Multiplicity of Binding by Lactate Dehydrogenases*

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ABSTRACT: We have studied the binding of nicotinamide–adenine dinucleotide (NADH) by the five forms of beef lactate dehydrogenase using fluorescence methods. Through the use of a dilution procedure in which the ratio of coenzyme to enzyme is kept constant and nearly stoichiometric, we have been able to evaluate the equilibrium concentrations of free protein, free ligand, and complex over nearly 95% of the titration range. Ten to twenty binary units of information were obtained in the different cases. Previous work covered only 30% of the titration curve and contained no more than three binary units of information. The different lactate dehydrogenases bind 4 moles of coenzyme/133,000–140,000 of protein. At pH 7.4, 0.05 M phosphate buffer, beef heart lactate dehydrogenase was found to have four independent, equal sites with $K = 3.9 \pm 0.4 \times 10^{-7}$ M.

Titrations of beef muscle lactate dehydrogenase showed marked changes in the order of the binding reaction (0.43–1.5). The most pronounced changed was observed upon the binding of the first molecule of NADH. Similar changes of reaction order were found for each of the hybrid lactate dehydrogenases. It is demonstrated that these cases of binding cannot be described by Adair's equation. Linked equilibria among protein tautomers are therefore an insufficient explanation. We believe that the observations may be explained in terms of molecular relaxation effects.

As multisubstrate enzymes, pyridine nucleotide–linked dehydrogenases offer the opportunity for the comparison of equilibrium binding studies with kinetic data. The binary complex of horse liver alcohol dehydrogenase and NADH[1] was first demonstrated fluorimetrically by Boyer and Theorell (1956). Fluorescence studies of coenzyme binding by other dehydrogenases, including lactate dehydrogenase (LDH)[1] from beef heart (Velick, 1958; Winer *et al.*, 1959; Winer and Schwert, 1959), rat liver (Vestling, 1963), rabbit muscle (Fromm, 1963), chicken muscle, chicken heart, and beef muscle (McKay and Kaplan, 1964), have since been reported.

The relationship between subunit structure and the chemical and physical properties of LDH has become of great interest with the discovery of the hybrid forms of LDH. The LDH molecule functions as a tetramer and the five electrophoretically distinct forms occurring in many animals are the combinations of two distinct types of subunit (Appella and Markert, 1961; Cahn *et al.*, 1962; Fondy *et al.*, 1964; Pesce *et al.*, 1964). The two types of subunit have been designated as H (heart type) and M (muscle type) (Cahn *et al.*, 1962).

Knowledge of the coenzyme binding properties of the dehydrogenases should elucidate some of the features of protein–ligand interaction and provide additional insight into the roles of the subunits of LDH. Using fluorescence measurements, we have studied the binding of NADH to several LDH's, including the five enzymes from beef, and horse liver alcohol dehydrogenase (ADH) to evaluate the relationship between protein subunit structure and protein–ligand interaction.

Experimental Procedures

Materials. NADH was purchased from Sigma Chemical Co. Solutions of NADH were prepared fresh each day and assayed enzymatically with excess pyruvate, at pH 7.0, in the presence of beef muscle LDH. The ratio of absorbancies at 260 and 340 mμ was 2.4. Oxidation by pyruvate was 98–99% complete. 3-Acetylpyridine–adenine dinucleotide was obtained from P & L Laboratories and reduced enzymatically by a modification of the method of Rafter and Colowick (1957). The reduced coenzyme was chromatographically purified by a variation of the procedure of Pastore and Friedkin (1961). The ratio of absorption at 260 mμ to that at 363 mμ was never in excess of 1.5 for the purified reduced 3-acetylpyridine–adenine dinucleotide.

Beef H_4 and H_3M LDH's were isolated chromatographically from a preparation of beef heart LDH obtained from Worthington Biochemical Corp. (Pesce *et al.*, 1964). Beef M_4 LDH was prepared from beef flank muscle by the procedure described by Pesce *et al.* (1964).

Preparation of the hybrids from pure beef M_4 and pure beef H_4 is based on the "quick-freeze" and "slow-thaw" method of Chilson *et al.* (1965). Beef H_4 (100 mg) and beef M_4 (150 mg) are thoroughly dialyzed against 0.1 M sodium phosphate, pH 7.0. Concentrations are then adjusted to give a solution of 0.1 M sodium phosphate, 0.5 M NaCl, 10^{-3} M β-mercaptoethanol,

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[1] Abbreviations used in this work: NADH, nicotinamide–adenine dinucleotide; LDH, lactate dehydrogenase; ADH, horse liver alcohol dehydrogenase.

pH 7.0, containing a total protein concentration of 1 mg/ml. The solution is poured into a chilled 2-liter vacuum flask. The contents of the flask are rapidly frozen over a mixture of dry ice and methanol. The mass is then allowed to thaw slowly, undisturbed, at room temperature. Freezing and thawing are repeated. About 85–90% of the initial enzyme activity remains. The solution is throughly dialyzed against 0.010 M Tris chloride, pH 7.3, containing 10^{-3} M β-mercaptoethanol. The dialyzed solution is then placed on a column of diethylaminoethylcellulose (2.5 × 60 cm) which has been previously equilibrated in the same buffer. The column is washed with 0.010 M Tris chloride until no more protein appears. The unbound enzyme is M_4. Then a gradient is applied. Tris chloride (0.010 M) and 0.22 M NaCl (500 ml, pH 7.3) are allowed to flow from overhead into 500 ml of 0.010 M Tris chloride, pH 7.3, in the mixing flask to establish an exponential gradient. Fractions containing 4 ml are collected. The enzymes are eluted from the column in the order: M_3H, H_2M_2, H_3M, and H_4. Fractions demonstrated by starch gel electrophoresis (Fine *et al.*, 1963) to be homogeneous are combined. H_2M_2 (75 mg) and H_3M (25 mg) are each concentrated by dialysis against saturated ammonium sulfate and stored as ammonium sulfate suspensions. Unlike the other four enzymes, M_3H is unstable in concentrated solutions of ammonium sulfate. The combined fractions of M_3H (60 mg) are placed in dialysis tubing (2.5 cm in diameter) and surrounded by powdered Aquacide II (obtained from Calbiochem). The tubing and powder are wrapped in a sheet of Parafilm and the solution is concentrated at 4° to give a final protein concentration of 15 mg/ml. The enzyme is then thoroughly dialyzed against 0.1 M potassium phosphate, 0.1 M ammonium sulfate, 10^{-3} M β-mercaptoethanol, pH 7.2, and stored at 4°. All experiments with M_3H were performed within a few days of its preparation.

