The Effect of Restricted Hydration on the Rate of Reaction of Glucose 6-Phosphate Dehydrogenase, Phosphoglucose Isomerase, Hexokinase and Fumarase

RELEVANCE FOR METABOLISM IN XERIC (NEAR-DRY) CONDITIONS

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A method is described for the measurement of enzyme activity under xeric conditions. The reaction mixtures had water contents ranging between 0.1 and 0.6 g/g of reaction mixture. For glucose 6-phosphate dehydrogenase, hexokinase and fumarase, enzyme activity became detectable (about 0.05 % of the fully hydrated rate) when the water content was about 0.2 g/g of reaction mixture, and for phosphoglucose isomerase, around 0.15 g/g of reaction mixture. With the water content raised to 0.3 g/g of reaction mixture the reaction rates were only increased to 0.1–3 % of the fully hydrated rate. When the combined rates for phosphoglucose isomerase and glucose 6-phosphate dehydrogenase were measured, reasonable agreement was found between the experimental data and those calculated from the individual experimentally determined rates on the assumption that diffusion was not further limiting. A method was devised for measuring the diffusion coefficients of low-molecular-weight substances in solutions having low water contents. The diffusion coefficients of riboflavin in sorbitol solution decreased by about 100-fold when the water content of the latter was reduced from 3 to 0.25 g/g of sorbitol. It is concluded that to detect enzyme activity a certain minimal amount of water is required and that above this minimum the rate is still restricted by diffusion limitation. The relevance of the results to the physical state of water in reaction mixtures and to metabolism in seeds and spores in xeric conditions is discussed.

Measurements of enzyme activity *in vitro* are usually made in the presence of excess solvent so that the enzyme protein itself is fully hydrated and the substrates, products and effectors can freely diffuse in the aqueous solvent. However, in many situations, for example, in fungal spores, in seeds in dry storage, in dehydrated foodstuffs, in membranes, and in concentrated protein solutions such as exist in the mitochondrial matrix, the total water content is reduced, or the water, although present, is unavailable to the enzymes concerned.

No attempt at a comprehensive study has been made to determine the effects of water limitation on enzyme reactions in general, although a few individual enzyme reactions have been studied in various ways (Linko & Milner, 1959; Acker, 1962; Skujins & McLaren, 1967; Potthast *et al.*, 1975; Stevens & Stevens, 1977). This is due, at least in part, to the practical difficulties entailed in such experiments. There are therefore at present insufficient data to make any quantitative generalizations about the effects of water limitation on enzyme activity.

As the usual methods used for measuring enzyme activity in aqueous solution are precluded for the

present purpose, we used a technique based on that devised by Skujins & McLaren (1967) to measure the effects of limiting the amounts of water on the rates of reaction of four different enzymes. This involves rapidly mixing and freezing the enzymes with their substrates in buffered solution, followed by freeze-drying. The dry reaction mixtures are allowed to take up measured amounts of water from saturated atmospheres, they are then sealed, and the subsequent reaction rate is measured. It is thus possible to ascertain what is the least water content required for an enzyme reaction to occur and above that limit the extent to which reaction is retarded by water restriction. With such knowledge a better understanding of the nature and rate of metabolic activity in various xeric situations, for example in dry resting seeds and fungal spores, should be gained. Such data are also relevant in an assessment of the importance of 'free' and 'bound' water (see Cooke & Kuntz, 1974) in enzyme reactions.

The four enzymes studied in this work, namely glucose 6-phosphate dehydrogenase (EC 1.1.1.49), phosphoglucose isomerase (EC 5.3.1.9), hexokinase (EC 2.7.1.1) and fumarase (EC 4.2.1.2), have been

chosen for the following reasons: (i) all are involved in glycolysis or the tricarboxylic acid cycle and thus indirectly in the provision of ATP, and so may be important in maintaining the very low metabolic rates in dry seeds and spores; (ii) each one represents a different class of enzyme: oxidoreductase, isomerase, transferase and lyase; (iii) three of the enzymes are metabolically linked, i.e. hexokinase, phosphoglucose isomerase and glucose 6-phosphate dehydrogenase, and so could be studied additionally as coupled enzymes; (iv) each is available commercially in purified state and the assays are straightforward.

