QMFCIAF REPORT NO. 19-62

MODIFIED MICROBIOLOGICAL PROCEDURES FOR VITAMIN ASSAYS

A MANUAL

Interim Report

July 1962

QUARTERMASTER FOOD AND CONTAINER INSTITUTE FOR THE ARMED FORCES
QUARTERMASTER RESEARCH AND ENGINEERING COMMAND, U.S. ARMY
CHICAGO 9, ILLINOIS

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by

Miriam H. Thomas

Nutrition Branch, Food Division

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CONTENTS

PREFACE

The purpose of this publication is to set forth the microbiological procedures for vitamin assays as they have been modified and are being employed by the Nutrition Branch. It is intended as a manual for the analyst. While many excellent procedures for vitamin methods have been published, there is also a wealth of unpublished information concerning modifications which extend the application of the methods to specific products, or to use with certain instruments, or to special conditions other than those for which the method was originally devised. Such modifications, when available, greatly influence the usefulness of the methods.

This manual includes assays for biotin, folic acid, pantothenic acid, niacin, and riboflavin. The basis for the procedures presented herein is Methods of Vitamin Assay, Assn. Vit. Chem., 2nd Ed. Interscience Publishers, Inc., 1951.

Appreciation is expressed to Mrs. Belle Rosler and Mr. Lawrence Wills for their assistance in compiling these procedures.

NOTE: Commercial products are named in this manual for identification only and do not imply indorsement of the particular products. Equivalent substitutes may be used.

GENERAL CONSIDERATIONS

Principle

Microbiological methods are based on observations that certain microorganisms require specific vitamins for growth. Growth responses of the organisms are compared quantitatively in standard and unknown solutions using a basal medium complete except for the vitamin under test. To determine the extent of growth and thereby the amount of vitamin in the test solution, the acid or the turbidity produced by the organism is measured.

There are certain time relations which exist in microbiological procedures. Active stabs of the stock culture, from one to four days old, must be available for the preparation of the inoculum. Whenever the stock culture has not been used for several weeks or months, obtain new cultures or revive the old ones by making daily successive stab transfers for at least three days before preparing the inoculum. Poor growth results from old cultures. Pure cultures can be obtained from the American Type Culture Collection, 2029 M Street, N. W., Washington 6, D.C.

The inoculum must be started 16 to 24 hours before it is used in the assay. Inoculum incubated longer than 24 hours should not be used as poor growth responses are encountered. In an emergency, an inoculum can be prepared in eight hours by adding a drop of active inoculum or incubated test sample medium not more than 24 hours old to a tube of inoculum medium and incubating at the usual temperature. This procedure

should not be repeated without returning to the stock stab culture. Also, in an emergency, the inoculum may be refrigerated for 24 hours before use. An old inoculum should never be used to prepare a new inoculum. This practice may alter the nutritive requirements of the test organisms making it unsuitable for the assay.

The time of inoculation of assay tubes should be chosen so that the titration can be carried out three days later. At the time of inoculation, all tubes must be the same temperature all the way through the assay, though small differences in temperature at the start of the assay will influence the rate of growth more than the extent of growth over a long period of time. The time of incubation can be extended by as much as 18 hours and shortened by as much as 12 hours, without appreciably affecting the results of the assay. After incubation the tubes may be refrigerated overnight and titrated the next day. The samples, however, may be extracted and the resulting test solution stored in the freezer indefinitely, provided contamination and evaporation are prevented.

Reagents

Many stock solutions and reagents are used in all the procedures presented here. They may be preserved at refrigerator temperature in dark bottles with toluene or toluene and chloroform. All chemicals should meet American Chemical Society specifications or be of reagent grade unless otherwise indicated. Distilled water is used in making all solutions unless otherwise specified.

Sampling

Although sampling technics vary in individual cases, in general, they may be classified according to the type of material to be assayed. As a rule, all samples should be mixed thoroughly immediately prior to sampling. It must be remembered, however, that certain materials are easily oxidized and aeration in the mixing is to be avoided.

For dry materials, the Wiley mill, burr mill, hammer mill, mortar and pestle, or even a rolling pin can be used to reduce the particle size sufficiently to permit ready mixing. Wet materials may be minced with a knife, ground through a food chopper, or blended in a Waring blendor. During such operations, changes in moisture must be considered and prevented. Losses of juices may change not only the vitamin content but vitamin concentration.

After comminution, the primary sample must be thoroughly mixed and stored under conditions most conducive to retention of the vitamins. Assays should be conducted as promptly as possible, but if delays are necessary, samples usually may be preserved by freezing. If the secondary sample is much larger than the amount required in the actual assay, then the entire sample or a portion of it is comminuted in a Waring blendor with liquid so that a uniform slurry will result. The weight of sample and liquid is required so that the composition of the slurry bears a known relation to that of the material sampled.

The size of the primary sample is controlled by practical considerations as well as the size of the lot of material which the sample is to represent.

A. Fruits and Vegetables

1. Raw

a. Take approximately 30 percent of the total raw sample from different parts of the whole being sure to get a representative sample. Blanch sample by steaming three to five minutes.

b. Mince, cut fine, or put through meat chopper and blend 200 gm. with 200 gm. of water (or extracting solution) to puree consistency.

or

Freeze-blanched sample. Mince frozen sample with food chopper in the presence of dry ice or in a cold room using a wooden pestle to crunch iced particles. Mix, weigh 200 gm. and blend with 200 gm. of water.

c. Get pH, proximate, moisture, etc. analyses.

