CHAPTER **10**

X-Ray Analysis

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10.1 PRELIMINARY REMARKS

There are a number of X-ray methods of analysis which are useful to the pharmaceutical scientist. Some of these bear little relationship to the others,

except for the common employment of X-radiation as an energy source. For the reader who is unfamiliar with these analytical methods, some preliminary remarks are pertinent.

X-Ray *diffraction* procedures apply only to *crystalline* materials, and the results disclose information about the material as a *compound*. Analyses by both X-ray *emission* (fluorescence) and X-ray *absorption* techniques may be applied to material in any physical state, solid, liquid, or gas. These methods provide analysis by chemical *elements*, rather than by compound, and are largely insensitive to the combination state of the element. They are applicable to all elements except those of low atomic number. Emission analysis is more sensitive than absorption analysis at trace levels, although at higher element concentration there are advantages to the absorption method.

The purpose of this chapter is to acquaint the pharmaceutical scientist with the various X-ray methods which he may find useful. No description has been made of the extensive array of instruments and accessories now available, nor of the mechanical or electronic principles involved in their operation. In general, the manufacturers' literature is the best source of this information.

Some of the figures used in this chapter have appeared before. The author is grateful to the editors of the *Journal of Pharmaceutical Sciences* for permission to reuse Fig. 10.6 and Figs. 10.8 through 10.14, which first appeared in that journal as part of original publications of the author.

10.2 X-RAY SAFETY

Two hazards are involved in working with X-rays. One stems from the high voltages associated with X-ray generators (20 to 60 kV). Such potentials can be lethal, but fortunately the manufacturers of modern-day equipment have built in many protective features, and thus risks from high voltages are virtually nonexistent with proper use of the equipment.

An additional hazard stems from the X-rays themselves. Although most X-ray equipment provides features to protect against radiation damage to personnel, the routine use of the equipment allows many opportunities for oversight on the part of the operator which result in radiation exposure. This can be from "scattered" radiation throughout the room as well as from the direct X-ray beam itself.

From sufficient exposure, somatic injuries (those to the individual operator) include leukemia and other malignant diseases, ocular lens opacities, impaired fertility, and shortening of life. Genetic injuries (those to the offspring of the irradiated individual) may not become apparent for many generations. Constant awareness and concern of the operator for the safety of himself and others is essential.

Convenient radiation monitoring devices in the form of "r-meters" are available commercially, and should be used to monitor the laboratory

routinely. It is also advisable for operating personnel to wear a "film badge," which is a small plastic device, clipped to the clothing, and which contains a strip of unexposed film. This film, usually a standard "dental pack," is replaced and developed regularly, providing evidence of accumulated exposure. The subject of the maximum permissible dose for a variety of circumstances has been thoroughly studied, and recommendations have been published.*)

10.3 PRODUCTION AND PROPERTIES OF X-RAYS

X-Ray tubes for analytical use are devices for bringing about, in a controlled fashion, the bombardment with fast-moving electrons of a pure target metal, which in turn emits the X-radiation. Several processes occur when the electrons impinge on the target metal. One is a quantum process in which an inner-shell electron of the target atom is displaced by collision, thereby ionizing the atom. The vacancy may then be filled by an outer-shell electron, a process accompanied by a release in energy. This energy is in the form of X-rays, of wavelength characteristic of the atom. A second quantum process occurring as a result of the electron bombardment of the target metal is simple decreasing of the velocity of the impinging electrons, due to the influence of the electric field near the atomic nucleus. The decrease in energy ΔE of the electron is compensated by an energy release in the form of an X-ray of frequency ν, according to the Einstein equation

$$h\nu = \Delta E \tag{10.1}$$

where h is Planck's constant. The X-rays thus produced are not characteristic of the bombarded atom, but consist of a wide range of continuously varying wavelengths, limited in range by the energy of the bombarding electrons.

Superposition of the two types of emitted X-rays gives an overall pattern similar to that shown in Fig. 10.1, which is an idealized drawing of the intensity of emitted radiation as a function of its wavelength, for sufficiently intense electron bombardment of a target atom. The two peaks represent X-radiation characteristic of the target element. The $K\alpha$ peak represents the energy emitted by an L-shell electron filling a K-shell vacancy; the $K\beta$ peak represents energy emitted by an M electron filling a K-shell vacancy. Similarly, the filling of L-shell vacancies by outer-shell electrons gives rise to an L series of peaks, and so forth. A relationship known as Moseley's law relates the wavelength of the characteristic radiation to the atomic number of the target element.

Monochromatic X-radiation is produced by effectively removing all radiation of wavelength lower than the $K\alpha$ radiation. This is accomplished by

* National Bureau of Standards Handbooks No. 76 and 93, Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402.

passing the radiation beam through a material whose mass absorption properties allow passage of wavelengths immediately greater than some specific value and effect absorption of wavelengths immediately below this value. The value is called the "absorption edge "for the filtering element; it is the wavelength at which a discrete discontinuity exists in a plot of the mass absorption coefficient vs. wavelength for the element. This discontinuity

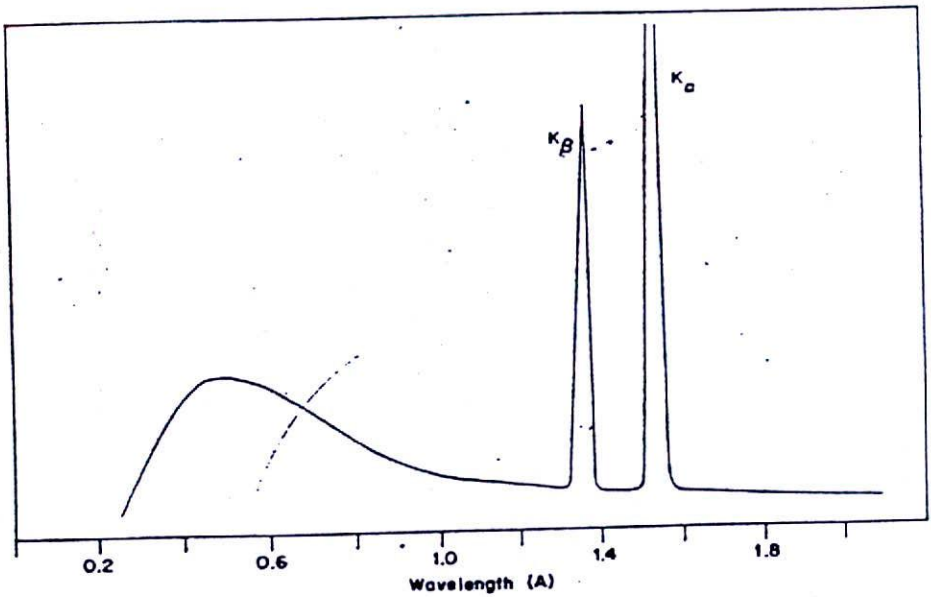

FIGURE 10.1: Intensity distribution in an X-ray beam from a copper target.

for nickel, for instance, is at a wavelength which is between the wavelength values for the $K\alpha$ and $K\beta$ radiation from a copper target. A thin film of nickel therefore selectively filters $K\beta$ and lower "white" wavelengths from copper radiation to produce fairly monochromatic X-rays of known wavelength. (Copper $K\alpha = 1.5418$ Å).

10.4 X-RAY DIFFRACTION

A. PRINCIPLES OF DIFFRACTION

As noted in preliminary remarks to this chapter, the analysis of materials by X-ray diffraction is limited to those materials which are crystalline. Ideal crystals are regular polyhedral forms bounded by smooth surfaces (faces)

which reflect an orderly internal arrangement of atoms or molecules. In the pharmaceutical field one seldom encounters large, ideal crystals with faces present, but the same internal arrangement persists through grinding or micronizing of the crystalline material, and it is this inner structure of crystals—a three-dimensional repeat pattern called a "space lattice"—which is responsible for the diffraction of X-rays.

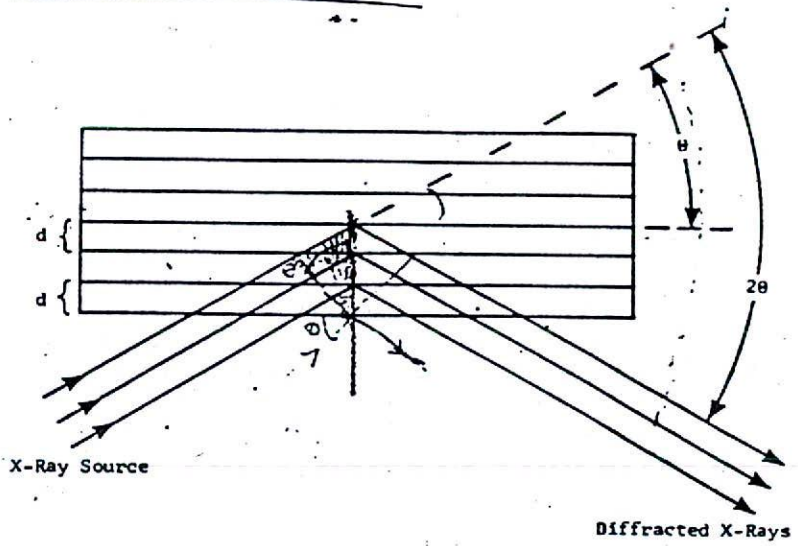

FIGURE 10.2: Diffraction of X-rays from an oriented crystal.

It is significant that the units of the space lattice are separated by distances of the same order of magnitude as the wavelength of X-rays. Upon impingement, the X-rays are scattered by the electrons within the atoms making up the space lattice. As the atoms are regularly arranged in a repeat pattern, the wave fronts emerging from each scattering center form a pattern. It is the reinforcement in specific directions of scattered X-rays which comprises the diffracted beam.

The atoms or molecules making up the internal structure of the crystal lie at intersections of the space lattice. This gives rise to a number of sets of planes, each set bearing a different angular relationship to the other sets. The planes within any one set are mutually parallel and are separated from each other by a fixed distance, called the interplanar spacing *d*. In general, each set of planes has a different *d* value from the other sets. (The sets of planes most densely populated with lattice points are most likely to have natural external crystal faces parallel to them.)

Consider a crystal whose edge view is shown in Fig. 10.2. The lines shown within the crystal are traces of planes which are parallel to one another and

are separated by the fixed spacing d. A beam of X-rays of wavelength λ is directed to these planes at an angle θ. A fundamental relationship, the Bragg equation, describes the condition for diffraction:

$$n\lambda = 2d \sin \theta \tag{10.2}$$

In this relationship n is a whole number. In Fig. 10.2, if the incident beam of X-rays is varied with respect to the fixed planes of the crystal through the angle θ, diffraction would occur only at specific relative positions (values of θ). These would be the positions at which the Bragg equation would be satisfied for values of $n = 1$, $n = 2$, etc. Thus, various *orders* of diffraction are possible from a given set of planes within a crystal. The intensity of diffracted rays differs for various orders.