Measurement of "low" to "high" pyruvate ratios (Pesce *et al.*, 1964) for each of these enzymes yields the following values when 10^{-4} M NADH serves as coenzyme: H_4, 3.0; H_3M, 2.38; H_2M_2, 2.00; M_3H, 1.60; and M_4, 0.93. Complement fixation studies have demonstrated that these hybrids are immunologically identical with the natural hybrids (O. P. Chilson, personal communication).

Chicken heart and muscle LDH's were gifts from Dr. Amadeo Pesce. Rat liver LDH was prepared by the procedure of Hsieh and Vestling (1965). Horse liver alcohol dehydrogenase was purchased from Worthington Biochemical Corp.

Protocol for Fluorescence Titration

1. The enzyme is removed from ammonium sulfate suspension by centrifugation, dissolved in a small volume of buffer, and dialyzed at 4° for 16 hr against 200 volumes of buffer with six changes of buffer. (The dialysis tubing has been previously washed by heating for several hours in 0.5 M acetic acid, followed by soaking in glass-distilled water containing 10^{-3} M EDTA.) Except for experiments at pH 10, the buffer is the same as that used in the titration. Any turbidity is removed by centrifugation. The enzyme is assayed by standard procedures and the concentration is determined from the molar extinction coefficients (based on a molecular weight of 140,000) at 280 mμ (Pesce *et al.*, 1964). The solution of enzyme, containing 5–15 mg/ml, is kept on ice.

2. Glass-distilled water is used in all of these experiments. At pH 7.4, a buffer containing 0.05 M potassium phosphate and 10^{-4} M EDTA is used. At pH 10.0, the buffer is 0.020 M glycine–0.020 M potassium phosphate containing 10^{-4} M EDTA. To avoid prolonged exposure of the enzyme to extremes of pH, enzyme for use in experiments at pH 10.0 is dialyzed against 0.010 M potassium phosphate, pH 8.0. The final protein concentration is 15 mg/ml. The sample is diluted at least 20-fold immediately before measurement to give a pH of 10.0. All pH values are determined at room temperature on a Beckman Model G pH meter.

3. The fluorometer cuvets (2 × 2 × 5 cm) are silicone coated by treatment with 1% dichlorodimethylsilane (obtained from the Aldrich Chemical Co.) in chloroform (Ferdinand, 1964). The volume of solution in the cuvet is 10.0 ml, corresponding to an area of 2.4 cm^2/ml. Possible losses of protein through adsorption to the walls of the cuvet are readily checked by determination of the protein fluorescence. Concentrated nitric acid is used for the cleaning of the cuvets and other glassware.

4. All measurements are made on the fluorometer described later in the paper. For NADH studies, the wavelength of the exciting light is 330 mμ. The emitted light passes through a liquid filter of 2 M $NaNO_2$ and Corning glass filters CS 3–73 and CS 5–58. The transmission maximum of this combination is 436 mμ. The fluorescence band examined is approximately that from 425 to 460 mμ.

5. The cuvet compartment is maintained at a constant temperature by means of circulating water from a constant temperature bath. Most experiments are performed in the temperature range 15–17°. The temperature is determined with a Tri-R thermistor thermometer.

6. The buffer, thermally equilibrated, is saturated with moist nitrogen to prevent photooxidation. A nitrogen atmosphere is maintained over the solution during the measurement. The background signal, arising from impurities in the water and buffer, is then determined.

7. Suitable portions of the stock enzyme solution and of the stock coenzyme solution are added manually to the cuvet with Hamilton microliter syringes so that the ratio of ligand to protein concentration is always constant and nearly stoichiometric. Excess solution is removed from the needles of the syringes and the tips of pipets by immersion in a beaker of distilled water. The solution is stirred gently with a plastic rod and the signal is graphically recorded for about 1 min. Instability, found only in the very dilute solutions, seems to arise primarily from photooxidation and is prevented through the use of nitrogen. The silicone coating helps to prevent losses of protein by adsorption

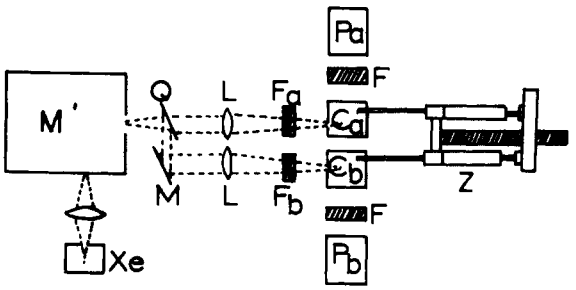

FIGURE 1: Plan of fluorescence titrator. Xe, 150 watt Xenon arc; M', grating monochromator; Q, aluminized quartz plate; M, aluminized mirror; F, F_a, and F_b, filters; C_a and C_b, cuvet holders; P_a and P_b, EMI 6255 photomultipliers; and Z, syringes.

to glass. The rate of loss of signal, in the most dilute solutions, is always less than 4% of the total signal/minute. Initial time readings are used. The solution is then discarded. This procedure is repeated with concentration increments of about 10% so that a series of independent measurements of fresh solutions is obtained. The most dilute solutions are always examined first to avoid contamination.

8. Calculations of the concentrations of free and bound coenzyme are based on the equations of Laurence (1952). Standard curves for free NADH fluorescence are obtained with the automatic titrator described.

The fluorescence titrator plan is shown in Figure 1. The exciting wavelength is isolated from the output of the 150 watt xenon arc X by means of the grating monochromator M (Bausch and Lomb: *f*/4.4, dispersion 16.6 A/mm at exit slit). The beam emerging from the exit slit S is split by the quartz plate Q, one of the surfaces of which is aluminized to reflect some 30% of the incident light. The reflected beam is rendered parallel to the transmitted by means of the aluminized mirror M, and both beams are focused at the center of the cuvet holders C_a and C_b by means of quartz lenses. Additional filters F_a and F_b may be used if it is desired to remove traces of parasitic light in the exciting beam. The fluorescence filters F are selected to transmit a broad band of frequencies represented in the fluorescence spectrum of the solutions while excluding the exciting light frequencies. The cuvet holders C_a and C_b are provided with independent jackets through which water at constant temperature is circulated. These holders are designed to take cuvets of 2 × 2 cm base dimensions and 5 cm height. Adaptors are used to accommodate cuvets of more conventional design. A small amount of concentrated solution may be delivered to the cuvets by means of narrow Teflon tubing connecting them to precision syringes, Z. The plungers of the syringes are advanced at constant selected speed by a screw device driven by a synchronous motor while the contents of the cuvets are made homogeneous by rapid magnetic stirring. P_a and P_b are small cathode EMI 6255 photomultipliers. They are supplied with stabilized dynode voltages of 1–1.5 kv according to the sensitivity desired. The output of the photomultipliers P_a and P_b may be directly recorded or subjected to electronic computing in real time. Up to the present time the instrument has been used mainly as a single-ended instrument.

