The Quaternary Structure of Lactate Dehydrogenase. I. The Subunit Molecular Weight and the Reversible Association at Acid pH*

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ABSTRACT: The monomer subunit molecular weight of lactate dehydrogenase has been shown to be 18,000 by sedimentation equilibrium studies in 6.2 M guanidine hydrochloride at pH 2.

It was demonstrated that 7 M guanidine hydrochloride at pH 7 was not sufficient to complete the dissociation. Lactate dehydrogenase undergoes a reversible association at pH 2. The association was analyzed in terms of a model containing monomers, dimers, tetramers, and octamers in equilibrium. A monomer molecular weight of 18,000, when coupled with an amino acid analysis, indicates lactate dehydrogenase must contain at least two nonidentical chains. Application of the above analysis to two restricted cases with two nonidentical chains is discussed.

One of the more interesting findings of the last decade was that lactate dehydrogenase existed in multiple catalytic forms (Wieland and Pfleiderer, 1957). Since then, a large body of evidence has accumulated dealing with the isolation, properties and hybridization of lactate dehydrogenase isozymes. At present, lactate dehydrogenase is regarded as a tetramer of four subunits, each of mol wt 35,000 (Appella and Markert, 1961; Cahn *et al.*, 1962). This theory has found support since it predicts that dissociation and random recombination of the two different homopolymers of lactate dehydrogenase, heart and muscle types, will lead to a mixture of five isozymes. In many cases, this prediction has been experimentally confirmed (Appella and Markert, 1961; Cahn *et al.*, 1962).

However, contrary to the theoretical consequences of the tetrameric theory are reports of the existence of more than five electrophoretic bands and subbands of lactate dehydrogenase (Fritz, 1963; Fritz and Jacobson, 1965; Costello and Kaplan, 1963; Koen and Shaw, 1964, 1965; Houssais, 1966), the observation of seven to eight amino-terminal end groups (Stegink and Vestling, 1966; Appella, 1964), and the production of tryptic digest peptides in amounts greater than those expected on the basis of the amino acid composition and the tetrameric theory (Appella, 1964). Apart from the reports themselves, the theoretical implications of these findings, regarding the subunit molecular weight and the mechanism of isozyme formation, have received little attention. Further, the challenge of existence of subbands to the tetramer theory, as originally pointed out by Fritz and Jacobson (1965), has not been resolved. Our experimental approach to resolve this problem is based on two observations: lactate dehydrogenase dissociates into units of 70,000 molecular weight at acid pH (Deal *et al.*, 1963), and hybridization between heart and muscle types results when lactate dehydrogenase is acidified (Anderson and Weber, 1966). The present paper reports the results of this investigation.

Experimental Section

Beef heart lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) was obtained as a crystalline suspension in 55% saturated ammonium sulfate from the Worthington Biochemical Corp., Freehold, N. J. A sedimentation velocity experiment revealed only a single homogeneous peak. However, as generally prepared, crystalline beef heart lactate dehydrogenase is a mixture of 80% H_4 and 20% H_3M isozymes (H = heart; M = muscle) (Millar, 1962).

Operationally, an aliquot of the stock enzyme suspension was spun down in a Spinco Model L ultracentrifuge at 15,000 rpm for 15 min at 4°. After the supernatant was removed, the packed enzyme was dissolved in a small volume of 5 mM Tris buffer (pH 7.0) and dialyzed overnight at 4° *vs.* 1 l. of solvent. Following this, dithiothreitol was added to the retentate to bring the dithiothreitol concentration to 0.01 M. The resulting solution was then rapidly stirred and titrated to a desired pH in the range 7–2 by dropwise additions of H_3PO_4. Polyethylene labware was used whenever possible in these operations. Necessary glass apparatus, with the exception of the pH electrodes, was thoroughly coated with 1:25 dilution of Beckman Desicote in chloroform. Finally the enzyme solution was dialyzed *vs.* a 0.1 M H_3PO_4–0.01 M dithiothreitol solution, the pH of which was adjusted with KOH to a value equal to that of the enzyme solution.

