1990

USP XXII
NF XVII

THE UNITED STATES PHARMACOPEIA
THE NATIONAL FORMULARY

By authority of the United States Pharmacopeial Convention, Inc., meeting at Washington, D.C., March 22–24, 1985. Prepared by the Committee of Revision and published by the Board of Trustees

Official from January 1, 1990

UNITED STATES PHARMACOPEIAL CONVENTION, INC.
12601 Twinbrook Parkway, Rockville, MD 20852

NOTICE AND WARNING

Concerning U. S. Patent or Trademark Rights

The inclusion in the Pharmacopeia or in the National Formulary of a monograph on any drug in respect to which patent or trademark rights may exist shall not be deemed, and is not intended as, a grant of, or authority to exercise, any right or privilege protected by such patent or trademark. All such rights and privileges are vested in the patent or trademark owner, and no other person may exercise the same without express permission, authority, or license secured from such patent or trademark owner.

Concerning Use of USP or NF Text

Attention is called to the fact that USP and NF text is fully copyrighted. Authors and others wishing to use portions of the text should request permission to do so from the Secretary of the USPC Board of Trustees.

12601 Twinbrook Parkway, Rockville, MD 20852

Library of Congress Catalog Card Number 83-640088
ISSN 0195-7996
ISBN 0-913595-37-3 (cloth)
0-913595-38-1 (leather)

Printed by Mack Printing Company, Easton, PA 18042

Guide to GENERAL CHAPTERS

(For complete alphabetic list of all general chapters in this Pharmacopeia, see under "General chapters" in the index.)

General Tests and Assays

General Information

GENERAL CHAPTERS

General Tests *and* Assays

General Requirements for Tests and Assays

⟨1⟩ INJECTIONS

Every care should be exercised in the preparation of all products intended for injection, to prevent contamination with microorganisms and foreign material. Good pharmaceutical practice requires also that each final container of Injection be subjected individually to a physical inspection, whenever the nature of the container permits, and that every container whose contents show evidence of contamination with visible foreign material be rejected.

Definitions—In this Pharmacopeia, the sterile preparations for parenteral use are grouped into five distinct classes, defined as follows: (1) medicaments or solutions or emulsions thereof suitable for injection, bearing titles of the form, ___ *Injection;* (2) dry solids or liquid concentrates containing no buffers, diluents, or other added substances, and which, upon the addition of suitable solvents, yield solutions conforming in all respects to the requirements for Injections, and which are distinguished by titles of the form, *Sterile* ___; (3) preparations the same as those described under (2) except that they contain one or more buffers, diluents, or other added substances, and which are distinguished by titles of the form, ___ *for Injection;* (4) solids which are suspended in a suitable fluid medium and which are not to be injected intravenously or into the spinal canal, distinguished by titles of the form, *Sterile* ___ *Suspension;* and (5) dry solids which, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for Sterile Suspensions, and which are distinguished by titles of the form, *Sterile* ___ *for Suspension.*

A Pharmacy bulk package is a container of a sterile preparation for parenteral use that contains many single doses. The contents are intended for use in a pharmacy admixture program and are restricted to the preparation of admixtures for infusion or, through a sterile transfer device, for the filling of empty sterile syringes.

The closure shall be penetrated only one time after constitution with a suitable sterile transfer device or dispensing set which allows measured dispensing of the contents. The *Pharmacy bulk package* is to be used only in a suitable work area such as a laminar flow hood (or an equivalent clean air compounding area).

Designation as a *Pharmacy bulk package* is limited to preparations from classes 1, 2, or 3 as defined above. *Pharmacy bulk packages*, although containing more than one single dose, are exempt from the multiple-dose container volume limit of 30 mL and the requirement that they contain a substance or suitable mixture of substances to prevent the growth of microorganisms.

Where a container is offered as a *Pharmacy bulk package*, the label shall (a) state prominently "Pharmacy Bulk Package—Not for direct infusion," (b) contain or refer to information on proper techniques to help assure safe use of the product, and (c) bear a statement limiting the time frame in which the container may be used once it has been entered, provided it is held under the labeled storage conditions.

Where used in this Pharmacopeia, the designation *Large-volume intravenous solution* applies to a single-dose injection that is intended for intravenous use and is packaged in containers labeled as containing more than 100 mL. The designation *Small-volume Injection* applies to an Injection that is packaged in containers labeled as containing 100 mL or less.

The Pharmacopeial definitions for sterile preparations for parenteral use generally do not apply in the case of the biologics, because of their special nature and licensing requirements (see *Biologics* ⟨1041⟩).

Aqueous Vehicles—The vehicles for aqueous Injections meet the requirements of the *Pyrogen Test* ⟨151⟩ or the *Bacterial Endotoxins Test* ⟨85⟩, whichever is specified. *Water for Injection* generally is used as the vehicle, unless otherwise specified in the individual monograph. Sodium chloride may be added in amounts sufficient to render the resulting solution isotonic; and *Sodium Chloride Injection*, or *Ringer's Injection*, may be used in whole or in part instead of *Water for Injection* unless otherwise specified in the individual monograph. For conditions applying to other adjuvants, see *Added Substances*, in this chapter.

Other Vehicles—Fixed oils used as vehicles for nonaqueous injections are of vegetable origin, are odorless or nearly so, and have no odor or taste suggesting rancidity. They meet the requirements of the test for *Solid paraffin* under *Mineral Oil*, the cooling bath being maintained at 10°, have a *Saponification value* of between 185 and 200 (see *Fats and Fixed Oils* ⟨401⟩), have an *Iodine value* of between 79 and 128 (see *Fats and Fixed Oils* ⟨401⟩), and meet the requirements of the following tests:

Unsaponifiable Matter—Reflux on a steam bath 10 mL of the oil with 15 mL of sodium hydroxide solution (1 in 6) and 30 mL of alcohol, with occasional shaking until the mixture becomes clear. Transfer the solution to a shallow dish, evaporate the alcohol on a steam bath, and mix the residue with 100 mL of water: a clear solution results.

Free Fatty Acids—The free fatty acids in 10 g of oil require for neutralization not more than 2.0 mL of 0.020 *N* sodium hydroxide (see *Fats and Fixed Oils* ⟨401⟩).

Synthetic mono- or diglycerides of fatty acids may be used as vehicles, provided they are liquid and remain clear when cooled to 10° and have an *Iodine value* of not more than 140 (see *Fats and Fixed Oils* ⟨401⟩).

These and other nonaqueous vehicles may be used, provided they are safe in the volume of injection administered, and also provided they do not interfere with the therapeutic efficacy of the preparation or with its response to prescribed assays and tests.

Added Substances—Suitable substances may be added to increase stability or usefulness, unless proscribed in the individual monograph, provided they are harmless in the amounts administered and do not interfere with the therapeutic efficacy or with the responses to the specified assays and tests. No coloring agent may be added, solely for the purpose of coloring the finished preparation, to a solution intended for parenteral administration (see also *Added Substances* under *General Notices*, and *Antimicrobial Preservatives—Effectiveness* ⟨51⟩).

Observe special care in the choice and use of added substances in preparations for injection that are administered in a volume exceeding 5 mL. The following maximum limits prevail unless otherwise directed: for agents containing mercury and the cationic, surface-active compounds, 0.01%; for those of the types of chlorobutanol, cresol, and phenol, 0.5%; and for sulfur dioxide, or an equivalent amount of the sulfite, bisulfite, or metabisulfite of potassium or sodium, 0.2%.

A suitable substance or mixture of substances to prevent the growth of microorganisms must be added to preparations intended for injection that are packaged in multiple-dose containers, regardless of the method of sterilization employed, unless otherwise directed in the individual monograph, or unless the active ingredients are themselves antimicrobial. Such substances are used in concentrations that will prevent the growth of or kill microorganisms in the preparations for injection (see also *Antimicrobial Preservatives—Effectiveness* ⟨51⟩ and *Antimicrobial Agents—Content* ⟨341⟩). Sterilization processes are employed even though such substances are used (see also *Parenteral and Topical Preparations* in the section, *Added Substances*, under *General Notices*, and *Sterilization and Sterility Assurance of Compendial Articles* ⟨1211⟩). The air in the container may be evacuated or be displaced by a chemically inert gas.

Containers for Injections—Containers, including the closures, for preparations for injection do not interact physically or chemically with the preparations in any manner to alter the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage, sale, and use. The container is made of material that permits inspection of the contents. The type of glass preferable for each parenteral preparation is usually stated in the individual monograph.

For definitions of single-dose and multiple-dose containers, see *Containers* under *General Notices*. Containers meet the requirements under *Containers* ⟨661⟩.

Containers are closed by fusion, or by application of suitable closures, in such manner as to prevent contamination or loss of contents. Closures for multiple-dose containers permit the withdrawal of the contents without removal or destruction of the closure. The closure permits penetration by a needle, and, upon withdrawal of the needle, at once recloses the container against contamination.

Containers for Sterile Solids—Containers, including the closures, for dry solids intended for parenteral use do not interact physically or chemically with the preparation in any manner to alter the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage, sale, and use.

A container for a sterile solid permits the addition of a suitable solvent and withdrawal of portions of the resulting solution or suspension in such manner that the sterility of the product is maintained.

Where the *Assay* in a monograph provides a procedure for *Assay preparation* in which the total withdrawable contents are to be withdrawn from a single-dose container with a hypodermic needle and syringe, the contents are to be withdrawn as completely as possible into a dry hypodermic syringe of a rated capacity not exceeding three times the volume to be withdrawn and fitted with a 21-gauge needle not less than 2.5 cm (1 inch) in length, care being taken to expel any air bubbles, and discharged into a container for dilution and assay.

Volume in Container—Each container of an Injection is filled with a volume in slight excess of the labeled "size" or that volume which is to be withdrawn. The excess volumes recommended in the accompanying table are usually sufficient to permit withdrawal and administration of the labeled volumes.

DETERMINATION OF VOLUME OF INJECTION IN CONTAINERS—Select 1 or more containers if the volume is 10 mL or more, 3 or more if the volume is more than 3 mL and less than 10 mL, or 5 or more if the volume is 3 mL or less. Take up individually the contents of each container selected into a dry hypodermic syringe of a rated capacity not exceeding three times the volume to be measured, and fitted with a 21-gauge needle not less than 2.5 cm (1 inch) in length. Expel any air bubbles from the syringe and needle, and then discharge the contents of the syringe, without emptying the needle, into a standardized, dry cylinder (graduated to contain rather than to deliver the designated volumes) of such size that the volume to be measured occupies at least 40% of its rated volume. Alternatively, the contents of the syringe may be discharged into a dry, tared beaker, the volume, in mL, being calculated as the weight, in g, of Injection taken divided by its density. The contents of two or three 1-mL or 2-mL containers may be pooled for the measurement, provided that a separate, dry syringe assembly is used for each container. The content of containers holding 10 mL or more may be determined by means of opening them and emptying the contents directly into the graduated cylinder or tared beaker.

	Recommended Excess Volume	
Labeled Size	For Mobile Liquids	For Viscous Liquids
0.5 mL	0.10 mL	0.12 mL
1.0 mL	0.10 mL	0.15 mL
2.0 mL	0.15 mL	0.25 mL
5.0 mL	0.30 mL	0.50 mL
10.0 mL	0.50 mL	0.70 mL
20.0 mL	0.60 mL	0.90 mL
30.0 mL	0.80 mL	1.20 mL
50.0 mL or more	2%	3%

The volume is not less than the labeled volume in the case of containers examined individually or, in the case of 1-mL and 2-mL containers, is not less than the sum of the labeled volumes of the containers taken collectively.

For Injections in multiple-dose containers labeled to yield a specific number of doses of a stated volume, proceed as directed in the foregoing, using the same number of separate syringes as the number of doses specified. The volume is such that each syringe delivers not less than the stated dose.

For Injections containing oil, warm the containers, if necessary, and thoroughly shake them immediately before removing the contents. Cool to 25° before measuring the volume.

Particulate Matter—All large-volume Injections for single-dose infusion, and those small-volume Injections for which the monographs specify such requirements, are subject to the particulate matter limits set forth under *Particulate Matter in Injections* ⟨788⟩. An article packaged as both a large-volume and a small-volume Injection meets the requirements set forth for *Small-volume Injections* where the container is labeled as containing 100 mL or less if the individual monograph includes a test for *Particulate matter;* it meets the requirements set forth for *Large-volume Injections for Single-dose Infusion* where the container is labeled as containing more than 100 mL. Injections packaged and labeled for use as irrigating solutions are exempt from requirements for *Particulate matter*.

Sterility Tests—Preparations for injection meet the requirements under *Sterility Tests* ⟨71⟩.

Labeling—[NOTE—See definitions of "label" and "labeling" under *Preservation, Packaging, Storage, and Labeling—Labeling* in the *General Notices*.]

The label states the name of the preparation; in the case of a liquid preparation, the percentage content of drug or amount of drug in a specified volume; in the case of a dry preparation, the

amount of *active* ingredient; the route of administration; a statement of storage conditions and an expiration date; the name of the manufacturer and distributor; and an identifying lot number. The lot number is capable of yielding the complete manufacturing history of the specific package, including all manufacturing, filling, sterilizing, and labeling operations.

Where the individual monograph permits varying concentrations of active ingredients in the large-volume parenteral, the concentration of each ingredient named in the official title is stated as if part of the official title, e.g., Dextrose Injection 5%, or Dextrose (5%) and Sodium Chloride (0.2%) Injection.

The labeling includes the following information, if the complete formula is not specified in the individual monograph: (1) In the case of a liquid preparation, the percentage content of each ingredient or the amount of each ingredient in a specified volume, except that ingredients added to adjust to a given pH or to make the solution isotonic may be declared by name and a statement of their effect; and (2) in the case of a dry preparation or other preparation to which a diluent is intended to be added before use, the amount of each ingredient, the composition of recommended diluent(s) [the name(s) alone, if the formula is specified in the individual monograph], the amount to be used to attain a specific concentration of active ingredient and the final volume of solution so obtained, a brief description of the physical appearance of the constituted solution, directions for proper storage of the constituted solution, and an expiration date limiting the period during which the constituted solution may be expected to have the required or labeled potency if it has been stored as directed.

Containers for Injections that are intended for use as dialysis, hemofiltration, or irrigation solutions and that contain a volume of more than 1 liter are labeled to indicate that the contents are not intended for use by intravenous infusion.

Injections intended for veterinary use are labeled to that effect.

The container is so labeled that a sufficient area of the container remains uncovered for its full length or circumference to permit inspection of the contents.

Packaging and Storage—The volume of Injection in single-dose containers provides the amount specified for parenteral administration at one time and in no case is more than sufficient to permit the withdrawal and administration of 1 liter.

Preparations intended for intraspinal, intracisternal, or peridural administration are packaged only in single-dose containers.

Unless otherwise specified in the individual monograph, no multiple-dose container contains a volume of Injection more than sufficient to permit the withdrawal of 30 mL.

Injections packaged for use as irrigation solutions or for hemofiltration or dialysis or for parenteral nutrition are exempt from the 1-liter restriction of the foregoing requirements relating to packaging. Containers for Injections packaged for use as hemofiltration or irrigation solutions may be designed to empty rapidly and may contain a volume of more than 1 liter.

Injections labeled for veterinary use are exempt from packaging and storage requirements concerning the limitation to single-dose containers and the limitation on the volume of multiple-dose containers.

CONSTITUTED SOLUTIONS

Sterile dosage forms from which constituted solutions are prepared for injection bear titles of the form, *Sterile* ____ or ____ *for Injection.* Since these dosage forms are constituted at the time of use by the health-care practitioner, tests and standards pertaining to the solution as constituted for administration are not included in the individual monographs on sterile dry solids or liquid concentrates. However, in the interest of assuring the quality of injection preparations as they are actually administered, the following nondestructive tests are provided for demonstrating the suitability of constituted solutions when they are prepared just prior to use.

Completeness and Clarity of Solution—Constitute the solution as directed in the labeling supplied by the manufacturer for the sterile dry dosage form.

A: The solid dissolves completely, leaving no visible residue as undissolved matter.

B: The constituted solution is not significantly less clear than an equal volume of the diluent or of Purified Water contained in a similar vessel and examined similarly.

Particulate Matter—Constitute the solution as directed in the labeling supplied by the manufacturer for the sterile dry dosage form: the solution is essentially free from particles of foreign matter that can be observed on visual inspection.

⟨11⟩ USP REFERENCE STANDARDS

USP Reference Standards are established and released under the authority of the USPC Board of Trustees upon recommendation of the USP Reference Standards Committee, which passes on the selection and suitability of each lot. The critical characteristics of each lot of specimen selected for the standard are usually determined independently in three or more laboratories. The USP Drug Research and Testing Laboratory (see *Preface*) and the Food and Drug Administration laboratories participate in testing almost all new Standards and replacements for existing Standards. In addition, laboratories throughout the nation, both academic and industrial, participate in the testing.

Reference Standards are specifically required in many Pharmacopeial assays and tests and are provided solely for such use; suitability for other non-official application(s) rests with the purchase. Originally introduced for the biological assays of USP X, reference standards are now required for numerous other procedures as well. This reflects the extensive use of modern chromatography and spectrophotometry, which require measurements relative to a reference standard to attain accurate and reproducible results.

USP Reference Standards are substances selected for their high purity, critical characteristics, and suitability for the intended purpose. Heterogeneous substances, of natural origin, also are designated "Reference Standards" where needed. Usually these are the counterparts of international standards.

Antibiotic reference standards distributed by the USPC have been designated by the U.S. Food and Drug Administration as identical to FDA working standards under the FDA certification procedures. USPC distributes both U.S. Reference Standards and USP Reference Standards for antibiotic substances. This difference in labeling the Standards is in effect only temporarily, and eventually all vials will bear the same title. Where a USP Reference Standard is called for, the corresponding substance labeled as a "U.S. Reference Standard" may be used, and vice versa.

Reference Standards currently labeled as "NF Reference Standards" will eventually all be designated and labeled as "USP Reference Standards" pursuant to the consolidation of USP and NF within the USPC as of January 2, 1975. Meanwhile, where a USP Reference Standard is called for, the corresponding substance labeled as an "NF Reference Standard" may be used.

Other Reference Substances

As a service, the USPC tests and distributes additional authenticated substances not currently required as USP or NF Reference Standards. These also are provided under the supervision of the USP Reference Standards Committee. These additional substances fall into three groups: (1) former USP and NF Reference Standards, not required in the current USP or NF but for which sufficient demand remains; (2) FCC Reference Standards, specified in the current edition of the Food Chemicals Codex; and (3) Authentic Substances (AS), which are highly purified samples of chemicals, including substances of abuse, that are collaboratively tested and made available as a service primarily to analytical, clinical, pharmaceutical, and research laboratories.

The distribution of controlled substances is subject to the regulations and licensing provisions of the Drug Enforcement Administration of the Department of Justice.

As an additional service, the USPC distributes several noncommercial reagents required in certain USP monographs. These reagents are specially prepared for their intended use and will be distributed by USPC only until they become commercially available.

A program to provide international biological standards and chemical reference substances is maintained by the World Health Organization, an agency of the United Nations. The WHO program is concerned with reference materials for antibiotics, bio-

logicals, and chemotherapeutic agents. As a rule, an International Standard for a material of natural origin is discontinued once the substance responsible for its characteristic activity has been isolated, identified, and prepared in such form that it can be completely characterized by chemical and physical means. The USP Reference Standards Committee collaborates closely with the WHO in order to minimize unavoidable differences in the actual units of potency, and in some cases to share in the preparation of a reference standard. Since some USP Reference Standards are standardized in terms of the corresponding International Standards, the relevant USP Units and the International Units of potency are generally identical.

Proper Use of USP Reference Standards—To serve its intended purpose, each USP Reference Standard must be properly stored, handled, and used. Generally, Reference Standards should be stored in their original stoppered containers away from heat and protected from light. Avoid humid storage areas in particular. Where special storage conditions are necessary, directions are given on the label.

Neither Reference Standards nor Authentic Substances are intended for use as drugs or as medical devices.

Many Pharmacopeial tests and assays are based on comparison of a test specimen with a USP Reference Standard. In such cases, measurements are made on preparations of both the test specimen and the Reference Standard. Where it is directed that a Standard solution or a *Standard preparation* be prepared for a quantitative determination by stepwise dilution or otherwise, it is intended that the Reference Standard substance shall be accurately weighed (see *Weights and Balances* ⟨41⟩ and *Volumetric Apparatus* ⟨31⟩). Due account should also be taken of the relatively large errors associated with weighing small masses (see also *Dilution* under *General Notices*).

Assay and test results are determined on the basis of comparisons of the specimen under test with a USP Reference Standard that has been freed from or corrected for volatile residues or water content as instructed on the label. The same directions are given in the *Reference standard(s)* sections of the applicable monographs. Where specific label instruction differ from the text in the applicable **Reference standard** section(s), the actual labe on the current distributed item will take precedence. such a situation reflects the ability to immediately effect a necessary change, on scientific ground in advance of the written change to the **Reference standard** section(s) via periodic official revisions. Where special drying requirements for Reference Standards are found in specific sections of USP or NF monographs, those supersede the usual instructions (see *Procedures* under *Tests and Assays* in the *General Notices*). Where a USP Reference Standard is required to be dried before using, transfer an amount, sufficient after drying, to a clean and dry vessel. Do not use the original container as the drying vessel, and do not dry a specimen repeatedly at temperatures above 25°. Where the titrimetric determination of water is required at the time a Reference Standard is to be used, proceed as directed for *Method I* under *Water Determination* ⟨921⟩. Instrumental or microanalytical methods are acceptable for this purpose. When using typical amounts, about 50 mg, of the Reference Standard as the test specimen, titrate with a fourfold dilution of the *Reagent*.

Unless a Reference Standard label states a specific potency or content, the Reference Standard is taken as being 100.0% pure for compendial purposes. The suitability of a USP Reference Stadard for noncompendial application is left up to the user.

Current Lots

It is the responsibility of each analyst to ascertain that his particular supply of USP Reference Standard is current. Only sufficient quantity for immedite use should be purchased, and long-term storage should be avoided.

To ensure ready access to the latest information, the USPC publishes the Official Catalog of Reference Standards and Authentic Substances, and the lot designations, bimonthly in *Pharmacopeial Forum*.* This system offers more positive control and flexibility in responding to revisions in Reference Standard usage than would expiration dates. The Catalog in the most recent *Pharmacopeial Forum* identifies items that are official in the USP Reference Standards collection at the time of publication.

Two columns appear in the Catalog to identify the current official lots. One column identifies the official lot currently being shipped by USPC. In some cases, the previous lot may still be considered official. If so, it is identified in the second column. Ordinarily the previous lot is carried in official status for about one year after the current lot entered distribution unless, because of a change in monograph requirements or stability limitations, the previous lot is found to be no longer suitable.

* For nonsubscribers, the most recent Official Catalog is available from: U.S. Pharamcopeial Convention, Inc., Reference Standards Order Department, 12601 Twinbrook Parkway, Rockville, MD 20852. Telephone 1-301-881-0666. FAX 1-301-881-5021. Toll-free telephone 1-800-227-USPC.

Apparatus for Tests and Assays

⟨16⟩ AUTOMATED METHODS OF ANALYSIS

Where a sufficiently large number of similar units are to be subjected routinely to the same type of examination, automated methods of analysis may be far more efficient and precise than manual methods. Such automated methods have been found especially useful in testing the content uniformity of tablets and capsules and in facilitating methods requiring precisely controlled experimental conditions. Many manufacturing establishments, as well as the laboratories of regulatory agencies, have found it convenient to utilize automated methods as alternatives to Pharmacopeial methods (see *Procedures*, under *General Notices*). In addition, the detection system and calculation of results for automated methods are often computerized.

Before an automated method for testing an article is adopted as an alternative, it is advisable to ascertain that the results obtained by the automated method are equivalent in accuracy and precision to those obtained by the prescribed Pharmacopeial method, bearing in mind the further principle stated in the General Notices that "where a difference appears, or in the event of dispute, only the result obtained by the procedure given in this Pharmacopeia is conclusive."

It is necessary to monitor the performance of the automated analytical system continually, by assaying standard preparations of known composition frequently interspersed among the test preparations. Where immiscible solvents are employed in the automated apparatus for rapid extractions, they are often separated for analysis before complete extraction is attained, and the chemical reactions utilized in automated methods rarely are stoichiometric. Both the accuracy and the precision of the determinations depend upon precise adjustment of the equipment, so maintained that all standard and test preparations are exposed to identical physical and chemical manipulations for identical time intervals. Excessive variability in the response of the standard preparations indicates that the analytical system is malfunctioning and that the test results are therefore invalid. However, where automated systems are shown to operate reliably, the precision of the automated method may surpass that of the manual procedure employing the same basic chemistry.

Many of the manual methods given in this Pharmacopeia can be adapted for use in automated equipment incorporating either discrete analyzers or continuous flow systems and operating under a variety of conditions. On the other hand, an analytical scheme devised for a particular automated system may not be readily transposable for use either in a manual procedure or in other types of automated equipment.

The apparatus required for manual methods is, in general, less complicated than the apparatus of automated systems, even those systems used for the direct automated measurement of a single analyte (i.e., the substance being determined or analyzed for) in a binary mixture. However, because of their versatility, automated systems designed for the rapid determination of a specified substance often can be readily modified by the addition of suitable modules and accessories to permit the determination of one or more additional substances in a dosage form. Such extended

systems have been utilized, for example, in the automated analysis of articles containing both estrogens and progestogens.

The accompanying pertinent diagrams represent examples of automated methods. Diagrams for official methods are reproduced here rather than in the individual monographs. The descriptions of the procedural details in these methods exemplify the general approach in automated analysis applicable to dosage forms. It should be noted that the diagrams, with many minutiae, are an indispensable part of the directions for conducting the analysis.

Antibiotics—Hydroxylamine Assay

The following procedure is applicable for the assay of those Pharmacopeial antibiotics, such as cephalosporins and penicillins, that possess the beta-lactam structure.

Apparatus—Automatic analyzer consisting of (1) a liquid sampler, (2) a proportioning pump, (3) suitable spectrophotometers equipped with matched flow cells and analysis capability at 480 nm, (4) a means of recording spectrophotometric readings, and/or computer for data retrieval and calculation, and (5) a manifold consisting of the components illustrated in the accompanying pertinent diagram.

Reagents—

Hydroxylamine hydrochloride solution—Dissolve 20 g of hydroxylamine hydrochloride in 5 mL of polyoxyethylene (23) lauryl ether solution (1 in 1000), and add water to make 1000 mL.

Acetate buffer—Dissolve 173 g of sodium hydroxide and 20.6 g of sodium acetate in water to make 1000 mL. Dilute 75 mL of this solution with water to 500 mL, and mix.

Ferric nitrate solution—Suspend 233 g of ferric nitrate in about 600 mL of water, add 2.8 mL of sulfuric acid, stir until the ferric nitrate is dissolved, add 1 mL of polyoxyethylene (23) lauryl ether, dilute with water to 1000 mL, and mix.

Reference Standard—Use the USP Reference Standard as directed in the individual monograph.

Standard Preparation—Unless otherwise directed in the individual monograph, dissolve an accurately weighed quantity of the Reference Standard in water, and dilute quantitatively with water to obtain a solution having a known concentration of about 1 mg per mL.

Assay Preparation—Unless otherwise directed in the individual monograph, using the specimen under test, prepare as directed under *Standard Preparation*.

Procedure—With the sample line pumping water, the other lines pumping their respective reagents, and the spectrophotometer set at 480 nm, standardize the system until a steady absorbance baseline has been established. Transfer portions of the *Standard Preparation* and the *Assay Preparation* to sampler cups, and place in the sampler. Start the sampler, and conduct determinations of the *Standard Preparation* and the *Assay Preparation* typically at the rate of 40 per hour, using a ratio of about 2:1 for sample and wash time. Calculate the potency by the formula given in the individual monograph, in which C is the concentration, in mg per mL, of USP Reference Standard in the *Standard Preparation*, P is the potency, in µg per mg, of the USP Reference Standard, and A_U and A_S are the absorbances, corrected for the absorbances of the respective blanks, of the solutions from the *Assay Preparation* and the *Standard Preparation*, respectively.

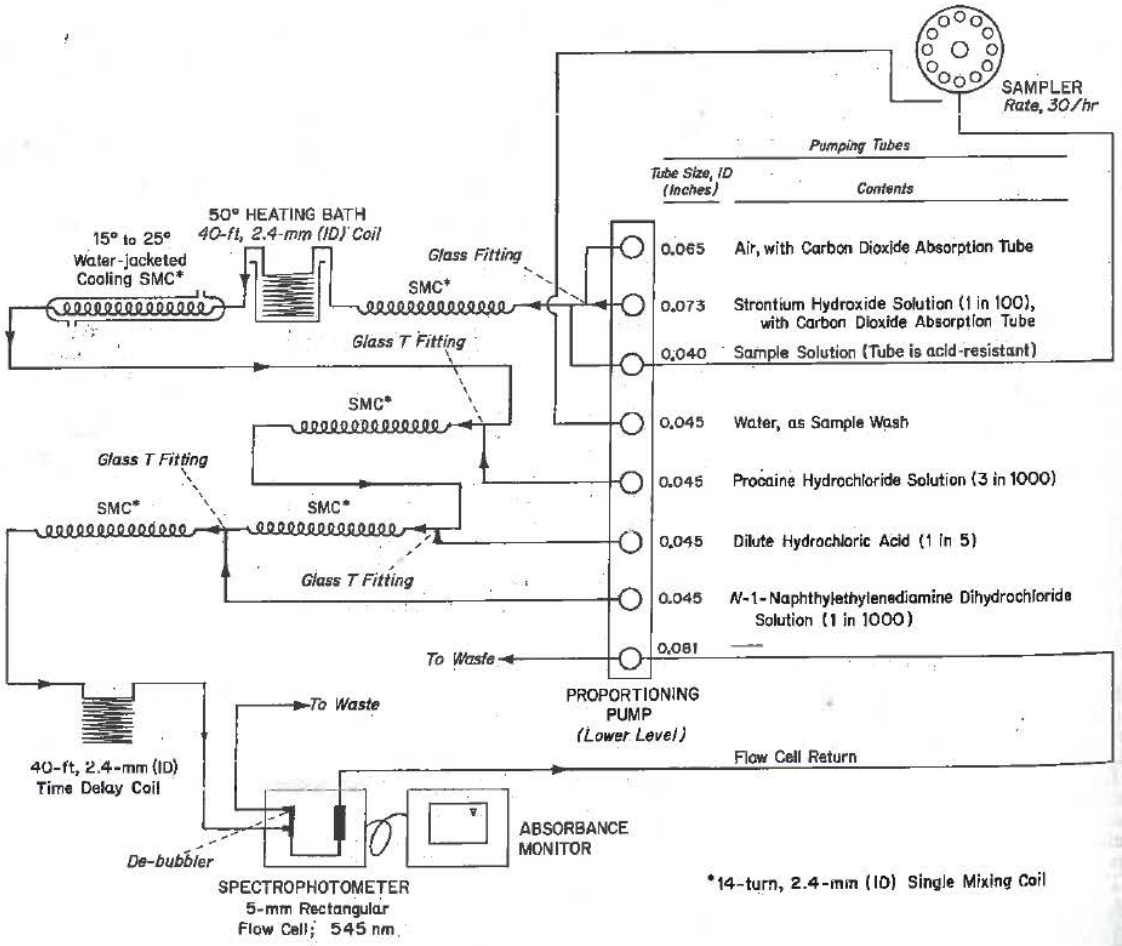


Diagram for Automated Assay for Nitroglycerin Tablets

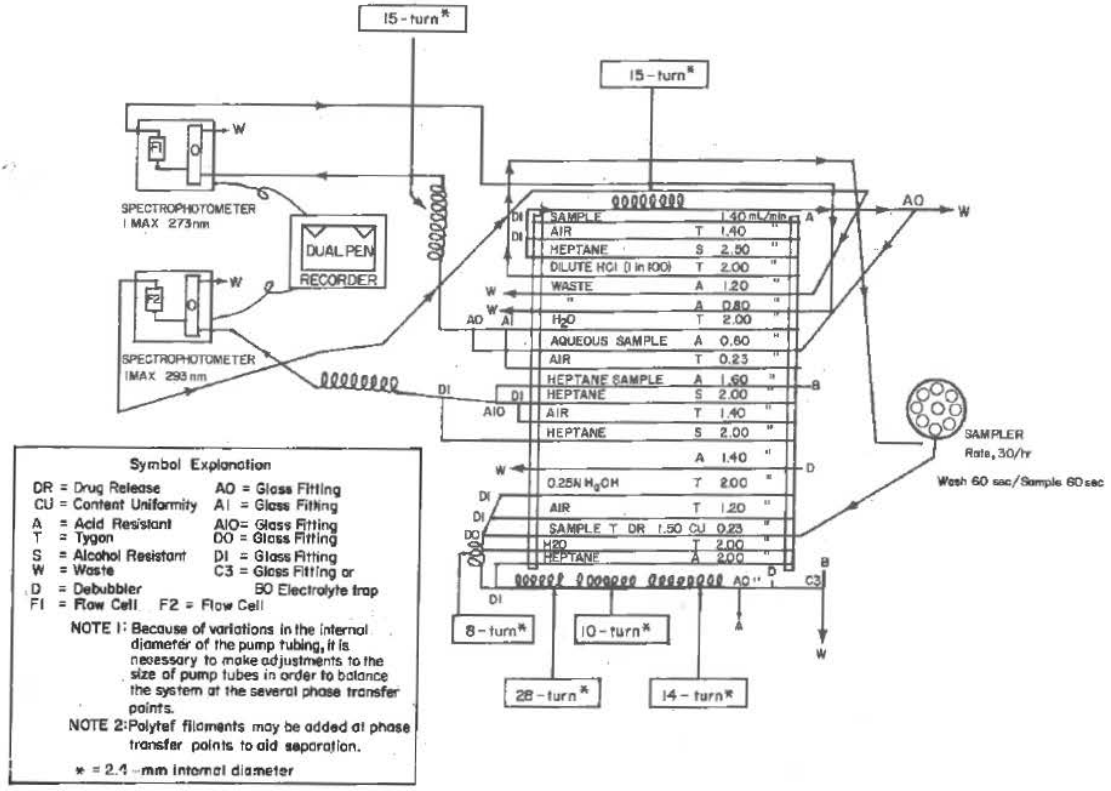
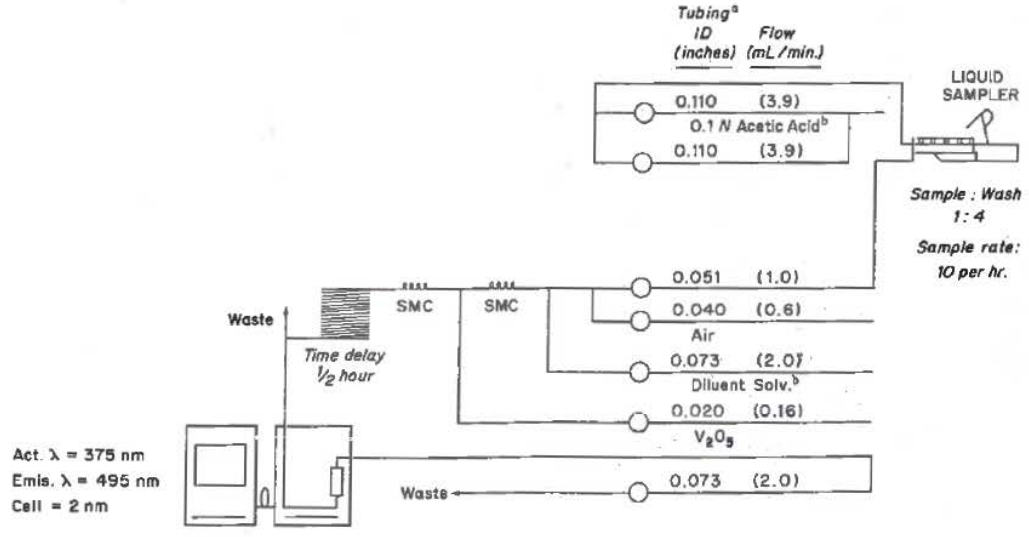


Diagram for Automated Drug Release and Content Uniformity Test for Propranolol Hydrochloride and Hydrochlorothiazide Extended-release Capsules



[a]All tubing is Tygon except for the 0.073-in. tubing, which is silicone.
[b]For the *Content Uniformity* test, use *Phosphoric acid–methanol solution*.

Diagram for Automated Dissolution and Content Uniformity Test for Reserpine Tablets

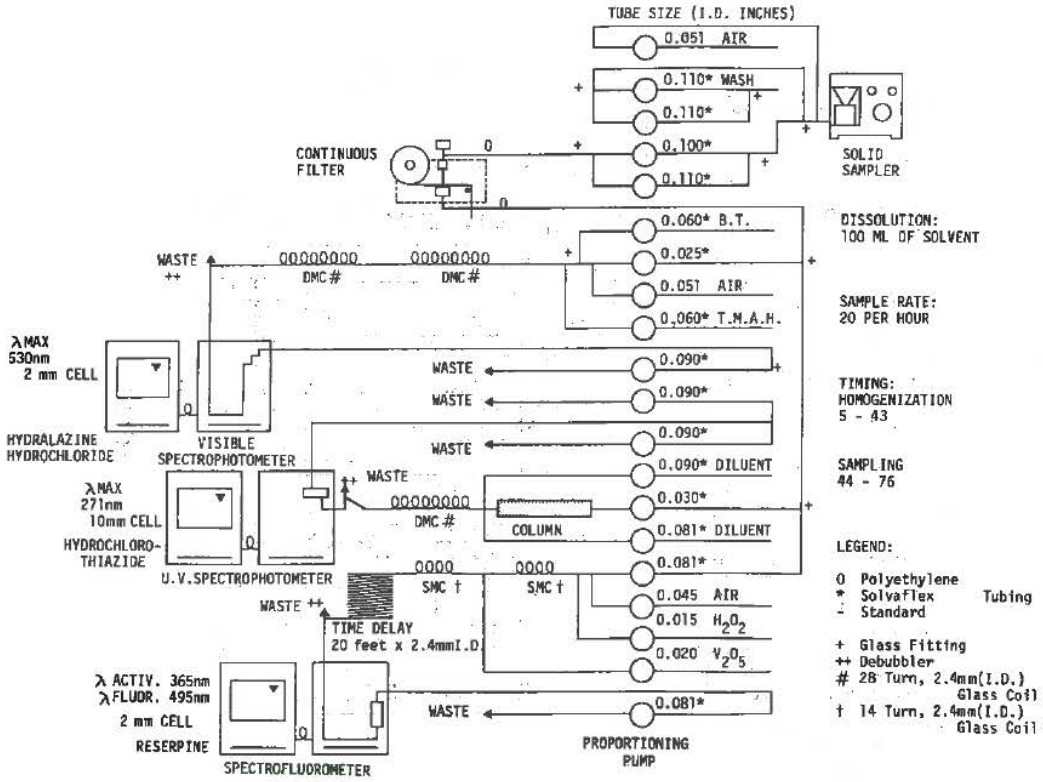


Diagram for Automated Content Uniformity Test for Reserpine, Hydralazine Hydrochloride, and Hydrochlorothiazide Tablets

Content Uniformity of Nitroglycerin Tablets

This is not to be considered as the official method. It is detailed here for further illustration of descriptions of automated methods.

Apparatus—Automatic analyzer consisting of (1) a liquid sampler, (2) a proportioning pump, (3) a heating bath, (4) a suitable spectrophotometer equipped with a 5-mm flow cell and analysis capability at 545 nm, (5) a means of recording spectrophotometric readings, and (6) a manifold consisting of the components illustrated in the accompanying pertinent diagram.

Reagents—

1 Percent strontium hydroxide solution—Dissolve 20.0 g of strontium hydroxide [$Sr(OH)_2.8H_2O$] in 1800 mL of carbon dioxide–free water, heating if necessary. Cool to room temperature, dilute with carbon dioxide–free water to 2000 mL, and mix. Allow to stand overnight, and filter. Store the clear solution in tightly closed containers, protected from carbon dioxide.

0.3 Percent procaine hydrochloride solution—Dissolve 3.0 g of procaine hydrochloride in water to make 1000 mL.

0.1 Percent N-1-naphthylethylenediamine dihydrochloride solution—Dissolve 1.0 g of *N*-1-naphthylethylenediamine dihydrochloride in water to make 1000 mL. Prepare fresh each week.

Standard Preparation—Dissolve an accurately weighed portion of 10 percent nitroglycerin–betalactose absorbate, previously standardized, in water, and dilute quantitatively and stepwise with water to obtain a solution having a known concentration of about 30 μg per mL.

Test Preparation—Dissolve 1 Nitroglycerin Tablet in water to obtain a solution having a concentration of about 30 μg of nitroglycerin per mL.

Procedure—With the sample line pumping water, the other lines pumping their respective reagents, and the spectrophotometer set at 545 nm, standardize the system by pumping until a

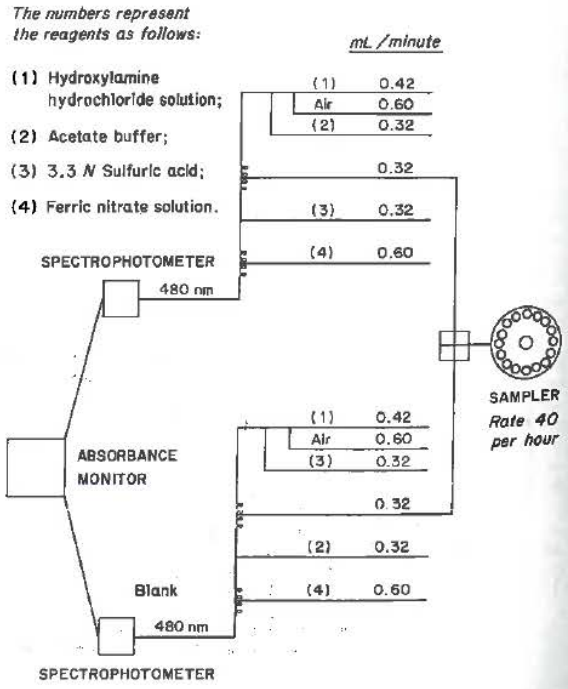


Diagram for Automated Hydroxylamine Assay for Antibiotics

steady absorbance baseline has been established. Transfer portions of the *Standard Preparation* and the *Test Preparation* to sampler cups, and place in the sampler. Start the sampler, and conduct determinations of the *Standard Preparation* and the *Test Preparation* at a rate of 30 per hour, using a ratio of 1:1 for sample and wash time. First, run 2 standards, discarding the first value, then continue the run using 1 standard after each 5 samples, recording the absorbance values. Calculate the quantity, in mg, of $C_3H_5N_3O_9$ in the Tablet by the formula:

$$(T/D)C(A_U/A_S),$$

in which T is the labeled quantity, in mg, of nitroglycerin in the Tablet, D is the concentration, in µg per mL, of nitroglycerin in the solution from the Tablet, based on the labeled quantity per Tablet and the extent of dilution, C is the concentration, in µg per mL, of nitroglycerin in the *Standard Preparation*, A_U is the absorbance of the *Test Preparation*, and A_S is the average of the absorbances of the two *Standard Preparations* that bracket the *Test Preparation*.

Diagrams

The preceding diagrams are arranged in alphabetic order by the name of the drug first mentioned, where the diagram is for a procedure for a specific article. Diagrams pertaining to general classes of articles, e.g., *Diagram for Automated Hydroxylamine Assay for Antibiotics*, appear after that alphabetic series.

⟨21⟩ THERMOMETERS

Thermometers suitable for Pharmacopeial tests conform to specifications of the American Society for Testing and Materials, ASTM Standards E 1, and are standardized in accordance with ASTM Method E 77.

The thermometers are of the mercury-in-glass type, and the column above the liquid is filled with nitrogen. Thermometers may be standardized for total immersion or for partial immersion. Insofar as practicable, each thermometer should be employed according to the condition of immersion under which it was standardized.

Standardization for total immersion involves immersion of the thermometer to the top of the mercury column, with the remainder of the stem and the upper expansion chamber exposed to ambient temperature. Standardization for partial immersion involves immersion of the thermometer to the indicated immersion line etched on the front of the thermometer, with the remainder of the stem exposed to ambient temperature. For use under other conditions of immersion, an emergent stem correction is necessary to obtain correct temperature readings.

In the selection of a thermometer, careful consideration of the conditions under which it is to be used is essential. The accompanying table lists specifications for a number of thermometers suitable for use in Pharmacopeial tests. The lower and upper limits of temperature range specified in the table are to be regarded as inclusive.

Thermometer Specifications

ASTM No. E 1	Temperature Range (°C)	Graduations (°C)	Immersion (mm)
Thermometers for General Use, Including Melting Range Determinations			
1C	−20 to 150	1	76
2C	−5 to 300	1	76
3C	−5 to 400	1	76
Thermometers for Boiling or Distilling Range or Temperature Determinations			
37C	−2 to 52	0.2	100
38C	24 to 78	0.2	100
39C	48 to 102	0.2	100
40C	72 to 126	0.2	100
41C	98 to 152	0.2	100
102C	123 to 177	0.2	100
103C	148 to 202	0.2	100
104C	173 to 227	0.2	100
105C	198 to 252	0.2	100
106C	223 to 277	0.2	100
107C	248 to 302	0.2	100
Thermometers for Congealing Range or Temperature Determinations			
89C	−20 to 10	0.1	76
90C	0 to 30	0.1	76
91C	20 to 50	0.1	76
92C	40 to 70	0.1	76
93C	60 to 90	0.1	76
94C	80 to 110	0.1	76
95C	100 to 130	0.1	76
96C	120 to 150	0.1	76

NOTE—The revised centigrade scale attributed to Celsius differs slightly from the so-called centigrade scale heretofore accepted. However, the magnitude of the difference is negligible, and the two scales are considered equally suitable for Pharmacopeial purposes.

⟨31⟩ VOLUMETRIC APPARATUS

Most of the volumetric apparatus available in the United States is calibrated at 20°, although the temperatures generally prevailing in laboratories more nearly approach 25°, which is the temperature specified generally for Pharmacopeial tests and assays. This discrepancy is inconsequential provided the room temperature is reasonably constant.

Use—To attain the degree of precision required in many Pharmacopeial assays involving volumetric measurements and directing that a quantity be "accurately measured," the apparatus must be chosen and used with care. A buret should be of such size that the titrant volume represents not less than 30% of the nominal volume. Where less than 10 mL of titrant is to be measured, a 10-mL buret or a microburet generally is required.

The design of volumetric apparatus is an important factor in assuring accuracy. For example, the length of the graduated portions of graduated cylinders should be not less than five times the inside diameter, and the tips of burets and pipets should restrict the outflow rate to not more than 500 µL per second.

Standards of Accuracy—The capacity tolerances for volumetric flasks, transfer pipets, and burets are those accepted by the National Institute of Standards and Technology (Class A),* as indicated in the accompanying tables found at the top of the next page.

The capacity tolerances for measuring (i.e., "graduated") pipets of up to and including 10-mL capacity are somewhat larger than those for the corresponding sizes of transfer pipets, namely, 10, 20, and 30 µL for the 2-, 5-, and 10-mL sizes, respectively.

Transfer and measuring pipets calibrated "to deliver" should be drained in a vertical position and then touched against the wall of the receiving vessel to drain the tips. Volume readings on burets should be estimated to the nearest 0.01 mL for 25- and 50-mL burets, and to the nearest 0.005 mL for 5- and 10-mL burets. Pipets calibrated "to contain" are called for in special cases, generally for measuring viscous fluids like syrups; however, a volumetric flask may be substituted for a "to contain" pipet. In such cases, the pipet or flask should be washed clean, after draining, and the washings added to the measured portion.

