Behaviour of hydrophobic pH indicators in aqueous–organic two-phase systems

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Very hydrophobic indicators can remain completely in an organic phase, even in ionized form, but nevertheless respond to the pH of an equilibrated aqueous solution. They are useful to monitor the inaccessible aqueous phase of low water media, such as may be employed in enzymic processes. We present a theoretical model for the indicator response, based on the presence of both free ions and ion pairs in the organic phase. This adequately describes the behaviour observed with C_{15} alkyl esters of fluorescein at concentrations around 10^{-5} mol dm^{-3}, as a function of aqueous pH and cation activity or concentration. Analysis of data for toluene suggests complete ion-pairing in the organic phase, with either tetraalkylammonium ions or dibenzo-18-crown-6 and Na^+. In more polar solvents Na^+ counter-ions can be extracted alone: fitting of the titration curves suggests complete dissociation in pentan-3-one and butan-1-ol, but partial ion-pairing in ethyl acetate. Fitted parameters obtained from the model may be related theoretically to other literature measurements, notably for picrate salt extraction, and are generally consistent.

Introduction

There is great interest at present in the use of mainly organic media for reactions catalysed by enzymes and whole biological cells (reviews, ref. 1). The systems usually contain only a little residual water, but nevertheless there is often a dilute aqueous phase of small volume; even though this may be confined to the pores of the catalyst particles and hence not obviously present as a second liquid phase.

It is expected that pH will affect enzyme activity in these reaction mixtures, as in bulk water systems; the failure of some attempted reactions has been attributed to the generation of unfavourable pH values in the catalyst phase (*e.g.* ref. 2). However, the inaccessibility of this phase has prevented direct measurements to confirm this explanation.

We have been studying the behaviour of very hydrophobic indicators, which may be used to measure pH in such systems. These compounds remain completely in the organic phase, in both protonated and unprotonated forms; hence their ionization state is readily measured, and the possibility of effects on the enzyme (in the aqueous phase) is minimized. Nevertheless, the position of their protonation equilibrium is affected by aqueous pH. We present here a description of their behaviour, both as theoretically predicted and experimentally observed, as a basis for their application in pH measurement.[3,4] Though ionic species in water-immiscible organic solvents have received some study, the titration of these *via* an equilibrated aqueous phase appears to be a novel system. Thus our findings make a contribution to understanding of equilibrium effects in such systems, including partitioning and ion-pairing.

Theory

The ionization state of a hydrophobic acid–base indicator in the organic phase will usually be affected by the pH of an equilibrated aqueous phase. However, the relationship will be less straightforward than in a simple one-phase aqueous system. The indicator will respond directly to H^+ in the organic phase, not that in the aqueous phase. Since H^+ is an ion, its thermodynamic activity is not straightforwardly definable, and there is no simple treatment of its equilibration between the phases; see Marcus (ref. 5) for a discussion of the problems of treating single ion activities in general and in two-phase systems in particular. The equilibration of H^+ will depend on the presence of other charged species in both aqueous and organic phases. These effects may be seen in terms of an equilibrium potential difference between the phases, or the need for some other ion to move across the interface with H^+ to ensure electroneutrality. In addition, the ionized indicator in the organic phase may exist significantly in the form of ion pairs, even at very dilute concentrations, because of the lower permittivity (dielectric constant) and solvating power of the solvent.

The treatment presented here provides a relationship between the observable ionization state of the indicator in the organic phase, and the activities or concentrations of H^+ and other ions, in the aqueous phase. Like our experiments so far, it is restricted to cases where a separate dilute aqueous phase is present, even if of very low volume (*i.e.* with thermodynamic water activity close to 1). In the treatment a variety of processes and associated equilibrium constants are used to derive the expected behaviour. It should be noted that, as in all equilibrium treatments, the correctness does not depend on these processes actually reflecting the mechanisms by which equilibrium is reached in real systems, where for example exchange processes at the phase interface may be important.

The processes assumed to occur in the treatment are shown in Fig. 1; the limiting assumptions that are necessary are set out below. The treatment presented is for an acid/anion indicator (HA/A^-) of the type we have studied experimentally; an analogous derivation is possible for a base/cation indicator.

In this derivation, we follow the convention of placing a bar over symbols that refer to the organic phase (unmarked ones are for the aqueous phase); [ ] indicates concentrations and γ the corresponding activity coefficients (infinite dilution based reference state). Single ion activity coefficients are used for convenience in intermediate mathematical manipulations.

We start with equilibrium expressions for two of the fundamental processes shown in Fig. 1. For indicator dissociation in the organic phase.

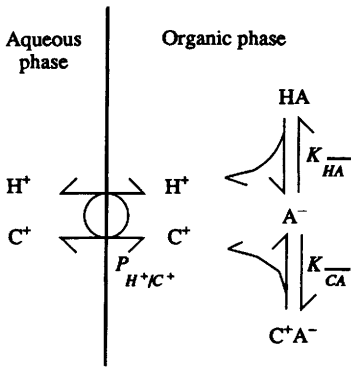

Fig. 1 Equilibria involved in ionization of indicator in the organic phase. The scheme is for an acid indicator represented as HA.

$$K_{\overline{HA}} = \frac{\gamma_{\overline{H^+}}\gamma_{\overline{A^-}}[\overline{H^+}][\overline{A^-}]}{\gamma_{\overline{HA}}[\overline{HA}]} \quad (1)$$

For exchange of H^+ and C^+ ions between the two phases:

$$P_{H^+/C^+} = \frac{\gamma_{C^+}\gamma_{\overline{H^+}}[C^+][\overline{H^+}]}{\gamma_{\overline{C^+}}\gamma_{H^+}[\overline{C^+}][H^+]} \quad (2)$$

In these equations concentrations and activity coefficients for A^- and C^+ refer to the free (dissociated) ions, even if these are only a low fraction of the total because of ion-pairing or other associations. By conventional, H^+ refers to protons bound to or solvated by the solvent, but not otherwise associated. (As discussed below, an equivalent possibility is that H^+ reflects H_3O^+ ions dissolved in the organic phase.) If more than one cation is present, we write an equation for each.

