Substrate-Inhibited Lactate Dehydrogenase. Reaction Mechanism and Essential Role of Dissociated Subunits*

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ABSTRACT: The mechanism of substrate inhibition of lactate dehydrogenase by excess pyruvate was studied. The kinetics of formation of the abortive ternary complex, lactate dehydrogenase–oxidized nucleotide–pyruvate, were determined as a function of the reactant concentrations, pH, ionic strength, and temperature. At pH 7, the initial velocity of complex formation is one-fourth order with respect to protein concentration above 0.15 mg/ml. For more dilute concentrations, the order of the initial rate with respect to lactate dehydrogenase changes gradually from one-fourth to first order. The molecular weight of the protein from sedimentation equilibrium measurements decreases significantly below the tetrameric value of 140,000 only for protein concentrations below 0.15 0.15 mg/ml. Above 1 mM nucleotide, the kinetics of ternary complex formation become independent of nucleotide concentration. However, the reaction is first order in pyruvate at least up to 0.1 M pyruvate. Our results suggest that the monomeric subunit of lactate dehydrogenase combines with oxidized nucleotide before reacting with pyruvate in a rate-limiting bimolecular step. The ternary complex monomers may then reaggregate to tetramers with either unreacted monomers or other ternary complex monomers. A detailed analysis of the ternary complex formation kinetics as well as of the effects of pH and ionic strength strongly support such a reaction scheme. Furthermore, since the Mg^{2+} catalysis of ternary complex formation parallels the Mg^{2+} catalysis of pyruvate enolization, it appears that the enol, not the keto, form of pyruvate is preferred. Reaction of the essential sulfhydryl groups of lactate dehydrogenase with $HgCl_2$ inhibits enzymatic activity to a much greater extent than it inhibits the kinetics or equilibrium of ternary complex formation. Pyruvate also reacts with oxidized nucleotide in the absence of enzyme. The rate law for the appearance of the first spectrally observable product of this reaction is: initial velocity equals (2.7×10^{-4} M^{-1} sec^{-1} + $[OH^-] \times 93$ M^{-2} sec^{-1})[NAD][pyruvate]. Thus, near neutral pH values, this nonenzymatically formed adduct is not relevant to ternary complex formation kinetics. The significant conclusion in this study is that monomers of lactate dehydrogenase are necessary for abortive ternary complex formation whereas both the active enzyme and the stable inhibited enzyme are tetrameric.

Substrate inhibition of lactate dehydrogenase (LDH)[1] from rabbit muscle by excess pyruvate is well documented (Plagemann *et al.*, 1961; Zewe and Fromm, 1962, 1965; Stambaugh and Post, 1966). The significance of such substrate inhibition of LDH has been discussed in terms of the different abilities of the two LDH isozymes, the heart and the muscle isozymes, to control the overall rate of glycolysis (Kaplan *et al.*, 1968). Evidence from *in vitro* studies strongly suggests that a ternary complex of LDH, oxidized coenzyme, and pyruvate is responsible for inhibition of LDH by excess pyruvate (Gutfreund *et al.*, 1968; Kaplan *et al.*, 1968; DiSabato, 1968a). Reports concerning the spectral properties of this ternary complex exist for LDH from: rabbit muscle (Fromm, 1961, 1963), beef heart (Winer, 1963; Kaplan *et al.*, 1968), rat liver (Vestling and Künsch, 1968), chicken heart (Kaplan *et al.*, 1968; DiSabato, 1968a), and dogfish muscle (Kaplan *et al.*, 1968). Some studies determine the dissociation constants of the ternary complex components (Fromm, 1963; Gutfreund *et al.*, 1968; Kaplan *et al.*, 1968). From one account concerning the isolation and stoichiometry of the ternary complex (DiSabato, 1968a), it appears that the complex contains one molecule of coenzyme and one molecule of pyruvate per subunit of 35,000 molecular weight units. The same report also shows that sodium borohydride treatment reduces the ternary complex. The kinetics of formation of the LDH–NAD–pyruvate complex is dealt with in one preliminary report (Gutfreund *et al.*, 1968).

Both the active form and the pyruvate-inhibited form of beef heart LDH are tetrameric in low ionic strength solutions (Hathaway and Criddle, 1966; Mire, 1969). However, ultracentrifugal studies of beef heart LDH (Hathaway and Criddle, 1966) and X-ray crystallographic studies of dogfish muscle LDH (M. Rossmann, personal communication) indicate that the ternary complex is structurally different from the binary LDH–coenzyme complex, from the enzymatically active tetramer, and from the apoenzyme.

In this article, we proceed to examine the detailed molecular mechanism by which the abortive ternary complex is formed using both kinetic and ultracentrifugal methods.

Pyruvate also reacts with NAD in the absence of enzyme to form an adduct(s), and various forms of this adduct react with LDH to yield an inhibited binary complex (Lee *et al.*, 1966). Here we report the kinetics of the initially formed NAD–

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[1] Abbreviation used is: LDH, lactate dehydrogenase.

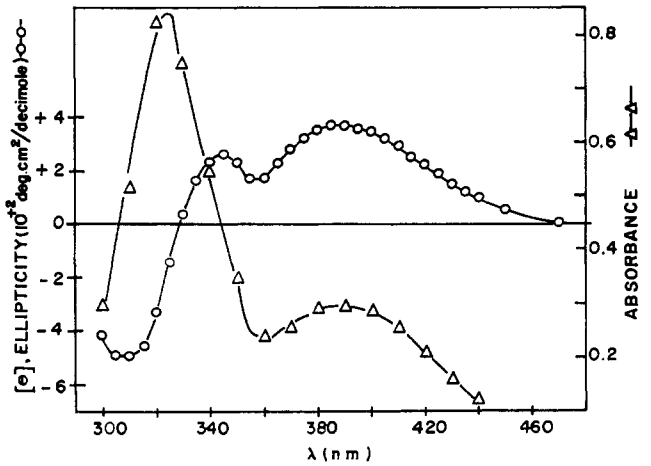

FIGURE 1: Circular dichroism and absorption difference spectra of ternary complex using 4.2 mg/ml of LDH, 1.0 mM NAD, and 10.0 mM pyruvate. Values of $[\theta]$ were calculated by assuming ϵ_{325} 7 $\times$ 10^3 cm²/35,000 molecular weight units of LDH for the ternary complex.

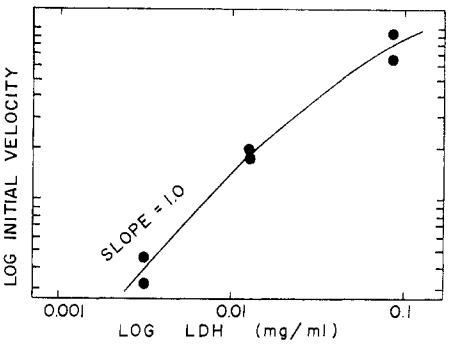

FIGURE 2: Effect of LDH on reaction order with respect to LDH. Pyruvate concentration was 10.0 mM and NAD concentration was 1.0 mM. The initial velocity is shown in relative units as $-\Delta$ (fluorescence) per unit time.

pyruvate adduct, and we assess its possible relevance to the LDH–NAD–pyruvate ternary complex formation.

Experimental Section

LDH (rabbit muscle, type II), bovine serum albumin, NAD, NADH, and sodium pyruvate were obtained from Sigma Chemical Co. All other chemicals were reagent grade and were used without further purification. Pyruvate concentrations were determined enzymatically with NADH and LDH, and LDH concentration was estimated spectophotometrically using the absorbancy index at 280 nm of 1.44 cm²/mg (Jaenicke and Knof, 1968).

