TIME RESOLVED FLUORESCENCE DEPOLARIZATION AND APPLICATION TO SOME MACROMOLECULAR SYSTEMS

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ABSTRACT

TIME RESOLVED FLUORESCENCE DEPOLARIZATION
AND APPLICATION TO STUDIES OF
SOME MACROMOLECULAR SYSTEMS

BY

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Recent developments in instrumentation now allow the direct observation of fluorescence decay on a nanosecond time scale. Rotations of macromolecules in low viscosity solution or small molecules in high viscosity solutions occur in the same relative time domain, 10^{-10} to 10^{-8} seconds, as the fluorescence lifetime of many molecules. By use of polarization of the exciting radiation, one is able to observe these molecular rotations through the fluorescence anisotropy decay. One is then able to study the effects of perturbants on molecular size and, to some degree, conformation, through the rotational relaxation time of the molecule. In the case of non-fluorescent molecules, fluorescent probes or labels can sometimes be attached to make the molecule

of interest observable by this technique. This paper discusses the theory of fluorescence depolarization, steady state and time resolved. The time resolved technique is applied to the protein systems of α-chymotrypsin labelled with 1-anilino-8-naphthalene sulfonate (ANS) and lysozyme labelled with dansyl-chloride. The rotational relaxation times and solvated volumes are calculated from experimental data. The effect of temperature change and addition of sucrose on rotational relaxation times is observed. Data are analyzed by conversion of observed intensities to anisotropy (time dependent) using the empirical relationship of Jablonski (4) and theoretical equations derived from the expressions of Chuang and Eisenthal (16) to describe the anisotropy decay of various model molecular shapes are fit to the experimentally determined anisotropy decay curves to obtain the rotational relaxation times. The equation of Einstein-Stokes is then used to calculate solvated volumes. The rotational relaxation time of Rhodamine B in glycerol is also calculated from time resolved depolarization data. The anisotropy decay is found to be double exponential with values of approximately 9 nsec and 96 nsec for rotation about the major and minor axes respectively. These are discussed in view of the molecular geometry and environment.

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By

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TO GAYANE, JACOB, AND MY PARENTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

The task of determining molecular structure and monitoring changes in molecular structure or conformation under various conditions is of primary importance in understanding the chemistry of many chemical reactions. When the molecules are proteins, this task is more difficult due to the complexity of the interacting species. Spectroscopic techniques are accepted as being invaluable in the study of molecular structure at many different levels, provided the system under investigation lends itself to study by these techniques. In the case of proteins, fluorescence spectroscopy is one of the few spectroscopic techniques which can be used to study the molecule in an environment approximating its natural environment. Until recently, however, the instrumentation available limited the application of fluorescence spectroscopic techniques to observation of changes in the steady state emission properties of molecules and steady state fluorescence depolarization studies. The recent development (10) of instrumentation which allows observation of the intensity of fluorescence emission directly as a function of time now permits the application of fluorescent spectroscopic techniques to study time dependent changes in fluorescent systems. These studies may involve the monitoring of the enhancement or decay of fluorescence intensity

at a particular wavelength as a function of time (time resolved fluorescence spectroscopy) or the study of the time dependent fluorescence anisotropy decay of a molecule (time resolved fluorescence deporarization.)

Extension of these techniques to non-fluorescent systems and systems with emission properties unsatisfactory for these types of analyses has been made possible by the development of the technique of attaching extrinsic or co-enzymatic fluorescent probes to protein molecules. (21) Weber first used this technique in the study of the steady state depolarization of proteins to determine their rotational relaxation times. More recently, time resolved depolarization studies have been used to investigate the flexibility of antibody molecules (19), the temperature dependence of rotational diffusion coefficients to estimate rotational energy barriers of probes bound to proteins (47), the freedom of twisting of DNA molecules under various conditions (26,28), and to estimate the degree of aggregation and monitor changes in protein conformation by measurement of the rotational relaxation times of proteins in solution (18).

In this study, the theory of fluorescence depolarization is discussed and the application of the time resolved technique of fluorescence depolarization is carried out for the proteins α-chymotrypsin and lysozyme by attachment of fluorescent probes to these molecules. A study of the rotational relaxation characteristics of Rhodamine B in glycerol is also discussed.

Rotational relaxation times are calculated for these systems using approximations as to molecular shape and the results compared to previously reported values of these parameters for these molecules. Possible interactions and pertubations that may occur in each of these systems which prevent relationship of the observed rotational relaxation times to hydrodynamic properties of the systems are also discussed.

II. THEORY

Principle of Photoselection

The theory underlying fluorescence depolarization is based on the concept of photoselection (1). This concept states that if one induces an electronic transition in a subensenble of molecules oriented in a specific direction with respect to the rest of the molecules in the ensemble, an anisotropic situation is produced. This is accomplished by means of excitation using monochromatic linearly polarized light. It is a state where molecules which had the absorption dipole for a particular transition oriented in a specific direction at the time of excitation are the ones which have most probably undergone electronic excitation. This creates in the system a subensemble of electronically excited molecules which are of very similar orientation with respect to the laboratory fixed axes and the rest of the molecules in the system. Analysis of the emission spectra of such a system may be used to investigate several parameters which depend on the environmental and physical properties of the system. The parameters of most interest to this investigation are the molecular volume and rotational relaxation times of molecules of various sizes and shapes in different media. It is first necessary to see how this anisotropic situation arises and how the irreversible tendency for restoration of an isotropic system can be used to obtain the desired information.

The theory of transition intensities and polarizations is well known. (2) In terms of internal electron and nuclear coordinates and utilizing the zeroth order Born-Oppenhimer approximation, a molecular state function can be written as the product of an electronic wave function, Φ, and a vibrational wave function, χ. For the m^{th} vibrational level of the n^{th} electronic state, one may write;

$$\Psi_{nm} = \Phi_n(x,Q)\chi_m^n(Q)$$

where ψ_{nm} is the molecular state function and x and Q are the internal molecular coordinates necessary to locate all of the electrons on all of the atoms. Absorption of light quanta arises from the interaction of the electric vector of the light radiation and electrons. To a first approximation, the electric field perturbs electrons in the same manner as the interaction of the $\vec{E}$ vector of light with the electric dipole moment of the molecule, $\vec{R}$. For an electronic transition to the s^{th} vibrational level of the r^{th} electronic state (r being the ground state) we may write the transition moment expression as

$$M_{rs,nm} = \iint \Phi_n \chi_m^n \, m(x) \Phi_r \chi_s^r \, dx dQ$$

where the vector, m(x), represents the electronic terms of the electric dipole moment operator, $\vec{R}$.

One can define the electronic transition moment at Q as

$$\Lambda_{r,n}(Q) = \int \Phi_n m(x) \Phi_r dx$$

and the Q dependence of the electronic wave functions, using the Herzberg-Teller development yields;

$$\Phi_n(x,Q) = \Phi_n^0 + \sum_p \lambda_{np}(Q) \Phi_p^0$$

Now the n^{th} electronic function at Q is written as a sum of the complete set of electronic wave functions evaluated at the equilibrium nuclear configuration of the ground electronic state (Q=0). The sum goes over all states but n; and the mixing coefficients, $\lambda_{np}(Q)$, mix the zeroth order function, Φ_n^0, with the Φ_p^0 through a vibrational motion of the proper symmetry. For the ground electronic state one may approximate

$$\Phi_r(x,Q) = \Phi_r(x,0) = \Phi_r^0 \qquad (I)$$

Which reduces the transition moment to

$$M_{rs,nm} = \Lambda_{r,n}^0 \int \chi_m^n \chi_s^r \, dQ + {}_p\Lambda_{r,p}^0 \int \chi_m^n \lambda_{np} \chi_s^r \, dQ \qquad (II)$$

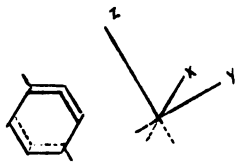
The first term in the equation (II) represents the pure electronic or allowed part of the transition moment. It represents a constant electronic transition moment modified by the Frank-Condon factor which involves the overlap of the combining vibrational wave functions. Since $M_{nm,rs}$ is the vector sense

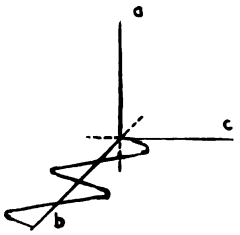
polarization of the nm→rs transition, it is determined by the sense of either term in equation (II), depending on their relative magnitudes.

When λ_{np} is non-totally symmetric, there are no interference effects between the components, i.e. vector addition of the transition moments of the components is allowed only if λ_{np} is totally symmetric or contains a totally symmetric component. If we therefore restrict the perturbing radiation to being polarized and monochromatic, the greatest probability for electronic transition will occur which gives a "photoselected" ensemble of molecules. These are molecules excited into energetically similar vibronic states having similarily oriented transition moments with respect to each other and the laboratory fixed axes.

Figure 1 illustrates the phenomenon of photoselection. This shows six molecules oriented in six principle directions to represent a random, iostropic sample. Illumination of this ensemble with monochromatic light, linearly polarized along the x axis and incident to the front face, will most probably result in the selective interaction of the exciting light with the electronic transition moment in each molecule which is oriented at the time of illumination to be coincident with the electric vector of the exciting radiation. The use of monochromatic radiation attempts to give the most probable excitation to similar vibronic states in all molecules which interact with the radiation. This results, upon relaxation, in emission corresponding to a particular electronic relaxation (3).

Figure 1. This illustrates the principle of photoselection. x, y, and z are the molecular fixed axes. a, b, and c are the laboratory fixed axes. Box A represents an isotropic distribution of molecules. If this is excited by monochromatic light of wavelength which results in absorption by the molecule along the z molecular axis and the exciting radiation is plane polarized with the electric vector parallel to the laboratory b axis, box I shows the molecules which will most probably be excited. If exciting radiation of similar polarization but with a wavelength necessary to excite the absorption dipole parallel to the y molecular axis, box II represents the molecules which are most likely to be excited.





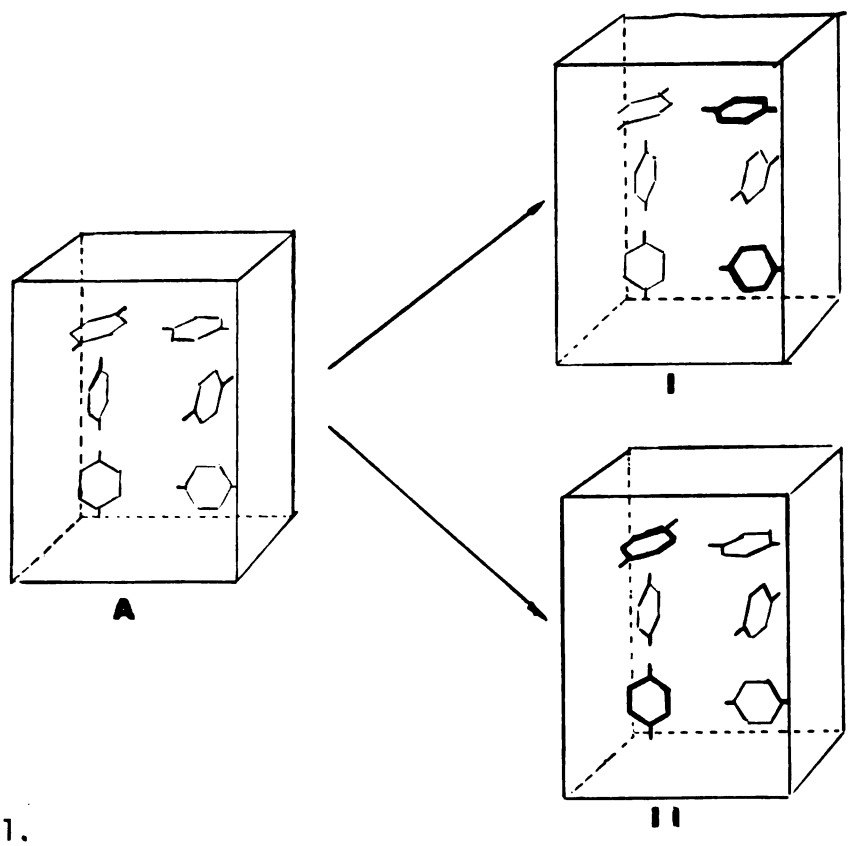


Figure 1.

Polarization of Fluorescence

The relationship of intrinsic polarization of transitions within a molecule to luminescence was established by Jablonski(4). The point of this theory is that by measuring the degree of polarization of fluorescence of a solution illuminated by a constant flux of plane polarized monochromatic light, physical information concerning the rotational freedom, hydrated volume, viscosity, and relative orientations of the absorbing and emitting dipoles of the molecule can be determined.

The polarization, p, of the emission under conditions of excitation by a constant source of plane polarized monochromatic light is defined as;

$$p = \frac{N_{\parallel} - N_{\perp}}{N_{\parallel} + N_{\perp}}$$

where $N_{\parallel}$ is the number of photons per unit time (intensity) emitted with their electric vector parallel to electric vector of the exciting radiation in the laboratory frame of reference, and $N_{\perp}$ is the number of photons per unit time with their electric vector perpendicular to the direction of the electric vector of the exciting radiation. These quantities are related to the transition moment vector of the emission dipole of the molecule by the equations;

$$N_{\parallel} = M^2 \cos^2\theta$$

and

$$N_{\perp} = M^2 \sin^2\phi \cos^2\theta$$

where M is the magnitude of the electronic transition vector in the molecular frame of reference which may be resolved into components projected in the direction of the electric vector of the exciting light and perpendicular to the direction of the electric vector of the exciting light. Theta, θ, is the angle between the directions of the transition moments of absorption and emission, assuming the absorption vector is coincident with the electric vector of the exciting radiation, and ϕ is the azimuthal angle the absorption vector makes with the direction of observation.

Theoretically, in the case of molecules in ordered polymers or single crystals in which the absorption and emission transition dipole moments of the fluorescent molecules are situated at some fixed angle β with respect to each other, the polarization may be described by the following equation;

$$p_0 = \frac{3\cos^2\beta - 1}{\cos^2\beta + 3}$$

The angle β becomes equivalent to θ when one uses plane polarized monochromatic radiation to give preferred excitation of molecules of specific orientation with respect to the laboratory fixed axes. In the case where molecules are randomly oriented crystals or in liquid media, the values of $N_{\parallel}$ and $N_{\perp}$ will be averaged

over all angles of θ and ϕ. For rigid, non-interacting molecules p may then vary between extrema of +1/2 and -1/3 for coincident and perpendicular absorption and emission transition dipole moments, respectively. The value of p at infinite viscosity is usually designated as p_0 corresponding to no molecular motion or interaction within the medium.

In solutions of low fluorochrome concentration and low solvent viscosity, molecular rotations can occur within the lifetime of the excited species (10^{-10} to 10^{-8} seconds). These rotations can lead to fluorescence depolarization,i.e. p changes with time and assumes values intermediate to the extrema depending on the speed of the molecular rotations relative to the decay time of the molecule's excited state. The speed of molecular rotations depends on the physical parameters of the environment (temperature, viscosity), the extent of solvation, and the particle volume. If the fluorchrome is present in large concentration, depolarization of the emission may also result from intermolecular energy transfer (trivial reabsorption and/or Forester transfer). By limiting the fluorochrome concentration to small values and using solvents of low viscosity, a study of the depolarization of fluorescence emission can be used to analyze the rotational motion of molecules in the solution. In the absence of intermolecular energy transfer, the steady state polarization is related to rotational diffusion phenomena according to Perrin's relationship (5,6);

$$p = \left[\left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{RT}{\eta}\left\{\frac{\tau}{V}\right\}\right) + \frac{1}{3}\right]^{-1}$$

This applies to a rigid, spherical molecule where R is the universal gas constant, T the Kelvin temperature, η is the solvent viscosity in poise, τ the excited state lifetime of the fluorochrome, and V is the solvated volume of the fluorescent particles in cubic centimeters per mole of solvated particles.

This relationship is generally used to construct normalized Perrin plots which are graphical representations of the expression:

$$\frac{U}{U_0} = \frac{\frac{1}{p} - \frac{1}{3}}{\frac{1}{p_0} - \frac{1}{3}} = 1 + \frac{RT\tau}{V\eta} = 1 + \frac{3\tau}{\rho_m}$$

where ρ_m is the mean rotational relaxation time of the molecules in the solution, p_o is the solution polarization at infinite viscosity, and p is the steady state polarization of the solution at the particluar temperature and viscosity conditions. Theoretically, plots of $\frac{U}{U_0}$ versus $\frac{T}{\eta}$ with knowledge of V could yield values of τ, the excited state lifetime of the excited molecules, or if τ is known, V and ρ may be determined. This method has been utilized in studies designed to determine rotational and binding properties of various fluorescent probe-protein systems (7,8).

Application of this technique to the study of protein size and micro-enviroments is limited because of several

variable quantities which may cause depolarization of the fluorescence emission at rates not indicative of the true physical state of the system (9). Among the most serious are;

1. A variation of the excited state lifetime, τ, with the temperature or with the composition of the medium which is varied to obtain different solvent viscosities.
2. Polydispersity (different states of aggregation of the molecule under investigation) resulting in a spectrum of relaxation times over the entire T/η range.
3. Appearance of new relaxation times as T/η is varied. This can result from dissociation into subunits, from additional rotations which arise as the temperature of the solution is changed, or from interactions produced by addition of substances to cause a variation in the solvent viscosity.

Steady state depolarization techniques are, therefore, of limited utility in systems where the dynamics of the mobility of the fluorochrome itself or with respect to the molecule under study are not well characterized.

Theory of Time Dependent Fluorescence Depolarization

Recent advances in technology have led to the development of instrumentation capable of recording the intensity of fluorescence emission of a molecule as a function of time in the nanosecond range (10). By use of polarizers, the direct observation of the time dependent intensity of emitted radiation which is plane polarized either parallel or perpendicular to the plane of polarization of the exciting radiation possible. Utilizing

the priciple of photoselection, one may then determine the fluorescence anisotropy of a system as a function of time. This is defined as;

$$A(t) = I_{||}(t) - I_{\perp}(t) \;/\; I_{||}(t) + 2I_{\perp}(t)$$

where A(t) is the time dependent fluorescence anisotropy of the system, $I_{||}(t)$ is the intensity of radiation emitted parallel, and $I_{\perp}(t)$ the intensity of radiation emitted perpendicular to direction of the polarization of the exciting radiation (4).

