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PROTEIN CRYSTAL NUCLEATION KINETICS USING RELATIVE LIGHT-SCATTERING TECHNIQUES – Center Director's Discretionary Fund Final Report

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TECHNICAL MEMORANDUM

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INTRODUCTION

Light-scattering intensity measurements are a sensitive method for following changes in the hydrodynamic radius of particles in solution. The goal of this project was to determine the equilibrium and kinetic constants, and to formulate a model for the initial stages of tetragonal lysozyme crystal nucleation. Light-scattering data, obtained while all components of the system were functioning, has given critical insight into the probable pathway by which tetragonal lysozyme crystals nucleate and grow.

The data are obtained from determination of the light-scattering intensity ratio:

$$\frac{I_{s2}}{I_{s1}} = \frac{C_2 M_{r2}}{C_1 M_{r1}} \qquad (1)$$

where I_{s2} and I_{s1} are the light-scattering intensities of the aggregating and monomeric systems, C is the weight/volume concentration of the scattering species, and Mr is their relative molecular masses, respectively. Assumptions are made concerning the second virial coefficient, the refractive index increment, and that all scattering species are roughly spherical, to reduce the classical light-scattering equations to this form. However, in this case both the aggregating and reference systems have the same gross component compositions, differing only in the aggregate distributions. All scattering species are assumed to be soluble (i.e., no post-critical nuclei are present). Shape factor effects will have to wait for more intimate knowledge of the actual aggregation pathway. However, unless aggregation and growth are by propagation of long rods, the assumption of spherical particles should not drastically alter the results.

This work was preceded by an extensive series of experiments to follow the gross protein crystal nucleation process through the disappearance of soluble protein to insoluble crystals over time. While a considerable number of experiments had been done, the data were variable, and not amenable to detailed analysis. However, it was determined that nucleation of tetragonal lysozyme crystals is not diffusionally, but rather a kinetically controlled process. A 50-fold change in the solution viscosity had no discernible effect on the gross nucleation rate.

Solubility determinations were to be done as required. However, at the time of proposing, this was anticipated to be a very minor effort due to extensive ongoing efforts in progress at the University of Alabama in Huntsville (UAH), and the prior determination of many points of interest in this laboratory.

RESULTS

A spectrofluorometer was assembled using commercially available components (Oriel Inc.). With the emission and excitation monochromators at the same wavelength, this instrument can be used for 90-deg light-scattering intensity measurements (Fig. 1). The photomultiplier outputs were connected via preamplifiers and a Keithley 500 data acquisition system to an IBM PC XT type computer used for data acquisition and processing.

Aggregation rate experiments were used for the initial trials of the system. Equal volumes of protein and precipitant solutions were injected into a thermostatted cuvette in the light-scattering apparatus. Data collection was manually initiated. These experiments were preliminary in nature, more to test the system than collect hard data. However, the results indicated that overall aggregation rates for the formation of tetragonal lysozyme nuclei is slow, conforming to the results previously obtained using precipitation rate experiments.

There was considerable difficulty in obtaining an accurate start point in the data set. However, the data appear useable, even accepting the inaccuracies in fixing the t=0 point. By numerically modeling the process, and fitting the model curves to the data (Fig. 2), a rate constant $k_1 = 135\ M^{-1}s^{-1}$ was estimated for the conditions given (pH 4.0, 5% NaCl, 15°C). This estimate is based upon a best fit to the data for the first 500 msec only. The model assumes that the initial event is the association of two monomers to form a dimer, and that the reverse reaction is negligible (a valid assumption under these conditions).

Following tests using a kinetic system, the procedural details for equilibrium mass ratio determinations were developed. Conditions chosen for the initial experiments were pH 4.0, 22°C, 3.0% NaCl, which gives a $C_{sat} = 10$ mg/ml for lysozyme. Monomeric lysozyme light-scattering measurements were done using a 1.0% NaCl concentration ($C_{sat} >> 100$ mg/ml), which has been previously shown to give a monodisperse protein solution [1,2]. For an aggregating system, a plot of I_{s2}/I_{s1} (I_{s2} = aggregating and I_{s1} = all monomer light-scattering intensities) versus the total molar protein concentration [M] in the system will give a curve which extrapolates to 1.0 as [M] → 0 (Fig. 3).

