Nature and Development of Lactic Dehydrogenases

The two major types of this enzyme form molecular hybrids which change in makeup during development.

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Many enzymes have been reported to exist in more than one form within the same species. By several methods a number of molecular forms of lactic dehydrogenase (LDH) have been found in differing amounts in the various organs of one individual *(1–4)*. This article describes studies which were undertaken in an attempt to elucidate the molecular nature of these multiple enzymatic forms and to follow their development. Most of the developmental studies were made on the chicken.

Two "pure" lactic dehydrogenases occur in the chicken. One of them (CM) is found principally in the breast muscle, and the other (CH) in the heart, of adult chickens. These two enzymes are separate entities as judged by physical, enzymatic, and immunochemical criteria *(1, 2, 5)*. During embryonic development the LDH enzymes of the chicken breast muscle shift from the enzymes related to CH, through several intermediate enzyme types, and appear in the adult as pure CM. The characterization of these intermediate enzyme types by several independent methods has led to the development of the hypothesis, which we present in this article, that the intermediate enzyme types which appear during embryonic development are "hybrid" enzymes, consisting of both CM and CH components. Furthermore, these "hybrids" also occur in the adult tissues of the chicken and in other animals.

Immunological Approach

In order to use immunological methods for the study of the development and structure of specific proteins, it is necessary that the immune systems be fully characterized. Two immune systems were used in this study: (i) CH-LDH rabbit anti-CH-LDH (anti-CH) and (ii) CM-LDH rabbit anti-CM-LDH (anti-CM). Anti-CM was shown to be homogeneous when measured by double diffusion in agar *(6)*, immunoelectrophoresis *(7)*, and quantitative precipitation analyses *(8, 9)*. Anti-CH was shown to be heterogeneous when measured by double diffusion in agar. After absorption with CM, two bands remained when tested with crude chicken heart extracts, only one of which showed LDH activity *(10, 11)*. This absorption did not decrease the complement (C′) fixation titer of CH-anti-CH (Fig. 1), nor did it remove antibody capable of neutralizing CH enzymatic activity. Thus, the heterogeneity in anti-CH reflected antibodies to impurities in the CH immunizing antigen (probably CM) and not anti-CH cross-reacting with CM.

Figure 1 shows the C′ fixation curves of CM and CH with anti-CM and anti-CH. After absorption with CM, anti-CH showed no reduction in titer against CH. Moreover, the C′ fixation curves were identical when either crystalline CH or crude heart extracts were used as antigen. At the dilutions of antisera used (1/5000 for anti-CH and 1/2200 for anti-CM) no cross reactions between CM and CH are detectable. Figure 2 demonstrates the ability of C′ fixation to resolve a mixture of pure CM and CH. The heights of the curves at peak fixation are the same in the mixture as in the two pure systems. By the shift in the abscissa at peak fixation it is possible to calculate the percentage of total enzyme activity which reacts with either anti-CM or anti-CH *(9, 12)*. In an artificial mixture of pure CM and CH (Fig. 2), the percentages calculated by C′ fixation are equal to the percentages obtained by enzymatic assay.

Figure 3 illustrates the results of enzyme inhibition with homologous and heterologous antisera. Anti-CM was found to have no inhibitory effect toward CH. Unabsorbed anti-CH, however, did neutralize CM, although much more antiserum was required than with CH. After absorption with crystalline CM, anti-CH was specific for CH (Fig. 3). No reduction in the enzyme neutralizing titer of anti-CH toward CH was effected by the absorption with CM (Fig. 3*B*)—a finding that confirmed our conclusion from earlier C′ fixation and gel diffusion analyses that anti-CH had a small amount of antibody to CM. Anti-CM, which was prepared against a highly purified recrystallized enzyme, showed absolute specificity for CM and appeared to be homogeneous.

Development of CM in Chick Embryos

Figures 4 and 5 represent the C′ fixation analyses of extracts of breast muscle of chick embryos of increasing age as measured by the reaction with anti-CH and anti-CM, respectively. Figure 4 shows that in the 6-day-old embryo essentially all of the LDH activity is identical with adult heart lactic dehydrogenase (CH). It should be noted that this same sample gave a very weak reaction with anti-CM. On the other hand, essentially all of the lactic dehydrogenase from breast muscle of an 8-day-old newly hatched chick was identical with the adult muscle lactic dehydrogenase (CM). At intermediate times during development (at 13.5, 16.5, 17.5, 19.5, and 22 days of incubation), reactivity with anti-CH decreases, whereas reactivity with anti-CM increases. These changes are most rapid just prior to and during hatching.

Quantitative C′ fixation is advantageous in that it can measure qualitative changes in protein structure as well as resolve quantitatively two independent antigens in admixture. As in quantitative precipitin analyses, maximum C′ fixation with a constant amount of antibody is a function of the extent of antigen-antibody aggregation. A protein whose determinant groups differ in

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number or structure from those of the homologous antigen will fix less C′ with the homologous antibody. Thus the decrease in the amount of C′ fixed at equivalence (peak fixation) (Fig. 4) and the increase in the same parameter (Fig. 5) reflect a change in LDH antigenic structure during development. The amount of lactic dehydrogenase (as measured by enzymatic activity) which is required to reach peak fixation, however, is a function of the fraction of the lactic dehydrogenase in the enzyme mixture which reacts with a particular antibody. For example, the lactic dehydrogenase in the breast muscle of the 6-day-old embryo gives maximum fixation with anti-CH at 0.08 enzyme units, essentially the same amount as that required for maximum fixation with pure CH. The same enzyme of the 6-day-old embryo reaches maximum fixation with anti-CM at 10 enzyme units; maximum fixation is reached with 0.1 enzyme unit with pure CM. From these data we have estimated the percentage of the extracts that react with either anti-CM or anti-CH (*11*, *13*). In Fig. 6 is presented a summary of these calculations: the percentages of lactic dehydrogenase reacting with anti-CH and anti-CM, respectively, in the chicken breast muscle during development. Figure 7 summarizes the changes in antigenic structure of the same extracts as measured by the maximum amount of C′ fixed as compared to the maximum amount for the homologous antigen (CM or CH). From an analysis of these data it can be shown that the lactic dehydrogenase of chicken breast muscle changes from CH to CM through intermediate antigenic enzyme types during development.

