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ON THE DETERMINATION OF ASSOCIATION CONSTANTS OF PROTEINS BY ELECTROPHORESIS MEASUREMENTS

H.P. MÜHLMANN and H. SCHÖNERT

Institut für Physikalische Chemie, Abt. für Biopolymere, Rheinisch-Westfälische Technische Hochschule Aachen, D-51 Aachen, Fed. Rep. Germany

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During the electrophoresis of a reversibly associating substance the concentration profile is determined by diffusion, migration and reaction. The influence of the diffusion can be eliminated by extrapolating the concentration profiles, taken at different times and suitably transformed, to infinite time. This leads to a profile which reflects migration and reaction only (Gilbert profile). From this the association constant can be deduced. Preliminary experiments with β-lactoglobulin A show the feasibility of the method.

1. Introduction

Since the pioneering papers of Gilbert [1,2] it is known that during the transport of an associating substance the concentration profiles show strong deviation from the gaussian behaviour, even giving rise to bimodal peaks. This anomalous behaviour has been used to determine association constants of proteins either by sedimentation or gel chromatography [3–7]. The theory of this effect has been taken to a point where the different factors (diffusion, mobility, nonideality of the solution, stoichiometry of the reaction) can be analyzed [3,4,5,8,9].

The method rests on two assumptions: the reaction must be fast compared to the transport, i.e. local chemical equilibria prevail; the mobilities of the species (monomer, polymer, ...) should be sufficiently different. These conditions are fulfilled for a wide class of reactions in protein solutions.

Furthermore, it is helpful in the analysis of the peaks if one has a relation between mobility and molar mass thus reducing the number of unknown parameters. Such a relation can be established for sedimentation and gel chromatography, but not for electrophoresis, a fact which is probably responsible for the scarce use of electrophoresis in studying associating systems.

This shortcoming may be outweighted by the following two factors: the electrophoresis cell (without supporting medium), being rectangular and infinite, allows of relatively simple mathematical boundary conditions; secondly, one can observe the movement of the boundary for quite a long time which, as will be seen later, can be used to eliminate the influence of the diffusional spreading of the boundary. This in turn gives experimental access to the Gilbert profile [1,2] which is a direct measure of the association process.

To see if this is a feasible method we have made some exploratory measurements on solutions of β-lactoglobulin A the association behaviour of which is known to a great extent [5,10–13].

2. Theory

The transport equation for the solute constituent is

$$\frac{\partial c}{\partial t} = \frac{\partial}{\partial x}\left[D\frac{\partial c}{\partial x} - c\bar{v}\right], \qquad (1)$$

where c is the constituent concentration of the solute (in mass per volume), D the diffusion coefficient and $\bar{v}$ the stationary migration velocity. As usual, t denotes the time and x the space coordinate

In case of the equilibrium

$$n\mathrm{M} \rightleftharpoons \mathrm{P}\,, \tag{2}$$

where n monomer molecules associate to a polymer P, we have

$$c = c_\mathrm{M} + c_\mathrm{P}\,, \tag{3}$$

$$\bar{v} = (c_\mathrm{M} v_\mathrm{M} + c_\mathrm{P} v_\mathrm{P})/c\,, \tag{4}$$

$$D = (c_\mathrm{M} D_\mathrm{M} + n c_\mathrm{P} D_\mathrm{P})/(c_\mathrm{M} + n c_\mathrm{P})\,, \tag{5}$$

$$K_n = c_\mathrm{P}/(c_\mathrm{M})^n\,. \tag{6}$$

Here the subscripts M and P refer to monomer and polymer. In formulating the mass action law with the association constant K_n, activity coefficients have been neglected or can be thought of as given by the assumption of Adams and Fujita [14].

Introducing the variables v and w [15]

$$v = x/t, \quad w = 1/t \tag{7}$$

eq. (1) is transformed into

$$-v\frac{\partial c}{\partial v} - w\frac{\partial c}{\partial w} = w\frac{\partial}{\partial v}D\frac{\partial c}{\partial v} - \frac{\partial}{\partial v}(c\bar{v})\,. \tag{8}$$

For infinite time, $w \to 0$, this equation reduces to [1,2]

$$v\frac{\partial c}{\partial v} = \frac{\partial}{\partial v}(c\bar{v})\,, \tag{9}$$

in which the diffusional boundary spreading is eliminated. The reason for this is of course that the distance travelled by migration is proportional to t whereas diffusional spreading is proportional to $(t)^{1/2}$. Thus diffusion dies out relative to migration.