⟨41⟩ WEIGHTS AND BALANCES

Pharmacopeial tests and assays require the use of balances that vary in capacity, sensitivity, and reproducibility. The accuracy needed for a weighing dictates the type of balance and the class of weights required for that weighing. Where substances are to be "accurately weighed," the weighing is to be performed so as to limit the error to not more than 0.1%. For example, a quantity of 50 mg is to be weighed so that the error does not exceed 50

* See "Testing of Glass Volumetric Apparatus," N.B.S. Circ. 602, April 1, 1959, and NTIS COM-73-10504, National Technical Information Service.

Volumetric Flasks

Designated volume, mL	10	25	50	100	250	500	1000
Limit of error, mL	0.02	0.03	0.05	0.08	0.12	0.15	0.30
Limit of error, %	0.20	0.12	0.10	0.08	0.05	0.03	0.03

Transfer Pipets

Designated volume, mL	1	2	5	10	25	50	100
Limit of error, mL	0.006	0.006	0.01	0.02	0.03	0.05	0.08
Limit of error, %	0.60	0.30	0.20	0.20	0.12	0.10	0.08

Burets

Designated volume, mL	10 ("micro" type)	25	50
Subdivisions, mL	0.02	0.10	0.10
Limit of error, mL	0.02	0.03	0.05

μg. A balance should be chosen such that the value of three times the standard deviation of the reproducibility of the instrument, divided by the amount to be weighed, does not exceed 0.001.

A weight classification should be chosen so as to limit the error to 0.1%. This generally means that Class P weights can be used for quantities greater than 100 mg, Class S-1 for quantities greater than 50 mg, Class S for quantities greater than 20 mg, and Class M for quantities greater than 10 mg. Quantities of less than 10 mg may be weighed on balances having appropriate reproducibilities and designed to afford electrical or optical methods for accurately subdividing a 10-mg, full-scale range, after calibration with a 10-mg, Class M weight.

The tolerances shown in the accompanying table are for new or newly adjusted weights. For weights that have been in use, the tolerances are somewhat larger, as follows:

Class M: 100-, 200-, 300-, and 500-mg denominations—10.5 μg; and 20.0 μg for the group.

Class S: 100-mg and heavier denominations—Twice the values shown in the accompanying table (for individual and group).

Class S-1: Same as shown in the accompanying table.

Class P: For all weights—Twice the values shown in the accompanying table.

Weights should be calibrated periodically, preferably against an absolute standard weight.

Tolerances for New Weights in Sets

Denomination g	Class M Individual μg	Class M Group μg	Class S Individual μg	Class S Group μg	Class S-1 Individual μg	Class P Individual μg
100	500		250		1000	2000
50	250		120		600	1200
30	150		74	154	450	900
20	100		74	"	350	700
10	50		74	"	250	500
5	34	65	54	105	180	360
3	34	"	54	"	150	300
2	34	"	54	"	130	260
1	34	"	54	"	100	200
mg						
500	5.4	10.5	25	55	80	160
300	5.4	"	25	"	70	140
200	5.4	"	25	"	60	120
100	5.4	"	25	"	50	100
50	5.4	10.5	14	34	42	85
30	5.4	"	14	"	38	75
20	5.4	"	14	"	35	70
10	5.4	"	14	"	30	60
5	5.4	10.5	14	34	28	55
3	5.4	"	14	"	26	52
2	5.4	"	14	"	25	50
1	5.4	"	14	"	25	50

NOTE—Not more than one-third of Class S-1 weights are in error by more than one-half of the tabulated tolerances.

Microbiological Tests

⟨51⟩ ANTIMICROBIAL PRESERVATIVES—EFFECTIVENESS

Antimicrobial preservatives are substances added to dosage forms to protect them from microbial contamination. They are used primarily in multiple-dose containers to inhibit the growth of microorganisms that may be introduced inadvertently during or subsequent to the manufacturing process. Antimicrobial agents should not be used solely to reduce the viable microbial count as a substitute for good manufacturing practice. Situations may arise, however, where their use may be required to minimize proliferation of microorganisms. It should be recognized that the presence of dead microorganisms or the metabolic by-products of living microorganisms may cause adverse reactions in sensitized persons.

Any antimicrobial agent may exhibit the protective properties of a preservative. However, all useful antimicrobial agents are toxic substances. For maximum protection of the consumer, the concentration of the preservative shown to be effective in the final packaged product should be considerably below the concentrations of the preservative that may be toxic to human beings.

The following tests are provided to demonstrate, in multiple-dose parenteral, otic, nasal, and ophthalmic products made with aqueous bases or vehicles, the effectiveness of any added antimicrobial preservative(s), the presence of which is declared on the label of the product concerned. The tests and standards apply only to the product in the original, unopened container in which it was distributed by the producer.[1]

Test Organisms—Use cultures of the following microorganisms:[2] *Candida albicans* (ATCC No. 10231), *Aspergillus niger* (ATCC No. 16404), *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027), and *Staphylococcus aureus* (ATCC No. 6538). Other microorganisms, in addition to those listed, may be included in the test on an optional basis, especially if it appears likely that such microorganisms may represent contaminants likely to be introduced during use of the article.

Media—For the initial cultivation of the test organisms, select an agar medium that is favorable to vigorous growth of the respective stock culture, such as Soybean-Casein Digest Agar Medium (see under *Microbial Limit Tests* ⟨61⟩).

Preparation of Inoculum—Preparatory to the test, inoculate the surface of a suitable volume of solid agar medium from a recently grown stock culture of each of the specified microorganisms. Incubate the bacterial cultures at 30° to 35° for 18 to

[1] For products made with nonaqueous (anhydrous) bases or vehicles, a suitable test may be feasible only at a particular stage in manufacture.

[2] Available from American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

24 hours, the culture of *C. albicans* at 20° to 25° for 48 hours, and the culture of *A. niger* at 20° to 25° for 1 week.

To harvest the bacterial and *C. albicans* cultures, use sterile saline TS, washing the surface growth into a suitable vessel, and add sufficient additional saline TS to reduce the microbial count to about 100 million microorganisms per mL. To harvest the *A. niger* culture, use sterile saline TS containing 0.05% of polysorbate 80, and adjust the spore count to about 100 million per mL by adding more sterile saline TS.

Alternatively, the stock culture organisms may be grown in a suitable liquid medium, and the cells may be harvested by centrifugation, washed, and resuspended in sterile saline TS to give the required microbial or spore count.

Determine the number of colony-forming units per mL in each suspension. This value serves to determine the size of inoculum to use in the test. If the standardized suspensions are not used promptly, periodically monitor the suspensions by the plate-count method to determine any loss of viability.

For the plate-count monitoring of inoculated test preparations, use an agar medium corresponding to that used for the initial cultivation of the respective microorganism. Where a specific inactivator of the preservative(s) is available, add a suitable amount of it to the agar plate count medium.

Procedure—Where the product container can be entered aseptically, such as with a needle and syringe through a rubber stopper, conduct the test in five original product containers. If the product container is such that it cannot be entered aseptically, transfer 20-mL samples of the product to each of five sterile, capped bacteriological tubes of suitable size. Inoculate each tube or product container with one of the standardized microbial suspensions, using a ratio equivalent to 0.10 mL of inoculum to 20 mL of product, and mix. A suitable concentration of test microorganisms should be added so that the concentration in the test preparation immediately after inoculation is between 100,000 and 1,000,000 microorganisms per mL. Determine the number of viable microorganisms in each inoculum suspension, and calculate the initial concentration of microorganisms per mL of product under test by the plate-count method.

Incubate the inoculated containers or tubes at 20° to 25°. Examine the containers or tubes at 7, 14, 21, and 28 days subsequent to inoculation. Record any changes observed in appearance, and determine by the plate-count procedure the number of viable microorganisms present at each of these time intervals. Using the theoretical concentrations of microorganisms present at the start of the test, calculate the percentage change in the concentration of each microorganism during the test.

Interpretation—The preservative is effective in the product examined if (a) the concentrations of viable bacteria are reduced to not more than 0.1% of the initial concentrations by the fourteenth day; (b) the concentrations of viable yeasts and molds remain at or below the initial concentrations during the first 14 days; and (c) the concentration of each test microorganism remains at or below these designated levels during the remainder of the 28-day test period.

⟨61⟩ MICROBIAL LIMIT TESTS

This chapter provides tests for the estimation of the number of viable aerobic microorganisms present and for freedom from designated microbial species in pharmaceutical articles of all kinds, from raw materials to the finished forms. An automated method may be substituted for the tests presented here, provided it has been properly validated as giving equivalent or better results. In preparing for and in applying the tests, observe aseptic precautions in handling the specimens. Unless otherwise directed, where a procedure specifies simply "incubate," hold the container in a place that is thermostatically controlled at a temperature between 30° and 35°, for a period of 24 to 48 hours. The term "growth" is used in a special sense herein, i.e., to designate *the presence and presumed proliferation of viable microorganisms.*

Preparatory Testing

The validity of the results of the tests set forth in this chapter rests largely upon the adequacy of a demonstration that the test specimens to which they are applied do not, of themselves, inhibit the multiplication, under the test conditions, of microorganisms that may be present. Therefore, preparatory to conducting the tests on a regular basis and as circumstances require subsequently, inoculate diluted specimens of the material to be tested with separate viable cultures of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella.* This can be done by adding 1 mL of not less than 10^{-3} dilution of a 24-hour broth culture of the microorganism to the first dilution (in pH 7.2 Phosphate Buffer, Fluid Soybean–Casein Digest Medium, or Fluid Lactose Medium) of the test material and following the test procedure. Failure of the organism(s) to grow in the relevant medium invalidates that portion of the examination and necessitates a modification of the procedure by (1) an increase in the volume of diluent, the quantity of test material remaining the same, or by (2) the incorporation of a sufficient quantity of suitable inactivating agent(s) in the diluents, or by (3) an appropriate combination of modifications (1) and (2) so as to permit growth of the inocula.

The following are examples of ingredients and their concentrations that may be added to the culture medium to neutralize inhibitory substances present in the sample: soy lecithin, 0.5%; and polysorbate 20, 4.0%. Alternatively, repeat the test as described in the preceding paragraph, using Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium to demonstrate neutralization of preservatives or other antimicrobial agents in the test material. Where inhibitory substances are contained in the product and the latter is soluble, a suitable, validated adaptation of a procedure set forth in the *Test Procedures Using Membrane Filtration,* under *Sterility Tests* ⟨71⟩, may be used.

If in spite of the incorporation of suitable inactivating agents and a substantial increase in the volume of diluent it is still not possible to recover the viable cultures described above and where the article is not suitable for employment of membrane filtration, it can be assumed that the failure to isolate the inoculated organism is attributable to the bactericidal activity of the product. This information serves to indicate that the article is not likely to be contaminated with the given species of microorganism. Monitoring should be continued in order to establish the spectrum of inhibition and bactericidal activity of the article.

Buffer Solution and Media

Culture media may be prepared as follows, or dehydrated culture media may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have similar ingredients and/or yield media comparable to those obtained from the formulas given herein.

In preparing media by the formulas set forth herein, dissolve the soluble solids in the water, using heat, if necessary, to effect complete solution, and add solutions of hydrochloric acid or sodium hydroxide in quantities sufficient to yield the desired pH in the medium when it is ready for use. Determine the pH at 25 ± 2°.

Where agar is called for in a formula, use agar that has a moisture content of not more than 15%. Where water is called for in a formula, use *Purified Water.*

pH 7.2 Phosphate Buffer

Stock Solution—Dissolve 34 g of monobasic potassium phosphate in about 500 mL of water contained in a 1000-mL volumetric flask. Adjust to pH 7.2 ± 0.1 by the addition of sodium hydroxide TS (about 175 mL), add water to volume, and mix. Dispense and sterilize. Store under refrigeration.

For use, dilute the *Stock Solution* with water in the ratio of 1 to 800, and sterilize.

MEDIA

Unless otherwise indicated, the media should be sterilized by heating in an autoclave (see *Steam Sterilization* under *Sterilization* ⟨1211⟩), the exposure time depending on the volume to be sterilized.

I. Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium

Pancreatic Digest of Casein	20 g
Soy Lecithin	5 g
Polysorbate 20	40 mL
Water	960 mL

Dissolve the pancreatic digest of casein and soy lecithin in 960 mL of water, heating in a water bath at 48° to 50° for about 30 minutes to effect solution. Add 40 mL of polysorbate 20. Mix, and dispense as desired.

II. Soybean-Casein Digest Agar Medium

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

pH after sterilization: 7.3 ± 0.2.

III. Fluid Soybean-Casein Digest Medium

Prepare as directed for *Soybean-Casein Digest Medium* under *Sterility Tests* ⟨71⟩.

IV. Mannitol-Salt Agar Medium

Pancreatic Digest of Casein	5.0 g
Peptic Digest of Animal Tissue	5.0 g
Beef Extract	1.0 g
D-Mannitol	10.0 g
Sodium Chloride	75.0 g
Agar	15.0 g
Phenol Red	0.025 g
Water	1000 mL

Mix, then heat with frequent agitation, and boil for 1 minute to effect solution.
pH after sterilization: 7.4 ± 0.2.

V. Baird-Parker Agar Medium

Pancreatic Digest of Casein	10.0 g
Beef Extract	5.0 g
Yeast Extract	1.0 g
Lithium Chloride	5.0 g
Agar	20.0 g
Glycine	12.0 g
Sodium Pyruvate	10.0 g
Water	950 mL

Heat with frequent agitation, and boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 10 mL of sterile potassium tellurite solution (1 in 100) and 50 mL of egg-yolk emulsion. Mix intimately but gently, and pour into plates. (Prepare the egg-yolk emulsion by disinfecting the surface of whole shell eggs, aseptically cracking the eggs, and separating out intact yolks into a sterile graduated cylinder. Add sterile saline TS to obtain a 3 to 7 ratio of egg yolk to saline. Add to a sterile blender cup, and mix at high speed for 5 seconds.)
pH after sterilization: 6.8 ± 0.2.

VI. Vogel-Johnson Agar Medium

Pancreatic Digest of Casein	10.0 g
Yeast Extract	5.0 g
Mannitol	10.0 g
Dibasic Potassium Phosphate	5.0 g
Lithium Chloride	5.0 g
Glycine	10.0 g
Agar	16.0 g
Phenol Red	25.0 mg
Water	1000 mL

Boil the solution of solids for 1 minute. Sterilize, cool to between 45° and 50°, and add 20 mL of sterile potassium tellurite solution (1 in 100).
pH after sterilization: 7.2 ± 0.2.

VII. Cetrimide Agar Medium

Pancreatic Digest of Gelatin	20.0 g
Magnesium Chloride	1.4 g
Potassium Sulfate	10.0 g
Agar	13.6 g
Cetyl Trimethylammonium Bromide (Cetrimide)	0.3 g
Glycerin	10.0 mL
Water	1000 mL

Dissolve all solid components in the water, and add the glycerin. Heat, with frequent agitation, and boil for 1 minute to effect solution.
pH after sterilization: 7.2 ± 0.2.

VIII. Pseudomonas Agar Medium for Detection of Fluorescin

Pancreatic Digest of Casein	10.0 g
Peptic Digest of Animal Tissue	10.0 g
Anhydrous Dibasic Potassium Phosphate	1.5 g
Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	1.5 g
Glycerin	10.0 mL
Agar	15.0 g
Water	1000 mL

Dissolve the solid components in the water before adding the glycerin. Heat, with frequent agitation, and boil for 1 minute to effect solution.
pH after sterilization: 7.2 ± 0.2.

IX. Pseudomonas Agar Medium for Detection of Pyocyanin

Pancreatic Digest of Gelatin	20.0 g
Anhydrous Magnesium Chloride	1.4 g
Anhydrous Potassium Sulfate	10.0 g
Agar	15.0 g
Glycerin	10.0 mL
Water	1000 mL

Dissolve the solid components in the water before adding the glycerin. Heat, with frequent agitation, and boil for 1 minute to effect solution.
pH after sterilization: 7.2 ± 0.2.

X. Fluid Lactose Medium

Beef Extract	3.0 g
Pancreatic Digest of Gelatin	5.0 g
Lactose	5.0 g
Water	1000 mL

Cool as quickly as possible after sterilization.
pH after sterilization: 6.9 ± 0.2.

XI. Fluid Selenite-Cystine Medium

Pancreatic Digest of Casein	5.0 g
Lactose	4.0 g
Sodium Phosphate	10.0 g
Sodium Acid Selenite	4.0 g
L-Cystine	10.0 mg
Water	1000 mL

Final pH: 7.0 ± 0.2.
Mix, and heat to effect solution. Heat in flowing steam for 15 minutes. *Do not sterilize.*

XII. Fluid Tetrathionate Medium

Pancreatic Digest of Casein	2.5 g
Peptic Digest of Animal Tissue	2.5 g
Bile Salts	1.0 g
Calcium Carbonate	10.0 g
Sodium Thiosulfate	30.0 g
Water	1000 mL

Heat the solution of solids to boiling. On the day of use, add a solution prepared by dissolving 5 g of potassium iodide and 6 g of iodine in 20 mL of water. Then add 10 mL of a solution of brilliant green (1 in 1000), and mix. Do not heat the medium after adding the brilliant green solution.

XIII. Brilliant Green Agar Medium

Yeast Extract	3.0 g
Peptic Digest of Animal Tissue	5.0 g
Pancreatic Digest of Casein	5.0 g
Lactose	10.0 g
Sodium Chloride	5.0 g
Sucrose	10.0 g
Phenol Red	80 mg
Agar	20.0 g
Brilliant Green	12.5 mg
Water	1000 mL

Boil the solution of solids for 1 minute. Sterilize just prior to use, melt the medium, pour into Petri dishes, and allow to cool.

pH after sterilization: 6.9 ± 0.2.

XIV. Xylose-Lysine-Desoxycholate Agar Medium

Xylose	3.5 g
L-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium Chloride	5.0 g
Yeast Extract	3.0 g
Phenol Red	80 mg
Agar	13.5 g
Sodium Desoxycholate	2.5 g
Sodium Thiosulfate	6.8 g
Ferric Ammonium Citrate	800 mg
Water	1000 mL

Final pH: 7.4 ± 0.2.

Heat the mixture of solids and water, with swirling, just to the boiling point. *Do not overheat or sterilize.* Transfer at once to a water bath maintained at about 50°, and pour into plates as soon as the medium has cooled.

XV. Bismuth Sulfite Agar Medium

Beef Extract	5.0 g
Pancreatic Digest of Casein	5.0 g
Peptic Digest of Animal Tissue	5.0 g
Dextrose	5.0 g
Sodium Phosphate	4.0 g
Ferrous Sulfate	300 mg
Bismuth Sulfite Indicator	8.0 g
Agar	20.0 g
Brilliant Green	25 mg
Water	1000 mL

Final pH: 7.6 ± 0.2.

Heat the mixture of solids and water, with swirling, just to the boiling point. *Do not overheat or sterilize.* Transfer at once to a water bath maintained at about 50°, and pour into plates as soon as the medium has cooled.

XVI. Triple Sugar-Iron-Agar Medium

Pancreatic Digest of Casein	10.0 g
Pancreatic Digest of Animal Tissue	10.0 g
Lactose	10.0 g
Sucrose	10.0 g
Dextrose	1.0 g
Ferrous Ammonium Sulfate	200 mg
Sodium Chloride	5.0 g
Sodium Thiosulfate	200 mg
Agar	13.0 g
Phenol Red	25 mg
Water	1000 mL

pH after sterilization: 7.3 ± 0.2.

XVII. MacConkey Agar Medium

Pancreatic Digest of Gelatin	17.0 g
Pancreatic Digest of Casein	1.5 g
Peptic Digest of Animal Tissue	1.5 g
Lactose	10.0 g
Bile Salts Mixture	1.5 g
Sodium Chloride	5.0 g
Agar	13.5 g
Neutral Red	30 mg
Crystal Violet	1.0 mg
Water	1000 mL

Boil the mixture of solids and water for 1 minute to effect solution.

pH after sterilization: 7.1 ± 0.2.

XVIII. Levine Eosin–Methylene Blue Agar Medium

Pancreatic Digest of Gelatin	10.0 g
Dibasic Potassium Phosphate	2.0 g
Agar	15.0 g
Lactose	10.0 g
Eosin Y	400 mg
Methylene Blue	65 mg
Water	1000 mL

Dissolve the pancreatic digest of gelatin, the dibasic potassium phosphate, and the agar in the water, with warming, and allow to cool. Just prior to use, liquefy the gelled agar solution, add the remaining ingredients, as solutions, in the following amounts, and mix: for each 100 mL of the liquefied agar solution—5 mL of lactose solution (1 in 5), 2 mL of the eosin Y solution (1 in 50), and 2 mL of methylene blue solution (1 in 300). The finished medium may not be clear.

pH after sterilization: 7.1 ± 0.2.

XIX. Sabouraud Dextrose Agar Medium

Dextrose	40 g
Mixture of equal parts of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein	10 g
Agar	15 g
Water	1000 mL

Mix, and boil to effect solution.

pH after sterilization: 5.6 ± 0.2.

XX. Potato Dextrose Agar Medium

Cook 300 g of peeled and diced potatoes in 500 mL of water prepared by distillation, filter through cheesecloth, add water prepared by distillation to make 1000 mL, and add the following:

Agar	15 g
Glucose	20 g

Dissolve by heating, and sterilize.

pH after sterilization: 5.6 ± 0.2.

For use, just prior to pouring the plates, adjust the melted and cooled to 45° medium with sterile tartaric acid solution (1 in 10) to a pH of 3.5 ± 0.1. Do not reheat the pH 3.5 medium.

Sampling

Provide separate 10-mL or 10-g specimens for each of the tests called for in the individual monograph.

Procedure

Prepare the specimen to be tested, by treatment that is appropriate to its physical characteristics and that does not alter the number and kind of microorganisms originally present, in order to obtain a solution or suspension of all or part of it in a form suitable for the test procedure(s) to be carried out.

For a solid that dissolves to an appreciable extent but not completely, reduce the substance to a moderately fine powder, suspend it in the vehicle specified, and proceed as directed under *Total Aerobic Microbial Count*, and under *Test for Staphylococcus aureus and Pseudomonas aeruginosa* and *Test for Salmonella Species and Escherichia coli.*

For a fluid specimen that consists of a true solution, or a suspension in water or a hydroalcoholic vehicle containing less than 30 percent of alcohol, and for a solid that dissolves readily and practically completely in 90 mL of *pH 7.2 Phosphate Buffer* or the media specified, proceed as directed under *Total Aerobic Microbial Count*, and under *Test for Staphylococcus aureus and Pseudomonas aeruginosa* and *Test for Salmonella Species and Escherichia coli.*

For water-immiscible fluids, ointments, creams, and waxes, prepare a suspension with the aid of a minimal quantity of a suitable, sterile emulsifying agent (such as one of the polysorbates), using a mechanical blender and warming to a temperature not exceeding 45°, if necessary, and proceed with the suspension as directed under *Total Aerobic Microbial Count*, and under *Test for Staphylococcus aureus and Pseudomonas aeruginosa* and *Test for Salmonella Species and Escherichia coli.*

For a fluid specimen in aerosol form, chill the container in an alcohol–dry ice mixture for approximately 1 hour, cut open the container, allow it to reach room temperature, permit the propellant to escape, or warm to drive off the propellant if feasible, and transfer the quantity of test material required for the procedures specified in one of the two preceding paragraphs, as appropriate. Where 10.0 g or 10.0 mL of the specimen, whichever is applicable, cannot be obtained from 10 containers in aerosol form, transfer the entire contents from 10 chilled containers to the culture medium, permit the propellant to escape, and proceed with the test on the residues. If the results of the test are inconclusive or doubtful, repeat the test with a specimen from 20 more containers.

Total Aerobic Microbial Count—For specimens that are sufficiently soluble or translucent to permit use of the *Plate Method*, use that method; otherwise, use the *Multiple-tube Method*. With

either method, first dissolve or suspend 10.0 g of the specimen if it is a solid, or 10 mL, accurately measured, if the specimen is a liquid, in pH 7.2 Phosphate Buffer, Fluid Soybean-Casein Digest Medium, or Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium to make 100 mL. For viscous specimens that cannot be pipeted at this initial 1:10 dilution, dilute the specimen until a suspension is obtained, i.e., 1:50 or 1:100, etc., that can be pipeted. Perform the test for absence of inhibitory (antimicrobial) properties as described under *Preparatory Testing* before the determination of *Total Aerobic Microbial Count*. Add the specimen to the medium not more than 1 hour after preparing the appropriate dilutions for inoculation.

PLATE METHOD—Dilute further, if necessary, the fluid so that 1 mL will be expected to yield between 30 and 300 colonies. Pipet 1 mL of the final dilution onto each of two sterile petri dishes. Promptly add to each dish 15 to 20 mL of Soybean-Casein Digest Agar Medium that previously has been melted and cooled to approximately 45°. Cover the petri dishes, mix the sample with the agar by tilting or rotating the dishes, and allow the contents to solidify at room temperature. Invert the petri dishes, and incubate for 48 to 72 hours. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as "less than 10 microorganisms per g or per mL of specimen."

Table 1. Most Probable Total Count by Multiple-tube Method.

Observed Combinations of Numbers of Tubes Showing Growth in Each Set			
No. of mg (or mL) of Specimen per Tube			Most Probable Number of Microorganisms per g or per mL
100 (100 μL)	10 (10 μL)	1 (1 μL)	
3	3	3	>1100
3	3	2	1100
3	3	1	500
3	3	0	200
3	2	3	290
3	2	2	210
3	2	1	150
3	2	0	90
3	1	3	160
3	1	2	120
3	1	1	70
3	1	0	40
3	0	3	95
3	0	2	60
3	0	1	40
3	0	0	23

MULTIPLE-TUBE METHOD—Into each of fourteen test tubes of similar size place 9.0 mL of sterile Fluid Soybean-Casein Digest Medium. Arrange twelve of the tubes in four sets of three tubes each. Put aside one set of three tubes to serve as the controls. Into each of three tubes of one set ("100") and into a fourth tube (*A*) pipet 1 mL of the solution or suspension of the specimen, and mix. From tube *A*, pipet 1 mL of its contents into the one remaining tube (*B*) not included in a set, and mix. These two tubes contain 100 mg (or 100 μL) and 10 mg (or 10 μL) of the specimen, respectively. Into each of the second set ("10") of three tubes pipet 1 mL from tube *A*, and into each tube of the third set ("1") pipet 1 mL from tube *B*. Discard the unused contents of tubes *A* and *B*. Close well, and incubate all of the tubes. Following the incubation period, examine the tubes for growth: the three control tubes remain clear and the observations in the tubes containing the specimen, when interpreted by reference to Table 1, indicate the most probable number of microorganisms per g or per mL of specimen.

Test for *Staphylococcus aureus* and *Pseudomonas aeruginosa*—To the specimen add Fluid Soybean-Casein Digest Medium to make 100 mL, mix, and incubate. Examine the medium for growth, and if growth is present, use an inoculating loop to streak a portion of the medium on the surface of Vogel-Johnson Agar Medium (or Baird-Parker Agar Medium, or Mannitol-Salt Agar Medium) and of Cetrimide Agar Medium, each plated on petri dishes. Cover and invert the dishes, and incubate. If, upon examination, none of the plates contains colonies having the characteristics listed in Tables 2 and 3 for the media used, the test specimen meets the requirements for freedom from *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

COAGULASE TEST (FOR *Staphylococcus aureus*)—With the aid of an inoculating loop, transfer representative suspect colonies from the agar surfaces of the Vogel-Johnson Agar Medium (or Baird-Parker Agar Medium, or Mannitol-Salt Agar Medium) to individual tubes, each containing 0.5 mL of mammalian, preferably rabbit or horse, plasma with or without suitable additives. Incubate in a water bath at 37°, examining the tubes at 3 hours and subsequently at suitable intervals up to 24 hours. Test positive and negative controls simultaneously with the unknown specimens. If no coagulation in any degree is observed, the specimen meets the requirements of the test for absence of *Staphylococcus aureus*.

OXIDASE AND PIGMENT TESTS (FOR *Pseudomonas aeruginosa*)—With the aid of an inoculating loop, streak representative suspect colonies from the agar surface of Cetrimide Agar Medium on the agar surfaces of Pseudomonas Agar Medium for Detection of Fluorescin and Pseudomonas Agar Medium for Detection of Pyocyanin contained in petri dishes. If numerous colonies are to be transferred, divide the surface of each plate into quadrants, each of which may be inoculated from a separate colony. Cover and invert the inoculated media, and incubate at 35 ± 2° for not less than three days. Examine the streaked surfaces under ultraviolet light. Examine the plates to determine

Table 2. Morphologic Characteristics of *Staphylococcus aureus* on Selective Agar Media.

Selective Medium	Vogel-Johnson Agar Medium	Mannitol-Salt Agar Medium	Baird-Parker Agar Medium
Characteristic Colonial Morphology	Black surrounded by yellow zone	Yellow colonies with yellow zones	Black, shiny, surrounded by clear zones 2 to 5 mm
Gram Stain	Positive cocci (in clusters)	Positive cocci (in clusters)	Positive cocci (in clusters)

Table 3. Morphologic Characteristics of *Pseudomonas aeruginosa* on Selective and Diagnostic Agar Media.

Medium	Cetrimide Agar Medium	Pseudomonas Agar Medium for Detection of Fluorescin	Pseudomonas Agar Medium for Detection of Pyocyanin
Characteristic Colonial Morphology	Generally greenish	Generally colorless to yellowish	Generally greenish
Fluorescence in Ultraviolet Light	Greenish	Yellowish	Blue
Oxidase Test	Positive	Positive	Positive
Gram Stain	Negative rods	Negative rods	Negative rods

Table 4. Morphologic Characteristics of *Salmonella* Species on Selective Agar Media.

Medium	Description of Colony
Brilliant Green Agar Medium	Small, transparent, colorless or pink to white opaque (frequently surrounded by pink to red zone)
Xylose-Lysine-Desoxycholate Agar Medium	Red, with or without black centers
Bismuth Sulfite Agar Medium	Black or green

Table 5. Morphologic Characteristics of *Escherichia coli* on MacConkey Agar Medium.

Characteristic Colonial Morphology	Brick-red; may have surrounding zone of precipitated bile
Gram Stain	Negative rods (cocco-bacilli)

whether colonies having the characteristics listed in Table 3 are present.

Confirm any suspect colonial growth on one or more of the media as *Pseudomonas aeruginosa* by means of the oxidase test. Upon the colonial growth place or transfer colonies to strips or disks of filter paper that previously has been impregnated with *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride: if there is no development of a pink color, changing to purple, the specimen meets the requirements of the test for the absence of *Pseudomonas aeruginosa*. The presence of *Pseudomonas aeruginosa* may be confirmed by other suitable cultural and biochemical tests, if necessary.

Test for *Salmonella* Species and *Escherichia coli*—To the specimen, contained in a suitable vessel, add a volume of Fluid Lactose Medium to make 100 mL, and incubate. Examine the medium for growth, and if growth is present, mix by gently shaking. Pipet 1-mL portions into vessels containing, respectively, 10 mL of Fluid Selenite-Cystine Medium and Fluid Tetrathionate Medium, mix, and incubate for 12 to 24 hours. (Retain the remainder of the Fluid Lactose Medium.)

TEST FOR *Salmonella* SPECIES—By means of an inoculating loop, streak portions from both the selenite-cystine and tetrathionate media on the surface of Brilliant Green Agar Medium, Xylose-Lysine-Desoxycholate Agar Medium, and Bismuth Sulfite Agar Medium contained in petri dishes. Cover and invert the dishes, and incubate. Upon examination, if none of the colonies conforms to the description given in Table 4, the specimen meets the requirements of the test for absence of the genus *Salmonella*.

If colonies of Gram-negative rods matching the description in Table 4 are found, proceed with further identification by transferring representative suspect colonies individually, by means of an inoculating wire, to a butt-slant tube of Triple Sugar-Iron-Agar Medium by first streaking the surface of the slant and then stabbing the wire well beneath the surface. Incubate. If examination discloses no evidence of tubes having alkaline (red) slants and acid (yellow) butts (with or without concomitant blackening of the butt from hydrogen sulfide production), the specimen meets the requirements of the test for the absence of the genus *Salmonella*.*

TEST FOR *Escherichia coli*—By means of an inoculating loop, streak a portion from the remaining Fluid Lactose Medium on the surface of MacConkey Agar Medium. Cover and invert the dishes, and incubate. Upon examination, if none of the colonies conforms to the description given in Table 5 for this medium, the specimen meets the requirements of the test for absence of *Escherichia coli*.

If colonies matching the description in Table 5 are found, proceed with further identification by transferring the suspect colonies individually, by means of an inoculating loop, to the surface of Levine Eosin–Methylene Blue Agar Medium, plated on petri dishes. If numerous colonies are to be transferred, divide the surface of each plate into quadrants, each of which may be seeded from a separate colony. Cover and invert the plates, and incubate. Upon examination, if none of the colonies exhibits both a characteristic metallic sheen under reflected light and a blue-black appearance under transmitted light, the specimen meets the requirements of the test for the absence of *Escherichia coli*. The presence of *Escherichia coli* may be confirmed by further suitable cultural and biochemical tests.

Total Combined Molds and Yeasts Count—Proceed as for the *Plate Method* under *Total Aerobic Microbial Count*, except for using the same amount of Sabouraud Dextrose Agar Medium or Potato Dextrose Agar Medium, instead of Soybean Casein Digest Medium, and except for incubating the inverted petri dishes for 5 to 7 days at 20° to 25°.

Retest—For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10.0-g specimen, a retest on a 25-g specimen of the product may be conducted. Proceed as directed under *Procedure*, but make allowance for the larger specimen size.

⟨71⟩ STERILITY TESTS

The following procedures are applicable for determining whether a Pharmacopeial article purporting to be sterile complies with the requirements set forth in the individual monograph with respect to the test for *Sterility*. (For the use of sterility test procedures as part of quality control in manufacture, see *Sterilization and Sterility Assurance of Compendial Articles* ⟨1211⟩.) In view of the possibility that positive results may be due to faulty aseptic techniques or environmental contamination in testing, provisions are included under *Interpretation of Sterility Test Results* for two stages of testing.

Alternative procedures may be employed to demonstrate that an article is sterile, provided the results obtained are at least of equivalent reliability. (See *Procedures* under *Tests and Assays* in the *General Notices*.) Where a difference appears, or in the event of a dispute, when evidence of microbial contamination is obtained by the procedure given in this Pharmacopeia, the result so obtained is conclusive of failure of the article to meet the requirements of the test. Similarly, failure to demonstrate microbial contamination by the procedure given in this Pharmacopeia is evidence that the article meets the requirements of the test. For additional interpretive information, see *Sterilization and Sterility Assurance of Compendial Articles* ⟨1211⟩.

Media

Media for the tests may be prepared as described below, or dehydrated mixtures yielding similar formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have growth-promoting properties equal or superior to those obtained from the formulas given herein.

I. Fluid Thioglycollate Medium

L-Cystine	0.5 g
Sodium Chloride	2.5 g
Dextrose ($C_6H_{12}O_6 . H_2O$)	5.5 g
Agar, granulated (moisture content not in excess of 15%)	0.75 g
Yeast Extract (water-soluble)	5.0 g
Pancreatic Digest of Casein	15.0 g
Sodium Thioglycollate	0.5 g
or Thioglycollic Acid	0.3 mL
Resazurin Sodium Solution (1 in 1000), freshly prepared	1.0 mL
Water	1000 mL

pH after sterilization: 7.1 ± 0.2.

Mix, and heat until solution is effected. Adjust the solution with 1 *N* sodium hydroxide so that, after sterilization, it will have a pH of 7.1 ± 0.2. Filter while hot through filter paper, if necessary. Place the medium in suitable vessels, which provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a color change indicative of oxygen uptake at the end of the incubation period. Sterilize in an autoclave. If more than the upper one-third has

* Additional, confirmatory evidence may be obtained by use of procedures set forth in *Official Methods of Analysis of the AOAC*, 12th ed. (1975), sections 46.013–46.026.

acquired a pink color, the medium may be restored once by heating on a steam bath or in free-flowing steam until the pink color disappears. When ready for use, not more than the upper one-tenth of the medium should have a pink color.

Use Fluid Thioglycollate Medium by incubating it under aerobic conditions.

II. Alternative Thioglycollate Medium for Devices Having Tubes with Small Lumina

L-Cystine	0.5 g
Sodium Chloride	2.5 g
Dextrose ($C_6H_{12}O_6 \cdot H_2O$)	5.5
Yeast Extract (water-soluble)	5.0
Pancreatic Digest of Casein	15.0
Sodium Thioglycollate	0.5 g
or Thioglycollic Acid	0.3 mL
Water	1000 mL

pH after sterilization: 7.1 ± 0.2.

Heat the ingredients in a suitable container until solution is effected. Mix, and, if necessary, adjust the solution with 1 *N* sodium hydroxide so that, after sterilization, it will have a pH of 7.1 ± 0.2. Filter, if necessary, place in suitable vessels, and sterilize by steam. The medium is freshly prepared or heated in a steam bath and allowed to cool just prior to use. Do not reheat.

Use Alternative Thioglycollate Medium in a manner that will assure anaerobic conditions for the duration of the incubation period.

III. Soybean-Casein Digest Medium

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose ($C_6H_{12}O_6 \cdot H_2O$)	2.5 g
Water	1000 mL

pH after sterilization: 7.3 ± 0.2.

Dissolve the solids in the water, warming slightly to effect solution. Cool the solution to room temperature, and adjust with 1 *N* sodium hydroxide, if necessary, to obtain a pH of 7.3 ± 0.2 after sterilization. Filter, if necessary, and dispense into suitable vessels. Sterilize by steam.

Use Soybean-Casein Digest Medium by incubating it under aerobic conditions.

Diluting and Rinsing Fluids

FLUID A—Dissolve 1 g of peptic digest of animal tissue (see *Reagent Specifications* in the section, *Reagents, Indicators, and Solutions*) in water to make 1 liter, filter or centrifuge to clarify, adjust to a pH of 7.1 ± 0.2, dispense into containers in 100-mL quantities, and sterilize by steam. NOTE—Where *Fluid A* is to be used in performing the test for *Sterility* on a specimen of the penicillin or cephalosporin class of antibiotics, aseptically add a quantity of sterile penicillinase to the *Fluid A* to be used to rinse the membrane(s) sufficient to inactivate any residual antibiotic activity on the membrane(s) after the solution of the specimen has been filtered.

FLUID D—If the test specimen contains lecithin or oil, or for device sterile pathway tests using membrane filtration, use *Fluid A* to each liter of which has been added 1 mL of polysorbate 80, adjust to a pH of 7.1 ± 0.2, dispense into flasks, and sterilize by steam.

FLUID K—

Peptic Digest of Animal Tissue (see *Reagent Specifications* in the section, *Reagents, Indicators, and Solutions*)	5.0 g
Beef Extract	3.0 g
Polysorbate 80	10.0 g
Water	1000 mL

pH after sterilization: 6.9 ± 0.2.

Sterilize by steam.

NOTE—A sterile fluid shall not have antibacterial or antifungal properties if it is to be considered suitable for dissolving, diluting, or rinsing an article under test for sterility.

Growth Promotion Test

Confirm the sterility of each lot of medium by incubation of representative containers, at the temperature and for the length of time specified in the test.

Test each autoclaved load of each lot of medium for its growth-promoting qualities by separately inoculating duplicate test containers of each medium with 10 to 100 viable microorganisms of each of the strains listed in the accompanying table, and incubating according to the conditions specified.

The test media are satisfactory if clear evidence of growth appears in all inoculated media containers within 7 days. The tests may be conducted simultaneously with the use of the test media for sterility test purposes. The sterility test is considered invalid if the test medium shows inadequate growth response.

If freshly prepared media are not used within 2 days, store them in the dark, preferably at 2° to 25°.

Finished media, if stored in unsealed containers, may be used for not more than one month, provided that they are tested within one week of the time of use and if the color indicator requirements are met. If stored in suitable sealed containers, the media may be used for not more than one year, provided they are tested for growth promotion every three months and if the color indicator requirements are met.

Bacteriostasis and Fungistasis

Before initiating direct transfer sterility tests on an article, determine the level of bacteriostatic and fungistatic activity by the following procedures. Prepare dilute cultures of bacteria and fungi from at least the strains of microorganisms cited under *Growth Promotion Test*. Inoculate the sterility test media with 10 to 100 viable microorganisms, employing volumes of medium listed in the table of Quantities for Liquid Articles under *Selection of Test Specimens and Incubation*. Add the specified portion of article to half of a suitable number of the containers already containing the inoculum and culture medium. Incubate the containers at the appropriate temperatures and under the conditions listed in the table for not less than 7 days.

If growth of the test organisms in the article-medium mixture is visually comparable to that in the control vessels, use the amounts

Medium	Test Microorganisms*	Incubation	
		Temperature (°)	Conditions
Fluid Thioglycollate	(1) *Bacillus subtilis* (ATCC No. 6633)†	30 to 35	
	(2) *Candida albicans* (ATCC No. 10231)	30 to 35	Aerobic
	(3) *Bacteroides vulgatus* (ATCC No. 8482)‡	30 to 35	
Alternative Thioglycollate	(1) *Bacteroides vulgatus* (ATCC No. 8482)‡	30 to 35	Anaerobic
Soybean-Casein Digest	(1) *Bacillus subtilis* (ATCC No. 6633)†	20 to 25	
	(2) *Candida albicans* (ATCC No. 10231)	20 to 25	Aerobic

* Available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

NOTE—Seed lot culture maintenance techniques should be employed so that the viable microorganisms used for inoculation are not more than 5 passages removed from the ATCC cultures.

† If a spore-forming organism is not desired, use *Micrococcus luteus* (ATCC No. 9341) at the incubation temperatures indicated in the table.

‡ If a spore-forming organism is desired, use *Clostridium sporogenes* (ATCC No. 11437) at the incubation temperature indicated in the table.

Quantities for Liquid Articles				
		Minimum Volume of Each Medium		
Container content (mL)	Minimum volume taken from each container for each medium	Used for direct transfer of volume taken from each container (mL)	Used for membrane or half membrane representing total volume from the appropriate number of containers (mL)	No. of containers per medium
Less than 10	1 mL, or entire contents if less than 1 mL	15	100	20 (40 if each does not contain sufficient volume for both media)
10 to less than 50	5 mL	40	100	20
50 to less than 100	10 mL	80	100	20
50 to less than 100, intended for intravenous administration	Entire contents	—	100	10
100 to 500	Entire contents	—	100	10
Over 500	500 mL	—	100	10

of article and medium regularly specified in the table of Quantities for Liquid Articles under *Selection of Test Specimens and Incubation.*

If the article is bacteriostatic and/or fungistatic when tested as described above, use a suitable sterile neutralizing agent, if available. Suitability of such an agent is determined as in the test described below. If a neutralizing agent is not available, establish, as described below, suitable amounts of article and medium to be used.

Repeat the tests set forth above, using the specified amount of article and larger volumes of the medium to determine the ratio of article to medium in which growth of the test organisms is not adversely affected.

If the specified amount of article is bacteriostatic or fungistatic in 250 mL of the medium, decrease the amount of the article to find the maximum amount that does not adversely affect the growth of the test organism in 250 mL of the medium. For liquids and suspensions, if this amount is less than 1 mL, increase the quantity of medium so that the 1 mL is sufficiently diluted to prevent inhibition of growth. For solids that are not readily soluble or dispersible, if the amount is less than 50 mg, increase the quantity of medium so that the 50 mg of the article is sufficiently diluted to prevent inhibition of growth. In either case, use the amounts of the article and medium established in this ratio for sterility testing.

Where membrane filtration is used, make similar comparisons using the specified portions of the article under test and similar quantities of a suitable diluting and rinsing fluid, rinsing the membrane in each case with three 100-mL portions of the diluting and rinsing fluid. Inoculate the stated quantities of viable microorganisms into each final portion of diluting and rinsing fluid used to filter the article under test and to filter the diluting and rinsing fluid only. The growth of the test organism in each case from the membrane(s) used to filter the article under test followed by the inoculated final diluting and rinsing fluid is visually comparable to that from the membrane(s) used to filter only the inoculated diluting and rinsing fluid.

General Procedure

The test procedures include (1) direct transfer to test media and (2) membrane filtration techniques. Sterility testing of Pharmacopeial articles using membrane filtration of the test specimens, where feasible, is the method of choice. The procedure is particularly useful for liquids and soluble powders possessing bacteriostatic or fungistatic properties, so as to permit separation of possible contaminating microorganisms from such growth inhibitors. The procedure is to be validated for such use. For similar reasons, it is very useful where the article is an oil, an ointment, or a cream that can be put into solution with non-bacteriostatic or non-fungistatic diluting fluids. Its use is also entirely appropriate and preferable in the sterility testing of non-bacteriostatic or non-fungistatic liquids or soluble powders. Certain devices also may be appropriately tested for sterility of surfaces or the critical pathways by the membrane filtration technique.

Because of diversity in the nature of articles to be tested and other factors affecting the conduct of the sterility test, it is important to observe the following considerations in performing sterility tests.

OPENING CONTAINERS

Cleanse the exterior surfaces of ampuls and closures of vials and bottles with a suitable decontaminating agent, and gain access to the contents in an aseptic manner. If the vial contents are packaged under vacuum, admit sterile air by means of a suitable sterile device, such as a needle attached to a syringe containing sterilizing grade filter material.

For purified cotton, gauze, surgical dressings, sutures, and related Pharmacopeial articles, open the package or container aseptically.

SELECTION OF TEST SPECIMENS AND INCUBATION

For liquid articles, use not less than the volumes of article and medium for each unit and the number of containers per medium specified in the table of Quantities for Liquid Articles, in this section. If the contents are of sufficient quantity, they may be divided so that portions are added to each of the two specified media. If each container does not contain sufficient volume for both media, use double the number of containers. For articles other than liquids, test 20 units of the article with each medium. For such articles in which only the lumen must be sterile, flush the lumen with a suitable quantity of appropriate medium to yield a recovery of not less than 15 mL of medium.

Unless otherwise directed in the individual monograph or in a section of this chapter, incubate the test mixture for 14 days with Fluid Thioglycollate Medium (or Alternative Thioglycollate Medium, where so indicated) at 30° to 35°, and with Soybean-Casein Digest Medium at 20° to 25°.

Test Procedures for Direct Transfer to Test Media

LIQUIDS

Remove liquids from test containers with a sterile pipet or with a sterile syringe and needle. Aseptically transfer the specified volume of the material from each test container to a vessel of culture medium. Mix the liquid with the medium, but do not aerate excessively. Incubate in the specified media as directed under *General Procedure*, for not less than 14 days. Examine the media visually for growth at least as often as on the third or fourth or fifth day, on the seventh or eighth day, and on the last day of the test period.

Where the material being tested renders the medium turbid, so that the presence or absence of microbial growth cannot be determined readily by visual examination, transfer suitable portions of the medium to fresh vessels of the same medium at least once during the period from the third to the seventh day after the test is started. Continue incubation of the original and of the transfer vessels for a total of not less than 14 days from the original inoculation.