Further evidence that the lactic dehydrogenase in the breast muscle is changing qualitatively as well as in overall composition (that is, in the proportions of CM to CH) was obtained from studies of the precipitation of enzyme activity by the specific antibodies. Figure 8 summarizes the results from several series of enzyme precipitation experiments. The pattern of change in the amount of enzyme activity precipitable with anti-CM and anti-CH is very similar to the summaries in Figs. 6 and 7. One striking fact is evident from an analysis of these data. More than 100 percent of the enzyme activity can be precipitated from embryos of intermediate stages (Table 1); that is, the sum of the amounts precipitated separately by

Table 1. Percentage of total enzyme activity precipitated. Each extract was tested separately against anti-CM and anti-CH. Tests on normal rabbit serum controls were made for each point, to check for nonspecific inhibition. Each value represents results for three tubes containing increasing amounts of anti-CM and anti-CH. After incubation at room temperature for 1 hour and overnight at 0° to 4°C, the tubes were spun and enzyme activity was determined on the supernatant fluid. The percentage of total activity was calculated at the point of maximum precipitation, on the basis of the normal-serum control for that amount of antiserum.

Tissue	Anti-CM	Anti-CH	Total
Adult heart extract	0	100	100
6-day breast muscle	14	100	114
11-day breast muscle	44	96	140
12.6-day breast muscle	54	95	149
13.8-day breast muscle	58	96	154
16.7-day breast muscle	64	96	160
17.3-day breast muscle	74	83	157
17.7-day breast muscle	83	67	150
19.5-day breast muscle	94	29	123
22-day breast muscle	94	18	112
Adult breast muscle extract	>98	<1	99
Mixture of breast-muscle and heart-muscle extracts (roughly 50–50)	43	57.5	100.5

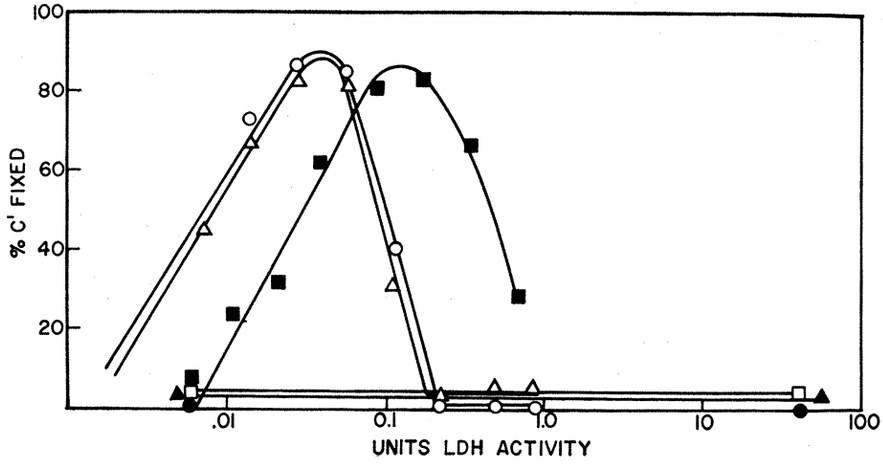


Fig. 1. Complement fixation by CM-anti-CM and CH-anti-CH. Open circles, CH-anti-CH; open triangles, CH-anti-CH absorbed with CM; solid squares, CM-anti-CM; open squares, CH-anti-CM; solid circles, CM-anti-CH; solid triangles, CM-anti-CH absorbed with CM.

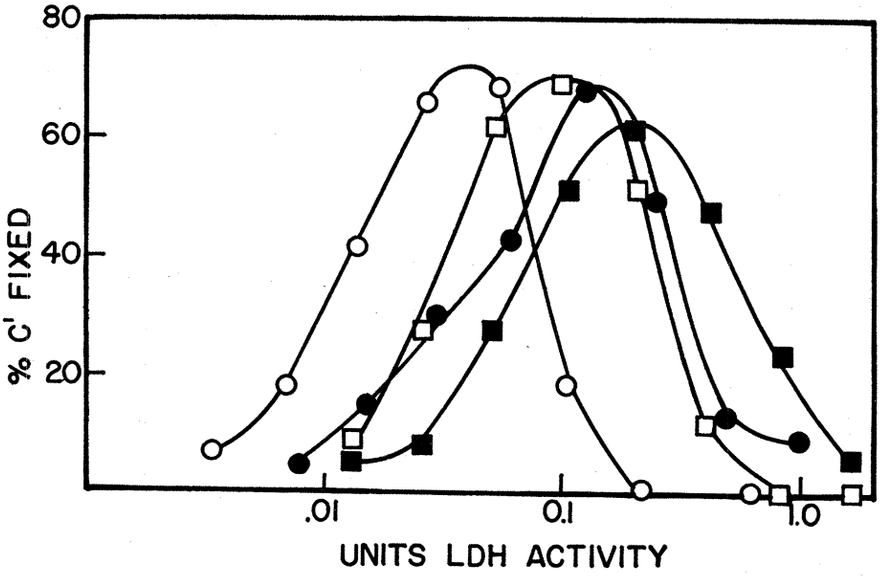


Fig. 2. Complement fixation analysis of a mixture of CM and CH. Open circles, CH-anti-CH; solid circles, CM-anti-CM; open squares, mixture of CM and CH-anti-CH; solid squares, mixture of CM and CH-anti-CM.

anti-CM and anti-CH is greater than the amounts of enzyme added. If a known mixture of pure CM and pure CH is treated with anti-CM and with anti-CH in a similar manner, the amounts of enzyme precipitated (Table 1) add up to exactly the amounts added. This is interpreted to mean that some enzyme molecules in the embryonic extracts are being precipitated by both anti-CM and anti-CH. Since no CM is ever precipitated under these conditions by anti-CH, nor any CH by anti-CM (Fig. 3), the only conclusion to be drawn is that, during development, LDH molecules arise which contain antigenic structures common to *both* CM and CH. These conclusions were confirmed directly when we looked at the change in the electrophoretic patterns of breast muscle from chick embryos of increasing age (Fig. 9).