Materials and Methods

Materials

NADP⁺, NADPH, yeast phosphoglucose isomerase and hexokinase were purchased from Boehringer, Lewes, Sussex, U.K. Tris, Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid), fructose 6-phosphate, L-malic acid, bovine serum albumin, ATP, freeze-dried glucose 6-phosphate dehydrogenase from *Torula* yeast, pig heart fumarase and bovine liver glutamate dehydrogenase were purchased from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K.

Method used for measurement of enzyme activities under conditions of water limitation

The appropriate reaction mixtures, lacking only either the enzyme or one of the substrates, were placed on ice in test tubes (120 mm length, 14 mm diameter). The composition of each reaction mixture is indicated below. The enzyme or substrate solution was added to each tube in turn, mixed for 2 s on a Whirlimixer and then frozen immediately in liquid N₂. The time from the addition of the last component of the reaction mixture until completion of freezing of the mixture was less than 15 s. The tubes were kept at -15°C until a batch could be freeze-dried overnight. After freeze-drying, each sample was rapidly transferred to preweighed weighing bottle (4.5 cm diameter) containing a glass spreader, and spread as evenly as possible over the bottom of the weighing bottle with the spreader. The weight of the transferred freeze-dried material was then determined by reweighing. Four weighing bottles, without lids, were then placed in a desiccator (15 cm diameter) containing 200 ml of water in the base. The samples were exposed to water vapour for periods of up to 3 h, although usually the desired water content was reached before 2 h. The water uptake was stopped by replacing the lid on the weighing bottle and the sample was reweighed to determine its water content. Reaction was then allowed to occur at a fixed water content for periods of 15 min to 24 h at room temperature (between 19 and 21°C). At appropriate intervals

reaction in a sample was stopped, and the amount of product was measured, thereby assessing the rate of reaction, and checking the linearity of the rate. A correction was made for any reaction that took place during the period of water uptake. Appropriate controls, including 'no-enzyme' controls, were also run in parallel with each experiment. The reaction mixtures and method of assay for each of the four enzymes are indicated below. One unit of each of the enzymes is defined as the amount that will convert 1 μ mol of substrate/min at 25°C.

(i) *Glucose 6-phosphate dehydrogenase*. The reaction mixture contained 1.75 μ mol of glucose 6-phosphate, 10 mg of albumin, 0.06 unit of glucose 6-phosphate dehydrogenase, 85 μ mol of Tes/HCl buffer, pH 7.6, in a total volume of 0.95 ml. The reaction mixture was completed by the addition of 0.05 ml of 22 mM-NADP⁺. After reaction had occurred, it was stopped by the addition of 1 ml of 1% (w/v) sodium dodecyl sulphate to the reaction mixture, and the A_{340} was measured in a 1 cm-path-length cuvette.

(ii) *Phosphoglucose isomerase*. The reaction mixture contained 1.5 μ mol of fructose 6-phosphate, 10 mg of albumin, 93 μ mol of Tes/HCl buffer, pH 7.6, in a total volume of 0.98 ml. The reaction mixture was completed by the addition of 0.02 ml of a solution containing 0.3 unit of phosphoglucose isomerase. After reaction had occurred, it was stopped by addition of 1 ml of 0.5 M-HClO₄. The ClO₄⁻ was removed by neutralization with K₂CO₃, and the glucose 6-phosphate formed was assayed by addition of glucose 6-phosphate dehydrogenase and NADP⁺.

(iii) *Hexokinase*. The reaction mixture contained 220 μ mol of glucose, 4.1 μ mol of ATP, 10 mg of albumin, 5 μ mol of MgCl₂, 74 μ mol of Tes/HCl buffer, pH 7.6, in a total volume of 0.95 ml. The reaction mixture was completed by the addition of 0.05 ml of a solution containing 0.06 unit of hexokinase. After reaction had occurred, it was stopped by the addition of 1 ml of 0.5 M-HClO₄, and the glucose 6-phosphate formed was measured as for the phosphoglucose isomerase reaction.

