Analytical Methods

TECHNICAL NOTE

View Article Online
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Cite this: *Anal. Methods*, 2017, **9**, 2744

Practical corrections for p(H,D) measurements in mixed H_2O/D_2O biological buffers†

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Mixtures of light and heavy water are used in NMR, small-angle neutron scattering (SANS), growth media for producing deuterated biological molecules, and analytical methods such as hydrogen–deuterium exchange (HDX) mass spectrometry. It is common to measure the pH of these solutions with a combination glass electrode with all chambers filled with aqueous (H_2O) potassium chloride solutions. In the daily measurement of samples containing mixtures of H_2O with D_2O in some ratio – call this measurement p(H,D) – we generally do not control for all of the contributions to the differences measured in carefully controlled electrochemical experiments. For example, the calibration solutions contain relatively low concentrations of the calibrant buffer with low or no added salt. Meanwhile the tested solutions can contain widely varying levels of any number of different salts as well as both polar and nonpolar organics and polymers and proteins. In this note, the p(H,D) behaviors of 50 mM solutions of five different buffers used in biological *in vitro* solutions were measured over the full range of H_2O : D_2O ratios in the open atmosphere. After calibration, pH measurements were made with the buffer solutions alone and with 100 mM KCl added to model a significant ionic strength difference. The solutions consisted of 1 : 1 volume mixtures of the acid and base forms of acetate, monobasic/dibasic phosphate, 2-amino-2-hydroxymethyl-propane-1,3-diol (tris), 2-amino-2-hydroxymethyl-propane-1,3-diol (HEPES), and glycine to span the common, full range for biological buffers. The pH values of the 1 : 1 mixtures mean that the measurements were, in fact, of their formal pK_a values. Each of the buffers exhibited a unique pattern of behavior, and none of them exhibited a measured $\Delta pK_a = pK_a^D - pK_a^H$ as large as 0.4, a value that has been suggested to be added to a pH measured in H_2O to match the equivalent pD measured in D_2O. The results do indicate that when a reasonable, required accuracy for pH measurement is ±0.1 units, three general guidelines apply: (1) where p(H,D) values are less than 8 for any D_2O content, no correction is needed for the p(H,D) measurement when comparing it to pH^H; (2) for less than 50% D_2O, if the 8 < p(H,D) < 10, again no correction is needed for the p(H,D) measurement compared to pH^H; (3) when the D_2O content is greater then 50% and the p(H,D) > 8, any corrections required will depend on the specific conditions and the specific buffer. Outside of the range 4 < p(H,D) < 10 or for needed greater accuracy, any corrections required will depend on the specific conditions and the identity of the buffer.

Received 13th March 2017
Accepted 20th April 2017

DOI: 10.1039/c7ay00669a

rsc.li/methods

Introduction

Mixtures of light and heavy water are used in NMR and small angle neutron scattering (SANS) samples and in growth media for producing deuterated biological molecules as well as analytical methods such as hydrogen–deuterium exchange (HDX) mass spectrometry. It is common to measure the pH of these solutions with a combination glass electrode with all its chambers filled with aqueous (H_2O) potassium chloride solutions. Often an adjustment is made for a measurement in D_2O by adding 0.4 pH units to the measured value. This correction was suggested in a 1968 paper by Covington *et al.*[1] that stated the "operational pH of buffer solutions in heavy water at 25 °C, measured with a glass electrode, can be converted to a pD value by adding 0.41 (molar scale) or 0.45 (molar scale) for 2 < pD < 9". This value was found by measuring the electrochemical potential difference between an H_2|Pt electrode in H_2O and a D_2|Pt electrode in D_2O. The relationship recommended for glass electrode measurements was pD = pH + 0.41. An earlier paper from Mikkelsen and Nielsen[2] reported similar results for a calomel electrode in H_2O compared to one in D_2O.

These differences were for measurement of p(H,D) for the specific electrochemical cells noted above (I use the nomenclature p(H,D) here for measurements in any ratio in the sample

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7ay00669a

of H_2O and D_2O). That correction should still occur if buffers are made 1 : 1 in their acid and base concentrations. In that case, the measurement of p(H,D) will be a measurement of the formal pK_a, which includes an assumption that the activity coefficients of the base and acid forms are equal.

Most of the literature of acid measurement in mixed-isotope water lists values of $\Delta pK_a = pK_a^D - pK_a^H = -\log(K_a{}^D/K_a{}^H)$, where $K_a{}^D$ and $K_a{}^H$ are the acid dissociation constants in D_2O and H_2O respectively. The papers referenced here report the values of ΔpK_a. That is the convention used in this study except for one set of samples that are not mixed 1 : 1 – where the equivalent measurement is $\Delta pH = pH^D - pH^H$.

