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AN EXPERIMENTAL STUDY OF THE DIELECTRIC DISPERSION OF SOLUTIONS OF CYTOCHROME *c* AT MEDIUM IONIC STRENGTHS

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In this paper, the results are presented of measurements of the dielectric dispersions of horse heart cytochrome *c* molecules in various buffers. The data are fitted to the Cole-Cole relaxation model. The influence of the concentration and the ionic strength on the parameters that result from the Cole-Cole model is determined. The measured data are compared with calculations based on the model presented previously. Good agreement is found between the model and the observed data.

1. Introduction

Since the first experiments of Oncley [1], a large number of investigators have studied the dielectric dispersion of proteins in solution. Particularly the dispersion in the megahertz region has been studied extensively [2–4]. South and Grant [5] discussed a number of mechanisms that could be responsible for the observed dispersion of solutions of myoglobin. They concluded that proton fluctuation [6] and Debye relaxation [7] are the only mechanisms that could explain the measurements.

As shown in the previous paper [8], the proton-fluctuation model cannot explain the large dispersion in the megahertz region. The results reported by South and Grant [5] should therefore be interpreted using the Debye model. We presented [8] a model, originating from the Debye model, for the dielectric relaxation of globular protein molecules. In this model the protein molecule is represented by a sphere with a spatially varying dielectric constant. The total dipole moment of the molecule is shown to be due to the charges of the protein, the polarization of the medium (due to the charges and to the external field), and the dipole moment arising from the distribution of the small ions in solution. In this paper, the observed dispersion curves of solutions of oxidized horse heart cytochrome *c* at various concentrations and pH values and dissolved in different buffers will be compared with the model calcuations [8].

2. Materials and methods

We investigated the dielectric constant of solutions of oxidized horse heart cytochrome *c* in the megahertz region. Cytochrome *c* was obtained from Sigma (type VI). Tris and HCl were from Baker (grade: Baker analyzed). H_2SO_4 and citric acid monohydrate were from Merck (grade: pro analysis), and sodium cacodylate was from Fluka (grade: purum). We used twice-distilled water for all our solutions.

For the dielectric measurements we used a 250B RX-meter from Hewlett Packard.

We used a coaxial liquid cell, similar to that described by Van Beek et al. [9]. We minimized the influence of the parasitic elements on the measured capacitance and resistance by measuring the

dielectric constant as a function of the height of the liquid column.

We determined the residual elements by doing measurements with the empty cell, the cell being filled with twice-distilled water and with salt solutions of various concentrations. In order to eliminate systematic errors arising from electrode polarization effects and incorrect knowledge of the residual elements, values for the dielectric constant of the buffered protein solutions were corrected with the measured values for solutions of the buffer according to:

$$\epsilon_p(f)=\epsilon'_p(f)-(\epsilon'_b(f)-\epsilon_w) \quad (1)$$

where $\epsilon'_p(f)$ and $\epsilon'_b(f)$ are the measured dielectric constants of the protein and buffer solutions, respectively, ϵ_w the dielectric constant of water (78.5 at 25°C), $\epsilon_p(f)$ the corrected dielectric constant for the protein solution and f the frequency. In this way, the dielectric constant can be determined within an error of about 1% in the frequency range from 3 to 100 MHz for protein solutions with specific conductivities of less than 0.35 Ω^{-1} m^{-1}. Since at lower frequencies the effects of the electrode polarization become comparable to those of the protein solutions, large experimental errors arise in the determination of the dielectric constant. The values of the dielectric constant were fitted to the empirical Cole-Cole equation [3,4,10]:

$$\hat{\epsilon}(\omega)=\epsilon_\infty+\frac{\epsilon_s-\epsilon_\infty}{1+(j\omega\tau)^{1-\alpha}} \quad (2)$$

where $\hat{\epsilon}(\omega)$ is the complex dielectric constant, $\omega=2\pi f$, τ the central relaxation time of the distribution, ϵ_s the static dielectric constant and ϵ_∞ the dielectric constant at frequencies much higher than $1/2\pi\tau$, and α a measure for the distribution of relaxation times. We fitted our measured ϵ values only to the real part of eq. 2; the imaginary part of $\hat{\epsilon}$ could not be determined accurately because of the large contributions of the ionic conductivity of the protein solutions.

The data were fitted numerically to the Cole-Cole equation [2] using a Marquardt algorithm [11,12]. All computations were performed on a PDP 11/05 mini-computer.