2. Cooked or Canned

a. Drain on #20 stainless steel sieve for three minutes.

b. Weigh cooked or canned sample and drained liquid separately. If an evaluation of the total can content is required, the sample analyzed must represent the same proportion of solid and liquid as present in the can.

c. Proceed as directed under A. 1. b and c.

d. Assay liquid for water soluble vitamins, if required.

3. Frozen

a. Proceed as directed for frozen samples under A. 1. b and c.

4. Dehydrated

a. Thoroughly mix and reduce sample size to degree of fineness that homogeneity is approached without changes in moisture taking place.

b. Weigh sample directly for analysis or preferably, after reconstitution by blending with four volumes of water.

B. Meats

1. Fresh, Frozen, and Cured

a. Grind the entire quantity through a meat grinder or chopper and mix the ground meat thoroughly.

b. Blend 100 gm of ground meat with 200 gm. of water or extractant such as 0.1N H_2SO_4 to make a slurry.

c. Withdraw sample with a piece of glass tubing while blendor is running, making several successive withdrawals rather than one for the whole portion.

2. Canned

a. Grind the entire contents of the can.

b. Blend a representative portion with two volumes of water or extractant to make a slurry.

c. Withdraw sample as described in B. 1. c.

3. Dehydrated

See A. 4.

C. Tissues

1. Organs

a. Weigh tissue accurately and grind with suitable apparatus (mortar and pestle, homogenizer, Waring blendor, etc.) using precaution not to introduce increased amounts of oxygen. A slurry may be prepared with a suitable extractant, chosen on the basis of the analyses to be made.

b. Filter or centrifuge and decant.

c. If sample is not to be analyzed immediately place in closed container which will prevent evaporation and store in deep-freeze.

2. Urine

a. Collect and refrigerate urine recording the period of time over which the sample was collected as well as the volume collected.

b. Add sufficient oxalic acid to bring the specimen to pH 3.0 to 5.0 (about 100 mg.) in order to stabilize the riboflavin, and N'-methylnicotinamide.

c. Analyze specimen for ascorbic acid immediately. The remaining vitamins should be determined within 24 hours, or an aliquot transferred into a small bottle for freezing.

3. Feces

a. Collect sample in large wide mouth jar and store in refrigerator (up to three days). Record number of collection days.

b. Add approximately 1500 ml. of water, 1 ml. of caprylic alcohol, and shake vigorously.

c. Completely homogenize sample in Waring blendor and make up to 2000 ml.

d. Transfer to brown bottles and freeze until used for analyses.

Equipment

The following equipment is used in all microbiological assays:

Incubator or Water Bath 30° to 37° ± 0.5°C.

Autoclave

Lipless Pyrex Tubes, 15 x 150 mm. to 25 x 200 mm.

Racks for tubes with 60 to 120 tube capacity

Cotton plugs, metal caps, and metal covers with edges 25 mm. deep and of sufficient area to cover two rows of tubes

Inoculating needle and loop

Hypodermic syringe and needle

Refrigerator

Centrifuge

Sterilizing can for pipets

pH Meter

Filter rack and support

Filter paper, quantitative grade

Cannon Automatic Dispenser-Titrator

Additional glassware, i.e., pipets, volumetric flasks, beakers, etc., is required. To prepare reagents, bottles, beakers, pipets, and the usual laboratory equipment are also needed

MICROBIOLOGICAL ASSAY FOR BIOTIN

Plantarum

(Lactobacillus arabinosus 17-5)

A. Reagents

1. Bacto Biotin Assay Medium, dehydrated. Commercial preparation of Difco Laboratories (B 419).

To rehydrate the medium, suspend 75 gm. in 1000 ml. of water and heat to boiling for two to three minutes. Keep the slight precipitate evenly distributed by shaking when dispensing.

2. Bacto Micro Assay Culture Agar, dehydrated. Commercial preparation of Difco Laboratoroes (B 319).

To rehydrate the agar, suspend 47 gm. in 1000 ml. of cold water and heat to boiling to dissolve the agar completely. Distribute in 10 ml. quantities in test tubes (16-20 mm. diameter) and plug. Sterilize in autoclave for 15 minutes at 15 pounds pressure. Cool tubes in upright position and store in refrigerator.

3. Culture Medium for Growing Inoculum.* Prepare by dissolving 7.5 gm. of Bacto Niacin Assay Medium (B 322) in water, adding 0.2 ml. of niacin stock solution and diluting to 200 ml. with water. Distribute in 10 ml. quantities in test tubes (16-20mm. diameter) and plug. Sterilize in autoclave for 15 minutes at 15 pounds pressure. Cool and store in refrigerator.

* Good growth unobtainable in Bacto Micro Inoculum Broth.

4. Isotonic Salt Solution. Weigh 0.9 gm. of NaCl and transfer to a 100 ml. volumetric flask. Dilute to volume with water and shake until salt has dissolved. Transfer 10 ml. quantities of this solution to culture tubes and 25 ml. quantities to 125 ml. Erlenmeyer flasks, plug with cotton and sterilize in the autoclave at 15 pounds pressure for 15 minutes. Store in refrigerator.