Making an adjustment to a p(H,D) measurement for changes that occur in even a relatively uncomplicated chemical solution using a combination glass electrode is not the same as comparing D_2O,D_2|Pt with H_2O,H_2|Pt electrodes or otherwise-matched calomel electrodes in H_2O and D_2O. This lack of comparability occurs because adding some fraction (call it a number fraction or volume fraction) of D_2O also may affect the surface acid–base chemistry of the glass electrode, the liquid–liquid interface with the salt bridge to the reference electrode, and the acid–base chemistry and binding equilibria of every component in the solution including the buffers, proteins, polymers, and biological components. In addition, most chemists and biochemists make p(H,D) measurements by first calibrating electrodes with a number of relatively low-ionic-strength standardizing buffers, *e.g.*, 4.0, 7.0, and 10.0 formulated in H_2O. Then the electrode is put into the test solution that has some combination of salts, sample, and buffer, and almost certainly also has an ionic strength that is different from the calibrants'.

It should be noted that a number of studies prior to the Covington *et al.* recommendation of a fixed 0.4 unit correction[1] disagreed with that declaration.[2–13] Reports subsequent to the Covington work[1] also did not agree with its claim[10,14–17] nor does this work. A brief expansion of this history is presented in the ESI.†

The rationale behind this study

A major fraction of the electrochemists who produced the data cited above sought a better understanding of the effects of isotopic substitution on the electrochemical potential. They used measurement systems that did not involve salt bridges since the bridges add an unknown interface potential to the measurement. However, on a daily basis we do not generally have that choice available, and instead use combination glass electrodes with double salt junctions. The cell notation for a double-junction combination electrode with a calomel reference half-cell is

$$\mathrm{Ag(s)|AgCl(s)|KCl(aq),\ 10^{-7}\ M\ H^+|test\ solution||KCl(aq)||KCl(aq)|Hg_2Cl_2(s)|Hg(l)|Pt(s)}$$

The central vertical single line is the H^+-selective glass membrane.

This work – describing the measurements of biochemically useful buffers – is a direct outgrowth of the early 1960's work reported by Salomaa *et al.*[11] and that by Glasoe and Long.[9] It was initiated to determine with more contemporary equipment what changes occur in the p(H,D) measured when different ratios of $H_2O : D_2O$ are present, what changes occur with changes in ionic strength, and observe any trends in ΔpK_a with the formal pK_a^H for some buffers commonly used in biochemistry. As will be seen, measured shifts in p(H,D) occur, and the measurements will allow us to choose whether the changes are within an allowable range to ignore or how to adjust the solution conditions so that the measured p(H,D) make the solutions the most closely equivalent between pure water as used for most biochemical measurements and the H_2O/D_2O mixture required for the chosen experimental techniques.

Materials and methods‡

Reagents, their sources, and assays

2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, (HEPES ≥ 99.5%, Acros, Thermo Fisher, NJ); HEPES sodium salt (≥99%, J. T. Baker, Center Valley, PA); Potassium Phosphate dibasic (K_2HPO_4 ≥ 99%, GFS, Columbus OH); Potassium Phosphate monobasic (KH_2PO_4 ≥ 99.5%, GFS, Columbus OH); Sodium Acetate, (NaOAc ≥ 99.5%, GFS, Columbus OH); Acetic acid, (HOAc 99.7%, Aldrich, Milwaukee, WI); Glycine ($H_2NCHCOOH$ UltraPure™, Life Technologies, Thermo Fisher, NJ); Glycine sodium salt, ($H_2NCH_2CO_2Na\cdot xH_2O$ 99%, Acros, Thermo Fisher, NJ); D_2O (99.9%, Cambridge Isotope Laboratories, Tewksbury, MA); 2-amino-2-hydroxymethyl-propane-1,3-diol (Tromethamine™, tris base USP 99+%, J. T. Baker, Center Valley, PA); tris(hydroxymethyl)aminomethane hydrochloride (tris·HCl 99.0+% Sigma, St. Louis, MO); KCl (≥99.5%, Bio-Refined, GFS, Columbus, OH).

Buffer preparation

The mixed H_2O–D_2O samples were made by combining the required proportions of 50 mM (1 mM = 1 mmol L^{-1}) buffer solutions made in H_2O and in D_2O. As illustrated in Fig. 1, each of these buffers were made by mixing 1.00 : 1.00 volumes of 50 mM solutions of the acid form and conjugate base form of that buffer; one acid–base set was made in H_2O and one set in D_2O. The acid and base solutions were made by weighing each buffer component and adding the correct amount of solvent to each. All measures of weight and volume had at most uncertainties of 1%. In addition, one set of sodium acetate/acetic acid solutions was mixed 6 : 5 in an acetate : acid ratio. For all sets, the final proportional mixing of H_2O and D_2O buffers yielded 11 samples of 1.00 mL each with D_2O content of (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100)%. The 0% and 100% are the two original buffer solutions. For those samples containing KCl, all the added H_2O and D_2O used to make the acid and base stock solutions from the weighed materials contained the nominal