The solution of eq. (9) has been given by Gilbert [1]:

$$\frac{\partial c}{\partial v} = \frac{(nK_n)^{1-n}}{(n-1)(v_\mathrm{P} - v_\mathrm{M})} \times \left(\frac{v_\mathrm{P} - v_\mathrm{M}}{v_\mathrm{P} - v}\right)^{(2n-1)/(n-1)} \left(\frac{v - v_\mathrm{M}}{v_\mathrm{P} - v_\mathrm{M}}\right)^{(2-n)/(n-1)} \tag{10}$$

The experimental concentration gradient extrapolated to infinite time, $(\partial c/\partial v)_{t\to\infty}$, can be fitted to this curve by choosing the appropriate values of the parameters. This means especially that the Gilbert curve starts at the point

$$v = v_\mathrm{M}\,, \tag{11}$$

and that for $n > 2$ the curve shows the well-known minimum which is located at

$$v_\mathrm{min} = \frac{(n-2)}{3(n-1)}(v_\mathrm{P} - v_\mathrm{M}) + v_\mathrm{M}\,. \tag{12}$$

So, assuming a value of n it is possible to evaluate from the start and the minimum position the two velocities v_M and v_P.

The area under the curve (10) up to the minimum position is given by

$$c_\mathrm{min} = \left(\frac{n-2}{nK_n(2n-1)}\right)^{1/(n-1)} \frac{2(n^2-1)}{n(2n-1)}, \tag{13}$$

which renders accessible the value of K_n.

This procedure can be checked by measuring $\bar{v}$ by means of the reduced first moment m_1/m_0 of the boundary:

$$m_1 = \int_{-\infty}^{+\infty} x\frac{\partial c}{\partial x}\,\mathrm{d}x\,, \tag{14}$$

$$m_0 = \int_{-\infty}^{+\infty} \frac{\partial c}{\partial x}\,\mathrm{d}x\,. \tag{15}$$

Introducing into

$$\frac{\partial m_1}{\partial t} = \int_{-\infty}^{+\infty} x\frac{\partial}{\partial x}\left(\frac{\partial c}{\partial t}\right)\mathrm{d}x$$

eq. (1) and integrating twice with the boundary conditions

$$\begin{aligned} \partial c/\partial x &= 0, \quad x \to \pm\infty\,, \\ c &= 0, \quad x \to -\infty\,, \\ c &= \mathrm{c}, \quad x \to +\infty\,, \end{aligned} \tag{16}$$

one finds

$$m_1/m_0 = \bar{v}\,t\,, \tag{17}$$

which in combination with eqs. (4) and (6) gives a check on v_M, v_P and K_n.

3. Materials and experimental methods

Part of the β-lactoglobulin A was a gift from Dr. G. Schmidt, Instituut voor Zuivelonderzoek, Ede (NL); another part was purchased from Serva, Heidelberg. It was used without further purification. Solutions

were made up in a Michaelis-buffer (NaOH = 0.1 M; CH_3COOH = 0.2 M; pH = 4.62 at 25°C) and filtered with Sartorius membranes (SM 11306, 0.45 μm).

Measurements were made in a standard Tiselius cell (Hellma, Müllheim). The cell was placed in a thermostat which kept the temperature at 2.5 ± 0.05°C.

The concentration gradient was observed with a schlieren optical system which was built with optical parts of Strübin, Basel. The schlieren picture was photographed and evaluated with a micro comparator (Nikon).

At the beginning of the experiment the boundary between buffer and solution was sharpened by the capillary technique of Kahn and Polson [16].

4. Results and discussion

In fig. 1 and 2 we give two examples of our measurements which clearly show the hypersharp leading boundary and the bimodality of the trailing boundary. Sometimes we have observed small peaks with a different velocity, which were probably due to minor impurities of the sample. They were neglected in the data processing of the gradient curves.