5. 0.1N Sodium Hydroxide. Since NaOH pellets often contain considerable water, amounts in excess (one to 15 percent depending upon water content of pellets) of the calculated NaOH may be used. To prepare one liter of 0.1N NaOH, 4.0 gram of NaOH are needed. It need not be exactly 0.1N, but it is well to have standardized solution so that results between laboratories can be compared.

6. 4 N Sulfuric Acid. To prepare one liter of 4 N H_2SO_4 add 112 ml. of concentrated H_2SO_4 slowly and with stirring to about 800 ml. of water. After cooling, make to one liter with water.

7. Standard Stock Solution of Biotin. Weigh 25 mg. of anhydrous d-biotin (free acid) and dilute to 500 ml. with 50 percent ethyl alcohol. Store in refrigerator in a dark, glass stoppered bottle. (50 mcg./ml.).

8. Working Biotin Standard Solution. Dilute five ml. of stock solution to 250 ml. with 50 percent ethyl alcohol. Dilute five ml. of this intermediate solution to 500 ml. with 50 percent ethyl alcohol. Dilute five ml. of this solution to 250 ml. with water (0.2 mmcg./ml.). Prepare on the day of use.

9. Microorganism. Plantarum (Lactobacillus Arabinosus 17-5)

B. Procedure

1. Preparation of Stab Culture. Prepare stab cultures in two or more agar stock culture tubes. Incubate for approximately 16 hours at 37°C. ± 0.5°C. Store in refrigerator under aseptic conditions not longer than two weeks, preferably one week, before transferring to a new stab. Reserve one stab culture unopened for use in the preparation of stock culture stabs. It is essential that three or four successive daily transfers are made before the organism is used for growing inoculum.

2. Preparation of Inoculum. On the day prior to use, transfer cells from the stab culture to a sterile tube of inoculum culture medium. (Do not use an old inoculum for preparing a new inoculum). Incubate the culture for approximately 16 hours at 37°C. Secure cotton plug and centrifuge. Decant the supernatant liquid and resuspend the cells in 10 ml. of isotonic salt solution. Centrifuge and repeat process once more. After two washings resuspend cells in 10 ml. of isotonic solution, dilute one to 10, and use at once.

3. Preparation of Samples. Weigh one gram of sample into a 125 ml. Erlenmeyer flask, add 25 ml. of 4N H_2SO_4, cover, and autoclave for one hour at 15 pounds pressure. Cool, adjust to pH 7.0, make up to 100 ml. with water, and filter. In the presence of large amounts of carbohydrate it is necessary to adjust samples to pH 4.0 with $Ba(OH)_2 \cdot 8 H_2O$, make up to 100 ml., and filter before making the pH adjustment to 7.0 with NaOH. Make suitable dilution so that the final dilution contains approximately 0.1 to 0.2 mmcg./ml. of biotin. A standard sample of dried yeast should be analyzed with each assay. (1 gm. to 200 ml.; 1 or 3 ml. to 100 ml.).

4. Preparation of Standard Tubes. To duplicate tubes add 0.0 to 5.0 ml. of working standard biotin solution in 0.5 ml. increments. Add sufficient water to bring the volume to 5.0 ml. in each tube. To each tube add 5.0 ml. of biotin assay basal medium.

5. Preparation of Assay Tubes. To duplicate tubes add 2.0, 3.0, and 5.0 ml. aliquots of the test solution. Add sufficient water to bring the volume in each tube to 5.0 ml. To each tube add five ml. of biotin assay medium.

6. Sterilization. Mix the contents of each tube thoroughly, cover, and autoclave at 15 pounds pressure for 15 minutes.

7. Inoculation and Incubation. Cool tubes to below incubation temperature, aseptically inoculate each tube with one drop of dilute inoculum and incubate at 37°C. for 72 hours.

8. Titration. Transfer the contents of each tube into a series of 100 ml. beakers, rinsing the tube several times with small amounts of water and adding the washings to the beaker. With the aid of a pH meter, titrate to pH 7.0 with 0.1N NaOH.

9. Calculations.

a. Draw a standard curve for the assay by plotting ml. of 0.1N NaOH used in titrating the standard tubes against mmcg. of biotin per tube in the standard series.

b. Determine the vitamin content of the tubes in the unknown series by interpolation of the titer values on the standard curve. Discard any values which show more than one mmcg. or less than 0.1 mmcg. of biotin per tube.

c. Calculate the vitamin content of each ml. of test solution for each of the duplicate sets of tubes.

d. Calculate the vitamin content of the test material from the average of the values for one ml. of test solution, obtained from tubes which do not vary by more than 10 percent from the average, using the following formula:

$$\left(\frac{\text{average mmcg, per ml.}}{\text{wt. of sample}}\right)(\text{volume})(\text{dilution factor}) = \text{mmcg. per gm.}$$

MICROBIOLOGICAL ASSAY FOR FOLIC ACID

Streptococcus faecalis

A. Reagents

1. Bacto Folic Acid Assay Medium, dehydrated. Commercial preparation of Difco Laboratories (B 318).

To rehydrate the medium, suspend 75 gm. in 1000 ml. of water and heat to boiling for two to three minutes. Cool. Keep the slight precipitate evenly distributed by shaking when dispensing.