We may consider the decay of fluorescence anisotropy to be an indicator of the rotational properties of the fluorescent species (assuming depolarization due to energy transfer is not present). This arises from considering the decay of A(t) in the following manner. If the molecules were rigidly held in the solvent medium and no rotation occured between the time of excitation and emission, all emitted radiation would be polarized parallel to exciting radiation (or at some fixed angle, θ, depending on the angle between the absorption and emission dipoles.) Since no rotation can occur, no depolarization will be observed. If the molecules are rotating very fast compared to the lifetime of the excited state, then the probability of emission polarized perpendicular to the excitation plane of polarization is comparable to the emission of parallel polarized radiation, and the solution will exhibit isotropic emission characteristics. If the rotational relaxation time is of the same order of magnitude

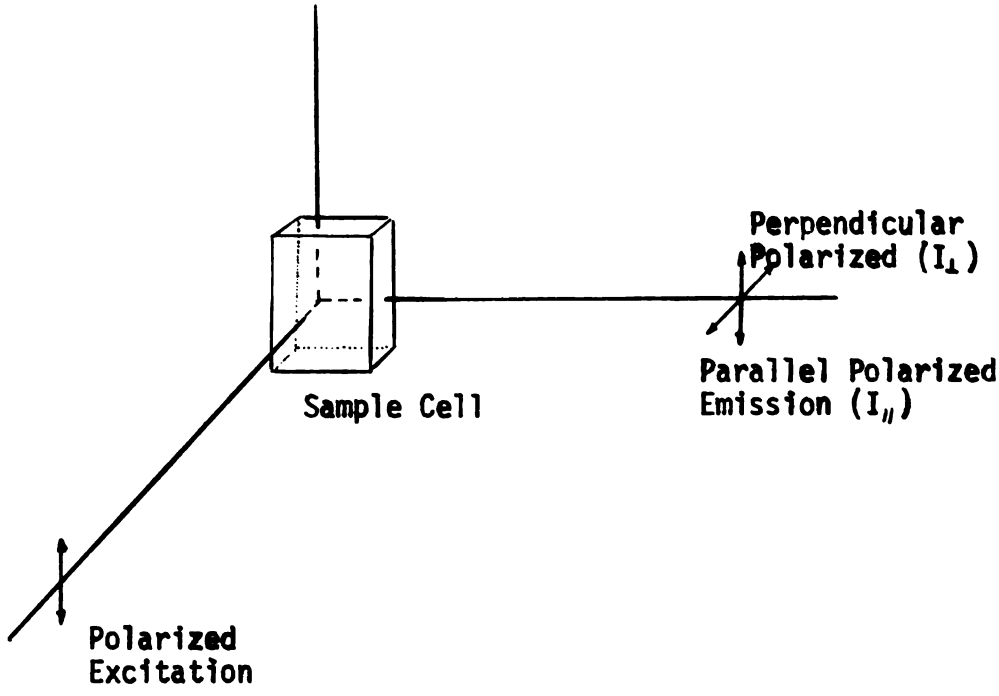


Figure 11. Diagram showing orientation of parallel and perpendicularly polarized emission with respect to plane of polarization of exciting radiation in the laboratory coordinate system.

as the excited state lifetime, then the emission polarized parallel and the emission polarized perpendicular to the plane of polarization of incident (exciting) radiation will exhibit time dependent intensities which are a function of the parameters that determine the rotational relaxation time of a molecule in solution. As time passes, these intensities which are initially non-equivalent will become equivalent at a rate dependent upon the rotational properties of the excited molecular species. Figure 2 shows this convergence for a typical time resolved depolarization experiment. The parameters which describe the rotation of particles in solution can be related by means of theoretical applications to the decay of the fluorescence anisotropy and , therefore characterized for the system under study.

Several derivations (11,12,13,14,15) of the theoretical decay of the fluorescence anisotropy have been published. These treat various 'special cases' of anisotropic decay. The most general treatment is given by Chuang and Eisenthal (16), and the results are outlined here.

Chuang and Eisenthal arrive at the following expressions for the time dependent change in the parallel and perpendicularly polarized intensities of emission;

$$
\begin{aligned}
I_{\parallel}(t) = P(t)\Big[&\frac{1}{9} + \frac{4}{15} q_x q_y \gamma_x \gamma_y \exp[-3(D_z + D)t] \\
&+ \frac{4}{15} q_y q_y \gamma_y \gamma_z \exp[-3(D_x + D)t] \\
&+ \frac{4}{15} q_z q_x \gamma_z \gamma_x \exp[-3(D_y + D)t] \\
&+ \frac{1}{15} (\beta + \alpha)\exp[-(6D + 2\Delta)t] \\
&+ \frac{1}{15} (\beta - \alpha)\exp[-(6D - 2\Delta)t]\Big]
\end{aligned}
$$

Figure 2. This is a photograph showing the parallel and perpendicular polarized emission intensities of lysozyme-dansyl chloride system. The lower trace is the perpendicularly polarized component. The vertical axis is relative intensity. The horizonal axis is time, spanning 0 to 100 nanoseconds.

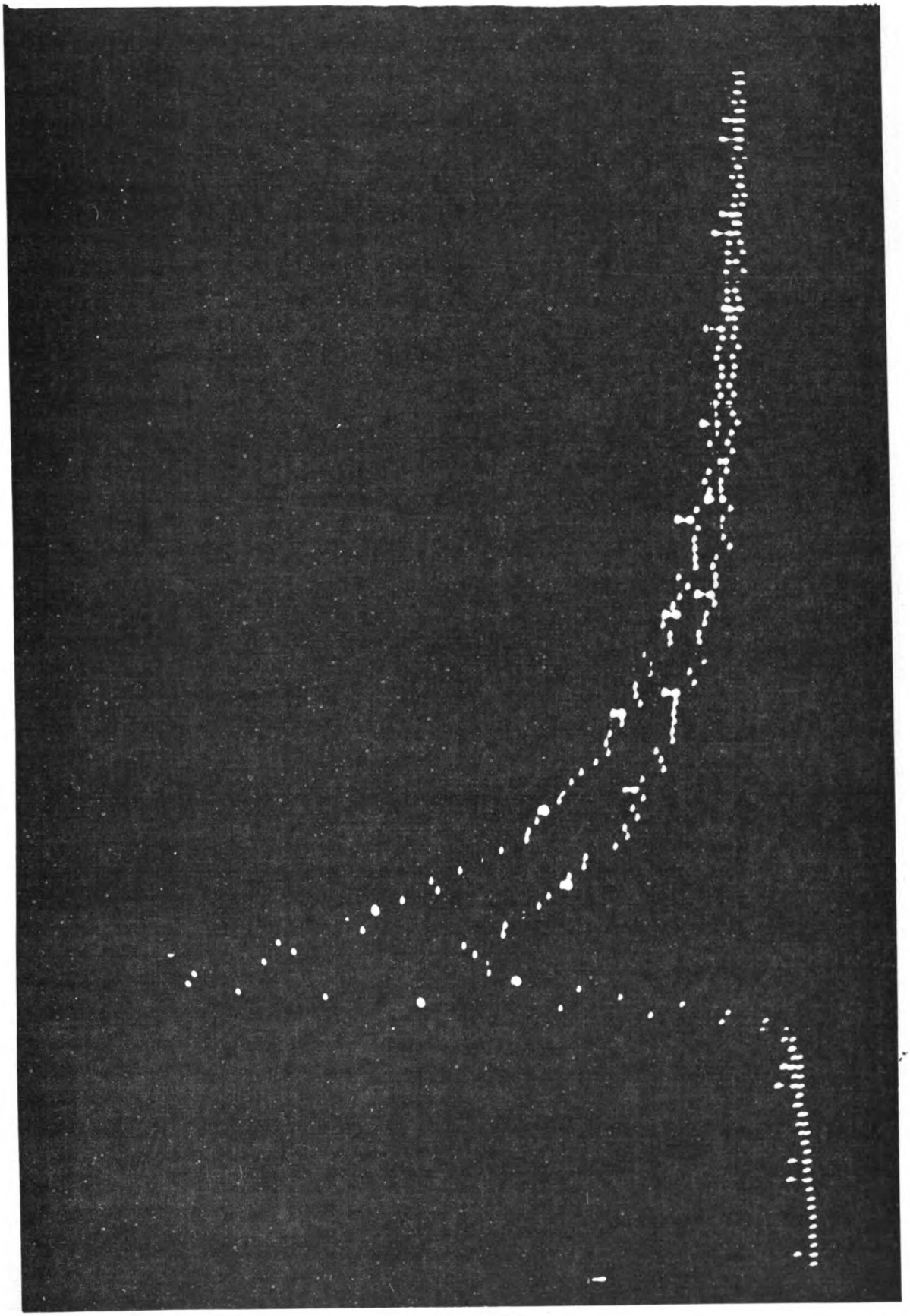


Figure 2.

and $$I_{\perp}(t) = \frac{1}{6} P(t) - \frac{1}{2} I_{\parallel}(t)$$

where $$\beta = q_x^2\gamma_x^2 + q_y^2\gamma_y^2 + q_z^2\gamma_z^2 - \frac{1}{3}$$

and $$\begin{aligned}\alpha = {} & (D_x/\Delta)(q_y^2\gamma_y^2 + q_z^2\gamma_z^2 - 2q_x^2\gamma_x^2 + \gamma_x^2 + q_x^2) \\ & + (D_y/\Delta)(q_z^2\gamma_z^2 + q_x^2\gamma_x^2 - 2q_y^2\gamma_y^2 + \gamma_y^2 + q_y^2) \\ & + (D_z/\Delta)(q_x^2\gamma_x^2 + q_y^2\gamma_y^2 - 2q_z^2\gamma_z^2 + \gamma_z^2 + q_z^2) - (2D/\Delta)\end{aligned}$$

with $$D = \frac{1}{3}(D_x + D_y + D_z)$$

and $$\Delta = (D_x^2 + D_y^2 + D_z^2 - D_xD_y - D_yD_z - D_zD_x)^{1/2}$$

The γ's are the projections of the unit absorption dipole vectors along the molecular axes, q's are the projections of the unit emission vectors along the molecular axes, and D_n is the rotational diffusion coefficient about the n'th molecular axis.

These most general expressions which apply to a completely asymmetric rotating particle yield a time dependent fluorescence anisotropy decay function of at least five exponentials (see Appendix A). Usually, the particle undergoing Brownian rotation is approximated to have higher symmetry. The two most useful approximations are that the particle has either spherical or elliptical symmetry.

In the case of spherical symmetry, the fluorescence anisotropy decay will reduce to a single exponential decay (6) of the form;

$$A(t) = A_0 e^{-3t/\rho}$$

where A_0 is the anisotropy if no rotation occured (the anisotropy at time zero), and ρ is the rotational relaxation time of the individual fluorescent molecules in the system. For a large solute-solvent particle size ratio where the solvent particles do not interact strongly at the solvated particle solvent boundary, hydrodymanic variables (17) can be used to relate the rotational relaxation time to experimental observables;

$$\rho = \frac{3kT}{V\eta}$$

where k is Boltzmann's constant, T is the Kelvin temperature, V is the hydrated volume of the solute molecules in cubic centimeters per molecule, and η is the viscosity of the solution in poise. This expression was used by Wahl and Timasheff (18) for studying the various states of association of β-Lactoglobulin-A. The anisotropy decay curves were characteristic of the monomeric, dimeric, and octameric species, as would be expected due to the changing volume of the rotating perticles.

For particles of approximately ellipsoidal symmetry in solution, the general expressions of Chuang and Eisenthal (III) reduce to the sum of three weighted exponentials of the form;

$$A(t) = A_0 \sum_{i=1}^{3} f_i \exp[-3t/\rho_i] = A_0 \sum_{i=1}^{3} f_i \exp[-t/\phi_i]$$

where ϕ is the rotational correlation time ($\phi = 3/\rho$) and the f_i are weighting coefficients which are a function of θ, the

angle between the emission transition moment and the major axis of the ellipsoid. The f_i's are given by the expressions;

$$f_1 = (3/2 \cos^2\theta - 1/2)^2$$
$$f_2 = 3 \cos^2\theta \sin^2\theta$$
$$f_3 = 3/4 \sin^4\theta$$

The rotational correlation times, ϕ_i, can be related to the diffusion coefficients about the major and minor axes of the ellipsoid ($D_{//}$ and $D_{\perp}$ respectively) by the expressions;

$$\phi_1 = (6D_{\perp})^{-1}$$
$$\phi_2 = (5D_{\perp} + D_{//})^{-1}$$
$$\phi_3 = (2D_{\perp} + 4D_{//})^{-1}$$

where $D_{//}$ and $D_{\perp}$ are related to ξ, the axial ratio of major to minor axis for a prolate ellipsoid, and to D, the rotational diffusion coefficient of a sphere with a volume equal to that of the ellipsoid, by the expressions;

$$D_{\perp}/D = \frac{3}{2} \frac{\xi[(2\xi^2 - 1)\beta - \xi]}{(\xi^4 - 1)}$$

$$D_{//}/D = \frac{3}{2} \frac{\xi(\xi - \beta)}{\xi^2 - 1}$$

where $$\beta = \frac{1}{\sqrt{\xi^2 - 1}} \log_e (\xi + \sqrt{\xi^2 - 1})$$

The expression for the anisotropic decay of an ellipsoid for random

but rigid arrangement of electronic transition dipoles of the fluorochrome becomes;

$$A(t) = \frac{A_0}{5} e^{-3t/\rho_1} + 2e^{-3t/\rho_2} + 2e^{-3t/\rho_3} \qquad (V)$$

where the ρ_i are the rotational relaxation times about the axes of the ellipsoid under study. This could in principle be used to fit graphs of $\log_e A(t)$ versus t for various values of ρ_i if the graph shows non-linear decay, (linear decay indicating spherical symmetry). Because the decay of the fluorescence anisotropy is usually only analyzed over a few decades of time (typically 1 to 50 nanoseconds), and the specific state of attachment is usually unclear, the ρ_i cannot usually be uniquely determined by equation V. The time dependent anisotropy decay function for an ellipsoid assuming rigid and non-random attachment can be expressed;

$$A(t)/A_0 = \sum_{i=1}^{3} f_i \exp[-t/\rho)\Omega_i]$$

where $\Omega_1 = D_\perp/D$

$\Omega_2 = (5D_\perp + D_{//})/6D$

$\Omega_3 = (2D_\perp + 4D_{//})/6D$

A plot of $\log_e A(t)/A_0$ versus t is now dependent on the axial ratio, and θ, but not V. Under these conditions one must also curve fit for two parameter fitting over a small range of data. In these cases where the volume,V, of the solvated species must

be obtained by other methods, one may calculate D, $D_{//}$, and $D_{\perp}$ for assumed values of axial ratios. The observed data can then be compared to these results to obtain optimum correlation or to results calculated assuming no prior knowledge of the volume of the solvated species. This method has been employed by Yguerabide et.al. (19) to study segmental flexability of antibodies of IgG molecules labeled with ε-dansyl-L-lysine at the active of the anti-dansyl antibody.

In the present study, values of V were obtainable from literature sources (obtained by methods other than time resolved fluorescence depolarization) for two of the systems studied, the protein systems. This allowed calculation of D, $D_{//}$ and $D_{\perp}$ for various axial ratios of the particle thought to have elliptical symmetry in solution (the lysozyme molecules). Analysis of the fluorescence anisotropy decay by means of curve fitting then allowed comparison of experimentally determined values of the solvated volume and rotational relaxation time with values calculated from the known data.

III. FLUORESCENT PROBES

The study of depolarization decay using fluorescent probes extends the utility of this technique to molecules that have a low quantum yield which makes detection of fluorescence difficult, to molecules which possess chromophores having unknown or unfixed orientations with respect to the entire molecule or to other chromophores within the molecule, and to molecules which are non-fluorescent. There are three broad classes of probes, intrinsic, extrinsic, and co-enzymatic.

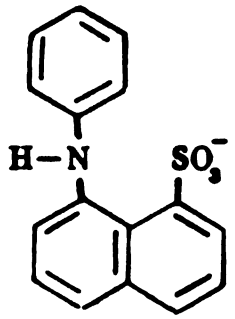
Intrinsic probes are the aromatic side chains of phenylalanine tyrosine, and tryptophan residues within the polypeptide chain which are fluorescent in the uv spectrum. A few proteins also contain a fluorescent co-enzyme such as reduced nicotinamide-adenine dinucleotide, flavin-adenine dinucleotide, or pyridoxyl-phosphate (20). In many cases, the residue or co-enzyme is attached at a site in the protein which may be attached within the molecule in such a manner as to not reflect the true rotational behavior of the entire molecule. There may also be several residues arranged such that energy transfer between them is possible, causing depolarization of the emission by non-rotational interactions. The presence of several residues of unknown relative orientations and locations within the protein may also, in the case of non-spherical molecules, exhibit fluorescence anisotropy decay characteristic of many rotational relaxation times, depending on the particular residue on each molecule

which is excited. The use of coenzymatic probes may also suffer from the possibility that enzyme-coenzyme interaction may induce conformational changes of the protein molecule under study.

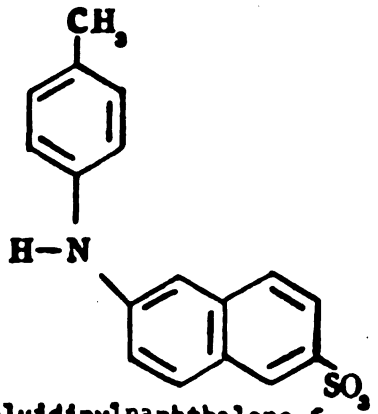
Weber (21) introduced the concept of attaching an extrinsic fluorescent probe to the molecule of interest. These extrinsic probes are widely used in the study of biological processes and properties of specific molecules (22-30). It is used here to study the rotational motions of proteins in solution. Table 1 contains the structure and nomenclature of four commonly used extrinsic fluorescent probes. These probes are attached to the molecule under study either by hydrophobic interaction, covalent attachment, or a combination of these mechanisms. For the study of protein molecules, attachment of a fluorescent probe is usually necessary.

Hydrophobic interactions arise from the fact that the non-polar side chains of various residues (especially valine, leucine, isoleucine, phenylalanine, proline, alanine, or tryptophan which comprise 35-45% of the residues of many proteins) may be brought into contact with each other through the folding of the polypeptide chain of the protein molecule. This close association creates micellular type regions which tend to exclude water and other highly polar substances. The tendency of non-polar side groups of protein residues to associate with the exclusion of polar substances is known as hydrophobic binding. The causes and effects of this type of association have been treated by several authors (31, 32).