The aim of this paper is to see, if the transformation given by eq. (7) can be applied to the experimental curves in such a way as to perform the limiting process which leads from eq. (8) to eq. (9). A look at fig. 2 shows at once that this may pose serious difficulties: on the one hand one should use the concentration gradient profiles at the end of the experiment in order to conform to the mathematical requirement of $w \to 0$; but these profiles tend to become identical with the baseline and they are therefore endowed with high experimental errors. On the other hand the profiles in the middle of the experiment which show less experimental errors may be so much distorted by the diffusional spreading that this effect is not just a small correction term to eq. (9). To put it in another way: are there regimes of time, diffusional spreading and experimental errors which make up to a good compromise?

At first the coordinate point $x = 0$ has to be evaluated in order to be able to use the transormation eq. (7). For this reason the reduced first moment, relative to an arbitrary point $x + a$ in the cell (one of the two markers on the cell which appear as vertical lines in

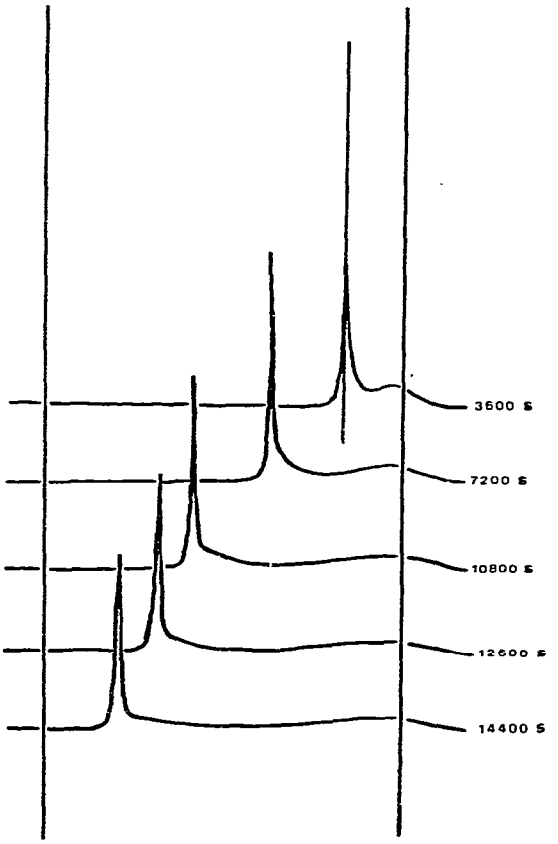

Fig. 1. Leading boundary at different times; $c = 7.2$ g dm^{-3}, $I = 20$ mA cm^{-2}. At the right marker one can see the electrolyte–electrolyte boundary at the starting position.

figs. 1,2), was calculated by numerical integration, plotted versus time t and extrapolated to t = 0. This gives the point $x = 0$, fig. 3, and according to eq. (17) the velocity $\bar{v}$ in the plateau region.

The velocity $\bar{v}$ is, as expected, proportional to the electrical current density I, fig. 4, thereby confirming the absence of convective disturbances.

We now have transformed the series of gradient curves of one run (for example those of fig. 2), i.e. $(\partial c/\partial x)$ versus x for $t = t_1, t_2, \ldots$, into a series of curves $t(\partial c/\partial x) = (\partial c/\partial v)$ versus v, fig. 5. The gradient $(\partial c/\partial v)$ is at constant v only a function of w. Therefore, it should be possible to extrapolate this series of curves

