Ultracentrifuge Time-Lapse Photography. Determination of Molecular Weights*

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ABSTRACT: Time-lapse motion picture photography has been widely employed in the study of slow processes in many fields. Application of this means of data acquisition to the analytical ultracentrifuge has apparently not been made previously. The practical advantages of the use of time-lapse photography are illustrated by the development of two methods which allow the determination of the molecular weights of macromolecules at low-speed sedimentation equilibrium without the necessity for either assumptions or ancillary experiments. The absolute concentration across the centrifuge cell, and, hence, the reduced molecular weight of the kinetic unit, are directly determined. These methods give excellent results in any solvent system, in contrast to previously available methodology. For the model systems employed, molecular weights obtained were: 13,600 for ribonuclease A in solutions of low ionic strength at pH 8.0; 13,250 for ribonuclease A in 5 M guanidine hydrochloride; and 25,500 for chymotrypsinogen in 5 M guanidine hydrochloride.

These approaches to molecular weight determination are suggested as particularly applicable to investigation of the subunit structure of proteins, and for the evaluation of the other methods which do require assumptions in calculation or ancillary experiments.

Recent experimental and theoretical developments have allowed the analytical ultracentrifuge to become the most widely used instrument for the accurate determination of the molecular weights of biological macromolecules. The employment of low-speed equilibrium techniques (Svedberg, 1925; Svedberg and Pedersen, 1940) has been facilitated by the use of short liquid columns (Van Holde and Baldwin, 1958), the development of reliable interferometric optical systems (Beams *et al.*, 1954; Richards and Schachman, 1959), and the use of overspeeding to reduce centrifugation times (*cf.* Richards *et al.*, 1968). Sedimentation to equilibrium at high speeds, with reduction of the concentration of solute at the meniscus to zero, has been utilized as an approach by Yphantis (1964). Further, the determination of molecular weights during the transient phase of approach to equilibrium has been discussed and widely employed (Archibald, 1947; Klainer and Kegeles, 1955; Ehrenberg, 1957; Labar, 1966a,b).

The methods previously available for the determination of molecular weights using the ultracentrifuge have been evaluated with dilute aqueous solutions of proteins or small molecules. When these methods are utilized for the determination of molecular weights in solvents containing, *e.g.*, high concentrations of guanidine hydrochloride or urea, or in nonaqueous solvents, many operational difficulties may appear. Thus, the accurate determination of the initial protein concentration, required for the low-speed method, is difficult to achieve, and the usage of differences, either in schlieren or Rayleigh optical systems (Nazarian, 1968) or their combination (Chervenka, 1966), can be subject to large errors, as well as being useful only at higher concentrations of the kinetic unit. High speed experiments (Yphantis, 1964) may be theoretically ambiguous due to significant redistribution of the solvent components in the necessarily high centrifugal fields (Casassa and Eisenberg, 1964). Approach-to-equilibrium methods may show marked time dependence of the apparent molecular weight, rendering interpretation difficult (Yphantis, 1959). Since many interesting questions in protein chemistry relate to the subunit structure of proteins, the determination of subunit molecular weights (usually done in such solvents) is a problem in principle of technique.

In this context, the present communication reports the development of methodology which allows the unambiguous determination of the reduced refractometric molecular weights of macromolecules in any solvent system. The theoretical, and potential practical, advantages of the use of time-lapse motion picture photography as the primary means for data acquisition in ultracentrifuge experiments have been detailed (Bethune, 1970). The present communication provides experimental verification for the predicted utility of this approach. A preliminary communication has been presented (Bethune and Simpson, 1969).

