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Aqueous solutions that model the cytosol: studies on polarity, chemical reactivity and enzyme kinetics †

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Concentrated solutions of a series of organic compounds have been prepared and the effects of these solutes on the properties of the solvent system assessed as a function of their concentration and nature. Polarity, as measured by Reichardt's $E_T(30)$ probe, exhibits a linear variation with both solute and water concentration for simple solutes. Non-linear behaviour was also observed and is associated with preferential solvation or binding of the $E_T(30)$ probe molecule by the added solute. The observed trends in polarity are mirrored in the effects of these solutes on chemical reactivity and enzyme kinetics. Environmental effects on the kinetics of hydrolysis of 4-nitrophenyl dichloroacetate, the hydronium-ion catalysed hydrolysis of 2-(4-nitrophenoxy)-tetrahydropyran, the acyl transfer reaction between 4-nitrophenyl acetate and TRIS, the Diels–Alder reaction between 1,4-naphthoquinone and cyclopentadiene and the trypsin-catalysed hydrolysis of 4-nitrophenyl acetate are reported and discussed in terms of the properties of the solutes and the mechanistic requirements of these reactions. Linear correlations were observed between the logarithms of the rate constants for the acetal hydrolysis, acyl transfer and Diels–Alder reactions with water concentration. Since the latter varies linearly with $E_T(30)$, this indicates a linear free energy relationship between solution polarity and chemical reactivity.

Introduction

The cytosol is an aqueous region, surrounded by a lipid bilayer membrane, crowded with macromolecules and other solutes totalling several hundred grams per litre.[1] This environment is markedly different to the dilute aqueous solutions generally used as background comparisons for biochemical processes,[2] hence is expected to have different solvent properties. In the cellular environment, the rate of diffusion, especially of large molecules, is reduced[3] and macromolecular crowding agents promote protein folding and association.[4]

The cytosol may be modelled by aqueous solutions of polar organic molecules, salts, sugars and their macromolecular analogues. This constitutes a reasonable analogue since the tertiary structure of proteins places hydrophilic groups outermost into solution, nucleic acids contain anionic phosphate groups and are generally associated with proteins, and bilayer structures similarly have polar headgroups disposed at the aqueous interface.[5] We have previously reported a study into the effects of added solutes at high concentrations on the trypsin-catalysed hydrolysis of 4-nitrophenyl acetate.[6] This report describes a more general investigation into the chemical properties of aqueous solutions containing large concentrations of solutes. The effects of the changing environment on polarity and chemical and enzyme reactivity have been assessed as a function of solute concentration. The general procedure followed was to prepare a series of solutions of the solute of interest, ranging from pure water to biologically significant concentrations of several hundred grams per litre. The change in a property such as polarity over this concentration range was measured and the dependence of this variation on the nature of the solute analysed. The solutes used in this study were selected to mimic the cellular environment as closely as possible whilst still allowing for controlled variation of properties.

Solutes were chosen that are freely soluble in water and unreactive towards the chemical probes used. The amide group is ubiquitous in biochemistry, hence a series of amides was chosen with the same basic functionality, but a progressive increase in hydrophobic character: urea, formamide, *N*,*N*-dimethylacetamide (DMA), *N*-*tert*-butyl acetoacetamide (NBAA) and *N*-cyclohexyl pyrrolidin-2-one (N–CHP). Sorbitol was chosen as a mimic for sugars which does not contain the reactive acetal functionality, and bovine serum albumin (BSA) was used as a model protein. Solutions of poly(ethylene glycol) (PEG), which has been widely employed as a solubilising agent and stabiliser in chemo-enzymatic synthesis,[7] were also studied. Whilst, obviously less appropriate as a mimic for biological systems, the constitution of PEG is roughly in-line with the other solutes studied and it is available in a range of well-defined molecular weights. This potentially allows the effects of solute size and macromolecular crowding to be assessed.

Results and discussion

Since the test systems generally contain a large proportion of water, the best measure of composition is the water concentration. This prevents distortion of the results by the size of the cosolute – for example, pure formamide has a formamide concentration of 25.2 M, whereas the concentration of pure *N*-cyclohexyl pyrrolidin-2-one is 6.0 M. Therefore a scale of solute concentration would have a different end-point depending on the solute. Using a water concentration scale reverses the direction of the trend relative to a solute concentration scale, but all solutions range in concentration from zero to 55.3 M at 25 °C. Using molar water concentration as the default scale for solution composition also removes the complication of how to treat mixtures containing different quantities of more than one solute. In practice, molar solute concentration and water concentration could be used interchangeably since, for the solutes and concentration ranges studied, they have an inverse linear relationship with each other. The solution density also increases

† Electronic supplementary information (ESI) available: further results and discussion and tables of experimental data. See http://www.rsc.org/suppdata/ob/b4/b402886d/

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linearly with solute concentration indicating that there is no significant change in volume associated with mixing.

Solution polarity

Polarity was measured using Reichardt's $E_T(30)$ probe.[8] The zwitterionic form of the molecule exhibits a charge transfer band in the visible region. The energy of this transition, the $E_T(30)$, is sensitive to the ability of the probe's environment to solvate hydrophobic groups, stabilise charges and donate hydrogen-bonds. Since the ground state of the molecule is charged and the excited state is effectively a diradical, an environment capable of stabilising charges will lower the ground state relative to the excited state, increasing the transition energy and shifting the charge transfer band to shorter wavelength.

Each aqueous test solution was made alkaline with the same concentration of NaOH and the $E_T(30)$ value was measured as a function of solution composition. Interestingly, simple solutes exhibited a linear variation of $E_T(30)$ with water concentration over a large concentration range (Fig. 1). This suggests that the solutes mix homogenously with water.

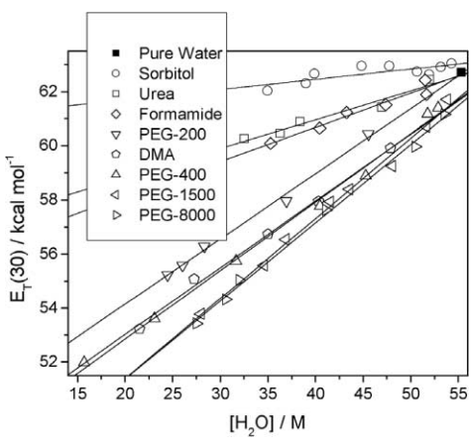


Fig. 1 Variation of $E_T(30)$ with water concentration for a series of solutions of simple organic solutes [PEG-number refers to the average molecular weight of the poly(ethylene glycol)].

