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Magnetization-transfer NMR Investigation of the Hydrogen Exchange in Mixtures of *N*-Methylacetamide and Water

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Hydrogen exchange rates in mixtures of *N*-methylacetamide and water are measured by the magnetization-transfer method. The exchange rate is studied as a function of pH, concentration and temperature. In mixtures with the mol ratio of NMA–H_2O=1:5.16, at 351 K, the exchange is catalyzed by protons and by hydroxyl ions; at pH=5.0, where the rate is minimal, the uncatalyzed exchange between the peptide group and H_2O amounts to 75 % of the total exchange measured. The results obtained are discussed with particular reference to the hydrogen exchange in protein solutions.

Measurements of the kinetics of hydrogen exchange in protein solutions are used as a tool to characterize protein conformations. In a recent review[1] on the acquisition and interpretation of hydrogen exchange data, the authors take a realistic attitude to the possibilities and the limitations of the method, and they state that "the interpretation of hydrogen exchange data has lagged somewhat behind the state of data acquisition and remains problematic for all but the simplest peptides". We can only agree with this statement except that we are inclined to include data on simple peptides in the problematic part. The aim of the present study of the hydrogen exchange in mixtures of *N*-methylacetamide (NMA) with water is to elucidate some fundamental aspects of the exchange reaction, and in this way to contribute to a strengthening of the basis of the interpretation of the exchange in protein solutions.

The exchange in NMA–H_2O mixtures is measured by the magnetization-transfer NMR method, as described in detail in Ref. 2. The pH dependence of the exchange rates, and the dependence on the water concentration, are studied at 351 K. This relatively high temperature is chosen in order to make the data directly comparable with available NMR measurements of the hydrogen-deuterium exchange in protein solutions.[3,4] The activation energy of the exchange reaction is estimated from measurements on three NMA-H_2O samples in the temperature range 307–370 K.

MATERIALS AND METHODS

N-Methylacetamide from Fluka (analytical grade) was distilled three times at 95 °C and 14 mmHg, using a Fischer Spaltrohr column with approximately 40 theoretical plates. The water used was glass distilled at atmospheric pressure.

Mixtures of *N*-methylacetamide and water were prepared by pipetting. The density of the components, used in the calculations of the concentrations, was 0.995 g cm^{-3} (H_2O) and 0.956 g cm^{-3} (NMA). The volume change of mixing was ignored in the calculations.

The pH was adjusted by the addition of (small amounts of) 0.5 M HCl or KOH. The pH was measured at room temperature by a Radiometer pH meter PHM 64 with a glass electrode GK 2320C, standardized on aqueous buffer solutions.

NMR EXPERIMENTS

Hydrogen exchange rates in the NMA-H_2O mixtures were measured by the magnetization-

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transfer NMR method using an unmodified Bruker HX-270 NMR spectrometer. The time dependence of the height of both the NH and the water proton signals was monitored after a selective inversion of either one of them, as illustrated in Fig. 1. In each experiment 4–40 accumulations per spectrum were employed, and the delay time between two consecutive accumulations was 4–5 times the longest relaxation time observed. The line width of the NH proton