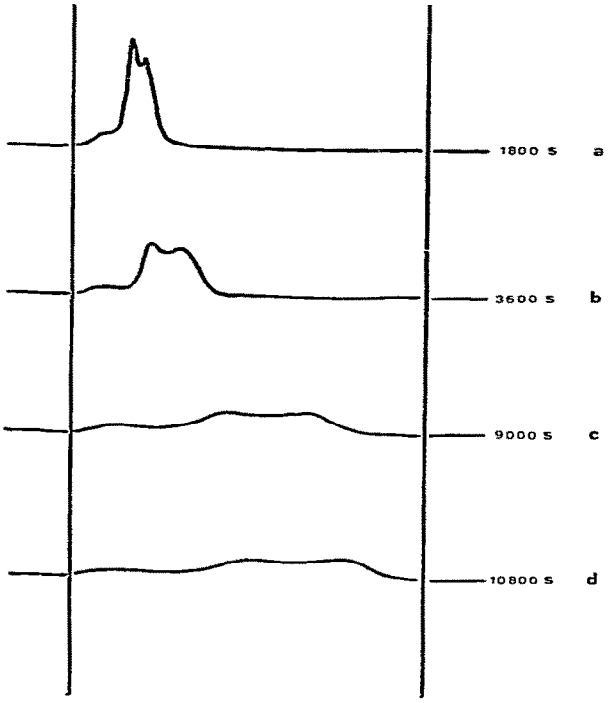

Fig. 2. Trailing boundary at different times; $c = 7.2$ g dm^{-3}, $I = 20$ mA cm^{-2}. The electrolyte–electrolyte boundary is near the left marker.

at constant v to the Gilbert profile, $w \to 0$. The exact nature of this limiting function is not known at the moment. Thus we had to rely on an empirical procedure which was as follows. The series of curves, like those in fig. 5, define within a given limit a start of the Gilbert profile, termed F_1, which was identified according to eq. (11) with the velocity of the monomer v_M. Likewise, the minimum position is fairly independent of w, thereby yielding with the help of eq. (12) a good estimate of the polymer velocity v_p. Furthermore, by integrating the curves of fig. 2 numerically up to v_{min}, we have found a series of values of c_{min}, which were slowly varying with time and which could be extrapolated to $w \to 0$ thus giving with eq. (13) the association constant K_n. So it was possible to construct the expected Gilbert curve which is included in fig. 5 as curve e. As can be seen, the series

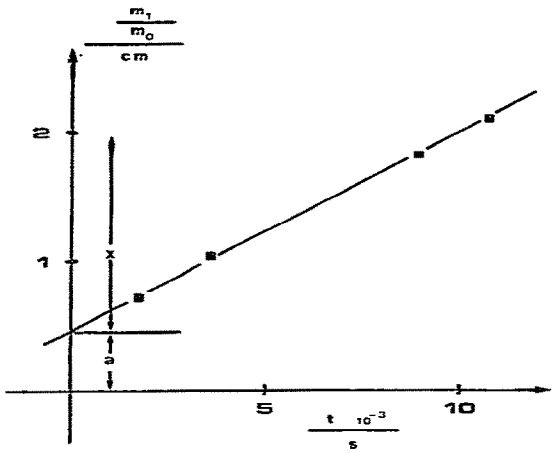

Fig. 3. The reduced first moment (eq. 17) as a function of time t defines the coordinate $x = 0$ and the plateau velocity $\bar{v}$. The four points correspond to the four gradient curves of fig. 2. More gradient curves have been evaluated but not included for the sake of clarity in fig. 2.

of curves does not converge to the Gilbert curve. Instead, with increasing time the distance between the Gilbert curve and the experimental curves increases. This unexpected behaviour was apparently irregular: the degree of divergence varied from experiment to experiment. The reason for this was found in the irregular starting conditions at $t = 0$. Ideally one should have a step function in the concentration at $t = 0$. But this cannot be achieved experimentally: the concentration gradient is not a δ-function but corresponds to a profile which has started from a δ-function a time lag τ before $t = 0$ at a position Δx beyond $x = 0$. These two corrections were determined in the following way: the gradient curves were integrated numerically to give $c(x, t)$. Then for c_i = const. they were plotted in an x-t-plane and extrapolated back to a common origin, fig. 6. This then gives τ and Δx.

