THE ROYAL SOCIETY

Published online 7 July 2004

The use of gas-phase substrates to study enzyme catalysis at low hydration

Rachel V. Dunn and Roy M. Daniel*

Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton, New Zealand

Although there are varying estimates as to the degree of enzyme hydration required for activity, a threshold value of *ca*. 0.2 g of water per gram of protein has been widely accepted. The evidence upon which this is based is reviewed here. In particular, results from the use of gas-phase substrates are discussed. Results using solid-phase enzyme–substrate mixtures are not altogether in accord with those obtained using gas-phase substrates. The use of gaseous substrates and products provides an experimental system in which the hydration of the enzyme can be easily controlled, but which is not limited by diffusion.

All the results show that increasing hydration enhances activity. The results using gas-phase substrates do not support the existence of a critical hydration value below which enzymatic activity is absent, and suggest that enzyme activity is possible at much lower hydrations than previously thought; they do not support the notion that significant hydration of the surface polar groups is required for activity. However, the marked improvement of activity as hydration is increased suggests that water does play a role, perhaps in optimizing the structure or facilitating the flexibility required for maximal activity.

Keywords: protein hydration; gas-phase catalysis; water

1. INTRODUCTION

The effect of hydration on protein structure has been well documented, both in the literature and in some of the research presented at this discussion meeting. From a biochemical point of view, the correct structure of an enzyme can be simply defined as the conformation that enables the display of good catalytic activity by the enzyme; it is from this view that the effect of hydration on enzyme activity is considered. It is generally accepted that protein hydration is essential for enzyme catalysis to occur, and that dry enzymes are inactive. Although there are varying estimates of the degree of hydration required for activity, a threshold value of *ca*. 0.2 g of water per gram of protein, i.e. 0.2 *h*, is generally accepted (Careri *et al.* 1979; Rupley *et al.* 1983; Rupley & Careri 1991; Franks 1993; Finney 1996). We review the evidence on which this acceptance is based, and in particular new evidence from the use of gas-phase substrates, which suggests that enzyme activity is possible at lower hydrations.

2. PROTEIN HYDRATION

The hydration of proteins can be described by a sorption isotherm, which is simply a measure of the amount of water bound to a protein as a function of the partial pressure of water in the vapour phase at constant temperature. It is useful to note that RH and water activity (a_w) can be related by the following equation:

$$a_w = P/P_0 = \mathrm{RH}/100,$$

where P is the equilibrium vapour pressure of water in the system, and P_0 is the vapour pressure of pure water at the same temperature. The binding of water to proteins produces a sigmoidal sorption isotherm, as shown in figure 1 (Rupley & Careri 1991). The sigmoidal shape is due to water interacting with different groups on the protein surface. Below *ca*. 0.05–0.1 *h* (g water g^{-1} protein), the water interacts primarily with the ionizable groups on the protein. In the linear region, between 0.1 and 0.25 *h*, water binds to polar sites. The 'upswing' in the plot above 0.25 *h* is due to water condensing onto the weakest binding sites on the protein surface to complete the hydration process.

The hydration of proteins is expressed in a variety of ways. One of the commonest is '*h*', as used above, which represents grams of water per gram of protein. The effect of water on protein function is likely to depend upon the surface coverage of the protein, and the same value of *h* will give significantly different surface coverage of proteins of different sizes (table 1). Therefore, comparing the effect of the same *h* value upon the activity of enzymes of different molecular masses may be misleading. The use of mole ratios has similar problems. The presentation of protein hydration as surface coverage might seem to be a solution to this difficulty but is complicated by the fact that there is not a good consensus as to the area covered by a single water molecule (the figure of 15 Å^2 used in table 1 is an arbitrary compromise value), and the accessible surface area is not always known. However, if the protein is roughly spherical the use of the formula

$$A_s = 6.3(M_r)^{0.73}$$

is probably an acceptable approximation (Creighton 1993). Many proteins have *ca*. 50% of their surface occupied by polar groups, which are preferentially hydrated.

* Author for correspondence (r.daniel@waikato.ac.nz).

One contribution of 16 to a Discussion Meeting Issue 'The molecular basis of life: is life possible without water?'.

© 2004 The Royal Society

Table 1. Relationship between protein hydration and percentage surface coverage, compared with lysozyme[a].

	percentage surface coverage at:				
protein M_r (and accessible surface area[b], A_s)	$h = 0.02$ (H_2O : protein mole ratio)	$h = 0.10$ (H_2O : protein mole ratio)	$h = 0.20$ (H_2O : protein mole ratio)	$h = 0.4$ (H_2O : protein mole ratio)	$h = 0.6$ (H_2O : protein mole ratio)
5000 ($A_s = 2823$ Å^2)	3.0% (5.6)	15% (27.8)	31% (56)	59% (111)	89% (167)
14 250 (lysozyme) ($A_s = 5670$ Å^2)	4.1% (16)	21% (79)	41% (158)	84% (317)	126% (475)
30 000 ($A_s = 9299$ Å^2)	5.2% (33)	27% (167)	54% (333)	109% (667)	162% (1000)
60 000 ($A_s = 14\,799$ Å^2)	6.7% (66)	33% (333)	67% (667)	134% (1333)	202% (2000)

[a] The percentage coverage was calculated assuming the surface coverage of a water molecule of 15 Å^2. The mole ratio of water : protein is also given in parentheses.
[b] The accessible surface area was calculated taking the accessible surface area of lysozyme as 5670 Å^2 (Golton 1980), and assuming an identical accessible surface area/radius for the other proteins, and that they are spherical, monomeric, and with a volume approximately equal to $1.27(M_r)$ Å^3 Da^{-1} (Creighton 1993). The figures are thus indicative and comparative only.

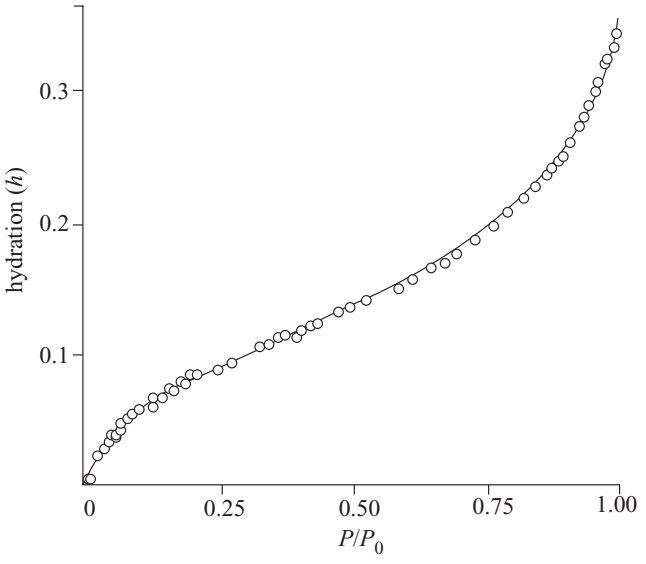

Figure 1. The D_2O sorption isotherm obtained for lysozyme at 27 °C as a function of RH (P/P_0). (Modified from Rupley & Careri (1991).)

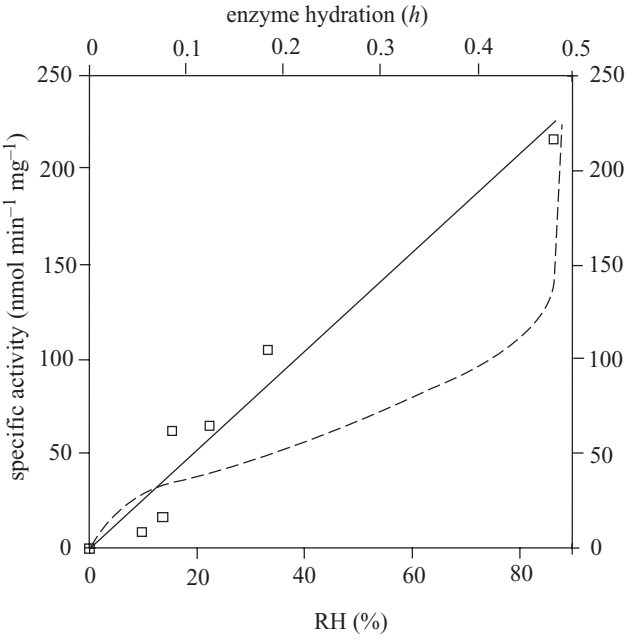

Figure 2. Effect of enzyme hydration on the vapour-phase hydrolysis activity of *Candida rugosa* lipase B against ethyl butyrate at 20 °C (open squares). The linear trend line fitted to the data has been redrawn against RH (dashed line). (R. V. Dunn, P. A. Lind, C. Monk and R. M. Daniel, unpublished results.)

