X-ray and Crystallographic Applications in Pharmaceutical Research II

Quantitative X-ray Diffraction

By JOHN W. SHELL†

In a comparison of quantitative methods, bioassays have an advantage of being specific for an intact molecule. Such assay methods, however, often lack a high degree of precision. Chemical methods usually offer high precision but are notoriously nonspecific. A general quantitative method which offers a combination of specificity and precision is highly desirable, particularly for use in describing pharmaceutical systems. This unique combination is found by the adaptation of quantitative X-ray diffraction to systems containing crystalline ingredients. This paper describes the general method and the procedures which have been found useful in the application of the method to the most commonly encountered situations. Illustrating the procedures are examples of the applications to specific drug systems.

ONE OF the most seldom used, and yet potentially powerful methods for describing some pharmaceutical systems is the method of quantitative X-ray diffraction. Of the numerous advantages of this method, the most striking is the offer of absolute specificity combined with a high degree of accuracy. This feature, which is particularly desirable for drug systems, is unique. Further, the method can often be applied directly to complex mixtures such as final drug formulations without the need for separations, is usually rapid, and does not necessarily require a knowledge of all of the ingredients of an unknown mixture. The method applies only to the determination of crystalline ingredients, but the system containing the ingredients may be in any physical state.

Some mention of the possibilities of quantitative diffraction was first made by Hull as early as 1919 (1), but the first work reported was by Clark and Reynolds on the analysis of mine dust in 1936 (2). This work, and other work following (3), 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 spectrometer (4) that truly quantitative diffraction became possible. Some early papers describing Geiger techniques were on the determination of quartz (5, 6) and heavy metal carbides (7).

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The mathematical relationships pertinent to quantitative diffraction analysis were derived and published in an important fundamental paper by Alexander and Klug appearing in 1948 (8). 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 (9). In 1958, Copeland and Bragg (10) described special conditions under which calibration curves could be sometimes eliminated and multiple components determined.

All of the above papers refer to investigations of inorganic systems. Published reports on quantitative diffraction applications to organic systems are almost nonexistent. A notable exception is the application to the determination of sodium penicillin G, by Christ, Barnes, and Williams (11).

It is the purpose of this paper to describe the application of quantitative diffraction methods to typical pharmaceutical systems. In making this application, several situations may be encountered and each situation requires a somewhat different procedure. The procedures found useful for each of the commonly encountered situations are described and illustrated by specific examples.

THEORY

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 absorption of 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.

The diffraction of X-rays by a crystal is a well-known phenomenon. It occurs because the wavelengths of X-rays are of the same order of magnitude as the spacings between planes of crystalline lattices. A fundamental relationship, the Bragg equation, is $N\lambda = 2d \sin \theta$. A monochromatic X-ray beam (wavelength λ) is diffracted by a set of parallel, equally spaced planes (spacing d) within a crystal. Diffraction occurs only in precise directions (the angle θ) with respect to the planes, for each of several orders of diffraction (N).

A single crystal has a number of sets of parallel planes, oriented differently from one another, each set having a constant d spacing between its parallel planes. Diffraction from any single set of parallel planes is possible when the crystal is properly oriented with respect to an X-ray beam. If a sample consists not of a single crystal but of a large number of small crystals packed together with random orientation, all possible orientations of single crystals will be presented to a fixed X-ray beam. Such an arrangement is found in the goniometer shown in Fig. 1 where S represents an edge view of a shallow tray containing a finely powdered crystalline sample with a planar surface. A proportional counter C rotates about S at an angle of 2θ, while the sample turns at θ, with respect to the fixed X-ray source. Whenever a crystal in the randomly packed powder sample falls into such a position that the Bragg equation is satisfied, diffraction will occur and the position (2θ) and intensity of the diffracted ray is measured by the scanning counter.