In all centrifugation experiments, cell centerpieces and sapphire windows were coated as described above. This precaution was taken since preliminary experiments indicated that some contaminant, possibly metal ions, either present as such

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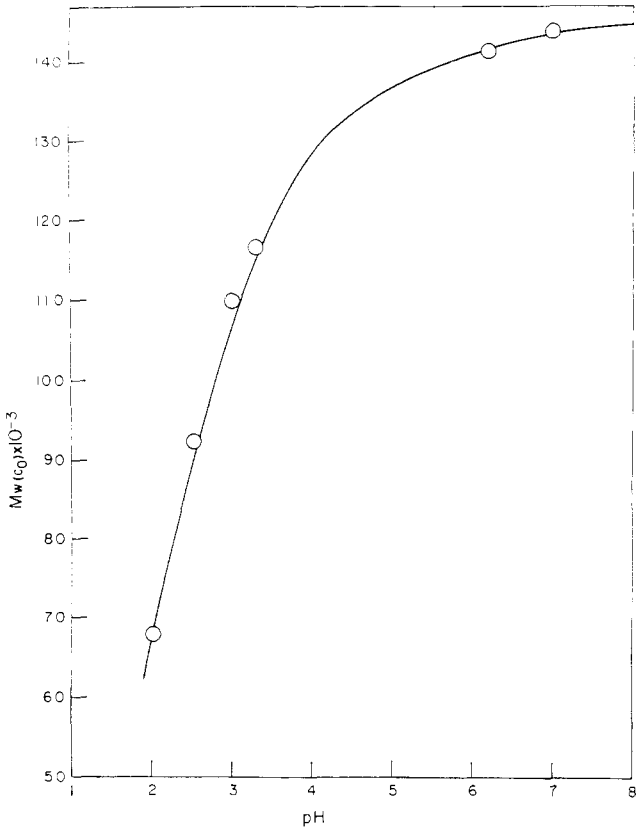

FIGURE 1: Dependence of the molecular weight, $M_{w(c_0)}$, of lactate dehydrogenase upon pH at a constant protein concentration of 2 mg/ml. The solvent was 0.1 M H_3PO_4–0.01 M dithiothreitol (adjusted to the desired pH by the addition of KOH), $T = 20°$.

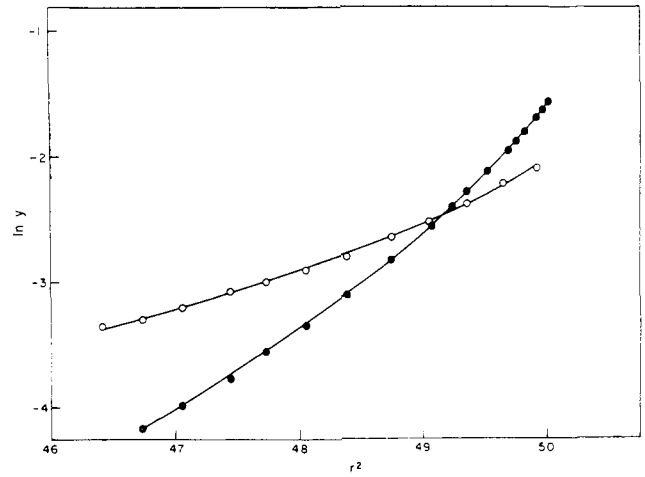

FIGURE 2: Plot of ln y (fringe height) *vs.* r^2 in a typical speed-shift experiment. The initial concentration of lactate dehydrogenase was 0.48 mg/ml. The solvent was 0.1 M H_3PO_4–KH_2PO_4–0.01 M dithiothreitol, pH 2.0, at 20°; (○) 12,590 and (●) 17,980 rpm.

or leached from the centerpiece or sapphire windows, resulted in time-dependent aggregation of the enzyme. In some extreme cases, omission of the Desicote treatment resulted in precipitation of the enzyme from solution. This phenomenon is likely related to the observations of Anderson and Weber (1966) who found that similar precautions were necessary for maximum restoration of enzymatic activity in their acid-induced hybridization experiments.

