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# Factors Affecting Tetramer Dissociation of Rabbit Muscle Lactate Dehydrogenase and Reactivity of Its Sulfhydryl Groups†

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ABSTRACT: Studies of the molecular size of rabbit muscle lactate dehydrogenase in 0.1 M phosphate buffers indicated that the tetramer undergoes a slow, concentration-dependent dissociation to a dimer. Dissociation is slight above 4–5 mg/ml at 20° and pH 7, but pronounced below 1 mg/ml. Increased dissociation occurs with storage of dilute solutions for periods up to 1 month. Reactivity of enzyme sulfhydryl groups could also be related to a slow, concentration-dependent dissociation. The number of groups reacting increased either with time after dilution with thiol reagent or simply with time following dilution. Dissociation in dilute solutions was prevented by NADH (0.5 μM) as evidenced by its effect in sedimentation equilibrium and sulfhydryl group reactivity studies. Storage for several days in dilute solutions resulted in oxidation to disulfide. Dithiothreitol protected against storage inactivation of dilute solutions but more concentrated solutions did not lose activity upon storage in either its presence or absence. Extensive modification of enzyme carboxyl groups or a large increase in net charge (pH 2) caused dissociation mostly to dimer but significant concentrations of monomer were detectable only after extensive disruption of structure. Dissociation and sulfhydryl group reactions may account for the previously unexplained sub-bands observed in gel electrophoresis experiments.

The quaternary structure of L-lactate dehydrogenase (EC 1.1.1.27) has been studied extensively during the past several years. Although an octameric structure has been proposed (Millar *et al.*, 1969), recent evidence has favored a tetrameric structure (Huston *et al.*, 1972). Several investigations have suggested a rapid dissociation equilibrium between protomers and the tetramer for both bovine heart (Millar, 1962) and rabbit muscle lactate dehydrogenases (Griffin and Criddle, 1970). However, conflicting observations have been made by others (*e.g.*, see Jaenicke and Knof, 1968). Recent studies of hybridization in dilute solution (Millar *et al.*, 1971) have supported the case for dissociation.

A suitable explanation for the sub-bands occurring in the region of each isozyme band upon gel electrophoresis has not yet been found although reaction of sulfhydry groups has been implicated (F itz and Jacobson, 1965). A thorough study of the reactivity of sulfhydryl groups of the isozymes from various species has been reported (Fondy *et al.*, 1965); however, studies of the rabbit muscle enzyme were not included.

We report here evidence suggesting that rabbit muscle lactate dehydrogenase tetramer dissociates slowly upon dilution in 0.1 M phosphate buffers, at a rate which is dependent on the preparation. This dissociation appears to be correlated with the reactivity of the enzyme's sulfhydryl groups.

## Experimental Section

*Materials.* Three lots (type II, No. 00C-9530, 90C-9520, and 51C-9580) of crystallized rabbit muscle lactate dehydrogenase were obtained from Sigma Chemical Co.[1] and used for this study. A crystallized preparation of the bovine heart enzyme (type III, lot No. 98B-0911) was also obtained from Sigma. Concentrations of the rabbit muscle enzyme were determined spectrophotometrically using an absorbancy index of 1.44 $cm^2$/mg at 280 nm (Jaenike and Knof, 1968). NADH[2] (grade III) and dithiothreitol were also obtained from Sigma.

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[1] The use of trade names in this publication does not imply endorsement of the product names by either the U. S. Department of Agriculture or the North Carolina Agricultural Experiment Station, nor criticism of similar ones not mentioned.

A purified grade (Sequanal) of Gdn·HCl containing no detectable interfering cations was purchased from Pierce Chemical Co. and used without further purification. All other chemicals were reagent grade.