From the Bragg equation, it can be seen that with a known wavelength of incident X-rays, and a measurable value for θ, the interplanar spacing d of the planes in the crystal of Fig. 10.2 can be calculated. This d value is a physical constant, characteristic for the one set of planes within the crystalline material. A repositioning of the crystal, such that a different set of planes is properly oriented to the incident X-ray beam, will allow the measurement of the d value for this second set of planes. Use of a single crystal, with known orientations to measure a few selected d values, constitutes the single crystal method of analysis, described in a later section.

One could continue this reorientation of a crystal and measurement until all possible d values, corresponding to all sets of planes, are measured. In practice, however, this multiple reorientation is accomplished in a simple manner by grinding the crystalline material to a fine powder, and exposing the packed powder to the incident X-ray beam in such a manner that *all possible orientations* of the crystal are presented. Whenever a crystal in the powder is in a position such that the Bragg equation is satisfied, diffraction will occur. Since all orientations are represented, diffractions at many values of θ—for the many d values—occur. This procedure was first described independently by Debye and Scherrer in Germany[1] and Hull in the United States,[2] and is the basis of the powder method, described next.

B. POWDER DIFFRACTION

1. Instrumentation

One method for the measurement of the diffraction angles, and hence the d spacings, from a powder specimen employs an X-ray *powder-diffraction camera*. Figure 10.3 depicts the use of this device in the popular Debye-Scherrer method. The camera is a light-tight container which holds a strip of X-ray film in the shape of a cylinder of known radius. Incident X-rays of known wavelength are collimated by a beam tunnel, and directed at the powdered sample contained in a thin-walled capillary. The specimen is

automatically rotated at a slow rate during the exposure time (2 to 4 hr. depending upon film characteristics and the nature of the sample), allowing a large number of crystallites within the sample to fall into a position relative to the incident beam such that the Bragg equation is satisfied. The diffraction from each set of planes occurs as a diverging cone from the sample, exposing the film strip in a series of arcs. Following development of the film, the distances between corresponding arcs are measured, converted to values of 2θ from the known camera radius, and recorded. The series of interplanar spacings, or d values, is calculated from the known wavelength of the incident X-rays and the measured values for 2θ.

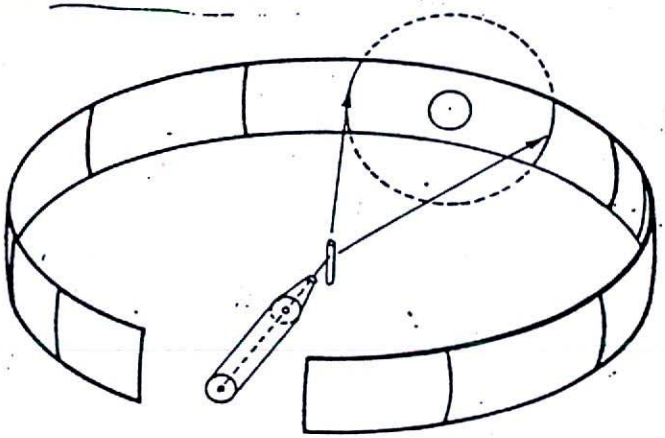

FIGURE 10.3: X-ray powder diffraction in a film camera.

Because of its high degree of versatility, the X-ray *diffractometer* (spectrometer) has considerable value to the pharmaceutical scientist. Among other features, the diffractometer permits measurement of the 2θ values from a powder sample without the use of film recording and the long film exposure and development times. X-Ray diffraction equipment produced by two of the several manufacturers who supply such instrumentation are pictured in Figs. 10.4 and 10.5. That portion of the diffractometer designed to measure angles is referred to as a "goniometer." Its operation is depicted in Fig. 10.6, where S represents an edge view of a shallow tray containing the powdered sample with a planar surface.

A proportional or other electronic counter C rotates about S at an angle of 2θ, while the sample turns at θ, relative to the fixed X-ray source. X-ray beam slitt (sl) define the beam parallel to the sample surface. While the scanning operation is in progress, a strip-chart recorder operates to plot the diffracted intensity (detected by the counter) as a function of the angle 2θ.

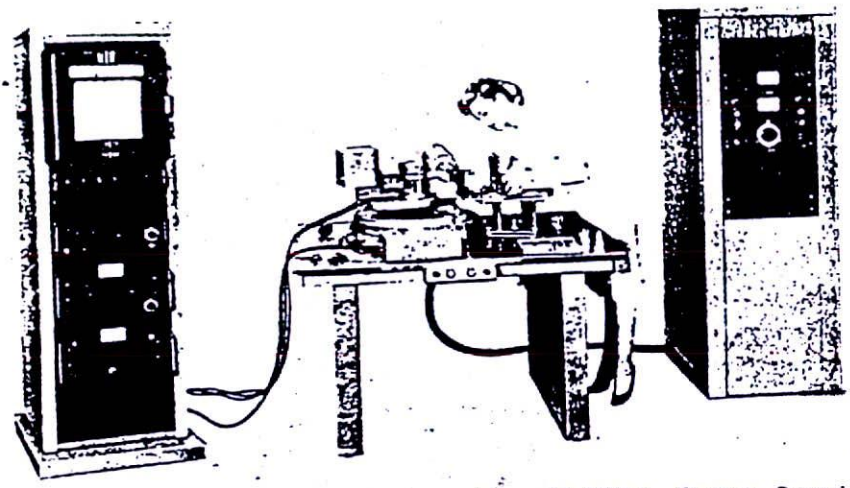

FIGURE 10.4: General Electric diffraction unit, model XRD-5. Courtesy General Electric Co.

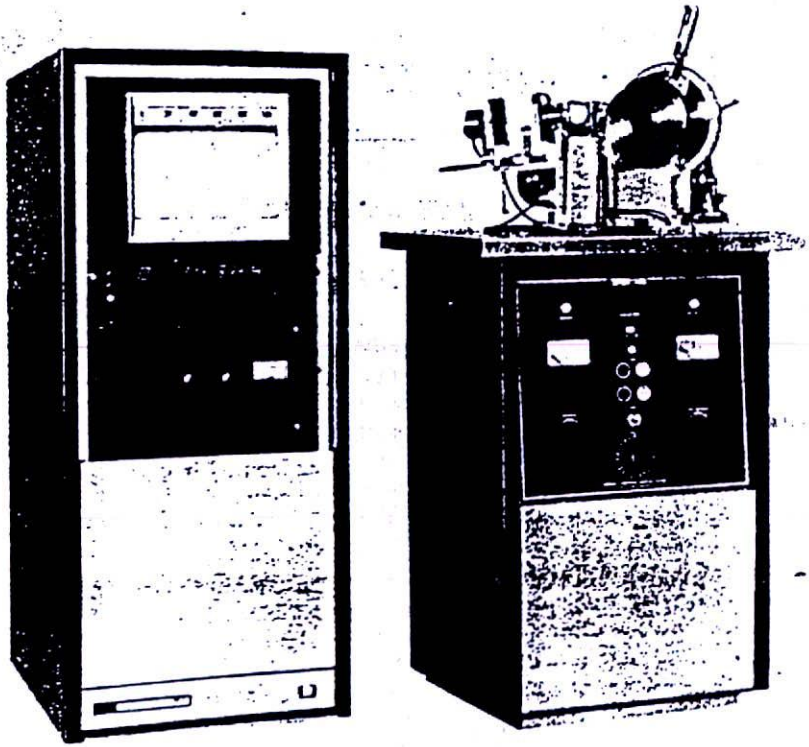

FIGURE 10.5: Norelco diffraction system. Courtesy Philips Electronic Instruments.

The intensity is recorded as counting rate, in counts per second (cps). Additional major components of commercially available equipment are the X-ray power source, the X-ray tube, and an electronic scaler and timer for quantitative measurement of diffracted intensity, in counts per second.

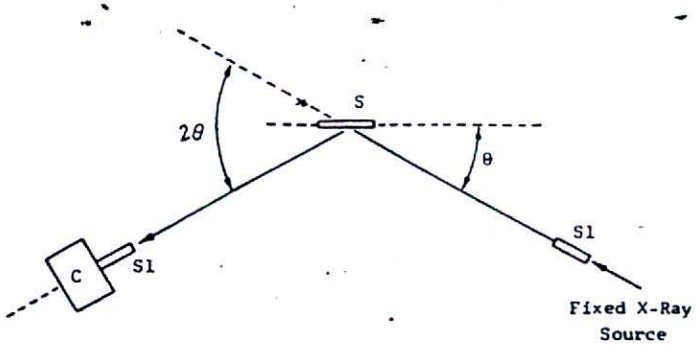

FIGURE 10.6: The sample (S) rotates through θ while the counter (C) scans through 2θ. An edge view of the planar surface sample is shown. X-ray beam slits (sl) define the beam parallel to the sample surface. Reprinted from Ref. (11), p. 25, by courtesy of *J. Pharm. Sci.*

2. Qualitative Analysis

Qualitative analysis by X-ray powder diffraction techniques is straightforward and fairly simple. Essentially it involves the measurement of a series of d spacings, the interplanar spacings, from the positions of the diffraction peaks (from the diffractometer) or the diffraction lines (from the powder camera film), as described in the foregoing section. As it is most practical from an instrumental point of view to record the diffraction angle in terms of 2θ, rather than θ, tables have been prepared from which all values of 2θ are readily converted to d values, expressed in Angstrom units (Å), for a given wavelength of X-rays. These tables are available from a variety of sources, including the equipment manufacturers.

At the time of recording the values of 2θ, the relative intensity of each line (peak) is noted. If the diffractometer is used, the intensities are measured as peak height above background, and expressed as percentages of the strongest line.

For purposes of identification, the table of measured d values, together with the relative intensities, is compared with similar listings for known compounds. A library consisting of a collection of data cards for known compounds, called the ASTM X-Ray Powder Data File, is commercially available.* This file contains powder diffraction data for essentially all metals,

* The American Society for Testing Materials, 1916 Race Street, Philadelphia, Pennsylvania.

minerals, and inorganic compounds, and an ever-increasing number of the more common organic compounds. Use of the file is made by entering the *d* values of the three strongest lines from the unknown. This usually produces a few cards representing possibilities. As each card also lists the complete table of *d* values and relative intensities, the positive identification readily follows.

