PHOTON AND FLUORESCENCE CORRELATION SPECTROSCOPY AND LIGHT SCATTERING OF EYE-LENS PROTEINS AT MODERATE CONCENTRATIONS

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ABSTRACT The bovine eye-lens protein, α_L-crystallin, has been studied with photon correlation spectroscopy to obtain the mutual diffusion coefficient, D_m, with fluorescence correlation spectroscopy to determine the tracer diffusion coefficient, D_T, and with light scattering to get the isothermal osmotic compressibility $(\delta\pi/\delta c)_{P,T}$. The concentration dependence of D_m, D_T, and $(\delta\pi/\delta c)_{P,T}$ up to a volume fraction ϕ of the protein of 2.5×10^{-2} has been interpreted on the basis of four different interaction potentials: (*a*) an extended hard-sphere potential; (*b*) a shielded Coulomb potential; (*c*) a shielded Coulomb interaction where the effect of counterions is included; (*d*) a simple mixed potential. The three parameters D_m, D_T, and $(\delta\pi/\delta c)_{P,T}$ have also been combined in the generalized Stokes-Einstein equation, $D_m = [(\delta\pi/\delta c)_{P,T} \cdot (1 - \phi) \cdot (D_T)]/(k_B \cdot T)$. Our results indicate that, in the case that photon correlation spectroscopy gives the mutual diffusion coefficient D_m, the applicability of the Stokes-Einstein equation can be questioned; or that, when one assumes the Stokes-Einstein equation to be valid, there is significant discrepancy between the result of photon correlation spectroscopy and D_m.

INTRODUCTION

Two physically distinct diffusion coefficients, which describe the translational motion of macromolecules in solution, can be defined. The tracer or self-diffusion coefficient, D_T, characterizes the random motion of individual solute molecules. The mutual diffusion coefficient, D_m, describes the relaxation of a concentration gradient. For an ideal solution, tracer and mutual diffusion coefficient are both given by the classical Stokes-Einstein relation $D_o = k_B \cdot T/f_o$, where k_B is the Boltzmann's constant, T is the absolute temperature, and f_o the hydrodynamical friction coefficient of a single molecule in pure solvent (Einstein, 1905; Einstein, 1908). For interacting systems, the diffusion coefficients become concentration dependent. The mutual diffusion coefficient is given by the generalized Stokes-Einstein equation

$$D_m = \frac{\left(\frac{\delta\pi}{\delta c}\right)_{P,T} \cdot (1 - \phi)}{f(c)} \qquad (1)$$

(Phillies, 1974). Here $(\delta\pi/\delta c)_{P,T}$ is the isothermal osmotic compressibility of the solute molecules and can be obtained by osmotic pressure measurements and also by light-scattering measurements; $f(c)$ is the friction coefficient of the solute molecules in the interaction system, and is related to the tracer diffusion coefficient by the relation $D_T = (k_B \cdot T)/f(c)$. Different expressions have been proposed for the concentration dependence of both $(\delta\pi/\delta c)_{P,T}$ and $f(c)$, depending on the interaction potential and the way this interaction potential defines the osmotic compressibility and friction coefficient (Hill, 1960; Batchelor, 1972; Van den Broeck et al., 1981).

Older diffusion measurements made use of a macroscopic concentration gradient (Gösting, 1956). Recently, much more accurate and practical techniques have become available. In this work, we make use of fluorescence correlation spectroscopy (FCS) and photon correlation spectroscopy (PCS). FCS detects the random motion of labeled molecules and permits the determination of the tracer diffusion coefficient, D_T (Phillies, 1975). PCS detects all molecules in the scattering volume and is assumed to give the mutual diffusion coefficient, D_m, as given by the generalized Stokes-Einstein equation (Phillies, 1974; Bloomfield and Lim, 1978) under the condition that the interaction length is smaller than the wavelength of the light.

Until now, few unambiguous tests have been performed to prove that PCS indeed measures the mutual diffusion coefficient because several techniques have to be combined. The diffusion coefficient from PCS was compared with different methods to obtain the two other quantities of

the generalized Stokes-Einstein equation. The isothermal osmotic compressibility was obtained from experimental osmotic pressure measurements or from theoretical calculations using a simple hard-sphere potential or using the Carnahan-Starling expression for the hard-sphere potential; the frictional coefficient was obtained from the tracer diffusion coefficient obtained with a diaphragm diffusion cell (DDC) method, from viscosity measurements, or from sedimentation measurements (Phillies et al., 1976; Cazabat et al. 1980; Hall et al., 1980; La Gattuta et al., 1981).

In this paper we present a combination of PCS, FCS, and classical light-scattering measurements on a bovine eye-lens protein, α_L-crystallin, as a test for the generalized Stokes-Einstein equation. For the description of the interparticle potential between the α_L-crystallins, we have accepted a hard-sphere interaction model, modulated by an electrostatic repulsion (Felderhof, 1978). The α_L-crystallin of bovine lens cortical fiber cells has been chosen to contribute to an understanding of the solution properties of the cytoplasma of the lens fiber cells. The major role of the cytoplasma of the vertebrate eye-lens cells is to form a highly refractive medium so that the lens can focus the images on the retina. The high refractive index (n = 1.4 to 1.5) is reached by an increased concentration (up to 40%) of structural proteins, the crystallins. The crystallins form a very complex system with three subclasses based on molecular weight and isoelectric point: α-, β-, and γ-crystallins (Bloemendal, 1982).

Clearly in order to maintain transparency, these molecules cannot act as independent scatterers of light, but have to create by their interactions some kind of local order or at least limit the density fluctuations in the medium (Benedek, 1971). To determine the precise conditions of absolute and relative concentration and ionic strength, which are necessary to maintain a transparent system, many biochemical analyses have been done both on normal and cataractous eye lenses (Harding and Dilley, 1976). But almost no quantitative information is available on the precise physical nature of the interaction between the crystallins and on the role of the different classes of proteins. Most physical measurements, e.g., diffusion and light-scattering, were performed on the total eye lenses or total cytoplasma, making the interpretation very difficult (Benedek et al., 1979; Delaye et al., 1982; Bettelheim, 1975, 1978; Bettelheim and Bettelheim, 1978; Bettelheim and Paunovic, 1979). Nonetheless these studies pointed out that protein-protein and protein-solvent interactions play an important role and give rise to the interesting phenomenon of cold cataract on lowering the temperature, which was interpreted as a phase separation (Tanaka and Benedek, 1975; Tanaka and Ishimoto, 1977; Tanaka et al., 1977). In certain cases, it could be proved that cataract was due to an increase of the phase-separation temperature above body temperature (Ishimoto et al., 1979). This paper reports on diffusion and light-scattering measurements on isolated α_L-crystallins in moderately concentrated solutions. This fraction was chosen because of its important role in the physical properties of the lens fiber cytoplasma, the abundance and homogeneity that can be reached during its isolation, and its suitability to be studied by light-scattering techniques due to its high molecular weight.