‡ Disclaimer: Certain commercial materials are identified in this paper, but such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

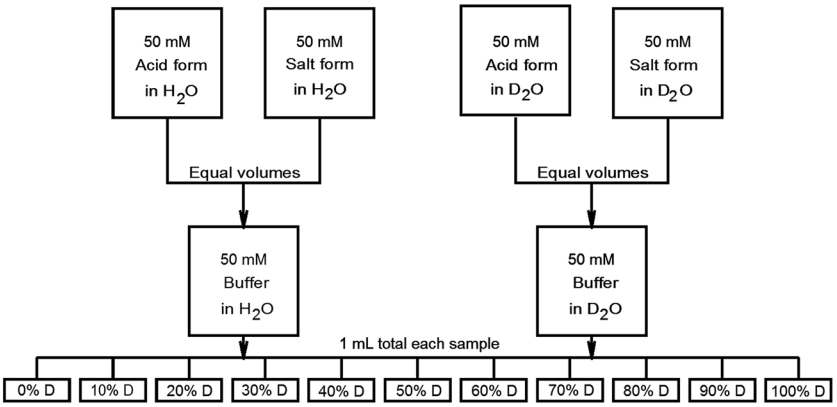

Fig. 1 The sample preparation protocol.

concentration of the salt. KCl was used to minimize perturbing the potential due to the salt bridge between the measured solution and the reference electrode.

No effort was made to exclude oxygen or carbon dioxide from the solutions when prepared or when the measurements were made. The 50 mM concentration of the buffers was chosen so that the presence of any level of atmospheric gases would have a negligible effect on the measurements and also to be in range of commonly utilized *in vitro* biochemical concentrations.

p(H,D) measurements

All p(H,D) measurements were made with an Accumet 13-620-95 double junction combination electrode (Thermo-Fisher, Waltham, MA). The electrode tip measures 3 mm in diameter with a 15 cm long Teflon tube stem fitted into a reservoir containing the reference electrode. Before the measurements were made, any air bubbles were removed from the stem, where persistent ones could be expelled using a hand cranked centrifuge modified to spin the electrode. The electrochemical notation for this electrode is that shown above.

The electrode was connected to a Hanna model HI 4211 pH meter – input impedance 10^{12} Ω (Hanna Instruments, Woonsocket, RI). Each 1.00 mL sample contained in a 1.5 mL Eppendorf tube was measured by inserting the electrode into the sample and mixing it with the electrode until the p(H,D) measured varied less than 0.01 units. The sample was then allowed to equilibrate while quiescent, and the readings noted each minute. The p(H,D)value recorded was either the reading after four minutes, as timed with a stopwatch, or earlier if the measured value did not change in the third decimal place for one minute. The 11 samples for each buffer were run in a more or less random order of their H : D content. The p(H,D) values measured are listed in their time order for each of the runs in the tables of ESI.† To test the uncertainties of the measurements, a few samples that were run near the beginning of a set were measured again near the end. These multiple measurement results are included in the graphs, where the spread of the measurements at each set of conditions indicates the uncertainties of that run.

Meter calibration was carried out using three standard buffers (certified, Fisher Scientific, Waltham, MA) run in the order 7.010, 4.010, and 10.005. The room and sample temperatures were between 20 °C and 21 °C, and the meter was set with compensation of temperature 21 °C. The measurements of the calibration buffers reached equilibration (no change in the third decimal point of pH over one minute) generally between 15 min and 30 min, at which time the values were accepted. The calibration buffer closest to the sample buffer measured was rechecked at the end of a run. If it lay more than 0.04 units from its initial value, the run was discarded.

Results

The experimental p(H,D) behavior

Each buffer will be discussed separately below, but all five buffers show a trend towards a higher measured ΔpK_a value with increasing % D_2O. The values of $\Delta pK_a = pK_a^D - pK_a^H$ vary from 0.05 (acetate and phosphate) to 0.30 (glycine). These are listed in Table 1.

Some observations are made in anticipation of the following graphs. For all but the acetate without KCl, the changes in the readings with D_2O percentage appear to be close to linear within the measurement uncertainties. The acetate readings remained flat until about 50% D_2O. The same behavior is seen for a 6 : 5 NaOAc : HOAc mix, which was used to make measurements where the solution pH ≠ pK_a.

Upon addition of KCl, three buffers (acetate, phosphate, and glycine) have a clear decrease in pK_a^H, one (tris) has an increase, and one (HEPES) remains unshifted for both 100 mM and 200 mM added KCl. In Table 1, when comparing each buffer with and without KCl, values that decrease are shown with light shading in the appropriate column, values that increase have a darker shading, and the unchanging values are unshaded.