The dimensions of the protein molecule can be estimated from the value of the central relaxation time. The rotational time τ of a spherical particle with radius a is given by the Stokes-Einstein relation:

$$\tau=\frac{4\pi a^3\eta}{kT} \quad (3)$$

where η is the intrinsic viscosity, k Boltzmann's constant and T the absolute temperature. η is given by:

$$\eta=\nu\cdot\rho \quad (4)$$

where ν is the kinematic viscosity and ρ density of the solution. The kinematic viscosity of the protein solution was measured as a function of the concentration with a Micro-KPG-Ubelohde capillary viscometer (Schott No. 24536) in the range 0–4 mmol/l. The density at the various concentrations was determined by measuring the weight of a known volume of the protein solution. In this way the intrinsic viscosity could be determined with an accuracy of about 0.3%. A linear relationship between viscosity and concentration was found in the observed concentration range.

The concentration of cytochrome c was determined from its absorption spectrum. The protein solution used for the dielectric measurements was diluted 100 times. Its absorption spectrum was measured on a Varian UV-VIS spectrophotometer Model 635. It appeared that about 5% of the proteins were in the reduced form. By adding a few grains of sodium dithionite (Baker analyzed reagents) the proteins became completely reduced and the absorption spectrum was measured once more. The concentration of the proteins was calculated from the differential absorption of the oxidized and reduced forms at 549.5 nm (the α-band). An extinction coefficient of 21.1 $mmol^{-1}$ cm^{-1} was used (E.C.I. Veerman, personal communication). The concentration could be determined with an accuracy of about 0.2 mmol/l.

The pH was measured with a Radiometer Copenhagen type PHM 28c pH meter. All measurements were performed at 298 K.

3. Results

Fig. 1 shows the experimentally determined dielectric dispersions of solutions of horse heart

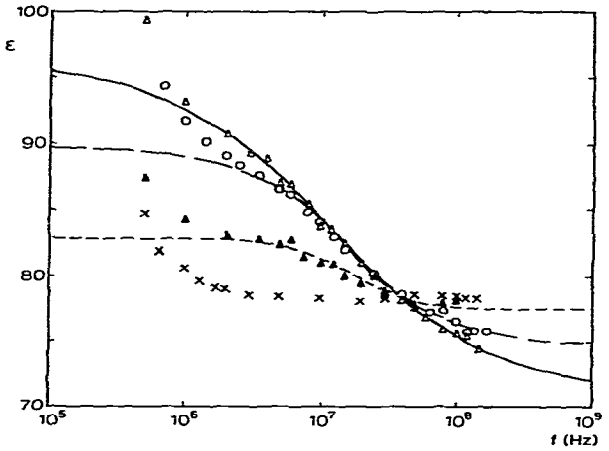

Fig. 1. Dielectric dispersion of solutions of horse heart cytochrome *c* at pH 5.2 in a cacodylic acid-citric acid buffer. The curves are the result of the fit to the Cole-Cole relaxation model. ×, buffer without proteins; ▲, $c_p = 1.0$ mmol/l, $\Delta\epsilon = 5.4$, $\tau = 9.5$ ns, $\alpha = 0.03$; ○, $c_p = 3.2$ mmol/l, $\Delta\epsilon = 15.0$, $\tau = 9.9$ ns, $\alpha = 0.22$; △, $c_p = 6.4$ mmol/l, $\Delta\epsilon = 25.3$, $\tau = 13.4$ ns, $\alpha = 0.40$.

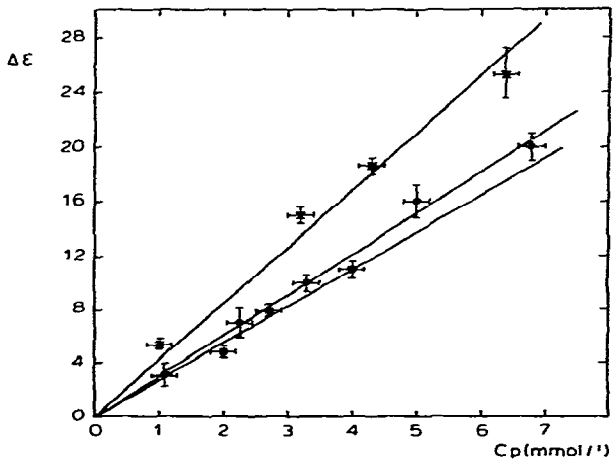

Fig. 2. Dielectric dispersion amplitude of solutions of horse heart cytochrome *c* as a function of the protein concentration at different pH values and dissolved in different buffers. ■, citric acid-HCl, pH 4.0; ×, cacodylic acid-citric acid, pH 5.2; ○, citric acid-Tris, pH 8.0.

cytochrome *c* at different concentrations (c_p) at pH 5.2. Also shown are the curves that are fitted to the dielectric data according to the Cole-Cole relaxation model.