It is apparent that those solutes with more hydrocarbon character give rise to a more pronounced decrease in $E_T(30)$ with water concentration, as would be expected from the $E_T(30)$ values of the pure compounds. The addition of sorbitol has a very small effect on solution polarity. A similar pattern is observed for solutions of sugars[9] and is consistent with the "camouflage effect".[10] The highly polar solutes urea and formamide exert only a small effect on polarity. Taken with the still greater effect of DMA, a picture emerges where solution polarity is dependent on the proportion by volume of polar and apolar functionality – the more alkyl group character in the molecule, the greater the decrease in polarity *per* volume of water replaced.

The results obtained with the series of PEG's indicate that the size of the solute is relatively unimportant, though it should be noted that the smaller PEG's have a much greater relative proportion by volume of terminal hydroxyl groups, hence a smaller effect on polarity.

The experimental $E_T(30)$ value of pure water (Fig. 1, closed square) was not always equal to that obtained by extrapolation to zero solute concentration. This is probably due to (weak) aggregation of the poorly water-soluble probe in the absence of organic cosolutes. Head-to-tail stacking of the probe molecule could give rise to additional stabilisation of charges hence a higher apparent $E_T(30)$ value. Similarly, an extrapolation to zero water concentration overestimates the $E_T(30)$ value of the pure solutes (not shown). This effect may be the result of preferential association of water to the charged ground state of the $E_T(30)$ probe at very low water concentrations.

Non-linear variation of $E_T(30)$ with concentration was observed for *N*-cyclohexyl pyrrolidin-2-one (N–CHP) (Fig. 2). This compound is capable of acting as a hydrotrope,[11] hence solubilises hydrophobic compounds in water. The concentrations of water and N–CHP in binary mixtures and the density of the solution are linearly dependent, again indicating that there is no appreciable change in molecular size. However, the non-linear variation in $E_T(30)$ of the mixture indicates non-specific binding of the $E_T(30)$ probe.

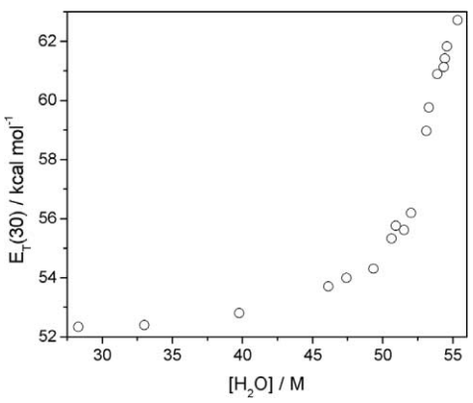


Fig. 2 Variation of $E_T(30)$ with water concentration for solutions of *N*-cyclohexyl pyrrolidin-2-one.

N–CHP may form micelle-like aggregates in aqueous solution above a critical concentration of around 750 mM (approximately 50 M in water). These aggregates would bind the hydrophobic $E_T(30)$ probe preferentially, giving rise to two regions in the $E_T(30)$-concentration profile. At low water concentrations, the probe is fully bound to the N–CHP aggregates and the small change in $E_T(30)$ on further increasing the N–CHP concentration is presumably the effect of the increasing proportion of N–CHP aggregates in the bulk solution. In the dilute region, the observed dependence of $E_T(30)$ on concentration is similar to that of the other solutes. The decline in $E_T(30)$ with water concentration in this region is much steeper than that of the other solutes studied, which may indicate hydrophobic association.

Actual binding of the $E_T(30)$ probe by the solute was observed when bovine serum albumin (BSA) was used in an attempt to mimic the presence of proteins in the cytosol (Fig. 3).

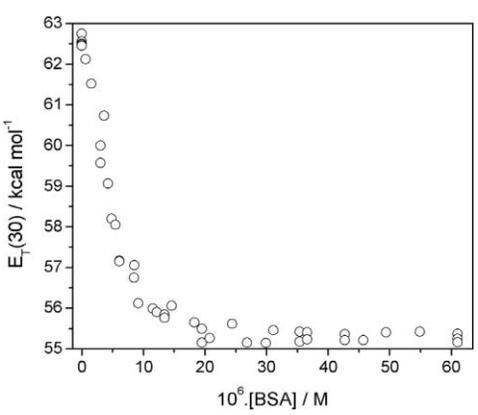


Fig. 3 $E_T(30)$ values of solutions of bovine serum albumin (BSA) at low concentration, indicating binding of the $E_T(30)$ probe molecule to the protein (probe concentration 28×10^{-6} M).

Initial studies on the polarity of BSA solutions showed that all the test solutions in the region of 50–200 g L^{-1} of BSA had essentially the same $E_T(30)$ value. It was necessary to study solutions of BSA in the micromolar concentration range in

order to follow the change in $E_T(30)$ value from that of pure water to this plateau value of 55.3 kcal mol^{-1}.

Several titrations of BSA into a solution of Reichardt's dye were carried out. A plateau on the $E_T(30)$-concentration profile was obtained above a BSA concentration of 2×10^{-5} M, indicating complete binding of the $E_T(30)$ probe to the protein. Given that saturation occurred at a concentration of BSA lower than the concentration of $E_T(30)$ probe (2.8×10^{-5} M), it is clear that one BSA molecule is capable of binding more than one $E_T(30)$ probe molecule. This is reasonable since serum albumins are known to contain multiple hydrophobic binding sites.[12] Attempts to ascertain the number of equivalents of bound $E_T(30)$ probe by a Job plot analysis[13] were generally unsatisfactory, though it is clear that more than two equivalents of $E_T(30)$ probe are bound per BSA (Fig. 4).

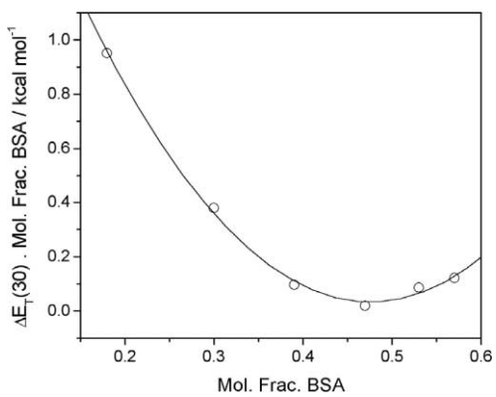


Fig. 4 Job-type plot for binding of the $E_T(30)$ probe to bovine serum albumin (BSA) [second-order polynomial fit].