With these corrections $(t + \tau)$ and $(x + \Delta x)$, fig. 5 was redrawn, see fig. 7. Now in all cases the series of curves could be extrapolated into the neighborhood of the expected Gilbert curve e. It was already stated, that the exact nature of this extrapolation is not known. So we have tried two procedures:

(1) the transformed and corrected gradient $(\partial c/\partial v)$

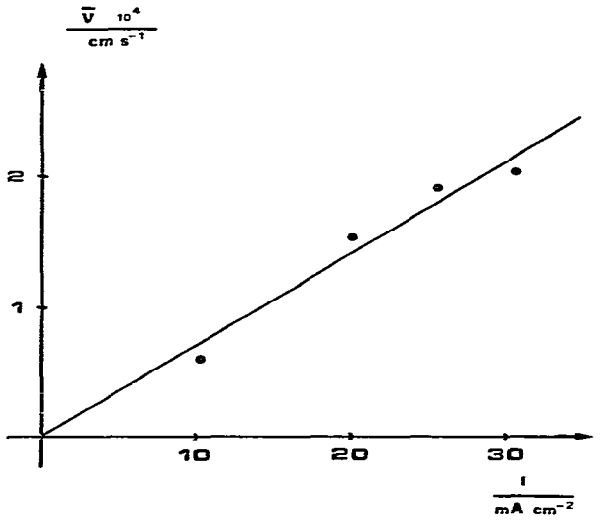

Fig. 4. The plateau velocity $\bar{v}$ as a function of the electrical current density I for trailing boundaries (c = 7.2 g dm^{-3}).

was plotted at constant v versus $w = 1/(t + \tau)$ and extrapolated to $w = 0$. Some representative values are shown in fig. 8. Their extrapolated points are included in fig. 7 as curve f;

(2) the same data are plotted versus $(w)^{1/2}$ and extrapolated to $(w)^{1/2} = 0$, thereby yielding curve g in fig. 7.

These two extrapolation procedures have been selected on the assumption that either migration (procedure 1) or diffusion (procedure 2) is the determining weighting factor. They give slightly different results and probably some yet unknown combination of both would be the solution to this problem.

The curves e, f and g gave the following values of K_n, using v_M and v_P as described, and eq. (10): K_n = 2.2 × 10^{-3} (e); 1.2 × 10^{-3} (f); 3.0 × 10^{-3} g^{-3} dm^3 (g). The stationary migration velocity $\bar{v}$ of the leading boundary (fig. 1) gave K_n = 4.3 × 10^{-3} g^3 dm^{-3}. Measurements at other concentrations and other electrical current densities placed K_n within the same limits. The literature [11] records the values K_n = 11.78 × 10^{-3} at 2°C and 4.29 × 10^{-3} g^{-3} dm^3 at 4.5°C.

This shows that it is possible in principle to deduce the association behaviour from electrophoresis measurements by a suitable extrapolation procedure. But

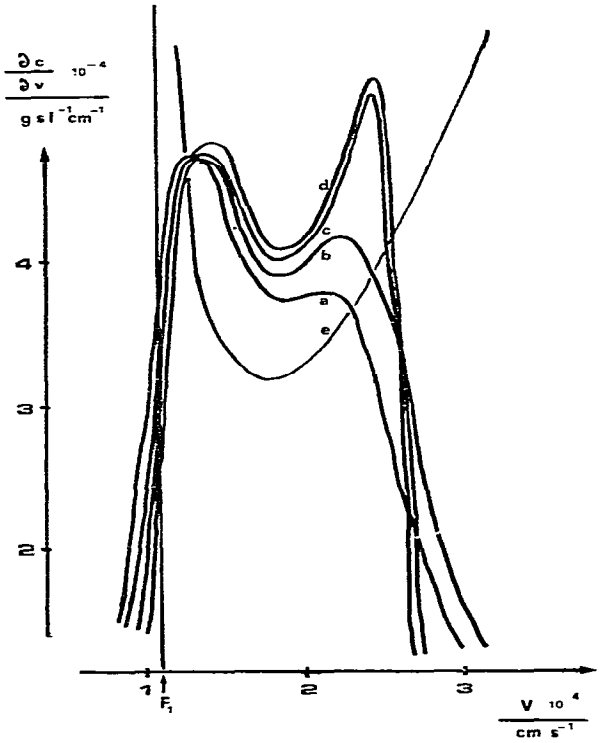

Fig. 5. Gradient curves a, b, c and d of fig. 2 replotted. Curve e is the expected Gilbert curve.

at this stage of the study four types of error limit the accuracy of the method:

(1) At the end of the experiment it is difficult to fix the base line, see fig. 2. So far we have used the constancy of the moment m_0 as a check on the proper choice of the base line. This could perhaps be improved by using absorption optics.