The proteins were dissolved in a buffer of 25 mmol/l citric acid and 25 mmol/l cacodylic acid. The ϵ values of the buffer alone (ϵ'_b) are also included in fig. 1. These values were inserted in eq. 1 to yield the corrected values for ϵ_p, which is the dielectric constant of the protein solutions.

In addition to the measured dispersion curves at pH 5.2, the dielectric dispersion was determined as a function of the concentration at pH 4.0 and 8.0. The values of the parameters obtained from the fit to the Cole-Cole relaxation model are presented in figs. 2–5.

Table 1

pH	$\Delta\epsilon / c_p$
4.0	2.8±0.6
5.2	4.2±0.5
8.0	3.0±0.1

It can be seen from table 1 and fig. 2 that the dielectric dispersion amplitude $\Delta\epsilon = \epsilon_s - \epsilon_\infty$ for horse heart cytochrome *c* is linearly dependent on the concentration for concentrations up to about 7 mmol/l and in the pH range 4–8. This is in agreement with the results of South and Grant [5] and Desnica [3].

Fig. 3 shows that ϵ_∞ is also linearly dependent on the concentration in the same pH and concentration ranges as for $\Delta\epsilon$. The large deviation from linearity of the point at pH 8.0 and $c_p = 6.8$ mmol/l probably arises from the lack of measurements at frequencies above 100 MHz. It can be seen from fig. 1 that the plateau in ϵ is not reached at high concentrations. It is possible, therefore, that the Cole-Cole fit overestimates the value of ϵ_∞ at high concentrations. The small value of α (for this point) given in fig. 4 also shows that the dispersion could be broader than is indicated by the fit to the Cole-Cole model. Table 2 lists the slopes of the lines shown in fig. 3. The slopes show no pH dependence within experimental errors.

Fig. 4 shows the concentration dependence of the parameter α of the Cole-Cole model. Only the values at pH 5.2 show a strong dependence of α on

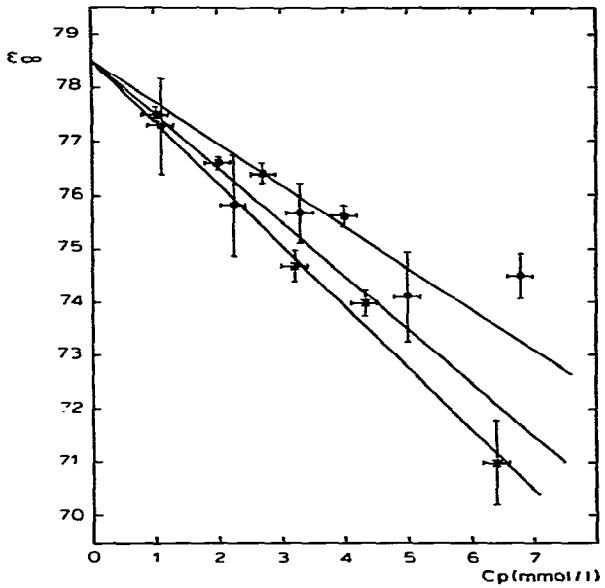

Fig. 3. ϵ_∞ of solutions of horse heart cytochrome c as a function of the protein concentration. ■, citric acid-HCl; ×, cacodylic acid-citric acid; ○, citric acid-Tris.

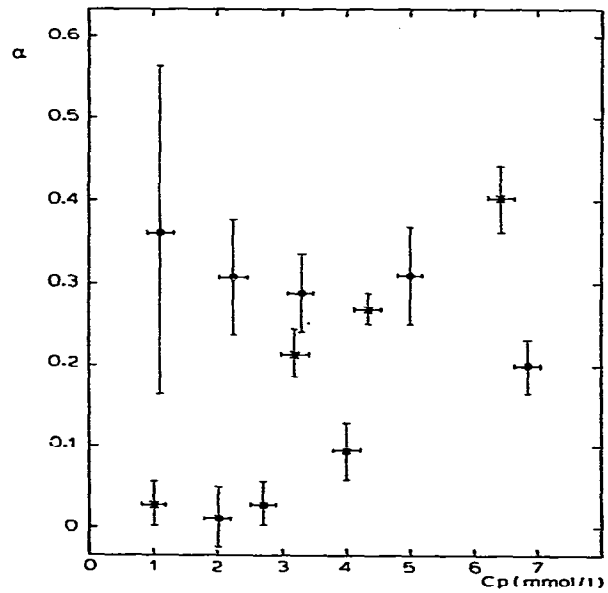

Fig. 4. The Cole-Cole parameter α of solutions of horse heart cytochrome c as a function of the protein concentration. ■, citric acid-HCl; ×, cacodylic acid-citric acid; ○, citric acid-Tris.