In the very young blastoderm the CH form of lactic dehydrogenase predominates, but there is a trace of a more slowly moving component. Gradually a third, even more positively charged, band appears, and these two intermediate bands increase slowly in intensity at the expense of CH, up to about 16 days. Then, suddenly, there is a rapid appearance of a band corresponding to CM and one slightly more negatively charged. This qualitative picture agrees remarkably well with the kinetics of development of CM and the disappearance of CH-related lactic dehydrogenase (Figs. 6–8), as judged by C′ fixation and enzyme neutralization with the specific antibodies. The sequential development of the more negatively migrating bands is mirrored in the increase in reactivity with anti-CM and the decrease in reactivity with anti-CH.

Table 2 summarizes the results of enzyme precipitations carried out with anti-CM and anti-CH on all five electrophoretic bands in the chicken. It may be seen that the intermediate bands arising during development are completely precipitated by both antisera, whereas there is no cross reaction between the extremes.

Still another method of approaching this problem has yielded the same answer as the immunochemical and electrophoretic approaches. The CM and CH forms of lactic dehydrogenase differ in their substrate saturation curves (Fig. 10) as well as in their ability to reduce coenzyme analogs (*1, 2, 5, 11*). The ratio of the rate of reduction of reduced diphosphopyridine nucleotide (DPNH) with 0.01*M* pyruvate to the rate of reduction of deamino DPNH with 0.00033*M* pyruvate can be used to measure the relative amounts of CM- and CH-related lactic dehydrogenases in a mixture. These data are plotted in Fig. 8. The ratio falls during the development of the chick breast muscle in a manner markedly similar to the way in which the amount of anti-CH-related lactic dehydrogenase changes (Figs. 6–8). Thus, all four of the methods used give the same result: the level of CM-related lactic dehydrogenase rises during the development of the breast muscle, and the level of CH-related lactic dehydrogenase falls. During the period in which both CM- and CH-like enzymes are present in about equal amounts, there is a large amount of enzyme present which is related to both CM and CH. We have called these intermediate enzyme types "hybrid" lactic dehydrogenases.

When cells from embryonic chicken tissues (heart, liver, leg muscle) are placed in tissue culture, their LDH pattern shifts from heart-related to muscle-related lactic dehydrogenases. These changes occur much faster than the normal, *in ovo*, shifts. We are at present investigating possible causes of these shifts (pO_2, pCO_2, yolk constituents, and so on).

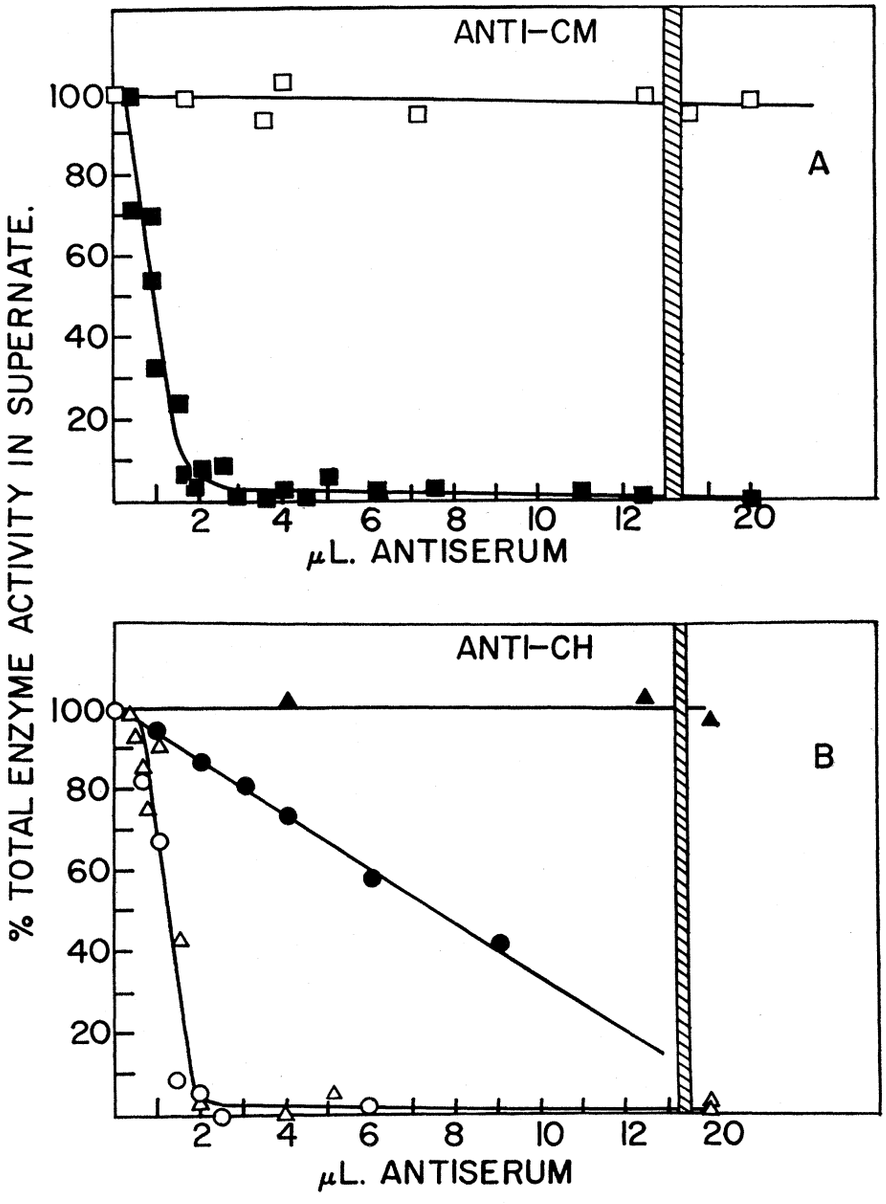


Fig. 3. Enzyme precipitation with anti-CM and anti-CH. Open circles, CH-anti-CH; open triangles, CH-anti-CH absorbed with CM; solid circles, CM-anti-CH; solid triangles, CM-anti-CH absorbed with CM; solid squares, CM-anti-CM; open squares, CH-anti-CM. Precipitations were carried out as described in Table 1.