LDH was prepared for all experiments by dialysis against buffer at 4° for 18 hr. All solutions of NAD and NADH were prepared freshly for each experiment while sodium pyruvate solutions were either freshly prepared or were frozen and then used within 6 hr after thawing.

Spectophotometric measurements were made using a Cary Model 14 spectrophotometer. Circular dichroism spectra were obtained with a Durrum-Jasco spectropolarimeter Model ORD/UV/CD-5. Path length varied from 10 to 50 mm. The molecular ellipticity, $[\theta]$, was calculated from $[\theta]_\lambda = 3300(\epsilon_l - \epsilon_r)$, where ϵ_l and ϵ_r are the absorptivities at wavelength λ of left and right circularly polarized light. Differences in optical density were measured directly and then converted into a difference in absorptivity. Fluorescence measurements were made with a Farrand spectrofluorometer Model 244.

Sedimentation equilibrium experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with a Spinco absorption scanning system. The meniscus depletion method of Yphantis (1964) was used as described recently (Hathaway *et al.*, 1969) except that a speed of 13,000 rpm at 20° was employed. Solvent densities were measured pycnometrically and the partial specific volume used in the calculations was 0.747 ml/g (Millar, 1962). Traces of absorbance at 280 nm *vs.* radial distance were recorded and the data were converted into ln (absorbance) *vs.* the square of the radial distance, r^2. Partially overlapping 15-point segments from the ln (absorbance) *vs.* r^2 data were processed by computer to yield a least-squares best-fit value of the slope at various distances from the center of rotation from which molecular weight values were obtained. Sedimentation velocity experiments were conducted with a Spinco Model E ultracentrifuge. Experimental conditions of 42,040 rpm at 23.5° were used. Data from photographs of Schlieren patterns were processed by computer to yield values of $s_{20,w}$.

The formation of the ternary complex, LDH–NAD–pyruvate, was initiated by adding a small aliquot (10–30 μl) of concentrated pyruvate solution on a Teflon swizzle stick to a cuvet containing LDH and NAD in buffer. The reaction mixture was quickly stirred, and either A_{325} or protein fluorescence, λ_{excit} 295 nm and λ_{emit} 345 nm, was recorded as a function of time.

Experiments to determine the effect of Mg^{2+} on ternary complex formation kinetics were carried out with 1.20 mg/ml of LDH, 1.0 mM NAD, 10 mM pyruvate, 0.1 M sodium phosphate (pH 7.2), and either 2 mM EDTA or 20 mM $MgCl_2$ at 23°.

For attempts to measure a LDH–NAD difference spectrum a double-tandem pair of matched cuvets with 1.0-cm path length in each section was used in a Cary Model 14 spectrophotometer equipped with a Cary 0–0.1 full-scale slidewire.

All experiments were conducted at 23 $\pm$ 1° in sodium phosphate buffer (0.1 M, pH 7.0) unless otherwise noted.

Results

Spectral Properties. Some spectral properties of the ternary complex, LDH–NAD–pyruvate, appear in Figure 1. The absorbance spectrum possesses maxima at 325 and 388 nm, similar to previous reports (Fromm, 1961; Winer, 1963; Vestling and Künsch, 1968). The circular dichroism difference spectrum exhibits a corresponding positive maximum at 388 nm. However, the molecular ellipticity shows a positive maximum at 345 nm, a negative maximum at 308 nm, and a value of zero at 329 nm. Mixtures of any two of the three reactants show no molecular ellipticity maxima above 300 nm. The solution of ternary complex under the conditions reported in Figure 1 shows three observable fluorescence excitations. The usual protein fluorescence, with excitation at 290 nm and emission at 340 nm, is 40 times as intense as each of the other two. Fluorescence spectra obtained with excitation maxima at 345 and 390 nm show emission maxima

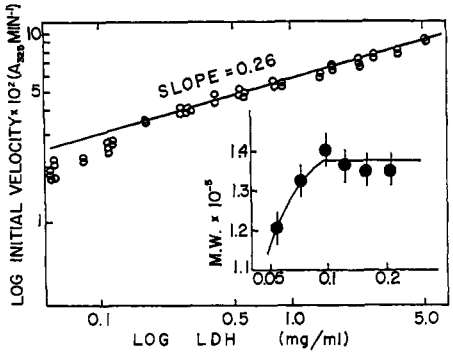

FIGURE 3: Effect of LHD on reaction order with respect to LDH and its relation to LDH molecular weight, MW. The value of 0.26 for the slope was obtained by computer calculations of the least-squares straight-line fit of the 31 data above 0.15 mg/ml of LDH. These data represent experiments done over a 6-month period which were conducted under identical solution conditions as described in Figure 2. For the molecular weight determinations, the standard buffer also contained 0.1 M sodium chloride (pH 7). Abscissa values record the actual LDH concentration.

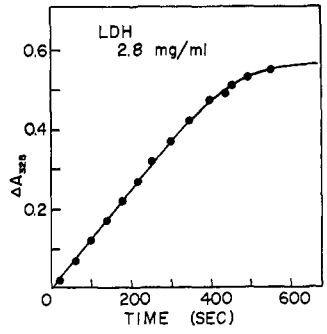

FIGURE 4: Time course of ternary complex formation under conditions described in Figure 2.

at 430 and 448 nm, respectively. Wavelength values from fluorescence experiments are accurate to ±3 nm.

A plot of ΔA_{325} *vs.* protein concentration is linear and gives a ϵ_{325} value of 7700 ± 500 per 35,000 molecular weight units. This estimate is similar to that given previously when similar complexes of beef heart LDH (Winer, 1963), chicken LDH (Kaplan *et al.*, 1968), or rat liver LDH (Vestling and Künsch, 1968) were reported.

LDH Concentration Effect. Formation of the ternary complex results in the quenching of 80% of the native protein fluorescence emitted at 345 nm (Fromm, 1963). The kinetics of fluorescence quenching at 345 nm and the kinetics of A_{325} appearance are indistinguishable when using 1 mM NAD and 10 mM pyruvate at LDH concentrations of 0.52, 0.26, 0.08, and 0.04 mg per ml. Hence, the kinetics of fluorescence quenching may reasonably allow following the time course of ternary complex formation at low LDH concentrations where absorbancy changes become too small to be measured. Figure 2 shows that the initial velocity of ternary complex formation is first order with respect to LDH concentration below 0.015 mg/ml. However, the initial velocity becomes less than first order as LDH concentration increases. Moreover, as depicted in Figure 3, the order of the initial velocity of ternary complex formation is 0.26 or one-fourth with respect to total LDH concentration above 0.15 mg/ml. Thus, initial velocity = $C[\text{LDH}]^{1/4}$ above 0.15 mg/ml of LDH, where C is a constant. If ϵ_{325} is 7700 cm²/mole of product, $C = 1.5 \times 10^{-4}$ $M^{3/4}$ min^{-1} under the conditions described in Figure 3.

The weight-average molecular weight of LDH from sedimentation equilibrium measurements as indicated in Figure 3 decreases observably below the tetrameric value of 139,000 (Jaenicke and Knof, 1968) only for LDH concentrations below 0.1 mg/ml, that is, just below the range of LDH concentration where the order of initial velocity with respect to LDH becomes greater than one-fourth order.