The equilibrium distribution was modeled assuming a monomer → dimer [Fig. 4(a)], then a monomer → dimer → tetramer [Fig. 4(b)] system. Model-derived light-scattering intensity ratio curves were matched to the data. An estimated $K_{eq(1)} \sim 5000\ L^{+1}M^{-1}$ was determined for the dimerization equilibrium constant, while $K_{eq(2)} \sim 20{,}000\ L^{+1}M^{-1}$ is estimated for the dimer → tetramer process.

A White light source
B Excitation monochromator
C Excitation slit
D Beam splitter
E Sample cell
F Emission slit
G Emission monochromator
H Sample photomultiplier
I Reference photomultiplier

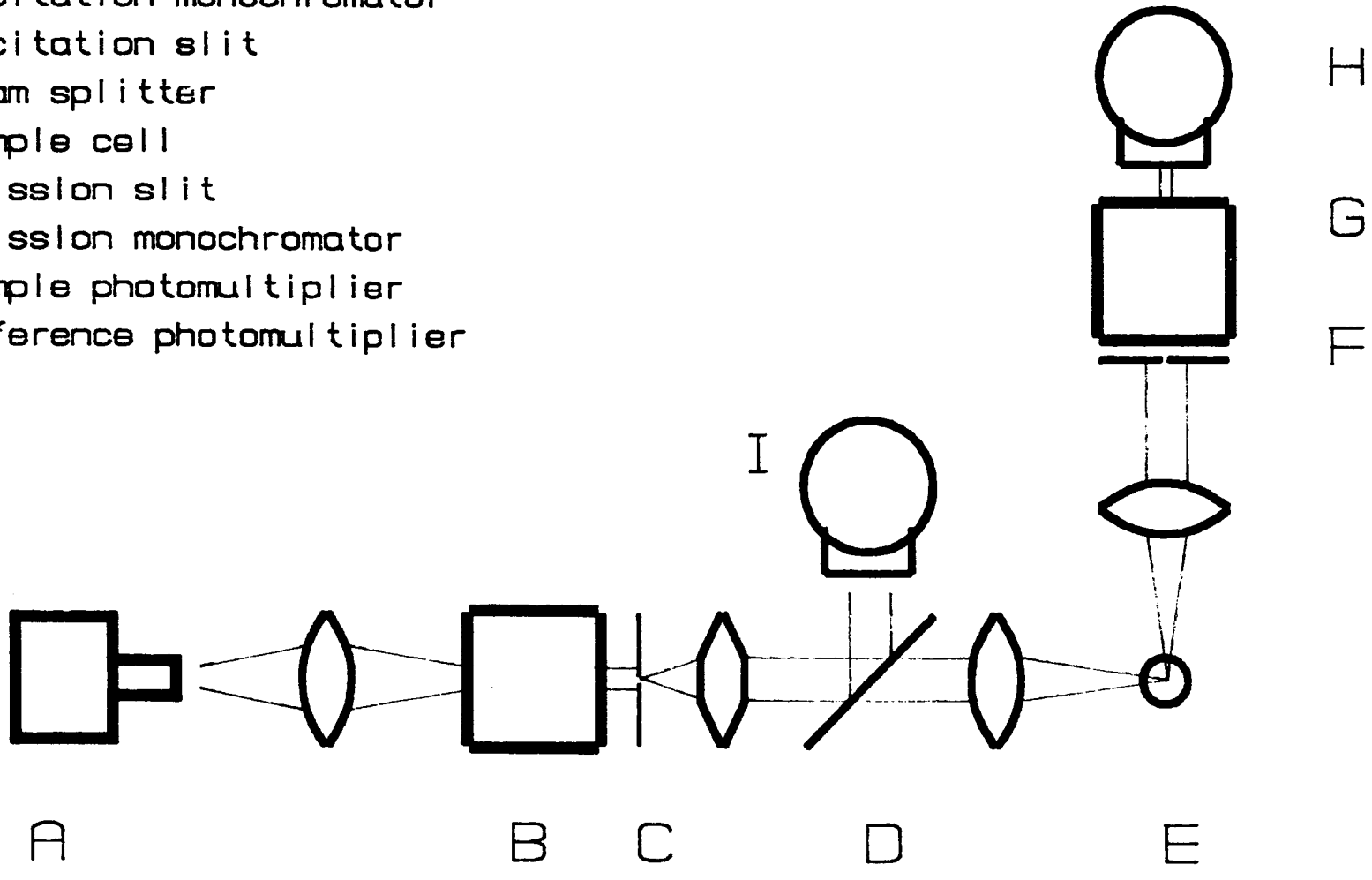

Figure 1. Schematic of light-scattering apparatus.