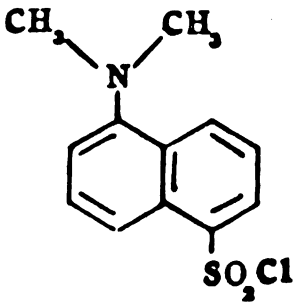
In 1954 Weber and Laurence discovered a class of fluorescent compounds which are practically non-fluorescent in polar media but



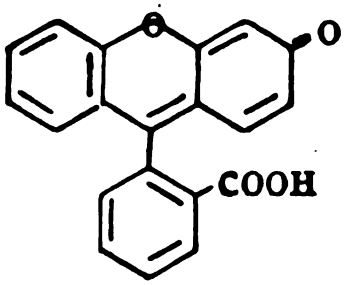
1-Anilino-8-naphthalene sulfonate (ANS)



2-p-Toluidinylnaphthalene-6-sulfonate (TNS)



1-dimethylaminonaphthalene 8-sulfonyl chloride (dansyl chloride)



Fluorescein

Figure 3. Commonly used Fluorescent Probes.

give strong emission in non-polar environments (22). These are especially useful in labeling proteins which contain hydrophobic binding sites. This effect is generally interpreted in terms of dipole-dipole interactions between the excited state of the probe molecule and solvent molecules (20). Fluorescent molecules which have a higher dipole moment in the excited state can, upon excitation in a polar medium, cause a realignment of the solvent molecules around the chromophore. A photon of lower energy is emitted by the hydrophobic probe in a polar medium since some of the solvation energy of the excited state is lost when the chromophore returns to the ground state. Consequently, the emission maximum is red shifted. The reason for a lower quantum yield in polar environments is not well understood. Camerman and Jensen (33) have attempted to interpret the changes in emission properties of the dye 2-p-toluidinyl-6-naphthalene sulfonate (TNS) in terms of geometric probe-solvent interactions which affect the ability for resonance delocalization throughout the molecule. The geometry of the protein binding site to which this type of probe is attracted may then be a factor in determining the characteristics of the associated probe.

A second type of extrinsic probe is one that chemically reacts with the protein molecule to attach itself to one of the residues or side chains of the protein's constituent amino acids. Fluorescein isothiocyanate and 1,1N-dimethylaminonaphthalene sulfonylchloride (dansyl chloride) are two of the most common probes of this type.

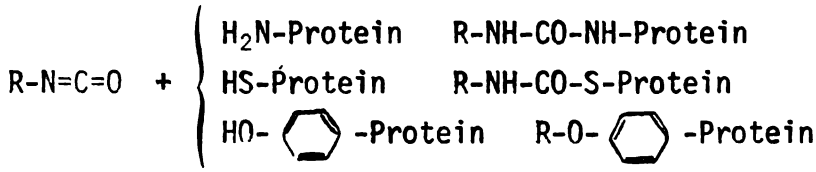
Fluorescein as the isocyanate or isothiocyanate derivative can react with the phenolic side chains of tyrosine, the sulfhydryl

group of cysteine, or the ε-amino group of lysine. Dansyl chloride can react with the phenolic groups of tyrosine, the sulfhdryl group of cysteine, or the amino groups of lysine (See Figure 4). The attachment of more than one probe molecule to the peptide chain is then possible if the chain contains several residues of the same type which react with the probe functional group, or if the chain contains a number of different residues which react with the probe. Selectivity in the degree of labeling of the protein may be accomplished by two methods; the control of the reaction conditions during the labeling reaction, and the fractionation of the resulting solution by methods which separate species by molecular weight (gel column and liquid chromatography) or total molecular charge differences (electrophoresis).

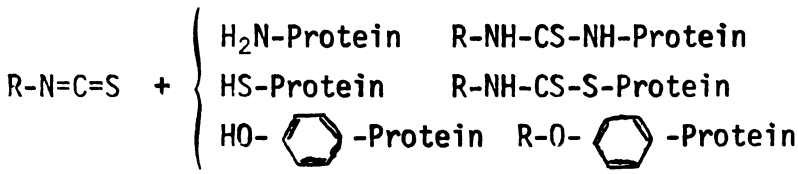
The first method is useful when labeling with dansyl chloride due to the different pK_a values of the side chains of the various residues, Table 1. Gros and Labouesse (34) studied the reaction of dansyl chloride with various amino acids and proteins. They found that above pH 9.5 to 10.0 the base catalyzed hydrolysis of dansyl chloride is very rapid, while below this range it is slower and remains essentially constant. Labeling of a protein such as lysozyme should be done at a pH which corresponds to low rates of hydrolysis of dansyl chloride and which is close to the pK_a value of the desired residue.

The second method of differentiation is based on the physical properties of the probe-protein molecules. Gel-column chromatography allows separation on the basis of different molecular weights. Gel electrophoresis allows differentiation on the basis of total

Isocyanates:



Isothiocyanates:



Arylsulfonyl Chlorides:

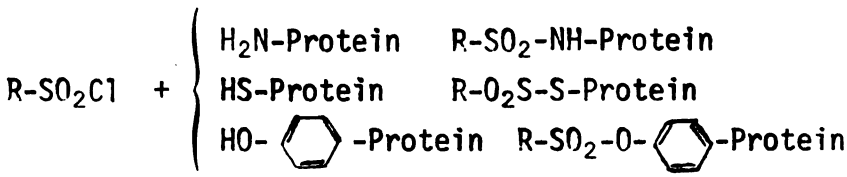


Figure 4. Labelling reactions of common probe functional groups with reactive protein residues.

Table 1. Values for Protonation Reactions of Various Amino Acids and Proteins.

Reactive Group	Apparent pK_a-protein
α-NH_2 (N-terminal of polypeptide chain)	8
ε-NH_2 (lysine)	10.5
Phenyl-OH (tyrosine)	11.9
Imidazole	7

electrical charge on the molecule. Both of these quantities will be a function of the degree of labeling of the protein molecule. (These are also a function of the pH, ionic strength of the solution, and temperature, factors which affect the state of aggregation of proteins) (35). Once separation has been effected, the average number of dye molecules bound per mole of protein can be determined experimentally using spectroscopic techniques. If v represents the average number of dye molecules bound per molecule of protein, the relationship (38)

$$v = \frac{A_1/\varepsilon_1 \times M.W. \times E_2^{1\%}}{10\,[A_2-(A_1 \times (\varepsilon_2/\varepsilon_1))]}$$

is used in determining the average labeling ratio of probe to protein molecules. Subscripts 1 and 2 represent the wavelengths of maximum absorption of bound dye and the protein respectively. A is the absorbance of the solution in question, ε is the molar extinction coefficient of bound dye, $E^{1\%}$ is the absorbance of a 1% solution of the protein (at 1 cm path length), and the assumption is made that the protein absorption is negligible at the absorption maximum of the bound dye. Several workers have used this method to investigate the degree of labeling protein molecules (36,37).

IV. EXPERIMENTAL

Reagents

The fluorescence depolarization of α-chymotrypsin was studied using the following solution: α-chymotrypsin from bovine pancrease which was salt free, 3x crystallized and lyophilized obtained from Sigma Chemical Co. , lot #15-C8150 was dissolved at a concentration of 10 mg/ml (38) in phosphate buffer of pH=7.8 made by mixing 8.6 ml of 1/15 molar KH_2PO_4 (monopotassium phosphate) with 91.4 ml of 1/15 molar disodium phosphate, Na_2HPO_4. This was labelled with 8-anilino-1-naphthalene sulfonate, ammonium salt from Sigma Chemical, lot #28B-0870 (ANS) by addition of the solid to the protein solution. Emission spectra of the resulting solution confirmed the binding of the ANS to the protein. Eight milligrams of ANS per gram of protein were used giving approximate concentrations of 1.3×10^{-4} M in ANS and 2.3×10^{-4} M in α-chymotrypsin. This ratio favors a binding ratio of one to one for probe protein molecules. Extensive characterization of the probe-protein ratio by chromatographic or spectroscopic methods was not permitted due to auto lysis (39-41) possibilities. All measurements were determined within two hours of initial mixing. The assumption was initially made that molecules which were lysed released ANS rendering the lysed protein incapable of contribution to the polarized fluorescence intensities. Because lysing seemed evident in some

cases, experiments were designed to study α-chymotrypsin in the absence of lysing. Solutions which were 0.3 M in Ca^{++} ($CaCl_2$) were mixed with α-chymotrypsin-ANS in 0.1 M TRIS [tris-(hydroxymethyl) aminomethane and HCl] buffer solution at pH=8.0. The resulting solution was 0.1 M in calcium ion. Calcium ion is known to prevent autolysis of α-chymotrypsin (39). The resulting solution was cloudy due to association of α-chymotrypsin at the relatively large ionic strength of the mixture. The fluorescence intensity of the solution was also reduced to low levels. Reduction of buffer concentrations resulted in insufficient capacity to maintain basic enough pH to prevent association of the α-chymotrypsin due to the acidity of the solution. Results from these experiments are not included in the Results section.

Since complexed α-chymotrypsin is known not to associate or to lyse, tosyl-α-chymotrypsin was dissolved at 10 mg/ml in pH=4.2 acetate buffer. The resulting solution was cloudy due to unknown factors. Dilution of the mixture to approximately one-tenth the original concentration gave a clear solution. The fluorescence intensity of bound ANS was enhanced due to the low pH of the solution (42). Results for this compound are included in the following section.

Lysozyme from chicken egg white purchased from Worthington Biochemical, salt free, 2x crystallized was dissolved in pH=7.8 phosphate buffer at a concentration of 20 mg/ml. Eight-tenths of a milligram of 5-dimethylamino-1-naphthalene sulfonyl chloride purchased from Sigma Chemical was dissolved in approximately 0.5 ml

of acetone (analytical reagent grade) and mixed with the lysozyme solution.(43) This was allowed to react for one hour. The mixture was then eluted on a column (45 cm length by 1 cm diameter) packed with Sephadex G-100 gel. The gel was swelled for six hours in boiling buffer (phosphate, pH=7.8). The dead volume was 11.5 ml as determined by elution of blue dextran through the column. Several volumes of eluant (phosphate buffer) were passed through the column, and a solution containg 5 mg/ml of ovaalbumin, 2x crystallized from Worthington Biochemical in phosphate buffer was eluted through the column to remove any traces of blue dextran remaining on the column. This was followed by passing several volumes of eluant through the column. The dansyl chloride-lysozyme solution was then eluted through the column at a flow rate of 0.25 ml/minute. Twelve fractions of approximately 4 ml each were collected, fraction 3 containing eluant which was directly following the dead volume. Fluorescence of this solution was very weak indicating very little labelled protein present. Fractions 4 through 8 showed fluorescence intensity indicative of the presence of labelled lysozyme. Absorption spectra were taken on these solution using a Cary Model 17 spectrophotometer against a blank consisting of the buffer solution. The spectrum of fraction 4 is shown in Figure 5. The labelling ratio of probe per protein as determined by the method described by Dandliker and Portmann was 0.38 molecules of probe molecules per molecule of protein using the molar absorbtivity of dansyl chloride at 280 nm to be 6670 cm^{-1} M^{-1}. This was determined by

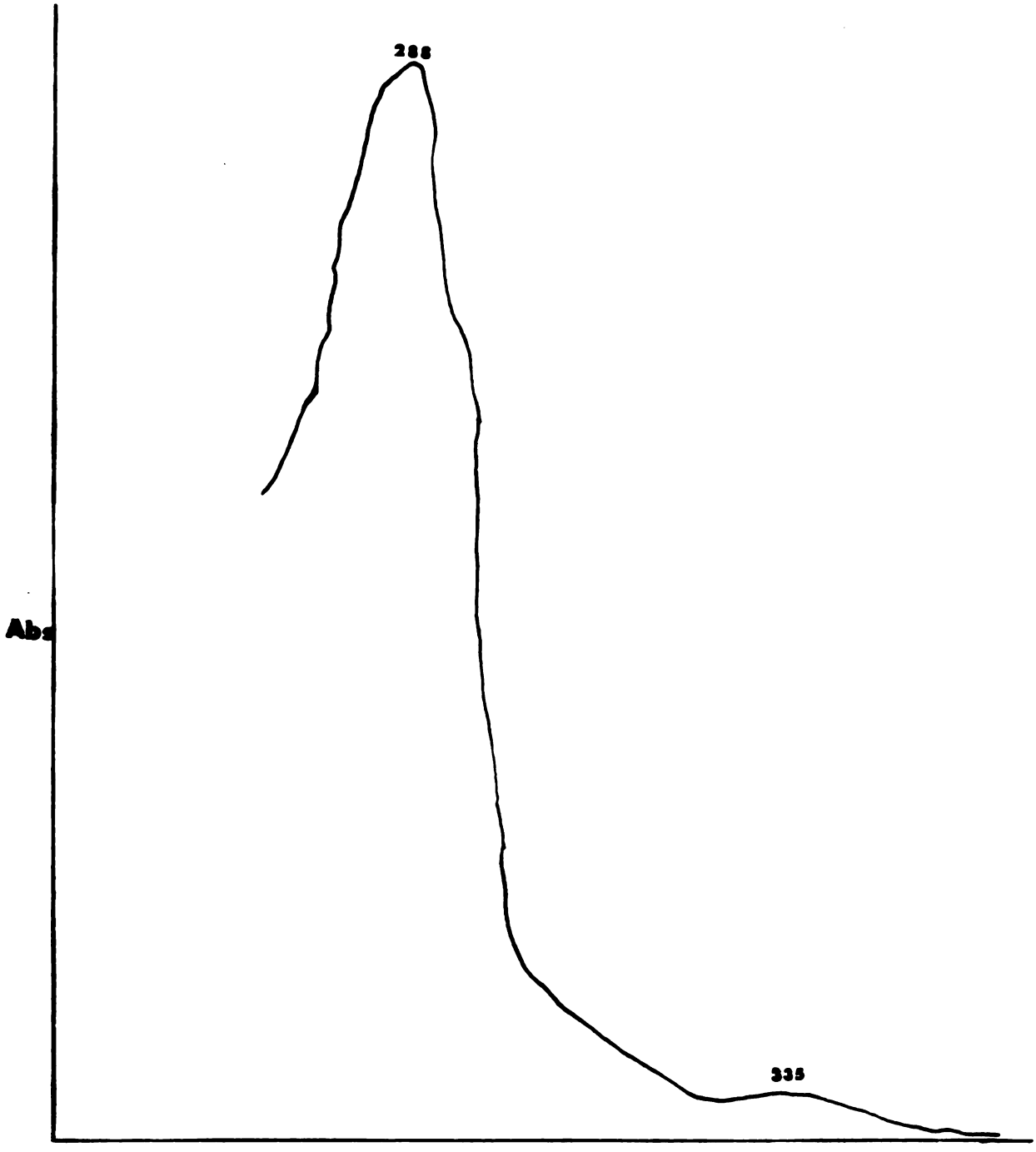


Figure 5. Absorption spectrum of lysozyme labelled with dansyl chloride at pH=7.8. The peak at 288 nm is protein absorption. The peak at 335 nm is bound dansyl absorption. Absorbance scale is normalized to one.

absorption experiments using 0.043 M dansyl chloride in 96% ethyl alcohol as a solvent and in spectrograde cyclohexane. This compares with a ratio of 0.39 probe/protein obtained using the molar absorbtivity of DAN at 280 nm to be 1250 cm^{-1} M^{-1} as calculated from the published (34) absorption spectra of dansyl-L-glycene. The above procedure was repeated for the experiments performed using lysozyme initially labelled at pH= 5.1. At this pH the labelling reaction was allowed to proceed for three hours.

A solution of 1.0 x 10^{-5} M Rhodamine B in spectrograde glycerol was used for the Rhodamine B depolarization experiments.

Depolarization Experiments.

The time dependent polarized intensity measurements were taken on an instrument similar to that developed and described by Ware (10). It is shown in Figure 6. This consists of a gated nanosecond pulse lamp, Model 501A of Photochemical Research Associates, Ontario, run at one half atmosphere pressure of deuterium(D_2) gas and 4.2 KV giving a flash frequency of approximately 30 KHz and a pulse width at half-height of about 2-3 nsec. The flash is detected by an RCA 1P28 phototube. The output of the phototube is delayed 124 nsec by cable and passed through an ORTEC Model 436 one hundred megaherz discriminator and is then used to gate the 'start' of the ORTEC model 457 Time to Pulse Height Converter. Corning Glass Filters and/or Wratten Diffraction Gratings were used to select radiation of appropriate frequency for excitation. The sample cell was

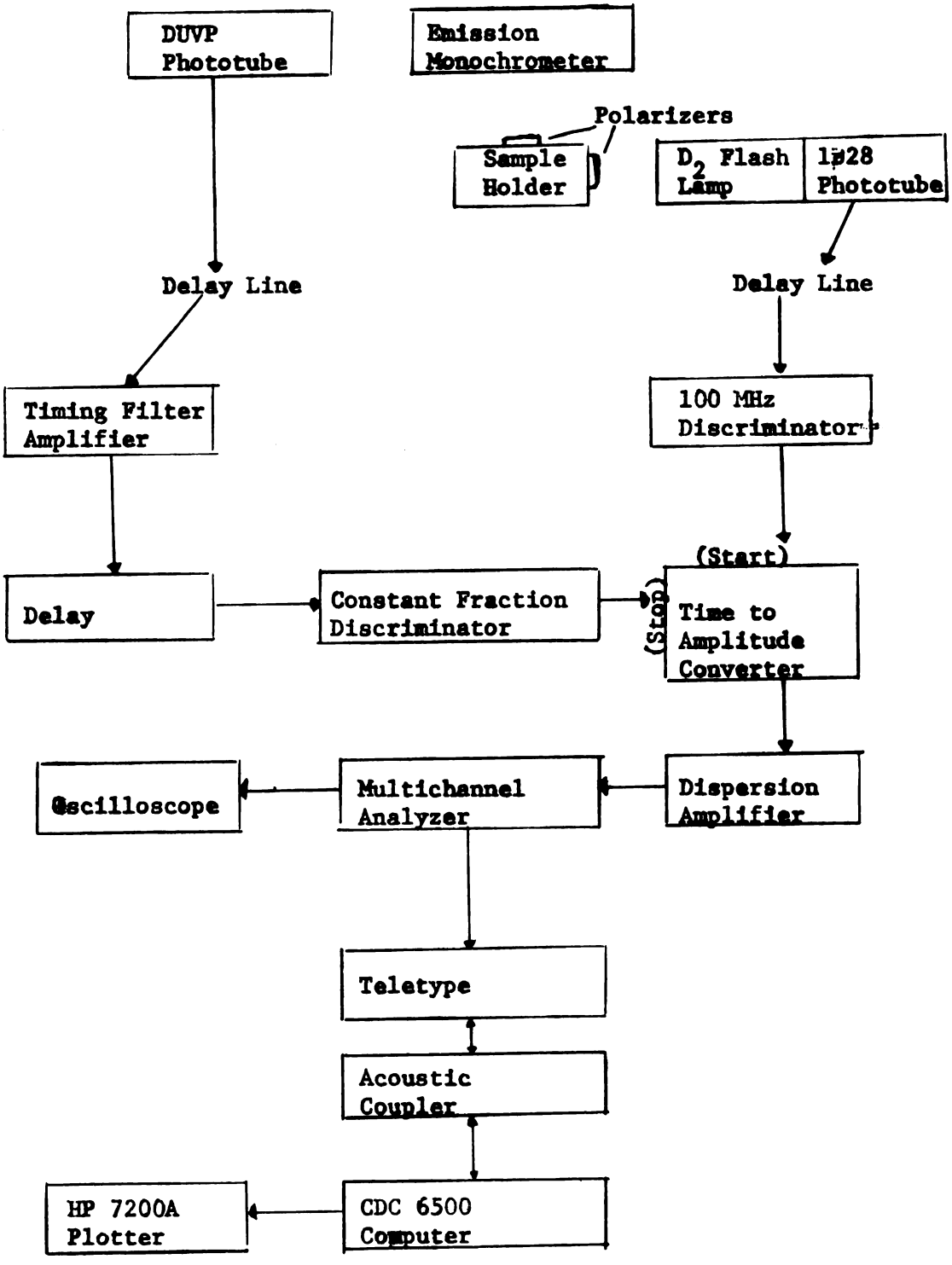


Figure 6. Diagram of the Time Resolved Spectroscopy Instrument.

an Aminco-Bowman with a temperature jacket to allow connection to a constant temperature control unit. Emission is viewed at 90^{o} to the excitation. A Bausch-Lomb Monochrometer served as the emission monochrometer. An Amperex 56DUVP/03 Photomultiplier tube served as the detector of fluorescence emission. The output pulse is passed through a 124 nsec delay cable and into an Ortec Model 454 Timing Filter Amplifier. The filtered signal is then passed through an ORTEC Delay Unit Model 425 giveing the pulse a constant 14 nsec delay before passing through an ORTEC Model 463 Constant Fraction Discriminator. The signal then gates the 'stop; input of the time to pulse height converter. The resulting time interval between the 'start' and 'stop' gating pulses is converted into an analog signal which is passed through a dispersion amplifier contained in the ORTEC Model 462 Time Calibration Unit, converted to a digital value by a Nuclear Data Systems Model ND560 Analog to Digital Converter, and is stored in a Nuclear Data Systems Model 1100 Multichannel Analyzer operated in the pulse height analysis mode. A count corresponding to some period of elapsed time is then stored in the appropriate channel of the multichannel analyzer corresponding to the time interval between the 'start' and 'stop' pulses. All components are mounted in an ORTEC Model 401A BIN power module.