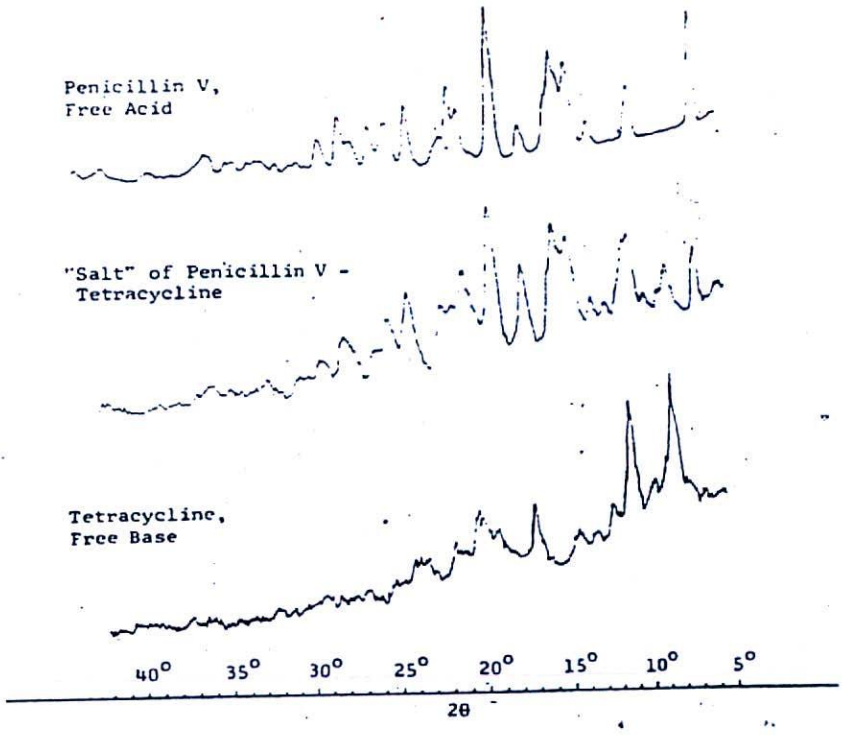

FIGURE 10.7: X-ray powder diffraction patterns of antibiotic materials.

Many investigators using the powder diffraction technique maintain a file of original diffraction patterns (or films) representing compounds of interest to them, and make identifications of unknowns by direct comparison of the patterns.

A major source of error in determining the relative intensities of diffraction lines is due to *preferred orientation* of the particles in the powder sample. If the material to be examined is made up of flat, micaceous crystals, and these are permitted to orient preferentially so that most of them lie flat in the sample holder, most of the resulting diffraction will be from the planes within the

crystals which are parallel to this flat face. This diffraction line will appear very strong, and other lines, requiring other crystal orientations for emergence, will be either missing or, at best, low in intensity. For a diffraction pattern reflecting true relative intensities, great care must be exercised to ensure random orientation of the crystals.

The most widespread use of X-ray powder-diffraction analysis is in the identification of unknown compounds. Moreover, since the method provides identification of a material by compound, rather than by its elements, it is very useful in the characterization of new compounds for purposes of patents or other publications. Of particular interest to the pharmaceutical chemist is the sensitivity of the diffraction pattern to polymorphic changes. Polymorphs are different crystal forms of the same chemical compound which possess different physical (and pharmaceutical) properties. Further, many drug compounds exist in more than one solvated crystal form; these forms often differ markedly in solubility and in other properties. Since solvates of a given compound are actually different compounds from one another as well as from the parent compound, their X-ray diffraction patterns are quite different. In addition, distinctions between different compositions of mixed crystals, or solid solutions, may be made by X-ray diffraction. It is interesting to observe that with systems in which mixed crystal formation obtains in all proportions, the composition can be followed by the measurable shift in 2θ value of a major diffraction line. Finally, the feasibility of the use of diffraction to identify the components of a complex mixture without the necessity of separation is worthy of note. Figure 10.7 shows the diffraction patterns of penicillin V (free acid), tetracycline (free base), and a material described in patent literature as the salt of these two antibiotics. A comparison of the three diffraction patterns allows the positive conclusion that the material represents a simple mixture of the reaction starting materials, rather than a true salt. Although this is a trivial example in one sense, the importance of the method becomes evident when one searches for alternative methods for proving—or disproving—the existence of a salt of large, complex molecules.

3. Quantitative Analysis

Among quantitative methods, those based on X-ray diffraction are unique in that they combine the absolute specificity usually found only with bioassays with the high precision of typical chemical assays. This fact is of particular interest to the pharmaceutical chemist who must develop assays to guide stability studies which are specific for the intact molecule. Further, such methods may often be applied directly to complex mixtures without separation or knowledge of the other ingredients.

Some mention of the possibilities of quantitative diffraction was first made by Hull as early as 1919,[3] but the first work reported was by Clark and Reynolds on the analysis of mine dust in 1936.[4] This work, and other work

following, was based upon microphotometric density measurements of X-ray film following exposure. This method of measuring the intensity of diffracted X-rays was highly inaccurate, and it was not until the advent of the Geiger counter diffractometer (spectrometer)[6] that truly quantitative diffraction became possible.

The mathematical relationships pertinent to quantitative diffraction analysis were derived and published in an important fundamental paper by Alexander and Klug appearing in 1948.[7] This paper described conditions under which standard curves alone could be used, and under which standard curves based on internal standards were required, depending upon absorption effects. Relationships permitting, in certain instances, quantitative analysis of differential absorption systems without the use of internal standards were described in 1953.[8] In 1958, Copeland and Bragg[9] described special conditions under which calibration curves could sometimes be eliminated and multiple components determined.

The papers just mentioned refer to investigations of inorganic systems, which, due to higher mass absorption coefficients of the ingredients, present special problems not so often encountered with organic systems. Only a few papers have appeared reporting applications to organic systems; some of these of pharmaceutical interest concerned applications to the determination of sodium penicillin G, published in 1948,[10] general applications to typical pharmaceutical systems, published in 1963,[11] and the analysis of intact tablets, published in 1964.[12]

The feasibility of quantitative X-ray diffraction stems from the fact that the intensity of a diffracted beam of X-rays is a function of the amount of diffracting material. The linearity of this response, for a fixed set of experimental conditions, depends upon the difference in the amount of absorption of X-ray energy between the compound of interest and its surrounding matrix. The energy absorption by any material depends upon the mass absorption coefficients of its constitutive atoms, which in turn are generally a function of atomic number for a given wavelength of radiant energy. Thus, as a compound differs chemically from its matrix, so will it differ in absorption for the radiant energy. The result of a large difference is a very nonlinear relationship between the amount of diffracting material and the diffracted intensity.

It is significant that in inorganic systems the probability of wide variation in X-ray absorption between the compound of interest and its surrounding matrix is high, whereas with organic systems the variation is usually low. This feature of organic systems results in an advantageous gain in linearity of response and allows, in many instances, the use of a simple calibration curve for the quantitative analysis of complex organic systems.

When diffraction from a powder sample occurs at a given 2θ value, it does so because a sufficient number of crystals have the same set of planes, whose d spacings correspond to the 2θ value, properly oriented with respect to the X-ray beam. The intensity of the diffracted ray is a function of the amount of

material so oriented. If truly random orientation is assured,* and except for absorption effects, the diffracted intensity becomes proportional to what may be termed the "specific lattice volume." It is highly significant that when the intensity of a single diffraction peak is measured at a fixed 2θ value, both additive and constitutive effects are being measured. This unique fact is the basis for the specificity of quantitative diffraction analysis.

In developing a method for a component of a given mixture, the procedure of choice will vary according to the complexity of the system. The complexity here refers more to the absorptive qualities of the matrix and the constancy of the matrix than to the number of ingredients present. If a diffraction assay is feasible at all, one of the procedures which follow should apply. These procedures assume the use of a modern X-ray diffractometer, equipped with counter, scaler, and timer circuits.

a. Use of Simple Calibration Curves. No errors due to changing absorption effects occur with simple, two-component mixtures, or with multicomponent mixtures whose composition, except for the component being analyzed, remains constant. The determination of a crystalline ingredient in such systems may be based on calibration curves prepared from synthetic mixtures.

The procedure begins with an examination of the diffraction pattern of the ingredient to be assayed. A major diffraction peak is first found in an area free from interferences from other components of the mixture (the matrix); synthetic mixtures are then prepared containing known amounts of the compound of interest, over the range of concentration of interest, and the intensity of the selected diffraction peak measured for each mixture.

The intensity measurement is made by setting the diffractometer to the 2θ position of maximum peak height, and engaging the timer and scaler to measure the peak height in counts per second. Using the diffraction pattern of the pure material for reference, the instrument is then set at a 2θ position for the measurement of the background intensity, usually near the base of the selected peak. Again, the intensity in counts per second is measured by use of the scaler and timer circuits. The background intensity for each sample is subtracted from the corresponding peak intensity, and this difference is plotted as a function of sample composition, to form the standard curve for use in subsequent assays.

Illustrating an assay based on a simple calibration curve is the determination of pamoic acid in a matrix of the pamoic acid salt of a basic antibiotic.[11] The method was found useful in testing for completeness of the salt-forming

* In all quantitative diffraction studies the effects of preferred orientation of the crystals must be minimized. Simple grinding, followed by careful packing in the sample tray, usually accomplishes this for organic compounds. In instances where an extreme of one crystal habit is represented, the advantageous use of 2% carbon black added to the mixture has been reported.[10]

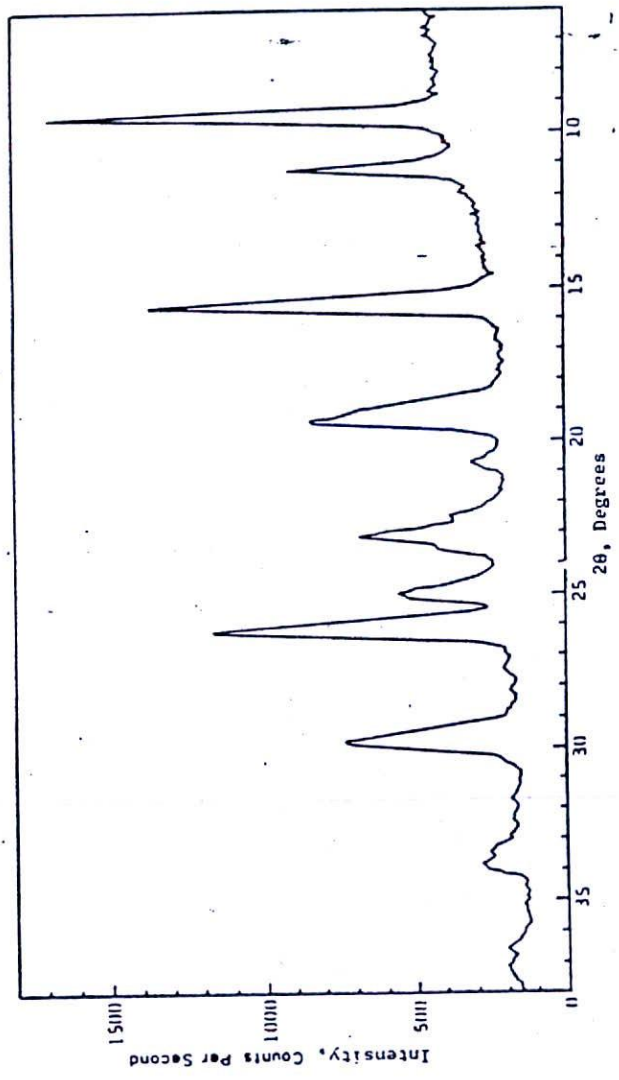

FIGURE 10.8: The pamoic acid diffraction pattern. Reprinted from Ref. 11, p. 27, by courtesy of *J. Pharm. Sci.*

reaction, and particularly in support of the long-term stability studies of a formulation of the antibiotic salt.

