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Effects of Phosphate on the Dissociation and Enzymic Stability of Rabbit Muscle Lactate Dehydrogenase†

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ABSTRACT: Frontal gel chromatography and sedimentation studies have been used to establish that rabbit muscle lactate dehydrogenase dissociates essentially completely into dimeric species (molecular weight ~70,000) in 0.2 *I* acetate–chloride (pH 5.0). Tetramer–dimer conversion, which occurred within a minute of adjusting the pH of an enzyme solution from 7 to 5, was reversed by restoring the solution to neutral pH, provided that exposure to the acidic environment was restricted to less than 4 hr at 4°. However, despite this re-formation of tetramer, some irreversible changes in enzyme structure were indicated by the recovery of only 70% of the original activity after exposure of the enzyme to pH 5 for 4 hr. Inclusion of phosphate (>30 mM) in the acetate–chloride solutions of rabbit muscle lactate dehydrogenase prevented any detectable dissociation into dimer; this increased stability of tetramer is allied with essentially complete retention of enzymic activity. Similar stabilizing effects were observed with lactate dehydrogenase solutions in which the phosphate was replaced by either reduced or oxidized nicotinamide adenine dinucleotide. Kinetic studies at pH 7 have established a pronounced activating effect of phosphate on the enzymic conversion of lactate to pyruvate. Under the same conditions the lactate dehydrogenase was shown to be tetrameric in the presence and absence of phosphate. Thus, although phosphate shares with the pyridine nucleotides the ability to stabilize the quaternary structure of rabbit muscle lactate dehydrogenase, the specific nucleotide-binding sites are not involved in these phosphate interactions unless phosphate and coenzyme can be accommodated simultaneously on the same sites.

In studies of the dissociation of lactate dehydrogenase (EC 1.1.1.27) by either sodium dodecyl sulfate or urea, Di Sabato and Kaplan (1964, 1965) observed that inclusion of pyridine nucleotides or phosphate in the dissociating medium led to increased recovery of enzymic activity on removal of the denaturant. Furthermore, some material with the sedimentation coefficient of tetrameric (native) LDH[1] was observed in the denatured samples that exhibited pronounced protection of enzymic activity (Di Sabato and Kaplan, 1964). Although these studies suggested a probable link between the effects of pyridine nucleotides and phosphate on the dissociation and enzymic stability of LDH, the situation is clouded by the relatively poor recovery of activity (<50%) and by the known tendency for denatured proteins to aggregate. More definitive evidence for the protective role(s) of pyridine nucleotides and phosphate clearly requires the selection of milder conditions for disruption of the quaternary structure of LDH. For this purpose the use of neutral buffers of moderate to high ionic strength (Millar, 1962; Hathaway and Criddle, 1966; Griffin and Criddle, 1970; Bartholmes *et al.,* 1973) or of acidic conditions (Deal *et al.,* 1963; Anderson and Weber, 1966; Jaenicke and Knof, 1968) seems plausible. Under the former conditions a rapidly established dimer–tetramer equilibrium is believed to operate, but there are also reports that dispute not only the rate of the dissociation reaction (Cho and Swaisgood, 1973) but also its existence (Wieland *et al.,* 1963; Jaenicke and Knof, 1968; Anderson, 1969; Mire, 1969). Because of the seemingly variable dissociation behavior of different LDH preparations at neutral pH, we have explored the acidic region for suitable conditions.

In previous studies a pH of 2.0–2.6 has been used for disruption of the tetrameric structure of LDH (Deal *et al.,* 1963; Anderson and Weber, 1966; Jaenicke and Knof, 1968). However, we have found essentially complete dissociation of rabbit muscle LDH into dimers in 0.2 *I* acetate–chloride (pH 5.0), conditions that were without effect on the beef heart enzyme. Rabbit muscle LDH has therefore been studied under these milder

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[1] Abbreviations used are: LDH, lactate dehydrogenase; NADH and NAD^+, reduced and oxidized nicotinamide adenine dinucleotide.

conditions in order to obtain further evidence for the effects of phosphate on the enzymic stability and polymeric state of lactate dehydrogenase.