2. Bacto Micro Assay Culture Agar, dehydrated. Commercial preparation of Difco Laboratories (B 319).

To rehydrate the agar, suspend 47 gm. in 1000 ml. of cold water and heat to boiling to dissolve the agar completely. Distribute in 10 ml. quantities in test tubes (16 to 20 mm. diameter) and plug. Sterilize in autoclave for 15 minutes at 15 pounds pressure. Cool tubes in upright position and store in refrigerator.

3. Bacto Micro Inoculum Broth, dehydrated. Commercial preparation of Difco Laboratories (B 320).

To rehydrate the broth, dissolve 37 gm. in 1000 ml. of water. Distribute in 10 ml. quantities in test tubes (16 to 20 mm. diameter) and plug. Sterilize in autoclave for 15 minutes at 15 pounds pressure. Cool and store in refrigerator.

4. Bacto Chicken Pancreas, dehydrated. Commercial preparation of Difco Laboratories (B 459).

This reagent is standardized desiccated chicken pancreas used in the enzymatic liberation of folic acid from its conjugated state. Enzyme preparations may contain sufficient amounts of folic

acid to necessitate correction of the calculated value. This correction is accomplished by assaying the enzyme (20 mg.) in the same manner as a sample. The amount of folic acid contained in the enzyme added to the sample divided by the weight of the sample gives the correction factor which must be subtracted from the value obtained for a gram of sample.

5. M/5 Phosphate Buffer. Dissolve 2,723 gm. of KH_2PO_4 and 0.560 gm. of NaOH in water and dilute to 100 ml.

6. Isotonic Salt Solution. Weight 0.9 gm. of NaCl and transfer to a 100 ml. volumetric flask. Dilute to volume with water and shake until salt has dissolved. Transfer 10 ml. quantitites of this solution to culture tubes and 25 ml. quantities to 125 ml. Erlenmeyer flasks, plug with cotton and sterilize in the autoclave at 15 pounds pressure for 15 minutes. Store in refrigerator.

7. 0.1N Sodium Hydroxide. Since NaOH pellets often contain considerable water, amounts in excess (one to 15 percent depending upon water content of pellets) of the calculated NaOH may be used. To prepare one liter of 0.1N NaOH, 4.0 gm. of NaOH are needed. It need not be exactly 0.1N, but it is well to have standardized solution so that results between laboratories can be compared.

8. Standard Stock Solution of Folic Acid. Dissolve 100 mg. of folic acid in 0.01N NaOH in 20 percent ethanol and make up to one liter. Cover the solution with toluene before storing in a dark glass bottle with ground glass stopper in the refrigerator. (100 mcg./ml.).

9. Working Folic Acid Standard Solution. Dilute one ml. of stock solution to one liter. Dilute this intermediate solution to concentration of 0.2 to 0.8 mmcg.ml. (based on the concentration of folic acid in the sample). Prepare on the day of use.

10. Microorganism. Stretococcus faecalis (ATCC No. 8043).

B. Procedure

1. Preparation of Stab Culture. Prepare stab cultures in two or more agar stock culture tubes. Incubate for approximately 16 hours at 37°C ± 0.5°C. Store in refrigerator under aseptic conditions not longer than two weeks, preferably one week, before transferring to a new stab. Reserve one stab culture unopened for use in the preparation of subsequent stock culture stabs. It is essential that three or four successive daily transfers are made before the organism is used for growing inoculum.

2. Preparation of Inoculum. On the day prior to use, transfer cells from the stab culture to a sterile tube of inoculum culture medium. (Do not use an old inoculum for preparing a new inoculum.) Incubate the culture for approximately 16 hours at 37°C. Secure cotton plug and centrifuge. Decant the supernatant liquid and resuspend the cells in 10 ml. of isotonic salt solution. Centrifuge and repeat process once more. After two washings resuspend cells in 10 ml. of isotonic solution, dilute one to 10, and use at once.

3. Preparation of Samples. Weigh one gram of sample into a 100 ml. beaker or 125 ml. Erlenmeyer flask. Add five ml. of phosphate buffer and steam for five minutes. Cool and add 20 mg. of chicken

pancreas. With the aid of a glass rod, thoroughly mix the sample with the enzyme. Cover with toluene and incubate 24 hours at 37°C. After incubation, add 50 ml. of boiling water; cool, adjust to pH 7.0 with NaOH, make up to 100 ml. with water and filter. Make suitable dilution so that the final dilution contains 0.4 to 0.8 mmcg. of folic acid/ml. A standard sample of dried yeast should be analyzed with each assay (one gram to 200; one ml. to 200).

4. Preparation of Standard Tubes. To duplicate tubes add 0.0 to 5.0 ml. of working standard folic acid solution in 0.5 ml. increments. Add sufficient water to bring the volume to 5.0 ml. in each tube. To each tube add 5.0 ml. of folic acid assay medium.

5. Preparation of Assay Tubes. To duplicate tubes add 2.0, 3.0, and 5.0 ml. aliquots of the test solution. Add sufficient water to bring the volume in each tube to 5.0 ml. To each tube add 5.0 ml. of folic acid assay medium.