(iv) *Fumarase*. The reaction mixture contained 10 μ mol or 50 μ mol of malate, 10 mg of albumin, 100 μ mol of Tes/HCl buffer, pH 7.6, in a total volume of 0.95 ml. The reaction mixture was completed by addition of 20 μ l of a solution containing 1.1 units of fumarase. After reaction had occurred, it was stopped by addition of 1 ml of 0.5 M-HClO₄. Protein was removed by centrifugation (1000 g for 5 min) and the supernatant was neutralized with 5 M-NaOH, and A_{253} was measured in a 1 cm-path-length cuvette.

(v) *Combined phosphoglucose isomerase and glucose 6-phosphate dehydrogenase*. The reaction mixture contained 1.5 μ mol of fructose 6-phosphate, 1.1 μ mol of NADP⁺, 10 mg of albumin, 83 μ mol of Tes/HCl buffer,

pH 7.6, in a total volume of 0.93 ml. The reaction mixture was completed by addition of 0.07 ml of a solution containing 0.3 unit of phosphoglucose isomerase and 0.06 unit of glucose 6-phosphate dehydrogenase. After reaction had occurred, it was stopped by addition of 1 ml of 1% (w/v) sodium dodecyl sulphate solution to the reaction mixture, and A_{340} was measured in a 1 cm-path-length cuvette.

Measurement of diffusion coefficients of riboflavin and NADPH

This method was devised in order to measure the diffusion coefficients of low-molecular-weight substances in viscous aqueous solutions. Either riboflavin or NADPH was dissolved in sorbitol solutions of various concentrations. Two sorbitol solutions at a required concentration, one containing riboflavin or NADPH, sufficient to give an absorbance of about 1.0 when measured in a polyacrylamide-gel tube (75 mm length $\times$ 5 mm internal diameter) placed in the linear-transport-scanning attachment of a Gilford 240 spectrophotometer, and one lacking riboflavin or NADPH, were layered, one on top of the other in a polyacrylamide-gel tube by using a thin-walled, wide-bored (4.5 mm diameter) Pasteur pipette. To obtain a good interface between the two layers a slight difference (about 1%) in the sorbitol concentrations was necessary. With the more concentrated solutions (70%, w/w, and above) it was necessary to heat them to 60°C in order to fill the gel tube, and with the more dilute solutions (40%, w/w, and below) it was necessary to cool the solutions on ice or in a freezer. Return to room temperature was then rapidly achieved. Once the gel tubes had been filled they were scanned in the vertical plane at various time intervals up to 24 h. From each scan of absorbance against distance, the slopes at various parts of the concentration gradients were determined and graphs of dA/dx against x (distance) were plotted. From these, the areas under the curve were determined and corrected for any expansion of the x -scale used on the chart recorder. Evaluation of the diffusion coefficient was made as described by Bowen (1970). This method proved satisfactory for sorbitol solutions of between 25 and 80% (w/w).

Results

Method used to achieve defined degrees of hydration of reaction mixtures

The method frequently used to hydrate a protein to a defined extent is to place it in a closed vessel containing either an appropriate mixture of H_2SO_4 and water, or a saturated salt solution, and to allow equilibration to take place in an atmosphere of pre-determined relative humidity. This method has been used by various workers to determine the water-

absorption isotherms of proteins (Bull, 1944; Bull & Breese, 1968; Breuer & Kennerly, 1971; Hansen, 1976). The method that we adopted of allowing water uptake to occur at 100% relative humidity considerably shortens the time required to reach a given degree of hydration. A short uptake time is important to minimize the reaction that occurs before the defined water content is achieved. Rapid uptake of water is also aided by spreading the freeze-dried sample over a large surface area and this also ensures a uniform uptake of water. The rates of water uptake for the different reaction mixtures were similar; for example, 20% water content required about 20 min exposure at 100% relative humidity.

The enzyme reactions were measured in reaction mixtures containing albumin. This was added for two reasons: (i) it was found to stabilize enzymes against freeze-drying; for example, with all four enzymes used the loss of activity on freeze-drying mixtures containing albumin was never more than 10%; (ii) it reduced the specific activities of the enzymes to values more comparable with those present in seeds; for example, specific activities in barley embryos are: glucose 6-phosphate dehydrogenase 0.018 unit, phosphoglucose isomerase 0.13 unit and hexokinase 0.007 unit, each per mg of protein (Stevens & Stevens, 1976, 1977).