All centrifugation experiments were conducted at 20°. In standard equilibrium experiments employing Rayleigh optics, solution columns of 2.6–3.0 mm were used. In such experiments, the concentration at the meniscus, c_a, was estimated by means of eq 1, where r is a radial distance; a and b are the

$$c_a = c_0 - \frac{b^2(c_b - c_a) - \int_a^b r^2 dy}{b^2 - a^2} \quad (1)$$

radial positions of the meniscus and base of the solution column, respectively; and c_0 is the initial concentration. The initial concentration was estimated using a synthetic boundary cell. The integral in eq 1 was evaluated by means of trapezoidal summation of fringe height, y, *vs.* r^2. In practice, the vertical fringe displacement, y, as a function of r, or the lateral fringe position as a function of fringe number, j, was measured and used directly in the equation since both are directly proportional to the concentration. The fringe number, j, is related to the vertical displacement as follows: $j = y/d$, where d is the distance between parallel fringes in a plateau region. To convert vertical fringe displacements into concentrations (LaBar, 1965), the factor, 7.7, was used. For this purpose, an average value of 1.85×10^{-3} dl/g for dn/dc, the refractive increment of the protein, was taken from Charlwood (1957). Readings of y *vs.* r were preferred rather than the customary observation of lateral fringe position since the former procedure provides considerably more information near the meniscus where low concentration gradients exist.

In experiments with the Yphantis (1964) meniscus-depletion method, 7-mm solution columns were used. To hasten attainment of equilibrium when guanidine hydrochloride was present in the protein solution, solvent was layered over solution in a synthetic boundary cell. In outlining the dependence of molecular weight upon pH at constant protein concentration, the Yphantis (1960) multichannel cell was used. With this simple, rapid equilibrium technique molecular weights are calculated from a point in the cell at which the concentration very closely approximates c_0. To distinguish the molecular weights calculated in this manner from those estimated by the more precise techniques described above, they are written as $M_{w(c_0)}$. The precision is approximately 5%.

Results

Dependence of the Molecular Weight of Lactate Dehydrogenase upon pH. Figure 1 shows the decrease in the weight-average molecular weight of lactate dehydrogenase as the pH is lowered from 7 to 2, for a 2-mg/ml solution of lactate dehydrogenase. The value obtained at pH 2 is in fair agreement with the value of Deal *et al.* (1963), who found a weight-average molecular weight of 70,000 for an initial concentration of 1.5 mg/ml at a pH of 2.6–2.9. Since the effect of acid is probably to suppress several association equilibria, it is not possible to draw any definite conclusions except to suggest that the form of the curve appears to indicate that the titration of side-chain carboxyls is involved.

The Dependence of the Molecular Weight of Lactate Dehydrogenase upon Protein Concentration. Since we desired to

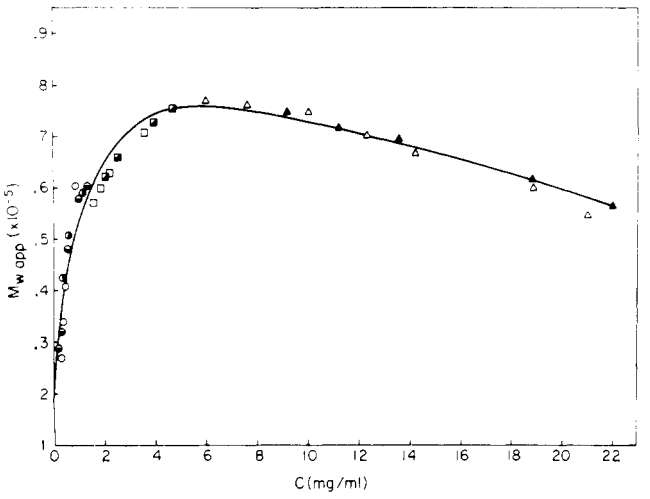

FIGURE 3: Dependence of the molecular weight of lactate dehydrogenase upon protein concentration at pH 2.0 and 20°. The solvent was the same as that given in Figure 2. Data points are experimental values; the line through the points is that calculated for a reversibly associating system as described in the text. (○, ◒, and ◑) 12,590, 15,220, and 17,980 rpm, respectively, at an initial protein concentration of 0.5 mg/ml; (□ and ◪) 8900 rpm at initial protein concentrations of 2 and 3 mg per ml, respectively; (△ and ▲) 9260 rpm at an initial protein concentration of 10 mg/ml.

