Biochimica et Biophysica Acta, 553 (1979) 342—350

BBA 78368

LIGHT SCATTERING TURBIDITY CHANGES AS A MEASURE OF THE KINETICS OF Ca^{2+}-PROMOTED AGGREGATION OF CHROMAFFIN GRANULE MEMBRANE GHOSTS

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(Received September 7th, 1978)

Key words Turbidity change, Ca^{2+}-promoted aggregation, Chromaffin granule, (Kinetics, Membrane ghost)

Summary

Changes in turbidity seen when chromaffin granule membrane ghosts are aggregated by Ca^{2+} can be modelled as dimerization of hollow spheres using Rayleigh-Gans-Debye light-scattering theory. The experimental changes agree well with the calculations. Thus, if shape or refractive index changes produced by osmotic perturbation, ion uptake, etc. can be excluded, turbidity readings can be used to follow the progress of the aggregation reaction of storage vesicles and other small particles or macromolecules.

Introduction

Chromaffin granules release their stored catecholamine in vivo by exocytosis. The release process is triggered by the influx of Ca^{2+} in response to cell depolarization [1]. Isolated granules aggregate and fuse when mixed with Ca^{2+} [2—4], thus studies of the aggregation process may help to explain granule-granule recognition in vitro and exocytosis in vivo. Several studies have used changes in turbidity to follow aggregation of granules or granule ghosts [3,5,6]. Similar observations using intensity fluctuations spectroscopy have recently been reported [7]. However, turbidity changes can also be produced by

Abbreviations used HEPES, *N*-2-hydroxylethylpiperazine-*N'*-2-ethanesulphonic acid, Me_2SO, dimethylsulphoxide.

changing the refractive index or the shape of the particles. Lysis and subsequent loss of the catecholamine, ATP and soluble protein stored in the granules results in a 15—20-fold decrease in turbidity [8—11]. Both the granules and the ghosts are excellent osmometers [12—14] and changes in turbidity may be used to assess swelling or shrinking of the particles due to ion fluxes or transport of materials across the membrane [13,15].

In this report we examine the assumption that changes in turbidity may be used to follow the progress of granule ghost aggregation in rapid mixing experiments.

Methods

Bovine adrenal medullary granules were prepared on sucrose step gradients as described previously [12] and the granule pellets subjected to 3 cycles of hypotonic lysis to produce resealed granule membrane ghosts [16].

Turbidity measurements of the Ca^{2+}-promoted aggregation of ghosts were made at 320 nm in a range which was linear with transmission changes, using an Aminco-Morrow Stopped Flow Apparatus (Cat. No. 4-8409) with a 0.1 mm slit setting. The progress of the monomer-dimer transition was determined from an analysis of the kinetics of the progress curve of τ in the stopped-flow experiment. Experimentally, the progress curve of the transmittance change, ΔT ($=T - T_0$), was used since this was proportional to $\Delta\tau$ ($=\tau - \tau_0$) for low values of τ (Fig. 1). The first phase of the transmittance change corresponding to the monomer to dimer transition was determined by the following trial and error method. The end point of the first phase was estimated and the slope of the curve at this point was determined. A 'baseline' was back-extrapolated to the point of zero time, and was used to correct for the contributions of the higher order reactions. The difference between the progress curve and the baseline was used to determine α, the degree of advancement of the reaction (cf. Eqns. 9—11 below). The values ($0 < \alpha < 1$) were plotted in second order form (Fig. 2).

The refractive index at 320 nm of the solvent for the stopped-flow experiments (10 mM HEPES, pH 7.2) was calculated to be 1.3552 by a three-parameter fit to the Couchy formula [17]

$$n_0 = K_1 + \frac{K_2}{\lambda_0^2} + \frac{K_3}{\lambda_0^4} \tag{1}$$

(where n_0 = refractive index of the solvent at wavelength λ_0), of 10 refractive index data points measured between 545 and 410 nm (the lower limit of visibility in our Abbe-type refractometer). The membrane refractive index was measured at 320 nm as ~1.592 by modifications of the method of Wallach et al. [18]. A series of 10 solvents were prepared consisting of 50 mM HEPES, pH 7.4 into which was incorporated either 5 or 10% Me_2SO (v/v) and varying concentrations of glycerol up to 20% (v/v). Previous experiments had established that both latter solvents would equilibrate across the granule membrane [12], thus giving the interior of the resealed ghost the same refractive index as the solvent. For each solvent mixture, triplicate samples consisting of 25 μl of concentrated suspension of granule ghosts in HEPES buffer was added to 2 ml of

