Multiple Molecular Forms of Lactate Dehydrogenase*

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ABSTRACT: Lactate dehydrogenase (LDH) is usually considered to exist in five forms, called isozymes, separable by electrophoresis. The present report describes for each isozyme multiple subbands which are separated by high voltage electrophoresis in polyacrylamide gel. Comparison of mouse muscle LDH and rabbit muscle LDH reveals two different patterns of subbands which can be altered by the presence of 2-mercaptoethanol during electrophoresis. In the mouse muscle LDH, changing the mercaptoethanol concentration from 0.001 to 0.004 M results in a redistribution of the subband pattern; changing further to 0.008 M results in yet another pattern. The rabbit muscle LDH pattern of subbands is not affected by changes of mercaptoethanol between 0.001 and 0.005 M.

The data are considered to indicate that a family of closely related charge forms of LDH exists for each individual isozyme. The pattern of subbands has not been reconciled with the tetramer hypothesis of LDH structure.

Lactate dehydrogenase has been shown to exist in five molecular forms in the organs of most vertebrates. An elegant physical explanation to account for this phenomenon has been provided by work from several laboratories (Appella and Markert, 1961; Markert, 1963; Cahn *et al.*, 1962). The observations support the idea that the enzyme in its active form is a tetramer composed of a random combination of two different polypeptides. The biosynthesis of the two polypeptides is apparently controlled by two separate genes.

It has been reported from this laboratory that, by use of high voltage electrophoresis in polyacrylamide gels, lactate dehydrogenase from mouse muscle homogenates can be resolved into at least fifteen enzymatically active fractions (Fritz and Jacobson, 1963). Similar experiments using crystalline rabbit muscle lactate dehydrogenase were reported to yield twelve fractions with enzyme activity (Fritz, 1963). This paper will serve further to supplement and document the previous observations and to describe how the multiple fractions are affected by various experimental conditions.

Methods

The polyacrylamide gels (5% acrylamide) were prepared according to the method of Raymond and Wang (1960), giving a gel 30 × 6.5 × 0.3 cm. The gels were made up in water and then soaked in the appropriate buffer at least 24 hours before use. The *p*H of each buffer was determined at 25°; in the case of Tris buffer, the *p*H is approximately one *p*H unit lower at 4°. Electrophoresis was accomplished by means of an E-C Apparatus Corp. Model 401 pressure-plate electrophoresis cell (migration chamber 20 × 30 cm) to cool the gel. The cell was modified in either of two ways: (1) The buffer vessels were plastic trays 14 × 19 × 3 cm and the electrodes were platinum grids 2 × 20 cm. (2) The buffer vessels were plastic trays 26 × 6 × 5 cm and the electrodes were platinum wires 6 cm long. The vessels each contained 500 ml of buffer. The gels were positioned on a sheet of polyvinylidene chloride (Saran wrap, Dow Chemical Co.) on the floor of the migration chamber. Samples (usually 5 μl) were applied to strips of Whatman 3MM filter paper (2.5 × 20 mm) and the strips were then placed against the cut edges of the gel. The cut portions of the gel were then rejoined, connections to the buffer vessels were made by means of five layers of Whatman No. 1 filter paper (6.5 × 7.5 cm), the gel was covered with Saran wrap, and the top cooling plate was put in place. Prestone at −5° was circulated through the cooling plates by means of a Wilkins-Anderson Co. Lo-Temp bath. The power supply was an E-C Model 453. Electrophoretic separation of the isozymes was accomplished using 800 v (26.6 v/cm) and from 30 to 50 ma. Under these conditions the temperature within the gels was around 12° as determined with a Tri-R Instruments electronic thermometer. Satisfactory separations were accomplished in 1.5–3 hours. After electrophoresis, the enzyme bands were revealed by the tetrazolium-staining technique of Dewey and Conklin (1960) except that no cyanide was used in the developing solution.