stay close to the pH range in which the hybridization experiments of Anderson and Weber (1966) were carried out, it was decided to examine the concentration dependence of the weight-average molecular weight, $M_{w\,app}$, as a function of c at pH 2. Figure 2 shows a plot of ln y *vs.* r^2 for a typical experiment, in which the solution was brought to successive equilibrium at two different speeds. The pronounced curvilinearity is typical of systems heterogeneous with respect to molecular weight. However, multicomponent systems in which there is no physical interaction between macromolecules could give rise to curves resembling those in Figure 2. This possibility was eliminated by varying initial lactate dehydrogenase concentrations and speeds. The fact that the resultant curve of $M_{w\,app}$ *vs.* c was *unique* (see below) demonstrated a reversible process (Adams and Fujita, 1963). Further, there appeared to be no slow, secondary, time-dependent irreversible aggregation occurring, such as has been observed with glutamate dehydrogenase (Willick, 1966), since analysis of runs made 24 hr after equilibrium had been obtained gave results identical within experimental error with those calculated at initial equilibrium. Accordingly, a complete molecular weight *vs.* concentration study was performed.

Two major assumptions have been made in the analysis of the data. Firstly, it has been assumed that the partial specific volume, $\bar{v}$, of lactate dehydrogenase in acid is equal to that of the native molecule at near-neutral pH, 0.74 ml/g (Millar, 1962). It is difficult to weigh the risks of this assumption, but Kauzmann (1958) has shown for ovalbumin and bovine serum albumin an acid environment results in a maximum increase in $\bar{v}$ of 0.01 ml/g. In the case of bovine serum albumin, it was, under experimental conditions, much less. Secondly, we assumed that all n-mers have the same $\bar{v}$. Although this assumption has been routinely made in the past, actual $\bar{v}$ divergence could be of considerable significance. In particular, it could give rise to a pressure dependence of the equilibrium in the centrifuge, *via* eq 2 (Kirkwood and Oppenheim, 1961), where

$$\left(\frac{\partial \ln K_n}{\partial P}\right)_T = -\frac{\Delta \bar{V}^0}{RT} \tag{2}$$

$\bar{V}^0$ = partial molal volume change for the reaction monomer–n-mer, and K_n is the associated equilibrium constant. Both TMV (Stevens and Lauffer, 1965) and hemocyanin (Cohen and Van Holde, 1964) show a decrease in $\bar{v}$ as the polymer dissociates. Such a change could be expected to give rise to a decrease in apparent weight-average molecular weight at a particular concentration as the speed of the centrifuge is increased. No such consistent trend was observed.

For a thermodynamically nonideal system, the apparent weight-average molecular weight, $M_{w\,app}$, as a function of the concentration, c, can be obtained from the tangents to a plot of ln c *vs.* r^2, according to eq 3, where $A = (1 - \bar{v}\rho)\omega^2/2RT$ and

$$M_{w\,app} = A\frac{d \ln c}{dr^2} \tag{3}$$

T have their customary meanings. It is often sufficient to express the nonideality as a one-term expansion of the logarithm of the activity coefficient

$$\ln y = \sum iBM_1c,\ i = 1, 2, \ldots \tag{4}$$

where B is the virial coefficient. Thus, $M_{w\,app}$ in eq 3 is defined by

$$\frac{M_1}{M_{w\,app}} = \frac{M_1}{M_{w(c)}} + BM_1c \tag{5}$$

where $M_{w(c)}$ is the true weight-average molecular weight at concentration c and is explicitly defined by

$$M_{w(c)} = \sum_i c_iM_i/c \tag{6}$$

In order to obtain the necessary tangents, the data was best fitted, in the least-squares sense, to a polynomial of the form

$$\ln c = \sum_{i=0}^{n} A_i(r^2)^i \tag{7}$$

where A_i is the coefficient of the polynomial. It was found that a third degree polynomial (n = 3) fitted the experimental data well and agreed within ± 3% with the values obtained with a fifth degree polynomial.