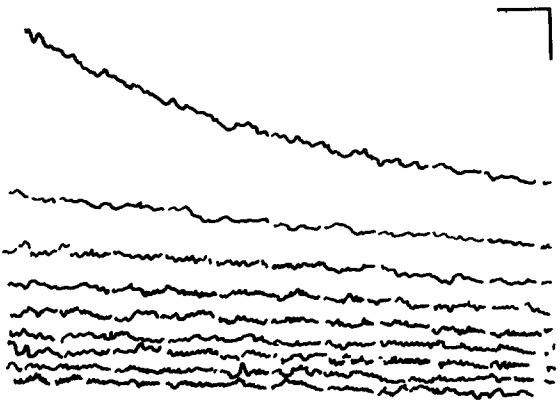

Fig. 1. Ca^{2+}-induced aggregation of chromaffin granule membranes. A stopped-flow progress curve showing the decrease in transmittance at 320 nm is presented. The ordinate is transmitted intensity, given in units of voltage (50 mV/division), and the abscissa is time after mixing (1 s/division). The reaction was initiated by mixing equal volumes of 0.10 mg/ml membrane protein in $5 \cdot 10^{-4}$ M EGTA and 10 mM HEPES buffer, pH 7.2 (Syringe A) with 9.0 mM $CaCl_2$ and 10 mM HEPES buffer, pH 7.2 (Syringe B). The oscilloscope was set in the repetitive sweep mode. The initial signal, extrapolated to zero time is 5.75 V. A scale division thus corresponds to 0.869% T. The turbidity of the initial reactants was calculated as $\log(T_{buffer}/(T_{endproducts} + \Delta T_{reaction})) = 0.0376$. Transmittance changes are proportional to turbidity changes in this range. The initial amplitude was not influenced by pH between 7 and 10. Repetition of the experiment measuring faster time domains (to 5 ms/division) revealed no faster processes. Repetition of the experiment with only buffer in Syringe B gave the same initial transmittance value and revealed no kinetic processes. The total aggregation behaviour is thus recorded in the figure presented.

solvent and incubated 14 h at 4° C. The turbidity of the suspension was read at 320 nm against a blank consisting of 25 μl of HEPES buffer added to 2 ml of the solvent. The refractive index for each of the 10 solvent blanks was estimated from the Couchy relationship as above. The membrane refractive index (n_2) ~ 1.592 was then determined graphically, as the x-intercept of the least squares straight line fit through a plot of $y = (Abs_{320})^{1/2}$ vs. $x = 1/n_{320}$. (dn/dc) was calculated for 320 nm to be 0.1874 using the Lorentz-Lorenz relationship [19] and a membrane density of 1.162 [12].

The particle concentration, c, was calculated as ~0.3 mg/ml from the measured protein concentration and a protein/lipid (w/w) ratio for granule membranes of 0.366 [20]. $K = 5.0$ was determined from the experimental data of Fig. 2. (τ/c) was calculated from Eqn. 3 using values for $P(\theta)$ derived from Eqn. 8, assuming ghosts to be hollow spheres of radius (R) = 150 nm (Morris, S.J. and Hellweg, M.A., unpublished data) and shell thickness ($R - r$) to be 6.5 nm (Morris, S.J. and Schober, R., unpublished data).

The semi-acceptance angle of the stopped flow absorption photomultiplier tube was calculated to be ~5°: therefore Q was integrated from 5—180°, using the QATR subroutine of the Scientific Subroutine Package for RT 11 Fortran and a PDP 11/40 computer.

Theory

Dean and Matthews [6] used Mie scattering theory to calculate the expected turbidity change for 5% conversion of monomeric spheres to multimers and

then used this value to extract initial rates for aggregation of intact granules. This approach is unsuitable for the present study since Mie theory cannot be extended to other than sphericals shapes. In the following discussion we use Rayleigh-Gans-Debye scattering theory. This approximation is limited by the assumption that the characteristic particle dimension is $<\lambda_0/2$ and that m is close to 1, where λ_0 = the wavelength of light in vacuo and m is the ratio of the refractive indices of the scattering particles and the solvent (n_2/n_0) [20,21].