The organs of various strains of mice were removed and either homogenized immediately or frozen in liquid nitrogen and stored at −20°. Homogenates were prepared using a Potter-Elvehjem glass homogenizer and 2 ml of Tris-HCl buffer [0.05 M, *p*H 8.5 (25°)] per gram of tissue. The homogenates were then centrifuged for 20 minutes at 30,000 × *g* in a Servall re-

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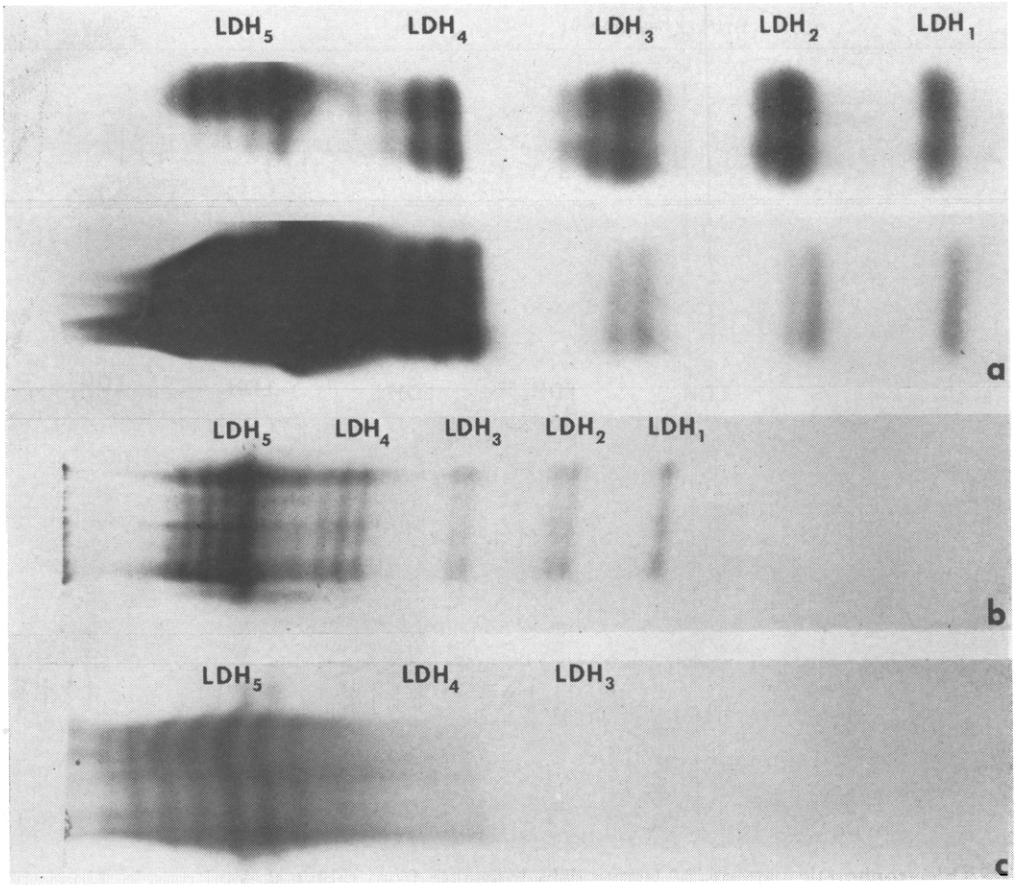

FIGURE 1: Electrophoretic patterns of lactate dehydrogenase prepared from C57BL mouse organs. (a) Electrophoresis for 4 hours at 16.6 v/cm; Tris-HCl buffer 0.038 M, *p*H 8.5; 5-μl samples of heart and skeletal muscle homogenates, 0.043 and 0.079 unit of LDH activity were used. Assay was for 3 hours. (b) Similar conditions, using skeletal muscle homogenates. Assay was for 1.5 hours. (c) Similar conditions, using less skeletal muscle homogenate. Assay was halted before the subbands of LDH_4 were apparent. The origin position and the cathode are to the left.

frigerated centrifuge. Zondag's observation (1963), that storage of human tissue homogenates at $-20°$ resulted in complete inactivation of LDH[1] isozymes 4 and 5, was not observed in mouse preparations, since homogenates thawed and refrozen repeatedly over a period of several months showed no apparent change in amount of LDH activity or in isozyme pattern.

Twice-recrystallized rabbit skeletal muscle lactate dehydrogenase was obtained from Worthington Biochemical Corp. The enzyme was sedimented from the ammonium sulfate suspension by centrifuging at 30,000 $\times$ *g* for 15 minutes and dissolved in glass-distilled water before use. Nitro blue tetrazolium was obtained from Dajac Laboratories. Phenazine methosulfate was obtained from Sigma Chemical Co. Nicotinamide adenine dinucleotide was obtained either from Sigma or from Pabst Laboratories.