The results of the experiments are shown in Figure 3. The symbols represent experimental data and the line joining them is calculated according to the procedures described below. The $M_{w\,app}$ *vs.* c curve has a maximum at about 5 mg/ml and shows a slow, monotonic decrease as the concentration increases above this level, such as would be expected for an association system with a small, positive virial coefficient. Below 5 mg/ml, $M_{w\,app}$ decreases with c, and drops well below 30,000 at the lowest concentrations studied. Linear extrapolation of the data from c = 0.5 to 0 mg per ml would lead to an intercept falling between 15,000 and 20,000. The wide spread

in values is due to the scatter of the data at these low concentrations. For a simple, self-association system

$$\lim_{c \to 0} M_{wapp} = M_1$$

where M_1 is the molecular weight of the monomeric subunit species. An accurate value for M_1 is necessary for an analysis of the association.

Molecular Weight of the Lactate Dehydrogenase Monomer Unit. Further experiments were carried out in an attempt to fully dissociate lactate dehydrogenase and obtain a value for M_1. Appella and Markert (1961) showed that the molecular weight of LDH drops to 35,000 in 5 M guanidine hydrochloride. Since this salt is usually considered to be a strong denaturant, we increased the concentration to 7 M and again determined the molecular weight (Figure 4). Although there is some evidence that the molecular weight is concentration dependent, the lowest value observed is still only about 35,000. This value is roughly the same as that at neutral pH at the same protein concentration in the absence of guanidine hydrochloride.[1] However, since the guanidine hydrochloride apparently completely suppressed the association above 35,000, it was decided to examine the molecular weight at pH 2 in the presence of the salt. The results are shown in Figure 5. The data extrapolate to a molecular weight of 17,000–19,000, utilizing the value for $\bar{v}$ of 0.74 ml/g estimated for lactate dehydrogenase in 5 M guanidine hydrochloride at pH 7 (Appella and Markert, 1961). Considering the potential error in $\bar{v}$, in addition to the inherent error of the high-speed equilibrium technique used for evaluating the molecular weight in these high salt concentrations, an error of $\pm 10\%$ is not unexpected in this extrapolation. Reversibility of the change was demonstrated by allowing a sample to stand for 24 hr at pH 2 in 6.2 M guanidine hydrochloride and then adjusting the pH to 7. The molecular weight was again about 35,000 (Figure 5).

The minimum molecular weight compatible with the amino acid analysis of lactate dehydrogenase (Millar, 1962) is 18,000. However, this implies that there are two or more nonidentical chains, since otherwise the bulk of the residues would be nonintegral. In view of the errors mentioned previously in the sedimentation equilibrium determination of the molecular weight, we have relied on the amino acid analysis to give us a value for M_1 of 18,000, with an estimated error of $\pm 3\%$. Further analysis of the present data must proceed on the basis of identical self-associating units. That is, we have *assumed* that the postulated different subunits have identical molecular weights. In principle, the association can be analyzed for the presence of all polymeric species from 1 to n, where n is the degree of polymerization necessary to adequately fit the maximum molecular weight data (Steiner, 1952). In view of the fact that the upper limiting molecular weight of lactate dehydrogenase at neutral pH is 144,000,[1] thus indicating octamers can be formed, it was decided to try to fit the association data at pH 2 with a simple model involving monomers, dimers, tetramers, and octamers. This model has been *assumed* on the basis of the apparent existence of octamer and tetramer (Figure 3) and the existence of dimer in guanidine solutions. Other more or less complex models may fit the data. However, on the basis of the