MATERIAL AND METHODS

Isolation and Preparation of Lens Proteins

Calf lenses were obtained from a local slaughterhouse and were processed in the cold room. Some lenses (between four and ten according to the amount of proteins that were needed) were gently mixed with a sixth-fold quantity (wt/wt) of buffer (containing 0.04 M Na^+ phosphate, pH 6.8, and 0.003 M mercaptoethanol). The first one-third part, which dissolved after ~4 h, was the cortical part. This suspension was centrifuged for 30 min at 9,000 g to remove all insoluble material and the supernatant contained the soluble material. If small amounts of α_L-crystallin proteins were needed, up to 10 ml of a solution containing up to 3 mg/ml, 1.5 ml of the cortical protein solution was applied to a Bio-Gel A 5m column (1 × 100 cm; Bio-Rad Laboratories, Richmond, CA) and the eluent was collected in 1.5-ml fractions. The α_L-crystallin eluted in a clearly resolved peak. If larger amounts were needed, a zonal centrifugation was performed. ~20 ml of the previously mentioned supernatant (containing ~2,000 $A_{280nm,1cm}$ units) were loaded on a 10–40% sucrose gradient in a Spinco Ti 14 zonal rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, CA) and centrifuged for 24 h at 43,000 rpm. The clearly resolved α_L-crystallin fractions were collected in fractions of 20 ml. As an alternative and more precise method to obtain large amounts of α_L-crystallins, a larger Bio-Gel A 5m column (2.5 × 100 cm; Bio-Rad Laboratories) was used for the separation of the eye-lens proteins. Whenever a protein solution had to be concentrated, e.g., after zonal centrifugation to obtain a protein concentration up to 50 mg/ml, the ultrafiltration method was applied using a concentration cell (model 52, Amicon Corp., Lexington, MA) and a XM 100 filter (Amicon Corp.). If small sample volumes were needed (order of magnitude of 1 ml), a sample concentrator (Minicon B; Amicon Corp.) was used as the last step of the concentration procedure. We distrusted polyethylen glycol or $(NH_4)_2SO_4$ precipitation as a concentrating method because of probable aggregation of the proteins during precipitation.

From PCS measurements, the gel filtration method results in a more homogeneous solution than the zonal centrifugation method where a slight contamination of higher molecular weight crystalllins is always present. All physical-chemical measurements were made in the same buffer, containing 0.04 M Na^+ phosphate, pH 6.8 (ionic strength of 0.08), and 0.003 M mercaptoethanol. Only when the latter could interfere with some chemicals, as for the fluorescein isothyocyanate (FITC) binding, it was omitted during the critical steps. Some measurements were made in higher ionic strength conditions. For that purpose KCl was added to obtain a 0.24 M concentration, so that the ionic strength was increased to 0.32 M.

Fluorescein Labeling of α_L-Crystallin

Fluorescein was used as labeling agent for the α_L-crystallin in the FCS. It was introduced as FITC (Sigma F 7250; Sigma Chemical Co., St. Louis, MO). 30 mg of celite and 3 mg of FITC were dissolved in 10 ml of the above-mentioned phosphate buffer. To this mixture, 5 ml of a α_L-crystallin solution in the same buffer and containing between 5 to 10 mg of proteins was added. After a few minutes, this mixture was centrifuged

to remove the celite. The unbound fluorescein was removed by putting the supernatant solution on a Bio-Gel A 5m column (1 × 100 cm; Bio-Rad Laboratories).

Analytical Boundary Sedimentation

The sedimentation coefficients were determined by boundary sedimentation, using an analytical ultracentrifuge (MSE Scientific Instruments, Crawley, Sussex, England) with double sector cells and absorption optics at 280 nm in combination with an automatic photoelectric scanning device (MSE Scientific Instruments). Rotor speeds ranged between 40,000 and 45,000 rpm and a six-hole rotor allowed simultaneous measurements on different samples. The concentration of proteins ranged from 0.2 to 1 mg/ml and no concentration effects were observed. Reduction of the sedimentation coefficients to standard conditions of water and 20°C yielded $s^\circ_{20,w}$ (Marshall, 1978).

PCS and Absolute Intensity Measurement of Scattered Light

The analysis of the intensity fluctuations of the light scattered by the proteins in Brownian motion is assumed to yield the mutual diffusion coefficient. During the preparation of the samples, several steps were taken to avoid dust particles in the light-scattering volume. Cylindrical glass-scattering cells, stored in an ethanol-HCl mixture, were washed with distilled water and further cleansed by flushing the surface with condensing acetone vapor, using an apparatus specially constructed for that purpose (Tabor, 1972). A centrifugation of the filled scattering cell up to 6,000 rpm in a specially constructed adaptor for a JA 20 or JS 13 Beckman rotor (Beckman Instruments Inc.) for 1 hr, removed the eventually present dust particles from the scattering volume. The temperature was maintained at 25 ± 0.1°C with a Malvern temperature controller RR56 (Malvern Instruments, Malvern, Worchestershire, England). A beam of light with a wavelength of $\lambda = 488$ nm, from an intensity-stabilized argon ion laser (Coherent Radiation, Palo Alto, CA), was focused in the cell. The scattered light was detected with a photomultiplier (FW130; ITT Electro Optical Products Div., International Telephone & Telegraph, Fort Wayne, IN). The single-clipped autocorrelation function of the photon counts was built up in a Malvern type 7023 24-channel high-speed digital correlator (Malvern Instruments), and was continuously displayed on an oscilloscope. A series of single-clipped photon-count autocorrelation spectra were taken at scattering angles of 45°, 90°, and 135°. At each angle, 20 to 30 correlation functions were taken. They were fed into a CBM 8000 minicomputer (Commodore Business Machines, Inc., Santa Clara, CA) and a first analysis was immediately made, fitting the correlation function to a cumulant series of second order (Koppel, 1972) $\ln |g^2(iT) - 1| = \ln A + \Gamma(iT) + X(iT)^2 + \ldots$. The data were stored and after completion of the measurements, a detailed analysis was made according to a procedure proposed by Nieuwenhuysen (1978). An unweighted and iteratively weighted linear and quadratic cumulant analysis is performed. A quality factor $Q = 2X/\Gamma^2$ is calculated and its deviation from 0 is accepted to be due to the presence of dust. We have accepted as the best experimental value of the diffusion coefficient, D, the intermediate value of the different fitting procedures, corresponding to a quality factor $Q = 0$.