Experimental Section

Materials. Crystalline preparations of LDH from rabbit muscle and also from beef heart were obtained as ammonium sulfate suspensions either from Sigma Chemical Co., St. Louis, Mo., or from Boehringer Mannheim GmbH, Mannheim, Germany, who also supplied pyruvate, NADH, and NAD^+. L-Lactic acid was a Sigma product. Starch gel electrophoresis in 0.03 M Tris-glycine (pH 9.0) (Georgiev *et al.*, 1970) revealed the presence of small amounts (5–10%) of muscle–heart hybrid isoenzymes in both LDH preparations. Removal of these contaminants by chromatography on DEAE-cellulose (Pesce *et al.*, 1964; Stambaugh and Post, 1966) yielded pure M_4 and H_4 isoenzymes, the association properties of which were indistinguishable experimentally from those of the respective commercial preparations. Glass-distilled water was used in the preparation of all buffers, their nominal compositions being as follows: (A) 0.020 M sodium acetate–0.008 M acetic acid–0.180 M NaCl (pH 5.0, 0.20 *I*); (B) 0.020 M sodium acetate–0.008 M acetic acid–0.120 M NaCl–0.060 M NaH_2PO_4 (pH 5.0, 0.20 *I*); (C) 0.015 M NaH_2PO_4–0.062 M Na_2HPO_4 (pH 7.4, 0.20 *I*); (D) 0.040 M imidazole–0.023 M HCl–0.377 M NaCl (pH 7.0, 0.40 *I*); (E) 0.040 M imidazole–0.023 M HCl–0.065 M NaH_2PO_4–0.104 M Na_2HPO_4 (pH 7.0, 0.40 *I*). EDTA (1 mM) and dithiothreitol (0.1 mM) were included routinely in all solutions, and buffers D and E also contained bovine serum albumin (20 μg/ml). Solutions of LDH in neutral (pH 6–8) buffers were prepared by dialysis at 4° against the appropriate buffer (4 × 500 ml) for 24 hr, but instability of the enzyme precluded use of this procedure at pH 5. The LDH was therefore dialyzed against unbuffered saline (0.2 M NaCl, pH 6–7) and the final solution obtained by suitable dilution of the dialyzed enzyme solution with buffer of the same ionic strength and the required pH, unless the sample was required for sedimentation equilibrium studies. Because of the need for dialysis equilibrium between sample and solvent in such experiments (Casassa and Eisenberg, 1964), solutions were prepared by zonal chromatography of the dialyzed enzyme in unbuffered saline (1 ml) on a Sephadex G-25 column (2.1 × 15 cm) equilibrated with acetate–chloride buffer (pH 5.0, 0.20 *I*).

Assay Procedure. LDH activity was assayed at 340 nm in a Unicam SP800 recording spectrophotometer, the cuvet being thermostated at 25°. In routine assays the reaction mixture contained 0.96 mmol of Tris-HCl (pH 8.0), 0.3 μmol of NADH, 5 μmol of sodium pyruvate, and enzyme in a total volume of 3 ml. Protein concentrations were estimated spectrophotometrically at 280 nm using extinction coefficients ($E_{1cm}^{1\%}$) of 14.4 (Jaenicke and Knof, 1968) and 15.0 (Pesce *et al.*, 1964) for rabbit muscle and beef heart enzymes, respectively. No change in the extinction coefficient of either LDH was observed in the pH range 2–7, behavior that contrasts with that reported for pig heart enzyme (Jaenicke and Knof, 1968).

Gel Chromatography. Solutions of rabbit muscle LDH (0.11 mg/ml) and also beef heart enzyme (0.12 mg/ml) were subjected to frontal gel chromatography (Winzor and Scheraga, 1963) on a 1.9 × 9.0 cm column of Sephadex G-200, preequilibrated with 0.2 *I* acetate–chloride (pH 5.0), and thermostated at 20°. The eluate from the column was maintained at a flow rate of 12 ml/hr and divided into 0.8-ml fractions which were analyzed spectrophotometrically at 280 nm.