The diffraction pattern of pamoic acid is shown in Fig. 10.8. Although major diffraction peaks occur at 2θ values of 9.50, 11.20, 15.60, and 19.40°, all of these peaks were at least partially obscured when superposed on the pattern of the matrix materials. For this reason the peak at 26.25° 2θ was chosen as a basis for the determination.*

For the preparation of the standard curve, use was made of synthetic mixtures containing known concentrations of pamoic acid in the antibiotic salt. Following thorough mixing and grinding in a mortar, each sample was poured into a standard 2-in. sample tray, which required about 200 mg of material. In order to assure random crystal orientation, the excess powder was carefully removed by use of the edge of a glass microscope slide, and the sample surface finally packed slightly by use of the surface of a rough, low-grade blotting paper. This procedure eliminates large errors due to preferred orientation of the crystals.

The diffraction peak intensity of each sample was determined in the following manner. After placing the sample in the diffractometer, the goniometer was set for 26.25° 2θ and the instrument set to record the time required for the accumulation of 20,000 counts. This operation was followed by a setting of the goniometer at 26.80° 2θ and a second recording of the time required to accumulate 20,000 counts. The diffraction intensity at 26.25° (peak intensity) in counts per second was calculated, and the intensity at 26.80° (background intensity) in counts per second was also calculated.† The background intensity was subtracted from the peak intensity and the resulting value for each of the standard samples representing synthetic mixtures was plotted vs. the known concentration. The results are shown in Fig. 10.9. The total counting time for each point was approximately 3 min.

Since organic molecules are large, organic crystals have large unit cells. Most interplanar spacings are, therefore, large. Such spacings give rise to diffraction peaks at small values of 2θ, which is the region of maximum response to scattering of the X-rays by the powdered sample. Thus it sometimes happens that a diffraction peak of interest occurs in an area of maximum background. This is particularly true when a sample contains a large amount of amorphous, or scattering material. Such a situation is illustrated by Fig. 10.10.[11] Under these conditions it has been found advantageous to

* One has a choice of several possibilities upon which to base a proposed analysis. If the matrix is not obtainable in pure form, and there is doubt as to the freedom from interference of diffraction peaks, two simultaneous methods may be developed, each using a separate peak. Freedom from interfering peaks is assured when results of the determinations agree.

† Since the statistical accuracy of a counting process depends upon the total number of counts taken, and not upon the time it takes to make the count, a preset count, rather than preset time, is used to determine the intensity in counts per second. This procedure allows all values, when compared later, to be of the same statistical accuracy.

determine the net peak height by a different procedure in order to maintain accuracy. Using the scaler and synchronous timer circuits, the intensity P (Fig. 10.10) is measured at 7.25° 2θ. The intensities at R_1 and R_2 are measured at 2θ values of 7.0 and 7.80°. The background B is calculated by interpolation:

$$B = R_2 + \frac{7.80 - 7.25}{7.80 - 7.00}(R_1 - R_2) = R_2 + 0.688(R_1 - R_2) \quad (10.3)$$

The net peak height $(P - B)$ can then be calculated for each sample.

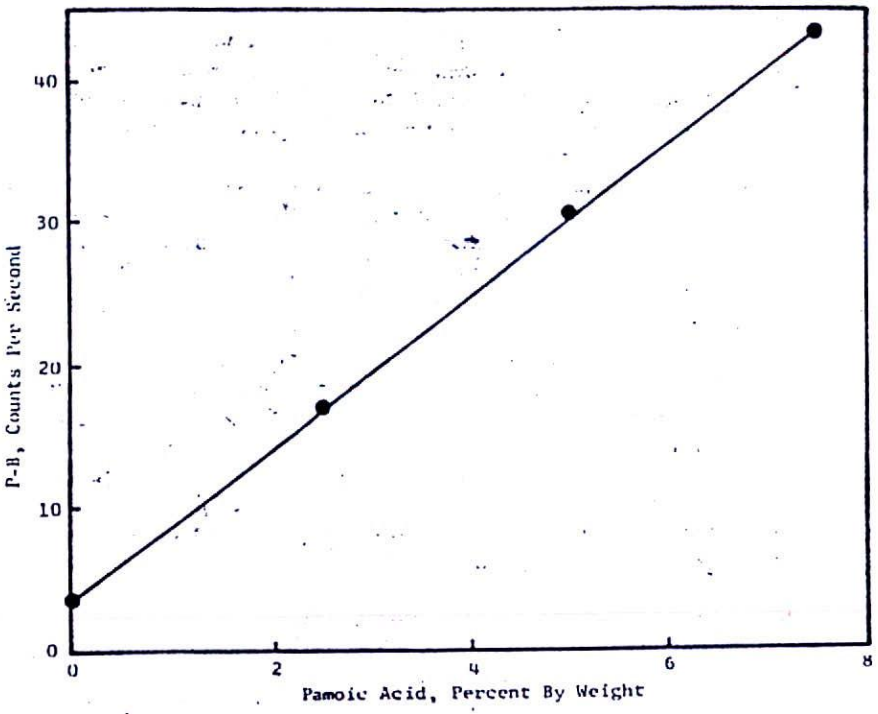

FIGURE 10.9: Intensity difference, peak minus background, as a function of pamoic acid concentration. Reprinted from Ref. 11, p. 27, by courtesy of *J. Pharm. Sci.*

If all variables such as instrumental drift and the density of sample packing could be held constant, the background correction could be eliminated. Its use, however, permits day-to-day validity of the standard curve with good accuracy.

In the two previous examples, peak intensities were corrected by subtracting background intensities. It is just as valid to use a peak-to-background intensity ratio in place of the arithmetical difference. Moreover, the choice

of position for the background measurement is not limited, an obvious advantage in instances of multiple peak overlapping.

b. Use of an Internal Standard. The most universally applicable method for quantitative diffraction involves the use of an internal standard. This method is free from all matrix effects as well as errors due to variations in sample packing density and variations in instrumental conditions. It is ideal for drug systems containing unknown mixtures, such as those containing possible degradation products.

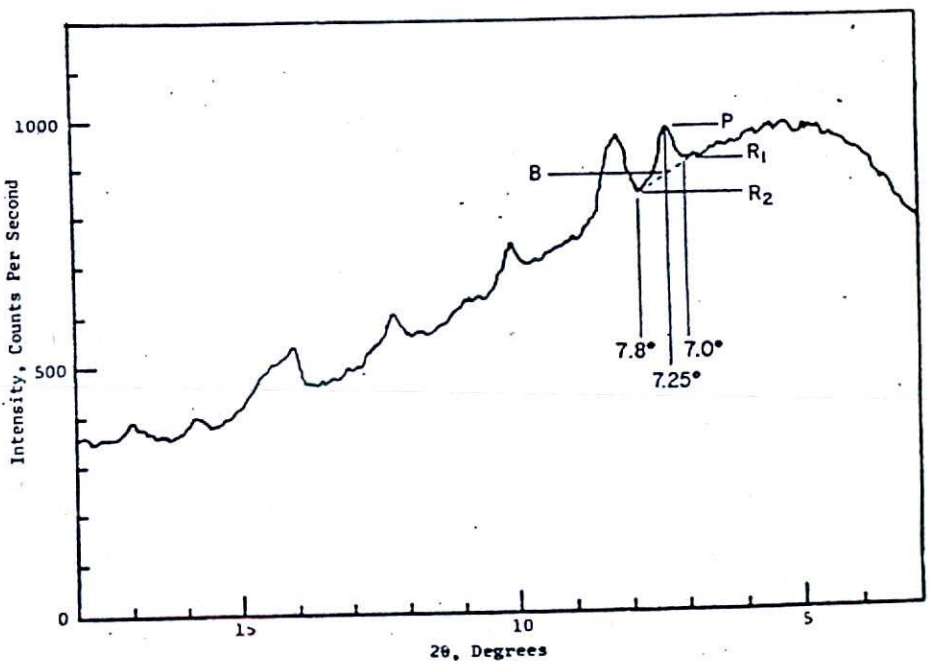

FIGURE 10.10: Partial diffraction pattern showing peak of interest over large background. Reprinted from Ref. (11), p. 27, by courtesy of *J. Pharm. Sci.*

The procedure involves examination of the diffraction patterns of the material to be assayed, and of the matrix in which it is found, and selecting a peak produced by the material which is free of interference from neighboring peaks. A suitable inert crystalline material, the internal standard, must then be selected which has a peak in a clear region with respect to the system to be analyzed.

When a suitable internal standard has been found, a standard curve is prepared from samples containing known but different concentrations of

the compound to be analyzed and a fixed concentration of the internal standard. The proper concentration of internal standard to establish for use in all samples is one which is found to give a diffraction peak about equal in intensity to that of the material to be analyzed when the latter is present in the usually expected concentration. The X-ray procedure consists simply of determining the ratio of intensities of the diffraction peaks of the unknown and the internal standard, without regard for the background. The ratio is linearly proportional to the concentration of the unknown.

Finding a suitable internal standard for each new system is often a problem. An ideal internal standard has several attributes: (1) It must have a diffraction peak which is not obscured by peaks of the system, and which will not interfere with a major peak of the material to be analyzed. (2) The internal standard peak should be near the usable peak of the analyzed ingredient. (3) It should be of high crystal symmetry, preferably isometric, so that strong but few diffraction peaks are produced and preferred orientation effects will be minimized (by the more equant crystals). (4) Because of absorption effects, it should contain only elements of low atomic number. (5) It should have a density not too far removed from those of the system ingredients (for aid in maintaining homogeneity in mixing); and (6) it should be chemically stable in the presence of the system. No one compound can qualify universally on all counts, but a material which has been found to have many of these attributes is hexamethylenetetramine, one of the few organic compounds of isometric (cubic) symmetry.

