centration at the interface, producing a greater voltage drop at the interface, which in turn causes the glycinate to catch up to the chloride. Under these conditions, a very sharp interface is maintained, and as it moves through the sample and spacer layers, the proteins in the sample tend to stack themselves at the interface in very thin layers in order of mobility. The process is called stacking and is the source of the disks which are separated.

When the stacked proteins reach the high-density separating gel, they are slowed down by a molecular sieving process. The higher pH encountered in the running gel also causes the glycinate to migrate faster, so that the discontinuous buffer interface overtakes the proteins and eventually reaches the bottom of the separating gel. During this period, the disks of protein continue to separate by electrophoresis and molecular sieving in the separating gel. At the end of the run, the pH of the separating gel will have risen above its original value of 8.9 to a value of about pH 9.5.

Relative Mobility—Bromophenol blue is often used as a standard for calculating the relative mobility of separated zones and to judge visually the progress of a run. It may be added to one of the sample wells, or mixed with the sample itself, or simply added to the buffer in the upper sample reservoir.

Relative mobility, M_B, is calculated as

$$M_B = \frac{\text{distance from origin to sample zone}}{\text{distance from origin to bromophenol blue zone}}.$$

Visualization of Zones—Since polyacrylamide is transparent, protein bands may be located by scanning in a densitometer with ultraviolet light. The zones may be fixed by immersing in protein precipitants such as phosphotungstic acid or 10% trichloroacetic acid. A variety of staining reagents including naphthalene black (amido black) and Coomassie brilliant blue R250 may be used. The fixed or stained zones may be conveniently viewed and photographed with transmitted light from an X-ray film illuminator.

SAFETY PRECAUTIONS

Voltages used in electrophoresis can readily deliver a lethal shock. The hazard is increased by the use of aqueous buffer solutions and the possibility of working in damp environments.

The equipment, with the possible exception of the power supply, should be enclosed in either a grounded metal case or a case made of insulating material. The case should have an interlock that deenergizes the power supply when the case is opened, after which reactivation should be prevented until activation of a reset switch is carried out.

High-voltage cables from the power supply to the apparatus should preferably be a type in which a braided metal shield completely encloses the insulated central conductor, and the shield should be grounded. The base of the apparatus should be grounded metal or contain a grounded metal rim which is constructed in such a way that any leakage of electrolyte will produce a short which will deenergize the power supply before the electrolyte can flow beyond the protective enclosure.

If the power supply contains capacitors as part of a filter circuit, it should also contain a bleeder resistor to ensure discharge of the capacitors before the protective case is opened. A shorting bar that is activated by opening the case may be considered as an added precaution.

Because of the potential hazard associated with electrophoresis, laboratory personnel should be completely familiar with electrophoresis equipment before using it.

⟨731⟩ LOSS ON DRYING

The procedure set forth in this chapter determines the amount of volatile matter of any kind that is driven off under the conditions specified. For substances appearing to contain water as the only volatile constituent, the procedure given in the chapter, *Water Determination* ⟨921⟩, is appropriate, and is specified in the individual monograph.

Mix and accurately weigh the substance to be tested, and, unless otherwise directed in the individual monograph, conduct the determination on 1 to 2 g. If the test specimen is in the form of large crystals, reduce the particle size to about 2 mm by quickly crushing. Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 minutes under the same conditions to be employed in the determination. Put the test specimen in the bottle, replace the cover, and accurately weigh the bottle and the contents. By gentle, sidewise shaking, distribute the test specimen as evenly as practicable to a depth of about 5 mm generally, and not more than 10 mm in the case of bulky materials. Place the loaded bottle in the drying chamber, removing the stopper and leaving it also in the chamber. Dry the test specimen at the temperature and for the time specified in the monograph. [NOTE—The temperature specified in the monograph is to be regarded as being within the range of ±2° of the stated figure.] Upon opening the chamber, close the bottle promptly, and allow it to come to room temperature in a desiccator before weighing.

If the substance melts at a lower temperature than that specified for the determination of *Loss on drying*, maintain the bottle with its contents for 1 to 2 hours at a temperature 5° to 10° below the melting temperature, then dry at the specified temperature.

Where the specimen under test is Capsules, use a portion of the mixed contents of not less than 4 capsules.

Where the specimen under test is Tablets, use powder from not less than 4 tablets ground to a fine powder.

Where the individual monograph directs that loss on drying be determined by thermogravimetric analysis, a sensitive electrobalance is to be used.

Where drying in vacuum over a desiccant is directed in the individual monograph, a vacuum desiccator or a vacuum drying pistol, or other suitable vacuum drying apparatus, is to be used.

Where drying in a desiccator is specified, exercise particular care to ensure that the desiccant is kept fully effective by frequent replacement.

Where drying in a capillary-stoppered bottle in vacuum is directed in the individual monograph, use a bottle or tube fitted with a stopper having a 225 ± 25 μm diameter capillary, and maintain the heating chamber at a pressure of 5 mm or less of mercury. At the end of the heating period, admit dry air to the heating chamber, remove the bottle, and with the capillary stopper still in place allow it to cool in a desiccator before weighing.

⟨733⟩ LOSS ON IGNITION

This procedure is provided for the purpose of determining the percentage of test material that is volatilized and driven off under the conditions specified. The procedure, as generally applied, is nondestructive to the substance under test; however, the substance may be converted to another form such as an anhydride.

Perform the test on finely powdered material, and break up lumps, if necessary, with the aid of a mortar and pestle before weighing the specimen. Weigh the specimen to be tested without further treatment, unless a preliminary drying at a lower temperature, or other special pretreatment, is specified in the individual monograph. Unless other equipment is designated in the individual monograph, conduct the ignition in a suitable muffle furnace or oven that is capable of maintaining a temperature within 25° of that required for the test, and use a suitable crucible, complete with cover, previously ignited for 1 hour at the temperature specified for the test, cooled in a desiccator, and accurately weighed.

Unless otherwise directed in the individual monograph, transfer to the tared crucible an accurately weighed quantity, in g, of the substance to be tested, about equal to that calculated by the formula:

$$10/L,$$

in which L is the limit (or the mean value of the limits) for *Loss on ignition*, in percentage. Ignite the loaded uncovered crucible, and cover at the temperature (±25°) and for the period of time designated in the individual monograph. Ignite for successive 1-hour periods where ignition to constant weight is indicated. Upon completion of each ignition, cover the crucible, and allow it to cool in a desiccator to room temperature before weighing.

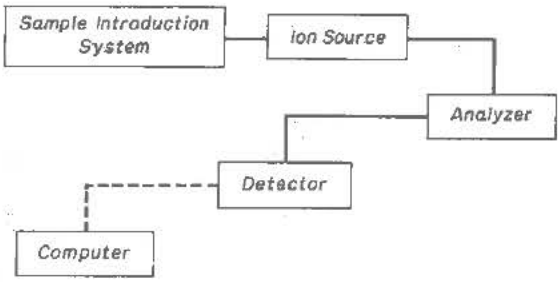
⟨736⟩ MASS SPECTROMETRY

Mass spectrometers can be used for the measurement of ionic mass-to-charge ratio, for the determination of ionic abundance, and for the study of the ionization process. In addition, studies of ionic reactions in the gas phase such as unimolecular decom-

position processes, and ion molecule reactions, are also possible.

A mass spectrometer is an instrument that produces a beam of ions from a substance under investigation, sorts these ions into a spectrum according to their mass-to-charge ratio (m/z), and records the relative abundance of each ionic species present. Traditionally only the positive ions have been studied, principally because the negative ion yield from electron impact (EI) sources is normally low. With the introduction of the chemical ionization (CI) and fast atom bombardment (FAB) techniques, both of which can produce a high negative ion yield, interest in the analysis of negative ions has increased.

In general, a mass spectrometer consists of three major components, as shown in the accompanying figure: an ion source for producing gaseous ions from the substance(s) being studied; an analyzer for resolving the ions into their characteristic mass components according to the mass-to-charge ratios of the ions present; and a detector system for recording the relative abundance or intensity of each of the resolved ionic species present. In addition, a sample introduction system is necessary in order to admit the samples to be studied to the ion source while still maintaining the high vacuum requirements ($\sim 10^{-6}$ to 10^{-8} torr) of the technique. As the accompanying figure indicates, most commercial instruments include a computer for conveniently handling the large amounts of data produced by these instruments.



Analyzers—The mass analyzer sorts the different masses present in the ionized sample, and this allows one to determine the mass and ultimately the abundance or relative intensity of each ionic species present. Four of the several methods that are commonly used for analysis are (1) the quadrupole, (2) the magnetic analyzer, (3) the time-of-flight analyzer, and (4) the fourier transform analyzer. Electrostatic analyzers are often used in conjunction with other mass analyzers.

In the quadrupole, mass separation may be achieved in an instrument composed of four coaxial rods; ideally each rod possesses a hyperbolic cross section, but in practice circular rods are commonly used. Two opposite rods have a fixed electric potential (U), the other two have a radio-frequency alternating potential (V,Ω). Under the action of these electric fields, all of the ions (except one selected mass) in the ion beam are deflected to the sides and lost; the selected mass (determined by the settings of U,V,Ω) is allowed through the rods. Thus, because all other m/z values but the selected one are rejected, the analyzer is sometimes called a *mass filter*. The theory is that as high mass ions take longer to traverse the analyzer and consequently have more opportunity both to fragment and to collide with residual gas molecules, sensitivity decreases with increasing mass: this phenomenon is called mass discrimination, and it is a fundamental characteristic of quadrupole analyzers.

In the presence of a magnetic field perpendicular to the motion of the positive ion beam, each ion experiences a force at right angles to both its direction of motion and the direction of the magnetic field, thereby deflecting the beam of ions. The following equation of motion applies:

$$m/z = H^2r^2/2V,$$

in which m is the mass in atomic mass units; z is the number of electronic charges; H is the magnetic field strength in gauss; r is the ion trajectory radius in centimeters; and V is the accelerating voltage. The mass spectrum is scanned by varying the strength of the magnetic field and detecting those ions passing through the exit slit as they come into "focus."

In the time-of-flight analyzer, separation of ions of different masses is based on all ions being given equal energy; therefore, ions of different masses have different velocities. If there is a fixed distance for the ions to travel, the time of their travel will vary with their mass, the lighter masses traveling more rapidly and thereby reaching the detector in a shorter period of time. The time of flight is given by:

$$t(f) = k\sqrt{m/z},$$

in which $t(f)$ is the time of flight in seconds, and m and z are the same as defined previously. Thus, the time-of-flight of the various ions is simply proportional to the square root of the mass-to-charge ratio of the ions.

Fourier transform mass spectrometry (FT–MS) is a technique based on the cyclotron motion of ions in a uniform magnetic field. In such a field of flux density B, ions are constrained to move in circular (cyclotron) orbits. The angular frequency, ω, of the cyclotron motion is given by the equation:

$$\omega = \frac{z \times B}{m}$$

In the cyclotron resonance mass spectrometer, the cyclotron orbits can be expanded by subjecting the ions to an alternating electric field. When the frequency of the signal generator matches the cyclotron frequency, the ions are steadily accelerated to larger and larger radii leading to a coherent motion (of an ensemble of ions) corresponding to a significant amount of kinetic energy. After excitation is turned off, the cyclotronic ions give rise to an alternating image current on the electrodes, which is amplified. A frequency analysis of the corresponding receiver signal yields the mass of the ions involved with high precision. Thus, the Fourier transform of the time domain transient signal yields the corresponding frequency spectrum from which the mass spectrum is computed.

Ionization Techniques—Positive ions may be produced by passing a beam of electrons through a gas at pressures of about 10^{-4} to 10^{-6} mm (Hg). Pressures other than these may be employed, but this range is the most common. The energy of the electron beam is usually controlled. If the energy is greater than the ionization potential of the gas, the electrons may cause ionization and/or fragmentation of the gas molecules, represented as follows:

$$e^- + M \rightarrow M^{+\bullet} + 2e^-.$$

Sources of this type are called electron bombardment (or electron impact, EI) sources. The electrons are usually emitted from a heated tungsten or rhenium filament.

Ions formed in the ionization chamber are accelerated through the source exit slit toward the analyzer region by a repeller/draw out field determined partly by field penetration through the source exit slit, and partly by a small potential applied to an ion repeller plate in the ionization chamber. The ions are further accelerated by the much larger field existing between the ionization chamber and the source exit slits, the final slits being at ground potential.

Conversion of a mass spectrometer to the negative ion mode is straightforward, and modern equipment is designed to execute the procedure automatically on selection of a single parameter. All that is required, in theory, is to reverse all operating voltages and fields. While negative ions are also formed in the various ionization processes discussed, the introduction of a sample with a high electron capture cross section leads to the formation of abundant negative ions. For this reason, multi-halide derivatives of compounds to be studied are often prepared.

Negative ion MS studies have been successfully applied to pesticide analyses, since their structures are favorable for the technique.

In the field ionization (FI) source ions are formed in the strong electrostatic field set up at the tip of a wire electrode to which a high voltage is applied. Ions formed from molecules present on the tip of the wire are almost all parent ions. The source is not widely used but is of considerable value in studying very unstable molecules or very complex mixtures and in surface reaction studies.

Field desorption (FD) may be considered as an extension of field ionization; the main difference is that the sample is coated on the field ion emitter tip and ionization occurs from the solid phase. The technique requires experience to obtain reliable results. Mass spectra consisting mainly of molecular ions may be recorded from highly nonvolatile and thermally labile compounds.

Chemical ionization (CI) is a popular secondary ionization technique, and most new instruments are purchased with this capability.

In chemical ionization, a reagent gas at a pressure of about 0.1 to 10 torr is admitted to the source and ionized. At this pressure, ion-molecule reactions occur and the primary reagent gas ions react further. The most commonly used reagent gases are methane, isobutane, and ammonia. Typical reactions for methane are shown in the following equations:

$$CH_4 + e^- \rightarrow CH_4^{+\bullet} + CH_3^{+\bullet} + CH_2^{+\bullet}$$

$$CH_4^{+\bullet} + CH_4 \rightarrow CH_5^+ + CH_3^\bullet$$

$$CH_3^+ + CH_4 \rightarrow C_2H_5^+ + H_2$$

The species CH_5^+ is a strong Bronsted acid and can transfer a proton to most organic compounds, as follows:

$$CH_5^+ + M \rightarrow MH^+ + CH_4$$

With methane, the protonated molecule ion $(MH)^+$ formed initially may be sufficiently energetic to dissociate further.

The fast atom bombardment (FAB) method uses a beam of fast (neutral) atoms to bombard the sample. Thus, the first requirement of this technique is a beam of fast-moving atoms, properly aimed at the target sample, which is dissolved in a nonvolatile liquid matrix. This is relatively easy to achieve, and methods for producing such beams are well developed. Essentially a fast atom gun consists of an ion gun with a collision cell in front of it. The ion gun is used to produce a xenon ion beam, which is then charge exchanged in the collision cell with xenon gas to produce the required beam of fast xenon atoms. The process is summarized in the following equations:

$$Xe \rightarrow Xe^+ + e^-$$

$$\underrightarrow{Xe^+} + Xe \rightarrow \underrightarrow{Xe} + Xe^+$$

in which the subscript arrow indicates the fast-moving particle.

Since FAB is a surface analysis technique, the preparation of the sample, in order to optimize the surface conditions, is of paramount importance. When the sample is coated on the probe by evaporation of a solution, the resultant sample ion beam is often transitory. Adduct ions are frequently produced. The preferential formation of $(M + Na)$ and $(M + K)$ adduct ions has some parallels in FD, especially in the ionization of sugars. This phenomenon can be used to good effect to assist in the ionization of these classes of compounds. Frequently, the sample surface is treated with sodium chloride solution to enhance the yield of the adduct ions. Heating the sample during analysis can sometimes increase the ion yield.

The suppression of sample ion is probably due to destruction of the sample surface, and a means of continuously replenishing the sample surface during the analysis is required. Dissolving the sample in a suitable, nonvolatile liquid and coating this mixture onto the probe tip achieves this. Using this approach, sample lifetimes of greater than 1 hour have been realized in the ion source, and the range of compounds amenable to FAB has expanded dramatically. These long sample lifetimes and higher sensitivity make FAB an important mass spectral technique for producing mass spectra from novel, difficult-to-handle, biochemicals, and also allow unequivocal identification of the elemental formula of the material through accurate mass determination. A further advantage of FAB, useful in structural determination, is the presence of fragment ions within the spectra.

Sample Introduction—The sample is to be admitted to the ionization chamber in the gaseous or vapor state. Since many samples are gases or liquids at room temperature and atmospheric pressure, a sample handling system and a leak arrangement to the ion source are all that are required.

To produce from solids a molecular beam directly within the vacuum system can be a simple matter of heating a solid sample in a crucible to a sufficiently high temperature. Several commercially available probes, or cartridges, are used, depending upon the particular instrument and applications involved.

Other analytical instruments are used as inlets into the mass spectrometer. The most popular and most successful early development in mass spectrometry was the combination of a gas chromatograph and mass spectrometer (GC/MS). This combined instrument was a ready success, since the effluent of the gas chromatograph was in the vapor state and the primary problem of the combined instrument was the task of selectively removing the unwanted carrier gas.

Combining the liquid chromatograph with the mass spectrometer (LC/MS) was a far more challenging problem. While the liquid chromatograph is a powerful separative instrument, the widely used eluting solvents are often quite polar, complex, and relatively nonvolatile. Nevertheless, the coupling of the two instruments has been achieved and LC/MS instruments are commercially available.

Finally, nearly all of the various combinations of one mass spectrometer being an inlet system with another mass spectrometer (MS/MS) have been developed and studied (e.g., TOF with Magnetic sector, two magnetic sectors, quadrupole with a magnetic sector, etc.). Application of this technique has been most successful for mixture analysis. It has been applicable also to structure analysis where it was necessary to ionize the molecule of interest by a technique yielding mostly parent ions, then introducing these parent ions into a second mass spectrometer in order to study fragmentation patterns.

Data Analysis and Interpretation—Although molecules are normally electrically neutral, if one electron is taken away or added, a molecular ion results. The mass of this ion is the molecular weight of the molecule under study. Furthermore, it is often possible to determine the accurate mass of this ion with sufficient precision to enable the calculation of the empirical formula of the compound. Accurate masses may be determined at high resolution by either scanning or by peak-matching measurements.

Fragment ions are those produced from the molecular ion by various bond cleavage processes. Numerous papers in the literature relate the bond cleavage patterns (fragmentation patterns) to molecular structure. Correlations of mass spectra and molecular structure are discussed for steroids, aromatics, aliphatics, and, recently, complex compounds arising from biotechnology.

The mass spectrum is often very complex and not all of the ions may be separated by the mass spectrometer. The limit of the ability of the instrument to separate two ions very close in mass is called the resolving power of the instrument. The most common definition of the resolving power of a mass spectrometer is the "10% valley" definition. This states that the resolving power of a mass spectrometer is the highest mass number at which peaks of adjacent molecular weight and equal heights have a valley between them of 10% of the peak height. In mass spectrometry, low resolution covers the range of about 100 to 2000, medium resolution 2000 to 10,000, and high resolution greater than 10,000.

Quantitative analysis in mass spectrometry is usually performed in one of two ways. The first is selective ion monitoring. In this technique the ions, or group of ions of interest, are individually focused on the detector and measured. Both sensitivity and selectivity are enhanced by this technique.

The second most popular quantitative technique is isotope dilution. This method may be applied through the use of either radioactive or stable isotopes, the latter is most popular for mass spectrometry. The isotope dilution technique has the unique advantage that it is not necessary to recover all of the original material being analyzed to obtain the quantitative information desired. The technique has been successfully applied in biological studies, often in combination with GC/MS or LC/MS.

⟨741⟩ MELTING RANGE OR TEMPERATURE

For Pharmacopeial purposes, the melting range or temperature of a solid is defined as those points of temperature within which, or the point at which, the solid coalesces and is completely melted, except as defined otherwise for *Classes II* and *III* below. Any apparatus or method capable of equal accuracy may be used. The accuracy should be checked frequently by the use of one or more of the six USP Melting Point Reference Standards, preferably the one that melts nearest the melting temperature of the compound to be tested (see *USP Reference Standards* ⟨11⟩).

Five procedures for the determination of melting range or temperature are given herein, varying in accordance with the nature of the substance. When no class is designated in the monograph, use the procedure for *Class Ia*.

The procedure known as the mixed-melting point determination, whereby the melting range of a solid under test is compared with that of an intimate mixture of equal parts of the solid and an authentic specimen of it, e.g., the corresponding USP Reference Standard, if available, may be used as a confirmatory identification test. Agreement of the observations on the original and the mixture constitutes reliable evidence of chemical identity.

Apparatus—An example of a suitable melting range apparatus consists of a glass container for a bath of transparent fluid, a suitable stirring device, an accurate thermometer (see *Thermometers* ⟨21⟩),* and a controlled source of heat. The bath fluid is selected with a view to the temperature required, but light paraffin is used generally and certain liquid silicones are well adapted to the higher temperature ranges. The fluid is deep enough to permit immersion of the thermometer to its specified immersion depth so that the bulb is still about 2 cm above the bottom of the bath. The heat may be supplied by an open flame or electrically. The capillary tube is about 10 cm long and 0.8 to 1.2 mm in internal diameter with walls 0.2 to 0.3 mm in thickness.

Procedure for Class I—Reduce the substance under test to a very fine powder, and, unless otherwise directed, render it anhydrous when it contains water of hydration by drying it at the temperature specified in the monograph, or, when the substance contains no water of hydration, dry it over a suitable desiccant for not less than 16 hours.

Charge a capillary glass tube, one end of which is sealed, with sufficient of the dry powder to form a column in the bottom of the tube 2.5 to 3.5 mm high when packed down as closely as possible by moderate tapping on a solid surface.

Heat the bath until the temperature is about 30° below the expected melting point. Remove the thermometer, and quickly attach the capillary tube to the thermometer by wetting both with a drop of the liquid of the bath or otherwise, and adjust its height so that the material in the capillary is level with the thermometer bulb. Replace the thermometer, and continue the heating, with constant stirring, sufficiently to cause the temperature to rise at a rate of about 3° per minute. When the temperature is about 3° below the lower limit of the expected melting range, reduce the heating so that the temperature rises at a rate of about 1° to 2° per minute. Continue heating until melting is complete.

The temperature at which the column of the substance under test is observed to collapse definitely against the side of the tube at any point is defined as the beginning of melting, and the temperature at which the test substance becomes liquid throughout is defined as the end of melting or the "melting point." The two temperatures fall within the limits of the melting range.

Procedure for Class Ia—Prepare the test substance and charge the capillary as directed for *Class I*. Heat the bath until the temperature is about 10° below the expected melting point and is rising at a rate of 1 ± 0.5° per minute. Insert the capillary as directed under *Class I* when the temperature is about 5° below the lower limit of the expected melting range, and continue heating until melting is complete. Record the melting range as directed for *Class I*.

Procedure for Class Ib—Place the test substance in a closed container and cool to 10°, or lower, for at least 2 hours. Without previous powdering, charge the cooled material into the capillary tube as directed for *Class I*, then immediately place the charged tube in a vacuum desiccator and dry at a pressure not exceeding 20 mm of mercury for 3 hours. Immediately upon removal from the desiccator, fire-seal the open end of the tube, and as soon as practicable proceed with the determination of the melting range as follows: Heat the bath until a temperature 10 ± 1° below the expected melting range is reached, then introduce the charged tube, and heat at a rate of rise of 3 ± 0.5° per minute until melting is complete. Record the melting range as directed for *Class I*.

If the particle size of the material is too large for the capillary, pre-cool the test substance as above directed, then with as little pressure as possible gently crush the particles to fit the capillary, and immediately charge the tube.

Procedure for Class II—Carefully melt the material to be tested at as low a temperature as possible, and draw it into a capillary tube, which is left open at both ends, to a depth of about 10 mm. Cool the charged tube at 10°, or lower, for 24 hours, or in contact with ice for at least 2 hours. Then attach the tube to the thermometer by suitable means, adjust it in a water bath so that the upper edge of the material is 10 mm below the water level, and heat as directed for *Class I* except, within 5° of the expected melting temperature, to regulate the rate of rise of temperature to 0.5° to 1.0° per minute. The temperature at which the material is observed to rise in the capillary tube is the melting temperature.

Procedure for Class III—Melt a quantity of the test substance slowly, while stirring, until it reaches a temperature of 90° to 92°. Remove the source of the heat and allow the molten substance to cool to a temperature of 8° to 10° above the expected melting point. Chill the bulb of a suitable thermometer (see *Thermometers* ⟨21⟩) to 5°, wipe it dry, and while it is still cold dip it into the molten substance so that approximately the lower half of the bulb is submerged. Withdraw it immediately, and hold it vertically away from the heat until the wax surface dulls, then dip it for 5 minutes into a water bath having a temperature not higher than 16°.

Fix the thermometer securely in a test tube so that the lower point is 15 mm above the bottom of the test tube. Suspend the test tube in a water bath adjusted to about 16°, and raise the temperature of the bath at the rate of 2° per minute to 30°, then change to a rate of 1° per minute, and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the test substance. If the variation of three determinations is less than 1°, take the average of the three as the melting point. If the variation of three determinations is greater than 1°, make two additional determinations and take the average of the five.

* ASTM Method E77 deal with "Verification and Calibration of Liquid-in-glass Thermometers."

⟨751⟩ METAL PARTICLES IN OPHTHALMIC OINTMENTS

The following test is designed to limit to a level considered to be unobjectionable the number and size of discrete metal particles that may occur in ophthalmic ointments.

Procedure—Extrude, as completely as practicable, the contents of 10 tubes individually into separate, clear, flat-bottom, 60-mm petri dishes that are free from scratches. Cover the dishes, and heat at 85° for 2 hours, increasing the temperature slightly if necessary to ensure that a fully fluid state is obtained. Taking precautions against disturbing the melted sample, allow each to cool to room temperature and to solidify.

Remove the covers, and invert each petri dish on the stage of a suitable microscope adjusted to furnish 30 times magnification and equipped with an eye-piece micrometer disk that has been calibrated at the magnification being used. In addition to the usual source of light, direct an illuminator from above the ointment at a 45° angle. Examine the entire bottom of the petri dish for metal particles. Varying the intensity of the illuminator from above allows such metal particles to be recognized by their characteristic reflection of light.

Count the number of metal particles that are 50 μm or larger in any dimension: the requirements are met if the total number of such particles in all 10 tubes does not exceed 50, and if not more than 1 tube is found to contain more than 8 such particles. If these results are not obtained, repeat the test on 20 additional tubes: the requirements are met if the total number of metal particles that are 50 μm or larger in any dimension does not exceed 150 in all 30 tubes tested, and if not more than 3 of the tubes are found to contain more than 8 such particles each.

⟨755⟩ MINIMUM FILL

The following tests and specifications apply to articles such as creams, gels, jellies, ointments, pastes, and powders that are packaged in containers in which the labeled net weight is not more than 150 g.

Select a sample of 10 filled containers, and remove any labeling that might be altered in weight during the removal of the container contents. Thoroughly cleanse and dry the outside of the containers by a suitable means, and weigh individually. Quantitatively remove the contents from each container, cutting the latter open and washing with a suitable solvent, if necessary, taking care to retain the closure and other parts of each container.

Dry, and again weigh each empty container together with its corresponding parts. The difference between the two weights is the net weight of the contents of the container. The average net weight of the contents of the 10 containers is not less than the labeled amount, and the net weight of the contents of any single container is not less than 90% of the labeled amount where the labeled amount is 60 g or less, or not less than 95% of the labeled amount where the labeled amount is more than 60 g but not more than 150 g. If this requirement is not met, determine the net weight of the contents of 20 additional containers. The average net weight of the contents of the 30 containers is not less than the labeled amount, and the net weight of the contents of not more than 1 of the 30 containers is less than 90% of the labeled amount where the labeled amount is 60 g or less, or less than 95% of the labeled amount where the labeled amount is more than 60 g but not more than 150 g.

⟨761⟩ NUCLEAR MAGNETIC RESONANCE

Nuclear magnetic resonance (NMR) spectroscopy is a useful analytical procedure because it is specific: every drug substance possesses a unique, characteristic NMR spectrum.

Since atomic nuclei are charged and may spin on their nuclear axes, the spinning nuclei create a magnetic dipole having a magnetic moment, μ, along this axis. The angular momentum of the spinning nucleus is characterized by a spin quantum number (I). If the mass number is odd, I is ½ or an integral multiple of ½. In contrast to those nuclei having an I of ½ and a spherical nuclear charge distribution, nuclei having $I \geq 1$ exhibit a nonspherical nuclear charge distribution that is characterized by a nuclear quadrupole moment and that results in demonstrable spectral perturbations.

Nuclei having a spin quantum number, I, when placed in an external uniform static magnetic field of strength, H_0, tend to be oriented similarly to a bar magnet in $(2I + 1)$ possible orientations. Thus, for nuclei with $I = ½$ there are two possible orientations, namely, $+½$ or $-½$, a lower or an upper energy state. Since two energy states exist, transitions from the lower to the higher state should be possible if the proper amount of energy is introduced. In a static magnetic field the nuclear magnetic axis spins or precesses (Larmor precession) about the external field axis. The precessional angular velocity, ω_0, is related to the external magnetic field strength through the equation:

$$\omega_0 = \gamma H_0,$$

in which γ is the magnetogyric ratio, a parametric constant for each nucleus. In addition, if energy from an oscillating radiofrequency field is introduced, the absorption of radiation takes place according to the relationship:

$$\Delta E = h\nu_0 = \mu H_0/I,$$

and

$$\nu_0 = \omega_0/2\pi = \gamma H_0/2\pi.$$

Thus, when the frequency (ν_0) of the external energy ($E = h\nu$) is the same as the precessional angular velocity, resonance is achieved, and the nucleus attains the upper state. The existence of two energy states ($I = ½$) introduces the question of relative population of the two states under usual conditions. With the use of the Boltzmann distribution law, it has been found that the lower level population exceeds that of the upper level by only a few parts per million because the separation of the energy levels is about 0.01 calorie at 10 to 15 kilogauss field strength—a small quantity compared to the Boltzmann energy unit kT. Because of this small lower energy level excess, any useful measurement scheme must have the capability to sense and to amplify weak signals.

NMR involves energy absorption by nuclei in the radiofrequency range, i.e., wavelengths of 1 to 100 m or frequencies of 3×10^8 to 3×10^6 Hz. In practice, NMR measurements are generally useful in studies of nuclei such as ^1H, ^{13}C, ^{19}F, ^{31}P, and ^{11}B, which have odd mass numbers and I values of ½ or integral multiples of ½. The proton, ^1H, is most often studied and most frequently used in quantitative analysis. A list of common elements studied and their significant NMR properties is presented in Table 1.

Table 1. Properties of Some Nuclei Amenable to NMR Study.

Nucleus	I	Natural Abundance, %	Sensitivity	Resonance Frequency (MHz) at 14.1 kilogauss
^1H	½	99.98	1.000	60.00
^{13}C	½	1.11	0.016	15.086
^{19}F	½	100	0.834	56.446
^{31}P	½	100	0.066	24.288
^{11}B	3/2	80.42	0.165	19.252

The Spectrum

The magnitude of the separation of the frequency of resonance of a proton from that of some standard (tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate) is called the chemical shift, which is proportional to the strength of the applied field. The latter is a composite of the external field and the field caused by the circulation of surrounding electrons about the protons. The conventional NMR spectrum is shown with the magnetic field strength increasing in the direction left to right, and a proton that resonates at a high magnetic field strength (near tetramethylsilane) is said to be more shielded (greater electron density) than a proton that resonates at a lower magnetic field strength and thus is said to be de-shielded (lower electron density).

