METHODS IN
Medical Research

VOLUME I

VAN R. POTTER, *Editor-in-Chief*

ASSAY OF ANTIBIOTICS, *Henry Welch, Editor*

CIRCULATION—BLOOD FLOW MEASUREMENT, *Harold D. Green, Editor*

SELECTED METHODS IN GASTROENTEROLOGIC RESEARCH, *A. C. Ivy, Editor*

CELLULAR RESPIRATION, *Van R. Potter, Editor*

PREFACE

IT SEEMS TO have become customary, in launching a new periodical or set of volumes in the field of medical research and its ancillary sciences, to adopt a faintly apologetic and deprecatory attitude; and it is particularly fitting to do so in the present case, which may be thought rather novel and ambitious both in plan and in objectives and which at best can hardly establish the general usefulness we hope for until several volumes have been distributed.

This series is to be devoted to methods and techniques, and there are four main reasons for our conclusion that such a series may be useful. In the first place, while the results of investigations are constantly subject to critical review, it is not usually easy to find anywhere an appraisal and discussion of the various methods that may have been proposed for the solution of some experimental problem. In the second place, it is becoming difficult, especially in physiology, to obtain publication of a paper dealing solely with a technique or even to include an adequate description of the technique in a paper describing the results obtained. Third, it frequently happens that a method is modified and improved in continued use, either in the laboratory whence it originated or elsewhere; such useful modifications find their way into print, if at all, only as brief and scattered indications and are to a great extent diffused by the uncertain process of personal communication. Fourth, many methods developed during the war have been described only in official reports.

Each volume will be divided into four or five principal, self-contained sections, each of which shall, for that volume, represent one of the broad fields of medical research: biochemistry, physiology and pharmacology, microbiology and immunology, and biophysics including radiobiology. Within each of these broad fields we shall try, year by year, to select narrower topics wherein a restatement of techniques seems timely. For example, the following topics have been considered among many others for inclusion in forthcoming volumes: methods related to acetylcholine; assay of hormones and their excretion products in urine; experimental surgery of the autonomic system; techniques of histochemistry; paper chromatography; design and use of stimulators; methods in the study of pulmonary function; methods in the study of bacterial viruses; and so forth.

When the topics have been selected, we shall try to find experts, like those who have so signally contributed to this first volume, willing to act as associate editors for their assigned topic for the year. The responsibilities of the associate editor are by no means light: it is for him

to select, within the topic and the space assigned, the methods most worthy of description and the contributors best fitted to describe. Obviously the methods most suitable for description in this form are those which are of wide actual or potential application and which have not been published in full or have been usefully modified since publication; obviously, too, the inclusion of a method stamps it as being convenient and reliable in the associate editor's expert estimation, but it does not conversely follow that omitted methods are of lesser value. The associate editor may also send each contribution to another experienced investigator for comment and review.

As members of the Governing Board, we are very conscious of the lightness of our own responsibilities in comparison with those of the associate editors and, still more, those of our Dr. V. R. Potter, who, to our great satisfaction, agreed to assume the further ungrateful task of acting as Editor-in-Chief for the year, charged among other things with the duty of distributing space among the sections. Any values which this volume may have must be credited to the editors, the contributors and the referees, rather than to us. It remains for us merely to select topics and to try to find equally competent and conscientious editors for the next volume and its successors. To this end we should most gratefully receive and consider any suggestions that readers may care to send us.

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SECTION I

Assay of Antibiotics

ASSOCIATE EDITOR—*Henry Welch*

INTRODUCTION

THE DISCOVERY of penicillin by Sir Alexander Fleming and its rapid commercial development in this country stimulated widespread interest in antibiotics. Production of penicillin increased from a few million units in the latter months of 1942 to over 7000 billion units per month during 1948. The amazing production record is the result of the efforts of 15 drug manufacturers in this country who, during the early part of World War II, were responsible for supplying great quantities of this valuable therapeutic agent to the armed forces of this country and to those of our allies. The therapeutic evaluation of penicillin was planned and arranged by the Committee on Medical Research of the Office of Scientific Research and Development who, with the co-operation of scientists throughout the country, demonstrated the efficacy of this drug for the treatment of a great variety of diseases.

In the early studies of penicillin, biologic assays were utilized exclusively, but during the past three years, with the isolation of crystalline penicillin, both chemical and physical methods have been developed. These have proved to be quite satisfactory. The demonstration that penicillin as produced by the mold was not necessarily one substance but a combination of at least five penicillins, F (Δ^2-pentenyl-penicillin), G (benzyl penicillin), X (P-hydroxybenzyl penicillin), K (n-heptyl penicillin) and dihydro F (n-amyl penicillin), has been of considerable value in the development of definitive methods.