Lactate dehydrogenase activity was assayed by use of a Zeiss Model PM QII spectrophotometer to follow the change in optical density at 340 mμ which accompanies the reduction of NAD. A unit of LDH activity is the international unit defined as the amount of enzyme which converts 1 μmole of substrate per minute at 25°.

Estimation of NAD associated with crystalline rabbit muscle LDH was according to Jacobson and Astrachan (1957). The enzyme was treated with 0.5 M $HClO_4$ and the acid-soluble material was assayed fluorometrically before and after its reaction with yeast alcohol dehydrogenase.

Results

The multiple subbands of the various isozymes of LDH can be seen in Figure 1. In Figure 1a, the mouse heart homogenate shows that LDH_5 has five subbands, LDH_4 has four subbands, LDH_3 has three subbands, LDH_2 has two subbands, and that LDH_1 exists as a single band. The skeletal muscle homogenate is also shown in Figure 1a, although overdeveloped for LDH_5 and LDH_4, to compare to the heart pattern.

[1] Abbreviation used in this work: LDH, lactic dehydrogenase.

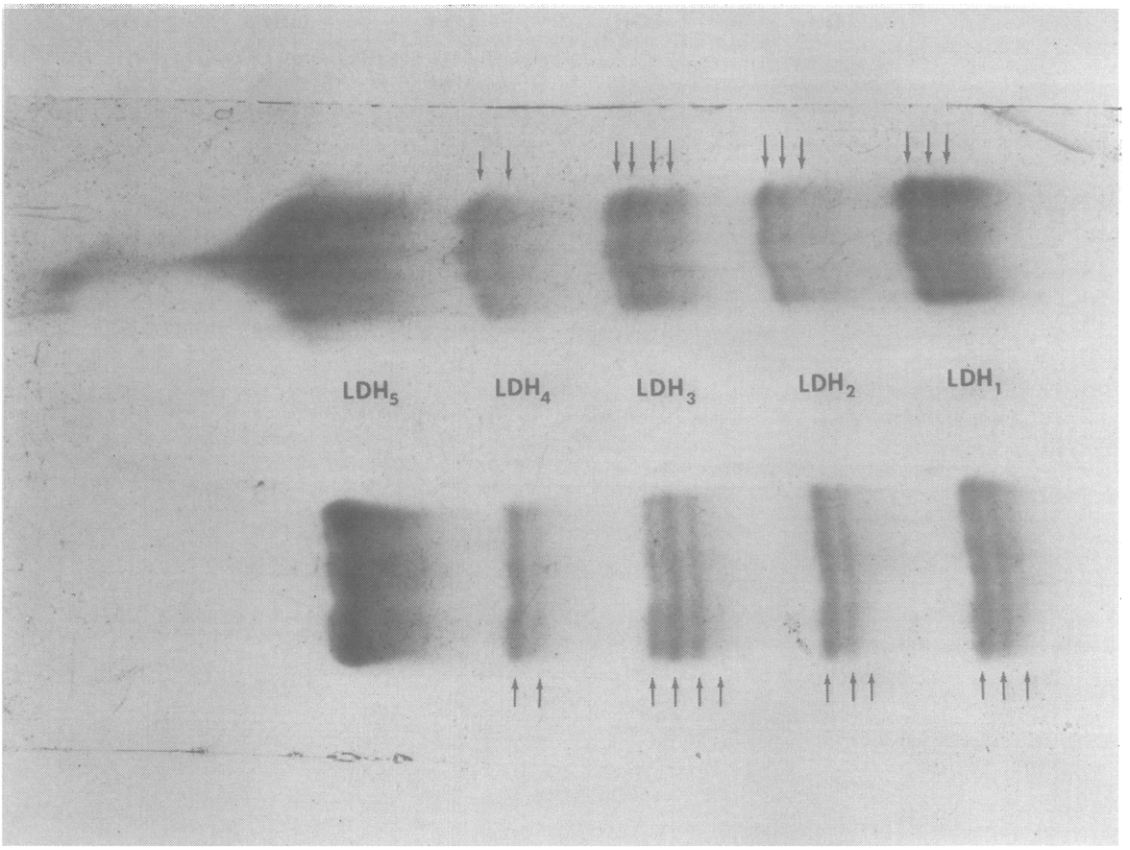

FIGURE 2: Electrophoretic patterns of lactate dehydrogenase from rabbit skeletal muscle. Upper pattern from homogenate, lower pattern from crystalline enzyme. Electrophoresis for 2 hours and 20 minutes at 26.6 v/cm; glycine-NaOH buffer, 0.05 M, *p*H 10.0; 5-μl samples, representing 0.079 LDH unit, were used. Origin position and cathode are to the left.

