ¹		Inoculum B ²	
	Aqueous	Serum-containing	Aqueous	Serum-containing
2.0	9.2 ³	7.2	6.2	4.8
4.0	16.0	13.0	14.5	8.9
6.0	21.8	17.7	19.5	12.5
8.0	26.4	21.2	24.9	16.7
10.0	29.6	25.1	30.1	20.5
12.0	32.8	28.2	33.1	23.9
% Recovery of B ₁₂ added to serum	71	94	59	94

¹A = washed and suspended in 20 ml.

²B = unwashed and made up to 20 ml. with culture supernatant.

³Mean growth in two experiments measured as O.D. units × 100. Inocula prepared from seven-day cultures.

organism became yellow. There was more growth throughout in the aqueous standards but the pattern was similar to that of the serum curves at the different temperatures.

Effect of washing the inoculum An unwashed culture was used as inoculum until Kristensen (1955, 1956) recommended washing the culture to remove the B₁₂-binding substance in the supernatant. The effect on growth and recovery of a washed and an

⁴As for the light curves, small corrections were made to the readings of the curves at 23.5°C., 26°C., 31°C., and 31° to 33°C. for true comparison with each other and the mean curve at 28.5°C.

FIG. 8. The effect of size of inoculum on growth in aqueous $\circ$ — $\circ$ and serum-containing $\bullet$ — $\bullet$ vitamin B_{12} standards. Curves 1, 2, and 3 represent growth when using inocula prepared from a seven-day washed culture diluted in 20, 10, and 5 ml. respectively. Each curve is the mean of two experiments.

unwashed inoculum was studied by comparing aqueous and serum-containing standards. The depression of growth using the unwashed inoculum was far greater in the serum-containing solutions with the result that recovery of B_{12} added to serum and estimated from the aqueous curve was only 59% compared with 71% using the washed inoculum (Table V).

Effect of density of inoculum The effect of varying inoculum density on the growth of the *Euglena* in aqueous and serum-containing standards was studied by using three different inocula of density equivalent to a 1 in 20, 1 in 10, and 1 in 5 dilution of the original culture respectively (Fig. 8.)

The more dense the inoculum, the greater was the growth at a particular concentration of B_{12} and less obvious the inhibitory effect of serum. In fact growth was greater in the serum-containing standards using the heaviest inoculum (1 in 5) in low concentrations of B_{12} (less than $2 \mu\text{g.}/\text{ml.}$).

Therefore recovery of B_{12} added to serum and estimated from the aqueous curve was greatest using the densest inoculum, but, except when readings were equivalent to very low concentrations of B_{12} , the recovery was still not complete. Recovery was approximately 100% for all inocula when estimated

from the serum curves. Readings were more regular and over several experiments the differentiation of growth at very low concentrations of B_{12} was better when the lightest inoculum (1 in 20) was used.

Effect of length of incubation This was studied by comparing the growth in aqueous and serum-containing standards after different lengths of incubation. Sets of standards were incubated for two, three, four, four and three-quarter, and six days. Four control sera were also set up and incubated for the same lengths of time and the values were estimated for each incubation time.

Though growth was greater in the aqueous standards, the pattern of growth for the different lengths of incubation was essentially similar for the aqueous and serum curves. The serum curves are compared in Figure 9. Provided that sera were set up at the appropriate dilutions, and growth was measured in a 1 cm. cell instead of the usual 0.25 cm. cell, there is sufficient differentiation of growth even at two or three days at the lower concentrations of B_{12} for the serum B_{12} to be accurately estimated, and there was a good agreement between the values of a serum estimated from two days onwards. Figure 10 shows a pair of aqueous and serum curves after three days incubation when growth was measured in a 1 cm. cell. Further, a low serum B_{12} can be detected as early as 24 hours, for the *Euglena*, in response to small amounts of B_{12} , quickly ceases to grow and settles within 24 hours in a button at the bottom of the assay tube.

NATURE OF MATERIAL IN SERUM AFFECTING GROWTH OF EUGLENA Growth of the *Euglena* in aqueous B_{12} standards was compared with that in standards containing trace amounts of (1) the supernatant after the extraction of proteins from serum, (2) serum, (3) albumin, (4) gamma globulin, (5) Tween 80⁷, and (6) serum + Tween 80. Serum and the individual proteins caused a similar marked inhibition of growth, but there was only a slight inhibitory effect by the protein-free supernatant. It seems likely that protein is responsible for this inhibition. Tween 80⁷ caused an inhibitory effect very similar to that of serum and proteins, but when Tween 80 was added to the serum-containing standards there was no further inhibition.

SERUM VITAMIN B_{12} CONCENTRATIONS IN CONTROL The serum vitamin B_{12} concentrations were assayed of 149 healthy control subjects, who were members of the hospital staff aged 20 to 50 years. The frequency distribution of the normal values is shown in

⁷The effect of Tween 80 was compared because it had also been found to exert an inhibitory effect on growth. It had been added to the medium in an attempt to diminish clumping and sticking of the organism to the bottom of the assay tube.