Polarization of the exciting and emitted radiations was achieved by use of Polaroid Type HNB'B uv polarizing film which was mounted on polarizer mounts and fixed directly over the entrance and exit ports of the cell holder, or by use of Glan-

Thompson type UGI-G55 UV Prism Polarizers. Temperature was controlled by use of Kahlsico Type U35 temperature control unit.

Viscosity determinations were made at constant temperatures ($\pm 0.2\ C^{o}$) using a Zeitfuchs Cross Arm Visciometer, size 2 by the method described in Moore (44). The viscosity of the protein solution was changed by adding various amounts of 30% sucrose (recrystallized from water-ethanol solution) solution.

Data Treatment

The intensity as a function of time data obtained for each polarization was normalized, the background was subtracted and the anisotropy as a function of time calculated empirically using computer programs supplied by W. Ware and modified to be compatable with the MSU CDC 6500 system. The data were plotted via Hewett-Packard Model 7200-A plotter interfaced with the computer. These plots represented the data as Log_e (anisotropy) vs. time. Rotational relaxation times and weighting coefficients corresponding to various model molecular shapes were obtained using the KINFIT curve fitting program developed by J. Dye and V. Nicely. (48)

The curve fitting program allows great latitude in calculation of the anisotropy at time zero, A_0, and rotational relaxation times, ρ_i, parameters from experimental data. For spherical particles, the expression relating rotational relaxation time to fluorescence anisotropy decay is;

$$A(t) = A_0 \times \exp(-3t/\rho)$$

The experimentally determined values of A(t) as a function of time over the period of interest are supplied in required form and fitted by least squares procedure to the above equation. If a sufficient number of data points are supplied, the program can find unique values of A_0 and ρ_i. The uniqueness of the values determined by KINFIT can be tested by comparing them to values obtained by linear regression analysis using a Texas Instruments SR-51 calculator, with the equation in the

form;

$$\log_e A(t) = \log_e A_0 + \exp(-3t/\rho)$$

The values may also be checked by entering different initial estimates of the parameters being determined by KINFIT and observing convergence to the same values obtained with the first set of initial estimates (48). Curve fits resulting in similar A_0 and ρ values using these different methods are an indication of the accuracy of the calculated value based on the data points supplied.

In the case of several exponential decays, one has the difficulty of determining the weighting coefficients, f_i, as well as the A_0 parameter and the ρ_i for rotation about the various molecular axes. In the case of the ellipsoidal model with non-random and rigid attachment of probe molecules, the decay of the fluorescence anisotropy as a function of time is given by the relationship;

$$A(t) = A_0 \sum_{i=1}^{3} f_i \exp(-3t/\rho_i)$$

where the f_i are the weighting coefficients for the exponential factors in the expression, and the ρ_i are the rotational relaxation times about the various axes. As seen in the theoretical discussion, the f_i are functions of θ, the angle between the absorption and emission dipoles of the fluorescent molecule. In time resolved determination of fluorescence dpeolarization, one need not consider the case of freely rotating probe molecules (with respect to the protein molecule) unless it is known the rotational relaxation time of the protein of some portion

of the protein in or on which the probe molecule may be attached may be very short. This is possible since the presence of freely rotating probe molecules will give a very fast component of the fluorescence anisotropy decay or , in the case of probes which have relatively long lifetimes, their emission would be completely depolarized and thus not contribute in the anisotropic condition produced over the time range of interest.

The curve fitting to the sum of two weighted exponentials can be somewhat simplified by inspection of the f_i as θ varies from zero to ninety degrees. Figure 7 shows this dependence. It is seen that only in the ranges of $35^\circ < \theta < 45^\circ$ and $70^\circ < \theta < 75^\circ$ do the values of all three weighting coefficients assume magnitudes of approximately equal value. For the remainder of the range (zero to ninety degrees) one or two of the f_i are relatively small such that only two or one rotational relaxation components is obtained by fluorescence polarization technique. This is true provided that the relative values of the ρ's are of similar magnitude, i.e. for ellipses with small axial ratios. Consequently, unless the system under study is completely uncharacterized as to the possible site and strength of probe attachment and the probes fluorescent properties, some approximation of the relative importance of the contributions of each component can be made and the method of curve fitting adjusted accordingly.

Hydrated volumes for the protein molecules were calculated using the Stokes-Einstein relationship for spherical particles

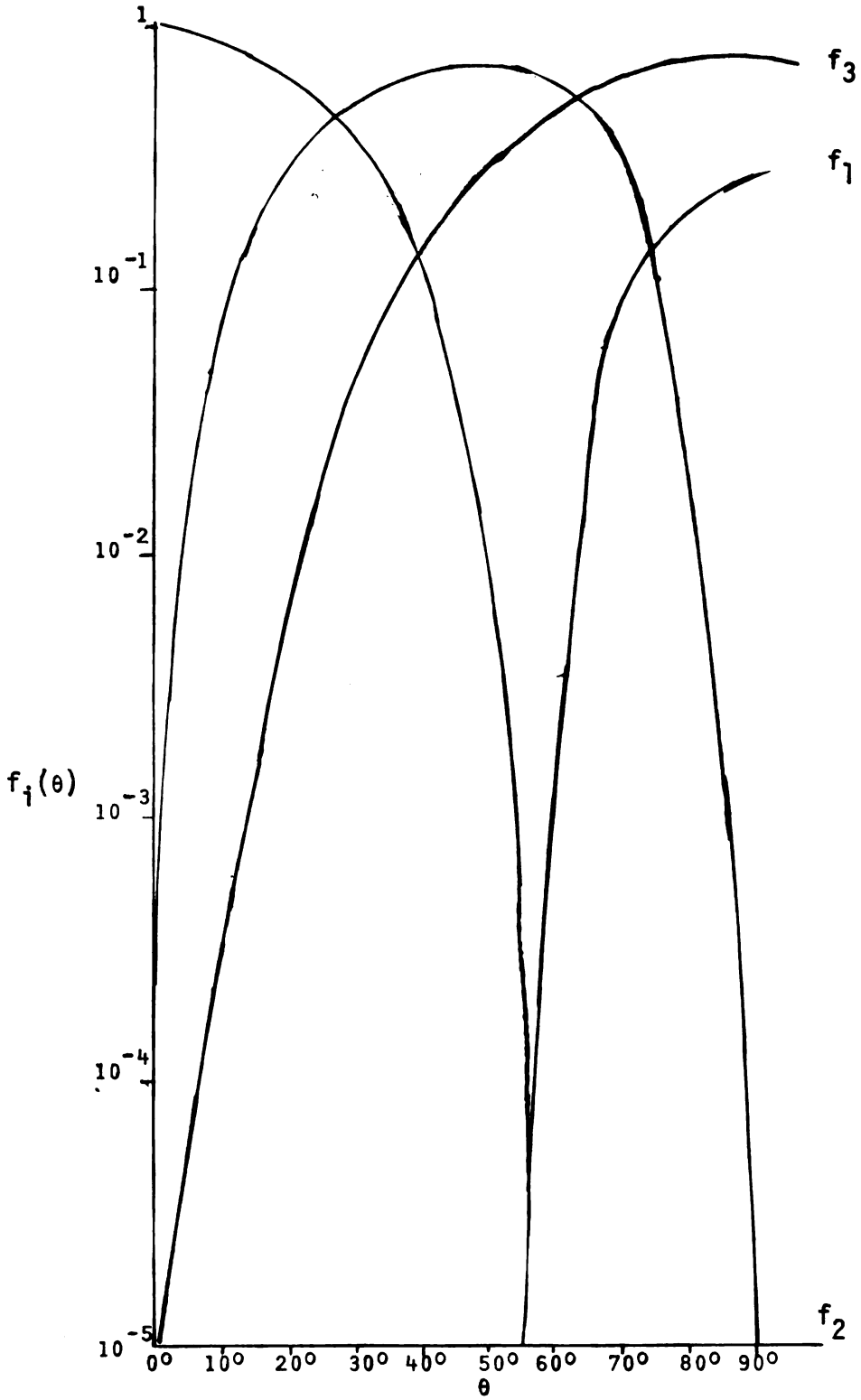


Figure 7. Plot of θ, the angle between the absorption and emission dipoles of a fluorochrome, vs. $f_i(\theta)$, the weighting coefficients for each component in the sum of exponentials describing anisotropy decay of an ellipsoid.

undergoing Brownian rotation in solution. (17) This assumes that hydrodymanic variables can be used to describe the solute behavior ,

$$V = \frac{\rho kT}{3\eta}$$

Where V is the hydrated volume, k is Boltzmann's constant, T is the Kelvin temperature, ρ is the rotational relaxation time of the sphere in the solution, and η is the viscosity of the solution in poise. For elliptically shaped molecules, the equations of Perrin (6) may be applied, if rotational relaxation times about each particular molecular axis is determined allowing the calculation of the length of the major and minor axes.

V. RESULTS

Alpha-chymotrypsin

Alpha-chymotrypsin is a proteolytic enzyme capable of catalyzing the hydrolytic cleavage of peptide and ester bonds, especially at bonds involving the carboxyl groups of aromatic amino acids. The crystal structure of α-chymotrypsin has been established by several workers (45,49). It has a molecular weight of approximately 25,000 g/mole and an axial ratio of 1.13:1.0 calculated form the x-ray crystallographic data, making it nearly spherical in the crystalline state. Table 2 shows the results obtained using time resolved fluorescence depolarization technique to determine the rotational relaxation times and the solvated molecular volumes of α-chymotrypsin conjugated with the hydrophobic probe ANS (see experimental) in various environments. The quantities were calculated assuming the solvated shape of the α-chymotrypsin as being spherical. The hydrated volume, V, was calculated using the equation;

$$V = \frac{\rho kT}{3\eta}$$

where the symbol representations are explained in the data treatment section. This expression is valid for dilute solutions of Newtonian liquids where the solute particles are much larger than the solvating molecules and the 'slip' condition is assumed valid

Table 2. Parameters Derived from the Study of α-chymotrypsin via Curve fitting and Linear Regression Analyses.

Temperature-C^{o}	Viscosity (poise)	A_o	ρ(nsec)	Volume(cm^3/molecule)
23.8	0.0091	.35±.02	27.5±2.0	$3.5x10^{-20}$
24.1	0.0090	.20±.004	56.7±2.5	$7.7x10^{-20}$
35.2	0.0072	.31±.015	19.2±0.8	$3.5x10^{-20}$
35.6	0.00715	.30±.007	54.4±2.7	$10.0x10^{-20}$
22.8+sucrose	0.0102	.47±.008	165.3±18.	$20.2x10^{-20}$
26.4+sucrose	0.0087	.50±.01	123.0±19.	$17.9x10^{-20}$
23.8	0.0091	.39	27.2	$3.9x10^{-20}$
24.1	0.0090	.21	57.0	$8.48x10^{-20}$
35.2	0.0072	.31	19.3	$3.71x10^{-20}$
35.6	0.00715	.25	54.2	$9.94x10^{-20}$
22.8+sucrose	0.0102	.47	166.7	$21.7x10^{-20}$
26.4+sucrose	0.0087	.51	124.5	$18.2x10^{-20}$

at the solvent-solute boundry. (This means that one is assuming the solvent particles associated with solute molecules do not interact with the solvent particles in the bulk of the solution.) Figure 8 shows a typical log A(t) versus time plot of the α-chymotrypsin-ANS system. Figure 9 shows the plots of the quantities Log ($I_{//}(t) - I_{\perp}(t)$) vs. time and log ($I_{//}(t) + 2I_{\perp}(t)$) vs. time for the same conditions. As can be seen form these figures, linearity of the logarithms of the component functions of the time dependent fluorescence anisotropy do not necessarily result in a linear or well defined decay of the log A(t) vs. time behavior.

Table 2 shows that the rotational relaxation times of α-chymotrypsin at various temperatures and in buffer solutions at a pH of approximately 7.3 can be grouped according to the values obtained at the temperatures of 23.8 and 35.2 C^{o} and another grouping for the trials at 24.1 and 35.6 C^{o}. These differences can be attributed to several factors. The most likely cause of the reduction of the apparent rotational relaxation time at the former temperatures is autolysis. Alpha-chymotrypsin is known to undergo autolysis under certain conditions. (39,40) The presence of labelled molecules in solution which have undergone autolysis should lead to observation of rotational relaxation times which are different (probably less) than that for the native protein molecule. In the event that the overall shape of the molecule is altered drastically or a protein molecule is produced which now has the labelled portion rotating independently of the

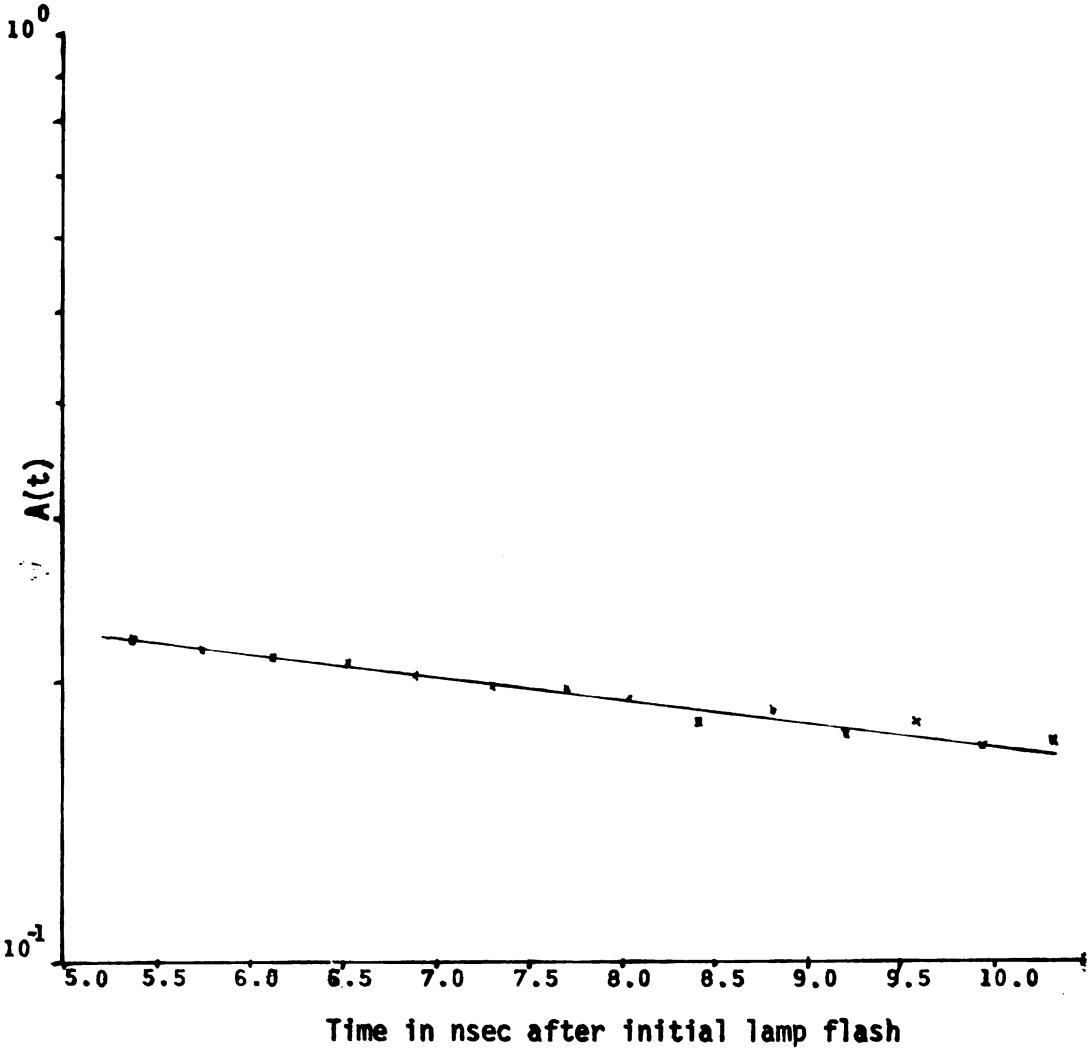


Figure 8. Typical plot of log A(t) versus time for α-chymotrypsin-ANS system.