These two muscle types have very different distributions of LDH activity in the major isozyme bands, but have very similar subband patterns in any given major band.

Figure 1b shows a clear resolution of subbands of LDH_4 of skeletal muscle. There are consistently four subbands of LDH_4 in skeletal muscle as well as in heart muscle.

Figure 1c shows LDH_5 of skeletal muscle resolved into eight subbands. This is the maximum number of subbands of LDH_5 observed, and frequently only five subbands are apparent. This variation is caused presumably by variation in the electrophoretic technique. A skeletal muscle homogenate has been stored for several months at $-20°$ and has yielded both five and eight subbands of LDH_5 at various times during storage.

Having observed these multiple subbands in the mouse, we then examined the rabbit. Figure 2 shows rabbit skeletal muscle LDH, a homogenate, and a highly purified commercial sample. The resolution of the homogenate sample is inferior, but sufficient to show a close resemblance to the pattern obtained from the purified enzyme. The lines point to each subband observed on the gel; very faint subbands do not reproduce well photographically. By using a more concentrated sample of the purified enzyme (45 mg/ml), seventeen subbands can be observed, distributed among the major bands as shown in Table I. Although both mouse and rabbit LDH show subbands in the usual five isozymes, the distribution of these subbands is rather different between these two animals.

NAD Effects on Subbands. We suggested that the NAD-enzyme complex could dissociate to various degrees, so that there could be from 4 to 0 NAD molecules per LDH tetramer (Fritz and Jacobson, 1963). The result would be a family of electrophoretic bands, closely spaced, for each isozyme. The evidence for this suggestion was that electrophoresis of mouse LDH in the presence of 0.01 M NAD did not yield subbands. Although subsequent experiments indicate that this observation is not readily reproducible, certain studies followed which, although they did not support the concept of partial dissociation of NAD, were a general test of the presence of a dissociable group.

Purified rabbit muscle LDH was subjected to electrophoresis in the presence of 0.01 M NAD. There was a more rapid migration of the various bands of LDH but no alteration in the pattern of subbands.

By two methods of charcoal treatment the purified rabbit muscle LDH was dissociated from NAD and some other orcinol-positive material and shown to

TABLE I: Distribution of Subbands within Major LDH Isozyme Bands in Mouse and Rabbit Skeletal Muscle.[a]

Isozyme	Number of Subbands	
	Mouse	Rabbit
LDH_5	5–8	1
LDH_4	4	3
LDH_3	3	5
LDH_2	2	4
LDH_1	1	4
	15–18	17

[a] Electrophoretic conditions were as described under Methods.

migrate on the acrylamide gel to give the same subband pattern as the untreated enzyme. Prior to the charcoal treatment the acid extraction of the LDH resulted in the detection of less than 0.01 mole NAD per 140,000 g of protein. In the acid extract there was also a substance which reacted with orcinol (Dische, 1955) to the extent of 0.1 mole ribose equivalents per 140,000 g of protein. Wieland *et al.* (1962) reported that extraction of purified LDH yielded a derivative of NAD which was inactive when tested with various dehydrogenases; this material may be the orcinol-positive substance described here although we attempted no further identification. The charcoal treatments were as follows: (1) A sample was dialyzed against 250 ml of buffer containing Norit A. This procedure removed 88% of the orcinol-positive substance but did not alter the subband pattern. (2) The purified LDH was percolated through a charcoal column to remove bound nucleotide (Fox and Dandliker, 1956). Again there was no change in the subband pattern. Thus all experiments with the effect of NAD on the LDH isozymes in the purified rabbit muscle preparation gave results which were not consistent with the theory of differential coenzyme binding. Purified LDH isozymes are now being prepared in order to test this point further in mouse organs.

Two-dimensional Electrophoresis. If the subbands of mouse LDH are the result of the presence of a dissociable, charged ligand, other than NAD, it should be possible to observe the dissociation of the ligand. If the ligand contributes a negative charge to the LDH, the fastest subband in LDH_5 should give rise to the other subbands when the ligand dissociates. To test this possibility, a sample of mouse muscle homogenate was subjected to electrophoresis for a longer time than usual (7 hours) to spread out the subbands. Longitudinal strips of the gel were cut (3–4 mm wide), and one was assayed to locate the subbands of LDH_5. Four strips of gel were stored for various periods of time and used as the origin for a second electrophoretic migration by butting the side of the strip against the end of another polyacrylamide gel slab, as suggested by Raymond and Aurell (1962).