The photomultiplier B was used to monitor continuously the exciting light to detect and correct for variations in intensity of the exciting source. P_a was directly attached to a sensitive recorder (Electro Instruments Model 400) with maximum sensitivity of 1 mv/in. A parallel combination of resistor of 50 kilohms and capacitor of 2 μF was used as anode load so as to give a time constant of 0.1 sec to the photooutput. This was intended to fall just under the slewing speed of the recorder (0.3 sec for full deflection). A solution of 1.25×10^{-7} M NADH in pH 7 buffer (quantum yield = 0.03) produced a photocurrent of 0.85 mv, when the total dynode potential applied was 1.2 kv. Under the same conditions a background signal of 0.85 mv was produced by the buffer alone. The signal:noise ratio under this circumstance was 17:1 so that detection of the fluorescence of NADH at a molar concentration of 1.5×10^{-8} was the useful limit of the instrument. This limit is largely determined by the magnitude of the background signal, since neither further amplification nor increase in the intensity of the exciting light can lead to a real improvement in sensitivity, when the signal to background ratio is small. To keep the background a minimum, the following devices were found of value: (a) use of large cuvets, which keeps the cuvet's edges (a source of background signal) well out of view of the detector; (b) limitation of the exciting beam to a narrow (2 mm) pencil traversing the center of the cuvet; (c) use of glass-distilled water to prepare all buffers. Small time fluctuations were found in the output of the xenon arc, usually no larger than 1 or 2% when observation was confined to a short period (minutes). It is our belief that much of the instability of fluorescence readings, attributed to exciting light fluctuations, is often due to poor common mode rejection by the amplifiers and recorders used.

Results

Stoichiometry Determinations. All LDH's studied have been found to have molecular weights of approximately 140,000 (Fondy and Kaplan, 1965). There is no clear evidence, however, that all of these enzymes possess the same number of coenzyme binding sites. Reported values range from four binding sites/mole for beef heart LDH (Velick, 1958; Winer *et al.*, 1959) to two binding sites/mole for other LDH's. To determine the number of binding sites, conditions of stoichiometric addition must be reached. It is necessary to use enzyme concentrations which are larger than, or at least equal to, the largest dissociation constant of the coenzyme. Use of a lower concentration will result in a lower number of binding sites than actually present. Since we have found that various LDH's have different average affinities for the coenzyme, minimum conditions for stoichiometric addition vary with the source

of enzyme. For the examples reported in this paper, stoichiometric addition is realized with protein concentrations of 1–2 mg/ml. The protein is titrated with NADH, both in the presence and absence of 0.1 M oxalate (Winer and Schwert, 1959) or with reduced 3-acetylpyridine–adenine dinucleotide. Table I contains the results. It is clear that all of the LDH's examined have combining weights of approximately 35,000, or bind 4 moles of coenzyme/140,000 g. This is consistent with the tetramer hypothesis. Part of the previous discrepancies have probably arisen from failure to achieve stoichiometric addition.

TABLE I: Combining Weights of Lactate Dehydrogenases.

Enzyme	$E_{1\text{ cm}}^{1\text{ mg/ml}}$	Grams LDH[a]/ Mole DPNH Bound $\times 10^3$	Molecular Weight Calcd for Tetramer $\times 10^3$	Published Molecular Weight $\times 10^3$
Beef H_4	1.50[b]	35.0	140	131,[b] 135[c]
Beef M_4	1.29[b]	34.2	137	151[b]
Chicken H_4	1.36[b]	33.3	133	151
Chicken M_4	1.56[b]	34.1	136	140[b]
Rat M_4	1.25[d]	34.0	136	126[d]

[a] Coefficient of variation is ±0.03. [b] Pesce *et al.* (1964). [c] Neilands (1952). [d] Gibson *et al.* (1953).

Titration Curves. Binding of NADH by horse liver ADH is shown in Figure 2. Seven to eight bits of information were obtained. The curve (solid line) calculated for a single dissociation constant of 2.9×10^{-7} M gives an adequate description of the observed behavior at pH 7.4 (circles). A span of 1.9 logarithmic units is covered in the range of 10–90% saturation. The coefficient of variation of K is ±0.1. Our dissociation constant is in excellent agreement with the fluorimetrically determined value of 3.1×10^{-7} M at pH 7 reported by Theorell and McKinley McKee (1961) and with the kinetically determined constant of 2.7×10^{-7} M at pH 7.4 of Dalziel (1963). In agreement with the recent report of Theorell and Yonetani (1963), we find that horse liver ADH maintains two binding sites throughout the pH range of 7 to 10.

A similar analysis of beef heart LDH is given in Figure 3A. Again, seven to eight bits of information were obtained. The Hill plot (Wyman, 1964) in Figure 3B indicates that j, the apparent reaction order (Weber, 1965), is 1.2. For independent, equivalent sites, j must equal one. For LDH, four is the maximum value of j

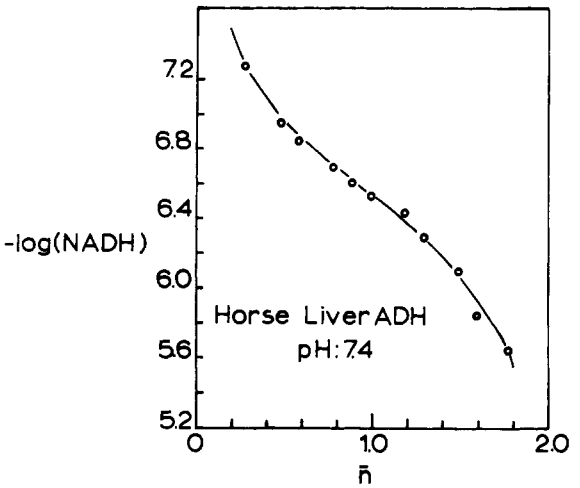

FIGURE 2: Titration of horse liver ADH with NADH. $\bar{n}$ = average number of moles of NADH bound/mole of protein; [NADH] = concentration of free NADH. Conditions: 15°, 0.042 M potassium phosphate, pH 7.4.

consistent with Adair's equation. Thus, under these conditions at pH 7.4, the binding of NADH by beef H_4 approaches the behavior predicted for identical sites with $K = 3.9 \pm 0.4 \times 10^{-7}$ M.

