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⟨171⟩ VITAMIN B_{12} ACTIVITY ASSAY

ASSAY

• **Procedure**

Assay preparation: Place a suitable quantity of the material to be assayed, previously reduced to a fine powder if necessary and accurately measured or weighed, in an appropriate vessel containing, for each g or mL of material taken, 25 mL of an aqueous extracting solution prepared just prior to use to contain, in each 100 mL, 1.29 g of disodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 minutes. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water so that the final test solution contains vitamin B_{12} activity approximately equivalent to that of the *Standard cyanocobalamin solution* which is added to the assay tubes.

Standard cyanocobalamin stock solution: To a suitable quantity of USP Cyanocobalamin (Crystalline) RS, accurately weighed, add sufficient 25 percent alcohol to make a solution having a known concentration of 1.0 µg of cyanocobalamin per mL. Store in a refrigerator.

Standard cyanocobalamin solution: Dilute a suitable volume of *Standard cyanocobalamin stock solution* with water to a measured volume such that after the incubation period as described for *Procedure*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard cyanocobalamin solution* is not less than that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 ng and 0.04 ng per mL of *Standard cyanocobalamin solution.* Prepare a fresh standard solution for each assay.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed, carefully dissolving the cystine and tryptophane in the hydrochloric acid before adding the next eight solutions in the resulting solution. Add 100 mL of water, mix, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary, add the polysorbate 80 solution, adjust the solution to a pH between 5.5 and 6.0 with 1 N sodium hydroxide, and add purified water to make 250 mL.

L-Cystine	0.1 g
L-Tryptophane	0.05 g
1 *N* Hydrochloric Acid	10 mL
Adenine–Guanine–Uracil Solution	5 mL
Xanthine Solution	5 mL
Vitamin Solution I	10 mL
Vitamin Solution II	10 mL
Salt Solution A	5 mL
Salt Solution B	5 mL
Asparagine Solution	5 mL
Acid-hydrolyzed Casein Solution	25 mL
Dextrose, Anhydrous	10 g
Sodium Acetate, Anhydrous	5 g
Ascorbic Acid	1 g

Polysorbate 80 Solution	5 mL

Acid-hydrolyzed casein solution: Prepare as directed under *Calcium Pantothenate Assay* ⟨91⟩.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed under *Calcium Pantothenate Assay* ⟨91⟩.

Xanthine solution: Suspend 0.20 g of xanthine in 30 mL to 40 mL of water, heat to about 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL. Add 2 drops of hydrochloric acid, and store under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid, and store under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution I: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N glacial acetic acid to make 400 mL. Store, protected from light, under toluene in a refrigerator.

Vitamin solution II: Dissolve 20 mg of para-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in dilute neutralized alcohol (1 in 4) to make 400 mL. Store, protected from light, in a refrigerator.

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend about 5 g per L of analytical filter-aid in the supernatant, and filter, with the aid of reduced pressure, through a layer of the filter-aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of water-soluble yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of potassium biphosphate in 60 mL to 70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution.* Adjust the solution with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 minutes. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed above for the *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0 g to 1.5 g of agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Place approximately 10-mL portions of the hot solution in test tubes, cover the tubes suitably, sterilize at 121° for 15 minutes in an autoclave (exhaust line temperature), and allow the tubes to cool in an upright position. Inoculate three or more of the tubes, by stab transfer of a pure culture of *Lactobacillus leichmannii*.* (Before first using a fresh culture in this assay, make not fewer than 10 successive transfers of the culture in a 2-week period.) Incubate 16 to 24 hours at any selected temperature between 30° and 40° but held constant to within ±0.5°, and finally store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the inoculum if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid inoculum can be observed 2 to 4 hours after inoculation. A slow-growing culture seldom gives a suitable response curve, and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an inoculum comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus leichmannii* to 2 sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16 to 24 hours at any selected temperature between 30° and 40° but held constant to within ±0.5°. Under aseptic conditions, centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1 in 20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1 in 400 dilution of the adjusted suspension using *Basal medium stock solution*, and use it for the test inoculum. (This dilution may be altered, when necessary, to obtain the desired test response.)

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Procedure: Cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 hours, hard-glass test tubes, about 20 mm × 150 mm in size, and other necessary glassware because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents.

To test tubes add, in duplicate, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, 4.0 mL, and 5.0 mL, respectively, of the *Standard cyanocobalamin solution.* To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and water to make 10 mL.

To similar test tubes add, in duplicate, respectively, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, and 4.0 mL of the *Assay preparation.* To each tube add 5.0 mL of *Basal medium stock solution* and water to make 10 mL. Place one complete set of standard and assay tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes suitably to prevent bacterial contamination, and sterilize the tubes and contents in an autoclave at 121° for 5 minutes, arranging to reach this temperature in not more than 10 minutes by preheating the autoclave, if necessary. Cool as rapidly as practicable to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling

conditions throughout the assay, since packing tubes too closely in the autoclave, or overloading it, may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard cyanocobalamin solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40° held constant to within ±0.5°, for 16 to 24 hours.

Terminate growth by heating to a temperature not lower than 80° for 5 minutes. Cool to room temperature. After agitating its contents, place the container in a spectrophotometer that has been set at a wavelength of 530 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the standard, calculate the response from the sum of the duplicate values of the transmittances (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard cyanocobalamin solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y, adding together the two transmittances for each level of the *Assay preparation.* Read from the standard curve the logarithm of the volume of the *Standard preparation* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Assay preparation* to obtain the difference, x, for each dosage level. Average the values of x for each of three or more dosage levels to obtain $\bar{x} = M'$, the log-relative potency of the *Assay preparation.* Determine the quantity, in µg, of USP Cyanocobalamin (Crystalline) RS corresponding to the cyanocobalamin in the portion of material taken for assay by the equation:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of cyanocobalamin that was assumed to be present in each mg (or capsule or tablet) of the material taken for assay

Replication: Repeat the entire determination at least once, using separately prepared *Assay preparations.* If the difference between the two log potencies M is not greater than 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Vitamin B_{12} Activity Assay* under *Design and Analysis of Biological Assays* ⟨111⟩). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of *M* that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

ADDITIONAL REQUIREMENTS

- **USP Reference Standards** ⟨11⟩
  USP Cyanocobalamin (Crystalline) RS

* Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110.

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

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