For the most frequently studied protein, lysozyme, these groups will be hydrated when h is *ca*. 0.2–0.3 (Finney & Poole 1984; Rupley & Careri 1991), but for larger proteins coverage of these groups will occur at significantly lower hydrations.

Many experimental papers express enzyme hydration as a function of the RH, or water activity, to which the protein has been exposed. While all protein hydration isotherms have the same general shape (figure 1), different proteins may have somewhat different degrees of hydration when equilibrated at the same RH. When inspecting graphs where enzyme activity is plotted against RH (or a_w), rather than a direct hydration measurement such as h, it is important to interpret these bearing in mind that there is a relatively small increase in hydration as the RH changes from 15% to 5%, but the increase is much more marked as the RH increases from 70% to 100%, and any curve will be quite different in shape if h or percentage hydration is used as an axis instead of RH. An example of an extreme case is shown in figure 2. The data shown are the rate of hydrolysis of gas-phase ethyl butyrate obtained with *Candida rugosa* lipase B as a function of enzyme hydration. A linear trend line has been fitted to the data. When this is replotted against RH, a curved trend line is then obtained, as indicated in the figure. For cases in which a transition in activity against RH is observed, a decrease in the markedness of the transition is more generally seen when replotted against h.

3. ROLE OF WATER IN ENZYME FUNCTION

Water can be considered as fulfilling four possible roles in respect of enzyme function.

First, the water may be required for the chemical reaction itself. This may be as a component of the chemical reaction itself, for example with hydrolysis reactions, or it may be in respect of free-energy considerations of the specific enzyme reaction involved. One contribution to the free energy is the entropy change that occurs during the reaction. When a substrate binds to an enzyme, water may be excluded from the active site and transferred to the bulk solution. The substrate and enzyme may therefore be more ordered, whereas the released water molecules will be more disordered, and hence have greater entropy. If there is an overall increase in entropy, this will lead to a more favourable reaction (Kornblatt & Kornblatt 1997). This water is likely to be located at or near the active site, with potentially only small amounts of water involved; it may not be a factor in all enzyme reactions (Kornblatt & Kornblatt 1997; Pocker 2000).

Second, water may play an internal structural role. These water molecules are viewed as integral parts of the protein structure and provide specific structural interactions, for example, by forming bridging hydrogen bonds (Franks 1993; Gronenborn & Clore 1997). Water molecules are also located in internal cavities and deep clefts in the protein structure (Williams *et al.* 1994; Gronenborn & Clore 1997). These internal structural water molecules appear to be important in stabilizing the native conformation of the enzyme (Baker & Hubbard 1984; Meyer 1992). Structural water molecules have been found to be highly conserved (e.g. Meyer 1992; Nakasako *et al.* 1999; Babor *et al.* 2002). The number and location of structural water molecules vary from protein to protein, and constitute a small proportion of the total number of water molecules interacting with the protein in some way.

Third, surface 'structural' water that is directly interacting with the protein surface has been implicated in protein function. There is evidence that many enzymes become active only once a 'threshold' hydration of the enzyme surface has been reached, typically *ca.* 0.2 *h* (for lysozyme) or a mole ratio of water : protein of greater than 150. For most small globular proteins this leads to 30–50% coverage of the surface (table 1), and coverage of most of the polar groups on the protein surface.

Finally, it is obvious that, *in vivo*, solvent water provides a fluid medium for the diffusion of substrate and product.

Which of these 'categories' of water is essential, and in which might water be replaced by another molecule? The requirement for water in the first two categories given above is well established for certain reactions and for particular enzymes. The requirement for water as a diffusion medium is not absolute as, for example, when organic solvents or gaseous substrates and products are used.

We focus on the significance of external, i.e. surface, structural water on enzyme activity. Unlike reaction water and internal water, which are known not to be universally required, the requirement for surface water is thought to be universal, based largely on the finding that enzyme activity is apparently always 'switched on' at a particular hydration. The experimental systems that will be discussed here are those where an alternative diffusion medium was not used. This is in contrast to work where an alternative solvent was used to allow substrate and product diffusion, for example an organic solvent. The restriction to these experimental systems allows the effect of hydration to be studied without any potentially complicating factors that may arise due to an effect of the altered solvent medium on the enzyme.

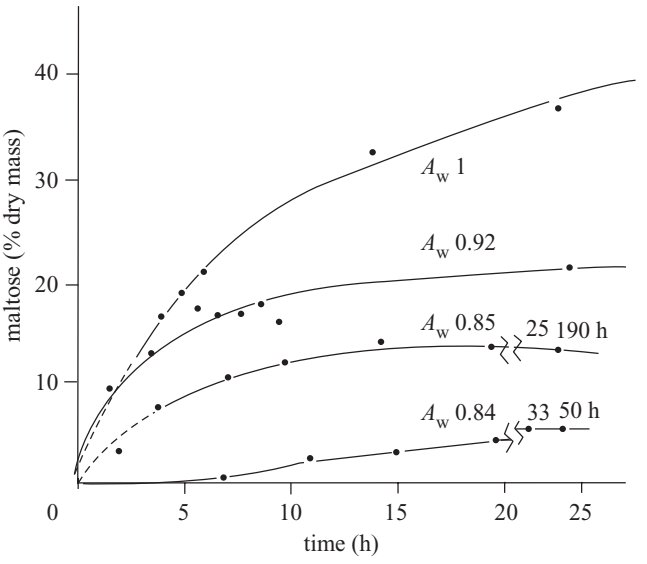

Figure 3. Extent of β-amylolysis in starch as a function of time and water activity at 30 °C. (Reproduced from Dapron (1985), fig. 4, with kind permission of Kluwer Academic Publishers.)

4. EFFECTS SEEN IN FOODS

Much of the early work on the activity of dry enzymes was motivated by food preservation concerns (Acker 1962). Many of the deleterious and also advantageous changes that occur in foods upon storage are the end result of the activity of 'contaminating' bacteria and enzymes. Although it is difficult to determine the exact hydration of the enzymes in foods, there seemed to be evidence that below a certain hydration level foods were 'safe', presumably due to the inactivity of the enzymes involved. However, it is not clear whether this is the result of a shut-off of enzyme activity below a particular hydration, or whether it is due to a decline in activity to a point where this is not significant during normal storage.

A good summary of the early work in food technology is given by Drapron (1985). Figure 3 shows the extent of amylolysis in starch as a function of time and water activity (Drapron 1985). The onset of enzymatic activity was seen to occur at an a_w of 0.65–0.7, which corresponds to a hydration of *ca.* 0.15 *h*. The rate of maltose formation is then seen to increase with an increase in water activity.

The effect of moisture content, or RH, on the activity of phospholipase in barley malt flour was investigated by pre-mixing the flour with the substrate lecithin (Acker 1962). The mixtures were then stored at specific RHs and the enzymatic reaction was followed by the amount of glycerol phosphocholine or choline released from the lecithin. The results of this study are shown in figure 4. The rate of enzymatic activity depends on the moisture content of the flour, increasing with increasing hydration. The onset

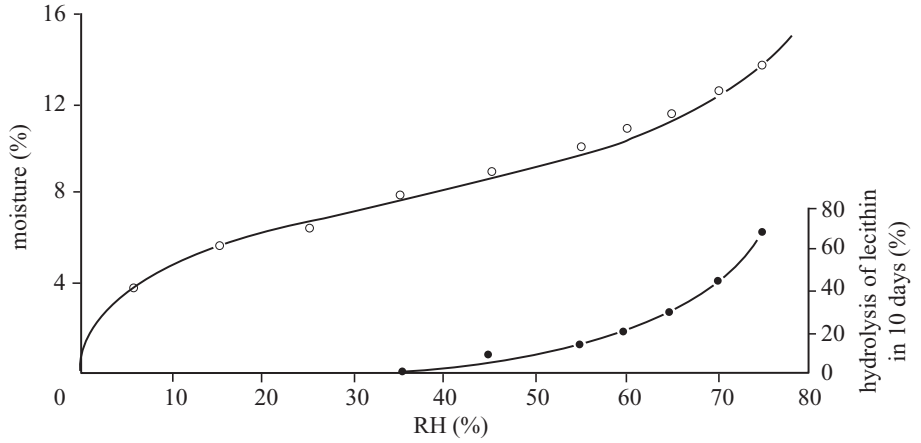

Figure 4. The sorption isotherm of a mixture of barley malt flour and lecithin (open circles) and its phospholipid-hydrolytic activity as a function of RH (filled circles). (Reprinted from Acker (1962) with permission from Elsevier.)

