Biochimica et Biophysica Acta, 410 (1975) 135—144

BBA 67638

BEHAVIOR OF SOLUBLE AND IMMOBILIZED ACID PHOSPHATASE IN HYDRO-ORGANIC MEDIA

HANNAH WAN and CSABA HORVATH

Bioengineering Group, Department of Engineering and Applied Science, Yale University, New Haven, Conn. 06520 (U.S.A.)

(Received May 20th, 1975)

Summary

The hydrolysis of *p*-nitrophenyl phosphate by wheat germ acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) has been investigated in mixtures of aqueous buffers with acetone, dioxane and acetonitrile. The enzyme was either in free solution or immobilized on a pellicular support which consisted of a porous carbonaceous layer on solid glass beads. The highest enzyme activity was obtained in acetone and acetonitrile mixed with citrate buffer over a wide range of organic solvent concentration. In 50% (v/v) acetone both V and K_m of the immobilized enzyme were about half of the values in the neat aqueous buffer, but the K_i for inorganic phosphate was unchanged. In 50% (v/v) mixtures of various solvents and citrate buffers of different pH, the enzymic activity was found to depend on the pH of the aqueous buffer component rather than the pH of the hydro-organic mixture as measured with the glass-calomel electrode. The relatively high rates of *p*-nitrophenol liberation in the presence of glucose even at high organic solvent concentrations suggest that transphosphorylation is facilitated at low water activity.

Introduction

The study of enzyme behavior in hydro-organic media is of interest for several reasons. For instance, further understanding of enzyme action can be gained from experiments in aqueous organic solvents [1—7] or at subzero temperatures, which necessitate the use of a hydro-organic medium [8—10]. On the other hand, there is substantial evidence that membrane-bound enzymes are embedded in a lipophilic microenvironment, which gives rise to an enzyme behavior quite different from that in aqueous buffers usually employed to study enzymic reactions in free solution [11]. Consequently, investigations with enzymes acting in hydro-organic media or in a matrix containing hydro-

phobic moieties can be useful to infer the behavior of enzymes in their natural environment.

Enzymic reactions in predominantly organic media could also expand the scope of enzyme catalysis in practical applications when the substrate is sparingly soluble in aqueous buffers and/or the enzyme specificity or the equilibrium point of the enzymic reaction can be shifted in the desired direction. Thus, it is conceivable that hydrolytic enzymes could be used for synthesis in a reaction medium of low water activity in view of the well established potential of certain hydrolases for transpeptidation and transesterification even in aqueous solution [12—14].

In the past most studies were made at relatively low concentrations of the organic solvent in the reaction mixture because of the low solubility of proteins in organic solvents and the denaturing effect of the medium. Recent works demonstrate, however, that the immobilization of enzymes on a suitable matrix not only provides a recoverable reagent of relatively high stability but also makes it possible to carry out enzymic reactions in contact with solutions of high organic solvent concentration [7]. A rigid porous support such as porous glass [15] or a pellicular material [16] are the preferred carriers for the immobilized enzyme under these conditions because the interior of hydrophilic gels such as agarose and cross-linked dextran, which have a high avidity for water, is less accessible to the organic solvent.

In this study the decomposition of *p*-nitrophenyl phosphate by wheat germ acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) [17,18] in various hydro-organic mixtures is investigated. The enzyme was used either in free solution or in immobilized form on a porous carbonaceous pellicular support.

Experimental

Materials and Equipment

Wheat germ acid phosphatase, Code AP, activity 0.17 unit/mg, was obtained from Worthington. Pellicular carbon beads, 270—325 mesh, were supplied by Northgate Laboratories. *p*-Nitrophenol phosphate and glutaraldehyde from K and K Laboratories and spectral grade solvents and and reagent grade chemicals from Fisher were used throughout the study. Radiometer Model 26 pH meter was employed with regular glass and calomel electrodes and standardized with aqueous buffer solutions. When the pH of hydro-organic mixtures was measured the needle of the pH meter was allowed to drift to the final reading.