Very dilute (0.01 μg/ml) solutions of rabbit muscle LDH in imidazole–chloride buffer (pH 7.0, 0.40 *I*) were also examined by frontal gel chromatography on a Sephadex G-200 column (2.1 × 26 cm). In order to minimize losses of enzyme due to adsorption, bovine serum albumin (20 μg/ml) was included in the buffer used for preequilibration of this column and also for preparation of the dilute enzyme solutions that were applied to the column. The eluate, maintained at a flow rate of 12 ml/hr, was collected in 0.9-ml fractions, which were then assayed for LDH activity.

Sephadex Partition Experiments. In an attempt to gain some insight into the rate of dissociation of rabbit muscle LDH at pH 5.0, a slurry of Sephadex G-200 (12 ml) equilibrated with either bovine serum albumin (0.88 mg/ml) or rabbit muscle LDH (0.8 mg/ml) in unbuffered sodium chloride (0.2 M, pH 6–8) was placed in a polypropylene tube and spun in a bench centrifuge at 500–1000 rpm for approximately 15 sec. A small aliquot (0.10 ml) was removed from the 3 ml of supernatant, and its protein concentration was estimated by the Lowry *et al.* (1951) procedure. At zero time 0.10 ml of concentrated acetate buffer (pH 5.0) (4.0 M sodium acetate–1.6 M acetic acid) was added to the tube and its contents mixed thoroughly. At specified time intervals the tube was recentrifuged for 15 sec and 0.10-ml aliquots were removed for estimation of the protein concentration in the liquid phase.

Sedimentation. Velocity and equilibrium sedimentation experiments were performed at 20° in a Spinco Model E ultracentrifuge using the schlieren optical system. The resulting photographic records were measured on a Nikon two-dimensional comparator fitted with projection screen and accurate to 2 μm. A value of 0.740 (Jaenicke and Knof, 1968) was used for the partial specific volume of rabbit muscle LDH. Weight-average sedimentation coefficients (Goldberg, 1953) were calculated from sedimentation velocity experiments at 50,000 rpm, and *z*-average molecular weights were determined from Lamm (1929) plots of equilibrium patterns in experiments conducted at 10,000 rpm. Enzyme concentrations in the range 0.5–0.8 mg/ml were used in all sedimentation experiments except for the equilibrium runs at neutral pH, where the initial concentration was increased to 1.4–1.6 mg/ml.

Reversibility of Acid-Induced Dissociation of Rabbit Muscle LDH. Enzyme (2 mg/ml) in unbuffered saline (0.2 M NaCl, pH 6–7) was mixed 1:1 with either an acetate–chloride buffer (0.04 M sodium acetate–0.016 M acetic acid–0.16 M NaCl), pH 5.0, or an acetate–chloride–phosphate buffer (0.04 M sodium acetate–0.016 M acetic acid–0.04 M NaCl–0.12 M NaH_2PO_4) of the same pH, so that the diluted solutions had the buffer compositions specified as A and B. After storage at 4° for specified time intervals, samples were removed and diluted 1:1 with 0.2 *I* phosphate (pH 7.4) (buffer C), in order to restore the pH to neutrality. Samples of the resulting solutions were then subjected to velocity sedimentation and also tested for recovery of enzymic activity.

Kinetic Studies. The effect of phosphate on the kinetics of the reaction catalyzed by rabbit muscle LDH was studied spectrophotometrically at 340 nm by observing the formation of NADH from mixtures of L-lactate and NAD^+. Rate measurements were made at 25 ± 0.1° in a Cary 14 recording spectrophotometer. The reaction was followed at pH 7.0 in imidazole–chloride and imidazole–chloride–phosphate buffers (D and E), and also in a 1:1 mixture of the two. A value of 0.03 for the maximal difference in pH was obtained from intercomparison of the three buffers. The enzyme concentration in the reaction mixtures was 0.01 μg/ml and the L-lactate concentration 0.010 M, with that of NAD^+ being varied from 0.45 to 4.50 mM. All reaction mixtures contained bovine serum albumin (20 μg/ml) to decrease losses of enzymic activity by adsorption.