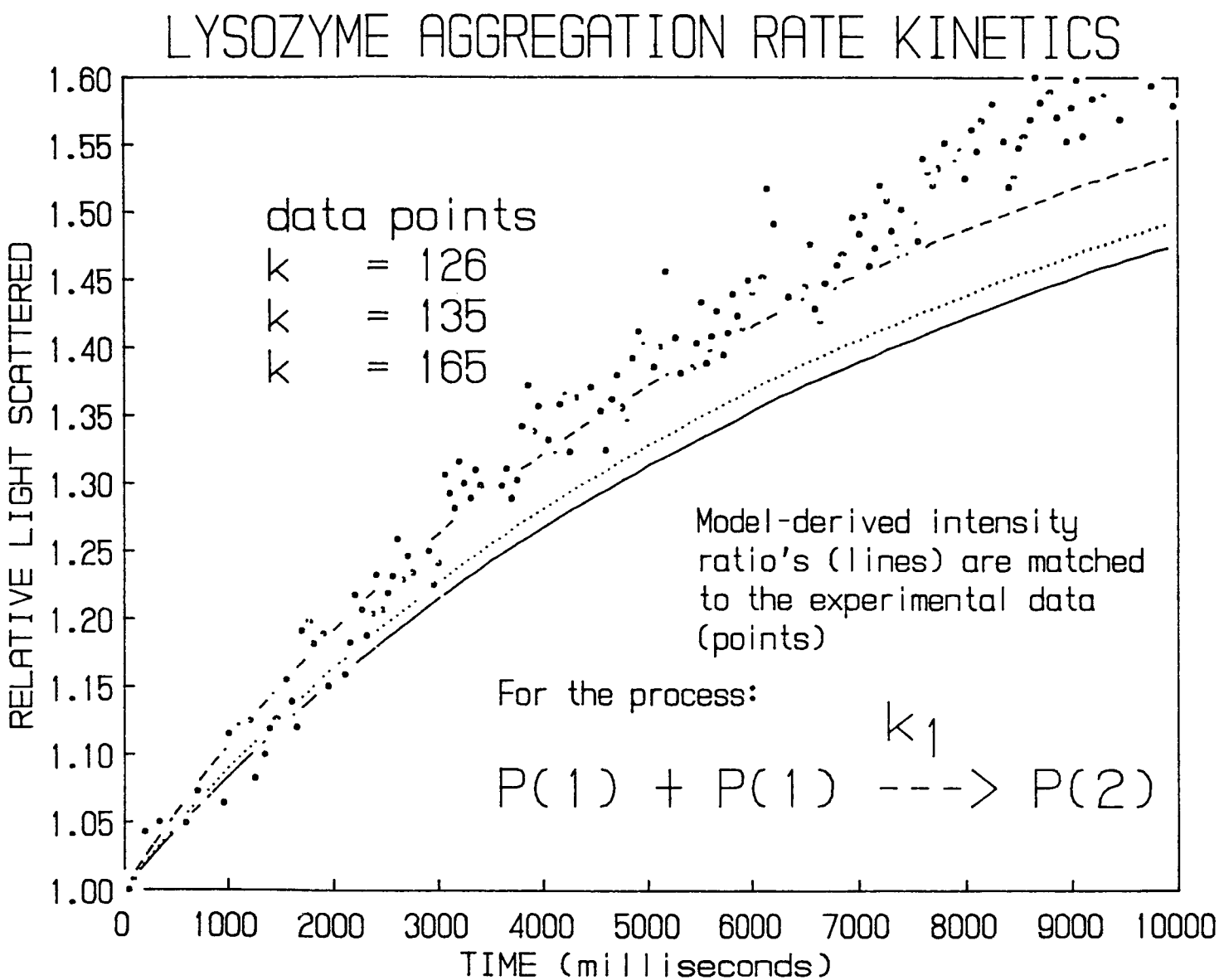

Figure 2. Lysozyme aggregation rate data and model curves for a monomer → dimer reaction.