Examples of more complex binding behavior are found in beef muscle LDH and in the hybrids. The information contained in each of these experiments was about twenty bits. The characteristic feature of NADH binding by beef M_4 and by the hybrids is the appearance of abrupt changes in the reaction order as $\bar{n}$ varies from 0 to 4 (see Figures 3A through 6B).

Before attempting an analysis of this behavior, it is important to rule out several sources of error. The most obvious possibility is that the fluorescence enhancement does not all arise from NADH. This is readily eliminated by the observation that the addition of appropriate amounts of sodium pyruvate results in the immediate disappearance of the fluorescence. Another possibility is that the quantum yields are not constant for the 4 molecules of coenzyme bound.

The Quantum Yield of Complexes of LDH with Coenzyme. Independence from $\bar{n}$. The constancy of the quantum yield of LDH–coenzyme complexes can easily be inferred from some of the experimental data presented here. Proof of constancy of quantum yield can be given by the existence of an isoemissive point (Daniel and Weber, 1965) in the system of LDH and stoichiometrically bound coenzyme. If a solution of two fluorescent substances (LDH and LDH–coenzyme complexes in our case) is excited at a wavelength at which the first component absorbs $(1 - \alpha)A$ quanta and the second component αA quanta, and the fluorescence spectrum of the mixture is recorded under conditions such that reabsorption of the radiation is negligible, then the total fluorescence at any given wavelength

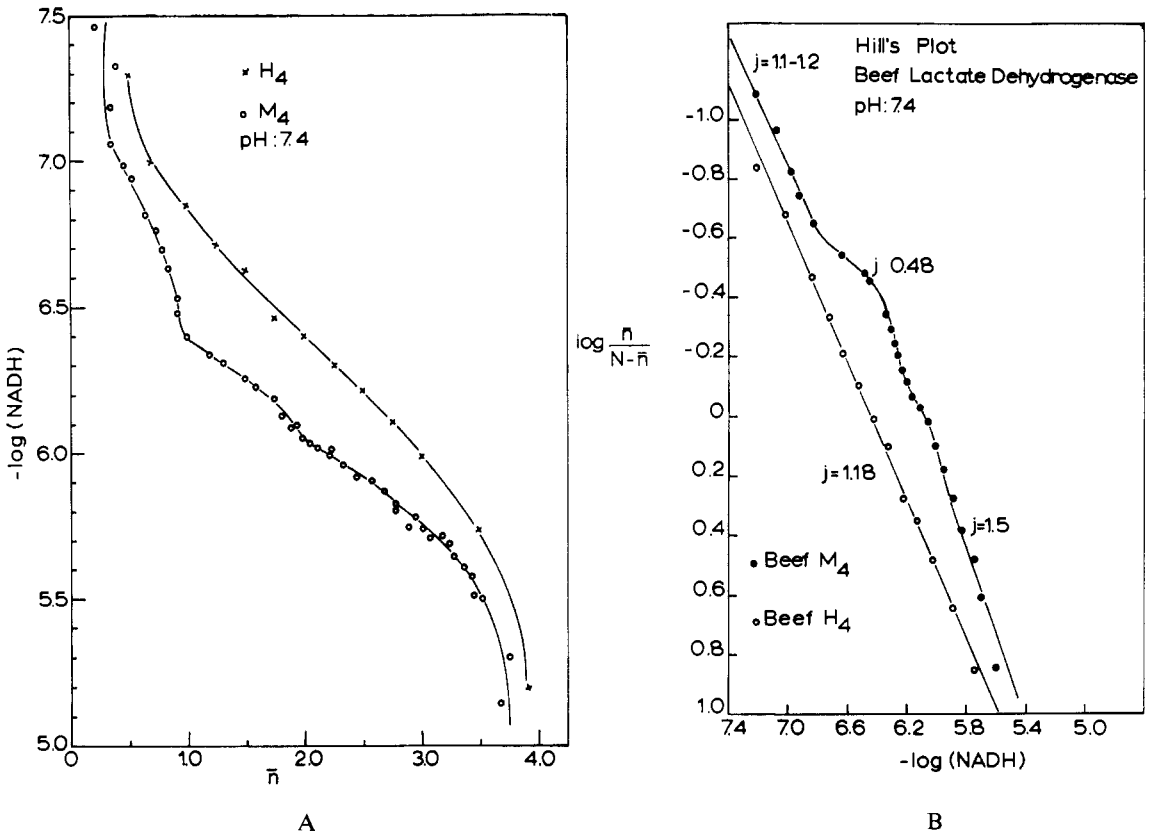

FIGURE 3: Titration and binding of beef heart and muscle. (A) Titration of beef muscle (M_4) and beef heart (H_4) LDH with NADH. O, Beef M_4; ×, beef H_4. Conditions: 17°, 0.050 M potassium phosphate, 1.0×10^{-4} M EDTA, pH 7.4; $\bar{n}$ and [NADH] are defined under Figure 2. (B) Hill plot of the binding of NADH by beef heart and beef muscle LDH. ●, Beef M_4; O, beef H_4; $\bar{n}$ is the average number of moles of NADH bound/mole of LDH; N is the maximum number of binding sites (in this case 4); [NADH] is the concentration of free coenzyme.

λ is the sum of two contributions:

$$F_1(\lambda) = A(1 - \alpha)(1 - f)q_1(\lambda)T(\lambda) \quad (1)$$

$$F_2(\lambda) = \alpha A q_2(\lambda)T(\lambda) + (1 - \alpha)Afq_2(\lambda)T(\lambda) \quad (2)$$

In the above equation f is the fraction of the excited states of LDH transferred to the coenzyme by radiationless transitions (Velick, 1961), $q_1(\lambda)$ and $q_2(\lambda)$ are the absolute fluorescence efficiencies of the components at wavelength λ, and $T(\lambda)$ is a factor depending upon monochromator transmission and detector response. The first term of $F_2(\lambda)$ represents the fluorescence of the second component (LDH–coenzyme complex) due to direct excitation of the coenzyme, the second term the fluorescence due to radiationless transfer from excited LDH to coenzyme. The two equations above give

$$F(\lambda) = F_1(\lambda) + F_2(\lambda) = Aq_1(\lambda)T(\lambda) - [q_1(\lambda) - q_2(\lambda)]T(\lambda)A(f + \alpha(1 - f)) \quad (3)$$

Equation (3) shows that if there is a wavelength λ at which $q_1(\lambda) = q_2(\lambda)$, the total fluorescence at this wavelength is proportional to A. If A remains constant, a series of fluorescence spectra of arbitrary mixtures of the two components (a fixed concentration of LDH and increasing concentrations of reduced 3-acetylpyridine–adenine dinucleotide in our case) will all cross at one wavelength if q_1 and q_2 are constant (complex fluorescence independent of $\bar{n}$ in our case). This unique wavelength is independent of $T(\lambda)$ and has therefore physical significance, much in the same way as the isosbestic point in absorption. We propose to call it the isoemissive point and from the above discussion it follows that there can be an isoemissive point only if the quantum yields of the two components are independent of their proportions.