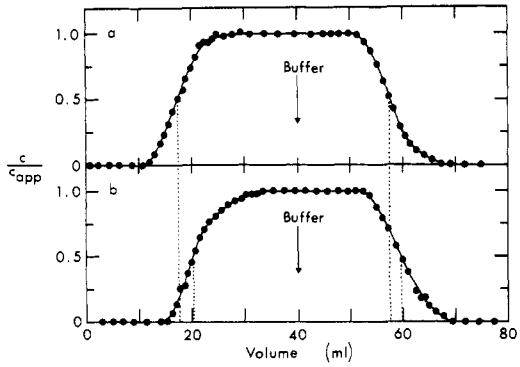

FIGURE 1: Frontal gel chromatography of LDH on a 1.9 × 9.0 cm column of Sephadex G-200 equilibrated with 0.2 *I* acetate-chloride (pH 5.0) and thermostated at 20°: (a) beef heart enzyme (0.12 mg/ml); (b) rabbit muscle LDH (0.11 mg/ml).

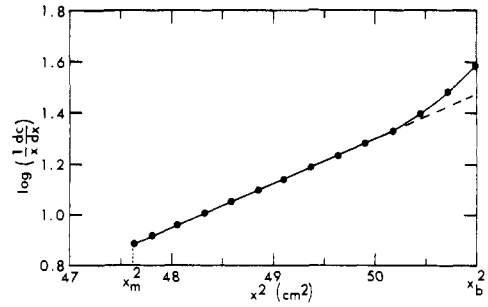

FIGURE 2: Lamm plot of a sedimentation equilibrium pattern obtained with rabbit muscle LDH at pH 5.0. x_m and x_b denote the extremities of the column of enzyme solution, the concentration of which was 0.7 mg/ml initially.

Results

Dissociation of Rabbit Muscle LDH. Elution profiles obtained in frontal gel chromatography of beef heart LDH (0.12 mg/ml) and rabbit muscle LDH (0.11 mg/ml) on Sephadex G-200 equilibrated with 0.2 *I* acetate-chloride (pH 5.0) are shown in Figures 1a and 1b, respectively, the ordinate scale having been normalized to facilitate comparisons. From Figure 1 it is evident that the rabbit muscle LDH is eluted slightly but consistently later than its counterpart from bovine heart. Such a difference was not observed in experiments at pH 7.4 (buffer C), conditions under which both enzymes are believed to be tetrameric (Jaenicke, 1964; Jaenicke and Knof, 1968). Furthermore, the profiles obtained for the two proteins at neutral pH were indistinguishable experimentally not only from each other but also from that for beef heart LDH at pH 5.0 (Figure 1a). These differences and similarities in the elution behavior of rabbit muscle and beef heart LDH under the two sets of conditions were confirmed by the layering technique of gel chromatography (Gilbert, 1966), which provides a more accurate estimate of elution volume differences. From these layering experiments the elution volumes of beef heart and rabbit muscle LDH at pH 5.0 were found to differ by 3.0 ml.

In order to establish the nature of the species responsible for the larger elution volume of rabbit muscle LDH at pH 5.0, the enzyme was subjected to velocity and equilibrium sedimentation at neutral pH (6.0–7.4) as well as at pH 5.0. Under the former conditions the observed values of 7.5–7.7 S and 142,000 for the sedimentation coefficient ($\bar{s}_{20,w}$) and molecular weight (M_z^{app} and $\bar{M}_z^{app}$) agree well with the respective values of 7.60 S and 138,000–144,000 daltons reported for the $s_{20,w}^0$ and molecular weight of tetrameric rabbit muscle LDH (Jaenicke and Knof, 1968). However, at pH 5.0 the weight average sedimentation coefficient had decreased to 4.0 S and $\bar{M}_z^{app}$ to 74,000 in experiments in which 0.7 mg/ml of LDH was used to fill the cells. Linearity of the Lamm plot of the equilibrium sedimentation pattern at pH 5.0 (Figure 2) establishes that these $\bar{s}_{20,w}$ and $\bar{M}_z^{app}$ values reflect essentially complete dissociation of the enzyme into dimeric species. Although Figure 2 only signifies stability of the dimer at concentrations as low as 0.3 mg/ml (the meniscus concentration), pronounced dissociation beyond dimer does not seem to have occurred at the still lower concentration (0.11 mg/ml) used in the gel chromatography experiment (Figure 1). The difference in elution volumes expressed as a fraction of the bed volume is 0.12, which is similar to the 0.11 that is inferred from Figure 1 of Andrews (1965) for the difference between the elution volumes of monomeric and dimeric serum albumin on Sephadex G-200. Thus a molecular weight change of approximately 70,000 is also indicated.

