

STUDIES WITH A MORE RAPID METHOD OF VITAMIN B₁₂ ASSAY UTILIZING *EUGLENA GRACILIS**

BY

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(RECEIVED FOR PUBLICATION JULY 7, 1958)

The use of microbiological assay techniques for the determination of vitamin B₁₂ concentrations in serum is well established. Various micro-organisms have been used for this purpose, each with specific advantages. The use of *Euglena gracilis* has become widespread since it was first introduced by Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin, and Jukes (1949). The reports of Mollin and Ross (1952), Ross (1952), Pitney and Beard (1953), Lear, Harris, Castle, and Fleming (1954), and others using this organism have established the ranges of vitamin B₁₂ concentration in the serum in the normal and in various pathological states, especially in pernicious anaemia, a disease characterized by a deficiency of vitamin B₁₂.

Hutner, Bach, and Ross (1956) reported on studies with a new strain of *E. gracilis*, the z strain. With it, assays were conducted in a new medium after five days of incubation at 25° to 30° C., instead of the seven days required by the older methods employing the *bacillaris* strain.

This new method has been compared with the older one in order to determine its reproducibility, and whether it represents a practical improvement in the assay of vitamin B₁₂ in serum.

Materials and Methods

The z strain of *E. gracilis*‡ was subcultured in the basal medium, as described by Hutner *et al.* (1956), with the addition of 0.2% tryptone§ and 50 µg. of cyanocobalamin per ml. The medium and organism for the *bacillaris* strain assay were as described by Mollin and Ross (1952). This strain was subcultured for maintenance in the basal medium with the addition of 150 µg. of cyanocobalamin per ml.

The preparation of the inoculum for both methods of assay was similar, but differed from the heavy,

washed inoculum recommended by Hutner. Instead, a four-day culture was diluted 10 to 15 times with double strength medium to a density corresponding to a reading of 50 to 60 on the Klett-Summerson photoelectric colorimeter. Each culture tube was then inoculated with 0.05 ml. of this inoculum using a 1.0 ml. serological pipette.

The assay was carried out as described by Lear *et al.* (1954). Serial halving dilutions to 1/128 were made of 2 ml. of serum in double strength medium. The final volume in each culture tube was 4 ml. Similar serial dilutions were made of a stock solution of cyanocobalamin containing 200 µg. of vitamin B₁₂ per ml. prepared from "cyanocobalamin" U.S.P. Both sets of dilutions, those of the sera to be assayed and those of the standard, were boiled for 15 minutes in a water-bath in order to free the bound vitamin. After cooling, they were inoculated with the test organisms as described above. One tube of medium was inoculated without added cyanocobalamin as a blank, control tube. The racks of culture tubes were placed on a glass shelf in an incubator illuminated from above and from below by white fluorescent lamps, and were moved about daily within the incubator in order to insure uniform lighting of all tubes. The temperature was maintained between 25° and 30° C.

The assays using the *bacillaris* strain were read after seven days of incubation, while those using the z strain were read after six days. The intensity of green colour in each tube was determined in a Klett-Summerson photoelectric colorimeter using a red filter ($\lambda=640-700$ m μ). The machine was adjusted to zero with the inoculated blank, control tube of each assay so as to read only the increase in colour above that of the inoculum.

With sterile, acid-washed syringes employed only for this purpose blood samples were taken from patients and allowed to clot in sterile, acid-washed, screw-capped tubes. The serum was transferred to similar sterile tubes and kept frozen until used. Except where specifically described, care was taken to exclude from the series described below any patients who had received vitamin B₁₂ therapy within two or three years.

*The expenses of this investigation were defrayed in part by the J. K. Lilly gift to the Harvard Medical School.

†Recipient of a National Research Fellowship in the Medical Sciences.

‡Obtained from the Culture Collection of Algae, Department of Botany, Indiana University.

§Obtained from Difco Laboratories, Detroit, Michigan.

||Obtained from the Board of Trustees, U.S. Pharmacopeial Convention, Inc.

Results

The average serum values obtained by one or the other method for hospital "normals," for patients with hepatic cirrhosis, and for patients with vitamin B₁₂ deficiency are compared in Table I. Equal amounts of samples of the serum of 27 of these patients were studied by both methods. The average values are shown in Table I, while the 27 individual serum values are plotted in Fig. 1. The hospital normals either had no clinical symptoms or signs of vitamin B₁₂ deficiency or did not respond later to vitamin B₁₂. The patients listed as having hepatic cirrhosis had both clinical and biochemical evidence of cirrhosis of the liver. Some of the hospital normals may also have had diseases of the liver, as no attempt was made to check all patients for hepatic dysfunction. Of the four patients who seemed to have borderline vitamin B₁₂ deficiency, one had