Note that when the five graphs and the values listed in Table 1 are compared for the two variables – D_2O content and the changing concentration of added KCl – each of the five buffers has a different pattern of response.

Sodium acetate/acetic acid

Unlike the other buffers, the acetate was run with two different formulations: the 1 : 1 mix and the 6 : 5 NaOAc : HOAc mix. The

Table 1 Values of pK_a^H and $\Delta pK_a = pK_a^D - pK_a^H$ for the buffers alone and with added 100 mM KCl[a]

	50 mM buffer[b]		Buffer + 100 mM KCl[b,c]	
Buffer	pK_a^H	ΔpK_a	pK_a^H	ΔpK_a
Acetate 1 : 1	4.68	0.05	4.62	0.13
Acetate 6 : 5	4.76	0.06	4.70	0.11
Phosphate	7.00	0.05	6.80	0.14
HEPES	7.57	0.21	7.60	0.21
Tris	8.32	0.18	8.43	0.16
Glycine	9.86	0.30	9.79	0.33

[a] Values are those of the experimental data points for pK_a or of the data differences ΔpK_a. Value uncertainties are 1 in the last place. [b] Where more than one p(H,D) value was measured at a specific D_2O percentage within a single run under a given set of conditions, chart entries were calculated from their averages. That is, $pK_a^H = \langle pK_a^H \rangle$, and $\Delta pK_a = \langle pK_a^D \rangle - \langle pK_a^H \rangle$. [c] Light fill indicates a decrease, darker fill an increase, and no fill indicates no change within experimental uncertainties.

latter mixture was carried out to investigate whether any differences showed up when the p(H,D) is not at the pK_a of the buffer. As seen in Fig. 2 and Table 1, other than the expected p(H,D) offset, both solutions are comparable. Both mixes of the buffer alone showed a negligible slope below 60% D_2O, after which the points showed a rise congruent with that occurring over the full D_2O range in the presence of KCl. The value of ΔpK_a for both 1 : 1 and 6 : 5 KCl-containing solutions are the same. In addition, the pK_a^H values shifted to lower values when KCl is present.

K_2HPO_4/KH_2PO_4

Two sets of measurements were made on the phosphate buffer solutions two days apart. As can be seen in Fig. 3, the dependence on the D_2O fraction was as small as that for acetate. Also, the pK_a^H dropped by 0.2 units, and the ΔpK_a increased by a factor of three in the presence of the added salt. In addition, in the presence of KCl, the system was much better behaved as seen by the smaller scatter of the data when multiple measurements were made at a specific D_2O percentage within each run.

NaHEPES/HEPES

The results here are unique among these five buffers. As seen in Fig. 4, in essence there was no change within the experimental variations in the measurements for the buffer alone, and with 100 mM and 200 mM KCl added at all D_2O concentrations. This consistency suggests that the liquid-junction potential's contribution to the measured values is not significant.

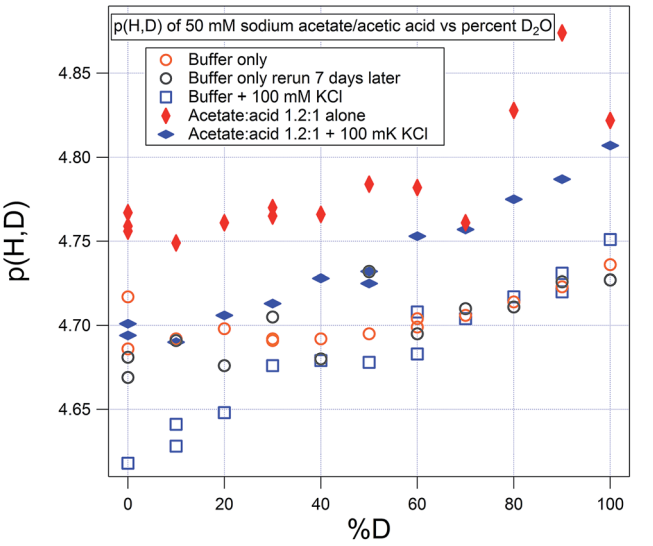

Fig. 2 Data plots for the measured p(H,D) for sodium acetate *versus* volume fraction D_2O. As for all the graphs of 1 : 1 acetate : acid, open circles are for runs of the buffer alone, and open squares show the data with 100 mM KCl added. Here, the filled diamonds are for the samples where acetate : acid is 6 : 5. See Table 1 for the values of pK_a^H and ΔpK_a that characterize the data sets. Here, as in all the graphs presented, where multiple data points appear at a specific D_2O percentage for a single run, the range of the points represents the overall uncertainty of the measurements for that run. The reasons for the deviations seen for the 6 : 5 buffer alone from 70% to 100% D_2O are unclear.