*Methods.* All sedimentation analyses were performed with a Beckman Model E analytical ultracentrifuge equipped with Schlieren and Rayleigh interference optics and a RTIC temperature control unit. Solutions were examined by sedimentation equilibrium in an AN-F rotor using regular, +0.6°, and −0.6° wedge centerpieces and sapphire windows. A standard solution column length of 3 mm was used in all cases. The interference patterns were recorded on Kodak Spectroscopic II-G plates and measured with a Gaertner microcomparator. Appropriate speeds for low-speed equilibrium were calculated according the Richards *et al.* (1968) and for high-speed equilibrium according to Yphantis (1964). The true fringe number of a reference fringe in low-speed equilibrium patterns was obtained by increasing the speed and measuring the fringe drop according to LaBar (1965). Cell-mass averages were calculated for low-speed data using $C_0$ obtained with a synthetic boundary cell and checked with $C_0$ values obtained from experiments corrected for radial dilution. These values were found to agree within experimental error. Analysis of low-speed data was accomplished with a program written for an IBM 360 computer by fitting fringe positions to a polynomial allowing weight and $Z$ averages to be evaluated at various radii or concentration values and also providing for the calculation of mass and volume averages. High-speed data were calculated using the method of Yphantis (Yphantis, 1964; Jeffrey and Pont, 1969) using a Wang 370 calculator program. A value of 0.74 for the partial specific volume was used for enzyme in neutral buffers (Millar, 1962) and 0.73 for the apparent specific volume in 6 M Gdn·HCl (Huston *et al.*, 1972). Densities were determined with a 25-ml pycnometer at 20.00 ± 0.01°.

Light-scattering measurements were performed as described previously (Swaisgood and Timasheff, 1968) using a Brice-Phoenix photometer. Solutions were rendered dust-free by repeated filtration through Gelman 0.2-$\mu$ filters in a closed Plexiglass box. A value of 0.183 for d$n$/d$c$ at 546 nm was used for the rabbit muscle enzyme (Jaenicke, 1963).

Columns of Sephadex G-200, 3 × 90 cm for zonal analysis and 1 × 30 cm for frontal analysis, were prepared by the standard procedures (Winzor, 1969). Elution for zonal analysis by descending chromatography was accomplished at a flow rate of 15 ml/hr with a Stalproduckter peristaltic pump. One-milliliter fractions were collected in a GME Escargot fractionator and read at 280 nm in a Shimadzu QV 50 spectrophotometer. Frontal analysis was performed as described by Winzor and Scheraga (1963). A flow rate of 5 ml/hr was maintained with the pump and effluent was passed through a 0.08-ml flow-through cell in a Gilford spectrophotometer and measured in a 50-ml buret.

Sulfhydryl groups were determined by reaction with DTNB as described by Ellman (1959). Zero time in these studies is the time of dilution of stock enzyme solution (usually ammonium sulfate suspensions dialyzed against 0.1 M phosphate, pH 7) with 0.1 M phosphate buffer, pH 8. Total sulfhydryl groups were determined by reaction at pH 8 in the presence of 1% sodium dodecyl sulfate.

Enzyme carboxyl groups were modified under mild conditions by addition of 200 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to 10 ml of enzyme solution (2 mg/ml) containing 0.1 M glycine methyl ester and 0.01 M dithiothreitol at pH 7 and 25°. The reaction was quenched after 15 min by dialysis against 0.05 M sodium acetate (pH 7) followed by extensive dialysis against water. The number of groups modified was determined by amino acid analysis. Extensive modification was accomplished by reaction of the enzyme with the carbodiimide and glycine methyl ester in the presence (Hoare and Koshland, 1967) or absence of 6 M Gdn·HCl. As before, the pH was maintained at 7.0; however, three additions of reagent were made and the glycine methyl ester concentration was 1.0 M.

Lactate dehydrogenase activities were assayed according to the procedure given in the Worthington Manual (Worthington Biochemical Corp., Freehold, N. J., p 7, 1962). NADH solutions were freshly prepared and protected from light and contact with air. Changes in absorbance at 340 nm were monitored with a Cary 15 spectrophotometer.