Glucose 6-phosphate dehydrogenase, phosphoglucose isomerase and combined phosphoglucose isomerase/glucose 6-phosphate dehydrogenase activities

In order to measure glucose 6-phosphate dehydrogenase activity in the presence of limited amounts of water, it is necessary to assay it discontinuously, and a suitable method for stopping the reaction was the addition of 1% (w/v) sodium dodecyl sulphate solution, after which A_{340} was measured. This avoids the problem arising from the instability of NADPH in acid solution, e.g. trichloroacetic acid or $HClO_4$. It is also necessary to measure phosphoglucose isomerase discontinuously, the glucose 6-phosphate formed being assayed with glucose 6-phosphate dehydrogenase. When measuring either glucose 6-phosphate dehydrogenase alone or combined with phosphoglucose isomerase under xeric conditions the choice of buffer is important. If controls comprising the freeze-dried reaction mixture but lacking enzyme were exposed to water vapour for the uptake of limited amounts of water, increases in the A_{340} in the region of the NADPH peak occurred. These increases were not due to NADPH production, since they could not be reversed by addition of NH_4Cl , 2-oxoglutarate and glutamate dehydrogenase. These were particularly marked when the buffer used was either triethanolamine/HCl or Tris/HCl, but were almost negligible if Tes buffer was used. The nature of these non-enzymic reactions is not clear, but

nicotinamide nucleotides are known to undergo addition reactions with a variety of reagents, giving rise to adducts having absorption spectra similar to those of reduced nicotinamide nucleotides (Kaplan, 1960). Tes buffer was adopted in the present work, and, by using 'no-enzyme' controls, satisfactory correction could be made for non-enzymic reactions. The results obtained for both glucose 6-phosphate dehydrogenase and phosphoglucose isomerase are shown in Fig. 1. Phosphoglucose isomerase activity was detectable in the presence of only 13% water, whereas, for glucose 6-phosphate dehydrogenase, activity was undetectable until the water content was around 22%. Both enzyme activities increased with increasing hydration of the reaction mixtures, so that both rates were approx. 1% of the fully hydrated rate when the water content reached 30%.

Having measured the rates of glucose 6-phosphate dehydrogenase and phosphoglucose isomerase activities under xeric conditions separately we measured

Fig. 1. Rate of reaction of glucose 6-phosphate dehydrogenase, phosphoglucose isomerase and hexokinase under xeric conditions

Glucose 6-phosphate dehydrogenase (●), phosphoglucose isomerase (■) and hexokinase (○) activities were measured as described in the Materials and Methods section.

$$\text{Water (\%)} = \frac{\text{weight of water}}{\text{weight of reaction mixture}} \times 100$$

Fig. 2. Comparison of the experimentally determined combined rate of reaction of phosphoglucose isomerase and glucose 6-phosphate dehydrogenase with the calculated combined rate

The experimentally determined rate (●) was measured as described in the Materials and Methods section. For the calculated combined rate (----) the experimentally determined rates for the individual reactions were used and calculated as described in the text. Water (%) was calculated as described in Fig. 1.

the combined activities of these enzymes, since, when considering whether a metabolic sequence of reactions can occur under xeric conditions *in vivo*, the rate of diffusion of metabolites from one enzyme to another may cause a further restriction on the overall rate. The combined enzymes were assayed under conditions similar to those used for the assay of glucose 6-phosphate dehydrogenase, phosphoglucose isomerase being present in approximately fivefold excess.