We now make the assumption that the organic phase concentrations of all other charge bearing species are negligibly low compared with C^+ or A^-. We have derived more complicated relationships that allow for significant concentrations of H^+, OH^- and of anions other than the indicator. However, the resulting equations did not give significantly better fits to our observed titration curves.

We also assume that only one cation makes a significant contribution. Once again, more complex equations involving summed contributions of several ions may be derived, but do not improve the fit to our data.

Hence we can write for electroneutrality of the organic phase eqn. (3).

$$[\overline{C^+}] = [\overline{A^-}] \quad (3)$$

Now we substitute in eqn. (3) for $[\overline{C^+}]$ [using (1) and (2)] to produce eqn. (4),

$$\frac{K_{\overline{HA}}\gamma_{\overline{HA}}[\overline{HA}]\gamma_{C^+}[C^+]}{\gamma_{\overline{A^-}}[\overline{A^-}]\gamma_{H^+}[H^+]P_{H^+/C^+}\gamma_{\overline{C^+}}} = [\overline{A^-}] \quad (4)$$

Which may be re-arranged to eqn. (5).

$$[\overline{A^-}] = \sqrt{\frac{K_{\overline{HA}}\gamma_{\overline{HA}}[\overline{HA}]\gamma_{C^+}[C^+]}{\gamma_{\overline{A^-}}\gamma_{H^+}[H^+]P_{H^+/C^+}\gamma_{\overline{C^+}}}} \quad (5)$$

We now consider the formation of ion pairs in the organic phase between the indicator anion and cations:

$$K_{\overline{CA}} = \frac{\gamma_{\overline{C^+}}\gamma_{\overline{A^-}}[\overline{C^+}][\overline{A^-}]}{\gamma_{\overline{CA}}[\overline{C^+A^-}]} \quad (6)$$

and substitute for $[\overline{C^+}]$ in this using eqns. (2) and (1), to give eqn. (7).

$$[\overline{C^+A^-}] = \frac{K_{\overline{HA}}\gamma_{\overline{HA}}[\overline{HA}]\gamma_{C^+}[C^+]}{\gamma_{H^+}[H^+]K_{\overline{CA}}P_{H^+/C^+}\gamma_{\overline{CA}}} \quad (7)$$

In some cases the ion pairs C^+A^- may show the same absorption spectrum as dissociated A^-, so that the measured anion fraction in the organic phase, f, is given by eqn. (8),

$$f = \frac{[\overline{A^-}] + [\overline{C^+A^-}]}{T} \quad (8)$$

where T is the total indicator concentration given by:

$$T = [\overline{A^-}] + [\overline{C^+A^-}] + [\overline{HA}]$$

Combining this with eqns. (5) and (7) gives eqn. (9).

$$f = \frac{K_{\overline{HA}}\gamma_{\overline{HA}}(1-f)\gamma_{C^+}[C^+]}{\gamma_{H^+}[H^+]K_{\overline{CA}}P_{H^+/C^+}\gamma_{\overline{CA}}} + \sqrt{\frac{K_{\overline{HA}}\gamma_{\overline{HA}}(1-f)\gamma_{C^+}[C^+]}{\gamma_{\overline{A^-}}\gamma_{H^+}[H^+]TP_{H^+/C^+}\gamma_{\overline{C^+}}}} \quad (9)$$

This equation can be expanded as a quadratic in f, and hence made explicit, but the form is very complex.

The two summed terms in the equation reflect the relative contributions of ion pairs and free ions to the indicator anion fraction. If the first term is dominant, then essentially all the indicator anion is ion-paired; while if the square root term is much larger, then ion pairing may be neglected and the indicator anion is present as a free dissociated ion. Note, however, that the relative values of the two terms depend on the value of $\gamma_{H^+} \times [H^+]$. Thus in a typical experiment where the pH of the aqueous phase varies and the response of the indicator is observed, a term that is insignificant at one part of the titration may become important at the other extreme.

If a complexing ligand (*e.g.* a crown ether) is added to aid solubilization of the cation in the organic phase, only a slight modification is needed. Assuming that in its presence all of the cations in the organic phase are complexed, the $[C^+]$ terms should be multiplied by $[\overline{L}]/K_{\overline{LC^+}}$ and appropriate activity coefficients; where $[\overline{L}]$ is the ligand concentration in the organic phase and $K_{\overline{LC^+}}$ the dissociation constant for the ligand–cation complex.

Discussion of assumptions made in this treatment

As noted above, several limiting assumptions have been made in the derivation of eqn. (9). In most cases more complex equations have also been derived without these assumptions. However, in no case was the improvement in the fit to our data by addition of extra adjustable parameters sufficient to justify their inclusion. The physical basis and meaning of these assumptions will now be discussed.

Anions other than the indicator in the organic phase. Note first that only free dissociated anions (not ion-pairs) will alter the predicted behaviour, by making eqn. (3) invalid. Where only simple inorganic ions like Na^+ and Cl^- are available, it seems very unlikely that these will partition together into the organic phase to reach concentrations significant with respect to the indicator anion at around 10^{-5} mol dm^{-3}. This is supported by estimates for free energies of transfer even to more polar solvents.[5] This may not be so certain in experiments with additions of relatively organic-soluble cations like tetraalkylammonium, or of solubilizing ligands like hydrophobic crown

ethers. However, inclusion of a term allowing for other anions in the organic phase did not significantly improve the fit with any of our data sets. In this context it is worth noting that the main anions present in our experiments, phosphate and pyrophosphate, are among the most difficult to extract into organic phases.[6,7]