The data does not fit two intersecting straight lines, but rather a non-linear distribution with a minimum at a mole fraction of BSA of approximately 0.46. This behaviour may be due to the fact that the binding sites of BSA are not all equivalent and also that a small concentration of the host was titrated into the guest rather than *vice versa*.

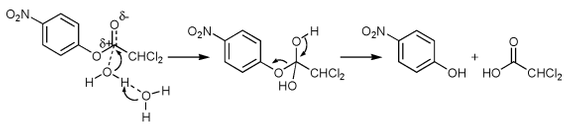
These experiments did not give insight into the bulk polarity of protein solutions, however, they did give the interesting result that the $E_T(30)$ values of the BSA binding sites are in the region of 55.3 kcal mol^{-1}. This is virtually the same polarity as neat methanol and gives an indication of the likely "solvent polarity" inside the active sites of enzymes.

Chemical reactivity

Chemical processes of interest in this research fall into three general categories: reactions that directly involve water, other reactions that are sensitive to their environment and reactions of biomolecules. Reactions that directly involve water would be expected to respond to the addition of solutes that interact with water. In conjunction with a study of the effects of solutes on medium-sensitive reactions in aqueous solution, such processes can be used to give insight into the nature of cellular water. The effects of added solutes on hydrolysis, acyl transfer and Diels–Alder reactions have been studied to this end. Furthermore, a study of environmental effects on biochemical reactions gives direct insight into *in vivo*-processes.

Initial studies on chemical reactivity focussed on the effect of amide solutes on the hydrolysis of the activated ester, 4-nitrophenyl dichloroacetate (4NPD). The neutral hydrolysis reaction in solutions of $2 < pH < 4$ involves two attacking water molecules in the rate-limiting step – one as a nucleophile and the other acting as a general base (Scheme 1).[14]

Changes in the rate of 4NPD hydrolysis upon addition of *N*,*N*-dimethylacetamide (DMA) or *N*-cyclohexyl pyrrolidin-2-one (N–CHP) mirrored the variation in polarity with water concentration observed for these solutes (Fig. 5), indicating



Scheme 1 Neutral hydrolysis of 4-nitrophenyl dichloroacetate (4NPD).

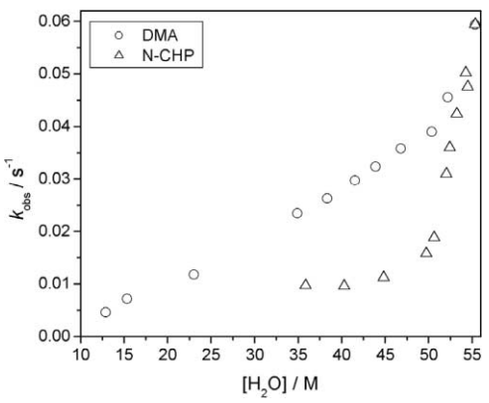


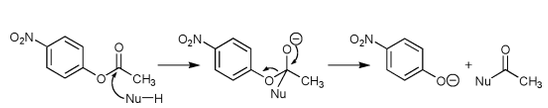
Fig. 5 Variation in the rate constants for hydrolysis of 4-nitrophenyl dichloroacetate with water concentration upon addition of *N*,*N*-dimethylacetamide (DMA, ○) and *N*-cyclohexyl pyrrolidin-2-one (N–CHP, △).

that the reaction rate is dependent on the polarity of the medium and that the relatively hydrophobic kinetic probe reports the same environmental characteristics as the $E_T(30)$ probe.

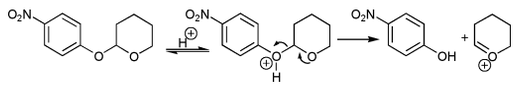
A free energy relationship between polarity and reactivity for a (pseudo)first-order process would require a correlation of log (k_{obs}/s^{-1}) with $E_T(30)$. This is indeed the case over part of the concentration range studied (not shown). Several complications emerge: firstly, the Gibbs energy of activation of the reaction depends on the chemical potential of water rather than its concentration (the solutions studied fall well outside the concentration range for which ideal behaviour can be assumed), hence a non-linear dependence of thermodynamic water activity on concentration is likely to contribute to the observed trend in reactivity. Secondly, it is possible that, at lower water concentrations, a hydrolysis mechanism involving only one water molecule dominates; it should be noted however that the solvent kinetic isotope effect ($k_{H_2O}/k_{D_2O} = 3.1$)[15] does not change over the DMA concentration range studied, implying that there is no change in mechanism. It is further possible that, at low water concentration, general base catalysis by the amide solutes occurs. Furthermore, the rate constant for hydrolysis and the pK_a's of the general base(s) are expected to vary with solution composition. Binding of the probe to N–CHP aggregates must also be considered in order to deconvolute the above hydrolysis data.[16] It was therefore decided to switch to a series of simpler test reactions and inert solutes in order to probe the changes in reactivity.

Three further probe reactions were studied: the acyl transfer reaction between 4-nitrophenyl acetate (4NPA) and *tris*(hydroxymethyl)aminomethane (TRIS) (Scheme 2), the hydronium-ion catalysed hydrolysis of 2-(4-nitrophenoxy)-tetrahydropyran (Scheme 3) and the Diels–Alder reaction between cyclopentadiene and 1,4-naphthoquinone (see Scheme 4).

Nucleophilic attack by TRIS on 4NPA[17] (Scheme 2, Nu–H = TRIS) is analogous to the hydrolysis of 4NPD (Scheme 1), with



Scheme 2 Nucleophilic displacement of 4-nitrophenolate from 4-nitrophenyl acetate.