Figure 1 shows the proton NMR spectrum of 2,3-dimethyl-2-butenyl methyl ether. This compound contains protons in a methylene group (marked *d* in the graphic formula) and in three methyl groups (*a*, *b*, and *c*). Because the three methyl groups are situated in different molecular environments, three different modes of methyl proton resonance are observed as spectral peaks in addition to the peak corresponding to methylene proton resonance. In some NMR spectra (see *a* and *c* in Figure 2), the observed signal corresponding to a particular proton is split into a set or multiplet of peaks. Such peak splitting is ascribed to the influence of nuclei that possess magnetic moments and are within several valence bonds of the nucleus being studied. This intramolecular effect is called coupling.

The coupling between two nuclei may be described in terms of the coupling constant, J, which is the separation (in Hz) between the individual peaks of the multiplet. Where two nuclei interact and cause reciprocal splitting, the measured coupling constants in the two resulting multiplets are equal. Furthermore, J is independent of magnetic field strength.

In a comparatively non-complex spin system, the number of individual peaks present in a multiplet and the relative peak in-

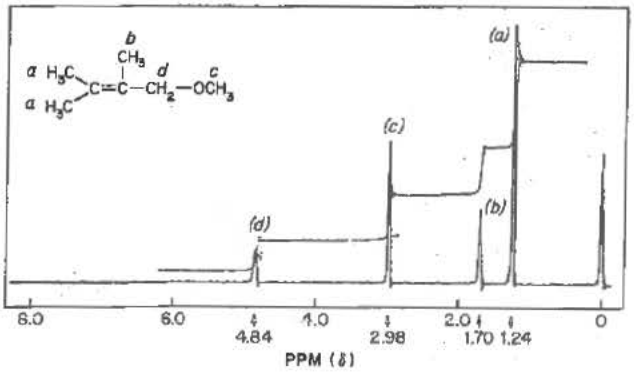


Fig. 1. NMR spectrum of 2,3-dimethyl-2-butenyl methyl ether (15% in CCl_4) showing four nonequivalent, uncoupled types of protons with a normal integral trace (peak area ratio from low H_0 to high H_0 of 2:3:3:6). (Tetramethylsilane, the NMR Reference, appears at 0 ppm.) The system of units represented by δ is defined under *Spectral Standards and Units of Measurement*, in this chapter.

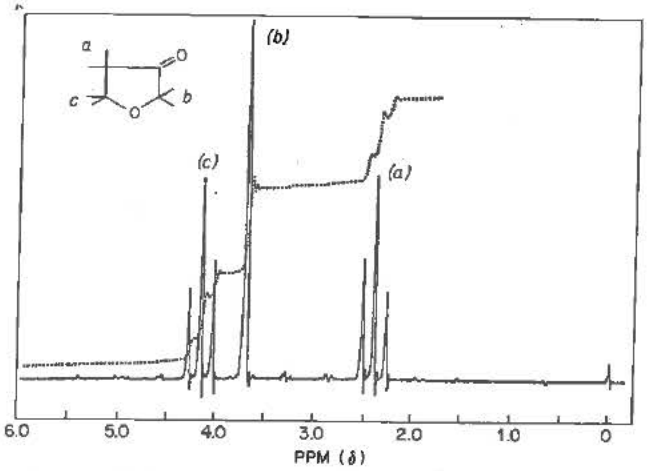


Fig. 2. NMR spectrum of 3-keto-tetrahydrofuran (10% in CCl_4) showing three nonequivalent types of protons with a normal integral trace (peak area ratio from low H_0 to high H_0 of 1:1:1). Note two sets of methylene groups coupled to each other at 4.2 and 2.4 ppm. (Tetramethylsilane, the NMR Reference, appears at 0 ppm.)

tensities are predictable. The peak number is determined by n, the number of protons on adjacent groups that are active in splitting. The total number of observed peaks is $(n + 1)$. Also, the relative intensity of each separate peak follows the coefficient of the binomial expansion $(a + b)^n$; these coefficients may be conveniently found by use of the Pascal triangle, which produces the following relative areas for the specified multiplets: doublet, 1:1; triplet, 1:2:1; quartet, 1:3:3:1; quintet, 1:4:6:4:1; sextet, 1:5:10:10:5:1; and septet, 1:6:15:20:15:6:1. This orderly arrangement, generally referred to as first-order behavior, may be expected when the ratio of $\Delta\nu$ to J is greater than about 10; $\Delta\nu$ is the chemical shift difference between two nuclei or two groups of equivalent nuclei, and J is the spin–spin coupling constant. Two examples of idealized spectra arising from first-order coupling are shown in Figure 3.

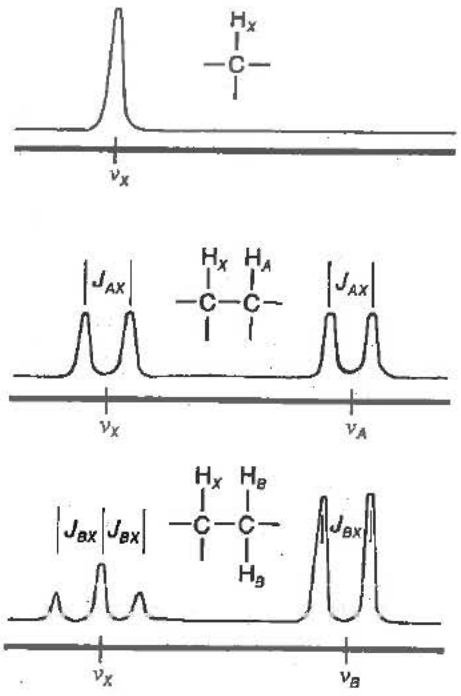


Fig. 3. Diagrammatic Representation of Simple First-order Coupling of Adjacent Protons.

The introduction of extra peaks not ascribable to NMR phenomena may complicate the spectrum. In an attempt to negate magnetic field inhomogeneity and increase resolution, the tube containing the test substance is spun. If this spinning frequency is too low, the desired field averaging is not complete, and an absorption signal is accompanied by aberrant signals of lower intensity called spinning side bands. These side bands are located symmetrically around the main signal, and the separation is equal to the spinning frequency or some integral multiple of that frequency. Thus, spinning side bands are readily identifiable, since their location changes with spinning frequency. Side bands can result also from uneven spinning.

Double resonance or spin decoupling is a useful means of simplifying spectra. This technique removes spin coupling between nuclei or groups of nuclei. For example, in a simple two-proton system, generally designated an AX system (see Figure 3), which is manifested as a pair of doublets, where a strong radiofrequency field at the frequency of X is introduced while the usual radiofrequency field is present to provide resonance possibilities for A, the coupling of X with A is removed, and A is no longer split but instead is manifested as a singlet. This technique provides a convenient way of establishing coupling relationships.

Another valuable property of the recorded spectrum is the peak area. During a single instrumental scan, the intensity of energy absorption is constant for all protons regardless of their nature. Thus the area of a single peak or a multiplet is directly proportional to the number of protons giving rise to that peak or multiplet. As a result, it is possible to determine the relative ratio of different kinds of protons in a molecule (note the integral traces in Figures 1 and 2). Moreover, if the solution of the analyte is prepared quantitatively and an Internal Standard is used, the peak areas, measured by instrumental integration, may be used for quantitative analysis.

Apparatus

Various instrument configurations are possible; the arrangement of a typical double-coil spectrometer is illustrated in Figure 4.

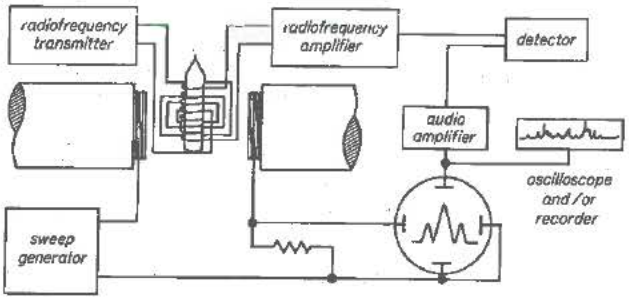


Fig. 4. Block Diagram of Typical NMR Spectrometer.

To detect variations in signal as a function of test substance constitution, NMR spectrometers can be operated either at fixed magnetic fields or at fixed oscillator (transmitter) frequency. In this manner, at a fixed magnetic field strength the test substance is scanned by varying the frequency over a relatively narrow region, or, if the frequency is fixed, the test substance is studied by varying the magnetic field.

The spectrometer must be able to reproduce line widths of a few tenths of a Hz at field strengths of the order of 100 MHz, and thus a stability in the range of 1 part in 10^8 is required. Some spectrometers maintain H_0 sufficiently constant so that line shape distortions do not occur when the instrument is operated at a fixed ν_0. However, problems in magnet stability make this difficult. As may be noted from $\nu_0 = \omega_0/2\pi = \gamma H_0/2\pi$, the ratio H_0/ν_0 is a constant for a particular nuclear resonance. This fact provides the foundation for maintaining reproducible spectra in terms of a combined field-frequency lock control system.

The field-frequency (H_0/ν_0) lock system requires a reference nucleus that is continuously irradiated at its exact resonance frequency with concomitant monitoring of the NMR signal. If the irradiation frequency does not match the resonance frequency (e.g., because of magnetic field changes) at any time, an error signal of an appropriate polarity is produced and taken into a

feed-back loop. In this way the magnet coils may react to correct the field, or, if it is appropriate, the modulation frequency may be adjusted in accordance with the manufacturer's instructions.

The reference nucleus may be part of the instrumental arrangement (external lock), or it may be placed in the test solution (internal lock). Spectrometers should be equipped with both external and internal lock systems. The external lock system, the most commonly used, requires a control compound (e.g., water) whose side-band frequency is used by the instrument feed-back loop to maintain a system stability of 1 part in more than 1×10^8/hour, in terms of field strength to frequency ratios. It is used with field sweep for easy operation, stable integration of peak areas, and easy scale expansion. The proton internal lock system utilizes two side-band frequencies, separated by at least a few Hz, derived from a sharp signal within the test substance, usually the internal reference. It is essential that internal lock stability be 1 part in more than 1×10^9/hour in order to be useful for accurate measurements and spin-decoupling (double resonance). The proton internal lock is not suitable for integration over very wide ranges on some instruments.

Some internal lock systems, using a separate radiofrequency oscillator, lock on the deuterium resonance of deuterated solvent. These systems allow easy scale expansion and integration over the entire spectral range.

The integrator in an NMR instrument is an electronic integrator generally present as part of the spectrometer. The integrator determines the relative areas of resonance peaks through connection with the spectrometer output stage and presents the areas as stepping horizontal lines when a tracing is made of the test specimen with the spectrometer in the integration mode (see Figures 1 and 2).

General Method

The method and procedures discussed herein refer specifically to ^1H (proton) and ^{19}F NMR. They are applicable, with modification, to other nuclei.

Useful interpretation of an NMR spectrum requires a relatively narrow Lorentzian peak. Line broadening severely hampers interpretation and may be caused by local inhomogeneities in the magnetic field. To decrease the effect of small variations in peak shape of nuclear signals, the tube containing the test substance is rotated about the vertical axis, perpendicular to the magnetic field, so that the nuclei in the effective field of the magnet are uniformly exposed to an average field strength. This tends to increase resolution and reduce line widths. The spinning speed (average spin speed is about 20 to 40 rps) should be sufficient to produce averaging and to remove spinning side bands, but not fast enough to produce an extended vortex in the specimen tube. A vortex extended near the region exposed to the radiofrequency coils decreases resolution.

The presence of paramagnetic substances tends to increase broadening. One of the most common paramagnetic species, dissolved oxygen, causes interferences where oxygenated solvents are used for dissolution and where extremely high resolution is required. To remove dissolved oxygen, immerse the test substance contained in a preconstricted, heavy-walled (about 0.5-mm) tube in liquid nitrogen until it is frozen. Evacuate the tube by applying a vacuum of about 10 mm of mercury for several minutes. Allow the tube to stand until thawed, then repeat the procedure twice. After the third operation, while the test substance is frozen, seal the tube at the constriction prior to inserting it into the spectrometer.

Line broadening is also often observed for single protons attached to nitrogen because of the quadrupole moment of the nitrogen nucleus.

Selection of Solvent—Since narrow Lorentzian peaks are possible only in solution NMR spectroscopy, the test substance must be either a liquid or a solution of the solid in a suitable solvent. Choice of solvent is critical for solubility purposes and for making use of the apparent resonance peaks, since solvent peaks, if present, tend to obscure structural features of the test substance. To avoid interferences, for proton NMR determinations, special deuterated solvents are employed, with at least 99.5% isotopic purity. Deuterium ($I = 1$) does not exhibit resonance under ^1H conditions (e.g., 14.1 kilogauss, 60.0 MHz), but a small amount of ^1H present causes a small resonance to be shown. Some solvents (e.g., D_2O) enter into fast exchange reactions with protons and may abolish resonance signals from —COOH, —OH, and —NH_2 structural groups present. The protons from alcohols and amines do not take part in rapid exchange, unless catalyzed by small concentrations of acid and base, except in the presence of D_2O. The most commonly used solvents for proton NMR are listed in Table 2.

Table 2. Solvents Commonly Used for Proton NMR.

Solvent	Residual Proton Signal, δ[a]
CCl_4[b]	—
CS_2[b]	—
SO_2 (liquid)	—
$(CF_3)_2CO$	—
$CDCl_3$	7.27
CD_3OD	3.35, 4.8[c]
$(CD_3)_2CO$	2.05
D_2O	4.7[c]
DMSO-d_6[d]	2.50[e]
C_6D_6	7.20
p-Dioxane-d_8	3.55
CD_3CO_2D	2.05, 8.5[c]
DMF-d_7[f]	2.77, 2.93, 8.05

[a] δ in ppm relative to tetramethylsilane arbitrarily taken as 0δ.
[b] Spectrophotometric grade.
[c] Highly variable; depends on solute and temperature.
[d] Dimethyl sulfoxide-d_6.
[e] Poor grades show an additional peak of water associate at about 3.7 ppm.
[f] Dimethylformamide-d_7.

For ^{19}F NMR, most solvents used in proton NMR may be employed, the most common ones being $CHCl_3$, CCl_4, H_2O, CS_2, aqueous acids and bases, and dimethylacetamide. In general, any nonfluorinated solvent may be used, provided that it is of spectral quality.

Spectral Standards and Units of Measurement—For uniformity, and to avoid unnecessary conversions of units, spectrometer outputs to recorders for proton NMR are designed and calibrated for spectral sweep in terms of ν and δ values relative to tetramethylsilane NMR Reference which has been given a spectral line value of 0 Hz (ν) or 0 ppm (δ). This appears at the far right end of the recorder or oscilloscope where the spectrometer is operated in the normal range and is in the diamagnetic (upfield) direction. The relationship of ν to δ is given by the equation:

$$\delta = (\nu_s - \nu_r)/\nu_0 ,$$

in which ν_s is the test substance line frequency, ν_r is the reference line frequency, and ν_0 is the instrument oscillator frequency.

Since ν_r generally is at arbitrary zero, and ν_0 is the specific resonance frequency, either δ_s or ν_s can be determined from the instrument recorder calibration of the other. For example, on a 60-MHz spectrometer, a resonance line signal of 420 Hz shows a δ value of 7 ppm, i.e., $\delta = [(420 - 0)(\text{Hz})]/60\ \text{MHz} = 420\ \text{Hz}/60{,}000{,}000\ \text{Hz} = 7 \times 10^{-6}$ parts = 7 ppm. Where the spectral scale is defined in terms of δ values, numerical values decrease from left to right (as does frequency). In order to have a scale that increases from left to right, a more conventional arrangement, a scale of τ values has been defined in which the tetramethylsilane resonance line has an assigned value of 10.00. The relationship between τ and δ is: $\tau = 10.00 - \delta$ (ppm). Thus, in the foregoing example, where $\delta = 7$ ppm, $\tau = 3.00$ (ppm).

The chemical shift value is obtained from the recorder trace and is specific for types of nuclei in a particular chemical environment. The NMR Reference tetramethylsilane (1% concentration) is mixed with the test solution where nonaqueous solvents are used or where chemical interaction between tetramethylsilane and the compound studied is not possible. For aqueous systems, an NMR Reference of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (1% concentration) is used because it has an absolute magnetic resonance value for its methyl groups closely approximating that of tetramethylsilane. The sodium 2,2-dimethyl-2-silapentane-5-sulfonate has the disadvantage, however, of showing a number of CH_2 multiplets at high gain and internal lock that may interfere with signals from the test substance. Where the use of NMR Reference tetramethylsilane is not possible because of solubility difficulties or interaction, an *external reference* is used.

For ^{19}F lock calibration, the most widely used NMR Reference is trichloromonofluoromethane, which, like tetramethylsilane, has an arbitrary shift value of 0.00 ppm (Φ^* scale). [NOTE—Φ is fluorine chemical shift in trichloromonofluoromethane extrapolated to infinite dilution; Φ^* is a nonextrapolated value. The difference $\Phi - \Phi^*$ is measurable, but generally not large.] Where operation is at subambient temperatures, trichloromonofluoromethane may be employed as both the reference and the solvent. Hexafluorobenzene, perfluorocyclobutane, and 1,4-dibromotetrafluorobenzene have been used also. Trifluoroacetic acid is the usual choice if an external reference is required. ^{19}F chemical shifts are much larger than ^1H values (−70 to about 250 ppm) and, as a result, instruments capable of use for ^{19}F have a wide sweep accessory in addition to a crystal oscillator, tunable to the appropriate fluorine (56.446 MHz at 14.1 kilogauss) resonance frequency.

The greatest difference in the recorder presentation of ^{19}F spectra with regard to ^1H is that the wide spectral range covered by ^{19}F scans makes it desirable to calibrate "zero" reference at some point paramagnetic (downfield) to the maximum field position. In this way, all the usable ^{19}F resonance signals are "on-chart" positive (upfield) to "lock-calibration zero."

Qualitative and Quantitative Analysis

NMR measurements are useful for a variety of analytical purposes. The various types of protons, fluorine atoms, carbon atoms, etc., each with a different environment, appear as different resonance signals with respect to their different chemical environments. The spectrum thus affords information about the molecular structure. The general multiplicity of each individual resonance (e.g., singlet, doublet, triplet) adds more structural information, and the combination of chemical shift and spin-coupling pattern enables the determination of (a) the number of the atoms being measured, (b) the chemical environment of each atom, (c) the structural and/or isomeric relationships, and (d) the presence of impurities. The integration of peak areas is an important step in interpretation since the ratio of areas yields the relative ratios of the various kinds of resonant nuclei. In addition, the integration may be extended to quantitative analysis.

The quantitative analysis of a compound by NMR exemplifies the use of a specific intimate property for measurement purposes. Once the position of a definite structural unit is known, the area of its resonance peak(s) can be related to that of other peaks, to obtain ratios of the various atoms represented in the spectrum. Quantitative estimation is limited largely by the accuracy and reproducibility of the built-in integrator that is usually part of the instrument recorder. By employing multiple integration tracings across the entire spectrum, as well as several independent analyses, a relative accuracy of ±2% can be achieved. If small parts of the scan yield the quantitative information of interest, these partial scans can be integrated at higher gain to improve the accuracy. The principal advantages of quantitative NMR are: (a) the intensity of a signal for a given nuclear isotope is proportional to the number of nuclei contributing to the signal but independent of its chemical nature; (b) a compound being analyzed in a mixture need not be available in pure form for use as a standard; and (c) resonance lines are narrow in relation to chemical shift differences (line positions). The likelihood of appreciable overlap of signals of different components of a mixture is thereby reduced.

For first-order interpretive qualitative usefulness, the spectrum should first be analyzed for total number of protons by area estimate with the integrator. [NOTE—Neighboring nonequivalent protons interact with the resonance field of the measured proton, producing multiple lines.]

Simple multiplet patterns may be recognized by their relative intensities, by a set of matched or coupled lines in a different part of the spectrum, or by observation of the spectrum on an instrument having a different field strength. In the last-mentioned case, the coupling constant (J) has the same value in Hz, but the field-dependent expression of the chemical shift (ν) changes. The easiest, most direct way is to search for a matched (equal J values) set of multiplets. Coupling usually occurs only over a distance of three chemical bonds, although in some instances long-range coupling (transmitted through four or five bonds) may occur. The coupling and intensity for first-order coupling follow the coefficients of the binomial law, as discussed previously.

Once the total number of protons has been ascertained, their field position may then be matched against the range of positions of known types of groups for the structure(s) present, e.g., $-CH_3$ types, $-CH_2$ types, and $-COCH_2$ types. In general, the closer the protons are to an electronegative group, the more de-shielded they are and thus they resonate at lower H_0 values (higher δ). Groups having significant alkyl character resonate at high H_0 values (0.9 to 2.4 ppm).

Valuable structural information may be obtained for test substances having —OH, $-NH_2$, or —COOH groups by taking advantage of the rapid exchange of these with D_2O. To determine the presence and position of these groups, scan the test substance in $CDCl_3$ or CCl_4, then add a few drops of D_2O to the sample tube, and scan again. Resonance peaks from —OH, $-NH_2$, and —COOH collapse in the second scan and are replaced by a singlet at 4.7 ppm from HDO.

More structural information is obtained from an inspection of the J values when coupling has occurred. The magnitude of J in the aromatic proton region (6.5 to 8.0 ppm) gives valuable data about the nature of aryl ring substituents, since *ortho*-, *meta*-, and *para*-protons show different coupling (8, 2, and about 0.5 Hz, respectively). A similar inspection of coupling in unsaturated systems gives data on isomer content (*cis*-HC=CH, 6 to 12 Hz; *trans*-HC=CH, 9 to 18 Hz), and conformational isomers have been successfully identified (ax–ax, 8 to 10 Hz; ax–eq, 2 to 5 Hz; and eq–eq, 1 to 4 Hz).

If alcoholic —OH is possibly present and has been noted by D_2O exchange, —OH coupling may be observed by scanning a test substance in DMSO-d_6. In this procedure, $-CH_2OH$ shows a triplet, —CHROH shows a doublet, and $-CR_2OH$ shows a singlet.

If a complex spectrum is not interpretable by usual first-order rules, double resonance, as previously discussed, may be employed. Instruments used at 60 MHz or above should be equipped with homonuclear spin decouplers.

The spectra of compounds containing ethers, esters, ketones, etc., often may have resonance lines grouped in a narrow region of the spectrum (1.0 to 3.0 ppm) and are difficult to analyze because of peak overlap. In these cases it is often advantageous to add a shift reagent such as the dipyridine adduct of tris(2, 2,-6, 6-tetramethyl-3, 5-heptanedionato)europium(III) [Eu(dpm)$_3$], or tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium(III) [Eu(fod)$_3$], or their corresponding praseodymium compounds. [Eu(fod)$_3$] is the most useful, and in its presence, coordination occurs between the functional group and the transition metal complex causing a downfield shift of the resonance lines. The relative shift indicates the type of groups proximal to the protons. In order to improve the spectrum, increments of reagent, not to exceed the weight of the test substance, may be added to the test substance.

Quantitative analysis can be done either in a relative manner or by use of an internal standard. In the former method, a resonance peak area attributed to the main absorbing group is compared proton for proton with a different area from a second compound or impurity, and a percentage value, in mole percent, is determined. For example, the partial spectrum (see Figure 5) shows the impurity A (one proton of type —CH=) in a specimen of B (one proton of type —CH<). The amount of A present is calculated in terms of the total areas of A and B. For the greatest accuracy, quantitative evaluation of this type should be done usually by comparing areas of equal numbers of protons. The areas should always be obtained not less than five times to average integrator variations.

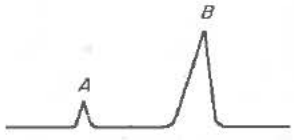


Fig. 5.

In the internal standard method a resonance peak area arising from the test substance is compared with a resonance peak area ascribable to part of an internal standard molecule. If both test substance and internal standard are accurately weighed, the absolute purity of the substance may be calculated. A good internal standard has the following properties: (a) it presents a reference resonance peak, preferably a singlet, at a field position at least

30 Hz removed from a sample peak; (b) it is soluble in the analytical solvent; (c) its proton equivalent weight, i.e., the molecular weight divided by the number of protons giving rise to the reference peak, is as small as possible; and (d) it does not interact with the compound being tested. Typical examples of useful standards are benzene and benzyl benzoate for comparing nonaromatic areas (—CH_2 of $C_6H_5CO_2CH_2C_6H_5$ at about 5.2 ppm) and maleic acid for nonalkene types. The utility and disadvantages of this method are the same as in gas chromatography, except that a standard of the measured unknown is not needed if some other pure reference standard is available.

Quantitative analysis by the internally relative method or the internal standard method, as well as detection of trace impurities, can be measurably improved by the use of a time-averaging accessory. This is a signal-to-noise booster that additively accumulates signals while averaging out randomly occurring noise level. An enhanced trace spectrum is obtained along with a digitalized count area, thus measurably improving accuracy.

For either qualitative or quantitative scans, coupling may occur between ^1H and other nuclei, especially ^{19}F, ^{31}P, and ^{199}Hg. In some cases, e.g., ^{31}P and ^{199}Hg, the coupling constants may be large enough so that part of the multiplet is off the chart at either the upfield or downfield end. Couplings of these types can occur over the normal "three-bond distance," as for ^1H-^1H coupling.

Qualitative Scans—Proceed as directed in the individual monograph, or weigh 60 to 90 mg of test substance into a 15- × 40-mm amber vial, and add about 500 µL of solvent, using a pipet or tuberculin syringe. For general organic solvents (such as $CDCl_3$, CCl_4, DMSO-d_6, and DMF-d_7), add about 5 µL of tetramethylsilane, mix, and transfer 400 to 500 µL of the solution to a 5-mm × 17.5-cm tube suitable for spinning. Take precautions to prevent evaporation of the tetramethylsilane prior to mixing with the solution. Place the cap on the tube. Using field sweep and external lock, scan the test substance from 0 ppm to about 8 ppm at a scan rate of about 1.5 to 4 minutes for a full-scale recording, adjusting amplification so that all peaks remain on scale. Adjust the spin rate so that no spinning side bands interfere with the peaks of interest. If the response is low at reasonable amplitude, increase the radiofrequency power to obtain the highest peak response without peak broadening.

In adjusting resolution, take care to ensure that the tetramethylsilane peak shows definite ringing. The phenomenon of "ringing" is the repeated excursion or "wiggling" of the recorder trace after the magnetic field has passed through a resonance value and the peak value has been recorded. The ringing noted on a number of the peaks in Figure 2 is seen during rapid scans and decays exponentially, finally reaching the baseline value. Ringing is a good indication of a homogeneous field.

After the initial scan, quickly check for peaks downfield of 8 ppm by offsetting the instrument response by 5 ppm. Record any peaks from 13 ppm to 5 ppm so that peak positions of 5 ppm to 8 ppm may be matched, since the tetramethylsilane position (0 ppm) may shift slightly in the higher range.

After scanning, set the recorder sweep time to about 1 minute, reset phase, and record the total integral using an integrator amplitude set to keep the total integrator response on chart scale. Compare the area of each set of peaks to the area of an assigned peak, and determine the number of nuclei contributing to each set.

Note peak positions, in ppm, from the tetramethylsilane peak, and measure the associated integrals. Check the spectrum for coupled peaks either visually or by spin decoupling. For $CDCl_3$, C_6D_6, $(CD_3)_2CO$, and CCl_4 solutions, add 2 drops of D_2O to the tube, shake for about 30 seconds, and again scan after resetting the 0-ppm position. Check the second scan for any disappearance of peaks, noting the additional peak for HDO.

For accurate peak matching, where accuracy of 0.5 to 1.0 Hz is needed, reset the instrument calibration on internal lock, and again scan.

Where shift reagents are specified, prepare a solution of the dipyridine adduct of [Eu(dpm)$_3$], or [Eu(fod)$_3$], or their corresponding praseodymium analogs in $CDCl_3$ or CCl_4 as directed in the individual monograph. In an amber vial, dilute the test solution with a specific volume of the shift solution, containing reagent equal to 0.2 times the weight of test substance, and reduce the volume to about 500 µL by using a thin stream of cold, dry nitrogen. Filter the solution through a fine-porosity, sintered-glass filter into the sample tube. Again scan the test substance, noting shifts in major peaks. Repeat using 0.4-, 0.6-, and 0.8-weight equivalent of the shift reagent. Assign the peak position by the nature of the shift. [NOTE—With shift reagent present, an additional peak results from the reagent and some degree of broadening occurs.]

Absolute Method of Quantitation—Where the individual monograph directs that the *Absolute Method of Quantitation* be employed, proceed as follows.

Solvent, *Internal Standard*, and *NMR Reference*—Use as directed in the individual monograph.

Test Preparation—Transfer a quantity of the article, containing about 4.5 proton mEq of the analyte, accurately weighed, to a glass-stoppered, graduated centrifuge tube. Transfer to the tube about 4.5 proton mEq of *Internal Standard*, accurately weighed, add 3.0 mL of *Solvent*, insert the stopper, and shake. When dissolution has been completed, add about 30 µL (30 mg if a solid) of *NMR Reference* if it will not interfere with subsequent measurements, and shake.

Procedure—Transfer about 0.4 mL of *Test Preparation* to a standard 5-mm NMR spinning tube, and record the spectrum, adjusting the spin-rate so that no spinning side bands interfere with the peaks of interest. Measure the area under each of the peaks specified in the individual monograph by integrating not fewer than five times. Record the average area of the *Internal Standard* peak as A_S and that of the *Test Preparation* peak as A_U.

Calculate the quantity, in mg, of the analyte in the *Test Preparation* by the formula:

$$W_S(A_U/A_S)(E_U/E_S),$$

in which W_S is the weight, in mg, of *Internal Standard* taken, and E_U and E_S are the proton equivalent weights (i.e., the molecular weights divided by the number of protons giving rise to the reference peak) of the analyte and the *Internal Standard*, respectively.

Relative Method of Quantitation—Where the individual monograph directs that the *Relative Method of Quantitation* be employed, proceed as follows.

Solvent, *NMR Reference*, and *Test Preparation*—Use as directed under *Absolute Method of Quantitation*.

Procedure—Transfer about 0.4 mL of *Test Preparation* to a standard 5-mm NMR spinning tube, and record the spectrum, adjusting the spin-rate so that no spinning side bands interfere with the peaks of interest. Measure the area under each of the peaks specified in the individual monograph by integrating not fewer than five times. Record the average areas resulting from the resonances of the groups designated in the individual monograph as A_1 and A_2.

Calculate the quantity, in mole percent, of the analyte in the *Test Preparation* by the formula:

$$100(A_1/n_1)/[(A_1/n_1) + (A_2/n_2)],$$

in which n_1 and n_2 are, respectively, the numbers of protons in the designated groups.

⟨771⟩ OPHTHALMIC OINTMENTS

Added Substances—Suitable substances may be added to ophthalmic ointments 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 an article intended for ophthalmic use (see also *Added Substances* under *General Notices*, and *Antimicrobial Preservatives—Effectiveness* ⟨51⟩).