Table 2

pH	$\{\epsilon_\infty - \epsilon_\infty(c_p=0)\}/c_p$
4.0	-0.8 ± 0.3
5.2	-1.1 ± 0.1
8.0	-0.9 ± 0.2

the concentration. No concentration dependence can be found at pH 4.0 and 8.0, within the experimental errors.

Fig. 5 shows the influence of the concentration on the quantity $\tau' = \tau/\eta$, where η is the measured absolute viscosity. According to eq. 3 one would expect τ' to be independent of the concentration. τ' is approximately constant at low concentrations but increases at higher concentrations.

In fig. 6 the measured dielectric dispersion per unit concentration is presented as a function of the ionic strength. The figure also shows theoretical

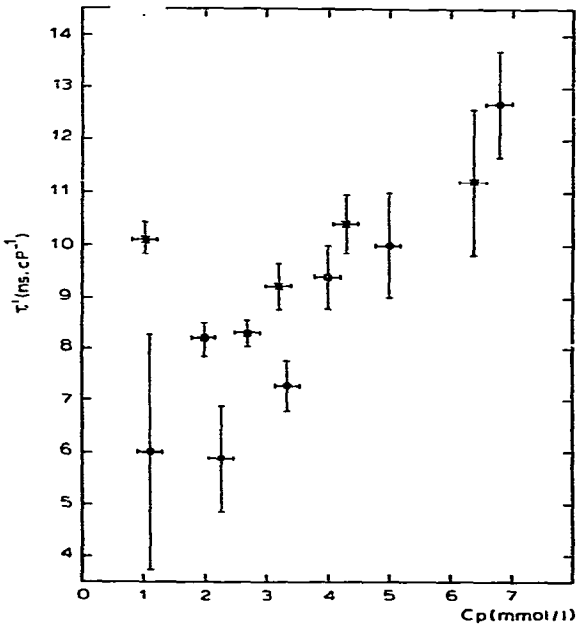

Fig. 5. τ' (see text) of solutions of horse heart cytochrome c as a function of the protein concentration. ■, citric acid-HCl; ×, cacodylic acid-citric acid; ○, citric acid-Tris.

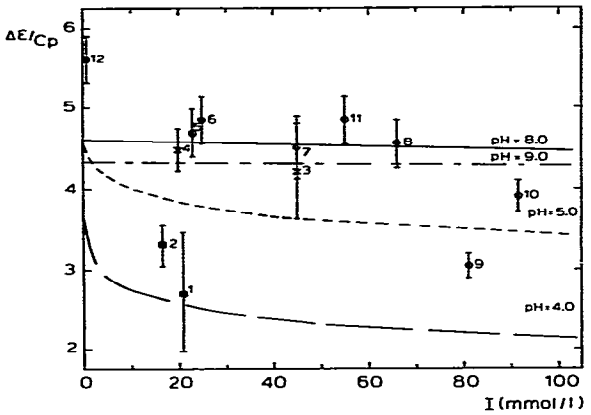

Fig. 6. Dielectric dispersion amplitude per unit of concentration for solutions of horse heart cytochrome *c* as a function of ionic strength. The curves are calculated according to the model presented in ref. 8. The numbers are related to table 3.

curves calculated at pH 4.0, 5.0, 8.0 and 9.0 according to the model presented previously [8].

It should be noted that the proteins were dissolved in different buffers. Table 3 summarizes the data for the solutions used in the experiments.

The ionic strength dependence of τ' and the Cole-Cole parameter α are shown in figs. 7 and 8, respectively. For all the concentration points the concentration is about 4 mmol/l and the numbers correspond to those given in table 3. The values show no direct dependence of the ionic strength, I, within the experimental errors.