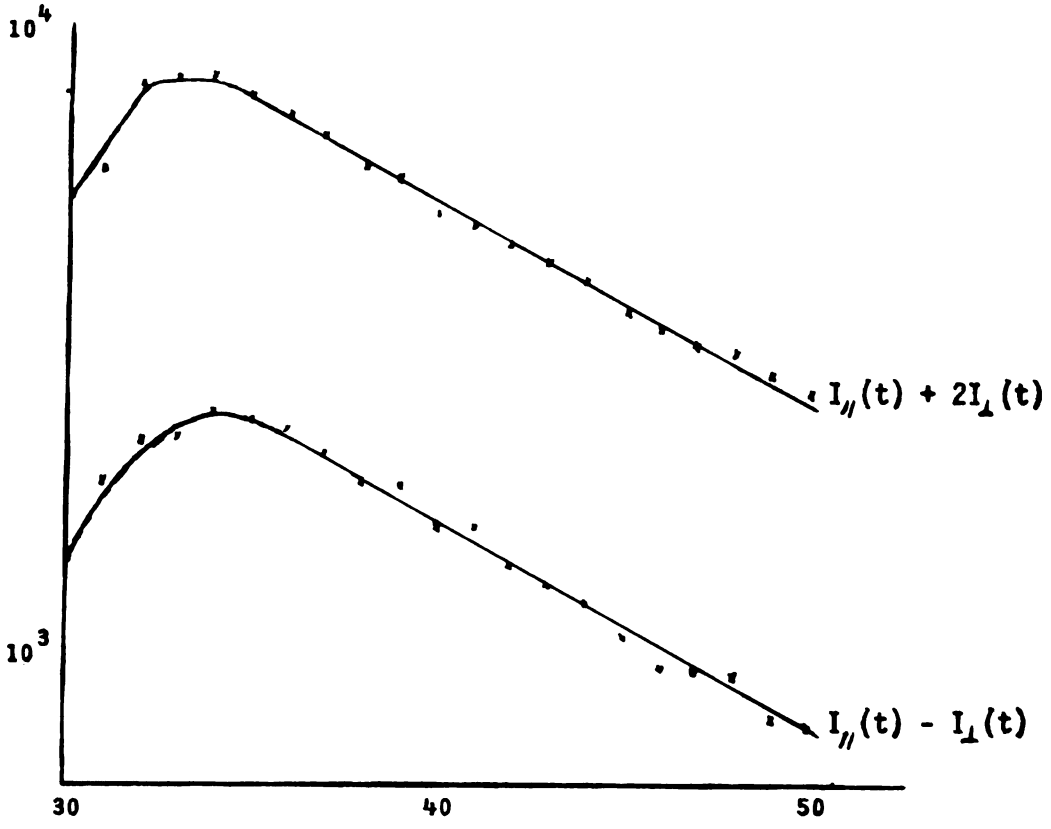


Figure 9. Log plots of the quantities $I_{//}(t) - I_{\perp}(t)$ and $I_{//}(t) + 2I_{\perp}(t)$ versus time for α-chymotrypsin ANS system. Horizonal is channel mumbers corresponding to channels in multichannel analyzer. Channel 30 is 1.52 nsec after initial lamp flash. Each channel corresponds to 0.383 nsec.

of the bulk portion of the molecule, the shape of the anisotropic decay curve will reflect this and a component indicating rapid rotation may become evident. If the cleavage results in the release of the probe molecule from the protein, a large decrease in the hydrophobic nature of the probe environment, the fluorescence quantum yield of the probe will be considerably lowered as it encounters a more hydrophilic environment, its emission maximum will be red shifted, and its contribution to the observed fluorescence will be negligible. The rotational relaxation times obtained for a solution which undergoes autolysis reflects relaxation times characteristic of protein molecules at various stages of cleavage.

In fresh solutions of low concentration, the effect of autolysis may not become apparent during the time of an experiment if the data collection is done within the first 30 minutes after mixing of the solutions. The measurements taken at 24.1 and 35.6 C^{o} satisfy this condition and give rotational relaxation times which are in fair agreement with those published for α-chymotrypsin by Stryer (20) and Kuzentsov (41). Stryer has reported a rotational relaxation time of 52 nsec for α-chymotrypsin complexed with p-nitrophenyl anthranilate. The measurements were made using time resolved fluorescence depolarization technique similar to that used in my studies. The derivative used shows no enzymatic activity and a conjugation ratio of one probe per protein molecule. Kuzentsov and his co-workers used the technique of ESR to determine the rotational relaxation time

of α-chymotrypsin. Attachment of a spin-probe was necessary to utilize this technique. They obtained a rotational relaxation time of approximately 45 nsec for this enzyme at similar conditions. It was thought that the spin probe is covalently attached at the active site. The smaller relaxation time may reflect a different site in which the probe is more free to rotate relative to the fluorescent probes used in my experiments.

To verify the possibility of autolysis, experiments were performed in which the system was modified to prevent autolysis. The two methods which were used to prevent the autolysis were addition of calcium ion (in the form of calcium chloride) to the solution, and complexation of the enzyme.

In the first method, the concentration of calcium ion used, 0.1 M (39) required use of a tris-hydroxymethyl amino methane- HCl buffer to effect solution of all species present. Unfortunately, the capacity of the buffer was not adequate at the suggested strength to maintain the pH of the solution above 6.0, a level necessary to prevent significant dimerization of the protein molecules. (The buffer was prepared to be pH = 8.0 at a concentration of 0.0059 M in TRIS.) An increase in the concentration of the buffer components to give adequate capacity to maintain the pH above 7.2 resulted in the precipitation of the protein upon addition of the calcium chloride. This was probably due to the relatively high ionic strength of the solution which caused a 'salting out' of the protein. Dilution to approximately one-tenth the original concentration

of all species gave a clear solution, but reduced the fluorescence intensity to low levels. Consequently intensity versus time measurements were very close to the noise level of the time resolved spectroscopy instrument and unsatisfactory results were obtained.

A second method for eliminating effects due to autolysis is to study the enzyme complexed to an inhibitor. This serves to prevent both autolysis and dimerization of the molecule at lower pH values of the solution, but does not prevent the binding of ANS to the protein molecule. The effect of preventing dimerization is useful in that it would apparently allow the collection of data at a pH where the fluorescence emission of the bound dye molecules is enhanced. Data obtained for the system of α-chymotrypsin complexed with tosylate is shown in Table 3. Resulting analysis of the anisotropic decay using KINFIT curve fitting to single exponential decay gave a rotational relaxation time at 22.4 C^{o} of 11.6 nsec. This is well below previous results obtained at pH = 7.2 using acitve α-chymotrypsin. This reduction in the apparent rotational relaxation time may be due to some form of energy transfer since the intense fluorescence enhancement at the lower pH may be a result of additional association of the ANS molecules per protein molecule. An alternate possibility is that additional protonation of nearby residues might change the binding affinity of ANS to the protein is questionable since the enhancement of the ANS fluorescence is substantial at the lower pH indicating a more hydrophobic

Table 3. Parameters Derived from the Study of Tosyl-α-chymotrypsin via Curve Fitting and Linear Regression Analysis.

Temperature-C^{o}	Viscosity (poise)	A_o	ρ(nsec)	Volume(cm^3/molecule)
22.4	0.0099	.22±.01	11.1±.3	1.49×10^{-20}
22.4	0.0099	.20	14.4	1.97×10^{-20}

environment for the ANS molecule. A decrease in the rotational relaxation time should occur only if the ANS has a greater rotational freedom with respect to the protein molecule in its new environment. Since the ANS is initially attracted and held to the protein by hydrophobic attraction, it would seem reasonable that an increase in the hydrophobicity of the environment of the probe binding site would increase its binding affinity. Our interpertation is that the reduction of the pH induces a conformational change such that the ANS finds itself in a more hydrophobic cavity which allow it to rotate more freely. This would explain the lowering of the rotational relaxation time while enhancing ANS fluorescence. Luminescence studies of the active enzyme and the tosyl derivative as a function of pH points to the importance of the pH change as being the important factor rather than complexation with the tosyl derivative.

Calculated values of the solvated volume of the active α-chymotrypsin using the data at 24.1 and 35.6 C^{o} are consistent with the known values of this parameter. This gives molecular radii of 26.2 A^{o} for molecules having solvated molecular volumes of 7.7×10^{-20} cm^3/molecule and 28.8 A^{o} for those with solvated molecular volumes of 10.0×10^{-20} cm^3/molecule. This compares with the value obtained for the crystals of α-chymotrypsin from x-ray diffraction analysis of 23.7 A^{o}. Thus a hydration shell with effective thickness of 3-6 A^{o} would be apparent.

Addition of sucrose to the solution to observe bulk viscosity effects on the rotational relaxation time gave increases which

were too large to explain in terms of bulk viscosity changes in the solution. Measurements using the Zeitfuchs Cross Arm Visciometer indicated the viscosity of the solution changed by a factor of approximately 1.1 at each temperature studied. The rotational relaxation time of the protein in these solutions would be expected to increase by a similar factor. The increase however is by a factor of approximately 2.5 to 3.0. This indicates interactions are occuring which lead to much longer rotational relaxation times of the protein molecules than expected.

A possible interaction is the preferred association of sucrose molecules with the α-chymotrypsin rather than becoming 'solvated' particles in the buffer medium and increasing the bulk viscosity of the solution. Using the observed rotational relaxation times and measured viscosity data, and assuming the shape of the protein to maintain its apparent spherical shape in the presence of the sucrose molecules, one calculates an effective hydrodymanic radius of the α-chymotrypsin molecule as approximately 34 A^{o}. From x-ray crystallographic studies the radius of the α-chymotrypsin was given as 23.7 A^{o}. This would mean that the effective solvation shell in the sucrose solution is approximately 10 A^{o}. Since the sucrose molecules are known to have effective diameters of 9.4 A^{o} (42) this would indicate that the protein molecules are being solvated by a layer of sucrose molecules at the surface, thus increasing the effective molecular radius by approximately 10 A^{o}. An increase in the anisotropy at time zero (A_{o}) parameter for these solutions to theoretically impossible

values also indicate some type of interaction is occuring other than just bulk viscosity effects.

Lysozyme from chicken egg white was used as a model compound of elliptical shape. From x-ray diffraction studies (58,59) the approximate dimensions of the crystalline lysozyme molecules are 30 A^o x 30 A^o x 45 A^o (A^o = Angstrom). This gives an axial ratio of 1.0:1.0:1.5. From the theory of Jablonski, the anisotropic decay as a function of time for an elliptical particle will be the sum of three exponential terms with relative contributions determined by the angle, θ, between the absorption and emission axes, i.e.

$$A(t) = A_0 \sum_{i=1}^{3} f_i \times \exp(-3t/\rho_i)$$

and with weighting coefficients, f_i, being given by the expressions;

$$f_1 = (3/2 \cos^2\theta - 1)^2$$
$$f_2 = 3 \cos^2\theta \sin^2\theta$$
$$f_3 = 3/4 \sin^4\theta$$

The fluorescence anisotropy decay, based on the above data, could show a decay characteristic of three exponentials. The lysozyme was tagged with the probe dimethylaminonaphthalene sulfonyl chloride and the preparation was carried out as described in the experimental section. Table 4 contains the results of measurements at several temperatures and different solution conditions. This compares with theoretical calculations of 33,30, and 25 nsec (nanoseconds) about each molecular axis and a volume determined as 2.12 x 10^{-20} cm^3/molecule from the x-ray diffraction

Table 4. Parameters Derived from the Study of Lysozyme via Curve Fitting and Linear Regression Analysis.

Temperature-C^{o}	Viscosity(poise)	A_o	ρ(nsec)	Volume(cm^3/ molecule)
23.5	0.0094	.29±.01	42.3±5.1	6.00×10^{-20}
25.5	0.0088	.33±.01	26.9±1.4	4.10×10^{-20}
29.4	0.0081	.32±.02	28.9±2.0	4.86×10^{-20}
25.5+sucrose	0.0098	.26±.01	26.7±2.4	2.21×10^{-20}
23.5	0.0094	.27	47.9	6.78×10^{-20}
25.5	0.0088	.32	29.3	4.47×10^{-20}
29.4	0.0081	.32	27.4	4.61×10^{-20}
25.5+sucrose	0.0098	.25	28.7	3.94×10^{-20}

data assuming a prolate ellipsoidal shape. A hydrated volume of approximately 3.3×10^{-20} cm^3/molecule is calculated from specific volume data supplied in the Handbook of Biochemistry (46). This table lists values which were calculated using the KINFIT curve fitting program of Dye and Nicely (48). Attempts to fit the data to a sum of three weighed exponentials were unsuccessful due to the limited number of data points at each set of conditions. Fitting to a double exponential was possible only when the f_i and A_0 parameters were known to within a small value of their values determined by the curve fitting program, these known values being inserted as the initial estimates of these parameters and fitting to the equation;

$$A(t) = A_0 \sum_{i=1}^{2} f_i \times \exp(-3t/\rho_i)$$

The values of A_0 and the f_i were determined in the following manner: assuming the principle of photoselection is valid, the values of the weighting coefficients, f_i, can be determined from a knowledge of A_0. Since this is the fluorescence anisotropy at time zero, it is a function only of the angle between the absorption and emission dipole, θ. This is seen by applying the expressions of Chuang and Eisenthal for the decay of the parallel and perpendicularly polarized components of radiation to the empirical relationship for the fluorescence anisotropy decay, and restricting the values to those at time zero. Assuming that no energy transfer processes (other than those giving non-radiative

decay occur, the value of A_0 can give an approximate estimate of the value of θ. Substituting the expressions of Chuang and Eisenthal (16) for the intensities and assuming the absorption dipole for the particular wavelength of exciting radiation of the molecule coincides with the z laboratory axis at the time of excitation, ($\gamma_z = 1$, $\gamma_x = \gamma_y = 0$) gives the expression;

$$A(0) = 0.6 \cos^2\theta - 0.2$$

This result is valid for any shape of molecule. Table 5 lists the value of A_0 for various values of theta. Hence, knowledge of A_0 from laboratory measurements allows one, in principle, to determine the angle between the absorption and emission dipoles (θ) and the f_i for the various components of the anisotropic decay function. Figure 7 shows the dependence of the f_i on theta. A_0 can be determined by linear regression analysis over the short time portion of the experimental anisotropy decay curve. It is apparent from figure 7 that only in the regions of $30^\circ < \theta < 45^\circ$ and $70^\circ < \theta < 80^\circ$ are all three weighting coefficients of relative improtance with respect to each other. For all other values of θ one and sometimes two of the f_i are less than ten percent of the magnitude of the other f_i, and are of little importance in determining the nature of the anisotropy decay curve. If the decay is observed to be single exponential, f_i for two of the exponential factors will be very nearly zero. Since single exponential decay can arise from the anisotropy decay of spherical

Table 5. A_o as a function of θ.

θ (degrees)	A_o
0	0.400
1	0.399
2	0.399
5	0.395
10	0.381
15	0.359
20	0.329
25	0.293
30	0.250
35	0.203
40	0.152
45	0.099
50	0.048
55	-0.003
60	-0.050
65	-0.093
70	-0.130
75	-0.160
80	-0.182
90	-0.200

molecules as well as form non-spherical particle rotation where two of the factors in the sum of exponentials which describe the fluorescence anisotropy decay are very small compared to the third, we must examine the conditions which can cause this to explain possible anomalus behavior observed experimentally. In the second case, the molecule may be of elliptical symmetry but the value of θ and the axial ratio such that the components of the sum of exponentials is simplified to one term as the other terms approach zero. For the case where f_2 or f_3 is approximately 1% of the magnitude of f_1, the corresponding value of ρ_2 or ρ_3 would have to be 2.3 times larger than ρ_1 to offset the small magnitude of the f_i for those components of the summation which are very small. For a given ρ_i corresponding to rotational relaxation about one axis of the ellipse one can show that if the f_i for that particular component of the ansiotropy decay is very small relative to the other components, the axial ratio r_i to r of the more important terms in the sum must be a factor 1.3 greater to give the i^{th} term an increase of one order of magnitude. This condition could, in principle, be used to determine possible allowed orientations of a dye molecule with respect to a protein molecule if the relative orientation is constant for every case.

Table 4 contains values of ρ and V obtained for lysozyme conjugated with dansyl chloride under various conditions. The values of ρ corresponding to temperatures 23.5 C^o, 25.5 C^o, and 29.4 C^o are seen to be approximately 42, 27, and 29 nanosec. respectively. The value of 42 nsec at 23,5 C^o is substantially

higher than that expected or previously observed by steady state depolarization methods. Rawitch (50) and Wahl (51) reported rotational relaxation times of 25 nsec for lysozyme labeled with dansyl chloride. Rawitch, however, did not account for the possibility of dimerization of lysozyme at the pH of the experiment (7.0) and both he and Wahl neglected the possible effects of addition of sucrose (50% solution) to vary the solution viscosity. My results would suggest some type of interaction of the lysozyme molecule leading to longer rotational relaxation times than expected. It is possible that one is seeing an effect of polymerization of the protein molecules into dimer form at this temperature. Sophianopoulous and Van Holde have reported the pH dependence of the dimerization of lysozyme. They also indicate that the extent of dimerization is dependent on the temperature of the solution and that at pH 8, complete dimerization does not occur unless the solution temperature is lowered to 5 C^{o}.

The values of ρ at 25.5 C^{o} with added sucrose to increase the viscosity was measured to be nearly the same as the rotational relaxation time for the solution at 25.5 C^{o} without sucrose. Since the solution viscosity is increased by a factor of 1.16 this anomaly could result for the interaction of the solvent with the conjugated dye molecules. Wang (52) investigated the effect of various solvent systems on dansyl chloride-lysozyme conjugates and found that changing the solvent using the series of 20% v/v dimethyl sulfoxide, ethylene glycol, and propylene glycol were sufficient to produce shifts in the absorption spectra.

He concluded that in the monomeric form, the dansyl chloride molecule is in a 100% exposed position on the lysozyme molecule. Since solvent pertubation technique often uses sucrose as a perturbing solvent medium (42) , we conclude that the sucrose molecules may interact with the protein molecules during solvation in such a manner as to influence the association or exposure of the fluorescent probe and may not reflect the true rotational character of the protein molecule. Emission spectra (figure 10) performed on labelled lysozyme at pH=5.1 before and after the addition of very small amounts of (5% v/v) of saturated (45%) sucrose solution show a significant shift, approximately 25 nanometers, in the emission maximum upon addition of the sucrose. This indicates some type of interaction between the sucrose and the bound dye molecules, or a change in the protein such that the bound dye no longer sees a similar environment.