Number of Multiple Forms

Although there have been a number of reports which suggest that more than five molecular forms of lactic dehydrogenase have been found on electrophoretic analysis, we believe that only five distinct types exist in any given animal species (Fig. 11). Under certain conditions, the most slowly migrating band appears to split into a major band and a minor satellite band. However, the catalytic and immunological characteristics of the two bands appear to be identical. Irwin Freedberg of our laboratory has found that many artifacts can be introduced if the gel is overloaded with lactic dehydrogenase, and more than five bands can be observed under these conditions. The additional bands do not occur, however, if the preparation is diluted before application to the gel. The bands detected in the undiluted solution were strong enough so that they could still have been detected after dilution if they had been genuine molecular species (*14*).

Physical Nature of Hybrid Lactic Dehydrogenase

In most vertebrates so far studied there are two chief categories of lactic dehydrogenase. One type predominates in heart, the other in muscle. In the chicken these heart and muscle lactic dehydrogenases are completely different. They have widely differing amino acid composition, electrophoretic mobility on starch grain and gel, substrate inhibition kinetics, and analog oxidation ratio, and they elicit antibodies in the rabbit that are non-cross-reacting (*1*, *2*, *5*, *11*). The heart enzymes of the various vertebrates studied (Table 3) show striking similarities, as do the muscle enzymes. There are much greater differences between the heart and muscle enzymes of the chicken than between beef-heart and chicken-heart enzymes (*1*, *2*, *5*, *11*).

The lactic dehydrogenases of most vertebrates can be separated into five equally spaced, distinct components by starch gel electrophoresis (*15*). In the chicken, pigeon, beef, rat, mouse, rabbit, and human the most positively moving of these bands is the heart type, whereas the most negatively moving is the muscle type (Fig. 11). The intervening bands show intermediate properties, as determined by all of the criteria mentioned above, and vary in a regular way from the heart band to the muscle band. (Tables 4 and 5). Different tissues from the same animal have characteristic distributions of the five bands, but these distributions are quite species specific. For example, in the human, band 1 (the muscle band) is predominant in the liver, whereas in the chicken, bands 4 and 5 (the hybrid and heart bands) are predominant in the liver. The fact that only five bands are found in most well-documented, careful studies with vertebrate tissues has suggested to us that there must be some fundamental significance in this number and that any hypothesis that attempts to explain "hybrid" enzymes must adequately account for these five bands. In an attempt to devise such a theory we have used the following facts.

The chicken muscle, chicken heart, beef muscle (beef M), and beef heart (beef H) forms of lactic dehydrogenase all bind four molecules of diphosphopyridine nucleotide (DPN) per molecule of enzyme. Recently, Appella and Markert (*3*) reported that beef H lactic dehydrogenase can be dissociated into four subunits by treatment with *5M* guanidine HC1 + 0.1*M* mercaptoethanol. In collaboration with Amadeo Pesce, we carried out this type of experiment on CM and CH. The results show that both of these enzymes can be bro-

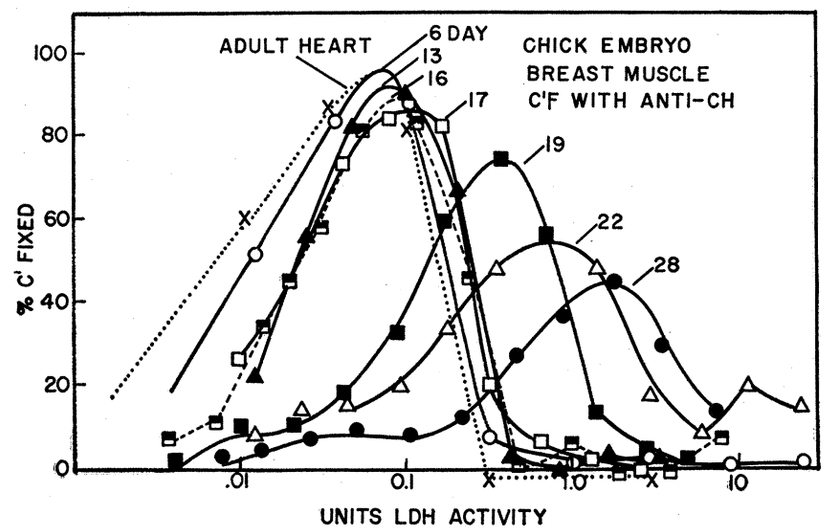


Fig. 4. Complement fixation by developing chicken breast muscle when tested with anti-CH. ×'s, adult heart; open circles, 6-day-old embryo breast muscle; solid triangles, 13-day-old embryo; half-solid squares, 16-day-old embryo; open squares, 17-day-old embryo; solid squares, 19-day-old embryo; open triangles, 22-day-old embryo; solid circles, 28-day-old embryo.

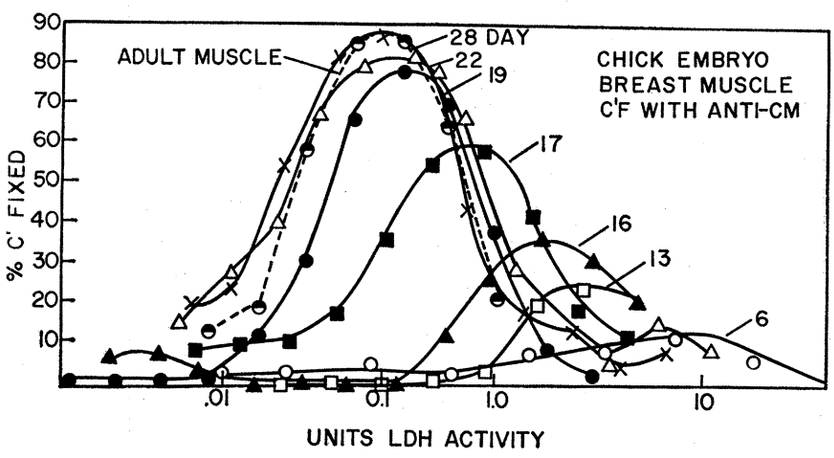


Fig. 5. Complement fixation by developing chicken breast muscle when tested with anti-CM. Half-solid circles, adult breast muscle; open circles, 6-day-old embryo breast muscle; open squares, 13-day-old embryo; solid triangles, 16-day-old embryo; solid squares, 17-day-old embryo; solid circles, 19-day-old embryo; open triangles, 22-day-old embryo; ×'s, 28-day-old embryo.