Figure 3a illustrates the results of a two-dimensional experiment using mouse skeletal muscle homogenate as the source of LDH. The figure depicts the resolution of LDH_5 only, and reveals that, in the first dimension, eight bands with enzyme activity can be seen. When this resolved band was used as the origin for a second electrophoresis at right angles to the first, the subbands moved the same distance, and a straight line drawn through the LDH spots passed through the origin for the first dimension. This result indicates that the subbands did not undergo alteration in the time between the first and second electrophoresis. In the experiment shown in Figure 3a, the second electrophoresis was carried out immediately after the first; if the subbands resolved in the first dimension were allowed to stand on the gel either at room temperature for 20 hours or at 0° for up to 4 days before the second electrophoresis was undertaken, the results were essentially the same as those pictured in Figure 4a; i.e., there was no indication of a conversion of one subband into another.

Figure 3b illustrates the results of a two-dimensional experiment using crystalline rabbit muscle LDH. The resolution of LDH_4, LDH_3, and LDH_2 into subbands is seen. In this experiment, bands 3 and 2 did not run quite as far in the second dimension as they did in the first; however, when the experiment was repeated these bands ran the same distance in both dimensions, again indicating that the subbands did not undergo alteration in the time between the first and second electrophoresis.

pH Effects on Subbands. The resolution of major bands into subbands is dependent on the *p*H at which electrophoresis is carried out. The optimum *p*H for detection of subbands in mouse muscle homogenates is around 8.6. At *p*H 7.2 it is difficult to detect subbands, at *p*H 7.5 and 8.0 LDH_5 becomes more diffuse, and at *p*H 8.6 it appears as a broad band well resolved into subbands. At *p*H 9.0, band 5 is still broad, but the resolution into subbands is poor.

Voltage Effects. The resolution of subbands was also a function of the voltage employed. Mouse organ homogenates or crystalline rabbit muscle lactate dehydrogenase run at *p*H 8.5 and 25 v/cm produced good resolution. When samples were run for 15 hours at 4 v/cm, no subbands could be detected in mouse muscle LDH. The lower voltage gradient and longer time for the electrophoretic separation allows greater diffusion of the proteins, thus obscuring the separation of the subbands.

To verify that no anomalies were being introduced by the electrophoretic conditions, the following experiment was carried out: A sample of mouse skeletal muscle homogenate was subjected to electrophoresis at *p*H 8.5 at 26 v/cm. After 1.5 hours the electrodes were reversed and the electrophoresis was carried out for 1.5 hours in the opposite direction. At this time the gel was assayed for LDH in the usual manner. The appearance of a dark purple band within 1 mm of each