Experimental Section

Ribonuclease A was obtained as a salt-free lyophilized preparation from Worthington Biochemical Corp. (lot RAF-7CA). Prior to use for molecular weight studies, the dissolved protein was heated at 60° for 10 min to dissociate

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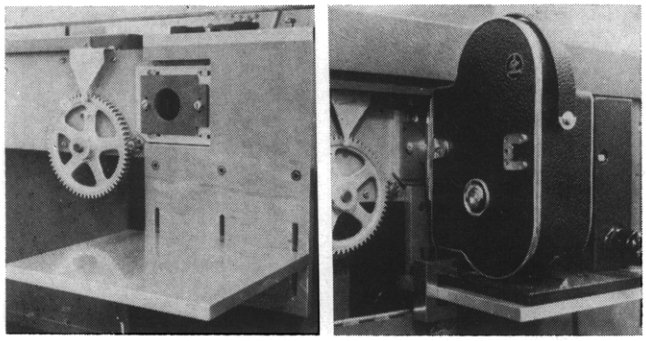

FIGURE 1: Modifications of the ultracentrifuge for application of time-lapse motion picture photography. Left: the aperture arrangement and a portion of the support table for the camera. Right: the camera in position on the support table.

dimers which may form during lyophilization. Guanidine hydrochloride (Eastman Organic Chemicals) was recrystallized from hot methanol–benzene, washed with acetone, and dried prior to use. Other chemicals employed were reagent grade.

The partial specific volume of ribonuclease employed in calculations was 0.695 in dilute salt solution (Harrington and Schellman, 1956) and 0.685 in the denaturing solvent (Kielley and Harrington, 1960). Similarly for chymotrypsinogen in 5 M guanidine hydrochloride, a partial specific volume of 0.714, 1% lower than that in water (Schwert, 1950), was utilized.

All centrifugal studies were performed in a Beckman Spinco Model E analytical ultracentrifuge. The optical system of the instrument was aligned according to Van Holde and Baldwin (1958). Monochromatic light was isolated with a Kodak 77A Wratten filter. The interference mask employed for all studies had parallel slits (1 mm wide). No modifications were made to the optical system of the centrifuge.

Certain physical modifications were made in the ultracentrifuge to permit the installation of a time-lapse camera unit. These modifications are described briefly here and illustrated in Figure 1. The metal covering at the camera end of the centrifuge was cut out to allow mounting of the components described. A square hole, 5.0 cm on a side and centered on the optic axis, was cut in the casette holder to allow direct observation of the fringe pattern. An aluminum plate with a similar opening was made to fit over the casette holder opening, and a covering plate of opaque plastic, drilled to accept the nosepiece extension of the camera, fitted to this (Figure 1). Slotted screw holes allowed horizontal movement of the aluminum plate and vertical movement of the plastic plate, to properly position the camera at any desired position in the fringe pattern.

An L-shaped table was fabricated from $^3/_8$-in. aluminum stock to support the camera behind the focal plane of the instrument. This support was fixed to the top of the casette holder, so that the entire assembly was fixed with reference to the optical axis (Figure 1). No significant deflection of the optical tube was noted on addition of the whole mass of the assembly to the end of the tube.

The camera employed was a 16-mm Bolex Paillard modified by Zeiler Instruments, Inc., for time-lapse photography, at intervals from 0 to 5 min, with exposure times up to 150 sec. No ancillary camera lens was employed; rather, a nosepiece fabricated from 2.9-cm diameter aluminum tubing, 1.7 cm long, was positioned to fix the film at the focal plane of the optical system.

Alignment of the camera is accomplished by placing the holes in the movable plates to center the fringe pattern both horizontally and vertically in the aperture. The camera assembly is then positioned without disturbing the movable plates, and is clamped to the support table. The camera is not moved during the course of a centrifuge run.

Motion pictures were taken on 16-mm Tri-X negative motion picture film, generally with exposure times of 100–150 sec. The film was developed in a manual tank-type developer with continuous agitation for 10 min at ~20° using either a 1:3 dilution of Kodak HC-110 developer or Kodak D-11 developer. After one wash with water, the films were fixed with a 1:3 dilution of Kodafix acid fixer for twice the time required for clearing, and then washed with tap water, and air dried. No distortion of the emulsion, compared with that obtained on Kodak IIG spectroscopic plates, was noted.

The data were evaluated using a modified Kodak Analyst 16-mm projector. This projector permits automatic, flicker-free projection at speeds down to 1 frame/sec, and is equipped with blower cooling so as to allow full light intensity even when stopped for single-frame projection. The pictures were projected onto a ground-glass screen with a rastor to allow quantification of movement of the interference fringes.