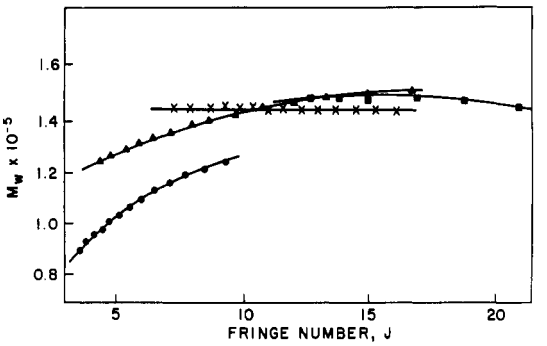


FIGURE 1: Low-speed sedimentation equilibrium data for rabbit muscle lactate dehydrogenase (lot OOC-9530). Initial concentrations of 1.1 mg/ml (●), 2.2 mg/ml (▲), and 3.4 mg/ml (■) in 0.1 M sodium phosphate buffer, pH 7, 0.01 M dithiothreitol, run simultaneously in an AN-F rotor at 6995 rpm, 20°. Initial concentration of 2.2 mg/ml (×) in 5 × $10^{-5}$ M NADH solution, other conditions being the same.

## Results

*Molecular Weight Studies.* Preparations were routinely examined by sedimentation velocity to check for characteristic behavior. Examination of freshly prepared solutions gave similar sedimentation rates in the presence (7.48 S) and absence of dithiothreitol and NADH (7.31 S), in excellent agreement with data previously reported (Fromm, 1963; Griffin and Criddle, 1970).

Freshly prepared solutions of the rabbit muscle enzyme in 0.1 M phosphate buffer, pH 7.0, were examined by low-speed sedimentation equilibrium. The dependence of point weight-average molecular weights obtained from sedimentation equilibrium on protein concentration is shown in Figure 1. Except in the presence of NADH, values were lower than the tetrameric value at lower concentrations. Actual values varied somewhat between preparations (compare, *e.g.*, data in Figure 1 and Figure 2); however, there was no correlation between extent of dissociation and specific activity of the preparation.

Effects of pH on dissociation were examined, and since the dissociation rate appeared to be slow, the effect of length of storage time in solution was also studied. As shown in Figure 2, the enzyme was extensively dissociated at pH 2. These values have been corrected for the Donnan effect (Huston *et al.*, 1972; Roark and Yphantis, 1971). When the protein concentration was about 1 mg/ml or less, every preparation ex-

[2] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Gdn·HCl, guanidine hydrochloride; NADH, reduced nicotinamide adenine dinucleotide.

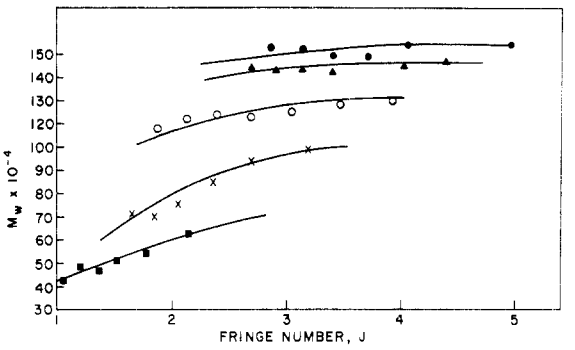


FIGURE 2: Effect of pH and storage in solution on the dissociation of rabbit muscle lactate dehydrogenase (lot 90C-9520). High-speed sedimentation equilibrium data for freshly prepared solutions at pH 8, 1.0 mg/ml (●), and pH 7, 1.0 mg/ml (▲), in 0.1 M phosphate buffer, 15,220 rpm, 20°. Data obtained when the solution at pH 7 was held for 16 days at 4°, 1.0 mg/ml (○), 15,220 rpm, 20°. Values for a solution at pH 7, 1.2 mg/ml, held for 1 month at 4° (×), 16,200 rpm, 20°. Data obtained at pH 2, 0.6 mg/ml (■), 35,600 rpm, 10°.