A suitable substance or mixture of substances to prevent the growth of microorganisms must be added to ophthalmic ointments that are packaged in multiple-use containers, regardless of the method of sterilization employed, unless otherwise directed in the individual monograph, or unless the formula itself is bacteriostatic. Such substances are used in concentrations that will prevent the growth of or kill microorganisms in the ophthalmic

ointments (see also *Antimicrobial Preservatives—Effectiveness* ⟨51⟩ and *Antimicrobial Agents—Content* ⟨341⟩). Sterilization processes are employed for the finished ointment or for all ingredients, if the ointment is manufactured under rigidly aseptic conditions, 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⟩). Ophthalmic ointments that are packaged in single-use containers are not required to contain antibacterial agents; however, they meet the requirements for *Sterility Tests* ⟨71⟩.

Containers—Containers, including the closures, for ophthalmic ointments 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 (see also *Containers for Articles Intended for Ophthalmic Use*, under *General Notices*).

Metal Particles—Follow the *Procedure* set forth under *Metal Particles in Ophthalmic Ointments* ⟨751⟩.

Leakage—Select 10 tubes of the Ointment, with seals applied when specified. Thoroughly clean and dry the exterior surfaces of each tube with an absorbent cloth. Place the tubes in a horizontal position on a sheet of absorbent blotting paper in an oven maintained at a temperature of 60 ± 3° for 8 hours. No significant leakage occurs during or at the completion of the test (disregard traces of ointment presumed to originate externally from within the crimp of the tube or from the thread of the cap). If leakage is observed from one, but not more than one, of the tubes; repeat the test with 20 additional tubes of the Ointment. The requirement is met if no leakage is observed from the first 10 tubes tested, or if leakage is observed from not more than one of 30 tubes tested.

⟨781⟩ OPTICAL ROTATION

Many drugs, in a pure state or in a solution, are optically active in the sense that they cause incident plane polarized light to emerge in a continuum of planes at different intensities such that the plane of maximum intensity forms a measurable angle with the plane of the incident light. Where this effect is large enough for precise measurement, it may serve as the basis for an assay or an identity test. The optical rotation is expressed in degrees, as either *angular rotation* (observed) or *specific rotation* (calculated with reference to the specific concentration of 1 g of solute in 1 mL of solution, measured under stated conditions). Substances that cause the light plane to rotate clockwise, as viewed toward the light source, are termed dextrorotatory and the *angular rotation* is designated (+); those that cause counter-clockwise rotation are termed levorotatory and the *angular rotation* is designated (−).

Specific rotation usually is expressed by the term:

$$[\alpha]_x^t,$$

in which t represents, in degrees centigrade (Celsius), the temperature at which the rotation is determined, and x represents the characteristic spectral line or wavelength of the light used. Unless otherwise specified, the values cited in this Pharmacopeia relate to measurements at 25° with the use of the D line of sodium (a doublet at 589.0 nm and 589.6 nm).

The accuracy and precision of optical rotatory measurements will be increased if they are carried out with due regard for the following general considerations.

The instrument itself must be in good condition. Optical elements must be brilliantly clean and in exact alignment. The match point should lie close to the normal zero mark. The light source should be rigidly set and well aligned with respect to the optical bench. It should be supplemented by a filtering system capable of transmitting light of a sufficiently monochromatic nature. Precision polarimeters generally are designed to accommodate interchangeable disks to isolate the D line from sodium light or the 546.1-mm line from the mercury spectrum. With polarimeters not thus designed, cells containing suitably colored liquids may be employed as filters. Temperature control of the solution and of the polarimeter requires special attention, since rotatory power varies appreciably with temperature. The temperature specified for the determination applies to the solution, and is maintained within 0.2° of the stated value. Accuracy is assured by calibration of the instrument with suitable standards.* Generally a polarimeter capable of giving replicate readings within 0.020° suffices for Pharmacopeial purposes.

Polarimeter tubes should be filled in such a way as to avoid creating or leaving air bubbles that interfere with the passage of the beam of light. Interference from bubbles is minimized with tubes in which the bore is expanded at one end. However, with tubes of uniform bore, such as semimicro or micro tubes, care is required for proper filling. Non-metallic tubes are recommended for use in testing corrosive articles or solutions of articles in corrosive solvents.

In closing tubes having removable end-plates fitted with gaskets and caps, the latter should be tightened only enough to ensure a leak-proof seal between the end-plate and the body of the tube. Excessive pressure on the end-plate may set up strains that result in interference with the measurement. In determining the specific rotation of a substance of low rotatory power, it is desirable to loosen the caps and tighten them again between successive readings in the measurement of both the rotation and the zero point. Differences arising from end-plate strain thus generally will be revealed, and appropriate adjustments to eliminate the cause may be made.

Procedure—Where the substance is a liquid, adjust its temperature to 25°, and transfer it to the polarimeter tube. Proceed as directed below, beginning with "Make at least five readings," but carry out the blank determination on the empty, dry tube.

Where the substance is a solid, accurately weigh a suitable portion and transfer it to a volumetric flask by means of water, or other solvent if specified, reserving a portion of the solvent for the blank determination. Add enough solvent to bring the meniscus close to but still below the mark, and adjust the flask contents to 25° as by suspending the flask in a constant-temperature bath. Add solvent to the mark, and mix. Transfer the solution to the polarimeter tube within 30 minutes from the time the substance was dissolved, taking care to standardize the elapsed time in the case of substances known to undergo racemization or mutarotation. During the elapsed time interval, maintain the solution at a temperature of 25°.

Make at least five readings, at 25°, of the observed rotation. Substitute the reserved solvent for the solution, and make an equal number of readings on it. The zero correction is the average of the blank readings, and is subtracted from the average observed rotation. It is necessary in this calculation to use the observed signs of rotation, whether positive or negative, to give the corrected observed rotation.

Where an automatic photoelectric polarimeter that possesses the necessary degree of accuracy and precision is employed, the need for five or more repetitive readings is obviated.

Calculation—Calculate the specific rotation of a liquid substance, or of a solid in solution, by application of one of the following formulas:

I. For liquid substances, $[\alpha]_x^t = \frac{a}{ld}$;

II. For solutions, $[\alpha]_x^t = \frac{100a}{lpd} = \frac{100a}{lc}$;

in which a is the corrected observed rotation, in degrees, at temperature t at wavelength x; l is the length of the polarimeter tube, in decimeters; d is the specific gravity of the liquid or solution at the temperature of observation; p is the concentration of the solution expressed as the number of g of substance in 100 g of solution; and c is the concentration of the solution expressed as the number of g of substance in 100 mL of solution.

⟨785⟩ OSMOLARITY

Osmotic pressure is fundamentally related to all biological processes that involve diffusion of solutes or transfer of fluids through membranes. Thus, knowledge of the osmolar concentrations of parenteral fluids is essential. The labels of Pharmacopeial solutions that provide intravenous replenishment of fluid, nu-

* Suitable calibrators are available from the Office of Standard Reference Materials, National Institute of Science and Technology, Washington, DC 20234, as Standard Reference Material 41b, Dextrose, and Standard Reference Material 17a, Sucrose.

trient(s), or electrolyte(s), as well as of the osmotic diuretic Mannitol Injection, are required to state the osmolar concentration.

The declaration of osmolar concentration on the label of a parenteral solution serves primarily to inform the practitioner whether the solution is hypo-osmotic, iso-osmotic, or hyper-osmotic. A quantitative statement facilitates calculation of the dilution required to render a hyper-osmotic solution iso-osmotic. It also simplifies many calculations involved in peritoneal dialysis and hemodialysis procedures. The osmolar concentration of an extemporaneously compounded intravenous solution prepared in the pharmacy (e.g., a hyperalimentation solution) from osmolar-labeled solutions also can be obtained simply by summing the osmoles contributed by each constituent.

The units of osmolar concentration are usually expressed as milliosmoles (abbreviation: mOsmol) of solute per liter of solution. In general terms, the weight of an osmole is the gram molecular weight of a substance divided by the number of ions or chemical species (n) formed upon dissolution. In ideal solutions, for example, $n = 1$ for glucose, $n = 2$ for sodium chloride or magnesium sulfate, $n = 3$ for calcium chloride, and $n = 4$ for sodium citrate.

The ideal osmolar concentration may be determined according to the formula:

$$\text{osmolar concentration (mOsmol/liter)} = \text{mOsM}$$

$$= \frac{\text{wt. of substance (g/liter)}}{\text{mol. wt. (g)}} \times \text{number of species} \times 1000.$$

As the concentration of the solute increases, interaction among solute particles increases, and actual osmolar values decrease when compared to ideal values. Deviation from ideal conditions is usually slight in solutions within the physiologic range and for more dilute solutions, but for highly concentrated solutions the actual osmolarities may be appreciably lower than ideal values. For example, the ideal osmolarity of 0.9% Sodium Chloride Injection is $9/58.4 \times 2 \times 1000 = 308$ milliosmoles per liter. In fact, however, n is slightly less than 2 for solutions of sodium chloride at this concentration, and the actual measured osmolarity of 0.9% Sodium Chloride Injection is about 286 milliosmoles per liter.

The theoretical osmolarity of a complex mixture, such as Protein Hydrolysate Injection, cannot be readily calculated. In such instances, actual values of osmolar concentration are to be used to meet the labeling requirement set forth in the individual monograph. They are determined by calculating the osmolarity from measured values of osmolal concentration and water content. Each osmole of solute added to 1 kg of water lowers the freezing point approximately 1.86° and lowers the vapor pressure approximately 0.3 mm of mercury (at 25°). These physical changes are measurable, and they permit accurate estimations of osmolal concentrations.

Where osmometers that measure the freezing-point depression are employed, a measured volume of solution (usually 2 mL) is placed in a glass tube immersed in a temperature-controlled bath. A thermistor and a vibrator are lowered into the mixture, and the temperature of the bath is decreased until the mixture is super-cooled. The vibrator is activated to induce crystallization of the water in the test solution, and the released heat of fusion raises the temperature of the mixture to its freezing point. By means of a Wheatstone bridge, the recorded freezing point is converted to a measurement in terms of milliosmolality, or its near equivalent for dilute solutions, milliosmolarity. The instrument is calibrated by using two standard solutions of sodium chloride that span the expected range of osmolarities.

Osmometers that measure the vapor pressures of solutions are less frequently employed. They require a smaller volume of specimen (generally about 5 µL), but the accuracy and precision of the resulting osmolality determination are comparable to those obtained by the use of osmometers that depend upon the observed freezing points of solutions.

Labeling—Where an osmolarity declaration is required in the individual monograph, the label states the total osmolar concentration in milliosmoles per liter. Where the contents are less than 100 mL, or where the label states that the article is not for direct injection but is to be diluted before use, the label alternatively may state the total osmolar concentration in milliosmoles per milliliter.

⟨788⟩ PARTICULATE MATTER IN INJECTIONS

Particulate Matter—Particulate matter consists of extraneous, mobile, undissolved substances, other than gas bubbles, unintentionally present in parenteral solutions. Injectable solutions, including solutions constituted from sterile solids intended for parenteral use, are essentially free from particles that can be observed on visual inspection. In the following tests, for large-volume and small-volume Injections, the results obtained in examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Statistically sound sampling plans based upon a known set of given operational factors must be elaborated if valid inferences are to be drawn from observed data to characterize the level of particulate matter in a large group of units. Two procedures for the determination of particulate matter are given herein, differing in accordance with the labeled volume of an article in a container. 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 for the test being applied.

LARGE-VOLUME INJECTIONS FOR SINGLE-DOSE INFUSION

Limits for particulate matter are prescribed herein for individual articles in containers that are labeled as containing more than 100 mL of a single-dose large-volume Injection intended for administration by intravenous infusion. The limits do not apply to multiple-dose Injections, to single-dose, small-volume Injections, nor to injectable solutions constituted from sterile solids.

This test for particulate matter is suitable for revealing the presence of particles whose longest axis, or effective linear dimension, is 10 µm or greater. Alternative procedures or procedural details may be employed to measure particulate matter, provided the results obtained are of equivalent reliability. However, where a difference appears, or in the event of a dispute, only the result obtained by the procedure given in this Pharmacopeia is conclusive.

PROCEDURE—[NOTE—Throughout this procedure, use suitable, nonpowdered gloves and scrupulously clean glassware and equipment that have been rinsed successively with a warm solution of detergent, hot water, water, and isopropyl alcohol. Apply the water as a strong jet back and forth across the surface of the vertically held object, working slowly from top to bottom. Perform the rinsing with isopropyl alcohol under a laminar flow hood equipped with ultra-HEPA (high-efficiency particulate air) filters. Permit the objects to dry under the hood upstream of all other operations. Preferably, locate the hood in a separate room that is supplied with filtered, air-conditioned air, and maintained under positive pressure with respect to the surrounding area. Prior to conducting the test, clean the laminar flow hood (except the surfaces of the filter media) with an appropriate solvent. Maintain airflow velocity at 90 ± 20 feet per minute.]

Membrane Filter and Assembly—Using forceps, remove a color contrast grid membrane filter from its container. Wash both sides of the membrane with a stream of water that has been further purified by filtration through a suitable membrane to remove particulate matter having an effective linear dimension greater than 5 µm, by holding the filter in a vertical position, and, starting at the top of the non-gridded side, sweeping the stream back and forth across the surface, working slowly from top to bottom so that particles will be rinsed downward off the filter, and repeating the process on the gridded side. Place the membrane (grid side up) on the filter holder base, and install the filtering funnel on the base without sliding the funnel over the membrane filter. Invert the assembled unit, and wash the inside of the funnel for about 10 seconds with a jet of filtered water. Allow the water to drain, and place the unit on the filter flask.

Test Preparation—Mix the solution by inverting the container 20 times. Thoroughly clean the outer surface of the container with a jet of water, and remove the closure carefully, avoiding contamination of the contents. Transfer 25 mL of the well-mixed solution to the funnel, allow to stand for 1 minute, and apply the vacuum and filter. Release the vacuum gently, and wash the

inner walls of the funnel with a jet of 25 mL of the filtered water. Direct the jet of filtered water in such manner as to wash the walls of the funnel free from any particles that may have become lodged on the walls, but avoid directing the stream onto the filter surface. After turbulence has dissipated, vacuum-filter the rinsing. Carefully remove the upper section of the filter assembly while maintaining vacuum. Release the vacuum, and remove the membrane filter with forceps. Place the filter in a plastic petri slide, using a very thin film of stopcock grease as pre-coating, if necessary to hold the filter flat and in place. Allow the filter to dry with the cover of the petri slide slightly ajar. Cover the slide carefully on the micrometer stage of the microscope, and count the particles on the filter as described below.

Determination—Examine the entire membrane filter in a suitable microscope under 100× magnification with the incident light at an angle of 10° to 20° with the horizontal. Count the number of particles having effective linear dimensions equal to or larger than 10 μm and equal to or larger than 25 μm. Perform a blank determination, using a *Membrane filter and assembly*, as directed under *Test preparation*, beginning with "wash the inner walls of the funnel with a jet." Subtract the total counts obtained in the blank determination from the uncorrected total counts for the *Test preparation*. [NOTE—For Dextrose-containing solutions, do not enumerate morphologically indistinct material showing little or no surface relief and presenting a gelatinous or film-like appearance. Since in solution this material consists of units of the order of 1 μm or less and is liable to be counted only after aggregation and/or deformation on the membrane, interpretation of enumeration may be aided by testing a specimen of the solution with a suitable electronic particle counter.]

Interpretation—Duplicate *Test preparations* and blanks may be examined as directed. If the blank determination yields more than 5 particles having effective linear dimension of 25 μm or greater, the operational environment is unsatisfactory and the test is invalid.

The large-volume Injection for single-dose infusion meets the requirements of the test if it contains not more than 50 particles per mL that are equal to or larger than 10 μm and not more than 5 particles per mL that are equal to or larger than 25 μm in effective linear dimension.

SMALL-VOLUME INJECTIONS

This test is applicable to all small-volume Injections, in containers that are labeled as containing 100 mL or less, single- or multiple-dose, either in solution or in solution constituted from sterile solids, wherever a requirement for a limit for particulate matter appears in the individual monograph. Injections packaged in prefilled syringes and cartridges are exempt from these requirements unless an individual monograph states specifically that prefilled syringes and cartridges are to be included. The requirement does not apply where the monograph specifies that the label shall state that the product is to be used with a final filter.

The test calls for the use of an electronic liquid-borne particle counter system utilizing a light-obscuration based sensor with a suitable sample feeding device. [NOTE—see *Tests and Assays, Apparatus*, page 5 under *General Notices and Requirements*].[1,2]

Acceptable resolution of the sensor, and accuracy of the sampling apparatus used, are critical to this test. The following two methods are intended to aid in assuring system suitability.

Determination of Sensor Resolution—The particle size resolution of the instrumental particle counter is dependent upon the particle sensor used. Determine the resolution of the particle counter for 10-μm particles. Use the monosized particle size standard. The relative standard deviation of the standard is less than 5%. Two acceptable methods of determining particle size resolution are: (1) manually generating a particle count versus particle size response curve, and (2) using an electronic method of measuring and sorting particle sensor voltage output with a multichannel analyzer.

[1] Equivalency may be determined by procedures outlined in ASTM F-660-83, available from the American Society for Testing and Materials, 1916 Race St., Philadelphia, PA 19103.

[2] Suitable apparatus is available from Pacific Scientific, Instrument Div., 2431 Linden Lane, Silver Spring, MD 20910. Suitable sensors are available also from Russell Laboratories, 3314 Rubio Crest Drive, Altadena, CA 91001.

Sensor Flow Rate—Verify that the flow rate is within the manufacturer's specifications for the sensor used.

MANUAL METHOD—Sample successive aliquots of the 10-μm particle size standard suspension at various particle size threshold settings (typically from 5 μm to 15 μm). The breadth of the particle size response range depends on the particle sensor resolution and the distribution of the particle size standard. Plot the particle counts versus the corresponding particle size thresholds to determine the observed size distribution (a Gaussian distribution results). Calculate the percentage resolution by the formula:

$$\% \text{ resolution} = (100/D)(\text{Var}_{\text{Obs}} - \text{Var}_{\text{Std}})^{1/2},$$

in which D is the mean particle diameter, and Var_{Obs} and Var_{Std} are the variances of the observed size distribution and the labeled distribution of the particle standard, respectively. The resolution is not greater than 10%.

ELECTRONIC METHOD—Record the voltage output distribution of the particle sensor, using a multi-channel analyzer while sampling a suspension of the 10-μm particle size standard. Proceed with the calculations as directed for the *Manual Method*. The resolution is not greater than 10%.

Sample Volume Accuracy—Since particle count varies directly with the volume of fluid sampled, it is important that the sampling accuracy be known (or known to be within a certain range). Fill all of the dead volume in the feeder with *Water for Injection*. Take 10 mL of *Water for Injection* in a tared container. Withdraw 5 mL through the sample feeding device, and again weigh the container. The accuracy of the volume sampled is ±5%.

PROCEDURE—[NOTE—Prepare the sample, glassware, closures, and other required equipment in an environment protected by HEPA (high-efficiency particulate air) filters. Particle-free garments and nonpowdered gloves preferably are worn throughout the preparation. Preferably locate the hood in a separate room supplied with HEPA-filtered, air-conditioned air, maintained under positive pressure with respect to the surrounding area.]

Use a pressure vessel capable of withstanding a pressure of 100 psi, having pressure tubing which does not shed particles and a hand-held spray nozzle with filter holder, for filtering water for cleaning and sample preparation. Use non-gridded filters of 5.0-μm pore size or less.

For standardization and sample preparation, use hardened, non-particle shedding, glass containers having openings of minimum size to reduce inadvertent contamination. Where closures are used, they are to have non-shedding liners such as polytef.

Glassware and Closure Cleaning—Cleanse glassware, closures, and other required equipment by immersing and scrubbing in warm, nonionic detergent solution, then rinsing in flowing warm tap water, followed by rinsing in flowing filtered water. Organic solvents may be used to facilitate cleaning. Finally, pressure-rinse in filtered water, using a hand-held pressure nozzle with final filter, or other appropriate equipment.

Particulate Control Test—Conduct this test to determine that the environment is suitable for the analysis and that the glassware is properly cleaned, and to assure the water to be used for analysis is particle-free.

Using filtered water and cleaned glassware, take 5 consecutive water samples of 5 mL each. Invert each sample 20 times. Degas by ultrasonication for 30 seconds, or by allowing to stand for 2 minutes. Stir each water sample by mechanical means at a speed sufficient to maintain a slight vortex throughout the analysis. If 5 particles of 25-μm or 25 particles of 10-μm or greater size are observed for the combined 25 mL, either the environment is not suitable for particulate analysis or the filtered water and glassware have not been properly prepared. Repeat the preparatory steps until environment, water, and glassware are suitable for this test.

Calibration—Calibrate the instrument with 3 standards, each consisting of monosized polystyrene spheres, approximately 10 μm, 20 μm, and 30 μm, in an aqueous vehicle.[3] When using particulate reference standards, take care to reduce particle agglomeration and assure particle purity. Suitable methods are

[3] See ASTM F322-80.

available for examining commercial spheres where desired.[4] Use the procedure in ASTM 658-87 (defining counting and sizing accuracy of a liquid-borne particle counter using near-mono disperse spherical particulate materials) to calibrate automated particle counters.

Test Preparation—Prepare the test specimens in the following sequence:

Remove outer closures, sealing bands, and any loose or shedding paper labels, wash the exterior of containers as described under *Glassware and Closure Cleaning*, and dry in a particle-free airflow. Withdraw the contents of the containers in the normal or customary manner of use, or as instructed in the package labeling, except that containers with removable stoppers may be sampled by removing the closure and emptying the contents into a clean container.

Determination—

(*A*) *Liquid Products*

(1) Mix by inverting 25 times within 10 seconds.

NOTE—Because of the small volume of some products, it is necessary to agitate the solution more vigorously in order to suspend the particles properly.

(2) Open and combine the contents of not less than 10 containers, to obtain a volume of not less than 20 mL, in a cleaned container.

(3) Degas by ultrasonication for 30 seconds or by allowing to stand for 2 minutes.

(4) Gently stir contents of containers by hand swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Stir continuously throughout the analysis.

(5) Take 3 consecutive portions, each not less than 5 mL. Discard the data from the first portion.

(*B*) *Dry or Lyophilized Products*

(1) Open the container, taking care not to contaminate the opening or cover.

(2) Constitute with a suitable volume of filtered water, or with the appropriate filtered diluent if water is not suitable.

(3) Replace cover, and agitate as in (*A*).

(4) Analyze as in (*A*).

(*C*) For products packaged in containers that are constructed to hold the drug product and a solvent in separate compartments, mix each unit as directed in the labeling. Analyze the solutions as in (*A*).

(*D*) For products labeled as "Pharmacy Bulk Package—Not for direct infusion," proceed as directed under (A) or (B) above, performing the test on a portion taken from pooled units that is equal to the maximum dose given in the labeling. For the calculations below, consider this portion to be the equivalent of the contents of one full container.

Calculations—Average the counts resulting from the 2 portions of the sample analyzed. Calculate the number of particles in each container, P_C, by the equation:

$$P_C = \overline{C}V_T/V_PN,$$

in which $\overline{C}$ is the average particle count obtained from the portions analyzed, V_T is the volume, in mL, of pooled sample, V_P is the volume, in mL, of each portion analyzed, and N is the number of containers pooled.

Interpretation—The small-volume Injection meets the requirements of the test if the average number of particles it contains is not more than 10,000 per container that are equal to or greater than 10 μm in effective spherical diameter and not more than 1000 per container equal to or greater than 25 μm in effective spherical diameter.

⟨791⟩ pH

For compendial purposes, pH is defined as the value given by a suitable, properly standardized, potentiometric instrument (pH meter) capable of reproducing pH values to 0.02 pH unit using an indicator electrode sensitive to hydrogen-ion activity, the glass electrode, and a suitable reference electrode such as calomel or silver–silver chloride. The instrument should be capable of sensing the potential across the electrode pair and, for pH standardization purposes, applying an adjustable potential to the circuit by manipulation of "standardization," "zero," "asymmetry," or "calibration" control, and should be able to control the change in millivolts per unit change in pH reading through a "temperature" and/or "slope" control. Measurements are made at 25 ± 2°, unless otherwise specified in the individual monograph or herein.

The pH scale is defined by the equation:

$$\text{pH} = \text{pHs} + (E - E_S)/k,$$

in which E and E_S are the measured potentials where the galvanic cell contains the solution under test, represented by pH, and the appropriate *Buffer Solution for Standardization*, represented by pHs, respectively. The value of k is the change in potential per unit change in pH and is theoretically $[0.05916 + 0.000198(t - 25°)]$ volts at any temperature t. This operational pH scale is established by assigning rounded pH values to the *Buffer Solutions for Standardization* from the corresponding National Bureau of Standards *molal* solutions.

It should be emphasized that the definitions of pH, the pH scale, and the values assigned to the *Buffer Solutions for Standardization* are for the purpose of establishing a practical, operational system so that results may be compared between laboratories. The pH values thus measured do not correspond exactly to those obtained by the classical definition, $\text{pH} = -\log [H^+(aq)]$. So long as the solution being measured is sufficiently similar in composition to the buffer used for standardization, the operational pH corresponds fairly closely to the theoretical pH. Although no claim is made with respect to the suitability of the system for measuring hydrogen-ion activity or concentration, the values obtained are closely related to the activity of the hydrogen ion in aqueous solutions.

Where a pH meter is standardized by use of an aqueous buffer and then used to measure the "pH" of a nonaqueous solution or suspension, the ionization constant of the acid or base, the dielectric constant of the medium, the liquid-junction potential (which may give rise to errors of approximately 1 pH unit), and the hydrogen-ion response of the glass electrode are all changed. For these reasons, the values so obtained with solutions that are only partially aqueous in character can be regarded only as apparent pH values. However, acidity may be accurately measured with the proper use of electrodes and instrument standardization.

Buffer Solutions for Standardization of the pH Meter—

Buffer Solutions for Standardization are to be prepared as directed in the accompanying table.* Buffer salts of requisite purity can be obtained from the National Institute of Science and Technology. Solutions may be stored in chemically resistant, tight containers, such as Type I glass bottles. Fresh solutions should be prepared at intervals not to exceed 3 months. The table indicates the pH of the buffer solutions as a function of temperature. The instructions presented here are for the preparation of solutions having the designated molal (m) concentrations. For convenience, and to facilitate their preparation, however, instructions are given in terms of dilution to a 1000-mL volume rather than specifying the use of 1000 g of solvent, which is the basis of the molality system of solution concentration. The indicated quantities cannot be computed simply without additional information.

Potassium Tetraoxalate, 0.05 *m*—Dissolve 12.61 g of $KH_3(C_2O_4)_2 \cdot 2H_2O$ in water to make 1000 mL.

Potassium Biphthalate, 0.05 *m*—Dissolve 10.12 g of $KHC_8H_4O_4$, previously dried at 110° for 1 hour, in water to make 1000 mL.

Equimolal Phosphate, 0.05 *m*—Dissolve 3.53 g of Na_2HPO_4 and 3.39 g of KH_2PO_4, each previously dried at 120° for 2 hours, in water to make 1000 mL.

[4] For example, ASTM F322-80. The National Bureau of Standards SRM 1960, approximately 10-μm spheres, is useful in this regard.

* Commercially available buffer solutions for pH meter standardization, standardized by methods traceable to the National Institute of Science and Technology (NIST), labeled with a pH value accurate to 0.01 pH unit, and provided with a table showing the pH values at various temperatures, may be used. Solutions prepared from ACS reagent grade materials or other suitable materials, in the stated quantities, may be used provided the pH of the resultant solution is the same as that of the solution prepared from the NBS certified material.

pH Values of Buffer Solutions for Standardization

Temperature, °C	Potassium Tetraoxalate, 0.05 *m*	Potassium Biphthalate, 0.05 *m*	Equimolal Phosphate, 0.05 *m*	Sodium Tetraborate, 0.01 *m*	Calcium Hydroxide, Saturated at 25°
10	1.67	4.00	6.92	9.33	13.00
15	1.67	4.00	6.90	9.28	12.81
20	1.68	4.00	6.88	9.23	12.63
25	1.68	4.01	6.86	9.18	12.45
30	1.68	4.02	6.85	9.14	12.29
35	1.69	4.02	6.84	9.10	12.13
40	1.69	4.04	6.84	9.07	11.98
45	1.70	4.05	6.83	9.04	11.84
50	1.71	4.06	6.83	9.01	11.71
55	1.72	4.08	6.83	8.99	11.57
60	1.72	4.09	6.84	8.96	11.45

Sodium Tetraborate, 0.01 *m*—Dissolve 3.80 g of $Na_2B_4O_7 \cdot 10H_2O$ in water to make 1000 mL. Protect from absorption of carbon dioxide.

Calcium Hydroxide, saturated at 25°—Shake an excess of calcium hydroxide with water, and decant at 25° before use. Protect from absorption of carbon dioxide.

Because of variations in the nature and operation of the available pH meters, it is not practicable to give universally applicable directions for the potentiometric determinations of pH. The general principles to be followed in carrying out the instructions provided for each instrument by its manufacturer are set forth in the following paragraphs. Examine the electrodes and, if present, the salt bridge prior to use. If necessary, replenish the salt bridge solution, and observe other precautions indicated by the instrument or electrode manufacturer.

To standardize the pH meter, select two *Buffer Solutions for Standardization* whose difference in pH does not exceed 4 units and such that the expected pH of the material under test falls between them. Fill the cell with one of the *Buffer Solutions for Standardization* at the temperature at which the test material is to be measured. Set the "temperature" control at the temperature of the solution, and adjust the calibration control to make the observed pH value identical with that tabulated. Rinse the electrodes and cell with several portions of the second *Buffer Solution for Standardization*, then fill the cell with it, at the same temperature as the material to be measured. The pH of the second buffer solution is within ±0.07 pH unit of the tabulated value. If a larger deviation is noted, examine the electrodes and, if they are faulty, replace them. Adjust the "slope" or "temperature" control to make the observed pH value identical with that tabulated. Repeat the standardization until both *Buffer Solutions for Standardization* give observed pH values within 0.02 pH unit of the tabulated value without further adjustment of the controls. When the system is functioning satisfactorily, rinse the electrodes and cell several times with a few portions of the test material, fill the cell with the test material, and read the pH value. Use carbon dioxide–free water (see *Water*, in the section, *Reagents, Indicators, and Solutions*) for solution or dilution of test material in pH determinations.

Where approximate pH values suffice, indicators and test papers (see *Indicators and Indicator Test Papers*, in the section, *Reagents, Indicators, and Solutions*) may be suitable.

For a discussion of buffers, and for the composition of standard buffer solutions called for in compendial tests and assays, see *Buffer Solutions*, in the section, *Reagents, Indicators, and Solutions*.

⟨801⟩ POLAROGRAPHY

Polarography is an electrochemical method of analysis based on the measurement of the current flow resulting from the electrolysis of a solution at a polarizable microelectrode, as a function of an applied voltage. The polarogram (see Figure 1) obtained by this measurement provides qualitative and quantitative information on electro-reducible and electro-oxidizable substances. The normal concentration range for substances being analyzed is from 10^{-2} molar to 10^{-5} molar.