Although amorphous penicillin, which was utilized in great quantities prior to March, 1946, had little or no toxicity, there has been a steady increase in the production of pure crystalline penicillin G since then, until now more than 90 per cent of the penicillin administered parenterally in clinical practice is crystalline material largely of the G type. There has been considerable reduction in the production of penicillin K

which, although more active than penicillin G in vitro, has only a fraction of its activity in vivo. Control of the quantity of penicillin K is maintained by regulation, as is the control of all penicillin and streptomycin preparations, under an amendment to the Federal Food, Drug and Cosmetic Act. Penicillin for use in the body must not contain more than 30 per cent penicillin K, while products labeled as containing crystalline penicillin G must contain at least 85 per cent of this fraction by weight. Although no penicillin X is available on the market at this time, the regulations require a product to contain at least 90 per cent of a salt of penicillin X to be labeled as this fraction. Methods for the assay of penicillin for G, K and X content are included in this section.

Although it may be said that penicillin was discovered by accident, the discovery of streptomycin, our second most important antibiotic, by Waksman and his co-workers was the result of a carefully designed investigation. These workers were searching for an antibiotic antagonistic to gram-negative organisms to complement the activity of penicillin which is selective for gram-positive organisms. Unlike penicillin, streptomycin has some toxicity, particularly when it is used in 1–4 g daily doses for 1–3 months, as is done in the treatment of certain types of tuberculosis. Deafness and vertigo due to eighth nerve damage may result even when crystalline streptomycin (the calcium chloride, trihydrochloride double salt of streptomycin) is used for treatment. In the treatment of other diseases for which streptomycin is effective (*Hemophilus influenzae* meningitis, tularemia and gram-negative urinary tract infections) the total dosage is so low that few if any toxic reactions are observed.

The manufacturing experience gained by industry in this country in the development of penicillin production has been of great value in the development of production methods for streptomycin. As a result, in a relatively short time production of streptomycin reached 1,000,000 g per month early in 1947 and was double that by the end of the year. One manufacturer (the largest at this time) is now producing only crystalline material. As with penicillin, early indications were that streptomycin was a single entity, but it soon became evident that it consists of at least three different "streptomycins," streptomycin A, streptomycin B and a third fraction unclassified. These streptomycins differ in their activity against sensitive organisms and occur in commercial streptomycin in varying concentrations, depending on the manufacturer and the extraction procedures utilized. It can be said, however, that streptomycin A (which is approximately five times as active as streptomycin B) constitutes about 70 per cent of commercial amorphous streptomycin. The methods described for the assay of streptomycin in this section are both biologic and chemical. However, the fact that commercial streptomycin has been found to be not a single entity has delayed development of accurate chemical methods, although the methods used are quite sufficient for clinical evaluation of this drug.

As compared to penicillin and streptomycin, bacitracin and tyrothricin are produced in relatively small amounts in this country. Because of their inherent toxicity, both are recommended for topical application only. There is a possibility that bacitracin, which is a polypeptide, may eventually be purified and utilized parenterally since conservative use of this drug systemically has already been successful in the hands of Meleney and his co-workers, the discoverers of this antibiotic. It is unlikely, however, that tyrothricin (a combination of 80 per cent tyrocidine and 20 per cent gramicidin), now produced in small amounts in crystalline form, will ever be satisfactory for parenteral use. The methods described here for both bacitracin and tyrothricin are tentative. In the case of the former substance, more accurate methods must await its eventual purification; tyrothricin preparations, on the other hand, frequently contain substances which have been found to interfere with the assay methods so far developed.

Test methods chosen for inclusion in this chapter obviously do not include all of the reliable methods to be found in the literature. The methods included have, in most cases, been given extensive trial and have proved satisfactory.

—HENRY WELCH.

ASSAY OF PENICILLIN POTENCY: A. BIOLOGIC METHODS

1. COMMERCIAL PREPARATIONS

Cylinder-Plate Assay[1]

PROCEDURE

a) Cylinders (cups).—Use stainless steel cylinders with outside diameter 8 mm (±0.1 mm), inside diameter 6 mm (±0.1 mm) and length 10 mm (±0.1 mm).

b) Culture media.—Use ingredients that conform to standards prescribed by the U.S.P. or N.F.

1. Make nutrient agar for the seed layer and for carrying the test organism as follows:

Peptone	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Glucose	1.0 g
Agar	15.0 g
Distilled water, q.s.	1000.0 ml

pH 6.5–6.6 after sterilization.