Since association studies seem to indicate that the dimerization of lysozyme may have been a factor in giving larger rotational relaxation times than expected for the three solutions at temperatures of 23.5, 25.5, and 29.4 C^{o}, experiments were performed to study the effect of dimerization of the lysozyme on the rotational relaxation times. In these experiments, the labeling reaction was carried out in a buffer medium (phosphate) which had a pH of 5.1. Sophianopoulos and Van Holde reported that the lysozyme existed totally in the monomer form at this pH. Since the labeling reaction should be much slower at this pH, the reaction was allowed to proceed for three hours before

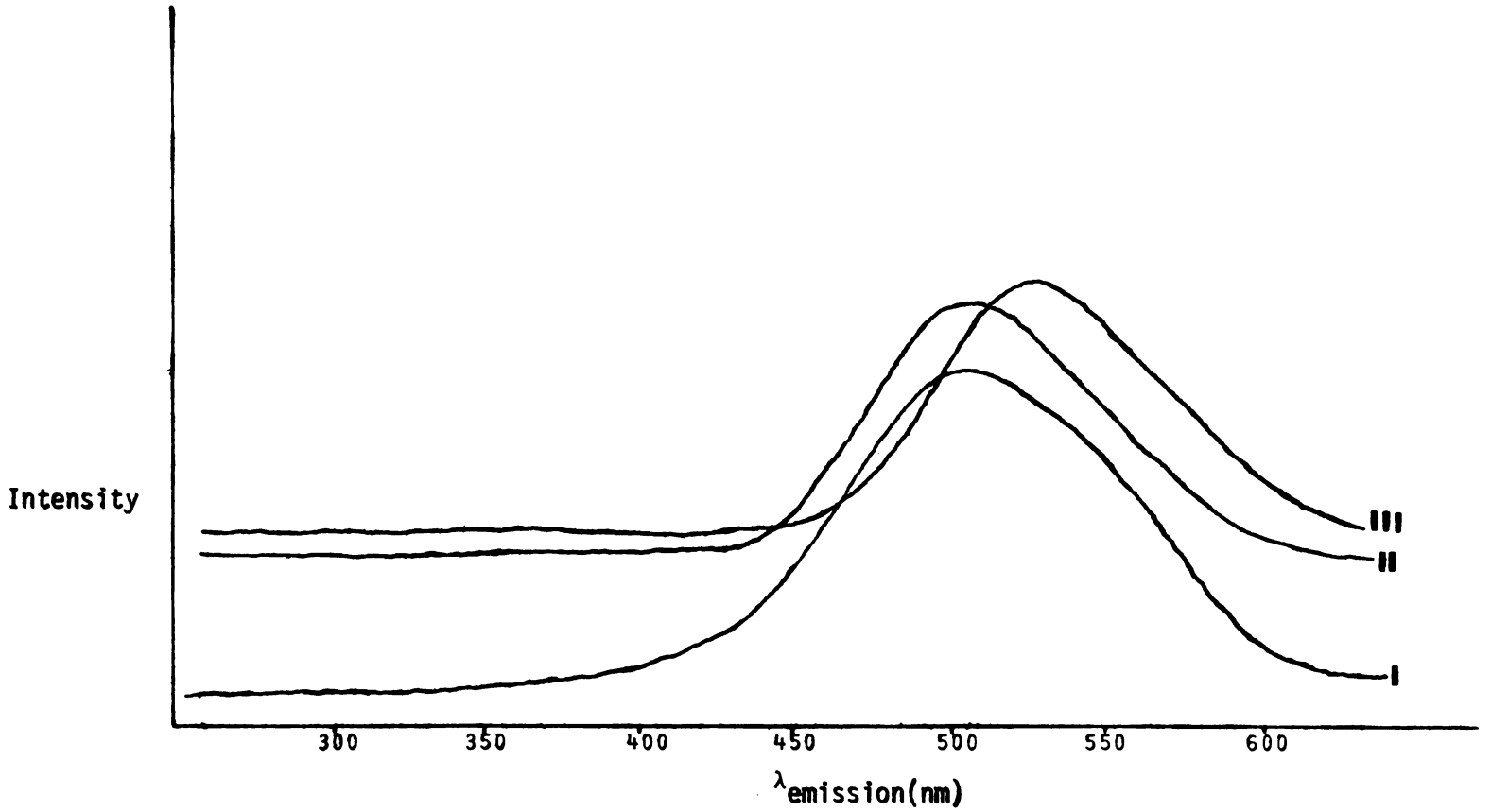


Figure 10. I is the emission spectrum of lysozyme-dansyl chloride labelled at pH 5.1.
II is the emission spectrum of lysozyme-dansyl chloride labelled at pH 9.1.
III is the emission spectrum of lysozyme-dansyl chloride labelled at pH 9.1 with sucrose added.

separation of the components on Sephadex G-100 gel was performed. Fluorescence depolarization experiments were performed on the fraction which was most lightly labeled as indicated by its abosrption and emission spectra. The results show a rotational relaxation time of 20 ±1 nsec which is well below that obtained at 23.5 C^{O} for the solution which was labelled at pH = 7.2. (The temperature of the solution run at pH 5.1 was 23.1 C^{O}.) This indicates that some association had probably taken place before labeling in the solution prepared at pH=7.6. The pH of the solution at 5.1 was then adjusted to 9.1 using 0.1 M NaOH. Depolarization experiments on this solution yielded a rotational relaxation time of 11 nsec, a value well below that expected since at this pH the lysozyme should have been completely dimerized. (35) The reason for this low rotational relaxation time value may be that dimerization of the protein was blocked by the presence of the probe molecules on the protein at the site of dimerization. The lowering of the value of the rotational relaxation time relative to that of the monomer, however, is not expected unless some interaction has occured as th pH is raised to 9.1 which results in the mobility of the probe with respect to the protein molecule being increased, or that dimerization of the labeled protein causes a change in the affinity of probe binding if it is present on the protein before dimerization. Further experimentation will be required to resolve this question.

Rhodamnine B in glycerol

A 1.0×10^{-5} M solution of ammonium-9-(o-carboxyphenyl)-6-(diethylamino)-3H-xanthen-3yldine)-diethyl chloride, (Rhodamine B, Figure 12) in glycerol, spectroscopic grade, was also investigated. Rhodamine B is an asymmetric molecule which should exhibit multiple rotational relaxation times, provided the relationship between the absorption and emission dipoles does not reduce one of the weighting coefficients to zero. Glycerol was chosen as a solvent because of its high viscosity (necessary to 'slow' the rotational motion of the small molecule in order that its rotational relaxation time be slow compared with the natural lifetime of the molecule and to insure that it be within the range of measurement of our instrument.) Table 6 contains the results of the investigation. The decay of the fluorescence anisotropy as a function of the time clearly shows non-single exponential decay character. (Figure 13) Values for attempted fits to single exponential as well as the sum of two exponentials are included in the table. The best fits of a sum of two exponentials give rotational relaxation times of approximatly five nanoseconds for the short time rotational relaxation and one hundred nanoseconds for the long lived component. The viscosity of glycerol at 22.4 C^{o} is 710 centipoise (53). This gives solvated volumes of 9.4×10^{-24} cm^3 per molecule and 187.0×10^{-24} cm^3/molecule respectively for the short and long-lived rotational modes. The single exponential rotational relaxation time was determined to be 76 nsec corresponding

Table 6. Parameters Derived from the Study of Rhodamine B via Curve Fitting and Linear Regression Analysis.

Temperature-C^{o}	Viscosity(poise)	A_o	ρ(nsec)	Volume(cm^3/molecule)
22.4	7.10	.33±.04	73.2±3.0	1.37×10^{-22}
22.4	7.10	.31±.02	96.8±20.	1.81×10^{-22}
		.55±.75	3.1±1.0	5.80×10^{-24}
22.4	7.10	.33	71.4	1.34×10^{-22}

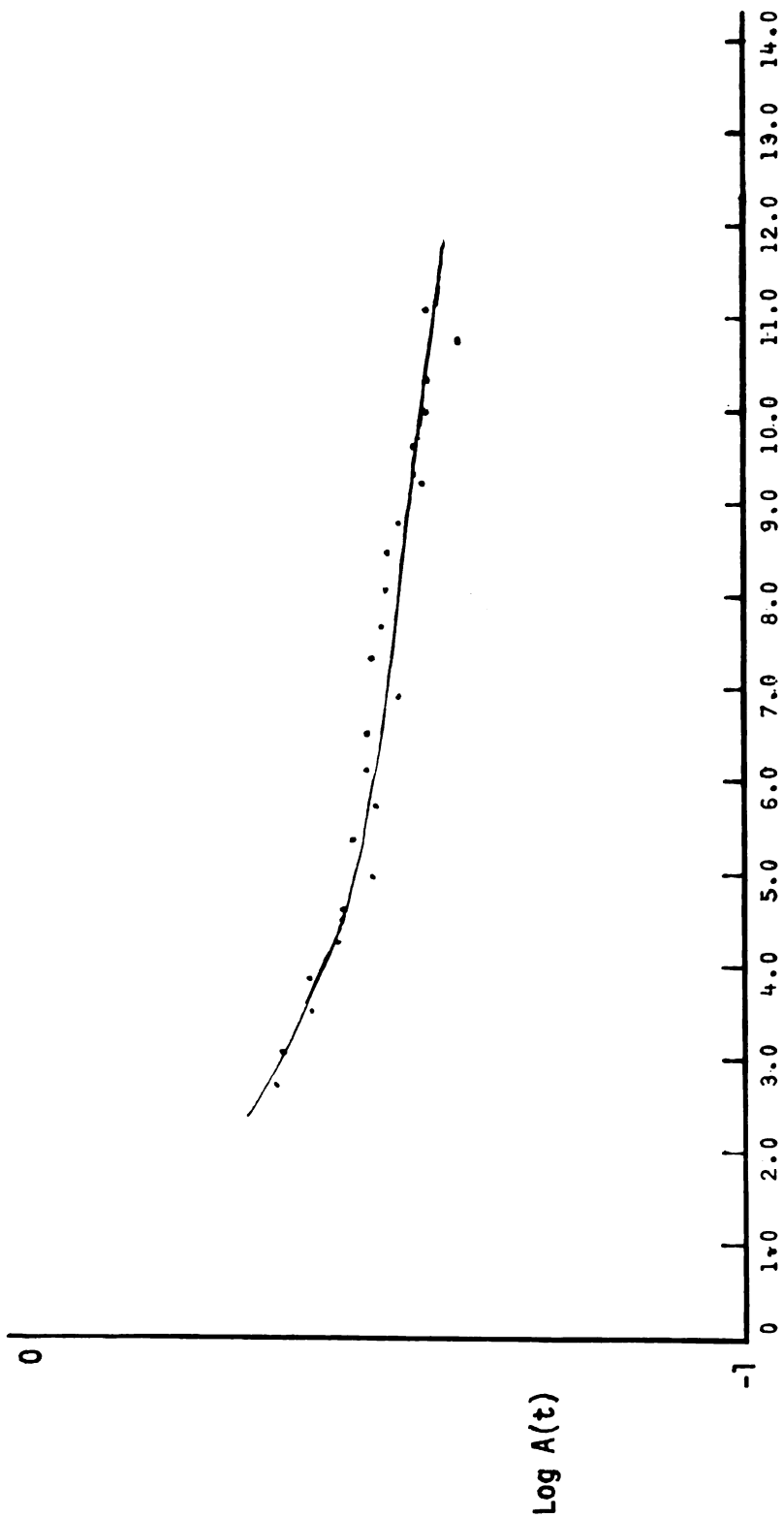


Figure 13. Plot of log A(t) versus time for Rhodamine B in glycerol.

to a solvated volume of 142.3 cm^3/molecule. Looking at the structure of Rhodamine B, one can attempt to explain these data within the framework of the theory. The anisotropy at time zero, A_o, was computed to be 0.33 in both linear regression and curve fitting analyses. A rotational relaxation time of 96 ± 17 nsec was calculated for the long time decay component of the rotation by curve fitting to the sum of two weighed exponentials. The weighting coefficient (f_i) for this decay was 1.02 ± 0.062 as determined by the program. The short time component was found to be in the range of 2 to 8 nanoseconds but required a weighting coefficient of 1.8 to fit to the curve, a value theoretically impossible to obtain without some interaction occuring within the solution or some property of the system which renders the theory inapplicable. The value of the long lived rotational relaxation can be compared with those reported by Cehelnik et. al (54) in their studies of the rotational relaxation of all-trans-1,6-diphenylhexa-1,3,5,-triene (DPH) in various media. They report a rotational relaxation time of approximately 14 nsec for DPH in paraffin oil at 25 C^o. The viscosity of the solution at this temperature was given as 134 centipoise. This would correspond to a rotational relaxation time of 77.0 nsec at 710 centipoise and 22.4 C^o, the conditions of temperature and viscosity used in my experiments. Cehelnik and his co-workers attributed this relaxation to the rotation of the molecule about its minor axis, see Fig. 14. This indicates that the component of the fluorescence anisotropy decay for the Rhodamine B which corresponds

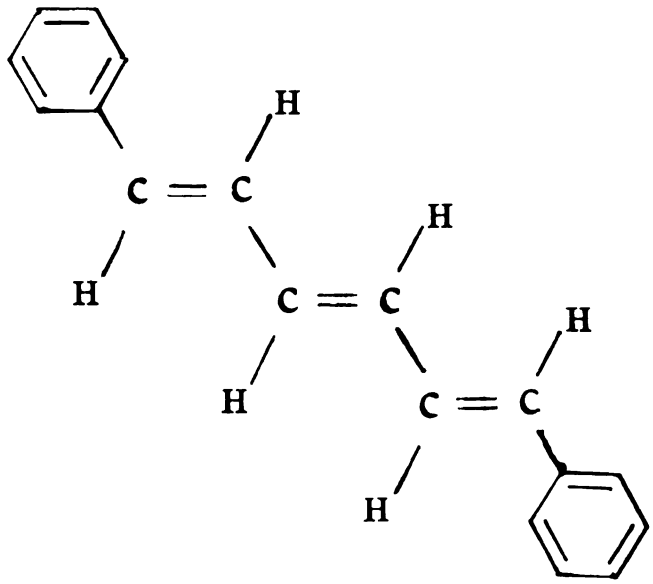


Figure 14. Structure of Diphenyl-hexatriene.

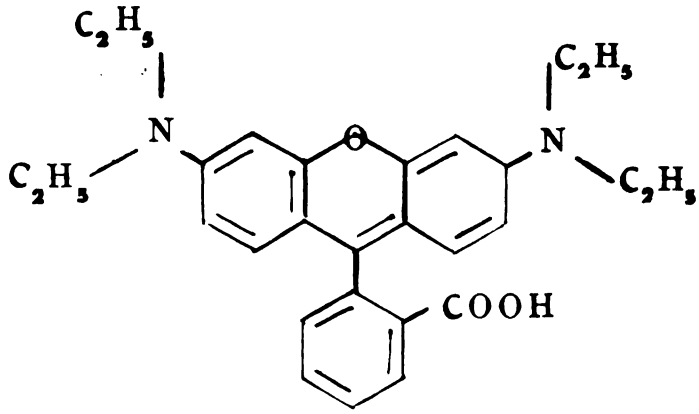


Figure 12. Structure of Rhodamine B.

to a rotational relaxation time of 95 nsec may reflect the rotation of the molecule about its minor axis. When one considers the molecular shape of Rhodamine B it is evident that the short anisotropy decay time of approximately 8 nsec found as the second component of the decay may reflect the rotation of the molecule about its major axis. Since the carboxyphenyl residue is 90^{o} out of the plane of the xanthen-3-ylidine ring system and thus presents only its 'edge' to restrict rotation about the major axis of the molecule, a value of the order of 10 nsec is not unreasonable for a rotational relaxation time corresponding to this rotation. The large value of the weighting coefficient, (f_i), for this component of the decay could be due to the excitation of different transitions of the molecules absorption dipole, or to its relative value compared with the other weighting coefficient being so small as to make its determination to within a small degree of error impossible without a large number of data points. The first possibility is discarded when one considers the data of several workers who have determined the steady state fluorescence anisotropy, (corresponds to the A_0 values determined in these experiments) for Rhodamine B in glycerol at micromolar concentrations. Ayres, et. al. found A_0 to vary between -0.15 and +0.30 for a range of exciting radiation between 300 and 500 nanometers. (55) Their data indicate however, when using excitation radiation above 500 nm that the fluorescence anisotropy of the system is constant and equal to a value of 0.30. This would indicate that in this region of excitation, one is exciting the same

electronic transition (lowest) and that the absorption and emission dipole moments of the molecule are at an angle of approximately 25^{o} with respect to each other. Dehrenlau (56) obtained results similar to those of Ayres and his co-workers. The value of A_0 obtained from these experiments was 0.33 ± 0.04 where the sample was excited at 540 nm using a Wratten diffraction grating. It is therefore doubtful that the anomously high value of the weighting coefficient for the short decay behavior of the Rhodamine B is due to excitation of multiple transitions. The second possibility, that of too few data points, must then be considered as a possible cause for the uncertainty in f_1. The collection of a greater number of data points in the region of interest would help to establish this as the cause. This would require collecting data using a shorter time scale for the Time to Amplitude converter such that each channel of storage now represented a smaller increment in time (increase in the precision of the intensity as a function of time data). Cooling of the single photon counting phototube would reduce the noise level of the system and allow collection of more accurate counts per channel, irrespective of the time precision factor.

For the Rhodamine B-glycerol system we must also consider the applicability of the various theories used in analyzing the rotational motion. Since we have a solution in which the solute molecules are not very much larger than the solvent molecules, this may lead to the case of solute and solvent not interacting in a strictly 'solvating' manner (i.e. no change in viscosity is

observed on addition of solute to the solvent) and the failure of the hydrodynamic approximations (17) used in the development of the data treatment for this type of experiment. In the event that non-hydrodymanic interactions are occuring, the rotation of the solute particles may only be an indication of some type of interaction between solute and solvent particles other than 'solvating' interactions, e.g. dipole-dipole, H- bonding. Should this be the case, the concept of a solvation sphere would become meaningless and the rotational relaxation time of the solute particles would not be a function of the bulk properties of the solvent (viscosity, temperature, etc.) but rather be influenced by specific intermolecular interactions between solvent and solute particles.

VI. FUTURE WORK

Some areas of this topic that may be extended by future work can be grouped into three major catagories. First is the instrumental development necessary for improvement of the quality and efficiency in obtaining data. Second is the treatment of the data. Third is the choice of system to be studied and which parameters of that system may be characterized.

The major problems with the instrument seem to be the signal to noise ratio, and the selection of the excitation frequency. The signal to noise ratio could be improved by use of a cooled DUVP photomultiplier tube. This would also allow measurement of lifetimes of systems with very low fluorescence quantum yields of emission. Several papers on this technique applied to steady state spectrofluorimeters have been published. (57)

In general, if a signal, S, is obtained during a measurement lifetime, t, and a wavelength range of $\lambda_i \pm \Delta\lambda_i$, the intensity of the signal may be represented as;

$$S = N_s(\lambda)t = N_m(\lambda_i)t - N_o(\lambda_i)t$$

where N_m represents the signal plus background and N_o represents the background alone. If we allow $\sigma(N_s,t)$ to represent the

deviation from the mean, to give the noise associated with the signal (thermal noise) we obtain for a signal to noise ratio;

$$\frac{S}{N} = \frac{N_s(\lambda)t}{(N_s(\lambda_i)t)} = \frac{N_s^{\frac{1}{2}}(\lambda_i)t^{\frac{1}{2}}}{\left[1 + 2\frac{N_o(\lambda_i)}{N_s(\lambda_i)}\right]^{\frac{1}{2}}}$$

and it is apparent that this may be improved by cooling the phototube (lowering N_o). Collection of data for long periods of time would theoretically improve the S/N ratio but this alternative is not always possible in many biological systems. A second alternative is use of electronic threshold values. This is not an acceptable alternative to cooling of the phototube because of the possibility that signals arising from white noise may be energetically equivalent to the true signal pulses. Electronic thresholds and bandpass filters may be useful in eliminating excess noise.