H^+ and equivalent ions in the organic phase. Free protons are unlikely to exist significantly in any solvent, but in our systems there are two possible bound, charged species. The solvent itself may be protonated (*e.g.* pentanone may give a Et_2COH^+ ion), or water molecules dissolved in it may be converted to H_3O^+. In the systems we have studied so far, the organic phase is always water saturated, so the activities of these two forms of 'H^+' will remain in a constant ratio, whose magnitude depends on the relative basicity of the solvent and of water dissolved in it (the latter will of course usually not be the same as that of pure water). In a similar way, if the solvent is more acidic than dissolved water, OH^- ions will not exist as such, but will remove protons from the solvent to give water and a solvent anion. Just as in water, H^+, OH^- ions and their equivalents will be in equilibrium with neutral water or solvent molecules. The product of their concentrations will be limited by the autoprotolysis constant of the solvent, or the dissociation constant of dissolved water. The autoprotolysis constants of the solvents we have used will be small: Reichardt[8] lists values of $10^{-22.83}$ for ethyl acetate and $10^{-25.94}$ for butan-2-one, while Rondinini *et al.*[9] give 10^{-31} for butan-2-one (all values for the anhydrous form). The dissociation constant of dissolved water can be expected to be less than in pure water, because of the general poorer solvation of ions. Hence at least one of H^+ or OH^- (or any equivalent) will always be present at only extremely low concentration. The arguments against the presence of simple inorganic anions in the organic phase (above) apply with even greater force to OH^-, though its equivalents in highly acidic solvents are less unlikely. For these, and for 'H^+', we have to rely again on our experimental findings; there was no significant improvement in the fit of our data to model equations that do not neglect these concentrations.

A significant contribution of 'H^+' to the total free cation concentration in the organic phase (most likely at low aqueous pH values) means that the indicator acid dissociates significantly even in the absence of other counter-ions. This would happen also under the conditions for the determination of the reference spectrum of the undissociated acid, so might be difficult to eliminate. However, the anions of the indicators we have studied show a long-wavelength absorption peak at around 540 nm. At this wavelength the spectrum of the acid standards is near to baseline at the extreme edge of a peak, with no evidence of a shoulder. Therefore it seems unlikely that there is significant spontaneous dissociation under these conditions.

Complex charged species in the organic phase. There is considerable evidence for the formation in organic solvents of species such as triple ions (*e.g.* $C^+A^-C^+$) or complexes of anions with neutral hydrogen bond donor species (*e.g.* $A^- \cdots HA$) (*e.g.* refs. 7, 10–12). Such species may be the dominant charge bearing forms in some cases, so our assumption may initially appear rather dubious. However, most of the literature studies have been made at much higher concentrations than apply in our experiments: in solvent extraction work, concentrations around 1 mol dm^{-3} are usual, while the indicator anion in our experiments does not exceed 3×10^{-5} mol dm^{-3}. Where association constants are known, they seem to be about 10^4 dm^3 mol^{-1} at most, and more usually around 100 dm^3 mol^{-1}, so that complex species would normally be insignificant under our conditions.

Indicator ion pairs and their spectra. Ion pairs, particularly where the ions are in contact (not solvent-separated), often show distinctive spectra,[13] compared with the free ions. In such cases our totalling of the concentrations of different forms of the indicator anion [eqns. (8) and (9)] may not reflect experiments. The concentrations of the free anions and of ion pairs with various cations may be measurable separately, so that eqns. (5) and (7) may be applied individually.

As described below, in our experiments so far, we have found only one case where the indicator anion seemed to be present in both free and ion-pair forms together (in ethyl acetate). In this case, the spectra showed no indication of differences between these two forms. Hence, we do not discuss this topic further.

Simplification of eqn. (9): activity coefficients

Eqn. (9) contains a number of activity coefficients. We may take $\gamma_{H^+} \times [H^+]$ as a function of the measured pH value. The activity coefficient for HA refers to a neutral species at low concentration, so we are confident it will normally be very close to 1. This is probably also true for that of the ion pair C^+A^-; literature data on ion pairing systems has been successfully correlated on the basis of this assumption.

The remaining activity coefficients refer to ionic species. That for C^+ in the aqueous phase is likely to be significantly different from 1 in the relatively high ionic strength buffers used in most of our experiments. For the cases where Na^+ serves as the cation, we have estimated its activity by direct electrochemical measurement (see below). With the added tetraalkylammonium cations used in a few of our experiments, nothing seems to be known about their activity coefficients, so a constant value was assumed; the difference from 1 will be reflected in a proportional change in the value of the fitted constants.

The other activity coefficients are for C^+ and A^- ions in the organic phase. There is little unambiguous experimental data for activity coefficients of ions in water-immiscible organic liquids, partly because observed reductions in salt activities can be equally ascribed to ion-pairing or to lower ion activity coefficients. Such systems are, however, normally analysed by assuming that the simple or extended Debye–Hückel equations correctly describe single ion activity coefficients, and data can be successfully correlated on this basis (*e.g.* refs. 10–12, 14, 15). The Debye–Hückel equation gives a mean ion activity coefficient of 0.943 for pentan-3-one at an ionic strength of 3×10^{-5} mol dm^{-3} (the maximum expected in our experiments, if the ionized indicator is the dominant anion in the organic phase). In ethyl acetate, where our data indicates only partial dissociation to free ions, a value of 0.843 is predicted at 10^{-5} mol dm^{-3} ionic strength.

Results and discussion

We have shown experimentally that the ionization of an indicator in the organic phase can be affected by changes in aqueous phase pH (see examples below, and refs. 3, 4). However, its ionization is also affected by aqueous phase activities of other ions. This may be shown by mathematical analysis of the various equilibria (a relatively simple one is shown above), but is equally apparent from the simple physical picture of the need for another ion movement to maintain electroneutrality if H^+ is exchanged with the organic phase. Hence, in order to measure aqueous pH, it is necessary to have some independent means of measuring or predicting the activity of at least one other ion in the aqueous phase. It is in principle possible to design the measuring system so that the secondary response is to any selected cation or anion, and this is an important part of method development.