The same samples and the same experimental set-up were used for absolute intensity measurements. To relate the intensity I, scattered by the solution, to the incident intensity, I_o, benzene was used as a standard scatterer. The results for benzene were corrected for dark current. After the sin θ correction at different angles for the difference in scattering volume was seen by the photomultiplier, the intensity of the laser light, scattered by the protein solution, was independent of the scattering angle; this can be expected for dust-free solutions of particles, which are small compared with the wavelength of the light. The results were analyzed according to the formula

$$\frac{K \cdot C}{R_\theta} = H_{\lambda,B} \frac{2\pi^2 n^2}{\lambda^4 N_A R_B} \cdot \left(\frac{\delta n}{\delta c}\right)^2 \cdot \frac{C}{\frac{I_{90,\alpha_L}}{I_{90,Ben}}} \cdot C_u(90) \cdot \frac{1 + \rho_{u,B}}{(1 + \rho_u)^2}$$

$$\frac{K \cdot C}{R_\theta} = \frac{1}{RT}\left(\frac{\delta \pi}{\delta c}\right)_{P,T},$$

where $H_{\lambda,B}$, the refraction correction, corrects for the unlike refractive index of the solution n and the calibrating liquid n_B, $H_{\lambda,B} = (n_B/n)^2$; λ represents wavelength, $\lambda = 488$ nm; N_A, Avogadro's number; R_B, Rayleigh ratio of benzene, $R_B = 27.8 \times 10^{-6}\ \mathrm{cm}^{-1}$ (Kratohvil et al., 1962; Cohen and Eisenberg, 1965; Billmeyer et al., 1971; Kaye and Havlik, 1971; Pike et al., 1975); $(\delta n/\delta c)$, refractive index increment, $(\delta n/\delta c) = 0.195\ \mathrm{ml\ g}^{-1}$, (Andries et al., 1982); C, concentration of protein in grams per liter; I_{90,α_L}, measured intensity at 90° of the α_L-crystallin solutions; $I_{90,Ben}$, measured intensity at 90° of benzene; ρ_u, depolarization ratio of solute in excess of the solvent; $C_u(90)$, Cabannes factor $C_u = (6 + 6\rho_u)/(6 - 7\rho_u)$; $\rho_{u,B}$, depolarization ratio of benzene, $\rho_{u,B} = 0.42$ (Kerker, 1969).

The depolarization ratio, ρ_u, of the solute in excess of the solvent for α_L-crystallin was determined experimentally in our laboratory and was very small, namely, $\rho_u = 0.0003$, so that we have $1 + \rho_u \sim 1$. The osmotic compressibility, $(\delta\pi/\delta c)_{P,T}$, derived from the light-scattering measurements, is equivalent to the value determined by the direct measurements of the osmotic pressure for monodisperse solutions. For polydisperse solutions, in the limit of zero concentration, the light-scattering osmotic compressibility gives the weight-average molecular weight when $(\delta n/\delta c)$ is constant for all components. The osmotic pressure measurements result in the number-average molecular weight, which is smaller than the weight-average molecular weight. But the homogeneity of our samples is sufficient as not to give such complications.

Measurement of the Friction Coefficient

The friction coefficient was measured by determining the tracer diffusion coefficient of fluorescently labeled molecules in a FCS setup. This rather original concept measures number fluctuations of labeled molecules in a well-defined laser spot, in the presence of a large excess of unlabeled molecules (typically at a ratio 1:1,000). In this way, one follows the random motion of single molecules in the uniform background interaction potential, created by the unlabeled molecules.

The FCS apparatus consists of an Ar^+-ion laser (SP 164; Spectra-Physics, Inc., Mountain View, CA) whose 488-nm output line is spatially filtered and directed into the fluorescence accessory of a microscope (AO 1–10; American Optical Scientific Instruments, Warner-Lambert Co., Buffalo, NY). The sample solution is kept in 200-μm optical pathlength microslides (Vitro Dynamics Inc., Rockaway, NJ) at the focal plane of the microscope objective. With the use of a 20× objective, the e^{-2} beam radius ω of the Gaussian exciting profile was ~2.45 μm, as measured by a far-field divergence method (Schneider and Webb, 1980) and by a convolution scan method (Magde et al., 1978). The fluorescence light is transmitted by a dichroic mirror and falls upon an EMI 9558 B photomultiplier (EMI, Middlesex, England) in photon counting mode. A small pinhole (400 μm diameter) is placed in front of the photocathode to reduce depth-of-focus.

The resulting pulse train is fed via a CAMAC 100-MHz scaler (CAMAC SR 1608; GEC-Elliot, Leicester, England) into a PDP 11-34 minicomputer (Digital Equipment Corp., Marlboro, MA), which builds up the autocorrelation function in real time and controls the experiment. Proper care of laser intensity fluctuations is taken by a monitor circuit. For this purpose a beam splitter deflects a fraction of the incident laser light to a PIN photodiode. The resulting voltage is transformed by a voltage/frequency converter into a pulse-train that controls the gate

signal of the CAMAC scaler. By means of this hardware correction, the fluorescent light is measured for a constant amount of incident laser light.

In the minicomputer, the autocorrelation function

$$G_j = \frac{\left(\frac{1}{N-j} \cdot \sum_{i=1}^{N-j} n_i \cdot n_{i+j} - \bar{n}^2\right)}{\bar{n}^2} \quad j = 0, \ldots N$$

is constructed, where n_i is the number of photon counts in the interval $(i-1)\,\tau \le t \le i\tau$; the time-window, τ, of the experiments is typically 20 or 30 ms; N is the maximal autocorrelation channel. The autocorrelation of the fluorescence signal is related to the dynamics of the number of fluctuations in the sample by the relationship (Elson and Magde, 1974)

$$G_j = \frac{\beta}{1 + \frac{4D \cdot j \cdot \tau}{\omega^2}} \quad j \neq 0,$$

where β^{-1} is the number of fluorescent molecules in the Gaussian exciting spot with e^{-2} radius ω. D is the tracer diffusion coefficient, D_T. For the measurement of the tracer diffusion coefficient, the number of labeled molecules, β^{-1}, has to be very low, a happy coincidence that tends to increase the autocorrelation signal (Koppel, 1974).