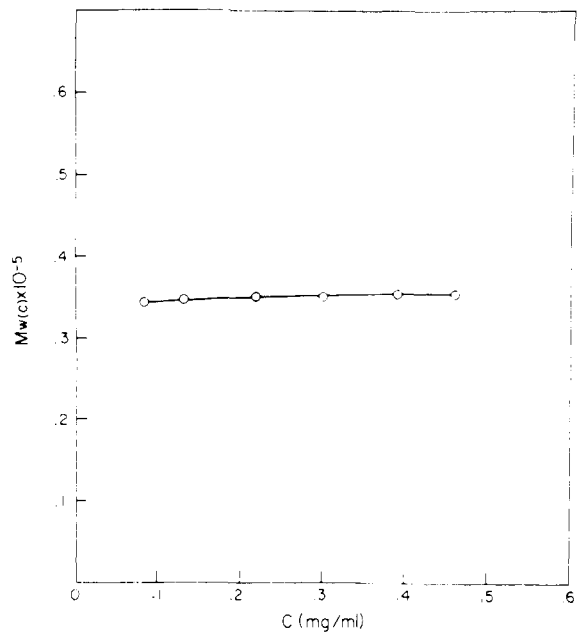

FIGURE 4: Molecular weight of lactate dehydrogenase in guanidine hydrochloride at pH 7. The solvent was 7 M guanidine hydrochloride, 0.05 M KH_2PO_4, and 0.01 M dithiothreitol, adjusted to pH 7.0 with KOH. Initial protein concentration = 0.4 mg/ml, solution column height = 7 mm, speed = 47,660 rpm, and $T = 20.4°$.

above reasoning we have chosen to analyze the data in terms of this model. For convenience of analysis, the equilibrium constants are chosen as follows

$$2H_1 \rightleftharpoons H_2 \qquad K_2 = \frac{c_2}{c_1^2}$$

$$4H_1 \rightleftharpoons H_4 \qquad K_4 = \frac{c_4}{c_1^4} \qquad (8)$$

$$8H_1 \rightleftharpoons H_8 \qquad K_8 = \frac{c_8}{c_1^8}$$

Here the c represents the concentrations, on a grams per liter basis of species H_i. The total concentration at any position, r, is then

$$c = c_1 + c_2 + c_4 + c_8 = c_1 + K_2 c_1^2 + K_4 c_1^4 + K_8 c_1^8 \qquad (9)$$

The weight fraction of monomer, x_1, at any c is defined as

$$x_1 = \frac{c_1}{c} \qquad (10)$$

Equations 9 and 10 lead to

$$c - x_1 c = K_2(x c_1)^2 + K_4(x_1 c)^4 + K_8(x_1 c)^8 \qquad (11)$$

The apparent weight fraction monomer, x_{app}, may be esti-

[1] G. E. Willick and D. B. Millar, in preparation.

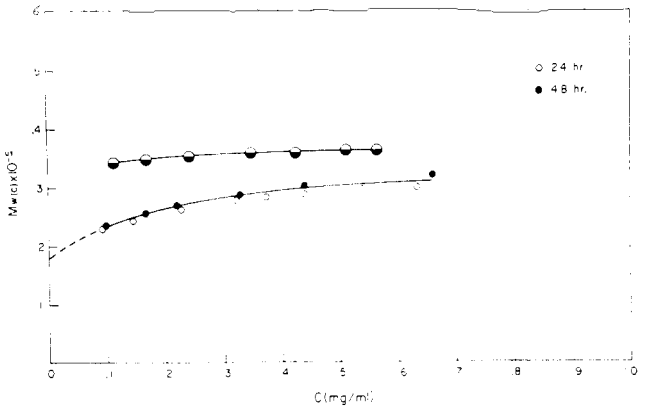

FIGURE 5: The molecular weight of lactate dehydrogenase in guanidine hydrochloride at pH 2 and 7. Lower curve: molecular weight of lactate dehydrogenase in 6.2 M guanidine hydrochloride, 0.1 M H_3PO_4–KH_2PO_4, and 0.01 M dithiothreitol, pH 2.0, 24 (○) and 48 (●) hr after layering. Upper curve: molecular weight obtained after lactate dehydrogenase in 6.2 M guanidine hydrochloride, pH 2.0, had been dialyzed *vs.* 6.2 M guanidine hydrochloride, 0.1 M KH_2PO_4–K_2HPO_4, and 0.01 M dithiothreitol (pH 7.0).