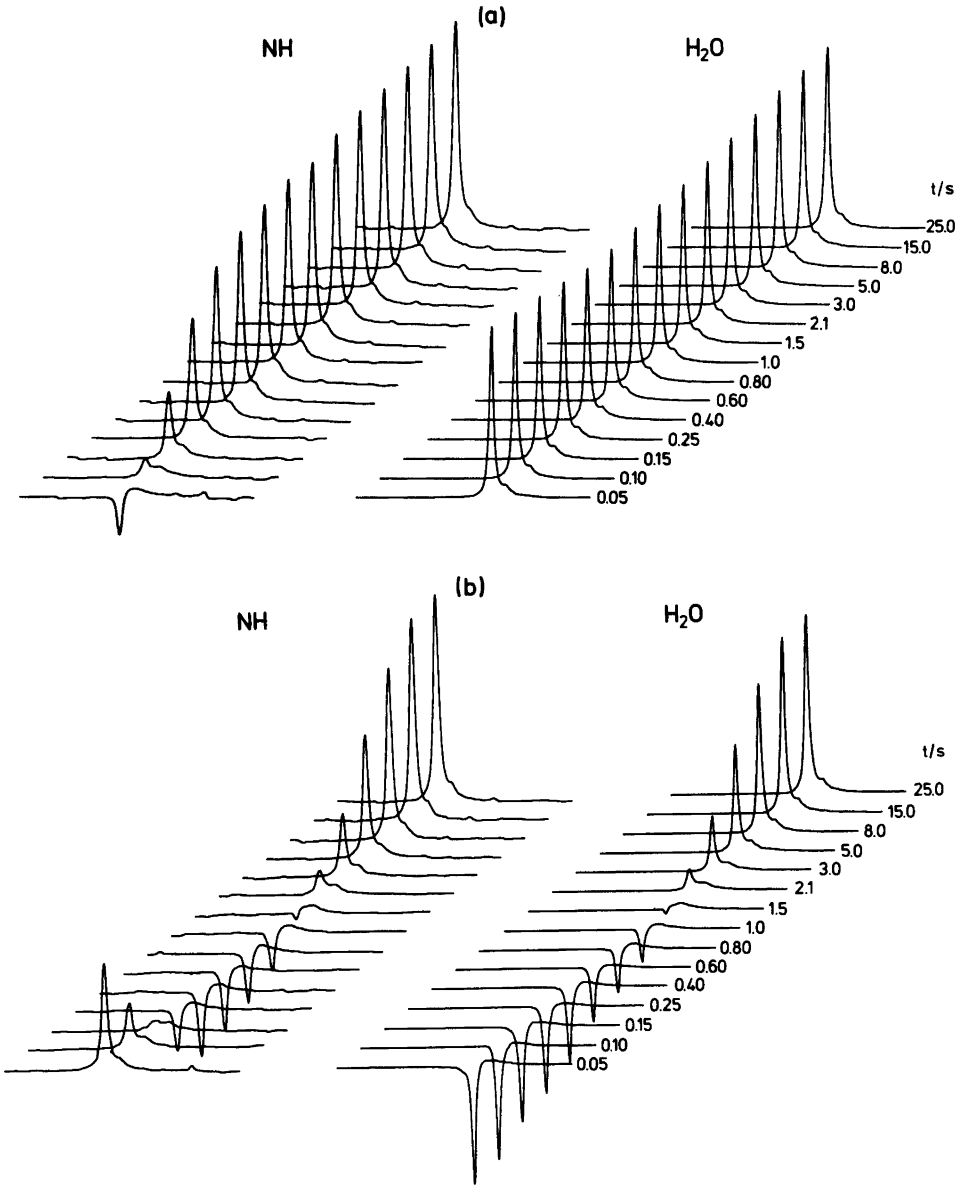

Fig. 1. Inversion transfer NMR spectra of a mixture of NMA and water. The mol ratio of the components is 1:5.16; pH=7.55, and T=339 K. In (a) the NH signal is inverted; in (b) the H_2O signal is inverted. Only every second spectrum of the experiment made is shown. The relative scaling of the NH and H_2O spectrum is ~1:20.