The data for the combined phosphoglucose isomerase/glucose 6-phosphate dehydrogenase reaction rates under conditions of water limitation are shown, together with the calculated combined rate, in Fig. 2. The calculated combined rate was determined as described by Webb (1963) for a partially reversible monolinear chain of a multienzyme system and assumes that diffusion steps are not rate-limiting:

Fructose 6-phosphate $\xrightleftharpoons{E_1}$ glucose 6-phosphate $\xrightarrow{E_2}$ 6-phosphogluconate
where E_1 is phosphoglucose isomerase and E_2 is glucose 6-phosphate dehydrogenase.

$$v_1 = \frac{V_1 K_{\text{Glc-6-P}} [\text{Fru-6-P}] - V_{-1} K_{\text{Fru-6-P}} [\text{Glc-6-P}]}{K_{\text{Fru-6-P}} K_{\text{Glc-6-P}} + K_{\text{Glc-6-P}} [\text{Fru-6-P}] + K_{\text{Fru-6-P}} [\text{Glc-6-P}]} \quad (1)$$

$$v_2 = \frac{V_2 [\text{Glc-6-P}]}{[\text{Glc-6-P}] + K_{\text{Glc-6-P}}} \quad (2)$$

where K is Michaelis constants for the appropriate forward and reverse reactions, V_1 and V_{-1} are the maximum velocities for phosphoglucose isomerase in the forward and reverse directions, and Glc-6-P and Fru-6-P are glucose 6-phosphate and fructose 6-phosphate respectively. Solution of eqns. (1) and (2) gives a quadratic expression for [Glc-6-P]. Knowing [Glc-6-P], the rate of formation of 6-phosphogluconate, i.e. the combined rate, may be calculated from eqn. (2) (Fig. 2). The Michaelis constants used in the calculation were: $K_{\text{Fru-6-P}}$, $0.11 \mu\text{M}$, $K_{\text{Glc-6-P}}$, $0.8 \mu\text{M}$ (Wurster & Schneider, 1970), $K_{\text{Glc-6-P}}$, $0.27 \mu\text{M}$ (Domagk *et al.*, 1969). V_{-1} was calculated from the Haldane relationship.

The calculated combined rates and the experimentally determined rates are in sufficiently close agreement to suggest that diffusion between the two enzymes does not further restrict the overall rate, particularly since, at the lower water contents, where diffusion would be slowest, the calculated rate appears less than the measured rate.

Hexokinase

Hexokinase was assayed by coupling it to glucose 6-phosphate dehydrogenase, though, as with phosphoglucose isomerase, it is necessary to assay discontinuously for the glucose 6-phosphate formed. The results (Fig. 1) are similar to those obtained for glucose 6-phosphate dehydrogenase. In both cases activity is first detectable when the water content is about 20% and both reach approx. 5% of their fully hydrated rates when the water contents lie between 35 and 40%.

Fumarase

Fumarase activity was studied under xeric conditions for two reasons: (i) when the reaction is measured in the direction of malate dehydration it is a single-substrate enzyme and therefore could be compared with the other single-substrate enzyme previously studied, i.e. phosphoglucose isomerase; (ii) since it is a dehydration reaction, it might be of particular interest to study the enzyme under xeric conditions. Its activity was measured at two malate concentrations, namely 0.05 and 0.01 M, these being the concentrations before the reaction mixtures were freeze-dried. Activity was detectable at 20% water content, but when compared with the other three enzymes the activities were much lower by comparison with the fully hydrated rates (Fig. 3). For example, 0.5% of the fully hydrated rate is not achieved in reaction mixtures containing 0.05 M-malate until the water content is between 40 and 50%. The reason for this difference is almost certainly that fumarase exhibits unusual kinetics and is inhibited by malate concentrations greater than 0.1 M (Alberty *et al.*, 1954; Rajender & McCulloch, 1967). Although the initial

Fig. 3. Rate of reaction of fumarase under xeric conditions. Fumarase activities were measured with 0.05 M-malate (●) and 0.01 M-malate (○) as substrate, as described in the Materials and Methods section. Water (%) was calculated as in Fig. 1.

Fig. 4. Diffusion coefficients of riboflavin and NADPH in sorbitol solutions.

The diffusion coefficients of riboflavin (●) and NADPH (■) were measured in sorbitol solutions as described in the Materials and Methods section. Water (%) was calculated as in Fig. 1, and sorbitol (%) was calculated similarly.

malate concentrations used in this experiment were less than 0.1 M, after freeze-drying followed by water uptake from moist air the malate concentration would undoubtedly be much higher. The lower rate of activity obtained with the higher malate concentration

suggests that inhibition occurs and that this explains the lower rate of activity shown by fumarase as compared with the other three enzymes.