6. Sterilization. Mix the contents of each tube thoroughly, cover, and autoclave at 15 pounds pressure for 15 minutes.

7. Titration. Transfer the contents of each tube into a series of 100 ml. beakers, rinsing the tube several times with small amounts of water and adding the washings to the beaker. With the aid of a pH meter, titrate to pH 7.0 with 0.1N NaOH.

9. Calculations.

a. Draw a standard curve for the assay by plotting ml. of 0.1N NaOH used in titrating the standard tubes against mmcg. of folic acid per tube in the standard series.

b. Determine the vitamin content of the tubes in the unknown series by interpolation of the titer values on the standard curve.

c. Calculate the vitamin content of each ml. of test solution for each of the duplicate sets of tubes.

d. Calculate the vitamin content of the test material from the average of the values for one ml. of test solution, obtained from tubes which do not vary by more than 10 percent from the average, using the following formula:

$$\left(\frac{\text{average mmcg. per ml.}}{\text{wt. of sample}}\right)(\text{volume})\left(\begin{array}{l}\text{dilution factor minus}\\ \text{correction factor for}\\ \text{enzyme}\end{array}\right) = \text{mmcg. per gm.}$$

MICROBIOLOGICAL ASSAY FOR NICOTINIC ACID

Plantarum

(Lactobacillus arabinosus 17-5)

A. Reagents

1. Bacto Niacin Assay Medium, dehydrated. Commercial preparation of Difco Laboratories (B 322).

To rehydrate the medium, suspend 75 gm. in 1000 ml. of water, and heat to boiling for two to three minutes. Cool. Keep the slight precipitate evenly distributed by shaking when dispensing.

2. Bacto Micro Assay Culture Agar, dehydrated. Commercial preparation of Difco Laboratories (B 319).

To rehydrate the agar, suspend 47 gm. in 1000 ml. of cold water and heat to boiling to dissolve the agar completely. Distribute in 10 ml. quantities in test tubes (16-20 mm. diameter) and plug. Sterilize in autoclave for 15 minutes at 15 pounds pressure. Cool tubes in upright position and store in refrigerator.

3. Culture Medium for Growing Inoculum.* Prepare by dissolving 7.5 gm. of reagent #1 above in water, adding 0.2 ml. of niacin stock solution and diluting to 200 ml. with water. Distribute in 10 ml. quantities in test tubes (16-20 mm. diameter) and plug. Sterilize in autoclave for 15 minutes at 15 pounds pressure. Cool and store in refrigerator.

4. Isotonic Salt Solution. Weigh 0.9 gm. of NaCl and transfer to a 100 ml. volumetric flask. Dilute to volume with water and shake until salt has dissolved. Transfer 10 ml. quantities of this solution

* Good growth unobtainable with Bacto Micro Inoculum Broth.

to culture tubes and 25 ml. quantities of this solution to culture tubes and 25 ml. quantities to 125 ml. Erlenmeyer flasks, plug with cotton and sterilize in the autoclave at 15 pounds pressure for 15 minutes. Store in refrigerator.

5. 10 N Sodium Hydroxide. Dissolve 400 gm. of NaOH in one liter of water; mix thoroughly.

6. 0.02N Sodium Hydroxide. Thoroughly mix 32 ml. of 10 NaOH in 16 liters of water. Protect from moisture with a lime drying tube. Standardize against an acid of known concentration.

7. 1N Sulfuric Acid. Add 28 ml. of concentrated H_2SO_4 to 800 ml. of water. Make up to one liter.

8. Standard Stock Solution of Niacin. Weight 50 mg. of U.S.P. Reference Standard, anhydrous, crystalline niacin (dried over P_2O_5 or concentrated H_2SO_4 in a vacuum desiccator for 24 hours), and dilute to 500 ml. with 50 percent ethyl alcohol. Store in refrigerator in a dark, glass stoppered bottle (100 mcg./ml.).

9. Working Niacin Standard Solution. Dilute one ml. of standard niacin stock solution to 100 ml. with water. Dilute further 10 ml. to 100 ml. (0.1 mcg./ml.). Prepare on the day of use.

10. Microorganism. Plantarum (Lactobacillus arabinosus 17-5).

B. Procedure

1. Preparation of Stab Culture. Prepare stab cultures in two or more agar stock culture tubes. Incubate for approximately 16 hours at $37^{\circ}C. \pm 0.5^{\circ}C.$ Store in the refrigerator under aseptic conditions not longer than two weeks, preferably one week, before transferring to a new stab. Reserve one stab culture unopened for use in the

preparation of subsequent stock culture stabs. It is essential that three or four successive daily transfers are made before the organism is used for growing inoculum.

2. Preparation of Inoculum. On the day prior to use, transfer cells from the stab culture to a sterile tube of inoculum culture medium. Incubate the culture for approximately 16 hours at 37°C. Secure cotton plug and centrifuge. Decant the supernatant liquid and resuspend the cells in 10 ml. of sterile isotonic salt solution. Repeat two times. Transfer cells into a 125 ml. Erlenmeyer flask containing 25 ml. of sterile isotonic salt solution, and use at once.