OINTMENTS AND OILS INSOLUBLE IN ISOPROPYL MYRISTATE

Select 20 representative containers, assign them to 2 groups of 10 containers, and treat each group as follows. Aseptically transfer 100 mg from each of the 10 containers to a flask containing 100 mL of a sterile, aqueous vehicle capable of dispersing the test material homogeneously throughout the fluid mixture. [NOTE—The choice of dispersing agent incorporated in the aqueous vehicle may differ according to the nature of the ointment or oil. Before initiating routine use of a given dispersing agent, test the dispersing agent to ascertain that in the concentration used it has no significant antimicrobial effects during the time interval for all transfers employing test procedures set forth under *Bacteriostasis and Fungistasis*.] Mix an aliquot of 10 mL of the fluid mixture so obtained with 80 mL of each medium, and proceed as directed under *Liquids*, beginning with "Incubate in the specified media."

SOLIDS

Take a quantity of the product in the form of a dry solid (or of a solution or a suspension of the product prepared by adding sterile diluent to the immediate container), corresponding to not less than 300 mg from each container being tested, or to the entire contents if each contains less than 300 mg of solids. Transfer it to not less than 40 mL of Fluid Thioglycollate Medium and to not less than 40 mL of Soybean-Casein Digest Medium, respectively, and mix, the number of containers and the conditions of incubation being the same as for liquids. Proceed as directed under *Liquids*, beginning with "Examine the media visually."

PURIFIED COTTON, GAUZE, SURGICAL DRESSINGS, SUTURES, AND RELATED ARTICLES

From each package of cotton, rolled gauze, or gauze bandage being tested, remove aseptically two or more portions of 100 mg to 500 mg each from the innermost part of the sample. From individually packaged single-use materials such as gauze pads, remove aseptically a single portion of 250 mg to 500 mg or the entire article in the case of small, i.e., 25- × 75-mm or smaller, adhesive absorbent bandages, or sutures.

Aseptically transfer these portions of the article to the specified number of containers of appropriate media and incubate as directed under *General Procedure*. Proceed as directed under *Liquids*, beginning with "Examine the media visually."

STERILIZED DEVICES

The following considerations apply to sterilized devices manufactured in lots, each consisting of a number of units. Special considerations apply to sterile devices manufactured in small lots or in individual units where the self-destructive nature of the Sterility Test renders the conventional Sterility Test impracticable. For these articles, appropriate and acceptable modifications to the Sterility Test must be made.

For articles of such size and shape as to permit complete immersion in not more than 1000 mL of culture medium, test the intact article, using the appropriate media, and incubate as directed under *General Procedure*. Proceed as directed under *Liquids*, beginning with "Examine the media visually."

For devices having hollow tubes, such as transfusion or infusion assemblies, or where the size of an item makes immersion impracticable and where only the fluid pathway must be sterile, flush the lumen of each of 20 units with a sufficient quantity of Fluid Thioglycollate Medium and the lumen of each of 20 units with a sufficient quantity of Soybean-Casein Digest Medium to yield a recovery of not less than 15 mL of each medium, and incubate with not less than 100 mL of each of the two media as directed under *General Procedure*. For devices in which the lumen is so small that Fluid Thioglycollate Medium will not pass through, substitute Alternative Thioglycollate Medium for Fluid Thioglycollate Medium, but incubate the medium anaerobically.

Where the entire intact article, because of its size and shape, cannot be tested for sterility by immersion in not more than 1000 mL of culture medium, expose that portion of the article most difficult to sterilize, and test that portion, or where practicable remove two or more portions each from the innermost portion of the article. Aseptically transfer these portions of the article to the specified number of vessels of appropriate media in a volume of not more than 1000 mL, and incubate as directed under *General Procedure*. Proceed as directed under *Liquids*, beginning with "Examine the media visually."

Where the presence of the test specimen in the medium interferes with the test because of bacteriostatic or fungistatic action, rinse the article thoroughly with a minimal amount of rinse fluid (see under *Diluting and Rinsing Fluids*). Recover the rinse fluid, and test as directed for *Devices* under *Test Procedures using Membrane Filtration*.

STERILE EMPTY OR PREFILLED SYRINGES

Sterility testing of prefilled syringes is performed by employing the same techniques used in testing sterile products in vials or ampuls. The direct transfer technique may be employed if the *Bacteriostasis and Fungistasis* determination has indicated no adverse activity under the test conditions. Where appropriate, the membrane filtration procedure may be employed. For prefilled syringes containing a sterile needle, flush the contained product through the lumen. For syringes packaged with a separate needle, aseptically attach the needle, and expel the product into the appropriate media. Pay special attention toward demonstrating that the outside of the attached needle (that portion which will enter the patient's tissues) is sterile. For empty sterile syringes, take up sterile medium or diluent into the barrel through the needle if attached, or if not attached, through a sterile needle attached for the purpose of the test, and express the contents into the appropriate media.

Test Procedures Using Membrane Filtration

Where the membrane filtration technique is used for liquid articles that may be tested by direct transfer to test media, test not less than the volumes and numbers specified under *Selection of Test Specimens and Incubation*.

Apparatus—A suitable membrane filter unit consists of an assembly that facilitates the aseptic handling of the test articles and that allows the processed membrane to be removed aseptically for inoculation of appropriate media or an assembly where sterile media can be added to the sealed filter and the membrane incubated in situ. A membrane generally suitable for sterility testing has a nominal porosity of 0.45 μm, a diameter of approximately 47 mm, and a flow rate of 55 to 75 mL of water per minute at a pressure of 70 cm of mercury. The entire unit may be assembled and sterilized with the membrane(s) in place prior to use in the test, or the membranes may be sterilized separately by any means that maintains the performance characteristics of the filter and assures the sterility of the filter and the assembly.

Where the article to be tested is an oil, the membrane may be sterilized separately, and after thorough drying, the unit assembled, using aseptic precautions.

LIQUIDS MISCIBLE WITH AQUEOUS VEHICLES

Aseptically transfer the volumes required for both media, as indicated in the table of Quantities for Liquid Articles under *Selection of Test Specimens and Incubation*, either directly into one or two separate membrane filter funnels or to separate sterile pooling vessel(s) prior to transfer. In the case of liquid articles in containers in which the volume of liquid is either less than 50 mL, or 50 mL to less than 100 mL, and not intended for intravenous administration, the required volumes from not less than 20 containers are thus represented by one membrane, or membrane half, transferred to each medium. If the volume of liquid in the article is 50 mL to less than 100 mL per container and is intended for intravenous administration, or is 100 mL or more up to 500 mL, aseptically transfer the entire contents of not less than 10 containers through each of two filter assemblies, or not less than 20 containers if only one filter assembly is used. If the volume of the liquid in the article is more than 500 mL, asep-

tically transfer not less than 500 mL from each of not less than 10 containers through each of two filter assemblies, or not less than 20 containers if only one filter assembly is used. Immediately pass each specimen through the filter with the aid of vacuum or pressure.

In some cases, where the liquid is highly viscous and not readily filterable through one or two membranes, more than two filter assemblies may be needed. In such cases, half the number of membranes used are incubated in each medium, provided that the volumes and requirements for numbers of containers per medium are complied with. If the product is bacteriostatic or fungistatic, rinse the membrane(s) with three 100-mL portions of *Fluid A*.

Aseptically remove the membrane(s) from the holder(s), cut the membrane in half (if only one is used), immerse the membrane, or one-half of the membrane, in 100 mL of Soybean-Casein Digest Medium, and incubate at 20° to 25° for not less than 7 days. Similarly, immerse the other membrane, or other half of the membrane, in 100 mL of Fluid Thioglycollate Medium, and incubate at 30° to 35° for not less than 7 days.

NOTE—Where the product under test has inherent bacteriostatic properties, use hydrophobic membrane filter disks, or after the specimen has been filtered, cut a disk comprising about one-half of the filtering area from the center of the membrane using a sterile cutting device, aseptically transferring the disk cut from the center of the membrane to Fluid Thioglycollate Medium, and aseptically transferring the remainder of the disk to Soybean-Casein Digest Medium.

LIQUIDS IMMISCIBLE WITH AQUEOUS VEHICLES (LESS THAN 100 mL PER CONTAINER)

Using the contents of not less than 20 containers (40 containers, if each one does not contain sufficient volume for both media), aseptically transfer the volumes required for both media, as indicated in the table of Quantities for Liquid Articles under *Selection of Test Specimens and Incubation*, either directly into one or two separate membrane filter funnels or to separate sterile pooling vessels prior to transfer. The required volumes from not less than 20 containers are thus represented by the membrane, or membrane half to be transferred to each medium. Immediately pass each specimen through the filter with the aid of vacuum or pressure.

If the substance is a viscous liquid or suspension and not adaptable to rapid filtration, aseptically add a sufficient quantity of diluting fluid to the pooled specimen prior to filtration to increase the flow rate.

If the product under test has inherent bacteriostatic or fungistatic properties or contains a preservative, wash the filter with from one to three 100-mL portions of *Fluid A*. If the substance under test contains lecithin or oil, substitute *Fluid D* for *Fluid A*.

Upon completion of the filtration, and rinsing, treat the membrane(s) as directed under *Liquids Miscible with Aqueous Vehicles*, beginning with "Aseptically remove the membrane(s)."

FILTERABLE SOLIDS

Take about 6 g of the product in the form of a dry solid (or a portion of a solution or suspension of the product, prepared by adding sterile diluent to the immediate container(s), corresponding to 6 g of solid), or not less than 300 mg from each container being tested, or the entire contents of each container if each contains less than 300 mg of solids, unless otherwise specified in the individual monograph, the number of containers being the same as specified for *Liquids Miscible with Aqueous Vehicles*. Transfer the specimen aseptically to a vessel containing 200 mL of *Fluid A*, and swirl to dissolve. If the specimen does not dissolve completely, use 400 mL of *Fluid A*, or divide the specimen aseptically into two portions and test each using 200 mL of *Fluid A*. Aseptically transfer the solution(s) into one or two membrane funnels, and immediately filter with the aid of vacuum or pressure. If the product under test has inherent bacteriostatic or fungistatic properties, rinse the membrane(s) with three 100-mL portions of *Fluid A*. Upon completion of the filtration and rinsing, treat the membrane(s) as directed under *Liquids Miscible with Aqueous Vehicles*, beginning with "Aseptically remove the membrane(s)."

OINTMENTS AND OILS SOLUBLE IN ISOPROPYL MYRISTATE

Dissolve not less than 100 mg from each of not less than 20 containers (40 containers, if each one does not contain sufficient volume for both media) in not less than 100 mL of isopropyl myristate with a pH of water extract not less than 6.5 (see under *Reagent Specifications* in the section, *Reagents, Indicators, and Solutions*) which previously has been rendered sterile by filtration through a 0.22-μm membrane filter. [NOTE—Warm the sterilized solvent, and if necessary the test material, to not more than 44° just prior to use.] Swirl the flask to dissolve the ointment or oil, taking care to expose a large surface of the material to the solvent. Filter the dissolved ointment promptly following dissolution. Aseptically transfer the mixture into one or two membrane filter funnels. Immediately pass each specimen through the filter with the aid of vacuum or pressure. Keep filter membrane(s) covered with liquid throughout the filtration for maximum efficiency of the filter.

Following filtration of the specimen, wash the membrane(s) with two 200-mL portions of *Fluid D*, then wash with 100 mL of *Fluid A*. Treat the test membrane(s) as directed under *Liquids Miscible with Aqueous Vehicles*, beginning with "Aseptically remove the membrane(s)," except to provide that the sterility test medium to be used contains 1 g of polysorbate 80 per liter.

If the substance under test contains petrolatum, use *Fluid K*. Moisten the membrane(s) with approximately 200 μL of the rinse medium before the filtration operation begins, and keep the membrane(s) covered with liquid throughout the filtration operation for maximum efficiency of the filter.

Following filtration of the specimen, wash the membrane(s) with three 100-mL portions of the rinse medium. Treat the test membrane(s) as directed above.

NOTE—For ointments and oils that are insoluble in isopropyl myristate, proceed as directed for *Ointments and Oils Insoluble in Isopropyl Myristate* under *Test Procedures for Direct Transfer to Test Media*.

NON-FILTERABLE SOLIDS

The sterility testing of these articles by membrane filtration is considered inadvisable unless it can be demonstrated that filter blockage does not occur. Proceed as directed for *Solids* under *Test Procedures for Direct Transfer to Test Media*.

DEVICES

Devices that are purported to contain sterile pathways may be tested for sterility by the membrane filtration technique as follows.

Aseptically pass a sufficient volume of *Fluid D* through each of not less than 20 devices so that not less than 100 mL is recovered from each device. Collect the fluids in aseptic containers, and filter the entire volume collected through membrane filter funnel(s) as directed under *Liquids Miscible with Aqueous Vehicles*, beginning with "Aseptically remove the membrane(s)."

Where the devices are large, and lot sizes are small, test an appropriate number of units as described for similar cases in the section, *Sterilized Devices*, under *Test Procedures for Direct Transfer to Test Media*.

Interpretation of Sterility Test Results

FIRST STAGE

At the prescribed intervals during and at the conclusion of the incubation period, examine the contents of all of the vessels for evidence of microbial growth, such as the development of turbidity and/or surface growth. If no growth is observed, the article tested meets the requirements of the test for sterility.

If microbial growth is found, but a review in the sterility testing facility of the monitoring, materials used, testing procedure, and negative controls indicates that inadequate or faulty aseptic technique was used in the test itself, the *First Stage* is declared invalid and may be repeated.

If microbial growth is observed but there is no evidence invalidating the *First Stage* of the test, proceed to the *Second Stage*.

SECOND STAGE

The minimum number of specimens selected is double the number tested in the *First Stage*. The minimum volumes tested from each specimen and the media and incubation periods are the same as those indicated for the *First Stage*. If no microbial growth is found, the article tested meets the requirements of the test for sterility. If growth is found, the result so obtained is conclusive that the article tested fails to meet the requirements of the test for sterility. If, however, it can be demonstrated that the *Second Stage* was invalid because of faulty or inadequate aseptic technique in the performance of the test the *Second Stage* may be repeated.

NOTE—Where sterility testing is used as part of an assessment of a production lot or batch or as one of the quality control criteria for release of such lot or batch, see *Sterilization and Sterility Assurance of Compendial Articles* ⟨1211⟩.

Biological Tests and Assays

⟨81⟩ ANTIBIOTICS—MICROBIAL ASSAYS

The activity (potency) of antibiotics may be demonstrated under suitable conditions by their inhibitory effect on microorganisms. A reduction in antimicrobial activity also will reveal subtle changes not demonstrable by chemical methods. Accordingly, microbial or biological assays remain generally the standard for resolving doubt with respect to possible loss of activity. This chapter summarizes these procedures for the antibiotics recognized in this Pharmacopeia for which microbiological assay remains the definitive method.

Two general methods are employed, the cylinder-plate or "plate" assay and the turbidimetric or "tube" assay. The first depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a petri dish or plate to an extent such that growth of the added microorganism is prevented entirely in a circular area or "zone" around the cylinder containing a solution of the antibiotic. The turbidimetric method depends upon the inhibition of growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favorable to its rapid growth in the absence of the antibiotic.

Apparatus

All equipment is to be thoroughly cleaned before and after each use. Glassware for holding and transferring test organisms is sterilized by dry heat, or by steam.

TEMPERATURE CONTROL

Thermostatic control is required in several stages of a microbial assay, when culturing a microorganism and preparing its inoculum, and during incubation in plate and tube assays. Maintain the temperature of assay plates at ±0.5° of the temperature selected. Closer control of the temperature (±0.1° of the selected temperature) is imperative during incubation in a tube assay, and may be achieved in either circulated air or water, the greater heat capacity of water lending it some advantage over circulating air.

SPECTROPHOTOMETER

Measuring transmittance within a fairly narrow frequency band requires a suitable spectrophotometer in which the wavelength of the light source can be varied or restricted by the use of a 580-nm filter for preparing inocula of the required density, or with a 530-nm filter for reading the absorbance in a tube assay. For the latter purpose, the instrument may be arranged to accept the tube in which incubation takes place, to accept a modified cell fitted with a drain that facilitates rapid change of content, or preferably, fixed with a flow-through cell for a continuous flow-through analysis; set the instrument at zero absorbance with clear, uninoculated broth prepared as specified for the particular antibiotic, including the same amount of test solution and formaldehyde as found in each sample.

NOTE—Either absorbance or transmittance measurement may be used for preparing inocula.

CYLINDER-PLATE ASSAY RECEPTACLES

For assay plates, use glass or plastic petri dishes (approximately 20 × 100 mm) having covers of suitable material. For assay cylinders, use stainless steel or porcelain cylinders with the following dimensions, each dimension having a tolerance of ±0.1 mm: outside diameter 8 mm; inside diameter 6 mm; and length 10 mm. Carefully clean cylinders to remove all residues. An occasional acid bath, e.g., with about 2 *N* nitric acid or with chromic acid (see *Cleaning Glass Apparatus* ⟨1051⟩) is needed.

TURBIDIMETRIC ASSAY RECEPTACLES

For assay tubes, use glass or plastic test tubes, e.g., 16 × 125 mm or 18 × 150 mm that are relatively uniform in length, diameter, and thickness and substantially free from surface blemishes and scratches. Tubes that are to be placed in the spectrophotometer are matched and are without scratches or blemishes. Cleanse thoroughly, to remove all antibiotic residues and traces of cleaning solution, and sterilize tubes that have been used previously, before subsequent use.

Media and Diluents

MEDIA

The media required for the preparation of test organism inocula are made from the ingredients listed herein. Minor modifications of the individual ingredients, or reconstituted dehydrated media, may be substituted, provided the resulting media possess equal or better growth-promoting properties and give a similar standard curve response.

Dissolve the ingredients in water to make 1 liter, and adjust the solutions with either 1 *N* sodium hydroxide or 1 *N* hydrochloric acid as required, so that after steam sterilization the pH is as specified.

Medium 1

Peptone	6.0 g
Pancreatic Digest of Casein	4.0 g
Yeast Extract	3.0 g
Beef Extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL

pH after sterilization: 6.6 ± 0.1.

Medium 2

Peptone	6.0 g
Yeast Extract	3.0 g
Beef Extract	1.5 g
Agar	15.0 g
Water	1000 mL

pH after sterilization: 6.6 ± 0.1.

Medium 3

Peptone	5.0 g
Yeast Extract	1.5 g
Beef Extract	1.5 g
Sodium Chloride	3.5 g
Dextrose	1.0 g
Dipotassium Phosphate	3.68 g
Potassium Dihydrogen Phosphate	1.32 g
Water	1000 mL

pH after sterilization: 7.0 ± 0.05.

Medium 5

Same as Medium 2, except that the final pH after sterilization is 7.9 ± 0.1.

Medium 8

Same as Medium 2, except that the final pH after sterilization is 5.9 ± 0.1.

Medium 9

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean	3.0 g
Sodium Chloride	5.0 g
Dipotassium Phosphate	2.5 g
Dextrose	2.5 g
Agar	20.0 g
Water	1000 mL

pH after sterilization: 7.2 ± 0.1.

Medium 10

Same as Medium 9, except to use 12.0 g of Agar instead of 20.0 g, and to add 10 mL of Polysorbate 80 after boiling the medium to dissolve the agar.

pH after sterilization: 7.2 ± 0.1.

Medium 11

Same as Medium 1, except that the final pH after sterilization is 8.3 ± 0.1.

Medium 13

Dextrose	20.0 g
Peptone	10.0 g
Water	1000 mL

pH after sterilization: 5.6 ± 0.1.

Medium 19

Peptone	9.4 g
Yeast Extract	4.7 g
Beef Extract	2.4 g
Sodium Chloride	10.0 g
Dextrose	10.0 g
Agar	23.5 g
Water	1000 mL

pH after sterilization: 6.1 ± 0.1.

Medium 32

Same as Medium 1, except for the additional ingredient 0.3 g of Manganese Sulfate.

Medium 34

Glycerol	10.0 g
Peptone	10.0 g
Beef Extract	10.0 g
Sodium Chloride	3.0 g
Water	1000 mL

pH after sterilization: 7.0 ± 0.1.

Medium 35

Same as Medium 34, except for the additional ingredient 17.0 g of Agar.

Medium 36

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

pH after sterilization: 7.3 ± 0.1.

Medium 37

Pancreatic Digest of Casein	17.0 g
Soybean Peptone	3.0 g
Dextrose	2.5 g
Sodium Chloride	5.0 g
Dipotassium Phosphate	2.5 g
Water	1000 mL

pH after sterilization: 7.3 ± 0.1.

Medium 38

Peptone	15.0 g
Papaic Digest of Soybean Meal	5.0 g
Sodium Chloride	4.0 g
Sodium Sulfite	0.2 g
L-Cystine	0.7 g
Dextrose	5.5 g
Agar	15.0 g
Water	1000 mL

pH after sterilization: 7.0 ± 0.1.

PHOSPHATE BUFFERS AND OTHER SOLUTIONS

Prepare as follows or by other suitable means the potassium phosphate buffers required for the antibiotic under assay. The buffers are sterilized after preparation, and the pH specified in each case is that after sterilization.

Buffer No. 1, 1 percent, pH 6.0—Dissolve 2.0 g of dibasic potassium phosphate and 8.0 g of monobasic potassium phosphate in 1000 mL of water. Adjust the pH with 18 *N* phosphoric acid or 10 *N* potassium hydroxide to 6.0 ± 0.05.

Buffer No. 3, 0.1 *M*, pH 8.0—Dissolve 16.73 g of dibasic potassium phosphate and 0.523 g of monobasic potassium phosphate in 1000 mL of water. Adjust the pH with 18 *N* phosphoric acid or 10 *N* potassium hydroxide to 8.0 ± 0.1.

Buffer No. 4, 0.1 *M*, pH 4.5—Dissolve 13.61 g of monobasic potassium phosphate in 1000 mL of water. Adjust the pH with 18 *N* phosphoric acid or 10 *N* potassium hydroxide to 4.5 ± 0.05.

Buffer No. 6, 10 percent, pH 6.0—Dissolve 20.0 g of dibasic potassium phosphate and 80.0 g of monobasic potassium phosphate in 1000 mL of water. Adjust the pH with 18 *N* phosphoric acid or 10 *N* potassium hydroxide to 6.0 ± 0.05.

Buffer No. 10, 0.2 *M*, pH 10.5—Dissolve 35.0 g of dibasic potassium phosphate in 1000 mL of water, and add 2 mL of 10 *N* potassium hydroxide. Adjust the pH with 18 *N* phosphoric acid or 10 *N* potassium hydroxide to 10.5 ± 0.1.

Buffer No. 16, 0.1 *M*, pH 7.0—Dissolve 13.6 g of dibasic potassium phosphate and 4.0 g of monobasic potassium phosphate in 1000 mL of water. Adjust with 18 *N* phosphoric acid or 10 *N* potassium hydroxide to a pH of 7.0 ± 0.2.

Other solutions—Use the substances specified under *Reagents, Indicators, and Solutions*. For water, use *Purified Water*. For saline, use *Sodium Chloride Injection*. Dilute formaldehyde is *Formaldehyde Solution* diluted 1:3 with water.

Units and Reference Standards

The potency of antibiotics is designated in either "Units" or "μg" of activity. In each case the "Unit" or "μg" of antibiotic activity is established and defined by the designated federal master standard for that antibiotic. The corresponding USP Reference Standard is calibrated in terms of the master standard. USP Reference Standards for antibiotic substances are held and distributed by the U.S. Pharmacopeial Convention, Inc.

The concept of "μg" of activity originated from the situation where the antibiotic preparation selected as the reference standard was thought to consist entirely of a single chemical entity

Table 1. Units of Potency of Reference Standards Available in 1988.

Antibiotic	Weight of Material (in μg) containing the Unit as defined by the master standard (and corresponding number of Units per mg)		Number of USP Units per mg of the USP Reference Standard (1988)
Bacitracin Zinc	13.51	(74)	58.5 (Lot L)
Nystatin	0.2817	(3550)	6044 (Lot L)
Penicillin G Sodium	0.600	(1667)	1590 (Lot G)
Penicillin V	0.590	(1695)	1520 (Lot F)
Polymyxin B Sulfate	0.1274	(7849)	8300 (Lot I)

NOTE—Each mg of penicillin G benzathine contains 1211 USP Penicillin G Units, and each mg of penicillin G procaine contains 1009 USP Penicillin G Units.

For any of the antibiotics listed, when stocks of a batch of USP Reference Standard are depleted, the replacement is calibrated to maintain continuity of the USP Unit. The number of USP Units per mg of the USP Reference Standard may therefore differ from that shown above.

Table 2. Preparation of Stock Solutions and Test Dilutions of Reference Standards.

Antibiotic and Type of Assay [Cylinder-plate (CP) or Turbidimetric (T)]	Prior Drying	Stock Solution: Initial Solvent (and initial concentration where specified); [Further Diluent, if different]	Stock Solution: Final Stock Concentration per mL	Stock Solution: Use Within	Test Dilution: Final Diluent	Test Dilution: Median Dose (μg of activity or Units per mL)
Amikacin (T)	No	Water	1 mg	14 days	Water	10 μg
Amphotericin B (CP)	Yes	Dimethyl sulfoxide	1 mg	Same day	*B.* 10	1.0 μg
Ampicillin (CP)	No	Water	100 μg	7 days	*B.* 3	0.1 μg
Bacitracin Zinc (CP)	Yes	0.01 *N* hydrochloric acid	100 U	Same day	*B.* 1	1.0 U
Bleomycin (CP)	Yes	*B.* 16	2 U	14 days	*B.* 16	0.04 U
Candicidin (T)	Yes	Dimethyl sulfoxide	1 mg	Same day	Water	0.06 μg
Capreomycin (T)	Yes	Water	1 mg	7 days	Water	100 μg
Carbenicillin (CP)	No	*B.* 1	1 mg	14 days	*B.* 1	20 μg
Cephalexin (CP)	No	*B.* 1	1 mg	7 days	*B.* 1	20 μg
Cephalothin (CP)	Yes	*B.* 1	1 mg	5 days	*B.* 1	1.0 μg
Cephapirin (CP)	No	*B.* 1	1 mg	3 days	*B.* 1	1.0 μg
Cephradine (CP)	No	*B.* 1	1 mg	5 days	*B.* 1	10 μg
Chloramphenicol (T)	No	Alcohol (10 mg/mL); [Water]	1 mg	30 days	Water	2.5 μg
Chlortetracycline (T)	No	0.01 *N* hydrochloric acid	1 mg	4 days	Water	0.06 μg
Clindamycin (CP)	No	Water	1 mg	30 days	*B.* 3	1.0 μg
Cloxacillin (CP)	No	*B.* 1	1 mg	7 days	*B.* 1	5.0 μg
Colistimethate Sodium (CP)	Yes	Water (10 mg/mL); [*B.* 6]	1 mg	Same day	*B.* 6	1.0 μg
Colistin (CP)	Yes	Water (10 mg/mL); [*B.* 6]	1 mg	14 days	*B.* 6	1.0 μg
Cycloserine (T)	Yes	Water	1 mg	30 days	Water	50 μg
Dactinomycin (CP)	Yes	Methanol (10 mg/mL); [*B.* 3]	1 mg	90 days	*B.* 3	1.0 μg
Demeclocycline (T)	Yes	0.1 *N* hydrochloric acid	1 mg	4 days	Water	0.1 μg
Dicloxacillin (CP)	No	*B.* 1	1 mg	7 days	*B.* 1	5.0 μg
Dihydrostreptomycin (CP)	Yes	*B.* 3	1 mg	30 days	*B.* 3	1.0 μg
Dihydrostreptomycin (T)	Yes	Water	1 mg	30 days	Water	30 μg
Doxycycline (T)	No	0.1 *N* hydrochloric acid	1 mg	5 days	Water	0.1 μg
Erythromycin (CP)	Yes	Methanol (10 mg/mL); [*B.* 3]	1 mg	14 days	*B.* 3	1.0 μg
Gentamicin (CP)	Yes	*B.* 3	1 mg	30 days	*B.* 3	0.1 μg
Gramicidin (T)	Yes	Alcohol 95%	1 mg	30 days	Alcohol 95%	0.04 μg
Kanamycin (T)	No	Water	1 mg	30 days	Water	10 μg
Lincomycin (T)	No	Water	1 mg	30 days	Water	0.5 μg
Methacycline (T)	Yes	Water	1 mg	7 days	Water	0.06 μg
Methicillin (CP)	No	*B.* 1	1 mg	4 days	*B.* 1	10 μg
Minocycline (T)	No	0.1 *N* hydrochloric acid	1 mg	2 days	Water	0.085 μg
Mitomycin (CP)	No	*B.* 1	1 mg	14 days	*B.* 1	1.0 μg
Nafcillin (CP)	No	*B.* 1	1 mg	2 days	*B.* 1	2.0 μg
Natamycin (CP)	No	Dimethyl sulfoxide	1 mg	Same day	*B.* 10	5.00 μg
Neomycin (CP)	Yes	*B.* 3	1 mg	14 days	*B.* 3	1.0 μg
Netilmicin (CP)	No	*B.* 3	1 mg	7 days	*B.* 3	0.1 μg
Novobiocin (CP)	Yes	Alcohol (10 mg/mL); [*B.* 3]	1 mg	5 days	*B.* 6	0.5 μg
Nystatin (CP)	Yes	Dimethylformamide	1,000 U	Same day	*B.* 6	20 U
Oxacillin (CP)	No	*B.* 1	1 mg	3 days	*B.* 1	5.0 μg
Oxytetracycline (T)	No	0.1 *N* hydrochloric acid	1 mg	4 days	Water	0.24 μg
Paromomycin (CP)	Yes	*B.* 3	1 mg	21 days	*B.* 3	1.0 μg
Penicillin G (CP)	No	*B.* 1	1,000 U	4 days	*B.* 1	1.0 U
Plicamycin (CP)	Yes	Water	100 μg	1 day	*B.* 1	1.0 μg
Polymyxin B (CP)	Yes	Water; [*B.* 6]	10,000 U	14 days	*B.* 6	10 U
Rifampin (CP)	No	Methanol	1 mg	1 day	*B.* 1	5.0 μg
Rolitetracycline (T)	Yes	Water	1 mg	1 day	Water	0.24 μg
Sisomicin (CP)	No	*B.* 3	1 mg	14 days	*B.* 3	0.1 μg
Spectinomycin (T)	No	Water	1 mg	30 days	Water	30 μg
Streptomycin (T)	Yes	Water	1 mg	30 days	Water	30 μg
Tetracycline (T)	No	0.1 *N* hydrochloric acid	1 mg	1 day	Water	0.24 μg
Ticarcillin (CP)	No	*B.* 1	1 mg	1 day	*B.* 1	5.0 μg
Tobramycin (T)	No	Water	1 mg	14 days	Water	2.5 μg
Troleandomycin (T)	Yes	Isopropyl alcohol–water (4:1)	1 mg	Same day	Water	25 μg
Vancomycin (CP)	No	Water	1 mg	7 days	*B.* 4	10 μg
Viomycin (T)	Yes	Water	1 mg	7 days	Water	100 μg

"*B*" denotes "buffer," and the number following refers to the potassium phosphate buffers defined in this chapter.

For Amphotericin B, Colistimethate Sodium, and Nystatin, prepare the reference standard solutions and the sample test solution simultaneously.

For Ampicillin, prepare the test dilutions of the reference standard and the sample simultaneously.

For Amphotericin B, further dilute the stock solution with dimethyl sulfoxide to give concentrations of 12.8, 16, 20, 25, and 31.2 μg per mL prior to making the test dilutions. The *Test Dilution* of the sample prepared from the *Assay Preparation* should contain the same amount of dimethyl sulfoxide as the test dilutions of the Standard.

For Bacitracin Zinc, each of the Standard test dilutions should contain the same amount of hydrochloric acid as the *Test Dilution* of the sample.

For Natamycin, further dilute the stock solution with dimethyl sulfoxide to give concentrations of 64.0, 80.0, 100, 125, and 156 μg per mL prior to making the test dilutions. Prepare the standard response line solutions simultaneously with dilutions of the specimen to be tested. Use red low-actinic glassware. The *Test Dilution* of the sample prepared from the *Assay Preparation* should contain the same amount of dimethyl sulfoxide as the test dilutions of the Standard.

For Nystatin, further dilute the stock solution with dimethylformamide to give concentrations of 256, 320, 400, 500, and 624 Units per mL prior to making the test dilutions. Prepare the standard response line solutions simultaneously with dilutions of the sample to be tested. The *Test Dilution* of the sample prepared from the *Assay Preparation* should contain the same amount of dimethylformamide as the test dilutions of the Standard. Use red low-actinic glassware.

When making the stock solution of Polymyxin B, add 2 mL of water for each 5 mg of weighed reference standard material.

For Sterile Penicillin G Procaine with Aluminum Stearate Suspension cylinder-plate assay only, use Penicillin G Reference Standard.

Where indicated, about 100 mg of the Reference Standard is dried before use in a vacuum oven at a pressure of 5 mm or less of mercury at a temperature of 60° for 3 hours, except in the case of Bleomycin and Plicamycin (dry at 25° for 4 hours), Capreomycin, Dihydrostreptomycin, and Novobiocin (dry at 100° for 4 hours), Gentamicin (dry at 110° for 3 hours), Candicidin (dry at 40° for 3 hours), and Nystatin (dry at 40° for 2 hours). The Sisomicin Reference Standard and the Netilmicin Reference Standard are not dried; the weight is calculated on the dried basis from the *Loss on drying* value of a separate portion taken concomitantly.

and was therefore assigned a potency of 1000 "μg" per mg. In several such instances, as a result of the development of manufacturing and purification methods for particular antibiotics, preparations became available that contained more than 1000 "μg" of activity per mg. It was then understood that such preparations had an activity equivalent to a given number of "μg" of the original reference standard. In most instances, however, the "μg" of activity is exactly equivalent numerically to the μg (weight) of the pure substance. Complications arise in some situations, e.g., where an antibiotic exists as the free base and in salt form, and the "μg" of activity has been defined in terms of one such form; where the antibiotic substance consists of a number of components having close chemical similarity but differing antibiotic activity; or where the potencies of a family of antibiotics are expressed in terms of a reference standard consisting of a single member which, however, might itself be heterogeneous. In such cases the "μg" of activity defined in terms of a "Master Standard" is tantamount to a "Unit." The "μg" of activity should therefore not be assumed necessarily to correspond to the μg (weight) of the antibiotic substance.

Table 3. Test Organisms for Antibiotics Assayed by the Procedure Indicated in Table 2.

Antibiotic	Test Organism	ATCC* Number
Amikacin	*Staphylococcus aureus*	29737
Amphotericin B	*Saccharomyces cerevisiae*	9763
Ampicillin	*Micrococcus luteus*	9341
Bacitracin	*Micrococcus luteus*	10240
Bleomycin	*Mycobacterium smegmatis*	607
Candicidin	*Saccharomyces cerevisiae*	9763
Capreomycin	*Klebsiella pneumoniae*	10031
Carbenicillin	*Pseudomonas aeruginosa*	25619
Cephalexin	*Staphylococcus aureus*	29737
Cephalothin	*Staphylococcus aureus*	29737
Cephapirin	*Staphylococcus aureus*	29737
Cephradine	*Staphylococcus aureus*	29737
Chloramphenicol	*Escherichia coli*	10536
Chlortetracycline	*Staphylococcus aureus*	29737
Clindamycin	*Micrococcus luteus*	9341
Cloxacillin	*Staphylococcus aureus*	29737
Colistimethate Sodium	*Bordetella bronchiseptica*	4617
Colistin	*Bordetella bronchiseptica*	4617
Cycloserine	*Staphylococcus aureus*	29737
Dactinomycin	*Bacillus subtilis*	6633
Demeclocycline	*Staphylococcus aureus*	29737
Dicloxacillin	*Staphylococcus aureus*	29737
Dihydrostreptomycin (CP)	*Bacillus subtilis*	6633
Dihydrostreptomycin (T)	*Klebsiella pneumoniae*	10031
Doxycycline	*Staphylococcus aureus*	29737
Erythromycin	*Micrococcus luteus*	9341
Gentamicin	*Staphylococcus epidermidis*	12228
Gramicidin	*Streptococcus faecium*	10541
Kanamycin	*Staphylococcus aureus*	29737
Lincomycin	*Staphylococcus aureus*	29737
Methacycline	*Staphylococcus aureus*	29737
Methicillin	*Staphylococcus aureus*	29737
Minocycline	*Staphylococcus aureus*	29737
Mitomycin	*Bacillus subtilis*	6633
Nafcillin	*Staphylococcus aureus*	29737
Natamycin	*Saccharomyces cerevisiae*	9763
Neomycin	*Staphylococcus epidermidis*	12228
Netilmicin	*Staphylococcus epidermidis*	12228
Novobiocin	*Staphylococcus epidermidis*	12228
Nystatin	*Saccharomyces cerevisiae*	2601
Oxacillin	*Staphylococcus aureus*	29737
Oxytetracycline	*Staphylococcus aureus*	29737
Paromomycin	*Staphylococcus epidermidis*	12228
Penicillin G	*Staphylococcus aureus*	29737
Plicamycin	*Staphylococcus aureus*	29737
Polymyxin B	*Bordetella bronchiseptica*	4617
Rifampin	*Bacillus subtilis*	6633
Rolitetracycline	*Staphylococcus aureus*	29737
Sisomicin	*Staphylococcus epidermidis*	12228
Spectinomycin	*Escherichia coli*	10536
Streptomycin (T)	*Klebsiella pneumoniae*	10031
Tetracycline	*Staphylococcus aureus*	29737
Ticarcillin	*Pseudomonas aeruginosa*	29336
Tobramycin	*Staphylococcus aureus*	29737
Troleandomycin	*Klebsiella pneumoniae*	10031
Vancomycin	*Bacillus subtilis*	6633
Viomycin	*Klebsiella pneumoniae*	10031

* American Type Culture Collection, 21301 Parklawn Drive, Rockville, MD 20852.

Preparation of the Standard

To prepare a stock solution, dissolve a quantity of the Reference Standard of a given antibiotic, accurately weighed, and previously dried where so indicated in Table 2, or the entire contents of a vial of Reference Standard, where appropriate, in the solvent specified in that table, and then dilute to the required concentration as indicated. Store in a refrigerator, and use within the period indicated. On the day of the assay, prepare from the stock solution 5 or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio of 1:1.25 for a cylinder-plate assay or smaller for a turbidimetric assay. Use the final diluent specified and a sequence such that the middle or median has the concentration designated.

Preparation of the Sample

From the information available for the preparation to be assayed (the "Unknown"), assign to it an assumed potency per unit weight or volume, and on this assumption prepare on the day of the assay a stock solution and test dilution as specified for each antibiotic but with the same final diluent as used for the Reference Standard. The assay with 5 levels of the Standard requires only one level of the Unknown at a concentration assumed equal to the median level of the Standard.

Organisms and Inoculum

TEST ORGANISMS

The test organism for each antibiotic is listed in Table 3, together with its identification number in the American Type Culture Collection. The method of assay is given for each in Table 2. Maintain a culture on slants of the medium and under the incubation conditions specified in Table 4, and transfer weekly to fresh slants. For *K. pneumoniae* use a non-capsulated culture.

PREPARATION OF INOCULUM

Preparatory to an assay, inoculate, from a recently grown slant or culture of the organism, the surface of 250 mL of the agar medium specified for that organism in Table 4 and contained in a Roux bottle except in the case of *Streptococcus faecium*, which is grown in a liquid medium. Spread the suspension evenly over the surface of the agar with the aid of sterile glass beads, and incubate at the temperature shown for approximately the indicated length of time. At the end of this period, prepare the stock suspension by collecting the surface growth in 50 mL of sterile saline, except for Bleomycin (use 50 mL of Medium 34) and for Ticarcillin (use 50 mL of Medium 37).

For the assay, dilute a portion of the stock suspension by adding a volume of sterile, purified water or sterile saline, in the dilution indicated in Table 4, and determine the transmittance of this trial dilution at 580 nm, with a spectrophotometer. Adjust the proportion in such a way that the *Inoculum* will have a transmittance of 25% against saline as the blank. For the turbidimetric assay, vary the composition of the stock suspension, if necessary, to obtain the optimum dose-response relationship.

For the cylinder-plate assay, determine by trial the proportions of stock suspension to be incorporated in the *Inoculum*, starting with the volumes indicated in Table 4, that result in satisfactory demarcation of the zones of inhibition of about 14 to 16 mm in diameter and giving a reproducible dose relationship. Prepare the inoculum by adding a portion of stock suspension to a sufficient amount of agar medium that has been melted and cooled to 45° to 50°, and swirling to attain a homogeneous suspension.

Table 4. Preparation of Inoculum.

Test Organism & (ATCC No.)	Incubation Conditions: Medium	Incubation Conditions: Temp. (°)	Incubation Conditions: Time	Dilution of Stock Suspension to obtain 25% Transmittance	Suggested Inoculum Composition: Medium	Suggested Inoculum Composition: Amount (mL per 100 mL)	Antibiotics Assayed
Bacillus subtilis (6633)	32	32 to 35	5 days		5	As required	Dactinomycin, Dihydrostreptomycin, Rifampin
					8	0.5	Mitomycin
					8	As required	Vancomycin
Bordetella bronchiseptica (4617)	1	32 to 35	24 hr.	1:20	10	0.1	Colistimethate Sodium, Colistin, Polymyxin B
Escherichia coli (10536)	1	32 to 35	24 hr.	1:20	3	0.7	Chloramphenicol
						0.1	Spectinomycin
Klebsiella pneumoniae (10031)	1	36 to 37.5	24 hr.	1:25	3	0.05	Capreomycin
						0.1	Streptomycin, Troleandomycin, Viomycin, Dihydrostreptomycin
Micrococcus luteus (9341)	1	32 to 35	24 hr.	1:40	11	0.5	Ampicillin, Clindamycin,
					11	1.5	Erythromycin
Micrococcus luteus (10240)	1	32 to 35	24 hr.	1:35	1	0.3	Bacitracin
Mycobacterium smegmatis (607)	36	36 to 37.5	48 hr.	As determined	35	1.0	Bleomycin
Pseudomonas aeruginosa (25619)	1	36 to 37.5	24 hr.	1:25	10	0.5	Carbenicillin
Pseudomonas aeruginosa (29336)	36	36 to 37.5	24 hr.	1:50	38	1.5	Ticarcillin
Saccharomyces cerevisiae (9763)	19	29 to 31	48 hr.	As determined	13	0.2	Candicidin
					19	1.0	Amphotericin B
					19	0.8	Natamycin
Saccharomyces cerevisiae (2601)	19	29 to 31	48 hr.	1:30	19	1.0	Nystatin
Staphylococcus aureus (29737)	1	32 to 35	24 hr.	1:20	1	0.05	Cephalexin, Cephradine
					1	0.1	Cephalothin, Cephapirin, Cloxacillin, Dicloxacillin
					1	0.3	Nafcillin, Oxacillin, Methicillin
					1	1.0	Penicillin G
					3	0.1	Amikacin, Chlortetracycline, Demeclocycline, Doxycycline, Lincomycin, Methacycline, Oxytetracycline, Rolitetracycline, Tetracycline
					3	0.2	Kanamycin, Minocycline
					3	0.4	Cycloserine
					3	0.15	Tobramycin
					8	0.1	Plicamycin
Staphylococcus epidermidis (12228)	1	32 to 35	24 hr.	1:14	11	0.25	Netilmicin
					1	4.0	Novobiocin
					11	0.03	Gentamicin, Sisomicin
					11	0.4	Neomycin
				1:25	11	2.0	Paromomycin
Streptococcus faecium (10541)	3	36 to 37.5	16 to 18 hr.	As determined	3	1.0	Gramicidin

The incubated test organism *Pseudomonas aeruginosa* (ATCC 29336) for the assay of Ticarcillin is suspended in Medium 37, instead of saline, for the determination of light transmittance.

For *Pseudomonas aeruginosa* (ATCC 25619) in the assay of Carbenicillin, use the dilution yielding 25% light transmission, rather than the stock suspension, for preparing the inoculum suspension.

The incubated test organism *Mycobacterium smegmatis* (ATCC 607) for the assay of Bleomycin is suspended in Medium 34, instead of saline, for the determination of light transmittance.

NOTE—The dilution of the Stock Suspension to obtain 25% transmittance is for checking the quality of the suspension and is not used for preparing the inoculum suspension, except as specified above. In all other cases, incorporate the stated amount of undiluted stock suspension in the Medium indicated by number, for preparing the inoculum of suggested starting composition.

Procedure

ASSAY DESIGNS

Microbial assays gain markedly in precision by the segregation of relatively large sources of potential error and bias through suitable experimental designs. In a cylinder-plate assay, the essential comparisons are restricted to relationships between zone diameter measurements within plates, exclusive of the variation between plates in their preparation and subsequent handling. To conduct a turbidimetric assay so that the differences in observed turbidity will reflect the differences in the antibiotic concentration requires both greater uniformity in the environment created for the tubes through closer thermostatic control of the incubator and the avoidance of systematic bias by use of a random place-

ment of replicate tubes in separate tube racks, each rack containing one complete set of treatments. The essential comparisons are then restricted to relationships between the observed turbidities within racks.

NOTE—For some purposes, the practice is to design the assay so that a set of treatments consists of not fewer than three tubes for each sample and standard concentration, and each set is placed in a single rack.

Within these restrictions, the assay design recommended is a 1-level assay with a standard curve. For this assay with a standard curve, prepare solutions of 5, 6, or more test dilutions, provided they include one corresponding to the reference concentration (S_3), of the Standard and a solution of a single median test level of the Unknown as described under *Preparation of Standard* and *Preparation of the Sample*. Consider an assay as preliminary if its computed potency with either design is less than 80 percent or more than 125 percent of that assumed in preparing the stock solution of the Unknown. In such a case, adjust its assumed potency accordingly and repeat the assay.

Microbial determinations of potency are subject to inter-assay as well as intra-assay variables, so that two or more independent assays are required for a reliable estimate of the potency of a given assay preparation or Unknown. Starting with separately prepared stock solutions and test dilutions of both the Standard and the Unknown, repeat the assay of a given Unknown on a different day. If the estimated potency of the second assay differs significantly, as indicated by the calculated standard error, from that of the first, conduct one or more additional assays. The combined result of a series of smaller, independent assays spread over a number of days is a more reliable estimate of potency than that from a single large assay with the same total number of plates or tubes.

CYLINDER-PLATE METHOD

To prepare assay plates using petri dishes, place 21 mL of Medium 2 in each of the required number of plates, and allow it to harden into a smooth base layer of uniform depth, except for Amphotericin B, Natamycin, and Nystatin, where no separate base layer is used. For Ampicillin, Clindamycin, Erythromycin, Gentamicin, Lincomycin, Neomycin B, Paromomycin, and Sisomicin, use Medium 11. For Bleomycin, use 10 mL of Medium 35. For Dihydrostreptomycin use Medium 5. For Dactinomycin, use 10 mL of Medium 5. For Plicamycin, Mitomycin, and Vancomycin, use 10 mL of Medium 8. For Carbenicillin, Colistimethate Sodium, Colistin, and Polymyxin B, use Medium 9. For Netilmicin, use 20 mL of Medium 11. Add 4.0 mL of seed layer inoculum (see *Preparation of Inoculum* and Table 4), prepared as directed for the given antibiotic, except for Bleomycin (use 6 mL), for Netilmicin (use 5 mL), and for Natamycin, Nystatin and Amphotericin B (use 8 mL), tilting the plate back and forth to spread the inoculum evenly over the surface, and allow it to harden. Drop 6 assay cylinders on the inoculated surface from a height of 12 mm, using a mechanical guide or other device to insure even spacing on a radius of 2.8 cm, and cover the plates to avoid contamination. After filling the 6 cylinders on each plate with dilutions of antibiotic containing the test levels specified below, incubate the plates at 32° to 35°, or at the temperature specified below for the individual case, for 16 to 18 hours, remove the cylinders, and measure and record the diameter of each zone of growth inhibition to the nearest 0.1 mm. Incubate the plates at 29° to 31° for Amphotericin B, Natamycin, Nystatin, and Rifampin. Incubate at 34° to 36° for Novobiocin. Incubate at 36° to 37.5° for Carbenicillin, Clindamycin, Colistimethate Sodium, Colistin, Dactinomycin, Dihydrostreptomycin, Gentamicin, Mitomycin, Neomycin, Netilmicin, Paromomycin, Polymyxin B, Sisomicin, Ticarcillin, and Vancomycin.