(2) The determination of τ and Δx has been purely empirical. Using the simulation technique of Cox [8] one has to find out the right way of evaluating these corrections.

In this connection it should be pointed out that τ decreases with increasing temperature T, using always the same sharpening technique. So we have found from diffusion measurements at different temperatures that $\tau(T)$ can be correlated with $D(T)$ by $\tau(T) = \beta/D(T)$, where β is a constant. Thus at 25°C the correction is

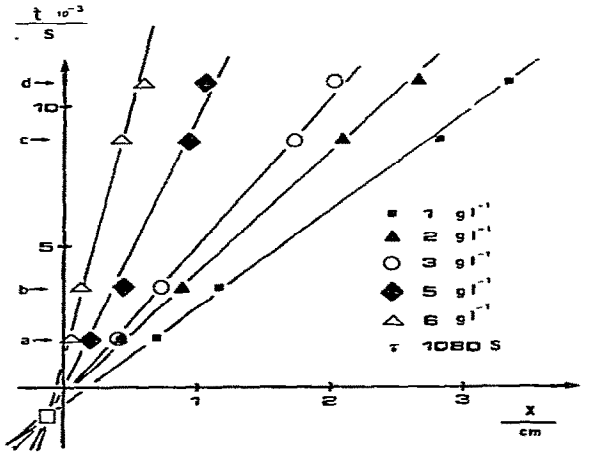

Fig. 6. At the five indicated concentrations the values of $c(x, t)$ = const. (those of fig. 2) are plotted in the $x - t$-plane for the determination of the time lag τ (= 1080 s) and Δx (= 0.16 cm).

not so influential as at 2.5°C, where we have performed the experiments.

(3) The limiting behaviour of $(\partial c/\partial v)$ as a function of w is not known. This introduces the most serious error and should be improved by using the simulation procedure, unless one can establish the next order of the asymptotic solution. Especially, one has to try to find out if according to eq. (8) a correction term $w\{(\partial c/\partial w) + D(\partial^2 c/\partial v^2)\}$ with an average value of D, applied to a plot of $v(\partial c/\partial v)$, would put the extrapolation on a firm basis. At the moment this would just give a third Gilbert curve besides the curves f and g in fig. 7, with no criterium as to which of these is the best one. Furthermore, a comparison of the curves in fig. 5 and fig. 7 shows, that the final results is sensibly dependent on the zerotime correction τ and Δx. So, the following experiments should be aimed at a) improwing the determination of τ and Δx, and b) establishing a criterium for the extrapolation procedure.

In this connection it should be remarked that the procedure of Gosting [17,18] for the evaluation of the heterogenity of non-associating solutes can not be used because the underlying assumptions are quite different from those embodied in eq. (8).

(4) Finally, we have neglected the concentration dependence of the species velocities v_i. It remains to

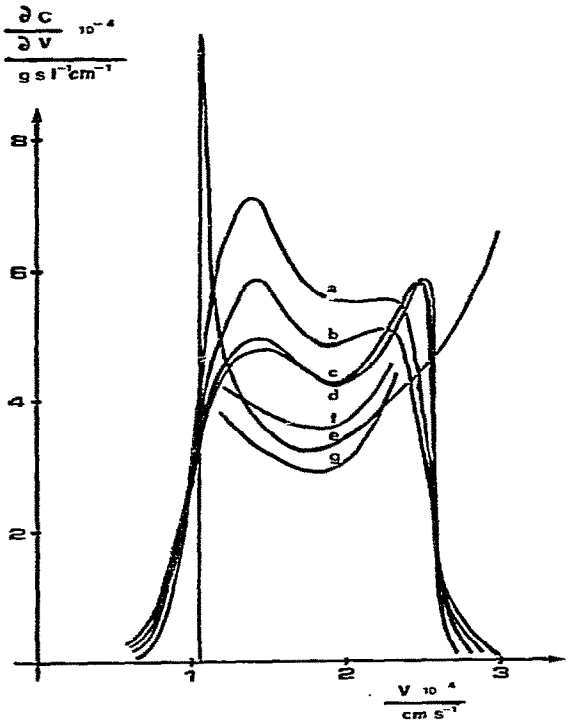

Fig. 7. The data of fig. 5, redrawn with the corrections τ and Δx. Curve f is from fig. 8, curve g from fig. 9.

find out how this dependence looks like.