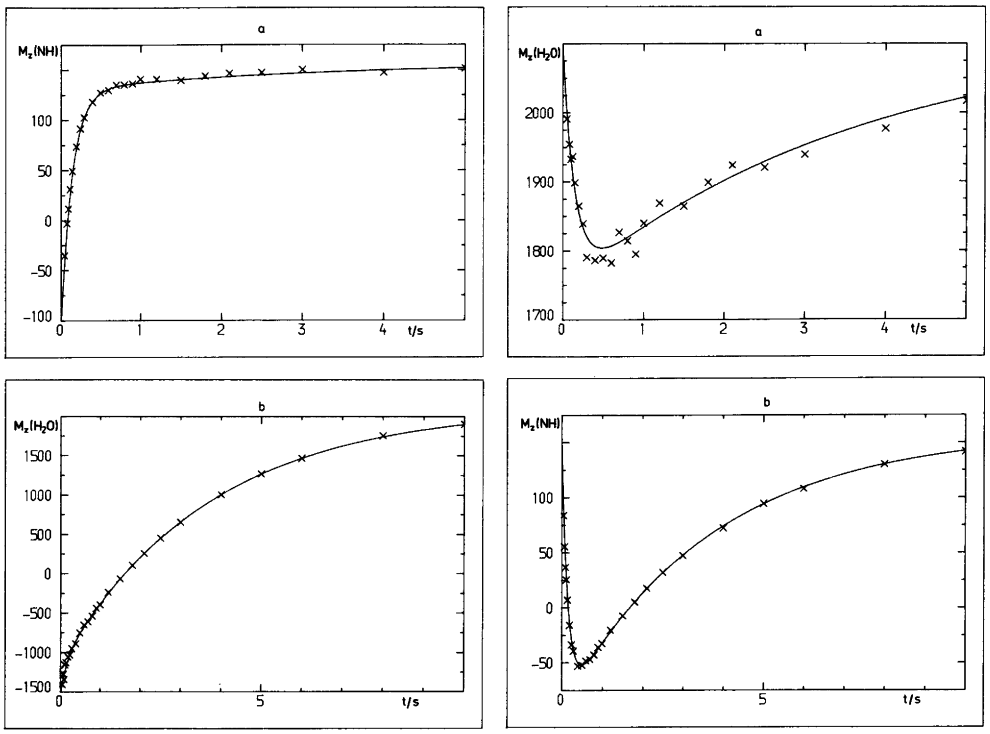

Fig. 2. Plots of the time-dependent peak heights of the set of complementary experiments in Fig. 1. The curves represent the best fit obtained in a simultaneous least squares analysis of the time dependence of the four variables.

signal was substantially decreased by a selective ^{14}N decoupling and the resulting narrowly spaced quartet was effectively averaged to a single signal by applying an exponential multiplication of the FID (free induction decay) signal corresponding to 15 Hz.

The temperature of a sample in the spectrometer was obtained from the chemical shift difference in ethylene glycol;[5] the temperature was measured before and after each experiment.

Further details about the experimental procedure are given in Ref. 2.

Data analysis. The basis of the data analysis was the McConnell equations, modified as previously described.[2] Exchange rates were retrieved by a simultaneous least squares analysis of all the data obtained in a set of complementary inversion-transfer experiments. This procedure allows an independent determination of all the parameters involved.[2] The fitting to the peak heights of the spectra in Fig. 1 is illustrated in Fig. 2.

NOE effects. While a possible static NOE effect caused by the ^{14}N-decoupling is accounted for in the experimental procedure and in the data analysis,[2] a possible transient NOE effect,[6] caused by a mutual dipolar interaction between the NH and H_2O protons, would make the estimated exchange rates smaller than the actual rates. However, in magnetization-transfer experiments performed on samples I, II and III (Table 3) at 293 K, *i.e.* under conditions where the exchange rates are too slow to be measurable, no change in the intensity of the non-inverted signal was observed. A transient NOE effect and NH–H_2O proton dipolar interactions are, therefore, not operative at 293 K. As explained previously[2] this, most likely, also holds at the higher temperatures of the present study.

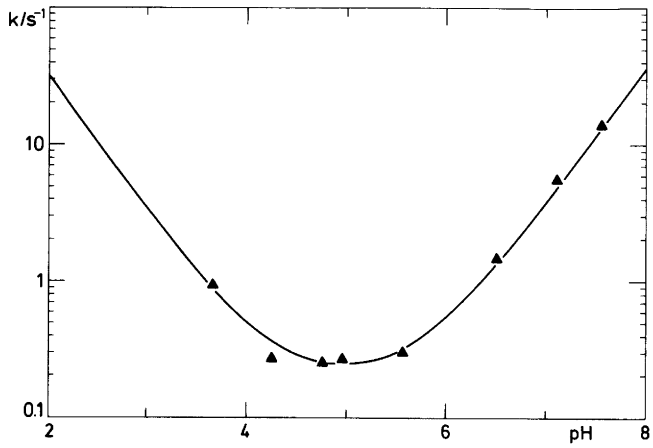

Fig. 3. The data in Table 1 plotted as log k *vs.* pH. The curve corresponds to the values of the parameters given in eqn. (4).

Table 1. Measured rates and 1σ standard deviations of the hydrogen exchange in NMA–H_2O mixtures at 351 K, and various values of pH. [NMA]=5.89(2) M; [H_2O]=30.4(2) M.

pH	k/s^{-1}
3.65	0.92(8)
4.25	0.26(3)
4.75	0.248(12)
4.95	0.267(13)
5.55	0.29(3)
6.49	1.78(18)
7.08	5.3(2)
7.55	13.2(3)

Table 2. Measured rates and 1σ standard deviations of the hydrogen exchange at 351 K in NMA–H_2O mixtures of various mol ratios.