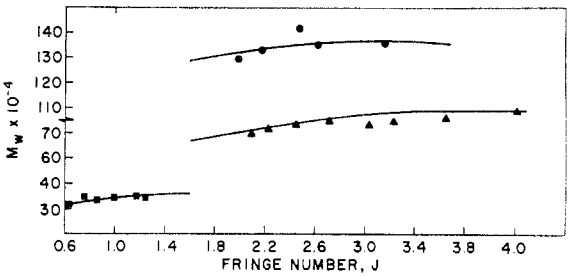


FIGURE 4: Effect of carboxyl group modification and concentrated Gdn·HCl on dissociation of rabbit muscle lactate dehydrogenase. High-speed sedimentation equilibrium results for lactate dehydrogenase with an average of five groups/tetramer modified (●), 16,200 rpm, 20°; extensively modified, 42 groups/tetramer (▲), 17,250 rpm, 20°; and extensively modified in 6 M Gdn·HCl, 92 groups/tetramer (■), 35,600 rpm, 20°. The solvent for the first two solutions was 0.1 M phosphate, pH 7, and the last, 6 M Gdn·HCl at pH 2.

amined exhibited more dissociation after dialysis against buffer for 1 week than did freshly prepared solutions. After standing for 1 month in dilute solution, the enzyme was considerably more dissociated (Figure 2). Data points lie on a straight line when plotted on a two-species plot (Roark and Yphantis, 1968) suggesting the presence of two species with molecular weights of *ca.* 156,000 and 78,000, corresponding to the tetramer and dimer, respectively (Figure 3).

Light scattering results yielded values of 143,400 and 136,200 for the molecular weights of fresh stock solutions and stock solutions aged for 1 month, respectively. The data showed that the curvature expected for systems in rapid equilibrium (*e.g.*, see Swaisgood and Timasheff, 1968) was not observed when measurements were made immediately on dilutions of the stock solutions.

The effect of modification of side-chain carboxyl groups on dissociation was also investigated. Slight modification did not cause much dissociation, but extensively modified enzyme gave molecular weights near that of the dimer (Figure 4). When enzyme was extensively modified in 6 M Gdn·HCl and analyzed in the same solvent adjusted to pH 2 a molecular weight corresponding to that of the monomer was obtained.

*Gel Chromatographic Studies.* In contrast to the results for bovine heart enzyme, more than one peak was obtained upon zonal gel chromatogrpahy of rabbit muscle enzyme (Figure 5). Although the initial enzyme concentration applied to the column in this experiment was greater than that applied in frontal gel chromatography, it should be noted that dilution occurs in the small zone experiment (in fact the concentration in the effluent is less than that for the frontal analysis experiment) and the time required for elution is about 5–6 hr. A slow dissociation of the enzyme was also indicated by analysis of frontal gel chromatography. Enzyme was chromatographed at concentrations of 0.03, 0.08, 0.1, 0.2, 1.0, and 2.5 mg/ml and typical results are shown in Figure 6. Graphical differentiation of the leading and trailing boundaries suggests the presence of at least two maxima in each boundary whose positions were not dependent on concentration. Furthermore, a hypersharp leading boundary and a more diffuse trailing boundary, as would be expected for a rapid association–dissociation equilibrium (Winzor, 1969), were not observed. Rather, the leading and trailing boundaries are enantiomorphic as shown by the first derivative curves and are typical of a paucidisperse solution. Column operation was checked by analysis of α-

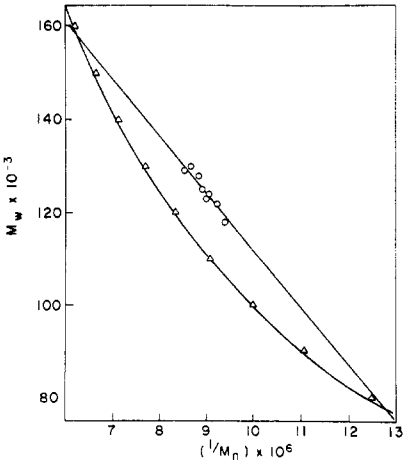


FIGURE 3: Two-species plot for rabbit muscle lactate dehydrogenase stored in 0.1 M phosphate buffer, pH 7, for 1 month at 4°. The hyperbola describes the equation $M_w(1/M_n) = 1$ and intersects the "two-species line" at values corresponding to the molecular weights of the species.