RESULTS

Characterization of the Labeled Proteins

To assure that the application of some physical techniques on unlabeled α_L-crystallins, such as light scattering and quasi-elastic light scattering and the measurement of FCS on labeled α_L-crystallins, can directly be compared, it has to be proven that both particles behave identically in solution. For that purpose, the sedimentation and translational diffusion coefficient of unlabeled and labeled α_L-crystallins was determined. We obtained for both proteins the same results, namely an $s_{20,w}$ of (18.9 ± 0.2) S and a $D_{20,w}$ of (2.50 ± 0.07) × 10^{-7}cm^2 s^{-1}. This proves that the labeling procedure and the label do not change the physical properties of the proteins, within determined experimental accuracy. In another experiment the limit value of D_T at infinite dilution was measured for the labeled proteins in the absence of unlabeled proteins with FCS. This should be equal to the mutual diffusion coefficient, D_m, at the limit of dilution of the unlabeled molecules. In the given experimental conditions, we found $D_{T,20,w}$ equals to (2.43 ± 0.3) × 10^{-7}cm^{2}s^{-1}.

FCS

The rather elegant concept of FCS has been used to determine the tracer diffusion coefficient. It is similar to PCS in that it does not need a macroscopic concentration gradient and therefore measures microscopic fluctuations. It is not able to, however, extract the same information as PCS in highly structured solutions (Weissman, 1980). Phillies (1975) has shown that in the case of a very small number, N_A, of fluorescently labeled molecules in the presence of a large excess, N_B, of unlabeled molecules, the photon current autocorrelation function is given by

$$G(\tau) = \frac{\langle I(0) \cdot I(\tau) \rangle - 1}{\langle I \rangle^2} = Q_A \cdot \frac{\beta}{1 + \frac{4\tau D_T}{\omega^2}},$$

where $I(\tau)$ is the photon current on the photocathode related to the number of photon counts, n_i (Koppel, 1974), and where Q_A is the product of the molar absorption, ϵ_A, the quantum yield Q, and geometrical efficiency g; β^{-1} is the number of molecules observed in the laser spot.

Problems could arise from the polydispersity of the sample, but other techniques (gel filtration, boundary sedimentation) have indicated a high degree of monodispersity. The presence of free FITC, due to the dissociation of the FITC-protein complex, has a negligible influence on the autocorrelation function (see Appendix). The contribution of molecules, diffusing along the optical axis and out of the plane of focus, is a very slow decaying function modulating the expected autocorrelation function. Its decay time is $T_2 = \sigma^2/4D$, where σ is given by (Koppel et al., 1976) $\sigma = \delta/M_o$ cotan α, where δ is the diameter of the image-plane field diagram, M_o is the magnification of the objective, and α is the collection half angle of the objective; σ is of the order of 20 to 40 μm. Furthermore, at the laser intensities used, the autocorrelation function was not affected by the bleaching of the fluorescence probe. Fig. 1 shows the concentration dependence of f_T on the total protein concentration at lower and higher ionic strength. Table I gives the results for a first-order concentration correction $f = f^\circ(a + \lambda\phi)$, where ϕ, the volume fraction, is given by $\phi = cv_2$, where c is the concentration in milligram per milliliter, and v_2 is the partial specific volume of the solute.

PCS

The correlation function for diluted and moderately concentrated solutions were monoexponential within experimental error ($Q < 0.06$). Fig. 2 *a* shows the mutual diffusion coefficient at $\theta = 90°$ and 135° as a function of the volume fraction ϕ. The volume fraction was calculated using $v_2 = 0.740$ cm^{3}g^{-1} for the partial specific volume (S. Coopman, unpublished results). Regression analysis of the data, obtained on two different preparations and at $\theta = 90°$ and 135°, resulted in

$$D_{20,w} = D^\circ_{20,w}(b + \alpha\phi) = 2.40 \times 10^{-7}\ \text{cm}^2\,\text{s}^{-1}\,[(0.97 \pm 0.05) + (8.56 \pm 1.11)\phi].$$

The same value of 2.40 × 10^{-7}cm^{2}s^{-1} has been obtained for $D_{20,w}$ on diluting the concentrated solution to the lowest value (~1 mg/ml), where still a reasonable signal-to-noise

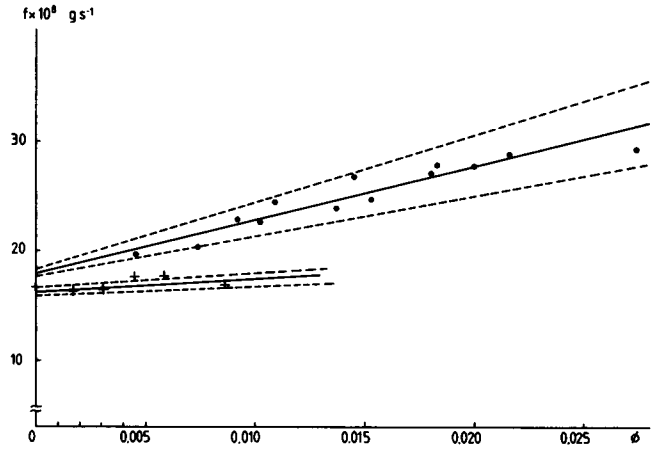

FIGURE 1 Dependence of the tracer friction coefficient, f_T, on the total volume fraction, ϕ; of the proteins at lower ionic strength (●) and at higher ionic strength (+). The solid lines (—) gives the first-order fit $f = f^\circ (a + \lambda\phi)$, calculated with the least-square method from the experimental points (● or +), and the dashed lines (--) give the uncertainty of this first-order fit (low ionic strength $I = 0.08$, higher ionic strength $I = 0.32$).

ratio can be obtained for the photon counts in reasonable time. This value is slightly lower than the optimal value of $2.50 \times 10^{-7}\text{cm}^2\text{s}^{-1}$, which can be obtained if all precautions are taken to obtain a perfectly homogeneous sample, and in this way resulting in a low yield.

