The Effects of Common Salts on the *Euglena gracilis* Bioassay of Vitamin B_{12}[1] (36306)

ROGER E. BENSON
(Introduced by C. A. Hall)

Hematology Research Laboratory, Veterans Administration Hospital and the Department of Medicine, Albany Medical College of Union University, Albany, New York 12208

This laboratory determines serum vitamin B_{12} (B-12) levels by bioassay following the basic method of Anderson (1) using *Euglena gracilis*, Z strain. We adapted this assay to the measurement of the B-12 content of eluate from the fractionation of human serum by gel filtration. The protein bound B-12 in the fractions are first released by the papain digestion technique of Hall and Allen (2), in contrast to the heat extraction technique used to free B-12 when assaying whole serum.

The eluting buffer of the Sephadex G-200 contained 500 m*M* NaCl. Hutner *et al.* (3) found inhibition of growth of *E. gracilis* at concentrations of NaCl of 0.5% (86 m*M*) or greater, but not at 0.25% (43 m*M*). We, in contrast, observed apparent stimulation of growth of *E. gracilis* by concentrations of NaCl of 100 m*M* or less.

The standard bioassay of B-12 has been established principally for the study of serum. If *E. gracilis* is used for the assay of B-12 in solutions containing varying concentrations of ions, such as serum fractions, it is imperative to evaluate the effect of these added ions on the response of the assay organism. The present study is such an evaluation of the effects of NaCl and NaH_2PO_4 on the growth of *E. gracilis*, Z strain.

Procedure. Since Sephadex and DEAE-cellulose eluates containing released B-12 (2) were diluted threefold before the bioassay, the effects of this dilution of Sephadex and phosphate buffers on the growth of *E. gracilis* were investigated.

Assay tubes contained 1 ml of cyanocobalamin (12 pg/ml), (Sigma Chemicals, St. Louis, MO), 1 ml of 2× media (General Biochemicals, Chagrin Falls, OH), and 1 ml of the buffer diluted three times with H_2O. The original NaCl concentration of the Sephadex buffer was 500 m*M*. (The final NaCl concentration in the assay tubes was 41.7 m*M*.) Before dilution the concentration of the three phosphate buffers used were 40, 100, and 400 m*M*. (The final molarities in the assay tubes were 3.3, 8.3, and 33.3 m*M*, respectively.) The control tubes contained 1 ml of H_2O instead of the dilute buffer. Each buffer concentration and the control were repeated in six samples. The cotton-plugged tubes were heated to 100° in a boiling H_2O bath for 15 min; no other extraction procedure was used.

The inoculum was prepared as described earlier (2). The inoculated assay tubes were placed in a constant temperature incubator at 28.5° for 4 days. The illumination was provided by three "warm white" fluorescent strip lights regulated to provide between 25 and 50 ft c. The optical density (OD) of the four-day growth was measured in a Bausch and Lomb Spectronic 20, spectrophotometer at 450 mμ. The OD's of the tubes with identical contents were in good agreement.

While both buffers contained several salts, sodium chloride was the primary ingredient of the Sephadex buffer and sodium phosphate was the principal salt in the DEAE-cellulose buffers. Therefore the effects of NaCl and NaH_2PO_4 on *E. gracilis* growth was investigated. Assay tubes were placed in 20 groups, with 6 tubes in each group, 2 ml of cyanocobalamin (6 pg/ml) and 1 ml of 2 × assay medium were added to every tube. The media added to each group of tubes con-

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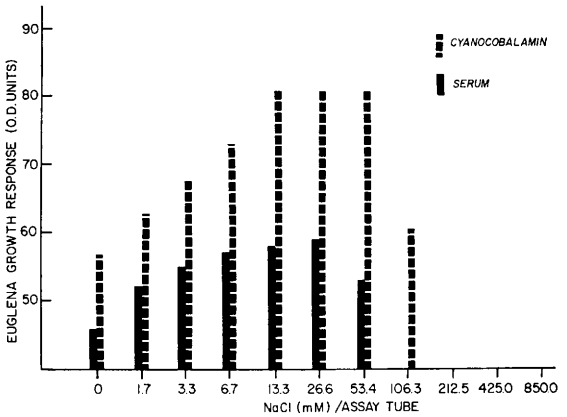

FIG. 1. The optical densities of *E. gracilis* growth in tubes containing constant amounts of cyanocobalamin or serum. The variable is the added concentration of NaCl. Growth responses less than the control are not represented.

tained a different added concentration of NaCl or NaH_2PO_4. The added concentrations of NaCl were 3400.0, 1700.0, 850.0, 425.0, 213.5, 106.3, 53.1, 26.6, 13.3, and 6.6 m*M*. The added concentrations of NaH_2PO_4 were 2000.0, 1000.0, 500.0, 250.0, 125.0, 62.5, 31.3, 15.6, and 7.8 m*M*. The final salt concentration in the assay tubes was one-fourth of the added molarity since the one ml of the salt supplemented media was diluted to 4 ml by the other contents of the tubes.