Methods

(a) Immobilization of acid phosphatase. 30 g of pellicular carbon beads were contacted with a solution of 1.5 g of acid phosphatase in 15 ml of 0.1 phosphate buffer, pH 4.5, at 5 °C for 1 h. The wet mass was reacted with 10 ml of 7% glutaraldehyde in the same buffer for 1 h. Subsequently the product was washed with 0.1 M phosphate buffer, pH 7.0, until the supernatant of the last washing showed no phosphatase activity and the product was dried. The pellicular carbon support itself and bovine serum albumin immobilized by the

above procedure showed no phosphatase activity in control experiments.

(b) Measurement of enzyme activity. Soluble or immobilized acid phosphatases were incubated in $5 \cdot 10^{-3}$ M *p*-nitrophenyl phosphate in 0.1 M acetate or citrate buffer, pH 5.0, at 25°C. The initial velocities were obtained by measuring the absorbance of the liberated *p*-nitrophenol at 410 nm after adding aliquots of the reaction mixture to an excess of 0.25 M borate buffer, pH 9.5, at various times. The molar absorptivity of the *p*-nitrophenolate was found to be 1.76×10^4 by calibration. In a typical experiment 0.3 mg of soluble enzyme and 100 mg of enzyme beads were used in a reaction volume of 10 and 20 ml, respectively.

When the concentration of the organic solvent was varied, the experiments were carried out in two different modes. In one case the organic solvent was mixed with different amounts of 0.1 M citrate buffer, pH 5.0, so that the citrate concentration varied with the concentration of the organic solvent. In other experiments, with acetone and acetate buffer, pH 5.0, the acetate concentration was maintained constant in the full range of acetone concentration investigated. The substrate solutions were prepared by diluting 1 ml of a 10^{-2} M *p*-nitrophenyl phosphate solution in 2 M acetate buffer, pH 5.0, to 20 ml with distilled water and acetone. The molar absorptivity of the *p*-nitrophenolate at 410 nm was the same in both the aqueous and hydro-organic mixtures.

In some experiments the rate of liberation of *p*-nitrophenol was measured with $2 \cdot 10^{-2}$ M D-glucose present in the hydro-organic reaction mixtures which were prepared with acetate buffer, pH 5.0, and contained $5 \cdot 10^{-2}$ M acetate as well as $5 \cdot 10^{-3}$ M *p*-nitrophenyl phosphate. Determination of the inorganic phosphate was attempted by using several variations of the molybdenum blue method [19] but in all cases the organic solvents severely interfered with the measurements.

(c) Measurement of the kinetic parameters and pH profiles. *p*-Nitrophenyl phosphate in 0.1 M acetate buffer, pH 5.0, was used in the concentration range from $5 \cdot 10^{-5}$ to $5 \cdot 10^{-3}$ M to measure initial velocities in aqueous media. Substrate solutions containing 50% (v/v) acetone were prepared by mixing acetone with phosphate solutions in 0.2 M acetate buffer, pH 5.0; the measured pH was 6.3. Product inhibition was measured with $1 \cdot 10^{-3}$ M *p*-nitrophenyl phosphate solutions in 0.1 M citrate buffer, pH 5.0. The initial concentration of inorganic phosphate was varied from 0 to 10^{-2} M. The respective concentrations were the same in the 50% (v/v) acetone solutions but the pH meter reading was 6.0.

Mixtures containing equal volumes of solvent and 0.1 M citrate buffer of pH values ranging from 3.2 to 6.2 were used to measure the pH profile of the enzyme activity with $5 \cdot 10^{-3}$ M substrate.

Results

Immobilization

As shown previously [16] the immobilization of acid phosphatase by glutaraldehyde on a variety of pellicular supports at pH 4.65 rather than 8.0

consistently yields about twice as high activity. Either the enzyme is more prone to deactivation by glutaraldehyde at high pH or the conformation of the protein is different so that the availability of peripheral primary amino groups for intermolecular cross-linking is drastically reduced. The latter phenomenon was found responsible for a similar effect of the pH on the immobilization of alkaline phosphatase by covalent bonds via the ϵ-amino groups of lysine [20].