Scheme 3 Hydronium-ion catalysed hydrolysis of 2-(4-nitrophenoxy)-tetrahydropyran (4NPTHP).

the attacking nucleophile taking the place of the two water molecules. The initial state is charge-neutral, hence nucleophilic attack is accompanied by separation of charge and is favoured by a more polar environment. The addition of solutes that are less polar than water would therefore be expected to retard the reaction. Lewis acid catalysis by hydrogen-bond donation to the carbonyl oxygen also stabilises the charge-separated transition state, therefore the addition of a solute that is a poorer hydrogen-bond donor than water or competes for hydrogen-bonds would also disfavour the reaction. Poly(ethylene glycol) (PEG) lowers solution polarity and is a hydrogen-bond acceptor only, hence is expected to decelerate the reaction *via* both effects.

As expected, the observed pseudofirst-order rate constants vary linearly with TRIS concentration, *i.e.* the reaction is first-order in TRIS, at all PEG concentrations studied (0–341 g L^{-1}) (Fig. 6, top). The intersection of the linear fits at a TRIS concentration of approximately 0.8 M is not indicative of an inversion in the trend in reactivity with respect to PEG concentration, but rather a reflection of the dependence of the background (zero-buffer) specific base-catalysed reaction on polarity. This may be related to an increase in the nucleophilicity of hydroxide with PEG concentration, or to a decline in the ionic product of water.

The second-order rate constants increase with water concentration (Fig. 6, bottom), which is proportional to the $E_T(30)$ polarity, *i.e.* the rate of acyl transfer increases with the polarity of the environment.

The logarithms of the second-order rate constants for the TRIS-catalysed reaction correlate linearly with water concentration. The former is proportional to the Gibbs energy of activation of the reaction and, since the $E_T(30)$ value of the solution varies linearly with water concentration (Fig. 1), the Gibbs energy of activation also correlates with solution polarity. There is therefore a linear free energy relationship between chemical reactivity and the polarity of the medium, as measured by the $E_T(30)$ probe (see Fig. 8). That the two properties – $E_T(30)$ polarity and reaction rate constant – show the same general trend with solution composition is not unexpected: chemical reactivity is quantified in terms of the Gibbs energy difference between the initial and transition states and the $E_T(30)$ value is the energy difference between a charged ground state and charge transfer excited state. However, the linear correlation is noteworthy.[18]

The hydronium-ion catalysed hydrolysis of 2-(4-nitrophenoxy)-tetrahydropyran (4NPTHP) (Scheme 3) in the presence of PEG (MW 400) was also studied. This reaction is known to be sensitive to its medium[19] and proceeds *via* an A1 mechanism,[20] hence does not involve water until after the rate-determining step. The first step is an equilibrium protonation, which is followed by rate-limiting elimination of 4-nitrophenol. Water then attacks the generated oxonium species at the 2-position forming a hemi-acetal that may undergo further hydrolysis.

The initial state of the reaction is charge-neutral, so although the transition state involves delocalisation of the formal positive charge on the phenolic oxygen, it is more polar than the initial state and hence is relatively stabilised by polar solvent systems.

The expected linear dependence of pseudofirst-order rate constant on acid concentration is observed at all concentrations of PEG studied (0–431 g L^{-1}) (Fig. 7, top). The second-order rate constants also follow a trend of increasing with water

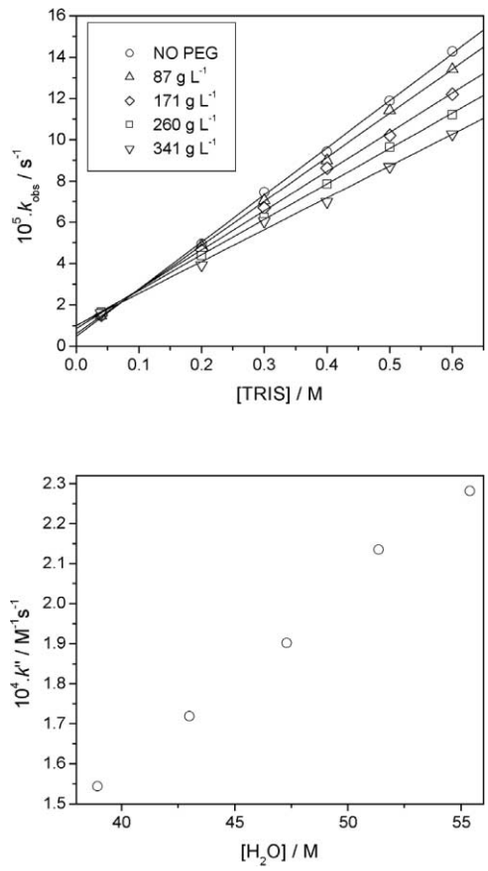


Fig. 6 Variation in pseudofirst-order rate constant with TRIS concentration (top) and second-order rate constant with water concentration (bottom) for the addition of TRIS to 4-nitrophenyl acetate at pH 7.7 upon addition of PEG (MW 400).

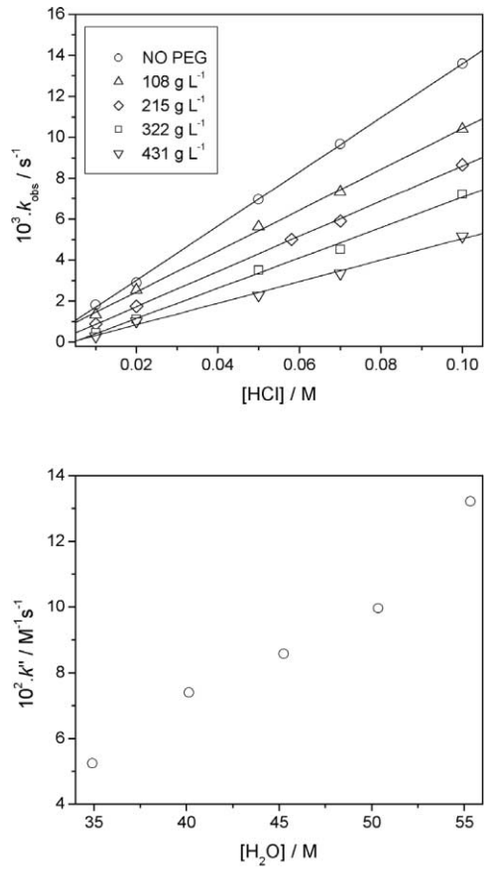


Fig. 7 Variation in pseudofirst-order rate constant with acid concentration (top) and second-order rate constant with water concentration (bottom) for the specific acid-catalysed hydrolysis of 2-(4-nitrophenoxy)-tetrahydropyran upon addition of PEG (MW 400).

concentration (Fig. 7, bottom). This indicates an analogous dependence of chemical reactivity on solution polarity to that of the acyl transfer reaction.