In direct current (dc) polarography, the microelectrode is a dropping mercury electrode (DME) consisting of small reproducible drops of mercury flowing from the orifice of a capillary tube connected to a mercury reservoir. A saturated calomel electrode (SCE) with a large surface area is the most commonly employed reference electrode. As the voltage applied to the cell increases, only a very small residual current flows until the substance under assay undergoes reduction or oxidation. Then the current increases, at first gradually, then almost linearly with voltage, and it gradually reaches a limiting value as is shown in Figure 1. On the initial rising portion of the polarographic wave, the increased flow of current results in a decrease in the concentration of the electro-active species at the electrode surface. As the voltage and current increase, the concentration of the reactive species decreases further to a minimal value at the electrode surface. The current is then limited by the rate at which the reacting species can diffuse from the bulk of the solution to the surface of the microelectrode. The final current rise is caused by the reaction of the supporting electrolyte. This large concentration of electrolyte is inert within the potential range used in the analysis, and it prevents the reactive species from reaching the electrode by electrical migration, thus assuring that the limiting current is diffusion-controlled.

Since, in the case of the DME, the electrode surface is being constantly renewed in a cyclic fashion, the current increases from a small value as the drop begins to form to a maximum value as the drop falls. By the use of a suitable recorder to measure the current, the characteristic saw-toothed record is obtained. The limiting current is the sum of the residual and the diffusion currents. The residual current is subtracted from the limiting current to give the wave height.

Ilkovic Equation—The linear relationship between the diffusion current (i_d) and the concentration of electro-active species is shown by the Ilkovic equation:

$$i_d = 708nD^{1/2}Cm^{2/3}t^{1/6},$$

in which i_d is the maximum current in microamperes, n is the number of electrons required per molecule of electro-active substance, D is its diffusion coefficient, in square cm per second, C is the concentration, in millimoles per liter, m is the rate of mercury flow from the DME, in mg per second, and t is the drop time, in seconds.

Modern polarographs are equipped with recorders capable of following the current during the latter portion of the drop life; consequently, the maximum of the oscillations is the measure of the current. When the current is measured only at the end of the drop life, the technique is termed sampled dc polarography. In this case, only the maximum currents are recorded and oscillations due to drop growth are not observed.

For instruments equipped with galvanometers to measure the current or recorders operated in a damped mode, the saw-toothed waves correspond to oscillations about the average current. In the latter case, the average of the oscillations is the measure of the current. For polarograms obtained in this manner, the i_d given by the Ilkovic equation is the average current in microamperes observed during the life of the drop, when the coefficient 708 is replaced by 607.

Control of the Diffusion Current—The Ilkovic equation identifies the variables that must be controlled to ensure that the diffusion current is directly proportional to the concentration of electro-active material. At 25° the diffusion coefficients for aqueous solutions of many ions and organic molecules increase

1% to 2% per degree rise in temperature. Thus the temperature of the polarographic cell must be controlled to within ±0.5°. The quantities m and t depend upon the dimensions of the capillary and the height of the mercury column above the electrode. Although results obtained with different capillaries can be compared if the product $m^{2/3}t^{1/6}$ is known, it is advisable to use the same capillary with a constant head of mercury during a series of analyses. The diffusion current is proportional to the square root of the height of the mercury column. A mercury reservoir with a diameter greater than 4 cm prevents any significant drop in the mercury level during a series of runs.

The capillary for the DME has a bore of approximately 0.04 mm and a length of 6 cm to 15 cm. The height of the mercury column, measured from the tip of the capillary to the top of the mercury pool, ranges from 40 cm to 80 cm. The exact length of the capillary and the height of the mercury column are adjusted to give a drop-time of between 3 and 5 seconds at open circuit with the capillary immersed in the test solution.

Equipment is available that allows controlled drop-times of fractions of a second to several seconds. As detail within a polarogram is related to the number of drops delivered during a given potential change, such short drop-times allow more rapid recording of the polarogram.

The current flowing through the test solution during the recording of a polarogram is in the microampere range. Thus, the current flow produces negligible changes in the test solution and several polarograms can be run on the same test solution without significant differences.

Half-wave Potential—The half-wave potential ($E_{1/2}$) occurs at the point on the polarogram one-half the distance between the residual current and the limiting current plateau. This potential is characteristic of the electro-active species and is largely independent of its concentration or the capillary used to obtain the wave. It is dependent upon the solution composition and may change with variations in the pH or in the solvent system or with the addition of complexing agents. The half-wave potential thus serves as a criterion for the qualitative identification of a substance.

The potential of the DME is equal to the applied voltage versus the reference electrode after correction for the iR drop (that potential need to pass the current, i, through the solution with a resistance R). It is especially important to make this correction for nonaqueous solutions, which ordinarily possess high resistance, if an accurate potential for the DME is needed. Correction of the half-wave potential is not required for quantitative analysis. Unless otherwise indicated, it is to be understood that potentials represent measurements made against the SCE.

Removal of Dissolved Oxygen—Inasmuch as oxygen is reduced at the DME in two steps, first to hydrogen peroxide and then to water, it interferes where polarograms are to be made at potentials more negative than about 0 volt versus SCE, and must be removed. This may be accomplished by bubbling oxygen-free nitrogen through the solution for 10 to 15 minutes immediately before recording the wave, the nitrogen first having been "conditioned" to minimize changes due to evaporation, by being passed through a separate portion of the solution.

It is necessary that the solution be quiet and vibration-free during the time the wave is recorded, to ensure that the current is diffusion-controlled. Therefore, the nitrogen aeration should be stopped and the gas be directed to flow over the surface of the solution before a polarogram is recorded.

In alkaline media, sodium bisulfite may be added to remove oxygen, provided the reagent does not react with other components of the system.

Measurement of Wave Height—To use a polarogram quantitatively, it is necessary to measure the height of the wave. Since this is a measure of the magnitude of the diffusion current, it is measured vertically. To compensate for the residual current, the segment of the curve preceding the wave is extrapolated beyond the rise in the wave. For a well-formed wave where this extrapolation parallels the limiting current plateau, the measurement is unambiguous. For less well-defined waves, the following procedure may be used unless otherwise directed in the individual monograph. Both the residual current and the limiting current are extrapolated with straight lines, as shown by the graph (Figure 1). The wave height is taken as the vertical distance between these lines measured at the half-wave potential.

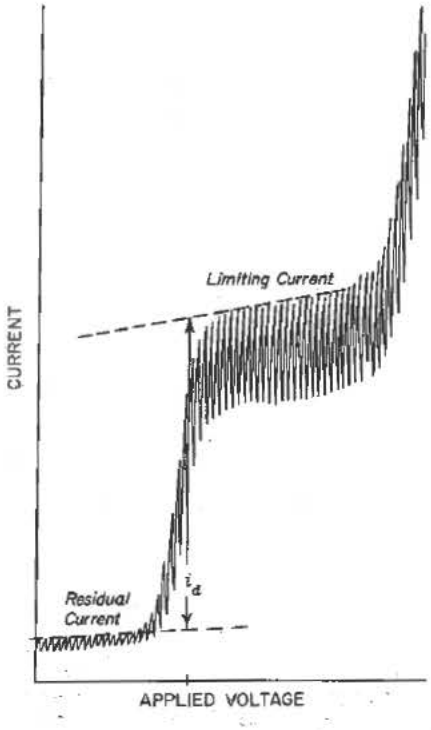


Fig. 1. Typical Polarogram Showing Change in Current Flow with Increasing Potential Applied to the Dropping Mercury Electrode.

Procedure—[*Caution—Mercury vapor is poisonous, and metallic mercury has a significant vapor pressure at room temperature. The work area in which mercury is used should be constructed in such a way that any spilled or spattered droplets of mercury can be completely recovered with relative ease. Scrupulously clean up mercury after each use of the instrument. Work in a well-ventilated laboratory, taking care to clean up any spilled mercury.*] Transfer a portion of the final dilution of the substance being assayed to a suitable polarographic cell immersed in a water bath regulated to 25 ± 0.5°. Pass a stream of nitrogen through the solution for 10 to 15 minutes to remove dissolved oxygen. Start the mercury dropping from the capillary, insert the capillary into the test solution, and adjust the height of the mercury reservoir. Switch the flow of nitrogen to pass over the surface of the solution, and record the polarogram over the potential range indicated in the individual monograph, using the appropriate recorder or galvanometer sensitivity to give a suitable wave. Measure the height of the wave, and unless otherwise directed in the monograph, compare this with the wave height obtained with the appropriate USP Reference Standard, measured under the same conditions.

Pulse Polarography—In conventional dc polarography, the current is measured continuously as potential is applied as a linear ramp (see Figure 2). This current is composed of two elements. The first, the diffusion (faradaic) current, is produced by the substance undergoing reduction or oxidation at the working electrode, and is directly proportional to the concentration of this substance. The second element is the capacitative current (charging of the electrochemical double layer). The changes in these currents as the mercury drop varies in size produce the oscillations present in typical dc polarograms.

In normal pulse polarography, a potential pulse is applied to the mercury electrode near the end of the drop life, with the drop being held at the initial potential during growth period (see Figure 3). Each succeeding drop has a slightly higher pulse applied to it, with the rate of increase being determined by the selected scan rate. The current is measured at the end of the pulse where the capacitative current is nearly zero, and thus primarily faradaic current is measured (see Figure 4). In addition, since the pulse is applied for only a short duration, the diffusion layer is not depleted as extensively as in dc polarography and larger current levels are obtained for equivalent concentrations. Concentrations as low as 10^{-6} M can be measured, providing approximately a ten-fold increase in sensitivity over that with dc polarography.

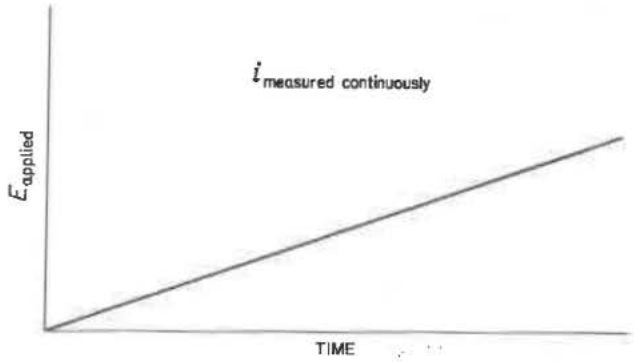


Fig. 2. Direct Current (dc) Polarography.

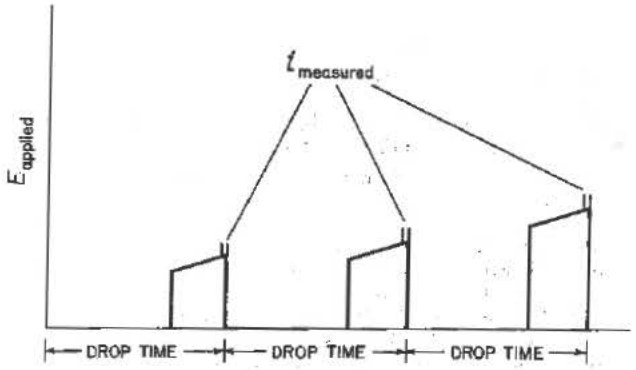


Fig. 3. Pulse Polarography.

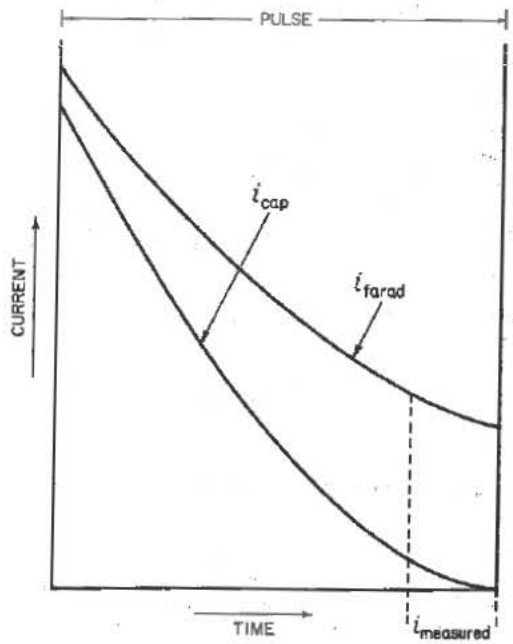


Fig. 4. Plot of Current Versus Time in Pulse Polarography.

Limiting current values are more easily measured, since the waves are free from oscillations.

Differential pulse polarography is a technique whereby a fixed-height pulse applied at the end of the life of each drop is superimposed on a linear increasing dc ramp (see Figure 5). Current flow is measured just before application of the pulse and again at the end of the pulse. The difference between these two currents is measured and presented to the recorder. Such a differential signal provides a curve approximating the derivative of the polarographic wave, and gives a peak presentation. The peak potential is equivalent to:

$$E_{1/2} - \Delta E/2,$$

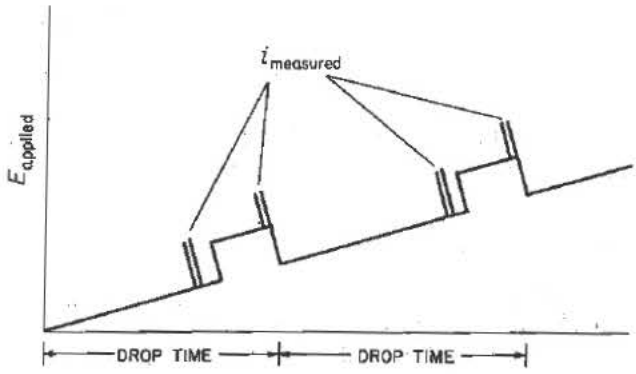


Fig. 5. Differential Pulse Polarography.

where ΔE is the pulse height. The peak height is directly proportional to concentration at constant scan rates and constant pulse heights. This technique is especially sensitive (levels of 10^{-7} M may be determined) and affords improved resolution between closely spaced waves.

Anodic Stripping Voltammetry—Anodic stripping voltammetry is an electrochemical technique whereby trace amounts of substances in solution are concentrated (reduced) onto an electrode and then stripped (oxidized) back into solution by scanning the applied voltage anodically. The measurement of the current flow as a function of this voltage and scanning rate provides qualitative and quantitative information on such substances. The concentration step permits analyses at 10^{-7} M to 10^{-9} M levels.

Basic instrumentation includes a voltage ramp generator; current-measuring circuitry; a cell with working, reference, and counter electrodes; and a recorder or other read-out device. Instruments having dc or pulse-polarographic capabilities are generally quite adequate for stripping application. The working electrode commonly used is the hanging mercury drop electrode (HMDE), although the mercury thin-film electrode (MTFE) has acquired acceptance. For analysis of metals such as silver, platinum, and gold, whose oxidation potentials are more positive than mercury, and mercury itself, the use of solid electrodes such as platinum, gold, or carbon is required. A saturated calomel electrode or a silver–silver chloride electrode serves as the reference except for the analysis of mercury or silver. A platinum wire is commonly employed as the counter electrode.

Test specimens containing suitable electrolyte are pipeted into the cell. Dissolved oxygen is removed by bubbling nitrogen through the cell for 5 to 10 minutes.

Generally, an electrolysis potential equivalent to 200 to 300 mV more negative than the half-wave potential of the material to be analyzed is applied (although this potential is to be determined experimentally), with stirring for 1 to 10 minutes. For reproducible results, maintain constant conditions (i.e., deposition time, stirring rate, temperature, specimen volume, and drop size if HMDE is used).

After deposition, the stirring is discontinued and the solution and electrode are allowed to equilibrate for a short period. The potential is then rapidly scanned anodically (10 mV/second or greater in dc polarography and 5 mV/second in differential pulse polarography). As in polarography, the limiting current is proportional to concentration of the species (wave height in dc and pulse; peak height in differential pulse), while the half-wave potential (dc, pulse) or peak potential (differential pulse) identifies the species. It is imperative that the choice of supporting electrolyte be made carefully in order to obtain satisfactory behavior. Quantitation is usually achieved by a standard addition or calibration method.

This technique is appropriate for trace-metal analysis, but has limited use in organic determinations, since many of these reactions are irreversible. In analyzing substances such as chloride, cathodic stripping voltammetry may be used. The technique is the same as anodic stripping voltammetry, except that the substance is deposited anodically and then stripped by a cathodic voltage scan.

Table 1. Classification of Powders by Fineness.

Classification of Powder	Vegetable and Animal Drugs			Chemicals		
	Nominal Designation No.[1] of Powder	Fineness Limit[2]		Nominal Designation No.[1] of Powder	Fineness Limit[2]	
		%	Sieve No.		%	Sieve No.
Very coarse	8	20	60			
Coarse	20	40	60	20	60	40
Moderately coarse	40	40	80	40	60	60
Fine	60	40	100	80	60	120
Very fine	80	100	80	120	100	120

[1] All particles of the powder pass through a sieve of the nominal designation.
[2] Designates the limit of the percentage that passes through a sieve of the size designated.

⟨811⟩ POWDER FINENESS

The fineness of powders in this Pharmacopeia, expressed in descriptive terms, is related to the number assigned to a standard (U. S. Series) sieve, as indicated in Table 1.

For practical reasons, sieves are the preferred means of measuring powder fineness for most pharmaceutical purposes; however, their applicability does not extend into the range of particle size that is of increasing interest with respect to the attainment of prompt and complete gastrointestinal absorption of administered drugs. For the measurement of particles less than 100 μm in nominal size, devices other than sieves may be more useful.

The efficiency and speed of particle separation by sieves vary inversely with the number of particles in the charge. The effectiveness of separation falls off rapidly when the depth of the charge exceeds a layer of 6 to 8 particles.

Sieves for Pharmacopeial Testing—Sieves for Pharmacopeial testing are of wire cloth woven, not twilled, except the cloth for the sizes Nos. 230, 270, 325, and 400, from brass, bronze, stainless steel, or other suitable wire, and are not coated or plated. Table 2 gives the average dimensions of the openings of woven wire cloth standard sieves.

For details on the standardization of sieves, reference may be made to Specification E11-70 of the American Society for Testing and Materials. For use in the evaluation of the effective opening of test sieves in the size range of No. 20 through No. 70, Standard Glass Spheres are available from the National Bureau of Standards as Standard Reference Material 1018.

Table 2. Openings of Standard Sieves.

Sieve Designation	
Nominal Designation No.	Sieve Opening
2[1]	9.5 mm
3.5	5.6 mm
4	4.75 mm
8	2.36 mm
10	2.00 mm
14	1.40 mm
16	1.18 mm
18	1.00 mm
20	850 μm
25	710 μm
30	600 μm
35	500 μm
40	425 μm
45	355 μm
50	300 μm
60	250 μm
70	212 μm
80	180 μm
100	150 μm
120	125 μm
200	75 μm
230	63 μm
270	53 μm
325	45 μm
400	38 μm

[1] Designated as ⅜ inch in ASTM Specification E11-70.

Powdered Vegetable and Animal Drugs—In determining the powder fineness of a vegetable or animal drug, no portion of the drug may be rejected during milling or sifting unless specifically permitted in the individual monograph.

Method for Determining Uniformity of Fineness—For determining uniformity of degree of fineness of powdered drugs and chemicals, the following process may be used, employing standard testing sieves that meet the requirements set forth above. Avoid prolonged shaking that would result in increasing the fineness of the powder during the testing.

For *very coarse, coarse,* and *moderately coarse powders,* place 25 to 100 g of the powder to be tested upon the appropriate standard sieve having a close-fitting receiving pan and cover. Shake the sieve in a rotary horizontal direction and vertically by tapping on a hard surface for not less than 20 minutes or until sifting is practically complete. Weigh accurately the amount remaining on the sieve and in the receiving pan.

In the case of *fine* or *very fine powders,* proceed as for *coarse powders,* except that the test specimen should not exceed 25 g, and except that the sieve is to be shaken for not less than 30 minutes or until sifting is practically complete.

In the case of oily or other powders that tend to clog the openings, carefully brush the screen at intervals during the test. Break up lumps that form during the sifting.

The fineness of a powdered drug or chemical may be determined also by screening through standard sieves in a *mechanical sieve shaker,* which reproduces the circular and tapping motion given to testing sieves in hand sifting but with a uniform mechanical action, following the directions provided by the manufacturer of the shaker.

⟨821⟩ RADIOACTIVITY

Radioactive pharmaceuticals require specialized techniques in their handling and testing in order that correct results may be obtained and hazards to personnel be minimized. All operations should be carried out or supervised by personnel having had expert training in handling radioactive materials.

The facilities for the production, use, and storage of radioactive pharmaceuticals are generally subject to licensing by the federal Nuclear Regulatory Commission, although in certain cases this authority has been delegated to state agencies. The federal Department of Transportation regulates the conditions of shipment of radioactive materials. State and local agencies often have additional special regulations. Each producer or user must be thoroughly cognizant of the applicable regulations of the federal Food, Drug, and Cosmetic Act, and any additional requirements of the U. S. Public Health Service and of state and local agencies pertaining to the articles concerned.

Definitions, special considerations, and procedures with respect to the Pharmacopeial monographs on radioactive drugs are set forth in this chapter.

GENERAL CONSIDERATIONS

Fundamental Decay Law

The decay of a radioactive source is described by the equation:

$$N_t = N_o e^{-\lambda t},$$

in which N_t is the number of atoms of a radioactive substance at elapsed time t, N_o is the number of those atoms when $t = 0$, and λ is the transformation or decay constant, which has a characteristic value for each radionuclide. The *half-life*, $T_{1/2}$, is the time interval required for a given activity of a radionuclide to decay to one-half of its initial value, and is related to the decay constant by the equation:

$$T_{1/2} = \frac{0.69315}{\lambda}.$$

The activity of a radioactive source (A) is related to the number of radioactive atoms present by the equation:

$$A = \lambda N,$$

from which the number of radioactive atoms at time t can be computed, and hence the mass of the radioactive material can be determined.

The activity of a pure radioactive substance as a function of time can be obtained from the exponential equation or from decay tables, or by graphical means based on the half-life (see Normalized Decay Chart, Figure 1).

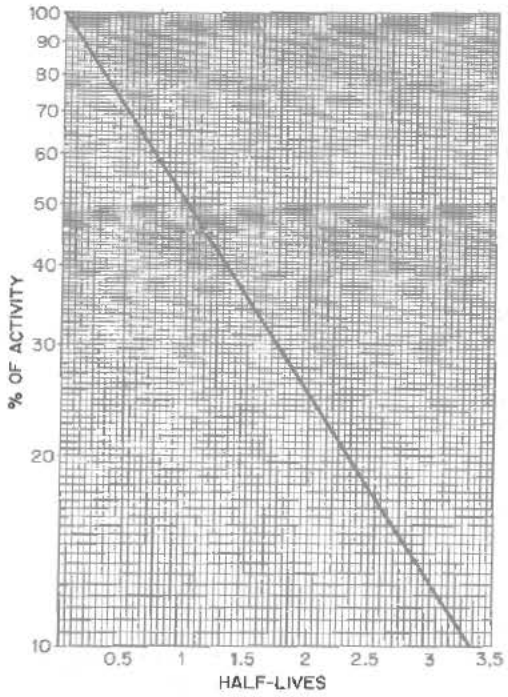


Fig. 1. Normalized Decay Chart.

The activity of a radioactive material is expressed as the number of nuclear transformations per unit time. The fundamental unit of radioactivity, the *curie* (Ci), is defined as 3.700×10^{10} nuclear transformations per second. The *millicurie* (mCi) and *microcurie* (μCi) are commonly used subunits. The "number of nuclear transformations per unit time" is the sum of rates of decay from all competing modes of disintegration of the parent nuclide. Before the activity of any given radionuclide in a measured specimen can be expressed in curies, it is often necessary to know the abundance(s) of the emitted radiation(s) measured.

Geometry

The validity of relative calibration and measurement of radionuclides is dependent upon the reproducibility of the relationship of the source to the detector and its surroundings. Appropriate allowance must be made for source configuration.

Background

Cosmic rays, radioactivity present in the detector and shielding materials, and radiation from nearby radioactive sources not properly shielded from the measuring equipment, all contribute to the background count rate. All radioactivity measurements must be corrected by subtracting the background count rate from the gross count rate in the test specimen.

Statistics of Counting

Since the process of radioactive decay is a random phenomenon, the events being counted form a random sequence in time. Therefore, counting for any finite time can yield only an estimate of the true counting rate. The precision of this estimate, being subject to statistical fluctuations, is dependent upon the number of counts accumulated in a given measurement and can be expressed in terms of the standard deviation σ. An *estimate* for σ is $\sqrt{n}$, where n is the number of counts accumulated in a given measurement. The probability of a single measurement falling within $\pm 100/\sqrt{n}\%$ of the mean of a great many measurements is 0.68. That is, if many measurements of n counts each were to be made, approximately two-thirds of the observations would lie within $\pm 100/\sqrt{n}\%$ of the mean, and the remainder outside.

Because of the statistical nature of radioactive decay, repeated counting of an undisturbed source in a counting assembly will yield count-rate values in accordance with the frequency of a normal distribution. Deviations in these values from the normal distribution conform to the χ^2 test. For this reason, the χ^2 test is frequently applied to determine the performance and correct operation of a counting assembly. In the selection of instruments and conditions for assay of radioactive sources, the figure of merit ϵ^2/B should be maximized (where ϵ = counter efficiency = observed count rate/sample disintegration rate, and B = background count rate).

Counting Losses

The minimum time interval that is required for the counter to resolve two consecutive signal pulses is known as the dead time. The dead time varies typically from the order of microseconds for proportional and scintillation counters, to hundreds of microseconds for Geiger-Müller counters. Nuclear events occurring within the dead time of the counter will not be registered. To obtain the corrected count rate, R, from the observed count rate, r, it is necessary to use the formula:

$$R = \frac{r}{1 - r\tau},$$

in which τ is the dead time. The foregoing correction formula assumes a nonextendable dead time. Thus, for general validity, the value of $r\tau$ should not exceed 0.1. The observed count rate, r, refers to the gross specimen count rate and is not to be corrected for background before use in the foregoing equation.

Calibration Standards

Perform all radioactivity assays using measurement systems calibrated with appropriately certified radioactivity standards. Such calibration standards may be purchased either direct from the National Institute of Standards and Technology or from other sources that have established traceability to the National Institute of Standards and Technology through participation in a program of inter-comparative measurements. Where such calibration standards are unavailable, the Pharmacopeia provides the nuclear decay data required for calibration. These data, as well as half-life values, are obtained from the Evaluated Nuclear Structure Data File of the Oak Ridge Nuclear Data Project, and reflect the most recent values at the time of publication.

Carrier

The total mass of radioactive atoms or molecules in any given radioactive source is directly proportional to the activity of the radionuclide for a given half-life, and the amount present in radiopharmaceuticals is usually too small to be measured by ordinary chemical or physical methods. For example, the mass of ^{131}I having an activity of 100 mCi is 8×10^{-7} g. Since such small amounts of material behave chemically in an anomalous

manner, carriers in the form of nonradioactive isotopes of the same radionuclide may be added during processing to permit ready handling. In many cases, adsorption can be prevented merely by increasing the hydrogen-ion concentration of the solution. Amounts of such material, however, must be sufficiently small that undesirable physiological effects are not produced. The term "carrier-free" refers only to radioactive preparations in which nonradioactive isotopes of the radionuclide are absent. This implies that radioactive pharmaceuticals produced by means of (n, γ) reactions cannot be considered carrier-free.

The activity per unit volume or weight of a medium or vehicle containing a radionuclide either in the carrier-free state or in the presence of carrier is referred to as the radioactive concentration, whereas the term specific activity is used to express the activity of a radionuclide per gram of its element.

Radiochemical Purity

Radiochemical purity of a radiopharmaceutical preparation refers to the fraction of the stated radionuclide present in the stated chemical form. Radiochemical impurities in radiopharmaceuticals may result from decomposition and from improper preparative procedures. Radiation causes decomposition of water, a main ingredient of most radiopharmaceuticals, leading to the production of reactive hydrogen atoms and hydroxyl radicals, hydrated electrons, hydrogen, hydrogen ions, and hydrogen peroxide. The last-mentioned is formed in the presence of oxygen radicals, originating from the radiolytic decomposition of dissolved oxygen. Many radiopharmaceuticals show improved stability if oxygen is excluded. Radiation may also affect the radiopharmaceutical itself, giving rise to ions, radicals, and excited states. These species may combine with one another and/or with the active species formed from water. Radiation decomposition may be minimized by the use of chemical agents that act as electron or radical scavengers. Electrons trapped in solids cause discoloration due to formation of F-centers and the darkening of glass containers for radiopharmaceuticals, a situation that typifies the case. The radiochemical purity of radiopharmaceuticals is determined by column, paper, and thin-layer chromatography or other suitable analytical separation techniques as specified in the individual monograph.

Radionuclidic Purity

Radionuclidic purity of a radiopharmaceutical preparation refers to the proportion of radioactivity due to the desired radionuclide in the total radioactivity measured. Radionuclidic purity is important in the estimation of the radiation dose received by the patient when the preparation is administered. Radionuclidic impurities may arise from impurities in the target materials, differences in the values of various competing production cross-sections, and excitation functions at the energy or energies of the bombarding particles during production.

Terms and Definitions

The *date of manufacture* is the date on which the manufacturing cycle for the finished product is completed.

The *date of assay* is the date (and time, if appropriate) when the actual assay for radioactivity is performed.

The *date of calibration* is an arbitrary assigned date and time to which the radioactivity of the product is calculated for the convenience of the user.

The *expiration date* is the date that establishes a limit for the use of the product. The expiration period (i.e., the period of time between the date of manufacture and the expiration date) is based on a knowledge of the radioactive properties of the product and the results of stability studies on the finished dosage form.

Labeling

Individual radiopharmaceutical monographs indicate the expiration date, the calibration date, and the statement, "Caution—Radioactive Material." The labeling indicates that in making dosage calculations, correction is to be made for radioactive decay, and also indicates the radioactive half-life of the radionuclide. Articles that are Injections comply with the requirements for *Labeling* under *Injections* ⟨1⟩, and those that are Biologics comply with the requirements for *Labeling* under *Biologics* ⟨1041⟩.

IDENTIFICATION AND ASSAY OF RADIONUCLIDES

Instrumentation

IONIZATION CHAMBERS

An ionization chamber is an instrument in which an electric field is applied across a volume of gas for the purpose of collecting ions produced by a radiation field. The positive ions and negative electrons drift along the lines of force of the electric field, and are collected on electrodes, producing an ionization current. In a properly designed well-type ionization chamber, the ionization current should not be too dependent on the position of the radioactive specimen, and the value of the current per unit activity, known as the calibration factor, is characteristic of each gamma-ray-emitting radionuclide.

The ionization current produced in an ionization chamber is related to the mean energy of the emitted radiation and is proportional to the intensity of the radiation. If standard sources of known disintegration rates are used for efficiency calibration, the ionization chamber may then be used for activity determinations between several microcuries and several hundred millicuries or more. The upper limit of activity that may be measured in an ionization chamber usually is not sharply defined and may be limited by saturation considerations, range of the amplifier, and design of the chamber itself. The data supplied with or obtained from a particular instrument should be reviewed to ascertain the useful ranges of energies and intensities of the device.

Reproducibility within approximately 5% or less can be readily obtained in about 10 seconds, with a deep re-entrant well-type chamber. The most commonly used form of ionization chamber for measurement of the activities of radiopharmaceuticals is known as a dose calibrator.