Acid phosphatase was also immobilized on porous silica beads having an average pore size of 125 nm, specific surface area of 25 m^2/g and particle diameter of 40 μm (Spherosil X B-015, Pechiney, Saint Gobain). By using the method described for porous glass [21] we obtained about the same enzymic activity per unit weight with both the fully porous and the superficially porous support. In view of the advantages of the pellicular configuration [22] our experiments were performed with the latter type of immobilized acid phosphatase.

Factors affecting the enzymic activity

The measurements of the maximum activity in neat aqueous buffers at pH 5.0 showed that in acetate the rate of *p*-nitrophenol liberation was 125 nmol · min^{-1} · mg^{-1} of the soluble enzyme and 200 nmol · min^{-1} · g^{-1} of the immobilized enzyme, whereas in citrate the corresponding rates were 63 and 170, respectively. Thus, the activity of the soluble enzyme was twice as high in acetate than in citrate buffer but the activity of the immobilized enzyme was only 15% lower in citrate than in acetate. The relatively low rates of *p*-nitrophenol liberation in citrate buffer were unexpected in view of the study of Nigam and Fishman [23] who found that hydroxylated substances in the reaction mixture can act as acceptors for phosphoryl transfer by acid phosphatase, thus, enhancing the rate of phenol liberation. It is possible that the interaction of citric acid with peripheral amino groups, which were largely eliminated or made inaccessible by glutaraldehyde in the immobilization process, is responsible for the relatively low activity of the soluble enzyme in citrate buffer.

The activity of both the soluble and immobilized acid phosphatase in mixtures of acetate buffer, pH 5.0, and acetone is illustrated in Fig. 1 which also shows that the measured pH rapidly increases with the acetone content. To some extent the decrease in the enzyme activity could be attributed to the changing pH of the reaction mixture and less decrease in the activity of both the soluble and immobilized acid phosphatase was found in mixtures containing acetone up to 50% (v/v) when the measured pH was maintained at 5.0 by addition of acetic acid.

Another set of experiments was carried out in mixtures of 0.1 M citrate buffer, pH 5.0, with acetone, dioxane and acetonitrile. Under these conditions the hydro-organic mixtures contained different concentrations of both the organic solvents and citrate. In each case the activity of both the free and immobilized enzymes monotonically decreased with increasing solvent concentration. The loss of enzymic activity with increasing organic solvent concentrations was less for the immobilized than for the soluble enzyme particularly in acetonitrile mixtures, where the activity in 95% (v/v) acetonitrile was 40% of that in the neat buffer. The monotonic decrease in enzyme activity is notewor-

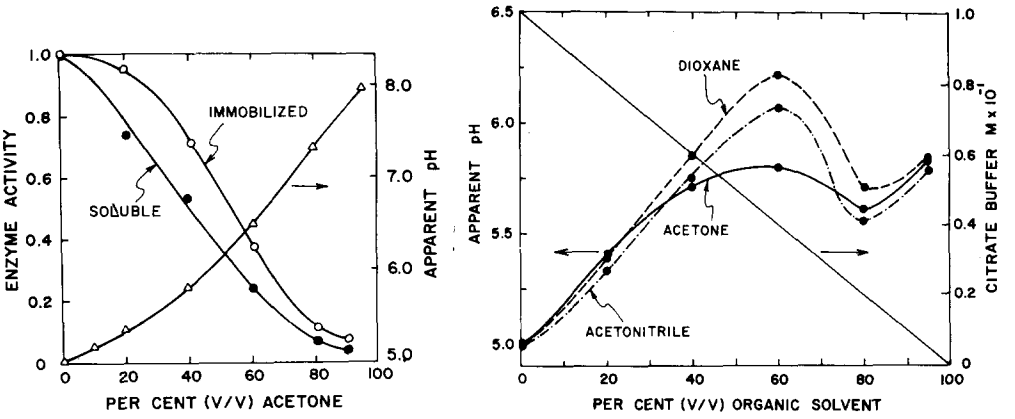

Fig. 1. Effect of acetone concentration on the hydrolysis of *p*-nitrophenyl phosphate by soluble and immobilized acid phosphatase as shown by plots of the relative enzyme activity against the organic solvent content. The concentrations of the acetate buffer and the substrate were 0.1 M and $5 \cdot 10^{-3}$ M, respectively, in all acetone/water mixtures. The pH as measured with the glass electrode is also shown as a function of the acetone concentration.