Additional photographs on Kodak Spectroscopic IIG plates were utilized for evaluation of equilibrium patterns. The plates were read on a Mann two-dimensional comparator, utilizing previously noted techniques (Bethune, 1965; Richards *et al.*, 1968).

A single centrifuge cell, with a 12-mm filled epon centerpiece and sapphire windows, was employed for all the studies presented. The cell was filled with 0.01 ml of FC-43 (Beckman Instruments) and 0.11 ml of solvent on the reference side; and 0.02 ml of FC-43 and 0.096 ml of solution on the protein side. Accurate loading of the cell was ensured through the use of a Hamilton microsyringe. Buffer densities were determined in a Fox (1955) pycnometer.

Data were evaluated using the differential form of the general equation for the distribution of solute c, at equilibrium, as a function of radial position in the cell, r. Thus

$$M_{app} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{d \ln c}{dr^2} \quad (1)$$

where M_{app} is the apparent molecular weight, $\bar{v}$ is the partial specific volume of the solute, ρ is the density of the solvent, ω is the angular velocity of the rotor, R is the gas constant, and T is the absolute temperature. Since the concentration distribution is measured refractometrically, concentrations are evaluated as fringe displacements, J, and the appropriate equation is

$$M_{app,ref} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{d \ln J}{dr^2} \quad (2)$$

where $M_{app,ref}$ is the refractometric molecular weight. In these studies it has been assumed that

$$c \propto J \quad (3)$$

and, therefore

$$M_{app} = M_{app,ref} \quad (4)$$

Since the methodology employed allows determination of the absolute meniscus concentration of protein, it is not necessary to utilize mass conservation, location of the white light fringe, or determination of the hinge point (Ginsburg *et al.*, 1956; Richards and Schachman, 1959) in calculation, although the position of the latter is readily determined on projection of the film. A single ultracentrifuge experiment itself provides all the information necessary for solution of eq 1 in terms of the reduced molecular weights, *i.e.*, $M_{app}(1 - \bar{v}\rho)$, either in the above terms or in the terms of J_0, the initial refractometric concentration, which is also directly determinable.

Data analysis was facilitated through utilization of two computer programs written for the Scientific Data Systems 940 time-sharing system. The first of these programs calculates the parameters of the least square equation for the plot of ln J *vs.* r^2 across the cell, yielding molecular weights averaged over the entire cell volume. In addition, the program includes as output molecular weights calculated between each pair of comparator readings, to detect both nonlinearity in the plot and erroneous comparator readings. This program uses either the meniscus concentration of solute, J_m, determined from the time-lapse studies or, alternatively, determines J_m by trapezoidal integration and mass conservation, using the values of the initial concentration of solute, J_0, determined separately in a synthetic boundary cell run or from the film record.

The second program proved particularly applicable for determinations in solvents containing high concentrations of denaturing agents, where the marked concentration dependence of M_{app}, if there is a significant concentration change across the cell, may give a high degree of error when the molecular weight is evaluated over the column height to yield a M_{app} at one concentration, *i.e.*, C_0, or $(C_m + C_b)/2$ (Williams *et al.*, 1958). This program calculates a least-squares fit of nine readings centered around every fourth reading through the cell. Thus, if the displacement across the cell is 15 fringes and readings are made at half-fringe intervals, the output would correspond to M_{app} calculated at the 2nd, 4th, 6th, 8th, 10th, and 12th fringes, *i.e.*, at $J_m + 2$, $J_m + 4$, etc., where J_m is the fringe number at the meniscus. In addition, molecular weights calculated between each pair of readings were included in the output to detect erroneous comparator readings. In systems where a high dependence of M_{app} on concentration is observed, this approach provides fuller utilization of the information inherently present in the data, resulting in smaller errors than does evaluation of the distribution across the column as a whole.

Results and Discussion

In contrast to sedimentation velocity experiments, where more nearly continuous observation of the liquid column in the centrifuge cell occurs, sedimentation equilibrium experiments are generally conducted with few recordings of the distribution of material within the cell. Thus, baseline photographs, taken either at the beginning or after completion of the experiment, and photographs taken at equilibrium, constitute the experimental record. With the introduction of time-lapse photography for the data acquisition in sedimentation experiments, it is possible to continuously monitor an experiment. The ability to continuously record the distribution of material within the ultracentrifuge cell has proven valuable as a general approach to this instrument.