TABLE 1: Effect of Dithiothreitol on Enzymatic Activity of Rabbit Muscle Lactate Dehydrogenase Stored at Low and High Concentrations.

| Enzyme Concn (μg/ml) | Dithiothreitol[a] | $\Delta A$/min[b] | Sp Act.[c] |
|---|---|---|---|
| 10 | | 0.46 | 222 |
| 10 | Added | 0.62 | 300 |
| 5000 | | 0.60 | 291 |
| 5000 | Added | 0.59 | 286 |

[a] Enzyme solutions containing dithiothreitol were prepared by dialyzing the ammonium sulfate precipitate against sodium phosphate buffer containing dithiothreitol. Activities were measured after 10 days storage. [b] Change in absorbance per minute at 340 nm. [c] One unit of activity is defined as an initial rate of oxidation of 1 μmol of NADH/min.

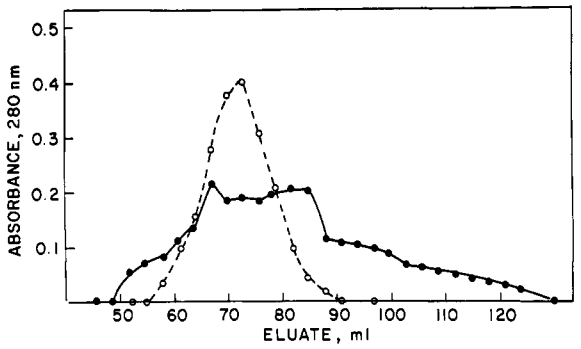


FIGURE 5: Zonal gel chromatography of rabbit muscle (solid line) and beef heart (dashed line) lactate dehydrogenase using Sephadex G-200. Eluting buffer was 0.1 M sodium phosphate, pH 7. A 2.0-ml sample volume (5 mg/ml) was applied to a 3 × 90 cm column.

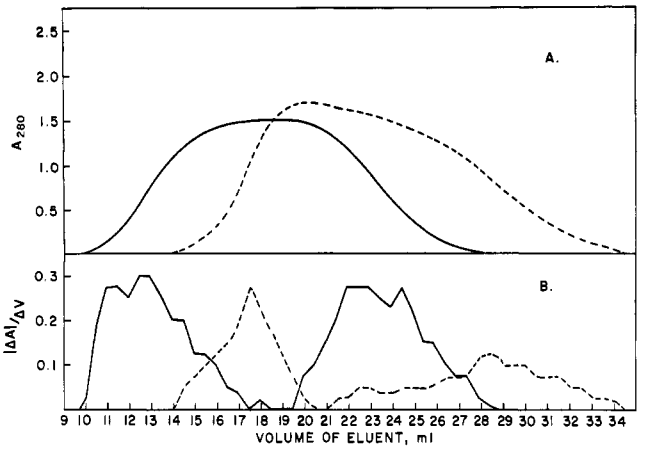


FIGURE 6: Frontal gel chromatography of rabbit muscle lactate dehydrogenase (solid line) and α-chymotrypsin (dashed line) using Sephadex G-200. Eluting buffer was 0.1 M sodium phosphate, pH 7 for the dehydrogenase and pH 8 for α-chymotrypsin. A 15-ml sample (0.6 mg/ml) was applied to a 1 × 30-cm column. Part A shows the elution profiles and part B the first-derivative curves, 0.5 ml having been used as the increment in volume.

chymotrypsin which is known to exist in a rapid monomer–polymer equilibrium. The results, as shown in Figure 6, are consistent with those reported by Winzor and Scheraga (1963).

*Reaction of the Sulfhydryl Groups.* Since Gelderman and Peacock (1965) had reported that mercaptoethanol affected the chromatography, the effect of reducing conditions on dissociation and activity was examined. Enzyme was stored for 1 month in dilute solution, in which dissociation is expected, and at high concentration, where dissociation does not occur (see Figure 1), in the presence and in the absence of dithiothreitol. Results of activity assays, as shown in Table I, demonstrate a loss of activity in the absence of reducing agent in the case of dilute solutions, but the concentrated solution did not lose activity even in the absence of reducing agent. Furthermore, addition of dithiothreitol to the dilute enzyme after storage but before assaying did not increase activity. Reactivity of the thiol groups and the effects of their reaction on enzymatic activity were further examined as a function of concentration and addition of NADH (Table II). These results show that, depending on the enzyme concentration, the number of thiol groups reacting ranges from about 3 to 13 without activity loss. Addition of NADH to solutions of low enzyme concentration decreased the accessibility of sulfhydryl groups; however, in all cases some enzymatic activity was lost in the presence of NADH. Reaction of one thiol group per tetramer of bovine heart lactate dehydrogenase resulted in a loss of one-fourth the original enzymatic activity.