Since the above studies provide no information on the rate of dissociation of rabbit muscle LDH at pH 5, an attempt was made to follow the reaction by observing time-dependent partitioning in experiments in which concentrated acetate (pH 5.0) was added to a slurry of Sephadex G-200 and protein in unbuffered saline (Figure 3). In an experiment with bovine serum albumin no change of protein concentration in the liquid phase was observed on altering the pH, a result consistent with the known stability of the hydrodynamic properties of this protein over the pH range 7–5. With the rabbit muscle LDH there was a decrease of concentration in the liquid phase, but no quantitative information on the rate of dissociation could be obtained beyond the fact that reaction was complete within the time required (1 min) to remove the first aliquot for assay.

In tests of the reversibility of the acid-induced dissociation of rabbit muscle LDH two criteria were used, *viz.*, re-formation of tetramer and extent of enzymic activity on restoring the pH to neutrality. Of these two criteria the second proved to be the more sensitive. Recovery of activity was decreased to 70% after exposure of the enzyme to acetate-chloride (pH 5.0) for a period of 4 hr at 4°, whereas sedimentation runs still indicated complete regain of tetrameric structure. Indeed, only after exposure to acetate-chloride for 12 hr did the measured weight-average sedimentation coefficient differ significantly from the value for native enzyme (6.9, *cf.* 7.6 S).

Effects of Phosphate on Rabbit Muscle LDH. The reported stabilizing effects of phosphate and pyridine nucleotides against denaturation of LDH by sodium dodecyl sulfate or urea (Di Sabato and Kaplan, 1964, 1965) prompted similar studies on the present system. Figure 4 summarizes the results of sedimentation velocity and sedimentation equilibrium runs on rabbit muscle LDH in buffers (pH 5.0, 0.20 *I*) containing different concentrations of NaH_2PO_4. The circles refer to $\bar{s}_{20,w}$

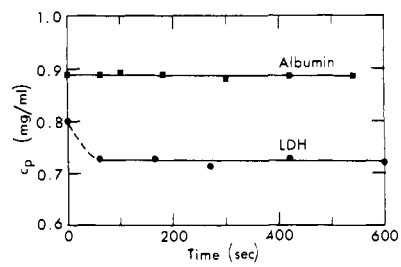

FIGURE 3: Time dependence of the concentration of bovine serum albumin (■) and rabbit muscle LDH (●) in the supernatant of a slurry (12 ml) of Sephadex G-200 on adjustment of the pH to 5.0 by addition of concentrated acetate buffer (0.10 ml).

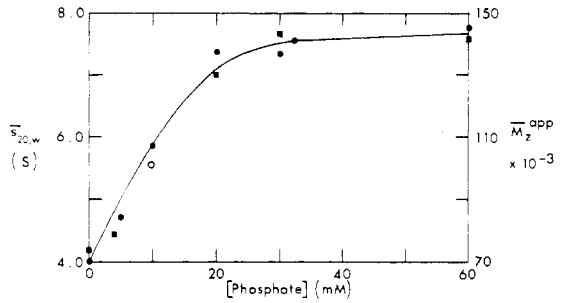

FIGURE 4: Dependence of the weight-average sedimentation coefficient (●) and apparent z-average molecular weight (■) of rabbit muscle LDH on the concentration of phosphate included in acetate-chloride buffers (pH 5.0) in which the ionic strength was maintained at 0.2 by adjusting the chloride concentration. The open circle refers to an experiment in which the concentration of sodium acetate was increased to 0.12 M at the expense of that of NaCl.