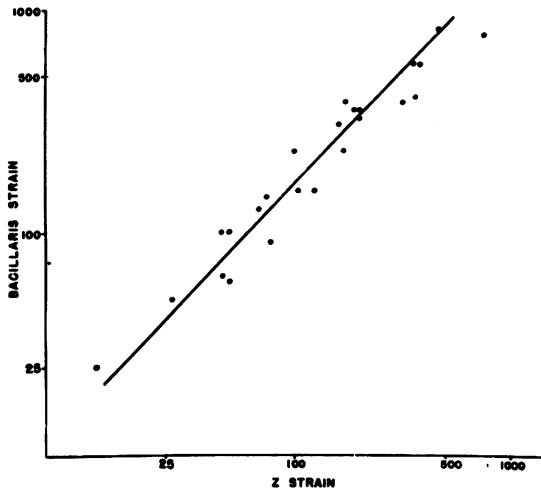


FIG. 1.—Relationship of vitamin B₁₂ values of 25 individual sera as obtained by assays using two different strains of *E. gracilis*. The vertical co-ordinate of each point represents the serum level according to the *bacillaris* strain assay; the horizontal co-ordinate is the value according to the *z* strain assay in $\mu\text{g. per ml.}$ Two additional points (not shown) extend the apparent trend beyond the limits of the figure with co-ordinates of 1,446–1,400 and 1,600–1,235 $\mu\text{g. per ml.}$, respectively.

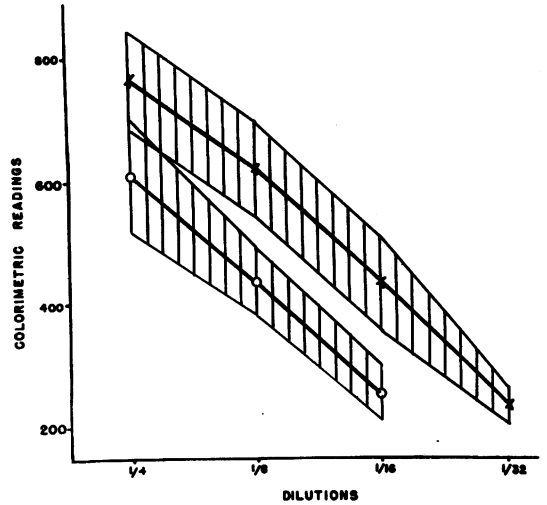


FIG. 2.—Comparison of average values of 20 standard curves from routine assays obtained with the *bacillaris* strain (O---O) and with the *z* strain (X---X) of *E. gracilis*, respectively. The cross-hatched areas represent ± 1 standard deviation. The vertical scale is for Klett-Summerson colorimeter readings; the horizontal scale indicates serial double dilutions of the vitamin B₁₂ standard containing 200 $\mu\text{g. per ml.}$

a delayed and another a suboptimal reticulocyte response to vitamin B₁₂ therapy. A third patient diagnosed as a case of pernicious anaemia had persistently low red cell and haemoglobin levels until given more than ordinarily adequate parenteral vitamin B₁₂ therapy. The fourth, diagnosed as early pernicious anaemia, was only slightly anaemic before therapy with vitamin B₁₂. The last two patients showed only occasional abnormal red cell precursors in the bone marrow. The patients considered to have manifest vitamin B₁₂ deficiency all had the blood and bone marrow changes and the response to parenteral vitamin B₁₂ administration characteristic of this deficiency.

As shown in Table I, the *z* strain of *E. gracilis* in the new medium gave values for serum vitamin B₁₂ which were consistently lower than those obtained with the *bacillaris* method. That the

TABLE I
COMPARATIVE RESULTS OF ASSAYS FOR SERUM VITAMIN B₁₂ BY BACILLARIS AND Z STRAINS OF *E. GRACILIS*

Type of Patient	Bacillaris Assay		Both Assays		Z Strain Assay		
	Number of Patients	Average B ₁₂ Level*	Number of Patients	Average B ₁₂ Levels		Number of Patients	Average B ₁₂ Level
				Bacillaris	Z Strain		
Hospital "normals" ..	32	499 ± 205	14	441 ± 176	282 ± 167	27	305 ± 229
Hepatic cirrhosis ..	4	1,840 ± 512	2	1,523 ± 77	1,318 ± 84	4	1,483 ± 224
B ₁₂ deficiency, borderline ..	4	144 ± 10	4	144 ± 10	93 ± 21	4	93 ± 21
" " manifest ..	22	61 ± 26	7	70 ± 26	43 ± 19	11	44 ± 15

* Average serum vitamin B₁₂ values in $\mu\text{g. per ml.}$ ± 1 standard deviation.

values obtained with the new method are proportional to those obtained with the old technique is shown in Fig. 1, in which the values obtained for equal samples of 27 sera assayed by both methods are plotted on log-log paper. A linear relationship is apparent.