Effects of time of DTNB reaction or dilution from freshly prepared concentrated enzyme solutions on accessibility of sulfhydryl groups were studied (Figure 7). In all cases, the number of reacting thiol groups increased with time when reaction was initiated immediately after dilution. The effect was not due to the presence of DTNB, since addition of the reagent to solutions, which had been allowed to stand for some time after dilution, resulted in significantly greater reactivity with DTNB than that for shorter times in the presence of DTNB. Furthermore, dilute enzyme solutions which had been stored for long periods prior to reaction were more

TABLE II: Reaction of Sulfhydryl Groups of Rabbit Muscle and Bovine Heart Lactate Dehydrogenases with DTNB.[a]

| Preparation | Concn (mg/ml) | NADH | mol of SH/mol of Enzyme[b] | % Orig Act.[c] |
|---|---|---|---|---|
| Muscle | 0.49 | − | 2.81 | 93 |
| Muscle | 0.49 | + | 3.43 | 73 |
| Muscle | 0.25 | − | 13.5 | 96 |
| Muscle | 0.25 | + | 7.9 | 72 |
| Heart | 0.56 | − | 0.9 | 72 |

[a] Reaction of the muscle enzyme with DTNB was allowed to proceed for 24 hr at 23° in 0.1 M phosphate buffer, pH 8. Reaction of the heart enzyme proceeded for 2 hr under the same conditions. Ammonium sulfate suspensions were dialyzed overnight against 4 l. of the buffer prior to dilution and addition of DTNB. [b] Amount of reaction based on a mol wt of 140,000; lactate dehydrogenase. [c] Activities were measured in 3-ml assay mixtures containing 100 μl of the enzyme solution.

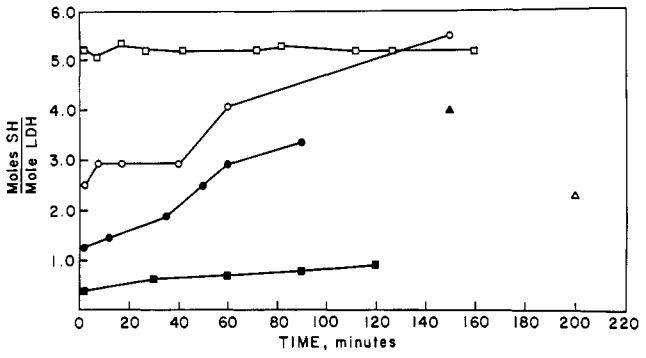


FIGURE 7: Number of sulfhydryl groups of rabbit muscle and bovine heart lactate dehydrogenases reacting with DTNB as function of reaction time. Ammonium sulfate suspensions were dialyzed against 0.1 M phosphate buffer, pH 7, giving a final stock concentration of ~5 mg/ml. At zero time, dilutions and reaction mixtures were made. Values for muscle enzyme: 0.49 mg/ml (●); 0.25 mg/ml (○); 0.15 mg/ml, stored for 1 month at a concentration of 0.75 mg/ml at 4° (□); 0.25 mg/ml, to which DNTB was added 150 min after dilution (▲); 0.25 mg/ml, containing NADH (0.033 mg/ml) to which DTNB was added 200 min after dilution (△). Values for bovine heart enzyme, 0.56 mg/ml (■).

reactive and did not exhibit a time dependence. The total number of sulfhydryl groups, as determined by reaction with DTNB in 1% sodium dodecyl sulfate, was 22.3 per tetramer for a freshly dialyzed solution, as compared to 16.4 for the dilute solution which had been stored for 1 month. Di Sabato *et al.* (1963) obtained a value of 17.3 per tetramer (mol wt = 140,000) by reaction with *p*-hydroxymercuribenzoate in concentrated urea.