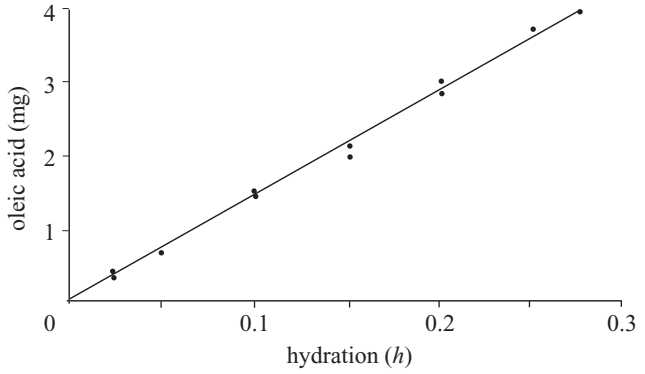

Figure 5. Effect of water activity on the lipolytic activity in olive oil as a function of water activity, expressed as milligrams of oleic acid formed from olive oil. (Reproduced from Dapron (1985), fig. 3, with kind permission of Kluwer Academic Publishers.)

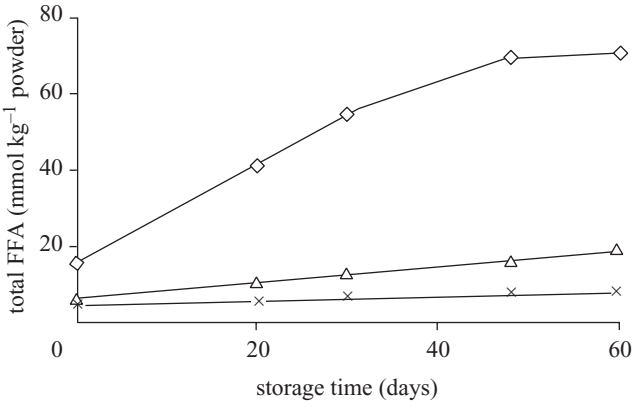

Figure 6. Effect of water activity on lipase activity in dry milk powders, expressed as the total FFAs released at 37 °C. The milk powders were: control (triangles); spiked with protease (crosses); spiked with lipase (diamonds) (Chen 2000).

of activity does not occur until a RH of 45%, corresponding to a moisture content of *ca*. 9%. This also occurs just before the 'upswing' region of the water sorption isotherm, also shown in figure 4. The 'upswing' region represents the condensation of water onto the surface of the material, and the availability of 'free' or solvent-like water. The activation of activity at this particular hydration has also been observed in a number of other food materials, including the amylolysis of starch as discussed above (Acker 1962; Drapron 1985).

A much lower hydration was found to be necessary for the onset of activity when the substrate was a liquid rather than a solid. The dependence of lipolysis in olive oil on water activity is shown in figure 5 (Drapron 1985). It was found that lipolysis can occur at an a_w of 0.025. As discussed by Drapron (1985) the increase in catalytic activity with the use of a liquid substrate may be due to a reduction in diffusion limitation of the substrate at low hydration.

Chen (2000) has investigated the storage properties of milk powders. The milk powders were spiked with lipase, and then freeze-dried or spray-dried. The enzymatic activity was followed by monitoring the release of FFAs during storage. It was found that in milk powders at hydrations of *ca*. 1% (corresponding to a maximum protein hydration of less than 2.5%) significant lipase activity was still detectable, as illustrated in figure 6. The chain length of the released fatty acids corresponded to the specificity of the added lipase (figure 7).

Although a substantial amount of work has been done with food products, the results are often difficult to interpret. This is due to the inhomogeneous nature of the materials concerned, and the uncertainty of the hydration of the different components.

5. SOLID-PHASE DRY ENZYME POWDER WORK

Coverage of early work on the effect of hydration on the activity of dry enzyme powders can be found in the review on protein hydration by Rupley & Careri (1991).

Skujins & McLaren (1967), in one of the earliest experiments with enzyme powders, looked at the effect of hydration on urease activity on ^{14}C-labelled urea. A well-mixed dry enzyme–substrate powder was achieved by mixing the substrate and enzyme in a cooled solution, followed by freeze-drying. After the powder had been equilibrated to a known hydration value, the evolution of $^{14}CO_2$ was monitored to detect enzymatic activity. It should be noted that as well as true catalytic turnover, the

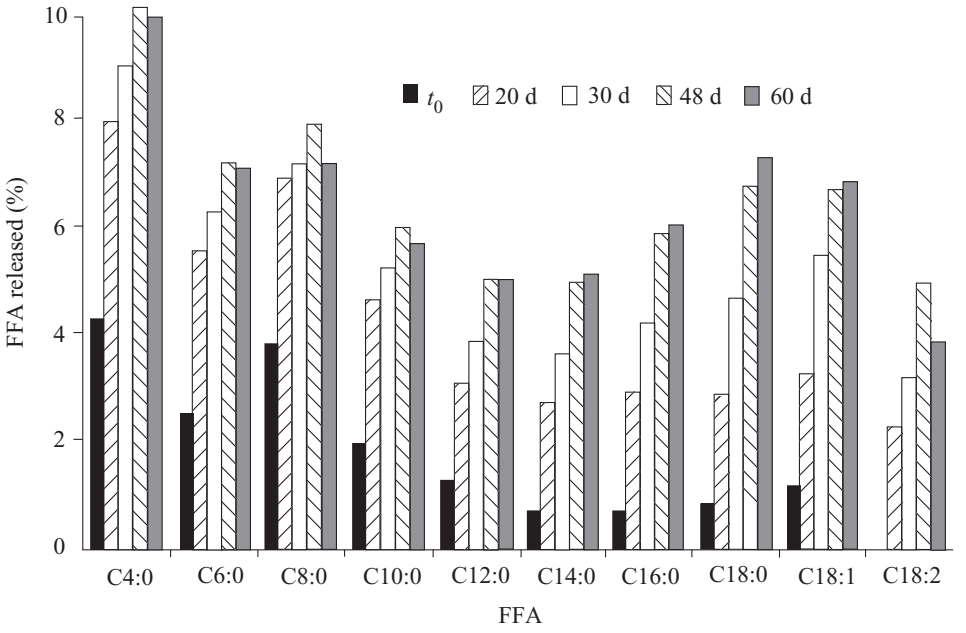

Figure 7. FFAs released in milk powder spiked with lipase during storage at 37 °C for up to 60 days. Each fatty acid is shown as the percentage of that acid available in the triacylglycerols in the powder (Chen 2000).

activity observed may simply be due to product release, since the enzyme–substrate complex (and concomitant enzyme–product complex) will form more rapidly than freezing can be accomplished. It was found that the onset of activity occurred at a RH of 60% ($h \approx 0.1$), and the rate of $^{14}CO_2$ release increased with increasing humidity. They explain their results in terms of the availability of water molecules to the active site of the enzyme, as a RH of 60% corresponds to a ratio of 1.3 mol of water per mol of polar side-chain groups. Therefore, only loosely bound water adsorbed in excess of the minimum amount required to hydrate the polar sites is able to take part in the hydrolysis reaction. However, the results are probably due to a mixture of effects including the effect on the K_m for water, and any other requirement of the enzyme for water.

Similar studies were performed by Khurgin *et al.* (1977), who investigated the effect of hydration on the rate of chymotrypsin acylation with the substrate *N*-succinyl-L-phenylalanine-*p*-nitroaniline. They determined that at a RH of 48% ($h \approx 0.12$; Lüscher-Mattli & Rüegg 1982) a critical hydration level was reached that enabled activity. However, the addition of an increasing amount of sodium acetate leads to a decrease in the critical hydration level, as shown in figure 8. This effect may have been due to the hydration of the salt, rather than a specific effect on the enzyme.

Stevens & Stevens (1979) investigated the hydration constraints in four different enzymes: fumarase, which catalyses a lyase reaction that is essentially a hydration reaction; phosphoglucose isomerase, which catalyses the isomerization of fructose 6-phosphate to glucose 6-phosphate; hexokinase, which catalyses a transferase reaction; and glucose 6-phosphate dehydrogenase, which is an oxidoreductase. The onset of activity for all these enzymes fell within the range of 0.1–0.2 *h*. However, even at quite high hydration levels only a small percentage of the fully hydrated rate was observed.

Rupley and colleagues have conducted similar experiments with lysozyme (Rupley *et al.* 1980, 1983; Careri *et al.* 1980). An equimolar amount of enzyme and substrate, the hexasaccharide of *N*-acetylglucosamine, was mixed under conditions of low activity and then freeze-dried. The pH of the solution was adjusted before freeze-drying, and the hydration of the resulting freeze-dried powder was set as required. The effect of hydration on activity for a range of initial solution pH values is shown in figure 9.

