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*LACTIC DEHYDROGENASES AND MUSCULAR DYSTROPHY IN THE CHICKEN**

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There are two distinct types of lactic dehydrogenases (LDH) in the tissues of most animals.[1-3] These two forms have been found to be completely different with respect to their catalytic characteristics, amino acid compositions, and immunological reactivity, as well as in other physical properties.[3, 4] The two separate types of enzyme also appear to be under the control of separate genes.[3, 5] Recent reports from our laboratory have demonstrated that hybrid forms of these two types of enzyme exist, presumably being formed in cells which are synthesizing both forms of the enzyme.[3] We have referred to the two pure types of the enzyme as M (muscle) and H (heart) LDH.[6] Since these LDH's appear to exist as tetramers,[3, 4, 6, 7] the two pure "parent" enzymes can be represented as M_4 and H_4; the three hybrids have been designated as M_3H_1, M_2H_2, and M_1H_3. We have recently presented evidence supporting our previous hypothesis that the hybrids are formed by random association of the monomer units within the cell. Since the subunits of the enzyme appear to maintain their specific catalytic and immunochemical identity while in the hybrids, it has been our view that the metabolic significance to a cell of the various forms of LDH lies in the relative proportions of H and M subunits in a given cell or tissue.

We have also recently reported[3] that the predominant embryonic form of LDH in the chicken is the H_4 LDH, and that progressive embryonic development is associated with the appearance and increase in relative and absolute amounts of the

M form. This is particularly true of the chicken breast muscle in which there is a complete change from the H_4 LDH in the early embryo to the M_4 enzyme in the adult chicken. Evidence has also been obtained indicating that there is both a quantitative and chronological difference among various tissues with regard to the kinetics of appearance of the M units.

In a recent short paper, Wieme and Herpol have presented electrophoretic patterns indicating differences in the composition of LDH in human dystrophic muscles when compared to normal muscles.[8] These patterns indicated that there was a higher per cent of H subunits in dystrophic muscle than was found in the normal human muscle tissue. Since we have observed that the human embryonic LDH is the H_4 form of LDH, in contrast to the embryonic patterns of other mammals (rabbit, rat, and beef),[9, 10] which have the M form as the embryonic type, we considered the possibility that human and avian muscular dystrophies might be related to an interference during development with the change-over in types of LDH. In the present paper, evidence obtained through studies on dystrophic chickens will be presented in support of this hypothesis.

Materials and Methods.—Preparation of extracts: Fertile eggs from "White Leghorn" chickens (probably containing some New Hampshire stock) were used as the source of the "normal" embryos and chickens for these studies. The eggs were incubated at 37.5°, 65–75 per cent relative humidity, in a standard David Bradley incubator (Model 73606, Sears, Roebuck and Co.). The embryos incubated for the required time were dissected out in cold 0.25 *M* sucrose or 0.1 *M* Tris (pH 7.5) and the muscles ground in a small all-glass Potter-Elvehjem homogenizer with 40 up-and-down strokes. The extract was then centrifuged for 30–60 min at 30–40,000 × *g* in a Spinco Model L ultracentrifuge. All measurements on any one extract were made within 48 hr. The "dystrophic" chicks were obtained from two sources: Chicks which appeared "dystrophic," as judged by their inability to right themselves when put on their backs, were selected from large populations of White Leghorn-New Hampshire chickens maintained in Northeast Massachusetts as flocks for commercial "broilers." Older "dystrophic" chickens were kindly supplied to us by W. Landauer, University of Connecticut, Agricultural Research Station, Storrs, Connecticut, from his stock of genetically dystrophic strains of chickens. This particular stock was originally developed at the University of California, Davis, California. The dystrophic chicken muscles were dissected out and treated in exactly the same way as were the muscles from the normal embryos. Final concentrations of extract were adjusted to 200 mg wet weight/ml, when possible, and 100 mg wet weight/ml, if sufficient material was not available for the former.

Starch gel electrophoresis was used to separate the LDH's of the crude muscle homogenates. The electrophoresis was carried out by a modification of the method of D. Lindsay, as follows: a 14 per cent starch gel (starch-hydrolyzed, Connaught Medical Research Laboratories, Toronto, Canada) was prepared in the following buffer: Citric acid, 0.2 *M*, 7.0 ml; Na_2HPO_4, 0.2 *M*, 43.0 ml, H_2O to 1.l, pH 7.0. Approximately 0.01–0.04 ml of enzyme solution was applied at the origin on 1–4 pieces of Whatman #1 filter paper, and the electrophoresis was carried out for 18 hr, 0.4°, 27 ma, 7.5–12.5 v/cm across the block. A Shandon constant amperage power supply (Colab, Chicago) was used with matching electrophoresis chamber. The blocks were 19.5 × 6.9 × 0.6 cm. The side well buffer was: Citric acid, 0.2 *M*, 20 ml; Na_2HPO_4, 0.2 *M*, 160 ml, H_2O to 1 liter, pH 7.0. Agar saturated-KCl bridges were used to connect the electrode chamber to the chamber in which the wicks, 8 thicknesses of Whatman #1 filter paper, were immersed.