Figure 4 presents a typical plot of A_{325} *vs.* time which is taken as a measurement of ternary complex concentration *vs.* time. Similar data have appeared previously (DiSabato, 1968a; Vestling and Künsch, 1968). For a reaction which is pseudo one-fourth order with respect to a reactant species, B, the rate law, $-d(B)/dt = k(B)^{1/4}$, is easily integrated to yield $0.75(B)^{3/4} = -kt$, where k is a constant and t is time. A plot of $(B)^{3/4}$ *vs.* time linear for a one-fourth-order reaction. The data of Figure 4 are apparently linear for the initial portion of the reaction. However, a plot of $(\Delta A_{325})^{3/4}$ *vs.* t also appears linear for the initial portion, though not for the terminal portion, of the reaction. It is concluded that integrated rate laws cannot distinguish between a zero-order reaction and a pseudo-one-fourth-order reaction in this case.

The nature of semilog plots of A_{325} *vs.* time varies markedly with LDH concentration as shown in Figure 5. Straight lines, indicative of a pseudo-first-order reaction, obtain only for LDH concentrations below 0.07 mg/ml. For LDH concentrations between 0.2 and 3.5 mg per ml, linear data are observed over the final 25% of the total absorbance change shown in Figure 5 for 2.8 mg/ml of LDH. That is, the final 25% of the reaction is pseudo first order in absorbancy change whereas the initial stage of the reaction is pseudo one-fourth order in absorbancy change. The kinetic data from experiments with LDH concentrations above 3.5 mg/ml show curvature on semilog plots through at least 90% of the

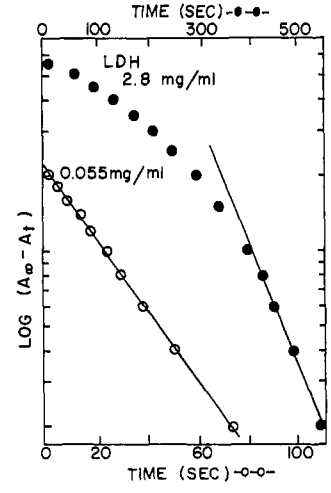

FIGURE 5: First-order reaction plot under conditions described in Figure 2. The linear terminal segments of such plots yield values for an observed psuedo-first-order rate constant, k_{obsd}. The values of k_{obsd} are 1.1×10^{-2} and 3.8×10^{-2} sec^{-1} for LDH concentrations of 2.8 and 0.055 mg per ml, respectively.

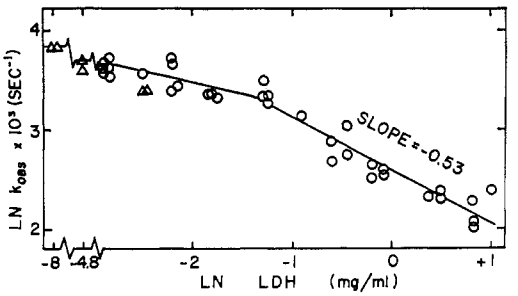

FIGURE 6: Relation of the observed pseudo-first-order rate constant to LDH concentration. Experimental conditions are described in Figure 3. Ordinate and abscissa record logarithm values. Computer least-squares fit of 20 points above 0.28 mg/ml of LDH yields a slope of −0.53, or one-half, while similar analysis of 15 points below 0.28 mg/ml of LDH shows a slope of −0.22. The open triangles represent values from fluorescence quenching experiments reported in Figure 3.

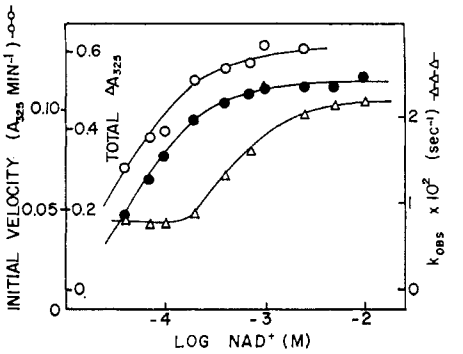

FIGURE 8: NAD effect on ternary complex formation using 2.5 mg/ml of LDH (7.1×10^{-5} of 35,000 molecular weight units/l.) and 20 mM pyruvate. Open circles indicate initial velocity values. Triangles indicate k_{obsd} values. Solid circles indicate total ΔA_{325}.

total absorbancy change. The effect of LDH concentration on k_{obsd} is indicated in Figure 6. The data are described by the equation $k_{obsd} = b/[\text{LDH}]^n$, where b is a constant and the exponent, n, an inverse reaction order, is one-half above 0.28 mg/ml of LDH. Figure 6 shows that n decreases to zero as LDH concentration decreases to the lowest measured value of 3 μg/ml where the value of k_{obsd} is determined to be 5×10^{-2} sec^{-1}. This corresponds to a half-time of 14 sec for ternary complex formation at 3 μg/ml of LDH. Figures 2, 3, and 6 emphasize that the half-time of ternary complex formation decreases upon dilution of LDH, *i.e.*, k_{obsd} increases while the initial velocity decreases.

Experiments at 35° show that the initial velocity and k_{obsd} are affected by LDH concentration in a manner similar to that depicted in Figures 3 and 6, although the total amount of ternary complex formed decreases by 30%.

The presence of 2 mg/ml of bovine serum albumin has no measurable effect on the kinetics of ternary complex formation for LDH concentrations of 0.1 or 0.6 mg per ml. This suggests that nonspecific protein–protein interactions are not important for interpreting the effects of LDH concentration on the ternary complex formation kinetics.

Pyruvate Concentration Effect. The influence of pyruvate on the kinetics of ternary complex formation is reported in Figure 7. Below 3×10^{-2} M pyruvate, both the initial velocity and k_{obsd} are first order with respect to pyruvate concentration. Deviations from linearity noted in Figure 7 for data above 3×10^{-2} M pyruvate are similar to the nonideal behavior of pyruvate described when Pedersen (1952) measured the dissociation constant of pyruvic acid. These deviations, therefore, are probably due to a decrease in activity coefficient rather than to a saturation of LDH. Half of the maximum total A_{325} change is attained for 1.3×10^{-3} M pyruvate under conditions described in Figure 7. Therefore, the overall dissociation constant of the ternary complex to LDH–NAD plus pyruvate may be considered equal to 1.3×10^{-3} M. However, this apparent dissociation constant may be the average of several different constants.

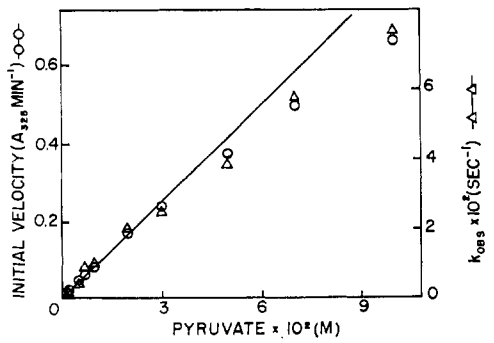

FIGURE 7: Pyruvate effect on ternary complex formation kinetics using 2.7 mg/ml of LDH and 1.0 mM NAD. Circles indicate initial velocity values. Triangles indicate k_{obsd} values.