The data have been replotted in figure 10 with an arbitrary scale for enzymatic activity. Enzymatic activity exhibits two transitions, the first beginning at *ca.* 0.18 weight fraction of water, equivalent to 0.22 *h*. The beginning of this transition coincides with the last stage in the hydration process as seen by heat capacity measurements, or the completion of the monolayer of water on the surface by condensation of water over the weakest interacting groups. During this first transition there is a 15th-order dependence of the reaction rate on water, leading the authors to suggest that the increase in activity is therefore independent of the role of water as a reactant. With increasing hydration above 0.5 *h*, the activity is seen to increase to the dilute solution value.

Overall, all the experiments that have been done with dry enzyme powders show similar trends. An onset of enzymatic activity is seen to occur at a critical hydration level, often near 0.2 *h*, followed by a significant increase in activity with hydration above this point.

The concept of a hydration 'threshold' for enzymatic activity, suggested by the above results, is very attractive, as it appears to correlate the change in activity with the change in the physical properties of the hydrated enzyme powder (Rupley & Careri 1991). For example, some of the time-averaged physical properties of lysozyme that change with hydration are shown in figure 11 (Careri *et al.* 1980). Several properties show discontinuities at a low hydration of 0.07 *h*, which probably represent changes in the water–water and water–protein interactions as shown by an effect

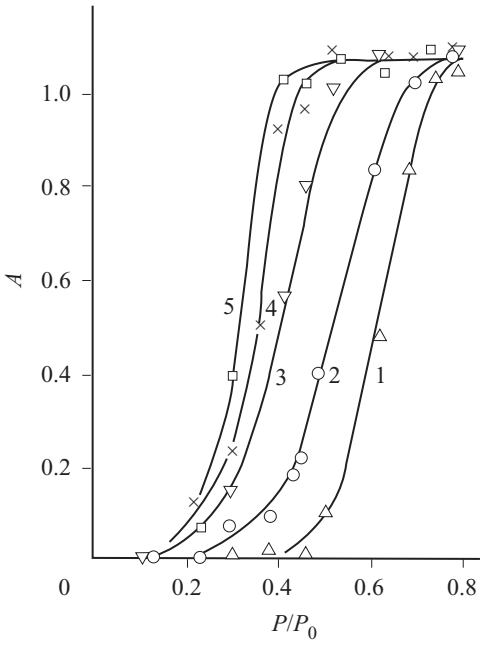

Figure 8. Effect of RH (P/P_0) on chymotrypsin acylation. The weight percentages of sodium acetate present in the powder were: 1, 0%; 2, 6.4%; 3, 12%; 4, 17%; and 5, 56.5% (Khurgin *et al.* 1977). The reaction is monitored by following the release of nitroaniline from the substrate *N*-succinyl-L-phenylalanine-*p*-nitroaniline, expressed as $A = A_{416}/A_{357}$. (Reprinted from Rupley & Careri (1991), with permission from Elsevier.)

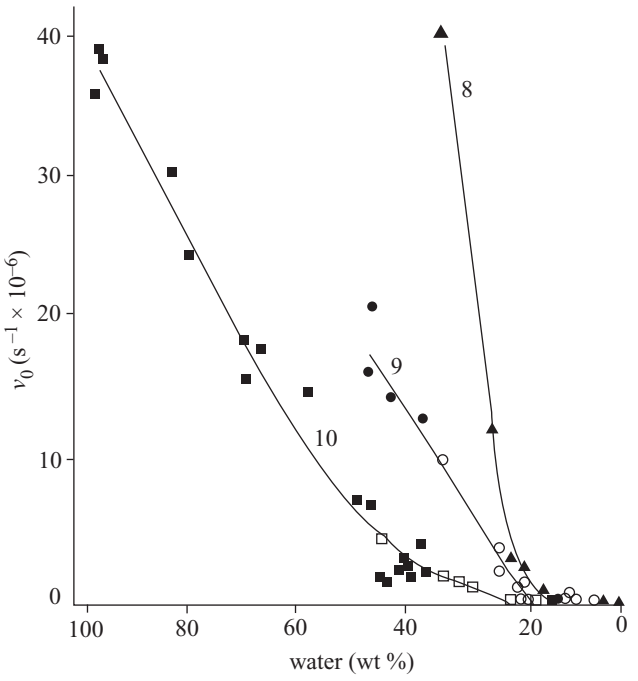

Figure 9. Enzymatic activity of lysozyme as a function of water content, at pH 8, 9 and 10. Open symbols: powders hydrated by isopiestic equilibration. Closed symbols: solvent added to powder. (Reprinted with permission from Rupley *et al.* (1980), American Chemical Society.)

on, for example, the IR spectroscopic properties of the water (figure 11*c*). At 0.2–0.25 *h* the carboxylate and carbonyl sites are fully saturated, as shown by the IR measurements (figure 11*a*,*b*). At this hydration there is also full coverage of the hydrogen-bonding sites as shown by the differential diamagnetic susceptibility measurements (figure 11*e*), which reach the value for liquid water at this hydration. Upon increasing hydration, from 0.25 to 0.38 *h*, the heat capacity is seen to rise and then fall to the dilute solution value (figure 11*d*). This behaviour has been suggested to be due to the coverage of the non-polar areas of the protein surface. Dynamic measurements of the protein surface have also been conducted, for example, spin-label studies of lysozyme powders containing Tempone. The electron spin resonance results showed changes with respect to hydration that agreed with the heat capacity and IR measurements (Rupley *et al.* 1980). The most significant change in motional properties coincided with the completion of amide hydration and the start of the condensation of water over the weakly interacting sites of the protein surface, as with the above time average results. A possibly relevant transition that occurs at a slightly lower hydration of 0.15 *h* is a transition in the water molecules themselves to become interconnected into one cluster that encompasses the whole protein surface (Careri 1999).

However, it may be that the results from this type of work are all influenced by the experimental difficulties associated with diffusional constraints. The fact that water is a reactant is an additional complication in many cases.

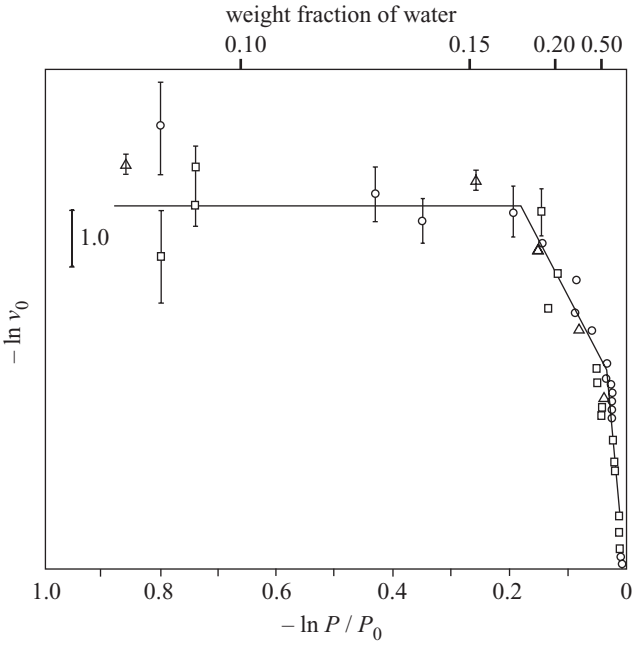

Figure 10. Logarithmic plot of data of figure 8, with arbitrary ordinate translations to bring curves for different pH into coincidence: pH 8 (triangles); pH 9 (circles); pH 10 (squares). (Reprinted with permission from Rupley *et al.* (1980), American Chemical Society.)

6. GAS-PHASE DRY ENZYME ACTIVITY RESULTS

Enzymes clearly have a requirement for a fluid medium in which substrate and product can diffuse to and from the enzyme. However, separating this functional role of water from any specific requirement for 'structural' water is not straightforward. There have been two solutions to