FIG. 9. The effect of different lengths of incubation on the growth in serum-containing vitamin B₁₂ standards. The growth was measured in a 0.25 cm. cell.

FIG. 10. Growth curves of aqueous $\circ$ — $\circ$ and serum-containing $\bullet$ — $\bullet$ vitamin B₁₂ standards after three days' incubation. The growth was measured in a 1 cm. cell.

Figure 11. The range of values determined from the aqueous curves was 125 to 765 (mean 402, S.D. 129 $\mu\text{g./ml.}$) and of these only five (3.4%) were less than 200; determined from the serum curves the range was 163 to 925 (mean 472, S.D. 151 $\mu\text{g./ml.}$) and only three (2.4%) were less than 200.

FIG. 11. The frequency of the serum B₁₂ concentrations of 149 normal subjects estimated from aqueous vitamin B₁₂ standards (A) and serum-containing vitamin B₁₂ standards (B).

Duplicate sera were taken from 125 of these normal subjects, mostly at intervals of two weeks, and were assayed together in three batches at one dilution in each batch. Assessed over six batches, the S.D. of estimations on aliquots of the same serum, assayed in the same batch under conditions similar to those used for the duplicate sera, was 3.1% of the mean. Hence the S.E. of the difference between a pair was $\sqrt{2} \times \text{S.D.}$ or 4.4% of the mean. Twenty-nine per cent of the duplicate sera varied from each other by more than twice the S.E. (8.8% of the mean) and therefore were significantly different at the 95%

level of significance. These pairs of sera varied from each other by 9.2 to 29.6% of the mean, average 14.4%.

SERUM VITAMIN B₁₂ CONCENTRATIONS IN PERNICIOUS ANAEMIA PATIENTS The serum vitamin B₁₂ concentrations were assayed of 59 patients with pernicious anaemia whose haemoglobin levels varied from 3 to 15.2 g./100 ml. The values ranged from 7 to 96 (mean 32, S.D. 20 μ g./ml.) when estimated from the aqueous curves and 9 to 113 (mean 38, S.D. 23 μ g./ml.) when estimated from the serum curves. In the latter range all but one were less than 95 μ g./ml.

DISCUSSION

The observations reported in this paper show that there can be a considerable variation from batch to batch in the assay results of sera estimated from an aqueous curve. Furthermore, recovery of B₁₂ added to serum was rarely complete and there was often a wide variation in recovery obtained in different batches, on occasions being as low as 60%. Other workers have also reported a wide variation in the results of repeated assays of the same serum in different batches (Ross, Hutner, and Bach, 1957; Killander, 1957a; Nicholas and Pitney, 1958; Shinton, 1959), but most workers have reported a 'satisfactory' mean recovery when B₁₂ was added to serum. The exception was Pitney, Beard, and Van Loon (1954) who at no time could obtain a recovery much greater than 60%. The considerable variation in recovery which may occur from batch to batch has not been reported.

The wide variation in results can be considerably reduced by using standard and optimal conditions but even under these conditions the recovery is not complete. Variation is more markedly reduced and recovery is complete if results are estimated from B₁₂ standards to which small amounts of serum have been added.

The presence of serum had an inhibitory effect on the growth of the *Euglena* and within limits this was independent of the amount of serum present (Figs. 2 and 3). Because of this inhibition the recovery of B₁₂ was poor when the growth in serum-containing solutions was compared to that in aqueous standards. The addition of trace amounts of serum to the aqueous standards corrected this by ensuring that growth in the standards was inhibited to the same degree as in the test solutions. In addition, the presence of serum in the standards caused the organism to react to small alterations in the assay conditions in a similar manner in both standard and test solutions. This reduced the variation in results from one batch to another, even when less than optimal conditions were used.

The depression of growth by serum was generally most obvious in assay batches in which the organism grew most prolifically and the aqueous standard curve was steepest (Table IV), and this fact was closely associated with the batch of medium used. It is interesting that Nicholas and Pitney (1958) also reported that their serum assay results were low when the aqueous standard curve was steepest.

However, in three observed instances the degree of inhibition was not related to the amount of growth. First, the presence of serum was less inhibitory if profuse growth was the result of a large inoculum. When a large inoculum was used, growth at very low concentrations of B₁₂ was in fact higher in the serum-containing standards than in the aqueous standards, so that recovery was greater than 100% (Fig. 8). Serum still inhibited growth at other concentrations of B₁₂ and recovery of B₁₂ added to serum was still usually incomplete if estimated from the upper portion of the aqueous curve. Secondly, there was markedly less growth in the serum-containing standards when poor growth was due to excessive light during incubation (Fig. 6) and recovery of B₁₂ added to serum was abnormally low when estimated from the aqueous curve. Thirdly, serum caused marked inhibition when poor growth was due to the use of an unwashed inoculum and recovery of B₁₂ added to serum was less than 60% when estimated from the aqueous curve (Table V). In all these three instances, recoveries estimated from serum curves were approximately 100%.