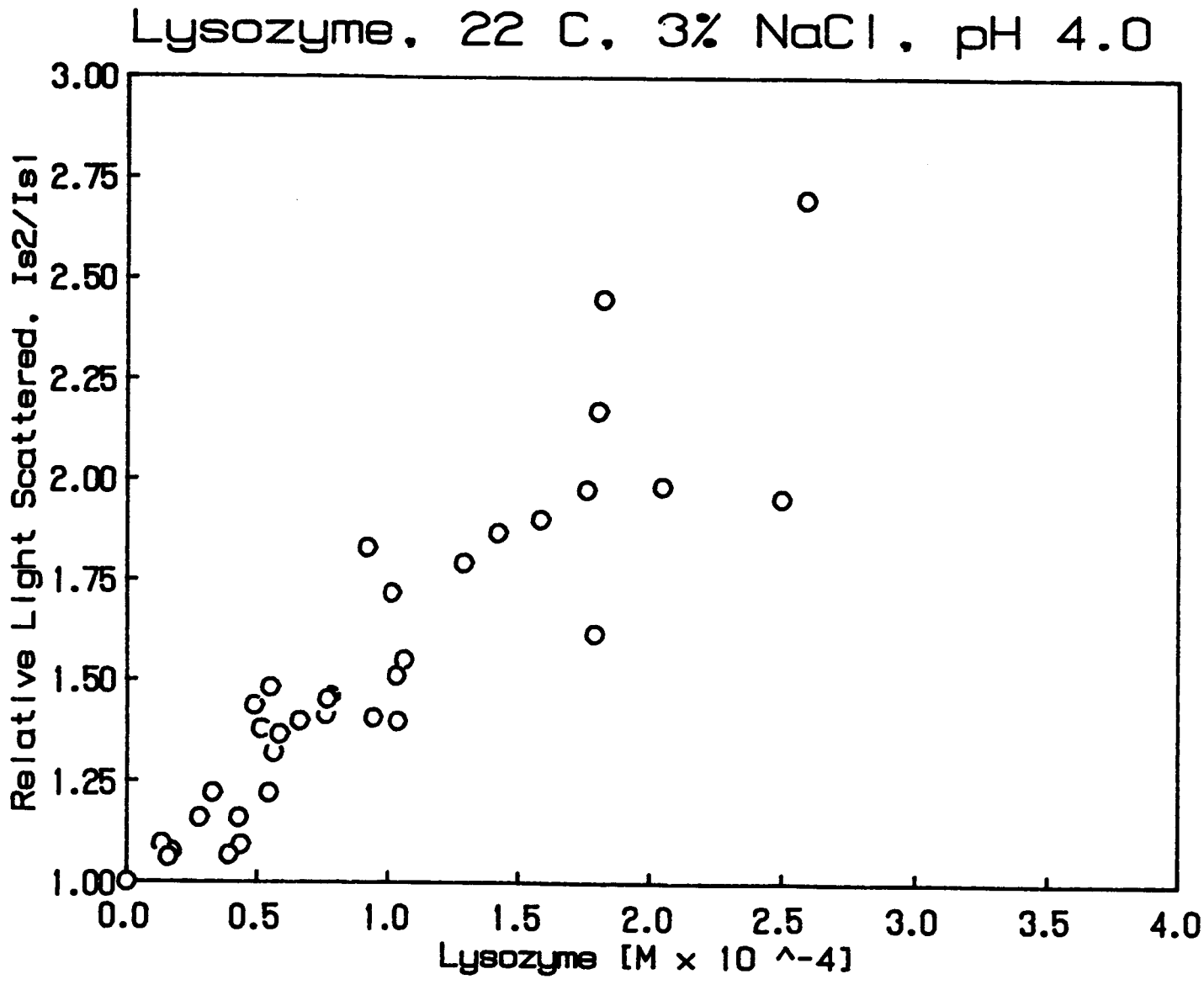

Figure 3. Summary of the equilibrium light-scattering intensity ratio data for lysozyme.