Experimentally, the existence of two components alone was ensured by keeping the concentration of LDH sufficiently high (5×10^{-6} M) so that free coenzyme was negligible at all values of $\bar{n}$. Since LDH has a large quantum yield (McKay and Kaplan, 1964), it is advantageous to use a coenzyme analog which gives a high quantum yield on binding to LDH. Thus, reduced 3-acetylpyridine–adenine dinucleotide was selected. The maximum concentration of coenzyme was 2×10^{-5} M. The value of A at wavelength of excitation (280 mμ) was 0.90 for LDH alone and 0.92 for the solution most concentrated in coenzyme. Thus A can be con-

sidered constant, as required. Reabsorption of the radiation at the isoemissive point was less than 1% at the highest concentration of coenzyme employed. The fluorescence tracings in Figure 7 were obtained by use of the spectrofluorometer described by Weber and Young (1964). Similar isoemissive point determinations indicate that the quantum yield is independent of n in each of these five enzymes. In addition, the unique slope found in recordings of fluorescence in which NADH, or the acetyl analog, is added to a solution of LDH under conditions of stoichiometric addition is only possible if the quantum yield is independent of $\bar{n}$. In the hybrids, the absolute quantum yields are intermediate between the values observed for M_4 and H_4 (Figure 8).

Independence of n from the Protein Concentration. It is possible that there are protein–protein interactions, or that the activity coefficient of the protein is not constant. If this were the case, dilution of the system at constant free ligand concentration ought to produce changes in $\bar{n}$. On the other hand, if protein activity coefficients are independent of concentration, there should be a unique value of $\bar{n}$ for each concentration of free NADH, regardless of the protein concentration. To rule out a dependence on protein concentration, the following experiment was performed. A solution containing 2.9×10^{-7} M beef muscle LDH and 1.45×10^{-6} M total NADH gives a value of two for $\bar{n}$, and the concentration of free NADH is 8.5×10^{-7} M. Various dilutions of this NADH–LDH mixture were made with a solution of 8.5×10^{-7} M NADH so as to maintain a constant concentration of free ligand.

Setting $[E_T]$ = total enzyme concentration, [E-NADH] = complex concentration, β/β_0 = fluorescence enhancement factor, F = total fluorescence, since $F = \beta_0[\text{NADH}] + \beta[\text{E-NADH}]$, and $[\text{E-NADH}]/[\text{E}]_T = 2$

$$F = \beta_0[\text{NADH}] + 2\beta[\text{E}_T]$$

Thus a linear relationship should be obeyed in a plot of F (observed fluorescence) *vs.* $[E_T]$ (the total protein concentration). The quotient slope × [NADH]/intercept = $2\beta/\beta_0$, or twice the enhancement factor (β/β_0).

The results of this experiment are given in Figure 9. Over the tenfold protein concentration range of 3×10^{-8} to 3×10^{-7} M there are no deviations from linearity. The value of the above quotient is 6.0; since the enhancement factor is 3.1 to 3.2, it is valid to conclude that the discontinuities in the titration curve do not arise from a dependence on the protein concentration.

Our observations on the binding of NADH to horse liver ADH and beef heart LDH give credence to the results obtained with the other enzymes. The concentration ranges examined are similar in each case and the same systematic errors apply. It is important to mention that similar results have been obtained with two independent preparations of beef muscle LDH.

Applicability of Adair's Equation. Large changes of slope corresponding to small increments in $\bar{n}$ are found in the Hill plots (Figures 3B–6B). Equation 27 of paper I

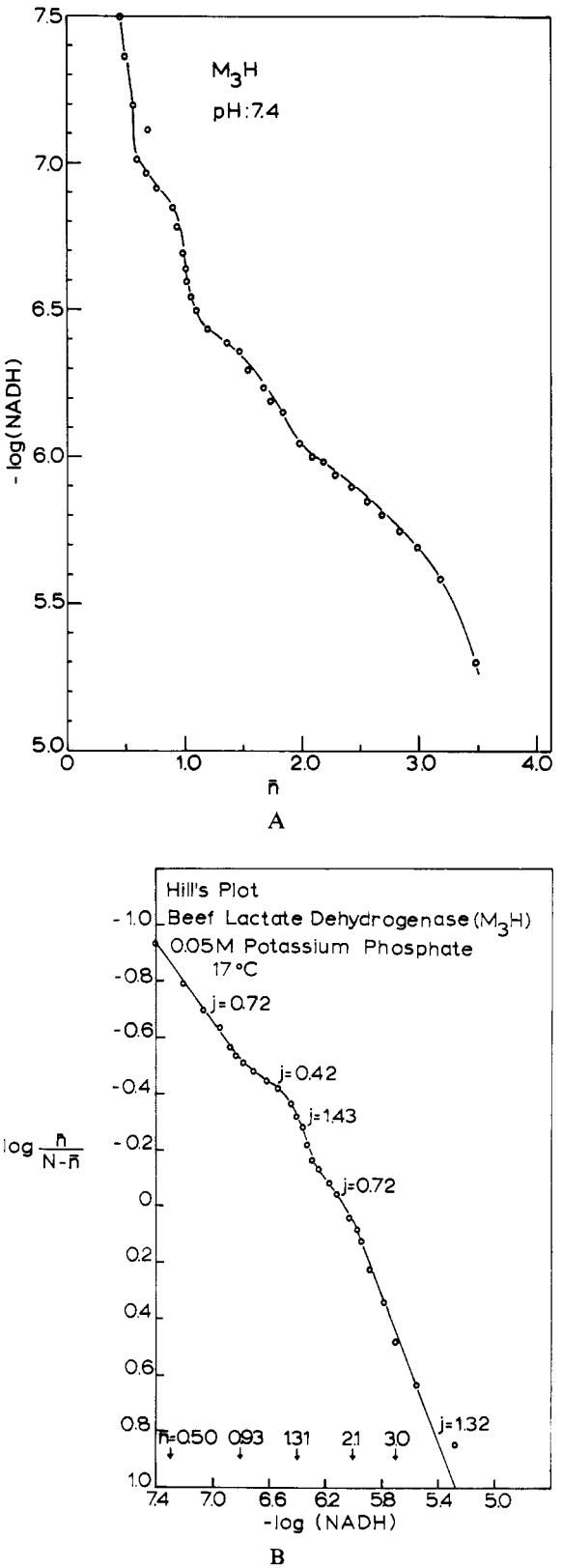

FIGURE 4: Titration and binding of beef M_3H. (A) Titration of beef M_3H with NADH. Conditions are those given under Figure 3A. (B) Hill plot of the binding of NADH by beef M_3H. For details, see Figure 3B.