Although the calibration factor for a radionuclide may be interpolated from an ionization chamber energy-response curve, there are a number of sources of error possible in such a procedure. It is therefore recommended that all ionization chamber calibrations be performed with the use of authentic reference sources of the individual radionuclides, as described hereinafter.

The calibration of a dose calibrator should be maintained by relating the measured response of a standard to that of a long-lived performance standard, such as radium 226 in equilibrium with its daughters. The instrument must be checked daily with the ^{226}Ra or other source to ascertain the stability over a long period of time. This check should include performance standard readings at all radionuclide settings employed. To obtain the activity (A_x) of the radionuclide being measured, use the relationship:

$$A_x = \frac{R_c R}{R_n},$$

in which R_n is the new reading for the radium or other source, R_c is the reading for the same source obtained during the initial calibration procedure, and R is the observed reading for the radionuclide specimen. Obviously, any necessary corrections for radioactive decay of the reference source must first be applied. Use of this procedure should minimize any effects due to drift in the response of the instrument. The recommended activity of the ^{226}Ra or other monitor used in the procedure described above is 75 to 150 μCi. It is recommended also that the reproducibility and/or stability of multirange instruments be checked for all ranges with the use of appropriate standards.

The size and shape of a radioactive source may affect the response of a dose calibrator, and it is often necessary to apply a small correction when measuring a bulky specimen.

SCINTILLATION AND SEMICONDUCTOR DETECTORS

When all or part of the energy of beta or gamma radiation is dissipated within scintillators, photons of intensity proportional to the amount of dissipated energy are produced. These pulses are detected by an electron multiplier phototube and converted to electrical pulses, which are subsequently analyzed with a pulse-height analyzer to yield a pulse-height spectrum related to the energy spectrum of the radiation emitted by the source. In general, a beta-particle scintillation pulse-height spectrum approximates the true beta-energy spectrum, provided that the beta-

particle source is prepared in such a manner that self-absorption is minimized. Beta-ray spectra may be obtained by using calcium fluoride or anthracene as the scintillator, whereas gamma-ray spectra are usually obtained with a thallium-activated sodium iodide crystal or a large-volume lithium-drifted germanium semiconductor detector. The spectra of charged particles also may be obtained using silicon semiconductor detectors and/or gas proportional counters. Semiconductor detectors are in essence solid-state ionization chambers, but the energy required to create an electron-hole pair or to promote an electron from the valence band to the conduction band in the semiconductor is about one-tenth the energy required for creation of an ion-pair in a gas-filled ionization chamber or proportional counter and is far less than the energy needed to produce a photon in a NaI(Tl) scintillation crystal. In gamma-ray spectrometry, a Ge(Li) detector can yield an energy resolution of 0.33% for 1.33 MeV gamma-rays from ^{60}Co, while a 3- × 3-inch NaI(Tl) crystal can give a value of 5.9% for the same gamma-ray energy. The energy resolution is a measure of the ability to distinguish the presence of two gamma rays closely spaced in energy and is defined by convention as the full width of the photopeak at its half maximum (FWHM), expressed in percentage of the photopeak energy.

Gamma-ray spectra exhibit one or more sharp, characteristic photopeaks, or full-energy peaks, as a result of total absorption in the detector of the full energy of gamma radiations from the source; these photopeaks are useful for identification purposes. Other secondary peaks are observed as a consequence of back-scatter, annihilation radiation, coincidence summing, fluorescent X-rays, etc., accompanied by a broad band known as the Compton continuum arising from scattering of the photons in the detector and from surrounding materials. Since the photopeak response varies with gamma-ray energy, calibration of a gamma-ray spectrometer should be achieved with radionuclide standards having well-known gamma-ray energies and emission rates. The shape of the gamma-ray spectrum is dependent upon the shape and size of the detector and the types of shielding materials used.

When confirming the identity of a radionuclide by gamma-ray spectrometry, it is necessary to make a comparison of the specimen spectrum with that of a specimen of known purity of the same radionuclide obtained under *identical instrument parameters and specimen geometry*. Where the radionuclides emit coincident X- or gamma-radiations, the character of the pulse-height distribution often changes quite dramatically because of the summing effect of these coincident radiations in the detector as the efficiency of detection is increased (e.g., by bringing the source closer to the detector). Such an effect is particularly evident in the case of iodine 125. Among the more useful applications of gamma-ray spectrometry are those for the identification of radionuclides and the determination of radionuclidic impurities.

Where confirmation of the identity of a given radionuclide by means of a direct comparison with the spectrum of a specimen of the same radionuclide of known purity is not possible, the identity of the radionuclide in question must then be established by the following method. Two or more of the following nuclear decay scheme parameters of the radionuclide specimen to be identified shall be measured, and agreement shall be within ± 10%: (1) half-life, (2) energy of each gamma- or X-ray emitted, (3) the abundance of each emission, and (4) E_{max} for those radionuclides that decay with beta-particle emissions. Such measurements are to be performed as directed in the *Identification* and *Assay* sections of this chapter. Agreement of two or more of the measured parameters with the corresponding published nuclear decay scheme data constitutes confirmation of the identity of the radionuclide.

LIQUID-SCINTILLATION COUNTERS

Alpha- and beta-emitting radionuclides may be assayed with the use of a liquid-scintillation detector system. In the liquid scintillator, the radiation energy is ultimately converted into light quanta that are usually detected by two multiplier phototubes so arranged as to count only coincidence radiation. The liquid scintillator is a solution consisting of a solvent, primary and secondary solutes, and additives. The charged particle dissipates its energy in the solvent, and a fraction of this energy is converted into fluorescence in the primary solute. The function of the secondary solute is to shift the fluorescence radiation to longer wavelengths that are more efficiently detected by the multiplier phototubes. Frequently used solvents are toluene and *p*-xylene; primary solutes are 2,5-diphenyloxazole (PPO) and 2-(4′-*tert*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (butyl-PBD); and secondary solutes are 2,2′-*p*-phenylenebis[4-methyl-5-phenyloxazole] (dimethyl-POPOP) and *p*-bis(*o*-methylstrylyl)benzene (bis-MSB). As a means of attaining compatibility and miscibility with aqueous specimens to be assayed, many additives, such as surfactants and solubilizing agents, are also incorporated into the scintillator. For an accurate determination of radioactivity of the specimen, care must be exercised to prepare a specimen that is truly homogeneous. The presence of impurities or color in solution causes a decrease in photon output of the scintillator; such a decrease is known as quenching. Accurate radioactivity measurement requires correcting for count-rate loss due to quenching.

The disintegration rate of a beta-particle source may be determined by a procedure in which the integral count rate of the specimen is measured as a function of the pulse-height discriminator bias, and the emission rate is then obtained by extrapolation to zero bias. Energetic alpha-particle emitters may be similarly measured by this method.

Identification

A radionuclide can be identified by its mode of decay, its half-life, and the energies of its nuclear emissions.

The radioactive half-life is readily determined by successive counting of a given source of the radionuclide over a period of time that is long compared to its half-life. The response of the counting assembly when employed for the decay measurement of long-lived radionuclides should be monitored with an even longer-lived reference source to assess and compensate for errors arising from electronic drift. In the case of short-lived radionuclides, when the counting period constitutes a significant fraction of the half-life of the radionuclide, the recorded count rate must be corrected to the time when the count is initiated, as follows:

$$R_i = \frac{r\lambda t}{1 - e^{-\lambda t}},$$

in which R_i is the count rate at the beginning of a counting period, r is the count rate observed over the entire counting period, t is the duration of the counting period, λ is the decay constant of the radionuclide, and e is the base of the natural logarithm. When t is small compared to the half-life of the radionuclide under study so that $\lambda t < 0.05$, then $(1 - e^{-\lambda t})$ approaches λt, and no such correction is necessary.

The energy of nuclear emissions is often determined by the maximum range of penetration of the radiation in matter (in the case of alpha- and beta-particles) and by the full-energy peak or photopeak in the gamma-ray spectrum (in the case of X- and gamma-rays). Since beta-particles are emitted with a continuous energy spectrum, the maximum beta-energy, E_{max}, is a unique index for each beta-emitting radionuclide. In addition to the maximum range and energy spectrum of the beta-particles, the absorption coefficient, when obtained under reproducible counting conditions, can serve as a reliable index for identification of a beta-emitter. Fortuitously, beta-particles are absorbed in matter in an approximately exponential manner, and a plot of the logarithm of the beta-particle count rate as a function of the absorber thickness is known as the absorption curve. The initial portion of the absorption curve shows linearity from which the absorption coefficient can be obtained. The maximum range is determined by the use of absorbers of varying thickness, and the energy spectrum is measured by beta-ray scintillation spectrometry.

The absorption of gamma-rays in matter is strictly exponential, but the half-value layers of attenuation have not been very useful for the purpose of radionuclide characterization. Gamma-rays from each isomeric transition are mono-energetic; their energy can be directly measured by gamma-ray spectrometry. Because of their high energy resolution, solid-state detectors [Ge(Li)] are vastly superior to scintillation detectors [NaI(Tl)] in gamma-ray spectrometry.

The activities of radiopharmaceutical solutions are frequently in the range of millicuries per mL. Such solutions usually must be extensively diluted before they can be accurately assayed. The diluent should be compatible with the radiopharmaceutical with respect to factors such as pH and redox potentials, so that no hydrolysis or change in oxidation state occurs upon dilution, which

could lead to adsorption and separation of the radionuclide from solution.

BETA-EMITTING RADIONUCLIDES

Mass Absorption Coefficient Procedure—Deposit and dry an aliquot of the radioactive phosphorus 32 solution on a thin plastic film to minimize backscattering, and place it under a suitable counter. Determine the counting rates successively, using not less than six different "thicknesses" of aluminum each between 20 and 50 mg/cm^2 and a single absorber thicker than 800 mg/cm^2, which is used to measure the background. (The absorbers are inserted between the test specimen and the counter but are placed nearer the counter window to minimize scattering.) Net beta-particle count rates are obtained after subtraction of the count rate found with the absorber having a thickness of 800 mg/cm^2 or greater. Plot the logarithm of the net beta-particle count rate as a function of the total absorber "thickness." The total absorber "thickness" is the "thickness" of the aluminum absorbers plus the "thickness" of the counter window (as stated by the manufacturer) plus the air-equivalent "thickness" (the distance in centimeters of the specimen from the counter window multiplied by 1.205 mg/cm^3 at 20° and 76 cm of mercury), all expressed in mg/cm^2. An approximately straight line results.

Choose two total absorber "thicknesses" that differ by 20 mg/cm^2 or more and that fall on the linear plot, and calculate the mass absorption coefficient, μ, by the equation:

$$\mu = \frac{1}{t_2 - t_1} \cdot \ln \left(\frac{N_{t_1}}{N_{t_2}}\right) = \frac{2.303}{t_2 - t_1} (\log N_{t_1} - \log N_{t_2})$$

in which t_1 and t_2 represent the total absorber "thicknesses," in mg/cm^2, t_2 being the thicker absorber, and N_{t_1} and N_{t_2} being the net beta-particle rates with the t_1 and t_2 absorbers, respectively.

For characterization of the radionuclide, the mass absorption coefficient should be within ±5% of the value found for a pure specimen of the same radionuclide when determined under identical counting conditions and geometry.

Other Methods of Identification—Other methods for determining the identity of a beta emitter also rely upon the determination of E_{max}. This may be accomplished in several ways. For example, (1) utilization of the range energy relationships of beta particles in an absorber, or (2) determination of E_{max} from a beta-particle spectrum obtained on an energy-calibrated beta-spectrometer using a thin source of the radionuclide (see *Scintillation and Semiconductor Detectors* in this chapter).

GAMMA-EMITTING RADIONUCLIDES

The gamma-ray spectrum of a radionuclide is a valuable tool for the qualitative identification of gamma-ray emitting radionuclides. The full-energy peak, or the photopeak, is identified with the gamma-ray transition energy that is given in the decay scheme of the radionuclide.

In determining radionuclidic identity and purity, the gamma-ray spectrum of a radioactive substance is obtained with either a NaI(Tl) crystal or a semiconductor Ge(Li) detector. The latter has an energy resolution more than an order of magnitude better than the former and is highly preferred for analytical purposes. The spectrum obtained shall be identical in shape to that of a specimen of the pure radionuclide, measured with the same detection system and in the same geometry. The gamma-ray spectrum of the radiopharmaceutical shall contain only photopeaks identifiable with the gamma-ray transition energies found in the decay scheme of the same radionuclide. For low geometrical efficiencies, the areas under the photopeaks, after correction for the measured detector efficiency, shall be proportional to the abundances or emission rates of the respective gamma-rays in the radionuclide.

RADIONUCLIDIC IMPURITIES

Because they are extremely toxic, alpha-emitting nuclides must be strictly limited in radiopharmaceutical preparations. Procedures for identifying beta- and gamma-active radionuclides as given in the foregoing text are applicable to the detection of gamma and usually beta contaminants.

The gross alpha-particle activity in radiopharmaceutical preparations can be measured by the use of a windowless proportional counter or a scintillation detector employing a silver-activated zinc-sulfide phosphor or by the techniques of liquid-scintillation counting.

The heavy ionization caused by alpha particles allows the measurement of alpha-emitting radionuclides in the presence of large quantities of beta- and gamma-active nuclides by the use of appropriate techniques for discriminating the amplitudes of signal pulses. In proportional counting, the operating voltage region for counting alpha particles, referred to as the "alpha plateau," is considerably lower than the "beta plateau" for counting beta and gamma radiations. Typical "alpha plateau" and "beta plateau" voltage settings with P-10 counting gas are 900 to 1300 and 1600 to 2000 volts, respectively.

When silver-activated zinc-sulfide phosphor is employed for alpha-particle detection, the alpha particles can be distinguished from other interfering radiation by pulse-height discrimination. Care must be exercised to minimize self-absorption at the source whenever specimens are prepared for alpha-particle counting.

Assay

BETA-EMITTING RADIONUCLIDES

Procedure—The disintegration rate (A) of a beta-particle-emitting specimen is obtained by counting a quantitatively deposited aliquot in a fixed geometry according to the formula:

$$A = \frac{R}{\epsilon \times f_r \times f_b \times f_s},$$

in which ϵ is the counting efficiency of the counter; f_r is the correction factor for counter dead time; f_b is the correction factor for backscatter; and f_s is the correction factor for self-absorption. The count rate for zero absorber is obtained by extrapolation of the initial linear portion of the absorption curve to zero absorber "thickness," taking into consideration the mg/cm^2 "thickness" of specimen coverings, counter window, and the intervening air space between specimen and the counter window. The counter efficiency, ϵ, is determined by use of a long-lived secondary standard with similar spectral characteristics. RaD + E has frequently been used for efficiency calibration of counters for phosphorus 32. By the use of identical measurement conditions for the specimen and the standard (and extrapolation to zero absorber), the ratio of the values of f_r, f_b, and f_s for the standard and the specimen approaches unity.

The previous relationship is valid also when the counter has been calibrated with a standard of the radionuclide to be assayed. In this case, however, the extrapolations to zero absorber "thickness" for the specimen and standard are not required, as the two absorption corrections cancel for a given geometry.

Another useful and frequently employed method for the determination of the disintegration rate of beta-emitting radionuclides is liquid-scintillation counting, which also utilizes an extrapolation of the specimen count rate to zero pulse-height discriminator bias.

GAMMA-EMITTING RADIONUCLIDES

For the assay of gamma-emitting radionuclides, three methods are provided. The selection of the preferred method is dictated by the availability of a calibration standard of the radionuclide to be assayed and the radionuclidic purity of the article itself.

Direct comparison with a calibration standard is required if a calibration standard of the radionuclide to be assayed is available and if the upper limit of conceivable error in the activity determination arising from the presence of radionuclidic impurities has been determined to be less than 3%. If the required calibration standard is not routinely available, as would probably be the case for a short-lived radionuclide, but was available at some time prior to the performance of the assay for determination of efficiency of the counting system for the radionuclide to be assayed, use a calibrated counting system, provided the radionuclidic impurity content of the specimen meets the requirements stated for the direct comparison method. If the requirements for either of the first two methods cannot be met, use the method for determination of activity from a calibration curve.

With the exception of the first method, the counting systems used are monitored for stability. This requirement is met by daily checks with a long-lived performance check source and weekly

checks with at least three sources covering a broad range of gamma-ray emission energies (e.g., ^{57}Co, ^{137}Cs, and ^{60}Co). If a discrepancy for any of the aforementioned measurements is found, either completely recalibrate or repair and recalibrate the system prior to further use.

Assay by Direct Comparison with a Calibration Standard—An energy selective measurement system (e.g., pulse-height analyzer) is not required for this procedure. Use either an ionization chamber or an integral counting system with a NaI(Tl) detector. A consistently reproducible geometrical factor from specimen to specimen is essential for accurate results. With proper precautions, the accuracy of this method approaches the accuracy with which the disintegration rate of the calibration standard is known.

Determine the counting rate of the detector system for a calibration standard of the radionuclide to be assayed (e.g., active enough to give good measurement statistics in a reasonable time, but not so active as to cause serious dead-time problems), selecting such a standard as to provide optimum accuracy with the particular assembly used. Place an accurately measured aliquot of the unknown assay specimen (diluted, if necessary) in a container identical to that used for the standard, and measure this specimen at approximately the same time and under the same geometrical conditions as for the standard. If the elapsed time between the measurements of the calibration standard and the specimen exceeds 12 hours, check the stability of the measurement system within 8 hours of the specimen measurement time with a long-lived performance check source. Record the system response with respect to the same check source at the time of calibration, and if subsequent checks exceed the original recorded response by more than ±3%, recalibration is required. Correct both activity determinations for background, and calculate the activity, in μCi per mL, by the formula:

$$SD(g/b),$$

in which S is the μCi strength of the standard, D is the dilution factor, and g and b are the measured values of counting rate for the specimen and the standard, respectively.

Assay with a Calibrated Integral Counting System—The procedure and precautions given for the preceding direct-comparison

Table of Nuclear Properties[1,2]

Principal Photon Emissions	Energy (keV)	Photons per 100 Disintegrations
^{129}I ($T_{1/2}$ = 1.57 × 10^7 years)		
$K_{\alpha 1}$[3]	29.8	37.0
$K_{\alpha 2}$	29.5	20.0
K_β	33.6	13.2
γ_1	39.6	7.52
Weighted Mean[4]	(31.3)	(77.80)
^{241}Am ($T_{1/2}$ = 432.2 years)		
ΣX_L	13.9	38.2
γ_1	26.4	2.5
γ_2	59.5	35.9
^{109}Cd ($T_{1/2}$ = 464 days)		
$K_{\alpha 1}$	22.2	55.1
$K_{\alpha 2}$	22.0	29.1
K_β	24.9	17.8
Weighted Mean[4]	(22.6)	(102.0)
γ_1	88.0	3.72
^{195}Au ($T_{1/2}$ = 183 days)		
$K_{\alpha 1}$	66.83	50
$K_{\alpha 2}$	65.12	29.0
K_β	75.7	21.7
Weighted Mean[4]	(68.25)	(100.7)
γ_1	30.88	0.83
γ_2	98.86	10.9
γ_3	129.7	0.89
^{57}Co ($T_{1/2}$ = 270.9 days)		
ΣX_K	6.5	56.0
γ_1	14.4	9.5
γ_2	122.1	85.6
γ_3	136.4	10.6
Weighted Mean ($\gamma_2 + \gamma_3$)[4]	(125.0)	(96.2)
^{139}Ce ($T_{1/2}$ = 137.7 days)		
$K_{\alpha 1}$	33.4	42.6
$K_{\alpha 2}$	33.0	23.1
K_β	37.8	15.6
Weighted Mean[4]	(34.1)	(81.3)
γ_1	165.8	80.0
^{203}Hg ($T_{1/2}$ = 46.6 days)		
ΣX_L	10.3	5.6
$K_{\alpha 1}$	72.87	6.27
$K_{\alpha 2}$	70.83	3.72
K_β	82.6	2.79
Weighted Mean[4]	(74.6)	(12.8)
γ_1	279.2	81.5
^{113}Sn-^{113m}In ($T_{1/2}$ = 115.1 days)		
ΣX_L	3.3	9.0
$K_{\alpha 1}$	24.2	51.5
$K_{\alpha 2}$	24.0	27.5
K_β	27.3	17.3
Weighted Mean[4]	(24.7)	(96.7)
γ_1	255.1	1.9
γ_2	391.7	64.6
^{85}Kr ($T_{1/2}$ = 10.72 years)		
γ_1	514.0	0.43
^{137}Cs-^{137m}Ba ($T_{1/2}$ = 30.0 years)		
$K_{\alpha 1}$	32.2	3.90
$K_{\alpha 2}$	31.8	2.11
K_β	36.4	1.42
Weighted Mean[4]	(32.9)	(7.43)
γ_1	661.6	89.9
^{94}Nb ($T_{1/2}$ = 2 × 10^4 years)		
γ_1	702	100.0
γ_2	871	100.0
^{22}Na ($T_{1/2}$ = 2.60 years)		
$h\nu$	511	179.78[5]
γ_1	1274.5	99.95
^{60}Co ($T_{1/2}$ = 5.27 years)		
γ_1	1173.2[6]	99.88
γ_2	1332.5[6]	99.98

(1) In measurements for gamma- (or X-)ray assay purposes, fluorescent radiation from lead shielding (specifically, lead K X-rays ~76 keV) may interfere with quantitative results. Allowance must be made for these effects, or the radiation suppressed; a satisfactory means of absorbing this radiation is covering the exposed lead with cadmium sheet 0.06 to 0.08 inch thick, and then covering the cadmium with copper 0.02 to 0.04 inch thick.

(2) Only those photon emissions having an abundance ≥1% are normally included.

(3) The K notation refers to X-ray emissions.

(4) The weighted mean energies and total intensities are given for groups of photons that would not be resolved by a NaI(Tl) detector.

(5) For this photon intensity to be usable, all emitted positrons must be annihilated in the source material.

(6) Cascade.

method apply, except that the efficiency of the detector system is determined and recorded for each radionuclide to be assayed, rather than simply recording the counting rate of the standard. Thus, the efficiency for a given radionuclide, x, is determined by $\epsilon_x = b_x/s_x$, in which b_x is the counting rate, corrected for background and dead-time, for the calibration standard of the radionuclide, x, and s_x is the corresponding activity of the certified calibration standard in nuclear transformations per second. For subsequent specimen assays, the activity is given by the formula:

$$A_x = Dg_x/\epsilon_x,$$

in which D is the dilution factor, g_x is the specimen counting rate (corrected for background and dead-time), and ϵ_x is the corresponding efficiency for the radionuclide.

Determination of Activity from a Calibration Curve—Versatility in absolute gamma-ray intensity measurements can be achieved by employing multi-channel pulse-height analysis. The photopeak efficiency of a detector system can be determined as a function of gamma-ray energy by means of a series of gamma-ray emission rate standard specimens, and the gamma-ray emission rate of any radionuclide for which no standard is available can be determined by interpolation from this efficiency curve. However, exercise care to ensure that the efficiency curve for the detector system is adequately defined over the entire region of interest by using a sufficient number of calibration points along the photopeak-energy axis.

Selection of a Counting Assembly—A gamma-ray spectrometer is used for the identification of radionuclides that emit X-rays or gamma rays in their decay. Requirements for an assembly suitable for identification and assay of the radionuclides used in radiopharmaceuticals are that (a) the resolution of the detector based on the 662-keV photopeak of ^{137}Cs-^{137m}Ba must be 8.0% or better, (b) the detector must be equipped with a specimen holder designed to facilitate exact duplication of counting geometry, and (c) the pulse-height analyzer must have enough channels to delineate clearly the photopeak being observed.

Procedure—Minimal requirements for the maintenance of instrument calibrations shall consist of weekly performance checks with a suitable reference source and a complete recalibration semi-annually. Should the weekly performance check deviate from the value determined at the time of calibration by more than 4.0%, a complete recalibration of the instrument is required at that time.

This method involves three basic steps, namely photopeak integration, determination of the photopeak efficiency curve, and calculation of the activity of the specimen.

PHOTOPEAK INTEGRATION—The method for the determination of the required photopeak area utilizes a Gaussian approximation for fitting the photopeak. A fixed fraction of the total number of photopeak counts can be obtained by taking the peak width, a, at some fraction of the maximum, where the shape has been experimentally found to be very close to Gaussian, and multiplying by the counting rate of the peak channel, P, after correction for any Compton and background contributions to the peak channel count rate. This background usually can be adequately determined by linear interpolation. This is illustrated in Figure 2.

The photopeak-curve shape is closest to a straight line at $0.606P$, and the contribution of the fractional channels to a can be accurately estimated by interpolation. Calculate a by the equation:

$$a = D' - D + \frac{d - 0.606P}{d - c} + \frac{d' - 0.606P}{d' - c'},$$

in which c and d and also c' and d' are the single *channel counting* rates on either side of $0.606P$, and D and D' are the channel *numbers* (locations) of d and d', respectively. The location of the required variables on the photopeak is illustrated in Figure 3.

From the known values for the counting rate in the peak channel of the photopeak, P, and the width of the peak at $0.606P$, a, a calibrated fraction of the photopeak area is then obtained from the product, (aP).

To summarize the procedures involved in obtaining a calibrated fraction of a photopeak area using this method, the necessary steps or calculations are presented below in a stepwise manner:

(1) Subtract any Compton and background contributions from the photopeak to be measured.

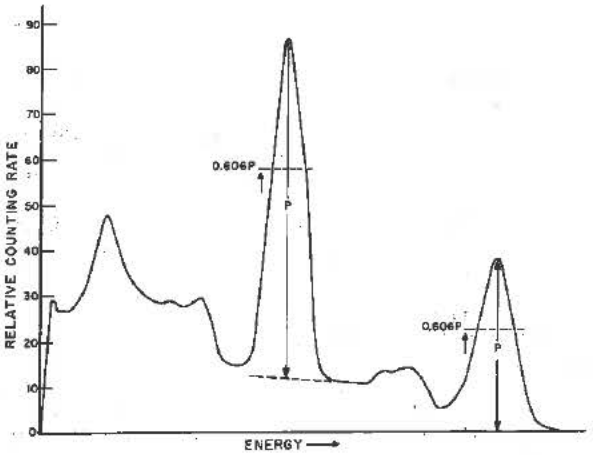


Fig. 2. Typical Gamma-ray Spectrum Showing the Selection of the Peak Channel Counting Rate, P, After Correction for Compton and Background Contributions.

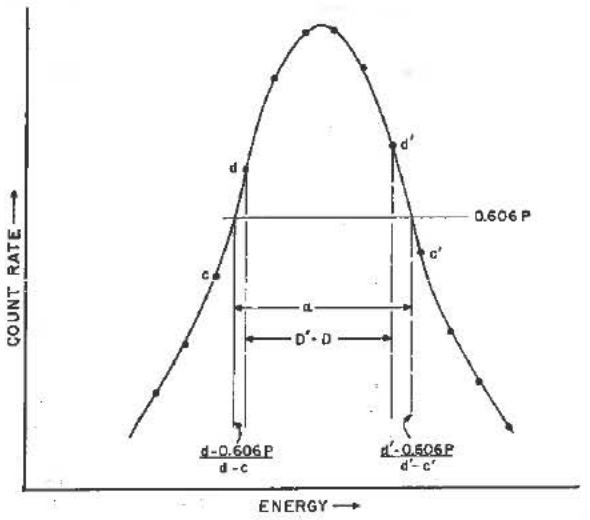


Fig. 3. Location of the Variables Required for the Determination of the Peak Width, a, at $0.606P$.

(2) Determine the counting rate of the peak channel (maximum channel counting rate after subtracting Compton and background), P.

(3) Multiply P by 0.606, and locate the horizontal line corresponding to the peak width, a.

(4) Obtain the peak width, a, by inserting the values of variables (obtained as shown in the preceding figure) into the equation defining a.

(5) The desired calibrated fraction of the peak area is then equal to the product of a times P or $F = aP$, where F is a fractional area of the peak proportional to the emission rate of the source.

This method provides a quick and accurate means of determining the gamma-ray emission rate of sources while avoiding, to a large extent, subjective estimates of the detailed shape of the tails of the peaks. The error due to using the maximum channel counting rate, rather than the theoretical maximum or peak channel rate, is of the order of 1.0% if a is 6 or greater.

PHOTOPEAK EFFICIENCY CALIBRATION—Radionuclides such as those listed in the accompanying table together with some of their nuclear decay data are available as certified reference standards.* A sufficient number of radioactive standard reference sources should be selected in order to obtain the calibration curve over the desired range. Where possible, standard sources of those radionuclides that are to be assayed should be included.

* These certified reference standards are obtainable from the National Institute of Standards and Technology, Washington, DC 20234.

Calculate the gamma-ray emission rate from the equation:

$$\Gamma = A_s b,$$

in which A_s is the activity, in disintegrations per second, of the standard used, and b is the number of gamma rays per disintegration at that energy. Accurately measure quantities of standard solutions of each radionuclide into identical containers, and determine the fractional photopeak area (F) for each of the standards.

Using the equation $\epsilon_p = F/\Gamma$, calculate the photopeak efficiency, ϵ_p, and construct a log–log plot of ϵ_p versus the gamma-ray energy as shown in Figure 4.

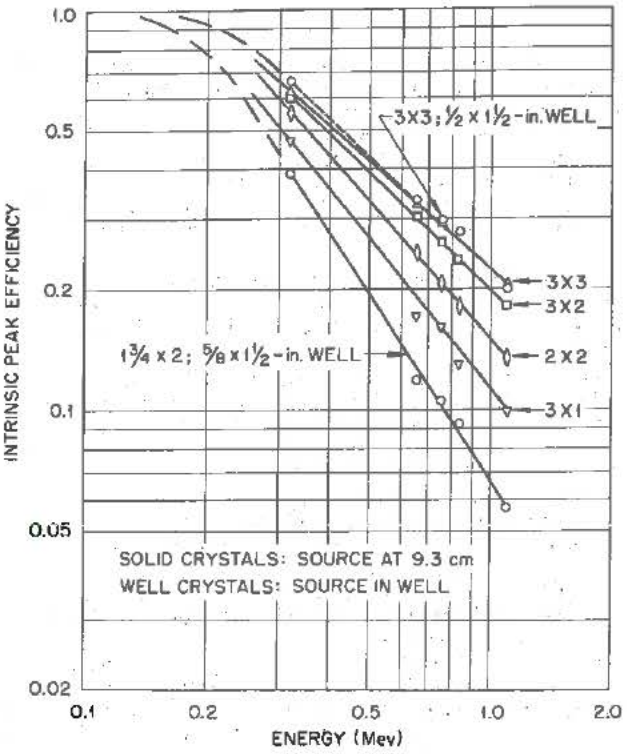


Fig. 4. Typical Photopeak Efficiency Calibration Curves for Various NaI(Tl) Detectors.

DETERMINATION OF SPECIMEN ACTIVITY—In the same manner as in the preparation of the calibration curve, determine the fractional area (F) of the principal photopeak of the specimen under assay or an accurately measured aliquot adjusted to the same volume in an identical container as used for the standards. From the calibration curve, find the value of ϵ_p for this radionuclide. Using the equation $\Gamma = F/\epsilon_p$, calculate the gamma-ray emission rate (Γ). Calculate the activity (A), in disintegrations per second, of the specimen using the equation $A = (\Gamma/b)(D)$, in which b is the number of gamma rays per disintegration and D is the dilution factor. To obtain the activity, in μCi or mCi, divide A by 3.7×10^4 or 3.7×10^7, respectively. The above relationship is equally valid for obtaining the activity of an undiluted specimen or capsule; in this case, the dilution factor, D, is unity.