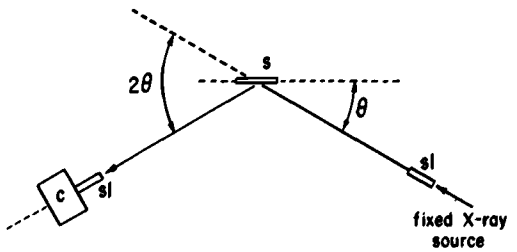

Fig. 1.—The sample (s) rotates through θ while the counter (c) scans through 2θ. An edge view of the planar-surface sample is shown. X-ray beam slits (sl) define the beam parallel to the sample surface.

Modern instrumentation permits this operation to be accomplished automatically. While the proportional counter is driven in its scanning operation at constant velocity, the sample is rotated at half this angular velocity, and a synchronized strip-chart recorder plots the diffracted intensity as a function of the angle, 2θ. For semiquantitative studies, strip-charts can be used directly but for truly quantitative work, scaler and timer circuits are employed, permitting the more accurate determination of the intensities of the diffracted rays.

When diffraction 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,[1] 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.

EXPERIMENTAL

For the experimental work reported, a General Electric XRD-5 unit and copper $K\alpha$ radiation were employed. The unit was equipped with a decade scaler and timer circuit, proportional counter, 1° beam slit, and 0.2° detector slit.

Simple Calibration Curves.—For the preparation of the standard curve for the determination of pamoic acid in a matrix of the pamoic acid salt of a basic antibiotic, 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. Since the statistical accuracy of a counting process is a function of the

[1] 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 (11).

total number of counts taken, a preset count, rather than preset time, was used to determine the intensities in counts-per-second. This procedure allows all values, when compared later, to be of the same statistical accuracy.

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 calibration curve for the determination of novobiocin in mixtures of tetracycline and amorphous suspending agent was prepared from aqueous suspensions containing known amounts of novobiocin in mixtures of these ingredients. Solids were separated from suspension by centrifugation and air dried.

Use of an Internal Standard and Integrated Diffraction Peak Areas.—A method for the determination of a crystalline sulfonamide in aqueous suspension illustrates the use of integrated peak areas and an internal standard.

In this method, exactly 3.00 Gm. of powdered $CaSO_4 \cdot 2H_2O$, the internal standard, was added to 50.0 ml. of the sulfonamide suspension. As the suspension was too viscous to filter and too stable to separate with reasonable centrifuge times, it was diluted 1:4 with water which was freshly saturated with the pure sulfonamide. Centrifugation and decantation was followed by a second suspension in the dilution medium. A second centrifugation and decantation step freed the total solids from all water-soluble materials. The sulfonamide itself was virtually insoluble. The dried residue was mixed by grinding and packed into a standard diffraction sample tray. A portion of the diffraction pattern of the mixed solids from a 5% suspension of the sulfonamide is shown in Fig. 2. The doublet at 10° is from the sulfonamide diffraction, and the peak at 11.5° is from the internal standard.

The integrated intensity values were determined 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. 2 by the lengths of the lines *AF*, *BE*, and *CD*.

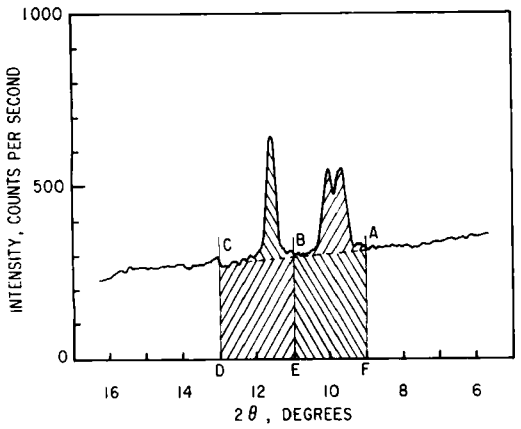

Fig. 2.—Partial diffraction pattern showing sulfonamide peaks (doublet at 10°) and internal standard peak (11½°).