A good linear correlation between the logarithm of the second-order rate constant and water concentration is also obtained for this reaction (Fig. 8). Both trends in reactivity may be rationalised in terms of the differential effects of the changing environment on an apolar initial state relative to a charge-separated transition state. However, that two mechanistically distinct reactions exhibit the same trend in reactivity with solution composition suggests that this property is general and the observed linear correlations are striking.

Interestingly, the relative effect of PEG on the acetal hydrolysis is greater than that on the acyl transfer reaction of the ester (Fig. 8). It is likely that the reduction in the ability of the medium to stabilise charges is the dominant factor. This is more important for the relatively polar transition state in the acetal hydrolysis than in the relatively early transition state for attack of TRIS at the ester carbonyl.

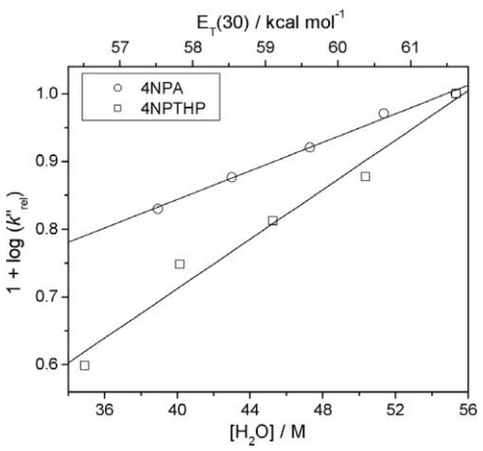
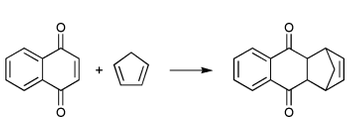


Fig. 8 Relative Gibbs energies of activation for the nucleophilic substitution of 4-nitrophenyl acetate (4NPA) and the specific acid-catalysed hydrolysis of 2-(4-nitrophenoxy)-tetrahydropyran (4NPTHP) in the presence of PEG (MW 400).

Both reactions exhibit linear free energy relationships with polarity. In principle, the gradient of a plot of Gibbs energy of activation or log (k) against $E_T(30)$ could be used as a measure of the polarity dependence of reactions where a linear correlation between the two is obtained. In practice, it may not be possible to apply this approach generally: for a particular reaction in a series of solvent systems, the correlation with $E_T(30)$ is unlikely to hold across a significant change in solvent properties such as going from a protic to an aprotic environment. However, the approach should be generally applicable to aqueous solutions.

The Diels–Alder reaction between 1,4-naphthoquinone and cyclopentadiene (Scheme 4) in the presence of PEG was also studied. Strong rate enhancements have been observed for the Diels–Alder reaction in protic solvents, and especially in water, relative to apolar organic solvents.[21] This rate acceleration has been attributed to enforced hydrophobic interactions and to the stabilisation of electron density at oxygen in the transition states for carbonyl-substituted dienophiles. Furthermore, a general trend of increasing reaction rate in more polar solvents has been observed.



Scheme 4 Diels–Alder reaction between 1,4-naphthoquinone and cyclopentadiene.

The reaction is therefore expected to exhibit a similar sensitivity to the addition of PEG as the activated ester hydrolysis discussed above. The replacement of a large concentration of water by a hydrogen-bond acceptor diminishes Lewis-acid catalysis and the decline in polarity upon addition of PEG would also be expected to lead to a decline in reactivity.

Addition of PEG does indeed diminish the reaction rate, but there is a break in the correlation at a water concentration of approximately 38 M (Fig. 9).

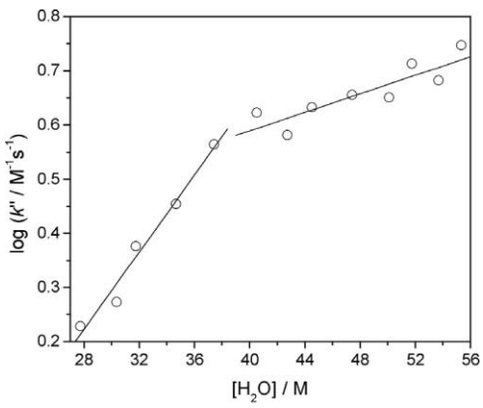


Fig. 9 Variation in Gibbs energy of activation for the Diels–Alder reaction between 1,4-naphthoquinone and cyclopentadiene with water concentration upon addition of PEG (MW 400).

A break in a linear free energy relationship can be indicative of a change in mechanism. However, it is hardly conceivable that this Diels–Alder reaction proceeds *via* anything other than a concerted electrocyclic mechanism. One possible explanation for this behaviour is that 1,4-naphthoquinone has two carbonyl groups conjugated to the dienophilic double bond, each of which has two oxygen lone pairs capable of accepting a hydrogen-bond. It is therefore possible that the observed rate depends on the contributions of five equilibrium hydrogen-bonded-states, each with different rate constants for the Diels–Alder reaction (although it is likely that only two of these states would be important in the observed kinetics). If this was the case, then an identical break would be expected in the rate constant-concentration profile of the retro-Diels–Alder reaction. However, no such break is present.[22] The significance of a water concentration of ~38 M could be that this is the critical concentration above which there are microscopic regions of "bulk" water. However, such partitioning of water would be expected also to cause an analogous break in the $E_T(30)$-water concentration profile, which is not observed. It appears likely that this behaviour is related to the large negative entropy of activation of the Diels–Alder reaction in the forward direction. A previous study into the effects of mixed aqueous-organic solvent systems on activated acetal hydrolysis attributed the decline in rate with decreasing water concentration to the greater entropic demands for solvating the transition state.[23] However, a satisfactory explanation of the origin of the observed behaviour requires a more detailed study into the kinetics of bimolecular reactions over this concentration range.

Interestingly, the above Diels–Alder reaction is slower in neat methanol than in a PEG solution with an $E_T(30)$ value of 55.4 kcal mol^{-1}, which is the $E_T(30)$ value of neat methanol. Methanol has a greater hydrogen-bond donating ability than the PEG solution, but a lower polarisibility,[24] indicating that the Diels–Alder reaction is more sensitive to the latter.