For particles such as granule ghosts, whose characteristic dimension is greater than approx. $\lambda/20$, the Rayleigh approximation for the specific turbidity (τ/c),

$$\frac{\tau}{c} = \frac{16\pi}{3} \cdot \frac{R_{90}}{c} \tag{2}$$

(where R_{90} = Rayleigh ratio at $\theta = 90°$), can no longer be used, due to the asymmetric distribution of the scattered light caused by interference between fixed scattering centers [22,23]. Thus, either direct turbidity measurements or measurement of the complete angular spectrum are required.

As shown by Chong and Colbow [24]

$$\frac{\tau}{c} = \frac{24\,\pi^3 n_0^3}{\lambda_0^4} \cdot \nu \cdot \left[\frac{dn}{dc}\right] \cdot \frac{(m+1)^2(m-1)}{(m^2+2)^2} \cdot Q \tag{3}$$

where ν = the volume of scattering material. The quantity (dn/dc) = specific refractive index increment (change in refractive index of the solution with increasing particle concentration) is discussed by Huglin [19]. The dissipation factor,

$$Q = \tfrac{3}{8} \int_0^{\pi} P(\theta)\,(1 + \cos^2\theta)\sin\theta\,d\theta. \tag{4}$$

Assuming that the chromaffin granule 'ghost' can be modelled as a spherical shell of inner and outer radii r and R,

$$\nu = \frac{4\pi}{3} \cdot (R^3 - r^3)\,, \tag{5}$$

and the particle scattering factor for a spherical shell [25]

$$P(\theta)_s = \left[\frac{3}{(1-t^3)(hR)^3}(\sin hR - hR\cos hR + hr\cos hr - \sin hR)\right]^2 \tag{6}$$

where $t = r/R$ and $h = \dfrac{4\pi n_0}{\lambda_0} \cdot \sin\dfrac{\theta}{2}$.

For dimerized hollow spheres [22],

$$P(\theta)_{2s} = P(\theta)_s\left(1 + \frac{\sin 2\,hR}{hR}\right). \tag{7}$$

assuming that the individual particles scatter independently [22], as the dimerization proceeds we can calculate a particle scattering factor for the mixture of monomers and dimers,

$$P(\theta)_{mix} = P(\theta)_s\left[1 + \left(1 - \frac{[V]}{[V]_0}\right)\frac{\sin 2\,hR}{hR}\right] \tag{8}$$

where $[V]$ is the concentration of monomers compared to the concentration at the beginning of the experiment ($=[V]_0$).

Results

Fig. 1 shows a typical time course of light transmission (T) changes at 320 nm resulting when chromaffin granule ghosts are mixed with Ca^{2+}. The rates and amplitudes of several such experiments, extracted from the raw data by the procedure outlined above, are presented in Table I.

As noted by Lansman and Haynes [23], the Ca^{2+}-promoted aggregation of phospholipid vesicle monomers (V) to dimers (V_2) can be fitted to the integrated second order rate equation,

$$[V]^{-1} - [V]_0^{-1} = 2\, k_{app} \cdot t \tag{9}$$

If we define the degree of the progress of the reaction α ($=1 - [V]/[V]_0$), rearrangement of Eqn. 9 yields,

$$\frac{\alpha}{1-\alpha} = \frac{t}{K}, \tag{10}$$

where

$K = 1/(2\, k_{app}[V]_0)$.

In a previous study of phospholipid vesicle aggregation [23], the turbidity changes could be described in terms of Eqn. 2. Here it was demonstrated that the monomer-dimer conversion produced the expected change in τ/c, and then assumed that α could be measured as the fraction of the maximal change in apparent absorption (turbidity) $\Delta\tau/\Delta\tau_{max}$. We will now present a more formal proof for this assumption and extend the method of analysis to particles larger than $\lambda_0/20$ by first showing that Eqn. 10 is a proper description of the raw data and then demonstrating that the time course and extent of the expected turbidity changes for the monomer-dimer reaction also are consistent with Eqn. 10.

TABLE I

EFFECT OF MEMBRANE CONCENTRATION ON THE MEASURED RATES AND AMPLITUDES OF Ca^{2+} AND Mg^{2+}-PROMOTED CHANGES IN THE TURBIDITY OF CHROMAFFIN GRANULE MEMBRANE GHOSTS

The solutions in both syringes were buffered with 10 mM HEPES, pH 7.2. The salts were added as chlorides. The reaction was carried out at 25°C by rapidly mixing equal volumes from syringes A and B The reaction amplitudes A_1 and A_2 are $\Delta\tau/\tau_0$ values, assuming that $\Delta\tau \sim \Delta T$