With the goniometer set at somewhat less than 9°, an automatic scan was begun at 2° per minute. 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 average of background counting rates measured at 9° and 11° (in counts-per-second) gives the area of background under the the sulfonamide 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 sulfonamide diffraction peak, expressed in total count. The operation was repeated between 11° and 13° to determine the area of the internal standard peak. The ratio of sulfonamide peak area to standard peak area was then computed.

RESULTS AND DISCUSSION

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. An example is the determination of pamoic acid in a matrix of the pamoic acid salt of a basic antibiotic. The method was found useful in testing for completeness of the salt-forming 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. 3. Although major diffraction peaks occur at 2Θ values of 9.50°, 11.20°, 15.60°, and 19.40°, all of these peaks are 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.[2] The results of a plot of intensity differences, peak minus background, for several known concentrations is shown in Fig. 4. The linearity shows the direct applicability to simple systems. 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. 5 which shows the superposition of diffraction peaks of novobiocin and tetracycline on a steep background slope. This diffraction pattern was measured using

[2] 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.

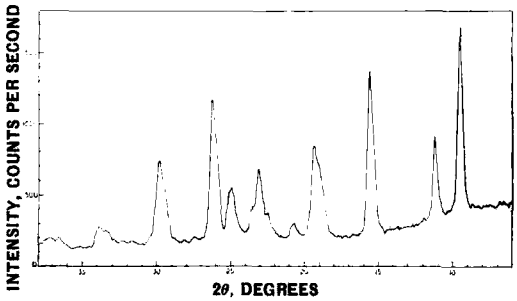

Fig. 3.—The pamoic acid diffraction pattern.

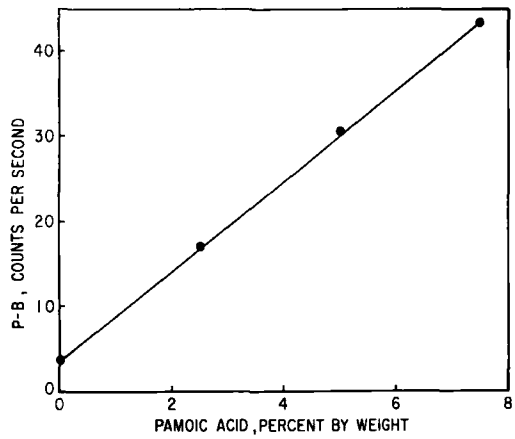

Fig. 4.—Intensity difference, peak minus background, as a function of pamoic acid concentration.

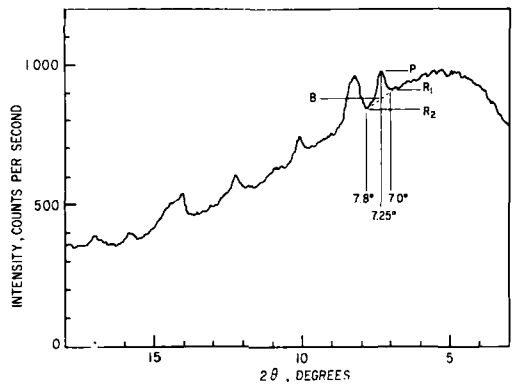

Fig. 5.—Partial diffraction pattern of an antibiotic mixture showing novobiocin peak at 7.25°.

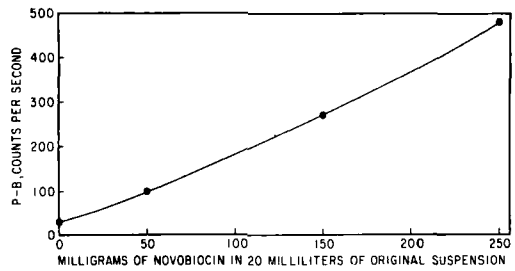

Fig. 6.—Intensity difference, peak minus background, as a function of novobiocin concentration.

the solids removed by centrifugation from a diluted aqueous suspension of calcium novobiocin, tetracycline, and an added known amount of crystalline novobiocin acid as a standard.