It is worth mentioning that in instances in which the system is of many variable components, requiring therefore an internal standard, but for which no suitable standard can be found, an alternative procedure exists. This consists of adding known amounts of the ingredient to be analyzed to the unknown system, plotting the peak intensity values as a function of the amount added, and extrapolating the curve. The intercept value gives a measure of the original amount of ingredient present.

c. Use of Integrated Diffraction Peak Areas. Some organic compounds, when produced in successive batches over a period of time, tend to vary in the degree of crystallinity possessed by any one batch. A decrease in the degree of crystallinity of a compound is accompanied by a drop in the apparent diffraction peak intensity as measured by the peak height. Such a drop in peak height, however, is accompanied by a peak broadening. It is significant that the area of the diffraction peak is relatively constant for a wide variation in crystallinity. It is, therefore, sometimes of value to base a diffraction assay on peak areas rather than on peak heights. Further, the use of peak areas is advantageous when the particle size of the crystalline ingredient is very small due, again, to line broadening and a significant drop in peak height. Measurable line broadening occurs in the particle size range below 0.2 μ. Finally, the peak-area method offers an advantage in being

free from errors due to apparent shifts in peak maxima. A standard curve for an assay based on the use of an internal standard and on integrated peak areas is always linear and intersects the origin.

To illustrate the method, a portion of a diffraction pattern is presented in Fig. 10.11, which shows a doublet at 10° from a sulfonamide from aqueous suspension, and a peak at 11.5° from an added internal standard, $CaSO_4 \cdot 2H_2O$.[11]

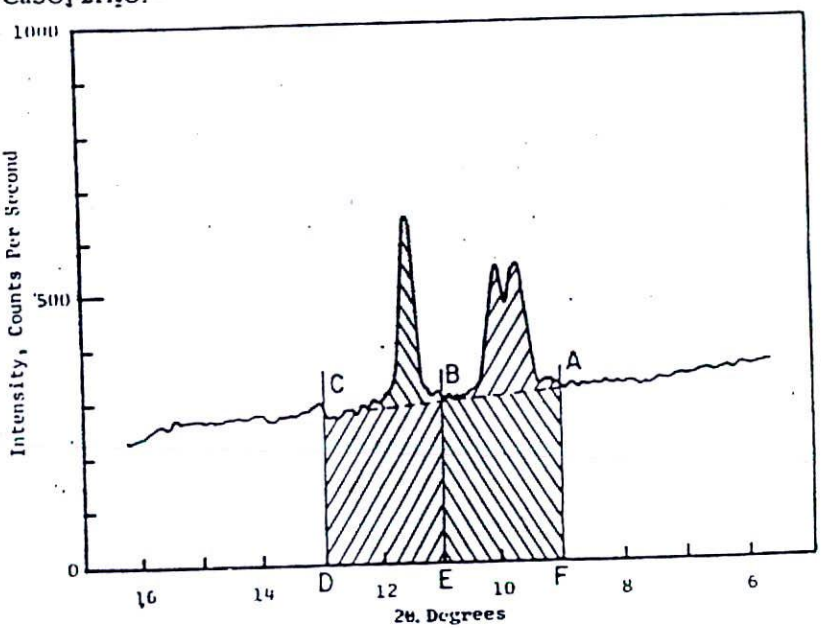

FIGURE 10.11: Partial diffraction pattern showing sulfonamide peaks (doublet at 10°) and internal standard peak (11½°). Reprinted from Ref. (11), p. 26, by courtesy of *J. Pharm. Sci.*

Integrated intensity values were determined from this system by the following procedure. With the scaler set for a preset time of 100 sec, the counting rate at 9° was determined in counts per second. This was repeated for positions at 11 and 13°. Diagrammatically these three values of background counting rates are represented in Fig. 10.11 by the lengths of the lines *AF*, *BE*, and *CD*. With the goniometer set at somewhat less than 9°, an automatic scan was begun at 2°/min. As the scan reached 9°, the counting switch was turned on, simultaneously starting the scaler and timer. As the scan crossed 11°, this switch was turned off. The total count and total elapsed time (approximately 60 sec) were recorded. The elapsed time (in seconds) is represented by *EF* on the diagram, and when multiplied by the

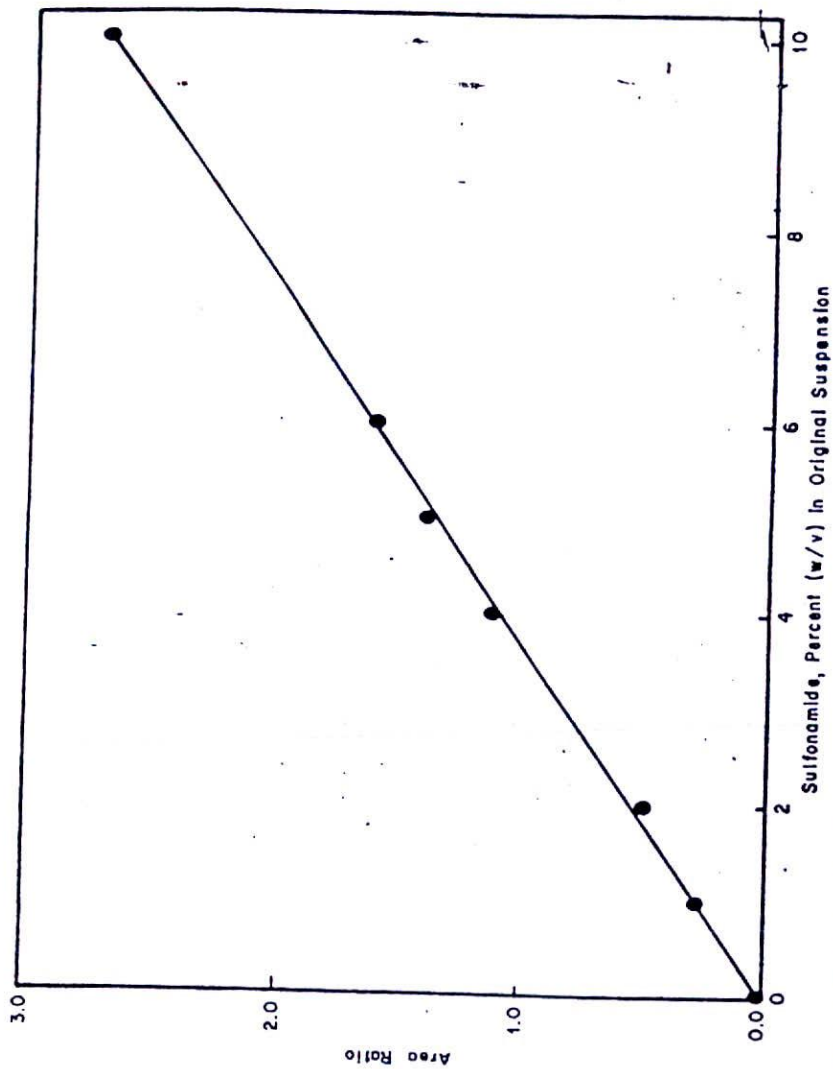

FIGURE 10.12: Ratio of peak areas, sulfonamide to internal standard, as a function of sulfonamide concentration. Reprinted from Ref. (11), p. 28, by courtesy of *J. Pharm. Sci.*

average of background counting rates measured at 9 and 11° (in counts per second) gives the area of background under the curve ($ABEF$) expressed in total count. This value was subtracted from the total count accumulated in the scan operation. The difference was the area of only the net sulfonamide diffraction peak, expressed in total count. The operation was repeated between 11 and 13° to determine the area of the internal standard peak, and the ratio of sulfonamide peak area to standard peak ares was then computed.

Figure 10.12 shows a least squares plot of values determined by this procedure for the ratios of sulfonamide peak areas to internal standard peak areas as a function of sulfonamide concentration in aqueous suspension.[11] Following sample preparation, X-ray analysis by this method required about 15 min/sample. The values indicate an accuracy of ±0.15% of the amount present. The method is specific for the intact sulfonamide molecule in the crystalline state and, therefore, sensitive to any product degradation within the aqueous suspension.

4. Particle-size Measurement

An X-ray powder-diffraction technique may be used to measure the mean dimensions of the crystallites composing a finely divided powder, provided the dimensions are small enough. The method is generally applicable to particle sizes from 20 to 2000 Å.

Most powders and suspensions of pharmaceutical interest contain particles in the micron-size range. Extensive research in the biopharmaceutical field has shown enhancement of drug dissolution rates and general drug availability from extremely fine particles, however, and with the promise of significant enhancement of these factors, some commercial formulations have emerged with particle sizes in the submicron range. Size-measuring methods which employ visible light are limited by the wavelength of the light in this range and are, therefore, not applicable.

The Bragg relationship predicts diffraction from an ideal set of planes within a crystal at a discrete angle θ. Truly ideal conditions do not exist, however, and in practice one finds diffraction from a set of planes occurring over a small range of angles near θ, resulting in an apparent broadening of the diffraction peak. The factors which contribute to this line broadening are mosaic structures within the crystal, nonuniform strain on the crystals, and small crystallite size. Fortunately, contributions to line broadening by the first two factors is often minimal from organic crystals, permitting a measurement of the broadening to reflect mean particle size.

The fundamental relationship was described by Scherrer[13]:

$$D = \frac{K\lambda}{\beta \cos \theta} \tag{10.4}$$

where

D = crystallite dimension
K = crystallite shape constant
λ = X-ray wavelength
β = corrected line breadth
θ = Bragg angle

In practice, one carefully records the X-ray powder-diffraction pattern of the crystalline powder of interest, and measures the observed peak breadths at half maximum intensity, in terms of angular degrees, of several diffraction peaks. The accuracy with which Eq. (10.4) can be applied is limited by the uncertainties within the K factor and the accuracy with which β can be deduced; for precise work corrections must be made.

Reference may be made to Jones[14] for the correction of the observed line width for unresolved K_α radiation. The resulting value must then be corrected for instrumental line broadening by reference to an experimentally determined plot of actual line breadth vs. diffracted angle, measured from a diffraction pattern produced by a powder sample of particles too large to produce line broadening. Finally, this value for β may be further refined by reference to a set of curves produced by Alexander[15] for use with diffractometers with narrow sources.

The value of the shape factor K approaches unity, and this value has been used for approximations. When nothing is known of the shape of the crystallites or of the indices of the prominent planes, the value of 0.9 is recommended for use for this factor.[16,17]

As noted previously, the applicability of X-ray line broadening to the determination of particle size is limited to the range of crystallite size from 20 to about 2000 Å. The sizes measured are average sizes, weighted toward the larger sizes present in a mixture. The accuracy of the determination is low; it diminishes rapidly with increasing size range.