Attempts to measure independently the aqueous phase activity of the second ion are likely to face the same problems as with pH. We have explored two other approaches to the estimation of a suitable ion activity or concentration: the addition of low, known levels of a counter-ion not otherwise

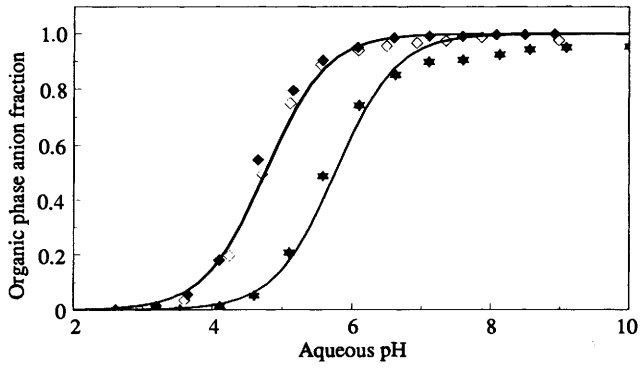

Fig. 2 Titration of tetrabromofluorescein C_{15} ester in toluene in the presence of tetrabutylammonion ions. All three theoretical lines are given by the single fitted parameter. The aqueous phase was prepared with: 3.09×10^{-3} mol dm^{-3} tetrabutylammonium phosphate, 0.4 mol dm^{-3} Na^+ (◆); 3.00×10^{-3} mol dm^{-3} tetrabutylammonium phosphate, 0.04 mol dm^{-3} Na^+ (◇); 3.01×10^{-4} mol dm^{-3} tetrabutylammonium phosphate, 0.4 mol dm^{-3} Na^+ (*). Indicator concentrations were 2.9–3.3 $\times 10^{-5}$ mol dm^{-3}.

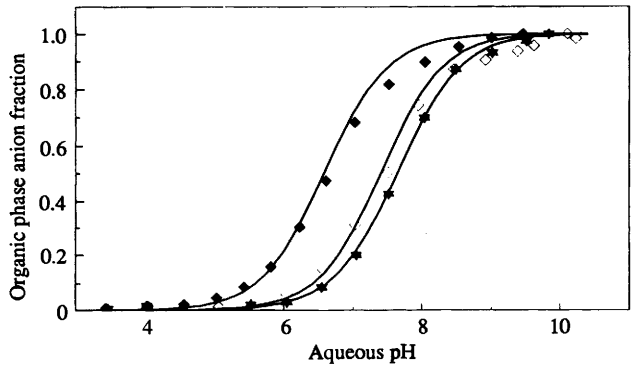

Fig. 3 Titration of tetrabromofluorescein C_{15} ester in toluene in the presence of dibenzo-18-crown-6. All three theoretical lines are given by the single fitted parameter. The aqueous phase normally contained 0.4 mol dm^{-3} Na^+, the organic phase 3.0×10^{-3} mol dm^{-3} crown (◆); 0.04 mol dm^{-3} Na^+ (◇); 3.0×10^{-4} mol dm^{-3} crown (*). Indicator concentrations in the organic phase were 3.0–3.4 $\times 10^{-5}$ mol dm^{-3}.

present in the system; or the selection of an ion present in the aqueous phase at reasonably predictable concentration (and activity).

The experiments described in detail here were performed with derivatives of fluorescein 3,7,11-trimethyldodecyl esters:[16] the 4,5-dibromo derivative ('dibromo indicator', **1**) and the 2,4,5,7-tetrabromo derivative ('tetrabromo indicator', **2**).

Added counter-ion with significant organic solubility

Experiments were carried out with addition of a tetraalkylammonium salt in 10 to 100-fold molar excess over the indicator, in both 1,1,1-trichloroethane and toluene. Fig. 2 shows the latter case, with theoretical curves fitted on the basis that $[C^+]$ was proportional to the total tetrabutylammonium concentration. Fitting gives a value for $K_{\overline{HA}}/(K_{\overline{CA}} \times P_{H^+/C^+})$ of $5.73 \pm 0.30 \times 10^{-3}$, with no evidence for a significant contribution from $K_{HA}/P_{H^+/C^+}$. (The parameter value has been calculated on the basis that the counter-ion present is predominantly in the aqueous phase, and has an activity coefficient of unity.) In physical terms the dominance of the first term implies that the indicator anion is essentially completely ion-paired with the tetraalkylammonium cation in these low polarity solvents. The ratio of the two model parameters gives an estimate for $K_{\overline{CA}}$. From the maximum possible value of $K_{\overline{HA}}/P_{H^+/C^+}$ we find $K_{\overline{CA}} < 10^{-7}$ mol dm^{-3}. This is in accord with the usual range of ion-pair association constants in solvents of similar low permittivity.[10,19]

These titration curves have the same form as found in simple aqueous solution. The titration curve was shifted to higher pH values (*i.e.* a lower value of $K_{\overline{HA}}/(K_{\overline{CA}} \times P_{H^+/C^+})$ by using a less acidic indicator (dibromo **1** instead of tetrabromo **2**, presumably an effect on $K_{\overline{HA}}$). However, unlike a simple aqueous titration, the position of the curve was also a function of the nature and concentration of the added cation, as the theory predicts *via* the influence of cation parameters. The transition pH was shifted to higher pH by using a less hydrophobic tetraalkylammonium cation (methyl > ethyl > butyl; not shown), or a lower concentration of the same species (Fig. 2). As expected with added hydrophobic counter-ions, the titration curve is not significantly affected by changing the aqueous $[Na^+]$ (Fig. 2).

With these added hydrophobic cations, there is some uncertainty over the concentration in the aqueous phase, as appears in eqn. (9). Some may partition into the organic phase as ion pairs with anions other than the indicator; because of essentially full ion-pairing, this will not affect the shape of the observed titration curves. Furthermore, as the aqueous phase volume fraction varies, and particularly becomes very small in the target systems for measurement, the aqueous phase concentration will become ever more difficult to predict from the amounts added. Hence, we do not believe that addition of counter-ions like this is a particularly promising approach for practical pH measurement.

