Peafowl Lactate Dehydrogenase: Problem of Isoenzyme Identification

Abstract. *Peafowl, like other vertebrates, contain multiple forms of lactate dehydrogenase. The electrophoretic properties of the peafowl isoenzymes are unusual in that the isoenzyme from heart tissue can be either more or less anodic than that of muscle, depending on the* p*H. This finding focuses attention on the problem of isoenzyme identification. It is suggested that isoenzymes be identified on the basis of properties that are chemically and biologically more significant than electrophoretic mobility.*

Multiple forms, or isoenzymes, of lactate dehydrogenase (*1*) occur in most vertebrates. It is now generally agreed that each form is composed of four subunits and that two major types of subunit (H and M) are present in most species. Thus, whenever random combination of subunits takes place (*2*), five tetrameric forms of lactate dehydrogenase result. The subunit composition of the five forms is indicated by the following formulas: H_4, H_3M, H_2M_2, HM_3, and M_4. This explanation of the nature of the five major forms of LDH (*3*) is based mainly on work from the laboratories of Markert (*4*) and Kaplan (*5, 6*).

The H_4 isoenzyme is most susceptible to pyruvate inhibition and usually predominates in heart muscle (*5*). The M_4 isoenzyme is least susceptible to pyruvate inhibition and usually predominates in skeletal muscle (*5*). Upon electrophoresis, the H_4 enzyme of mammals is the most anodic and the M_4 enzyme is the least anodic of the five forms. However, some bird species were found, after a survey of many vertebrates, in which the multiple forms of lactate dehydrogenase, detected by catalytic, immunological, and thermostability methods, did not differ in electrophoretic mobility (*7*). Some amphibians and fishes were found in which the least anodal form of lactate dehydrogenase predominated in heart muscle (*7, 8*) and was most susceptible to pyruvate inhibition (*7*). Cases of the latter type have now been found in a few birds, such as the peafowl (*Pavo cristatus*) and the Australian swamp quail (*Synoicus ypsilophorus*). Isoenzymes of the peafowl are described in this report.

Peafowl were obtained from several sources (*9*). Carcasses or tissues were preserved in the frozen state until needed for preparation of extracts which were made by homogenizing a 1-gram sample of tissue in 5 ml of cold 0.25*M* sucrose. Extracts were clarified by centrifugation (40,000*g* for 30 minutes) and stored frozen at −10°C. Crystalline chicken H_4 and M_4 LDH's were used as examples of well-characterized LDH's whose electrophoretic and catalytic properties are typical of birds (*5, 6, 10*). Lactate dehydrogenase activity was assayed spectrophotometrically at 23°C (*11*). Several properties of peafowl and chicken LDH's were compared; these included electrophoretic mobility, thermostability, catalytic characteristics, and immunological properties.

Electrophoresis was carried out in horizontal starch gels (*12*). Samples containing one or two units of LDH activity (*11*) were applied to the gels. After electrophoresis, LDH activity was located in the gels with a specific nitroblue tetrazolium staining mixture (*12*). Five types of LDH were detected in tissues such as neck muscle, leg muscle, and liver. Heart and breast muscle, however, each contained a single predominant isoenzyme. One of the extreme electrophoretic types predominated in the heart and the other in the breast muscle. All further studies were conducted with extracts of these two tissues.

After the extract of peafowl heart was subjected to electrophoresis at *p*H 7.0 in citrate-phosphate buffer (*12*) for 20 hours with a gradient of about 12 volt/cm, a single cathodal spot of LDH activity with a center of intensity 0.7 cm from the origin (Fig. 1) was observed. The breast extract yielded a single cathodal spot about 1.2 cm from the origin. Under the same conditions, the chicken H_4 enzyme moved 2.2 cm toward the anode, and chicken M_4 enzyme moved about 0.5 cm toward the cathode. Peafowl thus differs from chicken in that both isoenzymes migrated cathodally and were poorly separated at this *p*H. By contrast, when electrophoresis was performed at *p*H 8.6 in tris-borate-EDTA buffer (*13*) or at *p*H 9.7 in glycine buffer (*13*), the peafowl heart and breast enzymes both moved toward the anode, the breast enzyme moving faster than the heart enzyme as shown in Fig. 1. The peafowl LDH's not only changed their direction of migration, but their relative mobilities were reversed. Other properties of the peafowl isoenzymes were therefore investigated (*14*).

Table I. Catalytic properties of chicken and peafowl lactate dehydrogenases.

Chicken		Peafowl	
H_4	M_4	Heart	Breast
	$NADH_L/NADH_H$ at 23°C*		
3.4	0.86	3.9	0.75
	$NADH_L/NADH_H$ at 37°C		
1.1	0.36	1.3	0.26
	AcPyAD/NAD† at 23°C		
0.22	1.4	0.16	1.6

* Ratio of the activity with NADH at a low concentration of pyruvate ($3.3 \times 10^{-4}M$) to that at a high concentration of pyruvate ($1 \times 10^{-2}M$). † Ratio of the activity with the acetylpyridine analog of NAD to that with the natural coenzyme. The reaction mixtures contained one or the other of the two coenzymes ($2 \times 10^{-4}M$), lithium L-lactate (0.1*M*), sodium pyrophosphate buffer (0.1*M*, *p*H 8.9), and enzyme in a final volume of 3 ml.