3. Preparation of Samples. Weight 5.0 to 10 gm. of sample into a 250 ml. Erlenmeyer flask. (It is good practice to use 10 gm. samples and make necessary dilutions to obtain desired concentration.) Add 50 ml. of 1N H_2SO_4 and mix thoroughly. Autoclave the mixture at least 15 pounds pressure for 30 minutes. Cool, adjust to pH 6.8 with NaOH, make up to 100 ml., and filter. Dilute filtrate, if necessary, to obtain a concentration of 0.05 to 0.1 mcg. per ml. of niacin. A standard sample of dried yeast should be analyzed with each assay (1 gm. to 100 ml.; 2 ml. to 100 ml.).

4. Preparation of Standard Tubes. To duplicate tubes add 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml. of the working niacin standard solution. Add sufficient water to bring the volume in each tube to 1.0 ml. To each tube add 1.0 ml. of niacin assay medium.

5 Preparation of Assay Tubes. To duplicate tubes add 0.4, 0.6, and 1.0 ml. aliquots of the test solution. Add sufficient water to bring the volume in each tube to 1.0 ml. To each tube add 1.0 ml. of niacin assay medium.

6. Sterilization. Mix the contents of each tube thoroughly, cover, and autoclave at 15 pounds pressure for 15 minutes.

7. Inoculation and Incubation. Cool the tubes to the incubation temperature or below. Inoculate each tube with one drop of inoculum, and incubate at 37°C. for 72 hours.

8. Titration. Titrate with 0.02 N NaOH to pH 7.0 using an automatic titrator (Cannon).

9. Calculations.

a. Draw a standard curve for the assay by plotting number of counts per ml. from Cannon counter (ml. of 0.02N NaOH) against mcg. of niacin per tube in the standard series.

b. Determine the vitamin content of the tubes in the unknown series by interpolation of the titer values on the standard curve.

c. Calculate the vitamin content of each ml. of test solution for each of the duplicate sets of tubes.

d. Calculate the vitamin content of the test material from the average of the values for 1.0 ml. of test solution, obtained from tubes which do not vary by more than 10 percent from the average, using the following formula:

$$\left(\frac{\text{average mcg. per ml.}}{\text{wt. of sample}}\right)(\text{volume})(\text{dilution factor}) = \text{mcg. per gm.}$$

MICROBIOLOGICAL ASSAY FOR PANTOTHENIC ACID

Plantarum

(Lactobacillus arabinosus 17-5)

A. Reagents

1. Bacto Pantothenate Assay Medium, dehydrated. Commercial preparation of Difco Laboratories (B 323).

To rehydrate the medium, suspend 75 gm. in 1000 ml. of water and heat to boiling for two to three minutes. Keep the slight precipitate evenly distributed by shaking when dispensing.

2. Bacto Micro Assay Culture Agar, dehydrated. Commercial preparation of Difco Laboratories (B 319).

To rehydrate the agar, suspend 47 gm. in 1000 ml. of cold water and heat to boiling to dissolve the agar completely. Distribute in 10 ml. quantities in test tubes (16-20 mm. diameter) and plug. Sterilize in autoclave for 15 minutes at 15 pounds pressure. Cool tubes in upright position and store in refrigerator.

3. Culture Medium for Growing Inoculum.* Prepare by dissolving 7.5 gms. of Bacto Niacin Assay Medium (B 322) in water, adding 0.2 ml. of niacin stock solution and diluting to 200 ml. with water. Distribute in 10 ml. quantities in test tubes (16-20 mm. diameter) and plug. Sterilize in autoclave for 15 minutes at 15 pounds pressure. Cool and store in refrigerator.

* Good growth unobtainable in Bacto Micro Inoculum Broth.

4. Isotonic Salt Solution. Weigh 0.9 gm. of NaCl and transfer to a 100 ml. volumetric flask. Dilute to volume with water and shake until salt has dissolved. Transfer 10 ml. quantities of this solution to culture tubes and 25 ml. quantities to 125 ml. Erlenmeyer flasks, plug with cotton and sterilize in the autoclave at 15 pounds pressure for 15 minutes. Store in refrigerator.

5. 0.1N Sodium Hydroxide. Since NaOH pellets often contain considerable water, amounts in excess (1 to 15 percent depending upon water content of pellets) of the calculated NaOH may be used. To prepare one liter of 0.1N NaOH, 4.0 gm. of NaOH are needed. It need not be exactly 0.1N, but it is well to have standardized solution so that results between laboratories can be compared.

6. Acetate Buffer pH 4.5 to 4.7. Add one ml. of 2.5 N sodium acetate (205 gm. $NaC_2H_3O_2$ or 314 gm. $NaC_2H_3O_2 \cdot 3H_2O$ dissolved in sufficient water to make one liter) to 15 ml. of 0.1N sulfuric acid (2.8 ml. of concentrated H_2SO_4 to one liter) and mix. This solution may be made in 500 ml. quantities and kept until ready to use. Since mold may grow in this solution under certain conditions, it should be inspected for turbidity before each use.

7. Mylase P. Prepare the enzyme suspension by adding 0.1 gm. of Mylase P to 15 ml. of acetate buffer. In practice when 12 samples are assayed simultaneously 1.25 gm. of enzyme are added to 187.5 ml. of acetate buffer. Since different lots of enzyme contain varying amounts of pantothenic acid, the pantothenic acid content of each lot must be determined and a proper correction made in the calculation.