On the other hand the method seems to be promising because the extrapolated curve entails more information as we have used so far. Assuming that the experimental errors could be reduced sufficiently it might be possible to guess a value of n which could be refined (together with a refinement of v_i, K_i) by reconstructing with a simulation technique the whole series of curve $(\partial c/\partial x)$ as a function of x and t, once in a separate experiment D has been determined. This might also be applied to systems with two associating substances.

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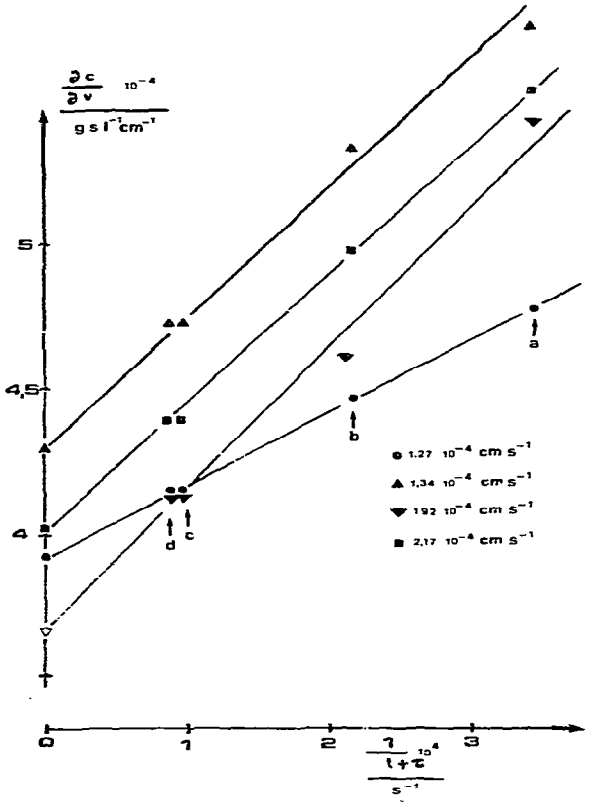

Fig. 8. Extrapolation of the data of fig. 7 at four representative values of v.

of a sample of β-lactoglobulin A and the Landesamt für Wissenschaft und Forschung, Nordrhein-Westfalen, for financial support.

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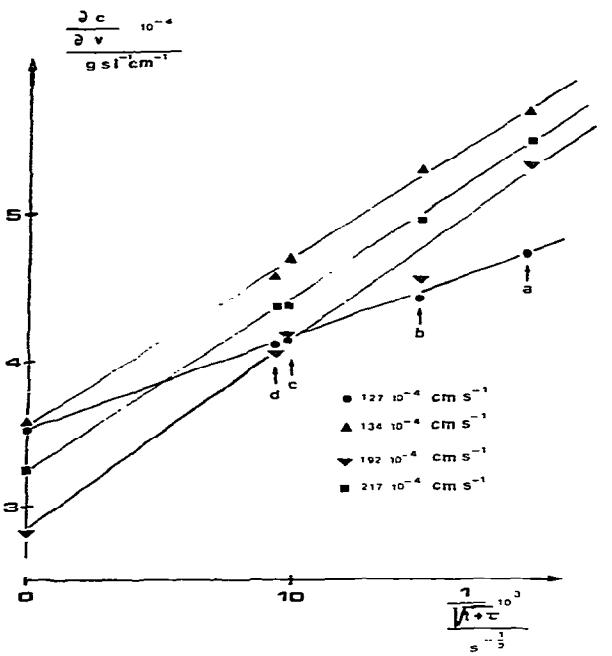

Fig. 9. The same data as in fig. 8, now versus $(w)^{1/2}$.

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