A more detailed presentation of this method has been presented by Klug and Alexander.[18]

5. Crystal Habit Quantitation

The symmetry of a crystal is fixed by the crystal system and class to which it belongs. Its relative dimensions, however, are independent of its symmetry. As a crystal grows from solution, a variety of factors, notably crystallization rate and the presence of impurities, tend to influence the relative amount of growth of the possible faces. Extremes of the possible conditions result in acicular, or needle-shaped, crystals as a consequence of unidimensional growth (bidimensional retardation) and tabular, or plate-shaped, crystals, as a consequence of bidimensional growth (unidimensional retardation).

Terms such as "acicular," "equant," and "tabular" describe crystal habit in a qualitative manner.

Crystal habit often exerts a dominant influence on some important pharmaceutical characteristics, such as suspension stability, suspension syringeability, and the behavior of powder mixes during a tablet-compressing process. In the case of suspension syringeability, the influence is mostly mechanical. A suspension of plate-shaped crystals, for instance, may be injected through a small needle with greater ease than one with needle-shaped crystals of the same overall dimensions.

When considering tableting behavior, however, the influence of the crystal habit of the active ingredient is more complex. The mechanical influence of crystal shape just mentioned is one factor, but there is another, sometimes dominant one, which results from the anisotropy of cohesion and of hardness which is possessed by organic (low symmetry) crystals and, therefore, of most pharmaceutically important compounds. It is significant that this anisotropy bears a fixed relation to the fundamental crystallographic directions. Therefore, as crystal habit varies, the dominant faces may vary in their relation to this anisotropy, and it is the influence of the dominant faces which tends to orient the crystals during a packing or compression process. Thus, major habit variations of an active ingredient can influence greatly the ease or the difficulty of making satisfactory compressed tablets. This is particularly true when the active ingredient makes up a large portion of the total tablet mass.

To evaluate tableting behavior as influenced by crystal habit, the habit must be expressed in quantitative terms which reflect some relationship between the dominant faces and the principal crystallographic directions. Qualitative terms describing shape are, in some instances, not sufficient.

For a typical pharmaceutical composition, it has been found that a quantitative description of crystal habit as it affects tableting behavior can be based upon measurements of preferred orientation. After relating habit extremes to tableting behavior by experimentation, optical* and X-ray crystallographic studies on representative single crystals allow the designation of the dominant faces by their Miller indices. An X-ray powder-diffraction pattern is then measured for crystals of each habit extreme on specially prepared samples. The samples are prepared in such a manner that preferred orientation effects are maximized. A ratio of the relative peak intensities of critical lines in this diffraction pattern serves to indicate the average habit of the crystals. The ratio is useful in predicting tableting behavior, as well as serving, when desired, as a manufacturing specification.

Details of this method and a general illustrative example have been published.[19]

* Methods for the measurement of optical properties of crystals are described by J. A. Biles in Chapter 9.

C. SINGLE-CRYSTAL DIFFRACTION

1. Determination of Molecular Weights

For the precise determination of molecular weights, the X-ray method, where applicable, has long been the method of choice. Applied with reasonable care, the accuracy of this method need be limited only by errors due to crystal imperfections, which are often of the order of 0.04% if due to void spaces and much less if due in part to inclusions.

The feasibility of the X-ray method for molecular weight determinations derives from the fact that an exact and determinable number of molecules occupies a *unit cell* which, by three-dimensional repetition, generates the space lattice of the crystal. The procedure involves the measurement of the unit cell dimensions by X-ray diffraction, and the calculation of the unit cell volume. Knowing the volume of the cell, its weight is then calculated from an experimentally determined value for the crystal density. The molecular weight M is then found by

$$M = \frac{Vd}{1.6604\,N} \tag{10.5}$$

where V is the cell volume in Å^3, d is the density, 1.6604 is a constant used to refer the cell weight to oxygen at 16, and N is the number of molecules per unit cell. The value for N can be determined directly if an approximate value for M is known; N is always some whole number, usually small, and limited to a few possibilities by the crystal symmetry.

The determination of the unit cell dimensions is most easily accomplished by the use of measurements from oriented, single crystals. Although the more easily acquired powder-diffraction pattern may be used to measure unit cell constants, this is readily accomplished only if the crystal is of high symmetry. Procedures for the indexing of powder patterns for this purpose have been described in detail.[20] Unfortunately, most compounds of pharmaceutical interest are organic, and these compounds most often crystallize in lower symmetry systems, requiring the single-crystal approach.

A suitable crystal, usually about 0.5 mm on an edge, is selected and cemented to the end of a fine glass fiber with the aid of a microscope. If a film camera is to be used, the crystal is oriented and then rotated in the X-ray beam about one of its crystallographic axes while the diffraction pattern is recorded on the film, arranged cylindrically about the rotation axis. Following development, "layer lines" appear on the film, the distances between which relate to and permit the calculation of the dimension of the unit cell along this rotation axis. The other dimensions of the cell are measured in similar fashion, following crystal reorientation and rotation about each of the other axes. If the crystal is of low symmetry (monoclinic or triclinic), the angular relationships of the crystal (and hence the cell)

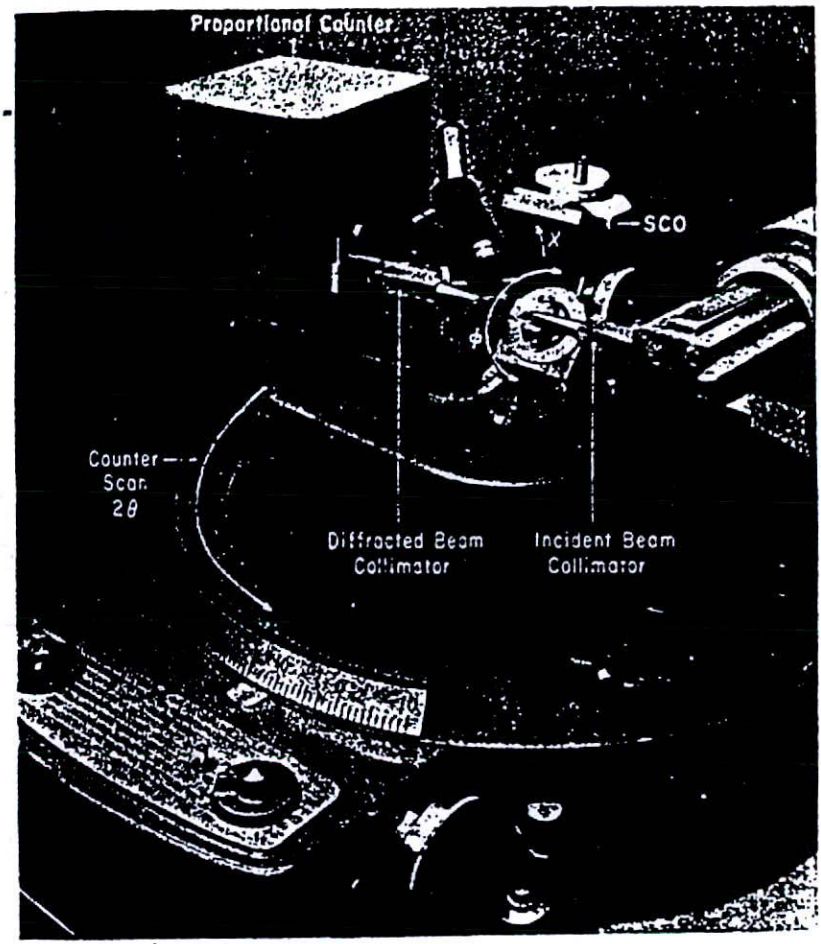

FIGURE 10.13: X-ray diffractometer and single-crystal orienter (SCO). Crystal rotation directions ϕ and χ are shown (radiation shields removed). Reprinted from Ref. (21), p. 155, by courtesy of *J. Pharm. Sci.*

must be determined; this may often be accomplished by use of the calibrated stage of a chemical microscope.

Several instrument manufacturers make available single-crystal diffractometers employing Geiger or proportional counters for rapid measurement of lattice parameters and relative diffraction intensities. One of these instruments is the single-crystal orienter, manufactured by General Electric Co., depicted in Fig. 10.13. In this photograph, the adjustments ϕ and χ

permit all possible orientations of the crystal from a single mounting. This allows rapid determination of all the dimensions of the unit cell (from 2θ measurements), as well as the angular relationships of the cell axes (from the ϕ and χ settings). Detailed procedures have been published[21] covering the use of such an instrument for the measurement of unit cell constants of crystals of all symmetries, particularly as applied to organic crystals.

Crystal densities are most accurately determined by flotation methods, wherein a liquid is generated whose density is precisely equal to that of the crystal. The liquid density is then determined by a standard pycnometer technique. Detailed descriptions and procedures for two such methods have been reported.[21]

2. Total Structure Determination

By far the most sophisticated X-ray methods are those whose objective is the elucidation of the total structure of complex molecules. From a suitable single crystal of a compound it is possible to discover and describe with precision the relative spacial arrangement of all of the atoms of the molecule. It is beyond the scope of this chapter to cover this area of endeavor; because of its immense importance, however, mention in at least summary fashion is pertinent.

From X-ray diffraction patterns of a single crystal, the *positions* of the diffracted rays disclose the dimensions of the unit cell, and the *intensities* of these rays are functions of the atomic distribution within this unit cell. The procedural steps in determining the total structure include measurement of the diffraction intensities, reduction of these values to structure amplitudes, discovering the phase of the scattering amplitudes, refinement of the structure, and the presentation of the results in terms of stereochemical information. The procedure involves extensive use of high-speed computer equipment.

Unfortunately, the process is not a direct one, due to lack of advanced knowledge of the phase angles of the scattering amplitudes. Sometimes the phase angles can be computed from an assumed approximate structure (or lucky guess), and these used to compute a Fourier series, which is a means of expressing periodic functions as summations of trigonometric terms. Other methods of obviating the phase problem exist; one involves the introduction into the molecules of a heavy (high atomic number) atom, whose coordinates may be determined, giving sufficient insight to the approximate structure for one to compute the phase angles and evaluate the series.

Much progress has been made in recent years in the development of equipment for more rapid and precise collection of single-crystal diffraction data, and for the automatic handling of these data by special computers. It is possible that the phase problem may be solved in time by a direct approach; the possibility of discovering the phase of the amplitude by simultaneous recording of the scattering amplitudes from two independent X-ray sources

and correlated detectors has been reported.[22] Possibly another approach may involve the use of extrapolated values from various wavelengths of X-rays.