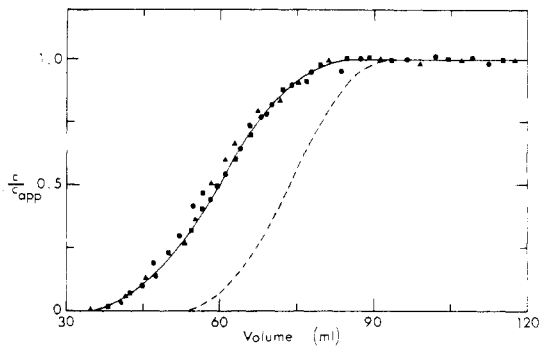

FIGURE 6: Advancing elution profiles obtained in frontal gel chromatography of 0.01 μg/ml solutions of rabbit muscle LDH in imidazole-chloride buffer (pH 7.0) (●), in imidazole-chloride buffer containing 0.17 M phosphate (■), and imidazole-chloride buffer containing 0.01 M lactate (▲); the ionic strength was 0.4 in each experiment and the pH 7.0. Solid and broken lines denote the elution profiles for tetrameric and dimeric enzymes, respectively, these having been deduced from experiments with higher enzyme concentrations at pH 7.0 and 5.0.

for solutions containing 1 mg/ml of enzyme, while the squares denote M_z^{app} at the midpoint of 1-mm columns. As noted by Yphantis (1960), such values may be assumed to apply to the initial enzyme concentration (2 mg/ml). The equilibrium and velocity results clearly provide mutual support for pronounced stabilization of tetramer by phosphate. Allied with this stabilization of tetrameric LDH is a marked enhancement of enzymic stability. In experiments with 60 mM NaH_2PO_4 present in the acetate medium 98% of the activity could be recovered after exposure of the enzyme to pH 5.0 for 4 hr, whereas the corresponding value in the absence of phosphate was 70%. Qualitatively similar sedimentation results were obtained when pyridine nucleotides replaced phosphate in the above experiments. For example, a value of 5.2 S was obtained for $\bar{s}_{20,w}$ for solutions initially containing 0.01 M NAD^+ or NADH, the duration of these experiments being such that about 20% of the NADH would have been degraded under the acidic conditions.

Since phosphate and the pyridine nucleotides could possibly stabilize the tetrameric form of LDH by similar mechanisms, it was of interest to determine whether phosphate competes with NAD(H) for active sites of the enzyme. The results of kinetic studies at pH 7.0 in the presence and absence of phosphate are shown in Figure 5, which indicates that phosphate exerts a pronounced activating effect on the enzymic reaction. Since the oligomeric state of the LDH at the enzymic concentrations (0.01 μg/ml) employed in the kinetic studies was not known, this information was obtained by frontal gel chromatography on Sephadex G-200, using enzymic assay for the detection of LDH in the elution profile and control assays to establish linearity between enzymic activity and concentration. In Figure 6 the circles refer to an experiment in 0.4 I imidazole-chloride (buffer D) and the squares to a corresponding run in 0.4 I imidazole-chloride-phosphate (buffer E). The elution profiles are indistinguishable experimentally from each other, and also from the pattern for tetrameric LDH. Furthermore, the presence of 0.01 M lactate, the concentration in the reaction mixtures used for the kinetic studies, also had no effect on the elution profile of rabbit muscle LDH (triangles in Figure 6). The activation is not, therefore, due to displacement of a dimer-tetramer equilibrium by preferential binding of NAD^+ to tetramer (Nichol *et al.*, 1967).