The enzyme is treated as a sample and diluted 2.5 gm. + 75 ml. of acetate buffer. Use 30 ml. aliquots diluted to 50 ml.; 15 ml. to 25 ml. The amount of pantothenic acid contained in the enzyme added to the sample divided by the weight of the sample gives the correction factor which must be subtracted from the value obtained for a gram of sample.

8. Standard Stock Solution of Calcium Pantothenate. Weigh 54.4 mg. of calcium pantothenate and transfer to a 500 ml. volumetric flask. Dilute to volume with 50 percent ethyl alcohol. Store in refrigerator. (100 mcg. of pantothenic acid per ml.).

9. Working Calcium Pantothenate Standard Solution. Dilute 5 ml. of stock solution to 250 ml. with water. Dilue one ml. of this intermediate solution to 100 ml. with water. (0.02 mcg./ml.). Prepare on the day of use.

10. Microorganism. Plantarum (Lactobacillus arabinosus 17-5).

B. Procedure

1. Preparation of Stab Culture. Prepare stab cultures in two or more agar stock culture tubes. Incubate for approximately 16 hours at 37°C. ± 0.5°C. Store in refrigerator under aseptic conditions not longer than two weeks, preferable one week, before transferring to a new stab. Reserve one stab culture unopened for use in the preparation of subsequent stock culture stabs. It is essential that three or four successive daily transfers are made before the organism is used for growing inoculum.

2. Preparation of Inoculum. On the day prior to use, transfer cells from the stab culture to a sterile tube of inoculum culture medium. (Do not use an old inoculum for preparing a new inoculum). Incubate the culture for approximately 16 hours at 37°C. Secure cotton plug and centrifuge. Decant the supernatant liquid and resuspend the cells in 10 ml. of isotonic salt solution. Centrifuge and repeat process once more. After two washings resuspend cells in 10 ml. of isotonic solution, dilute 1 to 10, and use at once.

3. Preparation of Samples. Weigh one gm. of sample into a 100 ml. beaker, add suspension of 0.1 gm. of Mylase P in 15 ml. of acetate buffer, cover with toluene and a watch glass and incubate for 18 to 24 hours at 37°C. After incubation, add 50 ml. of boiling water, cool, adjust to pH 7.0 with NaOH, make up to 100 ml. with water, and filter. Make suitable dilution so that the final dilution contains 0.01 to 0.02 mcg. of pantothenic acid/ml. A standard sample of dried yeast should be analyzed with each assay (1 gm. to 200 ml.; 5 ml. to 100 ml.).

4. Preparation of Standard Tubes. To duplicate tubes add 0.0 to 5.0 ml. of working standard calcium pantothenate solution in 0.5 ml. increments. Add sufficient water to bring the volume to 5 ml. in each tube. To each tube add 5 ml. of pantothenate assay medium.

5. Preparation of Assay Tubes. To duplicate tubes add 2.0, 3.0, and 5.0 ml. aliquots of the test solution. Add sufficient water to bring the volume in each tube to 5.0 ml. To each tube add 5 ml. of pantothenate assay medium.

6. Sterilization. Mix the contents of each tube thoroughly, cover, and autoclave at 15 pounds pressure for 15 minutes.

7. Inoculation and incubation. Cool tubes to below incubation temperature, aseptically inoculate each tube with one drop of dilute inoculum and incubate at 37°C. for 72 hours.

8. Titration. Transfer the contents of each tube into a series of 100 ml. beakers, rinsing the tube several times with small amounts of water and adding the washings to the beaker. With the aid of a pH meter, titrate to pH 7.0 with 0.1N NaOH.

9. Calculations

a. Draw a standard curve for the assay by plotting ml. of 0.1N NaOH used in titrating the standard tubes against mcg. of pantothenic acid per tube in the standard series.

b. Determine the vitamin content of the tubes in the unknown series by interpolation of the titer values on the standard curve.

c. Calculate the vitamin content of each ml. of test solution for each of the duplicate sets of tubes.

d. Calculate the vitamin content of the test material from the average of the values for 1.0 ml. of test solution, obtained from tubes which do not vary by more than 10 percent from the average, using the following formula:

$$\left(\frac{\text{average mcg. per ml.}}{\text{wt. of sample}}\right)(\text{volume})\left(\begin{array}{l}\text{dilution factor minus}\\ \text{correction factor for}\\ \text{enzyme}\end{array}\right) = \text{mcg. per gm.}$$

MICROBIOLOGICAL ASSAY FOR RIBOFLAVIN

Lactobacillus casei

A. Reagents

1. Bacto Riboflavin Assay Medium, dehydrated. Commercial preparation of Difco Laboratories (B 325).

To rehydrate the medium, suspend 48 gm. in 1000 ml. of water and heat to boiling for two to three minutes. Keep the slight precipitate evenly distributed by shaking when dispensing.

2. Bacto Micro Assay Culture Agar, dehydrated. Commercial preparation of Difco Laboratories (B319).

To rehydrate the agar, suspend 47 gm in 1000 ml. of cold water and heat to boiling to dissolve the agar completely. Distribute in 10 ml. quantities in test tubes (16-20mm. diameter) and plug. Sterilize in autoclave for 15 minutes at 15 pounds pressure. Cool tubes in upright position and store in refrigerator.