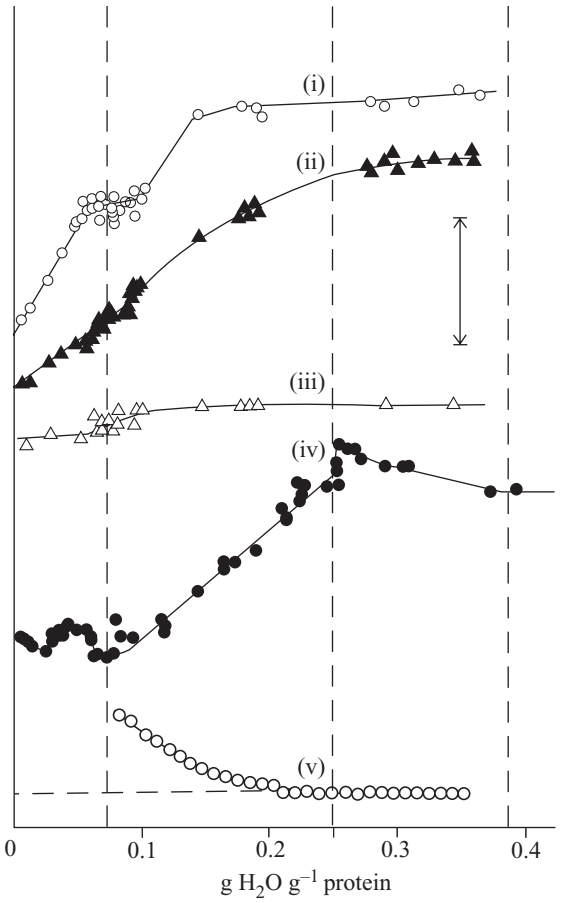

Figure 11. Effect of hydration on the properties of lysozyme. The curves are: (i) absorbance at the carboxylate band maximum (1580 cm^{-1}) at 37 °C; (ii) change in the amide I′ band at 38 °C; (iii) frequency of the highest intensity maximum of the OD stretching band of adsorbed D_2O at 38 °C; (iv) apparent specific heat capacity of lysozyme at 25 °C; (v) diamagnetic susceptibility measured at 25 °C. (Reprinted from Rupley *et al.* (1983), with permission from Elsevier.)

this problem, both as a result of the demands of biotechnology. The first is to replace water as a diffusion medium with the use of organic solvents. This approach has led to a greater understanding of the role of water and has shown that enzymatic activity is possible at very low hydrations (e.g. Valivety *et al.* 1992). However, it also has the potential drawback that the solvents themselves may be replacing water at sites on the enzyme surface, or distorting the protein structure.

The second approach, which avoids both the diffusional constraints and the potential complication of a new fluid constituent, is to study the enzymatic catalysis of gas-phase substrates. One of the earliest reports of this method was by Yagi *et al.* (1969), who investigated the activity of freeze-dried hydrogenase from *Desulfovibrio desulfuricans*. They observed that the dry enzyme still catalysed the conversion of *ortho*-H_2 to *para*-H_2. The difference between these two forms of molecular hydrogen is simply the orientation of the nuclear spins of the two protons; in *ortho*-H_2 the nuclear spins are parallel whereas in *para*-H_2 the nuclear spins are opposite.

Since that time, much work has been done using enzyme catalysis of gas-phase substrates, beginning in the late 1980s. Most work has been done using continuous-flow reactors, and with continued improvements in the control of RH (for reviews, see Lamare & Legoy 1993; Russell & Yang 1996). (Some work has also been done with immobilized whole cells, but this will not be dealt with here.)

The effect of hydration on the activity of alcohol oxidase adsorbed on DEAE-cellulose was investigated by Barzana *et al.* (1987), in a batch system. They showed that increasing the hydration of the enzyme preparation led to an increased rate of acetaldehyde production. For this experiment the enzyme was pre-hydrated at 20 °C to different water activities, by the use of saturated salt solutions, and then the gas-phase reactions were run at 50 °C. Thus, although the initial hydration of the samples was known, these may have changed when the temperature was raised. Owing to the nature of water binding, the protein is likely to have lost water at the higher temperature even if the RH was maintained, as at constant water proteins have been found to hold less water at higher temperatures (Lüscher-Mattli 1986; Bone 1996; Shamblin *et al.* 1998; Smith *et al.* 2002). In a later study with a similar reaction system, the hydration of the protein samples was more defined as the pre-hydration and gas-phase assays were performed at the same temperature (Barzana *et al.* 1989). They found that in the gas phase the activity of the immobilized enzyme increased by more than 10^4-fold as the water activity was increased from 0.11 to 0.84 (although the hydration of the enzyme itself is unknown). By comparing the activity obtained with enzyme adsorbed on DEAE-cellulose with that adsorbed on controlled pore glass, they were able to show that the enzyme was interacting by direct contact with vapour substrate molecules, rather than a condensed phase on the surface of the support or enzyme. Under optimal conditions, the rate of reaction in the gas phase was found to be comparable to that in solution. The use of immobilized alcohol oxidase in a continuous bioreactor system has also been reported (Hwang *et al.* 1993). Although several reaction parameters, such as temperature, were studied for their effect on reactor performance, the effect of hydration was not included.

Yang & Russell (1995, 1996) have studied the activity of alcohol dehydrogenase from baker's yeast in a continuous gas-phase reactor. They state that the enzyme required 0.16 *h* to become active over a temperature range of 22–50 °C. However, activity measurements made at hydrations as low as 0.05 *h* did show some activity. As data for a control reaction in the absence of enzyme are not shown, it is difficult to know if the small percentage conversion of substrate is due to enzymatic activity or not. There is a significant activation in activity at 0.16 *h*, but whether this level of hydration is an absolute requirement for activity is not clear. The authors calculated that this level of protein hydration corresponds to 46% surface coverage of the protein, or complete coverage of the hydrophilic regions of the molecule. They also noted significant hysteresis effects at RHs greater than 40%, which were also reflected in the activity results.

Much of the work on gas-phase substrates has been carried out on lipases and esterases, especially by the group of Legoy. The advantage of using esterases is that both substrates and products are gaseous, no cofactors are

required, and, using alcoholysis for example, no water need be involved in the reaction itself.

Porcine pancreatic lipase and *Fusarium solani* cutinase were used to catalyse transesterification reactions in a continuous gas–solid reactor (Parvaresh *et al.* 1992). The effect of the initial water activity on the rate of transesterification was studied by pre-equilibrating the enzyme preparation to a known hydration at 30 °C, and then monitoring the reaction rate at 65, 80 and 100 °C. As noted previously, there may be some uncertainty as to the exact hydration of the enzyme preparation during the gas-phase assays. The activity of porcine pancreatic lipase showed a maximum when previously equilibrated at a water activity of 0.29, decreasing at higher hydrations. The enzyme still displayed activity even when equilibrated at a water activity close to zero, by drying over P_2O_5. After 20 h of continuous use at 80 °C, the hydration of the porcine pancreatic lipase was only 0.002 *h*, after initial equilibration at a much higher hydration. Therefore, the lowest hydration at which transesterification was observed was probably at least equal to or below 0.002 *h*. The cutinase exhibited a completely different trend in behaviour, with a rather significant increase in rate and no apparent optimum in activity when the initial water activity was increased from 0.11 to 0.84, at 65 °C. The difference in behaviour was suggested to be due to the relative purities of the enzyme preparations: a crude porcine pancreatic lipase preparation as opposed to a pure cutinase preparation. The drop in activity seen at higher humidity with the crude enzyme preparation was suggested to be due to mass transfer limitations caused by the enzyme powder forming large clumps.

In a later study by Lamare & Legoy (1995), the effect of hydration on the rate of transesterification of free and immobilized cutinase and *Candida cylindracea* lipase was investigated. A continuous gas–solid system was used as in the earlier study by Parvaresh *et al.* (1992), but this time the activity of all components, including water, was controlled throughout the reaction. The rate of transesterification for cutinase and lipase adsorbed on Chromosorb P was shown to increase with the water activity of the system up to an a_w of 0.6 and 0.7, respectively. Above this hydration level, the rate was observed to decrease due to thermodenaturation of the enzyme samples. The effect of hydration on the activity of three different cutinase preparations was then investigated in more detail: free, adsorbed on Chromosorb P and covalently immobilized on a cation-exchange resin. The reaction rate was determined at 70 °C, whereas the sorption isotherms for each preparation were performed at 30 °C. However, the authors suggest the hydration state of the catalyst should be unchanged during the reaction unless a major structural change of the solid occurs. If any water is lost at the higher temperature, with constant RH, it is difficult to estimate. At $h = 0$, the rate was approximately zero for all three preparations, with activity continuously rising above zero hydration. However, there is no apparent 'critical' hydration after which activity is activated. For the free and adsorbed cutinase an optimum hydration was observed. An example of the results is shown in figure 12. The optimum occurs at the hydration just before the formation of free water, as determined from the sorption isotherm. However, no such optimum was seen with the immobilized cutinase preparation, as shown in figure 13. The water dependence appears to be biphasic, and follows the same trend as the sorption isotherm for the material. As noted by the authors, explaining this behaviour is difficult as the exact distribution of the water between the support and the enzyme is unknown.

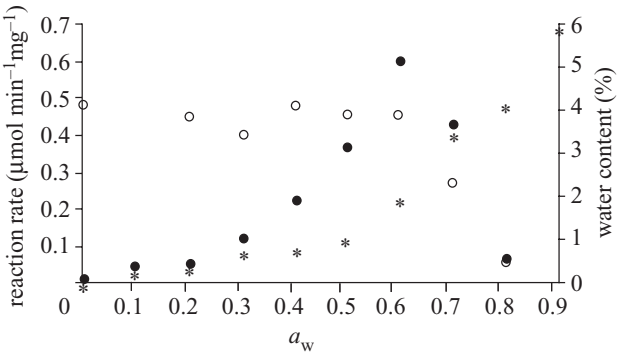

Figure 12. Effect of water activity on the transesterification reaction rate for cutinase adsorbed on Chromosorb P at 70 °C (filled circles). The residual activity after 24 h of continuous use at a particular a_w is plotted (open circles). The sorption isotherm determined at 30 °C is also shown (asterisks). (Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. (Lamare & Legoy 1995).)