Absolute Intensity Measurement

The light-scattering data yielded experimental values for the quantity

$$\frac{1}{RT}\left[\frac{\delta\pi}{\delta c}\right]_{P,T} = \frac{1}{k_B T}\left[\frac{\delta\pi}{\delta c_N}\right]_{P,T},$$

where R is the molar gas constant, and C_N, the number concentration of particles. They could be fitted into the following linear dependence of the volume fraction

$$\frac{1}{k_B T} \cdot \left(\frac{\delta\pi}{\delta c_N}\right)_{P,T} = d + C_v\phi,$$

where we obtained $d = 0.999$ and $C_v = 64.1 \pm 7.7$.

Fig. 2 *b* gives the experimental $1/k_BT \cdot (\delta\pi/\delta C_N)_{P,T}$ values as a function of the volume fraction. If applying the Svedberg relation

$$M = \frac{s^\circ_{20,w} \cdot RT}{D^\circ_{20,w}[1 - \bar{v}_2\rho]},$$

where ρ is the density of the solution, and taking into account the above-mentioned values for the experimental parameters, a molecular weight $M_r = 715{,}000 \pm 45{,}000$ can be calculated. Our absolute intensity light-scattering measurements allows also the calculation of the molecular weight. We obtained in that way, $M_r = 663{,}000 \pm 80{,}000$. Taking into account that different preparations and samples were used and that each final value results from at least four independent experimental sets of data, we can conclude that the agreement is very reasonable.

DISCUSSION

Concentration Dependence of Diffusion Parameters

The concentration dependence of the various diffusive parameters can be calculated for different interaction potentials, according to different schemes. (Ackerson, 1976; Felderhof, 1978; Altenberger, 1979). We choose the Felderhof approach for its numerical simplicity. The tracer diffusion coefficient essentially has only a hydrodynamical contribution

$$D_T = \frac{k_B T}{f_o}[1 + \lambda\phi],$$

while the osmotic compressibility has only a thermodynamical contribution

$$\left(\frac{\delta\pi}{\delta c_N}\right)_{P,T} = k_B T\,[1 + c_v\phi].$$

The mutual diffusion coefficient, as given by the generalized Stokes-Einstein relation, is then sensitive to all contributions. The various integrals have been evaluated numerically for the following choices of the interaction potentials: (*a*) an "extended hard-sphere" potential, where the interaction is given by

$$U(r) = \infty \text{ for } r \leq 2r_o(1 + \beta)$$

$$U(r) = 0 \text{ for } r > 2r_o(1 + \beta),$$

where r is the mean distance between the center of two interacting particles. r_o, the radius of the particles, and βr_o,

TABLE I
CONCENTRATION DEPENDENCE OF f_T ACCORDING TO THE RELATION $f_T = f_0\,(a + \lambda\phi)$

Ionic strength	$D_{T,20,w} = \frac{k_B T}{f_0}$	a	λ	Regression coefficient	Number of measurements
$I = 0.08$, pH 6.8	$2.43 \times 10^{-7}\text{cm}^2\,\text{s}^{-1}$	1.08 ± 0.02	29.5 ± 7.5	0.92	14
$I = 0.32$, pH 6.8	$2.44 \times 10^{-7}\text{cm}^2\,\text{s}^{-1}$	0.98 ± 0.02	7.15 ± 1.5	0.98	6

the extension of the hard-sphere value r_0 to take into account the electrostatic repulsion; (*b*) a shielded Coulomb potential, where the interaction potential is given by

$$U(r) = \infty \quad \text{for} \quad r \leq 2r_0$$

$$U(r) = kT\left[1 + \exp - \left(\frac{r - 2r_0}{\xi}\right)\right]^{-A} \quad \text{for} \quad r > 2r_0,$$

where A is a factor related to the surface charge of the molecule; it is physically defined in the following way, $\delta = \xi\ (\ln A + 0.577)$ is the distance where the interaction energy is of the order of kT. ξ is the Debye-Hückel length (Van den Broeck et al., 1981); (*c*) a shielded triangular potential where the effect of counterions is included; the interaction potentials are given by

$$U(r) = \infty \quad \text{for} \quad r \leq 2r_0$$

$$U(r) = \frac{Z^2e^2 \exp - \dfrac{(r - 2r_0)}{\xi}}{\epsilon k_B T \cdot \left(1 + \dfrac{r_0}{\xi}\right)^2 r} \quad \text{for} \quad r > 2r_0,$$

where Ze is the charge of the molecule and ϵ is the dielectric constant of the solvent (Phillies, 1974); (*d*) a simple mixed potential, where the interaction potential is given by

$$U(r) = \infty \qquad \text{for} \quad r \leq 2r_0$$

$$U(r) = \frac{w}{c}(r - 2r_0) - w \quad \text{for} \quad 2r_0 < r \leq 2(r_0 + c)$$

$$U(r) = 0 \qquad \text{for} \quad r > 2(r_0 + c),$$

where w is the depth of the attractive well in units of kT at $r = 2r_0$ and $2\ (r_0 + c)$ gives the extension of the interaction potential.

For each of the different interaction potentials, we adjusted the parameters to obtain a value for the concentration dependence of D_T, D_M, and $(\delta\pi/\delta c)_{P,T}$ that agrees with the experimentally determined values. Table II lists the parameters obtained for the four above-mentioned expressions of $U(r)$. To estimate the extent to which some parameters are realistic, and others not, it is useful to calculate the interparticle spacing at a moderate concentration of 25 mg/ml. This value equals $\rho_0^{-1/3}$ where ρ_0 is the number density of particles (cm^{-3}) and is given by $\rho_0 = CN/M$ (Brown et al. 1975), and a mean center-to-center distance of 380 Å is obtained. From the value of the diffusion coefficient at low concentration, accepting a spherical particle, a hydrodynamical radius, r_0 of 85 Å can be calculated for the α_L-crystallins, using the expression $D_m^o = D_T^o = (k_B T)/(6\pi\eta r_0)$, where η the viscosity of the solvents.