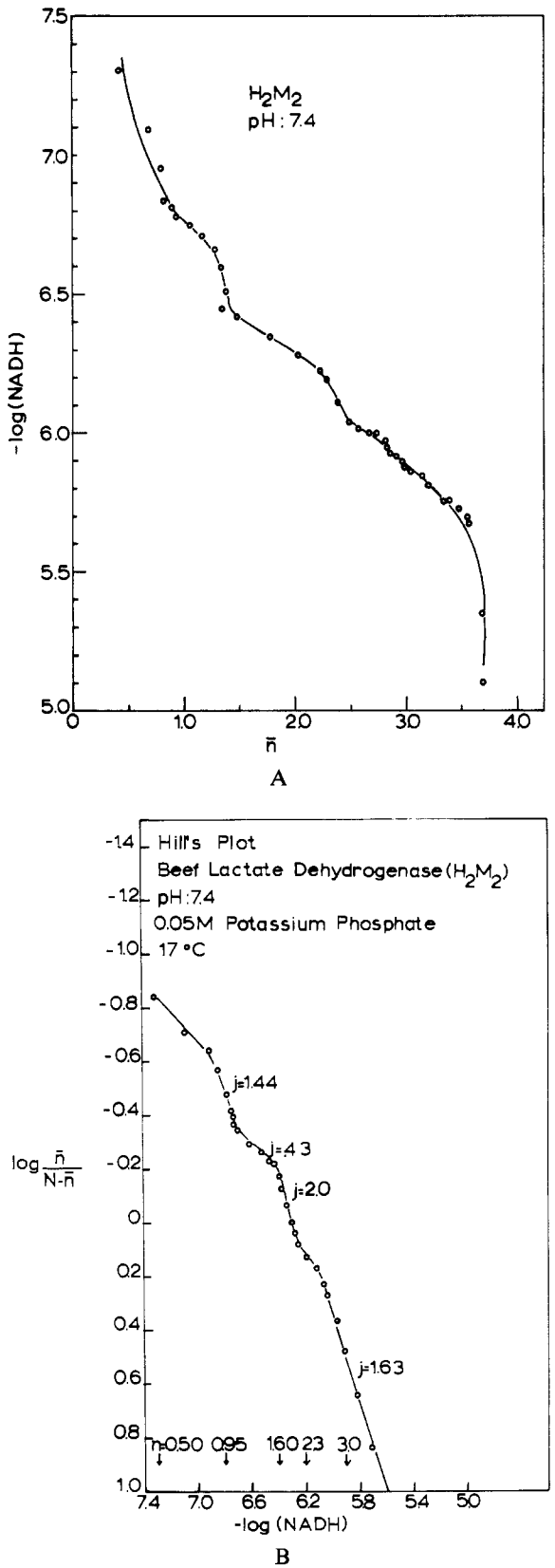

FIGURE 5: Titration and binding of beef H_2M_2. (A) Titration of beef H_2M_2 with NADH. Conditions are given under Figure 3A. (B) Hill plot of the binding of NADH by beef H_2M_2. See Figure 3B for details.

(Weber and Anderson, 1965) was applied to determine whether such changes in slope are at all consistent with the description of the system by Adair's equation. The test requires the determination of the ratio $\Delta S/\Delta\bar{n}$ from the Hill plots. The value of Δn selected must be sufficiently large to ensure that the values of $\bar{n}$ have the slopes attributed to them. Thus, although the maximum value of $[\Delta S/\Delta\bar{n}]$ cannot be determined with precision, a lower limit for this ratio can be ascertained.

For beef M_4, there is a change in reaction order near $n = 1$. From Figures 3A and 3B, we obtain the following pairs of values for $-$ log NADH and $\bar{n}$, respectively: 6.90, 0.61; 6.80, 0.75; 6.55, 0.95. From the first two pairs $S = 1.16$; from the last two pairs $S = 0.45$. Thus $\Delta S/\Delta\bar{n} \leq -2.1$. From equation (27) of the first paper, $\Delta S/\Delta\bar{n} \geq (S - N)/(N - \bar{n})$. Therefore for Adair's equation to apply, $\Delta S/\Delta\bar{n} \geq -1$. A similar analysis of M_3H and H_2M_2 yields the following values. For M_3H, $\Delta S/\Delta\bar{n} \leq -1.5$, whereas $(S - N)/(N - \bar{n}) = -1.04$; for H_2M_2, observed $\Delta S/\Delta\bar{n} \leq -2.5$, whereas $(S - N)/(N - \bar{n}) = -1.1$.

Thus it is clearly seen that the binding of NADH to M_4, M_3H, and H_2M_2 cannot be described by an equation of the Adair type. In each case, the absolute value of $\Delta S/\Delta\bar{n}$ is significantly larger than the predicted value of one at $n = 1$.

Titrations of M_4 enzymes from the chicken and the rat also indicate changes in the order of the binding reaction. The simple binding properties of beef heart LDH in 0.05 M potassium phosphate, pH 7.4, are not inflexible. At pH 10 (Figure 10) the titration curve of beef H_4 and NADH is no longer described by a single dissociation constant and is qualitatively very similar to the curves found for M_4.

Discussion

The experimental evidence presented in this paper is in our opinion conclusive in one respect: the binding of NADH by beef muscle and hybrid lactate dehydrogenases cannot be described by an equation of the Adair type. It can be demonstrated (Weber and Anderson, 1965) that Adair's equation will adequately describe those cases in which an indefinite number of tautomeric forms of protein exist, provided the proportions of the tautomers making up the free protein forms on the one hand, and the complexes with a given number of moles of ligand on the other, are the same for all values of $\bar{n}$. We are therefore entitled to conclude that this is not the case in the binding of NADH to these lactate dehydrogenases.