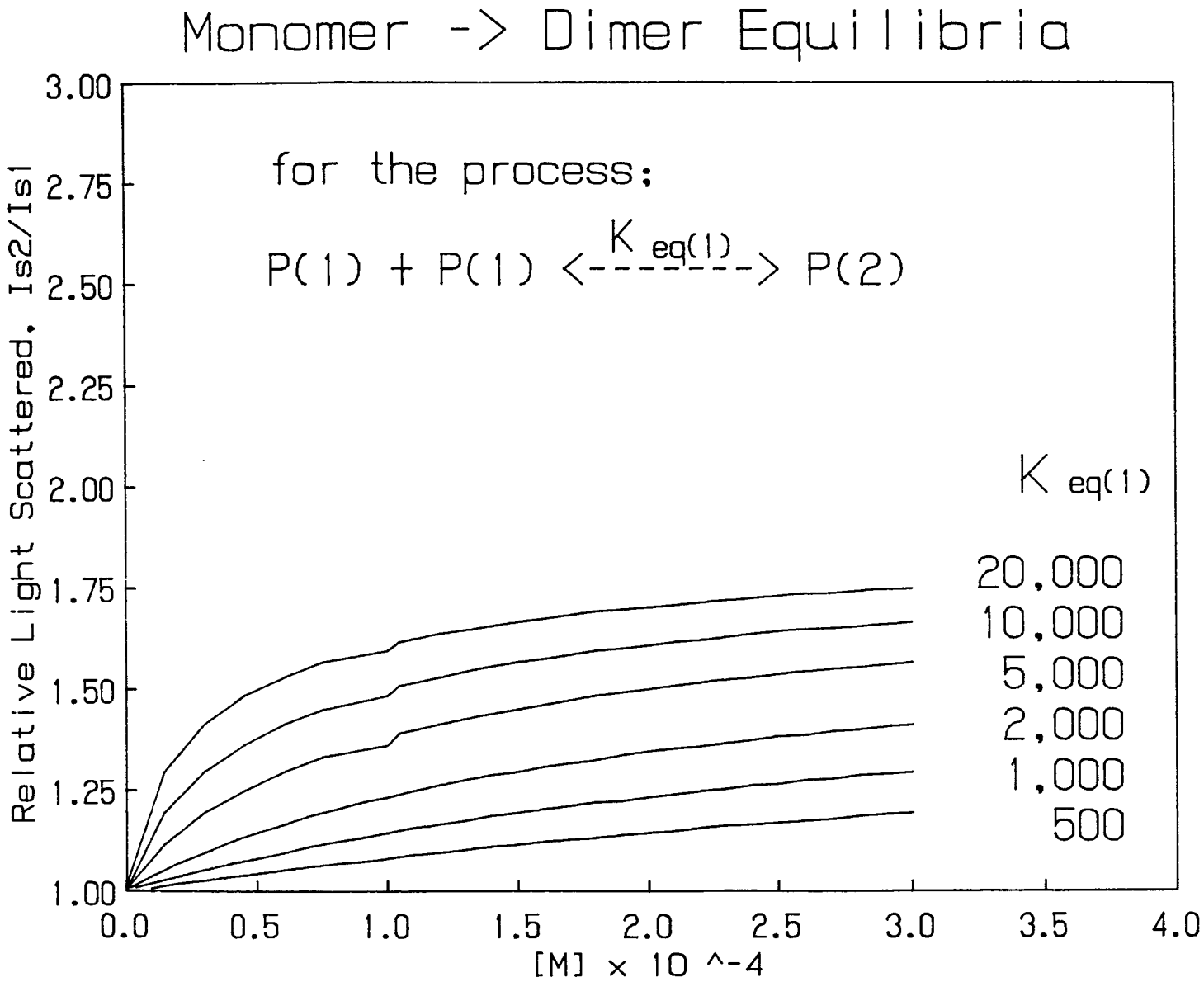

Figure 4(a). Calculated light-scattering intensity ratios assuming a monomer → dimer distribution only.