Use of counter-ion normally present in the aqueous phase

Instead of adding counter-ions, an alternative is to develop a system using a cation likely to be present in the aqueous phase of an enzyme catalyst at known concentration (or strictly activity). The cations Na^+ and/or K^+ will usually be present in the aqueous phase at reasonably high and constant concentrations. At least where this remains a dilute solution (even if of very low volume), their activities should be similarly reasonably predictable. In particular, their activities are unlikely to change much during the use of a given catalyst, so that an indicator response should be mainly to pH. We have investigated systems in which they are extracted into the organic phase either with the aid of a crown ether, or directly by the indicator anion. We should, however, recognize a general limitation to such systems for pH measurement: interference from any species present that can give cations of better organic solubility (*e.g.* an organic base). Unfortunately, many enzyme reactants or products would fit this description, so this is a significant limitation of the current method.

Use of chelating ligand to extract cations into organic phase.

The action of Na^+/K^+ as counter-ions may be promoted by addition of an appropriate ligand able to solubilize them in the organic phase. We have demonstrated the use of the hydrophobic crown ether, dibenzo-18-crown-6 in this way. Fig. 3 shows the titration of the tetrabromo indicator **2** in toluene in its presence (at 10 to 100-fold molar excess over the indicator). The theoretical curves shown were fitted on the basis that the effective $\gamma_{C^+} \times [C^+]$ terms were proportional to [crown] $\times$ a_{Na^+}, and that the crown ether is essentially completely partitioned into the organic phase.[20] As can be seen, this gives an acceptable fit to the experimental data when either [crown] or a_{Na^+} are varied. The curves shown are for the fitted $K_{\overline{HA}}/(K_{\overline{CA}} \times P_{H^+/C^+} \times K_{\overline{LC^+}})$ value of $4.34 \pm 0.22 \times 10^{-4}$ dm^3 mol^{-1}, with $K_{\overline{HA}}/(P_{H^+/C^+} \times K_{\overline{LC^+}})$ equal to zero. The fit was marginally, but probably not significantly, improved by using a non-zero value for $K_{\overline{HA}}/(P_{H^+/C^+} \times K_{\overline{LC^+}})$ (value definitely less than 5×10^{-10}). So once again it appears that the crown-complexed cation and indicator anion are (nearly) completely ion-paired in toluene, with a $K_{\overline{CA}}$ value $< 10^{-6}$ mol dm^{-3}.

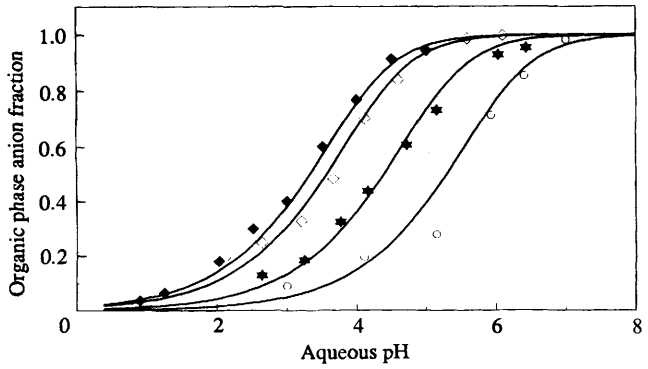

Fig. 4 Titration of tetrabromofluorescein C_{15} ester in pentan-3-one. All four theoretical lines are given by the single fitted parameter. Aqueous phase Na^+ and organic phase indicator concentrations were: 1.0 mol dm^{-3} Na^+, 1.27 × 10^{-5} mol dm^{-3} indicator (◆); 0.4 mol dm^{-3} Na^+, 1.18 × 10^{-5} mol dm^{-3} indicator (◇); 0.04 mol dm^{-3} Na^+, 1.18 × 10^{-5} mol dm^{-3} indicator (*); 0.004 mol dm^{-3} Na^+, 1.08 × 10^{-5} mol dm^{-3} indicator (○).

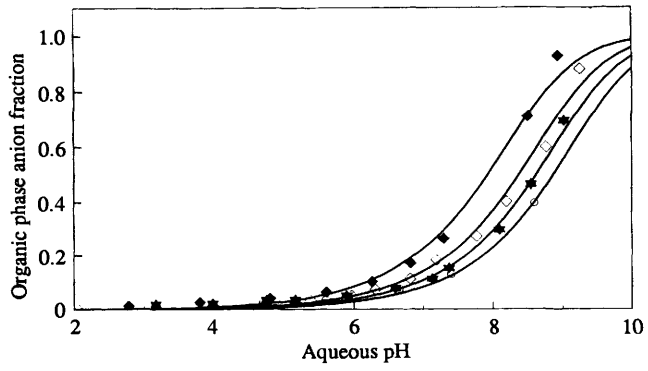

Fig. 5 Titration of dibromofluorescein C_{15} ester in pentan-3-one. All four theoretical lines are given by the single fitted parameter. Aqueous phase Na^+ and organic phase indicator concentrations were: 0.4 mol dm^{-3} Na^+, 3.73 × 10^{-5} mol dm^{-3} indicator (◆); 0.1 mol dm^{-3} Na^+, 3.79 × 10^{-5} mol dm^{-3} indicator (◇); 0.05 mol dm^{-3} Na^+, 3.81 × 10^{-5} mol dm^{-3} indicator (*); 0.025 mol dm^{-3} Na^+, 3.72 × 10^{-5} mol dm^{-3} indicator (○).

Direct extraction of counter-ions by the indicator

The simplest system is to use an indicator itself sufficiently hydrophobic to extract simple cations from the aqueous phase. We have demonstrated such a system with the most hydrophobic (C_{15}) fluorescein derivatives, and the relatively polar solvents ethyl acetate, pentan-3-one, and butan-1-ol. This has formed the basis of our first applications to pH measurement in enzyme reaction mixtures.[3,4]

In all these cases (Figs. 4–6), the indicator titration curve only superficially resembles that familiar in aqueous solution: it is quantitatively different, notably having a less steep slope. Such a slope is described by eqn. (9) when the first summed term is not dominant.