Attempts to study the environmental and binding effects of BSA on this Diels–Alder reaction failed since the protein reacts with the dienophile, presumably in a Michael addition, at a rate comparable to the desired reaction. General base catalysis of the Kemp elimination by BSA has previously been reported and exhibits Michaelis–Menten kinetics.[25]

Enzyme kinetics

The sensitivity of an enzyme to its environment has provoked much interest both for its immediate relevance to biochemistry and for its importance in the use of enzymes in chemical synthesis.[26] Previous studies into environmental effects on enzyme chemistry have highlighted the importance of water concentration,[27] water activity and co-solute nature[28] and dielectric constant with simultaneous competitive inhibition,[29] however no clear picture has emerged.

It is conventional to compare enzymatic rate enhancements to the uncatalysed reaction in dilute buffer solution. However, a fairer comparison would be that between the uncatalysed and enzyme-mediated reactions under biologically relevant conditions. It would be reasonable to assume that, as long as the active site of the enzyme is shielded from the bulk solution, the catalytic rate constant (k_{cat}) would be unaffected by the addition of co-solutes or crowding agents up until they cause denaturation of the enzyme or affect the diffusional rate-limit. The substrate binding constant (K_M) is expected to be sensitive to solvent composition since it depends on the chemical potential of the substrate free in solution. Substrate binding also requires the dehydration of both the active site and the substrate, hence might be dependent on water activity. However, our study into the trypsin-catalysed hydrolysis of 4-nitrophenyl acetate (4NPA) in the presence of PEG's (MW 8000 and 1500) and *N*-*tert*-butyl acetoacetamide (NBAA) showed that binding was essentially unaffected by the addition of these solutes, whereas k_{cat} decreased with water concentration (Figs. 10 and 11).[6] In order to confirm that our previous results were not the result of denaturation of the enzyme, we have carried out a further set of experiments using urea, a known denaturant, as the solute.

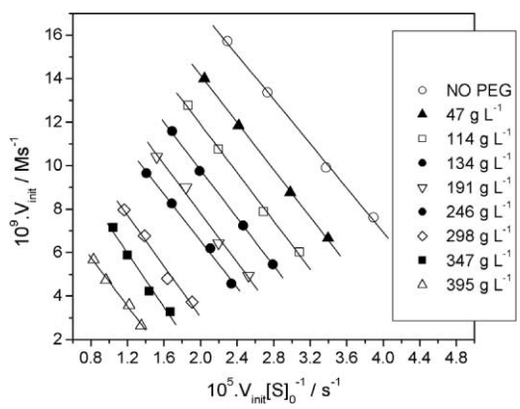


Fig. 10 Eadie–Hofstee plots for the trypsin-catalysed hydrolysis of 4-nitrophenyl acetate in a series of PEG (MW 400) solutions.

Trypsin catalysis was measured for a series of solutions ranging from no added solute up to 395 g L^{-1} of PEG, up to 269 g L^{-1} of NBAA and up to 522 g L^{-1} of urea. Monitoring the concentration of the product rather than the substrate allowed the use of four convenient initial substrate concentrations in the region of K_M, at the expense of having to calibrate against the extinction coefficient of 4-nitrophenolate in each test solution. Kinetic parameters k_{cat} and K_M were determined by the Eadie-Hofstee method.[30] Michaelis–Menten kinetics were observed for all solutes at all concentrations studied.

It is apparent that urea has a significantly different effect on the enzyme than do PEG and NBAA. The variation in Michaelis–Menten parameters for the latter two solutes is of the form classically expected for noncompetitive inhibition of an enzyme[31] (Fig. 10). However, it is unlikely that the two vastly different solutes have identical mechanisms of inhibition.

A crowding-induced change in enzyme conformation has previously been proposed to account for lowering in enzyme activity[32] but, unlike PEG, NBAA is not a crowding agent. The observed kinetics must therefore be attributable to an environmental effect on the enzyme, the substrate or both.

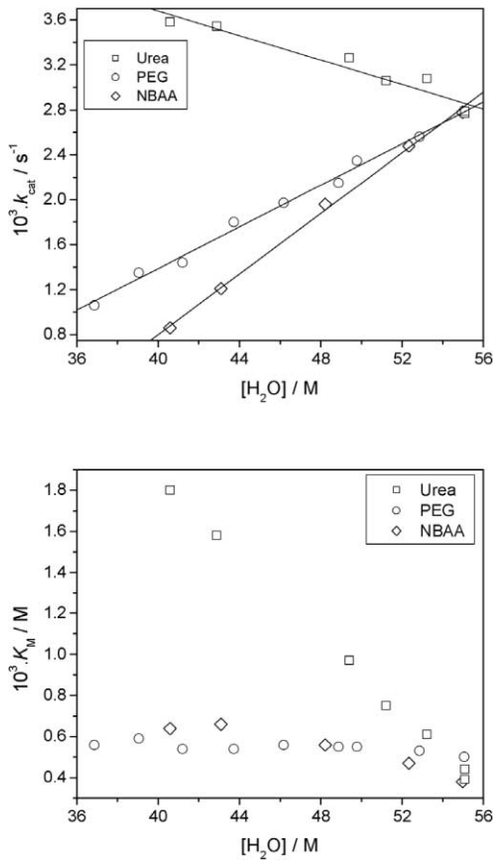


Fig. 11 Michaelis–Menten parameters for the trypsin-catalysed hydrolysis of 4-nitrophenyl acetate in the presence of different solutes.

That K_M is essentially unaffected by these solutes indicates strictly that any change in the chemical potentials of the free enzyme and substrate (relative to pure water) is equal to the change in chemical potential of the enzyme–substrate complex. If it is assumed as an approximation that any change in K_M results from the change in the chemical potential of the free substrate, it is clear that the concentrated test solutions of PEG and NBAA have a negligible effect on the neutral substrate (4NPA). However, a substantial effect on K_M could be expected for a charged substrate. Taking the $E_T(30)$ probe in pure water as a reference, the most concentrated PEG solution studied destabilises the zwitterionic state by ~16 kJ mol^{-1} relative to the neutral excited state. If this effect was mirrored on the chemical potential of the free substrate only, then a 1000-fold decrease in K_M would result.

