

# The Effective Charge Number and Diffusion Coefficient of Cationic Cytochrome *c* in Aqueous Solution

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The diffusion coefficient and the effective charge number of cytochrome *c* as a function of ionic strength, temperature and pH have been measured. The measurements were carried out using a method based on a convective diffusion process across a porous membrane. The effect of ionic strength was studied in an NaCl solution the concentration of which varied from 0.001 to 1.0 M. The temperature range studied was 10–50 °C, and the pH values studied were 4.0, 6.5 and 8.25.

The diffusion coefficient is fairly constant as a function of ionic strength and pH, and Walden's rule is valid in the temperature range studied. The effective charge number is practically constant (ca. 2) in the concentration range studied, except in 0.001 M solution, where it is the same as the titrated value. The charge number decreases slightly in the temperature range 10–30 °C, but seems to drop suddenly to zero at ca. 40 °C. Measurements using heavy water (D<sub>2</sub>O) as a solvent instead of water did not give zero charge at 40 °C for cytochrome *c*.

Cytochrome *c* is an interesting model compound of a biological macromolecule because it is stable, its structure is well known, and it has an important biological function.<sup>1</sup> The redox behaviour of cytochrome *c* has been widely studied by electrochemical methods at solid electrodes<sup>2–4</sup> and at liquid–liquid interfaces.<sup>5</sup>

A knowledge of their diffusion coefficients and the effective charge numbers is essential when trying to separate proteins (e.g. by chromatographic purification<sup>6</sup> or forced flow electrophoresis<sup>7</sup>), clarify the ion binding phenomenon, and understand their adsorption on interfaces. Also, the evaluation of the  $\zeta$ -potential is possible after measuring the charge number and diffusion coefficient, because from the latter the radius of a globular protein can be calculated according to Stokes' law, thus allowing the estimation of surface charge density with the aid of the charge number.

Recently, a method to determine the diffusion coefficient and effective charge number of a polyelectrolyte has been presented. This method is based on a convective diffusion process, and utilizes the Nernst–Planck equation in dilute solutions and equations derived from the thermodynamics of irreversible processes in concentrated solutions.<sup>8–11</sup> It has been applied to studies of diffusion coefficients and charge numbers of a polydisperse polyelectrolyte, lignosulphonate, while varying the pH,<sup>8,9</sup> the ionic strength,<sup>11</sup> the counter-ion and its valency,<sup>11</sup> the temperature,<sup>12</sup> the relative permittivity of the solvent<sup>13</sup> and the external electric field.<sup>14</sup> The charge number measured by this method is, in fact, the effective charge number ( $z_i$ ); i.e.  $z_i$  is the charge with which the polyelectrolyte (protein)

interacts with the solution. This is because the diffusion potential created by the supporting electrolyte is used as a probe to 'sense' the charge of the protein. Therefore, data on the diffusion coefficient, transport number and activity coefficient as a function of temperature and concentration are needed.<sup>11</sup> Surprisingly, those data are not available, even for an aqueous sodium chloride solution,<sup>15</sup> but fortunately existing data are sufficient to permit a critical estimation of the transport data needed in our calculations.

It is generally recognized that the charge obtained by titration is higher than the effective charge owing to ion binding.<sup>16</sup> In the present study the ion binding phenomenon is investigated by measuring the effective charge number of cytochrome *c* as a function of ionic strength, pH and temperature.

## Experimental

**Apparatus.** In Fig. 1 a side-view of the cell is presented. The cell is made of polycarbonate and is divided into two compartments  $\alpha$  and  $\beta$  by a porous membrane, Millipore BS, with pore size 2  $\mu\text{m}$ , thickness 0.15 mm and diameter 1.13 cm to give a surface area of 1 cm<sup>2</sup>. The volume of each compartment was 0.8 cm<sup>3</sup>. Stirring was carried out with two magnetic 'fleas'. The supporting electrolyte solutions were pumped with a peristaltic pump (Ismatec IPN) into the compartments at constant rates  $V^0$  and  $V^s$  and at constant concentrations  $c^0$  and  $c^s$ , respectively. The solution was pumped out from compartment  $\alpha$  with a flow rate  $V^\alpha$ , and since  $V^\alpha < V^0$  the convective flow through the porous membrane was given by  $V^c = V^0 - V^\alpha$ . The flow from compartment  $\beta$ ,  $V^\beta$ , was a free outlet, i.e.  $V^\beta = V^s + V^c$ .