Tris/tris·HCl

Two sets of measurements 16 days apart were made on the tris buffer solution sets. Values reported in Table 1 are averages of the two. As can be seen in Fig. 5, uniquely for

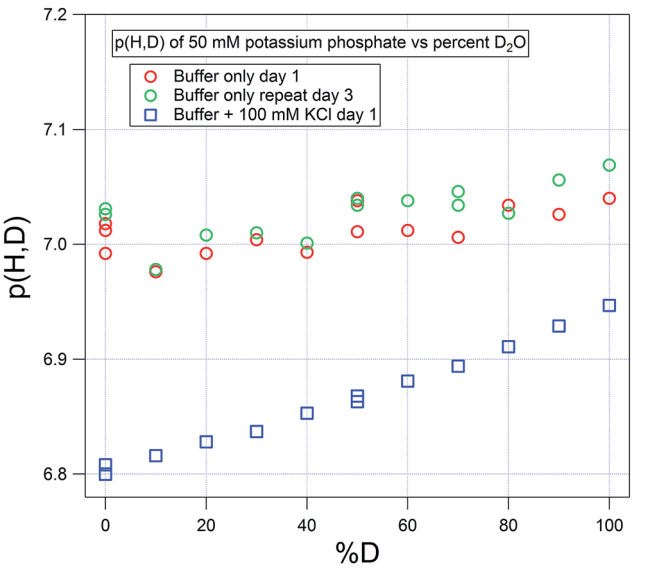

Fig. 3 Data plots for the measured p(H,D) for K_2HPO_4/KH_2PO_4 *versus* volume fraction D_2O. As for all the graphs of 1 : 1 K_2HPO_4/KH_2PO_4, open circles are for runs of the buffer alone, and open squares show the data with 100 mM KCl added. See Table 1 for the values of pK_a^H and ΔpK_a that characterize the data sets.

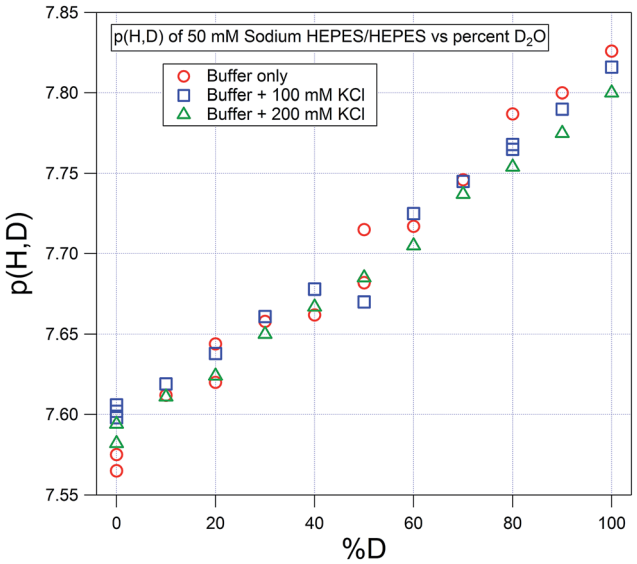

Fig. 4 Data plots for the measured p(H,D) for sodium HEPES : HEPES *versus* volume fraction D_2O. See Table 1 for the values of pK_a^H and ΔpK_a that characterize the data sets.

the tris, addition of KCl caused an increase of ≈ 0.1 pK_a^H units, and the slopes of the plots (the values of ΔpK_a) remain the same within the experimental variability. That variability is indicated by the range of p(H,D) values when remeasured at a single D_2O percentage during a single run.

Sodium glycinate/glycine

As seen in Fig. 6, the results for the glycine buffer system, discussed further below, shows the largest value of ΔpK_a of the set of five buffers. In the presence of KCl, the pK_a^H drops, and the slope of the data seems to be somewhat greater, although least-squares fitting of a straight line indicates that the slopes are the same within the fitting uncertainty.

Discussion

As is well understood, the changes in the ratio H_2O : D_2O can affect all of the chemistries involved in the p(H,D) measurement: the buffer equilibrium as indicated by the pK_a; the H^+,D^+ acid–base equilibrium at the electrode's silica surface; and the interface potential at the salt bridge leading to the reference electrode. In addition, the added KCl not only will have nonspecific ionic strength effects, but possibly specific interactions with the buffer components and the electrode's silica surface. A detailed description of the complexity of pH measurement appears in a recent review by de Levie.[18]

This empirical investigation was not initiated to probe the detailed chemistry of the mixed-isotope solutions, but only to find useful guidelines to ascertain whether a pH measurement made in a water solution and one made in a comparable solution containing some level of D_2O can be considered equivalent within some selected margin of error. However, the changes seen in the experimental measurements and the decades of discussion in the chemistry literature can allow some suggestions as to why the patterns of behavior seen here do occur.