Localization of LDH: LDH was localized in the gel with the following stain, modified after the method of Dewey and Conklin.[11] The gels were sliced horizontally into 3 pieces 2 mm thick. The top piece was discarded, and the middle piece used for the localization of LDH activity. This slice was incubated in the following solution for 15–120 min at 37°, in the dark (no anaerobiosis is necessary): lithium lactate, 2.0 *M*, 0.50 ml; Tris, pH 8.0, 0.1 *M*, 8.78 ml; phenazine methosulfate, 5 mg/ml, 0.04 ml; nitro blue tetrazolium, 10 mg/ml (Mann Biochemicals), 0.50 ml; nicotinamide adenine dinucleotide (NAD) (Sigma), 30 mg/ml, 0.20 ml. The pyruvate trapping reagent (hydrazine or cyanide) was omitted, since it was found to be responsible for the high back-

ground staining previously found and appeared to reduce the dye quite efficiently when in combination with hemoglobin or myoglobin, giving rise to several artifacts.

Enzyme assays with coenzyme analogs, varying pyruvate concentrations: This method is similar to the one described by Kaplan *et al.*[1, 2] The reactions were carried out with the final concentrations of reduced coenzyme of 1.5×10^{-4}, and potassium phosphate, pH 7.5, 0.1 *M*. The final concentration of sodium pyruvate was either 3.0×10^{-4} *M* for the low level of pyruvate or 1×10^{-2} *M* for the high level of pyruvate. The enzymatic reaction was initiated by adding the appropriate amount of the various extracts. Rates were taken for 2 min, and the rate was calculated from the decrease in the optical density at 340 mμ for 30–120 sec after the start of the reaction. The ratio $NHXDH_L/NADH_H$ signifies the rate using the reduced nicotinamide hypoxanthine dinucleotide (NHXDH) with the low level of pyruvate divided by the rate using the reduced nicotinamide adenine dinucleotide (NADH) with the high level of pyrvate. The percentage of H subunits was calculated from this ratio.[3, 9] It was assumed in these calculations that the ratios for the hybrids fall on the curve at a point corresponding to their compositions of M and H subunits. Although there were some minor deviations from this assumption in the case of the hybrids in the chicken, it was felt that this method gives a fairly accurate estimate of the makeup of any given mixture of LDH.[3, 9] The $NHXDH_L/NADH_H$ ratio for the M_4 at 26° is 0.5 and that for the H_4 is 3.10. Hence, a value of 1.8 would then be equivalent to 50 per cent H subunits, as a linear relationship exists which is shown in Figure 1.

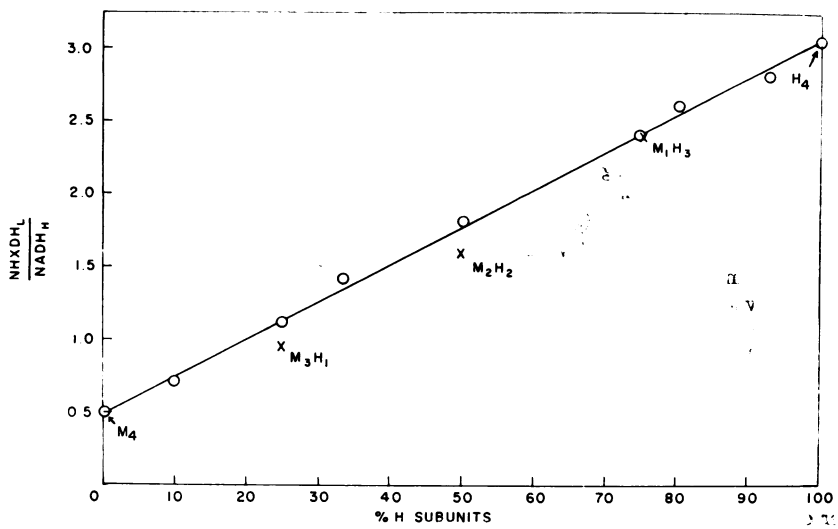

FIG. 1.—Relationship of the ratio $NHXDH_L/NADH_H$ to the amount of H subunits in the chicken. The circles represent values obtained when varying mixtures of H_4 and M_4 are added. The crosses are actual hybrids isolated from chicken tissues.