The reason for this consistent difference in the results of the two methods becomes apparent when the vitamin B₁₂ standard dilution curves of the assays performed by each are compared (Fig. 2). Here, the new technique gave consistently higher values than did the *bacillaris* method. However, the growth curves for aliquots of serum samples showed relatively small differences. Thus, the presence of serum seems to enrich the medium of the *bacillaris* strain technique, and so gives more growth than does a similar concentration of vitamin B₁₂ without serum. This apparently explains the higher calculated serum vitamin B₁₂ concentrations obtained with the *bacillaris* method.

Effect of Different Methods of Inoculation.—Hutner *et al.* (1956) recommend the use of a heavy, washed inoculum for the assay with the new medium. That washing tends to accelerate the growth of *E. gracilis* has been shown by Østergaard Kristensen (1955). Consequently, two sets of standard curves were run with the *z* strain of *E. gracilis*, using the medium recommended for this strain. One set of serial dilutions and a blank, control tube were inoculated with a dilute, unwashed culture of the organism as described under "methods." The other set and its blank control tube were inoculated as recommended by Hutner *et al.*, i.e., an eight-day culture of organisms in 15 ml. was washed in fresh culture medium and resuspended in 10 ml. of fresh medium. One drop of this suspension was added to each culture tube. Both sets of dilutions were incubated together, and each was assayed in quadruplicate. After first adjusting the colorimeter to zero with the blank tube of each series, duplicate sets of each assay were read after five days and after six days of incubation, respectively. The average density values of these duplicate dilutions are shown in Fig. 3.

The heavy, washed inoculum produced a considerable degree of colour in the blank, control tubes, reading 80–90 when compared with water. The blank tubes of the assays using the light inoculum read only 0–10 when compared with water. As shown in Fig. 3, the heavy inoculum grew sufficiently in five days to provide a usable growth curve, and showed approximately the same growth (new green colour) as did the

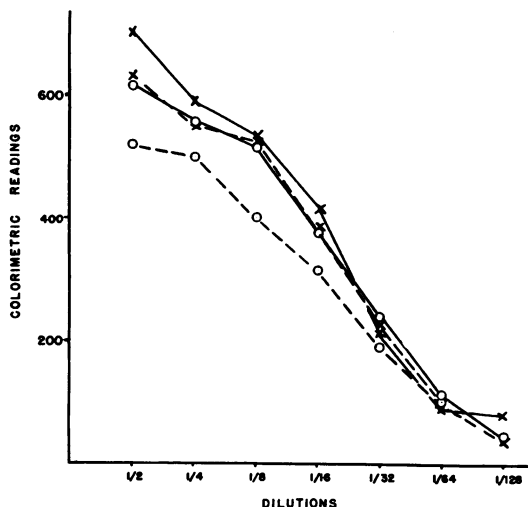


FIG. 3.—Comparison of average values of duplicate growth dilution curves of the *z* strain of *E. gracilis* using "light" (open dots) and "heavy" (crosses) inocula. The values were read after five days (dashed curves) and after six days (solid curves) of incubation, respectively. Vertical and horizontal scales are as in Fig. 2. Note that after six days the results with both light and heavy inocula are similar.

light inoculum after six days of incubation. Results similar to those obtained after six days with the light inoculum described above would thus be expected after five days with the heavy inoculum. This was found to be the case.

Effect of Lactic Acid.—One difficulty noted with the new assay was that the 1:4 dilutions of serum invariably coagulated during the boiling, and that the 1:8 dilutions often became cloudy during the period of growth of the organisms, giving erroneously high readings for these dilutions in the colorimeter. Dr. Hutner in a personal communication suggested that this might be due to alkalization of the medium as a result of heavy growth. At his recommendation, lactic acid was added to the medium to a concentration of 0.1%. Although the lactic acid did not alter the assay, it failed to prevent turbidity or coagulation.

Discussion

It is evident that the new method of assay using the *z* strain of *Euglena gracilis* and a new enriched medium will allow accurate determinations of vitamin B₁₂ levels in serum. It permits these determinations to be made in less time (five or six days) than is required for the assay employing the *bacillaris* strain of *E. gracilis* currently in use (seven or eight days). The range of calculated serum values obtained is consistently lower,

probably due to more growth in the absence of serum (standard curves) than was obtained with the medium of Mollin and Ross. These lower serum values tend to compress the range for vitamin B₁₂ deficiency, and to make more difficult the interpretation of results in the low normal range. This objection will probably become less important as more experience is obtained with the new method; and there is clearly a theoretical objection to the non-specific, growth-stimulating effect of serum in the older assay.

Summary

Comparative assays of serum for vitamin B₁₂ were carried out using the z strain of *Euglena gracilis* grown in a new enriched medium (Hutner), and also by the older method of Mollin and Ross

(1952), employing the *bacillaris* strain. The new method gives reproducible values proportional to, but lower than, those obtained with the older method. It shortens by one or two days the growth period required.

We are indebted to Mrs. Felicita Fradera for technical assistance.

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