From the experimental osmotic compressibility and tracer diffusion coefficient, the parameter β, if accepting the interaction potential 1, and the parameter δ, if accepting the interaction potential 2, can be calculated; for β and

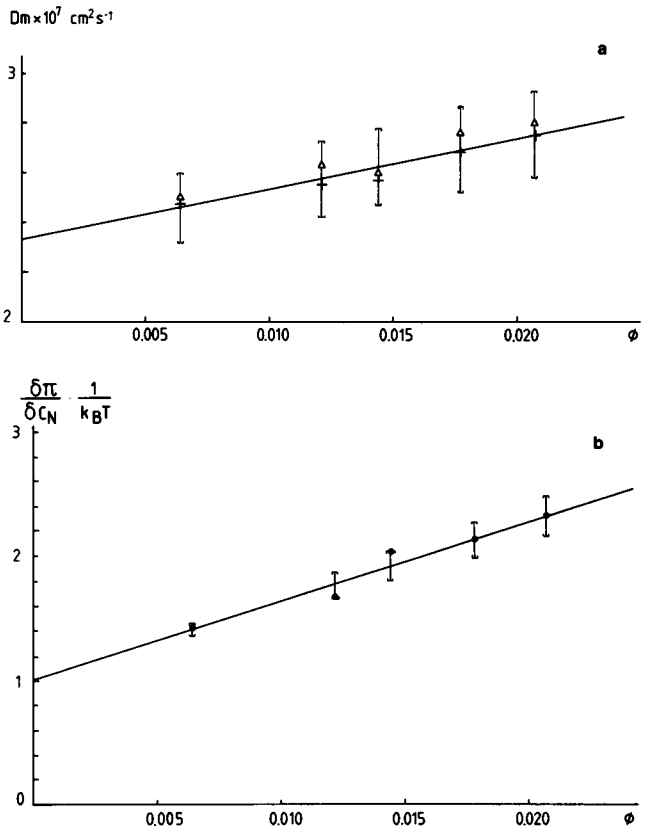

FIGURE 2 (a) Dependence of the mutual diffusion coefficient D_M deduced from PCS on the volume fraction of the proteins at a scattering angle of 90° (△) and at a scattering angle of 135° (+). The solid line (—) gives the first-order fit $D^\circ_{20,w} - D^\circ_{20,w}\ (b + \alpha\phi)$ calculated with the least-square method from the experimental points (△ and +). The brackets (⌶) indicate the uncertainty on this first-order fit. (*b*) Dependence of $\delta\pi/\delta c_N \cdot 1/k_B T$ on the volume fraction ϕ. ● indicates, mean value of the intensity at $\theta = 90°$. The solid line (—) gives the first-order concentration dependence $1/k_B T \cdot (\delta\pi/\delta c_n)_{P,T} = d + C_V\phi$, calculated with the least-square method from the experimental points, and the brackets (⌶) indicate the uncertainty of this first-order fit at the experimental volume fractions.

δ realistic and corresponding values are obtained. Indeed, for the lower ionic strength case, an expansion of the hard-sphere potential from the value $r_0 = 85$ Å to $r_0\ (1 + \beta) = r_0\ (1 + 1.06) = 175$ Å in potential 1, is almost identical to the distance $r_0 + \delta/2 = 85 + 175/2 = 172$ Å where the shielded Coulomb potential is felt in potential 2. At higher ionic strength, an expansion of $r_0 = 85$ Å to $r_0\ (1 + \beta) = r_0(1 + 0.11) = 94$ Å is close to the values of $r_0 + \delta/2 = 90$ Å of potential 2. For both potentials 1 and 2, the mutual diffusion coefficient gives an appreciably lower value for the parameters, describing the expanded hard-sphere or Coulomb expansion.

Starting from the modified Debye-Hückel potential 3 and accepting the Debye-Hückel length, which can be calculated from the ionic strength, the experimental osmotic compressibility and tracer diffusion coefficient give unrealistic values for the charge of the α_L-crystallin. Even the value of 75, which can be calculated from the

mutual diffusion coefficient expansion, seems too high (Siezen and Owen, 1982). For the mixed potential 4, an interaction energy of 1 kT at a distance $r_0 + C$ seems to confirm the results of potentials 1 and 2. The correspondence between the osmotic compressibility expansion C_v ($r_0 + C$ = 147 Å) and tracer diffusion coefficient expansion λ ($r_0 + C$ = 265 Å) is not as good as for the potentials 1 and 2, but can still be considered as convenient. For this potential, the mutual diffusion coefficient expansion, α, seems again to give rise to a value of $r_0 + C$ far outside the range of acceptable values.

Further evidence that suggests that a mixed interaction potential does not describe our situation very accurately is provided by an equality, derived by Van den Broeck and co-workers (1981), from tabulated values in the calculation of the first-order effects for pure repulsive or attractive potentials, namely,

$$\lambda_{int} = \lambda - 6.55 \leq \tfrac{1}{2} C_{int} = \tfrac{1}{2} (C_v - 8).$$

In our case, the experimental data yielded

$$29.5 - 6.55 = 22.95 \leq \tfrac{1}{2} (64 - 8) = 28.$$

The published values for the inorganic ion concentration of the lens can be used to calculate the ionic strength for the normal and cataractous lens. For the normal bovine lens, we obtain I = 0.12 (Duncan and Bushell, 1975). For cataractous lenses, the ionic strength can be as high as I = 0.25 and depends on the type of cataract. So our measurements at I = 0.08 and I = 0.32 cover the range of physiological conditions.

Our results show unambigously that under these conditions the interactions between the proteins play an important role in the light-scattering properties of the α_L-crystallin solutions. They also prove that the higher ionic strength of many cataractous lenses has a considerable influence on the protein interactions and could be partly responsible for increased light scattering. The use of moderately concentrated protein solutions made the characterization of the interactions as a function of a two-body potential possible. Further studies on highly concentrated α_L-crystallin solutions at physiological protein concentrations will give more direct information on the role of the α_L-crystallins in the transparence of the eye lens, but will be more difficult to interpret because the interaction becomes a many body problem with higher-order contributions.

Application of Generalized Stokes-Einstein Equation

To test if our measurements obey the generalized Stokes-Einstein equation, we compared two sets of D_m values, namely, the values obtained from photon correlation measurements and the values that can be calculated from the generalized Stokes-Einstein equation (Eq. 1)

$$D_m = \frac{\left(\frac{\delta \pi}{\delta c}\right)_{P,T} \cdot (1 - \phi)}{f(c)}.$$

The results of Table II indicate that the interaction length is much smaller than the wavelength of light, a condition that has to be satisfied for the application of Eq. 1 (Phillies 1974).