The salt-bridge interface potential

Even with the equipment available 60 years ago, Glasoe, *et al.*[9] found that the salt-bridge interface potential does not contribute to the differences seen between H_2O and D_2O. Similarly, here it appears that the interface potential contributes insignificantly to the measurements as seen for the

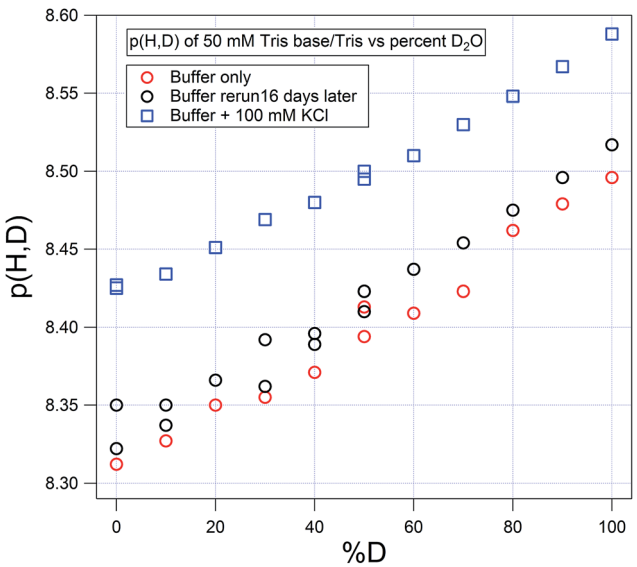

Fig. 5 Data plots for the measured p(H,D) for tris base/tris·HCl *versus* volume fraction D_2O. See Table 1 for the values of pK_a^H and ΔpK_a that characterize the data sets.

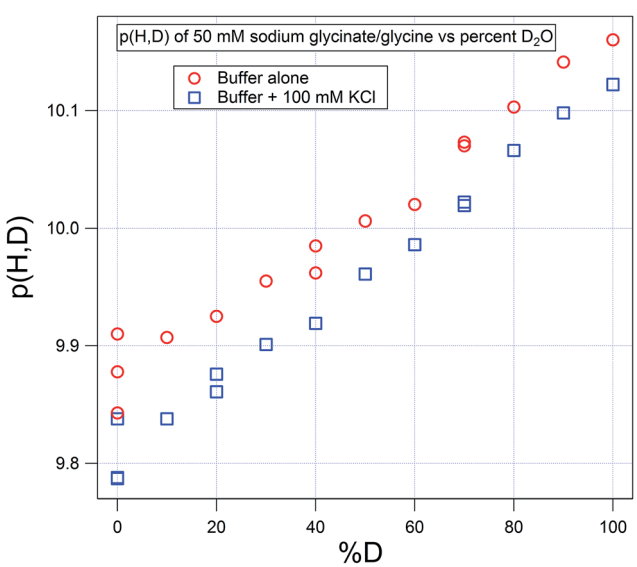

Fig. 6 Data plots for the measured p(H,D) for Na glycinate/glycine *versus* volume fraction D_2O. See Table 1 for the values of pK_a^H and ΔpK_a that characterize the data sets.

remarkable behavior for HEPES. The measured p(H,D) at each D_2O concentration remained the same with the buffer alone, which has 25 mM Na^+, and with 100 mM and 200 mM KCl present. The theory of the liquid junction potential tells us that to a first approximation when the transport numbers of the anion and cation are equal, even when they have different concentrations on either side of the interface, the interface potential will approach zero. Of course, KCl provides that condition. That there was no directional shift with these three conditions in the measured pK_a values at every D_2O volume fraction suggests that the interface potential makes a negligible contribution to the measurements.

The electrode surface chemistry

Martin and Butler[6] did an interesting competition experiment when they investigated the colorimetric indicator bromothymol blue in a buffer solution and found that it's dissociation behavior differed little between the buffers in H_2O and D_2O. The indicator's pK_a tracked that of the buffer with the changed solvent, so the competition between the indicator and the buffer remained the same. This result can be related to the p(H,D) measurements with a glass electrode in that a competitive equilibrium exists between the test solution and the surface silanols' acid–base equilibrium.[19,20] However, in this latter case of the surface chemistry changing, the measured buffers' equilibrium also changes. We should expect that to the extent that both the H/D dependences of the surface and the buffer equilibria match, they will track each other, and the measured value of $\Delta pK_a \approx 0$. Baucke[21] describes the chemistry of pH-electrode glass surfaces as they interact with the adjacent solution in both light and heavy waters. He differentiates a "direct isotope effect" from an "indirect isotope effect". The first results from the association of the acid groups on the surface with either protons or deuterons, and the latter from the differential binding of alkali metal ions at the surface between the H_2O and D_2O solutions. Baucke noted that the correction terms depend on the properties of the membrane glass and are not universal, although others[9,22] found that different commercial glass electrodes all gave the same results. Baucke noted that there existed an "interfacial equilibrium between surface groups of the membrane glass and hydronium and/or alkali ions in the solution involving dissociated surface groups whose charge causes the ion-activity-dependent potential of the glass".