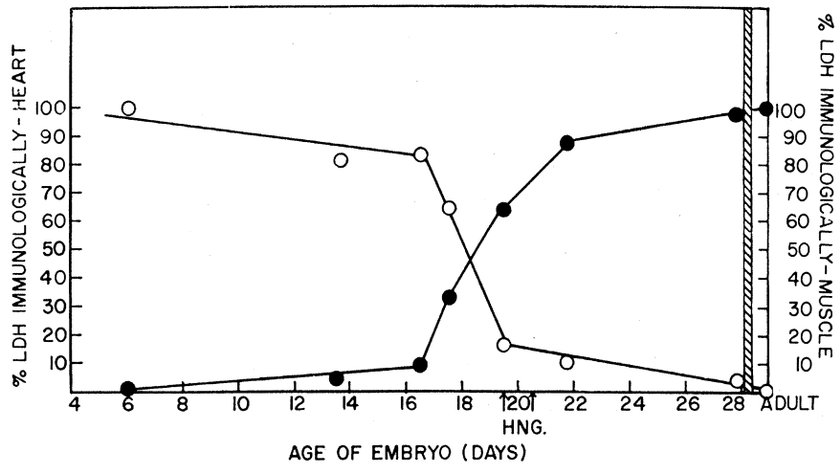


Fig. 6. Summary of LDH composition of developing chicken breast muscle, from complement fixation analyses (see text). Open circles, anti-CH; solid circles, anti-CM; *HNG*, hatching.

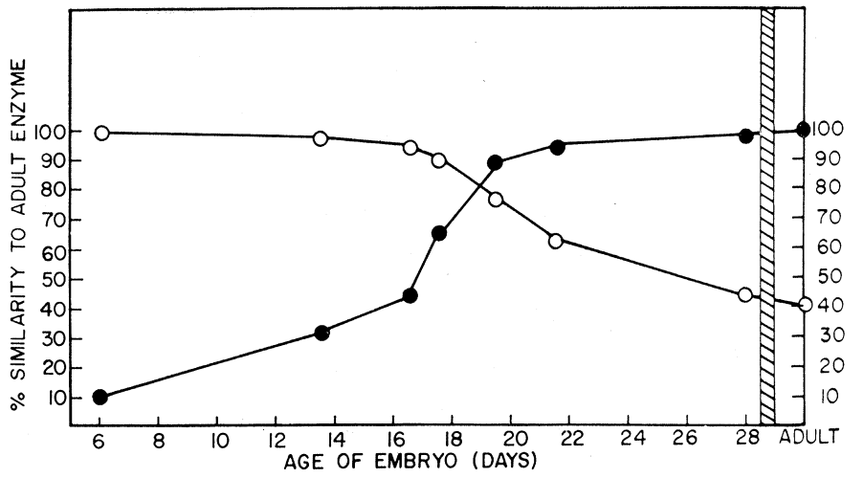


Fig. 7. Summary of change in immunochemical similarity to CM and to CH of LDH in developing chicken breast muscle, as shown in complement fixation analyses (see text). Open circles, anti-CH; solid circles, anti-CM.

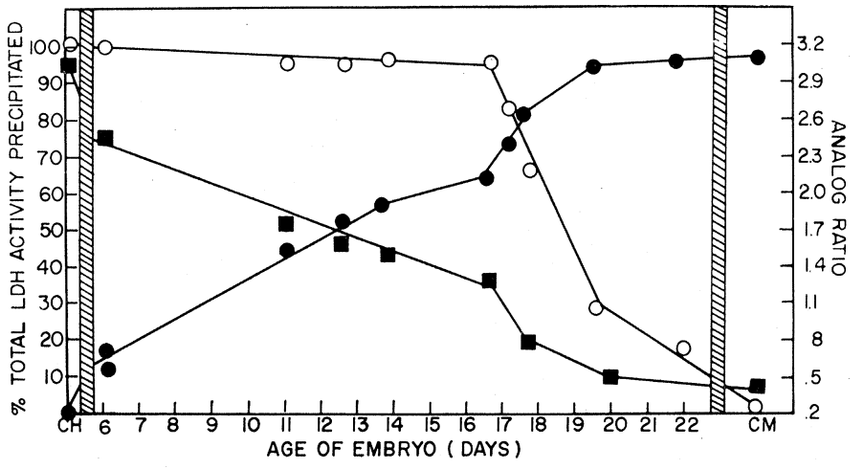
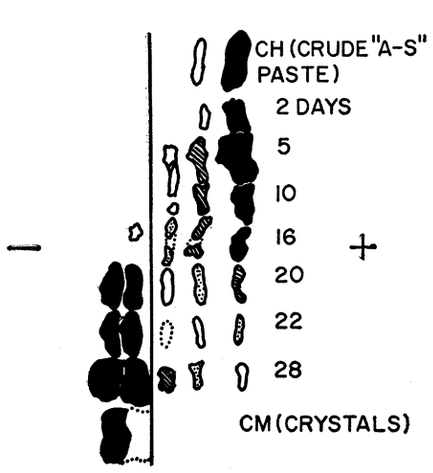


Fig. 8. Immunological and catalytic analysis of LDH in developing chicken muscle. Ordinate at left: Open circles, precipitation of enzyme wth anti-CH; solid circles, precipitation of enzyme with anti-CM. Ordinate at right; Solid squares, ratio of rate of oxidation, by breast muscle extracts, of deamino DPNH to rate of oxidation of DPNH, at concentrations of sodium pyruvate of $3.3 \times 10^{-4}M$ and $1.0 \times 10^{-2}M$, respectively.