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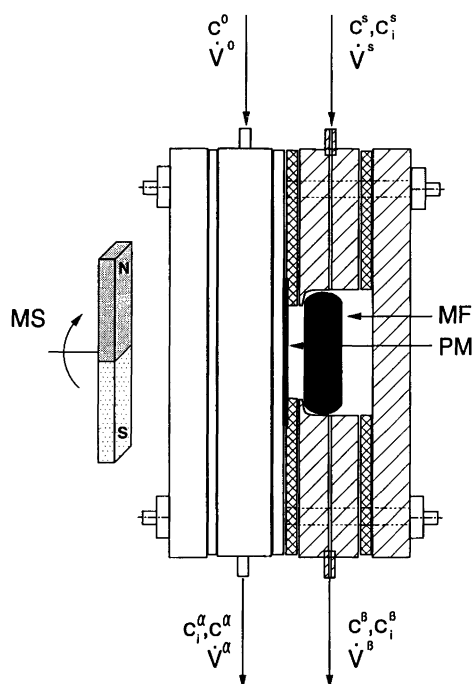


Fig. 1. A side view of the membrane cell. The system consists of two electrolyte solutions ( $\alpha$  and  $\beta$ ) separated by a thin porous membrane (PM). The solutions  $\alpha$  and  $\beta$  are kept homogeneous by stirring with magnetic fleas (MF). The supporting electrolyte solution is pumped into compartments  $\alpha$  and  $\beta$  at constant flow rates  $V^0$  and  $V^s$  and at constant concentrations  $c^0$  and  $c^s$ . Cytochrome  $c$  is added to the feed solution  $V^s$  with concentration  $c_i^s$ . A flow  $V^\alpha$  is pumped out from the  $\alpha$ -compartment, while  $V^\beta$  is the free outlet flow.  $c^\alpha$  and  $c^\beta$  are the stationary-state concentrations in compartments  $\alpha$  and  $\beta$ .

The whole cell was mounted in a thermostat bath which maintained the cell at the chosen temperature with an accuracy of  $\pm 0.05$  °C. The flow rates were measured by weighing the solution volumes and determining the densities (Anton Paar densitometer 40), and thus very accurate values for the flow rates were obtained. Also, leakages in the cell could be detected from the flow balances; the deviations were  $< 0.5\%$ .

The transient time of the measurements was estimated using a mathematical model and was found to be ca. 2 h using typical values of the measurements. Experimentally the stationary state was considered to be reached when no concentration changes took place in the flows  $V^\alpha$  and  $V^\beta$ . Experiments verified the calculations of transient behaviour.

**Materials and analysis.** Sodium chloride, potassium chloride, hydrochloric acid, sodium monohydrogen phosphate, potassium dihydrogen phosphate, disodium tetraborate, sodium acetate and acetic acid were of pro analysis grade. Cytochrome  $c$  was 95–100% pure grade (Sigma C 2506).

To determine the concentration of the supporting elec-

trolyte either the concentration of chloride ion was measured using ion chromatography or the concentration of sodium ion was measured by AAS. The internal standard  $K^+$  was analysed by AAS.

The concentration of cytochrome  $c$  was analysed by UV spectrometry using an analytical wavelength of 220 nm and a confirmational wavelength of 410 nm. The concentration of protein in  $V^\beta$  ( $200\text{--}800\text{ mg dm}^{-3}$ ) was diluted to approximately the same concentration as in  $V^\alpha$  ( $15\text{--}45\text{ mg dm}^{-3}$ ) with the supporting electrolyte solution in order to eliminate background effects.

**Measurements.** The influence of ionic strength on the diffusion coefficient and on the effective charge number was studied in six different supporting electrolyte concentrations (NaCl): 0.001, 0.01, 0.07, 0.1, 0.15 and  $1.0\text{ mol dm}^{-3}$ . The pH of the solutions varied between 6.2 and 6.7 and the temperature was 20 °C.