Futher refinements in the treatment of the data are also needed. The most useful and expedient would be to allow deconvolutions of the $I_{//}(t)$ and $I_{\perp}(t)$ data from lamp curves taken at the respective polarizer settings. This would eliminate any variance in the lamp function over the period of the measurement while compensating for any change in response of the system due to the presence of the polarizers. The program MAIN and associated subroutines which are already on the system could be modified to allow transfer of the data points from the deconvoluted curves directly to the anisotropy program. KINFIT

seems adequate for fitting the data to exponential decay laws.

The third area of work is more dependent upon the individual researcher's requirements. Theoretically, this technique can be used to study protein aggregation, subunit interaction, conformational change, micro-viscosity of local areas of the protein, or gross solvated volume of macromolecules. It may also be used to gain information about the internal or relative mobility of probes on proteins or between subunits of a protein.

APPENDICIES

APPENDIX A

The following graphs are the KINFIT curves of α-chymotrypsin for the various solution conditions used. In all curves, the vertical axis represents the time dependent fluorescence anisotropy and the horizonal axis represents time. The x indicate experimental points, the o indicate calculated points for the best fit curve, and the = indicate coincidence of an experimental and calculated point. The form of equation fit was;

$$A(t) = A(0)\ \exp(-3t/\rho)$$

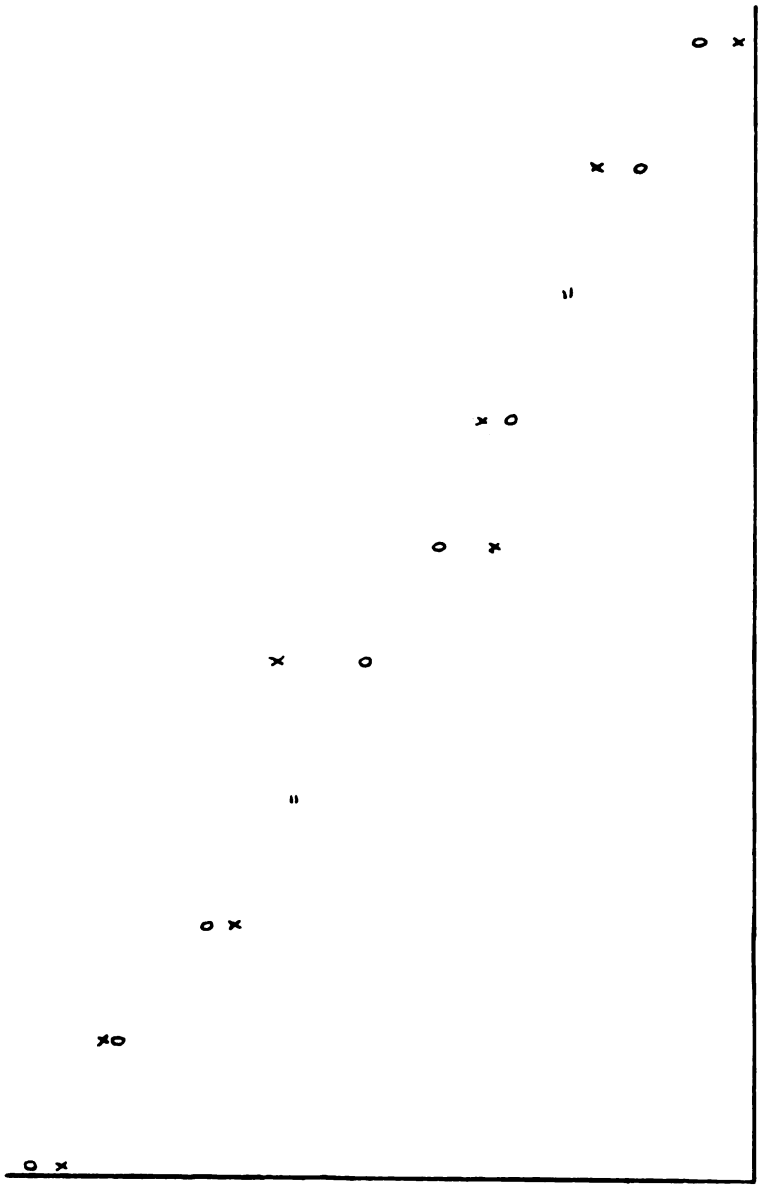


Figure 15. This is Alpha-chymotrypsin at 23.8 C°

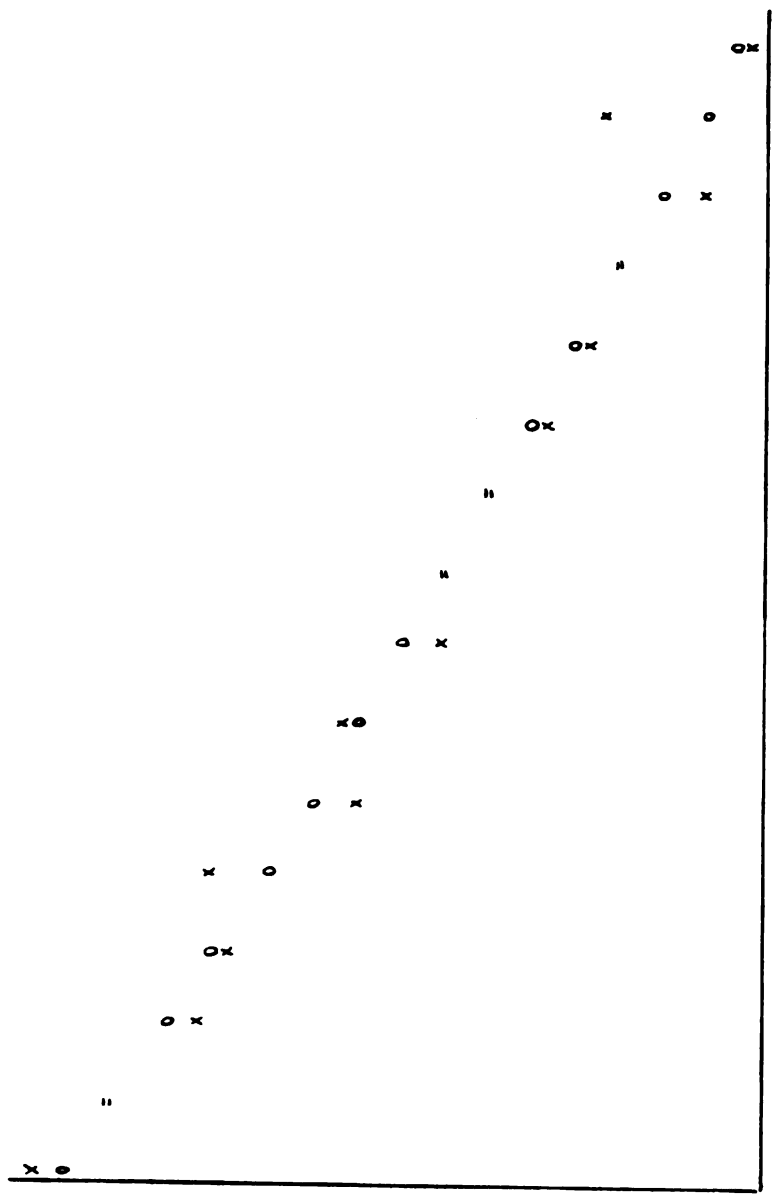


Figure 16. This is Alpha-chymotrypsin at 24.1 C°

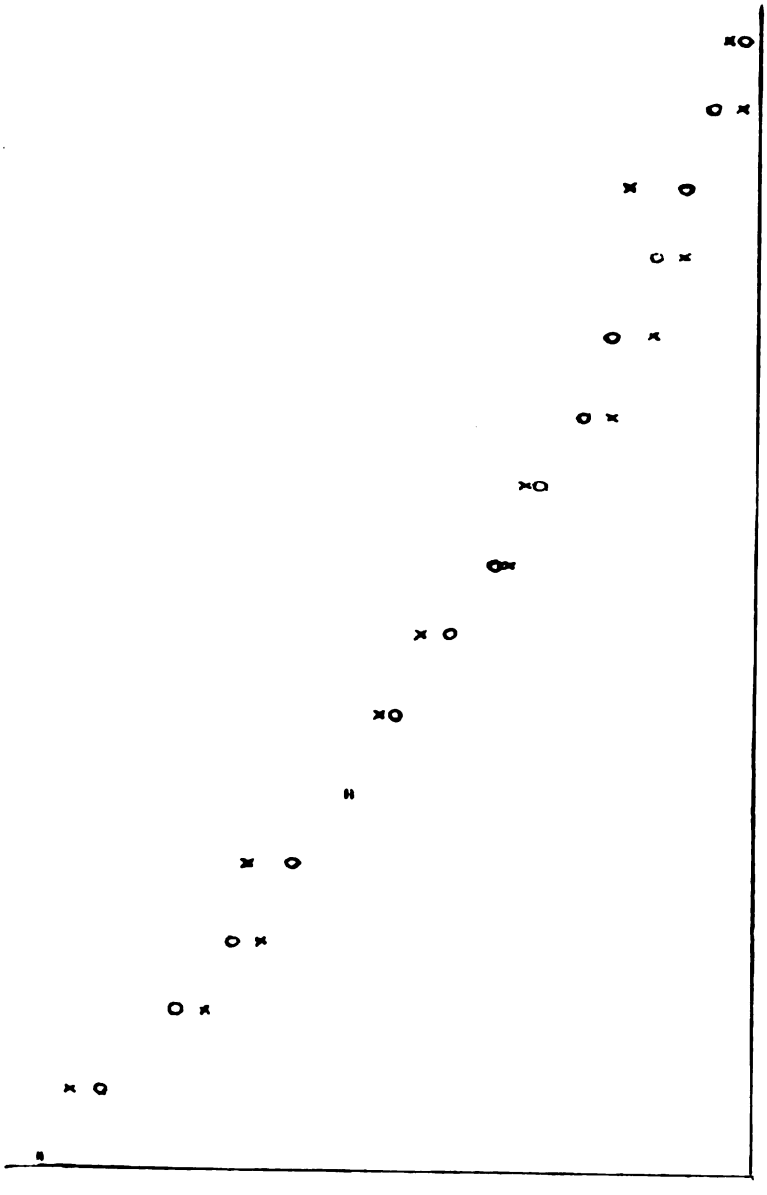


Figure 17. This is Alpha-chymotrypsin at 35.2 C°

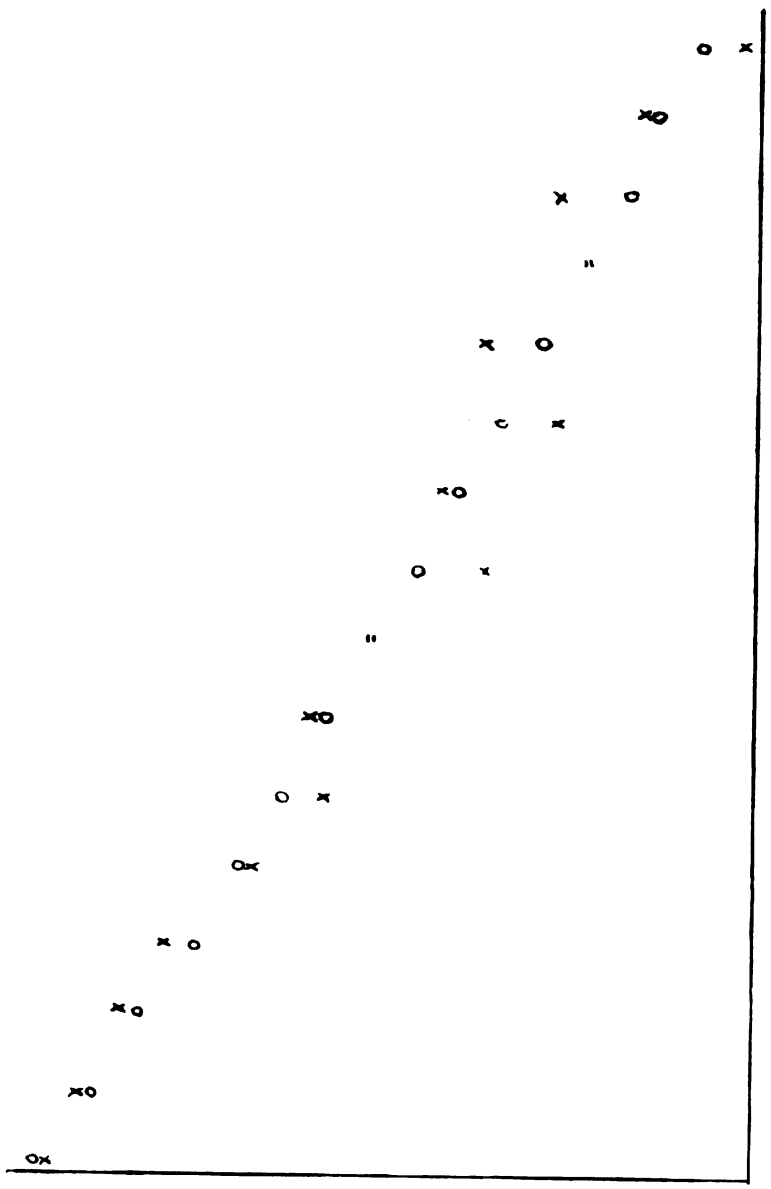


Figure 18. This is Alpha-chymotrypsin at 35.6 C°

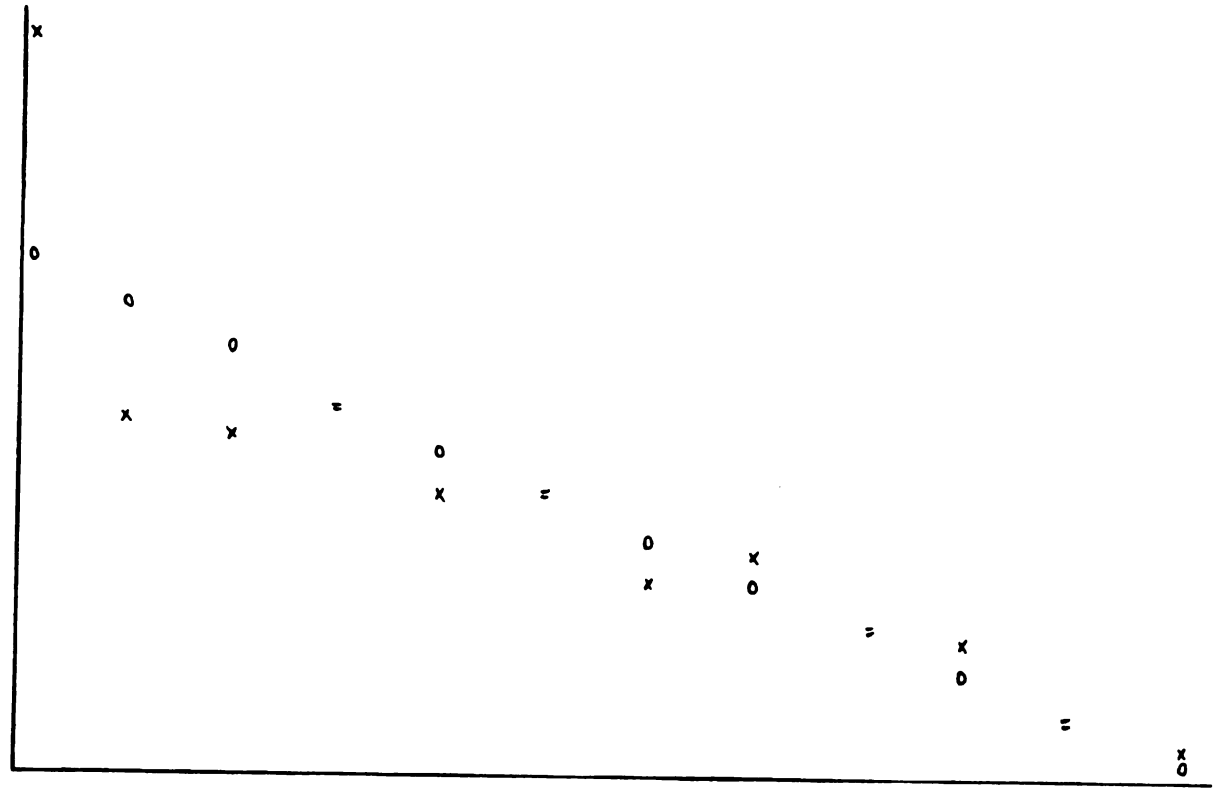


Figure 19. This is Alpha-chymotrypsin at 26.4 C^{o} in sucrose solution

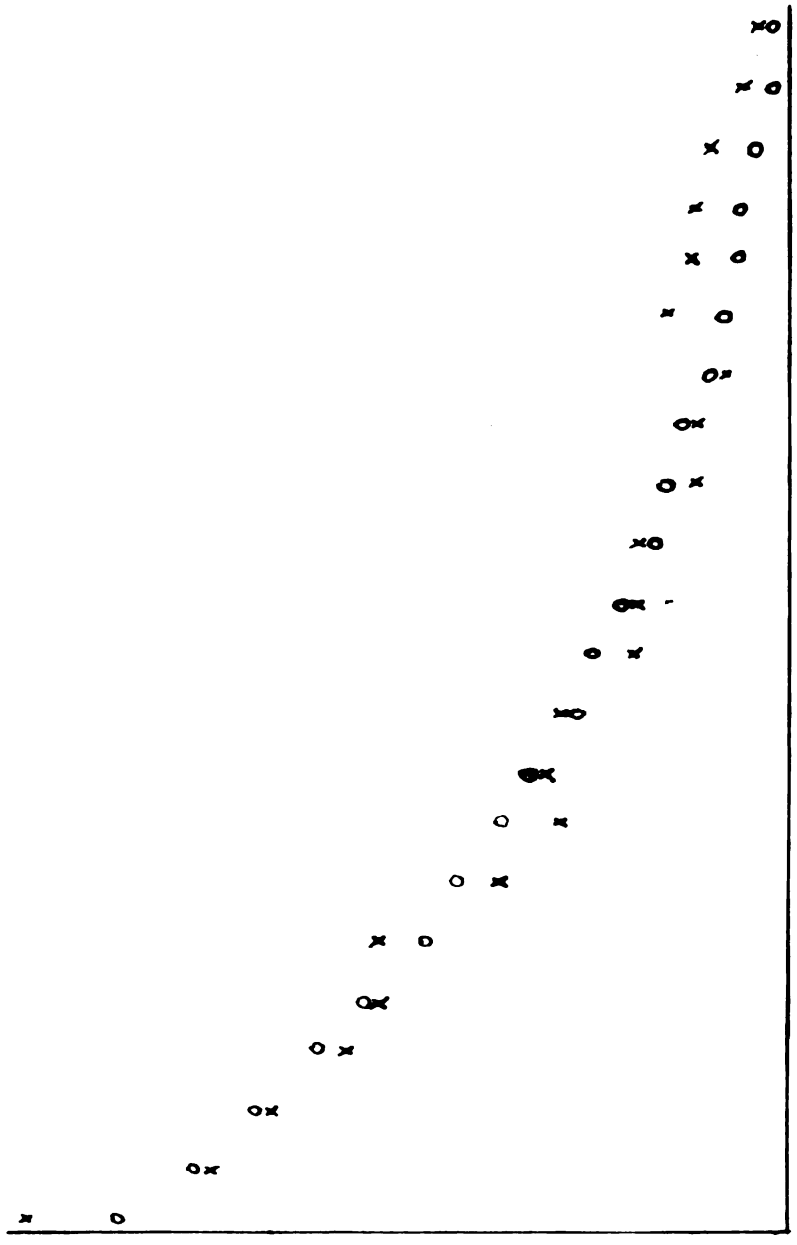


Figure 20. This is Tosyl-alpha-chymotrypsi at 22.5 C, pH=4.2.