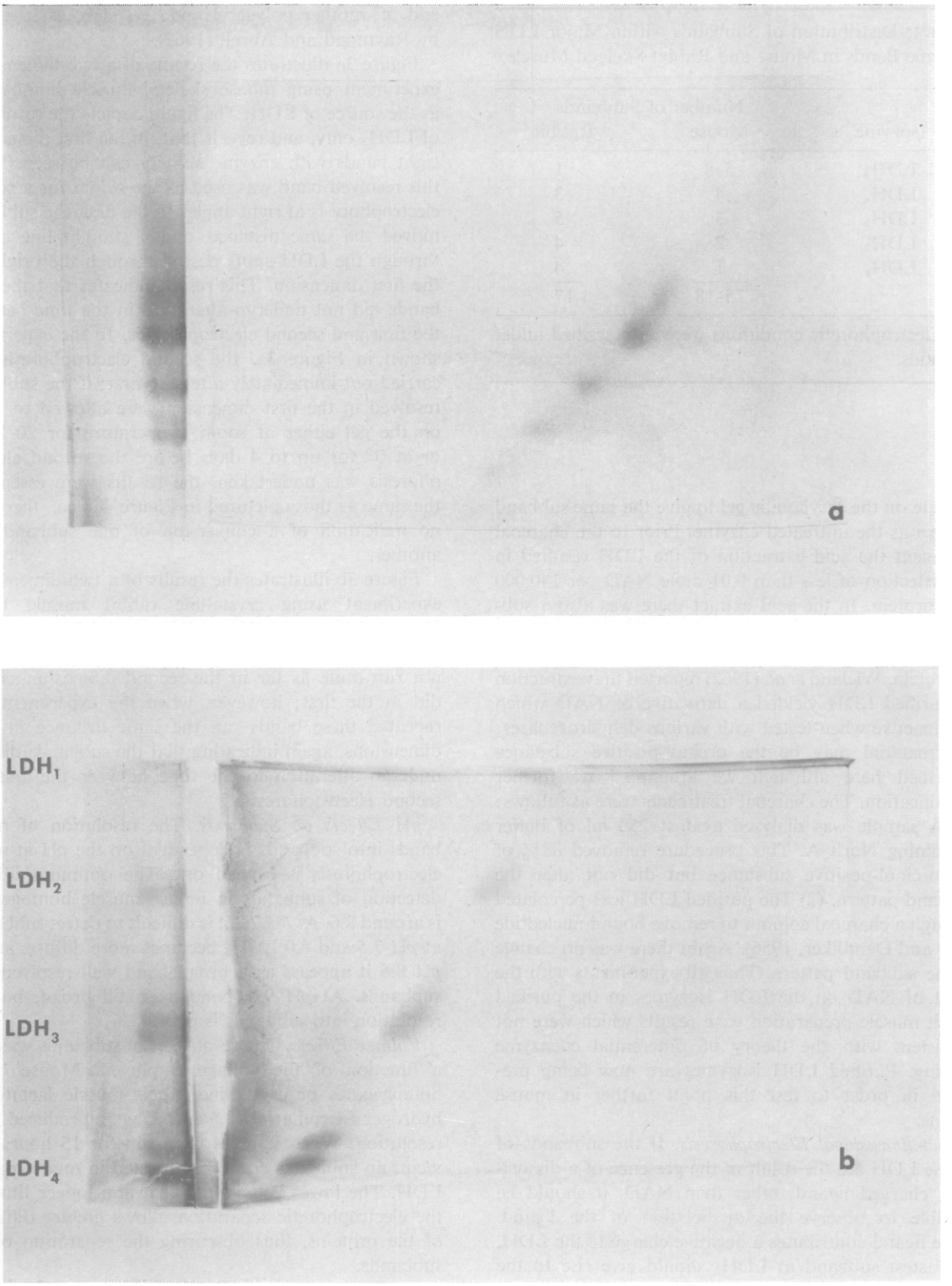

FIGURE 3: Two-dimensional electrophoresis. (a) Mouse skeletal muscle homogenate. Electrophoresis for 7 hours in both directions at 26.6 v/cm; Tris-HCl buffer 0.05 M, *p*H 8.5; 10 μl of sample, representing 0.30 LDH unit, was used. Assay was for 1 hour for first dimension, and for 3 hours for the second dimension. (b) Crystalline rabbit muscle LDH. Electrophoresis for 3 hours in both directions at 26.6 v/cm; Tris-HCl buffer 0.05 M, *p*H 8.5; 25 μl of sample, representing 0.8 LDH unit. Assay for 1 hour in first dimension and for 3 hours in the second dimension. The strip used for the origin in the second dimension is shown between the two gels. In the case of the rabbit, a small amount of LDH activity remained on the origin strip, as indicated in the photograph.