⟨831⟩ REFRACTIVE INDEX

The refractive index (n) of a substance is the ratio of the velocity of light in air to the velocity of light in the substance. It is valuable in the identification of substances and the detection of impurities.

Although the standard temperature for Pharmacopeial measurements is 25°, many of the refractive index specifications in the individual monographs call for determining this value at 20°. The temperature should be carefully adjusted and maintained, since the refractive index varies significantly with temperature.

The values for refractive index given in this Pharmacopeia are for the D line of sodium (doublet at 589.0 nm and 589.6 nm). Most instruments available are designed for use with white light but are calibrated to give the refractive index in terms of the D line of sodium light.

The Abbé refractometer measures the range of refractive index for those Pharmacopeial materials for which such values are given. Other refractometers of equal or greater accuracy may be employed.

To achieve the theoretical accuracy of ±0.0001, it is necessary to calibrate the instrument against a standard provided by the manufacturer and to check frequently the temperature control and cleanliness of the instrument by determining the refractive index of distilled water, which is 1.3330 at 20° and 1.3325 at 25°.

⟨841⟩ SPECIFIC GRAVITY

Unless otherwise stated in the individual monograph, the specific gravity determination is applicable only to liquids, and, unless otherwise stated, is based on the ratio of the weight of a substance in air at 25° to that of an equal volume of water at the same temperature. Where a temperature is specified in the individual monograph, the specific gravity is the ratio of the weight of the substance in air at the specified temperature to that of an equal volume of water at the same temperature. When the substance is a solid at 25°, determine the specific gravity at the temperature directed in the individual monograph, and refer to water at 25°.

Procedure—Select a scrupulously clean, dry pycnometer that previously has been calibrated by determining its weight and the weight of recently boiled water contained in it at 25°. Adjust the temperature of the substance to about 20°, and fill the pycnometer with it. Adjust the temperature of the filled pycnometer to 25°, remove any excess of the substance, and weigh. Subtract the tare weight of the pycnometer from the filled weight of the pycnometer.

The specific gravity of the substance is the quotient obtained by dividing the weight of the substance contained in the pycnometer by the weight of water contained, both determined at 25° unless otherwise directed in the individual monograph.

⟨851⟩ SPECTROPHOTOMETRY AND LIGHT-SCATTERING

ULTRAVIOLET, VISIBLE, INFRARED, ATOMIC ABSORPTION, FLUORESCENCE, TURBIDIMETRY, NEPHELOMETRY, AND RAMAN MEASUREMENT

Absorption spectrophotometry is the measurement of an interaction between electromagnetic radiation and the molecules, or atoms, of a chemical substance. Techniques frequently employed in pharmaceutical analysis include ultraviolet, visible, infrared, and atomic absorption spectroscopy. Spectrophotometric measurement in the visible region was formerly referred to as *colorimetry;* however, it is more precise to use the term "colorimetry" only when considering human perception of color.

Fluorescence spectrophotometry is the measurement of the emission of light from a chemical substance while it is being exposed to ultraviolet, visible, or other electromagnetic radiation. In general, the light emitted by a fluorescent solution is of maximum intensity at a wavelength longer than that of the exciting radiation, usually by some 20 nm to 30 nm.

Light-scattering involves measurement of the light scattered because of submicroscopic optical density inhomogeneities of solutions and is useful in the determination of weight-average molecular weights of polydisperse systems in the molecular weight range from 1000 to several hundred million. Two such techniques utilized in pharmaceutical analysis are *turbidimetry* and *nephelometry*.

Raman spectroscopy (inelastic light-scattering) is a light-scattering process in which the specimen under examination is irradiated with intense monochromatic light (usually laser light) and the light scattered from the specimen is analyzed for frequency shifts.

The wavelength range available for these measurements extends from the short wavelengths of the ultraviolet through the infrared. For convenience of reference, this spectral range is

roughly divided into the ultraviolet (190 nm to 380 nm), the visible (380 nm to 780 nm), the near-infrared (780 nm to 3000 nm), and the infrared (2.5 μm to 40 μm or 4000 cm^{-1} to 250 cm^{-1}).

Comparative Utility of Spectral Ranges

For many pharmaceutical substances, measurements can be made in the ultraviolet and visible regions of the spectrum with greater accuracy and sensitivity than in the near-infrared and infrared. When solutions are observed in 1-cm cells, concentrations of about 10 μg of the specimen per mL often will produce absorbances of 0.2 to 0.8 in the ultraviolet or the visible region. In the infrared and near-infrared, concentrations of 1 to 10 mg per mL and up to 100 mg per mL, respectively, may be needed to produce sufficient absorption; for these spectral ranges, cell lengths of from 0.01 mm to upwards of 3 mm are commonly used.

The ultraviolet and visible spectra of substances generally do not have a high degree of specificity. Nevertheless, they are highly suitable for quantitative assays, and for many substances they are useful as additional means of identification.

The near-infrared region is especially suitable for the determination of —OH and —NH groups, such as water in alcohol, —OH in the presence of amines, alcohols in hydrocarbons, and primary and secondary amines in the presence of tertiary amines.

The infrared spectrum is unique for any given chemical compound with the exception of optical isomers, which have identical spectra. However, polymorphism may occasionally be responsible for a difference in the infrared spectrum of a given compound in the solid state. Frequently, small differences in structure result in significant differences in the spectra. Because of the large number of maxima in an infrared absorption spectrum, it is sometimes possible to measure quantitatively the individual components of a mixture of known qualitative composition without prior separation.

The Raman spectrum and the infrared spectrum provide similar data, although the intensities of the spectra are governed by different molecular properties. Raman and infrared spectroscopy exhibit different relative sensitivities for different functional groups, e.g., Raman spectroscopy is particularly sensitive to C—S and C—C multiple bonds, and some aromatic compounds are more easily identified by means of their Raman spectra. Water has a highly intense infrared absorption spectrum, but a particularly weak Raman spectrum. Therefore, water has only limited infrared "windows" that can be used to examine aqueous solutes, while its Raman spectrum is almost completely transparent and useful for solute identification. The two major limitations of Raman spectroscopy are that the minimum detectable concentration of specimen is typically 10^{-1} *M* to 10^{-2} *M* and that the impurities in many substances fluoresce and interfere with the detection of the Raman scattered signal.

Optical reflectance measurements provide spectral information similar to that obtained by transmission measurements. Since reflectance measurements probe only the surface composition of the specimen, difficulties associated with the optical thickness and the light-scattering properties of the substance are eliminated. Thus, reflectance measurements are frequently more simple to perform on intensely absorbing materials. A particularly common technique used for infrared reflectance measurements is termed attenuated total reflectance (ATR), also known as multiple internal reflectance (MIR). In the ATR technique, the beam of the infrared spectrometer is passed through an appropriate infrared window material (e.g., KRS-5, a TlBr-TlI eutectic mixture), which is cut at such an angle that the infrared beam enters the first (front) surface of the window, but is totally reflected when it impinges on the second (back) surface (i.e., the angle of incidence of the radiation upon the second surface of the window exceeds the critical angle for that material). By appropriate window construction, it is possible to have many internal reflections of the infrared beam before it is transmitted out of the window. If a specimen is placed in close contact with the window along the sides that totally reflect the infrared beam, the intensity of reflected radiation is reduced at each wavelength (frequency) that the specimen absorbs. Thus, the ATR technique provides a reflectance spectrum that has been increased in intensity, when compared to a simple reflectance measurement, by the number of times that the infrared beam is reflected within the window. The ATR technique provides excellent sensitivity, but it yields poor reproducibility, and is not a reliable quantitative technique unless an internal standard is intimately mixed with each test specimen.

Fluorescence spectrophotometry is often more sensitive than absorption spectrophotometry. In absorption measurements, the specimen transmittance is compared to that of a blank; and at low concentrations, both solutions give high signals. Conversely, in fluorescence spectrophotometry, the solvent blank has low rather than high output, so that the background radiation that may interfere with determinations at low concentrations is much less. Whereas few compounds can be determined conveniently at concentrations below 10^{-5} *M* by light absorption, it is not unusual to employ concentrations of 10^{-7} *M* to 10^{-8} *M* in fluorescence spectrophotometry.

Theory and Terms

The power of a radiant beam decreases in relation to the distance that it travels through an absorbing medium. It also decreases in relation to the concentration of absorbing molecules or ions encountered in that medium. These two factors determine the proportion of the total incident energy that emerge. The decrease in power of monochromatic radiation passing through a homogeneous absorbing medium is stated quantitatively by Beer's law, $\log_{10}(1/T) = A = abc$, in which the terms are as defined below.

Absorbance [Symbol: *A*]—The logarithm, to the base 10, of the reciprocal of the transmittance (*T*). [NOTE—Descriptive terms used formerly include optical density; absorbancy; and extinction.]

Absorptivity [Symbol: *a*]—The quotient of the absorbance (*A*) divided by the product of the concentration, expressed in *g* per liter, of the substance and the absorption path length in cm. [NOTE—It is not to be confused with absorbancy index; specific extinction; or extinction coefficient.]

Molar Absorptivity [Symbol: ε]—The quotient of the absorbance (*A*) divided by the product of the concentration, expressed in *moles* per liter, of the substance and the absorption path length in cm. It is also the product of the absorptivity (*a*) and the molecular weight of the substance. [NOTE—Terms formerly used include molar absorbancy index; molar extinction coefficient; and molar absorption coefficient.]

For most systems used in absorption spectrophotometry, the absorptivity of a substance is a constant independent of the intensity of the incident radiation, the internal cell length, and the concentration, with the result that concentration may be determined photometrically.

Beer's law gives no indication of the effect of temperature, wavelength, or the type of solvent. For most analytical work the effects of normal variation in temperature are negligible.

Deviations from Beer's law may be caused by either chemical or instrumental variables. Apparent failure of Beer's law may result from a concentration change in solute molecules because of association between solute molecules or between solute and solvent molecules, or dissociation or ionization. Other deviations might be caused by instrumental effects such as polychromatic radiation, slit-width effects, or stray light.

Even at a fixed temperature in a given solvent, the absorptivity may not be truly constant. However, in the case of specimens having only one absorbing component, it is not necessary that the absorbing system conform to Beer's law for use in quantitative analysis. The concentration of an unknown may be found by comparison with an experimentally determined standard curve.

Although, in the strictest sense, Beer's law does not hold in atomic absorption spectrophotometry because of the lack of quantitative properties of the cell length and the concentration, the absorption processes taking place in the flame under conditions of reproducible aspiration do follow the Beer relationship in principle. Specifically, the negative log of the transmittance, or the absorbance, is directly proportional to the absorption coefficient, and, consequently, is proportional to the number of absorbing atoms. On this basis, calibration curves may be constructed to permit evaluation of unknown absorption values in terms of concentration of the element in solution.

Absorption Spectrum—A graphic representation of absorbance, or any function of absorbance, plotted against wavelength or function of wavelength.

Transmittance [Symbol: *T*]—The quotient of the radiant power transmitted by a specimen divided by the radiant power incident

upon the specimen. [NOTE—Terms formerly used include transmittancy and transmission.]

Fluorescence Intensity [Symbol: I]—An empirical expression of fluorescence activity, commonly given in terms of arbitrary units proportional to detector response. The *fluorescence emission spectrum* is a graphical presentation of the spectral distribution of radiation emitted by an activated substance, showing intensity of emitted radiation as ordinate, and wavelength as abscissa. The *fluorescence excitation spectrum* is a graphical presentation of the activation spectrum, showing intensity of radiation emitted by an activated substance as ordinate, and wavelength of the incident (activating) radiation as abscissa. As in absorption spectrophotometry, the important regions of the electromagnetic spectrum encompassed by the fluorescence of organic compounds are the ultraviolet, visible, and near-infrared, i.e., the region from 250 nm to 800 nm. After a molecule has absorbed radiation, the energy can be lost as heat or released in the form of radiation of the same or longer wavelength as the absorbed radiation. Both absorption and emission of radiation are due to the transitions of electrons between different energy levels, or orbitals, of the molecule. There is a time delay between the absorption and emission of light; this interval, the duration of the excited state, has been measured to be about 10^{-9} second to 10^{-8} second for most organic fluorescent solutions. The short lifetime of fluorescence distinguishes this type of luminescence from phosphorescence, which is a long-lived afterglow having a lifetime of 10^{-3} second up to several minutes.

Turbidance [Symbol: S]—The light-scattering effect of suspended particles. The amount of suspended matter may be measured by observation of either the transmitted light (turbidimetry) or the scattered light (nephelometry).

Turbidity [Symbol: τ]—In light-scattering measurements, the turbidity is the measure of the decrease in incident beam intensity per unit length of a given suspension.

Raman scattering activity—The molecular property (in units of cm^4 per g) governing the intensity of an observed Raman band for a randomly oriented specimen. The scattering activity is determined from the derivative of the molecular polarizability with respect to the molecular motion giving rise to the Raman shifted band. In general, the Raman band intensity is linearly proportional to the concentration of the analyte.

Use of Reference Standards

With few exceptions, the Pharmacopeial spectrophotometric tests and assays call for comparison against a USP Reference Standard. This is to ensure measurement under conditions identical for the test specimen and the reference substance. These conditions include wavelength setting, slit-width adjustment, cell placement and correction, and transmittance levels. It should be noted that cells exhibiting identical transmittance at a given wavelength may differ considerably in transmittance at other wavelengths. Appropriate cell corrections should be established and used where required.

The expressions, "similar preparation" and "similar solution," as used in tests and assays involving spectrophotometry, indicate that the reference specimen, generally a USP Reference Standard, is to be prepared and observed in a manner identical for all practical purposes to that used for the test specimen. Usually in making up the solution of the specified Reference Standard, a solution of about (i.e., within 10%) the desired concentration is prepared and the absorptivity is calculated on the basis of the exact amount weighed out; if a previously dried specimen of the Reference Standard has not been used, the absorptivity is calculated on the anhydrous basis.

The expressions, "concomitantly determine" and "concomitantly measured," as used in tests and assays involving spectrophotometry, indicate that the absorbances of both the solution containing the test specimen and the solution containing the reference specimen, relative to the specified test blank, are to be measured in immediate succession.

Apparatus

Many types of spectrophotometers are available. Fundamentally, most types, except those used for infrared spectrophotometry, provide for passing essentially monochromatic radiant energy through a specimen in suitable form, and measuring the intensity of the fraction that is transmitted. Fourier transform infrared spectrophotometers use an interferometric technique whereby polychromatic radiation passes through the analyte and onto a detector on an intensity and time basis. Ultraviolet, visible, and dispersive infrared spectrophotometers comprise an energy source, a dispersing device (e.g., a prism or grating), slits for selecting the wavelength band, a cell or holder for the test specimen, a detector of radiant energy, and associated amplifiers and measuring devices. Fourier transform infrared systems utilize an interferometer instead of a dispersing device and a digital computer to process the spectral data. Some instruments are manually operated, whereas others are equipped for automatic and continuous recording. Instruments that are interfaced to a digital computer have the capabilities also of co-adding and storing spectra, performing spectral comparisons, and performing difference spectroscopy (accomplished with the use of a digital absorbance subtraction method).

Instruments are available for use in the visible; in the visible and ultraviolet; in the visible, ultraviolet, and near-infrared; and in the infrared regions of the spectrum. Choice of the type of spectrophotometric analysis and of the instrument to be used depends upon factors such as the composition and amount of available test specimen, the degree of accuracy, sensitivity, and selectivity desired, and the manner in which the specimen is handled.

The apparatus used in atomic absorption spectrophotometry has several unique features. For each element to be determined, a specific source that emits the spectral line to be absorbed should be selected. The source is usually a hollow-cathode lamp, the cathode of which is designed to emit the desired radiation when excited. Since the radiation to be absorbed by the test specimen element is usually of the same wavelength as that of its emission line, the element in the hollow-cathode lamp is the same as the element to be determined. The apparatus is equipped with an aspirator for introducing the test specimen into a flame, which is usually provided by air-acetylene, air-hydrogen, or, for refractory cases, nitrous oxide–acetylene. The flame, in effect, is a heated specimen chamber. A detector is used to read the signal from the chamber. Interfering radiation produced by the flame during combustion may be negated by the use of a chopped source lamp signal of a definite frequency. The detector should be tuned to this alternating current frequency so that the direct current signal arising from the flame is ignored. The detecting system, therefore, reads only the change in signal from the hollow-cathode source, which is directly proportional to the number of atoms to be determined in the test specimen. For Pharmacopeial purposes, apparatus that provides the readings directly in absorbance units is usually required. However, instruments providing readings in percent transmission, percent absorption, or concentration may be used if the calculation formulas provided in the individual monographs are revised as necessary to yield the required quantitative results. Percent absorption or percent transmittance may be converted to absorbance, A, by the following two equations:

$$A = 2 - \log_{10}(100 - \%\ \text{absorption})$$

or:

$$A = 2 - \log_{10}(\%\ \text{transmittance}).$$

Depending upon the type of apparatus used, the readout device may be a meter, digital counter, recorder, or printer. Both single-beam and double-beam instruments are commercially available, and either type is suitable.

Measurement of fluorescence intensity can be made with a simple *filter fluorometer*. Such an instrument consists of a radiation source, a primary filter, a specimen chamber, a secondary filter, and a fluorescence detection system. In most such fluorometers, the detector is placed on an axis at 90° from that of the exciting beam. This right-angle geometry permits the exciting radiation to pass through the test specimen and not contaminate the output signal received by the fluorescence detector. However, the detector unavoidably receives some of the exciting radiation as a result of the inherent scattering properties of the solutions themselves, or if dust or other solids are present. Filters are used to eliminate this residual scatter. The primary filter selects short-wavelength radiation capable of exciting the test specimen, while the secondary filter is normally a sharp cut-off filter that allows the longer-wavelength fluorescence to be transmitted but blocks the scattered excitation.

Most fluorometers use photomultiplier tubes as detectors, many types of which are available, each having special characteristics with respect to spectral region of maximum sensitivity, gain, and electrical noise. The photocurrent is amplified and read out on a meter or recorder.

A *spectrofluorometer* differs from a filter fluorometer in that filters are replaced by monochromators, of either the prism or the grating type. For analytical purposes, the spectrofluorometer is superior to the filter fluorometer in wavelength selectivity, flexibility, and convenience, in the same way in which a spectrophotometer is superior to a filter photometer.

Many radiation sources are available. Mercury lamps are relatively stable and emit energy mainly at discrete wavelengths. Tungsten lamps provide an energy continuum in the visible region. The high-pressure xenon arc lamp is often used in spectrofluorometers because it is a high-intensity source that emits an energy continuum extending from the ultraviolet into the infrared.

In spectrofluorometers, the monochromators are equipped with slits. A narrow slit provides high resolution and spectral purity, while a large slit sacrifices these for high sensitivity. Choice of slit size is determined by the separation between exciting and emitting wavelengths as well as the degree of sensitivity needed.

Specimen cells used in fluorescence measurements may be round tubes or rectangular cells similar to those used in absorption spectrophotometry, except that they are polished on all four vertical sides. A convenient test specimen size is 2 to 3 mL, but some instruments can be fitted with small cells holding 100 to 300 μL, or with a capillary holder requiring an even smaller amount of specimen.

Light-scattering instruments are available and consist in general of a mercury lamp, with filters for the strong green or blue lines, a shutter, a set of neutral filters with known transmittance, and a sensitive photomultiplier to be mounted on an arm that can be rotated around the solution cell and set at any angle from −135° to 0° to +135° by a dial outside the light-tight housing. Solution cells are of various shapes, such as square for measuring 90° scattering; semioctagonal for 45°, 90°, and 135° scattering; and cylindrical for scattering at all angles. Since the determination of molecular weight requires a precise measure of the difference in refractive index between the solution and solvent, $[(n - n_0)/c]$, a second instrument, a differential refractometer, is needed to measure this small difference.

Raman spectrometers include the following major components: a source of intense monochromatic radiation (invariably a laser); optics to collect the light scattered by the test specimen; a (double) monochromator to disperse the scattered light and reject the intense incident frequency; and a suitable light-detection and amplification system. Raman measurement is simple in that most specimens are examined directly in melting-point capillaries. Because the laser source can be focused sharply, only a few microliters of the specimen is required.

Procedure

Spectrophotometry—Detailed instructions for operating spectrophotometers are supplied by the manufacturers. To achieve significant and valid results, the operator of a spectrophotometer should be aware of its limitations and of potential sources of error and variation. The instruction manual should be followed closely on such matters as care, cleaning, and calibration of the instrument, and techniques of handling absorption cells, as well as instructions for operation. The following points require special emphasis.

Check the instrument for accuracy of calibration. Where a continuous source of radiant energy is used, attention should be paid to both the wavelength and photometric scales; where a spectral line source is used, only the photometric scale need be checked. A number of sources of radiant energy have spectral lines of suitable intensity, adequately spaced throughout the spectral range selected. The best single source of ultraviolet and visible calibration spectra is the quartz-mercury arc, of which the lines at 253.7, 302.25, 313.16, 334.15, 365.48, 404.66, and 435.83 nm may be used. The glass-mercury arc is equally useful above 300 nm. The 486.13-nm and 656.28-nm lines of a hydrogen discharge lamp may be used also. The wavelength scale may be calibrated also by means of suitable glass filters, which have useful absorption bands through the visible and ultraviolet regions. Standard glasses containing didymium (a mixture of praseodymium and neodymium) have been used widely. Glass containing holmium[1] is considered superior. The wavelength scales of near-infrared and infrared spectrophotometers are readily checked by the use of absorption bands provided by polystyrene films, carbon dioxide, water vapor, or ammonia gas.

For checking the photometric scale, a number of standard inorganic glass filters as well as standard solutions of known transmittances such as potassium chromate or potassium dichromate are available.[2]

Quantitative absorbance measurements usually are made on solutions of the substance in liquid-holding cells. Since both the solvent and the cell window absorb light, compensation must be made for their contribution to the measured absorbance. Matched cells are available commercially for ultraviolet and visible spectrophotometry for which no cell correction is necessary. In infrared spectrophotometry, however, corrections for cell differences usually must be made. In such cases, pairs of cells are filled with the selected solvent and the difference in their absorbances at the chosen wavelength is determined. The cell exhibiting the greater absorbance is used for the solution of the test specimen and the measured absorbance is corrected by subtraction of the cell difference.

With the use of a computerized Fourier transform infrared system, this correction need not be made, since the same cell can be used for both the solvent blank and the test solution. However, it must be ascertained that the transmission properties of the cell are constant.

Comparisons of a test specimen with a Reference Standard are best made at a peak of spectral absorption for the compound concerned. Assays prescribing spectrophotometry give the commonly accepted wavelength for peak spectral absorption of the substance in question. It is known that different spectrophotometers may show minor variation in the apparent wavelength of this peak. Good practice demands that comparisons be made at the wavelength at which peak absorption occurs. Should this differ by more than ±1 nm from the wavelength specified in the individual monograph, re-calibration of the instrument may be indicated.

Test Preparation—For determinations utilizing ultraviolet or visible spectrophotometry, the specimen generally is dissolved in a solvent. Many solvents are suitable for these ranges, including water, alcohols, chloroform, lower hydrocarbons, ethers, and dilute solutions of strong acids and alkalies. Precautions should be taken to utilize solvents free from contaminants absorbing in the spectral region being used. It is usually advisable to use water-free methanol or alcohol, or alcohol denatured by the addition of methanol but not containing benzene or other interfering impurities, as the solvent. Solvents of special spectrophotometric quality, guaranteed to be free from contaminants, are available commercially from several sources. Some other analytical reagent-grade organic solvents may contain traces of impurities that absorb strongly in the ultraviolet region. New lots of these solvents should be checked for their transparency, and care should be taken to use the same lot of solvent for preparation of the test solution and the standard solution and for the blank.

No solvent in appreciable thickness is completely transparent throughout the near-infrared and infrared spectrum. Carbon tetrachloride (up to 5 mm in thickness) is practically transparent to 6 μm (1666 cm^{-1}). Carbon disulfide (1 mm in thickness) is

[1] Certified holmium glass filters are obtainable from the National Institute of Science and Technology (212.141g, 212.141h, and 212.141i). The same type of filter, without certification, is obtainable from Corning Glass Works, Corning, NY 14830 (Cat. No. 3130). The performance of an uncertified filter should be checked against one properly certified.

[2] For further detail regarding checks on both the wavelength and the photometric scales of a spectrophotometer, reference may be made to the following publications of the National Institute of Science and Technology, Department of Commerce, Washington, DC 20234: "Standards for Checking the Calibration of Spectrophotometers (200 to 1000 mμ)," Letter Circular LC-1017, January, 1955; "Spectrophotometry (200 to 1000 mμ)," Circular 484, September 15, 1949; "Calibrating Wave Lengths in the Region from 0.6 to 2.6 μ," *J. Research Natl. Bur. Standards* **49**, 13 (1952), RP 2338 and "Reference Wave Lengths for Calibrating Prism Spectrometers," *J. Research Natl. Bur. Standards* **58**, 195 (1957), RP 2752; "Liquid Absorbance Standards for UV-VIS," SRM #931.

suitable as a solvent to 40 μm (250 cm^{-1}) with the exception of the 4.2-μm to 5.0-μm (2381-cm^{-1} to 2000-cm^{-1}) and the 5.5-μm to 7.5-μm (1819-cm^{-1} to 1333-cm^{-1}) regions, where it has strong absorption. Other solvents have relatively narrow regions of transparency. For infrared spectrophotometry, an additional qualification for a suitable solvent is that it must not affect the material, usually sodium chloride, of which the cell is made. Where such a suitable solvent is not available, alternative methods for preparation of the test specimen include dispersing the finely ground solid specimen in mineral oil, or incorporating it in a transparent disk or pellet obtained by mixing it intimately with previously dried alkali halide salt (usually potassium bromide) and pressing the mixture in a die. A mineral oil dispersion is preferable where disproportionation between the alkali halide and the test specimen is encountered. For suitable materials the test specimen may be suspended neat as a thin film. For Raman spectrometry, most common solvents are suitable, and ordinary (nonfluorescing) glass specimen cells can be used. While the infrared region of the spectrum extends from about 2.5 μm to 40 μm, comparative spectra, obtained to ascertain compliance with a monograph specification for infrared absorption, are generally determined in the range from about 2.6 μm to 15 μm (3800 cm^{-1} to 650 cm^{-1}), unless otherwise specified in the individual monograph.

Where values for infrared line spectra are given in an individual monograph, the letters *s*, *m*, and *w* signify strong, medium, and weak absorption, respectively; *sh* signifies a shoulder, *bd* signifies a band, and *v* means very. The values may vary as much as 0.1 μm or 10 cm^{-1}, depending upon the particular instrument used. Polymorphism gives rise to variations in the infrared spectra of many compounds in the solid state. Therefore, when conducting infrared absorption tests, if a difference appears in the infrared spectra of the analyte and the standard, dissolve equal portions of the test substance and the standard in equal volumes of a suitable solvent, evaporate the solutions to dryness in similar containers under identical conditions, and repeat the test on the residues.

In atomic absorption spectrophotometry, the nature of the solvent and the concentration of solids must be given special consideration. An ideal solvent is one that interferes to a minimal extent in the absorption or emission processes and one that produces neutral atoms in the flame. If there is a significant difference between the surface tension or viscosity of the test solution and standard solution, the solutions are aspirated or atomized at a different rate, causing significant differences in the signals generated. The acid concentration of the solutions also affects the absorption processes. Thus, the solvents used in preparing the test specimen and the standard should be the same or as much alike in these respects as possible, and should yield solutions that are easily aspirated via the specimen tube of the burner-aspirator. Since undissolved solids present in the solutions may give rise to matrix or bulk interferences, the total undissolved solids content in all solutions should be kept below 2% wherever possible.

Calculations—The application of absorption spectrophotometry in an assay or a test generally requires the use of a Reference Standard. Where such a measurement is specified in an assay, a formula is provided in order to permit calculation of the desired result. A numerical constant is frequently included in the formula. The following derivation is provided to introduce a logical approach to the deduction of the constants appearing in formulas in the assays in many monographs.

The Beer's law relationship is valid for the solutions of both the Reference Standard (S) and the test specimen (U):

$$(1)\quad A_S = abC_S$$

$$(2)\quad A_U = abC_U$$

in which A_S is the absorbance of the Standard solution of concentration C_S, and A_U is the absorbance of the test specimen solution of concentration C_U. If C_S and C_U are expressed in the same units and the absorbances of both solutions are measured in matching cells having the same dimensions, the absorptivity, a, and the cell thickness, b, are the same; consequently, the two equations may be combined and rewritten to solve for C_U:

$$(3)\quad C_U = C_S(A_U/A_S).$$

Quantities of solid test specimens to be taken for analysis are generally specified in mg. Instructions for dilution are given in the assay and, since dilute solutions are used for absorbance measurements, concentrations are usually expressed for convenience in units of μg per mL. Taking a quantity, in mg, of a test specimen of a drug substance or solid dosage form for analysis, it therefore follows that a volume (V_U), in liters, of solution of concentration C_U may be prepared from the amount of test specimen that contains a quantity W_U, in mg, of the drug substance [NOTE—C_U is numerically the same whether expressed as μg per mL or mg per liter], such that:

$$(4)\quad W_U = V_UC_U.$$

The form in which the formula appears in the assay in a monograph for a solid article may be derived by substituting C_U of equation (3) into equation (4):

$$(5)\quad W_U = V_UC_S(A_U/A_S).$$

In summary, the use of equation (4), with due consideration for any unit conversions necessary to achieve equality in equation (5), permits the calculation of the constant factor (V_U) occurring in the final formula.

The same derivation is applicable to formulas that appear in monographs for liquid articles that are assayed by absorption spectrophotometry. For liquid dosage forms, results of calculations are generally expressed in terms of the quantity, in mg, of drug substance in each mL of the article. Thus it is necessary to include in the denominator an additional term, the volume (V), in mL, of the test preparation taken.

Spectrophotometry in the Visible Region—Assays in the visible region usually call for comparing concomitantly the absorbance produced by the *Assay preparation* with that produced by a *Standard preparation* containing approximately an equal quantity of a USP Reference Standard. In some situations, it is permissible to omit the use of a Reference Standard. This is true where spectrophotometric assays are made with routine frequency, and where a suitable standard curve is available, prepared with the respective USP Reference Standard, and where the substance assayed conforms to Beer's law within the range of about 75% to 125% of the final concentration used in the assay. Under these circumstances, the absorbance found in the assay may be interpolated on the standard curve, and the assay result calculated therefrom.

Such standard curves should be confirmed frequently, and always when a new spectrophotometer or new lots of reagents are put into use.

In spectrophotometric assays that direct the preparation and use of a standard curve, it is permissible and preferable, when the assay is employed infrequently, not to use the standard curve but to make the comparison directly against a quantity of the Reference Standard approximately equal to that taken of the specimen, and similarly treated.

NOTE—See *Calculations* under *Spectrophotometry*.

Visual Comparison—Where a color or a turbidity comparison is directed, color-comparison tubes that are matched as closely as possible in internal diameter and in all other respects should be used. For color comparison, the tubes should be viewed downward, against a white background, with the aid of a light source directed from beneath the bottoms of the tubes, while for turbidity comparison the tubes should be viewed horizontally, against a dark background, with the aid of a light source directed from the sides of the tubes.