NATURE OF MATERIAL CAUSING INHIBITION OF GROWTH

The substance causing inhibition of growth of the *Euglena* was fully active in amounts as little as 0.001 ml. of serum. The nature of the substance is uncertain but it is probably protein, for a protein-free supernatant had only a slight inhibitory effect and the addition of albumin or globulin at a concentration even less than in 0.01 ml. serum, had an inhibitory effect similar to that of serum. The material apparently acts by interfering with the growth of the *Euglena*. How it does this is uncertain but it may act by lowering the surface tension of the aqueous fluid. The addition of serum to aqueous solutions minimizes clumping and sticking of the organisms. Tween 80, an agent known to lower surface tension which is sometimes added to prevent clumping of organisms, also had an inhibitory effect on the growth of the organism similar to that of serum.

EFFECT OF CYANIDE The addition of cyanide markedly improves the yield of B₁₂ in an extraction procedure (Spray, 1955; Killander, 1957a; Girdwood, 1960; Matthews, 1962), apparently by preventing the adsorption of B₁₂ onto the denatured protein.

(Matthews, 1962). Hydroxocobalamin is known to bind more strongly to proteins than cyanocobalamin (Bauriedel, Picken, and Underkofler, 1956; Skeggs, Hanus, McCauley, and Rizzo, 1960), and it is probable that cyanide, by converting any hydroxocobalamin present in serum to cyanocobalamin, prevents the adsorption and precipitation of the hydroxocobalamin onto the denatured protein during extraction, for it has been shown that whereas a large proportion of hydroxocobalamin added to denatured protein adheres and is not recoverable in the supernatant, only a small proportion of added cyanocobalamin is not recoverable in the supernatant (Anderson and Pope, unpublished observations).

The *Euglena* method does not involve an extraction procedure and the results of previous workers suggested that the addition of cyanide to the medium did not improve the levels of B₁₂ in the serum (Hutner *et al.* 1956; Shinton, 1959). This would suggest that B₁₂ in the presence of the denatured protein in the assay tube is available to the *Euglena*. However, in spite of the fact that the *Euglena* reacted equally to aqueous solutions of cyanocobalamin and hydroxocobalamin, the recovery of hydroxocobalamin added to serum was slightly lower than that of cyanocobalamin, and was slightly but significantly improved by the addition of KCN. Furthermore, in my experience although the addition of cyanide did not have the striking effect on the yield of B₁₂ that it has in an extraction procedure, it did usually cause a very slight improvement in the serum B₁₂ concentration. The improvement was too small to justify including cyanide routinely in the medium.

SERUM CONCENTRATIONS OF VITAMIN B₁₂ WITH THE EUGLENA ASSAY Relatively small differences in assay conditions, for example, differences in medium, size of inoculum, amount of light, and the addition of Tween 80 to the medium, have been shown to affect the serum B₁₂ values when estimated from the aqueous curve. It is not surprising, therefore, that there is a great variation in the normal ranges reported from different laboratories. Reported mean concentrations have varied from 212 to 640 $\mu\text{g./ml.}$ (Table I).

In addition there are other factors which contributed to this variation. Before 1955, when Kristensen introduced the use of a washed inoculum, most workers used an unwashed inoculum. This in itself would have led to falsely low serum values because under these conditions serum had a greater inhibitory effect on growth (Table V). But also it is probable that the use of two strains of *Euglena* employing two different media contributed to this wide range of values reported from different labora-

tories. In detailed comparisons of the two strains, using a washed or diluted inoculum, the serum values obtained with the *bacillaris* were reported to be markedly higher than with the *z* strain (Nicholas and Pitney, 1958; Cooper, 1959). It was suggested that the higher values by the *bacillaris* were due to the fact that serum itself provided a growth factor not present in the incomplete *bacillaris* medium (Nicholas and Pitney, 1958; Cooper, 1959). But this could also be explained by the fact that the inhibitory effect of serum was likely to be less obvious in the *bacillaris* method where growth was considerably less than in the *z* strain method.

CONCLUSION

Although some variation is inherent in all microbiological assay work, the variations in the *Euglena* method as previously used were particularly serious because of the unsuspected and variable inhibitory effect of whole serum, and because small changes in growth conditions produced unexpected different effects on growth in aqueous and serum solutions. These difficulties could be overcome if an extraction procedure were used, but this would remove a great advantage of the *Euglena* method, namely, that because no extraction procedure is involved large numbers of sera can easily be assayed. The studies in this paper indicate that the variations can be greatly reduced, partly by establishing constant and optimal assay conditions, and especially by using serum-containing standards for estimation of B₁₂ in serum, which also ensures full recovery of B₁₂ added to serum. It is of interest that the new normal range reported in this paper is similar to that reported by Spray and Witts (1958) and Matthews (1962) assayed by the *Lactobacillus leichmanii*.

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