Diffusion of substrates

In xeric conditions, in addition to the possibility of the rates of enzyme catalysis at the active site being reduced, there may also be a further reduction in metabolism because of a lower rate of diffusion of metabolites to and from the active site of an enzyme or from one enzyme to another. We therefore attempted to make approximate measurements of diffusion coefficients in solutions that had water contents comparable with those used to measure enzyme activity under xeric conditions. The diffusion coefficient of riboflavin was measured, since its molecular weight is comparable with that of the substrates used and since the establishment of a satisfactory interface could be visually assessed. Sorbitol was used as the solute, since it is very soluble and has negligible absorption above 260 nm.

The diffusion coefficients of riboflavin were measured in sorbitol solutions having water contents ranging from 25 g/100 g of sorbitol to 300 g/100 g of sorbitol and were found to range from $0.77 \times 10^{-8} \text{ cm}^2/\text{s}$ to $5.29 \times 10^{-6} \text{ cm}^2/\text{s}$ (Fig. 4). With our method we were not able to measure diffusion below 25 g of water/100 g of sorbitol. The higher value of $5.29 \times 10^{-6} \text{ cm}^2/\text{s}$ obtained is in the range expected for a molecule of mol.wt. 500 diffusing in water (Webb, 1963). By reducing the water content to 25 g/100 g of sorbitol, the diffusion coefficient for riboflavin is reduced by two orders of magnitude. This would have the effect of increasing the transit time for diffusion between enzymes by 100-fold.

Discussion

Although it is generally accepted, *a priori*, that water is essential for enzyme reactions to occur, no

systematic study has been made on the effects of water limitation on enzyme reactions. Our results, based on studying four different enzymes, show that, when the water content is raised to approx. 20%, all show detectable activity. Activity increases by approximately an order of magnitude when the water content is raised to 30%.

A number of studies have been made by other workers (Linko & Milner, 1959; Skujins & McLaren, 1967; Wilson & Harris, 1968; Edwards, 1976) on enzyme activity or metabolism in xeric conditions, but it is difficult to make direct comparisons, since different experimental conditions have been used, and these will affect the results. For example, using our model system we showed that both the lowest detectable water content at which glucose 6-phosphate dehydrogenation occurred, and the rate of reaction at a given water content, depended on the proportion of albumin in the reaction mixture (Stevens & Stevens, 1977).

The physical state of water in protein solutions may be characterized as: (i) Type I 'bulk water', the properties of which are not appreciably changed by binding to macromolecules; (ii) Type II 'bound water', in which the rotations are hindered significantly by interaction with macromolecular surfaces; (iii) Type III 'irrotationally bound water' which is 'site-bound' to macromolecules for periods of micro-seconds (see Cooke & Kuntz, 1974). Type III may only constitute about 0.003 g of water/g of protein, whereas Type II constitutes from 0.3 to 0.6 g of water/g of protein, depending on the particular protein. Our data suggest that no enzyme activity is detectable in the presence of Type III water only, but becomes detectable in the presence of Type II. The latter is presumably sufficiently mobile to allow the movement of substrates and products and to allow the establishment of a normal environment about the active site. The accelerated increases in the rates

Table 1. Comparison of the enzyme activity ratios at different water contents with diffusion-coefficient ratios for riboflavin at different water contents

The activities of glucose 6-phosphate dehydrogenase, phosphoglucose isomerase, hexokinase and fumarase are expressed as the ratios of their rates at different water contents. The diffusion coefficients of riboflavin measured in sorbitol solutions of different water contents are also expressed as a comparable ratio.

Parameter ratio	Ratio of enzyme activities				Ratio of diffusion coefficient of riboflavin in sorbitol
	Glucose 6-phosphate dehydrogenase	Phosphoglucose isomerase	Hexokinase	Fumarase	
30% water					
25% water	3.3	1.3	2.1	2.1	3.5
35% water					
30% water	3.2	—	1.8	1.6	1.8
40% water					
35% water	1.4	—	—	1.5	1.6

of reaction that occurred when the water content was raised between 20 and 40% are probably largely due to the increased rate of diffusion of substrates and products to and from the active sites. In Table 1 the ratios of the reaction rates at different water contents are compared with the ratios of the diffusion coefficients of riboflavin in sorbitol, and it can be seen that both increase by similar amounts, thus supporting the above view.