Fig. 2. The apparent pH of hydro-organic mixtures as a function of the organic solvent content. 0.1 M citrate buffer, pH 5.0, was mixed with the organic solvent in different proportions so that the citrate concentration changed as indicated.

thy because under such conditions the apparent pH as measured with the pH meter is not a monotonic function of the organic solvent concentration as shown in Fig. 2.

Unlike in neat aqueous buffers, the relative enzymic activities were consistently higher in citrate than in acetate when acetone was present. This effect can be attributed to the higher apparent pH values in acetone/acetate mixtures than those in citrate/acetone mixtures as shown in Figs 1 and 2, or the relatively high rates of *p*-nitrophenol liberation were consequences of transphosphorylation [23] which may occur in citrate buffer when organic solvent is present.

Kinetic parameters and pH profiles

As obtained from Lineweaver-Burk plots, the K_m values in 0.1 M neat acetate buffer, pH 5, were 0.47 and 0.85 mM for the soluble and immobilized enzymes, respectively. On the other hand, the K_m for the immobilized enzyme was 0.30 mM in a mixture of equal volumes of 0.2 M acetate buffer, pH 5, and acetone. In the neat buffer the saturation rates of the soluble enzyme (60 μg) and the immobilized enzyme (100 mg) were 4.4 and 28 nmol $\cdot$ min^{-1}, respectively. The corresponding V of the immobilized enzyme in the acetone mixture was 12 nmol $\cdot$ min^{-1}. These data show that in the aqueous buffer the K_m value of the immobilized enzyme was twice as high as that of the soluble enzyme. In the hydro-organic medium, however, the K_m value of the immobilized enzyme was unexpectedly lower than the K_m values obtained in the neat buffer for both the soluble and immobilized enzymes. The V/K_m ratio for the immobilized enzyme was about the same in the absence as in the presence of acetone.

The study of inhibition by inorganic phosphate in neat citrate buffer and 50% (v/v) acetone/citrate mixtures showed that the K_i for the immobilized enzyme was the same in both media (K_i = 2.2 mM). This value is about twice of those measured with the soluble enzyme in neat citrate buffer (K_i = 0.85 mM) and in the acetone mixture (K_i = 1 mM).

The pH vs activity profile of immobilized acid phosphatase was investigated in mixtures containing equal volumes of 0.1 M citrate buffer and either acetone, dioxane or acetonitrile. The pH of the citrate solutions used in these mixtures ranged from 3.0 to 6.2. The pH of the hydro-organic solutions were measured with the pH meter and found in this domain to follow the relationship

$$pH^* = a\ pH + b \qquad (1)$$

where pH* is the meter reading, pH is the pH of the neat aqueous buffer. Whereas the constant a was about 0.9 in each case, the value of b was 1.70, 1.28 and 1.13 pH unit for mixtures containing dioxane, acetonitrile and acetone, respectively. It is noted that the observed pH values calculated from Eqn 1 for mixed acetate buffer/acetonitrile solutions are in very good agreement with recent literature data [24].