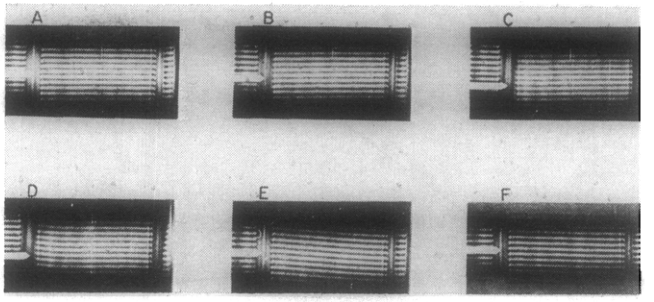

FIGURE 2: Fringe shifts in solvent *vs.* solvent experiments. The patterns are enlarged from individual frames of the 16-mm motion picture film taken during a blank run using 0.05 M Tris-Cl–0.10 M NaCl–5 M guanidine hydrochloride (pH 8.0) at 20°. The index at the left of each pattern is placed to identify a single fringe in all photographs. (A–C) Fringe shift on acceleration from 14,290 rpm (A) to 44,770 rpm (C). (C–F) Reversal of fringe shift on slowing rotor from 44,770 rpm (C) to 14,290 rpm. (F) The pattern returned completely to that shown in part A after 30 min at 14,290 rpm.

Thus, two experimental approaches to the determination of molecular weights have been devised. The first is analogous to that of LaBar (1965), modified in the operational approach of using motion picture photography to obtain the data, rather than graphical extrapolation from a few observations. Thus, after the attainment of low-speed sedimentation equilibrium, the rotor is accelerated slowly (at a driving current of about 3.9 ± 0.1 A) to a speed which ensures depletion of the kinetic unit at the meniscus. Usually this rate of acceleration requires about 8–10 hr to accelerate the rotor from the low-speed condition (10,000–20,000 rpm) to the high-speed condition (45,000–60,000 rpm). As the rotor accelerates, the meniscus is gradually depleted of protein, resulting in a fringe drop at that position. The total fringe drop then represents J_m for the low-speed run. Alternately, if the record is continuous, the first fringe drop, *i.e.*, that occurring from the time of initial imposition of the field to equilibrium, when added to J_m, gives J_0, the initial concentration.

Although in most cases buffer base lines show no deviation of the fringe pattern across the column greater than 0.1 fringe, the *entire* fringe pattern as a unit may shift up or down by as many as 6 fringes (Figures 2A–C).[1] The extent of this fringe shift is a function of the centerpiece and windows employed, the refractive index of the solvent, and the technique of assembly of the cell. The shifting is relatively minor until speeds greater than 30,000 rpm are attained, but becomes increasingly important above this speed. This artifact may

[1] Since methods of scientific communication do not presently allow presentation of a continuous record of an experiment, any illustrations must necessarily be still photographs selected individually from the total film record. Since these have been enlarged and processed several times to achieve these illustrations, some deterioration in quality from the original has occurred.

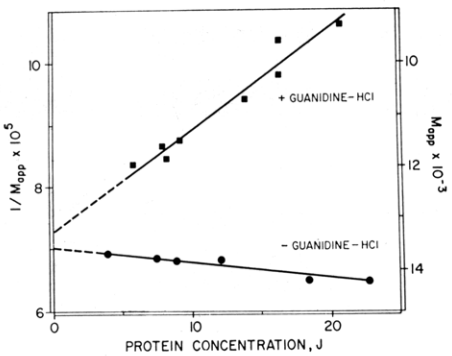

FIGURE 3: Molecular weight of ribonuclease A. The reciprocal apparent molecular weight of ribonuclease A as a function of protein concentration in fringes is shown, determined in 0.05 M Tris-Cl–0.1 M NaCl (pH 8.0), 20°, in the presence (■) and absence (●) of 5 M guanidine hydrochloride. Data were obtained by acceleration of the rotor after attainment of low-speed sedimentation equilibrium to determine the absolute meniscus concentration of protein, and evaluated with the first computer program described in the Experimental Section.