That equilibrium chemistry at the silica–water interface can be probed by second harmonic generation (SHG) spectroscopy. Using SHG, Ong *et al.*[23] observed the spectroscopic changes during a pH titration. Silica showed a titration curve representing a diprotic acid having 1/5 of the sites with a pK_a of 4.5 and 4/5 of the sites with pK_a of 8.5. More recent work[24] found that the ionic composition of the aqueous phase could modify these fractional amounts through specific ion effects and also modify both of the pK_as. For example, a 100 mM sodium concentration lowered the pK_as, and it was suggested that the origin was the sodium ions' stabilizing the siloxide sites. On the other hand, potassium chloride effectively stabilized the less charged silica surface in the higher pH region, while having little effect in the lower pH region.[24] Further studies from the same group has shown that for a silica surface, the structure is protean and the fractions with different pK_as depends on the history.[25] In addition, Yang, *et al.*[26] saw with infrared-visible sum frequency vibrational spectroscopy that the interfacial water structures changed at a silica surface with NaCl concentrations as low as 100 μM. The perturbation observed saturates in the range 10 mM to 100 mM, and potassium shows a similar effect. A great deal more work would be required to see whether such specific chemical interactions are causing some or all of the shifts seen between the buffers alone compared with the added KCl solutions. However, some caution is needed in connecting these hydrated silica surfaces probed with SHG methods with the pH-electrode surface since while the electrode is composed primarily of SiO_2, it is a mixed oxide with Na_2O and CaO as well.[27]

In addition, the buffer anions themselves may have a surface effect. For example, phosphate binds to silica even down to ppm phosphate in water.[28] The maximum binding occurs at pH 7, at the pK_{a2} of the phosphate buffer, which is the p(H,D) range measured here. The part anion binding could play in the changes seen both from H/D substitution and KCl addition remains indeterminate.

The buffer chemistry

As reviewed briefly in the introduction, a commonly mentioned relationship in p(H,D) measurement is that $\Delta pK_a \propto pK_a^H$. However, it is not a strong rule as can be seen comparing HEPES and tris in the series of buffers measured here and also by reviewing the paper by Robinson, Paabo, and Bates from 1969.[14]

On the other hand, the general tendency toward $\Delta pK_a \propto pK_a^H$ is seen here, and a consensus as to the cause is a change in the zero-point energy of the hydrogen bond. For example, Mora-Diez *et al.*[29] report calculations of the deuterium isotope effects on the pK_a values for 16 organic acids, the majority of which are phenols. The absolute values calculated did not agree with the experimental data, but the ΔpK_as were in moderate agreement with the experimental values. Again, dissecting the various contributions to the changes showed that the major contributing factor was the difference in zero point energy of the acidic H/D.

Note that some of the plots of pK_a *versus* the fraction of D_2O may appear to be curved (see glycine/glycinate alone and phosphate with 100 mM KCl). As shown by Quinn,[30] such curvature can be expected when the proton/deuteron equilibrium involves binding to more than one site. However, even though the measured p(H,D) dependence on the H/D ratio may be nonlinear for some of the buffers, within the practical limits of daily measurement and the imprecision of completely reproducible conditions, a linear approximation appears to be satisfactory.

Conclusions

From this limited survey of buffers and conditions, it seems clear that the generally used correction factor of 0.4 pH units

does not apply for measurements made with a light-water-containing combination pH electrode under ambient conditions for these biological buffers. Further, the values of ΔpK_a found for acetate and phosphate buffers are significantly smaller than have been reported before under other conditions. These small values appear to be at least partially due to the tracking of the solution buffers' response to a changing $H_2O : D_2O$ ratio by a parallel pK_a shift of the glass electrode's acid–base equilibrium.

The goal of this study was to determine how to adjust the measured acidity of an aqueous solution that has some known D_2O content so that its conditions most closely match – within some specified limit – a solution in H_2O containing the same biological species such as a protein, polysaccharide, or polynucleic acid. Toward that goal, based on the results shown in Fig. 2–6, three guidelines can be stated when calibration is done with standard (certified) buffers. Let us take the arbitrary limit for accuracy in the pH measurement to be 0.1 pH units. Then,

(1) Where $4 < p(H,D) < 8$ for any D_2O content, no correction is needed for the p(H,D) measurement when comparing it to pH^H;

(2) For less than 50% D_2O, if the $8 < p(H,D) < 10$, again no correction is needed for the p(H,D) measurement compared to pH^H;

(3) When the D_2O content is greater then 50% and the $p(H,D) > 8$, any corrections required will depend on the specific conditions and the specific buffer.