2. Make nutrient agar for base layer as follows:

Peptone	6.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Agar	15.0 g
Distilled water, q.s.	1000.0 ml

pH 6.5–6.6 after sterilization.

3. Make nutrient broth for preparing an inoculum of the test organism as follows:

Peptone	5.0 g
Yeast extract	1.5 g
Beef extract	1.5 g
Sodium chloride	3.5 g
Glucose	1.0 g
Dipotassium phosphate	3.68 g
Potassium dihydrogen phosphate	1.32 g
Distilled water, q.s.	1000.0 ml

pH 7.0 after sterilization.

[1] Food and Drug Administration, Federal Security Agency, Official Method, Federal Register, Apr. 4, 1947.

Instead of media prepared from the individual ingredients specified in paragraphs (*b*) (1), (2) and (3), media may be made from a dehydrated mixture which, when reconstituted with distilled water, has the same composition as such media. Minor modifications of the individual ingredients specified in paragraphs (*b*) (1), (2) and (3) are permissible if the resulting media possess growth-promoting properties at least equal to those of the media described.

c) Working standard.—Keep the working standard (obtained from the Food and Drug Administration) in tightly stoppered vials, which in turn are kept in larger stoppered tubes containing anhydrous calcium sulfate, constantly in the refrigerator at 15 C (59 F) or below. Weigh out carefully in an atmosphere of 50 per cent relative humidity or less between 4 and 5 mg of the working standard and dilute with sterile 1 per cent phosphate buffer (pH 6.0) to make a stock solution of any convenient concentration. Keep this solution at a temperature of about 10 C and use for 1 day only. From this stock solution make appropriate working dilutions.

d) Preparation of sample.—Dissolve aseptically, in sterile distilled water, the sample to be tested to make an appropriate stock solution.

e) Preparation of plates.—Add 21 ml of agar to each Petri dish (20 × 100 mm). Distribute the agar evenly in the plates and allow it to harden. Use the plates the same day they are prepared. The test organism is *Staphylococcus aureus* (FDA 209-P or American Type Culture Collection [ATCC] 9144). Maintain the test organism on agar slants and transfer to a fresh agar slant about once a week. Prepare an inoculum for the plates by transferring the culture from the agar slant into broth and incubate at 37 C. From 16 to 24 hr thereafter add 2.0 ml of this broth culture to each 100 ml of agar, which has been melted and cooled to 48 C. Mix the culture and agar thoroughly and add 4 ml to each of the plates containing the 21 ml of uninoculated agar. Tilt the plates back and forth to spread the inoculated agar evenly over the surface. Porcelain covers glazed on the outside are used. Place four cylinders on the agar surface so that they are at approximately 90° intervals on a 2.8 cm radius. In placing the cylinders drop them from a height of $\frac{1}{2}$ in., using a mechanical guide or device.

A suspension of the test organism may be used in place of the broth culture in preparing the inoculum for the seeding of plates. Prepare such a suspension as follows: Wash the organisms from an agar slant, which has been incubated for 24 hr at 37 C and stored for 24 hr at room temperature, with 2.0 ml of sterile physiologic saline onto a large agar surface such as that provided by a Roux bottle containing 300 ml of agar. Spread the suspension of organisms over the entire agar surface with the aid of sterile glass beads. Incubate 24 hr at 37 C and store for 24 hr at room temperature. Wash the resulting growth from the agar surface with about 50 ml of sterile physiologic saline. Standardize this suspension by determining the dilution which will permit 20 per cent light transmission