The diffraction peak at 7.25° 2θ (Fig. 5) is due to crystalline novobiocin acid. Its intensity is not influenced by the other peaks, which are due to tetracycline, but it is influenced by the unusually high background caused by the large percentage of amorphous suspending agent which was concentrated by the centrifugation.

A large part of the apparent novobiocin peak intensity is actually due to the background intensity. The true net peak intensity, upon which accuracy is based, is smaller. Moreover, reproducibility of the background intensity at the base of the peak is poor due to the steepness of the background slope.

Under these conditions it has been found advantageous to determine the net peak height by a different procedure in order to maintain accuracy. This procedure resulted in a close approach to linearity of response when the net peak intensity was plotted against concentration of crystalline novobiocin, for a series of prepared standard samples.[3] Using the scaler and synchronous timer circuits, the intensity P was measured at 7.25° 2θ (Fig. 4). The intensities at R_1 and R_2 were measured at 2θ values of 7.00° and 7.80°. The background B was calculated by interpolation

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

The net peak height (P-B) was then calculated for each sample. These values were plotted against concentration of crystalline novobiocin. The results are shown in Fig. 6.

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. In the formation of a standard curve for the determination of mucic acid in mixtures of mucic acid and tetracycline,[3] peak-to-background ratios were used and the 2θ position for the background determination (29.00°) was necessarily some distance removed from the mucic acid peak of choice (19.48°). The resulting plot of intensity ratios *vs.* known concentration over the range to which interest was limited is shown in Fig. 7.

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 possible degradation products.

[3] The author is grateful to Drs. L. C. Schroeter and W. Morozowich, of The Upjohn Co., who prepared the samples for these standard curves.

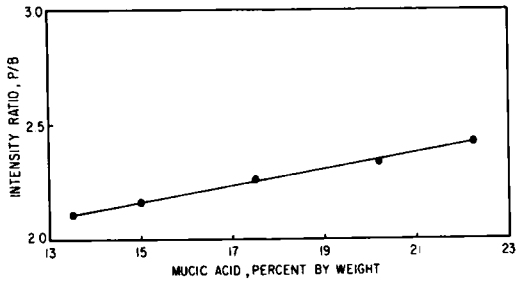

Fig. 7.—Intensity ratio, peak to background, as a function of per cent mucic acid in tetracycline.

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 found 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. Samples for the standard curve contain 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 then 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. Typical results of the use of an internal standard are shown in an example to follow.

Finding a suitable internal standard for each new system is often a problem. An ideal internal standard has several attributes: It must not have a diffraction peak which is obscured by matrix peaks, nor which will interfere with that of the material to be analyzed; the internal standard peak should be near the usable peak of the analyzed ingredient; 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; because of absorption effects, it should contain only elements of low atomic number; it should have a density not too far removed from those of the system ingredients for aid in maintaining homogeneity in mixing; and it should be chemically stable in the presence of the system. No one compound can qualify universally on all counts, but in addition to the compound used in an example to follow later, a material which has been found to have many of the above attributes is hexamethylenetetramine, one of the few organic compounds of isometric (cubic) symmetry. Another compound, beryllium acetate, has been proposed as an ideal standard since it meets most of the above specifications. Its extreme toxicity, however, would tend to disqualify it for use in pharmaceutical areas.

It is worth mentioning that in instances in which the system is of many variable components, and would therefore require an internal standard, but for which no suitable standard can be found, an alternative procedure exists. This consists of using known additions 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.

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 at any one time. 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 range of 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.