For the titrations with the tetrabromo indicator **2** in pentan-3-one (Fig. 4), the data fitted adequately to a $K_{\overline{HA}}/P_{H^+/C^+}$ value of 7.45 ± 0.55 × 10^{-9} mol dm^{-3}, with $K_{\overline{HA}}/(K_{\overline{CA}} \times P_{H^+/C^+})$ equal to zero. The fit was not significantly improved by a non-zero value of $K_{\overline{HA}}/(K_{\overline{CA}} \times P_{H^+/C^+})$ (upper limit can be set at 10^{-6}, with $K_{\overline{CA}} > 5 \times 10^{-3}$ mol dm^{-3}). Physically this implies that the indicator anion is not significantly ion-paired under these conditions. Literature values for $K_{\overline{CA}}$ in comparable systems are slightly lower: 2.4 × 10^{-4} mol dm^{-3} for Na-picrate in methyl isobutyl ketone;[15] 1.35 × 10^{-3} mol dm^3 for Na-picrate in acetone;[21] 1.03 × 10^{-3} mol dm^{-3} for $Me_4N^+Br^-$ in butanone.[19] The reduced ion-pairing of the fluorescein nucleus might result from a greater delocalization of charge, giving a larger effective distance between the ions in the pair. A small but probably not significant improvement in fit was obtained with a more complicated equation with $K_{\overline{HA}}$ as an independent parameter (physically this means a significant 'H^+' concentration in the organic phase). Based on this, we can, however, set an upper limit for $K_{\overline{HA}}$ at 5 × 10^{-8} mol dm^{-3}.

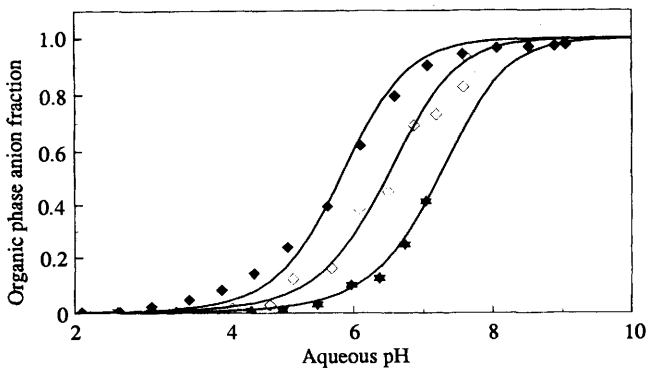

Fig. 6 Titration of terabromofluorescein C_{15} ester in ethyl acetate. All three theoretical lines are given by the same two fitted parameters. Aqueous phase Na^+ and organic phase indicator concentrations were: 0.4 mol dm^{-3} Na^+, 1.64 × 10^{-5} mol dm^{-3} indicator (◆); 0.05 mol dm^{-3} Na^+, 0.56 × 10^{-5} mol dm^{-3} indicator (◇); 0.008 mol dm^{-3} Na^+, 0.62 × 10^{-5} mol dm^{-3} indicator (*).

With the dibromo indicator **1** in pentan-3-one (Fig. 5), there is again no need for a non-zero value of $K_{\overline{HA}}/(K_{\overline{CA}} \times P_{H^+/C^+})$ ($< 2.5 \times 10^{-10}$), and the curves shown are for the fitted $K_{\overline{HA}}/P_{H^+/C^+}$ value of 1.28 ± 0.09 × 10^{-12} mol dm^{-3}. As before, we can set a limit for $K_{\overline{CA}} > 5 \times 10^{-3}$.

For the titrations in ethyl acetate (Fig. 6), the fit is significantly better if non-zero values are used for both $K_{\overline{HA}}/P_{H^+/C^+}$ and $K_{\overline{HA}}/(K_{\overline{CA}} \times P_{H^+/C^+})$: 3.24 ± 1.13 × 10^{-12} mol dm^{-3} and 5.61 ± 0.85 × 10^{-6}, respectively. (The errors are relatively high here, because the quality of the fit changed little if the two parameters were changed together in opposite directions.) Physically, this implies that both ion-pairs and dissociated ions make a significant contribution in this solvent. The ratio of the fitted parameters gives an estimate for $K_{\overline{CA}}$ of 5.8 × 10^{-7} mol dm^{-3}. The smaller value than in pentanone is as expected for the lower permittivity of ethyl acetate (6.0 compared with 17). Schill[7] gives $K_{\overline{CA}}$ as 5.4 × 10^{-6} mol dm^{-3} for Me_3EtN^+-picrate in water-saturated ethyl acetate. One consequence of this partial ion-pairing is a slope of pH dependence intermediate between that found in pentanone, and the steeper one of aqueous titrations. Again, a maximum value of 10^{-10} mol dm^{-3} can be set for $K_{\overline{HA}}$.

With butan-1-ol as solvent, the titration shows the lower slope characteristic of completely dissociated ions (data presented).[4]

Values of fitted parameters

Although parameters like these have not been previously measured, as far as we know, they can be related to findings from partition experiments. Extraction or partition coefficients are usually reported for ion pairs in the organic phase (P_{ip}), but sometimes for free dissociated ions (P_{fi}). These are defined by:

$$P_{ip} = \frac{[\overline{C^+A^-}]}{[C^+] \times [A^-]} \qquad P_{fi} = \frac{[\overline{C^+}] \times [\overline{A^-}]}{[C^+] \times [A^-]}$$

In the case of an anion detectably protonated in aqueous solution, a simple partition coefficient may also be measured:

$$P_{HA} = \frac{[\overline{HA}]}{[HA]}$$

Both P_{ip} or P_{fi} and P_{HA} tend to vary by the same proportion when the structure of A is altered remote from the site of ionization. (This is reflected in the fact that log P values may be correlated well on a group contribution basis.)[22] Hence, the ratio P_{ip}/P_{HA} or P_{fi}/P_{HA} will vary little with such changes.