Results and Discussion.—Table 1 compares the LDH composition of the various muscles of a dystrophic chicken, representative of the dystrophic strain, with that of a normal chicken of the same age. The relative amounts of the two types of LDH have been expressed as per cent composition of the H type (see *Methods*). The total LDH activities per gram wet weight are also given in the table. As can be readily seen, there appear to be considerably more H type of LDH subunits in all of the muscles of the dystrophic chickens, when compared with the normal muscles. Only in the *M. Latissimus dorsi, pars anterior*, the muscle which in the normal animal has almost completely the H_4 type of LDH, is there no difference between the normal and dystrophic birds. This is significant, since in the chicken the embryonic

TABLE 1

LDH COMPOSITION OF STRIATED MUSCLES OF NORMAL AND DYSTROPHIC CHICKENS*

Muscle	Normal H Type (%)	Normal Units of LDH†	Dystrophic H Type (%)	Dystrophic Units of LDH
Pectoralis (superficial)	<1	4300	23.0	226
Dorsalis scapulae	2.4	290	16.3	73
Scapulotriceps	3.8	350	20.7	130
Tibialis anterior	5.3	1045	28.1	136
Iliotibialis posterior	7.0	1490	24.8	225
" middle	7.0	1350	26.1	260
Deltoid	8.1	80	27.4	42
Rhomboid	12.3	553	32.0	105
Humerotriceps	13.2	140	35.4	82
Iliotibialis anterior	13.3	1120	36.1	540
Biceps femoris and semitendinosus	14.7	560	27.0	217
Iliotrochantericus	16.5	2275	37.5	780
Gastrocnemius	16.6	795	26.2	128
Deep neck	20.4	655	56.3	390
Latissimus dorsi, pars posterior	23.0	115	55.7	85
Large medial neck	30.4	1170	42.5	820
Lateral neck	48.5	325	75.0	210
Latissimus dorsi, pars anterior	>99	235	>99	290

* The chickens used for this study were 8 weeks old. The dystrophic chicken was from a genetic strain kindly supplied by Walter Landauer.

† Values are expressed as units per gram fresh weight. A unit is that amount of LDH which will produce an optical density change of 1.0 in a volume of 3 ml at 26° using NADH as coenzyme and the low level of pyruvate.

type of LDH is the H type. Thus, this skeletal muscle retains the embryonic LDH composition.

Another interesting aspect of Table 1 is the finding that the composition of the LDH in the skeletal muscles of the normal chicken varies considerably, ranging from almost pure H type to pure M type. Hence, when examining skeletal muscles for comparison, one must be certain to indicate which muscle has been analyzed. The data in Table 1 clearly indicate that the difference in composition of the LDH (a relative increase of H-LDH) is a characteristic of nearly all of the muscles of the dystrophic chicken. Observations similar to the data in Table 1 have been made on several other chickens of this genetic strain. Furthermore, we have found that there is no significant difference in the composition or in the amount of LDH in the heart of the dystrophic chicken when compared to the normals. The liver also appears to be roughly the same in normal and dystrophic birds, although the livers of dystrophic chickens may have slightly more M-LDH than those of the normals. It is of interest to point out that in the dystrophic chickens not only is the predominant type of enzyme different from that of normals but also the absolute level of the enzyme is decreased very significantly in most dystrophic muscles.

The question can be raised as to whether the marked decrease in LDH levels in the dystrophic muscles is due to the presence of connective tissue. We have considered this possibility, but we believe that the infiltration of connective tissue cells is not a factor contributing to the lowered LDH levels. For example, the amount of connective tissue in different dystrophic muscles seems to be the same. However, the amount of LDH is considerably different in the various muscles. The amount of infiltration of connective tissue cells is about the same in the *M. latissimus dorsi* as in other muscles. The connective tissue appears also to represent a small percentage of the weight of the muscle. Furthermore, connective tissue LDH in

the chicken is largely of the M type. Details of a histological study will be presented elsewhere.

Figure 2 gives an example of the differences in electrophoretic patterns obtained when the dystrophic muscles are compared to those of normal birds. It can be seen that the predominant LDH in the normal deep *M. pectoralis* ("*pectoralis minor*") is the M_4 enzyme. However, the electrophoretic pattern from the same muscle of a dystrophic bird is quite different and resembles more closely the embryonic muscle, in which the H_4 and H_3M_1 types of enzymes predominate. (Like results have been obtained recently in humans, although it is not known which muscles are involved.) In the chicken, we have found similar qualitative changes in the composition of the LDH, judged from the electrophoretic patterns, in most of the muscles of the dystrophic birds.