NAD Concentration Effect. The relation of NAD concentration to the kinetics and equilibrium of ternary complex formation is depicted in Figure 8. Since both the initial velocity and k_{obsd} reach a plateau at higher NAD concentrations, NAD saturates LDH in the reaction sequence prior to the rate-limiting step of ternary complex formation. Thus, a LDH–NAD binary complex is required for ternary complex formation. Values of NAD concentration in Figure 8 reflect the total amount of nucleotide in solution without regard to whether the NAD is LDH bound or free in solution. Since the concentration of nucleotide binding sites is 7.1×10^{-5} M for these experiments, appreciable corrections should be made to find the concentration of free NAD. When such calculations are performed, a value of $2 \pm 0.5 \times 10^{-5}$ M is obtained for the concentration of free NAD necessary for obtaining half of the maximum initial velocity. For half of the maximum total change in A_{325} the concentration of free NAD is $4 \pm 0.5 \times 10^{-5}$ M. Below 0.1 and above 1.0 mM NAD the value of k_{obsd} is independent of NAD concentration. However, k_{obsd} varies from 0.0008 to 0.022 sec^{-1} when the total A_{325} change varies from 0.40 to its maximal value of 0.52. That is, k_{obsd} varies only in that region of NAD concentration over which the final 25% of the maximum total change in A_{325} is obtained. The midpoint for the variation of k_{obsd} corresponds to $4.5 \pm 0.5 \times 10^{-4}$ M NAD.

Beef heart LDH and AcNAD form a spectrally observable binary complex (Winer, 1963) with λ_{max} at 355 nm at pH 8.15. Rat liver LDH and NAD or AcNAD form similar binary complexes with λ_{max} at 335 and 355 nm, respectively, at pH 7.8 in the presence of 1 mM mercaptoethanol (Vestling

TABLE I: Dependence of Kinetic Parameters on LDH Concentration as a Function of pH.[a]

pH	Init Velocity Ratio[b]	k_{obsd} Ratio[c]
6.0	2.1	2.9
6.5	2.0	2.6
7.0	2.3	2.5
7.5	2.2	1.8
8.0	2.4	1.6

[a] Data from Figure 10 were used for these calculations. [b] Initial velocity ratio is defined as the ratio of the velocity at 2.5 mg/ml of LDH to the velocity at 0.25 mg/ml. [c] k_{obsd} ratio is defined, however, as the ratio of k_{obsd} at 0.25 mg/ml of LDH to k_{obsd} at 2.5 mg/ml.

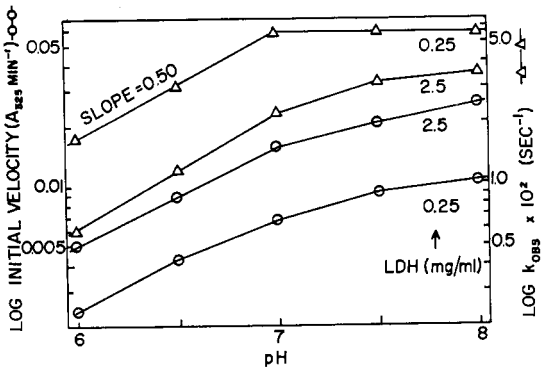

FIGURE 9: Effect of pH on ternary complex formation kinetics using 1.0 mM NAD, 20 mM pyruvate, and LDH and pH as indicated in 0.1 M sodium phosphate buffer. Circles indicate initial velocity values. Triangles indicate k_{obsd} values.

and Künsch, 1968). However, all of our attempts to observe any spectrum above 300 nm of a rabbit muscle LDH–NAD binary complex have failed. When LDH was varied from 3 to 5 mg per ml in 0.1 M sodium phosphate buffer at pH 6 or 8 with 1 mM NAD, no observable difference spectrum (±0.001 optical density unit) was recorded. However, in the presence of 1 mM mercaptoethanol at pH 8 but not at pH 6, a difference spectrum with λ_{max} 330 nm developed slowly (A_{330} min^{-1} = 0.00015). Since this spectral change occurred only in the presence of mercaptoethanol and since a mercaptoethanol–NAD adduct has a spectrum with λ_{max} 330 nm (Van Eys and Kaplan, 1957), this slowly developing spectrum most likely represents the LDH-catalyzed formation of a mercaptoethanol–NAD adduct.

Mg^{2+} Catalysis. Experiments run in triplicate show that the initial velocity of ternary complex formation is 0.053 ± 0.0001 A_{325} min^{-1} in the presence of 2 mM EDTA, while in the presence of 20 mM $MgCl_2$, it is 0.177 ± 0.005 A_{325} min^{-1}. Reaction conditions are given in the Experimental Section. The ratio of the initial velocity in the presence of $MgCl_2$ to the initial velocity in the presence of EDTA is 3.34 ± 0.15. Values of k_{obsd} are quantitatively affected in the same manner by 20 mM $MgCl_2$. Experiments using 1, 2, 5, and 20 mM $MgCl_2$ show that the catalysis of ternary complex formation is a linear function of $MgCl_2$ concentration. The overall equilibrium of ternary complex formation is only slightly affected by 20 mM $MgCl_2$ since the total A_{325} change is decreased 5% in its presence. Control experiments show that 2 mM EDTA exerts no significant effects on the ternary complex formation kinetics. Finally, since 0.1 M NaCl does not alter the initial velocity (*vide infra*), Mg^{2+} clearly catalyzes ternary complex formation.

pH Effect. Figure 9 summarizes the effect of pH on the initial velocity of ternary complex formation and on k_{obsd} at two different LDH concentrations. From the indicated slope of one-half, it appears that k_{obsd} = constant × $[OH^-]^{1/2}$ between pH 6 and 7. Above pH 7, the kinetics become increasingly independent of pH. For 0.25 mg/ml of LDH, Figure 10 shows clearly that the initial velocity and k_{obsd} are affected differently by increasing pH; however, this point is less clear for 2.5 mg/ml of LDH. The data of Figure 9 are tabulated in Table I to evaluate the influence of LDH concentration on the kinetics as a function of pH. For a tenfold increase in LDH concentration, the initial velocity ratio, defined in Table I, remains constant ranging between 2.0 and 2.4. However, for a tenfold decrease in LDH concentration, the k_{obsd} ratio, defined in Table I, decreases smoothly in going from pH 6 to 8. This variation in the k_{obsd} ratio, from 2.9 to 1.6, falls in the same range as going from the square root of 10, 3.1, to the fourth root of 10, 1.8. It is suggested, therefore, that at pH 6 k_{obsd} is inverse one-half order in LDH, whereas at pH k_{obsd} is inverse one-fourth order in LDH under the experimental conditions of Figure 9. The ratio of the total A_{325} change for the tenfold change in LDH concentration is 10 ± 0.4 at all pH values studied. pH has no effect on the equilibrium position of the reaction above pH 6.5 since the total change in A_{325} is independent of pH. However, over the pH range from 6.0 to 6.5, the total A_{325} change increases by 10%.

The $s_{20,w}$ of LDH at 5.0 mg/ml changes from 7.45 to 6.90 S when the pH changes from 6.0 to 8.0. This difference in $s_{20,w}$ of 0.55 ± 0.2 S is similar to results obtained for pig heart LDH (R. S. Criddle and J. H. Griffin, and R. Cantwell and H. Gutfreund, unpublished results).

Effect of Blocking Essential Sulfhydryl Groups. The essential thiol group of pig heart LDH can be reversibly blocked by $HgCl_2$ (Pfleiderer and Jeckel, 1967) resulting in the total loss of enzymatic activity. Treatment of the LDH–Hg^{2+} complex with cysteine fully restores the original enzymatic activity. Table II summarizes our results from experiments carried out to test the effect of blocking the essential sulfhydryl group of rabbit muscle LDH with $HgCl_2$ on ternary complex formation. Since 0.75 mg/ml of LDH is equivalent to 2.1 × 10^{-5} M monomeric subunits, preincubation of LDH with 1.4 × 10^{-5} M $HgCl_2$ could yield a maximum of 67% of monomeric LDH–Hg^{2+} complex. Table II shows that under such conditions LDH activity is inhibited 74%. On the other hand, the LDH–Hg^{2+} complex exhibits a significantly smaller decrease in the initial velocity of ternary complex formation, in k_{obsd}, and in the total A_{325} change. Table II shows that a 300-fold molar excess of cysteine over Hg^{2+} eliminates the Hg^{2+} inhibition of enzymatic activity. Thus, the ternary complex formation is much less inhibited than the enzymatic activity by the reaction of $HgCl_2$ with the essential sulfhydryl group of LDH.