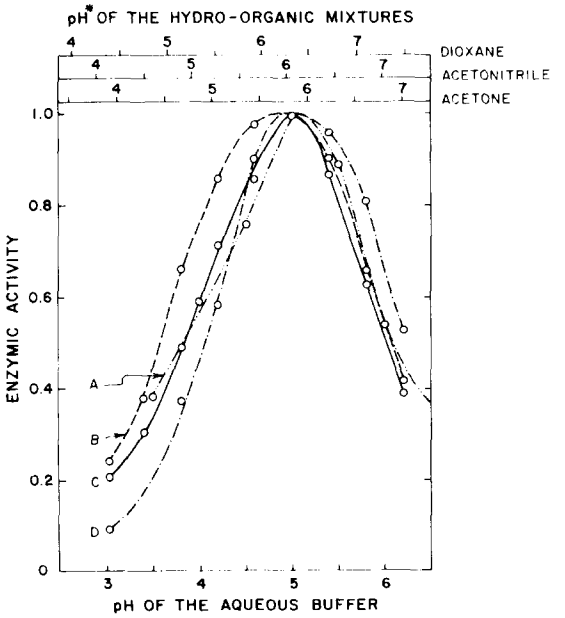

Fig. 3. pH/activity profiles of immobilized acid phosphatase in hydro-organic media prepared by diluting 0.1 M aqueous citrate solutions of different pH with equal volumes of organic solvents. The bottom scale shows that pH of the neat 0.1 M citrate buffer without organic solvent. The measured pH values, pH*, of the corresponding hydro-organic mixtures are shown on the scales at the top. Curve A is the pH profile of the immobilized enzyme in 0.1 M citrate buffer without organic solvent. Curves B, C and D represent the activity profiles of the immobilized enzyme in hydro-organic media containing 50% (v/v) dioxane, acetone and acetonitrile, respectively. The reaction mixtures contained 100 mg of immobilized enzyme and $5 \cdot 10^{-3}$ M *p*-nitrophenyl phosphate at 25°C.

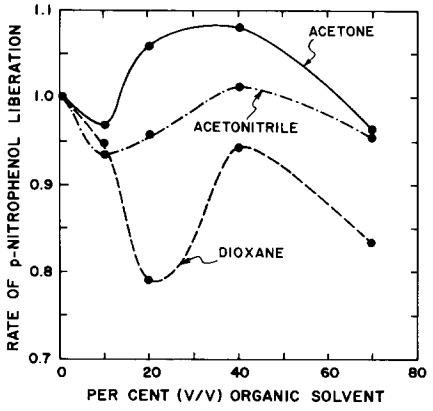

Fig. 4. The rate of liberation of *p*-nitrophenol by soluble acid phosphatase as a function of the organic solvent concentration in hydro-organic media containing $2 \cdot 10^{-2}$ M D-glucose. Acetate buffer, pH 5.0, was mixed with the solvents so that the buffer concentration was $5 \cdot 10^{-2}$ M in all reaction mixtures which contained $5 \cdot 10^{-3}$ M *p*-nitrophenyl phosphate.

The activity of the immobilized enzyme in aqueous citrate buffers and in their mixtures with equal volumes of organic solvents as a function of the pH of the neat buffer is shown in Fig. 2. The scales at the top of the graph show the measured pH*. It is seen that the maximum activity of the immobilized enzyme is determined by the pH of the citrate buffer used to prepare the hydro-organic mixtures rather than by the measured pH*.

Liberation of p-nitrophenol in the presence of added glucose

In view of the well established property of phosphatases [23] to catalyze phosphoryl transfer reactions, experiments were carried out with the soluble enzyme in various hydro-organic mixtures in the presence of D-glucose which is a potential phosphoryl acceptor [25]. As the organic solvents interfered with the molybdenum blue reaction the inorganic phosphate could not be measured, thus, the rate of transphorylation could not be evaluated. As shown in Fig. 4 the rate of phenol liberation goes through maxima and minima with increasing organic solvent concentration in the presence of D-glucose, whereas the rate of reaction monotonically decreases in the absence of the sugar as shown previously. The relatively high rate of phenol liberation in the presence of the added nucleophile at high organic solvent concentrations suggests that transphosphorylation is augmented in the hydro-organic media. The organic solvent may reduce the rate of *p*-nitrophenyl phosphate hydrolysis and increase the rate of transphosphorylation at the same time. The interplay of these two phenomena is strongly dependent on the nature of the organic solvent.

Discussion

This study demonstrates that both the soluble and immobilized acid phosphatase have substantial catalytic activity in hydro-organic mixtures even at high organic solvent concentrations and the immobilized enzyme is generally more active. It is assumed that the immobilized enzyme is less exposed to the

organic solvent because the actual water concentration is higher in the immediate vicinity of the immoblized protein molecules than in the bulk solution. Furthermore, the covalent inter- and possibly intramolecular linkages may stabilize the enzyme structure.