Table 2 presents some data on the enzyme composition of a representative young dystrophic chicken which appeared spontaneously in a population of "normal" chickens. This chick had a severe case of dystrophy, and chicks of this type usually do not live very long. Table 2 gives a comparison of the LDH from the dystrophic muscles with those from a normal 7-day-old chick. Here again, one can

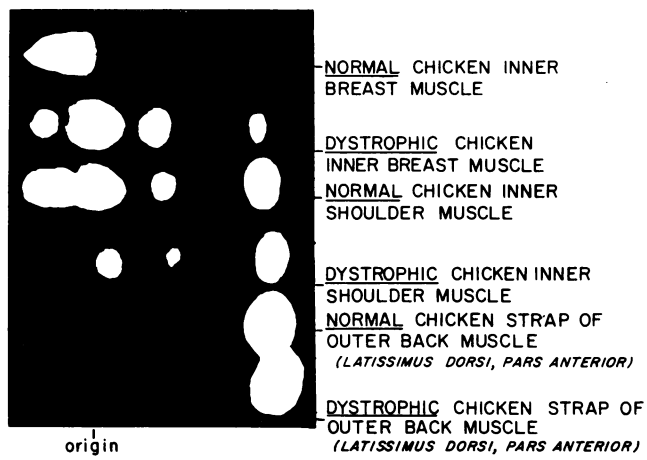

FIG. 2.—Tracing of starch gel electrophoresis of normal and dystrophic chicken muscles.

TABLE 2

LDH COMPOSITION OF ONE-WEEK-OLD NORMAL AND DYSTROPHIC CHICKS

	Normal		Dystrophic	
Muscle	H Type (%)	Units of LDH*	H type (%)	Units of LDH
Scapulotriceps	9.7	485	31.9	84
Pectoralis (superficial)	12.9	148	42.8	34
Iliotibialis anterior	15.0	670	71.8	167
Pectoralis (deep)	15.5	57	56.7	4
Iliotibialis posterior	16.1	915	51.6	121
Sartorius	30.7	358	63.3	118
Tibialis anterior	34.3	775	53.9	220
Neck muscles	40.0	550	78.4	95
Gastrocnemius	41.2	485	83.7	165
Iliotrochantericus	68.6	430	100.0	60

* See footnote, Table 1, for explanation. Both the dystrophic and normal chickens were obtained from the same flock of chickens.

see that there is much more H type of LDH present in the dystrophic chicken muscles. The results also demonstrate a striking difference in the absolute amounts of LDH present in the dystrophic muscles—the enzyme levels are greatly reduced in the diseased birds. Other dystrophic chickens found in the same population of chickens have a similar decrease in levels of LDH and in M type of enzyme, compared to normal birds of the same age.

The data in Tables 1 and 2 indicate that in the muscles of chickens afflicted with muscular dystrophy the predominant type of LDH changes. This alteration is also accompanied by a decrease in the intramuscular level of LDH.

In the development of the chick, the H_4 type of LDH appears first in all muscles and the M-containing LDH appears subsequently. As development continues in the normal chicken, there is an increase in amount of M type, as illustrated with the pectoralis muscle (see Table 3). It is of interest that this change in type of enzyme

TABLE 3

CHANGES IN COMPOSITION AND AMOUNT OF LDH IN CHICKEN BREAST MUSCLE (PECTORALIS)

	H Type (%)	Total units per gram fresh weight
	Embryo	
6 days	92	—
14 "	63	18
20 "	24	35
22 "	16	52
	After hatching	
1 day	19	48
7 "	13	148
9 "	6	445
15 "	3	1570
9 weeks	<1	4820

is also associated with an increase in the level of enzyme on a per gram basis. Even after hatching, there is a great rise in amount of LDH in the breast muscle. This rise suggests that the repressor for the M synthesis may be completely removed and, at least in this muscle, such removal may be dependent on the number of divisions of the muscle cells.

Thus, it appears that in muscular dystrophy, at least in the chicken, there is a block in the functioning of a gene which controls the synthesis of the M unit. This block may be due to the continued presence of a repressor which prevents the M gene from synthesizing M subunits (i.e., the failure of a normal process of derepression). Our data, although preliminary, indicate that the more severe the disease in the chicken the more embryonic are the LDH patterns of all the muscles of the dystrophic birds.