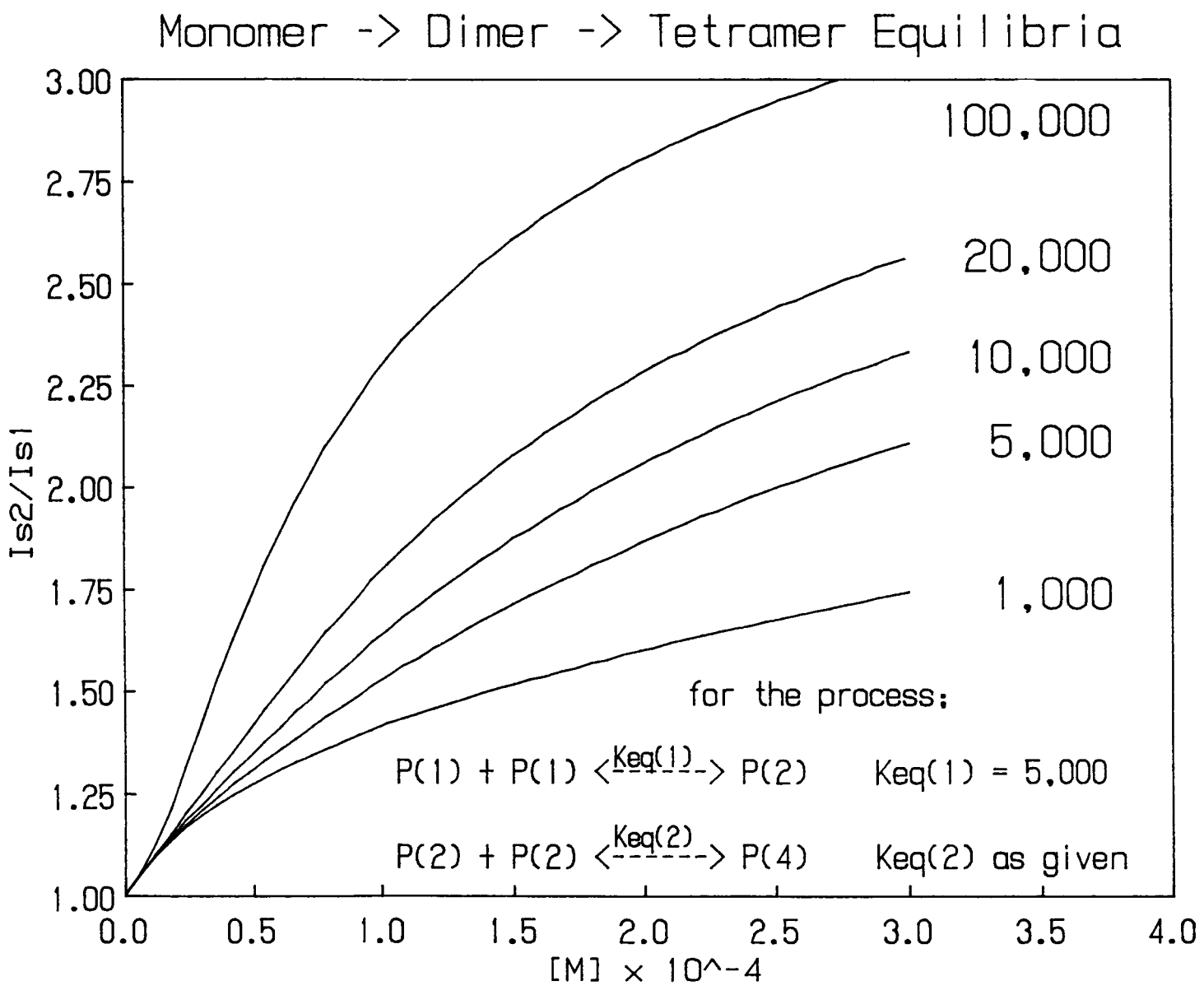

Figure 4(b). Calculated light-scattering intensity ratios assuming a monomer → dimer → tetramer distribution.

The optimal experimental procedure was to start at the highest protein concentration to be used. After determination of the light-scattering intensity at this point, the system was diluted by removal of an aliquot (used to determine the protein concentration), followed by addition of a protein-free buffer solution to the cuvette. Equilibration times after dilution were typically $\geq$ 1/2 hour, and therefore only 5 to 7 data points could be collected per day. Also, because of instrumentation drift the baseline had to be redetermined every 2 to 3 data points.

Data collection came to a standstill due to problems with the protein and the light-scattering system. The appearance of atypical light-scattering data led to an investigation of the lysozyme purity. The material, as supplied, was 3x crystallized, and was routinely recrystallized and extensively dialyzed in the laboratory prior to use. Size exclusion chromatography gave an unsymmetrical single major peak with several smaller peaks at higher molecular masses. Repeated chromatography of the leading and trailing edges of the major peak showed it to be composed of two closely co-chromatographing species. Both fractions also gave anomalous low-scattering data. When commercial lysozyme was freshly prepared, and chromatographed using cation exchange chromatography, five or more peaks were observed depending upon the elution protocol used. Also, after several runs, a band of yellow was found accumulating at the top of the column. This material was eluted when the column was cleaned, and found to have a molecular mass of ca 45 to 60 K (lysozyme MW = 14.4 K) by size exclusion chromatography.