The influence of temperature was measured in the solution in which the supporting electrolyte concentration (NaCl) was  $0.1\text{ mol dm}^{-3}$ , and the temperatures studied were 10, 20, 30, 35, 40, 45 and 50 °C. The measurements were also carried out using deuterium oxide (i.e. heavy water) as a solvent in 0.1 M NaCl solution and at the temperatures 20, 30 and 40 °C.

The influence of pH at 20 °C was measured in 0.15 M supporting electrolyte solution which was buffered with  $10^{-4}$  M sodium tetraborate at pH 8.25 and with  $10^{-4}$  M sodium acetate–acetic acid at pH 4.0.

The concentration of cytochrome  $c$  in the feed  $V^s$  was  $900\text{ mg dm}^{-3}$  in all measurements except that for which the supporting electrolyte concentration was  $0.001\text{ mol dm}^{-3}$ , when it was  $250\text{ mg dm}^{-3}$ . Diffusion coefficients were determined from measurements for which the concentration of the supporting electrolyte was homogeneous, i.e. the concentrations  $c^0$  and  $c^s$  of the supporting electrolyte in the feeds  $V^0$  and  $V^s$  were equal. In the case of determining the effective charge number the concentration of the supporting electrolyte was kept non-homogeneous, i.e.  $c^0$  and  $c^s$  were not equal. The concentration ratio of the supporting electrolyte  $c^0/c^s$  was then between 1.5 and 1.9.

The membrane constant  $A/l$  was determined for each measurement using KCl as an internal standard, but it could also be calculated from the diffusion flux of the supporting electrolyte during the non-homogeneous experiment. The latter procedure was more accurate because of the lack of accurate tracer diffusion coefficients of  $K^+$  in various NaCl solutions and temperatures used in the measurements.

To give some idea of the flow rates and  $A/l$  we give the ranges of the different quantities used in our experiments:  $V^0 = 1.2\text{--}1.7\text{ cm}^3\text{ h}^{-1}$ ,  $V^\alpha = 1.3\text{--}1.6\text{ cm}^3\text{ h}^{-1}$ ,  $V^c = 0.06\text{--}0.12\text{ cm}^3\text{ h}^{-1}$  and  $A/l = 20\text{--}26\text{ cm}$ .

All solutions were sterilized using Sartorius Minisart NML disposal filter units (pore size  $0.2\text{ }\mu\text{m}$ ).

## Results

The diffusion coefficient of cytochrome  $c$  was determined from the measurements where the concentration of the supporting electrolyte was homogeneous throughout the diffusion process. After reaching the stationary state, the concentration ratio of cytochrome  $c$ ,  $c_i^\beta/c_i^\alpha$ , was measured and the diffusion coefficient could then be calculated from eqn. (1), where  $A/l$  is the membrane constant measured

$$c_i^\beta/c_i^\alpha = (1 + V^\alpha/V^c) \exp [V^c/(A/l)D_i] - V^\alpha/V^c \quad (1)$$

by an internal standard or from a separate measurement of the non-homogeneous supporting electrolyte.<sup>11</sup>

The effective charge number of cytochrome  $c$  was determined from measurements in which the concentration of

$$\frac{c_i^\beta}{c_i^\alpha} = \left(1 + \frac{V^\alpha}{V^c} - \frac{V^0 c^0}{V^c c^\alpha}\right) \exp\left(\frac{V^c}{(A/l)D_M}\right) - \frac{V^\alpha}{V^c} + \frac{V^0 c^0}{V^c c^\alpha} \quad (2)$$

the supporting electrolyte was non-homogeneous, i.e. different in compartments  $\alpha$  and  $\beta$ . After measuring the concentration ratios of cytochrome  $c$ ,  $c_i^\beta/c_i^\alpha$ , and that of the supporting electrolyte,  $c^\beta/c^\alpha$ , the membrane constant,  $A/l$ , and the effective charge number,  $z_i$ , were calculated from eqns. (2) and (3), respectively, where  $c(x)$  is given by eqn. (4).