The simplest hypothesis to explain a change in the proportions of the conformations with the degree of saturation of the system is that the conformations are the result of protein–protein interactions of some kind, most likely of those that result from a rapid, reversible equilibrium between protein tetramers and lower order polymers. The latter possibility is ruled out by several independent observations. Hybridization does not take place in dilute solutions, or indeed under any conditions

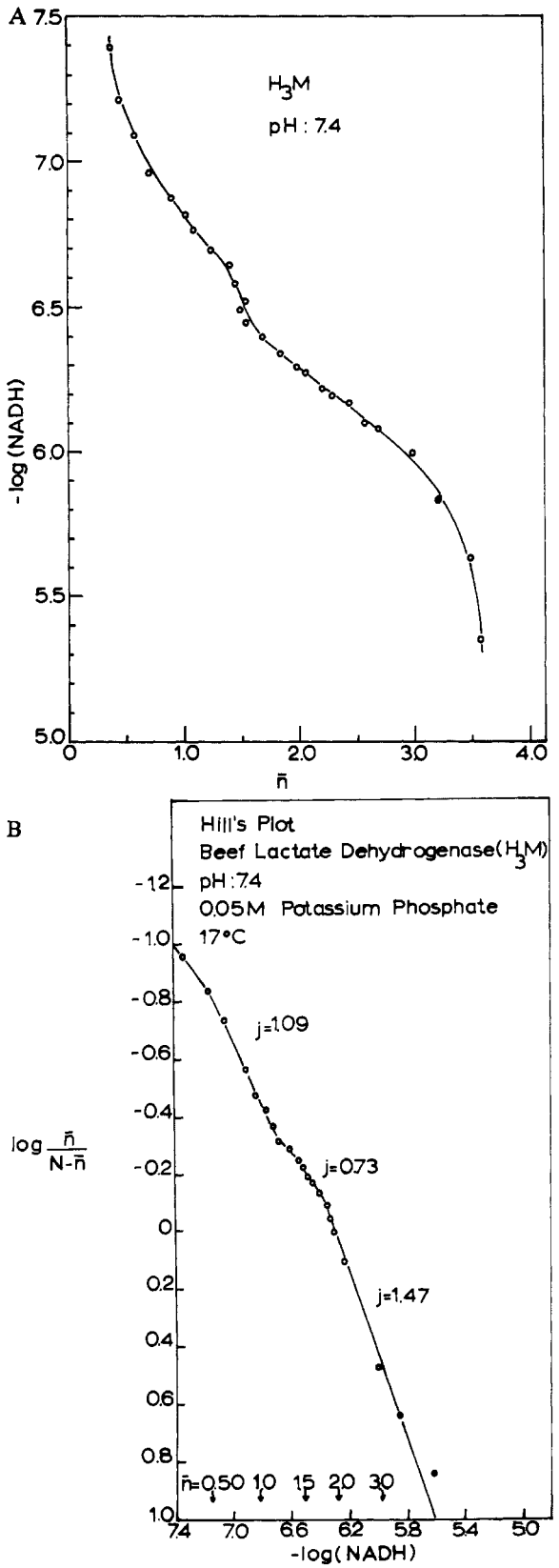

FIGURE 6: Titration and binding of beef H_3M. (A) Titration of beef H_3M with NADH. Conditions are given under Figure 3A. This H_3M is the natural hybrid isolated from beef heart. (B) Hill plot of the binding of NADH by beef H_3M. Details are given in Figure 3B.

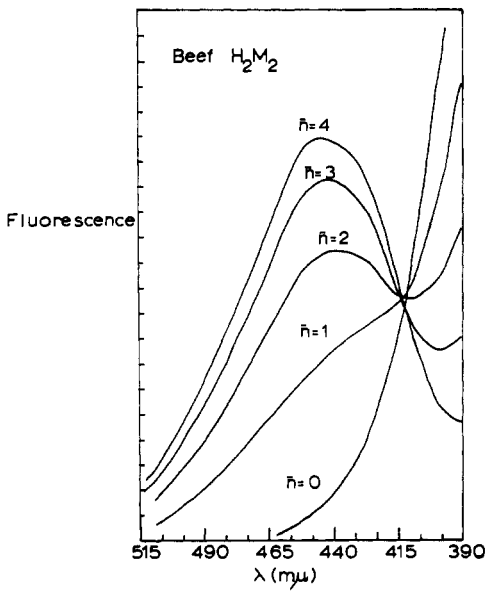

FIGURE 7: Isoemissive point determination of beef H_2M_2 and reduced 3-acetylpyridine-adenine dinucleotide. Conditions are 5.0×10^{-6} M LDH in 0.05 M phosphate buffer, pH 7.4, 25°. Spectrum is excited with 280 mμ light. Fluorescence is given in arbitrary units. $\bar{n}$ is defined in Figure 2.

used in these experiments (Chilson *et al.*, 1965). Sedimentation studies have shown constancy in molecular weight over a wide range of protein concentrations (D. N. Raval and H. K. Schachman, personal communication). The clear electrophoretic and chromatographic separation of the hybrids is only possible if the time for the achievement of the equilibrium is much longer than the experimental times. Finally, our own experiment showing the constancy of the degree of saturation under constant free ligand concentration over a wide range of protein concentrations (Figure 8) shows that there is no reversible protein dissociation relevant to the observed anomalies of binding. This experiment also rules out other less obvious kinds of protein–protein interactions. The fact that the binding anomalies are observed at protein concentrations of 10^{-6} M and less, at which intermolecular distances are on the average greater than 500 A, and that they remain, or are even enhanced by increase in ionic strength, would in any case discredit a protein–protein interaction hypothesis.