Cameron *et al.* (2002) also studied the effect of water activity on the rate of transesterification by porcine pancreatic lipase. The enzyme was immobilized on glass wool that was then used in reactors of various configurations. The rate of transesterification between ethyl acetate and isoamyl alcohol was measured with a 'continuous-loop recycle' reactor. The enzyme showed the expected increase in thermostability at low hydrations, as well as an increase in activity as hydration increased. The pseudo-first-order rate constant for the reaction was found to increase from 0.26 to 1.97 l h^{-1} when the RH was increased from 0% to 75% at 25 °C. The water content of the immobilized material was found by Karl–Fischer titration to be 23 and 134 mg water g^{-1} packing at 0% and 75% RH, respectively. We have found that little water adsorbs on glass fibres at low humidity; however, even if it is assumed that most of the water is bound to the enzyme rather than the support, activity is observed at a maximum hydration of the protein of 2%. This is also in agreement with the results of Parvaresh *et al.* (1992) for the same enzyme discussed previously (see § 6).

The effect of water on the rate of alcoholysis catalysed by *Candida antarctica* lipase B adsorbed on silanized Chromosorb P was studied in a continuous solid–gas reactor by Bousquet-Dubouch *et al.* (2001). As with the study by Cameron *et al.* (2002), a significant amount of activity, determined from initial rate measurements, was seen at a RH equal to zero. The rate of reaction was then seen to decrease with increasing water activity, as shown in figure 14. It was proposed that the decreased rate was due to inhibition by water in competition with the alcohol n-propanol, for the acyl-enzyme intermediate. A later study by Graber *et al.* (2003) investigated the effect of water activity on the enthalpic and entropic contributions to the Gibbs free energy of activation (ΔG^*) in a solid–gas reactor with immobilized *Candida antarctica* lipase B. They found that

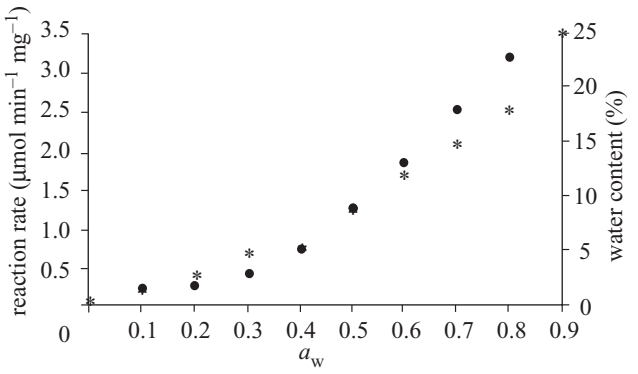

Figure 13. Effect of water activity on the transesterification rate for cutinase covalently immobilized on a cation-exchange resin at 70 °C (filled circles). The sorption isotherm determined at 30 °C is also shown (asterisks). (Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. (Lamare & Legoy 1995).)

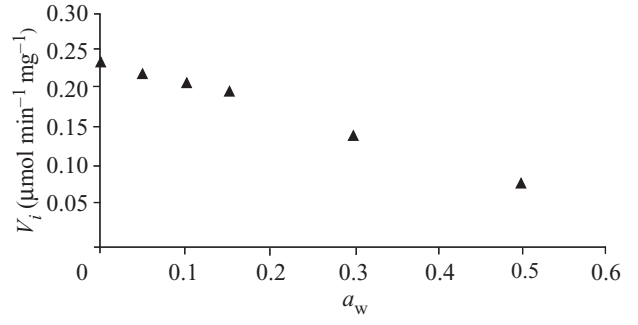

Figure 14. Effect of water activity on the initial rate of *Candida antarctica* lipase B adsorbed on silanized Chromosorb P at 70 °C. (Reprinted from Bousquet-Dubouch *et al.* (2001), with permission from Elsevier.)

with increasing water activity there was a decrease in the reaction energetic barrier, and a supposed increase in enzyme flexibility. It was suggested that increasing hydration led to the ground-state enzyme–substrate complex occupying a broader distribution of conformational states, and therefore a greater entropy compared with the less hydrated states. The resulting decrease in the entropy of activation (ΔS^*) with increasing hydration was determined to be the predominant contribution to the ΔG^* in the gas phase. This led to a decrease in ΔG^* with increasing hydration, which agreed with the kinetic results discussed above that showed a decrease in initial rate with increasing hydration (Bousquet-Dubouch *et al.* 2001).

In our laboratory, we have performed studies with similar enzymes, lipase B from *Candida rugosa* and pig liver esterase. Our experimental set-up was simpler than that mentioned above, and consisted of a batch-type reactor, as shown in figure 15. Typically, equilibration of the enzyme hydration was achieved by the use of an appropriate salt solution on one side of the reaction vessel, which was then isolated before the reaction was started. The effect of hydration on enzymatic activity was monitored by measuring the initial rate of ester hydrolysis. As it is a hydrolysis reaction, water will be consumed during the reaction; however, this was always a small fraction of the total water in the system. For example, at the lowest level at which activity was measured, the total water in the system was approximately distributed as follows: in the atmosphere, at RH 7.2%, 12.0 μg (670 nmol); bound to the 500 μg (8 nmol) pig liver esterase, 14.6 μg (800 nmol); consumed in the ester hydrolysis during the reaction, 0.02 μg (1 nmol). Therefore, even if all the water consumed during catalysis were removed from the enzyme surface, it would have a very small effect on the hydration of the enzyme.

The results obtained for lipase B are shown in figure 2. As can be seen, activity can be detected at even the lowest hydration investigated, which corresponds to 0.054 and 0.029 *h* for lipase B and pig liver esterase, respectively. The rate of hydrolysis increases with increasing hydration, with an approximately linear dependence for lipase B (R. V. Dunn, P. A. Lind, C. Monk and R. M. Daniel, unpublished results).

Dravis *et al.* (2000) used a similar batch gas-phase experimental set-up to study the effect of hydration on the activity of haloalkane dehalogenase. The results are shown in figure 16, and show an approximately linear dependence of rate on RH. The lack of an apparent critical hydration level for activity agrees with our results for lipase B given above.

7. CONCLUSIONS

Most of the results from gas-phase systems show that enzyme hydration enhances activity, but do not support the existence of any clear 'cut-off' hydration value below which activity declines dramatically. However, we need to note that very few enzyme 'types' have been examined, and there is at least one set of results that runs counter to this. So the main conclusions from gas-phase studies are as follows.

(i) Evidence for a 'threshold' hydration, or a very sharp increase in activity as hydration increases, is absent for most enzymes examined. Concomitantly, there is no support for the notion that extensive hydration of the surface polar groups, which cover approximately half the surface of most proteins, is required for activity.

(ii) There is evidence for activity at very low or zero hydration; this is in line with experiments carried out in dry organic solvents, i.e. in the other potentially anhydrous non-diffusion-limited system for examining dry enzyme activity (e.g. Zaks & Klibanov 1988; Valivety *et al.* 1992).

(iii) Activity increases with hydration. Thus, although a structure sufficiently close to the native structure to permit activity is possible at zero or very low hydration, the marked improvement of activity as hydration increases suggests that hydration may optimize structure and/or facilitate flexibility.