Thermostability studies were conducted (Fig. 2, legend) with extracts or pure enzymes diluted in a buffer containing bovine serum albumin (*15*). The chicken H_4 enzyme is much more resistant to thermal denaturation than is the chicken M_4 enzyme (*16*). Figure 2 shows that, at *p*H 7.5, peafowl heart LDH was almost as thermostable as the chicken H_4 enzyme, whereas LDH from peafowl breast extract resembled chicken M_4 LDH in being relatively thermolabile. When thermostability experiments were conducted at

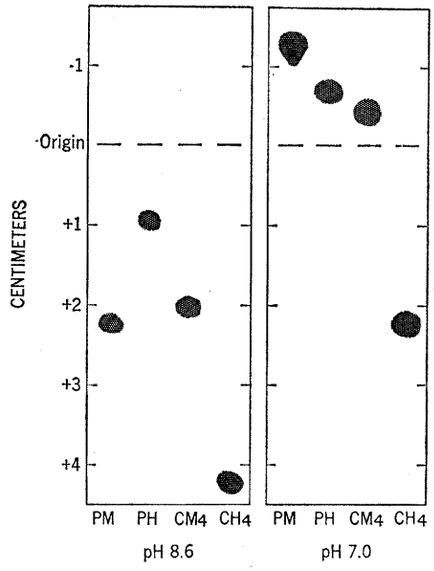

Fig. 1. Electrophoresis at different *p*H's. Gels were made of 14 percent starch (Connaught, Toronto) in either citrate-phosphate buffer at *p*H 7.0 (*12*) or tris-borate-EDTA buffer at *p*H 8.6 (*13*). The ionic strength was 0.025 in both cases. The dimensions of the gel slabs were 20.3 × 7.7 × 0.6 cm. Other conditions: 12 volt/cm; current, 18 ma; 20 hours; 10°C. After electrophoresis, slices of the gels were treated with LDH-specific stain (*12*), and tracings were made to indicate the location of LDH.

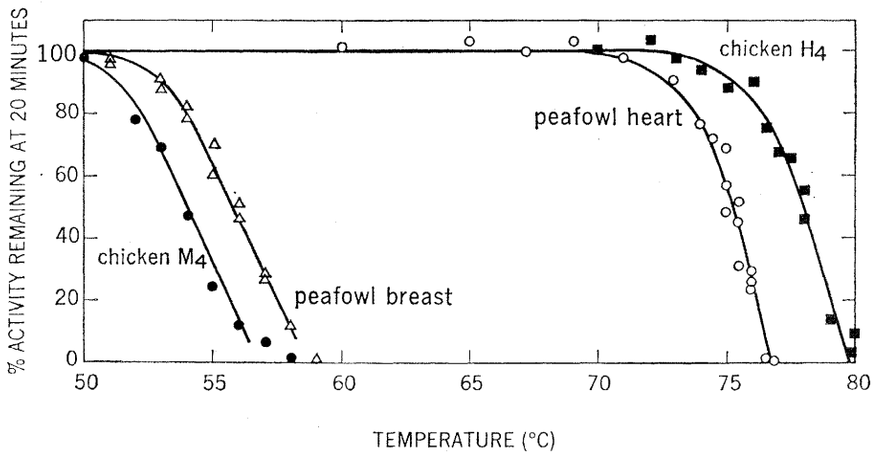

Fig. 2. Thermal denaturation of LDH. Extracts or pure enzymes were diluted at least 100-fold in BSA buffer (*15*) at *p*H 7.5 to a concentration of about one unit of LDH activity per milliliter (about 1 μg of LDH per milliliter). Portions (0.5 ml) were incubated in test tubes in a water bath at the temperature indicated for 20 minutes. After the tubes were rapidly cooled to 0°C, samples were taken from the tubes for assay of LDH activity at 25°C (*11*). Under these conditions, extracts of chicken heart and breast muscle behave exactly like the pure H_4 and M_4 enzymes, respectively, with regard to thermal denaturation of LDH.

*p*H 7.0 or 8.6, essentially similar results were obtained.

Two catalytic properties were examined next, (i) substrate inhibition and (ii) utilization of a coenzyme analog. The inhibitory effect of high concentration of pyruvate on the conversion of pyruvate to lactate, catalyzed by the H_4 enzyme, has long been known (*17*) and has been interpreted as being of biological significance. Thus, the functional difference which is believed to exist between the H_4 and M_4 enzymes is thought to depend on the fact that the M_4 enzyme is less susceptible than the H_4 enzyme to pyruvate inhibition (*18*).