Effect of NaCl Concentration. The influence of salt on

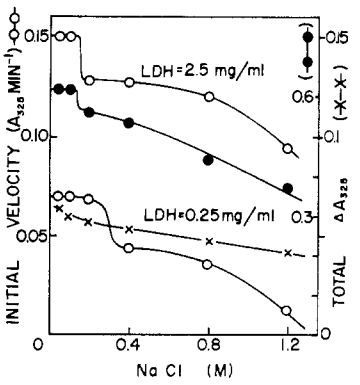

FIGURE 10: Effect of NaCl on initial velocity of ternary complex formation and on total A_{325} change. Two different LDH concentrations, as indicated, were studied with 20 mM pyruvate and 1 mM NAD. True values of NaCl concentration are 10% less than those indicated.

ternary complex formation is presented in Figures 10–12. The initial velocity shows a clear discontinuity in its dependence on NaCl concentration in Figure 11. This abrupt behavior is registered between 0.1 and 0.2 M salt for 2.5 mg/ml of LDH and between 0.2 and 0.4 M NaCl for 0.25 mg/ml of LDH. The total A_{325} behaves similarly, though this point is not as clear at the lower LDH concentration. The semilogarithmic plots in Figure 12 for 0.25 mg/ml of LDH suggest that at 0.8 M NaCl the reaction is a monophasic pseudo-first-order reaction whereas the typical curved plot for 0.05 M NaCl reflects a more complex process. The semilogarithmic plots for the experiments reported in Figure 10 possess curvature for all values of NaCl at 2.5 mg/ml of LDH. However, as is seen for 0.8 M salt in Figure 11, the first-order-reaction plot for 1.2 M NaCl at the lower LDH concentration is linear. Values of the logarithm of k_{obsd} for the experiments of Figure 10 are displayed in Figure 12 as a function of NaCl concentration. Here again the kinetics at 2.5 mg/ml of LDH exhibit a singular discontinuity near 0.2 M salt. However, the curve for 0.25 mg/ml of LDH is not so clearly biphasic. The data of Figures 10 and 12 are tabulated in Table III to evaluate the influence of LDH concentration on the kinetics as a function of NaCl. For a tenfold increase in LDH concentration, the initial velocity ratio at the three lower salt levels is 2.0 ± 0.1 which approximates very well the fourth root of 10, 1.8, indicative of a reaction one-fourth order in LDH. However, as NaCl concentration increases above 0.2 M, the initial velocity ratio increases markedly with values indicative of a one-half order reaction in LDH for NaCl between 0.4 and 0.8 M, and at 1.2 M salt, a ratio of 7.7 is fast approaching 10, a value indicative of a reaction first order in LDH. For the tenfold decrease in LDH concentration, the k_{obsd} ratio in Table III appears to decrease especially above 0.4 M NaCl until a value near 1.0 is obtained at 1.2 M NaCl. The ratio of the total A_{325} change for the tenfold change of LDH concentration is 10 ± 1 at all NaCl concentrations studied. Since the total A_{325} change in Figure 10 decreases as NaCl increases, the salt may affect either the equilibrium of ternary complex formation or the spectral properties of the complex or of its components.

TABLE II: Effect of Blocking Essential Sulfhydryl Groups on Ternary Complex Formation.[a]

	$-HgCl_2$	$+HgCl_2$	% Decrease
Initial velocity[b]	0.062	0.045	27
$k_{obsd} \times 10^3$ (sec^{-1})	12	6.8	43
Total A_{325} change	0.118	0.105	11
Activity (−cysteine)[c]	2.04	0.53	74
Activity (+cysteine)[c,d]	2.38	2.44	0

[a] Experiments involving ternary complex formation were conducted in 0.1 M sodium pyrophosphate buffer, pH 8, 23°, using 0.75 mg/ml of LDH, 10 mM pyruvate, 1 mM NAD. When indicated, 1.4×10^{-5} M $HgCl_2$ was preincubated with LDH for 10–30 min. [b] Initial velocity units are A_{325} min^{-1}. [c] Enzymatic activity, reported as A_{340} min^{-1}, was measured in 0.1 M sodium phosphate buffer, pH 8, with 0.24 mM NADH–1 μg/ml of LDH–1 mM pyruvate, at 23°. [d] Aliquots of ternary complex were preincubated for 30 min with cysteine such that [cysteine]/[$HgCl_2$] > 300.

TABLE III: Dependence of Kinetic Parameters on LDH Concentration as a Function of NaCl Concentration.[a]

NaCl (M)	Init Velocity Ratio[b]	k_{obsd} Ratio[c]
0.05	2.1	2.4
0.1	2.1	2.3
0.2	1.9	2.6
0.4	2.9	2.1
0.8	3.4	1.8
1.2	7.7	0.8

[a] Data from Figures 10 and 12 were used for these calculations. [b] See Table I. [c] See Table I. Maximum error limits are ±10%.

Kinetics of the Nonenzymatic Formation of a Pyruvate–NAD Adduct. Under alkaline solution conditions in the absence of enzyme, pyruvate, and NAD react to form new covalent bonds (Lee *et al.*, 1966). The initial observable spectral change above 300 nm during the course of the reaction of NAD with pyruvate is the appearance of a single peak with a maximum at 340 nm (A. D. Winer, personal communication; Ozols and Marinetti, 1969). The initial rate of absorbancy change at 340 nm was studied in 0.1 M glycine (pH 9.5). When pyruvate concentration varies from 2.5 to 50 mM and NAD concentration from 0.25 to 5 mM, the initial velocity is first order in each reactant (Griffin, 1969). Assuming a molar absortivity at 340 nm of 6.2×10^3 M^{-1} cm^{-1} for the adduct (Burton and Kaplan, 1954), the second-order rate constant for adduct formation at pH 9.5 is 3.0×10^{-4} M^{-1} sec^{-1}. Experiments conducted at pH values from 8.0 to 9.5 at constant NAD and pyruvate concentrations show that the reaction is approximately first order in hydroxide ion, and the overall rate law is that the initial rate of NAD–pyruvate adduct formation equals (2.7×10^{-4} M^{-1} sec^{-1} + [OH$^-$] 93 M^{-2} sec^{-1}) [NAD][pyruvate].

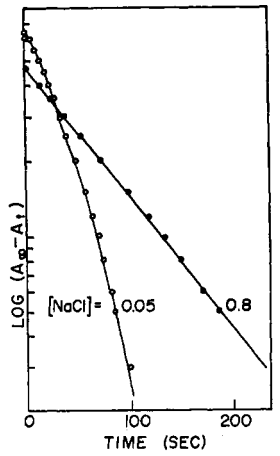

FIGURE 11: Effect of NaCl on first-order reaction plots at 0.25 mg/ml of LDH for experiments described in Figure 11.