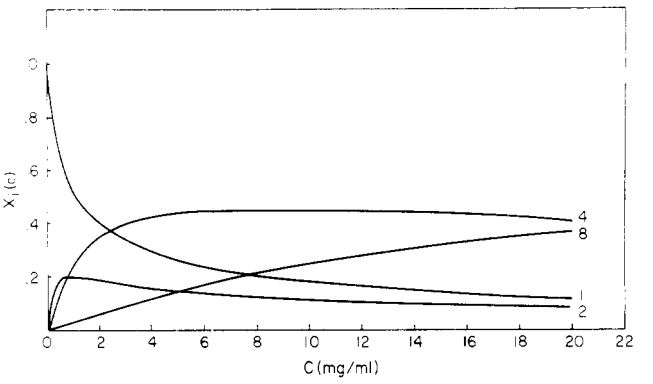

FIGURE 6: The equilibrium variation in the mole fraction, $X_i(c)$, of monomer (1), dimer (2), tetramer (4), and octamer (8), with total protein concentration in 0.1 M H_3PO_4–KH_2PO_4 and 0.01 M dithiothreitol, pH 2.0, at 20°.

mated by the following equation (Steiner, 1952; Adams and Williams, 1964)

$$\ln x_{app} = \int_0^c \left(\frac{M_1}{M_{wapp}} - 1\right)\frac{dc}{c} = \ln x_1 + BM_1c \quad (12)$$

In order to reduce errors due to data scatter, particularly at low concentrations, a smooth curve was drawn through the data of Figure 3 and data pairs of M_{wapp} *vs.* c taken from it for numerical evaluation of the integral in eq 12. The values of the integrand of eq 12 were calculated and extrapolated to give the value at $c = 0$. Values of X_{app} were then calculated, with the integral values approximated using the trapezoidal rule. Three sets of x_{app} and c values judiciously chosen from the region in Figure 3 between 0 and 4 mg per ml, where nonideality effects are minimal, were substituted in eq 11. In this way, rough values of K_2, K_4, and K_8 were obtained. Final values for the equilibrium constants and the nonideality term, BM_1c, were obtained by successive approximations with eq 5 using a computer. The value of BM_1 was 0.007. Equilibrium constants values are given in Table I. As is seen in Figure 3, the curve calculated with the procedures and assumptions detailed above fits the experimental data quite well.

To give a graphical display of the relative amounts of each species present, the *mole* fractions were calculated as a function of the concentration (Figure 6). One perhaps surprising result is that the fraction of monomer molecules is quite high throughout, constituting over 10% of the molecules when the total concentration is 20 mg/ml. As expected, the tetramer is also a dominant species through much of the concentration range. It should again be pointed out that these calculations are based on an assumed model. Information of this type, coupled with a study of the kinetics of hybrid formation, could be useful in determining more precisely the nature of the hybrid-forming reaction.

pH Reversibility. Attempts were made to reverse the acid-induced dissociation, by either direct titration or dialysis to neutral pH. However, all attempts eventually resulted in heavy aggregation. If the same experiment was done in the presence of guanidine, the results shown in Figure 5 were obtained. As shown, the acid-reversed material has a molecular weight of about 36,000 and is homogeneous. Apparently guanidine hydrochloride prevents the secondary aggregation which takes place in its absence.

TABLE I: Apparent Equilibrium Constants for the Association of Lactate Dehydrogenase at pH 2.0 for Identical Chains.[a]

i	K_i[b]	K_i[c]
2	3.0	2.70×10^4
4	120	2.75×10^{14}
8	3800	2.90×10^{32}

[a] For definition of constants, see text. [b] A gram per liter concentration scale. [c] A moles per liter concentration scale is used.