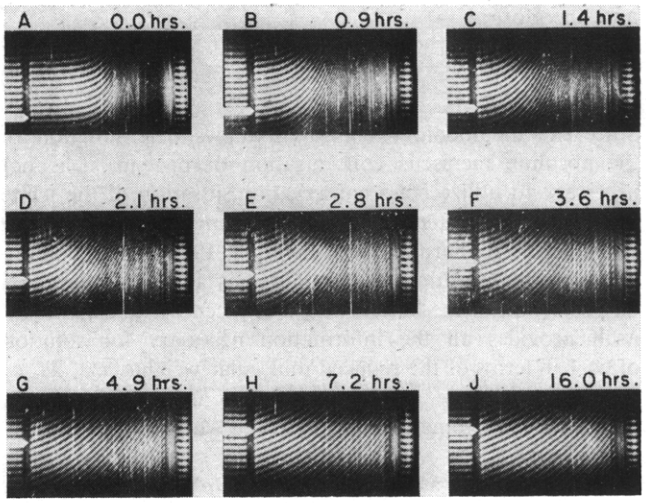

FIGURE 4: Determination of absolute protein concentrations at low-speed equilibrium during diffusion to equilibrium from the condition of meniscus depletion. The patterns are enlarged from individual frames of the 16-mm motion picture films taken during an experiment with chymotrypsinogen at a concentration of 4 mg/ml in 0.05 M Tris-Cl–0.1 M NaCl (pH 8.0), 20°. The index at the left of each pattern is placed to identify a single fringe in all photographs. Meniscus depletion was carried out at a rotor speed of 44,770 rpm and then the rotor was slowed to 14,290 rpm. The times indicated are the times after the attainment of the lower rotor speed.

be due to wedging of the cell centerpiece, since the effect is entirely analogous to that obtained with water-wedge cells, where the prismatic effect of constantly increasing or decreasing cell depth with radial distance raises or lowers the fringe pattern in the solution solvent region as a whole. It is fully reversible on deceleration in an essentially instantaneous fashion (Figures 2C–F). A blank was run every time the cell was assembled, and appropriate corrections were made to the fringe shift observed in the experimental run.

The results of a number of molecular weight determinations on ribonuclease A in two solvent systems are shown in Figure 3. In dilute salt solutions the data are of high precision, and extrapolate to a molecular weight of 13,600 ± 100, in acceptable agreement with the molecular weight of 13,683 calculated from amino acid analysis (Hirs *et al.*, 1956). A small positive concentration dependence of M_{app} is observed. It should be noted that these studies are performed at a pH different from the isoelectric pH for the protein. Previous investigations of the molecular weight of ribonuclease have demonstrated little or no concentration dependence of the apparent molecular weight, when investigated at the isoelectric pH (Richards *et al.*, 1968). The possibility that the present results reflect a pH dependent association process are under study.

In the presence of 5 M guanidine hydrochloride, the scatter of the experimental data around the least-squares line is somewhat greater (Figure 3). However, the extrapolated molecular weight, 13,250 ± 500, agrees almost exactly with that determined in the absence of guanidine hydrochloride. These results clearly demonstrate the applicability of this methodology to determination of the molecular weight of even small protein subunits which could be present in a complex native molecule.

In part, decreased precision in determination of the molecular weight of ribonuclease in 5 M guanidine hydrochloride is due to the high speeds (>50,000 rpm) which must be employed to deplete the meniscus of this relatively small species. Thus, the correction due to the solvent blank fringe shift may become relatively large, and the reading of the moving picture films may become difficult as a result of some blurring of the fringe pattern.

For most proteins studied, the molecular weight of the smallest subunit detected thus far is between 20,000 and 40,000 daltons (Sund and Weber, 1966). Therefore, chymotrypsinogen was selected as a suitable test protein for the evaluation of the second method of molecular weight determination. In this method, the solution of protein is centrifuged at a high speed until the meniscus is fully depleted. Then, the rotor is decelerated to an appropriate speed for the equilibrium run, and allowed to equilibrate by diffusion. The appearance of protein at the meniscus, reflected as a fringe rise, rather than its disappearance, is followed in the film record (Figure 4). Since the artefactual shifting of fringes under the centrifugal field is reversed rapidly upon deceleration (Figures 2C–F) while diffusion of protein to the meniscus is a much slower process, no correction is necessary for wedging in this type of experiment. Moreover, the field-imposed distribution of the solvent components at the high speed relaxes to that characteristic of the lower speed much more rapidly than does the protein. Thus the protein is diffusing through a much more homogeneous solvent than is at first apparent, allowing full definition of the kinetic unit.