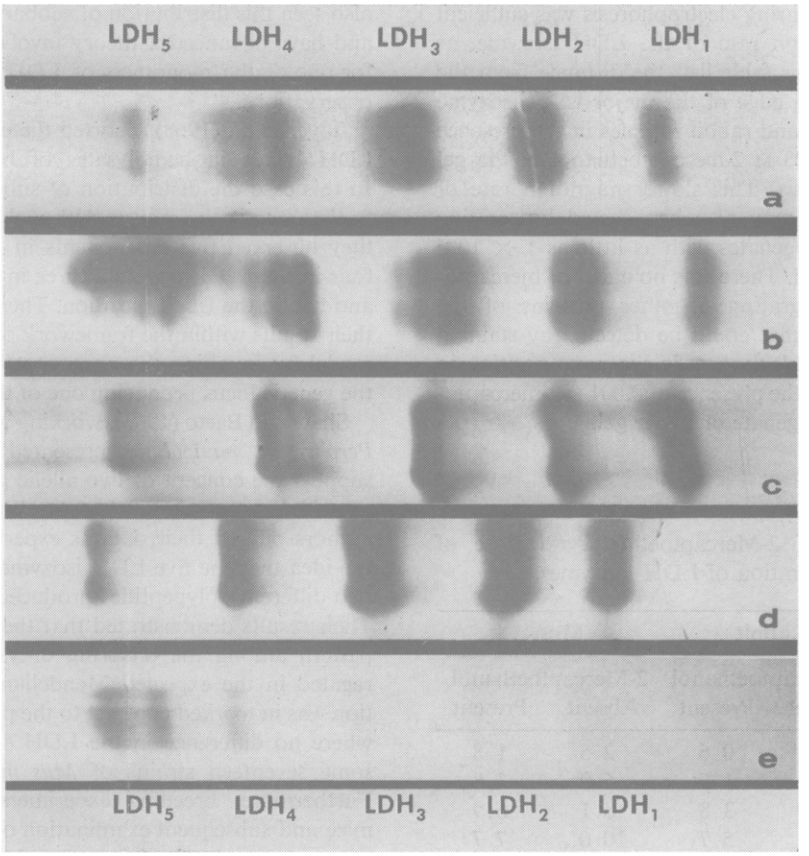

FIGURE 4: Subbands of heart muscle and skeletal muscle LDH at different concentrations of 2-mercaptoethanol. In (a–d) heart muscle homogenate was subjected to electrophoresis in acrylamide gel containing 0.035 M Tris, *p*H 8.5, and 2-mercaptoethanol at the following concentrations: (a) 0; (b) 0.001 M; (c) 0.004 M; (d) 0.008 M. In (e) a skeletal muscle homogenate was used under the conditions of (d). The distribution of subbands of skeletal muscle LDH was the same as those of heart muscle under the conditions of (a–c). Origin and cathode are to the left.

side of the origin indicated that the LDH isozymes had migrated the same distance in the two 1.5-hour periods.

2-Mercaptoethanol Effects. For demonstration of the subbands, the presence of 0.001 M 2-mercaptoethanol in the gel during electrophoresis is advantageous (Fritz and Jacobson, 1963), although not essential. The subbands were influenced in different ways depending upon the 2-mercaptoethanol concentration, and there was a marked difference between the response of the mouse and rabbit samples to this reagent.

Incorporation of 2-mercaptoethanol into the gel was accomplished by soaking the gel in the desired concentration for at least 2 hours prior to electrophoresis. After the soaking period, the concentration of 2-mercaptoethanol in the solution external to the gel was verified by assay (Boyer, 1954), since at these levels it was rather unstable. Concentrations of 0.001 M 2-mercaptoethanol enhanced the resolution of subbands in mouse organ homogenates. When the electrophoresis was carried out in the absence of 2-mercaptoethanol, the largest amount of LDH activity was seen in the two fastest subbands of LDH in mouse heart muscle homogenates (Figure 4). However, if the 2-mercaptoethanol concentration is about 0.004 M, a shift in the intensity of the bands results, so that the slowest subband of each major isozyme is the most active. At concentrations of 0.008 M it is no longer possible to detect multiple subbands within LDH_5 in the mouse skeletal muscle samples, but the activity is localized into two distinct regions of approximately equal intensity (see Figure 4e). On the other hand, the mouse heart LDH did not yield such a condensed pattern at 0.008 M 2-mercaptoethanol. This progressive shifting of activity among the subbands was not seen in the crystalline rabbit muscle LDH samples; indeed, there was no effect on this enzyme until the concentration of 2-mercaptoethanol was 0.007 M, whereupon the subbands were no longer apparent. Concentrations above 0.008 M could not be tested because the tetrazolium salt was nonspecifically reduced, causing the whole gel to be stained dark purple.

Yet another effect of 2-mercaptoethanol was upon the electrophoretic mobility of the LDH isozymes in both mouse and rabbit samples. The presence of this

reagent in the gel during electrophoresis was sufficient to alter the migration rate of the LDH isozyme, as seen in Table II. This table lists the distance from the origin to the trailing edge of the major LDH isozyme bands in the mouse and rabbit samples in the presence and absence of 0.003 M 2-mercaptoethanol in the gel during electrophoresis. This slower migration rate of the LDH isozymes may also be effected by treating mouse muscle homogenates with as little as 1×10^{-4} M 2-mercaptoethanol. There was no effect of mercaptoethanol on the migration of other proteins of the homogenate; those that could be detected by staining with naphthol blue black were in the same position in the gel regardless of the presence of 0.001 M 2-mercaptoethanol in the homogenate or in the gel.