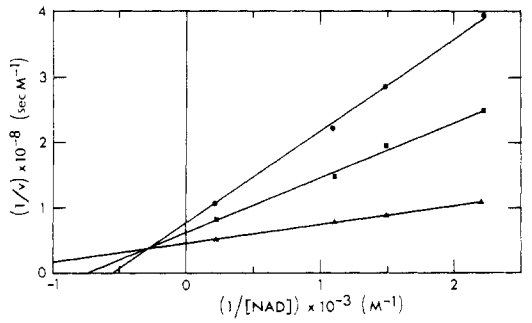

FIGURE 5: Effect of phosphate on the double reciprocal plot of kinetic data obtained with rabbit muscle LDH (0.01 μg/ml) in 0.4 I imidazole-chloride buffers (pH 7.0): (●) no phosphate; (■) 0.085 M phosphate; (▲) 0.17 M phosphate.

Discussion

The present studies of rabbit muscle LDH provide substantial evidence for dissociation of the enzyme at pH 5.0, conditions milder than those used in earlier investigations (Deal *et al.*, 1963; Jaenicke and Knof, 1968). However, whereas dissociation into monomers with molecular weights of 35,000 occurs at pH 2.0 (Anderson and Weber, 1966; Jaenicke and Knof, 1968; Cho and Swaisgood, 1973), dissociation does not appear to proceed beyond the dimer stage under the present conditions. The reaction at pH 5.0 is very rapid, being essentially complete in less than 1 min.

Measurements of enzymic activities and sedimentation coefficients have established reversibility of the dissociation reaction, but prolonged exposure of the dimeric enzyme to acetate-chloride (pH 5) does lead to some irreversible inactivation of the enzyme, despite retention of its ability to regain a tetrameric form. From the viewpoint of reversibility with regard to enzymic activity, it should be noted that the recovery of activity on restoring the pH to neutrality is significantly better in the present study than in earlier investigations using either denaturants (Di Sabato and Kaplan, 1964, 1965) or acid (Deal *et al.* 1963; Anderson and Weber, 1966) as the dissociating agent. For example, more than 90% of the original activity could be regained after incubation of the enzyme at pH 5.0 for 60 min, whereas Anderson and Weber (1966) report the loss of 80–90% of the coenzyme-binding capacity of LDH after exposure for 10 min to conditions causing appreciable dissociation into monomeric subunits. The present finding that inclusion of phosphate in the acetate-chloride medium (pH 5.0) leads to virtual elimination of irreversible inactivation and to stability of the tetrameric form of rabbit muscle LDH is more compelling evidence than earlier observations (Di Sabato and Kaplan, 1964, 1965) that suggested a possible link between the protective ef-

fects of phosphate on LDH structure and enzymic stability.

Although pyridine nucleotides exert stabilizing effects on LDH similar to those observed with phosphate (Di Sabato and Kaplan, 1964, 1965; Jago *et al.*, 1971; Cho and Swaisgood, 1973; Bartholmes *et al.*, 1973), the phosphate interactions that are responsible for protection of tetrameric integrity are not competitive with specific binding of coenzyme. Our finding that phosphate causes activation, not inhibition, of LDH (Figure 5) has also been observed in kinetic studies of beef heart enzyme (Hakala *et al.*, 1956; Winer and Schwert, 1958). However, the only acknowledgment of the phenomenon was a comment on the incompatibility but reproducibility of results obtained in Tris and phosphate buffers (Winer and Schwert, 1958). Comparison of the kinetic constants obtained in phosphate (Hakala *et al.*, 1956) and Tris (Winer and Schwert, 1958) shows that phosphate causes pronounced increases not only in the rates but also in the apparent association constants for the equilibrium reaction between LDH and the reduced and oxidized pyridine nucleotides. Thus, in the case of beef heart LDH the effect of phosphate stems at least in part from activation of the coenzyme-binding sites.

In summary, the present investigation has shown that phosphate exerts a stabilizing effect on the enzymic activity and tetrameric integrity of rabbit muscle LDH, as well as causing activation of the enzyme. However, the nature and location of the phosphate binding that is the common cause of these phenomena cannot be deduced from this study, since the methods used provide information on the consequences of phosphate binding, and not on the molecular interactions of phosphate with the enzyme. Clearly, the availability of the three-dimensional structure of LDH (Adams *et al.*, 1970) gives added incentive to the search for an explanation of the effects of phosphate at the molecular level.

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