This comparison has to show if the use of the terms "mutual" and "tracer" diffusion coefficient for the measured coefficients by PCS and FCS is justified. Fig. 3 shows the mutual diffusion coefficient, in function of concentration, for the two sets of independent measurements and calculations. Comparison shows that both the absolute values and the slope of the experimental D_m values are lower than the combined results of isothermal osmotic compressibility and FCS, but the differences just fall within the experimental error. Because of this result, a critical examination of the experimental evidence, presented in the literature, is necessary.

Phillies and co-workers performed photon correlation measurements on bovine serum albumin and combined their results with osmotic pressure measurements and tracer diffusion values from the literature (Phillies et al., 1976). Their values for D_m, predicted by the generalized

TABLE II
PARAMETERS OF THE DIFFERENT INTERACTION POTENTIALS THAT YIELD THE EXPERIMENTAL VALUES OF THE COEFFICIENTS λ, a, AND C_v

Experimental method and ionic strength conditions	Potential 1	Potential 2		Potential 3		Potential 4	
	β	ξ (Å)	δ (Å)	Z	ξ (Å)	$w(kT)$	C(Å)
I = 0.08 ξ = 11.9 Å							
Osmotic compressibility, C_v	$1.06^{1.13*}_{0.98}$	12	175^{181}_{165}	$20{,}000^{27{,}000}_{14{,}000}$	12	1.0	62^{78}_{45}
Tracer diffusion coefficient, λ	$1.16^{1.45}_{0.91}$	12	187^{206}_{170}	$32{,}000^{90{,}000}_{13{,}000}$	12	1.0	180^{350}_{70}
Mutual diffusion coefficient, a	$0.58^{0.64}_{0.54}$	12	48^{55}_{42}	75^{105}_{58}	12	1.0	0.1^{1}_{0}
I = 0.32 ξ = 6.2 Å							
Tracer diffusion coefficient	0.11	6	10	30	6	1.0	15

*The number above, $^{1.13}$, and below, $_{0.98}$, the value 1.06 indicate the upper and lower limit of this parameter, taking the uncertainties of the experimental data into account.

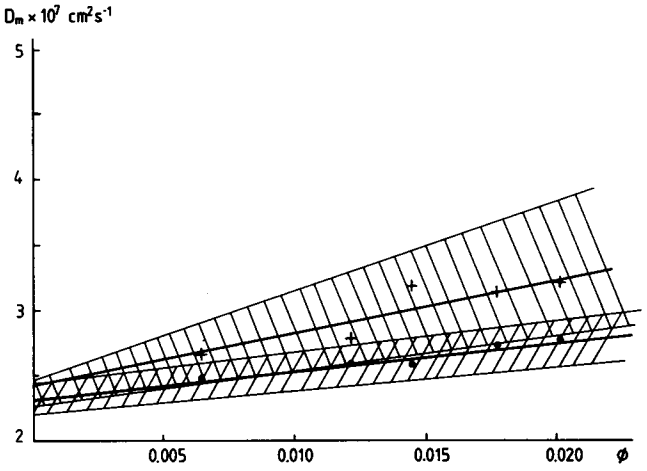

FIGURE 3 Comparison between the experimental mutual diffusion coefficient D_m (●), as deduced from PCS, and the theoretical values (+), which can be calculated from the Stokes-Einstein relation based on the experimental values of the tracer diffusion coefficient and the osmotic compressibility. The solid lines (—) give the first-order concentration fit for both cases $D_m = D_m^o (x + y \phi)$, calculated with the least-square method from the values (● or +). The zone for the experimental values ▨ and the zone for the calculated values ▩ give the uncertainty region for these first-order relationships.

Stokes-Einstein equation at high concentration and acidic pH, are systematically too small, but at higher pH values (5.9–6.1) and intermediate concentrations (30–100 g/l) the predicted values are systematically too high. Nonetheless, they conclude that their results roughly confirm the predictions of the generalized Stokes-Einstein equation, but their data are hampered by their use of different batches of BSA so that an adjustable parameter for D_o had to be used.

Alpert and Banks (1976) compared the PCS on hemoglobin, close to the isoelectric point, with tracer diffusion data (DDC and a radioactive detection technique) from the literature, accepting the hard-sphere expression for the osmotic compressibility. They found that their photon correlation results were greatly overestimated; agreement could be found with the DDC measurements by introducing an additional hydrodynamic correction factor

$$D_m = \frac{\frac{\delta\pi}{\delta c}(1 - \phi)}{f(1 - 6\phi)}$$

for the concentration dependence of the friction factor (Phillies, 1975).

Veldkamp and Votano (1976) performed photon correlation, osmotic pressure, and viscosity measurements on hemoglobin in high salt solutions (Veldkamp and Votano, 1976). A comparison was made between the measured diffusion coefficient and the prediction of the generalized Stokes-Einstein equation by substituting the Stokes relation $f = 6\pi \cdot \eta(c) \cdot r$ for the concentration dependence of the friction coefficient f. The experimental values are systematically lower except at very high concentrations (larger than 140 g/l), but as the authors point out it is questionable if the combination of the Stokes and generalized Stokes-Einstein relation can be used for higher concentrations. Also the use of literature data for the tracer diffusion coefficient of hemoglobin results in an underestimation of the experimental D_m as compared with the calculated one. Cazabat and co-workers (1980) used their absolute and dynamic light-scattering data on water-oil micro-emulsions to calculate the friction coefficient. Comparison with the values found in the literature for the friction coefficients from sedimentation concludes once more that the photon correlation diffusion coefficient is overestimated by the generalized Stokes-Einstein equation, although in this case it is not clear whether the friction coefficient deduced from sedimentation and light-scattering photon correlation are the same physical parameters. Hall and co-workers (1980) compared their photon correlation data on hemoglobin with viscosity, sedimentation, and tracer diffusion measurements by diaphragm diffusion cell (DDC) from the literature, and they found excellent agreement although no estimate for the experimental error is given.

La Gattuta and co-workers (1981) described their photon correlation diffusion measurements using data from the literature on the friction coefficient (DDC) and the theoretical Carnahan-Starling expression for hard spheres for the isothermal osmotic compressibility. Their calculations, where an adjustable parameter is introduced to account for the deviation from a pure hard sphere, seem to overestimate the experimental mutual diffusion coefficient for concentrations below 80 g/l, but the scatter on the data is considerable. Also theoretical considerations resulted in the restriction of the conditions under which the generalized Stokes-Einstein relation is valid.