To cover a range of protein concentrations across the liquid column, the rotor may be run at several speeds, allowed to attain equilibrium at each, and thereby provide a significantly wider range of protein concentration within a single experiment. The data obtained from two such experiments, analyzed by the second computation method outlined in the Experimental Section, are detailed in Figure 5.

The absence of the necessity for correction of the fringe shifts due to wedging and solvent redistribution had reduced the scatter among the experimental points. The extrapolated weight of 25,500 ± 400 compares very favorably with the

molecular weight of 26,000 derived from the known amino acid composition of the protein (Hartley, 1964), or one of 25,600 derived from sedimentation equilibrium studies in aqueous solutions (LaBar, 1965).

The application of time-lapse motion picture photography has been widely made in a variety of scientific endeavors, with notable advantages in the study of slow processes. However, such application to the field of physical biochemistry has not been previously reported. The results obtained in this initial experimental study suggest that this operational approach to the analytical ultracentrifuge and potentially to other physicochemical instruments will be as profitable as it has been in other fields.

As an illustration of the potential of this means of data acquisition, the present communication details two methods of determination of the molecular weights of biological macromolecules. In contrast to the theoretical and/or practical difficulties in the application of the previously available means of determination of molecular weights of subunits of proteins in solvent systems necessary for their dissociation, the present methodology gives reliable results in any solvent system.[2]

Several comments concerning the relative advantages and disadvantages of the two methods presented are appropriate. The second method, that of initial meniscus depletion, followed by attainment of equilibrium through diffusion in a low centrifugal field, would appear to lead to significantly higher precision, in large measure due to elimination of corrections for cell wedging and solvent redistribution. However, certain requirements in the protein–solvent system under study must be met for this method to be applicable. Thus, the occurrence of a very slowly reversible precipitation of protein at the base of the cell at high speeds would lead to impractically long times for the attainment of equilibrium at the lower speed. Secondly, in the study of large molecules, characterized by low values of the diffusion coefficient, inconveniently long times again would be necessary for attainment of equilibrium.

The first method, *i.e.*, sedimentation to equilibrium, followed by meniscus depletion on imposition of a higher field also suffers from operational disadvantages. Thus, when high speeds are necessary for depletion to occur fully, problems of cell wedging and solvent redistribution necessitate significant corrections in the determination of the concentration of sedimenting material present at the meniscus at equilibrium, or redefinition of the kinetic unit, the concentration distribution of which is determined.

For systems where it is applicable, the second method would seem to offer more advantages, and would be suggested as the method of choice. Fortunately, as noted above, the use of time-lapse photography as a monitor during the ultracentrifuge experiment allows the facile detection of various problems in the use of the instrument. Thus, the problems possibly encountered in these methods of molecular weight determination, *i.e.*, base-line fringe shifts, nonattainment of equilibrium, and loss of solute due to irreversible aggregation, are all readily detectable on simple viewing of the motion pictures taken during the run.