The apparently linear decline in k_{cat} with water concentration for these solutes mirrors the previously observed dependence of reactivity on polarity. [Over this relatively small change in k_{cat}, it is impossible to distinguish between a linear change in k_{cat} and in log (k_{cat}/s^{-1}) for these results]. This dependence can be rationalised since the active site of trypsin is relatively exposed to the solvent,[33] hence a similar dependence of the enzyme-catalysed reaction can be expected to that of the acyl transfer reaction discussed above.[34]

The effect of urea on the enzyme-catalysed reaction was significantly different to those of the other two solutes. Surprisingly, urea concentrations in excess of 500 g L^{-1} do not completely inhibit the enzyme. Binding and catalysis are observed, however there is a large decline in binding (*i.e.* an increase in K_M) on addition of urea (Fig. 10, bottom), but a concurrent increase in k_{cat} (Fig. 10, top). The change in K_M can be rationalised in terms of partial denaturation of the enzyme. This could potentially open up the structure of the enzyme, exposing amine or carboxylate amino acid side chains that could catalyse

the hydrolysis of, or be alkylated by the substrate, releasing 4-nitrophenolate and leading to an apparent increase in k_{cat}. It should be noted that the background TRIS-catalysed reaction is essentially independent of urea concentration (not shown).

Most importantly, we have shown that so long as the structure of the enzyme is not perturbed by the altered environment, the presence of large concentrations of inert solutes such as PEG and NBAA has only a modest effect on enzyme activity – less than an order of magnitude reduction in k_{cat} and a negligible effect on K_M. Apparently, the catalytic process hardly reacts to the large reduction in the concentration of water in the model system. This indicates that the enzyme is relatively stable to changes in its environment, which is presumably related to its role *in vivo* as a digestive enzyme. The insignificant effect on K_M indicates that these solutes have a minimal effect on the chemical potential of the neutral substrate in the bulk solution and do not compete strongly with the enzyme for solvating water. It is noteworthy that the decline in k_{cat} is linear with increasing additive concentration. Comparison to solvent polarity data and the nucleophilic reaction of the substrate with TRIS indicates that this appears to be a medium effect on enzyme chemistry.

Conclusions

For simple solutes, $E_T(30)$ polarity varied linearly with both solute and water concentration, indicating homogeneous mixing and a linear dependence of polarity on volume composition of the test solution over the concentration range studied. As expected, the least polar solutes gave the largest changes in $E_T(30)$ as water concentration diminished.

The $E_T(30)$-concentration data for *N*-cyclohexyl pyrrolidin-2-one indicated aggregation of the solute and binding of the probe molecule, hence the probe reported the polarity of a hydrophobic domain rather than that of the solution as a whole.

It was not possible to obtain the bulk polarity of solutions of bovine serum albumin, since the probe molecule was preferentially bound by the protein. However, the measured $E_T(30)$ value of the BSA binding sites – in the region of 55.3 kcal mol^{-1} – gives an indication of the polarity that can be expected inside the active site of an enzyme. This region is "methanol-like" in terms of polarity.

The acyl transfer reaction of 4-nitrophenyl acetate with TRIS and the specific-acid catalysed hydrolysis of 2-(4-nitrophenoxy)-tetrahydropyran exhibited linear relationships between the logarithms of the rate constants and water concentration or $E_T(30)$ *i.e.* linear free energy relationships between reactivity and polarity. The effect on reaction rate on going from pure water to biologically significant solute concentrations of around 400 g L^{-1} was within one order of magnitude for both reactions.

The Diels–Alder reaction between 1,4-naphthoquinone and cyclopentadiene also showed a linear correlation between the logarithm of the rate constant and water concentration, but there was a break in the linear free energy relationship at a water concentration of ~38 M in water. This reaction is retarded more by methanol (relative to the reaction in pure water) than by a poly(ethylene glycol) solution with the same $E_T(30)$ polarity as methanol, indicating a stronger dependence of this reaction on the polarisability of the solvent than on hydrogen-bond donating ability. The gradient of a log (k)/$E_T(30)$ graph for mixed aqueous solutions such as those described here may prove a useful tool for the characterisation of chemical reactions.

Taking the trends in polarity and reactivity with solution composition together, it is clear that varying the concentration of an additive can have a pronounced effect on chemical reactivity. We suggest that further research is merited into the effects of such changes on competing processes and the use of additives to facilitate organic chemistry in aqueous solutions.

The trypsin-catalysed hydrolysis of 4-nitrophenyl acetate was remarkably insensitive to the addition of large concentrations of solute. The substrate binding constant (K_M) was essentially unaffected by the addition of poly(ethylene glycol) and *N*-*tert*-butyl acetoacetamide, but the catalytic rate constant (k_{cat}) decreased linearly with solute concentration, by less than one order of magnitude up to 400 g L^{-1}. This indicates a small polarity-dependence of the reaction, presumably due to the active site being partially exposed to the bulk solution. The addition of urea disfavoured substrate binding and increased the apparent catalytic rate constant, again linearly with concentration, but in the case of urea, these effects may be attributable to partial denaturation of the enzyme. It is likely that the effect of the environment on enzyme binding will be far more pronounced for a charged substrate binding to an active site that is well shielded from the bulk solvent. Further research is called for, ideally using an intracellular enzyme with an ionic substrate, to assess the effects of the environment on enzyme chemistry *in vivo*.

Experimental

Materials

4-Nitrophenol, 4-nitrophenyl acetate, dichloroacetyl chloride, deuterium oxide, dextrans, *N*,*N*-dimethylacetamide, *N*-cyclohexyl pyrrolidin-2-one and *N*-*tert*-butylacetoacetamide, were purchased from Aldrich. 1,4-Naphthoquinone, poly(ethylene glycol)s and acetonitrile were purchased from Acros. Trypsin (hog pancreas), bovine serum albumin, hen egg-white albumin and TRIS buffer solution were purchased from Fluka. Urea was purchased from J. T. Baker.

Methods

UV-visible spectra and absorbance-time measurements were recorded using a Perkin-Elmer Lambda 12 spectrophotometer, or a Shimadzu Multispec-1501 UV-visible spectrophotometer fitted with diode array detector, attached to a Haake K15/DC50 thermostatted water bath. All masses were measured on a Sartorius BP211D five decimal place balance and densities were measured using a Mettler Toledo KEM DA-100M specific gravity meter. Sonication was carried out using a Bandelin Sonotex RK255 bath sonicator. Water activity was measured using an AquaLab 3TE vapour pressure osmometer.