For the 1-level assay with a standard curve, prepare dilutions representing 5 test levels of the Standard (S_1 to S_5) and a single test level of the Unknown U_3 corresponding to S_3 of the standard curve, as defined under *Preparation of the Standard* and *Preparation of the Sample*. For deriving the standard curve, fill alternate cylinders on each of 3 plates with the median test dilution (S_3) of the Standard and each of the remaining 9 cylinders with one of the other four dilutions of the Standard. Repeat the process for the three dilutions of the Standard. For each Unknown, fill alternate cylinders on each of 3 plates with the median test dilution of the Standard (S_3), and the remaining 9 cylinders with the corresponding test dilution (U_3) of the Unknown.

TURBIDIMETRIC METHOD

On the day of the assay, prepare the necessary doses by dilution of stock solutions of the Standard and of each Unknown as defined under *Preparation of the Standard* and *Preparation of the Sample*. Add 1 mL of each dose to each of 3 prepared test tubes, and place the 3 replicate tubes in a position selected at random, in a tube rack. Include similarly in each rack 1 or 2 control tubes containing 1 mL of the test diluent (see Table 4) but no antibiotic. Upon completion of the rack of test solutions (with candicidin within 30 minutes of the time when water is added to the methyl sulfoxide stock solution), add 9.0 mL of inoculum to each tube in the rack in turn, and place the completed rack immediately in an incubator or a water bath maintained at 36° to 37.5° for 2 to 4 hours, except for Candicidin (incubate at 27° to 29° for 16 to 18 hours). After incubation add 0.5 mL of dilute formaldehyde to each tube, taking one rack at a time, and read its transmittance or absorbance in a suitable spectrophotometer fitted with a 530-nm filter.

For the 1-level assay with a standard curve, prepare dilutions representing 5 test levels of the Standard (S_1 to S_5) and a single test level (U_3) of each of up to 20 Unknowns corresponding to S_3 of the Standard. Prepare also an extra S_3 as a test of growth. Add 1 mL of each test dilution, except for Gramicidin (use 0.1 mL) to 3 tubes and 1 mL of antibiotic-free diluent to 6 tubes as controls. Distribute one complete set, including 2 tubes of controls, to a tube rack, intermingling them at random. Add 9.0 mL of inoculum, incubate, add 0.5 mL of dilute formaldehyde, and complete the assay as directed above. Determine the exact duration of incubation by observation of growth in the reference concentration (median dose) of the dilutions of the standard (S_3).

Calculation

To calculate the potency from the data obtained either by the cylinder-plate or by the turbidimetric method, proceed in each case as directed under *Potencies Interpolated from a Standard Curve* (see *Design and Analysis of Biological Assays* ⟨111⟩), using a log transformation, straight-line method with a least squares fitting procedure, and a test for linearity. Where a number of assays of the same material are made with the same standard curve, calculate the coefficient of variation of results of all of the assays of the material. Where more than one assay is made of the same material with different standard curves, average the two or more values of the potency.

⟨85⟩ BACTERIAL ENDOTOXINS TEST

This chapter provides a test for estimating the concentration of bacterial endotoxins that may be present in or on the sample of the article(s) to which the test is applied using Limulus Amebocyte Lysate (LAL) which has been obtained from aqueous extracts of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*, and which has been prepared and characterized for use as a LAL reagent for gel-clot formation.

Where the test is conducted as a limit test, the specimen is determined to be positive or negative to the test judged against the endotoxin concentration specified in the individual monograph. Where the test is conducted as an assay of the concentration of endotoxin, with calculation of confidence limits of the result obtained, the specimen is judged to comply with the requirements if the result does not exceed (a) the concentration limit specified in the individual monograph, and (b) the specified confidence limits for the assay. In either case the determination of the reaction end-point is made with dilutions from the material under test in direct comparison with parallel dilutions of a reference endotoxin, and quantities of endotoxin are expressed in defined Endotoxin Units.

Since LAL reagents have also been formulated to be used for turbidimetric (including kinetic assays) or colorimetric readings, such tests may be used if shown to comply with the requirements for alternative methods. These tests require the establishment of a standard regression curve and the endotoxin content of the test material is determined by interpolation from the curve. The procedures include incubation for a pre-selected time of reacting endotoxin and control solutions with LAL Reagent and reading of the spectrophotometric light absorbance at suitable wave-

lengths. In the case of the turbidimetric procedure the reading is made immediately at the end of the incubation period, or in the kinetic assays, the absorbance is measured throughout the reaction period and rate values are determined from those readings. In the colorimetric procedure the reaction is arrested at the end of the pre-selected time by the addition of an appropriate amount of acetic acid solution, prior to the readings. A possible advantage in the mathematical treatment of results, if the test be otherwise validated and the assay suitably designed, could be the application of tests of assay validity and the calculation of the confidence interval and limits of potency from the internal evidence of each assay itself (see *Design and Analysis of Biological Assays* ⟨111⟩).

Reference Standard and Control Standard Endotoxins

The reference standard endotoxin (RSE) is the USP Endotoxin Reference Standard which has a defined potency of 10,000 USP Endotoxin Units (EU) per vial. Constitute the entire contents of 1 vial of the RSE with 5 mL of LAL Reagent Water,[1] vortex for not less than 20 minutes, and use this concentrate for making appropriate serial dilutions. Preserve the concentrate in a refrigerator, for making subsequent dilutions, for not more than 14 days. Allow it to reach room temperature, if applicable, and vortex it vigorously for not less than 5 minutes before use. Vortex each dilution for not less than 1 minute before proceeding to make the next dilution. Do not use stored dilutions. A control standard endotoxin (CSE) is an endotoxin preparation other than the RSE that has been standardized against the RSE. If a CSE is a preparation not already adequately characterized, its evaluation should include characterizing parameters both for endotoxin quality and performance (such as reaction in the rabbit), and for suitability of the material to serve as a reference (such as uniformity and stability). Detailed procedures for its weighing and/or constitution and use to assure consistency in performance should also be included. Standardization of a CSE against the RSE using a LAL Reagent for the gel-clot procedure may be effected by assaying a minimum of 4 vials of the CSE or 4 corresponding aliquots, where applicable, of the bulk CSE and 1 vial of the RSE, as directed under *Test Procedure*, but using 4 replicate reaction tubes at each level of the dilution series for the RSE and 4 replicate reaction tubes similarly for each vial or aliquot of the CSE. If all of the dilutions for the 4 vials or aliquots of the CSE cannot be accommodated with the dilutions for the 1 vial of the RSE on the same rack for incubation, additional racks may be used for accommodating some of the replicate dilutions for the CSE, but all of the racks containing the dilutions of the RSE and the CSE are incubated as a block. However, in such cases, the replicate dilution series from the 1 vial of the RSE are accommodated together on a single rack and the replicate dilution series from any one of the 4 vials or aliquots of the CSE are not divided between racks. The antilog of the difference between the mean $\log_{10}$ end-point of the RSE and the mean $\log_{10}$ end-point of the CSE is the standardized potency of the CSE which then is to be converted to and expressed in Units per ng under stated drying conditions for the CSE, or in Units per container, whichever is appropriate. Standardize each new lot of CSE prior to use in the test. Calibration of a CSE in terms of the RSE must be with the specific lot of LAL Reagent and the test procedure with which it is to be used. Subsequent lots of LAL Reagent from the same source and with similar characteristics need only checking of the potency ratio. The inclusion of one or more dilution series made from the RSE when the CSE is used for testing will enable observation of whether or not the relative potency shown by the latter remains within the determined confidence limits. A large lot of a CSE may, however, be characterized by a collaborative assay of a suitable design to provide a representative relative potency and the within-laboratory and between-laboratory variance.

A suitable CSE has a potency of not less than 2 Endotoxin Units per ng and not more than 50 Endotoxin Units per ng, where in bulk form, under adopted uniform drying conditions, e.g., to a particular low moisture content and other specified conditions of use, and a potency within a corresponding range where filled in vials of a homogeneous lot.

Preparatory Testing

Use a LAL reagent of confirmed label or determined sensitivity. In addition, where there is to be a change in lot of CSE, LAL Reagent or another reagent, conduct tests of a prior satisfactory lot of CSE, LAL and/or other reagent in parallel on changeover. Treat any containers or utensils employed so as to destroy extraneous surface endotoxins that may be present, such as by heating in an oven at 250° or above for sufficient time.[2]

The validity of test results for bacterial endotoxins requires an adequate demonstration that specimens of the article, or of solutions, washings, or extracts thereof to which the test is to be applied do not of themselves inhibit or enhance the reaction or otherwise interfere with the test. Validation is accomplished by testing untreated specimens or appropriate dilutions thereof, concomitantly with and without known and demonstrable added amounts of RSE or a CSE, and comparing the results obtained. Appropriate negative controls are included. Validation must be repeated if the LAL Reagent source or the method of manufacture or formulation of the article is changed.

Test for confirmation of labeled LAL Reagent sensitivity—Confirm the labeled sensitivity of the particular LAL reagent with the RSE (or CSE) using not less than 4 replicate vials, under conditions shown to achieve an acceptable variability of the test, viz., the antilog of the geometric mean $\log_{10}$ lysate gel-clot sensitivity is within 0.5λ to 2.0λ, where λ is the labeled sensitivity in Endotoxin Units per mL. The RSE (or CSE) concentrations selected in confirming the LAL reagent label potency should bracket the stated sensitivity of the LAL reagent. Confirm the labeled sensitivity of each new lot of LAL reagent prior to use in the test.

Inhibition or Enhancement Test—Conduct assays with standard endotoxin, of untreated specimens in which there is no endogeneous endotoxin detectable, and of the same specimens to which endotoxin has been added, as directed under *Test Procedure*, but using not less than 4 replicate reaction tubes at each level of the dilution series for each untreated specimen and for each specimen to which endotoxin has been added. Record the end-points (E, in Units per mL) observed in the replicates. Take the logarithms (e) of the end-points, and compute the geometric means of the log end-points for the RSE (or CSE), for the untreated specimens and for specimens containing endotoxin by the formula antilog:

$$\Sigma e/f,$$

in which Σe is the sum of the log end-points of the dilution series used and f is the number of replicate end-points in each case. Compute the amount of endotoxin in the specimen to which endotoxin has been added. The test is valid for the article if this result is within twofold of the known added amount of endotoxin. Alternatively, if the test has been appropriately set up, the test is valid for the article if the geometric mean end-point dilution for the specimen to which endotoxin has been added is within one 2-fold dilution of the corresponding geometric mean end-point dilution of the standard endotoxin.

If the result obtained for the specimens to which endotoxin has been added is outside the specified limit, the article is unsuitable for the *Bacterial Endotoxins Test*, or, in the case of Injections or solutions for parenteral administration, it may be rendered suitable by diluting specimens appropriately.

Repeat the test for inhibition or enhancement using specimens diluted by a factor not exceeding that given by the formula:

$$x/\lambda$$

(see *Maximum Valid Dilution*, below). Use the least dilution sufficient to overcome the inhibition or enhancement of the known added endotoxin, for subsequent assays of endotoxin in test specimens.

If endogeneous endotoxin is detectable in the untreated specimens under the conditions of the test, the article is unsuitable

[1] LAL Reagent Water—Sterile Water for Injection or other water that shows no reaction with the specific LAL Reagent with which it is to be used, at the limit of sensitivity of such reagent.

[2] For a test for validity of procedure for inactivation of endotoxins, see "Dry-heat Sterilization" under *Sterilization and Sterility Assurance of Compendial Articles* ⟨1211⟩. Use a LAL Reagent having a sensitivity of not less than 0.15 Endotoxin Unit per mL.

for the *Inhibition or Enhancement Test*, or, it may be rendered suitable by removing the endotoxin present by ultra-filtration, or by appropriate dilution. Dilute the untreated specimen (as constituted, where applicable, for administration or use), to a level not exceeding the maximum valid dilution, at which no endotoxin is detectable. Repeat the test for *Inhibition or Enhancement* using the specimens at those dilutions.

Test Procedure

In preparing for and applying the test, observe precautions in handling the specimens in order to avoid gross microbial contamination. Washings or rinsings of devices must be with LAL Reagent Water in volumes appropriate to their use and, where applicable, of the surface area which comes into contact with body tissues or fluids. Use such washings or rinsings if the extracting fluid has been in contact with the relevant pathway or surface for not less than 1 hour at controlled room temperature (15° to 30°). Such extracts may be combined, where appropriate. The ultimate rinse or wash volume is such as to result in possible dilution of any contained endotoxin to a level not less than that suitable for use in the *Pyrogen Test* ⟨151⟩ under *Transfusion and Infusion Assemblies* ⟨161⟩.

For validating the test for an article, for endotoxin limit tests or assays, or for special purposes where so specified, testing of specimens is conducted quantitatively to determine response end-points for gel-clot readings. Usually graded strengths of the specimen and standard endotoxin are made by multifold dilutions. Select dilutions so that they correspond to a geometric series in which each step is greater than the next lower by a constant ratio. Do not store diluted endotoxin, because of loss of activity by adsorption. In the absence of supporting data to the contrary, negative and positive controls are incorporated in the test.

Use not less than 2 replicate reaction tubes at each level of the dilution series for each specimen under test. Whether the test is employed as a limit test or as a quantitative assay, a standard endotoxin dilution series involving not less than 2 replicate reaction tubes is conducted in parallel. A set of standard endotoxin dilution series is included for each block of tubes, which may consist of a number of racks for incubation together, provided the environmental conditions within blocks are uniform.

Preparation—Since the form and amount per container of standard endotoxin and of LAL reagent may vary, constitution and/or dilution of contents should be as directed in the labeling. The pH of the test mixture of the specimen and the LAL Reagent is in the range 6.0 to 7.5 unless specifically directed otherwise in the individual monograph. The pH may be adjusted by the addition of sterile, endotoxin-free sodium hydroxide or hydrochloric acid or suitable buffers to the specimen prior to testing.

Maximum Valid Dilution (MVD)—The Maximum Valid Dilution is appropriate to Injections or to solutions for parenteral administration in the form constituted or diluted for administration, or where applicable, to the amount of drug by weight if the volume of the dosage form for administration could be varied. Where the endotoxin limit concentration is specified in the individual monograph in terms of volume (in EU per mL), divide the limit by λ, which is the labeled sensitivity (in EU per mL) of the lysate employed in the assay, to obtain the MVD factor. Where the endotoxin limit concentration is specified in the individual monograph in terms of weight or of Units of active drug (in EU per mg or in EU per Unit), multiply the limit by the concentration (in mg per mL or in Units per mL) of the drug in the solution tested or of the drug constituted according to the label instructions, whichever is applicable, and divide the product of the multiplication by λ, to obtain the MVD factor. The MVD factor so obtained is the limit dilution factor for the preparation for the test to be valid.

Procedure—To 10- × 75-mm test tubes add aliquots of the appropriately constituted LAL reagent, and the specified volumes of specimens, endotoxin standard, negative controls, and a positive product control consisting of the article, or of solutions, washings or extracts thereof to which the RSE (or a standardized CSE) has been added at a concentration of endotoxin of 2λ for that LAL reagent (see under *Test for confirmation of labeled LAL Reagent sensitivity*). Swirl each gently to mix, and place in an incubating device such as a water bath or heating block, accurately recording the time at which the tubes are so placed. Incubate each tube, undisturbed, for 60 ± 2 minutes at 37 ± 1°, and carefully remove it for observation. A positive reaction is characterized by the formation of a firm gel that remains when inverted through 180°. Record such a result as positive (+). A negative result is characterized by the absence of such a gel or by the formation of a viscous gel that does not maintain its integrity. Record such a result as negative (−). Handle the tubes with care, and avoid subjecting them to unwanted vibrations, or false negative observations may result. The test is invalid if the positive product control or the endotoxin standard does not show the end-point concentration to be within ±1 twofold dilutions from the label claim sensitivity of the LAL Reagent or if any negative control shows a gel-clot end-point.

Calculation and Interpretation

Calculation—Calculate the concentration of endotoxin (in Units per mL or in Units per g or mg) in or on the article under test by the formula:

$$\rho S/U,$$

in which S is the antilog of the geometric mean $\log_{10}$ of the end-points, expressed in Endotoxin Units (EU) per mL for the Standard Endotoxin, U is the antilog of Σef, where e is the $\log_{10}$ of the end-point dilution factors, expressed in decimal fractions, f is the number of replicate reaction tubes read at the end-point level for the specimen under test, and ρ is the correction factor for those cases where a specimen of the article cannot be taken directly into test but is processed as an extract, solution, or washing.

Where the test is conducted as an assay with sufficient replication to provide a suitable number of independent results, calculate for each replicate assay the concentration of endotoxin in or on the article under test from the antilog of the geometric mean log end-point ratios. Calculate the mean and the confidence limits from the replicate logarithmic values of all the obtained assay results by a suitable statistical method (see *Calculation of Potency from a Single Assay* ⟨111⟩).

Interpretation—The article meets the requirements of the test if the concentration of endotoxin does not exceed that specified in the individual monograph, and where so specified in the individual monograph or in this chapter, the confidence limits of the assay do not exceed those specified.

⟨87⟩ BIOLOGICAL REACTIVITY TESTS, IN-VITRO

The following tests are designed to determine the biological reactivity of mammalian cell cultures following contact with the elastomeric plastics and other polymeric materials with direct or indirect patient contact or of specific extracts prepared from the materials under test. It is essential to make available the specific surface area for extraction. When the surface area of the specimen cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every mL of extraction fluid. Also it is essential to exercise care in the preparation of the materials to prevent contamination with microorganisms and other foreign matter.

Three tests are described; i.e., the *Agar Diffusion Test*, the *Direct Contact Test*, and the *Elution Test*.* The decision as to which type of test or the number of tests to be performed to assess the potential biological response of a specific sample or extract depends upon the material, the final product, and its intended use. Other factors that may also affect the suitability of sample for a specific use are the polymeric composition; processing and cleaning procedures; contacting media; inks; adhesives; absorption, adsorption, and permeability of preservatives; and conditions of storage. Evaluation of such factors should be

* Further details are given in the following publications of the American Society for Testing and Materials, 1916 Race St., Philadelphia, PA 19103: "Standard Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity," ASTM Designation F 895-84; "Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices," ASTM Designation F 813-83.

made by appropriate additional specific tests before determining that a product made from a specific material is suitable for its intended use.

Reference Standards—*USP Negative Control Plastic Reference Standard. USP Positive Bioreaction Solid Reference Standard. USP Positive Bioreaction Extract Reference Standard.*

Cell Culture Preparation—Prepare multiple cultures of L-929 (ATCC cell line CCL 1, NCTC clone 929) mammalian fibroblast cells in serum-supplemented minimum essential medium having a seeding density of about 10^5 cells per mL. Incubate the cultures at 37 ± 1° for not less than 24 hours in a 5 ± 1% carbon dioxide atmosphere, until a monolayer, with greater than 80% confluence, is obtained. Examine the prepared cultures under a microscope to ensure uniform, near-confluent monolayers. [NOTE—The reproducibility of the *In-vitro Biological Reactivity Tests* depends upon obtaining uniform cell culture density.]

Extraction Solvents—Sodium Chloride Injection (see monograph—use Sodium Chloride Injection containing 0.9 percent of NaCl); Alternatively, serum-free mammalian cell culture media or serum-supplemented mammalian cell culture media may be used. Serum supplementation is used when extraction is done at 37° for 24 hours.

Apparatus—

Autoclave—Employ an autoclave capable of maintaining a temperature of 121 ± 2°, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about 20°, but not below 20°, immediately following the heating cycle.

Oven—Use an oven, preferably a mechanical convection model, that will maintain operating temperatures in the range of 50° to 70° within ±2°.

Incubator—Use an incubator capable of maintaining a temperature of 37 ± 1° and an atmosphere of 5 ± 1% carbon dioxide in air. [NOTE—If capped culture tubes are used, it is unnecessary to maintain a carbon dioxide atmosphere in the incubator.]

Extraction Containers—Use only containers, such as ampuls or screw-cap culture test tubes, or their equivalent, of Type I glass. If used, culture test tubes, or their equivalent, are closed with a screw cap having a suitable elastomeric liner. The exposed surface of the elastomeric liner is completely protected with an inert solid disk 50 to 75 μm in thickness. A suitable disk can be fabricated from polytetrafluoroethylene (polytef).

Preparation of Apparatus—Cleanse all glassware thoroughly with chromic acid cleansing mixture and, if necessary, with hot nitric acid followed by prolonged rinsing with Sterile Water for Injection. Make containers and devices used for extraction, transfer, or administration of test material sterile and dry by a suitable process. If ethylene oxide is used as the sterilizing agent, allow not less than 48 hours for complete degassing.

Procedure—

Preparation of Sample for Extracts—Follow the procedure in chapter ⟨88⟩ *Biological Reactivity Tests, In-Vivo.*

Preparation of Extracts—Prepare as directed for *Preparation of Extracts* in chapter ⟨88⟩, *Biological Reactivity Tests, In-vivo* using either Sodium Chloride Injection (0.9 percent NaCl) or serum-free mammalian cell culture media as *Extraction Solvents.* [NOTE—If extraction is done at 37° for 24 hours, in an incubator, use cell culture media supplemented by serum. The extraction conditions should not in any instance cause physical changes such as fusion or melting of the material pieces other than a slight adherence.]

Agar Diffusion Test

This test is designed for elastomeric closures in a variety of shapes. The agar layer acts as a cushion to protect the cells from mechanical damage while allowing the diffusion of leachable chemicals from the polymeric specimens. Extracts of materials that are to be tested are applied to a piece of filter paper.

Sample Preparation—Use extracts, prepared as directed or use portions of the test specimens having flat surfaces not less than 100 mm^2 in surface area.

Procedure—Prepare the monolayers in 60-mm diameter plates using 7 mL of *Cell Culture Preparation.* Aspirate the culture medium from the monolayers, and replace it with serum-supplemented culture medium containing not more than 2% of agar. Place the flat surfaces of *Sample Preparation*, USP Negative Control Plastic RS (to provide a *Negative Control*), and either USP Positive Bioreaction Extract RS or USP Positive Bioreaction Solid RS (to provide a *Positive Control*) in duplicate cultures in contact with the solidified agar surface. Incubate all cultures for not less than 24 hours at 37 ± 1°, preferably in a humidified incubator containing 5 ± 1% of carbon dioxide. Examine each culture around each *Sample*, *Negative Control*, and *Positive Control*, under a microscope, using cytochemical stains, if desired.

Interpretation of Results—The biological reactivity (cellular degeneration and malformation) is described and rated on a scale of 0 to 4 (see Table 1). Measure the responses obtained from the *Negative Control* and the *Positive Control*. The test system is suitable if the observed response corresponds to the labeled biological reactivity grade of the relevant Reference Standard. Measure the response obtained from the *Sample Preparation*. The *Sample* meets the requirements of the test if none of the cell culture exposed to the *Sample* shows greater than a mild reactivity (Grade 2). Repeat the test if the suitability of the system is not confirmed.

Direct Contact Test

This test is designed for materials in a variety of shapes. The procedure allows for simultaneous extraction and testing of leachable chemicals from the specimen with a serum-supplemented medium. The procedure is not appropriate for very low- or high-density materials that could cause mechanical damage to the cells.

Sample Preparation—Use portions of the test specimen having flat surfaces not less than 100 mm^2 in surface area.

Procedure—Prepare the monolayers in 35-mm diameter plates using 2 mL of cell suspension. Aspirate the culture medium from the cultures, and replace it with 0.8 mL of fresh culture medium. Place a single *Sample Preparation*, USP Negative Control Plastic RS (to provide a *Negative Control*), and USP Positive Bioreaction Solid RS (to provide a *Positive Control*) in each of duplicate cultures. Incubate all cultures for not less than 24 hours at 37 ± 1° in a humidified incubator preferably containing 5 ± 1%

Table 1. Reactivity Grades for Agar Diffusion Test.

Grade	Reactivity	Description of Reactivity Zone
0	None	No detectable zone around or under specimen
1	Slight	Zone limited to area under specimen
2	Mild	Zone extends less than 0.5 cm beyond specimen
3	Moderate	Zone extends 0.5 to 1.0 cm beyond specimen
4	Severe	Zone extends greater than 1.0 cm beyond specimen but does not involve entire dish

Table 2. Reactivity Grades for Direct Contact Test and for Elution Test.

Grade	Reactivity	Conditions of all Cultures
0	None	Discrete intracytoplasmic granules; no cell lysis
1	Slight	More than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present
2	Mild	More than 50% of the cells are round and devoid of intracytoplasmic granules; extensive cell lysis and empty areas between cells
3	Moderate	Greater than 70% of the cell layers; contain rounded cells and/or are lysed
4	Severe	Nearly complete destruction of the cell layers

of carbon dioxide. Examine each culture around each *Sample*, *Negative Control*, and *Positive Control*, under a microscope, using cytochemical stains, if desired.

Interpretation of Results—Proceed as directed for *Interpretation of Results* under *Agar Diffusion Test* using Table 2. The *Sample* meets the requirements of the test if none of the cultures treated with the *Sample* shows greater than a mild reactivity (Grade 2). Repeat the test if the suitability of the system is not confirmed.

Elution Test

This test is designed for the evaluation of extracts of polymeric materials. The procedure allows for extraction of the specimens at physiological or non-physiological temperatures for varying time intervals. It is appropriate for high-density materials and for dose-response evaluations.

Sample Preparation—Prepare as directed in *Preparation of Extracts*, using ether Sodium Chloride Injection (0.9 percent NaCl) or serum-free mammalian cell culture media as *Extraction Solvents*. If the size of the *Sample* cannot be readily measured, a mass of not less than 0.1 g of elastomeric material or 0.2 g of plastic or polymeric material per mL of extraction medium may be used. Alternatively, use serum-supplemented mammalian cell culture media as the extracting medium to simulate more closely physiological conditions. Prepare the extracts by heating for 24 hours in an incubator preferably containing 5 ± 1% of carbon dioxide. Maintain the extraction temperature at 37 ± 1°, because higher temperatures may cause denaturation of serum proteins.

Procedure—Prepare the monolayers in 35-mm diameter plates using 2 mL of *Cell Culture Preparation*. Aspirate the culture medium from the monolayers, and replace it with either extracts of the *Sample*, USP Negative Control Plastic RS (to provide a *Negative Control*), or USP Positive Bioreaction Extract RS (to provide a *Positive Control*). The serum-supplemented and serum-free cell culture media extracts are tested in duplicate without dilution (100%). The Sodium Chloride Injection extract is diluted with serum-supplemented cell culture medium and tested in duplicate at 25% extract concentration. Incubate all cultures for 48 hours at 37 ± 1° in an incubator preferably containing 5 ± 1% of carbon dioxide. Examine each culture at 48 hours, under a microscope, using cytochemical stains, if desired.

Interpretation of Results—Proceed as directed for *Interpretation of Results* under *Agar Diffusion Test* but using Table 2. Repeat the test if the suitability of the system is not confirmed. The *Sample* meets the requirements of the test if the cultures treated with the *Samples* show not greater than a mild reactivity (Grade 2). If the cultures treated with the *Sample* show a significantly greater reaction than the cultures treated with the *Negative Control*, repeat the test with several quantitative dilutions of the extracts.

⟨88⟩ BIOLOGICAL REACTIVITY TESTS, IN-VIVO

The following tests are designed to determine the biological response of animals to elastomeric plastics and other polymeric material with direct or indirect patient contact, or by the injection of specific extracts prepared from the material under test. It is essential to make available the specific surface area for extraction. When the surface area of the specimen cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every mL of extraction fluid. Also it is essential to exercise care in the preparation of the materials to be injected or instilled to prevent contamination with microorganisms and other foreign matter.

Four tests are described. The *Systemic Injection Test* and the *Intracutaneous Test* are used for elastomeric materials, especially to elastomeric plastics for which the appropriate *Biological Reactivity Tests, In-vitro* ⟨87⟩ have indicated significant biological, reactivity. These two tests are used for plastics and other polymers in addition to a third test, the *Implantation Test*, to test the suitability of these materials intended for use in fabricating containers and accessories thereto, for use in parenteral preparations, and for use in medical devices, implants, and other systems.

The fourth test that is used for plastics and other polymeric material for containers and medical devices for ophthalmics is the *Eye Irritation Test*. It consists of the instillation of extracts of materials under test into the eye of a rabbit and a comparison of its effect with that of a rabbit eye treated with control blank extracts.

For the purpose of this chapter, these definitions apply: The *Sample* is the specimen under test or an extract prepared from such a specimen. A *Blank* consists of the same quantity of the same extracting medium that is used for the extraction of the specimen under test, treated in the same manner as the extracting medium containing the specimen under test. A *Negative Control** is a specimen that gives no reaction under the conditions of the test.

On the basis of response to the biological test procedures (see *Systemic Injection Test*, *Intracutaneous Test*, *Implantation Test*), six general classes of plastics are defined in Table 1. This class-

* USP Negative Control Plastic RS.

Table 1. Classification of Plastics.

Plastic Classes[a]						Tests To Be Conducted			
I	II	III	IV	V	VI	Test Material	Animal	Dose	Procedure[b]
x	x	x	x	x	x	Extract of *Sample* in *Sodium Chloride Injection*	Mouse	50 mL/kg	A (iv)
x	x	x	x	x	x		Rabbit	0.2 mL/animal at each of 10 sites	B
	x	x	x	x	x	Extract of *Sample* in *1 in 20 Solution of Alcohol in Sodium Chloride Injection*	Mouse	50 mL/kg	A (iv)
	x	x	x	x	x		Rabbit	0.2 mL/animal at each of 10 sites	B
		x		x	x	Extract of *Sample* in *Polyethylene Glycol 400*	Mouse	10 g/kg	A (ip)
				x	x		Rabbit	0.2 mL/animal at each of 10 sites	B
		x	x	x	x	Extract of *Sample* in *Vegetable Oil*	Mouse	50 mL/kg	A (ip)
			x	x	x		Rabbit	0.2 mL/animal at each of 10 sites	B
			x		x	Implant strips of *Sample*	Rabbit	4 strips/animal	C

[a] Tests required for each class are indicated by "x" in appropriate columns.

[b] Legend: A (ip)—Systemic Injection Test (intraperitoneal); A (iv)—Systemic Injection Test (intravenous); B—Intracutaneous Test intracutaneous); C—Implantation Test (intramuscular implantation).

ification does not apply to plastics that are intended for use as containers for oral or topical products, or that may be used as an integral part of a drug formulation. Extracts of elastomers to be tested are in Sodium Chloride Injection and in vegetable oils.

Factors such as material composition, processing and cleaning procedures, contacting media, inks, adhesives, absorption, adsorption and permeability of preservatives, and conditions of storage may also affect the suitability of a material for a specific use. Evaluation of such factors should be made by appropriate additional specific tests to determine the suitability of a material for its intended use.

With the exception of the *Implantation Test*, the procedures are based on the use of extracts which, depending on the heat resistance of the material, are prepared at one of three standard temperatures: 50°, 70°, and 121°. Therefore, the class designation of a plastic must be accompanied by an indication of the temperature of extraction; e.g., IV–121°, which represents a class IV plastic extracted at 121°, or I–50°, which represents a class I plastic extracted at 50°.

The *Systemic Injection Test* and the *Intracutaneous Test* are designed to determine the systemic and local, respectively, biological responses of animals to plastics and other polymers by the single-dose injection of specific extracts prepared from a *Sample*. The *Implantation Test* is designed to evaluate the reaction of living tissue to the plastic and other polymers by the implantation of the *Sample* itself into animal tissue. The proper preparation and placement of the specimens under aseptic conditions are important in the conduct of the *Implantation Test*.

These tests are designed for application to plastics and other polymers in the condition in which they are used. If the material is to be exposed to any cleansing or sterilization process prior to its end-use, then the tests are to be conducted on a *Sample* prepared from a specimen pre-conditioned by the same processing.

Extracting Media—

SODIUM CHLORIDE INJECTION (see monograph). Use Sodium Chloride Injection containing 0.9 percent of NaCl.

1 in 20 SOLUTION OF ALCOHOL IN *Sodium Chloride Injection*.

POLYETHYLENE GLYCOL 400 (see monograph).

VEGETABLE OIL—Use freshly refined Sesame Oil (see monograph) or Cottonseed Oil (see monograph) or other suitable vegetable oils.

DRUG PRODUCT VEHICLE (where applicable).

WATER FOR INJECTION (see monograph).

[NOTE—The Sesame Oil or Cottonseed Oil or other suitable vegetable oil meets the following additional requirements: Obtain, if possible, freshly refined oil. Use three properly prepared animals and inject the oil intracutaneously in a dose of 0.2 mL into each of 10 sites per animal, and observe the animals at 24, 48, and 72 hours following injection. Rate the observations at each site on the numerical scale indicated in Table 5. For the 3 rabbits (30 injection sites), at any observation time, the average response for erythema is not greater than 0.5 and for edema is not greater than 1.0, and no site shows a tissue reaction larger than 10 mm in overall diameter. The residue of oil at the injection site should not be misinterpreted as edema. Edematous tissue blanches when gentle pressure is applied.]

Apparatus—The apparatus for the tests includes the following.

AUTOCLAVE—Use an autoclave capable of maintaining a temperature of 121 ± 2.0°, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about, but not below, 20° immediately following the heating cycle.

OVEN—Use an oven, preferably a forced-circulation model, that will maintain operating temperatures of 50° or 70° within ±2°.

EXTRACTION CONTAINERS—Use only containers, such as ampuls or screw-cap culture test tubes, of Type I glass. If used, culture test tubes are closed with screw caps having suitable elastomeric liners. The exposed surface of the elastomeric liner is completely protected with an inert solid disk 0.05 mm to 0.075 mm in thickness. A suitable disk may be fabricated from a polytetrafluoroethylene (polytef) resin.

Preparation of Apparatus—Cleanse all glassware thoroughly with chromic acid cleansing mixture, or if necessary with hot nitric acid, followed by prolonged rinsing with water. Clean cutting utensils by an appropriate method (e.g., successive cleaning with acetone and methylene chloride) prior to use in subdividing a specimen. Clean all other equipment by thorough scrubbing with a suitable detergent and prolonged rinsing with water.

Render containers and equipment used for extraction, and in transfer and administration of test material, sterile and dry by a suitable process. [NOTE—If ethylene oxide is used as the sterilizing agent, allow adequate time for complete degassing.]

Procedure—

Preparation of Sample—Both the *Systemic Injection Test* and the *Intracutaneous Test* may be performed using the same extract, if desired, or separate extracts may be made for each test. Select and subdivide into portions a *Sample* of the size indicated in Table 2. Remove particulate matter, such as lint and free particles, by treating each subdivided *Sample* or *Negative Control* as follows: Place the *Sample* into a clean, glass-stoppered, 100-mL graduated cylinder of Type I glass, and add about 70 mL of Water for Injection. Agitate for about 30 seconds, and drain off the water, repeat this step, and dry those pieces prepared for the extraction with *Vegetable Oil* in an oven at a temperature not exceeding 50°. [NOTE—Do not clean the *Sample* [with a dry or wet cloth or by rinsing or washing with an organic solvent, surfactant, etc.]

Preparation of Extracts—Place a properly prepared *Sample* to be tested in an extraction container, and add 20 mL of the appropriate extracting medium. Repeat these directions for each extracting medium required for testing. Also prepare one 20-mL blank of each medium for parallel injections and comparisons.

Table 2. Surface Area of Specimen To Be Used.[1]

Form of Material	Thickness	Amount of *Sample* for Each 20 mL of Extracting Medium	Subdivided Into
Film or sheet	<0.5 mm	Equivalent of 120 cm^2 total surface area (both sides combined)	Strips of about 5 × 0.3 cm
	0.5 to 1 mm	Equivalent of 60 cm^2 total surface area (both sides combined)	
Tubing	<0.5 mm (wall)	Length (in cm) = 120 cm^2/(sum of ID and OD circumferences)	Sections of about 5 × 0.3 cm
	0.5 to 1 mm (wall)	Length (in cm) = 60 cm^2/(sum of ID and OD circumferences)	
Slabs, tubing, and molded items	>1 mm	Equivalent of 60 cm^2 total surface area (all exposed surfaces combined)	Pieces up to about 5 × 0.3 cm
Elastomers	>1 mm	Equivalent of 25 cm^2 total surface area (all exposed surfaces combined)	Do not subdivide[2]

[1] When surface area cannot be determined due to the configuration of the specimen, use 0.1 g of elastomer or 0.2 g of plastic or other polymers for every 1 mL of extracting fluid.

[2] Molded elastomeric closures are tested intact.

Extract by heating in an autoclave at 121° for 60 minutes, in an oven at 70° for 24 hours, or at 50° for 72 hours. Allow adequate time for the liquid within the container to reach the extraction temperature.

[NOTE—The extraction conditions should not in any instance cause physical changes such as fusion or melting of the *Sample* pieces, which result in a decrease in the available surface area. A slight adherence of the pieces can be tolerated. Always add the cleaned pieces individually to the extracting medium. If culture tubes are used for autoclave extractions with *Vegetable Oil*, seal screw caps adequately with pressure-sensitive tape.]

Cool to about room temperature but not below 20°, shake vigorously for several minutes, and decant each extract immediately, using aseptic precautions, into a dry, sterile vessel. Store the extracts at a temperature between 20° and 30°, and do not use for tests after 24 hours. Of importance are the contact of the extracting medium with the available surface area of the plastic and the time and temperature during extraction, the proper cooling, agitation, and decanting process, and the aseptic handling and storage of the extracts following extraction.

Systemic Injection Test

This test is designed to evaluate systemic responses to the extracts of materials under test following injection into mice.

Test Animal—Use healthy, not previously used albino mice weighing between 17 and 23 g. For each test group use only mice of the same source. Allow water and food, commonly used for laboratory animals and of known composition, ad libitum.

Procedure—[NOTE—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter. However, visible particulates should not be injected intravenously.] Inject each of the five mice in a test group with the *Sample* or the *Blank* as outlined in Table 3, except to dilute each g of the extract of the *Sample* prepared with *Polyethylene Glycol 400*, and the corresponding blank, with 4.1 volumes of *Sodium Chloride Injection* to obtain a solution having a concentration of about 200 mg of polyethylene glycol per mL.

Observe the animals immediately after injection, again 4 hours after injection, and then at least at 24, 48, and 72 hours. If during the observation period none of the animals treated with the extract of the *Sample* shows a significantly greater biological reactivity than the animals treated with the *Blank*, the *Sample* meets the requirements of this test. If any animals treated with the *Sample* show only slight signs of biological reactivity, and not more than one animal shows gross symptoms of biological reactivity or dies, repeat the test using groups of 10 mice. On the repeat test, all 10 animals treated with the *Sample* show no significant biological reactivity above the *Blank* animals during the observation period.

Intracutaneous Test

This test is designed to evaluate local responses to the extracts of materials under test following intracutaneous injection into rabbits.

Test Animal—Select healthy, thin-skinned albino rabbits whose fur can be clipped closely and whose skin is free from mechanical irritation or trauma. In handling the animals, avoid touching the injection sites during observation periods, except to discriminate between edema and an oil residue. [NOTE—Rabbits previously used in unrelated tests, such as the *Pyrogen Test* ⟨151⟩, and that have received the prescribed rest period, may be used for this test provided that they have clean, unblemished skin.]

Procedure—[NOTE—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter.] On the day of the test, closely clip the fur on the animal's back on both sides of the spinal column over a sufficiently large test area. Avoid mechanical irritation and trauma. Remove loose hair by means of vacuum. If necessary, swab the skin lightly with diluted alcohol, and dry the skin prior to injection. More than one extract from a given material can be used per rabbit, if you have determined that the test results will not be affected. For each *Sample* use two animals and inject each intracutaneously, using one side of the animal for the *Sample* and the other side for the *Blank*, as outlined in Table 4. [NOTE—Dilute each g of the extract of the *Sample* prepared with *Polyethylene Glycol 400*, and the corresponding *Blank*, with 7.4 volumes of *Sodium Chloride Injection* to obtain a solution having a concentration of about 120 mg of polyethylene glycol per mL.]

Examine injection sites for evidence of any tissue reaction such as erythema, edema, and necrosis. Swab the skin lightly, if necessary, with diluted alcohol to facilitate reading of injection sites. Observe all animals at 24, 48, and 72 hours after injection. Rate the observations on a numerical scale for the extract of the *Sample* and for the *Blank*, using Table 5. Reclip the fur as necessary during the observation period.

If each animal at any observation period shows an average reaction to the *Sample* that is not significantly greater than to the *Blank*, the *Sample* meets the requirements of this test. If at any observation period the average reaction to the *Sample* is questionably greater than the average reaction to the *Blank*, repeat the test using three additional rabbits. On the repeat test, the average reaction to the *Sample* in any of the three animals is not significantly greater than the *Blank*.

Table 3. Injection Procedure—Systemic Injection Test.

Extract or Blank	Dose per kg	Route*	Injection Rate, µL per second
Sodium Chloride Injection	50 mL	IV	100
1 in 20 solution of Alcohol in Sodium Chloride Injection	50 mL	IV	100
Polyethylene Glycol 400	10 g	IP	—
Drug product vehicle	50 mL	IV	100
(where applicable)	50 mL	IP	—
Vegetable Oil	50 mL	IP	—

* IV = intravenous (aqueous sample and blank); IP = intraperitoneal (oleaginous sample and blank).

Table 4. Intracutaneous Test.

Extract or Blank	Number of Sites (per animal)	Dose, µL per site
Sample	5	200
Blank	5	200

Table 5. Evaluation of Skin Reactions.

Erythema and Eschar Formation	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet-redness) to slight eschar formation (injuries in depth)	4
Edema Formation	**Value**
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

Implantation Test

The implantation test is designed for the evaluation of plastic materials and other polymeric materials in direct contact with living tissue. Of importance are the proper preparation of the implant strips and their proper implantation under aseptic conditions. Prepare for implantation 8 strips of the *Sample* and 4 strips of USP Negative Control Plastic RS. Each strip should measure not less than 10 × 1 mm. The edges of the strips should

be as smooth as possible to avoid additional mechanical trauma upon implantation. Strips of the specified minimum size are implanted by means of a hypodermic needle (15- to 19-gauge) with intravenous point and a sterile trocar. Use either pre-sterilized needles into which the sterile plastic strips are aseptically inserted, or insert each clean strip into a needle, the cannula and hub of which are protected with an appropriate cover, and then subjected to the appropriate sterilization procedure. [NOTE—Allow for proper degassing if agents such as ethylene oxide are used.]

Test Animal—Select healthy, adult rabbits weighing not less than 2.5 kg, and whose paravertebral muscles are sufficiently large in size to allow for implantation of the test strips. Do not use any muscular tissue other than the paravertebral site. The animals may be anesthetized with a commonly used anesthetic agent to a degree deep enough to prevent muscular movements, such as twitching.

Procedure—Perform the test in a clean area. On the day of the test or up to 20 hours before testing, clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum. Swab the skin lightly with diluted alcohol and dry the skin prior to injection.

Implant four strips of the *Sample* into the paravertebral muscle on one side of the spine of each of 2 rabbits, 2.5 to 5 cm from the midline and parallel to the spinal column, and about 2.5 cm apart from each other. In a similar fashion implant 2 strips of USP Negative Control Plastic RS in the opposite muscle of each animal. Insert a sterile stylet into the needle to hold the implant strip in the tissue while withdrawing the needle. If excessive bleeding is observed after implantation of a strip, place a duplicate strip at another site.

Keep the animals for a period of not less than 72 hours, and sacrifice them at the end of the observation period by administering an overdose of an anesthetic agent or other suitable agents. Allow sufficient time to elapse for the tissue to be cut without bleeding. Examine macroscopically the area of the tissue surrounding the center portion of each implant strip. Use a magnifying lens if necessary. The tissue immediately surrounding the USP Negative Control Plastic RS strips appears normal and entirely free from hemorrhage, film, or encapsulation. The requirements of the test are met if, in each rabbit, the reaction to not more than 1 of the 4 *Sample* strips is significantly greater than that to the strips of USP Negative Control Plastic RS.

Eye Irritation Test

This test is designed to evaluate responses to the instillation of extracts of materials under testing in the eye of a rabbit.

Extracting Media—Use (1) Sodium Chloride Injection and (2) Vegetable Oil.

Test Animal—Select healthy, albino rabbits having no visible eye irritation and not previously used for an eye irritation test. The animal facilities should be designed and maintained as to exclude sawdust, wood chips, or other extraneous materials that might produce eye irritation. Examine both eyes of the animals before testing, and use only those animals without eye defects or eye irritation.

Procedure—Use three albino rabbits for each extract to be tested. Restrain the animals firmly but gently until quiet. Gently pull and lower lid away from the eyeball to form a cup, and instill about 200 µL of the *Blank* prepared as directed under *Systemic Injection Test*. Hold the lid together for about 30 seconds. Instill into the other eye 200 µL of the *Sample* extract prepared as directed under *Systemic Injection Test*. Examine the eyes at 24-, 48-, and 72-hour intervals. Examination is facilitated by the use of a binocular loupe and a hand slit-lamp. The requirements of the test are met if the *Sample* extract shows no significant irritation over the *Blank* extract during the observation period. If significant irritation is observed in the eye treated with the *Blank extract*, repeat the test using three additional rabbits. On the repeat test, all rabbits meet the test requirement.

SAFETY TESTS—GENERAL

The general safety test set forth here is intended to detect in an article any unexpected, unacceptable, biological reactivity. This in-vivo test is provided for the safety assessment of biologics (see *Biologics* ⟨1041⟩) and any article in *Transfusion and Infusion Assemblies* ⟨161⟩, *Elastomeric Closures for Injections* ⟨381⟩, and *Containers* ⟨661⟩.

Safety Test

Select five healthy mice not previously used for testing, weighing between 17 and 23 g, unless otherwise directed in the individual monograph or elsewhere in this chapter, and maintained on an adequate balanced diet. Prepare a test solution as directed in the individual monograph. Unless otherwise directed in the individual monograph or elsewhere in this chapter, inject intravenously a dose of 0.5 mL of the test solution into each of the mice, using a 26-gauge needle of suitable length, or of the length specified below as applicable. Observe the animals over the 48 hours following the injection. If, at the end of 48 hours, all of the animals survive and not more than one of the animals shows outward symptoms of a reaction not normally expected of the level of toxicity related to the article, the requirements of this test are met. If one or more animals die or if more than one of the animals shows signs of abnormal or untoward toxicity of the article under test, repeat the test using at least another 10 mice similar to those used in the initial test, but weighing 20 ± 1 g. In either case, if all of the animals survive for 48 hours and show no symptoms of a reaction indicative of an abnormal or undue level of toxicity of the article, the requirements of the test are met.