Fitted parameters like $K_{\overline{HA}}/P_{H^+/C^+}$ or $K_{\overline{HA}}/(K_{\overline{CA}} \times P_{H^+/C^+})$ from our experiments (now referred to as K_{ex1} and K_{ex2}) are approximately equal to:

$$K_{ex1} = \frac{[\overline{C^+}] \times [\overline{A^-}] \times [H^+]}{[\overline{HA}] \times [C^+]} \quad K_{ex2} = \frac{[\overline{C^+A^-}] \times [H^+]}{[\overline{HA}] \times [C^+]}$$

Division by the aqueous dissociation constants of HA gives:

$$\frac{K_{ex1}}{K_{HA}} = \frac{[\overline{C^+}] \times [\overline{A^-}] \times [HA]}{[C^+] \times [A^-] \times [\overline{HA}]} \quad \frac{K_{ex2}}{K_{HA}} = \frac{[\overline{C^+A^-}] \times [HA]}{[C^+] \times [A^-] \times [\overline{HA}]}$$

which are equal to the ratios described above, P_{fi}/P_{HA} and P_{ip}/P_{HA}, respectively.

Our indicators are too insoluble in aqueous media to measure K_{HA}, but it may be estimated from literature[23] values for the short-chain homologues in 50% aqueous methanol: $10^{-2.46}$ for tetrabromofluorescein (eosine) ethyl ester and $10^{-6.36}$ for ethyl fluorescein. We would expect dissociation to be more favoured as the medium becomes more polar and better able to solvate the resulting ions. Hence the true K_{HA} is likely to be somewhat larger than these values, while $K_{\overline{HA}}$ is much smaller, as our results indicate. For the estimates that follow, we have used 10^{-2} mol dm^{-3} for K_{HA}.

We know of no data on the distribution of fluorescein ions in aqueous–organic systems. There have, however, been fairly extensive studies on 2,4,6-trinitrophenolate (picrate) ions, which also have a charge formally on a phenolic O but delocalized over an extensive system.

Iwachido[15] measured the distribution of simple alkali picrates between methyl isobutyl ketone and water. These were found to be significantly dissociated in the organic phase, and P_{fi} was 0.0017 for the Na^+ salt. Using their measurement of the distribution of picric acid (P_{HA} = 500) gives P_{fi}/P_{HA} of 3.4×10^{-6}. This may be compared with an estimated K_{ex1}/K_{HA} value of 7×10^{-7} for our tetrabromo indicator **2** in pentanone.

Gustavii[24] reported P_{ip} of 3.9×10^3 dm^3 mol^{-1} for Bu_4N^+-picrate between benzene and water, with P_{HA} equal to 107, giving P_{ip}/P_{HA} of 36 dm^3 mol^{-1}. This may be compared with our studies in toluene with the same cation, which give K_{ex2}/K_{HA} as 0.57 dm^3 mol^{-1}.

Essentially the same treatment can be applied to experiments with dibenzo-18-crown-6, with the organic phase concentration of the crown ether included in the denominator of both P_{ip} and K_{ex2}. For Na^+-picrate with this crown ether, Sadakane *et al.*[12] reported P_{ip} of 1.6×10^2 dm^6 mol^{-2} for benzene, while Danesi *et al.*[25] give the same value with 5% nitrobenzene in toluene as the solvent. Using Gustavii's[24] value for P_{HA} in benzene we estimate P_{ip}/P_{HA} of 1.5 dm^6 mol^{-2}. From our data value in toluene we estimate K_{ex2}/K_{HA} to be 4×10^{-2} dm^6 mol^{-2}.

Thus in each case the values for the tetrabromo fluorescein ester **2** are within two orders of magnitude for those calculated from the corresponding picrate system. All the fluorescein values are lower, suggesting that the contribution of the ionized portion of the molecule may be less favourable to partitioning into organic media than the picrate nucleus.

Since the value of P_{H^+/C^+} should not change when only the nature of the indicator is altered, the same value should apply for the tetrabromo and dibromo compounds in pentanone. Therefore we can say that $K_{\overline{HA}}$ is about 6×10^3 greater for the former. This difference is rather greater than the effect that might be expected on K_{HA}, based on the data for ethyl esters cited above, though there is no measurement for the dibromo derivative in this case.

Using the maximum value for $K_{\overline{HA}}$ of the tetrabromo indicator in pentan-3-one we can set a maximum value for P_{H^+/C^+} of *ca.* 7. Similarly, a maximum value of *ca.* 100 can be set for ethyl acetate. P_{H^+/C^+} reflects the relative affinities of the two phases for H^+ and other cations. Collected data for a variety of solvents[5] shows that free energies for such exchanges of H^+ and Na^+ are generally within the range -10 to $+10$ kJ mol^{-1}, with some notable exceptions such as propylene carbonate and acetonitrile. However, none of the solvents for which data is available are similar in both polarity and structure to ours. Nevertheless, our value seems to be generally in line.

Mechanisms of aqueous phase pH change in two-phase systems

During studies of the behaviour of these indicators in two-phase systems, we observed two effects that can lead to a change in pH of the aqueous phase, particularly when this is a small volume fraction and/or restricted to the pores of solid particles. (These pH changes were reflected in the ionization of the indicator, and also measured by other methods.) Both of these may be significant in real enzyme reaction mixtures of this type.

(*i*) Ethyl acetate as solvent can cause a significant acidification of the aqueous phase, presumably by hydrolysis releasing acetic acid. As expected the effect is greater the more alkaline the initial aqueous pH, and at values above 8 is significant even at equal phase volumes.

(*ii*) Very small aqueous phases trapped within particles were prepared by adjusting the pH in a slurry, drying, and then adding back a smaller quantity of pure water. (This is the usual approach with enzyme catalysts themselves.) With high initial buffer concentrations, one of the buffer salts may exceed its solubility when reconstituted with water, giving a different pH value. (Protein inactivation during freeze-drying is sometimes attributed to a similar effect of differential precipitation.)

Conclusions

The ionization of very hydrophobic indicators in organic solvents, in response to the pH of an equilibrated aqueous phase, can be described by an equilibrium model. This takes account of partitioning between phases, and (partial) ion-pairing in the organic phase. In toluene, the indicator anion is completely ion-paired, even at the concentrations around 10^{-5} mol dm^{-3} used. In pentanone, however, the ions are essentially completely free. The fitted equilibrium parameters are consistent with literature data on related systems.