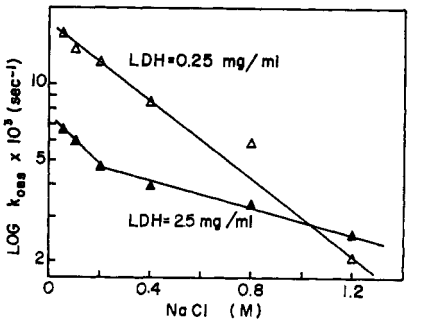

FIGURE 12: Effect of NaCl on log k_{obsd} from experiments reported in Figure 11.

At pH 9 the initial rate of adduct formation in 0.1 M sodium pyrophosphate buffer is twice the initial rate in 0.1 M glycine buffer. Thus, as previously suggested (DiSabato, 1968b), anions other than hydroxide ion may also catalyze the adduct formation.

Discussion

Spectral Properties. NAD, nucleophilic compounds, and a number of dehydrogenases form ternary complexes having spectral properties similar to the enzyme–NADH binary complexes (Van Eys *et al.*, 1958). In addition it is observed that NAD may also react with the nucleophile in the absence of enzyme to yield an adduct with a NADH-like spectrum. It is further observed that the spectral peak of the adduct is shifted 10–30 nm toward the blue in the ternary complex. Since the reduced NAD–pyruvate adduct absorbs maximally at 340 nm and the ternary complex with LDH exhibits an absorption maximum at 325 nm, it is possible that the pyruvate in the ternary complex is covalently linked to the NAD at the 4 position of the nicotinamide ring, *i.e.*, where the β carbon of the pyruvate is linked to NAD in the nonenzymatically formed adduct (A. D. Winer and H. A. Lee, unpublished results; Ozols and Marinetti, 1969). The observed value of ϵ_{325}, 7700 per nucleotide binding site, is similar to that of DPNH-like adducts (Burton and Kaplan, 1954).

The positive and negative circular dichroism peaks on either side of the sharp absorption band at 325 nm are not amenable to simple interpretation. However, one possibility is that a rotatory strength couplet arises here due to the dipole–dipole interactions of two closely located identical chromophores (*cf.* Schellman, 1968).

The broad absorption band of the ternary complex centered at 388 nm has a complementary broad circular dichroism band and also a fluorescence excitation band at the same wavelength. Model charge-transfer complexes of amino acids with NAD exhibit an extinction coefficient just slightly below that of the ternary complex at 388 nm (Shifrin, 1968), and the value of the molecular ellipticity of the ternary complex at 388 nm is approximately twice that of the value for the suggested charge-transfer complex of NAD–glyceraldehyde phosphate dehydrogenase (DeVijldner and Harmsen, 1969). Thus, the chromophore responsible for the 388-nm transition may be a charge-transfer complex between NAD and LDH in the ternary complex. Since the charge-transfer complex requires oxidized coenzyme, a covalent link of pyruvate to a reduced nicotinamide ring would seem precluded in such a case. However, the extinction coefficient at 388 nm of 2000 M^{-1} cm^{-1} is also a reasonable value for an imino bond and this or other possibilities cannot be ruled out. On the other hand, it is improbable that this absorption band is due to the presence of some bicyclic NAD–pyruvate adduct similar to the adduct(s) studied in Winer's laboratory since the spectral results of Vestling and Künsch (1968) show an absorption shoulder near 390 nm when AcNAD is used in place of NAD for ternary complex formation.

LDH Concentration Effect. The most reasonable interpretation of the effect of LDH concentration on the initial rate of ternary complex formation involves protein association–dissociation phenomena. Let L denote a LDH monomer and LP a ternary complex monomer. For tetrameric LDH, L_4, in dynamic equilibrium with dimers, L_2, and monomers of LDH, one writes that $K' = [L_2]^2/[L_4]$ and $K'' = [L]^4/[L_2]^2$, where K' and K'' are equilibrium constants. Hence, $[L]^4 = K'K''[L_4]$. Thus, the concentration of monomer is proportional to the one-fourth root of the concentration of tetrameric LDH, $[L] = (K'K'')^{1/4}[L_4]^{1/4}$. If greater than 95% of the total LDH is tetrameric, then it is a good approximation that $[L] = (K'K'')^{1/4}[\text{total LDH}]^{1/4}$. Note also that under such conditions the weight-average molecular weight would not be detectably lower than the tetrameric value. Since Figure 3 shows clearly that the initial rate of complex formation is one-fourth order with respect to LDH concentration when the observed molecular weight is indistinguishable from its tetrameric value, it is concluded that a LDH monomer is required for ternary complex formation. The approximation that LDH monomer concentration is proportional to $[\text{total LDH}]^{1/4}$ breaks down when significant percentages, on the order of >10%, of dissociated species are present. The weight-average molecular weight measured by centrifugal techniques become observably lower than the tetrameric value when the dissociated species exceed 10% of the total protein. If LDH monomers are required for ternary complex formation, a significant decrease in molecular weight and a deviation of the initial velocity from one-fourth order in *total LDH concentration* to a higher reaction order should both occur within

a narrow LDH concentration range. The data of Figure 3 confirm this prediction.

The observations that k_{obsd} is inversely proportional to the square root of total LDH concentration when k_{obsd} is derived from the terminal 25% of total A_{325} change can be easily rationalized by considering the association–dissociation of LDH. Let LP, $(LP)_2$, and $(LP)_4$ denote, respectively, a monomer, dimer, and tetramer of ternary complex. If hybrid tetramers, $L_3(LP)$, $L_2(LP)_2$, and $L(LP)_3$, were much less stable than either L_4 or $(LP)_4$, then the ternary complex formation kinetics should remain pseudo-one-fourth order in A_{325} as long as L_4 concentration exceeds 0.15 mg/ml. Such behavior is not observed as Figure 5 shows that the change in A_{325} is first order for the terminal 25% of the reaction. On the other hand, if all tetrameric aggregates are of equal probability, then when 75% of the available LDH has reacted to form ternary complex, the predominant tetramer would be $L(LP)_3$. The kinetic scheme is then formulated to be

$$L(LP)_2 \rightleftharpoons (LP)_2 + L(LP) \rightleftharpoons (LP) + L \xrightarrow{k_{obsd}} LP$$

For the linked preequilibria when most of the protein is tetrameric

$$[L] = K_i \frac{[L(LP)_3]}{[(LP)_2][(LP)]} \cong \frac{K_i A_{325}}{3\epsilon_{325}}$$

where K_i is a product of several distinct equilibrium constants and $[L(LP)_3] \gg [(LP)_2]$ or $[(LP)]$. Hence, the concentration of reactive monomer, L, is directly proportional to A_{325}, and ternary complex formation, represented as the conversion of L to LP, is first order in A_{325} as experimentally observed. Here we must emphasize that the absorbancy change is pseudo first order albeit the reaction is not first order in protein concentration. Another feature of this kinetic scheme is that $(LP)_2$ and (LP) are inhibitors of the reaction. The concentration of either monomer, L or LP, is proportional to the fourth root of total LDH concentration while the concentration of the $(LP)_2$ dimer is proportional to the square root of total LDH concentration. The reaction rate is proportional to reactant concentration divided by inhibitor(s) concentration(s), and this is in turn proportional to $[L]/[(LP)_2][(LP)]$ or $[\text{total LDH}]^{1/4}/[\text{total LDH}]^{1/2}[\text{total LDH}]^{1/4}$. Consequently, k_{obsd} is inversely related to the square root of total LDH concentration as experimentally observed for higher concentrations of LDH. When LDH concentration decreased, k_{obsd} is less affected by the presence of the inhibitory dimer $(LP)_2$, and it is observed that the value of k_{obsd} is less sensitive to total protein concentration. Thus, the kinetic scheme for ternary complex formation postulating dissociation of LDH tetramers to reactive monomers and random reaggregation of reacted and unreacted subunits adequately explains the unusual one-fourth-order initial rate law, the the relation of k_{obsd} to A_{325} changes, and the complicated dependence of k_{obsd} and of the initial rate on the total LDH concentration. The postulated kinetic scheme also accurately predicts the concentration of LDH for which significant decreases in the weight-average molecular weight become observable.