[H_2O]/M	NMA/M	pH	k/s^{-1} (obs.)	k/s^{-1} (calc.)
1.47	12.7	7.55	0.011(2)	0.009
5.02	11.9	7.10	0.018(6)	0.019
11.4	10.4	5.30	0.056(8)	0.084
18.6	8.68	5.17	0.150(6)	0.137
23.8	7.46	5.10	0.182(5)	0.184
30.4	5.89	4.25	0.26(3)	0.25
41.4	3.27	4.25	0.48(4)	0.44
44.2	2.62	4.30	0.51(6)	0.45
48.3	1.64	4.25	0.60(5)	0.50
51.8	0.82	4.32	0.64(2)	0.65

RESULTS

Proton exchange rates, $k=\tau^{-1}$ (s^{-1}), measured at 351 K in NMA–H_2O mixtures with a mol ratio 1:5.16, and at various values of pH, are presented in Table 1 and illustrated in Fig. 3 by a plot of log k *vs.* pH.

Measurements at 351 K of the dependence of the exchange rate of the NMA–H_2O mol ratio are reported in Table 2.

In conformity with general practice, the pseudo first order exchange rate constants measured are expressed as eqn. (1),

$$k=k_{H^+}[H^+]+k_{OH^-}[OH^-]+k_w \quad (1)$$

where k_w is the contribution to k due to the uncatalyzed exchange between a peptide group and water. Assuming that this reaction is of first order with respect to the concentration of water, *i.e.* $k_w=k_{H_2O}[H_2O]$, eqn. (1) can be rewritten as

$$k=k_{H^+}10^{-pH}+k_{OH^-}K_w10^{pH}+k_{H_2O}[H_2O] \quad (2)$$

Here K_w is the ionization constant of water, $K_w=[H^+][OH^-]=K_{H_2O}[H_2O]$, where K_{H_2O} is the dissociation constant.

Values of K_w for pure water are available at temperatures up to 333 K,[7] and extrapolation of these data gives the value $K_w=25.1\ 10^{-14}$ M^2 at 351 K, where [H_2O]=55.25 M. In accordance with Fig. 15-6-1 and Table 15-6-2A in Ref. 8 the

Table 3. Measured rates and 1σ standard deviations of the hydrogen exchange as functions of temperature in three mixtures of NMA and H_2O.
I: $[H_2O]$=11.4 M; [NMA]=10.4 M pH=5.3.
II: $[H_2O]$=48.3 M; [NMA]=1.64 M pH=4.3.
III: $[H_2O]$=30.4 M; [NMA]=5.89 M pH=7.6.

Sample	*T*/K	k/s^{-1}
I	339.2	0.026(8)
	351.5	0.058(2)
	362.0	0.149(4)
	369.6	0.228(5)
II	322.1	0.068(5)
	327.2	0.102(4)
	339.2	0.270(12)
	351.5	0.60(5)
	362.0	1.20(3)
	369.6	2.41(18)
III	307.5	0.163(4)
	313.5	0.466(8)
	322.1	1.274(12)
	329.6	2.40(4)
	339.2	6.07(7)
	351.5	14.1(2)
	362.0	30.0(15)
	369.6	44.9(8)

concentration dependence of K_w at 351 K is expressed as

$$K_w=([H_2O]/55.25)\times25.1\times10^{-14}\exp[a(x_{H_2O}-1)] \quad (3)$$

Here x_{H_2O} is the mol fraction of water, and a is a parameter estimated in the numerical fitting to the experimental data.

The measurements in Table 1 and 2 were analyzed simultaneously using eqn. (2), and with K_w as given in eqn. (3). The result of the least squares analysis of the data is the following

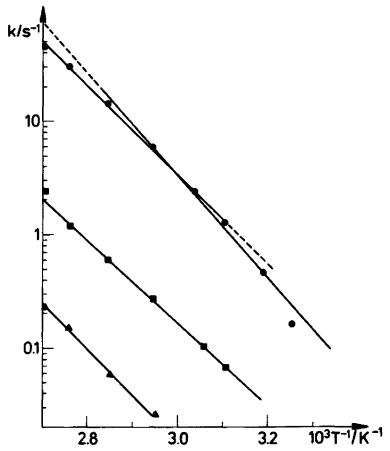

Fig. 4. The data in Table 3 plotted as log k *vs.* T^{-1}. ▲, sample I; ■, sample II; ●, sample III. The specifications of the samples are given in Table 3.