For biologics, perform the test according to the procedures prescribed in the *Federal Regulations* (see *Biologics* ⟨1041⟩, Section 610.11, using not less than 2 mice similar to those described above but weighing less than 22 g and not less than 2 healthy guinea pigs weighing less than 400 g. Unless otherwise directed in the individual monograph, for a liquid product or a freeze-dried product that has been constituted as directed in the labeling, inject a volume of 0.5 mL intraperitoneally into each mouse, and inject a volume of 5.0 mL intraperitoneally into each guinea pig. For freeze-dried products for which the volume of constitution is not indicated in the label, or for nonliquid products other than freeze-dried products, perform the test using the route of administration, test dose, and diluent approved by the Center For Biologics Evaluation and Research (FDA), on the basis of substantial evidence demonstrating that the test variation will assure sensitivity equal to or greater than that of the test described above. Observe the animals for a minimum observation period of 7 days. If all of the animals survive the test period, do not exhibit any response that is not specific for or expected from the product and that may indicate a difference in such product quality, and weigh no less at the end of the test period than at the time of injection, the requirements of the test are met. If the article fails to meet the requirements, the test may be repeated as in the initial test, in the one or both species in which the requirements were not met. If the animals fulfill the criteria specified for the initial test, the article meets the requirements of the test. If the article fails to meet the requirements after the first repeat test, and not less than 50% of the total number of animals of the species in which the requirements of the test were not met in the combined initial and first retests have survived, a second retest may be performed. Use twice the number of animals of the relevant species used in the initial test. If the animals fulfill the criteria specified for the initial test, the requirements of the test are met.

⟨91⟩ CALCIUM PANTOTHENATE ASSAY

Reference standard—*USP Calcium Pantothenate Reference Standard*—Dry at 105° for 3 hours before using.

Standard Stock Solution of Calcium Pantothenate—Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and accurately weighed while protected from absorption of moisture during the weighing, in about 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 *N* acetic acid and 100 mL of sodium acetate solution (1 in 60), then dilute with water to volume. Each mL represents 50 µg of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard Preparation—On the day of the assay, dilute a measured volume of *Standard Stock Solution of Calcium Pantothenate* with sufficient water so that it contains, in each mL, between 0.01 μg and 0.04 μg of calcium pantothenate, the exact concentration being such that the responses obtained as directed under *Procedure*, 2.0 and 4.0 mL of the *Standard Preparation* being used, are within the linear portion of the log-concentration response curve.

Assay Preparation—Proceed as directed in the individual monograph for preparing a solution expected to contain approximately the equivalent of the calcium pantothenate concentration in the *Standard Preparation*.

Basal Medium Stock Solution—

Acid-hydrolyzed Casein Solution	25	mL
Cystine-Tryptophane Solution	25	mL
Polysorbate 80 Solution	0.25	mL
Dextrose, Anhydrous	10	g
Sodium Acetate, Anhydrous	5	g
Adenine-Guanine-Uracil Solution	5	mL
Riboflavin–Thiamine Hydrochloride–Biotin Solution	5	mL
Para-aminobenzoic Acid–Niacin–Pyridoxine Hydrochloride Solution	5	mL
Salt Solution A	5	mL
Salt Solution B	5	mL

Dissolve the anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 *N* sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL, and mix.

Acid-hydrolyzed Casein Solution—Mix 100 g of vitamin-free casein with 500 mL of 6 *N* hydrochloric acid, and reflux the mixture for 8 to 12 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 *N* sodium hydroxide to a pH of 3.5 ± 0.1, and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-Tryptophane Solution—Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophane (or 2.0 g of D,L-tryptophane) in 700 to 800 mL of water, heat to 70° to 80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a refrigerator at a temperature not below 10°.

Adenine-Guanine-Uracil Solution—Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 *N* hydrochloric acid, cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 Solution—Dissolve 25 g of polysorbate 80 in alcohol to make 250 mL.

Riboflavin–Thiamine Hydrochloride–Biotin Solution—Prepare a solution containing, in each mL, 20 μg of riboflavin, 10 μg of thiamine hydrochloride, and 0.04 μg of biotin, by dissolving riboflavin, thiamine hydrochloride, and biotin in 0.02 *N* acetic acid. Store, protected from light, under toluene in a refrigerator.

Para-aminobenzoic Acid–Niacin–Pyridoxine Hydrochloride Solution—Prepare a solution in neutral 25 percent alcohol to contain 10 μg of para-aminobenzoic acid, 50 μg of niacin, and 40 μg of pyridoxine hydrochloride in each mL. Store in a refrigerator.

Salt Solution A—Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Salt Solution B—Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Stock Culture of Lactobacillus plantarum—Dissolve 2.0 g of water-soluble yeast extract in 100 mL of water, add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Add approximately 10-mL portions of the hot solution to test tubes, suitably close or cover the tubes, sterilize at 121°, and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus plantarum*,* incubating for 16 to 24 hours at any selected temperature between 30° and 37° but held constant to within ±0.5°, and finally store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for inoculum if the culture is more than 1 week old.

Culture Medium—To each of a series of test tubes containing 5.0 mL of *Basal Medium Stock Solution* add 5.0 mL of water containing 0.2 μg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121°, and cool.

Inoculum—Make a transfer of cells from the stock culture of *Lactobacillus plantarum* to a sterile tube containing 10 mL of culture medium. Incubate this culture for 16 to 24 hours at any selected temperature between 30° and 37° but held constant to within ±0.5°. The cell suspension so obtained is the inoculum.

Procedure—To similar test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL, respectively, of the *Standard Preparation*. To each tube and to 4 similar tubes containing no *Standard Preparation* add 5.0 mL of *Basal Medium Stock Solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Assay Preparation* corresponding to 3 or more of the levels listed above for the *Standard Preparation*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal Medium Stock Solution* and sufficient 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 of both series suitably to prevent contamination, and heat in an autoclave at 121° for 5 minutes. Cool, add 1 drop of inoculum to each tube, except 2 of the 4 tubes containing no *Standard Preparation* (to serve as the uninoculated blanks), and mix. Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16 to 24 hours of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of standard during a 2-hour period.

Determine the transmittance of the tubes in the following manner: Mix the contents of each tube, and transfer to an optical container if necessary. Place the container in a spectrophotometer that has been set at a specific wavelength between 540 nm and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer 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 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation—Prepare a standard concentration-response curve as follows: For each level of the standard, calculate the response from the sum of the duplicate values of the transmittance as the difference, $y = 2.00 - \Sigma$ (of transmittance). Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard Preparation* 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 fall 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 mg, of USP Calcium Pantothenate RS corresponding

* American Type Culture Collection No. 8014 is suitable. This strain formerly was known as *Lactobacillus arabinosus* 17–5.

to the calcium pantothenate in the portion of material taken for assay as antilog:

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

in which R is the number of mg of calcium pantothenate 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, M, is the assayed log-potency of the test material (see *The Confidence Interval and Limits of Potency* ⟨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.

⟨101⟩ DEPRESSOR SUBSTANCES TEST

Reference Standard—*USP Histamine Dihydrochloride Reference Standard*—Keep container tightly closed and protected from light. Dry over silica gel for 2 hours before using.

Standard Solution of Histamine—Dissolve a suitable quantity of USP Histamine Dihydrochloride RS, accurately weighed, in water, and dilute with water to obtain a solution having a known concentration of the equivalent of 1.0 μg of histamine base per mL.

The Animal—Weigh and anesthetize a healthy, and if female, nonpregnant adult cat by intraperitoneal injection of an anesthetic substance, such as sodium phenobarbital, that is favorable to maintenance of uniform blood pressure. Immobilize the animal, and make provisions to prevent excess loss of body heat. If preferable, insert a tracheal cannula. Expose a carotid or other suitable artery, separate it from surrounding tissues, and arrange for continuous blood-pressure recording with a manometer or other apparatus of at least equivalent sensitivity. Then expose a femoral vein to facilitate intravenous injection.

Determine the sensitivity of the animal to histamine by injecting, at uniform time intervals of not less than 5 minutes, doses of the *Standard Solution of Histamine* corresponding to 0.05, 0.1, and 0.15 μg of histamine base per kg of body weight of the animal. Repeat these injections, and disregard the first set of responses. Determine the extent of variation in depressor response to the same dose by repeating the injection of 0.1 μg per kg. Use the animal for the test only if the responses to the graded doses are clearly different and the responses to several injections of the dose of 0.1 μg per kg are approximately the same and correspond to a decrease in pressure of not less than 20 mm of mercury. If in this test the doses of the *Standard Solution of Histamine* and of the solution under test are to be given through a single common cannula, each injection in the preliminary test and in the succeeding test is to be followed immediately by an injection of about 2.0 mL of Sodium Chloride Injection to flush in any residual activity.

Procedure—Dissolve the substance under test in the diluent designated so as to give the concentration specified in the individual monograph. Follow the same time schedule established during the injection of the *Standard Solution of Histamine*. Inject a series of three doses, of which two doses of 0.1 μg of histamine base per kg are alternated with an intervening dose of the solution under test in the dosage specified in the individual monograph. Measure the change in blood pressure following each of the three injections. The depressor response to the solution under test is not greater than one-half the mean depressor response to the two associated doses representing 0.1 μg of histamine base per kg. If this requirement is not met, continue the series of injections similarly until it consists of five doses, of which three doses of 0.1 μg of histamine base per kg are alternated with two doses of the solution under test in the dosage specified in the individual monograph. Measure the change in blood pressure following each of the additional injections. The depressor response to each dose of the solution under test is not greater than the mean of the respective depressor responses to the associated doses, representing 0.1 μg of histamine base per kg.

If the depressor response to either dose of the solution under test is greater than the mean of the depressor responses to the associated doses representing 0.1 μg of histamine base per kg, the test may be continued in the same animal, or in another animal similarly prepared and tested for responses to the *Standard Solution of Histamine*. If the test is continued in the same animal, after the last dose of the *Standard Solution of Histamine* of the initial series, administer four more injections, of which two are doses of the solution under test and two are doses representing 0.1 μg of histamine base per kg, alternately in sequence. If the test is continued in another animal, prepare a fresh solution of the substance under test from an independent container or containers of test substance, and inject a series of five doses comprising the *Standard Solution of Histamine* and the solution under test in accordance with the initial injection sequence. Measure the change in blood pressure following each of the additional injections. Compute the difference between each response to the dose of the solution under test and the mean of the associated doses representing 0.1 μg of histamine base per kg in the entire series, initial and additional, and calculate the average of all such differences. The requirements of the test are met if the average of the differences is such that in the specified dose the depressor response to the solution under test is not greater than the depressor response to the dose representing 0.1 μg of histamine base per kg, and if not more than one-half of the depressor responses to the solution under test are greater than the mean of the respective depressor responses to the associated doses, representing 0.1 μg of histamine base per kg.

⟨111⟩ DESIGN AND ANALYSIS OF BIOLOGICAL ASSAYS

General

The potency of several Pharmacopeial drugs must be determined by bioassay. A controlling factor in assay design and analysis is the variability of the biological test system, which may vary in its mean response from one laboratory to another, and from time to time in the same laboratory. To control this type of variation, the response to a Pharmacopeial drug is compared with that to a USP Reference Standard or other suitable standard. For convenience, each such preparation will be called the "Standard" and each preparation under assay, or Sample, the "Unknown," and these will be designated respectively by the symbols S and U. (The Sample is sometimes referred to as the "test preparation.")

After elimination of extraneous variables from the comparison of the Standard and the Unknown, an error variance is computed from the remaining variation, which, while uncontrolled, can nevertheless be measured. The error variance is required in calculating the confidence interval of the assayed potency. The confidence interval, known also as the fiducial interval, is so computed that its upper and lower limits are expected to enclose the true potency of the Unknown in 19 out of 20 assays. Many assay procedures fix the acceptable width of the confidence interval, and two or more independent assays may be needed to meet the specified limit. The confidence limits of the individual component assays usually overlap.

The aim of this chapter is to present a concise account of biometrical procedures for the USP bioassays. Its various sections are interrelated. Although the procedures are planned primarily for the assay of a single Unknown, equations for the joint assay of several Unknowns are given in context throughout the chapter and are summarized in the last section. Proof that an assayed potency meets its required confidence limits may be based also upon other recognized biometric methods that have a precision equivalent to that of the methods outlined herein.

A glossary of the terms used in the equations is provided at the end of this chapter.

Steps Preceding the Calculation of Potency

Designs for Minimizing the Error Variance—Variation in response is reduced as much as is practicable by the limitations

imposed on body weight, age, previous handling, environment, and similar factors. In a number of assays, the test animals or their equivalent are then assigned at random but in equal numbers to the different doses of the Standard and Unknown. This implies an objective random process, such as throwing dice, shuffling cards, or using a table of random numbers. Assigning the same number of individuals to each treatment simplifies the subsequent calculations materially, and usually leads to the shortest confidence interval for a given number of observations.

In some assays, the potential responses can be assembled into homogeneous sets in advance of treatment. The differences between sets are later segregated, so that they do not affect adversely either the computed potency or its confidence interval. One unit within each set, picked at random, receives each treatment. Examples of randomized sets are the cleared areas on a single plate in the plate assay of an antibiotic, and four successive paired readings in the same rat in the Vasopressin Injection assay. Sets of two occur where each test animal is used twice, as in the assays of Tubocurarine Chloride Injection and Insulin Injection. In these cases, neither the average differences between individuals nor the order of treatment can bias the potency or precision. In the microbial assays for vitamin B_{12} activity and for calcium pantothenate, replicate tubes are assigned to two or more separate, complete sets, preferably with the tubes arranged at random within each set. This restricts the variation due to position or order within a set to the differences within each complete replicate.

Rejection of Outlying or Aberrant Observations—A response that is questionable because of failure to comply with the procedure during the course of an assay is rejected. Other aberrant values may be discovered only after the responses have been tabulated, but can then be traced to assay irregularities, which justify their omission. The arbitrary rejection *or* retention of an apparently aberrant response can be a serious source of bias. In general, the rejection of observations solely on the basis of their relative magnitudes is a procedure to be used sparingly. When this is unavoidable, each suspected aberrant response or outlier may be tested against one of two criteria:

1. The first criterion is based upon the variation within a single group of supposedly equivalent responses. On the average, it will reject a valid observation once in 25 or once in 50 trials, provided that relatively few, if any, responses within the group are identical. Beginning with the supposedly erratic value or outlier, designate the responses in order of magnitude from y_1 to y_N, where N is the number of observations in the group. Compute the relative gap $G_1 = (y_2 - y_1)/(y_N - y_1)$ when $N = 3$ to 7, $G_2 = (y_3 - y_1)/(y_{N-1} - y_1)$ when $N = 8$ to 13, or $G_3 = (y_3 - y_1)/(y_{N-2} - y_1)$ when $N = 14$ to 24. If G_1, G_2, or G_3 exceeds the critical value in Table 1 for the observed N, there is a statistical basis for omitting the outlier.

This criterion is applicable also in a microbial assay where each treatment is represented by a transmittance in each of two separate complete sets. Subtract each transmittance in the first set from its paired value in the second set, and record each difference with its sign, either plus or minus. Beginning with the most divergent difference, designate the N differences in order of magnitude from y_1 to y_N and compute the relative gap G_1, G_2, or G_3. If this exceeds its critical value in Table 1, one of the two transmittances giving the aberrant difference is suspect and may be identified on inspection or by comparison with its expectation (see next column). Repeat the process with the remaining differences if an outlier is suspected in a second pair.

2. The second criterion compares the ranges from a series of $k = 2$ or more groups. Different groups may receive different treatments, but all f responses within each group represent the same treatment. Compute the range from each group by subtracting the smallest response from the largest within each of the k groups. Divide the largest of the k ranges by the sum of all the ranges in the series. Refer this ratio R_* to Table 2. If k is not larger than 10, use the tabular values in the upper part of Table 2; if k is larger than 10, multiply R_* by $(k + 2)$ and interpolate, if necessary, between the tabular values in the lower

Table 1

Test for outliers. In samples from a normal population, gaps equal to or larger than the following values of G_1, G_2, and G_3 occur with a probability $P = 0.02$ where outliers can occur only at one end, or with $P = 0.04$ where they may occur at either end.

N	3	4	5	6	7						
G_1	.976	.846	.729	.644	.586						
N	8	9	10	11	12	13					
G_2	.780	.725	.678	.638	.605	.578					
N	14	15	16	17	18	19	20	21	22	23	24
G_3	.602	.579	.559	.542	.527	.514	.502	.491	.481	.472	.464

Table 2

Test for groups containing outliers. Compute the range from the f observations in each of k groups, where all groups in the series are equal in size. The observed ratio R_* of the largest range to the sum of the k ranges will equal or exceed the following critical values at a probability of $P = 0.05$.

No. of Ranges k	Critical R_* for Ranges Each from f Observations								
	2	3	4	5	6	7	8	9	10
2	0.962	0.862	0.803	0.764	0.736	0.717	0.702	0.691	0.682
3	.813	.667	.601	.563	.539	.521	.507	.498	.489
4	.681	.538	.479	.446	.425	.410	.398	.389	.382
5	.581	.451	.398	.369	.351	.338	.328	.320	.314
6	0.508	0.389	0.342	0.316	0.300	0.288	0.280	0.273	0.267
7	.451	.342	.300	.278	.263	.253	.245	.239	.234
8	.407	.305	.267	.248	.234	.225	.218	.213	.208
9	.369	.276	.241	.224	.211	.203	.197	.192	.188
10	.339	.253	.220	.204	.193	.185	.179	.174	.172
No. of Ranges k	Critical $(k + 2)R_*$ for Ranges Each from f Observations								
	2	3	4	5	6	7	8	9	10
10	4.06	3.04	2.65	2.44	2.30	2.21	2.14	2.09	2.05
12	4.06	3.03	2.63	2.42	2.29	2.20	2.13	2.07	2.04
15	4.06	3.02	2.62	2.41	2.28	2.18	2.12	2.06	2.02
20	4.13	3.03	2.62	2.41	2.28	2.18	2.11	2.05	2.01
50	4.26	3.11	2.67	2.44	2.29	2.19	2.11	2.06	2.01

part of Table 2. If R_* exceeds the tabular or interpolated value, the group with the largest range is suspect and inspection of its components will usually identify the observation, which is then assumed to be aberrant or an outlier. The process may be repeated with the remaining ranges if an outlier is suspected in a second group.

Replacement of Missing Values—As directed in the monographs and in this section, the calculation of potency and its confidence interval from the total response for each dose of each preparation requires the same number of observations in each total. When observations are lost or additional responses have been obtained with the Standard, the balance may be restored by one of the following procedures, so that the usual equations apply.

1. Reduce the number of observations in the larger groups until the number of responses is the same for each treatment. If animals have been assigned at random to each treatment group, either omit one or more responses, selected at random, from each larger group, or subtract the mean of each larger group from its initial total as often as may be necessary. The latter technique is preferred when extra animals have been assigned deliberately to the Standard. When the assay consists of randomized sets, retain only the complete sets.

2. Alternatively, an occasional smaller group may be brought up to size when the number of missing responses is not more than one in any one treatment or 10% in the entire assay. Estimate a replacement for each missing value by either method *a* or method *b*. One degree of freedom (n) is lost from the error variance s^2 for each replacement by either method, except in a microbial assay where each response is based on the sum of two or more transmittances and only one transmittance is replaced.

(*a*) If animals have been assigned to treatments at random, add the mean of the remaining responses in the incomplete group to their total. In a microbial assay, when one of two transmittances is missing for a given treatment, add the mean difference between sets, computed from all complete pairs, to the remaining transmittance to obtain the replacement.

(*b*) If the assay consists of randomized sets, replace the missing value by

$$y' = \frac{fT_r' + kT_t' - T'}{(f-1)(k-1)}, \qquad (1)$$

where f is the number of sets, k is the number of treatments or doses, and T_r', T_t', and T' are the incomplete totals for the randomized set, treatment, and assay from which an observation is missing.

If the assay consists of n' Latin squares with k rows in common, replace a missing value by

$$y' = \frac{k(n'T_c' + T_r' + T_t') - 2T'}{(k-1)(n'k-2)}, \qquad (1a)$$

where n' is the number of Latin squares with k rows in common, k is the number of treatments or doses, and T_c', T_r', T_t', and T' are respectively the incomplete totals for the column, row, treatment, and assay from which an observation is missing.

If more than one value is missing, substitute the treatment mean temporarily in all but one of the empty places, and compute y' for the other by Equation 1. Replace each of the initial substitutions in turn by Equation 1, and repeat the process in successive approximations until a stable y' is obtained for each missing observation.

Calculation of Potency from a Single Assay

Directions for calculating potency from the data of a single assay are given in the individual monographs. In those assays which specify graphical interpolation from dosage-response curves but which meet the conditions for assay validity set forth herein, potency may be computed alternatively by the appropriate method in this section.

Planning the assay involves assigning to the Unknown an assumed potency, to permit administering it in dosages equivalent to those of the Standard. The closer the agreement between this original assumption and the result of the assay, the more precise is the calculated potency. The ratio of a given dose of the Standard, in μg or in USP Units, to the corresponding dose of the Unknown, measured as specified in the monograph, is designated uniformly by R. The log-relative potency in quantities assumed initially to equal those of the Standard is designated as M'. Ideally, M' should not differ significantly from zero. The log-potency is

$$M = M' + \log R \qquad (2)$$

or

$$\text{Potency} = P_* = \text{antilog } M = (\text{antilog } M')R.$$

Assay from Direct Determinations of the Threshold Dose—Tubocurarine Chloride Injection and Metocurarine Iodide are assayed from the threshold dose that just produces a characteristic biological response. The ratio of the mean threshold dose for the Standard to that for the Unknown gives the potency directly. The threshold dose is determined twice in each animal, once with the Standard and once with the Unknown. Each dose is converted to its logarithm, the difference (x) between the two log-doses is determined for each animal, and potency is calculated from the average of these differences.

In the *Bacterial Endotoxins Test* ⟨85⟩, the geometric mean dilution end-point for the Unknown corresponding to the geometric mean dilution end-point for the Standard (multiplied by a dilution factor, where applicable) gives the concentration of endotoxin in the test material.

In these assays, the confidence interval depends upon the variability in the threshold dose.

Indirect Assays from the Relationship between the Log-dose and the Response—Generally, the threshold dose cannot be measured directly; therefore, potency is determined indirectly by comparing the responses following known doses of the Standard with the responses following one or more similar doses of the Unknown. Within a restricted dosage range, a suitable measure of the response usually can be plotted as a straight line against the log-dose, a condition that simplifies the calculation of potency and its confidence interval. Both the slope and position of the log-dose response relationship are determined in each assay by the use of two or more levels of the Standard, or, preferably, of both the Standard and the Unknown.

In the assay of Heparin Sodium, the interval between the dose at which clotting occurs and that which produces no clotting is so small that the dosage-response curve is not determined explicitly. Moving averages are used instead to interpolate the log-dose corresponding to 50% clotting for both the Standard and the Unknown, leading to the log-potency (see *Calculation* under *Heparin Sodium*). The precision of the potency is estimated from the agreement between independent assays of the same Unknown.

For a drug that is assayed biologically, the response should plot as a straight line against the log-dose over an adequate range of doses. Where a preliminary test is required or the assay depends upon interpolation from a multi-dose Standard curve, plot on coordinate paper the mean response of the *Standard* at each dosage level on the ordinate against the log-dose x on the abscissa. If the trend is basically linear over the required dosage range, the initial response unit may be used directly as y; if, instead, the trend is clearly curvilinear, a suitable transformation of each initial reading may bring linearity.

One possible transformation is to logarithms; another, in microbial tube assays, where y = (100 − % transmittance) does not plot linearly against the log-dose x, is to probits. In this case, if absorbance cannot be read directly, the % transmittance for each tube or test solution is first converted to absorbance, A = 2 − log(% transmittance). Each absorbance value, in turn, is converted to % reduction in bacterial growth as

$$\%\ \text{reduction} = 100(\bar{A}_c - A)/\bar{A}_c,$$

where $\bar{A}_c$ is the mean density for the control tubes (without antibiotic or with excess of vitamin) in the same set or tube rack. Percent reduction is then transformed to a probit (see Table 3) to obtain a new y for all later calculation. The probit transformation offers the advantage of extending the working range of linearity even where a portion of the dosage-response relationship is non-linear in the original units of percent transmittance, provided that the incubation period does not extend beyond the logarithmic phase of growth of the control tubes.

The LD_{50} in the *Safety* test for Iron Dextran Injection is calculated with log-doses and probits. The four doses of the Injection

Table 3

Probits (normal deviates + 5) corresponding to percentages in the margins.

	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Table 4

Coefficients x_* for computing the responses Y_L and Y_H predicted by least squares at the lowest and highest of k log-doses when these are spaced at equal intervals.

No. of Doses	Predicted End Y	Coefficient x_* for Mean Response $\bar{y}_t$ at Log-Dose 1	2	3	4	5	6	Divisor
3	Y_L	5	2	−1				6
	Y_H	−1	2	5				6
4	Y_L	7	4	1	−2			10
	Y_H	−2	1	4	7			10
5	Y_L	3	2	1	0	−1		5
	Y_H	−1	0	1	2	3		5
6	Y_L	11	8	5	2	−1	−4	21
	Y_H	−4	−1	2	5	8	11	21

in mg of iron per kg of body weight are transformed to $x_1 = 2.574$, $x_2 = 2.699$, $x_3 = 2.875$, and $x_4 = 3.000$. The probits corresponding to the number of deaths observed in each group of 10 mice are designated y_1, y_2, y_3, and y_4, respectively, and are given in Table 3 for mortalities from 10 to 90 percent. For observed deaths of 0 and 10 adjacent to doses giving an intermediate mortality, use the approximate probits 3.02 and 6.98, respectively; omit the end value (at x_1 or x_4) if not adjacent to an intermediate mortality. Since the information in a probit varies with its expectation, assign each probit an approximate relative weight w for computing the LD_{50} of the Injection, as shown in the accompanying table.

No. of Deaths	0 or 10	1 or 9	2 or 8	3 or 7	4 to 6
Weight, w	0.3	0.7	1.0	1.2	1.3

Calculate the weighted means

$$\bar{x} = \Sigma(wx)/\Sigma w$$

and (2a)

$$\bar{y} = \Sigma(wy)/\Sigma w$$

from the sum of the weights, Σw, of the four (or three) acceptable responses and the corresponding weighted sums of the log-doses, $\Sigma(wx)$, and of the probits, $\Sigma(wy)$. From the sums of the weighted products, $\Sigma(wxy)$, and of the weighted squares, $\Sigma(wx^2)$, compute the slope b of the log-dose-probit line as

$$b = \frac{\Sigma(wxy) - \bar{x}\Sigma(wy)}{\Sigma(wx^2) - \bar{x}\Sigma(wx)}. \quad (2b)$$

The LD_{50} for this safety test, in mg of iron per kg of body weight, is calculated as

$$LD_{50} = \text{antilog}[\bar{x} + (5 - \bar{y})/b]. \quad (2c)$$

In quantal assays not included in this Pharmacopeia, such as the mouse assay for insulin, the calculation with probits involves other adjustments that are omitted here.

When the mean response $\bar{y}_t$ for each dose of Standard plots linearly against the log-dose, and the k doses are spaced at equal intervals on the logarithmic scale, the predicted responses (Y_L and Y_H) at the extreme ends of the line of best fit can be computed directly with the coefficients x_* in Table 4, which correspond to the k successive log-doses, as

$$Y_L = \Sigma(x_*\bar{y}_t)/\text{divisor}$$

and (3)

$$Y_H = \Sigma(x_*\bar{y}_t)/\text{divisor},$$

where Σ stands uniformly for "the sum of" the values that follow it. When Y_L and Y_H are plotted against the low and high log-doses, X_L and X_H, respectively, they may be connected by a straight line with the slope

$$b = (Y_H - Y_L)/(X_H - X_L). \quad (4)$$

At any selected log-dose x of *Standard*, the predicted response is

$$Y = \bar{y} + b(x - \bar{x}), \quad (5)$$

where $\bar{x} = \Sigma x/k$, and $\bar{y} = (Y_L + Y_H)/2$, or, for predictions within a set, $\bar{y}$ is the mean response for the *Standard* within the set.

When the log-dose response relationship is linear, but the k doses (expressed in mL) are spaced substantially in an arithmetic sequence as in Table 5 (which refers to the microbial assays set forth under *Antibiotics—Microbial Assays* ⟨81⟩), the slope b of the straight line of best fit may be computed with the terms in Table 5 and the mean response at each dose $\bar{y}_t$, or $T_t = f\bar{y}_t$ where the number of y's(f) is constant at each dose, as

$$b = \Sigma(x_t\bar{y}_t)/e_b'i = \Sigma(x_tT_t)/fe_b'i. \quad (6)$$

The coefficients x_t are convenient multiples of the differences $(x - \bar{x})$ about the mean log-dose $\bar{x}$, and $e_b'i$ is the corresponding multiple of $\Sigma(x - \bar{x})^2$. The predicted response Y at a given log-dose x may be computed by substitution of the assay slope b in Equation 5 and of the mean $\bar{y}$ either of all the responses on the *Standard* in the entire assay or of those for each set separately.

Table 5

Coefficients x_i for computing the slope b of a log-dose response curve when the doses are spaced on an arithmetic scale as shown.

No. of Doses	Coefficients x_i for Computing b from the Responses y at Doses, in mL, of						Divisor $e_b'i$	Mean Log-dose $\bar{x}$
	1	1.5	2	3	4	5		
4	—	−29	−12	12	29	—	14.4663	0.38908
5	−34	—	−9	5	15	23	24.7827	0.41584
5	—	−20	−11	2	11	18	13.3249	0.45105
6	−15	−8	−3	4	9	13	14.1017	0.37588

POTENCIES INTERPOLATED FROM A STANDARD CURVE—Where the log-dose response curve of the *Standard* in a given assay is curvilinear and is fitted graphically to the plotted points, the amount of *Standard* that would be expected to produce each observed response y of an *Unknown* is estimated by interpolation from the curve and then adjusted for the known concentration of its test solution.

When the response to the *Standard* can be plotted linearly against the log-dose, it is fitted numerically by a straight line, as described in the preceding section. For assays in randomized sets, a standard curve is computed with b for the assay and $\bar{y}$ for each set and the response y_U in each tube of a given *Unknown* in that set is converted to an estimated log-relative potency,

$$X = (y_U - Y_S)/b, \qquad (7)$$

where Y_S is the response predicted by the standard curve at the assumed log-dose x of the *Unknown*. The average of the separate estimates from each of f sets, $M' = \Sigma X/f$, is the assayed log-relative potency of the *Unknown*.

Factorial Assays from the Response to Each Treatment—When some function of the response can be plotted linearly against the log-dose, the assayed potency is computed from the total response for each treatment, and its precision is measured in terms of confidence intervals. This requires that (1) in suitable units the response (y) depends linearly upon the log-dose within the dosage range of the assay, and (2) the number (f) of responses be the same at each dosage level of both Standard and Unknown. The y's are totaled at each dosage level of each preparation. In different combinations, these totals, T_i, lead directly to the log-relative potency and to tests of assay validity. The factorial coefficients in Tables 6, 7, and 8 determine how they are combined. In a given row, each T_i is multiplied by the corresponding coefficient and the products summed to obtain T_i. The T_i's in the successive rows carry the same meaning in all assays.

T_a in the first row measures the difference in the average response to the Standard and to the Unknown. T_b in the second row leads directly to the combined slope of the dosage-response curves for both Standard and Unknown. The third to the fifth rows (*ab*, *q*, and *aq*) provide tests for the validity of an assay, as described in a later section. From the totals T_a and T_b, compute the log-relative potency of the Unknown, before adjustment for its assumed potency, as

$$M' = ciT_a/T_b, \qquad (8)$$

where i is the interval in logarithms between successive log-doses of both the Standard and the Unknown, and the constant c is given separately at the bottom of each table. Each M' is corrected to its log-potency M by Equation 2.

When doses are spaced unequally on a log scale, as in Table 8, use instead the constant ci at the bottom of the table.

In a fully balanced assay, such as the assay for corticotropin, compute M' with the coefficients in Table 6. If one preparation has one less dose than the other but the successive log-doses of both Standard and Unknown differ by a constant interval i, use the factorial coefficients in Table 7, correcting for the actual difference between the observed mean log-doses, $\bar{x}_S$ and $\bar{x}_U$, by computing

$$M = \bar{x}_S - \bar{x}_U + M'. \qquad (9)$$

In assays where the successive doses are not spaced at equal log-intervals, the log-relative potency of a single Unknown may be computed by Equation 8 with the factorial coefficients and ci in Table 8.

In an assay of two or more Unknowns against a common Standard, all with dosage-response lines that are parallel within the

Table 6

Factorial coefficients x_i for analyzing a balanced bioassay, in which successive log-doses of Standard (S_i) and of Unknown (U_i) are spaced equally, each with the same number (f) of responses totaling T_i.

Design	Row	Factorial Coefficients x_i for Each Dose								e_i	T_i
		S_1	S_2	S_3	S_4	U_1	U_2	U_3	U_4		
2,2	*a*	−1	−1			1	1			4	T_a
	b	−1	1			−1	1			4	T_b
	ab	1	−1			−1	1			4	T_{ab}
3,3	*a*	−1	−1	−1		1	1	1		6	T_a
	b	−1	0	1		−1	0	1		4	T_b
	ab	1	0	−1		−1	0	1		4	T_{ab}
	q	1	−2	1		1	−2	1		12	T_q
	aq	−1	2	−1		1	−2	1		12	T_{aq}
4,4	*a*	−1	−1	−1	−1	1	1	1	1	8	T_a
	b	−3	−1	1	3	−3	−1	1	3	40	T_b
	ab	3	1	−1	−3	−3	−1	1	3	40	T_{ab}
	q	1	−1	−1	1	1	−1	−1	1	8	T_q
	aq	−1	1	1	−1	1	−1	−1	1	8	T_{aq}

For Computing	Equation No.	Constant	Value of Constant for Design		
			2,2	3,3	4,4
M'	8, 10	c	1	4/3	5
L	26, 29	c'	1	8/3	5

Table 7

Factorial coefficients x_i for analyzing a partially balanced assay, in which successive log-doses of Standard (S_i) and of Unknown (U_i) are spaced equally, each with the same number (f) of responses totaling T_i. If the number of successive doses of the Unknown exceeds by one the number on the Standard, interchange S_i and U_i in the heading and reverse all signs in rows *a*, *ab*, and *aq*.

		Factorial Coefficients x_i for Each Dose								
Design	Row	S_1	S_2	S_3	S_4	U_1	U_2	U_3	e_i	T_i
2,1	*a*	−1	−1			2			6	T_a
	b	−1	1			0			2	T_b
3,2	*a*	−2	−2	−2		3	3		30	T_a
	b	−2	0	2		−1	1		10	T_b
	ab	1	0	−1		−2	2		10	T_{ab}
	q	1	−2	1		0	0		6	T_q
4,3	*a*	−3	−3	−3	−3	4	4	4	84	T_a
	b	−3	−1	1	3	−2	0	2	28	T_b
	ab	3	1	−1	−3	−5	0	5	70	T_{ab}
	q	3	−3	−3	3	2	−4	2	60	T_q
	aq	−1	1	1	−1	1	−2	1	10	T_{aq}

For Computing	Equation No.	Constant	Value of Constant for Design 2,1	3,2	4,3
M'	8, 10	c	1/2	5/6	7/6
L	26, 29	c'	3/4	25/12	49/12

Table 8

Factorial coefficients x_i for analyzing assays with a 3- or 4-dose sequence of 1.5, 2.0, 3.0, and 4.0, each dose having the same number (f) of responses.

		Dose of Standard				Dose of Unknown					
Design	Row	1.5	2.0	3.0	4.0	1.5	2.0	3.0	4.0	e_i	T_i
4,4	*a*	−1	−1	−1	−1	1	1	1	1	8	T_a
	b	−29	−12	12	29	−29	−12	12	29	3940	T_b
	ab	29	12	−12	−29	−29	−12	12	29	3940	T_{ab}
	q	1	−1	−1	1	1	−1	−1	1	8	T_q
	aq	−1	1	1	−1	1	−1	−1	1	8	T_{aq}
3,3	*a*	−1	−1	−1		1	1	1		6	T_a
	b	−25	−3	28		−25	−3	28		2836	T_b
	ab	25	3	−28		−25	−3	28		2836	T_{ab}
	q	31	−53	22		31	−53	22		8508	T_q
	aq	−31	53	−22		31	−53	22		8508	T_{aq}
3,3	*a*		−1	−1	−1		1	1	1	6	T_a
	b		−28	3	25		−28	3	25	2836	T_b
	ab		28	−3	−25		−28	3	25	2836	T_{ab}
	q		22	−53	31		22	−53	31	8508	T_q
	aq		−22	53	−31		22	−53	31	8508	T_{aq}

For Computing	Equation No.	Constant	Value of Constant for Design 4,4	3,3
M'	8, 10	ci	7.2332	5.3695
L	26, 29	$c'i^2$	0.10623	0.06100

experimental error, each log-relative potency may be computed with the same assay slope as follows: For each preparation, determine the slope factor $T_b' = \Sigma(x_i T_i)$ or $\Sigma(x_i y)$, where the values of x_i are the factorial coefficients for the Standard in the appropriate row *b* of Table 6 or 8. The log-relative potency of each Unknown is

$$M' = cih' T_a / 2\Sigma T_b', \qquad (10)$$

where h' is the number of values of T_b' summed in the denominator.

Assays from Differences in Response—When doses of the Standard and Unknown are paired and the difference in response is computed for each pair, these differences are not affected by variations in the average sensitivity of the paired readings. The paired 2-dose insulin assay corresponds to the first design in Table 6, and requires four equal groups of rabbits each injected twice (see *Insulin Assay* ⟨121⟩). The difference (y) in the blood sugar response of each rabbit to the two treatments leads to the log-relative potency M' (see the first two paragraphs of the section, *Calculation of Potency from a Single Assay*). The Vasopressin Injection assay follows a similar design, substituting two or more randomized sets of four successive pairs of injections into rats for the four treatment groups of rabbits in the insulin assay.

Oxytocin Injection is assayed from blood pressure changes in a single test animal following alternating injections of a single dose of Standard and of one of two doses of the Unknown. The calculation of potency from the differences in the response of the Unknown and to the average of the two adjacent responses to the Standard is equivalent to the first design in Table 7 with S and U reversed, where i is the log-interval between the two dosage levels of the Unknown.

Experimental Error and Tests of Assay Validity

As the term is used here, "experimental error" refers to the residual variation in the response of biological indicators, not to a mistake in procedure or to an outlier that needs replacement. It is measured in terms of the error variance of a single response or other unit, which is designated uniformly as s^2, despite differences in the definition of the unit. It is required in tests of assay validity and in computing the *confidence interval*.

Error Variance of a Threshold Dose—The individual threshold dose is measured directly in some assays. In a Digitalis assay, designate each individual threshold dose by the symbol z, the number or frequency of z's by f, and the total of the z's for each preparation by T, with subscripts S and U for Standard and Unknown, respectively. Compute the error variance of z as

$$s^2 = [\Sigma z^2 - T_S^2/f_S - T_U^2/f_U]/n, \qquad (11)$$

with $n = f_S + f_U - 2$ degrees of freedom. In the assay of Tubocurarine Chloride Injection, each log-threshold dose of the Unknown is subtracted from the corresponding log-dose of the Standard in the same rabbit to obtain an individual difference x. Since each x may be either positive or negative (+ or −), it is essential to carry the correct sign in all sums. Designate the total of the x's for the animals injected with the Standard on the first day as T_1, and for those injected with the Standard on the second day as T_2. Compute the error variance of x with $n = N - 2$ degrees of freedom as

$$s^2 = \{\Sigma x^2 - (T_1^2 + T_2^2)/f\}/n, \qquad (12)$$

where N is the total number of rabbits that complete the assay, excluding any replacement for a missing value to equalize the size of the two groups.

Error Variance of an Individual Response—In the Pharmacopeial assays, differences in dose that modify the mean response are assumed not to affect the variability in the response. The calculation of the error variance depends upon the design of the assay and the form of the adjustment for any missing values. Each response is first converted to the unit y used in computing the potency. Determine a single error variance from the combined deviations of the y's around their respective means for each dosage level, summed over all levels. Doubtful values of y may be tested as described under *Rejection of Outlying or Aberrant Observations*, and proved outliers may be replaced as missing values (see *Replacement of Missing Values*).

In the simplest design, the units of response are assigned at random to each dosage level, as in the assay for corticotropin. If a missing value is replaced by adding the mean of the remaining y's at any given dosage level to their total, the degrees of freedom (n) in the error variance are reduced by one for each replacement but no other change is needed in the calculation. Assuming that f is then the same for all doses or groups, compute the error variance from the variation within doses of all the y's as

$$s^2 = \{\Sigma y^2 - \Sigma T_t^2/f\}/n, \qquad (13)$$

where T_t is the total at each dose of the f values of y, there are k totals T_t and the degrees of freedom $n = \Sigma f - k$, with Σf diminished by 1 for each replacement.

If variations in f are adjusted by subtracting a group mean from its group total, compute the error variance from the observed y's and the *unadjusted* totals T_t as

$$s^2 = \{\Sigma y^2 - \Sigma(T_t^2/f)\}/n, \qquad (14)$$

where $n = \Sigma f - k$.

In the calculation of the result of an assay using the coefficients of Table 6 or 8, s^2 may be computed from the response y for each of the h' preparations, including the h Unknowns and the corresponding dosage levels of the Standard. For each preparation, compute $T' = \Sigma y$ and the slope factor $T_b' = \Sigma(x_1 y)$ where the values of x_1 are the factorial coefficients for the Standard in the appropriate row b of Table 6 or 8. The error variance for the assay is

$$s^2 = \{\Sigma y^2 - \Sigma T'^2/k - 2(\Sigma T_b')^2/h' e_b f\}/n, \qquad (15)$$

where the degrees of freedom $n = h'(k - 1) - 1$, and e_b is the e_i from the same table and row as the coefficients x_1.

The Error Variance in Restricted Designs—In some assays, the individual responses occur in randomized sets of three or more. Examples of sets are litter mates in the assay of vitamin D, the cleared areas within each plate in an antibiotic assay, and the responses following four successive pairs of injections in the vasopressin assay. Arrange the individual y's from these assays in a 2-way table, in which each column represents a different treatment or dose and each row a randomized set. Losses may be replaced as described under *Replacement of Missing Values*. The k column totals are the T_t's required for the analysis of balanced designs. The f row totals (T_r) represent a source of variation that does not affect the estimated potency and hence is excluded from the assay error. Compute the approximate error variance from the squares of the individual y's and of the marginal totals as

$$s^2 = \{\Sigma y^2 - \Sigma T_r^2/k - \Sigma T_t^2/f + T^2/N\}/n, \qquad (16)$$

where $T = \Sigma T_r = \Sigma T_t$, and the $n = (k - 1)(f - 1)$ degrees of freedom must be diminished by one for any gap in the original table that has been filled by computation.

When the order of treatment is an additional potential source of variation, its effect can be corrected by the dose regimen for a series of n' Latin squares with k rows in common, such as that for the two Latin squares in the dose regimens 1 to 4 and 5 to 8 in the assay of Glucagon for Injection. List the observed responses y of each test animal in a separate column in the order of dosing. The responses to each of the k doses then occur equally often in each of the k rows and of the $n'k$ columns, where n' is the number of Latin squares. Total the responses y in each row (T_r) in each column (T_c), and, in a separate listing, for each dose or treatment (T_t). An occasional lost reading may be replaced by Equation 1a as described under *Replacement of Missing Values*. Compute the error variance from the squares of the individual y's and of the marginal and treatment totals as

$$s^2 = \{\Sigma y^2 - \Sigma T_r^2/n'k - \Sigma T_c^2/k - \Sigma T_t^2/n'k + 2T^2/N\}/n, \qquad (16a)$$

where $T = \Sigma y = \Sigma T_r = \Sigma T_c = \Sigma T_t$, $N = n'k^2$, and the $n = (k - 1)(n'k - 2)$ degrees of freedom must be diminished by one for any gap in the original table that has been filled by computation.

In assays where the reactions occur in pairs, the differences between test animals or paired reactions are segregated automatically by calculating the assay with the difference within a pair as the response. With insulin, the response is the difference y in the blood sugar of a single rabbit following two injections (see *Insulin Assay* ⟨121⟩). After adjustment for rabbits lost during the assay, compute the error variance of y from the responses in all four groups and from the group totals $T_t = T_1$ to T_4 as

$$s^2 = \{\Sigma y^2 - \Sigma T_t^2/f\}/n, \qquad (17)$$

where the number of rabbits f is the same in each group and the degrees of freedom, $n = 4(f - 1)$, are reduced by one for each replacement of a rabbit lost during the assay. In the Oxytocin Injection assay, each y represents the difference between the blood pressure response to a dose of the Unknown and the average for the two adjacent doses of Standard. Compute the error variance of y as

$$s^2 = \{\Sigma y^2 - (T_1^2 + T_2^2)/f\}/n \qquad (18)$$

with $n = 2(f - 1)$ degrees of freedom, where T_1 is the total of the y's for the low dose of the Unknown and T_2 that for the high dose.

In a microbial assay calculated by interpolation from a standard curve, convert each difference between two paired responses to units of log-dose, X, by the use of Equation 7. With each difference X as the unit, a composite s^2 is computed from the variation in the f values of X for each *Unknown*, totaled over the h *Unknowns* in the assay, as

$$s^2 = \{\Sigma X^2 - \Sigma(T_x^2/f)\}/n, \qquad (19)$$

where $T_x = \Sigma X$ for a single *Unknown* and the degrees of freedom $n = \Sigma f - h$.

Tests of Assay Validity—In addition to the specific requirements in each monograph and a combined log-dose response curve with a significant slope (see the statistic C in the next section),

two conditions determine the validity of an individual factorial assay: (1) the log-dose response curve for the Unknown must parallel that for the Standard within the experimental error, and (2) neither curve may depart significantly from a straight line. When the assay has been completely randomized or consists of randomized sets, the necessary tests are computed with the factorial coefficients for *ab*, *q*, and *aq* from Tables 6 to 8 and the treatment totals T_t. Sum the products of the coefficients in each row by the corresponding T_t's to obtain the product total T_i, where the subscript *i* stands in turn for *ab*, *q*, and *aq*, respectively. Each of the three ratios, $T_i^2/e_i f$, is computed with the corresponding value of e_i from the table and with f equal to the number of *y*'s in each T_i. That in row *ab* tests whether the dosage-response lines are parallel, and is the only test available in a 2-dose assay. With three or more doses of both preparations, that in row *q* is a test of combined curvature in the same direction, and in row *aq* of separate curvatures in opposite directions. If any ratio in a 3- or 4-dose assay exceeds s^2 as much as three-fold, compute

$$F_3 = \Sigma(T_i^2/e_i f)/3s^2. \qquad (20)$$

For a 2-dose assay, compute instead

$$F_1 = T_{ab}^2/e_{ab} f s^2, \qquad (21)$$

and for a 3,2 assay (Table 7) determine

$$F_2 = \Sigma(T_i^2/e_i f)/2s^2. \qquad (22)$$

For a valid assay, F_1, F_2, or F_3 does not exceed the value given in Table 9 (at odds of 1 in 20) for the degrees of freedom *n* in s^2.