Mixing configuration		First reaction ($V \rightarrow V_2$)		Second reaction ($V \rightarrow V_n$)	
Syringe A	Syringe B	$t_{1/2}$	A_1	$t_{1/2}$	A_2
(0.050 mg/ml vesicles) vs.	(40 mM Ca^{2+})	5.0 ± 1.0	+0.28 ± 0.02	140 ± 40	+0.19 ± 0.03
(0.10 mg/ml vesicles) vs.	(40 mM Ca^{2+})	2.3 ± 0.2	+0.31 ± 0.03	150 ± 20	+0.98 ± 0.15
(0.050 mg/ml vesicles) vs.	(40 mM Mg^{2+})	4.8 ± 0.7	+0.27 ± 0.03	125 ± 15	+0.20 ± 0.03
(0.10 mg/ml vesicles) vs.	(40 mM Mg^{2+})	2.3 ± 0.02	+0.31 ± 0.03	125 ± 15	+0.98 ± 0.15

For very dilute suspensions, ΔT is proportional to $\Delta\tau$. Therefore:

$$\alpha = \frac{\Delta T}{\Delta T_{max}} = \frac{\Delta\tau}{\Delta\tau_{max}}. \tag{11}$$

Thus, the raw data (Fig. 1), when transformed by Eqn. 11 and plotted as in Eqn. 10 should yield a straight line of slope $1/K$ and $y_0 = 0$. Such a plot of transformed experimental data for Ca^{2+}-aggregated granule ghosts is presented in Fig. 2 (filled circles). The deviation from the straight line is assumed to be due to formation of trimers and higher aggregates as the reaction proceeds. Table I shows that the slope of such a plot is proportional to $[V]_0$ as predicted by Eqn. 10.

If Eqn. 11 is a valid measure of the progress of the reaction then the following should be true:

(1) Plots of $1 - [V]/[V]_0$ vs. $\Delta\tau/\Delta\tau_{max}$ (where the latter is calculated from Eqn. 3) should yield straight lines of slope = 1 and y-intercept = 0;

(2) Plots of τ/c (calculated from Eqn. 3) vs. % dimerization ($=\alpha$) should be linear, and the total change in turbidity $[(\tau_{max} - \tau_0)/\tau_{max}]$, approximately equal to the experimentally measured change;

(3) Plots of $\alpha/(1-\alpha)$ versus t should be linear, where the former is calculated as $(\Delta\tau/\Delta\tau_{max})/(1 - \Delta\tau/\Delta\tau_{max})$ and the latter from the former using equation [10].

Results are tabulated in Table II and graphed in Figs. 2 and 3. The calculated total change in turbidity for the monomer-dimer conversion $(\tau_{max} - \tau_0)/\tau_{max} = 0.287 \sim 0.28$ that was seen experimentally. All calculations are consistent with the predictions [1—3] above.

TABLE II

TURBIDITY PARAMETERS CALCULATED FROM A MODEL FOR THE SCATTERING OF DIMERIZING HOLLOW SPHERES

τ/c was calculated from Eqn. 3 by substituting the appropriate value of $\alpha = ([V]_0 - [V])/[V]_0$ into Eqn. 8 as described in the Materials and Methods section. $\Delta\tau/\Delta\tau_{max}$ was calculated from the results of column 2. The results in column 4 were calculated from those of column 3 as $\left[\frac{\Delta\tau}{\Delta\tau_{max}} \Big/ \left(1 - \frac{\Delta\tau}{\Delta\tau_{max}}\right)\right]$ (cf. Eqn. 11), where $\Delta\tau_{max} = \tau_\alpha = 1 - \tau_\alpha = 0$. The equivalent times ($t$) were then calculated from Eqn. 10.

(1) $\frac{[V]_0 - [V]}{[V]_0}$	(2) $\tau/c \cdot 10^3$	(3) $\frac{\Delta\tau}{\Delta\tau_{max}}$	(4) $\frac{\alpha}{1-\alpha}$	(5) t (min)
0.0	0.2798	0.0	0.0	0.0
0.10	0.2798	0.0993	0.11	0.55
0.20	0.3023	0.1996	0.25	1.25
0.30	0.3136	0.2993	0.427	2.135
0.40	0.3248	0.3993	0.665	3.325
0.50	0.3361	0.4992	0.997	4.985
0.60	0.3473	0.5991	1.494	7.47
0.70	0.3585	0.6990	2.322	11.66