When the experimentally determined coupled rate of the phosphoglucose isomerase and glucose 6-phosphate dehydrogenase reaction was compared with the combined rate, calculated on the assumption that diffusion between the two enzymes was not rate-limiting (Fig. 2), the data agreed sufficiently well to suggest that diffusion between the enzymes was not, in fact, rate-limiting. The most likely explanation of these findings is, we believe, that the rate of diffusion of metabolites between the two enzymes does not restrict the overall rate further than it is already being restricted by the rate of diffusion of substrates and products to and from the individual enzymes.

Although the trends observed with each enzyme studied show a common pattern, there are individual differences, but with this limited study it is too early to say what the differences are due to. A more extensive study with further related enzymes will be necessary before a general pattern will emerge.

Such studies probably have practical application. Air-dry seeds and fungal spores have a very low rate of metabolism. This is undoubtedly in part due to low water contents [in the range of 5–25% for air-dry seeds and fungal spores (Roberts & Roberts, 1972; Gottlieb, 1978)]. This being so, comparison with the results described in the present paper suggests that enzyme activity in air-dry seeds and fungal spores is very low indeed, especially when the water content is below 20%: when the water content rises to between 20 and 30%, the rate of enzymic reaction may appreciably increase to around 1% of the fully hydrated rate. Such considerations obviously have a bearing on storage conditions of seeds used by man.

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References

- Acker, L. (1962) *Adv. Food Res.* **11**, 263–330
- Alberty, R. A., Massey, V., Frieden, C. & Fuhlbrigge, A. R. (1954) *J. Am. Chem. Soc.* **76**, 2485–2493
- Bowen, T. J. (1970) *An Introduction to Ultracentrifugation*, pp. 44–46, Wiley-Interscience, London
- Breuer, M. M. & Kennerly, M. G. (1971) *J. Colloid Interface Sci.* **37**, 124–131
- Bull, H. B. (1944) *J. Am. Chem. Soc.* **66**, 1499–1507
- Bull, H. B. & Breese, K. (1968) *Arch. Biochem. Biophys.* **128**, 488–496
- Cooke, R. & Kuntz, I. D. (1974) *Annu. Rev. Biophys. Bioeng.* **3**, 95–126
- Domagk, G. F., Chilla, R., Domschke, W., Engel, H. J. & Sørensen, N. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 626
- Edwards, M. (1976) *Plant Physiol.* **58**, 237–239
- Goettlieb, D. (1978) *The Germination of Fungus Spores*, p. 20, Meadowfield Press, Co. Durham
- Hansen, J. R. (1976) *Agric. Food Chem.* **24**, 1135–1141
- Kaplan, N. O. (1960) *Enzymes 2nd Ed.* **3B**, 105–169
- Linko, P. & Milner, M. (1959) *Plant Physiol.* **34**, 392–396
- Pothast, K., Hamm, R. & Acker, L. (1975) in *Water Relations of Foods* (Duckworth, R. B., ed.), pp. 365–377, Academic Press, London and New York
- Rajender, S. & McCulloch, R. J. (1967) *Arch. Biochem. Biophys.* **118**, 279–283
- Roberts, E. H. & Roberts, D. L. (1972) in *Viability of Seeds* (Roberts, E. H., ed.), pp. 424–437, Chapman and Hall, London
- Skujins, J. J. & McLaren, A. D. (1967) *Science* **158**, 1569–1570
- Stevens, E. & Stevens, L. (1976) *Biochem. Soc. Trans.* **4**, 1109–1110
- Stevens, E. & Stevens, L. (1977) *J. Exp. Bot.* **28**, 292–303
- Webb, J. L. (1963) *Enzyme and Metabolic Inhibitors*, vol. 1, chapter 7, Academic Press, London and New York
- Wilson, A. M. & Harris, G. A. (1968) *Plant Physiol.* **43**, 61–65
- Wurster, B. & Schneider, F. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 961–966