$$k_{H^+}=(2.90\pm0.65)\times10^3\ M^{-1}s^{-1}$$

$$k_{OH^-}=(1.54\pm0.28)\times10^7\ M^{-1}s^{-1}$$

$$k_{H_2O}=(6.00\pm0.43)\times10^{-3}\ M^{-1}s^{-1} \quad (4)$$

$$a=\mathrm{d}\ln K_{H_2O}/\mathrm{d}\,x_{H_2O}=10.85\pm1.2$$

The variation of the exchange rate with the temperature was measured in the temperature range 307–370 K on three samples with the mol ratio NMA–H_2O of 1:1.057 (I), 1:29.67 (II) and 1:5.16 (III), and pH values of 5.30 (I), 4.25 (II) and 7.55 (III), respectively. The rates obtained including the 1σ standard deviations are reported in Table 3 and presented in Fig. 4 as a plot of log k *vs.* T^{-1}. The apparent activation energy of the exchange reaction, estimated as $E^{\#}_{app}=-R\ \mathrm{d}\ln k/\mathrm{d}(T^{-1})$, is given in Table 4.

Table 4. The apparent activation energy of the exchange reaction.

	$E^{\#}_{app}$/kJ mol^{-1}	$E^{\#}_{H^+}$/kJ mol^{-1}	$E^{\#}_{OH^-}$/kJ mol^{-1}	$E^{\#}_{H_2O}$/kJ mol^{-1}
Sample I[a]	80 (6)			
Sample II[a]	70 (2)	97 (18)	25 (1)	48 (8)
Sample III[a]	75 (2) (322–370 K) 88 (1) (307–351 K)			

[a] The specifications of the samples are given in Table 3.

DISCUSSION

In attempts to explain the hydrogen exchange in aqueous protein solutions, it is usually assumed that the exchange in a given peptide group of a protein occurs as a direct proton transfer between the peptide group and water. Exchange rates measured in protein solutions are tentatively taken as a tool to estimate the conditions of solvent exposure of peptide groups in protein molecules, but the mechanisms governing this exposure are at present a subject of some controversy.[9,10] Part of the problems involved in a meaningful interpretation of data on protein hydrogen exchange is related to our limited knowledge of the basic exchange reaction, *i.e.* the hydrogen exchange in a peptide group exposed to water.

The pH dependence of the exchange. Measurements at or below room temperature of the exchange in dilute aqueous solutions of amides or randomly coiled peptides have shown that the exchange of peptide hydrogen atoms is catalyzed by protons and by hydroxyl ions.[11–15] The pH dependence of the pseudo first order rate constants measured is found to be in accordance with eqn. (1), and the data indicates that the uncatalyzed exchange reaction is negligible ($k_w \ll 2(K_w k_{H^+} k_{OH^-})^{1/2}$), so that

$$k \simeq k_{H^+} 10^{-pH} + k_{OH^-} K_w 10^{pH} \qquad (5)$$

This feature of the exchange reaction has been of importance in interpretations of the kinetics of the exchange in protein solutions.[1,9,10,16–18] It seems to be a generally accepted assumption, underlying discussions of the exchange, that under all conditions the pH dependence of the rate of hydrogen exchange in a solvent-exposed peptide group is in accordance with eqn. (5), with pH and K_w being the pH measured in the protein solution, and the ionization constant of pure water, respectively.

However, considerations of dynamic aspects of protein conformations have shown that access of water molecules to interior regions of protein molecules can be made through channels created by "mobile defects",[19] or volume fluctuations of cavities[20] in the conformations. Such channels hold only a limited number of water molecules, and it is to be expected that the ionization constant of water present in the interior of protein molecules may be different from the ionization constant of pure water. In an evaluation of the importance of this mechanism of exposure of protein peptide groups to water it is, therefore, of interest to know the order, with respect to water, of the exchange reaction, and to estimate the ionization constant of water within a protein molecule. Although mixtures of NMA with water are, indeed, imperfect models of protein molecules, measurements of the hydrogen exchange in such mixtures may throw some new light on the mechanisms governing the exchange in protein solutions.

In a preliminary fitting of the parameters in eqn. (1) to the data in Tables 1 and 2 k_w was expressed as $k_w = k_{H_2O}[H_2O]^n$, which resulted in the value of $n = 1.3(2)$. In the subsequent analysis of the data, which led to the results in eqn. (4), it was assumed that $n = 1$.