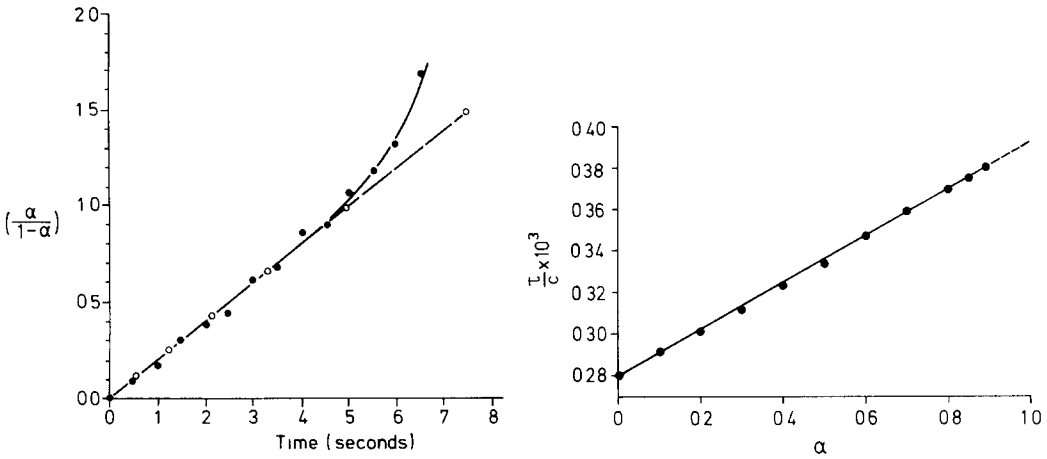

Fig. 2. Second order plot of the progress of dimerization of granule membrane ghosts. •, data transformed from the curve given in Fig. 1. The end point of the dimerization was taken as the value at 25 s after initiation (2.5 sweeps) and the slope between 25 s and 30 s was used for the baseline correction as outlined in the Methods section. The total amplitude of the change is −0.12 V which corresponds to $\Delta\tau$ = +0.0100 which corresponds to a 28% increase in τ. ○, calculated from Eqn. 3 as noted in Table II.

Fig. 3. Change in specific turbidity (τ/c) as a function of the progress of dimerization (α). The values, taken from Table II, lie on a straight line and extrapolate to τ/c = 0.391. The expected change in turbidity is 28.8%.

Discussion

As noted above, turbidity is a multivariable parameter; changes in the size, shape and/or refractive index of the particles will be reflected in changes in τ. Since both the granules and the granule ghosts are good osmometers, mixing experiments which involve changing the final concentration of impermeant solutes may cause expansion or collapse of the particles in addition to aggregation or disaggregation. Turbidity measurements have been used to follow the swelling of resealed chromaffin granule ghosts due to Mg^{2+}-ATPase dependent transport of protons and anions across the membrane [15].

The maximal change in scattering experienced by a granule ghost collapsing under increased osmolality was modelled as the transition of a hollow sphere into a thin disc of the same surface area. For this case

$$P(\theta)_{\text{disc}} = \frac{2}{hR}\left[1 - \frac{1}{hR} \cdot J_1(2\,hR)\right] \tag{12}$$

where R = radius of the disc and J_1 represents the J-Bessel-function of order 1 [22].

Eqn. 12 was substituted into Eqn. 4 and the resulting turbidity calculated from relation [3]. For a 150 nm hollow sphere the maximal expected change in τ/c is about 35% compared to about 29% for dimerization. Thus partial collapse of the ghosts could easily be mistaken for aggregation. A Ca^{2+}-promoted osmotic shape change in these experiments was ruled out by the following:

(1) The experimental observations which show that $K \propto [V]_0$ (Fig. 2 and

Table I). Osmotic pressure changes would not be expected to show a dependence upon $[V]_0$.

(2) Controls which showed that mixing the granule ghosts with solution of monovalent ions or sucrose of osmolality equivalent to that of the Ca^{2+}-solution produced negligible changes in turbidity.

(3) Osmotic pressure changes produce changes in granule shape which occur much faster than those seen during aggregation (Morris, S.J. and Hellweg, M.A., unpublished data).

Lysis of intact granules produces a 15—20-fold decrease in turbidity without appreciable change in the overall diameter of the particle. This takes place as a result of the loss of the relatively high refractive index contents of the granule core. The resulting changes in turbidity have been shown to follow first order kinetics [8—10].