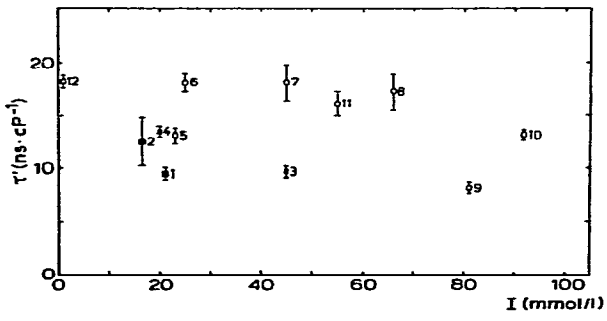

Fig. 7. τ' for solustions of horse heart cytochrome *c* as a function of ionic strength. The numbers are related to table 3.

Table 3

Ionic strength and pH of the various buffers

No.	Buffer	pH	I (mmol/l)
1	citric acid-HCl	4.0	21
2	H_2SO_4	4.2	17
3	cacodylic acid-citric acid	5.2	45
4	H_2SO_4	5.6	20
5	H_2SO_4-Tris	7.9	23
6	H_2SO_4-Tris	8.4	25
7	H_2SO_4-Tris	8.4	45
8	H_2SO_4-Tris	8.5	66
9	citric acid-Tris	8.0	81
10	citric acid-Tris	8.3	92
11	citric acid-Tris	8.4	55
12	no buffer	8.9	≈0

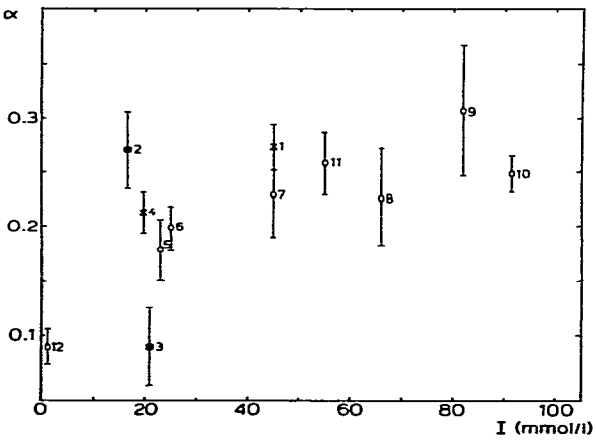

Fig. 8. The Cole-Cole parameter α of solutions of horse heart cytochrome *c* as a function of ionic strength. The numbers are related to table 3.

4. Discussion

The model presented in the previous paper [8] for the calculation of the dielectric dispersion of solutions of globular protein molecules is based on the assumption that protein molecules do not interact (infinite dilution). This assumption can be justified on the basis of the experimental evidence that the dielectric dispersion amplitude of horse heart cytochrome *c* is linearly dependent on the

concentration for concentrations up to 7 mmol/l in the pH range 4–8. These results are in agreement with those given by South and Grant [5] for sperm whale myoglobin and by Desnica [3] for bovine oxyhaemoglobin.

In our model we proposed ϵ_∞ to be linearly dependent upon the concentration. The parameters τ' and α obtained from the fit to the Cole-Cole equation are found to be dependent on the concentration, which suggests that these parameters are more sensitive to intermolecular interactions than are $\Delta\epsilon$ and ϵ_∞. A similar behaviour was found previously by Desnica [3] for haemoglobin solutions. Our results indicate that the interactions between the protein molecules could become important at concentrations higher than 2 mmol/l.

From eq. 3 we see that the observed values for τ' correspond to a protein radius ranging from 130 to 160 nm. The higher limit agrees reasonably well with the expected value of 170 nm [13].

It should be noted that the relaxation times are sensitive to the nature of the buffer or to the pH. This effect could in part be due to the binding of buffer ions to the protein. However, it is not possible to determine whether the buffer effects are specific.

It should be emphasized that the Cole-Cole relaxation model which we used to interpret our results is an empirical one and is not specifically for solutions of globular proteins. The physical significance of parameters α and τ is therefore open to doubt. However, it can be seen that the dispersion amplitude $\Delta\epsilon$ is model independent.

Table 2 shows that the linear dependence of ϵ_∞ on the concentration c_p of protein molecules is not influenced by the nature of the buffer or the pH of a solution within experimental error. ϵ_∞, the dielectric constant at frequencies too high for the molecules to follow the oscillations of the electric field, is due to a random distribution of protein molecules in the solution. This implies that ϵ_∞ is independent of the charge configuration of the protein, and hence of the pH.

Calculations, based on our model [8], suggest for $d\epsilon_\infty/dc_p$ a value of 0.36 l/mmol whereas its experimental value lies between 0.8 and 1.1 l/mmol (fig. 3). In order to improve the agreement between theory and experiment it is necessary to assume much lower values for the dielectric constant in the model calculations. On the other hand, such a decrease in the model parameters leads to a marked deterioration in the agreement between theory and experiment for all the other properties of the molecule discussed in this work. The reasons for the discrepancy are not clear.