The values of the parameters in eqn. (4), and Fig. 3, show that under the conditions of the experiments reported, the contribution to the exchange due to the uncatalyzed reaction of NMA with H_2O is substantial. In the mixtures the ionization constant of water, calculated according to eqn. (3), is $K_w = 1.70 \times 10^{-14}$ M^2. The minimal exchange rate is observed at pH=5.0 ($[H^+]_{min} = (K_w k_{OH^-}/k_{H^+})^{1/2}$), and at this pH the uncatalyzed reaction amounts to about 75 % of the total exchange ($k_{H^+}[H^+]_{min} = k_{OH^-}[OH^-]_{min} = (K_w k_{H^+} k_{OH^-})^{1/2} = 2.7 \times 10^{-2}$ s^{-1}, and $k_w = k_{H_2O}[H_2O] = 18 \times 10^{-2}$ s^{-1}).

This result may be of interest in relation to recent NMR measurements of the exchange rates of individual peptide groups of the bovine pancreatic trypsin inhibitor (BPTI),[3,4,18] where pronounced deviations from the "normal" pH dependence, as expressed in eqn. (5), were observed. A comparison of the protein data with the results of the present investigation of the exchange in NMA–H_2O mixtures indicates that the uncatalyzed exchange reaction contributes significantly to the exchange rates observed in the protein experiments.

Since the uncatalyzed reaction is normally assumed to be negligible in peptide hydrogen exchange with bulk water,[3] the data on BPTI may suggest that the observed exchange occurs partly in reactions with "channel" water, characterized by a smaller ionization constant.

The temperature dependence of the exchange. As illustrated in Fig. 4 the exchange rates

measured show a simple exponential dependence on T^{-1}, except for sample III, where the largest temperature range is covered. The apparent activation energy of the exchange reaction, $E^{\#}_{app}$, estimated by a least squares fit of the expression $E^{\#}_{app} = -R \, d \ln k/d \, T^{-1}$ to the experimental data, is reported in Table 4. The values are within the range of the values in the literature,[1] obtained by a similar procedure.

The apparent activation energy may be considered as a sum of three contributions

$$E^{\#}_{app}(pH,[H_2O]) = (k_{H^+}/k)10^{-pH}E^{\#}_{H^+} + (k_{OH^-}/k)K_W \times 10^{pH}(E^{\#}_{OH^-} + \Delta H^{\circ}_W) + (k_{H_2O}[H_2O]/k)E^{\#}_{H_2O} \quad (6)$$

where ΔH°_W is the ionization enthalpy of water. In this expression it is assumed that the individual activation energies and ΔH°_W are independent of the temperature.

At pH=7.6 (sample III) the first two terms of eqn. (6) are negligible, so that $E^{\#}_{app} \simeq E_{OH^-} + \Delta H^{\circ}_W$. The observed deviation from a linear relationship between ln k and T^{-1}, may be due to change in ΔH°_W with changes in the temperature. In pure water ΔH°_W varies only about 10 % [7] in the observed temperature region ($\Delta H^{\circ}_W \simeq 50$ kJ mol^{-1}), but the strong solvation of peptide groups by water [21] may influence the ionization enthalpy of water in the mixtures studied, as well as its temperature dependence.

In an attempt to estimate the individual activation energies, $E^{\#}_{H^+}$, $E^{\#}_{OH^-}$ and $E^{\#}_{H_2O}$, by a numerical analysis based on a combination of eqns. (2) and (6), we have tentatively made the following approximations:

(1) Measurements made below 314 K (sample III) are ignored and (2) the ionization enthalpy of water in the mixtures is assumed to be equal to the ionization enthalpy of pure water at 333.15 K, $\Delta H^{\circ}_W = 52$ kJ mol^{-1},[7] and independent of the temperature. The results of the analysis are presented in Table 4. The relatively small value of $E^{\#}_{H_2O}$, compared with $E^{\#}_{H^+}$ and $(E^{\#}_{OH^-} + \Delta H^{\circ}_W)$, indicates that the uncatalyzed exchange reaction is of increasing importance with decreasing temperature, or that the plateau in a plot of ln k *vs.* pH becomes broader when the temperature is lowered. This behaviour is in contrast to the general view that the plateau is absent at room temperature.[1] It may, however, be noted that the values of $E^{\#}_{H^+}$, $E^{\#}_{OH^-}$ and $E^{\#}_{H_2O}$, reported in Table 4, are based on measurements above 314 K, and that the data on sample III in Fig. 4 suggest that a straight-lined extrapolation of ln k *vs.* T^{-1} below 314 K may not be justified.

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