ken down into four subunits of equal molecular weight by treatment with $5.5M$ guanidine HCl plus $0.1M$ mercaptoethanol (*16*). Electrophoretic-chromatographic "fingerprints" of tryptic digests of CM and CH show only roughly 35 amino acid spots. Furthermore, there are only one-quarter the number of arginine plus lysine spots as there are residues of these amino acids in the entire molecule. These data, taken together with the results, cited above, indicating the presence of several species of LDH molecules with antigenic groupings common to both CM and CH, have led us to formulate the following hypothesis: CH is a tetramer, composed of four identical subunits (HHHH) of molecular weight about 31,000; CM also is a tetramer, composed of four identical subunits (MMMM) that differ in many respects (including net charge at *p*H 7.0) from the subunits that compose CH; H and M are probably elaborated by two different genes. If both the H and M genes are active in the same cell, then the primary gene products (monomer units) recombine in some manner in groups of four, yielding five different molecular species (HHHH, HHHM, HHMM, HMMM, and MMMM) all possessing LDH activity. The order of the arrangement of the subunits may affect the antigenic specificity of the molecule, but the subunits, according to the hypothesis, operate independently in respect to their substrate inhibition and rates of reaction with DPN analogs. This hypothesis predicts *five* electrophoretically distinct bands of lactic dehydrogenase on starch gel electrophoresis if, and only if, subunits H and M differ in net charge at the *p*H used. It also predicts that the five bands should be spaced at approximately equal intervals and should show regular changes in their antigenic and enzymatic properties. It is conceivable that a species may possess subunits H and M with identical net charges, so that only one band can be detected, and yet that there might still exist the basic heterogeneity predicted. We propose to call the three intermediate lactic dehydrogenases "hybrid" enzymes, since they differ quantitatively and qualitatively from the "parental" types much as genetic hybrids do. We feel that this categorization is much more suitable than the terms previously used—*isoenzymes* or *isozymes*—since the enzymes are not the same but show properties differing in a regular way from one extreme to the other. It is



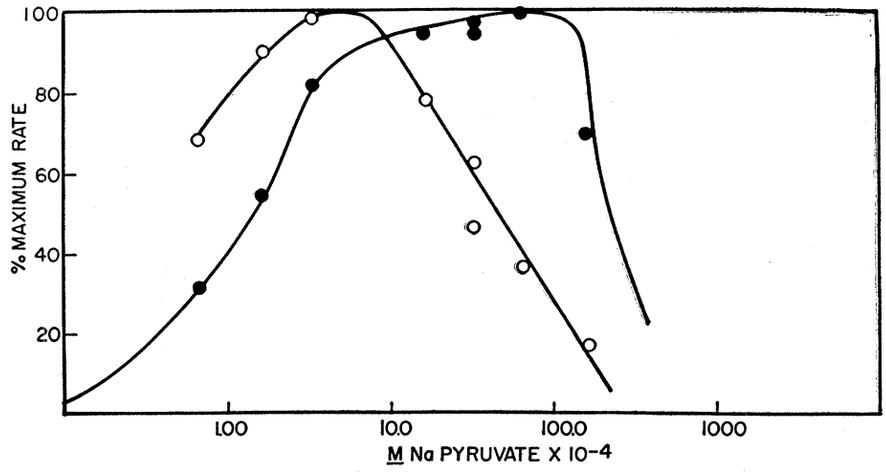


Fig. 9 (left). Electrophoretic patterns of LDH during the development of chicken breast muscle. Tracing of starch gels (Connaught, 14 percent) stained for LDH with nitro blue tetrazolium (*10, 11*). Blocks, 19.5 by 6.9 by 0.6 centimeters; run, 15 hours; temperature, 0°C; current, 27 milliamperes, 7.5 to 12.5 volts per centimeter across the block. Citrate-phosphate buffer, *p*H 7.0 (*11*). Solid bands, very intense staining; striped bands, intense staining; speckled bands, moderate staining; clear bands, weak staining; broken outline, barely visible. *A–S*, ammonium sulfate. Fig. 10 (right). Effect of pyruvate concentration on the activity of CM and CH. Open circles, CH; solid circles, CM.

likely that many other cases where enzymes exist as "hybrid" enzymes will come to light, but there are also cases where electrophoretically distinct bands may represent other modifications of enzyme molecules (*17*). We wish to emphasize that when one observes that a given enzyme has multiple bands, this does not necessarily imply that hybrids are involved. To have hybrids, it is essential that at least two distinct types of enzymes be present.

Various studies by Markert and Appella, as well as by us, have indicated that there are no distinguishable catalytic differences between the two electrophoretically separated lactic dehydrogenases found in the crystalline beef heart preparation. We have now been able to show, through more detailed studies with the coenzyme analogs, that the two bands do have some significantly different characteristics. The major band is the pure heart type (HHHH), whereas the minor component has the properties of the hybrid (MHHH) (Table 4).

Significance of Multiple Forms

The data presented in this article support the suggestion, made previously, that there are two types of lactic dehydrogenase (*1, 2*). Our view that the synthesis of the two types of subunits is controlled by two separate genes has also been supported by comparative studies. We have reported previously that the heart lactic dehydrogenase in the Heterosomata or flatfish (halibut, sole, flounder) differs greatly from that in other teleosts. We have now compared the properties of a number of skeletal muscle lactic dehydrogenases in members of several other orders of teleost fish and in the flatfish. An example of such a comparison is given in Table 6, where the ratios of the rates of reaction of tuna and flounder lactic dehydrogenase with some coenzyme analogs are summarized. It may be seen from the data that the skeletal muscle lactic dehydrogenases are quite similar in tuna and flounder. However, the heart enzymes are very different in the two species. From the characterizations given in Table 6, it appears that the skeletal muscle enzymes and the heart enzymes of the flounder are identical. It is of interest to note (Table 6) that an antibody to the purified halibut muscle lactic dehydrogenase completely precipitates muscle lactic dehydrogenases of both tuna and flounder. The antibody, however, reacts with the flounder heart enzyme but not with the tuna heart enzyme. We have obtained similar data indicating that the heart and muscle lactic dehydrogenases of the sole and halibut are identical. The flatfish are the only group of vertebrates for which we have found identical types of lactic dehydrogenase in heart and muscle in the adult stage. Furthermore, we have been unable to detect evidence of more than one type of lactic dehydrogenase in any of the tissues of the adult flatfish; on