APPENDIX B

The following graphs are the KINFIT curves of lysozyme for the various solution conditions used. In all curves, the vertical axis represents the time dependent fluorescence anisotropy and the horizonal axis represents time. The x indicate experimental points, the o incidate calculated points for the best fit curve, and the = incidate coincidence of an experimental and calculated point. The form of the equation fit was;

$$A(t) = A(o)\ \exp(-3t/\rho)$$

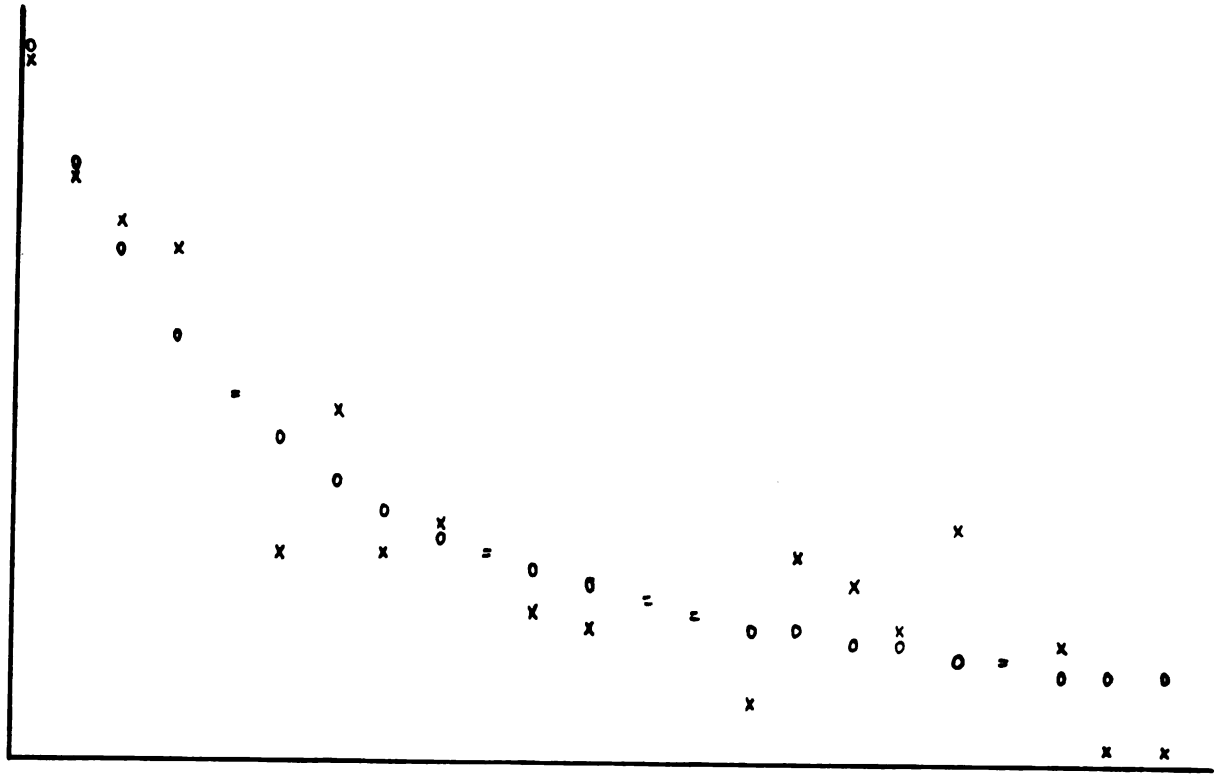


Figure 21. This is Lysozyme at 23.5 C°

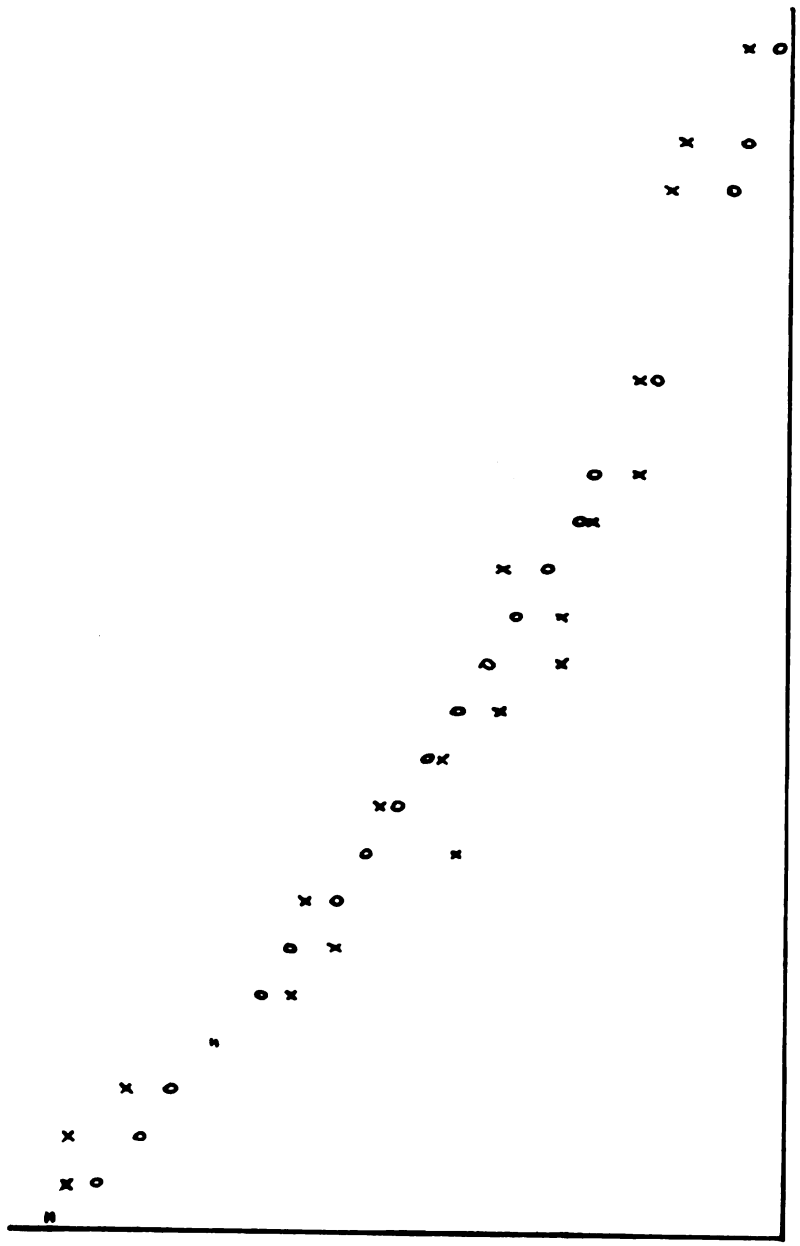


Figure 22. This is Lysozyme at 25.5 C°

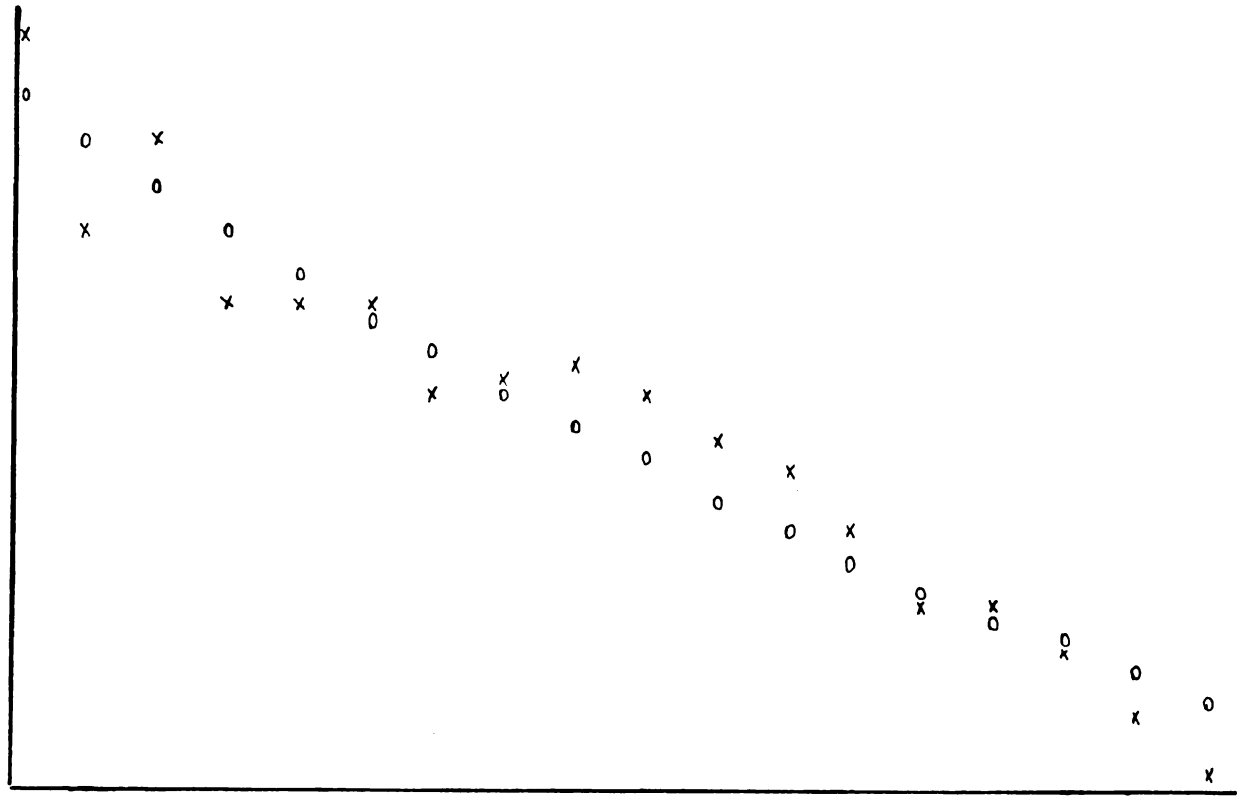


Figure 23. This is Lysozyme at 29.4 C°

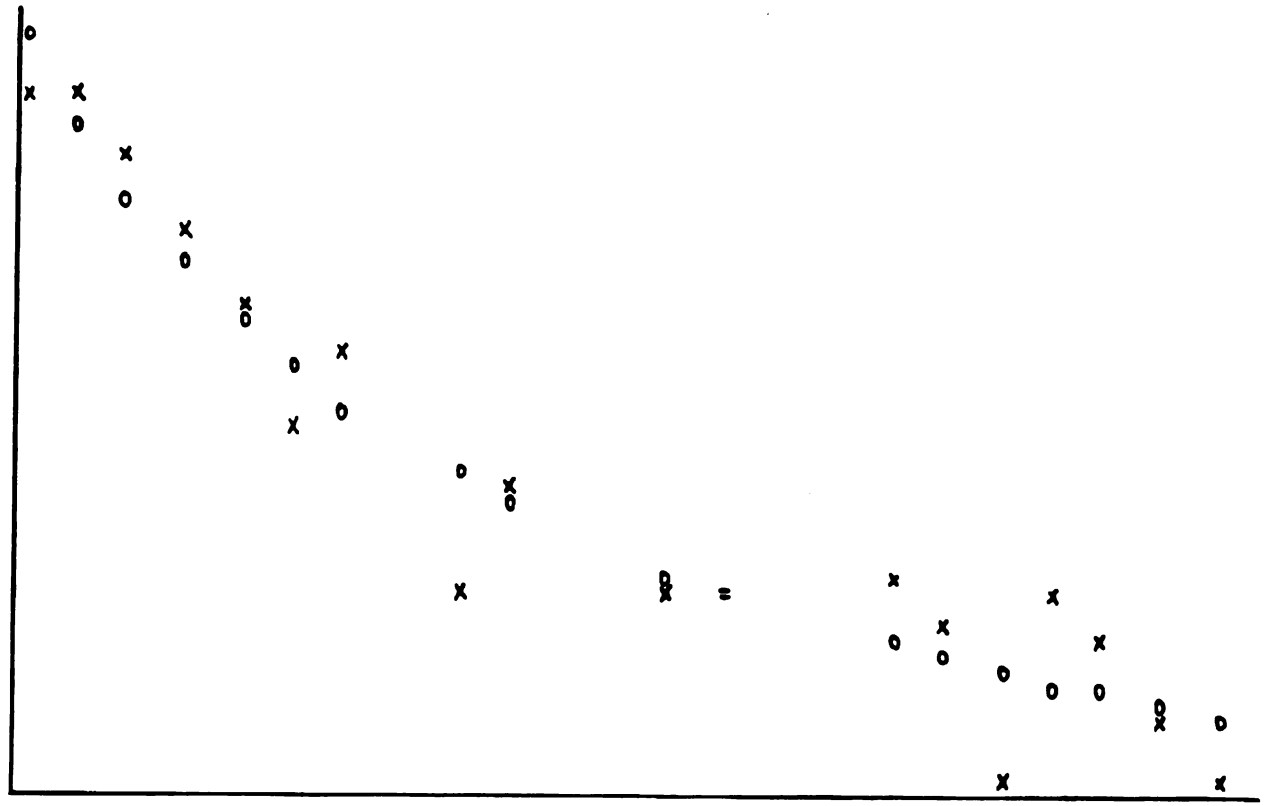


Figure 24. This is Lysozyme at 25.5 C° in sucrose solution.

APPENDIX C

The following graphs are the KINFIT curves for Rhodamine B in glycerol. In these curves, the vertical axis represents the time dependent fluorescence aniostropy and the horizonal axis represents time. The x indicate experimental points, the o indicate calculated points for the best fit curve, and the = indicate coincidence of an experimental and calculated point. The form of equation fit was;

$$A(t) = A(o) \sum_{i=1}^{2} f_i \exp(-3t/\rho_i)$$

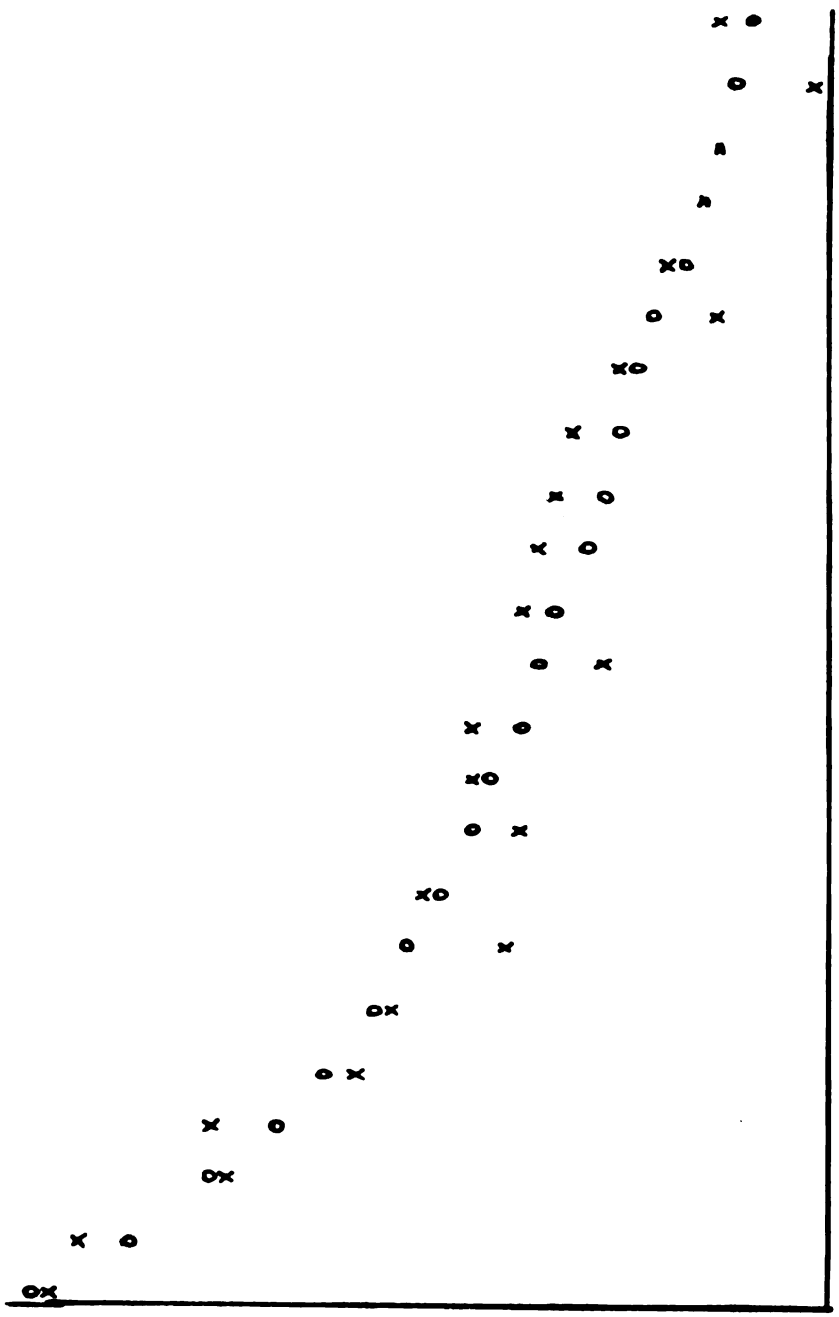


Figure 25. This is Rhodamine B in glycerol at 23.5 C°.

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