Despite the obvious impurity of the protein, the continued atypical data suggested that (part of) the problem was elsewhere. Surface induced effects from the cuvettes are now thought to be a prime suspect. Treatment with surfactants (silanization) has altered the type of anomalous data (atypically low versus atypically high). The effects are usually reversed after rigorous cleaning. New cuvettes were ordered, but they immediately gave atypically low results when tried.

ANALYSIS AND DISCUSSION

Assuming that the initial process in the formation of tetragonal lysozyme nuclei is a bimolecular association reaction, then a rate of constant of $k_1 \sim 135\ M^{-1}s^{-1}$ is estimated at pH 4.0, 5.0% NaCl, 22°C. In the absence of a known t=0 point, this data can only be considered an approximation at best. This rate is strikingly low when one considers that typical collisional rates for particles of equal size are $\sim 10^9$ - 10^{10} [3]. No attempts have been made at calculation of the second association rate constant k_2 due to the absence of a definite model for the association pathway and of an accurate t=0 point.

The slowness of the equilibration process became especially evident during the collection of the equilibrium data. Whether the cuvette concentrations were adjusted by addition of protein or by dilution with buffer, considerable time was required before the system reached equilibrium. This served to keep down the amount of data which could be collected at any one time. Some instrumentation drift occurred, resulting in the discarding of many data points, and requiring periodic redetermination of the baseline signal. This drift had greatest impact on the low concentration data, precisely where the greatest accuracy was required.