$$\frac{c_i^\beta}{c_i^\alpha} = \exp\left(\frac{V^c}{(A/l)D_i}\right) \left[ \left(\frac{c^\alpha}{c^\beta}\right)^{z_i \alpha} \left(\frac{D}{D_{0+}} - 1\right) + \frac{V^\alpha}{(A/l)D_i} \int_0^1 \left(\frac{c(x)}{c^\beta}\right)^{z_i \alpha} \left(\frac{D}{D_{0+}} - 1\right) \exp\left(-\frac{V^c x}{(A/l)D_i}\right) dx \right] \quad (3)$$

$$c(x) = c^\alpha \left[ -\frac{V^\alpha}{V^c} + \frac{V^0 c^0}{V^c c} + \left(1 + \frac{V^\alpha}{V^c} - \frac{V^0 c^0}{V^c c^\alpha}\right) \times \exp\left(\frac{V^c x}{(A/l)D_M}\right) \right] \quad (4)$$

In eqn. (2)  $D_M$  is the measured diffusion coefficient of the supporting electrolyte (NaCl in water). The parameters in eqn. (3) are as follows:  $D$  and  $D_{0+}$  are the diffusion coefficients due to the thermodynamic force, i.e. the gradient of the chemical potential. They can be derived as described earlier,<sup>11</sup> and in the Appendix we present estimated values. The values of  $\alpha = 1 + d \ln y_\pm / d \ln c$  are also presented in the Appendix. Some of the values presented in the Appendix are estimated because of the limited number of measured data available. However, in the case of sodium chloride in water, sufficient data were presented in the literature to carry out a critical evaluation of the parameters needed in the calculations.

The diffusion coefficient and effective charge number of cytochrome  $c$  are presented in Fig. 2 as a function of temperature in 0.1 M NaCl solution and in Fig. 3 as a function of ionic strength at 20°C.

In 0.1 M NaCl solution the diffusion coefficient increases and the effective charge number decreases slightly with increasing temperature until about 40°C, at which point  $z_i$  suddenly drops to zero (cf. Fig. 2).

At 20°C the diffusion coefficient is practically constant as a function of ionic strength as is also the charge number, except at very low concentrations of the supporting electrolyte ( $1.0 \times 10^{-3}$  M), where the charge number reaches the titrated value (cf. Fig. 3). Thus cytochrome  $c$  is completely dissociated at an ionic strength of 0.001 M.

In order to elucidate the behaviour of the charge number at 40°C and above, measurements using heavy water as solvent at a sodium chloride concentration of 0.1 M were carried out. However, the charge number remained constant (ca. 2) with increasing temperature from 20 to 40°C, indicating that water as a solvent is likely to be responsible

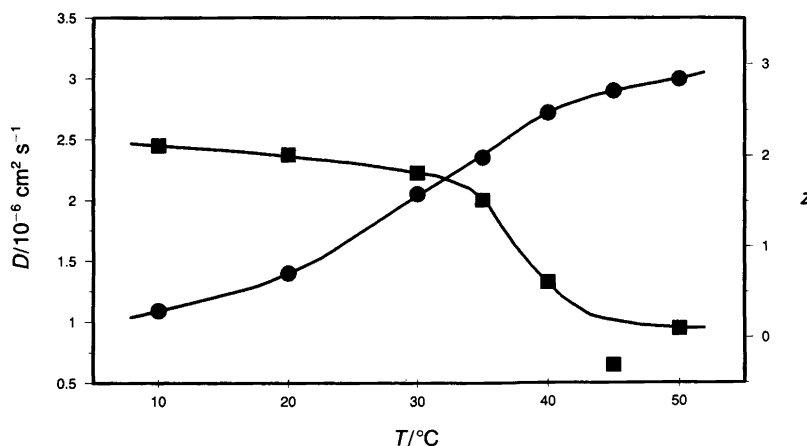


Fig. 2. The diffusion coefficient  $D$  (●) and effective charge number  $z$  (■) of cytochrome  $c$  in 0.1 M NaCl solution as a function of temperature.

Fig. 3. The diffusion coefficient  $D$  (●) and effective charge number  $z$  (■) of cytochrome  $c$  at 20 °C as a function of ionic strength.