## Discussion

Results from low-speed and high-speed sedimentation equilibrium, light scattering, and gel chromatography measurements indicate that rabbit muscle lactate dehydrogenase undergoes a slow dissociation starting at the time of dilution from ammonium sulfate suspensions into neutral buffers. In contrast to the behavior of bovine heart lactate dehydrogenase, as studied by Millar (1962), a rapid equilibrium between protomers was not observed for the rabbit muscle enzyme. The degree or rapidlty of dissociation varied slightly between preparations, an observation made by other investigators (Millar, personal communication). Jaenicke's (1963) light scattering results for purified human lactate dehydrogenase isozymes are considerably lower than the accepted tetrameric molecular weight suggesting that these isozymes also undergo a slow dissociation. Later, Jaenicke and Knof (1968) reported molecular weights for the rabbit muscle enzyme which indicated that the tetramer did not dissociate; however, if as stated, 1–2% solutions were dialyzed and dilutions made just prior to measurement, sufficient time would not have elapsed to allow for a detectable amount of dissociation. We have obtained results similar to theirs when stock solutions were diluted immediately prior to light scattering measurements. Furthermore, the observation that very little dissociation occurred when a 6.2-mg/ml stock solution was stored for 1 month is consistent with the results of sedimentation, gel chromatography, thiol reactivity, and enzymatic activity studies which indicate that at concentrations above *ca.* 1 mg/ml dissociation is very slow.

Addition of NADH to the solutions prevents dissociation, thereby yielding a tetrameric molecular weight of 141,000 ± 4000. This value is consistent with that of 139,000 reported for bovine heart lactate dehydrogenase tetramer (Huston *et al.*, 1972), a value of 72,000 for the dimer (Millar, 1962), and a value of 35,000 for the monomer (Appella and Markert, 1961; Huston *et al.*, 1972) and is in excellent agreement with a value of 142,000 obtained by Jaenicke and Knof (1968) for the rabbit muscle enzyme. A monomeric molecular weight of 34,400 ± 1700 was obtained, after correction of the pH 2 and 6 M Gdn·HCl data for the charge effect (Roark and Yphantis, 1971; Huston *et al.*, 1972). This is in agreement with the data cited above for the bovine heart enzyme and indicates that the low molecular weight impurity (Huston *et al.*, 1972) was not present in these preparations.

Protection of the enzyme from dissociation by NADH binding was not unexpected since a ligand-induced conformational change has been detected by X-ray crystallography (Adams *et al.*, 1969). Furthermore, Di Sabato and Kaplan (1964) observed that NADH prevented dissociation of the bovine heart enzyme in the presence of sodium dodecyl sulfate; Gelderman and Peacock (1965) found that NADH prevented mercaptoethanol-dependent changes in the rabbit muscle enzyme; Zondag (1963) found that NADH protected against cold inactivation; and Levi and Kaplan (1971) observed that NADH greatly increased the rate of reactivation of dogfish $M_4$ lactate dehydrogenase. We conclude that binding of NADH to the rabbit muscle enzyme results in conformational changes within the protein which strengthen subunit interactions.

The dimer was the smallest species which could be detected by extrapolation of low-speed sedimentation equilibrium data or by direct measurement of high-speed sedimentation equilibrium patterns. These observations have been made on three different rabbit muscle enzyme preparations, even after storage in dilute solution for periods up to 1 month. Moreover, a linear two-species plot was obtained for sedimentation equilibrium data yielding values corresponding to a dimer and tetramer. Millar (1962) has observed dissociation of the bovine heart enzyme to a dimer under similar conditions, although this isozyme apparently exists in rapid equilibrium. A slow dissociation of rabbit muscle lactate dehydrogenase to dimer, however, is consistent with the recent hydridization studies by Millar *et al.* (1971), who found that the formation of $M_3H$, $M_2H_2$, and $MH_3$ isozymes after mixing dilute solutions of rabbit muscle and bovine heart lactate dehydrogenases, or purified $M_4$ and $H_4$, was time dependent. Furthermore, $M_2H_2$ was the first detected and always "the dominant heteropolymeric band throughout incubation."