An assay may fail the test for validity and still provide a contributory estimate of potency that can be combined profitably with the result of a second assay of the same Unknown, as described in a later section. An end dosage level for either the Standard or the Unknown, or both, may fall outside the linear zone. With three or more dosage levels and relatively large values of T_a, T_{ab}, and T_{aq}, the total response T_t at an end dose of one preparation may approach an upper or lower limit and be responsible for the large values of T_{ab} and T_{aq}. This T_t may be omitted and the assay recomputed with the appropriate design in Table 7. If the assay then meets the test in Equation 20, or 22, the resulting potency, *M*, may be combined with that of a second assay in computing the log-potency of the Unknown (see under *Combination of Independent Assays*). If T_a is not significant but T_q shows significant combined curvature, the largest (or smallest) dose of both preparations may be too large (or too small). Their omission may lead to a valid assay with the factorial coefficients for the next smaller design in Table 6 or 8. A statistically significant T_q or $\Sigma T_q'$ may be neglected and all dosage levels retained without biasing the computed log-potency *M'* and its confidence interval by more than 5% when the following inequality is true:

$$T_b^2/e_b > 100T_q^2/e_q$$

or

$$(\Sigma T_b')^2/e_b > 100(\Sigma T_q')^2/e_q, \qquad (23)$$

where each T_b' and T_q' is computed with the T_t's (or *y*'s) for a single preparation multiplied by the coefficients for the Standard in rows *b* and *q*, respectively. If both T_a and T_{ab} are significant in a 2-dose assay, one T_t may be outside the linear zone. Sometimes a preliminary or contributory estimate of potency can be computed from the remaining three values of T_t and the first design in Table 7. In assays of insulin and of other drugs in which the responses are paired, the test for parallelism is so insensitive that it is omitted. If the tubes in each set are arranged systematically instead of at random in a microbial assay, the tests for validity may be subject to bias from positional effects.

The Confidence Interval and Limits of Potency

A bioassay provides an estimate of the true potency of an Unknown. This estimate falls within a confidence interval, which is computed so that the odds are not more than 1 in 20 ($P = 0.05$) that the true potency either exceeds the upper limit of the confidence interval or is less than its lower limit. Since this interval is determined by a number of factors that may influence the estimate of potency, the required precision for most bioassays is given in the monograph in terms of the confidence interval, related either to the potency directly or to its logarithm.

General Calculation—Despite their many forms, bioassays fall into two general categories: (1) those where the log-potency is computed directly from a mean or a mean difference, and (2) those where it is computed from the ratio of two statistics.

(1) When the log-potency of an assay is computed as the mean of several estimated log-potencies that are approximately equal in precision, the log-confidence interval is

$$L = 2st\sqrt{k}, \qquad (24)$$

where *s* is the standard deviation of a single estimated log-potency, *t* is read from Table 9 with the *n* degrees of freedom in *s*, and *k* is the number of estimates that have been averaged. The same equation holds where the log-potency is computed as the mean $\bar{x}$ of *k* differences *x*, with *s* the standard deviation of a single *x*. In either case, the estimated log-potency *M* is in the center of its confidence interval, so that its confidence limits are

$$X_M = M + \tfrac{1}{2}L \text{ and } M - \tfrac{1}{2}L, \text{ or } X_M = M \pm \tfrac{1}{2}L. \qquad (25)$$

Table 9

Values of *t*, t^2, F_1 and χ^2 for different degrees of freedom *n* that will be exceeded with a probability $P = 0.05$ (or 0.95 for confidence intervals).†

n	*t*	$t^2 = F_1$	F_2	F_3	χ^2	*n*	*t*	$t^2 = F_1$	F_2	F_3	χ^2
1	12.706	161.45	—	—	3.84	19	2.093	4.381	3.52	3.13	30.1
2	4.303	18.51	19.00	19.16	5.99	20	2.086	4.351	3.49	3.10	31.4
3	3.182	10.128	9.55	9.28	7.82	21	2.080	4.325	3.47	3.07	32.7
4	2.776	7.709	6.94	6.59	9.49	22	2.074	4.301	3.44	3.05	33.9
5	2.571	6.608	5.79	5.41	11.07	23	2.069	4.279	3.42	3.03	35.2
6	2.447	5.987	5.14	4.76	12.59	24	2.064	4.260	3.40	3.01	36.4
7	2.365	5.591	4.74	4.35	14.07	25	2.060	4.242	3.38	2.99	37.7
8	2.306	5.318	4.46	4.07	15.51	26	2.056	4.225	3.37	2.98	38.9
9	2.262	5.117	4.26	3.86	16.92	27	2.052	4.210	3.35	2.96	40.1
10	2.228	4.965	4.10	3.71	18.31	28	2.048	4.196	3.34	2.95	41.3
11	2.201	4.844	3.98	3.59	19.68	29	2.045	4.183	3.33	2.93	42.6
12	2.179	4.747	3.89	3.49	21.03	30	2.042	4.171	3.32	2.92	43.8
13	2.160	4.667	3.81	3.41	22.36	40	2.021	4.085	3.23	2.84	55.8
14	2.145	4.600	3.74	3.34	23.68	60	2.000	4.001	3.15	2.76	79.1
15	2.131	4.543	3.68	3.29	25.00	120	1.980	3.920	3.07	2.68	146.6
16	2.120	4.494	3.63	3.24	26.30	∞	1.960	3.841	3.00	2.60	
17	2.110	4.451	3.59	3.20	27.59						
18	2.101	4.414	3.55	3.16	28.87						

† Adapted from portions of Tables III to V of "Statistical Tables for Biological, Agricultural and Medical Research," by R. A. Fisher and F. Yates, published by Oliver and Boyd, Ltd., Edinburgh.

The upper and lower limits are converted to their antilogarithms to obtain the limits as explicit potencies.

(2) More often, the log-potency or potency is computed from a ratio, and in these cases the length of the confidence interval is typified by the log-interval in the equation

$$L = 2\sqrt{(C-1)(CM'^2 + c'i^2)}, \quad (26)$$

where M' is the log-relative potency as defined (see *Calculation of Potency from a Single Assay*), i is the log-interval between successive doses, and c' is a constant characteristic of the assay procedure. The remaining term C depends upon the precision with which the slope of the dosage-response curve has been determined. (This is sometimes expressed in terms of $g = (C-1)/C$.) In factorial assays, it is computed as

$$C = T_b^2/(T_b^2 - e_b f s^2 t^2), \quad (27)$$

where s^2 is the error variance of a single observation, t^2 is read from Table 9 with the degrees of freedom in s^2, f is the number of responses in each T_t used in calculating T_b, and T_b and e_b are computed with the factorial coefficients for row b in Tables 6 to 8. The s^2 in Equation 26 depends upon the design of the assay, as indicated for each drug in the next section. In a valid assay, C is a positive number.

In an assay of two or more Unknowns against a common Standard, all with dosage-response curves that are parallel within the experimental error, C may be computed with the error variance s^2 for the assay and with the assay slope as

$$C = (\Sigma T_b')^2/\{(\Sigma T_b')^2 - e_b f h' s^2 t^2/2\}. \quad (28)$$

The slope factor $T_b' = \Sigma(x_1 T_t)$ or $\Sigma(x_1 y)$ for each of the h' preparations, including the Standard, is computed with the factorial coefficients x_1 for the Standard in the appropriate row b of Table 6 or 8. If a treatment total T_t includes one or more replacements for a missing response, replace $e_b f$ in Equation 27, or $e_b f h'/2$ in Equation 28, by $f'^2\Sigma(x_1^2/f')$, where each x_1 is a factorial coefficient in row b of Tables 6 to 8, in this chapter, and f' is the number of responses in the corresponding T_t *before* adding the replacement. With this C, compute the confidence interval as

$$L = 2\sqrt{(C-1)(CM'^2 + c'i^2 h'/2)}. \quad (29)$$

In assays computed from a ratio, the most probable log-potency M is not in the exact center of the confidence interval. The upper and lower confidence limits in logarithms are

$$X_M = \log R + CM' + \tfrac{1}{2}L \text{ and } \log R + CM' - \tfrac{1}{2}L. \quad (30)$$

C is often very little larger than unity, and the more precise the assay, the more nearly C approaches 1 exactly. $R = z_S/z_U$ is the ratio of corresponding doses of the Standard and of the Unknown or the assumed potency of the Unknown. The upper and lower confidence limits in log-potencies are converted separately to their antilogarithms to obtain the corresponding potencies.

Confidence Intervals for Individual Assays—Since the confidence interval may vary in detail from the above general patterns, compute it for each assay by the special directions given under the name of the substance in the paragraphs following.

Antibiotic Assays—The confidence interval may be computed by Equations 24 and 25.

Calcium Pantothenate—For log-potencies obtained by interpolation from the Standard curve, the confidence interval may be computed with Equations 19 and 24. For log-potencies calculated with Equation 8 or 10, s^2 may be computed with Equation 15, C with Equation 27 or 28, and the confidence interval L with Equation 26 or 29.

Corticotropin Injection—Compute the log confidence interval by Equations 26 and 27, with the coefficients and constants in Table 6 for a 3-dose assay, and s^2 as determined by Equation 13 or 14.

Digitalis—Compute the confidence interval as

$$L = 2\sqrt{(C-1)\{C(\bar{z}_S/\bar{z}_U)^2 + f_U/f_S\}}, \quad (31)$$

where f_U and f_S are the number of observations on the Unknown and on the Standard, and

$$C = \bar{z}_U^2/(\bar{z}_U^2 - s^2 t^2/f_U) \quad (32)$$

is determined with s^2 from Equation 11. The confidence limits for the potency in USP Units are then

$$X_{P_*} = R\{C(\bar{z}_S/\bar{z}_U) \pm \tfrac{1}{2}L\}, \quad (33)$$

in which R is as defined in the *Glossary of Symbols*.

Glucagon for Injection—Compute the error variance s^2 by Equation 15a, C by Equation 27 with $e_b f = 16n'$, and the log confidence interval L by Equation 26 with $c'i^2 = 0.09062$.

Chorionic Gonadotropin—Proceed as directed under *Corticotropin Injection*.

Heparin Sodium—If two independent determinations of the log-potency M differ by more than 0.05, carry out additional assays and compute the error variance among the N values of M as

$$s^2 = \{\Sigma M^2 - (\Sigma M)^2/N\}/n \quad (34)$$

with $n = N - 1$ degrees of freedom. Given this value, determine the confidence interval in logarithms (L) by Equation 24.

Insulin Injection—Compute the error variance (s^2) of y by Equation 16 and C as

$$C = T_b^2/(T_b^2 - s^2 t^2 N), \quad (35)$$

where t^2 from Table 9 depends upon $n = 4(f-1)$ degrees of freedom in s^2 and $N = 4f$ is the total number of differences in the four groups. By Equation 26, compute the confidence interval L in logarithms, where $c'i^2 = 0.09062$. The upper and lower confidence limits in USP Units of insulin are given by the antilogarithms of X_M from Equation 30.

Oxytocin Injection—Compute the approximate log confidence interval by Equation 26, in which

$$C = (T_2 - T_1)^2/\{(T_2 - T_1)^2 - 4(f+1)s^2 t^2/3\}, \quad (36)$$

where s^2 is defined by Equation 18, and

$$c' = (4f-1)/8(f+1). \quad (37)$$

Tubocurarine Chloride Injection—Compute the error variance by Equation 12, and the confidence interval by Equation 24.

Vasopressin Injection—Compute the error variance s^2 by Equation 16, C by Equation 35, and the log confidence interval by Equation 26, where $c' = 1$ and i is the log-interval separating the two dosage levels.

Vitamin B_{12} Activity—Proceed as directed under *Calcium Pantothenate*.

Combination of Independent Assays

When the method permits, additional animals can be added to an insufficiently precise assay until the combined results reduce the confidence interval within the limits specified in the monograph. Where two or more independent assays are required, each leading to a log-potency M, the M's are combined in determining the *weighted mean potency* of the Unknown. Except in the Heparin Sodium assay, where the log-potencies are weighted equally, the relative precisions of the two or more independent M's determine the weight assigned to each value in computing their mean and its confidence interval.

Before combining two or more separate estimates of M, test their mutual consistency. If the M's are consistent, their respective confidence intervals will overlap. Where the intervals do not overlap or where the overlap is small, compute an approximate χ_M^2. Assign each of the h individual assays a weight w, defined as

$$w = 4t^2/L^2, \quad (38)$$

where the length of the confidence interval L is computed with the appropriate equation from the preceding section, and t^2 is read from Table 9 for the degrees of freedom n in the error variance of the assay. Sum the individual weights to obtain Σw. Then an approximate χ^2 with $h - 1$ degrees of freedom is determined as

$$\text{Approx. } \chi_M^2 = \Sigma(wM^2) - \{\Sigma(wM)\}^2/\Sigma w. \quad (39)$$

For two assays with log-potencies M_1 and M_2 and weights w_1 and w_2, Equation 35 reduces to

$$\text{Approx. } \chi_M^2 = \frac{w_1 w_2 (M_1 - M_2)^2}{w_1 + w_2}, \qquad (40)$$

with one degree of freedom. If the approximate χ_M^2 is well under the critical value for χ^2 in Table 9, use the weights w in computing the mean log-potency $\overline{M}$ and its confidence interval, L. If χ_M^2 approaches or exceeds this critical value, use instead the semi-weights w' (Equation 47) when computing $\overline{M}$.

Compute the mean log-potency $\overline{M}$ of two or more mutually consistent assays as

$$\overline{M} = \Sigma(wM)/\Sigma w. \qquad (41)$$

This is the most probable single value within a combined confidence interval of length L_c, defined as the square root of

$$L_c^2 = \frac{4t_L^2}{\Sigma w}\left\{1 + \frac{4}{\Sigma^2 w}\sum\frac{w(\Sigma w - w)}{n'}\right\}, \qquad (42)$$

where each $n' = n - 4(h-2)/(h-1)$ and t_L^2 is interpolated from Table 9 with the degrees of freedom

$$n_L = \Sigma^2 w/\Sigma(w^2/n).$$

For two assays ($h = 2$) with log-potencies M_1 and M_2 and weights w_1 and w_2, respectively, the above equation may be rewritten as

$$L_c^2 = \frac{4t_L^2}{\Sigma w}\left\{1 + \frac{4w_1 w_2}{\Sigma^2 w}\left[\frac{1}{n_1} + \frac{1}{n_2}\right]\right\}, \qquad (43)$$

where $\Sigma w = w_1 + w_2$. Where L_c, the confidence interval for a combined estimate, does not exceed the requirement in a monograph, upper and lower confidence limits are taken $\frac{1}{2}L_c$ above and below M, to obtain approximately a 95% confidence interval.

Where the variation in the assayed potency between the h independent determinations, as tested by χ_M^2, approaches or exceeds $P = 0.05$, the several estimates are assigned semi-weights w'. From the weight w, compute the variance of each M as

$$V = 1/w = L^2/4t^2. \qquad (44)$$

Calculate the variance of the heterogeneity between assays as

$$v = \frac{\Sigma M^2 - (\Sigma M)^2/h}{h-1} - \frac{\Sigma V}{h}, \qquad (45)$$

or if $h = 2$,

$$v = \frac{(M_1 - M_2)^2}{2} - \frac{V_1 + V_2}{2}. \qquad (46)$$

Where V varies so markedly that v calculated as above is a negative number, compute instead an approximate v by omitting the term following the minus sign in Equations 45 and 46. A semi-weight is defined as

$$w' = 1/(V + v). \qquad (47)$$

Substitute w' and $\Sigma w'$ for w and Σw in Equation 41 to obtain the semi-weighted mean $\overline{M}$. This falls near the middle of a confidence interval of approximate length L_c', where

$$L_c'^2 = 4t^2/\Sigma w' \qquad (48)$$

and t^2 from Table 9 has Σn degrees of freedom.

Where χ_M^2 in Equation 39, from $h = 4$ or more estimates of M, exceeds the critical level in Table 9 by more than 50%, and the weights w differ by less than 30%, the h estimates of M may be checked for a suspected outlier with Table 1. Where significant, the outlying M may be omitted in computing $\overline{M}$ with w'.

Where the potency of a drug is determined repeatedly in a given laboratory by the same bioassay method, successive determinations of both the slope b and the error variance s^2 may scatter randomly within the sampling error about a common value for each parameter. Plotting estimates from successive assays on a quality control chart for each statistic and computing the mid-value and control limits defining the allowable random variation make it possible to check continuously the consistency of an assay technique. Where estimates of b and s^2 from a single assay fall within the control limits, they may be replaced by their laboratory means. Reject any assay in which these statistics fall outside the control limits, or accept it only after close scrutiny with respect to its validity.

Joint Assay of Several Preparations

Each monograph describes the assay of a single Unknown against the Standard. Although not provided explicitly, several different Unknowns are often included in the same assay and each is compared separately with the same responses to the Standard. This fact may warrant increasing the number of observations with the Standard. Given f observations at each dosage level of each of h different Unknowns, the number of observations at each dosage level of the Standard may be increased advantageously, if h is large, to $f\sqrt{h}$. This rule can be applied only approximately where litter differences or their equivalent must be segregated, and in any case is merely suggestive.

If all of several assays conducted concurrently meet the requirements for validity, and have linear log-dose response curves with the same slope b and the same error variance s^2 about these lines, these two statistics may be considered as characteristic of the assay. Combining all of the evidence from the same assay into a single value of the assay slope results in a more stable and reliable estimate of b than if each Unknown were analyzed independently. The degrees of freedom and reliability of the error variance s^2 can be increased similarly. Confidence intervals computed with these composite values for b and s^2 are smaller on the average than if based upon only part of the relevant data. For the calculation or application of such assay estimates, see Equations 10, 15, 16, 19, 28, and 29. The potency estimated with a slope computed from a single Unknown and the Standard agrees within a fraction of the confidence interval with that computed from the combined slope for the entire assay. Since it is based upon more evidence, the latter is considered the better estimate.

GLOSSARY OF SYMBOLS

- A absorbance for computing % reduction in bacterial growth from turbidimetric readings.
- b slope of the straight line relating response (y) to log-dose (x) [Equations 2b, 4, 5, 6].
- c constant for computing M' with Equations 8 and 10.
- c' constant for computing L with Equations 26 and 29.
- ci constant for computing M' when doses are spaced as in Table 8.
- $c'i^2$ constant for computing L when doses are spaced as in Table 8.
- C term measuring precision of the slope in a confidence interval [Equations 27, 28, 35, 36].
- χ^2 statistical constant for testing significance of a discrepancy [Table 9].
- χ_M^2 χ^2 testing the disagreement between different estimates of log-potency [Equations 39, 40].
- e_b e_i from row b in Tables 6 to 8.
- $e_b'i$ multiple of $\Sigma(x - \bar{x})^2$ [Table 5; Equation 6].
- e_i sum of squares of the factorial coefficients in each row of Tables 6 to 8.
- e_q e_i from row q in Tables 6 to 8.
- f number of responses at each dosage level of a preparation; number of replicates or sets.
- f_S number of observations on the Standard.
- f_U number of observations on the Unknown.
- F_1 to F_3 observed variance ratio with 1 to 3 degrees of freedom in numerator [Table 9].
- G_1, G_2, and G_3 relative gap in test for outlier [Table 1].
- h number of Unknowns in a multiple assay.
- h' number of preparations in a multiple assay, including the Standard and h Unknowns; i.e., $h' = h + 1$.
- i interval in logarithms between successive log-doses, the same for both *Standard* and *Unknown*.
- k number of estimated log-potencies in an average [Equation 24]; number of treatments or doses [Table 4; Equations 1, 13, 15, 16]; number of ranges or groups in a series [Table 2]; number of rows, columns, and doses in a single Latin square [Equations 1a, 16a].
- L length of the confidence interval in logarithms [Equations 24, 26, 29, 38], or in terms of a proportion of the

relative potency of the dilutions compared [Equations 31, 33].

L_c length of a combined confidence interval [Equations 42, 43].

L_c' length of confidence interval for a semi-weighted mean $\overline{M}$ [Equation 48].

LD_{50} lethal dose killing an expected 50% of the animals under test [Equation 2c].

M log-potency [Equation 2].

M' log-potency of an Unknown, relative to its assumed potency.

$\overline{M}$ mean log-potency.

n degrees of freedom in an estimated variance s^2 or in the statistic t or χ^2.

n' number of Latin squares with rows in common [Equations 1a, 16a].

N number; e.g., of observations in a gap test [Table 1], or of responses y in an assay [Equation 16].

P probability of observing a given result, or of the tabular value of a statistic, usually $P = 0.05$ or 0.95 for confidence intervals [Tables 1, 2, 9].

P_* potency, $P_* =$ antilog M or computed directly.

R ratio of a given dose of the Standard to the corresponding dose of the Unknown, or assumed potency of the Unknown [Equations 2, 30, 33].

R_* ratio of largest of k ranges in a series to their sum [Table 2].

$s = \sqrt{s^2}$ standard deviation of a response unit, also of a single estimated log-potency in a direct assay [Equation 24].

s^2 error variance of a response unit.

S_l a log-dose of Standard [Tables 6, 7].

Σ "the sum of."

t Student's t for n degrees of freedom and probability $P = 0.05$ [Table 9].

T total of the responses y in an assay [Equation 16].

T' incomplete total for an assay in randomized sets with one missing observation [Equation 1].

T_1 $\Sigma(y)$ for the animals injected with the Standard on the first day [Equations 18, 36].

T_2 $\Sigma(y)$ for the animals injected with the Standard on the second day [Equations 18, 36].

T_a T_l for the difference in the responses to the Standard and to the Unknown [Tables 6 to 8].

T_{ab} T_l for testing the difference in slope between Standard and Unknown [Tables 6 to 8].

T_{aq} T_l for testing opposed curvature in the curves for Standard and Unknown [Tables 6 to 8].

T_b T_l for the combined slope of the dosage-response curves for Standard and Unknown [Tables 6 to 8].

T_b' $\Sigma(x_1 T_l)$ or $\Sigma(x_1 y)$ for computing the slope of the log-dose response curve [Equations 10, 23, 28].

T_l sum of products of T_l multiplied by the corresponding factorial coefficients in each row of Tables 6 to 8.

T_q T_l for testing similar curvature in the curves for Standard and Unknown [Tables 6 to 8].

T_r row or set total in an assay in randomized sets [Equation 16].

T_r' incomplete total for the randomized set with a missing observation in Equation 1.

T_t total of f responses y for a given dose of a preparation [Tables 6 to 8; Equations 6, 13, 14, 16].

T_t' incomplete total for the treatment with a missing observation in Equation 1.

U_l a log-dose of Unknown [Tables 6 to 8].

v variance for heterogeneity between assays [Equation 45].

$V = 1/w$ variance of an individual M [Equations 44 to 47].

w weight assigned to the M for an individual assay [Equation 38], or to a probit for computing an LD_{50} [Equations 2a, 2b].

w' semi-weight of each M in a series of assays [Equations 47, 48].

x a log-dose of drug in a bioassay [Equation 5]; also the difference between two log-threshold doses in the same animal [Equation 12].

x_* coefficients for computing the lowest and highest expected responses Y_L and Y_H in a log-dose response curve [Table 4; Equation 3].

x_1 a factorial coefficient that is a multiple of $(x - \overline{x})$ for computing the slope of a straight line [Table 5; Equation 6].

$\overline{x}$ mean log-dose [Equation 5].

$\overline{x}_S$ mean log-dose for Standard [Equation 9].

$\overline{x}_U$ mean log-dose for Unknown [Equation 9].

X log-potency from a unit response, as interpolated from a standard curve [Equations 7a, 7b, 19].

X_M confidence limits for an estimated log-potency M [Equations 25, 30].

X_{P_*} confidence limits for a directly estimated potency P_* (see *Digitalis* assay) [Equation 33].

y an observed individual response to a dose of drug in the units used in computing potency and the error variance [Equations 13 to 16]; a unit difference between paired responses in 2-dose assays [Equations 17, 18].

$y_1 \ldots y_N$ observed responses listed in order of magnitude, for computing G_1, G_2, or G_3 in Table 1.

y' replacement for a missing value [Equation 1].

$\overline{y}$ mean response in a set or assay [Equation 5].

$\overline{y}_t$ mean response to a given treatment [Equations 3, 6].

Y a response predicted from a dosage-response relationship, often with qualifying subscripts [Equations 3 to 5].

z threshold dose determined directly by titration (see *Digitalis* assay) [Equation 11].

$\overline{z}$ mean threshold dose in a set (see *Digitalis* assay) [Equations 31, 32, 33].

⟨115⟩ DEXPANTHENOL ASSAY

The following procedure is provided for the determination of dexpanthenol as an ingredient of multiple-vitamin preparations. It is applicable also to the determination of the dextrorotatory component of racemic panthenol and of other mixtures containing dextrorotatory panthenol.

Media may be prepared as described hereinafter, or dehydrated mixtures yielding similar formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have growth-promoting properties equal to or superior to those obtained from the formulas given herein.

Reference Standard—*USP Dexpanthenol Reference Standard.*

Standard Stock Solution of Dexpanthenol—Dissolve an accurately weighed quantity of USP Dexpanthenol RS in water, dilute with water to obtain a solution having a known concentration of about 800 µg per mL, and mix. Store in a refrigerator, protected from light, and use within 30 days.

Standard Preparation—On the day of the assay, prepare a water dilution of the *Standard Stock Solution of Dexpanthenol* to contain 1.2 µg of dexpanthenol per mL.

Assay Preparation—Proceed as directed in the individual monograph for preparing a solution expected to contain approximately the equivalent of the dexpanthenol concentration in the *Standard Preparation.*

Modified Pantothenate Medium—

Acid-hydrolyzed Casein Solution	25	mL
Cystine-Tryptophane Solution	25	mL
Polysorbate 80 Solution	0.25	mL
Dextrose, Anhydrous	10	g
Sodium Acetate, Anhydrous	5	g
Adenine-Guanine-Uracil Solution	5	mL
Riboflavin–Thiamine Hydrochloride–Biotin Solution	5	mL
Para-aminobenzoic Acid–Niacin–Pyridoxine Hydrochloride Solution	5	mL
Salt Solution A	5	mL
Salt Solution B	5	mL
Pyridoxal–Calcium Pantothenate Solution	5	mL
Polysorbate 40–Oleic Acid Solution	5	mL

Dissolve the anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 *N* sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL, and mix.

Double-strength Modified Pantothenate Medium—Prepare as directed under *Modified Pantothenate Medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Acid-hydrolyzed Casein Solution—Mix 100 g of vitamin-free casein with 500 mL of 6 *N* hydrochloric acid, and reflux the

mixture for 8 to 12 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 *N* sodium hydroxide to a pH of 3.5 ± 0.1, and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-Tryptophane Solution—Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophane (or 2.0 g of D,L-tryptophane) in 700 mL to 800 mL of water, heat to 75 ± 5°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, add water to make 1000 mL, and mix. Store under toluene in a refrigerator at a temperature not below 10°.

Adenine-Guanine-Uracil Solution—Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 *N* hydrochloric acid, cool, add water to make 200 mL, and mix. Store under toluene in a refrigerator.

Polysorbate 80 Solution—Dissolve 25 g of polysorbate 80 in alcohol to make 250 mL, and mix.

Riboflavin–Thiamine Hydrochloride–Biotin Solution—Prepare a solution containing, in each mL, 20 μg of riboflavin, 10 μg of thiamine hydrochloride, and 0.04 μg of biotin, by dissolving riboflavin, thiamine hydrochloride, and biotin in 0.02 *N* acetic acid. Store, protected from light, under toluene in a refrigerator.

Para-aminobenzoic Acid–Niacin–Pyridoxine Hydrochloride Solution—Prepare a solution in neutral 25 percent alcohol to contain 10 μg of para-aminobenzoic acid, 50 μg of niacin, and 40 μg of pyridoxine hydrochloride in each mL. Store in a refrigerator.

Salt Solution A—Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, mix, and store under toluene.

Salt Solution B—Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, mix, and store under toluene.

Pyridoxal–Calcium Pantothenate Solution—Dissolve 40 mg of pyridoxal hydrochloride and 375 μg of calcium pantothenate in 10 percent alcohol to make 2000 mL, and mix. Store in a refrigerator, and use within 30 days.

Polysorbate 40–Oleic Acid Solution—Dissolve 25 g of polysorbate 40 and 0.25 g of oleic acid in 20 percent alcohol to make 500 mL, and mix. Store in a refrigerator, and use within 30 days.

Stock Culture of *Pediococcus acidilactici*—Dissolve in about 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 *N* sodium hydroxide or 0.1 *N* hydrochloric acid to a pH between 6.5 and 6.6, adjust the volume with water to 1000 mL, and mix. Add approximately 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize at 121° for 15 minutes. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici** on a slant of this medium. Incubate at 35° for 20 to 24 hours, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum—Inoculate three 250-mL portions of *Modified Pantothenate Medium* from a stock culture slant, and incubate at 35° for 20 to 24 hours. Centrifuge the suspension from the combined portions, and wash the cells with *Modified Pantothenate Medium*. Resuspend the cells in sufficient *Modified Pantothenate Medium* so that a 1:50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Procedure—Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0 mL, 4.5 mL, 4.0 mL, 3.5 mL, 3.0 mL, 2.0 mL, 1.0 mL, and 0.0 mL. To these same tubes, and in the same order, add 0.0 mL, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, 4.0 mL, and 5.0 mL of the *Standard Preparation*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0 mL, 3.5 mL, 3.0 mL, 2.0 mL, and 1.0 mL. To these same tubes, and in the same order, add 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, and 4.0 mL of the *Assay Preparation*.

Add 5.0 mL of *Double-strength Modified Pantothenate Medium* to each tube, and mix. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 minutes. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 hours. Terminate growth by heating to a temperature not below 80°, such as by steaming at atmospheric pressure in a suitable sterilizer, for 5 to 10 minutes. Cool, and concomitantly determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer, at 530 nm.

Calculation—Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percent transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, in terms of dexpanthenol, of each tube containing portions of the *Assay Preparation*. Divide the potency of each tube by the amount of *Assay Preparation* added to it, to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by not more than 15%, using not less than half the total number of tubes. Calculate the potency of the portion of the material taken for assay, in terms of dexpanthenol, by multiplying the mean response by the appropriate dilution factor.

* American Type Culture Collection No. 8042 is suitable.

⟨121⟩ INSULIN ASSAY

The most prominent manifestation of insulin activity, an abrupt decrease in blood glucose, was the basis for biologic assay from the time of the first clinical use of insulin. The procedure, although relatively cumbersome, has the great merit of accurately reflecting the effect on the diabetic patient. Another attribute of insulin, that of reacting under in-vitro conditions to specific antibodies, the amounts of which are rendered measurable by means of radioactive isotopes, is the basis for a procedure that makes possible the rapid measurement of minute amounts of insulin.

Rabbit Blood-sugar Method

Reference Standard—*USP Insulin Reference Standard*—Preserve in a refrigerator, and after opening the ampul, store in a tight container.

Standard Solution—Dissolve a suitable quantity of USP Insulin RS, accurately weighed, in sufficient water, containing 0.1% to 0.25% (w/v) of either phenol or cresol, 1.4% to 1.8% (w/v) of glycerin, and sufficient hydrochloric acid to make a *Standard Solution* containing 40 USP Insulin Units per mL and having a pH between 2.5 and 3.5, unless otherwise directed in the individual monograph. Store in a cold place, protected from freezing, and use within 6 months.

Standard Dilutions—Dilute portions of the *Standard Solution* to make two solutions, one to contain 1.0 USP Insulin Unit per mL (*Standard dilution 1*), and the other to contain 2.0 USP Insulin Units per mL (*Standard dilution 2*). Use as a diluent a solution containing 0.1% to 0.25% (w/v) of either cresol or phenol, 1.4% to 1.8% (w/v) of glycerin, and sufficient hydrochloric acid to produce a pH between 2.5 and 3.5, unless otherwise directed in the individual monograph.

Assay Dilutions—Employing the same diluent used in preparing the *Standard Dilutions*, make two dilutions of the preparation to be assayed, one of which may be expected, on the basis of the assumed potency, to contain 1.0 USP Insulin Unit per mL (*Assay dilution 1*), and the other to contain 2.0 USP Insulin Units per mL (*Assay dilution 2*). In the case of neutral Insulin Injection, adjust to a pH of 2.5 to 3.5 prior to making the dilutions.

Doses of the Dilutions To Be Injected—Select on the basis of trial or experience the dose of the dilutions to be injected, the volume of which usually will be between 0.30 mL and 0.50 mL. For each animal the volume of the *Standard dilution* shall be the same as that of the *Assay dilution.*

The Animals—Select suitable, healthy rabbits each weighing not less than 1.8 kg. Keep the rabbits in the laboratory for not less than 1 week before use in the assay, maintaining them on an adequate uniform diet, with water available at all times except during the assay.

Procedure—Divide the rabbits into four equal groups of preferably not less than six rabbits each. On the preceding day, approximately 20 hours before the assay, provide each rabbit with an amount of food that will be consumed within 6 hours. Follow the same feeding schedule before each test day. During the assay, withhold all food and water until after the final blood specimen is taken. Handle the rabbits with care in order to avoid undue excitement, and inject subcutaneously the doses indicated in the following design, the Second Injection being made on the day after the First Injection or not more than 1 week later.

At 1 hour and 2½ hours after the time of injection obtain from each rabbit a suitable blood specimen from a marginal ear vein.

Determine the dextrose (glucose) content of the blood specimens by a suitable procedure, preferably one that depends upon the reduction of ferricyanide and is adapted to automatic handling. The following method may be used, all steps being carried out in a pre-determined sequence accurately reproduced for each blood specimen and for prepared solutions of dextrose of known concentration.

Pipet into separate, suitable vessels 0.8 mL of each blood specimen and 0.8 mL each of standard solutions containing, respectively, the following concentrations of dextrose: 0.25, 0.50, 0.75, 1.0, and 1.25 mg per mL. Into each vessel pipet 2.4 mL of saline TS, and mix. Place each vessel in a water bath maintained at 38°, and subject the diluted blood to dialysis across a semipermeable membrane for a sufficient time for a definite proportion of the dextrose to pass through the membrane into a solution, in saline TS, of potassium ferricyanide (1 in 1670) and sodium carbonate (1 in 50). Add a measured portion of potassium cyanide solution (1 in 200). Heat at a temperature of 95° for 5 minutes, cool to 40°, and determine the absorbance, at 420 nm, in a recording colorimeter. In a similar manner, determine the absorbances of solutions obtained from one or more standard solutions of dextrose of known concentration in the range of 25 mg to 125 mg per 100 mL.

Calculation—Calculate the response of each rabbit to each injection from the sum of the two blood-sugar values, and subtract its response to *Dilution 1* from that to *Dilution 2*, disregarding the chronological order in which the responses were observed, to obtain the individual differences, y, shown in the accompanying table.

Group	First Injection	Second Injection
1	Standard dilution 2	Assay dilution 1
2	Standard dilution 1	Assay dilution 2
3	Assay dilution 2	Standard dilution 1
4	Assay dilution 1	Standard dilution 2

Group	Differences	Individual Response (y)	Total Response (T)
1	Standard 2 − Assay 1	y_1	T_1
2	Assay 2 − Standard 1	y_2	T_2
3	Assay 2 − Standard 1	y_3	T_3
4	Standard 2 − Assay 1	y_4	T_4

When the data for one or more rabbits are missing in an assay, allow for differences in the sizes of the groups by suitable means (see *Replacement of Missing Values* ⟨111⟩).

When the number of rabbits, f, carried through the assay is the same in each group, total the y's in each group and compute $T_a = -T_1 + T_2 + T_3 - T_4$ and $T_b = T_1 + T_2 + T_3 + T_4$. The logarithm of the relative potency of the test dilutions is $M' = 0.301T_a/T_b$. The potency of the Injection in USP Units per mL equals the antilog (log $R + M'$), where $R = v_S/v_U$, in which v_S is the number of USP Units per mL of the Standard dilution and v_U is the number of mL of Injection per mL of the Assay dilution.

Determine the confidence interval of the log-relative potency M' (see *Confidence Intervals for Independent Assays* ⟨111⟩). If the confidence interval is more than 0.082, which corresponds at $P = 0.95$ to confidence limits of about ±10% of the computed potency, repeat the assay until the combined data of the two or more assays, re-determined as described under *Combination of Independent Assays* ⟨111⟩, meet this acceptable limit.

⟨141⟩ PROTEIN—BIOLOGICAL ADEQUACY TEST

This test is intended for the evaluation of the biological adequacy, as an index to the completeness of the mixture of amino acids contained, of Protein Hydrolysate Injection.

Depletion Diet—

	Parts by Weight
Dextrin	83.9
Corn Oil	9.0
Salt Mixture	4.0
Agar	2.0
Cod Liver Oil	1.0
Choline Chloride	0.15
Inositol	0.10
Calcium Pantothenate	0.002
Niacinamide	0.0015
Riboflavin	0.0003
Pyridoxine	0.00025
Thiamine	0.0002
p-Aminobenzoic Acid	0.0002
Folic Acid	0.0002
Menadione	0.0002
Biotin	0.00002

Salt Mixture—Prepare the salt mixture specified in the *Depletion Diet* as follows:

Sodium Chloride	139.3 g
Potassium Biphosphate	389.0 g
Magnesium Sulfate, Anhydrous	57.3 g
Calcium Carbonate	381.4 g
Ferrous Sulfate	27.0 g
Manganese Sulfate	4.01 g
Potassium Iodide	0.79 g
Zinc Sulfate	0.548 g
Cupric Sulfate	0.477 g
Cobaltous Chloride	0.023 g

Place a portion of the weighed quantity of sodium chloride in a suitable mortar and add, with grinding, the potassium iodide. Set aside the mixture, and mix in a similar manner all the other salts with the remainder of the sodium chloride, adding finally the previously mixed sodium chloride and potassium iodide. Reduce the entire mixture to a fine powder (see *Powder Fineness* ⟨811⟩).

Control Nitrogen Supplement Mixture—Place 50 g of calcium caseinate and 46 g of anhydrous dextrose in a beaker, add sufficient water to make a paste, and finally add 1000 mL of water. Heat the solution between 70° and 82° for 5 minutes with stirring, and cool. Determine nitrogen on an aliquot using *Nitrogen Determination—Method I* or *Method II* ⟨461⟩. Store in a refrigerator. Mix before removing portions for analysis or use.

Depletion and Control Periods—Select a group of not less than six male rats 2 to 4 months of age and each weighing between 190 g and 225 g. Place the rats in individual cages with free access to water and the *Depletion Diet* for 12 days. Weigh the depleted rats, and discard any rat that weighs more than 90% of its starting weight.

For the next 3 days substitute as drinking water the *Control Nitrogen Supplement Mixture* in a quantity equivalent to 0.12 g of nitrogen per rat per day, diluted with water to 20 mL, and offered at the same time each morning either in a dish suitable for preventing spillage or in a reservoir fitted with a drinking tube. Remove all drinking water from the cages of the depleted

rats during each feeding, and return it after the supplement has been consumed or is removed. On the third day, weigh each rat. Discard any rats that have not consumed all of the *Control Nitrogen Supplement Mixture.*

For the next 3 days, replace the *Control Nitrogen Supplement Mixture* with water ad libitum, and continue the rats on the *Depletion Diet.* Weigh the rats, and discard any that have not lost weight since the previous weighing.

Procedure—Assemble not less than six rats that have completed the depletion and control periods. For 5 days maintain the assembled rats on the *Depletion Diet* with a daily supplement of 20 mL, accurately measured, of a solution containing the Protein Hydrolysate Injection in an amount equivalent to 0.12 g of nitrogen offered each morning in the same way as the *Control Nitrogen Supplement Mixture* was offered previously. Withhold water for at least 2 hours prior to offering the supplement and for 4 hours afterward. Then if the supplement has been consumed, offer water ad libitum.

On the afternoon of the fifth day, weigh each rat, and compare the respective final and starting weights. Not fewer than 80% of the group of rats used gain weight or maintain their weight during the test.

⟨151⟩ PYROGEN TEST

The pyrogen test is designed to limit to an acceptable level the risks of febrile reaction in the patient to the administration, by injection, of the product concerned. The test involves measuring the rise in temperature of rabbits following the intravenous injection of a test solution and is designed for products that can be tolerated by the test rabbit in a dose not to exceed 10 mL per kg injected intravenously within a period of not more than 10 minutes. For products that require preliminary preparation or are subject to special conditions of administration, follow the additional directions given in the individual monograph or, in the case of antibiotics or biologics, the additional directions given in the federal regulations (see *Biologics* ⟨1041⟩).

Apparatus and Diluents—Render the syringes, needles, and glassware free from pyrogens by heating at 250° for not less than 30 minutes or by any other suitable method. Treat all diluents and solutions for washing and rinsing of devices or parenteral injection assemblies in a manner that will assure that they are sterile and pyrogen-free. Periodically perform control pyrogen tests on representative portions of the diluents and solutions for washing or rinsing of the apparatus. Where Sodium Chloride Injection is specified as a diluent, use Injection containing 0.9 percent of NaCl.

Temperature Recording—Use an accurate temperature-sensing device such as a clinical thermometer, or thermistor probes or similar probes that have been calibrated to assure an accuracy of ±0.1° and have been tested to determine that a maximum reading is reached in less than 5 minutes. Insert the temperature-sensing probe into the rectum of the test rabbit to a depth of not less than 7.5 cm, and, after a period of time not less than that previously determined as sufficient, record the rabbit's body temperature.

Test Animals—Use healthy, mature rabbits. House the rabbits individually in an area of uniform temperature between 20° and 23° and free from disturbances likely to excite them. The temperature varies not more than ±3° from the selected temperature. Before using a rabbit for the first time in a pyrogen test, condition it not more than seven days before use by a sham test that includes all of the steps as directed under *Procedure* except injection. Do not use a rabbit for pyrogen testing more frequently than once every 48 hours, nor prior to 2 weeks following a maximum rise of its temperature of 0.6° or more while being subjected to the pyrogen test, or following its having been given a test specimen that was adjudged pyrogenic.

Procedure—Perform the test in a separate area designated solely for pyrogen testing and under environmental conditions similar to those under which the animals are housed and free from disturbances likely to excite them. Withhold all food from the rabbits used during the period of the test. Access to water is allowed at all times, but may be restricted during the test. If rectal temperature-measuring probes remain inserted throughout the testing period, restrain the rabbits with light-fitting neck stocks that allow the rabbits to assume a natural resting posture. Not more than 30 minutes prior to the injection of the test dose, determine the "control temperature" of each rabbit: this is the base for the determination of any temperature increase resulting from the injection of a test solution. In any one group of test rabbits, use only those rabbits whose control temperatures do not vary by more than 1° from each other, and do not use any rabbit having a temperature exceeding 39.8°.

Unless otherwise specified in the individual monograph, inject into an ear vein of each of three rabbits 10 mL of the test solution per kg of body weight, completing each injection within 10 minutes after start of administration. The test solution is *either* the product, constituted if necessary as directed in the labeling, *or* the material under test treated as directed in the individual monograph and injected in the dose specified therein. For pyrogen testing of devices or injection assemblies, use washings or rinsings of the surfaces that come in contact with the parenterally administered material or with the injection site or internal tissues of the patient. Assure that all test solutions are protected from contamination. Perform the injection after warming the test solution to a temperature of 37 ± 2°. Record the temperature at 1, 2, and 3 hours subsequent to the injection.

Test Interpretation and Continuation—Consider any temperature decreases as zero rise. If no rabbit shows an individual rise in temperature of 0.6° or more above its respective control temperature, and if the sum of the three individual maximum temperature rises does not exceed 1.4°, the product meets the requirements for the absence of pyrogens. If any rabbit shows an individual temperature rise of 0.6° or more, or if the sum of the three individual maximum temperature rises exceeds 1.4°, continue the test using five other rabbits. If not more than three of the eight rabbits show individual rises in temperature of 0.6° or more, and if the sum of the eight individual maximum temperature rises does not exceed 3.7°, the material under examination meets the requirements for the absence of pyrogens.

RADIOACTIVE PHARMACEUTICALS

Test Dose for Preformulated, Ready-to-use Products Labeled with Radioactivity

AGGREGATED ALBUMIN AND OTHER PARTICLE-CONTAINING PRODUCTS

For the rabbit pyrogen test, dilute the product with Sodium Chloride Injection to not less than 100 μCi per mL, and inject a dose of 3 mL per kg of body weight into each rabbit.

OTHER PRODUCTS

Where Physical Half-life of Radionuclide Is Greater Than 1 Day—Calculate the maximum volume of the product that might be injected into a human subject. This calculation takes into account the maximum recommended radioactive dose of the product, in μCi, and the radioactive assay, in μCi per mL, of the product at its expiration date or time. Using this information, calculate the maximum volume dose per kg to a 70-kg human subject.

For the rabbit pyrogen test, inject a minimum of 10 times this dose per kg of body weight into each rabbit. If necessary, dilute with Sodium Chloride Injection. The total injected volume per rabbit is not less than 1 mL and not more than 10 mL of solution.

Where Physical Half-life of Radionuclide is Less Than 1 Day—For products labeled with radionuclides having a half-life of less than 1 day, the dosage calculations are identical to those described in the first paragraph under *Other Products.* These products may be released for distribution prior to completion of the rabbit pyrogen test, but such test shall be initiated at not more than 36 hours after release.

Test Dose for Pharmaceutical Constituents or Reagents to Be Labeled

The following test dose requirements pertain to reagents that are to be labeled or constituted prior to use by the direct addition of radioactive solutions such as Sodium Pertechnetate Tc 99m Injection, i.e., "cold kits."

Assume that the entire contents of the vial of nonradioactive

reagent will be injected into a 70-kg human subject, or that 1/70 of the total contents per kg will be injected. If the contents are dry, constitute with a measured volume of Sodium Chloride Injection.

For the rabbit pyrogen test, inject 1/7 of the vial contents per kg of body weight into each rabbit. The maximum dose per rabbit is the entire contents of a single vial. The total injected volume per rabbit is not less than 1 mL and not more than 10 mL of solution.

⟨161⟩ TRANSFUSION AND INFUSION ASSEMBLIES

The requirements apply to medical devices that are labeled nonpyrogenic in contact directly or indirectly with the cardiovascular system or other soft body tissues. This includes, but is not limited to, solution administration sets, extension sets, transfer sets, blood administration sets, intravenous catheters, implants extracorporeal oxygenator tubings and accessories, dialysers and dialysis tubing and accessories, heart valves, vascular grafts, intramuscular drug delivery catheters, and transfusion and infusion assemblies. These requirements do not apply to orthopedic products, latex gloves, or wound dressings.

Sterility—Proceed as directed for *Sterilized Devices* under *Sterility Tests* ⟨71⟩.

Bacterial Endotoxins (see *Bacterial Endotoxins Test* ⟨85⟩)—*Transfusion and Infusion Assemblies and Similar Devices*—Through the tubing of each of 10 assemblies, pass a separate 40-mL portion of Limulus Amoebocyte Lysate (LAL)-negative *Sterile Water for Injection* at a temperature between 37° and 40°, at a flow rate of approximately 10 mL per minute. The pooled effluent meets the requirements of the *Bacterial Endotoxins Test* ⟨85⟩ with an endotoxin limit not to exceed 0.5 USP Endotoxin Unit per mL.

Implants—For implants as well as other medical devices that are intended to be sterile internally as well as externally, select not less than 3 implants and not more than 10 implants. Cut in small pieces or, if modular, separate the pieces, and soak for not less than 40 minutes and not more than 60 minutes in 400 mL of LAL-negative *Sterile Water for Injection* at a temperature between 37° and 40°, with swirling at 10-minute intervals. The supernatant liquid meets the requirements of the *Bacterial Endotoxins Test* ⟨85⟩ with an endotoxin limit not to exceed 0.5 USP Endotoxin Unit per mL.