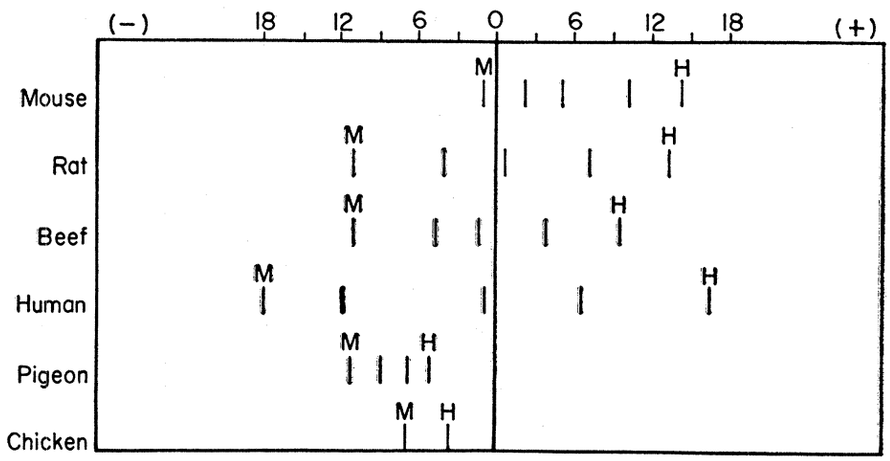


Fig. 11. Starch-grain electrophoresis of lactic dehydrogenases of different animals. *M*, most negatively migrating band, or muscle LDH; H, most positively migrating band, or heart LDH. Electrophoresis was continued for 18 hours at 10 volts per centimeter, with sodium veronal buffer, 0.05*M*, *p*H 8.6, at 2° to 4°C.

electrophoresis, only one band has been observed in any of the tissues studied. These results suggest that the flatfish may represent a mutant in which lactic dehydrogenase of the heart type is lost, at least in the adult stage.

Further evidence suggesting that the two types of lactic dehydrogenase are under the control of different genes has been obtained from studies with amphibians. Through analysis with coenzyme analogs we have found that the skeletal muscle lactic dehydrogenase of several species of frog and toad appear to be almost identical. However, the heart enzyme of the bullfrog (*Rana clamitans*) appears to differ distinctly from that of the other species studied. Not only does it differ with respect to reaction with the analogs but it differs greatly from the heart enzymes of the other amphibians studied in electrophoretic mobility on starch grain. However, the muscle enzyme of the bullfrog has electrophoretic characteristics almost identical to those of the other species studied. Differences in immunological and thermal stability have also been found between the heart enzyme of the bullfrog and that of other amphibian species. From its catalytic, physical, and immunological properties, it appears that the lactic dehydrogenase of the bullfrog heart may be a mutant enzyme. However, the lactic dehydrogenase of the bullfrog skeletal muscle appears to be very similar to that of the skeletal muscle of other amphibian species. This indicates that a modification of one type of enzyme can take place without an alteration in the second type. It seems reasonable to assume from such data that the two types of lactic dehydrogenase are controlled by different genes.

It should be emphasized that the electrophoretic migration, on starch, of the hybrids depends on the migration of the two pure types. In the case of the bird enzymes, the two pure types migrate relatively close together toward the negative pole at *p*H 8.6, and observation of the hybrid forms under these conditions is very difficult. On the other hand, the two types of lactic dehydrogenase in mammals usually differ greatly in their migratory characteristics, and the hybrids in mammals therefore can be detected more easily as electrophoretic entities. It can be said with considerable certainty that the more widely the two basic enzymes differ in charge, the more separable the hybrids will be after electrophoresis on starch.

Each species appears to possess both a heart and a muscle type of lactic dehydrogenase. For example, the muscle enzyme from the rat migrates considerably more negatively than the corresponding enzyme from the mouse (Fig. 11). Hence, the locations of the hybrid enzymes of the two rodents on electrophoresis are somewhat different. Although the muscle enzymes of the rat and mouse differ electrophoretically, they still appear to be closely related, as indicated by their reaction with the coenzyme analogs (Tables 5 and 7). The electrophoretic mobilities of the lactic dehydrogenases are not accurate criteria of the relationship between species and should not be used as indicators in evolutionary studies. In this connection, it is valuable to note that we have found some slight but significant differences in the migration of the same LDH moiety in different tissues of the same animal.

We have suggested previously (*11, 18*) that the differences in properties of the heart and muscle lactic dehydrogenases are of functional significance. There is a great difference in the degree to which pyruvate inhibits activity of the chicken heart and of the chicken muscle enzymes (see Fig. 10). A similar difference was found previously for purified bovine skeletal muscle and heart enzymes (*2*). Our view is that these marked differences are not *in vitro* curiosities but are physiologically important factors in the regulation of cellular metabolism. In evaluating the role of lactic dehydrogenase in skeletal and cardiac muscle, it is important to emphasize the difference in metabolic

Table 2. Percentage of enzyme activity precipitated. The assays were carried out as described in Table 1.

Type	Anti-CH	Anti-CM
MMMM (band 1)	0	100
MMMH (band 2)	95	100
MMHH (band 3)	100	70*
MHHH (band 4)	100	43*
HHHH (band 5)	100	0

* Since very little of these two bands was available, the enzyme precipitations may have been incomplete, due to the fact that the proportion of antibody to antigen was 7 to 10 times that in assays of the other bands. This reaction is inhibited when there is antibody excess (*11*) as well as when there is antigen excess, and this probably accounts for the low values observed (see *22*).

Table 3. Ratios of rates of reduction of $TNDPN_1$ to $APDPN_3$* in crude extracts of vertebrate heart and skeletal muscle.

Animal	Skeletal muscle	Heart
Turkey	0.48	5.3
Chicken	0.50	5.2
Pigeon	0.63	5.0
Tuna	0.22	8.2
Toad	1.60	6.2
Green frog	1.62	6.8
Rabbit	0.73	5.4
Human	2.2	5.7
Mouse	1.7	5.6
Beef	1.2	5.8

* APDPN, 3-acetylpyridine DPN; TNDPN, thionicotinamide DPN. Subscript 1 or 3 indicates that 3.3 × $10^{-4}M$ lactate, or 1.0 × $10^{-2}M$ lactate, respectively, was used in the assay.

Table 4. Comparison of the rates of reduction of $DeDPNH_1/DPNH_3$* ratio for molecular forms of lactic dehydrogenase from beef chest muscle and heart.

Type	Chest muscle	Heart
MMMM (band 1)	0.53	
MMMH (band 2)	0.70	0.78
MMHH (band 3)	1.23	1.29
MHHH (band 4)	1.83	1.74
HHHH (band 5)	2.78	2.50

* DeDPNH, nicotinamide hypoxanthine dinucleotide; DPNH, reduced DPN. Subscript 1 or 3 indicates that 3.3 × $10^{-4}M$ pyruvate, or 1.0 × $10^{-2}M$ pyruvate, respectively, was used in the assay.