In mixed organic solvent systems even relatively simple chemical phenomena are poorly understood [26], consequently, very little is known about solvent interactions with the catalytically active protein and other participants of the reaction. The difficulties in the study of such effects commence with the definition of pH and maintenance of a constant pH in both the mixed aqueous solvent and the enzymic microenvironment. It is recalled that the recognition of the role of the H^+ concentration in enzyme catalysis and introduction of buffers by Sörensen [27] have been essential to study enzyme action in a meaningful fashion. In hydro-organic media there is no general pH scale and the pH as obtained with the pH meter does not have the same meaning as in aqueous solutions since "the interpretation of measured pH values is limited to water solutions, and, in fact, to dilute water solutions, if the pH is to retain its full significance in terms of H^+" [28]. Although different pH scales have been established for mixtures of an aqueous buffer solution and organic solvents, the significance of such scales with respect to the behavior of enzymes is still unknown. It is recognized that the organic solvents affects also the properties of the buffer whose pK value changes due to the reduced dielectric constant of the hydro-organic medium.

The results of this study shown in Fig. 3 indicate that at a fixed concentration of the organic solvent the maximum enzyme activity is obtained when the pH of the buffer used for making the hydro-organic mixture is the same as the pH of the maximum enzymic activity in the neat buffer per se. In mixtures of citrate buffers and 50% (v/v) of organic solvents this effect was observed irrespective of the nature of organic solvent and the pH measured with the glass electrode. We conclude, therefore, that under such conditions the immobilized enzyme senses only the pH of the aqueous citrate buffer, which is used for making the hydro-organic mixture, and the pH of maximum activity is independent of the measured pH of the bulk solution. This phenomenon conforms with the recent observation of Jordan [24] that the effect of pH of mixed water/acetonitrile buffer solutions can be calculated from the concentration of the acid in the aqueous portion of the solvent, neglecting the cosolvent altogether.

According to the theoretical study by Bass and McIlroy [29] the reduced dielectric constant of the hydro-organic media should have resulted in pH profiles significantly narrower in shape than that obtained in aqueous buffers. Fig. 3 shows, however, that the widths of the pH profiles at half of the maximum activity are only slightly smaller in the solvent mixtures. This observation suggests that the organic solvent in the bulk solution has little effect on the dissociation constant of the acidic and basic groups on the protein surface under the conditions investigated.

It is known that in heterogeneous enzyme systems the concentration of the various species in the enzymic microenvironment can be different from that in the bulk solution. The microenvironmental pH can be affected, for instance, by the fixed charges in the matrix [30] or diffusion resistances [31]. In

hydro-organic media the situation is even more complicated as the concentration of the organic solvent can also be different in the enzymic micro- and macroenvironment. The observed pH profiles can be explained by assuming that the organic solvent is largely excluded from the surroundings of the enzyme molecules so that the local pH approximates that of the aqueous buffer component of the mixture.

The variation of the measured pH at changing concentration of the organic solvent as shown in Fig. 2 is in agreement with the frequently occurring periodicity of chemical phenomena in mixed solvents [26]. The maximum of the measured pH is probably caused by the same effect that is responsible for the observed maximum of the acidity function in ethanol/water mixtures [32,33], whereas the minimum can be explained by the pH increase at high solvent concentrations due to vanishing buffer concentration. On the other hand the activity of both the soluble and the immobilized enzyme shows a monotonous decrease with increasing concentration of the organic solvent which is another indication that the measured bulk pH is not representative for the microenvironmental pH relevant to the enzyme behavior.

Even if the concentration of water is greater in the immediate surroundings of the enzyme than in the bulk solution, the rate-reducing effect of the organic solvent can be significant due to differences in the free energies, enthalpies and entropies of solvation of the substrate and products or simply due to the reduced availability of water for the hydrolytic reaction. In turn, the relatively high rate of *p*-nitrophenol liberation in the presence of glucose, which is less soluble in the hydro-organic mixtures than in the aqueous buffer, could be caused by the increased availability of the nucleophile in the enzymic microenvironment.