10.5 X-RAY EMISSION (FLUORESCENCE)

A. QUALITATIVE ANALYSIS

In the section of this chapter relating to the production and properties of X-rays, a description of the origin of X-ray spectra was presented. It was noted that several processes occur when matter is bombarded by intense radiation energy. One of these is a quantum process wherein atomic electrons are displaced, with the vacancies thus caused filled by electrons from an outer shell. Such a process must be accompanied by a release of energy, and it is significant that this energy is in the form of X-rays. As there are several energy levels associated with the various electron shells, quantum theory predicts a number of possible transitions, some more likely than others. Thus an element, when bombarded by sufficiently intense radiant energy, will emit several X-ray "lines," each at a specific wavelength, characteristic of the element. Qualitative analysis of a sample for the elements present is based upon the measurement of the wavelength of the emission lines.

In practice, the radiant energy directed to the sample is supplied by an X-ray tube. The determination of wavelength of the emitted X-ray lines is accomplished by diffraction using a large, single analyzing crystal of known orientation and known interplanar spacing; the wavelength of each emission line is then given by solution of the Bragg equation. The commercially available spectrometers make this process easy by producing an automatic plot of the diffracted intensity against the Bragg angle 2θ. The "peaks" from this spectrum can then be read off in terms of 2θ, and converted not only to wavelength, but directly to the element by use of prepared tables. These tables are available from the various equipment manufacturers, and provide the conversion for each of several standard analyzing crystals.

The same commercially available equipment used for X-ray diffraction (see Fig. 10.4, and 10.5) may also be used for emission analysis, simply by the use of added accessories. The conversion to a spectrometer (spectrogoniometer) consists of replacement of the lower rated diffraction tube by an X-ray tube of higher intensity output (a tungsten tube is commonly used); the placement of a sample holder in the primary X-ray beam; and the positioning of an analyzing crystal in the path of the collimated rays from the sample holder. The conversion may involve changing the electronic counter, depending upon the analytical wavelength of interest.

Several advantages of the X-ray emission method make it especially adaptable for pharmaceutical problems. Due to the low atomic number of carbon, hydrogen, and oxygen constituting organic systems, matrix component interferences, encountered in X-ray emission applications to other

systems, are often absent. Moreover, the method is applicable directly to samples, either liquid or solid, and usually without need for separations, permitting assays on final product formulations. Further advantages are speed, specificity, the nondestructive nature of the method, the inherent simplicity of X-ray spectra, and sensitivity for trace amounts of material.

The probability that an excited atom will emit radiation depends upon many factors; it is related to what has been termed the "fluorescent yield," a quantity which rises precipitously with increasing atomic number of the element excited. Presently, X-ray emission analysis is limited in its application to elements of higher atomic number: elements of atomic number less than 12 are not detectable with present-day equipment; those elements of atomic number from 12 to 22 are detectable by use of vacuum paths or helium-filled paths, and a detector (such as a gas-flow proportional counter) for long wavelength emission. Elements of atomic number greater than 22 are detected with no special conditions being required. The nonapplicability to low atomic number elements is a disadvantage when considering X-ray emission as a general method, but is a decided advantage when the method is used for the detection or determination of heavy elements in a light (organic) matrix.

B. QUANTITATIVE ANALYSIS

Whereas the emission of X-ray lines at specific wavelengths from an irradiated element serves as a basis for qualitative analysis, the fact that the intensity of any selected line is proportional to, among other factors, the number of emitting atoms is the basis for a quantitative method. The method generally requires independent calibration from standards of known concentration.

As noted in the preceding section, samples for X-ray emission analysis may be in any physical state. For quantitative work, it is only essential that samples for the development of the required standard curve and for the analyses be prepared and analyzed under identical conditions. For solid samples, powdering and briquetting has been found to be an ideal method for maintaining reproducibility. A variety of holders for liquid samples are available; most of these employ a thin Mylar window to pass the incident and emitted radiation.

In practice, the spectrometer is set to measure the emission intensity of a selected "line" from previous knowledge of the 2θ value of the line. The signal is maximized by slight adjustments of the spectrometer, and the intensity measured by use of the counter and scaler circuits. The peak intensity, in counts per second, is corrected by either subtracting the background counting rate, as determined by counting at a convenient 2θ setting near the base of the peak of interest, or computing the peak-to-background ratio. In systems comprising a light matrix (low atomic number elements) containing

a heavy element, a valid analysis for the heavy element may be based simply on the net peak height (or the peak-to-background ratio) thus determined.

Figure 10.14 shows a typical plot of the ratio quantity obtained by counting 0.5 g, 0.5 in. diameter briquettes of a steroid at the 2θ position for the selenium K_α line for several concentrations of selenium.[23] The detector was set to record the time required to accumulate 200,000 counts, and the

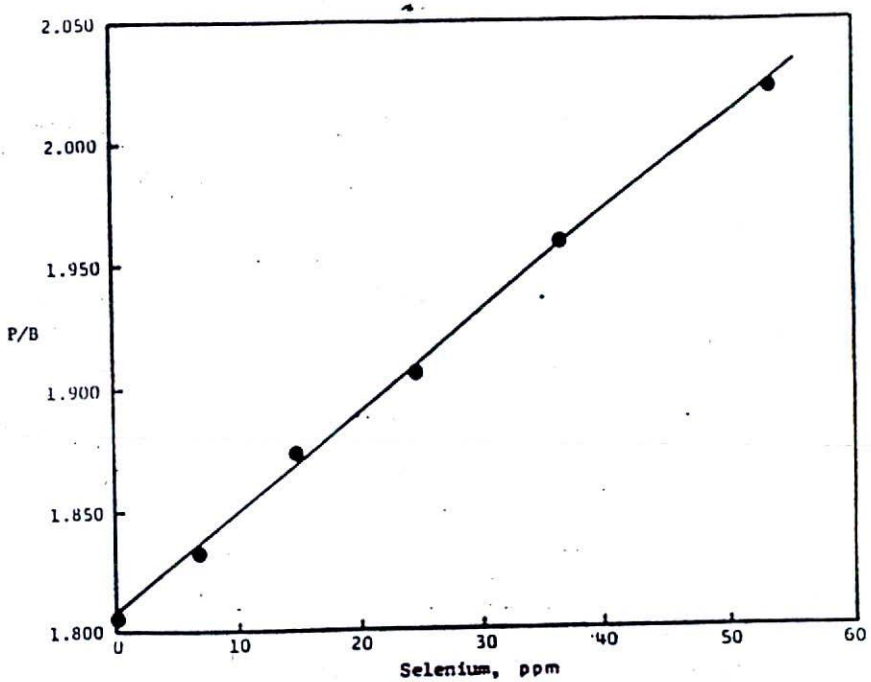

FIGURE 10.14: Selenium, peak to background ratio vs. concentration. Reprinted from Ref. (23), p. 733, by courtesy of *J. Pharm. Sci.*

counting rate was calculated from this quantity. This required, for the highest concentration sample, 153 sec. This particular assay was of interest due to the use of this toxic material as a dehydrogenation catalyst in the steroid synthesis.

If the element of interest is contained in a matrix which is not made exclusively of light elements, a variety of effects, collectively referred to as "absorption effects," occur. Their overall effect destroys the linearity of the response elicited by simpler systems. The most practical way to overcome these effects is through use of an internal standard, selected for an appropriate emission wavelength of one of its lines, and added in a fixed amount

to all standard curve samples and unknown samples. The ratio of the peak height (counts per second) of the element of interest to the peak height (counts per second) of the internal standard element, without background corrections, is then plotted against concentration to form the standard calibration curve, and measured on the unknown samples for their analysis.

In instances where high background-counting rates, or interferences from an emission line of another element present problems, use may be made of an electronic circuit called a "pulse-height selector," which is available for most commercial spectrometers. This device permits electronic discrimination against undesired wavelengths, with an overall gain in peak-to-background ratio.

Application of quantitative X-ray emission analyses appearing in the literature are numerous, and their number is steadily increasing. Of particular interest to the pharmaceutical chemist is the application of the method to the determination of selected elements in biological materials,[24] and the application to trace elements in general pharmaceutical systems.[23] Extension of the range of applicability to the gamma concentration range in solutions through use of ion exchange membranes has been reported.[25] Finally, two reference texts are selected[26,27] which cover the fundamentals of X-ray emission analysis in detail.

10.6 X-RAY ABSORPTION

When the intensity of X-rays which have passed through a pure element is measured as a function of the X-ray wavelength, several abrupt discontinuities are observed at specific wavelength values. These critical absorption wavelengths are called "absorption edges" for the element, and occur because the mass absorption coefficient of the element suffers abrupt discontinuities at the excitation potentials of the various spectra (*K* series, *L* series, etc.).

Several facts regarding the absorption edge make it of interest to the analytical chemist. The position of the edge is characteristic for only the one element producing it; the change in transmitted intensity at the edge is related to the amount of the element present; and the change in transmitted intensity is not influenced by other elements which might be present.

For a monochromatic X-ray beam transmitted through a sample containing an element of interest, it has been shown[28] that

$$\ln I''/I' = (\mu'_M - \mu''_M)WG \qquad (10.6)$$

where I'' and I' are transmitted intensities on either side of the absorption edge of the element, μ'_M and μ''_M are the mass absorption coefficients at these positions, W is the weight fraction of the element, and G is mass thickness in grams per square centimeter of the sample (thickness in centimeters $\times$ density).

In practice, one may use a standard X-ray diffractometer, and produce continuous monochromatic radiation by placing a standard analyzing crystal in the sample space. Samples for absorption analysis may be solids in the form of briquettes or liquids in special cells,[29] placed in the X-ray beam. Wavelength of the radiation is determined from the 2θ settings of the diffractometer, related by the Bragg equation to the known spacings of the particular analyzing crystal being used.

As the quantitative feature is strictly valid only at the absorption edge, it is practical to determine the transmitted intensities on either side of the edge,

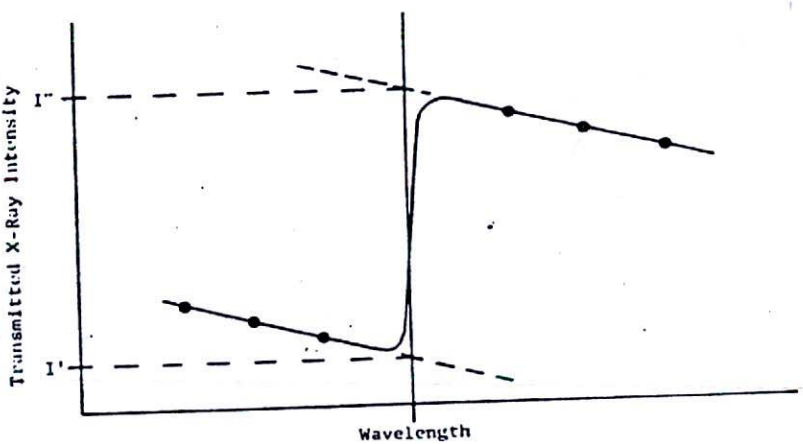

FIGURE 10.15: X-ray absorption near the absorption edge for the element.

and extrapolate to the edge. The values I'' and I' are thus determined in Fig. 10.15 by extrapolation from three experimentally determined points on either side of the absorption edge. The wavelengths of the absorption edges of a given element are handbook values, but they may be determined experimentally as well.