Other Medical Devices—Pass equal portions of 400 mL of LAL-negative *Sterile Water for Injection* at a temperature between 37° and 40°, at a flow rate of approximately 10 mL per minute through a number of devices representative of a lot (not less than 3 devices and not more than 10). The 400 mL effluent meets the requirements of the *Bacterial Endotoxins Test* ⟨85⟩ with an endotoxin limit not to exceed 0.5 USP Endotoxin Unit per mL.

Medical Devices in Contact with Cerebrospinal Fluid—Proceed as directed under *Transfusion and Infusion Assemblies, Other Medical Devices*, or *Implants*, whichever is applicable. The 400 mL pooled effluent meets the requirements of the *Bacterial Endotoxins Test* ⟨85⟩ with an endotoxin limit of not more than 0.06 USP Endotoxin Unit per mL.

A *Bacterial Endotoxins Test* failure can be retested once by another *Bacterial Endotoxins Test*. If the cause of the initial failure is due to a level of endotoxin of less than 0.5 USP Endotoxin Unit per mL, the *Pyrogen Test* ⟨151⟩ can be used to retest.

Pyrogen—For samples that meet the requirements of the *Bacterial Endotoxins Test* retest and for samples that cannot be tested by the *Bacterial Endotoxins Test* because of nonremovable inhibition or enhancement of the test, the *Pyrogen Test* ⟨151⟩ is applied. A pooled effluent is obtained by passing a separate 40-mL portion of sterile pyrogen-free saline TS at a flow rate of approximately 10 mL per minute through the tubing of each of 10 Transfusion and Infusion Assemblies, or through 3 to 10 Medical Devices, or by soaking the cut or separated pieces of *Implants* in 400 mL of the Saline TS, whichever is appropriate. The requirements of the *Pyrogen Test* ⟨151⟩ are met.

Safety—It meets the requirements of the *Safety Test* under *Safety Tests—General* in chapter ⟨88⟩, *Biological Reactivity Tests, In-vivo*, or in chapter ⟨87⟩, *Biological Reactivity Tests, In-Vitro*.

Other Requirements—The portions of medical devices that are made of plastics or other polymers meet the requirements under *Containers—Plastics* ⟨661⟩; those made of elastomers meet the requirements under *Elastomeric Closures for Injection* ⟨381⟩.

⟨171⟩ VITAMIN B_{12} ACTIVITY ASSAY

Reference Standard—*USP Cyanocobalamin Reference Standard*—Keep container tightly closed and protected from light. Dry over silica gel for 4 hours before using.

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 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 under *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 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 liter of analytical filter-aid in the supernatant liquid, 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 liquid. 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*

* Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852.

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 RS corresponding to the cyanocobalamin in the portion of material taken for assay by the equation antilog M = antilog (M' + log R), in which R is the 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, $\bar{M}$, 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.

Chemical Tests and Assays

IDENTIFICATION TESTS

⟨181⟩ IDENTIFICATION—ORGANIC NITROGENOUS BASES

This test is for the identification of tertiary amine compounds.

Dissolve 50 mg of the substance under test, if in bulk, in 25 mL of 0.01 *N* hydrochloric acid, or shake a quantity of powdered tablets or the contents of capsules equivalent to 50 mg of the substance with 25 mL of 0.01 *N* hydrochloric acid for 10 minutes. Transfer the liquid to a separator, if necessary filtering it and washing the filter and the residue with several small portions of water. In a second separator dissolve 50 mg of the corresponding USP Reference Standard in 25 mL of 0.01 *N* hydrochloric acid. Treat each solution as follows: Add 2 mL of 1 *N* sodium hydroxide and 4 mL of carbon disulfide, and shake for 2 minutes. Centrifuge if necessary to clarify the lower phase, and filter it through a dry filter, collecting the filtrate in a small flask provided with a glass stopper.

Determine the absorption spectra of the filtered solutions of both standard and sample without delay, in 1-mm cells between 7 µm and 15 µm, with a suitable infrared spectrophotometer, using carbon disulfide in a matched cell as the blank. The spectrum of the solution prepared from the sample shows all of the significant absorption bands present in the spectrum of the solution prepared from the Reference Standard.

⟨191⟩ IDENTIFICATION TESTS—GENERAL

Under this heading are placed tests that are frequently referred to in the Pharmacopeia for the identification of official articles.

NOTE—The tests are not intended to be applicable to mixtures of substances unless so specified.

Acetate—When acetic acid or an acetate is warmed with sulfuric acid and alcohol, ethyl acetate, recognizable by its characteristic odor, is evolved. With neutral solutions of acetates, ferric chloride TS produces a deep red color which is destroyed by the addition of mineral acids.

Aluminum—Solutions of aluminum salts yield with 6 *N* ammonium hydroxide a gelatinous, white precipitate that is insoluble in an excess of 6 *N* ammonium hydroxide. 1 *N* sodium hydroxide or sodium sulfide TS produces the same precipitate, which dissolves in an excess of either of these reagents.

Ammonium—Ammonium salts are decomposed by the addition of an excess of 1 *N* sodium hydroxide, with the evolution of ammonia, recognizable by its odor and by its alkaline effect upon moistened red litmus paper exposed to the vapor. Warming the solution accelerates the decomposition.

Antimony—Solutions of antimony (III) compounds, strongly acidified with hydrochloric acid, yield with hydrogen sulfide an orange precipitate of antimony sulfide which is insoluble in 6 *N* ammonium hydroxide, but is soluble in ammonium sulfide TS.

Barium—Solutions of barium salts yield a white precipitate with 2 *N* sulfuric acid. This precipitate is insoluble in hydrochloric and in nitric acid. Barium salts impart a yellowish green color to a nonluminous flame, which appears blue when viewed through green glass.

Benzoate—In neutral solutions, benzoates yield a salmon-colored precipitate with ferric chloride TS. In moderately concentrated solutions, benzoates yield a precipitate of benzoic acid upon acidification with 2 *N* sulfuric acid. This precipitate is readily soluble in ether.

Bicarbonate—See *Carbonate*.

Bismuth—When dissolved in a slight excess of nitric or hydrochloric acid, bismuth salts yield a white precipitate upon dilution with water. This precipitate is colored brown by hydrogen sulfide, and the resulting compound dissolves in a warm mixture of equal parts of nitric acid and water.

Bisulfite—See *Sulfite*.

Borate—To 1 mL of a borate solution, acidified with hydrochloric acid to litmus, add 3 or 4 drops of a saturated solution of iodine and 3 or 4 drops of polyvinyl alcohol solution (1 in 50): an intense blue color is produced. When a borate is treated with sulfuric acid, methanol is added, and the mixture is ignited, it burns with a green-bordered flame.

Bromide—Solutions of bromides, upon the addition of chlorine TS, dropwise, liberate bromine, which is dissolved by shaking with chloroform, coloring the chloroform red to reddish brown. Silver nitrate TS produces in solutions of bromides a yellowish white precipitate, which is insoluble in nitric acid and is slightly soluble in 6 *N* ammonium hydroxide.

Calcium—Solutions of calcium salts form insoluble oxalates when treated as follows: To a solution of the calcium salt (1 in 20) add 2 drops of methyl red TS, and neutralize with 6 *N* ammonium hydroxide. Add 3 *N* hydrochloric acid, dropwise, until the solution is acid to the indicator. Upon the addition of ammonium oxalate TS, a white precipitate is formed. This precipitate is insoluble in 6 *N* acetic acid but dissolves in hydrochloric acid. Calcium salts moistened with hydrochloric acid impart a transient yellowish red color to a nonluminous flame.

Carbonate—Carbonates and bicarbonates effervesce with acids, evolving a colorless gas, which when passed into calcium hydroxide TS produces a white precipitate immediately. A cold solution of a soluble carbonate is colored red by phenolphthalein TS, while a similar solution of a bicarbonate remains unchanged or is only slightly colored.

Chlorate—Solutions of chlorates yield no precipitate with silver nitrate TS. The addition of sulfurous acid to this mixture produces a white precipitate which is insoluble in nitric acid, but is soluble in 6 *N* ammonium hydroxide. Upon ignition, chlorates yield chlorides, recognizable by appropriate tests. When sulfuric acid is added to a dry chlorate, decrepitation occurs and a greenish yellow gas is evolved. [*Caution—Use only a small amount of chlorate for this test and exercise extreme caution in performing it.*]

Chloride—Solutions of chlorides yield with silver nitrate TS a white, curdy precipitate, which is insoluble in nitric acid, but is soluble in a slight excess of 6 *N* ammonium hydroxide. When testing alkaloidal hydrochlorides, add 6 *N* ammonium hydroxide, filter, acidify the filtrate with nitric acid, and proceed as directed above. Dry chlorides, when mixed with an equal weight of manganese dioxide, moistened with sulfuric acid, and gently heated, evolve chlorine which is recognizable by the production of a blue color with moistened starch iodide paper.

Citrate—To 15 mL of pyridine add a few mg of a citrate salt,

dissolved or suspended in 1 mL of water, and shake. To this mixture add 5 mL of acetic anhydride, and shake: a light red color is produced.

Cobalt—Solutions of cobalt salts (1 in 20) in 3 *N* hydrochloric acid yield a red precipitate when heated on a steam bath with an equal volume of a hot, freshly prepared solution of 1-nitroso-2-naphthol (1 in 10) in 9 *N* acetic acid. Solutions of cobalt salts, when saturated with potassium chloride and treated with potassium nitrite and acetic acid, yield a yellow precipitate.

Copper—Solutions of cupric compounds, acidified with hydrochloric acid, deposit a red film of metallic copper upon a bright, untarnished surface of metallic iron. An excess of 6 *N* ammonium hydroxide, added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue-colored solution. With potassium ferrocyanide TS, solutions of cupric salts yield a reddish brown precipitate, insoluble in diluted acids.

Hypophosphite—When strongly heated, hypophosphites evolve spontaneously flammable phosphine. Hypophosphites in solution yield a white precipitate with mercuric chloride TS. This precipitate becomes gray when an excess of hypophosphite is present. Solutions of hypophosphites, acidified with sulfuric acid, and warmed with cupric sulfate TS yield a red precipitate.

Iodide—Solutions of iodides, upon the addition of chlorine TS, dropwise, liberate iodine, which colors the solution yellow to red. When the solution is shaken with chloroform, the latter is colored violet. The iodine thus liberated gives a blue color with starch TS. Silver nitrate TS produces in solutions of iodides a yellow, curdy precipitate, which is insoluble in nitric acid and in 6 *N* ammonium hydroxide.

Iron—Ferrous and ferric compounds in solution yield a black precipitate with ammonium sulfide TS. This precipitate is dissolved by cold 3 *N* hydrochloric acid with the evolution of hydrogen sulfide.

FERRIC SALTS—Acid solutions of ferric salts yield a dark blue precipitate with potassium ferrocyanide TS. With an excess of 1 *N* sodium hydroxide, a reddish brown precipitate is formed. Solutions of ferric salts produce with ammonium thiocyanate TS a deep red color which is not destroyed by dilute mineral acids.

FERROUS SALTS—Solutions of ferrous salts yield a dark blue precipitate with potassium ferricyanide TS. This precipitate is insoluble in 3 *N* hydrochloric acid, but is decomposed by 1 *N* sodium hydroxide. Solutions of ferrous salts yield with 1 *N* sodium hydroxide a greenish white precipitate, the color rapidly changing to green and then to brown when shaken.

Lactate—When solutions of lactates are acidified with sulfuric acid, potassium permanganate TS is added, and the mixture is heated, acetaldehyde, recognizable by its distinctive odor, is evolved.

Lead—Solutions of lead salts yield with 2 *N* sulfuric acid a white precipitate which is insoluble in 3 *N* hydrochloric or 2 *N* nitric acid, but is soluble in warm 1 *N* sodium hydroxide and in ammonium acetate TS. With potassium chromate TS, solutions of lead salts, free or nearly free from mineral acids, yield a yellow precipitate which is insoluble in 6 *N* acetic acid, but is soluble in 1 *N* sodium hydroxide.

Lithium—Moderately concentrated solutions of lithium salts, made alkaline with sodium hydroxide, yield with sodium carbonate TS a white precipitate on boiling. The precipitate is soluble in ammonium chloride TS. Lithium salts moistened with hydrochloric acid impart an intense crimson color to a nonluminous flame. Solutions of lithium salts are not precipitated by 2 *N* sulfuric acid or soluble sulfates (*distinction from strontium*).

Magnesium—Solutions of magnesium salts in the presence of ammonium chloride yield no precipitate when neutralized with ammonium carbonate TS, but on the subsequent addition of dibasic sodium phosphate TS, a white, crystalline precipitate, which is insoluble in 6 *N* ammonium hydroxide, is formed.

Manganese—Solutions of manganous salts yield with ammonium sulfide TS a salmon-colored precipitate, which dissolves in acetic acid.

Mercury—Solutions of mercury salts, free from an excess of nitric acid, when applied to bright copper foil, yield a deposit, which, upon rubbing, becomes bright and silvery in appearance. With hydrogen sulfide, solutions of mercury compounds yield a black precipitate, which is insoluble in ammonium sulfide TS and in boiling 2 *N* nitric acid.

MERCURIC SALTS—Solutions of mercuric salts yield a yellow precipitate with 1 *N* sodium hydroxide. They yield also, in neutral solutions with potassium iodide TS, a scarlet precipitate, which is very soluble in an excess of the reagent.

MERCUROUS SALTS—Mercurous compounds are decomposed by 1 *N* sodium hydroxide, producing a black color. Solutions of mercurous salts yield with hydrochloric acid a white precipitate which is blackened by 6 *N* ammonium hydroxide. With potassium iodide TS, a yellow precipitate, which may become green upon standing, is formed.

Nitrate—When a solution of a nitrate is mixed with an equal volume of sulfuric acid, the mixture is cooled, and a solution of ferrous sulfate is superimposed, a brown color is produced at the junction of the two liquids. When a nitrate is heated with sulfuric acid and metallic copper, brownish red fumes are evolved. Nitrates do not decolorize acidified potassium permanganate TS (*distinction from nitrites*).

Nitrite—When treated with dilute mineral acids or with 6 *N* acetic acid, nitrites evolve brownish red fumes. The solution colors starch-iodide paper blue.

Oxalate—Neutral and alkaline solutions of oxalates yield a white precipitate with calcium chloride TS. This precipitate is insoluble in 6 *N* acetic acid, but is dissolved by hydrochloric acid. Hot acidified solutions of oxalates decolorize potassium permanganate TS.

Permanganate—Solutions of permanganates acidified with sulfuric acid are decolorized by hydrogen peroxide TS and by sodium bisulfite TS, in the cold, and by oxalic acid TS, in hot solution.

Peroxide—Solutions of peroxides slightly acidified with sulfuric acid yield a deep blue color upon the addition of potassium dichromate TS. On shaking the mixture with an equal volume of ether and allowing the liquids to separate, the blue color is found in the ether layer.

Phosphate—Neutral solutions of orthophosphates yield with silver nitrate TS a yellow precipitate, which is soluble in 2 *N* nitric acid and in 6 *N* ammonium hydroxide. With ammonium molybdate TS, a yellow precipitate, which is soluble in 6 *N* ammonium hydroxide, is formed. Pyrophosphates obtained by ignition yield with silver nitrate TS a white precipitate, which is soluble in 2 *N* nitric acid and in 6 *N* ammonium hydroxide. With ammonium molybdate TS, a yellow precipitate, which is soluble in 6 *N* ammonium hydroxide, is formed.

Potassium—Potassium compounds impart a violet color to a nonluminous flame, but the presence of small quantities of sodium masks the color unless the yellow color produced by sodium is screened out by viewing through cobalt glass. In neutral, concentrated or moderately concentrated solutions of potassium salts (depending upon the solubility and the potassium content), sodium bitartrate TS produces a white crystalline precipitate, which is soluble in 6 *N* ammonium hydroxide and in solutions of alkali hydroxides and carbonates. The formation of the precipitate, which is usually slow, is accelerated by stirring or rubbing the inside of the test tube with a glass rod. The addition of a small amount of glacial acetic acid or alcohol also promotes the precipitation.

Salicylate—In moderately dilute solutions of salicylates, ferric chloride TS produces a violet color. The addition of acids to moderately concentrated solutions of salicylates produces a white, crystalline precipitate of salicylic acid, which melts between 158° and 161°.

Silver—Solutions of silver salts yield with hydrochloric acid a white, curdy precipitate, which is insoluble in nitric acid, but is readily soluble in 6 *N* ammonium hydroxide. A solution of a silver salt to which 6 *N* ammonium hydroxide and a small quantity of formaldehyde TS are added deposits, upon warming, a mirror of metallic silver upon the sides of the container.

Sodium—Solutions of sodium compounds after conversion to chloride or nitrate yield, with five times their volume of cobalt–uranyl acetate TS, a golden yellow precipitate, which forms after agitation for several minutes. Sodium compounds impart an intense yellow color to a nonluminous flame.

Sulfate—Solutions of sulfates yield with barium chloride TS a white precipitate, which is insoluble in hydrochloric acid and in nitric acid. With lead acetate TS, sulfates yield a white precipitate, which is soluble in ammonium acetate solution. Hydro-

chloric acid produces no precipitate when added to solutions of sulfates (*distinction from thiosulfates*).

Sulfite—When treated with 3 *N* hydrochloric acid, sulfites and bisulfites yield sulfur dioxide, recognizable by its characteristic, pungent odor. This gas blackens filter paper moistened with mercurous nitrate TS.

Tartrate—Dissolve a few mg of a tartrate salt in 2 drops of sodium periodate solution (1 in 20). Add a drop of 1 *N* sulfuric acid, and after 5 minutes add a few drops of sulfurous acid followed by a few drops of fuchsin–sulfurous acid TS: a reddish pink color is produced within 15 minutes.

Thiocyanate—Solutions of thiocyanates yield with ferric chloride TS a red color, which is not destroyed by moderately concentrated mineral acids.

Thiosulfate—Solutions of thiosulfates yield with hydrochloric acid a white precipitate which soon turns yellow, and sulfur dioxide, recognizable by its odor, is liberated. The addition of ferric chloride TS to solutions of thiosulfates produces a dark violet color which quickly disappears.

Zinc—In the presence of sodium acetate, solutions of zinc salts yield a white precipitate with hydrogen sulfide. This precipitate is insoluble in acetic acid, but is dissolved by 3 *N* hydrochloric acid. Ammonium sulfide TS produces a similar precipitate in neutral and in alkaline solutions. Zinc salts in solution yield with potassium ferrocyanide TS a white precipitate, which is insoluble in 3 *N* hydrochloric acid.

⟨193⟩ IDENTIFICATION—TETRACYCLINES

The following chromatographic procedures are provided to confirm the identity of Pharmacopeial drug substances that are of the tetracycline type, such as doxycycline, oxytetracycline, and tetracycline, and to confirm the identity of such compounds in their respective Pharmacopeial dosage forms. Two procedures are provided, one based on paper chromatography (*Method I*) and the other on thin-layer chromatography (*Method II*). *Method I* is to be used unless otherwise directed in the individual monograph.

Standard Solution—Unless otherwise directed in the individual monograph, dissolve the USP Reference Standard for the drug substance being identified in the same solvent and at the same concentration as for the *Test solution*.

Test Solution—Prepare as directed in the individual monograph.

Method I

pH 3.5 Buffer—Dissolve 13.4 g of anhydrous citric acid and 16.3 g of dibasic sodium phosphate in 1000 mL of water, and mix.

Developing Solvent—On the day of use, mix 10 volumes of chloroform, 20 volumes of nitromethane, and 3 volumes of pyridine.

Mixed Test Solution—Mix equal volumes of the *Standard solution* and the *Test solution*.

Chromatographic Sheet—Draw a spotting line 2.5 cm from one edge of a 20-cm × 20-cm sheet of filter paper (Whatman No. 1, or equivalent). Impregnate the sheet with *pH 3.5 Buffer* by passing it through a trough filled with *pH 3.5 Buffer*, and remove the excess solvent by firmly pressing the sheet between non-fluorescent blotting papers.

Procedure—To a suitable chromatographic chamber, prepared for ascending chromatography (see *Chromatography* ⟨621⟩), add *Developing Solvent* to a depth of 0.6 cm. On the spotting line of the *Chromatographic Sheet* apply at 1.5-cm intervals 2 µL each of the *Standard Solution*, the *Test Solution*, and the *Mixed Test Solution*. Allow the sheet to dry partially, and while still damp place it in the chromatographic chamber with the bottom edge touching the *Developing Solvent*. When the solvent front has risen about 10 cm, remove the sheet from the chamber, and expose the sheet to ammonia vapor. Examine the chromatogram under long-wavelength ultraviolet light. Record the positions of the major yellow fluorescent spots: the R_f value of the principal spot obtained from the *Test Solution* and from the *Mixed Test Solution* corresponds to that obtained from the *Standard Solution*.

Method II

Resolution Solution—Prepare as directed in the individual monograph.

Developing Solvent—Prepare a mixture of 0.5 *M* oxalic acid, previously adjusted with ammonium hydroxide to a pH of 2.0, acetonitrile, and methanol (80:20:20).

Chromatographic Plate—Use a suitable thin-layer chromatographic plate (see *Thin-layer Chromatography* under *Chromatography* ⟨621⟩), coated with a 0.25-mm layer of octylsilanized chromatographic silica gel mixture. Activate the plate by heating it at 130° for 20 minutes, allow to cool, and use while still warm.

Procedure—On the *Chromatographic plate* separately apply 1 µL each of the *Standard Solution*, the *Test Solution*, and the *Resolution Solution*. Allow the spots to dry, and develop the chromatogram in the *Developing Solvent* until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow to air-dry. Expose the plate to ammonia vapors, for 5 minutes, and promptly locate the spots on the plate by viewing under long-wavelength ultraviolet light: the chromatogram of the *Resolution Solution* shows clearly separated spots, and the principal spot obtained from the *Test Solution* corresponds in R_f value, intensity, and appearance to that obtained from the *Standard Solution*.

⟨201⟩ THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

The following procedure is applicable as an aid in verifying the identities of many compendial drug substances as such and in their respective dosage forms.

Prepare a test solution as directed in the individual monograph. On a line parallel to and about 2 cm from the edge of a suitable thin-layer chromatographic plate, coated with a 0.25-mm layer of chromatographic silica gel with a suitable fluorescing substance (see *Chromatography* ⟨621⟩), apply 10 µL of this solution and 10 µL of a Standard solution prepared from the USP Reference Standard for the drug substance being identified, in the same solvent and at the same concentration as the test solution, unless otherwise directed in the individual monograph. Allow the spots to dry, and develop the chromatogram in a solvent system consisting of a mixture of chloroform, methanol, and water (180:15:1), unless otherwise directed in the individual monograph, until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Unless otherwise directed in the individual monograph, locate the spots on the plate by examination under short-wavelength ultraviolet light. The R_f value of the principal spot obtained from the test solution corresponds to that obtained from the Standard solution.

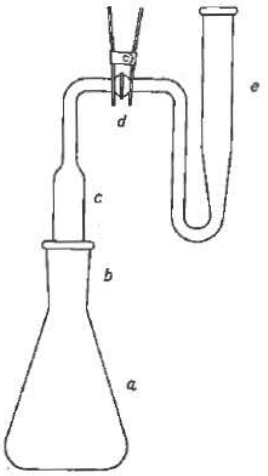
LIMIT TESTS

⟨211⟩ ARSENIC

This procedure is designed to determine the presence of trace amounts of arsenic (As) by converting the arsenic in a substance under test to arsine, which is then passed through a solution of silver diethyldithiocarbamate to form a red complex. The red color so produced is compared, either visually or spectrophotometrically, to the color produced similarly in a control containing an amount of arsenic equivalent to the limit given in the individual monograph. Limits are stated in terms of arsenic (As). The content of arsenic does not exceed the limit given in the individual monograph.

Two methods are provided, the methods differing only in the preliminary treatment of the test substance and the standard. Generally, *Method I* is used for inorganic materials, while *Method II* is used for organic materials.

Apparatus—The apparatus (see illustration)* consists of an arsine generator (*a*) fitted with a scrubber unit (*c*) and an absorber tube (*e*) with standard-taper or ground glass ball-and-socket joints (*b* and *d*) between the units. However, any other suitable apparatus, embodying the principle of the assembly described and illustrated, may be used.



Arsenic Test Apparatus

Arsenic Trioxide Stock Solution—Dissolve 132.0 mg of arsenic trioxide, previously dried at 105° for 1 hour and accurately weighed, in 5 mL of sodium hydroxide solution (1 in 5) in a 1000-mL volumetric flask. Neutralize the solution with 2 *N* sulfuric acid, add 10 mL more of 2 *N* sulfuric acid, then add recently boiled and cooled water to volume, and mix.

Standard Arsenic Solution—Transfer 10.0 mL of *Arsenic Trioxide Stock Solution* to a 1000-mL volumetric flask, add 10 mL of 2 *N* sulfuric acid, then add recently boiled and cooled water to volume, and mix. Each mL of *Standard Arsenic Solution* contains the equivalent of 1 μg of arsenic (As). Keep this solution in an all-glass container, and use within 3 days.

Method I

Standard Preparation—Pipet 3.0 mL of *Standard Arsenic Solution* into a generator flask, and dilute with water to 35 mL.

Test Preparation—Unless otherwise directed in the individual monograph, transfer to the generator flask the quantity, in g, of the test substance calculated by the formula:

$$3.0/L,$$

in which L is the arsenic limit in ppm, dissolve in water, and dilute with water to 35 mL.

Procedure—Treat the *Standard Preparation* and the *Test Preparation* similarly as follows: Add 20 mL of 7 *N* sulfuric acid, 2 mL of potassium iodide TS, 0.5 mL of stronger acid stannous chloride TS, and 1 mL of isopropyl alcohol, and mix. Allow to stand at room temperature for 30 minutes. Pack the scrubber tube (*c*) with two pledgets of cotton that have been soaked in saturated lead acetate solution, freed from excess solution by expression, and dried in vacuum at room temperature, leaving a 2-mm space between the two pledgets. Lubricate the joints (*b* and *d*) with a suitable stopcock grease designed for use with organic solvents, and connect the scrubber unit to the absorber tube (*e*). Transfer 3.0 mL of silver diethyldithiocarbamate TS to the absorber tube. Add 3.0 g of granular zinc (No. 20 mesh) to the mixture in the flask, immediately connect the assembled scrubber unit, place the generator flask (*a*) in a water bath maintained at a temperature of 25 ± 3°, and allow the evolution of hydrogen and the color development to proceed for 45 minutes, swirling the flask gently at 10-minute intervals. Disconnect the absorber tube from the generator and scrubber units, and transfer the absorbing solution to a 1-cm absorption cell. Any red color produced by the *Test Preparation* does not exceed that produced by the *Standard Preparation*. If necessary or desirable, determine the absorbance at the wavelength of maximum absorbance between 535 nm and 540 nm, with a suitable spectrophotometer or colorimeter, using silver diethyldithiocarbamate TS as the blank.

* A suitable apparatus is obtainable from Fisher Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.

Method II

NOTES—

(1) *Caution—Some substances may react with explosive violence when digested with hydrogen peroxide. Exercise safety precautions at all times.*

(2) If halogen-containing compounds are present, use a lower temperature while heating the test specimen with sulfuric acid, avoid boiling the mixture, and add the hydrogen peroxide with caution, before charring begins, to prevent loss of trivalent arsenic.

(3) If the test substance reacts too rapidly and begins charring with 5 mL of sulfuric acid before heating, use instead 10 mL of cooled dilute sulfuric acid (1 in 2), and add a few drops of the hydrogen peroxide before heating.

Standard Preparation—Pipet 3.0 mL of *Standard Arsenic Solution* into a generator flask, add 2 mL of sulfuric acid, mix, and add the total amount of 30 percent hydrogen peroxide used in preparing the *Test Preparation*. Heat the mixture to strong fuming, cool, add cautiously 10 mL of water, and again heat to strong fumes. Repeat this procedure with another 10 mL of water to remove any traces of hydrogen peroxide. Cool, and dilute with water to 35 mL.

Test Preparation—Unless otherwise directed in the individual monograph, transfer to a generator flask the quantity, in g, of the test substance calculated by the formula:

$$3.0/L,$$

in which L is the arsenic limit in ppm. Add 5 mL of sulfuric acid and a few glass beads, and digest in a fume hood, preferably on a hot plate and at a temperature not exceeding 120°, until charring begins. (Additional sulfuric acid may be necessary to wet some specimens completely, but the total volume added should not exceed 10 mL.) Cautiously add, dropwise, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops. Add the first few drops very slowly with sufficient mixing, in order to prevent a rapid reaction. Discontinue heating if foaming becomes excessive. When the reaction has abated, heat cautiously, rotating the flask occasionally to prevent the specimen from caking on glass exposed to the heating unit. *Maintain oxidizing conditions at all times during the digestion by adding small quantities of the hydrogen peroxide solution whenever the mixture turns brown or darkens.* Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate until fumes of sulfur trioxide are copiously evolved, and the solution becomes colorless or retains only a light straw color. Cool, add cautiously 10 mL of water, mix, and again evaporate to strong fuming, repeating this procedure to remove any trace of hydrogen peroxide. Cool, add cautiously 10 mL of water, wash the sides of the flask with a few mL of water, and dilute with water to 35 mL.

Procedure—Proceed as directed for *Procedure* under *Method I*.

Interfering Chemicals—Metals or salts of metals such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver may interfere with the evolution of arsine. *Antimony*, which forms stibine, produces a positive interference in the color development with silver diethyldithiocarbamate TS; when the presence of antimony is suspected, the red colors produced in the two silver diethyldithiocarbamate solutions may be compared at the wavelength of maximum absorbance between 535 nm and 540 nm, with a suitable colorimeter, since at this wavelength the interference due to stibine is negligible.

⟨216⟩ CALCIUM, POTASSIUM, AND SODIUM

The flame photometer characteristically is equipped with a photomultiplier phototube detector for determination of calcium or sodium, a red-sensitive phototube detector for the determination of potassium, a monochromator, an adjustable exit slit, sensitivity controls, and an oxyacetylene burner. An oxyhydrogen burner is necessary for the determination of potassium in the presence of large amounts of calcium.

Standard Calcium Ion Solution—Transfer 249.7 mg of calcium carbonate, previously dried at 300° for 3 hours and cooled in a desiccator for 2 hours, to a 100-mL volumetric flask, dissolve in a mixture of 20 mL of water and 5 mL of 3 *N* hydrochloric acid, dilute with water to volume, and mix. Each mL contains 1.00 mg of calcium ion (Ca).

Standard Potassium Ion Solution—Transfer 190.7 mg of potassium chloride, previously dried at 105° for 2 hours, to a 100-mL volumetric flask, dissolve in water, dilute with water to volume, and mix. Each mL contains 1.00 mg of potassium ion (K).

Standard Sodium Ion Solution—Transfer 254.2 mg of sodium chloride, previously dried at 105° for 2 hours, to a 100-mL volumetric flask, dissolve in water, dilute with water to volume, and mix. Each mL contains 1.00 mg of sodium ion (Na).

Standard Preparation—Transfer a 50-mL aliquot of the *Test Preparation* to a 100-mL volumetric flask, add the volume(s) of *Standard Ion Solution(s)* specified in the individual monograph, dilute with water to volume, and mix. Quantitatively dilute aliquots of this solution with water as necessary to bring the concentration of the ion to be determined into the proper range for the flame photometer used.

Test Preparation—Unless otherwise directed in the individual monograph, transfer 2.000 g of test specimen to a 100-mL volumetric flask, chill in an ice bath, add 5 mL of nitric acid, swirl to dissolve, and allow to warm to room temperature. Heat gently, if necessary, to obtain a clear or just slightly turbid mixture. Cool to room temperature, if necessary, dilute with water to volume, and mix. Filter or centrifuge if necessary, to obtain a clear solution.

Adjust the flame photometer to give a reading as near as possible to 100 percent transmittance with the *Standard Preparation* at the wavelength setting giving maximum emission corresponding to the designated characteristic wavelength as shown in the accompanying table. Use an exit slit width corresponding as nearly as possible to the designated bandwidth. Record the transmittance reading, labeling it as *S*.

Dilute aliquots of the *Test Preparation* with water as necessary to prepare a solution in which the concentration is similar to that in the *Standard Preparation*. Without changing any of the adjustments of the flame photometer, determine the emission of the solution as percent transmittance, and record the reading, labeling it as *T*. Readjust only the monochromator to the designated wavelength for background determination. Determine the emission of the solution at this wavelength as percent transmittance, and record the reading, labeling it as *B*.

The requirements of the test are met if the value of *T* minus *B* is less than or equal to the value of *S* minus *T*.

Ion	Wavelength (nm)		Bandwidth (nm)
	Characteristic	Background	
Calcium	422.7	430	0.8
Potassium	766.5	750	12
Sodium	589	580	0.8

⟨221⟩ CHLORIDE AND SULFATE

The following limit tests are provided as general procedures for use where limits for chloride and sulfate are specified in the individual monographs.

Perform the tests and the controls in glass cylinders of the same diameter and matched as closely as practicable in other respects (see *Visual Comparison* ⟨851⟩). Use the same quantities of the same reagents for both the solution under test and the control solution containing the specified volume of chloride or sulfate. If, after acidification, the solution is not perfectly clear, filter it through a filter paper that gives negative tests for chloride and sulfate. Add the precipitant, silver nitrate TS or barium chloride TS as required, to both the test solution and the control solution in immediate sequence.

Where the individual monograph calls for applying the test to a specific volume of a solution of the substance, and the limit for chloride or sulfate corresponds to 0.20 mL or less of 0.020 *N* hydrochloric acid or sulfuric acid, respectively, apply the test to the solution without further dilution. In such cases maintain the same volume relationships for the control solution as specified for the solution under test. In applying the test to the salts of heavy metals, which normally show an acid reaction, omit the acidification and do not neutralize the solution. Dissolve bismuth salts in a few mL of water and 2 mL of nitric acid before treating with the precipitant.

Chloride—Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with nitric acid to litmus. Add 1 mL each of nitric acid and of silver nitrate TS, and sufficient water to make 50 mL. Mix, allow to stand for 5 minutes protected from direct sunlight, and compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 *N* hydrochloric acid specified in the monograph.

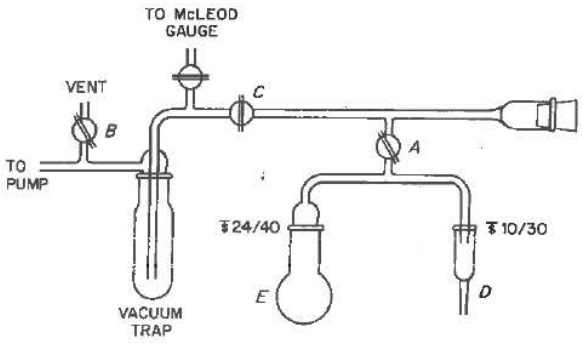
Sulfate—Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with hydrochloric acid to litmus. Add 1 mL of 3 *N* hydrochloric acid, 3 mL of barium chloride TS, and sufficient water to make 50 mL. Mix, allow to stand for 10 minutes, and compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 *N* sulfuric acid specified in the monograph.

⟨224⟩ DIOXANE

The following limit test is provided as a general procedure, where specified in the individual monograph, for the gas chromatographic determination of traces of 1,4-dioxane that may be separated from compendial articles.

Apparatus—Assemble a closed-system vacuum distillation apparatus, employing glass vacuum stopcocks (*A*, *B*, and *C*), as shown in the accompanying diagram. The concentrator tube (*D*)* is made of borosilicate or quartz (not flint) glass, graduated precisely enough to measure the 0.9 mL or more of distillate collected and marked so that the analyst can dilute accurately to 2.0 mL.

Standard preparation—Prepare a solution of dioxane in water having a known concentration of 100 µg per mL. Use a freshly prepared solution.



Closed-system Vacuum Distillation Apparatus for Dioxane

* A suitable tube is available as Chromaflex concentrator tube, Kontes Glass Co., Vineland, NJ (Catalog No. K42560-0000).

Test preparation—Unless otherwise specified in the individual monograph, transfer 20 g of the substance to be tested, accurately weighed, to a 50-mL round-bottom flask (*E*) having a 24/40 ground-glass neck joint. Liquefy semisolid or waxy test specimens by heating on a steam bath before making the transfer. Add 2.0 mL of water to the flask for crystalline specimens, and 1.0 mL for liquid, semisolid, or waxy specimens. Place a small polytef-covered stirring bar in the flask, insert the stopper, and stir to mix. Immerse the flask in an ice bath, and chill for about 1 minute.

Wrap heating tape around the tube connecting the concentrator tube (*D*) and the round-bottom flask (*E*), and apply about 10 volts to the tape. Apply a light coating of high-vacuum silicone grease to the ground-glass joints, and connect the tube *D* to the 10/30 joint and the round-bottom flask *E* to the 24/40 joint. Immerse the vacuum trap in a Dewar flask filled with liquid nitrogen, close stopcocks *A* and *B*, open stopcock *C*, and begin evacuating the system with a vacuum pump. Prepare a slurry bath from powdered dry ice and methanol, and raise the bath to the neck of the round-bottom flask. After freezing the contents of the flask for about 10 minutes, and when the vacuum system is operating at 0.05-mm pressure or lower, open stopcock *A* for 20 seconds, then close it. Remove the slurry bath, and allow the flask to warm in air for about 1 minute. Immerse the flask in a water bath maintained at a temperature of 20° to 25°, and after about 5 minutes warm the water bath to 35° to 40° (sufficient to liquefy most specimens) while stirring slowly but constantly with the magnetic bar. Cool the water in the bath by adding ice, and chill for about 2 minutes. Replace the water bath with the slurry bath, freeze the contents of the flask for about 10 minutes, then open stopcock *A* for 20 seconds, and close it. Remove the slurry bath, and repeat the heating steps as before, this time reaching a final temperature of 45° to 50° or a temperature necessary to melt the specimen completely. If there is any condensation in the tube connecting the round-bottom flask to the concentrator tube *D*, slowly increase the voltage to the heating tape, and heat until condensation disappears.

Stir with the magnetic stirrer throughout the following steps. Very slowly immerse the tube *D* in a Dewar flask containing liquid nitrogen. [*Caution*—When there is liquid distillate in tube *D*, immerse the tube in the liquid nitrogen *very slowly* or the tube will break.]

Water will begin to distil into the concentrator tube. As ice forms in the tube, raise the Dewar flask to keep the liquid nitrogen level only slightly below the level of ice in the tube. When water begins to freeze in the neck of the 10/30 joint, or when liquid nitrogen reaches the 2.0-mL graduation mark on the tube *D*, remove the Dewar flask, and allow the ice to melt without heating. After the ice has melted, check the volume of water that has distilled, and repeat the sequence of chilling and thawing until not less than 0.9 mL of water has been collected. Freeze the tube once again for about 2 minutes, and release the vacuum first by opening stopcock *B*, followed by opening stopcock *A*. Remove the tube *D* from the apparatus, close it with a greased stopper, and allow the ice to melt without heating. Mix the contents of the tube by swirling, note the volume of distillate, and dilute with water to 2.0 mL, if necessary. Use this *Test preparation* as directed under *Procedure*.

Procedure—Use a gas chromatograph equipped with a flame-ionization detector. Under typical conditions, the instrument contains a 2-mm × 1.8-m glass column packed with 80- to 100- or 100- to 120-mesh support S10. The column is maintained isothermally at a temperature of about 140°, the injection port at 200°, and the detector at 250°. Nitrogen or helium is the carrier gas, flowing at a rate of about 35 mL per minute. Install an oxygen scrubber between the carrier gas line and the column. Condition the column for about 72 hours at 230° with 30 to 40 mL per minute carrier flow. [NOTE—Support S10 is oxygen-sensitive. Flush both new and used columns with carrier gas for 30 to 60 minutes before heating each time they are installed in the gas chromatograph.]

Inject a volume of the *Standard preparation*, accurately measured, to produce about 20% of maximum recorder response. Where possible, keep the injection volume in the range of 2 µL to 4 µL, and use the solvent-flush technique to minimize errors associated with injection volumes. In the same manner, inject an equal volume of the *Test preparation*. The height of the peak produced by the *Test preparation* is not greater than that produced by the *Standard preparation*. The limit is 10 ppm, unless otherwise specified in the individual monograph.

⟨226⟩ 4-EPIANHYDROTETRACYCLINE

This chromatographic procedure is provided to demonstrate that the content of 4-epianhydrotetracycline, a degradation product of tetracycline, does not exceed the limit given in the individual monograph.

EDTA Buffer—Dissolve 37.2 g of disodium ethylenediaminetetraacetate in 800 mL of water, adjust with ammonium hydroxide to a pH of 7.8, dilute with water to 1000 mL, and mix.

Support Phase—Add 5 mL of *EDTA buffer* to 10 g of acid-washed chromatographic siliceous earth for column chromatography, and mix until the siliceous earth is uniformly moistened.

Test Solution—Prepare as directed in the individual monograph.

Procedure—Prepare a 15-mm × 170-mm chromatographic tube with a 4-mm × 50-mm outlet by packing it, in increments, with *Support phase*, firmly tamping down each increment, until the tube is filled to a height of about 10 cm. In a beaker, prepare a mixture of 1 g of acid-washed chromatographic siliceous earth for column chromatography and 1 mL of *Test solution*. Transfer the mixture to the top of the column. Dry-wash the beaker with *Support phase*, and transfer to the column to provide an additional 1-cm layer on top of the mixture containing the *Test solution*. Within 30 minutes, pass chloroform through the column, and collect successive fractions of 5.0 mL, 5.0 mL, 10.0 mL, 10.0 mL, and 5.0 mL. Observe the column during elution, and note the appearance of two separate yellow bands. The fraction or fractions containing the first yellow band contain the anhydrotetracyclines. Discard these fractions. The fractions after the first yellow band contain the 4-epianhydrotetracycline. Determine the absorbance of each 4-epianhydrotetracycline fraction at the wavelength of maximum absorbance at about 438 nm, with a suitable spectrophotometer, diluting each fraction, if necessary, with chloroform, and using chloroform as the blank. Calculate the quantity, in mg, of 4-epianhydrotetracycline in each fraction by the formula:

$$AVD/20.08,$$

in which A is the absorbance, V is the volume, in mL, of the fraction taken, D is the dilution factor, if the fraction was diluted, and 20.08 is the absorptivity of 4-epianhydrotetracycline at 438 nm. From the sum of the quantities of 4-epianhydrotetracycline found in the fractions, calculate the percentage of 4-epianhydrotetracycline in relation to the tetracycline hydrochloride equivalent contained in the *Test solution*.

⟨231⟩ HEAVY METALS

This test is provided to demonstrate that the content of metallic impurities that are colored by sulfide ion, under the specified test conditions, does not exceed the *Heavy metals* limit specified in the individual monograph in terms of the percentage (by weight) of lead in the test substance, as determined by concomitant visual comparison (see *Visual Comparison* in the section *Procedure* under *Spectrophotometry and Light-scattering* ⟨851⟩) with a control prepared from a *Standard Lead Solution*.

Determine the amount of heavy metals by *Method I*, unless otherwise specified in the individual monograph. *Method I* is used for substances that yield clear, colorless preparations under the specified test conditions. *Method II* is used for substances that do not yield clear, colorless preparations under the test conditions specified for *Method I*, or for substances that, by virtue of their complex nature, interfere with the precipitation of metals by sulfide ion, or for fixed and volatile oils. *Method III*, a wet-digestion method, is used only in those cases where neither *Method I* nor *Method II* can be utilized.

Special Reagents

Lead Nitrate Stock Solution—Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid,

then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.

Standard Lead Solution—On the day of use, dilute 10.0 mL of *Lead Nitrate Stock Solution* with water to 100.0 mL. Each mL of *Standard Lead Solution* contains the equivalent of 10 μg of lead. A comparison solution prepared on the basis of 100 μL of *Standard Lead Solution* per g of substance being tested contains the equivalent of 1 part of lead per million parts of substance being tested.

Method I

Standard Preparation—Into a 50-mL color-comparison tube pipet 2 mL of *Standard Lead Solution* (20 μg of Pb), and dilute with water to 25 mL. Adjust with 1 *N* acetic acid or 6 *N* ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH indicator paper as external indicator, dilute with water to 40 mL, and mix.

Test Preparation—Into a 50-mL color-comparison tube place 25 mL of the solution prepared for the test as directed in the individual monograph; or, using the designated volume of acid where specified in the individual monograph, dissolve and dilute with water to 25 mL the quantity, in g, of the substance to be tested, as calculated by the formula:

$$2.0/(1000L),$$

in which L is the *Heavy metals* limit, in percentage. Adjust with 1 *N* acetic acid or 6 *N* ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH indicator paper as external indicator, dilute with water to 40 mL, and mix.

Monitor Preparation—Into a third 50-mL color-comparison tube place 25 mL of a solution prepared as directed for *Test Preparation*, and add 2.0 mL of *Standard Lead Solution*. Adjust with 1 *N* acetic acid or 6 *N* ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH indicator paper as external indicator, dilute with water to 40 mL, and mix.

Procedure—To each of the three tubes containing the *Standard Preparation*, the *Test Preparation*, and the *Monitor Preparation*, respectively, add 10 mL of freshly prepared hydrogen sulfide TS, mix, allow to stand for 5 minutes, and view downward over a white surface: the color of the solution from the *Test Preparation* is not darker than that of the solution from the *Standard Preparation*, and the intensity of the color of the *Monitor Preparation* is equal to or greater than that of the *Standard Preparation*. [NOTE—If the color of the *Monitor Preparation* is lighter than that of the *Standard Preparation*, use *Method II* instead of *Method I* for the substance being tested.]

Method II

Standard Preparation—Prepare as directed under *Method I*.

Test Preparation—Use a quantity, in g, of the substance to be tested as calculated by the formula:

$$2.0/(1000L),$$

in which L is the *Heavy metals* limit, in percentage. Transfer the weighed quantity of the substance to a suitable crucible, add sufficient sulfuric acid to wet the substance, and carefully ignite at a low temperature until thoroughly charred. (The crucible may be loosely covered with a suitable lid during the charring.) Add to the carbonized mass 2 mL of nitric acid and 5 drops of sulfuric acid, and heat cautiously until white fumes no longer are evolved. Ignite, preferably in a muffle furnace, at 500° to 600°, until the carbon is completely burned off. Cool, add 4 mL of 6 *N* hydrochloric acid, cover, digest on a steam bath for 15 minutes, uncover, and slowly evaporate on a steam bath to dryness. Moisten the residue with 1 drop of hydrochloric acid, add 10 mL of hot water, and digest for 2 minutes. Add 6 *N* ammonium hydroxide dropwise, until the solution is just alkaline to litmus paper, dilute with water to 25 mL, and adjust with 1 *N* acetic acid to a pH between 3.0 and 4.0, using short-range pH indicator paper as external indicator. Filter if necessary, rinse the crucible and the filter with 10 mL of water, combine the filtrate and rinsing in a 50-mL color-comparison tube, dilute with water to 40 mL, and mix.

Procedure—To each of the tubes containing the *Standard Preparation* and the *Test Preparation*, respectively, add 10 mL of freshly prepared hydrogen sulfide TS, mix, allow to stand for 5 minutes, and view downward over a white surface: the color of the solution from the *Test Preparation* is not darker than that of the solution from the *Standard Preparation*.