The experiments using gas-phase substrates help us to separate the effect of diffusional limitation from any requirement of the enzyme for water to maintain its function. However, they do not allow us to distinguish between the three probable types of requirement for water, namely as: (i) a direct or indirect reaction constituent (some enzymes); (ii) internal structural water (some enzymes); (iii) surface structural water. In particular, none of the

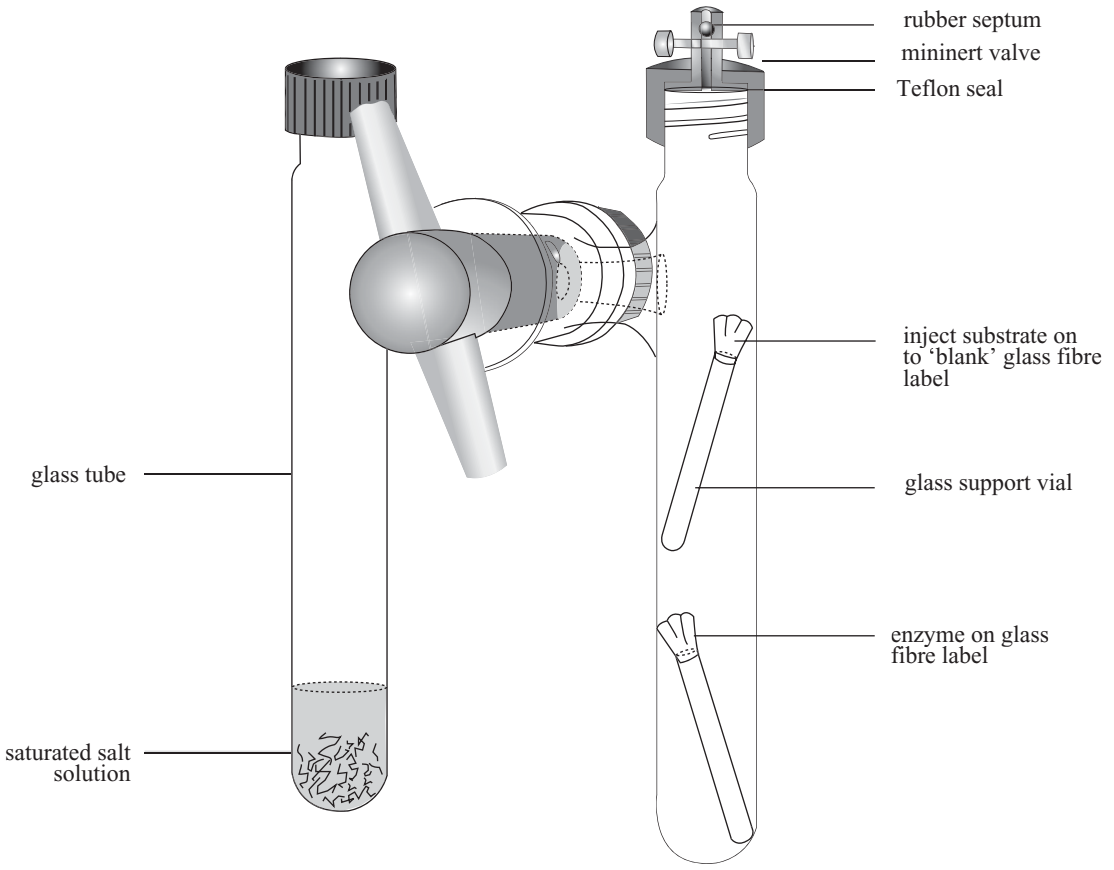

Figure 15. Experimental set-up for batch gas-phase *Candida rugosa* lipase B assays. The assay is started by the injection of the appropriate volatile substrate onto the upper glass fibre label. The saturated salt solution, used to equilibrate the enzyme to the required hydration, is isolated before starting the reaction by closing the connecting tap.

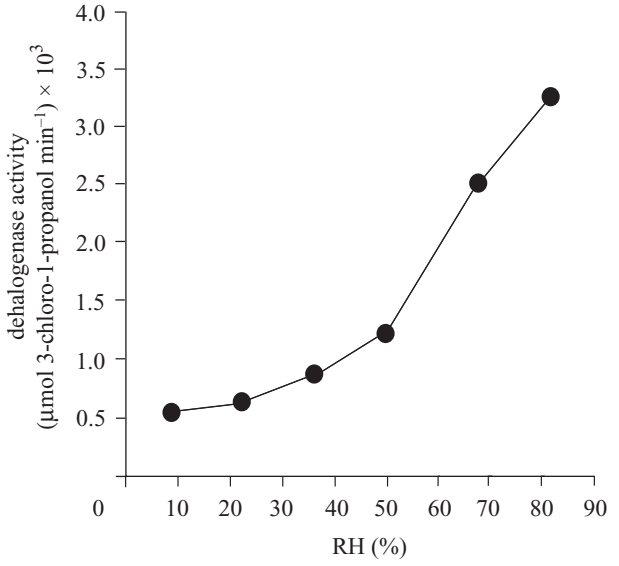

Figure 16. Dependence of haloalkane dehalogenase activity on RH. Reactions were conducted as 35 °C in a vapour phase saturated with the substrate, 1,3-dichloropropane. (Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. (Dravis *et al.* 2000).)

experiments carried out presents evidence for the absence of internal structural water, and the present state of our analytical techniques for water would make such evidence hard to obtain. In structural terms, therefore, a positive result for function at low hydration, such as has been found here for some enzymes, may be taken as good evidence only for the absence of a requirement for 'surface' structural water for enzyme activity. It may follow from this that activity is also independent of the dynamic motions that are observed only at higher hydrations (Schinkel *et al.* 1985; Rupley & Careri 1991). It is possible that the 'threshold' hydration for activity seen in some dry powder/solid substrate enzyme experiments is due to the easing of diffusional constraints occurring at the threshold hydration.

It is less clear that this relative independence of surface water for a functional enzyme structure is a general one. Most of the results that demonstrate this independence are from studies of lipases or esterases, partly because of the difficulty of finding other enzymes that are good subjects for this type of work.

The authors thank the Marsden Fund for financial support.

REFERENCES

Acker, L. 1962 Enzymic reactions in foods of low moisture content. *Adv. Food Res.* **11**, 263–330.

Babor, M., Sobolev, V. & Edelman, M. 2002 Conserved positions for ribose recognition: importance of water bridging interactions among ATP, ADP and FAD–protein complexes. *J. Mol. Biol.* **323**, 523–532.

Baker, E. & Hubbard, R. 1984 Hydrogen bonding in globular proteins. *Prog. Biophys. Mol. Biol.* **44**, 97–179.

Barzana, E., Klibanov, A. & Karel, M. 1987 Enzyme-catalyzed, gas-phase reactions. *Appl. Biochem. Biotechnol.* **15**, 25–34.

Barzana, E., Karel, M. & Klibanov, A. 1989 Enzymatic oxidation of ethanol in the gaseous phase. *Biotechnol. Bioengng* **34**, 1178–1185.

Bone, S. 1996 Dielecrric and gravimetric studies of water binding to lysozyme. *Phys. Med. Biol.* **41**, 1265–1275.

Bousquet-Dubouch, M.-P., Graber, M., Sousa, N., Lamare, S. & Legoy, M.-D. 2001 Alcoholysis catalyzed by *Candida antarctica* lipase B in a gas/solid system obeys a Ping Pong Bi Bi mechanism with competitive inhibition by the alcohol substrate and water. *Biochim. Biophys. Acta* **1550**, 90–99.

Cameron, P., Davison, B., Frymier, P. & Barton, J. 2002 Direct transesterification of gases by 'dry' immobilized lipase. *Biotechnol. Bioengng* **78**, 251–256.

Careri, G. 1999 Collective effects in hydrated proteins. In *Hydration processes in biology: theoretical and experimental approaches* (ed. M.-C. Bellissent-Funel), pp. 143–155. The Netherlands: IOS Press.

Careri, G., Giansanti, A. & Gratton, E. 1979 Lysozyme film hydration events: an IR and gravimetric study. *Biopolymers* **18**, 1187–1203.

Careri, G., Gratton, E., Yang, P.-H. & Rupley, J. 1980 Correlation of IR spectroscopic, heat capacity, diamagnetic susceptibility and enzymatic measurements on lysozyme powder. *Nature* **284**, 572–573.

Chen, L. 2000 Thermophilic enzymes and their impact on milk powders during storage. PhD thesis, University of Waikato, Hamilton.

Creighton, T. 1993 *Proteins—structures and molecular properties*. New York: Freeman.

Drapron, R. 1985 Enzyme activity as a function of water activity. In *Properties of water in foods* (ed. D. Simatos & J. Multon), pp. 171–190. Dordrecht, The Netherlands: Martinus Nijhoff Publishers.

Dravis, B., LeJeune, K., Hetro, A. & Russell, A. 2000 Enzymatic dehalogenation of gas phase substrates with haloalkane dehalogenase. *Biotechnol. Bioengng* **69**, 235–241.

Finney, J. 1996 Hydration processes in biological and macromolecular systems. *Faraday Discuss.* **103**, 1–18.

Finney, J. & Poole, P. 1984 Protein hydration and enzyme activity: the role of hydration-induced conformation and dynamic changes in the activity of lysozyme. *Comments Mol. Cell. Biophys.* **2**, 129–151.

Franks, F. 1993 Protein hydration. In *Protein biotechnology* (ed. F. Franks), pp. 437–465. Totawa, NJ: The Humana Press Inc.

Golton, I. 1980 *An experimental and theoretical study of the interaction between water and a globular protein*. University of London.