Table 5. Comparison of the rates of reduction of $DeDPNH_1/DPNH_3$* ratio for various bands from mouse and rat tissues (*23*).

Type	Rat	Mouse
MMMM (band 1)	0.73	0.83
MMMH (band 2)	1.25	1.12
MMHH (band 3)	1.57	1.42
MHHH (band 4)	1.80	2.02
HHHH (band 5)	2.40	2.58

* DeDPNH, nicotinamide hypoxanthine dinucleotide; DPNH, reduced DPN. Subscript 1 or 3 indicates that 3.3 × $10^{-4}M$ pyruvate, or 1.0 × $10^{-2}M$ pyruvate, respectively, was used in the assay.

Table 6. Analog and immunological properties of lactic dehydrogenase in tuna and flounder.

Part	$APDPN_1/TNDPN_1$*	Percentage precipitated by antibody to halibut skeletal-muscle LDH
	Tuna	
Heart	0.34	0
Muscle	11.5	100
	Flounder	
Heart	11.2	100
Muscle	12.0	100

* APDPN, 3-acetylpyridine DPN; TNDPN, thionicotinamide DPN. The subscript 1 indicates that 3.3 × $10^{-4}M$ lactate was used in the assay.

Table 7. Analog reactivities of MMMM type lactic dehydrogenase from mouse and rat.

Ratio*	Mouse	Rat
$DPNH_3/DPNH_1$	0.80	0.87
$DPNH_3/APDPNH_1$	0.62	0.45
$DPN_3/TNDPN_1$	3.2	2.8
$TNDPN_3/APDPN_3$	3.5	6.2

* APDPNH, 3-acetylpyridine DPN; TNDPN, thionicotinamide DPN. Subscript 1 or 3 indicates that 3.3 × $10^{-4}M$ pyruvate, or 1.0 × $10^{-2}M$ pyruvate, respectively, was used in the assay.

properties of the two tissues. Skeletal muscle depends particularly on the anaerobic breakdown of carbohydrates for utilizable energy during exercise, and this results in a large production of lactic acid. This generation of lactic acid is essential for anaerobic metabolism; otherwise, reduced DPN would cease to be formed by the oxidation of triose phosphate. Hence, in skeletal muscle, the activity of lactic dehydrogenase must be compatible with the formation of lactate from pyruvate. Therefore, one might expect the skeletal muscle enzyme to operate in the presence of relatively high levels of pyruvate. On the other hand, heart activity depends more on aerobic metabolism than on anaerobic processes. There is no sudden requirement for energy in the heart. Pyruvate metabolism in the heart is directed toward oxidation rather than toward reduction to lactate. The marked inhibitory effect, on heart lactic dehydrogenase, of increasing concentrations of pyruvate permits pyruvate metabolism to proceed toward oxidation through the citric acid cycle. We suggest that the characteristic catalytic properties of the two lactic dehydrogenases are important in regulating the physiological activities of the tissues in which they are found (*11, 18*). We believe that the heart enzyme is a catalyst geared for activity in an aerobic environment, whereas the muscle enzyme functions in an anaerobic environment.

It is of interest that the heart enzyme appears first in the chick, even in the breast muscle. Grabowski (*19*) has reported that lactic acid is toxic to the early chick embryo; hence, one might expect that the embryonic tissues would have a system for keeping the formation of lactate low. The heart enzyme allows for formation of less lactate than the muscle enzyme. Therefore, it is possible that the chick tissues rely more on an aerobic than on an anaerobic metabolism, since the embryo may have no mechanism for removing the lactate.

In contrast to the chick embryo, the fetal rat heart appears to develop the skeletal muscle enzyme first (*20*). After birth, there is a changeover from the primarily muscle type to the heart type of enzyme. It appears likely that metabolism in the fetal tissues is more anaerobic than in the chick embryo, and thus one might expect the enzyme of the muscle type to be the primary embryonic form. Furthermore, lactate probably can be removed quite efficiently by diffusion through the placenta.

The variation in lactic dehydrogenase which has been described suggests that an intriguing type of molecular lability may have been evolved, somewhat similar to that seen in hemoglobins and visual opsins, which provides considerable selective advantage to organisms. The lactic dehydrogenase in a given organ may differ in different species (for example, the liver of most mammals—among them rat, mouse, and human—contains the muscle type of the enzyme, whereas beef liver and liver of other ruminants contains primarily the heart type), and, as emphasized in this article, different LDH molecules may predominate at various times during the development of a given structure. It is possible that particular molecular configurations are most efficient for given sets of metabolic conditions. While little is known of the significance of LDH hybrids in the metabolism of the cell, it may be speculated that a tissue whose metabolism is intermediate between that of heart and that of muscle may function best with a mixture of both types of lactic dehydrogenase. At any rate, the evolution of a system in which there are two basic molecular units and a mechanism for altering the relative proportions not only of the "pure" molecules but of molecules with intermediate properties (hybrid molecules) as well, offers distinct adaptive advantage to an organism. We may presume that such alterations in response to metabolic requirements have occurred with respect to two time scales: that of the evolution of species and that of the development of an individual.

We are not certain of the precise role of the LDH hybrids. It is possible that they are not important as distinct entities, since the individual subunits appear to be operating independently of each other within an enzyme molecule. This is indicated by the intermediate catalytic characteristics. We have as yet obtained no evidence to suggest that a single subunit has catalytic activity by itself. All four subunits seem to be necessary for any enzymatic activity. The subunits appear to be held together by hydrogen or hydrophobic bonds, and it seems likely that the interaction of the four units is essential for stability as well as to give the molecule the tertiary structure that may be necessary for the catalytic function. What may be of importance to the cell is the relative amounts of muscle and heart subunits present at any one time (*21*).

References and Notes

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21. Part of this paper has been taken from a Ph.D. dissertation submitted by R. D. Cahn to the faculty of Brandeis University. We acknowledge the excellent assistance of Shirley White, Howard Fine, and Natalie Grimes. This article is publication No. 158 of the graduate department of biochemistry, Brandeis University, Waltham, Mass. The study was aided in part by a training grant in developmental biology of the National Institutes of Health (CRT-5043 and 2G-883) and by grants from the American Cancer Society and the National Institutes of Health.
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23. The work summarized in Table 5 was carried out in collaboration with Barbara Cunningham of this laboratory.