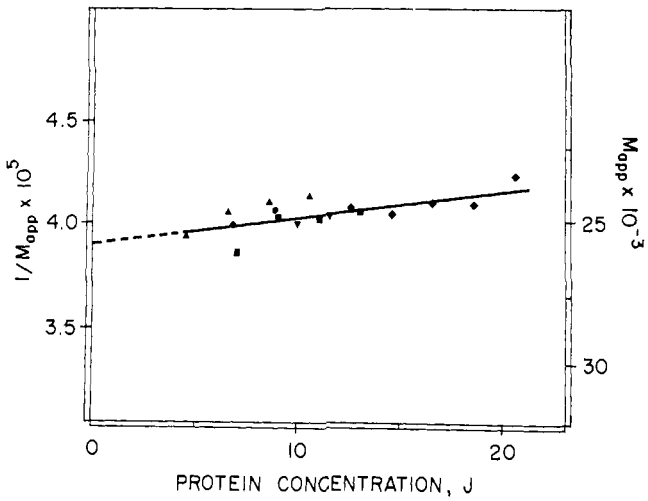

FIGURE 5: Molecular weight of α-chymotrypsinogen. The reciprocal apparent molecular weight of chymotrypsinogen, as a function of protein concentration in fringes is shown, as determined in 0.05 M Tris-Cl–0.1 M NaCl–5 M guanidine hydrochloride (pH 8.0), 20°. Data were obtained by deceleration of the rotor from the high-speed condition of meniscus depletion to a state of low-speed equilibrium and were analyzed by the second computer program detailed in the Experimental Section. The different groups of points indicate different speeds and/or experiments.

These results suggest that this approach might prove to be the method of choice for the ultracentrifugal determination of the molecular weights of protein subunits in the solvent systems conventionally employed for dissociation of the parent molecule.

Further, the avoidance of any assumptions in calculations of molecular weights suggest the employment of this method in the evaluation of other methods which do invoke assumptions in calculation or require ancillary experiments. Finally, as previously noted (Bethune, 1970), other applications of the method of data acquisition described here are apparent. Thus, the time derivative formulation of the equations for the approach to equilibrium should be readily approachable experimentally, a supposition presently under examination.

[2] The present methodology is highly complementary to the automatic split-beam photoelectric scanner system (Schachman and Edelstein, 1966; Edelstein and Schachman, 1967). Thus, this latter method allows the absolute determinations of concentrations throughout the liquid column at low concentrations of the kinetic unit. The present method is applicable to solutes which do not absorb radiation in the accessible range of the scanner, and can be extended to protein concentrations much higher than those to which the scanner is limited. Further, at present, the higher practical accuracy of the interferometric method of data measurement lends greater accuracy to the methods proposed here. Additionally, there is a practical consideration: the cost of installation of the time lapse system is about 5% that of the scanning system.

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pH Dependence of the Fluorescence Decay of Tryptophan*

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ABSTRACT: The fluorescence decay of tryptophan was measured as a function of pH using a nanosecond flash apparatus. Within the pH range 2–10.6 variations in the lifetimes could be understood in terms of the mixtures of different ionic species present in solution. Each species has a characteristic lifetime or contributes to the total decay in proportion to their molar fractions. Within the pH range 10.6–11.5, the fluorescence lifetimes decreased with increased pH due to quenching by hydroxyl ions. It is shown that the average fluorescence lifetime as a function of pH follows the relative quantum yield–pH curve as expected. The results of fluorescence lifetime and quantum yield measurements of some tryptophan derivatives are also reported and quantum yields are proportional to the lifetimes.

The effect of pH on the fluorescence of tryptophan and its derivatives has been the subject of numerous investigations (White, 1959; Cowgill, 1963; Weber, 1961; Konev, 1967). White (1959) was the first to report on the variations in the quantum yield of tryptophan as a function of pH. The quantum yield of tryptophan was found to be constant in the pH range 4–8, to decrease when the pH was lowered below pH 4, to progressively increase as the pH is raised above 8, to reach a maximum and then decrease as the alkalinity is still further increased. Subsequent investigations by several other workers confirmed those results (Cowgill, 1963; Weber, 1961; Konev, 1967). Bridges and Williams (1968) have reported pH–fluorescence curves which showed evidence for five distinct molecular species of tryptophan which were fluorescent.

Previous studies on the pH dependence of tryptophan fluorescence have been confined to measurements of fluorescence emission spectra and quantum yield. Several workers have reported the results of decay-time measurements of tryptophan in polar solvents (Chen *et al.*, 1967; Badley and Teale, 1969; Weinryb, 1969; Eisinger and Navon, 1969), but no literature is available on measurements of the pH dependence of the fluorescence decay of tryptophan. The present study consisted of measurements of fluorescence decay times in order to determine the effect of pH on that parameter. Since tryptophan is an ampholyte, it is capable of dissociation in aqueous solution as either an acid or base

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