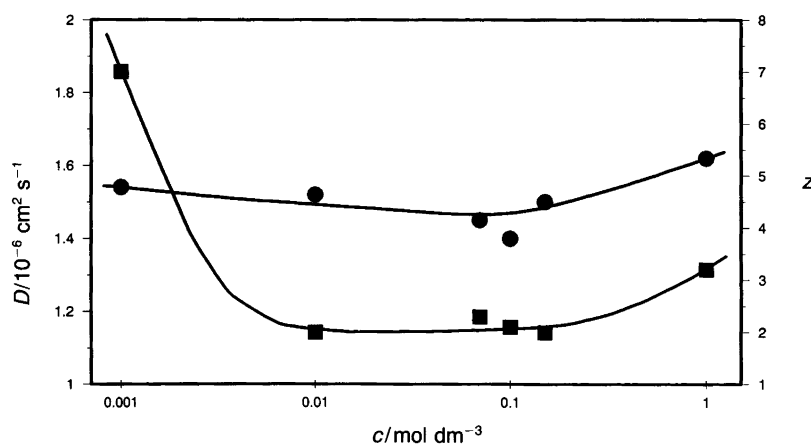


Table 1. Diffusion coefficient  $D$  and effective charge number  $z$  of cytochrome  $c$  as a function of pH. The concentration of the supporting electrolyte (NaCl) is 0.15 mol dm<sup>-3</sup>.

pH	$D / 10^{-6} \text{ cm}^2 \text{ s}^{-1}$	$z$
8.25	1.65	1.4
6.5	1.50	2.0
4.0	1.59	2.6

for the loss of charge. The transport parameters for NaCl in heavy water were calculated from the transport number data (25 °C) and interpolated values of molar conductance (0.1 M NaCl),<sup>17</sup> and the activity coefficient was taken to be the same as in water, which is a good assumption in a 0.1 M NaCl solution.<sup>18</sup>

The effect of pH on the diffusion coefficient and effective charge number is shown in Table 1. The diffusion coefficient and effective charge number are practically constant; however, at pH 8.25 the charge number decreases slightly, which is quite reasonable because the isoelectric point of cytochrome  $c$  is ca. 10.

The reproducibility of the measurements was ca. 5%. Owing to the low value of the charge number its error is relatively large ( $\pm 0.3$ ).

## Discussion

From the results for the diffusion coefficient of cytochrome  $c$  it can be stated that: (1) Walden's rule is valid (Fig. 4); (2) the diffusion coefficient is not significantly dependent on the supporting electrolyte concentration; and (3) the pH has a minor effect on it. Thus it can be concluded that cytochrome  $c$  does not undergo noticeable structural changes under the conditions studied, i.e. while changing the temperature from 10 to 50 °C, the supporting electrolyte concentration from 10<sup>-3</sup> to 1 M, and the pH from 4 to 8.

All our results give a radius of ca. 15 Å for the cytochrome  $c$  molecule, which is in accordance with the crystal-

lographic data<sup>4</sup> (25 Å × 25 Å × 37 Å). The values of the diffusion coefficient obtained by the present method are somewhat greater than those obtained by other methods.<sup>19–21</sup> Most of the diffusion data for proteins have been evaluated from measurements not directly related to the diffusion process, which could be an explanation for the deviation. Using cyclic voltammetry, which resembles our measuring method except that in the former an external electric field is applied, the diffusion coefficient is reported to be  $1.2 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ .<sup>3</sup> This value is ca. 20% lower than ours.

The results for the effective charge number ( $z_i$ ) of cytochrome  $c$  are more puzzling. In a 0.1 M solution of sodium chloride, cytochrome  $c$  becomes neutral, i.e.  $z_i = 0$ , when the temperature exceeds 40 °C. At the temperatures below 40 °C the values of effective charge clearly indicate considerable ion binding. This can be concluded from the early results by Teorell and Åkesson,<sup>22</sup> which showed that the titrated charge is 7 in the pH range 8–4.