Where Phillies (1974) considered only one restriction, namely that the wavelength λ of the light has to be larger than the interaction length, Ackerson (1978) claims that this relation is valid under much more limited conditions. He concluded that the scattering vector has to be zero and that the hydrodynamic interactions have to be neglected to obtain the generalized Stokes-Einstein equation; if one does not accept these restricting conditions, the correlation function becomes nonexponential with a K-dependent diffusion coefficient. From this literature overview, we can conclude that for the majority of macromolecular solutions where this problem has been examined, there is a clear tendency for the photon correlation diffusion coefficient to be lower than the mutual diffusion coefficient as defined by the generalized Stokes-Einstein equation, but most studies are not conclusive because of the large experimental uncertainties.

In view of the results obtained for the different interaction parameters, starting from the experimental measurements of light scattering, FCS, and PCS, clearly PCS results give a systematic underestimation of the parame-

ters characterizing the interaction potentials, as compared with the realistic values from light scattering and FSC (see Table II). From this it is very tempting to conclude that the failure of the generalized Stokes-Einstein equation is indeed due to the fact that the use of the term "mutual diffusion" for the PCS is not justified. So, the correspondence between the mutual diffusion coefficient and the photon correlation diffusion coefficient is not an established point and deserves further investigation.

APPENDIX

A possible artifact is the presence of free FITC molecules dissociating from the protein complex. Although the equilibrium is very much shifted towards the bound form, it is worthwhile investigating this effect more quantitatively. Indeed, a second process of fluorescence fluctuations interferes with the Brownian motion, namely, the association and dissociation of the free molecule. If c_j is the concentration of α_L-crystallin proteins with j molecules FITC bound to it ($0 \le j \le 7$), and if C_B is the concentration of free FITC (initially zero), the relaxation matrix describing the concentration kinetics becomes very complicated and we have to incorporate not less than 27 contributions to the fluorescence correlation signal.

On the other hand, if we assume that only the last step, i.e.,

$$C_{n-1} + B \rightleftharpoons C_n \tag{A1}$$

is the most important (this is a good assumption for the experimental situation), we can refer to an analytically solved problem (Elson and Magde, 1974). The final autocorrelation function is composed of two contributions

$$G(\tau) = G_0(\tau) + G_1(\tau) \tag{A2}$$

where

$$G_0(\tau) = A \frac{1}{1 + \frac{4D_0\tau}{\omega^2}} \cdot \left[C_{n-1}\left(3 - \frac{2kc_B}{1 + kc_B}\right) + c_n\left(\frac{2kc_B - 1}{1 + kc_B}\right)\right]. \tag{A3}$$

$$G_1(\tau) = A \int_{-\infty}^{+\infty} dv_x \int_{-\infty}^{+\infty} dv_y e^{-(v_x^2+v_y^2)\omega^2/4} \times \frac{c_B}{n^2}\left(\frac{F^- e^{\lambda^- \tau} - F^+ e^{\lambda^+ \tau}}{F^- - F^+} + 2\frac{c_n - c_{n-1}}{1 + kc_B} \cdot \frac{Fe^{-\lambda^+\tau} - Fe^{+\lambda^-\tau}}{F^- - F^+}\right) \tag{A4}$$

with $F^{\pm} = (V^2 D + \lambda^{\pm})/(V^2 D_B + \lambda^{\pm})$; $\lambda^{\pm} = V^2 (D + D_B) + R \pm [(V^2\Delta + R)^2 - 4V^2 \Delta k_f C_n]^{1/2}$; $R = k_f (C_n + C_B) + k_b$; $\Delta = D - D_B$; V = Fourier parameter; A is a constant taking into account experimental parameters; k is the equilibrium binding constant $k = k_b/k_f$, where k_b is the binding rate constant and k_f the dissociation rate constant; D and D_B are the diffusion coefficients of the α_L-crystallin-FITC complex and free FITC, respectively. The first contribution (Eq. A3) is the relaxation of the labeled protein molecules, with the decay time $\tau = \omega^2/4D$; the second term Eq. (A4) takes care of the association-dissociation process and the diffusion of free FITC.

Introducing reasonable values for k, n, C_n and C_{n-1}, we can integrate numerically the relative importance of the two contributions. It turns out that the ratio

$$\frac{Z_1}{Z_0} = \frac{\int_0^\infty G_1(\tau) \cdot d\tau}{\int_0^\infty G_0(\tau) \cdot d\tau}$$

varies between 0.03 and 0.1, so that most of the power spectrum of the fluctuations comes from the proteins. Moreover, as Fig. 4 shows, the additional autocorrelation function, $G_1(\tau)$, decays more rapidly than the autocorrelation function due to the proteins; so that the long time tail of the signal is uniquely determined by the protein parameters. The time windows, referred to in the abscissa of Fig. 4, are 30 ms. This conclusion could be reached in a much simpler way by considering that the decay of the term (Eq. A4) is certainly determined by the diffusion D_B of the free FITC. Indeed the characteristic time for diffusion $T_B = \omega^2/4D \sim 0.006$ s is much smaller than the characteristic rate for the chemical reaction, which is of the order of $1/R \sim 0.1$ s. Therefore, the total fluorescence autocorrelation function has contributions from the diffusing proteins and from the free FITC, the latter contribution dying off more than ten times faster.

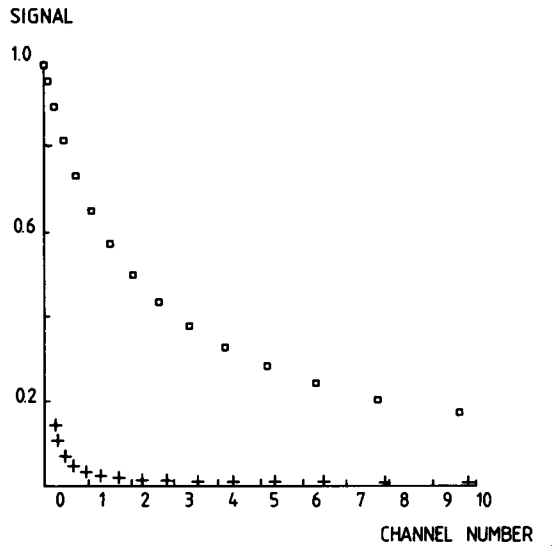

FIGURE 4 Correlation function $G_0(\tau)$ for the FITC α-crystallin complex (□), and $G_1(\tau)$ for the free FITC molecules (+). The amplitude of $G_1(\tau)$ is small and its relaxation time short, so that the influence of $G_1(\tau)$ is, negligible.

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