Method III

Standard Preparation—Transfer a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid to a clean, dry, 100-mL Kjeldahl flask, and add a further volume of nitric acid equal to the incremental volume of nitric acid added to the *Test Preparation*. Heat the solution to the production of dense, white fumes, cool, cautiously add 10 mL of water and, if hydrogen peroxide was used in treating the *Test Preparation*, add a volume of 30 percent hydrogen peroxide equal to that used for the substance being tested, and boil gently to the production of dense, white fumes. Again cool, cautiously add 5 mL of water, mix, and boil gently to the production of dense, white fumes and to a volume of 2 to 3 mL. Cool, dilute cautiously with a few mL of water, add 2.0 mL of *Standard Lead Solution* (20 μg of Pb), and mix. Transfer to a 50-mL color-comparison tube, rinse the flask with water, adding the rinsing to the tube until the volume is 25 mL, and mix.

Test Preparation—

If the substance is a solid—Transfer the quantity of the test substance specified in the individual monograph to a clean, dry, 100-mL Kjeldahl flask [NOTE—A 300-mL flask may be used if the reaction foams excessively], clamp the flask at an angle of 45°, and add sufficient of a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid to moisten the substance thoroughly. Warm gently until the reaction commences, allow the reaction to subside, and add additional portions of the same acid mixture, heating after each addition, until a total of 18 mL of the acid mixture has been added. Increase the amount of heat, and boil gently until the solution darkens. Cool, add 2 mL of nitric acid, and again heat until the solution darkens. Continue the heating, followed by addition of nitric acid until no further darkening occurs, then heat strongly to the production of dense, white fumes. Cool, cautiously add 5 mL of water, boil gently to the production of dense, white fumes, and continue heating until the volume is reduced to a few mL. Cool, cautiously add 5 mL of water, and examine the color of the solution. If the color is yellow, cautiously add 1 mL of 30 percent hydrogen peroxide, and again evaporate to the production of dense, white fumes and a volume of 2 to 3 mL. If the solution is still yellow in color, repeat the addition of 5 mL of water and the peroxide treatment. Cool, dilute cautiously with a few mL of water, and rinse into a 50-mL color-comparison tube, taking care that the combined volume does not exceed 25 mL.

If the substance is a liquid—Transfer the quantity of the test substance specified in the individual monograph to a clean, dry, 100-mL Kjeldahl flask [NOTE—A 300-mL flask may be used if the reaction foams excessively], clamp the flask at an angle of 45°, and cautiously add a few mL of a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid. Warm gently until the reaction commences, allow the reaction to subside, and proceed as directed under *If the substance is a solid*, beginning with "add additional portions of the same acid mixture."

Procedure—Treat the *Test Preparation* and the *Standard Preparation* as follows: Adjust the solution to a pH between 3.0 and 4.0, using short-range pH indicator paper as external indicator, with ammonium hydroxide (a dilute ammonia solution may be used, if desired, as the specified range is approached), dilute with water to 40 mL, and mix.

To each tube add 10 mL of freshly prepared hydrogen sulfide TS, mix, allow to stand for 5 minutes, and view downward over a white surface: the color of the *Test Preparation* is not darker than that of the *Standard Preparation*.

⟨241⟩ IRON

This limit test is provided to demonstrate that the content of iron, in either the ferric or the ferrous form, does not exceed the limit for iron specified in the individual monograph. The determination is made by concomitant visual comparison with a control prepared from a standard iron solution.

Special Reagents—

STANDARD IRON SOLUTION—Dissolve 863.4 mg of ferric ammonium sulfate [$FeNH_4(SO_4)_2.12H_2O$] in water, add 10 mL of 2 *N* sulfuric acid, and dilute with water to 100.0 mL. Pipet 10 mL of this solution into a 1000-mL volumetric flask, add 10 mL of 2 *N* sulfuric acid, dilute with water to volume, and mix. This solution contains the equivalent of 0.01 mg (10 μg) of iron per mL.

AMMONIUM THIOCYANATE SOLUTION—Dissolve 30 g of ammonium thiocyanate in water to make 100 mL.

Standard Preparation—Into a 50-mL color-comparison tube pipet 1 mL of *Standard Iron Solution* (10 μg of Fe), dilute with water to 45 mL, add 2 mL of hydrochloric acid, and mix.

Test Preparation—Into a 50-mL color comparison tube place the solution prepared for the test as directed in the individual monograph and if necessary dilute with water to 45 mL; or, dissolve in water, and dilute with water to 45 mL the quantity, in g, of the substance to be tested, as calculated by the formula:

$$1.0/(1000L),$$

in which *L* is the *Iron* limit in percentage. Add 2 mL of hydrochloric acid, and mix.

Procedure—To each of the tubes containing the *Standard Preparation* and the *Test Preparation* add 50 mg of ammonium peroxydisulfate crystals and 3 mL of *Ammonium Thiocyanate Solution*, and mix: the color of the solution from the *Test Preparation* is not darker than that of the solution from the *Standard Preparation*.

⟨251⟩ LEAD

The imposition of stringent limits on the amounts of lead that may be present in pharmaceutical products has resulted in the use of two methods, of which the one set forth following depends upon extraction of lead by solutions of dithizone. For determination of the content of heavy metals generally, expressed as a lead equivalent, see *Heavy Metals* ⟨231⟩.

Select all reagents for this test to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Rinse thoroughly all glassware with warm dilute nitric acid (1 in 2), followed by water.

Special Reagents—

AMMONIA-CYANIDE SOLUTION—Dissolve 2 g of potassium cyanide in 15 mL of ammonium hydroxide, and dilute with water to 100 mL.

AMMONIUM CITRATE SOLUTION—Dissolve 40 g of citric acid in 90 mL of water. Add 2 or 3 drops of phenol red TS, then cautiously add ammonium hydroxide until the solution acquires a reddish color. Remove any lead that may be present by extracting the solution with 20-mL portions of *Dithizone Extraction Solution* (see below), until the dithizone solution retains its orange-green color.

DILUTED STANDARD LEAD SOLUTION—Dilute an accurately measured volume of *Standard Lead Solution* (see *Heavy Metals* ⟨231⟩) [containing 10 μg of lead per mL], with 9 volumes of dilute nitric acid (1 in 100) to obtain a solution that contains 1 μg of lead per mL.

DITHIZONE EXTRACTION SOLUTION—Dissolve 30 mg of dithizone in 1000 mL of chloroform, and add 5 mL of alcohol. Store the solution in a refrigerator.

Before use, shake a suitable volume of the dithizone extraction solution with about half its volume of dilute nitric acid (1 in 100), discarding the nitric acid.

HYDROXYLAMINE HYDROCHLORIDE SOLUTION—Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make approximately 65 mL. Transfer to a separator, add 5 drops of thymol blue TS, then add ammonium hydroxide until the solution assumes a yellow color. Add 10 mL of sodium diethyldithiocarbamate solution (1 in 25), mix, and allow to stand for 5 minutes. Extract this solution with successive 10- to 15-mL portions of chloroform until a 5-mL portion of the chloroform extract does not assume a yellow color when shaken with cupric sulfate TS. Add 3 *N* hydrochloric acid until the solution is pink (if necessary, add 1 or 2 drops more of thymol blue TS), and then dilute with water to 100 mL.

POTASSIUM CYANIDE SOLUTION—Dissolve 50 g of potassium cyanide in sufficient water to make 100 mL. Remove the lead from this solution by extraction with successive portions of *Dithizone Extraction Solution*, as described under *Ammonium Citrate Solution* above, then extract any dithizone remaining in the cyanide solution by shaking with chloroform. Finally dilute the cyanide solution with sufficient water so that each 100 mL contains 10 g of potassium cyanide.

STANDARD DITHIZONE SOLUTION—Dissolve 10 mg of dithizone in 1000 mL of chloroform. Keep the solution in a glass-stoppered, lead-free bottle, suitably wrapped to protect it from light, and store in a refrigerator.

NOTE—The following special reagents are called for in the test for *Lead* under *Ferrous Sulfate*.

CITRATE-CYANIDE WASH SOLUTION—To 50 mL of water add 50 mL of *Ammonium Citrate Solution* and 4 mL of *Potassium Cyanide Solution*, mix, and adjust the pH, if necessary, with ammonium hydroxide to 9.0.

pH 2.5 BUFFER SOLUTION—To 25.0 mL of 0.2 *M* potassium biphthalate add 37.0 mL of 0.1 *N* hydrochloric acid, and dilute with water to 100.0 mL.

DITHIZONE–CARBON TETRACHLORIDE SOLUTION—Dissolve 10 mg of dithizone in 1 liter of carbon tetrachloride. Prepare this solution on the day of use.

pH 2.5 WASH SOLUTION—To 500 mL of dilute nitric acid (1 in 100) add 6 *N* ammonium hydroxide until the pH of the mixture is 2.5, then add 10 mL of *pH 2.5 Buffer Solution*, and mix.

AMMONIA-CYANIDE WASH SOLUTION—To 35 mL of *pH 2.5 Wash Solution* add 4 mL of *Ammonia-Cyanide Solution*, and mix.

Test Preparation—[NOTE—If, in the following preparation, the substance under test reacts too rapidly and begins charring with 5 mL of sulfuric acid before heating, use instead 10 mL of cooled dilute sulfuric acid (1 in 2), and add a few drops of the hydrogen peroxide before heating]. Where the monograph does not specify preparation of a solution, prepare a *Test Preparation* as follows:

Caution—Exercise safety precautions in this procedure, as some substances may react with explosive violence when digested with hydrogen peroxide. Transfer 1.0 g of the substance under test to a suitable flask, add 5 mL of sulfuric acid and a few glass beads, and digest on a hot plate in a hood until charring begins. Other suitable means of heating may be substituted. (Add additional sulfuric acid, if necessary, to wet the substance completely, but do not add more than a total of 10 mL.) Add, dropwise and with caution, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops. Add the first few drops very slowly, mix carefully to prevent a rapid reaction, and discontinue heating if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls of the flask. [*NOTE—Add peroxide whenever the mixture turns brown or darkens.*] Continue the digestion until the substance is completely destroyed, copious fumes of sulfur trioxide are evolved, and the solution is colorless. Cool, cautiously add 10 mL of water, evaporate until sulfur trioxide again is evolved, and cool. Repeat this procedure with another 10 mL of water to remove any traces of hydrogen peroxide. Cautiously dilute with 10 mL of water, and cool.

Procedure—Transfer the *Test Preparation*, rinsing with 10 mL of water, or the volume of the prepared sample specified in the monograph to a separator, and, unless otherwise directed in the monograph, add 6 mL of *Ammonium Citrate Solution* and 2 mL of *Hydroxylamine Hydrochloride Solution*. (For the determination of lead in iron salts use 10 mL of *Ammonium Citrate Solution*.) Add 2 drops of phenol red TS, and make the solution just alkaline (red in color) by the addition of ammonium hydroxide. Cool the solution if necessary, and add 2 mL of *Potassium Cyanide Solution*. Immediately extract the solution with 5-mL portions of *Dithizone Extraction Solution*, draining off each extract into another separator, until the dithizone solution retains its green color. Shake the combined dithizone solutions for 30 seconds with 20 mL of dilute nitric acid (1 in 100), and discard the chloroform layer. Add to the acid solution 5.0 mL of *Standard Dithizone Solution* and 4 mL of *Ammonia-Cyanide Solution*, and shake for 30 seconds: the color of the chloroform

layer is of no deeper shade of violet than that of a control made with a volume of *Diluted Standard Lead Solution* equivalent to the amount of lead permitted in the sample under examination, and the same quantities of the same reagents and in the same manner as in the test with the sample.

⟨261⟩ MERCURY

Method I

[NOTE—Mercuric dithizonate is light-sensitive. Perform this test in subdued light.]

Reagents—

Dithizone Stock Solution—Dissolve 40 mg of dithizone in 1000 mL of chloroform.

Dithizone Titrant—Dilute 30.0 mL of *Dithizone Stock Solution* with chloroform to 100.0 mL. This solution contains approximately 12 mg of dithizone per liter.

Mercury Stock Solution—Transfer 135.4 mg of mercuric chloride to a 100-mL volumetric flask, and dilute with 1 *N* sulfuric acid to volume. This solution contains the equivalent of 100 mg of Hg in 100 mL.

Mercury Solution for Standardizing Dithizone Titrant—Transfer 2.0 mL of *Mercury Stock Solution* to a 100-mL volumetric flask, and dilute with 1 *N* sulfuric acid to volume. Each mL of this solution contains the equivalent of 20 μg of Hg.

The following solutions are called for in the limit test for mercury that is specified in the monographs on Ferrous Fumarate, Ferrous Sulfate, and Dried Ferrous Sulfate.

HYDROXYLAMINE HYDROCHLORIDE SOLUTION—Prepare as directed in the test for *Lead* ⟨251⟩.

STANDARD MERCURY SOLUTION—On the day of use, quantitatively dilute 1.0 mL of *Mercury Stock Solution* with 1 *N* sulfuric acid to 1000 mL. Each mL of the resulting solution contains the equivalent of 1 μg of mercury.

DITHIZONE EXTRACTION SOLUTION—Prepare as directed in the test for *Lead* ⟨251⟩.

DILUTED DITHIZONE EXTRACTION SOLUTION—Just prior to use, dilute 5 mL of *Dithizone Extraction Solution* with 25 mL of chloroform.

Standardization of Dithizone Titrant—Transfer 1.0 mL of *Mercury Solution for Standardizing Dithizone Titrant* to a 250-mL separator, and add 100 mL of 1 *N* sulfuric acid, 90 mL of water, 1 mL of glacial acetic acid, and 10 mL of hydroxylamine hydrochloride solution (1 in 5). Titrate the solution with *Dithizone Titrant* from a 10-mL microburet, shaking the mixture 20 times after each addition and allowing the chloroform layer to separate, then discarding the chloroform layer. Continue until a final addition of *Dithizone Titrant* is green in color after shaking. Calculate the quantity, in μg, of Hg equivalent to each mL of *Dithizone Titrant* by the formula:

$$20/V,$$

in which V is the volume, in mL, of *Dithizone Titrant* added.

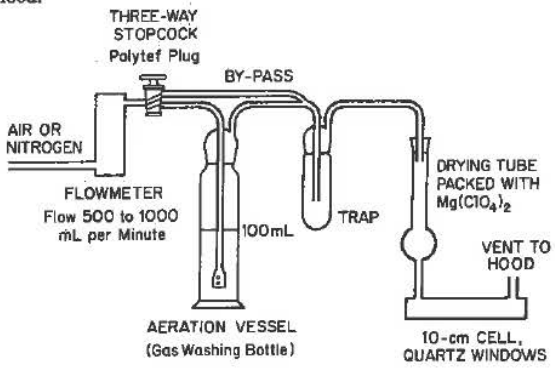
Test Preparation—Transfer about 2 g of the substance under test, accurately weighed, to a glass-stoppered, 250-mL conical flask, add 20 mL of a mixture of equal volumes of nitric acid and sulfuric acid, attach a suitable condenser, reflux the mixture for 1 hour, cool, cautiously dilute with water, and boil until fumes of nitrous acid no longer are noticeable. Cool the solution, cautiously dilute with water, transfer to a 200-mL volumetric flask, dilute with water to volume, mix, and filter.

Procedure—Transfer 50.0 mL of *Test Preparation* to a 250-mL separator, and extract with successive small portions of chloroform until the last chloroform extract remains colorless. Discard the chloroform extract, and add to the extracted *Test Preparation* 50 mL of 1 *N* sulfuric acid, 90 mL of water, 1 mL of glacial acetic acid, and 10 mL of hydroxylamine hydrochloride solution (1 in 5). Proceed as directed under *Standardization of Dithizone Titrant*, beginning with "Titrate the solution." Calculate the amount of mercury.

Method IIa and Method IIb

Mercury Detection Instrument—Use any suitable atomic absorption spectrophotometer equipped with a fast-response recorder and capable of measuring the radiation absorbed by mercury vapors at the mercury resonance line of 253.6 nm. [NOTE—Wash all glassware associated with the test with nitric acid, and rinse thoroughly with water before use.]

Aeration Apparatus—The apparatus (see accompanying diagram) consists of a flowmeter capable of measuring flow rates from 500 to 1000 mL per minute, connected via a three-way stopcock fitted with a polytef plug to an aeration vessel (250-mL gas washing bottle), followed by a trap, a drying tube packed with magnesium perchlorate, a 10-cm × 25-mm flow-through cell with quartz windows, and terminating with a vent to a fume hood.



Connections are glass or polyvinyl chloride

Mercury Acration Apparatus

Reagents—

Potassium Permanganate Solution—Dissolve 5 g of potassium permanganate in 100 mL of water.

Hydroxylamine Hydrochloride Solution—Dissolve 10 g of hydroxylamine hydrochloride in 100 mL of water.

Stannous Chloride Solution—Dissolve 10 g of $SnCl_2.2H_2O$ in 20 mL of warm hydrochloric acid, and add 80 mL of water. Prepare fresh each week.

Standard Mercury Solution—Prepare from *Mercury Stock Solution* as directed under *Method I*. Each mL of the *Standard Mercury Solution* contains the equivalent of 1 μg of mercury.

Test Preparation—Unless otherwise directed in the individual monograph, use the quantity, in g, of the test substance calculated by the formula:

$$2.0/L,$$

in which L is the mercury limit, in ppm.

Method IIa

Standard Preparation—Pipet 2.0 mL of *Standard Mercury Solution* into a 100-mL beaker, and add 35 mL of water, 3 mL of sulfuric acid, and 1 mL of potassium permanganate solution. Cover the beaker with a watch glass, boil for a few seconds, and cool.

Test Preparation—Transfer the calculated amount of the test substance to a 100-mL beaker, and add 35 mL of water. Stir, and warm to assist solution, if necessary. Add 2 drops of phenolphthalein TS, and, as necessary, slowly neutralize with constant stirring, using 1 *N* sodium hydroxide or 1 *N* sulfuric acid. Add 3 mL of sulfuric acid and 1 mL of *Potassium Permanganate Solution*. Cover the beaker with a watch glass, boil for a few seconds, and cool.

Procedure—Assemble the *Aeration apparatus* as shown in the accompanying diagram, with the aeration vessel and the trap empty, and the stopcock in the bypass position. Connect the apparatus to the absorption cell, and adjust the air or nitrogen flow rate so that, in the following procedure, maximum absorption and reproducibility are obtained without excessive foaming in the

test solution. Obtain a smooth baseline reading at 253.6 nm, following the manufacturer's instructions for operating the instrument.

Treat the *Standard Preparation* and the *Test Preparation* similarly, as follows: Destroy the excess permanganate by adding *Hydroxylamine Hydrochloride Solution*, dropwise, until the solution is colorless. Immediately wash the solution into the aeration vessel with water, and dilute with water to 100 mL. Add 2 mL of *Stannous Chloride Solution*, and immediately reconnect the aeration vessel to the aeration apparatus. Turn the stopcock from the bypass position to the aerating position, and continue the aeration until the absorption peak has been passed and the recorder pen returns to the baseline. Disconnect the aeration vessel from the apparatus, and wash with water after each use. After correcting for any reagent blank, any absorbance produced by the *Test Preparation* does not exceed that produced by the *Standard Preparation*.

Method IIb

[*Caution—Some substances may react with explosive violence when digested with hydrogen peroxide. Exercise safety precautions at all times.*]

Standard Preparation—Pipet 2.0 mL of *Standard Mercury Solution* into a 125-mL conical flask, add 3 mL each of nitric acid and sulfuric acid, mix, and add an amount of 30 percent hydrogen peroxide equal to the total amount used in preparing the *Test Preparation*. Attach a suitable water-cooled condenser with a standard-taper joint to fit the flask, and reflux the mixture in a fume hood for 1 hour. Turn off the water circulating through the condenser, and heat until white fumes appear in the flask. Cool, and cautiously add 10 mL of water through the condenser, while swirling the flask. Again heat until white fumes appear, cool, and add an additional 15 mL of water. Remove the condenser, and rinse the sides of the flask to obtain a volume of 35 mL. Add 1 mL of *Potassium Permanganate Solution*, boil for a few seconds, and cool.

Test Preparation—Transfer the calculated amount of the test substance to a 125-mL conical flask. Add 5 mL each of nitric acid and sulfuric acid and a few glass beads. Attach a suitable water-cooled condenser with a standard-taper joint to fit the flask, and digest in a fume hood, preferably on a hot plate, and at a temperature not exceeding 120°, until charring begins. (If additional sulfuric acid is necessary to wet the specimen completely, add it carefully through the condenser, but do not allow the total volume added to exceed 10 mL.) After the test substance has been decomposed by the acid, cautiously add, dropwise through the condenser, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops (add the first few drops very slowly with sufficient mixing, in order to prevent a rapid reaction; discontinue heating if foaming becomes excessive). When the reaction has abated, heat cautiously, rotating the flask occasionally to prevent the specimen from caking on glass exposed to the heating unit. Maintain oxidizing conditions at all times during the digestion by adding small quantities of the hydrogen peroxide solution whenever the mixture turns brown or darkens. Continue the digestion until the organic matter is destroyed, and then reflux the mixture for 1 hour. Turn off the water circulating through the condenser, and heat until fumes of sulfur trioxide are copiously evolved and the solution becomes colorless or retains only a light straw color. Cool, and cautiously add 10 mL of water through the condenser, while swirling the flask. Again heat until white fumes appear. Cool, and cautiously add 15 mL of water. Remove the condenser, and rinse the sides of the flask with a few mL of water to obtain a volume of 35 mL. Add 1 mL of *Potassium Permanganate Solution*, boil for a few seconds, and cool.

Procedure—Proceed as directed for *Procedure* under *Method IIa*.

⟨271⟩ READILY CARBONIZABLE SUBSTANCES TEST

In tests for readily carbonizable substances, unless otherwise directed, add the specified quantity of the substance, finely powdered if in solid form, in small portions to the comparison container, which is made of colorless glass resistant to the action of sulfuric acid and contains the specified volume of sulfuric acid TS (see under *Test Solutions*).

Stir the mixture with a glass rod until solution is complete, allow the solution to stand for 15 minutes, unless otherwise directed, and compare the color of the solution with that of the specified matching fluid in a comparison container which also is of colorless glass and has the same internal and cross-section dimensions, viewing the fluids transversely against a background of white porcelain or white glass.

When heat is directed in order to effect solution of the substance in the sulfuric acid TS, mix the sample and the acid in a test tube, heat as directed, and transfer the solution to the comparison container for matching with the designated Matching Fluid (see *Color and Achromicity* ⟨631⟩).

Special attention is directed to the importance of the concentration of sulfuric acid used in this test. The reagent of the required strength, i.e., 95.0 ± 0.5 percent of H_2SO_4, is designated as a "Test Solution."

⟨281⟩ RESIDUE ON IGNITION

Weigh accurately 1 to 2 g of the substance, or the amount specified in the individual monograph, in a suitable crucible that previously has been ignited, cooled, and weighed. Heat, gently at first, until the substance is thoroughly charred, cool, then, unless otherwise directed in the individual monograph, moisten the residue with 1 mL of sulfuric acid, heat gently until white fumes no longer are evolved, and ignite at 800 ± 25° until the carbon is consumed. Cool in a desiccator, weigh, and calculate the percentage of residue. If the amount of the residue so obtained exceeds the limit specified in the individual monograph, again moisten the residue with 1 mL of sulfuric acid, heat and ignite as before, and again calculate the percentage of residue. Continue the ignition until constant weight is attained, unless otherwise specified.

Conduct the ignition in a well-ventilated hood, but protected from air currents, and at as low a temperature as is possible to effect the complete combustion of the carbon. A muffle furnace may be used, if desired, and its use is recommended for the final ignition at 800 ± 25°.

Calibration of the muffle furnace may be carried out using an appropriate digital temperature meter and a working thermocouple probe calibrated against a standard thermocouple traceable to the National Bureau of Standards.

Verify the accuracy of the measuring and controlling circuitry of the muffle furnace by checking the positions in the furnace at the control set point temperature of intended use. Select positions that reflect the eventual method of use with respect to location of the specimen under test. The tolerance is ±25° at each position measured.

⟨291⟩ SELENIUM

Stock Solution—Dissolve 40.0 mg of metallic selenium in 100 mL of dilute nitric acid (1 in 2) in a 1000-mL volumetric flask, warming gently on a steam bath if necessary to effect solution, add water to volume, and mix. Pipet 5 mL of this solution into a 200-mL volumetric flask, add water to volume, and mix. Each mL of the resulting solution contains the equivalent of 1 μg of selenium (Se).

Diaminonaphthalene Solution—Dissolve 100 mg of 2,3-diaminonaphthalene and 500 mg of hydroxylamine hydrochloride in 0.1 *N* hydrochloric acid to make 100 mL. Prepare this solution fresh on the day of use.

Standard Solution—Pipet 6 mL of *Stock Solution* into a 150-mL beaker, and add 25 mL of dilute nitric acid (1 in 30) and 25 mL of water.

Test Solution—Clean combustion of the test material is an important factor in conducting the test. For compounds that burn poorly and produce soot, the addition of magnesium oxide usually results in more thorough combustion and reduces soot formation. Where the need to add magnesium oxide has been identified, it is specified in the individual monograph. Using a 1000-mL com-

bustion flask and using 25 mL of dilute nitric acid (1 in 30) as the absorbing liquid, proceed as directed under *Oxygen Flask Combustion* ⟨471⟩. Upon completion of the combustion, place a few mL of water in the cup, loosen the stopper, and rinse the stopper, the specimen holder, and the sides of the flask with about 10 mL of water. Transfer the solution with the aid of about 20 mL of water to a 150-mL beaker, and heat gently to the boiling temperature. Boil for 10 minutes, and allow the solution to cool to room temperature.

Procedure—Treat the *Standard Solution*, the *Test Solution*, and the reagent blank consisting of 25 mL of dilute nitric acid (1 in 30) and 25 mL of water, concomitantly and in parallel, as follows: Add ammonium hydroxide solution (1 in 2) to adjust to a pH of 2.0 ± 0.2. Dilute with water to 60.0 mL, and transfer to a low-actinic separator with the aid of 10.0 mL of water, adding the 10.0 mL of rinsings to the separator. Add 200 mg of hydroxylamine hydrochloride, swirl to dissolve, immediately add 5.0 mL of *Diaminonaphthalene Solution*, insert the stopper, and swirl to mix. Allow the solution to stand at room temperature for 100 minutes. Add 5.0 mL of cyclohexane, shake vigorously for 2 minutes, and allow the layers to separate. Discard the aqueous layer, and centrifuge the cyclohexane extract to remove any dispersed water. Determine the absorbances of the cyclohexane extracts of the *Test Solution* and the *Standard Solution* in a 1-cm cell at the wavelength of maximum absorbance at about 380 nm, with a suitable spectrophotometer, using the cyclohexane extract of the reagent blank as the blank, and compare the absorbances: the absorbance of the *Test Solution* is not greater than that of the *Standard Solution* where a 200-mg test specimen has been taken, or is not greater than one-half that of the *Standard Solution* where a 100-mg test specimen has been taken.

OTHER TESTS AND ASSAYS

⟨301⟩ ACID-NEUTRALIZING CAPACITY

NOTE—All tests shall be conducted at a temperature of 37 ± 3°.

Standardization of pH Meter—Standardize a pH meter using the 0.05 *m* potassium biphthalate and 0.05 *m* potassium tetraoxalate standardizing buffers as described under *pH* ⟨791⟩.

Magnetic Stirrer—Transfer 100 mL of water to a 250-mL beaker containing a 40- × 10-mm magnetic stirring bar that is coated with solid perfluorocarbon and has a spin ring at its center. Adjust the power setting of the magnetic stirrer to produce a stirring rate of 300 ± 30 rpm when the stirring bar is centered in the beaker, as determined by a suitable optical tachometer.

Test Preparation—

Powders—Transfer the accurately weighed portion of the substance specified in the individual monograph to a 250-mL beaker, add 70 mL of water, and mix on the *Magnetic Stirrer* for 1 minute.

Effervescent Solids—Transfer an accurately weighed quantity, equivalent to the minimum labeled dosage, to a 250-mL beaker, add 10 mL of water, and swirl the beaker gently while allowing the reaction to subside. Add another 10 mL of water, and swirl gently. Wash the walls of the beaker with 50 mL of water, and mix on the *Magnetic Stirrer* for 1 minute.

Suspensions and Other Liquids—Shake the container until the contents are uniform, and determine the density. Transfer an accurately weighed quantity of the uniform mixture, equivalent to the minimum labeled dosage, to a 250-mL beaker, add water to make a total volume of about 70 mL, and mix on the *Magnetic Stirrer* for 1 minute.

Non-chewable Tablets—Weigh not less than 20 tablets, and determine the average tablet weight. Grind the tablets to a fine powder, mix to obtain a uniform mixture, and transfer an accurately weighed quantity of it, equivalent to the minimum labeled dosage, to a 250-mL beaker. If wetting is desired, add not more than 5 mL of alcohol (neutralized to an apparent pH of 3.5), and mix to wet the specimen thoroughly. Add 70 mL of water, and mix on the *Magnetic Stirrer* for 1 minute.

Chewable Tablets—Prepare as directed for *Non-chewable Tablets*.

Tablets That Are Required To Be Chewed—Transfer 1 Tablet to a 250-mL beaker, add 50 mL of water, and mix on the *Magnetic Stirrer* for 1 minute.

Capsules—Weigh accurately not less than 20 capsules. Remove the capsule contents completely, with the aid of a cotton swab if necessary. Accurately weigh the empty capsules, and determine the average weight of the contents per capsule. Mix the combined capsule contents to obtain a uniform mixture, and proceed as directed for *Non-chewable Tablets*, beginning with "transfer an accurately weighed quantity of it."

Procedure for Powders, Effervescent Solids, Suspensions and Other Liquids, Non-chewable Tablets, Chewable Tablets, and Capsules—Pipet 30.0 mL of 1.0 *N* hydrochloric acid VS into the *Test Preparation* while continuing to stir with the *Magnetic Stirrer*. [NOTE—Where the acid-neutralizing capacity of the specimen under test is greater than 25 mEq, use 60.0 mL of 1.0 *N* hydrochloric acid VS.] Stir for 15 minutes, accurately timed, after the addition of the acid, begin to titrate immediately, and in a period not to exceed an additional 5 minutes, titrate the excess hydrochloric acid with 0.5 *N* sodium hydroxide VS to attain a stable (for 10 to 15 seconds) pH of 3.5. Calculate the number of mEq of acid consumed, and express the result in terms of mEq of acid consumed per g of the substance tested. Each mL of 1.0 *N* hydrochloric acid is equal to 1 mEq of acid consumed.

Procedure for Tablets That Are Required To Be Chewed—Pipet 30.0 mL of 1.0 *N* hydrochloric acid VS into the *Test Preparation* while continuing to stir with the *Magnetic Stirrer* for 10 minutes, accurately timed, after the addition of the acid. Discontinue stirring briefly, and without delay remove any gum base from the beaker using a long needle. Promptly rinse the needle with 20 mL of water, collecting the washing in the beaker, and resume stirring for 5 minutes, accurately timed, then begin to titrate immediately, and in a period not to exceed an additional 5 minutes, titrate the excess hydrochloric acid with 0.5 *N* sodium hydroxide VS to attain a stable (for 10 to 15 seconds) pH of 3.5. Calculate the number of mEq of acid consumed by the Tablet tested. Each mL of 1.0 *N* hydrochloric acid is equal to 1 mEq of acid consumed.

⟨311⟩ ALGINATES ASSAY

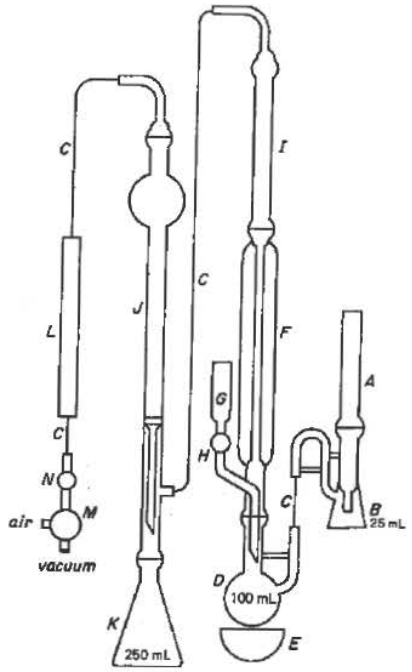
Apparatus—The apparatus required is shown in the accompanying diagram. It consists essentially of a soda lime column, *A*, a mercury valve, *B*, connected through a side arm to a reaction flask, *D*, by means of a rubber connection, *C*. Flask *D* is a 100-mL round-bottom, long-neck boiling flask, resting in a suitable heating mantle, *E*.

The reaction flask is provided with a reflux condenser, *F*, to which is fitted a delivery tube, *G*, of 40-mL capacity, having a stopcock, *H*. The reflux condenser terminates in a trap, *I*, containing 25 g of 20-mesh zinc or tin, which can be connected with an absorption tower, *J*.

The absorption tower consists of a 45-cm tube fitted with a medium-porosity, sintered-glass disk sealed to the inner part above the side arm and having a delivery tube sealed to it extending down to the end of the tube. A trap, consisting of a bulb of approximately 100-mL capacity, is blown above the sintered-glass disk, and the outer portion of a ground spherical joint is sealed on above the bulb. A 250-mL conical flask, *K*, is connected to the bottom of the absorption tower. The top of the tower is connected to a soda lime tower, *L*, which is connected to a suitable pump to provide vacuum and air supply, the selection of which is made by a three-way stopcock, *M*. The volume of air or vacuum is controlled by a capillary-tube regulator or needle valve, *N*.

All joints are size $^{35}/_{25}$, ground spherical type.

Procedure—Unless otherwise directed, transfer a specimen of about 250 mg, previously dried in vacuum for 4 hours at 60° and accurately weighed, into the reaction flask, *D*, add 25 mL of dilute hydrochloric acid (1 in 120), insert several boiling chips, and connect the flask to the reflux condenser, *F*, using phosphoric acid as a lubricant. [NOTE—Stopcock grease may be used for the other connections.] Check the system for air leaks by forcing



Apparatus for Alginates Assay

mercury up into the inner tube of the mercury valve, *B*, to a height of about 5 cm. Turn off the pressure using the stopcock, *M*. If the mercury level does not fall appreciably after 1 to 2 minutes, the apparatus may be considered to be free from leaks. Draw carbon dioxide–free air through the apparatus at a rate of 3000 to 6000 mL per hour. Raise the heating mantle, *E*, to the flask, heat the specimen to boiling, and boil gently for 2 minutes. Turn off and lower the mantle, and allow the specimen to cool for 15 minutes. Charge the delivery tube, *G*, with 23 mL of hydrochloric acid. Disconnect the absorption tower, *J*, rapidly transfer 25.0 mL of 0.25 *N* sodium hydroxide VS to the tower, add 5 drops of butyl alcohol, and again connect the absorption tower. Draw carbon dioxide–free air through the apparatus at the rate of about 2000 mL per hour, add the hydrochloric acid to the reaction flask through the delivery tube, raise the heating mantle, and heat the reaction mixture to boiling. After 2 hours, discontinue the current of air and heating. Force the sodium hydroxide solution down into the flask, *K*, using gentle air pressure, and then rinse down the absorption tower with three 15-mL portions of water, forcing each washing into the flask with air pressure. Remove the flask, and add to it 10 mL of barium chloride solution (1 in 10). Insert the stopper in the flask, shake gently for about 2 minutes, add phenolphthalein TS, and titrate with 0.1 *N* hydrochloric acid VS. Perform a blank determination (see *Residual Titrations* under *Titrimetry* ⟨541⟩). Each mL of 0.25 *N* sodium hydroxide consumed is equivalent to 5.5 mg of carbon dioxide (CO_2).

⟨321⟩ ALKALOIDAL DRUG ASSAYS; PROXIMATE ASSAYS

Most alkaloids are slightly or very slightly soluble in water, but soluble in certain organic solvents immiscible with water, such as chloroform, ether, amyl alcohol, and benzene, or mixtures of these. Salts of alkaloids, however, are usually soluble in water, but in most cases very slightly soluble or practically insoluble in nearly all of the organic solvents. The process of assay by immiscible solvents, generally known as the "shaking out" process, is based on these partitioning properties of alkaloids. It is carried out by treating the drug, or a concentrated liquid extract of it, with a solvent immiscible with water, in the presence of an excess of alkali which liberates the alkaloid. The free alkaloid is dissolved by the immiscible solvent from which it is subsequently removed by means of an excess of dilute aqueous acid. The acid solutions are then extracted with an immiscible solvent in the presence of an excess of alkali, and the immiscible solvent is evaporated to obtain the alkaloid which is either weighed or determined volumetrically.

Preparation of Drug for Assay—Grind the drug to be extracted to a powder of the fineness designated (see *Powder Fineness* ⟨811⟩). Care should be taken to avoid the loss of water during the powdering of the drug. If it is impossible to avoid this loss, dry the drug at a low temperature before powdering, note the loss of water, and make a correction in the final calculations.

Weighing for Assay—In weighing bulky, crude drugs for the assay, an accuracy to within 10 mg for quantities of 5 g and over is sufficient. Portions of pilular extracts or ointments may be weighed on a tared piece of waxed or parchmentized paper, the surplus paper cut away, and the paper with the specimen dropped into the vessel containing the solvent. In transferring weighed portions to a separator, thoroughly rinse the vessel in which the material to be assayed was weighed, and add the rinsings to the separator.

Extraction of Drugs—The alkaloidal content of alkaloid-bearing drugs is usually extracted by one of the following methods:

A. Maceration—Treat an accurately weighed portion of the ground drug with the specified solvent or mixture of solvents, made alkaline with ammonia TS, and thoroughly mixed. Allow to macerate for 12 to 24 hours with occasional agitation or for a shorter period with continuous agitation. At the end of this period, allow the drug to settle, decant an aliquot of the solvent, and treat as directed for *Purification of Alkaloids*.

B. Percolation—Place an accurately weighed quantity of the ground drug in a suitable container, saturate it with the specified solvent or mixture of solvents, and allow to stand for 5 minutes. Add a quantity of ammonia TS sufficient to make the mixture distinctly alkaline, and mix thoroughly with the drug. Transfer the mixture to a cylindrical percolator, previously prepared by packing the outlet with purified cotton. Use a small amount of the solvent to rinse the container, and add the rinsing to the percolator. Allow the drug to macerate for a suitable period of time (from 1 to 12 hours or overnight, depending upon the drug to be assayed). Then pack the drug firmly, place a pledget of purified cotton above it, and percolate slowly with the solvent until the drug is completely exhausted of its alkaloid content. Determine the completeness of extraction of the alkaloid by evaporating about 4 mL of the last percolate to dryness, dissolving the residue in 500 µL of approximately 0.5 *N* acid, and adding a drop of mercuric iodide TS (Valser's Reagent): not more than a slight turbidity is produced. Treat the percolate as directed for *Purification of Alkaloids*.

C. Continuous Extraction—Place an accurately weighed portion of the ground drug in an extraction thimble, and insert the thimble into a suitable extractor (a Soxhlet extractor of appropriate size is satisfactory). Moisten the drug with the specified solvent, mix by means of a stirring rod, and allow to stand for about 5 minutes. Render the mixture alkaline with the specified quantity of ammonia TS, and mix. Rinse the stirring rod with a small portion of the solvent, and allow the drug to macerate for 6 to 12 hours or overnight. Then pack the drug in the thimble, cover it with a pledget of purified cotton, add a sufficient quantity of solvent, and extract the drug for a specified period of time or until extraction is complete.

Purification of Alkaloids—The alkaloidal solution obtained by any of the extraction methods is usually contaminated with other extractives that interfere with the quantitative determinations of the alkaloids. To effect their purification remove the alkaloids from the immiscible solvent by shaking out with an acid, then render the acid solution alkaline, usually with an alkali hydroxide, and extract with an immiscible solvent.

The volume and strength of the acid to be used are usually left to the discretion of the operator. It is best, however, to keep the total volume as small as possible. For the first extraction, use not less than 10 mL of approximately 1 *N* acid or sufficient to render the mixture distinctly acid. When the drug contains a large amount of fat, use a smaller volume of more concentrated acid to prevent the formation of emulsions in the first extraction.

For succeeding extractions, use a dilution of 5 mL of the acid with 5 mL of water. In all assays, continue the extraction until 500 µL of the last acid washing shows not more than a slight turbidity on the addition of a drop of mercuric iodide TS. The acid extracts, before proceeding with the next step, should be clear or practically so. If not clear, filter or treat as follows: Shake the combined acid extracts with one or more 10-mL portions of the appropriate immiscible solvent until the acid solution is clear or practically so. Then wash the immiscible solvent extracts with one or more 5-mL portions of water acidified with hydrochloric or sulfuric acid, and add these washings to the acid solution.

Render the acid solution alkaline, in most cases with ammonia TS, and extract it with several successive portions of the appropriate immiscible solvent. Use a volume of the latter in each operation not less than half that of the water solution, and repeat the operation as long as any alkaloid is extracted by the immiscible solvent. To determine the completeness of extraction, evaporate 1 mL of the last extraction, and dissolve the residue in 0.5 mL of approximately 0.5 *N* hydrochloric acid: the resulting solution shows not more than a slight turbidity on the addition of a drop of mercuric iodide TS. The number of extractions required depends largely on the partitioning character of the alkaloid. With most alkaloids, extract several times before testing.

Washing—Carefully wash the stems of separators and funnels and the lips of flasks, separators, and graduates from which solvents containing alkaloids have been drawn or poured with some of the solvent to prevent loss and to remove any of the alkaloids left by evaporation. Add these washings to the other extractions containing the alkaloids.

Determination of Alkaloids—Evaporate the solution of the purified alkaloids in the immiscible solvent on a steam bath or with a current of air to dryness. When the alkaloidal residue is to be determined volumetrically, soften it by the addition of about 1 mL of neutralized alcohol or ether, add an accurately measured volume of standard acid, equivalent to about one and one-half to two times the volume estimated for the quantity of alkaloid present, and warm the mixture gently to ensure the complete solution of the alkaloid. If preferred, dissolve the alkaloidal residue in chloroform, add the standard acid, and remove the chloroform completely by evaporation. Then add water to make the volume of the mixture measure not less than 25 mL, and titrate the excess of acid with standard alkali, using the appropriate indicator.

If the alkaloidal residue is to be weighed, dry it at 105° to constant weight. If the final solvent has been chloroform, remove the last traces of that solvent by the addition of a few mL of neutralized ether or alcohol, followed by evaporation. Avoid loss by decrepitation, especially when evaporating chloroform solutions of alkaloids, by the addition of a little alcohol after the solution has been reduced to a volume of 1 or 2 mL, and evaporate at a low temperature, rotating the container during the evaporation.

Indicators—Unless otherwise directed in the individual monograph, use methyl red TS as the indicator in volumetric determinations and for standardizing volumetric solutions.

Aliquots—When using aliquots, measure the solvent and the aliquot at the same temperature. When handling volatile liquids, a lower temperature and a more quickly conducted operation reduce the loss by evaporation.

Adsorbants—In assaying fluidextracts, tinctures, and other preparations of alkaloid-bearing drugs, it is often necessary to evaporate these to dryness and, to avoid loss and to aid in the evaporation, they are usually added to some adsorbent material. For this purpose use paper pulp, previously acid- and alkali-washed, then made neutral by washing with water, and dried before use.

Emulsions—Shake or rotate a water solution with an immiscible solvent in a separator for about 1 minute. Avoid long or violent agitation as emulsions are likely to form, especially in alkaline solutions. Belladonna leaves sometimes contain saponins that cause troublesome emulsions. If emulsions prove persistent, draw off the emulsified portion, and add an excess of either solvent. This usually breaks the emulsion and permits a complete separation. A separated emulsion may sometimes be broken by the addition of a small amount of anhydrous sodium sulfate. If this is done, wash the residue with additional solvent to remove the alkaloid completely.

Emulsification may sometimes be prevented by increasing the volume of the water or of the immiscible solvent. Chloroform and ether solutions of drugs that contain large proportions of fat may form troublesome emulsions. In such cases, add sufficient sulfuric acid to acidify, and evaporate the volatile solvent, while stirring with a glass rod. When the resinous and fatty matter has been agglutinated, cool the acid solution, and filter through a small, wetted filter into a separator. Redissolve the residue in 15 mL of ether, add 5 to 10 mL of 0.1 *N* acid, evaporate the ether as before, with continued stirring, and pour the acid solution through the filter into the separator. Repeat the extraction of the fatty residue with dilute acid two or three times, and finally wash the filter free from alkaloids.

⟨331⟩ AMPHETAMINE ASSAY

Reference Standard—*USP Dextroamphetamine Sulfate Reference Standard*—Keep container tightly closed and protected from light. Dry at 105° for 2 hours before using.

Standard Preparation—Dissolve a suitable quantity of USP Dextroamphetamine Sulfate RS, accurately weighed, in 2 *N* sulfuric acid (saturated with chloroform), and dilute quantitatively with the same solvent to obtain a solution having a known concentration of about 0.5 mg of dextroamphetamine sulfate per mL.

Assay Preparation—Prepare as directed in the individual monograph.

Preparation of Chromatographic Column (see *Chromatography* ⟨621⟩)—Pack a pledget of fine glass wool in the base of a 25- × 300-mm chromatographic tube. Place 2 g of purified siliceous earth in a 100-mL beaker, add 1 mL of 0.1 *N* hydrochloric acid, and mix until a fluffy mixture is obtained. Transfer the mixture to the column, and tamp moderately to compress the material into a uniform mass. Transfer the *Assay Preparation* to the column, dry-rinse the beaker with 1 g of purified siliceous earth, and transfer to the column. Tamp a pledget of fine glass wool into place at the top of the column.

Procedure—Wash the column with 100 mL of chloroform previously saturated with water, and discard the washings. Place under the column, as a receiver, a 125-mL separator containing 10.0 mL of 2 *N* sulfuric acid previously saturated with chloroform. Pass through the column 35 mL of ammoniacal chloroform, prepared by equilibrating 2 mL of ammonium hydroxide and 100 mL of chloroform, and complete the elution with 70 mL of chloroform previously saturated with water. Remove the separator, shake vigorously for 1 minute, allow the layers to separate, discard the chloroform layer, and use the 10.0-mL acid solution of the sulfate salt of the amphetamine as the *Assay Solution*. Concomitantly determine the absorbance of the solution from the *Standard Preparation* and that of the *Assay Solution* in 1-cm cells at 280 nm and at the wavelength of maximum absorbance at about 257 nm, with a suitable spectrophotometer, using 2 *N* sulfuric acid previously saturated with chloroform as the blank. Record the absorbance of the solution from the *Standard Preparation* as A_S and that of the *Assay Solution* as A_U, and calculate as directed in the individual monograph.

⟨341⟩ ANTIMICROBIAL AGENTS—CONTENT

An essential component of Injections preserved in multiple-dose containers is the agent or agents present to reduce the hazard of having introduced, in the course of removing some of the contents, accidental microbial contamination of the contents remaining. It is a Pharmacopeial requirement that the presence and amount added of such agent(s) be declared on the label of the container. The methods provided herein are to be used to demonstrate that the declared agent is present but does not exceed the labeled amount.

The concentration of an antimicrobial preservative added to a multiple-dose and single-dose parenteral, otic, nasal, and ophthalmic preparation may diminish during the shelf-life of the product. The quantitative label statement of the preservative content is not intended to mean that the labeled quantity is retained during the shelf-life of the product; rather, it is a statement