The results when varying the pH show that the  $pK$  values of the individual ionizing groups cannot explain the measured charge numbers. The effective charge numbers are clearly dependent on ionic strength, and in such a manner that activities cannot be used as an explanation. Therefore, ion binding is an interpretation for the measured behaviour, and, for example, from the measurements of  $z_i$  as a function of temperature when the supporting electrolyte concentration is 0.1 M and the temperature below 40 °C, the degree of ion binding is ca. 0.7.

It is common practice to present the  $\zeta$ -potential instead of the effective charge. When doing so it must be recognized that, strictly speaking, the  $\zeta$ -potential is not a measurable quantity. According to Newman<sup>23</sup> the  $\zeta$ -potential is given for a flat surface by eqn. (5), where  $q_2$  is the surface

$$q_2 = -\epsilon \zeta / \lambda \quad (5)$$

charge density in the diffuse layer,  $\epsilon$  is the permittivity and  $\lambda$  is the Debye length. An equation corresponding to eqn. (5) for small spheres has also been derived,<sup>16</sup> but is less

Fig. 4. Verification of Walden's rule for cytochrome *c* when the concentration of supporting electrolyte (NaCl) is 0.001 M (●), 0.1 M (▲) and 1.0 M (■), respectively.

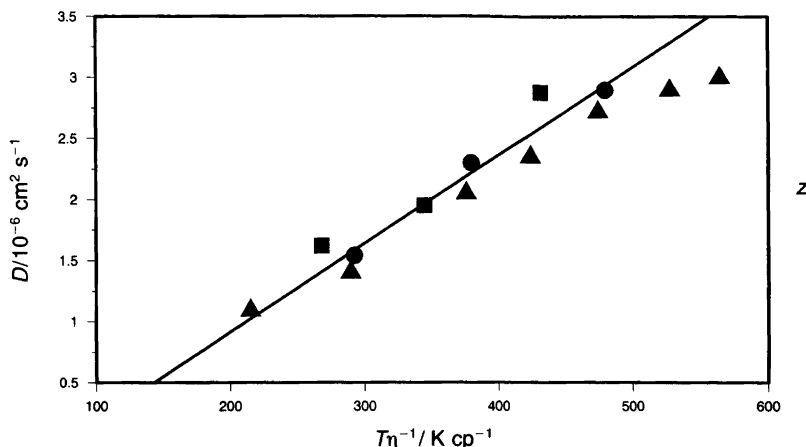


Table 2. Calculated values of the  $\zeta$ -potential for cytochrome *c* at 20 °C.

$c/\text{mol dm}^{-3}$	$\zeta/\text{mV}$
0.001	-610
0.01	-56
0.07	-24
0.1	-18
0.15	-14
1.0	-2.8

accurate in our case than that for a flat surface. Therefore, the  $\zeta$ -potential is calculated using the above equation. When the surface charge density was estimated using measured  $z_i$  values, and the calculated radius of the cytochrome *c* molecule, the values of the  $\zeta$ -potential presented in Table 2 were obtained.

A problematic behaviour is connected with the temperature dependence of the effective charge number. As shown in Fig. 2, in a 0.1 M solution of NaCl  $z_i$  reaches a very low value when the temperature exceeds 40 °C. In practice we can say that  $z_i = 0$ , resembling the behaviour of lignosulphonate under the same conditions.<sup>24</sup> A similar behaviour has not been observed for polystyrene sulphate,<sup>25</sup> which is considered to be a non-free-draining coil, while lignosulphonate and cytochrome *c* are considered to be compact spheres.

In order to obtain an explanation of the loss of charge at elevated temperatures a set of measurements was carried out in heavy water (deuterium oxide), i.e. an attempt was made to determine if the phenomenon is due to the properties of the solvent. The results of the experiments described above clearly showed that in  $\text{D}_2\text{O}$  the charge is not lost at 40 °C. Therefore, it is concluded that the loss of charge at ca. 40 °C is due to the solvent (water). It must be recognized that Robinson and Stokes<sup>26</sup> pointed out the unexceptional behaviour of water in the neighbourhood of 40 °C. That is why we, resorting to evidence obtained from measurements with lignosulphonate,<sup>24</sup> propose that the loss of

charge is somehow due to changes in structured water at the surface of the protein.