In conducting limit tests that involve a comparison of colors in two like containers (e.g., matched color-comparison tubes), a suitable instrument, rather than the unaided eye, may be used.

Fluorescence Spectrophotometry—The measurement of fluorescence is a useful analytical technique. *Fluorescence* is light emitted from a substance in an excited state that has been reached by the absorption of radiant energy. A substance is said to be *fluorescent* if it can be made to fluoresce. Many compounds can be assayed by procedures utilizing either their inherent fluorescence or the fluorescence of suitable derivatives.

Test specimens prepared for fluorescence spectrophotometry are usually one-tenth to one-hundredth as concentrated as those used in absorption spectrophotometry, for the following reason. In analytical applications, it is preferable that the fluorescence signal be linearly related to the concentration; but if a test spec-

imen is too concentrated, a significant part of the incoming light is absorbed by the specimen near the cell surface, and the light reaching the center is reduced. That is, the specimen itself acts as an "inner filter." However, fluorescence spectrophotometry is inherently a highly sensitive technique, and concentrations of 10^{-5} *M* to 10^{-7} *M* frequently are used. It is necessary in any analytical procedure to make a working curve of fluorescence intensity versus concentration in order to establish a linear relationship. All readings should be corrected for a solvent blank.

Fluorescence measurements are sensitive to the presence of dust and other solid particles in the test specimen. Such impurities may reduce the intensity of the exciting beam or give misleading high readings because of multiple reflections in the specimen cell. It is, therefore, wise to eliminate solid particles by centrifugation; filtration also may be used, but some filter papers contain fluorescent impurities.

Temperature regulation is often important in fluorescence spectrophotometry. For some substances, fluorescence efficiency may be reduced by as much as 1% to 2% per degree of temperature rise. In such cases, if maximum precision is desired, temperature-controlled specimen cells are useful. For routine analysis, it may be sufficient to make measurements rapidly enough so that the specimen does not heat up appreciably from exposure to the intense light source. Many fluorescent compounds are light-sensitive. Exposed in a fluorometer, they may be photo-degraded into more or less fluorescent products. Such effects may be detected by observing the detector response in relationship to time, and may be reduced by attenuating the light source with filters or screens.

Change of solvent may markedly affect the intensity and spectral distribution of fluorescence. It is inadvisable, therefore, to alter the solvent specified in established methods without careful preliminary investigation. Many compounds are fluorescent in organic solvents but virtually nonfluorescent in water; thus, a number of solvents should be tried before it is decided whether or not a compound is fluorescent. In many organic solvents, the intensity of fluorescence is increased by elimination of dissolved oxygen, which has a strong quenching effect. Oxygen may be removed by bubbling an inert gas such as nitrogen or helium through the test specimen.

A semiquantitative measure of the strength of fluorescence is given by the ratio of the fluorescence intensity of a test specimen and that of a standard obtained with the same instrumental settings. Frequently, a solution of stated concentration of quinine in 0.1 *N* sulfuric acid or fluorescein in 0.1 *N* sodium hydroxide is used as a reference standard.

Light-scattering, Turbidimetry, and Nephelometry—Turbidity can be measured with a standard photoelectric filter photometer or spectrophotometer, preferably with illumination in the blue portion of the spectrum. Nephelometric measurements require an instrument with a photocell placed so as to receive scattered rather than transmitted light; this geometry applies also to fluorometers, so that, in general, fluorometers can be used as nephelometers, by proper selection of filters.

In practice, it is advisable to ensure that settling of the particles being measured is negligible. This is usually accomplished by including a protective colloid in the liquid suspending medium. It is important that results be interpreted by comparison of readings with those representing known concentrations of suspended matter, produced under precisely the same conditions.

Turbidimetry or nephelometry may be useful for the measurement of precipitates formed by the interaction of highly dilute solutions of reagents, or other particulate matter, such as suspensions of bacterial cells. In order that consistent results may be achieved, all variables must be carefully controlled. Where such control is possible, extremely dilute suspensions may be measured.

Light-scattering—The specimen solute is dissolved in the solvent at several different accurately known concentrations, the choice of concentrations being dependent on the molecular weight of the solute and ranging from 1% for M_w = 10,000 to 0.01% for M_w = 1,000,000. Each solution must be very carefully cleaned before measurement by repeated filtration through fine filters. A dust particle in the solution vitiates the intensity of the scattered light measured. A criterion for a clear solution is that the dissymmetry, 45°/135° scattered intensity ratio, has attained a minimum.

The turbidity and refractive index of the solutions are measured. From the general 90° light-scattering equation, a plot of HC/r versus C is made and extrapolated to infinite dilution, and the weight-average molecular weight, M, is calculated from the intercept, $1/M$.

⟨861⟩ SUTURES—DIAMETER

The gauge for determining the diameter of sutures is of the dead-weight type, mechanical or electrical, and equipped with a direct-reading dial, a digital readout, or a printed readout. Use a gauge graduated to 0.002 mm (to 0.0001 inch if the gauge is in English units) or smaller. The anvil of the gauge is about 50 mm in diameter, and the presser foot is 12.70 ± 0.02 mm in diameter. The presser foot and moving parts connected therewith are weighted so as to apply a total load of 210 ± 3 g to the specimen. The presser foot and anvil surfaces are plane to within 0.005 mm and parallel to each other to within 0.005 mm. For measuring the diameter of sutures of metric size 0.4 and smaller, remove the additional weight from the presser foot so that the total load on the suture does not exceed 60 g.

Collagen Absorbable Surgical Suture—Determine the diameter immediately after removal from the immediate container and without stretching. Lay the strand across the center of the anvil and presser foot, and gently lower the foot until its entire weight rests upon the suture. Measure the diameter of each strand at three points corresponding roughly to one-fourth, one-half, and three-fourths of its length.

Synthetic Absorbable Surgical Suture—Proceed as directed for *Nonabsorbable Surgical Suture*.

Nonabsorbable Surgical Suture—Lay the strand across the center of the anvil and presser foot, and gently lower the foot until its entire weight rests upon the suture. Measure nonabsorbable sutures, whether packaged in dry form or in fluid, immediately after removal from the container, without prior drying or conditioning.

Measure the diameter of the suture at three points corresponding roughly to one-fourth, one-half, and three-fourths of its length. In the case of braided suture of sizes larger than 3–0 (metric size 2), make two measurements at each point at right angles to each other, and use the average as the observed diameter at that point.

In measuring multifilament sutures, attach a portion of the designated section of the strand in a fixed clamp in such a way that the strand lies across the center of the anvil. While holding the strand in the same plane as the surface of the anvil, place the strand under tension by suitable means, such as by passing the free end of the strand around a cylinder or a pulley and attaching to the free end a weight of about one-half of the knot-pull limit for the non-sterilized Class I suture of the size concerned, taking care not to permit the strand, if twisted, to untwist. Measure the diameter at the designated points on the strand, and calculate the average diameter likewise as directed.

⟨871⟩ SUTURES—NEEDLE ATTACHMENT

Absorbable (collagen) surgical sutures and nonabsorbable surgical sutures with *Standard Needle Attachment* are such that the needles are firmly attached and are not intended to be separated. Sutures supplied with eyeless needles attached fall into either the category of *Standard Needle Attachment* or the category of *Removable Needle Attachment*. *Removable Needle Attachment* of both absorbable and nonabsorbable surgical sutures is such that the needle may be deliberately separated from the suture by means of a quick tug. Both types of attachments are tested on equipment as specified under *Tensile Strength* ⟨881⟩.

Procedure—Clamp each of 5 sutures in the tensilometer so that the needle is in the fixed clamp with all of the swaged portion exposed and in line with the direction of force applied to the suture by the moving clamp. Determine the force required to detach the suture from the needle. In the case of *Standard Needle Attachment*, the suture may break without needle detachment.

STANDARD NEEDLE ATTACHMENT—The requirements are met if neither the average of the 5 values nor any individual value is less than the limit given for the designated size in Table 1.

Table 1. Standard Needle Attachment for Absorbable and Nonabsorbable Sutures.

Metric Size (Gauge No.)			Limits on Needle Attachment	
Absorbable (Collagen) Suture	Nonabsorbable and Synthetic Absorbable Suture	USP Size	Average (kgf) (Min.)	Individual (kgf) (Min.)
	0.1	11–0	0.007	0.005
	0.2	10–0	0.014	0.010
0.4	0.3	9–0	0.021	0.015
0.5	0.4	8–0	0.050	0.025
0.7	0.5	7–0	0.080	0.040
1	0.7	6–0	0.17	0.08
1.5	1	5–0	0.23	0.11
2	1.5	4–0	0.45	0.23
3	2	3–0	0.68	0.34
3.5	3	2–0	1.10	0.45
4	3.5	0	1.50	0.45
5	4	1	1.80	0.60
6 and larger	5 and larger	2 and larger	1.80	0.70

Table 2. Removable Needle Attachment for Absorbable and Nonabsorbable Sutures.

Metric Size			Attachment Limits	
Absorbable (Collagen)	Nonabsorbable and Synthetic Absorbable	USP Size	Minimum (kgf)	Maximum (kgf)
1.5	1	5–0	0.028	1.59
2	1.5	4–0		
3	2	3–0		
3.5	3	2–0		
4	3.5	0		
5	4	1		
6	5	2		

REMOVABLE NEEDLE ATTACHMENT—The requirements are met if the individual values of the 5 sutures are within the limits shown in Table 2.

NOTE—For either type of attachment, if not more than 1 of the individual values falls outside the prescribed limits, repeat the test on an additional 10 sutures: the requirements of the test are met if none of the 10 additional values falls outside the individual limit requirements.

⟨881⟩ TENSILE STRENGTH

Devices for measurement of tensile strength used in the United States may be calibrated in the English units of measure. The following directions are given in metric units with the understanding that the corresponding English equivalents may be used.

Surgical Sutures

Equipment—Determine the tensile strength of surgical sutures on a motor-driven tensile strength testing machine using the principle of the constant specimen-rate-of-load, having suitable clamps for holding the specimen firmly.

This description applies specifically to that known as the Incline Plane Tester.

The carriage used in any test is of a weight such that when the break occurs, the position of the recording pen on the chart is between 20% and 80% of the capacity that may be recorded on the chart. The friction in the carriage is low enough to permit the recording pen to depart from the zero line of the chart at a point not exceeding 2.5% of the capacity of the chart when no specimen is held in the clamps.

For surgical sutures of intermediate and larger sizes, the clamp for holding the specimen is of the roll type, with a flat gripping surface. The roll has a diameter of 19 mm and the flat gripping surface is not less than 25 mm in length. The length of the specimen, when inserted in the clamps, is at least 127 mm from nip to nip. The speed of inclination of the plane of the tester is such that it reaches its full inclination of 30° from the horizontal in 20 ± 1 seconds from the start of the test.

For surgical sutures of small sizes, the suitable clamp has a flat gripping surface of not less than 13 mm in length. The length of the specimen, when inserted in the clamps, is at least 127 mm from clamp to clamp or 35 mm less than the labeled length, whichever distance is shorter. In the event that a labeled length is less than 47 mm, use a clamp to clamp distance of 12 mm. The speed of inclination of the plane is such that it reaches its full inclination of 30° from the horizontal in 60 ± 5 seconds from the start of the test.

Procedure—Determine the tensile strength of sutures, whether packaged in dry form or in fluid, promptly after removal from the container, without prior drying or conditioning.

Except where straight pull (no knot required) is indicated in the suture monograph, tie the test suture into a surgeon's knot with one turn of suture around flexible rubber tubing of 6.5-mm inside diameter and 1.6-mm wall thickness. The surgeon's knot is a square knot in which the free end is first passed twice, instead of once, through the loop, and pulled taut, then passed once through a second loop, and the ends are drawn taut so that a single knot is superimposed upon a compound knot. Start the first knot with the left end over the right end, exerting sufficient tension to tie the knot securely. Where the test specimen includes a knot, place the specimen in the testing device with the knot approximately midway between the clamps.

Attach one end of the suture in the clamp at the load end of the machine, pass the other end through the opposite clamp, with sufficient tension so that the specimen is taut between the clamps, and close the second clamp. Perform as many breaks as are specified in the individual monograph. If the break occurs outside the central 80% of the specimen length, discard the reading on the specimen. If the labeled length of the strand exceeds 7 meters (25 feet), take 2 meters from each of five strands, selected at random from the lot, rejecting the first 30 cm (12 inches), and make at least two breaks on each strand, about 60 cm to 100 cm apart.

Textile Fabrics and Films

Equipment—Determine the tensile strength of textile fabrics, including adhesive tape, on a constant-speed or pendulum type of testing machine, of the following general description.

The clamps for holding the specimen are smooth, flat, parallel jaws that are not less than 25 mm in length in the dimension parallel to the direction of application of the load. When the width of the strip being tested does not exceed 19 mm, the jaws of the clamp should be at least 25 mm wide. If the width of the strip is greater than 19 mm and not greater than 44 mm, the width of the jaws of the clamp should be at least 50 mm. If the width of the specimen is greater than 44 mm, cut a 25-mm strip, and use a clamp with jaws not less than 50 mm wide. Round all edges that might have a cutting action on the specimen to a radius of 0.4 mm (1/64 inch). The jaws are 76.2 mm apart at the beginning of the test, and they separate at the rate of 30.5 cm ± 13 mm per minute. The machine is of such capacity that when the break occurs, the deviation of the pendulum from the vertical is between 9° and 45°.

⟨891⟩ THERMAL ANALYSIS

Precisely determined thermodynamic events, such as a change of state, can indicate the identity and purity of drugs. Compendial standards have long been established for the melting or boiling temperatures of substances. These transitions occur at characteristic temperatures, and the compendial standards therefore contribute to the identification of the substances. Because impurities affect these changes in predictable ways, the same compendial standards contribute to the control of the purity of the substances.

Thermal analysis in the broadest sense is the measurement of physical-chemical properties of materials as a function of temperature. Instrumental methods have largely supplanted older methods dependent on visual inspection and on measurements

under fixed or arbitrary conditions, because they are objective, they provide more information, they afford permanent records, and they are generally more sensitive, more precise, and more accurate. Furthermore, they may provide information on crystal perfection, polymorphism, melting temperature, sublimation, glass transitions, dehydration, evaporation, pyrolysis, solid-solid interactions, and purity. Such data are useful in the characterization of substances with respect to compatibility, stability, packaging, and quality control. The measurements used most often in thermal analysis, i.e., transition temperature, thermogravimetry, and impurity analysis, are described here.

Transition Temperature—As a specimen is heated, its uptake (or evolution) of heat can be measured [differential scanning calorimetry (DSC)] or the resulting difference in temperature from that of an inert reference heated identically [differential thermal analysis (DTA)] can be measured. Either technique provides a record of the temperature at which phase changes, glass transitions, or chemical reactions occur. In the case of melting, both an "onset" and a "peak" temperature can be determined objectively and reproducibly, often to within a few tenths of a degree. While these temperatures are useful for characterizing substances, and the difference between the two temperatures is indicative of purity, the values cannot be correlated with subjective, visual "melting-range" values or with constants such as the triple point of the pure material.

A complete description of the conditions employed should accompany each thermogram, including make and model of instrument; record of last calibration; specimen size and identification (including previous thermal history); container; identity, flow rate, and pressure of gaseous atmosphere; direction and rate of temperature change; and instrument and recorder sensitivity.

It is appropriate to make a preliminary examination over a wide range (room temperature to decomposition temperature) at a high (10° to 20° per minute) heating rate in order to reveal unusual effects, then to make replicate examinations over a narrow range bracketing the transition of interest at lower heating rates (about 2° per minute). As the reliability of the measurements varies from one substance to another, statements of the number of significant figures to be used in the reporting, of intralaboratory repeatability, and of interlaboratory reproducibility cannot be given here, but should be included in the individual monograph.

Thermogravimetric Analysis—Thermogravimetric analysis involves the determination of the mass of a specimen as a function of temperature, or time of heating, or both, and when properly applied, provides more useful information than does loss on drying at fixed temperature, often for a fixed time and in what is usually an ill-defined atmosphere. Usually, loss of surface-absorbed solvent can be distinguished from solvent in the crystal lattice and from degradation losses. The measurements can be carried out in atmospheres having controlled humidity and oxygen concentration to reveal interactions with the drug substance, between drug substances, and between active substances and excipients or packaging materials.

While the details depend on the manufacturer, the essential features of the equipment are a recording balance and a programmable heat source. Equipment differs in the ability to handle specimens of various sizes, the means of sensing specimen temperature, and the range of atmosphere control. Calibration is required with all systems, i.e., the mass scale is calibrated by the use of standard weights; calibration of the temperature scale, which is more difficult, involving either variations in positioning of thermocouples and their calibration; or in other systems, calibration involves the use of standard materials because it is assumed that the specimen temperature is the furnace temperature.

Procedural details are specified in order to provide for valid interlaboratory comparison of results. The specimen weight, source, and thermal history are noted. The equipment description covers dimensions and geometry, the materials of the test specimen holder, and the location of the temperature transducer. Alternatively, the make and model number of commercial equipment are specified. In all cases, the calibration record is specified. Data on the temperature environment include the initial and final temperatures and the rate of change or other details if non-linear. The test atmosphere is critical; the volume, pressure, composition, whether static or dynamic, and if the latter, the flow rate and temperature are specified.

Eutectic Impurity Analysis—The basis of any calorimetric purity method is the relationship between the melting and freezing point depression, and the level of impurity. The melting of a compound is characterized by the absorption of latent heat of fusion, ΔH_f, at a specific temperature, T_o. In theory, a melting transition for an absolutely pure crystalline compound should occur within an infinitely narrow range. A broadening of the melting range, due to impurities, provides a sensitive criterion of purity. The effect is apparent visually by examination of thermograms of specimens differing by a few tenths percent in impurity content. A material that is 99% pure is about 20% molten at 3° below the melting point of the pure material (see accompanying figure).

The parameters of melting (melting range, ΔH_f, and calculated eutectic purity) are readily obtained from the thermogram of a single melting event using a small test specimen, and the method does not require multiple, precise actual temperature measurements. Thermogram units are directly convertible to heat transfer, millicalories per second.

The lowering of the freezing point in *dilute solutions* by molecules of nearly equal size is expressed by a modified van't Hoff equation:

$$\frac{dT}{dX_2} = \frac{RT^2}{\Delta H_f} \cdot (K - 1), \qquad (1)$$

in which T = absolute temperature in degrees Kelvin (°K), X_2 = mole fraction of minor component (solute; impurity), ΔH_f = molar heat of fusion of the major component, R = gas constant, and K = distribution ratio of solute between the solid and liquid phases.

Assuming that the temperature range is small and that no solid solutions are formed ($K = 0$), integration of the van't Hoff equation yields the following relationship between mole fraction of impurity and the melting-point depression:

$$X_2 = \frac{(T_o - T_m)\Delta H_f}{RT_o^2}, \qquad (2)$$

in which T_o = melting point of the pure compound, in °K, and T_m = melting point of the test specimen, in °K.

With no solid solution formation, the concentration of impurity in the liquid phase at any temperature during the melting is inversely proportional to the fraction melted at that temperature, and the melting-point depression is directly proportional to the mole fraction of impurity. A plot of the observed test specimen temperature, T_s, versus the reciprocal of the fraction melted, $1/F$, at temperature T_s, should yield a straight line with the slope equal to the melting-point depression $(T_o - T_m)$. The theoretical melting point of the pure compound is obtained by extrapolation to $1/F = 0$:

$$T_s = T_o - \frac{RT_o^2X_2(1/F)}{\Delta H_f}. \qquad (3)$$

Substituting the experimentally obtained values for $T_o - T_m$, ΔH_f, and T_o in Equation 2 yields the mole fraction of the total eutectic impurity, which, when multiplied by 100, gives the mole percentage of total eutectic impurities.

Deviations from the theoretical linear plot also may be due to solid solution formation ($K \neq 0$), so that care must be taken in interpreting the data.

To observe the linear effect of the impurity concentration on the melting-point depression, the impurity must be *soluble* in the liquid phase or melt of the compound, but *insoluble* in the solid phase, i.e., no solid solutions are formed. Some chemical similarities are necessary for solubility in the melt. For example, the presence of ionic compounds in neutral organic compounds and the occurrence of thermal decomposition may not be reflected in purity estimates. The extent of these theoretical limitations has been only partially explored.

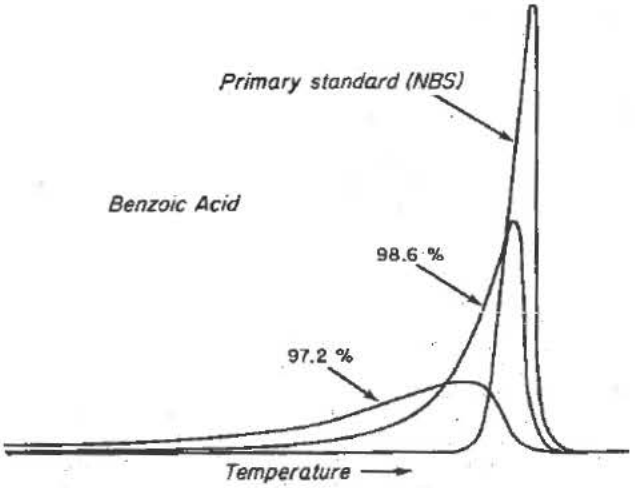
Impurities present from the synthetic route often are similar to the end product, hence there usually is no problem of solubility in the melt. Impurities consisting of molecules of the same shape, size, and character as those of the major component can fit into the matrix of the major component without disruption of the lattice, forming solid solutions or inclusions; such impurities are

not detectable by DSC. Purity estimates are too high in such cases. This is more common with less-ordered crystals as indicated by low heats of fusion.

Impurity levels calculated from thermograms are reproducible and probably reliable within 0.1% for ideal compounds. Melting-point determinations by scanning calorimetry have a reproducibility with a standard deviation of about 0.2°. Calibration against standards may allow about 1° accuracy for the melting point, so that this technique is comparable to other procedures.

Compounds that exist in polymorphic form cannot be used in purity determination unless the compound is completely converted to one form. On the other hand, DSC and DTA are inherently useful for detecting, and therefore monitoring, polymorphism.

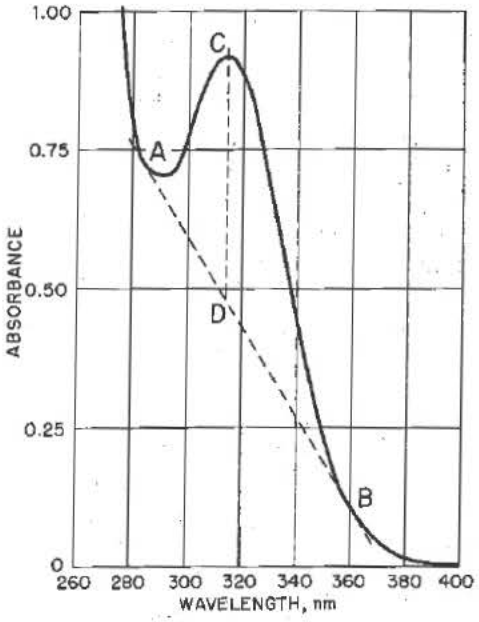
Procedure—The actual procedure and the calculations to be employed are dependent on the particular instrument used. Consult the manufacturer's literature and/or the thermal analysis literature for the most appropriate technique for a given instrument. In any event, it is imperative to keep in mind the limitations of solid solution formation, insolubility in the melt, polymorphism, and decomposition during the analysis.



Superimposed Thermograms Illustrating the Effect of Impurities on DSC Melting Peak Shape

⟨901⟩ ULTRAVIOLET ABSORBANCE OF CITRUS OILS

Place about 250 mg, accurately weighed, of the oil in a 100-mL volumetric flask, add alcohol to volume, and mix. Determine the ultraviolet absorption spectrum of this solution in the range from 260 nm to 400 nm in a 1-cm cell, with a suitable recording or manual spectrophotometer, using alcohol as the blank. If a manual instrument is used, read the absorbances at 5-nm intervals from 260 nm to a point about 12 nm from the expected maximum absorbance, then at 3-nm intervals for 3 readings, and at 1-nm intervals to a point about 5 nm beyond the maximum, and then at 10-nm intervals to 400 nm; and from these data, plot the absorbances as ordinates against wavelength on the abscissa, and draw the absorption spectrum or spectrogram. Draw a base-line tangent to the latter, as shown in the accompanying figure (which is typical of Lemon Oil), between points *A* and *B*. Locate the point of maximum absorbance (*C*), and from it drop a vertical line, perpendicular to the abscissa, that intersects line *AB* at *D*. Read from the ordinate the absorbances corresponding to points *D* and *C*, subtract the former from the latter, and calculate the difference on the basis of a 250-mg sample.



Typical Spectrum of Lemon Oil

⟨905⟩ UNIFORMITY OF DOSAGE UNITS

The uniformity of dosage units can be demonstrated by either of two methods, weight variation or content uniformity. The requirements of this chapter apply both to dosage forms containing a single active ingredient and to dosage forms containing two or more active ingredients.

Weight Variation requirements may be applied where the product is a liquid-filled soft capsule, or where the product to be tested contains 50 mg or more of an active ingredient comprising 50% or more, by weight, of the dosage-form unit. Uniformity with respect to other active ingredients, if present in lesser proportions, is demonstrated by *Content Uniformity* requirements. *Weight Variation* requirements may be applied to solids (including sterile solids) that contain no inactive or active added substances.

Weight Variation requirements may be applied to solids (including sterile solids), with or without inactive or active added substances, that have been prepared from true solutions and freeze-dried in the final containers, and labeled to indicate this method of preparation.

Content Uniformity requirements may be applied in all cases. The test for *Content Uniformity* is required for all coated tablets, including film-coated tablets, for transdermal systems, and for suspensions in single-unit containers or in soft capsules. The test for *Content Uniformity* is required for solids (including sterile solids) that contain inactive or active added substances, except that the test for *Weight Variation* may be applied for special situations as stated above.

WEIGHT VARIATION

For the determination of dosage-form uniformity by weight variation, select not less than 30 units, and proceed as follows for the dosage form designated. [NOTE—Specimens other than these test units may be drawn from the same batch for *Assay* determinations.]

UNCOATED TABLETS—Weigh accurately 10 tablets individually, and calculate the average weight. From the result of the *Assay*, obtained as directed in the individual monograph, calculate the content of active ingredient in each of 10 tablets, assuming homogeneous distribution of the active ingredient.

HARD CAPSULES—Weigh accurately 10 capsules individually, taking care to preserve the identity of each capsule. Remove the contents of each capsule by a suitable means. Weigh accurately the emptied shells individually, and calculate for each capsule the net weight of its contents by subtracting the weight of the shell from the respective gross weight. From the results of the *Assay*, obtained as directed in the individual monograph, cal-

culate the content of active ingredient in each of the capsules, assuming homogeneous distribution of the active ingredient.

SOFT CAPSULES—Determine the net weight of the contents of individual capsules as follows: Weigh accurately the 10 intact capsules individually to obtain their gross weights, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 minutes, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. From the results of the *Assay*, obtained as directed in the individual monograph, calculate the content of active ingredient in each of the capsules, assuming homogeneous distribution of the active ingredient.

SOLIDS IN SINGLE-UNIT CONTAINERS and STERILE SOLIDS FOR PARENTERAL USE—Proceed as directed under *Hard Capsules*, treating each unit as described therein.

CONTENT UNIFORMITY

For the determination of dosage-form uniformity by assay of individual units, select not less than 30 units, and proceed as follows for the dosage form designated.

UNCOATED AND COATED TABLETS, HARD AND SOFT CAPSULES, TRANSDERMAL SYSTEMS, SUSPENSIONS IN SINGLE-UNIT CONTAINERS, INHALATIONS IN SINGLE-UNIT CONTAINERS, and SOLIDS (INCLUDING STERILE SOLIDS) IN SINGLE-UNIT CONTAINERS—Assay 10 units individually as directed in the *Assay* in the individual monograph, unless otherwise specified in the test for *Content uniformity*. Where the amount of active ingredient in a single dose unit is less than required in the *Assay*, adjust the degree of dilution of the solutions and/or the volume of aliquots so that the concentration of the active ingredients in the final solution is of the same order as that obtained in the *Assay* procedure; or, in the case of a titrimetric assay, use a more dilute titrant, if necessary, so that an adequate volume of titrant is required (see *Titrimetry* ⟨541⟩); see also *Procedures* under *Tests and Assays* in the *General Notices*. If any such modifications are made in the *Assay* procedure set forth in the individual monograph, make the appropriate corresponding changes in the calculation formula and titration factor.

Where a special procedure is specified in the test for *Content uniformity* in the individual monograph, make any necessary correction of the results obtained as follows.

(1) Prepare a composite specimen of a sufficient number of dosage units to provide the amount of specimen called for in the *Assay* in the individual monograph plus the amount required for the special procedure given in the test for *Content uniformity* in the monograph by finely powdering tablets or mixing the contents of capsules or suspensions or solids in single-unit containers to obtain a homogeneous mixture. If a homogeneous mixture cannot be obtained in this manner, use suitable solvents or other procedures to prepare a solution containing all of the active ingredient, and use appropriate aliquot portions of this solution for the specified procedures.

(2) Assay separate, accurately measured portions of the composite specimen of capsules or tablets or suspensions or inhalations or solids in single-unit containers, both (a) as directed in the *Assay*, and (b) using the special procedure given in the test for *Content uniformity* in the monograph.

(3) Calculate the weight of active ingredient equivalent to 1 average dosage unit, by (a) using the results obtained by the *Assay* procedure, and by (b) using the results obtained by the special procedure.

(4) Calculate the correction factor, F, by the formula:

$$F = A/P,$$

in which A is the weight of active ingredient equivalent to 1 average dosage unit obtained by the *Assay* procedure, and P is the weight of active ingredient equivalent to 1 average dosage unit obtained by the special procedure.

(5) If F is between 0.970 and 1.030 no correction is required.

(6) If F is not within 0.970 and 1.030, calculate the weight of active ingredient in each dosage unit by multiplying each of the weights found using the special procedure by F.

Calculation of the Relative Standard Deviation

The use of pre-programmed calculators or computers is acceptable. A manual mathematical method is as follows:

s = sample standard deviation.

RSD = relative standard deviation (the sample standard deviation expressed as a percentage of the mean).

$\overline{X}$ = mean of the values obtained from the units tested, expressed as a percentage of the label claim.

n = number of units tested.

$x_1, x_2, x_3 \ldots x_n$ = individual values (x_i) of the units tested, expressed as a percentage of the label claim.

$$s = \left[\frac{\Sigma(x_i - \overline{X})^2}{n-1}\right]^{1/2}$$

$$RSD = \frac{100s}{\overline{X}}.$$

Criteria

Apply the following criteria, unless otherwise specified in the individual monograph.

(A) If the Average of the Limits Specified in the Potency Definition in the Individual Monograph is 100.0 Percent or Less—

COMPRESSED TABLETS (COATED OR UNCOATED), SUSPENSIONS IN SINGLE-UNIT CONTAINERS, SOLIDS (INCLUDING STERILE SOLIDS) IN SINGLE-UNIT CONTAINERS, and STERILE SOLIDS FOR PARENTERAL USE—Unless otherwise specified in the individual monograph, the requirements for dose uniformity are met if the amount of the active ingredient in each of the 10 dosage units as determined from the *Weight variation* or the *Content uniformity* method lies within the range of 85.0 percent to 115.0 percent of the label claim and the *Relative standard deviation* is less than or equal to 6.0 percent.