Experimental

Synthesis of indicator molecules

This was performed as described in ref. 16.

Titration curves

The aqueous phase was buffered using $Na_4P_2O_7$ adjusted with H_3PO_4. Hence $[Na^+]$ remained constant at all pH values in the titration.

The activity of Na^+ in the buffers used was estimated using a Na^+-sensitive glass electrode (Philips G15-Na) with a calomel reference electrode (Philips RE1). The electrodes were calibrated using 0.001, 0.01, 0.1 and 1 mol kg^{-1} NaCl solutions.[17] The activity coefficient for Na^+ in our buffers was found to fall with increasing concentration; but also with increasing pH, probably because of the rising concentration of

multiply charged anions. For interpolation and smoothing, the measured activity coefficients were fitted using the semi-empirical equations

$$\alpha = K/(K + 10^{-n \times pH})$$

$$I = c \times (a + b \times \alpha)$$

$$\log \gamma = -A\sqrt{I}/(1 + B\sqrt{I})$$

where c is the concentration of $Na_4P_2O_7$ used. After initial trials the values of n, a, b and A were set at the (semi-)theoretical values of 1, 4, 6 and 0.51, respectively. The remaining two parameters K and B were treated as adjustable and fitted as 4.3×10^{-8} and 0.266 respectively. This fitted the data within experimental error (about 0.1 pNa units); there was no significant improvement from allowing more parameters to be adjustable.

Titration curves were determined as described in refs. 3 and 4. In outline, aqueous buffer samples were then equilibrated by shaking with organic solutions of the indicators. The more polar solvents were pre-saturated with water. The extent of ionization of the indicator in the organic phase was then measured spectrophotometrically.

In our earliest studies we had assumed that the pH of a well-buffered aqueous phase would not be significantly affected by equilibration with an equal volume of an organic phase. While investigating the cause of certain 'anomalies' that could not be accounted for by the theoretical model, we found that this assumption was not always reliable. (This itself has some consequences for pH in enzymic reaction mixtures, as noted below.) All the quantitative data now presented are based on aqueous pH values measured after equilibration. The pH electrode was calibrated in purely aqueous buffers, and the readings were not corrected for the effect of organic solvent dissolved in the aqueous layer in our experiments. The maximum concentration involved would be 0.68 mol dm^{-3} (the value for ethyl acetate in pure water; the buffer ions present will reduce this by salting-out). Therefore the effective pH scale is likely to be close to that in pure water.

Fitting of experimental data to a theoretical equation

The experimental data from the test titration system consisted of sets of measured indicator anion fraction values (f) as a function of aqueous phase pH. For each set the $[Na^+]$ and the total indicator concentration were constant; the latter was also used as a further check on the calculated concentrations of the indicator acid and anion. Where present, the total amounts of hydrophobic counter-ion or crown ether were also constant. We could consider together two or more sets of data from titrations with only one parameter altered: aqueous phase cation concentration, indicator or solvent identity.

Where Na^+ was the only cation, we have assumed that $\gamma_{C^+} \times [C^+]$ is given by the interpolated activity of this ion (as a function of concentration and pH). With the added tetraalkylammonium cations, we assume constant values of $\gamma_{C^+} \times [C^+]$ through each titration, proportional to the added ion concentration.

Eqn. (9) (and more complicated variants allowing for additional processes) is non-linear. A number of non-linear least squares regression algorithms were tried, and reliable convergence to the best fits was eventually obtained using Powell's method.[18] The algorithm described in this book was slightly altered to try alternate range expansions in each direction during the initial attempt to bracket the 'line minimum'; when the parameter values are very wrong, the theoretical equation can predict f values of 0 or 1 across the whole pH range studied. As a result the weighted sum of squares (chi-square) has a high value that is (within machine accuracy) independent of small changes in parameter values. So the original algorithm can search off to infinity in the wrong direction without finding an increase in the already maximal value of chi-square to make it turn back.

As may be seen from eqn. (9), the predicted shape of the titration curve is determined by the values of the groups $K_{HA}^{\overline{}}/(K_{CA}^{\overline{}} \times P_{H^+/C^+})$ and $K_{HA}^{\overline{}}/P_{H^+/C^+}$, rather than by the individual constants. So it is only these groups that can be determined by fitting experimental data. If $K_{HA}^{\overline{}}$ reaches a sufficiently high value, then a significant anion fraction is predicted to persist even at the lowest aqueous pH values, as shown by a more complex form of the equation. No certain evidence for this was found in any of the cases studied, but the relevant model does allow us to estimate an upper limit for the possible magnitude of this parameter (and hence also for $K_{CA}^{\overline{}} \times P_{H^+/C^+}$ and P_{H^+/C^+}.

While the theoretical equation could always offer a good fit to a single titration, a more discriminating test (giving better parameter estimates) was to fit several together as a group. For a series of titrations with the same indicator and organic solvent, we would expect the fitted parameters to be proportional to the known activities of the relevant cation in the aqueous phase (multiplied by that of crown ether complexing agent if used). If this is assumed, the whole set of titrations may be fitted, using these activities and the total indicator concentrations as additional independent variables.

In many cases the fitted value for one of the parameters in eqn. (9) (or for one or more parameters in more complex variants) became sufficiently small that it had little effect on the theoretical titration curves. In these cases, regression was then tried with the relevant value(s) fixed at zero. The significance of including a non-zero value for these parameters was then judged by comparison of the resulting chi-square and Q-likelihood[18] values; a 50% reduction in the former, or a 10^3-fold increase in the latter, was judged probably significant.

Errors in fitted parameter values were estimated by Monte-Carlo simulation of data sets, to which parameters were re-fitted. About 100 data sets in each case were used to estimate 68% confidence limits (approximately standard errors).

In some cases, the deviations of some experimental points from the fitted curves seem to be possibly significant. An improved fit was not possible by incorporating any of the effects discussed in this paper. If these deviations are real, they must reflect additional factors we have not considered.

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