TABLE II: Effect of 2-Mercaptoethanol on Rate of Electrophoretic Migration of LDH Isozymes.[a]

	Rabbit		Mouse	
	2-Mercaptoethanol		2-Mercaptoethanol	
Isozyme	Absent	Present	Absent	Present
LDH_5	0.6	0.6	2.5	1.8
LDH_4	2.5	1.6	6.0	3.8
LDH_3	4.9	3.8	8.1	6.9
LDH_2	7.5	5.7	10.0	7.7
LDH_1	9.8	7.5	11.9	9.3

[a] Distance (cm) from the origin to trailing edge of isozymes from crystalline rabbit muscle LDH and mouse homogenate in presence of 0.003 M 2-mercaptoethanol and in the absence of this reagent. Electrophoresis at *p*H 8.5 in Tris-phosphate buffer (0 05 M Tris) for 3 hours at 26.6 v./cm.

Discussion

It has been demonstrated that lactate dehydrogenase isozymes exist in multiple fractions in extracts of the different organs of the mouse as well as in crystalline preparations from rabbit skeletal muscle. Evidence from several laboratories mentioned in the introduction supports a hypothesis to explain the existence of the major isozymes: that the LDH molecule consists of four polypeptide subunits, of which there are two types distinguishable by charge. Arrangement of these two types in all possible combinations of four yields five separate products if sequential order is not considered. The discovery that each of these five isozymes can be further subdivided presents a challenge to the theory as presently proposed.

Our original observation that the major isozymes in mouse organ homogenates were resolved into fifteen subbands with a 5, 4, 3, 2, 1 distribution was rationalized within the framework of the tetramer hypothesis as previously mentioned. Costello and Kaplan (1963) have also seen this distribution of subbands in mouse tissues, and have advanced a theory involving duplicate genes for one of the monomers of LDH to account for our observations.

Boyer *et al.* (1963) reported the existence of multiple LDH bands in hemolysates of human erythrocytes. In this case the distribution of subbands was opposite to that seen in the mouse. Although band 5 was absent, they observed five components in the LDH_1 position, four in the LDH_2 position, three in the LDH_3 position, and two in the LDH_4 position. These authors explained their results within the framework of the LDH tetramer model by invoking the concept of a mutant allele at the genetic locus producing one of the subunits.

Shaw and Barto (1963), working with the deer mouse, *Peromyscus maniculatus*, presented genetic evidence to support the concept of two allelic genes producing the observed subbands of lactate dehydrogenase. These authors offered their data as experimental support for the idea that the five LDH isozymes were the result of two different polypeptides produced by separate genes. Their results demonstrated that the variations in LDH pattern among the offspring of various matings segregated in the expected Mendelian ratios. This situation was in marked contrast to the present observations, where no difference in the LDH subband patterns of some seventeen strains of *Mus musculus* was noted. Furthermore, breeding experiments with dystrophic mice and subsequent examination of the LDH patterns of parents and offspring revealed no difference in the distribution of subbands within the major isozyme bands.

Further genetic evidence for the control of subbanding, in human erythrocytes, is presented by Kraus and Neeley (1964). In this case there were two types of subband patterns for LDH_3, both of which were subject to hereditary control.

The current theories to explain the origin of the subbands within the framework of the LDH tetramer concept depend upon a 5, 4, 3, 2, 1 distribution of subbands among the major isozymes, progressing from slowest to fastest or vice versa. This pattern appears to be the case for mouse muscle LDH, although we have observed, irregularly and inconsistently, eight bands in LDH_5. The observation that the distribution of subbands in rabbit muscle LDH is 1, 3, 5, 4, 4, progressing from LDH_5 to LDH_1, cannot be reconciled with the LDH tetramer model by assuming a single alteration in one of the monomers of LDH. The differences in response of the subbands to various experimental conditions in the mouse and rabbit suggest that a single explanation is not adequate to account for the observed phenomena in the two species. At present we have no satisfactory explanation for the observations in either mouse or rabbit. It appears that SH groups may somehow be involved, since the optimum *p*H for resolution of subbands is around 8.6, which is the approximate *pK* of SH groups in proteins. An additional argument for SH-group involvement is the observation that the resolution of subbands is noticeably affected by 2-mercaptoethanol. The insensitivity of the rabbit

muscle enzyme to low concentrations of 2-mercaptoethanol, in contrast to the susceptibility of the mouse samples, was one reason for suspecting that the explanation for the subband phenomena in the two different species might not be the same.

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