It is not clear as yet whether the LDH changes themselves represent a contributory factor to the disease, or whether these changes only reflect blocks in the development of the muscle cells which have occurred earlier. Whatever the role of the LDH *per se* in the disease, it does give a clue as to the time at which a developmental block may have occurred. Recognition of the composition of the LDH in the dystrophic cells may be important in pinpointing the time when some metabolic abnormality occurred, interrupting the processes of normal development. This may be important not only for muscular dystrophy but also for other developmental diseases as well.

The dystrophy observed in chickens has certain similarities to the dystrophic diseases of humans. Both species appear to have early hypertrophy of the muscles. If the preliminary results of Wieme[8] are correct, then one might expect that similar alterations of the patterns of LDH would be present in the human disease. (This has also been very recently observed by Dreyfus *et al.*[12]) As we have indicated, the human embryonic type also appears to be the H form.[9, 10] In developing and maturing human muscles, there is also a shift from the synthesis of H to M LDH polypeptide chains. In humans, too, muscular dystrophy varies in its severity. There are some forms of the disease which occur early in life and are quite severe; those occurring later are usually somewhat milder and nonfatal. It is possible that the early form of the disease in humans is like that of the spontaneously arising disease in the chicken. We are now investigating this possibility.

There are several genetic strains of mice which have muscular dystrophy. We have been able to find very little difference between the composition of LDH of the muscles of normal mice and those of dystrophic mice. It should be pointed out that the mouse appears to have the M type of enzyme as the embryonic form[13] and that most of its muscles have little or no H type of enzyme; hence, one would not expect to find changes of the same magnitude or direction in the composition of LDH of the muscles of the mouse as those found in the human and chicken muscles, both of which have the H form as the embryonic LDH. Whether this is the reason for not detecting changes in LDH in the mouse remains to be investigated.

Summary.—It has been shown that in dystrophic chickens the muscles contain a higher per cent of H (heart) type LDH than do the muscles of normal chickens. This change appears to represent a block in the development of the normal enzymatic pattern of the muscle cells, since the H type of enzyme is the embryonic form in the chicken. Some correlation has been obtained between the severity of the dystrophy in the chicken and the composition of the LDH's in most of the muscles. The more severe the disease the more embryonic is the LDH pattern in the muscles of the dystrophic animal. It has also been found that in the normal chicken different skeletal muscles have widely varying compositions of M and H forms of LDH. Not only is there a difference in composition of the LDH in the skeletal muscles of dystrophic chickens but also there is a significant decrease in total level of LDH per unit wet weight in the diseased birds. Furthermore, nearly all of the skeletal muscles of the dystrophic chickens have an increased per cent of H enzyme, which is comparable to normal embryonic muscles. The disease in the chicken is compared to that occurring in humans and in mice, and a possible mechanism of its genesis is proposed.

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*THE INHIBITION OF A BIOLOGICAL CLOCK BY ACTINOMYCIN D**

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Persistent daily rhythms have been studied in organisms ranging from unicellular to mammalian forms.[1] Characteristically, such rhythms involve a daily variation in some biological function, with a maximum occurring at a particular time of day. In *Gonyaulax polyedra*, the marine dinoflagellate used in the present study, a rhythm of bioluminescence exhibits a maximum late at night, whereas photosynthetic capacity, which is also rhythmic, is greatest during the day.[2]

Evidence from numerous investigations indicates that rhythms are a manifestation of a biological time-measuring system, exerting temporal control over the capacities of specific biochemical processes. Under constant conditions (Fig. 1) diurnal rhythmicity in *Gonyaulax* persists undamped. The period, although approximately equal to the solar day, is actually slightly different from 24 hours.[3] Similar observations have been reported from studies of rhythms in many other organisms.

In spite of the compelling evidence concerning the existence of a "metabolic cellular clock," substantial understanding concerning the chemical pathways involved in its operation and control has been lacking. In fact, some features of rhythms are not altogether characteristic of a metabolically dependent physiological function. First, the frequency is essentially temperature-independent; that is to say, the period of a rhythm is relatively unaffected when organisms are placed in different (but constant) temperatures.[4, 5] Second, rhythms have been reported to be insensitive to a variety of metabolic inhibitors. In the studies reported it was not possible to alter appreciably either the timing (phase) of a rhythmic function or to abolish its expression by various agents tested.[6–8]

These observations concerning temperature and inhibitor effects have been taken by some workers as evidence that biological timing mechanisms must be related to an exogenous "signal."[9] These same facts, however, are consistent with the hypothesis that rhythms relate to a truly functional biological clock, since the utility of a feature such as temperature independence for a timekeeping mechanism is evident.