Graph-fitting was done using GraFit v3.00 (Erithacus Software Ltd) or Origin v. 6.0 (Microcal Software Inc.). Wavelengths of maximal absorption and absorbance values were estimated using UV WinLab v. 2.80.03 (Perkin-Elmer Corporation).

All solvents were distilled prior to use. Aqueous solutions were prepared from de-ionised water, doubly distilled in an all-quartz apparatus. Stock solutions of HCl, NaOH and NaCl were prepared by appropriate dilutions of Merck Titrisol concentrates in volumetric glassware. Sugar, sorbitol and PEG's were dried over P_2O_5 prior to use; formamide, *N*,*N*-dimethylacetamide and *N*-cyclohexyl pyrrolidin-2-one were distilled off CaO; urea was recrystallised from water–ethanol and dried over P_2O_5. Cyclopentadiene was prepared by thermal cracking of its dimer and used immediately. Other compounds were used as supplied. 4-Nitrophenyl dichloroacetate was prepared by the method of Fife and McMahon.[35] 2-(4-Nitrophenoxy)-tetrahydropyran was prepared by a procedure based on that of Woods and Kramer.[36]

Test solutions for $E_T(30)$, water activity and chemical reactivity measurements were prepared by mass and their density measured. Test solutions for enzyme studies were prepared by dilution of stock solutions to keep the concentrations of

enzyme, buffer and salts constant across the range of added solute concentration.

General method for the determination of $E_T(30)$

Two samples of test solution (2 × 2 cm^3) were preheated to 25 °C and a baseline wavelength scan was recorded.[37] Solutions of the $E_T(30)$ probe (10 μL, 7.0 mM in EtOH) and NaOH (10 μL, 2.00 M in water) were added to each and their UV-visible spectra recorded in triplicate at 240 nm per min with 6 nm smoothing and the $E_T(30)$ obtained using the equation:[8] $E_T(30) = 28591/\lambda_{max}(CT)$.

$E_T(30)$ probe-BSA binding experiment

$E_T(30)$ probe (10 μL, 7.0 mM in EtOH) and NaOH (10 μL, 1.00 M in water) were added to water (2.5 cm^3) and the solution preheated to 25 °C. BSA solution (105 g L^{-1} in water) was added in aliquots from a microlitre syringe and the UV-visible spectrum was recorded in triplicate after each addition. The $E_T(30)$ value was calculated as described above. The combined data from four titrations is presented in Fig. 3.

General method for chemical reactivity measurements

A stock solution of kinetic probe (*ca.* 4 × 10^{-3} M in acetonitrile) was prepared. Samples of test solution (2 cm^3) were pre-heated to 25 °C and the kinetic probe solution (5 μL) injected. An appropriate wavelength at which to follow the kinetics was selected by recording repetitive absorbance-wavelength scans at a number of time intervals until >95% completion of the probe reaction in a sample of test solution. A suitable wavelength is one at which there was a large change in absorbance over the course of the reaction in a relatively flat region of the absorbance-wavelength profile. (All repetitive absorbance-wavelength scans exhibited at least one isosbestic point). Absorbance-time data was then recorded at that wavelength and fitted to a first-order rate law.

Neutral hydrolysis of 4-nitrophenyl dichloroacetate

As described in the above general procedure, except 2.5 cm^3 of test solution was used and acidified with 5–30 μL of 0.1 M HCl. The increase in absorbance at 322.2 nm was followed for a minimum of six half-lives at a minimum of four different acid concentrations to verify that all measurements were taken in the region of neutral hydrolysis.

Acyl-transfer reaction between 4-nitrophenyl acetate and TRIS

As described in the above general procedure. Six different TRIS (50% free base) concentrations in the range 0.04–0.60 M were used (pH 7.7 ± 0.1). The ionic strength was not adjusted. The increase in absorbance at 400.0 nm was followed in triplicate for a minimum of six half-lives.

Hydronium-ion catalysed hydrolysis of 2-(4-nitrophenoxy)-tetrahydropyran

As described in the above general procedure. Five different acid concentrations in the range 0.01–0.10 M were used and the ionic strength adjusted to 0.10 M with NaCl. The increase in absorbance at 330.0 nm was followed in triplicate for a minimum of six half-lives.

Diels–Alder reaction between 1,4-naphthoquinone and cyclopentadiene

As described in the above general procedure. Test solutions contained 430 μM in cyclopentadiene. The decline in absorbance at 340.0 nm was followed in triplicate for a minimum of six half-lives.

Determination of kinetic parameters for the trypsin-catalysed hydrolysis of 4-nitrophenyl acetate

Accurate stock solutions of 4-nitrophenol and 4-nitrophenyl acetate were prepared (*ca.* 3 mg and *ca.* 7 mg in 3.00 cm^3 acetonitrile, respectively). An accurate stock solution containing trypsin (*ca.* 800 mg L^{-1}), $CaCl_2.2H_2O$ (10.0 g L^{-1}) and TRIS buffer solution (0.04 M, pH 7.7) was prepared and stored in the freezer. All trypsin solutions were used within two days of preparation. Test solutions were made by five-fold dilution of this stock. Two samples of test solution (2 × 2 cm^3) were preheated to 25 °C and a baseline UV-visible scan recorded. 4-Nitrophenol stock solution (10 μL) was injected, the solution was briefly sonicated to ensure mixing and the UV-visible spectrum recorded in triplicate. The wavelength of maximal absorbance of 4-nitrophenolate (between 400 and 410 nm) and the extinction co-efficient at that wavelength were determined. Four further samples of test solution (4 × 2 cm^3) were preheated to 25 °C and 4-nitrophenyl acetate stock solution (20, 40, 50 and 70 μL, respectively) injected into each. The solutions were briefly sonicated to ensure mixing and the absorbance was recorded for a minimum of 3600 s at the wavelength of maximal absorbance of 4-nitrophenolate in that test solution. Initial rates were determined by linear regression of the first 2000 s of absorbance-time data, converting to concentration units using the previously determined extinction co-efficient of the product.

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