In a third experiment, 2 ml of 1:80 serum dilution was substituted for cyanocobalamin. Tubes with H_2O instead of a B-12 source were also prepared with the varying salt concentrations. The tubes were then inoculated and incubated as above.

A 10-day growth curve of *E. gracilis* when cultured with standard media and added salts was also prepared. Each tube contained 1 ml of cyanocobalamin (10 pg/ml), 1 ml of 1:3 dilute Sephadex buffer and the standard media and inoculum. A control growth curve with no added salts was also prepared substituting H_2O for the dilute buffer. The experimental and control tubes were prepared in triplicate and the OD determined on days 2, 4, 6, 8, and 10 after the beginning of incubation.

In a final study, eight normal sera were diluted 1:25 with H_2O. One milliliter of the dilute serum was assayed in quadruplicate to determine the B-12 level by comparison with cyanocobalamin standards (1). Heat was used to free the B-12 from serum. An identical assay was repeated but an ion concentration similar to 1 ml of 1:3 dilute Sephadex buffer was also added to both the tubes containing the dilute serum and cyanocobalamin standards. Both serum assays contained the same inoculum and were incubated together.

Results. The 1:3 dilution of the buffers increased the growth response over the control in all four cases. The average OD of the six control tubes was 69, while the average OD of the tubes containing the dilute Sephadex buffer was 98. The tubes containing the diluted 40, 100 and 400 m*M* phosphate buffers gave average OD of 83, 82, and 91, respectively.

The *E. gracilis* growth response in tubes with NaCl added was greater than the salt-free control, between 1.7 and 53.4 m*M* for serum B-12 and greater between 1.7 and 106.3 m*M* for cyanocobalamin (Fig. 1). Likewise, NaH_2PO_4 promoted greater growth than the control from 2.0 to 62.5 m*M* for both sources of B-12. Those tubes which contained no B-12 with and without added salts gave similar OD. Concentrations of and greater than 212.5 m*M* NaCl and 125.0 m*M* NaH_2PO_4 inhibited or eliminated growth of the test organism in all cases.

During the 10-day growth period the OD of the *E. gracilis* cultures containing the added salts were higher than the controls at

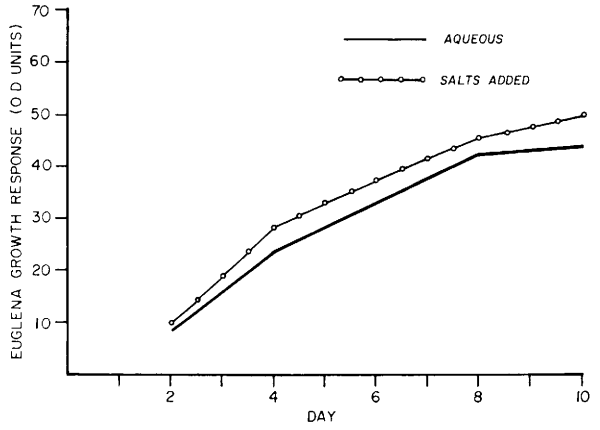

FIG. 2. The comparison over a 10 day period of two growth response curves of *E. gracilis* to 2.5 pg/ml of cyanocobalamin. One set contained low concentrations of added salts while the second did not.

every observation (Fig. 2). The experimental and control cultures also showed an increase in OD throughout the observation period.

When the ions were added to the dilute serum, the assayed B-12 levels were increased by a factor of approximately 2.5 when compared to the aqueous cyanocobalamin standard. If the standard with the added ions was used as a reference (Fig. 3), the serum B-12 levels were approximately the same as the aqueous serum B-12 levels compared to the aqueous standard. Table I shows the average B-12 levels of the sera with ions added determined using the aqueous standard and the ion supplemented standard. The average B-12 level of the sera when assayed by the established procedure (1) is also given in the bottom row.