In this way, a mixed D_2O/H_2O buffered solution can be made as close as possible to the same solution containing only light water. In quantitative terms, when the measured p(H,D) is within 0.1 p(H,D) unit of the value of pH for the same sample mixture in H_2O, $[H^+,D^+]_{mixed}$ lies within a factor of 1.25 of $[H^+]_{H_2O}$.

Acknowledgements

I am pleased to thank Marlon Walker, Philippe Buhlmann, Faye Rubinson, and Thomas Stevenson for comments on the manuscript and the NCNR for hospitality and support.

Notes and references

1 A. K. Covington, M. Paabo, R. A. Robinson and R. G. Bates, *Anal. Chem.*, 1968, **40**, 700–706.
2 K. Mikkelsen and S. O. Nielsen, *J. Phys. Chem.*, 1960, **64**, 632–637.
3 S. Korman and V. K. La Mer, *J. Am. Chem. Soc.*, 1936, **58**, 1396–1403.
4 G. Schwarzenbach, A. Epprecht and H. Erlenmeyer, *Helv. Chim. Acta*, 1936, **19**, 1292–1304.
5 C. K. Rule and V. K. La Mer, *J. Am. Chem. Soc.*, 1938, **60**, 1974–1981.
6 D. C. Martin and J. A. V. Butler, *J. Chem. Soc.*, 1939, 1366–1369.
7 R. W. Kingerley and V. K. La Mer, *J. Am. Chem. Soc.*, 1941, **63**, 3256–3262.
8 R. B. Fischer and R. A. Potter, *USAEC MDDC-715*, Oak Ridge, TN, 1945, MDDC-715.
9 P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, 1960, **64**, 188–190.
10 V. Gold and B. M. Lowe, *J. Chem. Soc. A*, 1968, 1923–1932.
11 P. Salomaa, L. L. Schaleger and F. A. Long, *J. Am. Chem. Soc.*, 1964, **86**, 1–7.
12 R. Gary, R. G. Bates and R. A. Robinson, *J. Phys. Chem.*, 1965, **69**, 2750–2753.
13 E. L. Wehry and L. B. Rogers, *J. Am. Chem. Soc.*, 1966, **88**, 351–354.
14 R. A. Robinson, M. Paabo and R. G. Bates, *J. Res. NBS*, 1969, **73**, 299–308.
15 B. M. Lowe and D. G. Smith, *J. Chem. Soc., Faraday Trans. 1*, 1975, 389–397.
16 S. P. Dagnall, D. N. Hague, M. E. McAdam and A. D. Moreton, *J. Chem. Soc., Faraday Trans. 1*, 1985, **81**, 1483–1494.
17 A. Krężel and W. Bal, *J. Inorg. Biochem.*, 2004, **98**, 161–166.
18 R. de Levie, *Electrochim. Acta*, 2014, **135**, 604–639.
19 R. G. Bates, *Determination of pH Theory and Practice*, Wiley, New York, 2nd edn, 1964, ch. 10.
20 R. G. Bates, *Determination of pH Theory and Practice*, Wiley, New York, 2nd edn, 1973, ch. 11.
21 F. G. K. Baucke, *J. Phys. Chem. B*, 1998, **102**, 4835–4841.
22 B. M. Lowe and D. G. Smith, *Anal. Lett.*, 1973, **6**, 903–907.
23 S. Ong, X. Zhao and K. B. Eisenthal, *Chem. Phys. Lett.*, 1992, **191**, 327–335.
24 M. S. Azam, A. Darlington and J. M. Gibbs-Davis, *J. Phys.: Condens. Matter*, 2014, **26**, 244107.
25 A. M. Darlington and J. M. Gibbs-Davis, *J. Phys. Chem. C*, 2015, **119**, 16560–16567.
26 Z. Yang, Q. Li and K. C. Chou, *J. Phys. Chem. C*, 2009, **113**, 8201–8205.
27 D. Hubbard, G. W. Cleek and G. F. Rynders, *J. Res. NBS*, 1950, **44**, 247–257.
28 P. Delaney, C. McManamon, J. P. Hanrahan, M. P. Copley, J. D. Holmes and M. A. Morris, *J. Hazard. Mater.*, 2011, **185**, 382–391.
29 N. Mora-Diez, Y. Egorova, H. Plommer and P. R. Tremaine, *RSC Adv.*, 2015, **5**, 9097–9108.
30 D. M. Quinn, in *Isotope Effects in Chemistry and Biology*, ed. A. Kohen and H.-H. Limbach, CRC, Boca Raton, 2006, pp. 995–1018.