Graber, M., Bousquet-Dubouch, M.-P., Sousa, N., Lamare, S. & Legoy, M.-D. 2003 Water plays a different role on activation thermodynamic parameters of alcoholysis reaction catalyzed by lipase in gaseous and organic media. *Biochim. Biophys. Acta* **1645**, 56–62.

Gronenborn, A. & Clore, M. 1997 Water in and around proteins. *Biochemist* **19**, 18–21.

Hwang, S., Trantolo, D. & Wise, D. 1993 Gas phase acetaldehyde production in a continuous bioreactor. *Biotechnol. Bioengng* **42**, 667–673.

Khurgin, Y., Medvedeva, N. & Roslyakov, V. 1977 Solid-state enzymatic reactions. II. Chymotrypsin hydrolysis of N-succinyl-L-phenylalanine n-nitroanilide. *Biofizika* **22**, 1010–1014.

Kornblatt, J. & Kornblatt, J. 1997 The role of water recognition and catalysis by enzymes. *Biochemist* **19**, 14–17.

Lamare, S. & Legoy, M.-D. 1993 Biocatalysis in the gas phase. *Trends Biotechnol.* **11**, 413–418.

Lamare, S. & Legoy, M. 1995 Working at controlled water activity in a continuous process: the gas/solid system as a solution. *Biotechnol. Bioengng* **45**, 387–397.

Lüscher-Mattli, M. 1986 Thermodynamic parameters of biopolymer–water systems. In *Thermodynamic data for biochemistry and biotechnology* (ed. H.-J. Hinz), pp. 276–296. Berlin: Springer.

Lüscher-Mattli, M. & Rüegg, M. 1982 Thermodynamic function of biopolymer hydration. I. Their determination by vapor pressure studies, discussed in an analysis of the primary hydration process. *Biopolymers* **21**, 403–418.

Meyer, E. 1992 Internal water molecules and H-bonding in biological macromolecules: a review of structural features with functional implications. *Protein Sci.* **1**, 1543–1562.

Nakasako, M., Odaka, M., Yohda, M., Dohmae, N., Takio, K. N. & Endo, I. 1999 Tertiary and quarternary structures of photoreactive Fe-type nitrile hydratase from *Rhodococcus* sp. N-177: roles of hydration water molecules in stabilizing the structures and the structural origin of the substrate specificity of the enzyme. *Biochemistry* **38**, 9887–9898.

Parvaresh, F., Robert, H., Thomas, D. & Legoy, M.-D. 1992 Gas phase transesterification reactions catalyzed by lipolytic enzymes. *Biotechnol. Bioengng* **39**, 467–473.

Pocker, Y. 2000 Water in enzyme reactions: biophysical aspects of hydration-dehydration processes. *Cell. Mol. Life Sci.* **57**, 1008–1017.

Rupley, J. & Careri, G. 1991 Protein hydration and function. *Adv. Protein Chem.* **41**, 37–172.

Rupley, J., Yang, P.-H. & Tollin, G. 1980 Thermodynamic and related studies of water interacting with proteins. In *Water in polymers—ACS Symp. Ser. 127* (ed. S. Rowland), pp. 111–132. Washington, DC: American Chemical Society.

Rupley, J., Gratton, E. & Careri, G. 1983 Water and globular proteins. *Trends Biochem. Sci.* **8**, 18–22.

Russell, A. & Yang, F. 1996 Catalyse gas-phase reactions with enzymes. *CHEMTECH* **26**, 24–27.

Schinkel, J., Downer, N. & Rupley, J. 1985 Hydrogen exchange of lysozyme powders. Hydration dependence of internal motions. *Biochemistry* **24**, 352–366.

Shamblin, S., Hancock, B. & Zografi, G. 1998 Water vapor sorption by peptides, proteins and their formulations. *Eur. J. Pharm. Biopharm.* **45**, 239–247.

Skujins, J. & McLaren, A. 1967 Enzyme reaction rates at limited water activities. *Science* **158**, 1569–1570.

Smith, A., Shirazi, H. & Mulligan, S. 2002 Water sorption isotherms and enthalpies of water sorption by lysozyme using the quartz crystal microbalance/heat conduction calorimeter. *Biochim. Biophys. Acta* **1594**, 150–159.

Stevens, E. & Stevens, L. 1979 The effect of restricted hydration on the rate of reaction of glucose 6-phosphate dehydrogenase, phosphoglucose isomerase, hexokinase and fumarase. *Biochem. J.* **179**, 161–167.

Valivety, R., Halling, P. & Macrae, A. 1992 *Rhizomucor miehei* lipase remains highly active at water activity below 0.0001. *FEBS Lett.* **301**, 258–260.

Williams, M., Goodfellow, J. & Thornton, J. 1994 Buried waters and internal cavities in monomeric proteins. *Protein Sci.* **3**, 1224–1235.

Yagi, T., Tsuda, M., Mori, Y. & Inokuchi, H. 1969 Hydrogenase activity in the dry state. *J. Am. Chem. Soc.* **91**, 2801.

Yang, F. & Russell, A. 1995 The role of hydration in enzyme activity: 1. Water adsorption by alcohol dehydrogenase in a continuous gas phase reactor. *Biotechnol. Bioengng* **49**, 700–708.

Yang, F. & Russell, A. 1996 The role of hydration in enzyme activity and stability: 2. Alcohol dehydrogenase activity and stability in a continuous gas phase reactor. *Biotechnol. Bioengng* **49**, 709–716.

Zaks, A. & Klibanov, A. 1988 Enzymatic caalysis in nonaqueous solvents. *J. Biol. Chem.* **263**, 3194–3201.

Discussion

M. W. Ho (*Institute of Science in Society, PO Box 32097, London NW1 0XR, UK*). My comment applies to you and the previous speaker as well. It seems to me that a lot of effects of solvents and gas phase catalysis on enzymes may be confounded with effective local concentrations of substrates. A substrate that is water soluble will be greatly concentrated with the protein in organic solvent.

R. M. Daniel. This does not seem to be the case; several workers have determined K_m values for gas-phase substrates, and for substrates in organic solvents, and although the values often vary from those in aqueous solution, there is no evidence for a systematic decrease.

P. J. Halling (*Department of Chemistry, University of Strathclyde, Glasgow GL1 1XL, UK*). In discussing water effects we often forget how we would test any other species. You showed some examples of a low dependence of hydrolysis rates on water activity. For any other substrate we would explain this as a mass action effect when K_m is very high. Is there any reason to look for a different explanation just because it is water?

R. M. Daniel. I agree. Because water is involved as a reactant or in the energetics of so many reactions, and low hydration has therefore slowed reactions, this has lent unjustified weight to the contention that water is an essential surface structural component.

M. F. Chaplin (*Food Research Centre, London South Bank University, London SE1 0AA, UK*). Why is the surface structural water so discounted when it is clear that the rates of reaction depend upon it absolutely, in the same way as they depend on the enzyme concentration?

R. M. Daniel. First, I agree that in nearly all cases the reaction rate increases as the enzyme hydration increases. However, the evidence from both experimental systems that are not diffusion limited (i.e. gas-phase substrates and organic solvents) is very clear that appreciable enzyme rates are possible at very low hydrations—and in some cases the hydration is so low that we must doubt that the role of the water is as 'surface structural water'.

So while I agree we must not discount the role of surface water, and that reaction rates depend upon it, the evidence seems to be that this dependence is not universal, or absolute.

G. Zaccai (*Institut de Biologie Structurale, 38027 Grenoble Cedex 1, France*). Using pressure as a probe in the gas phase enzymology, you would not have to vary it by much; a factor of two would give effectively a factor of two in concentration.

R. M. Daniel. Yes. We have not done anything ourselves along these lines, but I suspect those working on applications in this area must have this in mind.

J. L. Finney (*Department of Physics and Astronomy, University College London, Gower Street, London WC1E 6BT, UK*). Looking at your linear lines of activity versus hydration, why should the activity go to zero at zero water? If it does, what does it tell us about the effects of the first few molecules of water?

R. M. Daniel. In our experimental system observing ester hydrolysis, it is because water is a reaction substrate. In some other experimental systems the reaction rate does not seem to be zero at zero hydration: but as Peter Halling has pointed out, our analytical methods for the determination of water are not very sensitive, so it is hard to be sure the hydration is really zero, and in many experiments the work has been applications-directed, and low or zero hydration data have not been very rigorously determined; so there is a tendency to conservatively assume that if there is a low enzyme rate at 'zero' hydration, this may be because the hydration is not in fact zero. So we have some difficulty in determining the effect of the 'first few' molecules of water. Nevertheless, it is clear that enzyme activity is perfectly possible at hydrations that are (conservatively) less than 1%, and represent surface coverages of less than 3%.

GLOSSARY

DEAE: diethylaminoethyl
FFA: free fatty acid
IR: infrared
M_r: relative molecular mass
RH: relative humidity