The molecular weight data of Figure 3 are derived from sedimentation equilibrium experiments employing an absorption scanning system. That such data do not contain significant artifacts is verified by similar experiments on rabbit muscle LDH in 0.1 M Tris buffer (pH 7) employing Rayleigh interference optics. These experiments show that significant dissociation, indicated by weight-average molecular weights below 130,000, occurs just below 0.15 mg/ml of protein (V. D. Hoagland, Jr., and D. C. Teller, unpublished results). These molecular weight results from absorption measurements or from interference optics contradict the work of Jaenicke and Knof (1968) which concluded that dissociation–association equilibria between the native apoenzyme and its protomers may be excluded. The kinetic data and the ultracentrifugal experiments taken together strongly emphasize the important presence of dynamic dissociation–association equilibria of LDH subunits.

The observation that dilution of LDH increases k_{obsd} although it decreases the initial velocity of ternary complex formation clarifies the preliminary work of Gutfreund *et al.* (1968) that the initial velocity of the reaction is inversely related to LDH concentration.

NAD and Pyruvate Concentration Effect. Variations of NAD and pyruvate concentrations indicate that the rate-limiting step of ternary complex formation is a bimolecular reaction between a NAD–LDH monomer binary complex and a pyruvate molecule. These results indicate that oxidized coenzyme is bound to a monomer of LDH. The obligatory order of the reaction requiring coenzyme before pyruvate in forming the ternary complex is also noted for the enzymatic reactions of LDH (Zewe and Fromm, 1962, 1965; Silverstein and Boyer, 1964).

The apparent dissociation constant for pyruvate of 1.3×10^{-3} M is approximately four times the value determined by Fromm (1963). It is also four times the inhibition constant determined for pyruvate inhibition of rabbit muscle LDH (Stambaugh and Post, 1966). However, it has been suggested that a "second" molecule of inhibitory pyruvate may add to the enzyme (Stambaugh and Post, 1966). Thus, the value of 1.3 mM obtained here for converting all of the LDH tetramer into ternary complex may represent the average of several dissociation constants for the inhibitory pyruvate, and such a multiplicity of dissociation constants would constitute an example of negative cooperativity (*cf.* Levitzki and Koshland, 1969). It should be noted that abortive LDH–NAD–pyruvate ternary complexes which do not possess observable spectral properties might arise. One such possible case for pig heart LDH at pH 6 has been reported from rapid reaction studies (Criddle *et al.*, 1968). Thus, both rapidly formed ternary complexes, not spectrally detectable, and slowly formed spectrally observable ternary complexes may be important to the inhibition of LDH.

An apparent dissociation constant, K_{diss}, of NAD from LDH–NAD binary complex has been measured by fluorescence techniques to be 0.9 mM at pH 6.9 in 0.05 M phosphate buffer. However, the observation that half the maximal initial velocity of ternary complex formation is achieved at 2×10^{-5} M NAD means that NAD binds tightly to either the first of four nucleotide sites on tetrameric LDH or to the first LDH monomer required for the reaction. However, the NAD concentration necessary for obtaining half of the maximal total A_{325} change is 4×10^{-5} M. Thus, there exists at least one other nucleotide binding site with a K_{diss} higher than 4×10^{-5} M such that the overall apparent K_{diss} of $4 \times$

10^{-5} M results from the individual contributions of sites with a K_{diss} of 2×10^{-5} M and of sites with a K_{diss} greater than 4×10^{-5} M. Since k_{obsd} varies only for the final 25% of the maximum total A_{325} change, we suggest that the fourth LDH subunit, *i.e.*, the unreacted monomer in the tetramer, $L(LP)_3$, has a K_{diss} of 4.5×10^{-4} M (Figure 8). Such could be regarded as evidence of negative cooperativity of NAD binding as shown for rabbit muscle glyceraldehyde phosphate dehydrogenase (Conway and Koshland, 1968). However, this does not show whether the L_4 tetramer exhibits any cooperativity in NAD binding. This difference in K_{diss} for the L subunit in the tetramer, $L(LP)_3$, may be due to a structural change of the LDH tetramer caused by ternary complex formation. Thus, LDH in the ternary complex may be significantly structurally different from LDH in the binary enzyme–coenzyme complex.

The slow spectral changes occurring in the solution of LDH, NAD, and mercaptoethanol at pH 8 emphasize that caution must be exercised in interpreting the spectrum of any solution containing oxidized nucleotide and a nucleophile since a wide range of nucleophiles react with NAD (Van Eys *et al.*, 1958). The spectral properties of a solution containing rat liver LDH, NAD, and mercaptoethanol which were ascribed to binary LDH–NAD complex formation may merit reevaluation (Vestling and Künsch, 1968).

Mg^{2+} Catalysis. The rate of enolization of pyruvate at pH 7.2 is enhanced by a factor of 3.15 in the presence of 20 mM $MgCl_2$ as measured by nuclear magnetic resonance measurements (Kosicki, 1968). Similarly, the initial velocity of ternary complex formation is increased by a factor of 3.34 by 20 mM $MgCl_2$. This similarity suggests that the enol, rather than keto, form of pyruvate is required for ternary complex formation and, thus, for inhibition of LDH. A recent communication by Coulson and Rabin (1969) discussed the role of the enol form in the inhibition. On the other hand, it appears that the keto form of pyruvate is required for the enzymatic reactions of LDH (Loewus *et al.*, 1955).

Blocking of Essential Sulfhydryl Groups. Hg^{2+}-treated LDH from pig heart binds four molecules of NAD–sulfite complex although all enzymatic activity is lost (Pfleiderer and Jeckel, 1967), and the dissociation constant of the NAD–sulfite adduct from the enzyme is somewhat increased. LDH treated with *p*-mercuribenzoate does not lose its NADH binding ability (Velick, 1958). Similarly, the ternary complex formation equilibrium is only slightly affected by treating rabbit muscle LDH with $HgCl_2$, a treatment which readily blocks sulfhydryl groups essential for enzymatic activity. These experiments show that the essential sulfhydryl groups of rabbit muscle LDH are not required for binding either NAD or pyruvate in the ternary complex.

pH Effect. From pH 6 to 8 the initial velocity ratio (Table I) is constant. This suggests that the dependency of initial rate on LDH concentration is the same for each pH value. Therefore, as shown at pH 7, the conversion of the first subunit into ternary complex requires NAD–(monomeric LDH) at each pH. Hyroxide ion could increase the initial rate of the reaction at saturating levels of NAD concentration by affecting the protein. For the kinetic scheme, $L_4 \rightleftharpoons L_2 + L_2 \rightleftharpoons L + L \rightleftharpoons LP$, hydroxide ion could increase the reaction rate by enhancing the reactivity of L or by favoring the dissociation of L_4 and/or of L_2. Consider the stoichiometry of each of these possibilities. One hydroxide ion per monomer would be required for the enhancement of the reactivity of L. If the four subunits of LDH interact with one another in a symmetrical manner, then one hydroxide ion per monomer would be necessary for an increased dissociation. However, if identical subunits interact in an unsymmetrical structure, then the dissociation of either L_4 or L_2 could be selectively enhanced by pH such that two hydroxide ions per four monomers are involved. Our results indicate that over the range from pH 6 to 7 the initial rate and k_{obsd} are one-half order in hydroxide ion, and on this basis it is suggested that the LDH tetramer is composed of unsymmetrical dimers. The smooth variation in the k_{obsd} ratio of Table I shows that the relation between k_{obsd} and LDH concentration changes as pH increases from 6 to 8. Since k_{obsd} is a kinetic parameter for the reaction involving hybrid tetramers composed of L and LP subunits, it is suggested that the pH change alters the subunit interactions in the hybrid tetramers.