Acknowledgement

This study was supported by a grant No. GM-20993 from the National Institutes of Health, U.S. Public Health Service.

References

1 Applewhite, T.H., Martin, R.B. and Niemann, C. (1958) J. Am. Chem. Soc. 80, 1457—1464
2 Singer, S.J. (1962) Advances in Protein Chemistry (Anfinsen, Jr, C.B., Anson, M.L., Bailey, K. and Edsall, J.T., eds), Vol. 17, pp. 1—68, Academic Press, New York
3 Clement, G.E. and Bender, M.L. (1963) Biochemistry 2, 836—843
4 Inagami, T. and Sturtevant, J.M. (1964) Biochem. Biophys. Res. Commun. 14, 69—74
5 Lake, A.W. and Lowe, G. (1966) Biochem. J. 101, 402—410
6 Sinnott, M.L. and Viratelle, O.M. (1973) Biochem. J. 133, 81—87
7 Tanizava, K. and Bender, M.L. (1974) J. Biol. Chem. 249, 2130—2134
8 Douzou, P., Sireix, R. and Travers, F. (1970) Proc. Natl. Acad. Sci. U.S. 66, 787—792
9 Hui Bon Hoa, G. and Douzou, P. (1973) J. Biol. Chem. 248, 4649—4654
10 Fink, A.L. (1973) Biochemistry 12, 1736—1742
11 Katchalski, E., Silman, I. and Goldman, R. (1971) Advances in Enzymology (Nord, F.F., ed.), Vol. 34, pp. 445—536, Wiley-Interscience, New York
12 Desnuelle, P. (1972) The Enzymes (Boyer, P.D., ed.), 3rd edn, Vol. 7, pp. 601—602, Academic Press, New York
13 Fruton, J.S. (1971) The Enzymes (Boyer, P.D., ed.), 3rd edn, Vol. 3, pp. 156—157, Academic Press, New York

14 Hollander, V.P. (1971) The Enzymes (Boyer, P.D., ed.), 3rd edn, Vol. IV, pp. 449—498, Academic Press, New York
15 Weetall, H.H. and Hersh, L.S. (1969) Biochim. Biophys. Acta 185, 464—465
16 Horvath, C. (1974) Biochim. Biophys. Acta 338, 164—177
17 Singer, T.P. (1948) J. Biol. Chem. 174, 11—21
18 Joyce, B.K. and Grisolia, S. (1969) J. Biol. Chem. 235, 2278—2281
19 Crouch, S.R. and Malmstadt, H.V. (1967) Anal. Chem. 39, 1084—1089
20 Zingaro, R.A. and Uziel, M. (1970) Biochim. Biophys. Acta 213, 371—379
21 Weetall, H.H. (1970) Biochim. Biophys. Acta 212, 1—7
22 Horvath, C. and Engasser, J.M. (1973) Ind. Eng. Chem. Fundamentals 12, 229—235
23 Nigam, V.N. and Fishman, W.H. (1959) J. Biol. Chem. 234, 2394—2398
24 Jordan, F. (1973) J. Phys. Chem. 77, 2681—2683
25 Morton, R.K. (1955) Disc. Faraday Soc. 20, 149—156
26 Amis, E.S. and Hinton, J.F. (1973) in Solvent Effects on Chemical Phenomena, Vol. 1, pp. 182—206, Academic Press, New York
27 Sörensen, S.P.L. (1909) Biochem. Z. 21, 131—304
28 Bates, R.G. (1973) Determination of pH, 2nd edn, p. 170, Wiley, New York
29 Bass, L. and McIlroy, D.K. (1968) Biophys. J. 8, 99—108
30 Engasser, J.M. and Horvath, C. (1975) Biol. J. 145, 431—435
31 Engasser, J.M. and Horvath, C. (1974) Biochemistry 13, 3845—3859
32 Bates, R.G. and Schwarzenbach, G. (1955) Helv. Chim. Acta 38, 699—712
33 Braude, E.A. and Stern, E.S. (1948) J. Chem. Soc. 1976—1981