Modification of all available carboxyl groups on the enzyme surface in cold, pH 7 buffers causes nearly complete dissociation to a dimer, but again a smaller subunit was not detectable. Extensive denaturation by extreme pH or Gdn·HCl was required to produce the monomer. These data indicate that all of the subunit interactions are not of equal energy. Our data support the contention that this enzyme is also a "dimer of dimers" as has been suggested for rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Fenselau, 1972), CTP-synthetase, and other enzymes (Levitzki *et al.*, 1971). Griffin and Criddle (1970) arrived at a similar conclusion from the effect of pH on ternary complex formation. Similarly, Saito (1972) has suggested that two protomers act in cooperation as an active dimer in the tetramer based on data from heat inactivation rate studies of isozymes.

Reactivity of the enzyme's sulfhydryl groups appears to be correlated with the spontaneous dissociation of the tetramer. More sulfhydryl groups were available for reaction when the enzyme was exposed at low concentrations where physical studies indicate more extensive dissociation. Furthermore, NADH did not affect the amount of sulfhydryl reaction at high enzyme concentrations, whereas, at low enzyme concentrations, NADH protected against both sulfhydryl group reaction and dissociation. If enzyme is stored in dilute solution for long periods prior to reaction with DTNB, the amount of reaction does not vary with time as would be expected in the case of a slow dissociation. However, the total reaction is somewhat less than that of a fresh solution of similar concentration. This discrepancy could be explained by partial oxidation of exposed sulfhydryl groups during storage. In fact, comparison of the total sulfhydryl groups (reaction in 1% sodium dodecyl sulfate) of a fresh solution with those of a stored solution indicates that oxidation must have occurred. We have also observed that dithiothreitol prevents loss of activity during storage of dilute solutions; however, activity is not lost when concentrated enzyme solutions are stored either in the presence or absence of dithiothreitol. Perhaps the greatest support for the proposed correlation between thiol group exposure and subunit dissociation comes from X-ray crystallographic data of Adams *et al.* (1969) which have indicated a nonessential thiol group to be very close to the contact surface between subunits.

Reaction of sulfhydryl groups with DTNB at pH 7 did not affect its enzymatic activity. This result contrasts with that for the bovine heart enzyme which showed a direct relation between the amount of reaction and loss of activity. The latter result agrees with that for reaction of this enzyme with *p*-hydroxymercuribenzoate (Di Sabato and Kaplan, 1963). Fondy *et al.* (1965) have classified various lactate dehydrogenases into two groups according to their reactivities with *p*-hydroxymercuribenzoate; in the first group essential thiol groups are the first to react with this reagent, whereas in the second group nonessential thiols react first. The rabbit muscle enzyme thus falls into the second group along with many other $M_4$ types.

Dube *et al.* (1963) and Holbrook *et al.* (1966) have studied the inactivation of rabbit muscle enzyme by various sulfhydryl reagents; however, they did not determine the extent of sulfhydryl group reaction. The latter workers found that heart-type subunits were much more reactive (based on activity loss as a function of reaction time) than muscle-type, in agreement with our observations. Surprisingly, our data suggest that some activity is lost by reaction with DTNB in the presence of NADH, whereas this coenzyme protects the activity of the bovine heart enzyme. A ligand-induced conformational change, making the essential thiol groups more accessible, could explain the results observed.

The observation of sub-bands around the five major isozyme bands obtained by gel electrophoresis has never been adequately explained (Fritz and Jacobson, 1965). A slow, concentration-dependent dissociation and concomitant exposure of thiol groups could produce these extra bands if protomers and/or oxidized forms exhibit enzymatic activity. Other investigators (Markert, 1962; Koen, 1967) have already suggested that oligomers smaller than the tetramer are active, and studies in our laboratory (Cho and Swaisgood, 1972) have supported this possibility. Koen (1967) found that when a single sub-band was isolated and re-electrophoresed other sub-bands formed including some new bands near other isozyme regions. Since our data indicated that on standing some of the exposed thiol groups may oxidize, it seems possible that new bands represent oxidized forms of the enzyme. Furthermore, Fritz and Jacobson (1965) noted that the presence of low concentrations of mercaptoethanol enhanced the appearance of sub-bands, but when the concentration was more than 0.007 M no sub-bands were observed.

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