If 1 unit is outside the range of 85.0 percent to 115.0 percent of label claim and no unit is outside the range of 75.0 percent to 125.0 percent of label claim, or if the *Relative standard deviation* is greater than 6.0 percent, or if both conditions prevail, test 20 additional units. The requirements are met if not more than 1 unit of the 30 is outside the range of 85.0 percent to 115.0 percent of label claim and no unit is outside the range of 75.0 percent to 125.0 percent of label claim and the *Relative standard deviation* of the 30 dosage units does not exceed 7.8 percent.

CAPSULES, TRANSDERMAL SYSTEMS, INHALATIONS, AND MOLDED TABLETS—Unless otherwise specified in the individual monograph, the requirements for dose uniformity are met if the amount of the active ingredient in not less than 9 of the 10 dosage units as determined from the *Weight variation* or the *Content uniformity* method lies within the range of 85.0 percent to 115.0 percent of label claim and no unit is outside the range of 75.0 percent to 125.0 percent of label claim and the *Relative standard deviation* of the 10 dosage units is less than or equal to 6.0 percent.

If 2 or 3 dosage units are outside the range of 85.0 percent to 115.0 percent of label claim, but not outside the range of 75.0 percent to 125.0 percent of label claim, or if the *Relative standard deviation* is greater than 6.0 percent or if both conditions prevail, test 20 additional units. The requirements are met if not more than 3 units of the 30 are outside the range of 85.0 percent to 115.0 percent of label claim and no unit is outside the range of 75.0 percent to 125.0 percent of label claim, and the *Relative standard deviation* of the 30 dosage units does not exceed 7.8 percent.

(B) If the Average of the Limits Specified in the Potency Definition in the Individual Monograph is Greater than 100.0 Percent—

(1) If the average value of the dosage units tested is 100.0 percent or less, the requirements are as in (*A*).

(2) If the average value of the dosage units tested is greater than or equal to the average of the limits specified in the potency definition in the individual monograph, the requirements are as in (*A*), except that the words "label claim" are replaced by the words "label claim multiplied by the average of the limits specified in the potency definition in the monograph divided by 100."

(3) If the average value of the dosage units tested is between 100 percent and the average of the limits specified in the potency definition in the individual monograph, the requirements are as

in (*A*), except that the words "label claim" are replaced by the words "label claim multiplied by the average value of the dosage units tested (expressed as a percent of label claim) divided by 100."

⟨911⟩ VISCOSITY

Viscosity is a property of liquids that is closely related to the resistance to flow. It is defined in terms of the force required to move one plane surface continuously past another under specified steady-state conditions when the space between is filled by the liquid in question. It is defined as the shear stress divided by the rate of shear strain. The basic unit is the *poise;* however, viscosities commonly encountered represent fractions of the poise, so that the *centipoise* (1 poise = 100 centipoises) proves to be the more convenient unit. The specifying of temperature is important because viscosity changes with temperature; in general, viscosity decreases as temperature is raised. While on the absolute scale viscosity is measured in poises or centipoises, for convenience the kinematic scale, in which the units are *stokes* and *centistokes* (1 stoke = 100 centistokes) commonly is used. To obtain the kinematic viscosity from the absolute viscosity, the latter is divided by the density of the liquid at the same temperature, i.e., kinematic viscosity = (absolute viscosity)/(density). The sizes of the units are such that viscosities in the ordinary ranges are conveniently expressed in centistokes. The approximate viscosity in centistokes at room temperature of ether is 0.2; of water, 1; of kerosene, 2.5; of mineral oil, 20 to 70; and of honey, 10,000.

Absolute viscosity can be measured directly if accurate dimensions of the measuring instruments are known, but it is more common practice to calibrate the instrument with a liquid of known viscosity and to determine the viscosity of the unknown fluid by comparison with that of the known.

Many substances, such as the gums employed in pharmacy, have variable viscosity, and most of them are less resistant to flow at higher flow rates. In such cases, a given set of conditions is selected for measurement, and the measurement obtained is considered to be an apparent viscosity. Since a change in the conditions of measurement would yield a different value for the apparent viscosity of such substances, the instrument dimensions and conditions for measurement must be closely adhered to by the operator.

Measurement of Viscosity—The usual method for measurement of viscosity involves the determination of the time required for a given volume of liquid to flow through a capillary. Many capillary-tube viscosimeters have been devised, but Ostwald and Ubbelohde viscosimeters are among the most frequently used. Several types are described, with directions for their use, by the American Society for Testing and Materials (ASTM, D-445). The viscosity of oils is expressed on arbitrary scales that vary from one country to another, there being several corresponding instruments. The most widely used are the Redwood No. I and No. II, the Engler, the Saybolt Universal, and the Saybolt Furol. Each of these instruments uses arbitrary units that bear the name of the instrument. Standard temperatures are adopted as a matter of convenience with these instruments. For the Saybolt instruments, measurements usually are made at 100°F and 210°F; Redwood instruments may be used at several temperatures up to 250°F; and values obtained on the Engler instrument usually are reported at 20°C and 50°C. A particularly convenient and rapid type of instrument is a rotational viscosimeter, which utilizes a bob or spindle immersed in the test specimen and measures the resistance to movement of the rotating part. Different spindles are available for given viscosity ranges, and several rotational speeds generally are available. Other rotational instruments may have a stationary bob and a rotating cup. The Brookfield, Rotouisco, and Stormer viscosimeters are examples of rotating-bob instruments, and the MacMichael is an example of the rotating-cup instrument. Numerous other rotational instruments of advanced design with special devices for reading or recording, and with wide ranges of rotational speed, have been devised.

Where only a particular type of instrument is suitable, the individual monograph so indicates.

For measurement of viscosity or apparent viscosity, the temperature of the substance being measured must be accurately controlled, since small temperature changes may lead to marked changes in viscosity. For usual pharmaceutical purposes, the temperature should be held to within ±0.1°.

Procedure for Cellulose Derivatives—Measurement of the viscosity of solutions of the high-viscosity types of methylcellulose is a special case, since they are too viscous for the commonly available viscosimeters. The Ubbelohde viscosimeter may be adapted (cf. ASTM, D-1347) to the measurement of the ranges of viscosity encountered in methylcellulose solutions.

Calibration of Capillary-Type Viscosimeters—Determine the viscosimeter constant, *k*, for each viscosimeter by the use of an oil of known viscosity.*

Ostwald-Type Viscosimeter—Fill the tube with the exact amount of oil (adjusted to 20.0 ± 0.1°) as specified by the manufacturer. Adjust the meniscus of the column of liquid in the capillary tube to the level of the top graduation line with the aid of either pressure or suction. Open both the filling and capillary tubes in order to permit the liquid to flow into the reservoir against atmospheric pressure. [NOTE—Failure to open either of these tubes will yield false values.] Record the time, in seconds, for liquid to flow from the upper mark to the lower mark in the capillary tube.

Ubbelohde-Type Viscosimeter—Place a quantity of the oil (adjusted to 20.0 ± 0.1°) in the filling tube, and transfer to the capillary tube by gentle suction, taking care to prevent bubble formation in the liquid by keeping the air vent tube closed. Adjust the meniscus of the column of liquid in the capillary tube to the level of the top graduation line. Open both the vent and capillary tubes in order to permit the liquid to flow into the reservoir against atmospheric pressure. [NOTE—Failure to open the vent tube before releasing the capillary tube will yield false values.] Record the time, in seconds, for the liquid to flow from the upper mark to the lower mark in the capillary tube.

Calculations—

Calculate the viscosimeter constant, *k*, from the equation:

$$k = v/dt,$$

in which *v* is the known viscosity of the liquid in centipoises, *d* is the specific gravity of the liquid tested at 20°/20°, and *t* is the time in seconds for the liquid to pass from the upper mark to the lower mark.

If a viscosimeter is repaired, it must be recalibrated, since even minor repairs frequently cause significant changes in the value of its constant, *k*.

⟨921⟩ WATER DETERMINATION

Many Pharmacopeial articles either are hydrates or contain water in adsorbed form. As a result, the determination of the water content is important in demonstrating compliance with the Pharmacopeial standards. Generally one of the methods given below is called for in the individual monograph, depending upon the nature of the article. In rare cases, a choice is allowed between two methods. When the article contains water of hydration, the Titrimetric Method, the Azeotropic Method, or the Gravimetric Method is employed, as directed in the individual monograph, and the requirement is given under the heading, *Water*.

The heading, *Loss on drying* (see ⟨731⟩), is used in those cases where the loss sustained on heating may be not entirely water.

I—Titrimetric Method

Principle—The titrimetric determination of water is based upon the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer which reacts with hydrogen ions.

In the original titrimetric solution, known as Karl Fischer Reagent, the sulfur dioxide and iodine are dissolved in pyridine and methanol. The test specimen may be titrated with the *Reagent* directly, or the analysis may be carried out by a residual titration

* Oils of known viscosities may be obtained from the Cannon Instrument Co., Box 16, State College, PA 16801. For methylcellulose, choose an oil the viscosity of which is as close as possible to that of the type of methylcellulose to be determined.

procedure. In the residual titration, excess *Reagent* is added to the test specimen, sufficient time is allowed for the reaction to reach completion, and the unconsumed *Reagent* is titrated with a standard solution of water in a solvent such as methanol. The residual titration procedure is applicable generally and avoids the difficulties that may be encountered in the direct titration of substances from which the bound water is released slowly.

The stoichiometry of the reaction is not exact, and the reproducibility of a determination depends upon such factors as the relative concentrations of the *Reagent* ingredients, the nature of the inert solvent used to dissolve the test specimen, and the technique used in the particular determination. Therefore, an empirically standardized technique is used in order to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system. The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen; however, other suitable solvents may be used for special or unusual test specimens.

Apparatus—Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and determination of the end-point. In the case of a colorless solution that is titrated directly, the end-point may be observed visually as a change in color from canary yellow to amber. The reverse is observed in the case of a test specimen that is titrated residually. More commonly, however, the end-point is determined electrometrically with an apparatus employing a simple electrical circuit that serves to impress about 200 mV of applied potential between a pair of platinum electrodes (about 5 square mm in area and about 2.5 cm apart) immersed in the solution to be titrated. At the end-point of the titration a slight excess of the reagent increases the flow of current to between 50 and 150 microamperes for 30 seconds to 30 minutes, depending upon the solution being titrated. The time is shortest for substances that dissolve in the reagent. With some automatic titrators, the abrupt change in current or potential at the end-point serves to close a solenoid-operated valve that controls the buret delivering the titrant. Commercially available apparatus generally comprises a closed system consisting of one or two automatic burets and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant such as phosphorus pentoxide, and the titration vessel may be purged by means of a stream of dry nitrogen or current of dry air.

Reagent—

Prepare the Karl Fischer Reagent as follows:

Add 125 g of iodine to a solution containing 670 mL of methanol and 170 mL of pyridine, and cool. Place 100 mL of pyridine in a 250-mL graduated cylinder and, keeping the pyridine cold in an ice bath, pass in dry sulfur dioxide until the volume reaches 200 mL. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake well to dissolve the iodine, transfer the solution to the apparatus, and allow to stand overnight before standardizing. One mL of this solution when freshly prepared is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 hour before use, or daily if in continuous use. Protect from light while in use. Store any bulk stock of the reagent in a suitably sealed, glass-stoppered container, fully protected from light, and under refrigeration.

A commercially available, stabilized solution of Karl Fischer type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine and/or alcohols other than methanol may be used also. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted *Reagent* called for in some monographs should be diluted as directed by the manufacturer. Either methanol or other suitable solvent, such as ethylene glycol monomethyl ether, may be used as the diluent.

Test Preparation—Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 10 to 250 mg of water.

Where the specimen under test is an aerosol with propellant, store it in a freezer for not less than 2 hours, open the container, and test 10.0 mL of the well-mixed specimen. In titrating the specimen, determine the end-point at a temperature of 10° or higher.

Where the specimen under test is capsules, use a portion of the mixed contents of not less than 4 capsules.

Where the specimen under test is tablets, use powder from not less than 4 tablets ground to a fine powder in an atmosphere of about 10% relative humidity.

Where the monograph specifies that the specimen under test is hygroscopic, using a dry syringe inject an appropriate volume of methanol, or other suitable solvent, accurately measured, into the container, previously accurately weighed, and shake to dissolve the specimen. Using the same syringe, remove the solution from the container and transfer it to a titration vessel prepared as directed under *Procedure*. Repeat the procedure with a second portion of methanol, or other suitable solvent, accurately measured, add this washing to the titration vessel, and immediately titrate. Determine the water content, in mg, of a portion of solvent of the same total volume as that used to dissolve the specimen and to wash the container and syringe, as directed under *Standardization of Water Solution for Residual Titrations*, and subtract this value from the water content, in mg, obtained in the titration of the specimen under test. Dry the container and its closure at 100° for 3 hours, allow to cool in a desiccator, and weigh. Determine the weight of specimen tested from the difference in weight from the initial weight of the container.

Standardization of the Reagent—Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient *Reagent* to give the characteristic end-point color, or 100 ± 50 microamperes of direct current at about 200 mV of applied potential.

For determination of trace amounts of water (less than 1%), sodium tartrate may be used as a convenient water reference substance. Quickly add 150 to 350 mg of sodium tartrate ($C_4H_4Na_2O_6 \cdot 2H_2O$), accurately weighed by difference, and titrate to the end-point. The water equivalence factor F, in mg of water per mL of reagent, is given by the formula:

$$2(18.02/230.08)(W/V),$$

in which 18.02 and 230.08 are the molecular weights of water and sodium tartrate dihydrate, respectively. W is the weight, in mg, of sodium tartrate dihydrate, and V is the volume, in mL, of the *Reagent* consumed in the second titration.

For the precise determination of significant amounts of water (more than 1%), use purified water obtained by distillation as the reference substance. Quickly add between 25 mg and 250 mg of water, accurately weighed by difference, from a weighing pipet or from a pre-calibrated syringe or micropipet, the amount taken being governed by the reagent strength and the buret size, as referred to under *Volumetric Apparatus* ⟨31⟩. Titrate to the end-point. Calculate the water equivalence factor, F, in mg of water per mL of reagent, by the formula:

$$W/V,$$

in which W is the weight, in mg, of the water, and V is the volume, in mL, of the reagent required.

Standardization of Water Solution for Residual Titration—Prepare a *Water Solution* by diluting 2 mL of water with methanol or other suitable solvent to 1000 mL. Standardize this solution by titrating 25.0 mL with the *Reagent*, previously standardized as directed under *Standardization of the Reagent*. Calculate the water content, in mg per mL, of the *Water Solution* by the formula:

$$V'F/25,$$

in which V' is the volume of the *Reagent* consumed, and F is the water equivalence factor of the *Reagent*. Determine the water content of the *Water Solution* weekly, and standardize the *Reagent* against it periodically as needed.

Procedure—Determine the water by *Method Ia*, unless otherwise specified in the individual monograph.

Method Ia (*Direct Titration*)—Unless otherwise specified, transfer 35 to 40 mL of methanol or other suitable solvent to the titration vessel, and titrate with the *Reagent* to the electrometric or visual end-point to consume any moisture that may be present. (Disregard the volume consumed, since it does not enter into the calculations.) Quickly add the *Test Preparation*, mix, and again titrate with the *Reagent* to the electrometric or visual end-point. Calculate the water content of the specimen, in mg, by the formula:

$$SF,$$

in which S is the volume, in mL, of the *Reagent* consumed in the second titration, and F is the water equivalence factor of the *Reagent*.

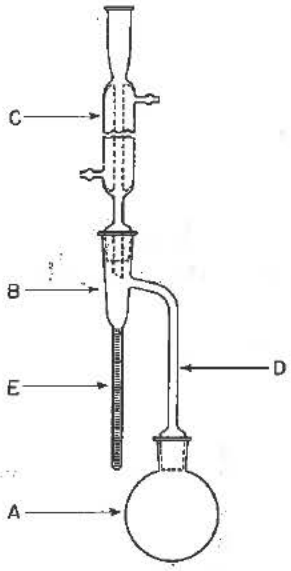
Method Ib (*Residual Titration*)—Where the individual monograph specifies that the water content is to be determined by *Method Ib*, the *residual titration* procedure, transfer 35 to 40 mL of methanol or other suitable solvent to the titration vessel, and titrate with the *Reagent* to the electrometric or visual end-point. Quickly add the *Test Preparation*, mix, and add an accurately measured excess of the *Reagent*. Allow sufficient time for the reaction to reach completion, and titrate the unconsumed *Reagent* with standardized *Water Solution* to the electrometric or visual end-point. Calculate the water content of the specimen, in mg, by the formula:

$$F(X' - XR),$$

in which F is the water equivalence factor of the *Reagent*, X' is the volume, in mL, of the *Reagent* added after introduction of the specimen, X is the volume, in mL, of standardized *Water Solution* required to neutralize the unconsumed *Reagent*, and R is the ratio, $V'/25$ (mL *Reagent*/mL *Water Solution*), determined from the *Standardization of Water Solution for Residual Titration*.

II—Azeotropic (Toluene Distillation) Method

Apparatus—Use a 500-mL glass flask *A* connected by means of a trap *B* to a reflux condenser *C* by ground glass joints (see illustration).



Toluene Moisture Apparatus

The critical dimensions of the parts of the apparatus are as follows: The connecting tube *D* is 9 to 11 mm in internal diameter. The trap is 235 to 240 mm in length. The condenser, if of the straight-tube type, is approximately 400 mm in length and not less than 8 mm in bore diameter. The receiving tube *E* has a 5-mL capacity and its cylindrical portion, 146 to 156 mm in length, is graduated in 0.1-mL subdivisions, so that the error of reading is not greater than 0.05 mL for any indicated volume. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the connecting tube may be insulated with asbestos.

Clean the receiving tube and the condenser with chromic acid cleansing mixture, thoroughly rinse with water, and dry in an oven. Prepare the toluene to be used by first shaking with a small quantity of water, separating the excess water, and distilling the toluene.

Procedure—Place in the dry flask a quantity of the substance, weighed accurately to the nearest centigram, which is expected to yield 2 to 4 mL of water. If the substance is of a pasty character, weigh it in a boat of metal foil of a size that will just pass through the neck of the flask. If the substance is likely to cause bumping, add enough dry, washed sand to cover the bottom of the flask, or a number of capillary melting-point tubes, about 100 mm in length, sealed at the upper end. Place about 200 mL of toluene in the flask, connect the apparatus, and fill the receiving tube *E* with toluene poured through the top of the condenser. Heat the flask gently for 15 minutes and, when the toluene begins to boil, distil at the rate of about 2 drops per second until most of the water has passed over, then increase the rate of distillation to about 4 drops per second. When the water has apparently all distilled over, rinse the inside of the condenser tube with toluene while brushing down the tube with a tube brush attached to a copper wire and saturated with toluene. Continue the distillation for 5 minutes, then remove the heat, and allow the receiving tube to cool to room temperature. If any droplets of water adhere to the walls of the receiving tube, scrub them down with a brush consisting of a rubber band wrapped around a copper wire and wetted with toluene. When the water and toluene have separated completely, read the volume of water, and calculate the percentage that was present in the substance.

III—Gravimetric Method

Procedure for Chemicals—Proceed as directed in the individual monograph preparing the chemical as directed under *Loss on Drying* ⟨731⟩.

Procedure for Biologics—Proceed as directed in the individual monograph.

Procedure for Vegetable Drugs—Place about 10 g of the drug, prepared as directed (see *Vegetable Drugs—Methods of Analysis* ⟨561⟩) and accurately weighed, in a tared evaporating dish. Dry at 105° for 5 hours, and weigh. Continue the drying and weighing at 1-hour intervals until the difference between two successive weighings corresponds to not more than 0.25%.

⟨941⟩ X-RAY DIFFRACTION

Every crystal form of a compound produces its own characteristic X-ray diffraction pattern. These diffraction patterns can be derived either from a single crystal or from a powdered specimen (containing numerous crystals) of the material. The spacings between and the relative intensities of the diffracted maxima can be used for qualitative and quantitative analysis of crystalline materials. Powder diffraction techniques are most commonly employed for routine identification and the determination of relative purity of crystalline materials. Small amounts of impurity, however, are not normally detectable by the X-ray diffraction method, and for quantitative measurements it is necessary to prepare the sample carefully to avoid preferred orientation effects.

The powder methods provide an advantage over other means of analysis in that they are usually nondestructive in nature (specimen preparation is usually limited to grinding to ensure a randomly oriented sample, and deleterious effects of X-rays on solid pharmaceutical compounds are not commonly encountered). The principal use of single-crystal diffraction data is for the determination of molecular weights and analysis of crystal structures at the atomic level. However, diffraction established for a single crystal can be used to support a specific powder pattern as being truly representative of a single phase.

Solids—A solid substance can be classified as being crystalline, noncrystalline, or a mixture of the two forms. In crystalline materials, the molecular or atomic species are ordered in a three-dimensional array, called a lattice, within the solid particles. This ordering of molecular components is lacking in noncrystalline material. Noncrystalline solids sometimes are referred to as glasses or amorphous solids when repetitive order is nonexistent in all three dimensions. It is also possible for order to exist in only one or two dimensions, resulting in mesomorphic phases (liquid crystals). Although crystalline materials are usually considered to have well-defined visible external morphologies (their habits), this is not a necessity for X-ray diffraction analysis.

The relatively random arrangement of molecules in noncrystalline substances makes them poor coherent scatterers of X-rays, resulting in broad, diffuse maxima in diffraction patterns. Their X-ray patterns are quite distinguishable from crystalline specimens, which give sharply defined diffraction patterns.

Many compounds are capable of crystallizing in more than one type of crystal lattice. At any particular temperature and pressure, only one crystalline form (polymorph) is thermodynamically stable. Since the rate of phase transformation of a metastable polymorph to the stable one can be quite slow, it is not uncommon to find several polymorphs of crystalline pharmaceutical compounds existing under normal handling conditions.

In addition to exhibiting polymorphism, many compounds form crystalline solvates in which the solvent molecule is an integral part of the crystal structure. Just as every polymorph has its own characteristic X-ray patterns, so does every solvate. Sometimes the differences in the diffraction patterns of different polymorphs are relatively minor, and must be very carefully evaluated before a definitive conclusion is reached. In some instances, these polymorphs and/or solvates show varying dissolution rates. Therefore, on the time scale of pharmaceutical bioavailability, different total amounts of drug are dissolved, resulting in potential bioinequivalence of the several forms of the drug.

Fundamental Principles—A collimated beam of monochromatic X-rays is diffracted in various directions when it impinges upon a rotating crystal or randomly oriented powdered crystal. The crystal acts as a three-dimensional diffraction grating to this radiation. This phenomenon is described by Bragg's law, which states that diffraction (constructive interference) can occur only when waves that are scattered from different regions of the crystal, in a specific direction, travel distances differing by integral numbers (n) of the wavelength (λ). Under such circumstances, the waves are in phase. This condition is described by the Bragg equation:

$$\frac{n\lambda}{2 \sin \theta} = d_{hkl},$$

in which d_{hkl} denotes the interplanar spacings and θ is the angle of diffraction.

A family of planes in space can be indexed by three whole numbers, usually referred to as Miller indices. These indices are the reciprocals, reduced to smallest integers, of the intercepts that a plane makes along the axes corresponding to three nonparallel edges of the unit cell (basic crystallographic unit). The unit cell dimensions are given by the lengths of the spacings along the three axes, a, b, c, and the angles between them, α, β, and γ. The interplanar spacing for a specific set of parallel planes hkl is denoted by d_{hkl}. Each such family of planes may show higher orders of diffraction where the d values for the related families of planes nh, nk, nl are diminished by the factor $1/n$ (n being an integer: 2, 3, 4, etc.). Every set of planes throughout a crystal has a corresponding Bragg diffraction angle associated with it (for a specific λ).

The amplitude of a diffracted X-ray beam from any set of planes is dependent upon the following atomic properties of the crystal: (1) position of each atom in the unit cell; (2) the respective atomic scattering factors; and (3) the individual thermal motions. Other factors that directly influence the intensities of the diffracted beam are: (1) the intensity and wavelength of the incident radiation; (2) the volume of crystalline specimen; (3) the absorption of the X-radiation by the specimen; and (4) the experimental arrangement utilized to record the intensity data. Thus, the experimental conditions are especially important for measurement of diffraction intensities.

Only a limited number of Bragg planes are in a position to diffract when monochromatized X-rays pass through a single crystal. Techniques of recording the intensities of all of the possible diffracting hkl planes involve motion of the single crystal and the recording media. Recording of these data is accomplished by photographic techniques (film) or with radiation detectors.

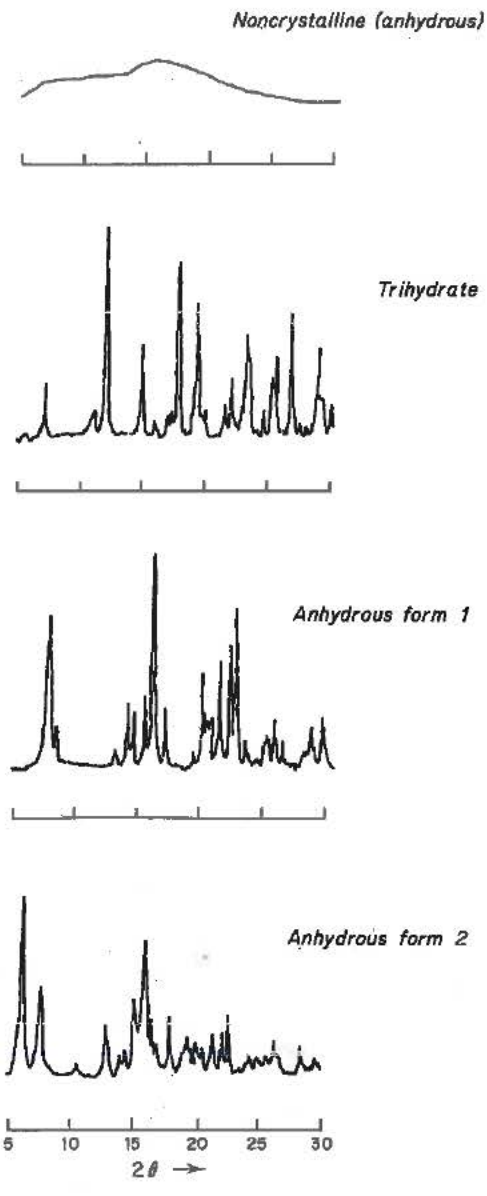
A beam passing through a very large number of small, randomly oriented crystals produces continuous cones of diffracted rays from each set of lattice planes. Each cone corresponds to the diffraction from various planes having a similar interplanar spacing. The intensities of these Bragg reflections are recorded by either film or radiation detectors. The Bragg angle can be measured easily from a film, but the advent of radiation detectors has made possible the construction of diffractometers that read this angle directly. The intensities and d spacings are more conveniently determined with powder diffractometers employing radiation detectors than by film methods. Microphotometers are frequently used for precise intensity measurements of films.

An example of the type of powder patterns obtained for four different solid phases of ampicillin are shown in the accompanying figure. These diffraction patterns were derived from a powder diffractometer equipped with a Geiger-Müller detector; nickel-filtered Cu $K\alpha$ radiation was used.

Radiation—The principal radiation sources utilized for X-ray diffraction are vacuum tubes utilizing copper, molybdenum, iron, and chromium as anodes; copper X-rays are employed most commonly for organic substances. For each of these radiations there is an element that will filter off the $K\beta$ radiation and permit the $K\alpha$ radiation to pass (nickel is used, in the case of copper radiation). In this manner the radiation is practically monochromatized. The choice of radiation to be used depends upon the absorption characteristics of the material and possible fluorescence by atoms present in the specimen.

Caution—Care must be taken in the use of such radiation. Those not familiar with the use of X-ray equipment should seek expert advice. Improper use can result in harmful effects to the operator.

Test Preparation—In an attempt to improve randomness in the orientation of crystallites (and, for film techniques, to avoid a grainy pattern), the specimen may be ground in a mortar to a fine powder. Grinding pressure has been known to induce phase



Typical Powder Patterns Obtained for Four Solid Phases of Ampicillin

transformations; therefore, it is advisable to check the diffraction pattern of the unground sample.

In general, the shapes of many crystalline particles tend to give a specimen that exhibits some degree of preferred orientation in the specimen holder. This is especially evident for needle-like or plate-like crystals where size reduction yields finer needles or platelets. Preferred orientation in the specimen influences the relative intensities of various reflections.

Several specialized handling techniques may be employed to minimize preferred orientation, but further reduction of particle size is often the best approach.

Where very accurate measurement of the Bragg angles is necessary, a small amount of an internal standard can be mixed into the specimen. This enables the film or recorder tracing to be calibrated. If comparisons to literature values (including compendial limits) of d are being made, calibrate the diffractometer. NBS standards are available covering to a d-value of 0.998 nm. Tetradecanol[1] may be used (d is 3.963 nm) for larger spacing.

The absorption of the radiation by any specimen is determined by the number and kinds of atoms through which the X-ray beam passes. An organic matrix usually absorbs less of the diffracted radiation than does an inorganic matrix. Therefore, it is important in quantitative studies that standard curves relating amount of material to the intensity of certain d spacings for that substance be determined in a matrix similar to that in which the substance will be analyzed.

In quantitative analyses of materials, a known amount of standard usually is added to a weighed amount of specimen to be analyzed. This enables the amount of the substance to be determined relative to the amount of standard added. The standard used should have approximately the same density as the specimen and similar absorption characteristics. More important, its diffraction pattern should not overlap to any extent with that of the material to be analyzed. Under these conditions a linear relationship between line intensity and concentration exists. In favorable cases, amounts of crystalline materials as small as 10% may be determined in solid matrices.

Identification of crystalline materials can be accomplished by comparison of X-ray powder diffraction patterns obtained for known[2] materials with those of the unknown. The intensity ratio (ratio of the peak intensity of a particular d spacing to the intensity of the strongest maxima in the diffraction pattern) and the d spacing are used in the comparison. If a reference material (e.g., USP Reference Standard) is available, it is preferable to generate a primary reference pattern on the same equipment used for running the unknown sample, and under the same conditions. For most organic crystals, it is appropriate to record the diffraction pattern to include values for 2θ that range from as near zero degrees as possible to 40 degrees. Agreement between sample and reference should be within the calibrated precision of the diffractometer for diffraction angle (2θ values should typically be reproducible to ± 0.10 or 0.20 degrees), while relative intensities between sample and reference may vary up to 20 percent. For other types of samples (e.g., inorganic salts), it may be necessary to extend the 2θ region scanned to well beyond 40 degrees. It is generally sufficient to scan past the ten strongest reflections identified in the JCPDS[2] file.

[1] Brindley, GW and Brown, G, eds., *Crystal Structures of Clay Minerals and their X-ray Identification*, Mineralogical Society Monograph No. 5, London, 1980, pp. 318 ff.

[2] The Joint Committee on Powder Diffraction Standards, 1601 Park Lane, Swarthmore, PA 19081, maintains a file on more than 21,000 crystalline materials, both organic and inorganic, suitable for such comparisons.