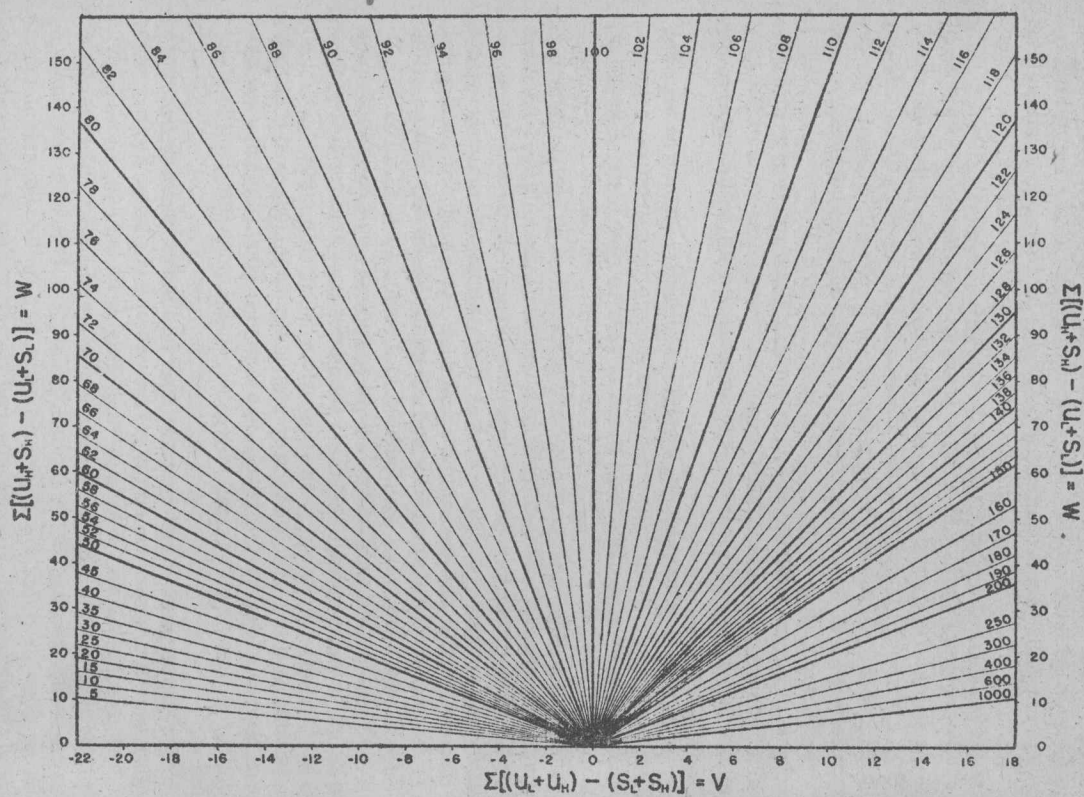

FIG. 1.—Penicillin assay. Chart for determining potency as percent of standard from two-dose four-plate method, ratio of doses 4:1. H = high dose; L = low dose; $H/L = 4$.

through a filter at 6500 A in a photoelectric colorimeter. Add 1.5–2.0 ml of this resulting dilution to each 100 ml of agar which has been melted and cooled to 48 C to prepare the inoculum for the plates. The suspension may be used for 1 week.

f) Assay.—Use four plates for each sample. Fill one cylinder on each plate with a 1.0 unit/ml dilution, and one with a 0.25 unit/ml dilution, of the working standard. Add the estimated dilutions of 1.0 unit/ml and 0.25 unit/ml of the sample under test to the remaining two cylinders on each plate. Carefully place the plates in racks and incubate 16–18 hr at 37 C. After incubation, measure the diameter of each circle of inhibition to the nearest 0.5 mm, using a colony counter with a millimeter scale etched into the supporting glass over the light source. Other measuring devices of equal accuracy may be used.

g) Estimation of potency and error.—1. Use the chart (Fig. 1) and nomograph (Fig. 2) for estimating potency and its standard error. To use the chart for estimating potency two values, namely, V and W, are required. For each plate calculate two values.

$$v = (U_L + U_H) - (S_L + S_H)$$

and

$$w = (U_H + S_H) - (U_L + S_L),$$

where S_H and S_L are the diameters of the zones of inhibition in millimeters of the 1.0 unit and 0.25 unit dilutions of the standard, respectively, and U_H and U_L refer similarly to the corresponding dilutions of the sample under test. The value of V is the sum of the v values for all plates and W is the sum of the w values for all plates. To estimate potency, locate the point on the chart corresponding to the values of V and W; the potency can be read from the radial lines on the chart.

2. The standard error of the assay is estimated by using the nomograph (Fig. 2) which requires five values, namely, the potency, V, W, Rv and Rw. Rv (range of the v's) is the highest value of v minus the lowest value of v obtained from the individual plates. Similarly, Rw is the difference between the highest and the lowest w value. After obtaining these five values, connect with a straightedge the points corresponding to v and w on the respective scales on the right nomograph (Fig. 2*B*). Mark with a pin or sharp-pointed pencil the intersection of the straightedge and the diagonal line of the nomograph. Move the straightedge so that it connects the value of Rw on its scale and the diagonal line at the point of the pin. The value for Q is thus determined by the scale value where the straightedge crosses the line labeled "Q." T is obtained by adding the squares of Q and Rv. On the left nomograph (Fig. 2*A*) connect the values of T and W with the straightedge and read the value of the ratio (standard error of assay–potency) where the straightedge intersects the scale of values for the ratio. This value multiplied by the potency equals the percentage standard error of the assay. The standard error of the assay calculated here estimates only how closely one assayist can check himself on