## Appendix

The diffusion coefficients of NaCl were calculated up to 0.05 M by the Nernst–Hartley equation with the thermodynamic correction term, because the electrophoretic correction is bound to be small.<sup>26</sup> At other concentrations Walden's rule in the form  $D\eta/T$  was applied, based on the measured diffusion coefficients ( $D_M$ ) at 25 °C. The measured data on diffusion of NaCl at other temperatures is surprisingly limited. The viscosity ( $\eta$ ) data for Walden's rule were obtained from Ref. 27. The thermodynamic term ( $\alpha = 1 + \text{dln } \gamma_{\pm} / \text{dln } c$ ) in the Nernst–Hartley equation was calculated by Debye–Hückel theory up to 0.07 M. In other concentrations the data from Ref. 15 were interpolated to suitable concentrations and the necessary temperatures. The transport numbers were calculated by an equation from conductivity theory (cf. Ref. 26). The proposed values for the measured diffusion coefficient,  $D_M$ , for the diffusion coefficient based on the thermodynamic force,  $D$ , and for the ionic diffusion coefficient for the sodium ion based on the thermodynamic force  $D_{0+}$ , are presented in Table A1. The calculations were performed as described earlier<sup>11</sup> using the values as described above.

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Table A1. The proposed values for the transport properties.

$t/^\circ\text{C}$	10	15	20	25	30	35	40	45	50
0.002 M NaCl									
$D_M$	1.05	1.21	1.39	1.57	1.78	1.98	2.21	2.44	2.68
$D$	1.08	1.24	1.42	1.61	1.82	2.03	2.26	2.50	2.75
$D_{O^+}$	0.88	1.02	1.17	1.33	1.51	1.69	1.88	2.09	2.31
$\alpha$	0.977	0.977	0.977	0.977	0.976	0.976	0.976	0.976	0.975
0.01 M NaCl									
$D_M$	1.03	1.19	1.36	1.54	1.73	1.94	2.16	2.38	2.62
$D$	1.08	1.24	1.42	1.61	1.82	2.03	2.26	2.50	2.75
$D_{O^+}$	0.88	1.02	1.17	1.32	1.50	1.68	1.88	2.08	2.30
$\alpha$	0.955	0.955	0.955	0.954	0.954	0.953	0.953	0.952	0.952
0.07 M NaCl									
$D_M$	0.97	1.31	1.31	1.50	1.70	1.91	2.14	2.38	2.55
$D$	1.06	1.23	1.43	1.63	1.85	2.09	2.34	2.61	2.80
$D_{O^+}$	0.85	1.00	1.16	1.33	1.52	1.71	1.92	2.15	2.32
$\alpha$	0.916	0.916	0.915	0.915	0.914	0.913	0.912	0.912	0.910
0.1 M NaCl									
$D$	0.96	1.12	1.30	1.48	1.68	1.90	2.12	2.36	2.53
$D$	1.05	1.23	1.42	1.62	1.84	2.07	2.31	2.57	2.75
$D_{O^+}$	0.85	0.99	1.15	1.32	1.50	1.69	1.89	2.12	2.28
$\alpha$	0.912	0.914	0.914	0.915	0.915	0.916	0.916	0.917	0.918
0.15 M NaCl									
$D_M$	0.96	1.12	1.29	1.48	1.67	1.89	2.11	2.35	2.52
$D$	1.05	1.22	1.41	1.61	1.83	2.06	2.30	2.56	2.74
$D_{O^+}$	0.85	0.99	1.14	1.31	1.49	1.68	1.88	2.10	2.26
$\alpha$	0.909	0.911	0.912	0.913	0.913	0.914	0.914	0.914	0.915
1 M NaCl									
$D_M$	0.98	1.14	1.30	1.48	1.68	1.88	2.10	2.33	2.49
$D$	0.98	1.12	1.28	1.45	1.64	1.83	2.04	2.56	2.41
$D_{O^+}$	0.77	0.89	1.02	1.16	1.31	1.47	1.64	1.82	1.96
$\alpha$	0.987	0.995	0.999	1.003	1.006	1.009	1.011	1.013	1.015

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