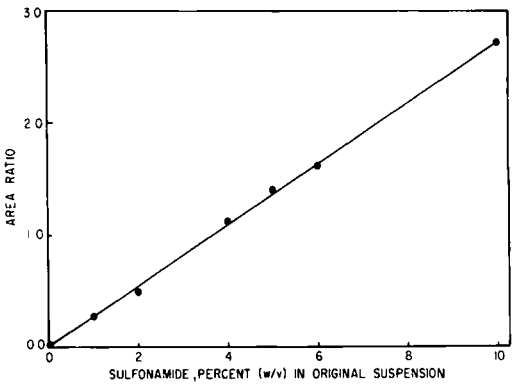

Fig. 8.—Ratio of peak areas, sulfonamide to internal standard, as a function of sulfonamide concentration.

A method for the determination of a sulfonamide in aqueous suspension illustrates the use of an internal standard, as well as integrated diffraction peak areas. Figure 8 shows a least squares plot of values for the ratios of sulfonamide peak areas to internal standard peak areas as a function of sulfonamide concentration. Following sample preparation, X-ray analysis required about 15 min. per sample. The values indicate an accuracy of $\pm 0.15\%$ of the amount present. The method is specific for the intact sulfonamide molecule in the crystalline state and, therefore, sensitive to any product degradation.

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Dissolution Rate Studies I

Continuous Recording Technique for Following Rapid Reactions in Solution

By PAUL J. NIEBERGALL† and JERE E. GOYAN

An automatic recording apparatus was developed to follow the process of dissolution. Results were obtained as a recording of per cent transmittance *vs.* time. The recorder was equipped with various speed chart drive motors that permitted readings to be taken from the graph at intervals down to 0.375 sec. Several experiments were performed to demonstrate the applicability of this apparatus to the determination of dissolution rates, and constants obtained agreed well with those obtained by the usual method of removing samples with a pipet.

THE FIRST quantitative study of the dissolution process was made by Noyes and Whitney in 1897 (1). Since that time numerous workers have evaluated the effect of different variables on the dissolution rate constant. The majority of these studies have been done using a single large tablet or disk. In a few instances, particularly in attempts to obtain a measure of mixing efficiency, multiparticulate systems have been used.

Initial dissolution rates in multiparticulate systems are rapid, and previous studies have been handicapped by the lack of an accurate method for sampling the solution in the early stages of the process. Therefore, the object of this study was to develop an automatic recording technique which would furnish continuous data and obviate the need for hand sampling in studies of rapid reactions.

THEORY

Calculation of Dissolution Rate Constants.—Hixson and Crowell (2) derived an equation for the dissolution of a single particle in which the surface area was allowed to change with time, using the following form of the Noyes-Whitney equation

$$V\frac{dw}{dt} = -KS(w_s - w_o + w) \quad \text{(Eq. 1)}$$

in which w is the weight of the particle at time t, K is a rate constant, S is the surface area of the particle, w_s is the weight of solid needed to saturate the volume, V, of solvent at a given temperature, and w_o is the initial weight of the particle. Using the property of geometrically similar solids, Hixson and Crowell replaced the surface area by

$$S = kw^{2/3} \quad \text{(Eq. 2)}$$

in which k is a constant containing the shape factor and the density of the particle.[1]

The basic equation of Hixson and Crowell has been extended for use in multiparticulate systems by assuming a system of N equal-sized particles. The total surface area, A, would then be equal to $N \times S$ or

$$A = kNw^{2/3} \quad \text{(Eq. 3)}$$

The total weight, W, for a sample of N equal sized particles would be

$$W = Nw \quad \text{(Eq. 4)}$$

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[1] The particles used in this study had a height, h, greater than the diameter, d, which may result in a change in the h/d ratio during the process of dissolution, and the Hixson-Crowell equations would theoretically not be applicable. However, the salicylamide crystals were followed for a total change in weight which was felt to represent a negligible change in the h/d ratio. The benzoic acid crystals were followed to a much greater change in weight. However, the Hixson-Crowell equations did yield straight lines, and it was felt that the apparent rate constants obtained from these equations would be suitable for use in a comparison study of this nature. [See also the paper by Blumberg, *J. Phys. Chem.*, **63**, 1129(1959).]