Discussion

Our evidence suggests strongly that the basic subunit of lactate dehydrogenase has a molecular weight of about 18,000, and not 35,000 as previously reported (Appella and Markert, 1961). The data indicate that the molecular weight drop which occurs in 6.2 M guanidine hydrochloride as the pH is altered from 7 to 2 is reversible (Figure 5). This experiment apparently rules out an acid-induced cleavage of a covalent bond as being responsible for the molecular weight drop, unless cleavage occurred and the resulting products still associated in 7 M guanidine hydrochloride at neutral pH. While possible, this explanation has been discounted by us due to the reports mentioned previously concerning the existence of 7–8 end groups which clearly point to the existence of a subunit with the molecular weight reported here. The possibility of the reduction of a disulfide bond or bonds between chains had previously been ruled out, since there are no disulfide bonds in lactate dehydrogenase (Di Sabato *et al.*, 1963). In addition, we have evidence for the existence of molecular weights of from 25,000 to 30,000 at neutral pH,[1] where no such reaction would be expected to occur. The existence of a subunit of mol

wt 18,000 is in agreement with the observation that there are 8 amino-terminal groups/144,000 g of lactate dehydrogenase (Stegink and Vestling, 1966; Appella and Zito, 1968).

The fact that lactate dehydrogenase is not fully dissociated at pH 7 in guanidine hydrochloride concentrations of up to 7 M is of interest. The noncovalent interactions which presumably give rise to the formation of a dimer are apparently not particularly affected by high concentrations of guanidine hydrochloride at neutral pH (Figure 4). It has been suggested that a concentration of 6.2 M guanidine hydrochloride results in complete elimination of the noncovalent intramolecular interactions in proteins (Tanford *et al.*, 1967). One might expect that intermolecular interactions would be still easier to disrupt than intramolecular ones. These results with lactate dehydrogenase would seem to indicate that the assumption that high concentrations of guanidine hydrochloride completely denature proteins at neutral pH should be reconsidered.

In the analysis of the equilibrium data, we have assumed a model in which there is one component, an associating heart-type isozyme. We have ignored the presence of approximately 5% of the muscle-type isozyme. The fact that the muscle-type isozyme interacts with itself to form homopolymers, as well as with the heart-type isozyme to form hybrids (Anderson and Weber, 1966), indicates that it, too, is probably in reversible equilibrium at acid pH. Consequently, it would not be expected to seriously affect the shape of the association curve when present in amounts of about 5% of the total enzyme mass. However, there is apparently more than one heart-type chain. If one reduces the amino acid analysis (Millar, 1962) to the number of residues equivalent to an 18,000 molecular weight unit, the great bulk of the residues become significantly nonintegral. Since only 4 equiv of carboxy-terminal amino acid are released per 144,000 g of lactate dehydrogenase (Appella and Zito, 1968) with carboxypeptidase, it appears likely that there are four chains less reactive than the first four, thus giving rise to an apparently low value. Just such a problem has been experienced with aldolase (Morse *et al.*, 1967).

For the present, at least the important point is that a relatively simple model, which assumes only monomers, dimers, tetramers, and octamers of a basic heart-type subunit, has been sufficient to satisfy all of the data. A more detailed examination would require the physical separation of the unlike chains.

Finally, the relevance of these data to the problem of lactate dehydrogenase isozyme distribution and formation should be considered. Assuming, one has four nonidentical chains (two in each of the heart- and muscle-type isozyme), the maximum number of bands that can be calculated is 165 arising from the random combination of four nonidentical chains into an octameric unit. Successively moderate degrees of combinatorial restrictions lead to 45, 15, and then finally to the 5 bands commonly seen. The intermediate numbers are compatible with the observation of Stambaugh and Buckley (1967) and Fritz and Jacobson (1965). This suggests that the association under certain conditions is highly specific, and that the formation of hybrids by otherwise forbidden associations requires relatively strong denaturing conditions, such as are provided by low pH or high concentrations of urea or guanidine hydrochloride.

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