In setting up a working equation for routine use, the value in Eq. (10.6) of $(\mu'_M - \mu''_M)$ may be determined experimentally from a known concentration of the element of interest. For example, Rose and Flick[30] developed a working formula for the determination of iodine in thyroid extract:

$$\% \text{ iodine} = \frac{\log I''/I'}{\text{wt. sample}} \times 24.8 \qquad (10.7)$$

in which the constant 24.8 represented, in addition to conversion factors, the term $(\mu'_M - \mu''_M)$ as determined experimentally from potassium iodide solutions.

Like X-ray emission methods, X-ray absorption analysis is limited to elements of higher atomic number. It is not so sensitive as the emission method for low concentrations of the element of interest, but when applied to higher concentrations, it may be preferred because of its freedom from interference from other elements present.

10.7 LOW-ANGLE X-RAY ANALYSIS

A. MEASUREMENT OF EXCEPTIONAL PERIODICITIES

A consideration of the Bragg equation $n\lambda = 2d \sin \theta$ [Eq. (10.2)] reveals that very large spacings d must correspond to extremely small values of θ. It will only be mentioned here that modifications of diffraction equipment is possible for accurate measurement in the areas of $2\theta < 2°$ for large periodic spacings, from 50 to 1000 Å. This is the range of spacings found in certain muscle tissues, high polymers, and fibered proteins. This method for the detection and characterization of viruses holds great promise.

For the accurate recording of diffraction from large periodicities, several approaches are useful. They involve increasing the degree of divergence at the low angle by increasing the specimen-to-detector distance, using fine beam slits for maintaining resolution; employing longer wavelengths to increase the diffraction angle; the use of staining of the tissue with selective agents; and the use of longer exposures.

B. MEASUREMENT OF LARGE MOLECULES IN SOLUTION: RADIUS OF GYRATION

With the simple addition of special elongated beam slits, commercially available for the purpose, and a readily fashioned liquid sample holder, a standard X-ray diffractometer may be adapted for the determination of the radius of gyration of large molecules in solution by low-angle X-ray scattering.

The radius of gyration is defined as the root mean square of the distance from all of the electrons in the molecule to its center of gravity. While not too definitive in itself, it is of interest because it is a readily measured value which is sensitive to change as the molecular configuration changes. It is a parameter by which complex molecular changes may be followed, when the changes cannot be followed by other methods. Hence, the pH at which abrupt configurational changes occur, or the ionic strength at which such changes occur with certain proteins, may readily be found. Of particular interest to the pharmaceutical chemist is the sensitivity of the method to changes in macromolecules in the presence of drugs, due to binding. It is a useful method for evaluating the effects of agents on protein binding of drugs.

Use is made of the theory as developed by Guinier[31] according to which

the intensity of X-rays scattered by a group of identical particles at sufficiently small angles is

$$I_{(\phi)} = Nn^2 I_e \exp(-4\pi^2 R^2 \phi^2 / 3\lambda^2) \tag{10.8}$$

where

N = total number of particles irradiated
n = number of electrons per particle
I_e = Thomson scattering at zero angle for one electron
R = radius of gyration of the particle
ϕ = scattering angle in radians
λ = wavelength of X-rays used

R is defined with respect to the electron density, rather than the mass density. It may be obtained readily from the slope of a plot of log $I_{(\phi)}$ vs. the square of the scattering angle:

From Eq. 10.8, let $Nn^2 I_e$ = a constant A.

Then

$$I_{(\phi)} = Ae^{-(4\pi^2 R^2 \phi^2 / 3\lambda^2)} \tag{10.9}$$

Let

I_1 = intensity at low angle of plot
I_2 = intensity at high angle of plot
ϕ_1 = angle at lower angle of plot
ϕ_2 = angle at higher angle of plot

(all from the slope of the line, log I vs. ϕ^2), then

$$\ln I_1 = \ln A - \frac{4\pi^2 R^2 \phi_1^2}{3\lambda^2} \tag{10.10}$$

$$\ln I_2 = \ln A - \frac{4\pi^2 R^2 \phi_2^2}{3\lambda^2} \tag{10.11}$$

Combining (10.10) and (10.11),

$$\ln I_1 - \ln I_2 \left[= \ln \frac{I_1}{I_2} \right] = \frac{4\pi^2 R^2}{3\lambda^2} [\phi_2^2 - \phi_1^2] \tag{10.12}$$

where ϕ is in radians, or

$$2.303 \log \frac{I_1}{I_2} = \frac{4\pi^2 R^2}{3\lambda^2} (\phi_2^2 - \phi_1^2) \times \frac{\pi^2}{(180)^2} \tag{10.13}$$

where ϕ is in degrees, then

$$R^2 = \frac{2.303 \log \frac{I_1}{I_2} \times 3\lambda^2 \times (180)^2}{4\pi^2 (\phi_2^2 - \phi_1^2) \pi^2} \tag{10.14}$$

For copper K_α radiation ($\lambda = 1.539$ Å),

$$R = 36.89 \sqrt{\frac{\log \frac{I_1}{I_2}}{\phi_2^2 - \phi_1^2}} \text{ Å} \tag{10.15}$$

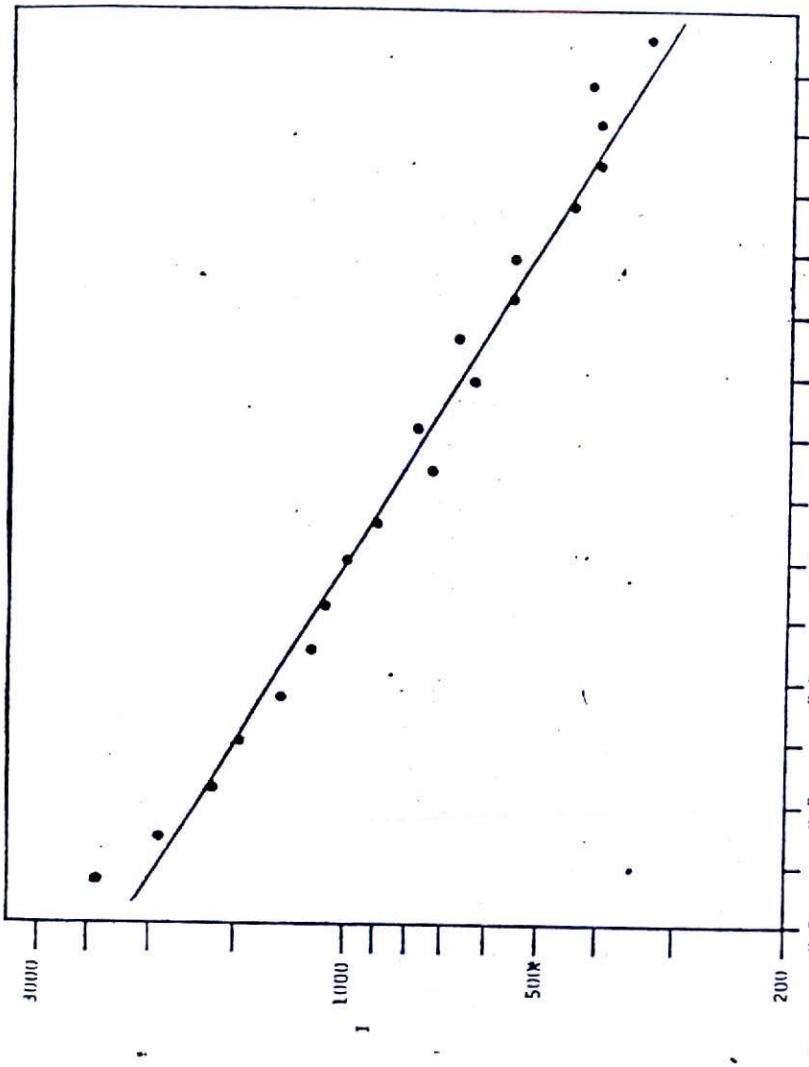

FIGURE 10.16: A plot of log I (scattered intensity) as a function of ϕ^2 (scattering angle) for bovine serum albumin.

In practice, use is conveniently made of plastic, cylindrical test tubes for liquid sample holders. Rectangular cells with Mylar windows may be used as well, as the path length of the cell does not effectively change with the small angular changes involved. Concentrations must be kept below about 20 to 30%, as the plot of log I vs. ϕ^2 produces an S-shaped curve, rather than a straight line, at higher concentrations. Unlike solutions for light-scattering experiments, solutions for X-ray scattering do not have to be so scrupulously free from all dust particles, which is a major advantage of the X-ray method.

Intensity determinations are made by counting for approximately 200 sec at a number of 2θ settings, from 2θ values of 0.25° to 1.2°. Some 15 to 20 intermediate settings should be made for counting, spaced closer together at larger 2θ settings so that the points for the ϕ^2 plot will be equidistant. A goniometer zero correction should be applied to the 2θ values to produce values for ϕ. The pure solvent is run in the same cell to determine a blank value in counts per second (cps), which is subtracted from each measured solution intensity value. The log I values are then plotted against the ϕ^2 values.

Figure 10.16 shows a plot of these values as determined from a 5% solution of bovine serium albumin using copper radiation.[22] From Eq. (10.15), taking values from Fig. 10.16,

$$R = 36.89\sqrt{\frac{\log \frac{2380}{348}}{1.40}}\ \text{Å}$$

$$= 28.3\ \text{Å}$$

QUESTIONS

Q10.1. In what physical state must a sample be in order that X-ray diffraction procedures may be applied? X-ray emission? X-ray absorption?

Q10.2. Provide a definition for the X-ray powder-diffraction pattern. Let your answer disclose what a single "line" or "peak" represents. Make use of the Bragg relationship.

Q10.3. Describe the principle of the commonly used method for obtaining monochromatic energy for use in X-ray diffraction.

Q10.4. State the particle size range over which X-ray line broadening occurs, sufficient for size analysis.

Q10.5. Give an equation showing the relationship of constants and measurable parameters relating to the determination of molecular weights by X-ray diffraction. Define each term in the equation.

Q10.6. Write a short paragraph describing the origin of X-ray spectra.

Q10.7. What is the major contributing factor responsible for the nonlinearity of the calibration curve used for the X-ray emission analysis of complex mixtures?

Q10.8. State the principle advantages of the X-ray absorption method of analysis.

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