Computer modeling of the equilibrium distributions given an estimate of $K_{eq(1)} = 5000\ L^{+1}M^{-1}$ for the initial dimerization reaction. In contrast, Feher and Kam [4] estimated the first dimerization equilibrium constant at $K_{eq} \sim 50\ L^{+1}\ M^{-1}$ (after conversion from their units) for the conditions pH 4.2, 5% NaCl, 20°C (C_{sat} determined at 5 mg/ml, while we find C_{sat} = 2 mg/ml at pH 4.0). As their data is collected at a higher NaCl concentration, with an attendant lower saturation concentration, it would be expected that values for K_{eq} will increase with increasing NaCl concentrations (decreasing C_{sat}). An estimated 200 fold difference lies between the two values. Also, using a rapid solubility technique devised during this project [5], we find that the C_{sat} value used by Feher and Kam [4] is ca 2 x greater than the actual value. Finally, they determined that 95 percent of the protein was monomeric at their C_{sat}, while the data reported here (see below) suggest that only a small percentage of the protein is monomeric at saturation.

By the 50 percent saturation point, the average mass/particle had risen to ∿3 x the monomeric mass. When the data are extrapolated to the saturation concentration, the average mass/particle is >4 x the monomeric mass. This implies that by the saturation point there is probably very little monomeric protein left relative to the total concentration. From precipitation rate studies and crystal nucleation theory the nucleation concentration is probably well past the saturation concentration. Average mass/particle at this point would be ≥6 x the monomeric mass, and the population of monomer probably approaching zero. The crystal face growth rate also increases with increasing supersaturation. However, one can easily show that because of the equilibrium constants (which will not change with increasing concentration), the aggregate size distributions will be shifted upward with increasing protein concentration. Monomer concentration, both net and as a percentage of the total protein concentration, will decrease rapidly with the appearance of higher aggregates in the system. In fact, this holds true for the succeeding lower order aggregate concentrations as well. A working theory of the tetragonal lysozyme nucleation process has been developed based on these results. This evidence argues that protein crystal nucleation and growth must occur, at least for tetragonal lysozyme, by higher order aggregate addition. While the correct pathway has not been determined, the favored model is for a monomer → dimer → tetramer → octamer series, with crystal growth and nucleation occurring by octamer (the unit cell of tetragonal lysozyme) addition.

As data collection began in earnest, problems arose which have yet to be satisfactorily resolved. First was atypical aggregation behavior, with relatively high relative molecular masses/particle at low concentrations, going to low masses/particle at high concentrations. This is exactly the reverse of what was originally observed and expected. Also, equilibration times became somewhat longer, reducing the number of data points obtained per day. Freshly prepared protein solutions also gave these anomalous results. However, in all cases the protein solution still crystallizes in a reproducible manner.

The sample cuvette had been rigorously washed just prior to the onset of these problems. Suspecting possible surface mediated effects, the cuvette was treated with a siliconizing solution and the light-scattering experiments repeated. At this point, the second set of anomalous results appeared with a decrease in the amount of light-scattering per particle. After re-washing the cuvette, the high-scattering anomalous

data were again observed. New cuvettes were obtained. However, these have only given low scattering data to date. The atypical low-scattering data extend to kinetic experiments. Solutions as much as 10 x supersaturated had no increase in light-scattering intensities over the monomeric system with time.

Finally, it was recently noted that the reference light intensity signal is increasing with increasing scattering. This phenomena had not been previously observed, and it is not yet known if it is due to a fault in the optical or electronic components of the system.

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APPROVAL

PROTEIN CRYSTAL NUCLEATION KINETICS USING
RELATIVE LIGHT-SCATTERING TECHNIQUES –
Center Director's Discretionary Fund Final Report

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The information in this report has been reviewed for technical content. Review of any information concerning Department of Defense or nuclear energy activities or programs has been made by the MSFC Security Classification Officer. This report, in its entirety, has been determined to be unclassified.

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16. ABSTRACT

Light-scattering intensity measurements are a sensitive method for following changes in the hydrodynamic radius of particles in solution. The approach used in this report utilizes the light-scattering intensity ratios of a polydisperse to a monodisperse system. By numerically modeling the process, and fitting the model curves to the data, estimates have been obtained for the dimerization equilibrium constant, the dimer + dimer → tetramer equilibrium constant, and the association rate constant for the dimerization process.

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