NaCl Concentration Effect. Experiments conducted with 0.25 mg/ml of LDH show that the ternary complex formation kinetics gradually become simple pseudo first order as NaCl concentration increases. The increase in the initial velocity ratio in Table III and the fact that high ionic strength enhances the dissociation of LDH (Jaenicke and Knof, 1968) indicate that the reaction becomes pseudo first order as the percentage of monomeric subunits increases. These results, quite separately from the effects of LDH concentration on the kinetics, suggest that LDH monomers are required for ternary complex formation. Discontinuities in the data of Figure 10 and the biphasic curve in Figure 12 imply a highly specific effect of NaCl concentration on LDH structure and/or dissociability. Such a specific effect also explains the observation that increasing concentrations of NaCl markedly decrease the $s_{20,w}$ of LDH before significant decreases in the weight-average molecular weight are detectable (Hathaway, 1967).

NAD–Pyruvate Adduct Kinetics. Calculations of the initial rate of NAD–pyruvate adduct formation in the absence of enzyme using the observed rate law yield a velocity of 1.7×10^{-7} M min^{-1} or a maximal absorbance change of less than 0.001 min^{-1}. This rate is small with respect to rates reported for the ternary complex kinetics, and, thus, nonenzymatic formation of NAD–pyruvate adducts is not deemed relevant to the interpretation of the kinetic data reported here.

Ternary Complex Crystallographic Model. X-Ray crystallographic studies on dogfish muscle LDH from Rossmann's laboratory (Adams *et al.*, 1969; M. Rossmann, personal communication) furnish a model for the structure of the tetramer to 5-Å resolution. Although the monomers appear chemically (Pesce *et al.*, 1967) and crystallographically identical, the tetramer is a dimer of dimers in accordance with the suggestion of our pH studies. Such an arrangement of dimers has also been suggested for glyceraldehyde phosphate dehydrogenase on the basis of kinetic experiments (Malhotra and Bernhard, 1968). Each LDH subunit is divided into two parts by a narrower neck. The essential thiol group of the dogfish LDH is in this neck region while the nucleotide binding site is located of the wider part with the nicotinimide moiety approximately 13 Å from the essential sulfhydryl group. Our observation that blocking the essential sulfhydryl group with Hg^{2+} only slightly inhibits ternary complex formation is consistent with the finding that the position of the essential thiol is separated from the NAD binding site. Rossmann's results also show that the tetramer of LDH–NAD–pyruvate ternary

complex is structurally different from either apoenzyme or enzyme–coenzyme binary complex. The comparison of ternary complex crystals to apoenzyme crystals shows a movement of one dimer with respect to the other dimer on the order of 15 Å. It is also observed that formation of the crystalline ternary complex is not accomplished by diffusing pyruvate into crystals of LDH–NAD. Ternary complex crystals must be formed by crystallization in the presence of all three reactants. This situation may arise because the pyruvate site for ternary complex formation is inaccessible in the tetramer or dimer or because the structurally altered ternary complex tetramers can be formed only by the association or the reaction of structurally altered monomers. The X-ray studies on LDH show that the 4 position of the nicotinamide in the NAD–LDH binary complex is closely surrounded by portions of the LDH molecule and appears inaccessible to solvent (M. Rossmann, personal communication). NADH fluorescence studies are consistent with this model (Velick, 1958). If pyruvate combines covalently with NAD in the ternary complex or if the inhibitory pyruvate binding site is near the nicotinamide 4 position between the closely aligned segments of polypeptide, then the requirement for LDH monomers for ternary complex formation is reasonable on the basis of the crystallographic picture of the NAD binding site. Attachment of pyruvate to the nicotinamide 4 position at the subunits' interface resulting in dissociation of the tightly packed surrounding protein could thus alter the quaternary structure of LDH in the ternary complex as seen crystallographically. It should be noted here that the X-ray work utilized dogfish muscle LDH while rabbit muscle LDH was employed for ternary complex formation, and a total synthesis of the results from each study may be open to question.

In Vivo Significance. The concentration of "soluble" LDH in rabbit muscle (R. S. Criddle, unpublished results) and in rat muscle (Fritz *et al.*, 1969) is estimated on the order of 0.2 mg/ml. However, the possible particulate nature of LDH in the cell (Green *et al.*, 1965; Hultin and Westort, 1966; Arnold and Pette, 1968) constricts meaningful extrapolation of *in vitro* results to *in vivo* significance, and the presence of cellular compartmentalization of LDH also complicates interpretations (Güttler and Clausen, 1967). However, if the spectrally observable ternary complex is requisite for LDH inhibition, and if monomeric LDH is required *in vivo*, as it is *in vitro*, to form the ternary complex, then LDH would be highly resistant *in vivo* to pyruvate inhibition by reason of its extensive particulate state which would preserve tetrameric integrity.

Allosteric Models. The phenomena of allosteric effects (see Koshland and Neet, 1968) has usually been ascribed to conformational changes induced solely by the binding of ligands. The model of Monod *et al.* (1965) is based on the existence of two conformational states of a protein which differ in their affinities for ligand. The more general case of equilibria among sequentially altered intermediate conformational forms has been developed by Koshland and collaborators (*cf.* Koshland and Neet, 1968). However, the presence of a polymerizing protein system in which polymer and dissociated species differ in kinetic or binding properties has been shown capable of yielding sigmoidal saturation curves (Frieden, 1967; Nichol *et al.*, 1967; Wyman, 1967). In fact, dissociation–association equilibria among liganded and nonliganded forms of hemoglobin seem essential to explain the sigmoidal oxygenation of the molecule (Benesch *et al.*, 1965, 1966; Guidotti, 1967; Wyman, 1967). That is, protein–protein subunit interactions can be affected and facilitated by dissociation–association equilibria. The specific activity of rabbit muscle LDH decreases as LDH is diluted (Agatova and Kurganov, 1966; Kurganov *et al.*, 1968). Both the active form and the stable pyruvate-inhibited form of LDH are tetrameric even at 0.1 μg/ml of enzyme on the basis of active enzyme centrifugation experiments (Hathaway and Criddle, 1966; G. M. Hathaway, J. H. Griffin, and R. S. Criddle, in preparation; Mire, 1969) although inactive LDH dimers exist at very low protein concentrations (Kurganov, 1968). However, formation of ternary complex requires monomers of LDH. Hybrids such as $L(LP)_3$ and others exist and possess decreased affinities for NAD and for pyruvate as well as enhanced reactivity, *i.e.*, higher k_{obsd} values. Thus, reversible aggregation of subunits is essential for achieving altered quaternary structure and kinetic properties of LDH. The mechanism of ternary complex formation emphasizes the importance of assessing the role that association–dissociation equilibria may play in providing the molecular basis for allosteric phenomena.

Acknowledgments

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