The dielectric dispersion amplitude per unit of concentration is presented in fig. 6 as a function of ionic strength. At pH 9 the calculated dispersion is almost independent of the ionic strength [8]. At pH values lower than 9 the dispersion decreases with increasing ionic strength. The observed dispersions correspond reasonably well with the theoretical curves.

In our theoretical calculations we neglected the effects of binding of ions to specific groups of the protein. Osheroff et al. [14] presented a map of ion-binding sites on cytochrome *c* molecules. We studied the influence of the binding of an anion to the Lys-72 group of the molecules. In our programme we left Lys-72 uncharged over the complete pH range by assigning a value of 0 to the pK_{Int} of this group. $\Delta\epsilon/c_p$ was calculated at an ionic strength of 50 mmol/l. Under this condition the dielectric dispersion amplitude was found to decrease drastically. $\Delta\epsilon/c_p$ decreases by 1.74, 1.81, 2.26 and 0.87 at pH 9, 8, 5 and 4, respectively. This large effect is brought about because Lys-72 is situated very close to the electric dipole axis of the cytochrome *c* molecule. It is known that H_2SO_4 and Tris, in contrast to citric acid, do not bind strongly to cytochrome *c* [15,16]. On comparing the experimental results obtained at pH 8 with H_2SO_4 on the one hand and with citric acid on the other, one can conclude that the proteins dissolved in the latter buffer show smaller dispersions than in the case of H_2SO_4. This might be attributed to binding of citric acid to cytochrome *c*.

As can be seen from ref. 8, the dielectric dispersion amplitude is sensitive to the choice of the parameters *a* and *b*, which indicate the variation of the dielectric constant in the protein molecule. The values of 50 nm for *a* and 170 nm for *b* that gave the best fit to the proton titration curve [8] also show good agreement with the experimentally determined dielectric dispersions. Furthermore, the ionic strength dependence of the measured dielec-

tric dispersion amplitudes is in reasonable agreement with the calculated dependence, although the potential used in ref. 8 to determine the ionic strength dependence is strictly valid only for buffer molecules with spherically symmetric charge distributions. The molecules of the buffers used for the measurements, however, have fairly large dipole moments.

Thus, the model presented in ref. 8 provides an adequate description of the principal features of the dielectric dispersions of solutions of horse heart cytochrome *c*.

References

1 J.L. Oncley, in: Proteins, amino acids and peptides, eds. E.J. Cohn and J.T. Edsall (Reinhold, New York, 1943) Ch. 22.
2 R. Lumry and R.H.-S. Yue, J. Phys. Chem. 69 (1965) 1162.
3 D. Desnica, Biopolymers 18 (1979) 1685.
4 P. Schlecht, A. Mayer, G. Hettner and H. Vogel, Biopolymers 7 (1969) 963.
5 G.P. South and E.H. Grant, Proc. R. Soc. Lond. A 328 (1972) 371.
6 J.G. Kirkwood and J.B. Shumaker, Proc. Natl. Acad. Sci. U.S.A. 38 (1952) 855.
7 P. Debye, in: Polar molecules (Reinhold, New York, 1929).
8 F.J.M. Mofers, G. Casteleijn and Y.K. Levine, Biophys. Chem. 16 (1982) 9.
9 W.M. van Beek, F. van der Touw and M. Mandel, J. Phys. E 9 (1976) 385.
10 C.J.F. Böttcher and P. Bordewijk, in: Theory of electric polarization (Elsevier, Amsterdam, 1978) vol. 2.
11 D.W. Marquardt, J. Soc. Ind. Appl. Math. 11 (1963) 431.
12 P. McClellan, in: Non-linear least-squares using the Gauss-Newton and Marquardt algorithms (Tektronix Technical Notes, 1978).
13 N. Mandel, G. Mandel, B.L. Trus, J. Rosenberg, G. Carlson and R.E. Dickerson, J. Biol. Chem. 252 (1977) 4619.
14 N. Osheroff, D.L. Brautigan and E.G. Margoliash, Proc. Natl. Acad. Sci. U.S.A. 77 (1980) 4439.
15 G.H. Barlow and E. Margoliash, J. Biol. Chem. 241 (1966) 1473.
16 P. Nicholls, Biochim. Biophys. Acta 346 (1974) 261.