3. Bacto Micro Inoculum Broth, dehydrated. Commercial preparation of Difco Laboratories (B 320).

To rehydrate the broth, dissolve 37 gm. in 1000 ml. water. Distribute in 10 ml. quantities in test tubes (16-20 mm. diameter) and plug. Sterilize in autoclave for 15 minutes at 15 pounds pressure. Cool tubes and store in refrigerator.

4. Isotonic Salt Solution. Weigh 0.9 gm. of NaCl and transfer to a 100 ml. volumetric flask. Dilute to volume with water and shake until salt has dissolved. Transfer 10 ml. quantitites of this solution to culture tubes and 25 ml. quantities to 125 ml. Erlenmeyer flasks,

plug with cotton and sterilize in the autoclave at 15 pounds pressure for 15 minutes. Store in refrigerator.

5. 10 N Sodium Hydroxide. Dissolve 400 gm. of NaOH in one liter of water; mix thoroughly.

6. 0.02 N Sodium Hydroxide. Thoroughly mix 32 ml. of 10 N NaOH in 16 liters of water. Protect from moisture with a lime drying tube. Standardize against an acid of known concentration.

7. 0.1 N. Hydrochloric Acid. Dilute 8.5 ml. of concentrated HCl to one liter with water.

8. Standard Stock Solution of Riboflavin. Weigh 50 mg of U.S.P. Reference Standard, anhydrous, crystalline riboflavin (dried over P_2O_5 or concentrated H_2SO_4 in a vacuum desiccator for 24 hours), dissolve in 10 ml. of dilute acetic acid (60 ml. of glacial acetic acid to 100 ml. of water) and make up to two liters with water. Cover with toluene, refrigerate in a dark bottle, and protect from light at all times (25 mcg./ml.).

9. Working Riboflavin Standard Solution. Dilute 10 ml. of stock solution to 25 ml. with water. Dilute further 10 ml. of the diluted solution to one liter with water (0.1 mcg./ml.). Prepare on the day of use.

10. Microorganism. Lactobacillus casei.

B. Procedure

1. Preparation of Stab Culture. Prepare stab cultures in two or more agar stock culture tubes. Incubate for approximately 16 hours at $37^{\circ}C. \pm 0.5^{\circ}C.$ Store in refrigerator under aseptic conditions not

longer than two weeks, preferably one week, before transferring to a new stab. Reserve one stab culture unopened for use in the preparation of subsequent stock culture stabs.

2. Preparation of Inoculum. On the day prior to use transfer cells from the stab culture to a sterile tube of inoculum culture medium. (Do not use an old inoculum for preparing a new inoculum.) Incubate the culture for approximately 16 hours at 37°C. Secure cotton plug and centrifuge. Decant the supernatant liquid and resuspend the cells in 10 ml. of isotonic salt solution. Centrifuge and repeat process twice more. Transfer cells into 125 ml. flask containing 25 ml. of sterile isotonic salt solution, and use at once.

3. Preparation of Samples. Weigh 5 to 10 gm. of sample (to contain approximately 0.1 mg of riboflavin) into a 250 ml. Erlenmeyer flask. (It is good practice to use 10 gm. samples and make necessary dilutions to obtain the desired concentration.) Add 50 ml. of 0.1 N HCl and mix thoroughly. Autoclave for 30 minutes at 15 pounds pressure. Cool, adjust to pH 4.5 with NaOH, make up to 100 ml. with water and filter. Take aliquot for assay, adjust to pH 6.8 with NaOH, and make up to volume so that one ml. is equivalent to approximately 0.05 to 0.10 mcg. of riboflavin. If a precipitate forms, refilter. A standard sample of dried yeast should be analyzed with each assay (1 gm. to 100 ml.; 10 ml. to 50 ml.).

4. Preparation of Standard Tubes. To duplicate tubes add 0.0 to 1.0 ml. of working standard riboflavin solution in 0.1 ml. increments. Add sufficient water to bring the volume to one ml. in each tube. To each tube add one ml. of riboflavin assay medium.

5. Preparation of Assay Tubes. To duplicate tubes add 0.4, 0.6, 1.0 ml. aliquots of the test solution. Add sufficient water to bring the volume in each tube to 1.0 ml. To each tube add 1.0 ml. of riboflavin assay medium.

6. Sterilization. Mix the contents of each tube thoroughly, cover, and autoclave at 15 pounds pressure for 15 minutes.

7. Inoculation and Incubation. Cool tubes to below incubation temperature aseptically inoculate each tube with one drop of dilute inoculum and incubate at 37°C. for 72 hours.

8. Titration. Titrate with 0.02 N NaOH to pH 7.0 using automatic titrator (Cannon).

9. Calculations.

a. Draw a standard curve for the assay by plotting number of counts per ml. from the Cannon counter (ml. of 0.02 N NaOH) against concentration of riboflavin per tube in the standard series.

b. Determine the vitamin content of the tubes in the unknown series by interpolation of the titer values on the standard curve.

c. Calculate the vitamin content of each ml. of test solution for each of the duplicate sets of tubes.

d. Calculate the vitamin content of the test material from the average of the values for 1.0 ml. of test solution, obtained from tubes which do not vary by more than 10 percent from the average, using the following formula:

$$\left(\frac{\text{average mcg. per ml.}}{\text{wt. of sample}}\right)(\text{volume})(\text{dilution factor}) = \text{mcg. per gm.}$$

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