In the experiments reported here, lysed granule ghosts were used to prevent misinterpretation of the results. If it is necessary to work with whole granules further precautions to reduce or control for lysis must be instituted. These would include working at low temperatures to avoid lysis [8] and experiments to show that the mixing or handling of the granules produce negligible lysis. The perturbation should not activate mechanisms which lead to lysis. Intact granules contain 120—200 mM ATP [20] as well as 20 mM Ca^{2+} and 4 mM Mg^{2+} [26] some of which inevitably finds its way into the suspension medium from damaged granules. Mixing granules with divalent or monovalent cations may activate the proton translocating Mg^{2+}-ATPase leading to lysis of granules [10] or may lyse the granules if permeant anions or ionophores are used [11,17].

If competing reactions can be eliminated or properly subtracted from the raw data, then analysis of the turbidity changes by the method presented here provides a rapid simple and positive method for assessing the effects of ions, drugs, etc., on the aggregation of liposomes, storage vesicles, mitochondria viruses and other particles whose light scattering properties can be described by Rayleigh-Gans-Debye theory. Applications include polymerization or depolymerization of macromolecules, microfilaments or microtubuli. We have also used the methodology to probe the surface properties of chromaffin granule and synaptic vesicle membranes [27].

Acknowledgements

D.H.H. was supported by N.I.H. grants AM 20086, HL 23392 and HL 16117. M.A.H. was supported by a fellowship from the Max-Planck-Gesellschaft.

References

1 Smith, A.D. and Winkler, H. (1972) Handbook of Exp. Pharmacol. 33, 538—617
2 Edwards, W., Phillips, J.H. and Morris, S.J. (1974) Biochim. Biophys. Acta 356, 164—173
3 Morris, S.J., Edwards, W. and Phillips, J.H. (1974) FEBS Lett. 44, 217—223
4 Morris, S.J. and Schober, R. (1977) Eur. J. Biochem. 75, 1—12
5 Creutz, C.E., Pazoles, C.J. and Pollard, H.B. (1978) J. Biol. Chem. 253, 2858—2866
6 Dean, P.M. and Matthews, E.K. (1975) J. Theoret. Biol. 54, 309—321
7 Green, D.J., Westhead, E.W., Langley, K.H. and Sattelle, D.B. (1978) Biochim. Biophys. Acta 539, 364—371

8 Morris, S.J., Schober, R. and Schultens, H.A. (1977) Biochim. Biophys. Acta 464, 65—81
9 Morris, S.J., Schultens, H.A., Hellweg, M.A., Striker, G. and Jovin, T.M. (1979) Applied Optics, in the press
10 Casey, R.P., Njus, D., Radda, G.K. and Sehr, P.A. (1976) Biochem. J. 153, 583—588
11 Papadopolou-Daifotis, Z., Morris, S.J. and Schober, R. (1977) Neuroscience 2, 609—619
12 Morris, S.J. and Schovanka, I. (1977) Biochim. Biophys. Acta 464, 53—65
13 Johnson, R.G. and Scarpa, A. (1976) J. Gen. Physiol. 68, 601—631
14 Morris, S.J., Schultens, H.A. and Schober, R. (1977) Biophys. J 20, 33—45
15 Phillips, J.H. and Allison, Y. (1978) Biochem. J. 170, 661—672
16 Phillips, J.H. (1974) Biochem. J. 144, 311—318
17 Johnson, B.L. and Smith, J. (1972) in Light Scattering From Polymer Solutions (Huglin, M.B., ed.), Chapter 2, Academic Press, New York
18 Wallach, D.F.H., Kamat, V.B. and Gail, M.H. (1966) J. Cell Biol. 30, 601—621
19 Huglin, M.B. (1972) in Light Scattering From Polymer Solutions (Huglin, M.B., ed.), Chapter 6, Academic Press, New York
20 Winkler, H. (1976) Neuroscience 1, 65—80
21 Tanford, C. (1961) Physical Chemistry of Macromolecules, John Wiley and Sons, New York,
22 Kerker, M. (1969) The Scattering of Light and Other Electromagnetic Radiation, Academic Press, New York
23 Lansman, J. and Haynes, D.H. (1975) Biochim. Biophys. Acta 394, 335—347
24 Chong, C.S. and Colbow, K. (1976) Biochim. Biophys. Acta 436, 260—282
25 Oster, G. and Riley, D.P. (1952) Acta Cryst. 5, 1—6
26 Phillips, J.H., Allison, Y. and Morris, S.J. (1977) Neuroscience 2, 147—157
27 Morris, S.J., Chiu, V.K.C. and Haynes, D.H. (1979) Memb. Biochem. 2, 163—201