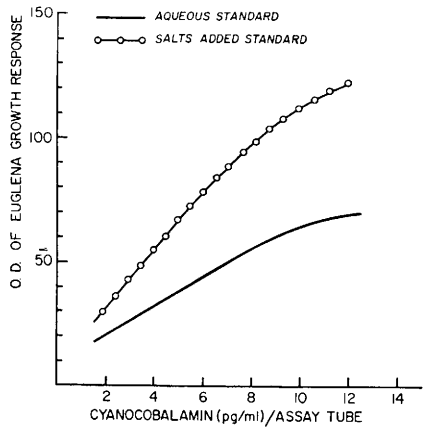

FIG. 3. The comparison of two growth response curves of *E. gracilis* to cyanocobalamin. One set contained low concentrations of added salts while the second did not.

TABLE I. Comparison of the Average B-12 Levels of Eight Ion Supplemented Sera Determined Using Aqueous and Ion Supplemented Standards.

Serum preparation	Standard preparation	Serum B_{12} (pg/ml)
Ions added	Aqueous	1645
Ions added	Ions added	705
Aqueous	Aqueous	637

Discussion. The promotion or inhibition of *E. gracilis* growth by varying concentrations of NaCl and NaH_2PO_4 illustrates the sensitivity of this assay organism to concentrations of ions in the growth medium. The use of this bioassay to evaluate B-12 levels in tissues and serum fractions is therefore complicated by the fact that salt solutions in varying concentrations are often used in the processing of such samples. Depending on the final concentration of the added ions in the fraction, the determined B-12 levels may be either erroneously high or low.

NaCl and NaH_2PO_4 will not replace the B-12 requirement of *E. gracilis*, as there was no increased growth response with the addi-

tion of the salts unless there was a source of B-12 available.

The ten day growth curve of *E. gracilis* cultures exposed to added salts demonstrated that the increased growth response began as early as the second day and continued to at least the tenth day after inoculation. If the growth curves had intersected, it would have been possible to determine the B-12 levels of the ion containing samples by comparison to an aqueous standard at the day of intersection. This would have been the time when the growth response in aqueous and ion supplemented media was the same for equal concentrations of B-12.

The fact that salts affected *E. gracilis* growth when either serum or cyanocobalamin was the B-12 source led to an attempt to accurately determine the B-12 when salts were present. Serum was used as a B-12 source in these studies, but a change of serum B-12 assay technique is not suggested since salt levels in diluted serum are probably too low to affect *E. gracilis* growth.

It appeared that the addition of ions to the standards of growth in amounts equal to the concentration in the unknown corrected for the effects of the ions. A similar approach is also being used for determination of B-12 content of serum fractions; the ions used in the fractionation procedure were also added to the cyanocobalamin standards. Since an attempt to remove the added ions from fractions by dialysis against H_2O often led to precipitation of serum proteins, this technique was discarded.

No attempt was made in these experiments to determine the mechanism responsible for the action of these salts on growth of *E. gracilis*, although evidence of increased growth over a period of 10 days was demonstrated (Fig. 2). Rather this study was undertaken to demonstrate the need for evaluation of the effect of ions used in the fractionation of serum proteins on B-12 levels determined by *E. gracilis* bioassay.

Summary. Euglena gracilis cultures were grown in standard media with the addition of varying concentrations of NaCl and NaH_2PO_4. The growth response was greatest between 1.7 and 106.3 m*M* NaCl and 2.0 and 62.5 m*M* NaH_2PO_4. The growth potentiation was only observed in the presence of cyanocobalamin. Concentrations of and greater than 212.5 m*M* NaCl and 125.0 m*M* NaH_2PO_4 inhibited or eliminated growth of the test organism. Since it often is desirable to determine the vitamin B-12 level by *E. gracilis* bioassay of chromatographed serum fractions, the assayed B-12 levels may be either erroneously high or low because of the ions present in the eluting buffers. It appeared that addition of ions to the standards of growth in amounts equal to the concentration in the unknown corrected for the effect of the ions.

1. Anderson, B. B., J. Clin. Pathol. 17, 14 (1964).
2. Hall, C. A., and Allen, E. S., J. Lab. Clin. Med. 63, 517 (1964).
3. Hutner, S. H., Bach, M. K., and Ross, G. L., J. Protozool. 3, 10 (1956).

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