As discussed in the previous paper, we are thus lead to put forward the existence of relaxation effects in the protein molecule as the most probable cause of the phenomenon. By assuming the existence of relaxation processes in binding, we are compelled to deny the existence of detailed balance among the participants in the ligand–protein equilibrium. It has been recognized (Landsberg, 1961) that the existence of a steady state does not imply by itself that detailed balance must exist among the molecular species concerned. Because of the limited stability of proteins in solution,

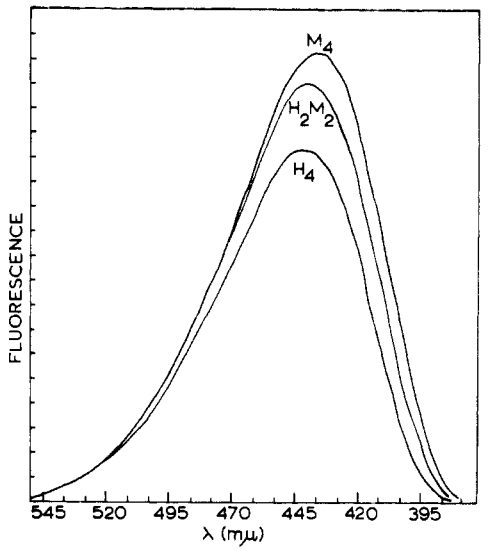

FIGURE 8: Fluorescence spectra of reduced 3-acetylpyridine–adenine dinucleotide bound to LDH. The concentration of LDH was 5.0×10^{-6} M and that of coenzyme 1.5×10^{-5} M. Wavelength of exciting light is 355 mμ. Note difference of 4–5 mμ in emission spectra of complexes of M_4 and of H_4. Fluorescence is in arbitrary units. Other conditions are given under Figure 7.

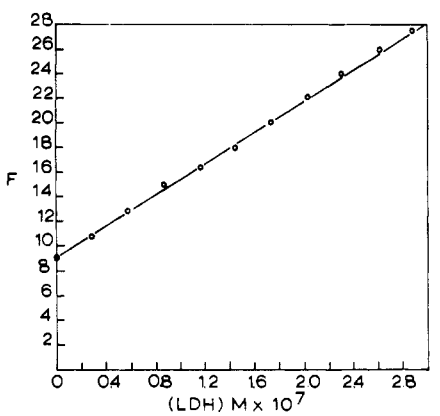

FIGURE 9: Effect of dilution on beef muscle LDH–NADH complex. $\bar{n} = 2$; [NADH] is held constant at 8.5×10^{-7} M. For other conditions, see Figure 3A.

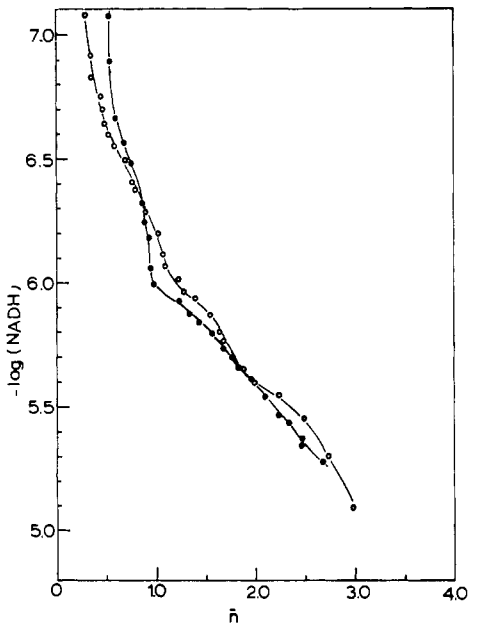

FIGURE 10: NADH binding in alkaline solutions. ●, M_4; ○, H_4: 0.020 M glycine–0.020 M potassium phosphate, pH 10.0, 17°.

it may be that we ought to consider all cases of binding of ligands to proteins as examples of stationary states rather than as true thermodynamic equilibria.

The phenomena discussed by us are by no means the only ones in the literature that point to our conclusion. Rossi-Fanelli *et al.* (1961) have demonstrated that at high salt concentrations hemoglobin exists in the dimer form rather than the usual tetramer, yet the slope in the Hill plot is 2.7–3. The system cannot therefore be described by Adair's equation. Spirographis chlorocruorin exhibits abrupt changes of slope in the Hill plot (Antonini *et al.*, 1962). Gibson (1959) has directly observed relaxation phenomena in the combination of O_2 and hemoglobin. When hemoglobin is released from combination with carbon monoxide by flash photolysis, the rate of combination with O_2 is much higher during the first millisecond after flashing than it is afterwards. The significance of this is much enhanced by the fact that hemoglobin H, which does not show cooperative effects in binding, maintains a constant high rate of oxygenation after decomposition of the CO derivative by light (Benesch *et al.*, 1964). The observations of Gibson just mentioned, those of Rossi-Fanelli *et al.* (1961), and our present experiments are the results of entirely different techniques. A different kind of artifact would have to be discovered to discredit each in turn. On the other hand, all three observations are simply explained as resulting from molecular relaxation effects.

Examination of previously published titrations of dehydrogenases with NADH reveals that, almost without exception, insufficient information was obtained to warrant conclusions about the multiplicity of binding. Resolution of the binding process into its components requires a nearly continuous distribution of measurements from $n = 0$ to $n = 4$. The obtention of less information may result in failure to detect the deviations present. For example, we have obtained 20 binary units of information in the titration of beef muscle LDH. Previously published titrations of LDH generally contain 1–3 bits of information. The work of Theorell and his associates with horse liver ADH is the only previous work comparable to ours in completeness.

The observations of Theorell on horse liver ADH are in excellent agreement with our own.

It has been found that the hybrids of chicken heart and muscle LDH's have catalytic properties intermediate between those observed for the two parent types (Fondy *et al.*, 1964). Similarly, each of the beef hybrids exhibits mixed properties. The binding anomalies found at pH 7.4 in 0.05 M potassium phosphate are associated with the presence of a beef M-type subunit. Each hybrid exhibits, in varying degrees, the anomalous binding characteristic of beef M_4. In addition, the absolute quantum yields for the bound coenzyme are intermediate. The most obvious explanation for the intermediate behavior of the hybrids is that of simple additivity of properties. However, the fact that additivity of properties implies intermediate behavior does not mean that the converse is true. Proof of additivity requires evaluation of each of the processes contributing to the over-all properties observed. The statistical nature of a measurement must be kept in mind.

It is interesting to consider the catalytic properties of a complex system such as beef muscle LDH. What are the effects of the anomalous coenzyme binding behavior on the kinetic properties? What additional effect does another molecule, such as lactate or pyruvate, have? What of all the possible combinations of binary and ternary complexes which can exist within a single LDH molecule? Do the sites make unequal contributions to reaction velocities? It is not possible to answer these questions without analysis of the individual processes involved. The kinetic constants are complex functions of many rate constants; thus, it is difficult to make deductions concerning individual steps from kinetic data. At the present, it seems best not to attempt detailed correlations of kinetic data with the results of equilibrium binding experiments. It seems profitable to select for kinetic study those examples where coenzyme binding is described by a single dissociation constant.

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