Pyruvate inhibition may be expressed, as in Table 1, by the ratio $NADH_L/NADH_H$, which is the reaction rate at a low concentration of pyruvate ($3.3 \times 10^{-4}M$) divided by the reaction rate at high concentration ($1 \times 10^{-2}M$). Table 1 shows that peafowl heart LDH was strongly inhibited by pyruvate, as was chicken H_4 LDH. Peafowl breast LDH, like the chicken M_4 enzyme, was only slightly inhibited. When measurements were made in reaction mixtures at 37°C, lower values of $NADH_L/NADH_H$ were obtained, as expected (*19*), but the catalytic difference between heart and muscle LDH's was maintained (*20*). Pyruvate inhibition was also measured at *p*H 7.0 and at *p*H 8.6, with 0.1*M* tris-chloride as buffer. At both these *p*H's, peafowl heart LDH was more susceptible than the breast muscle enzyme to pyruvate inhibition.

Coenzyme analogs have been used before (*5*, *21*) to demonstrate differences between LDH isoenzymes. For our study, the analog AcPyAD was substituted for NAD, and the rate of the lactate to pyruvate reaction was measured spectrophotometrically at 23°C. As shown in Table 1, the analog reacted rapidly with peafowl breast LDH and chicken M_4 LDH, but it reacted slowly with the peafowl heart enzyme and chicken H_4 LDH.

Immunological experiments were done with two rabbit antiserums, one directed to pure chicken H_4 LDH and the other directed to pure chicken M_4 LDH. The antiserums were prepared and characterized as described (*5*). To test for reactivity of these serums with peafowl lactate dehydrogenases, two methods were used: (i) the Ouchterlony double-diffusion method and (ii) quantitative complement fixation ("micro" method). The Ouchterlony method was applied (Fig. 3) as recommended by Stollar and Levine (*22*). The antiserum to M_4 reacted strongly with both chicken M_4 LDH and peafowl breast extract (Fig. 3*a*). No line of precipitation was obtained with chicken H_4 LDH or peafowl heart extract. Conversely, when antiserum H_4 was used (Fig. 3*b*), a strong precipitin line was obtained with chicken H_4 LDH and peafowl heart extract. No reaction was detected between chicken M_4 LDH or peafowl breast extract and antiserum to H_4. In all cases, the precipitin lines stained strongly when the Ouchterlony plates were treated with the LDH-specific, nitroblue tetrazolium staining mixture (*12*).

Quantitative microcomplement fixation was performed as described by Wasserman and Levine (*23*, footnote 3), but with 7-ml reaction volumes. Antiserum to M_4 reacted well with chicken M_4 LDH and peafowl breast

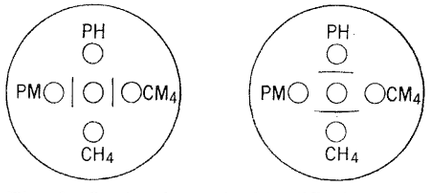

Fig. 3. Ouchterlony double-diffusion tests with antiserum to chicken H_4 LDH (right) and with antiserum to chicken M_4 LDH (left). The wells contained 0.1 ml of antigen or antiserum and were 0.8 cm apart. The central well contained undiluted antiserum. The surrounding wells contained 10 units of LDH activity (approximately 10 μg LDH), *CH*$_4$ (chicken H_4 LDH), *CM*$_4$ (chicken M_4 LDH), *PH* (peafowl heart extract), and *PM* (peafowl breast muscle extract). Diffusion was allowed to proceed for 24 hours, and then tracings were made to indicate the position of the precipitin lines.

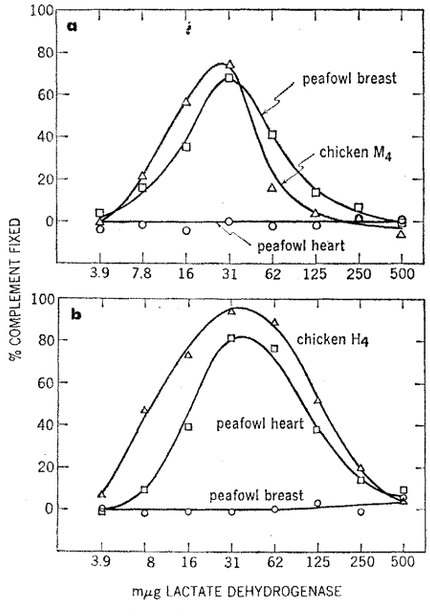

Fig. 4. Results of "micro" complement fixation tests with antiserums to chicken H_4 LDH and to chicken M_4LDH. All reagents were diluted in BSA buffer (*15*). (a) Antiserum to M_4 (antiserum No. 11B6), testing pure chicken M_4LDH (△), peafowl breast muscle extract (□), and peafowl heart extract (○). The antiserum dilutions used were 1/18,000, 1/18,000, and 1/5000, respectively. (b) Antiserum to H_4 (antiserum No. 174B5), versus pure chicken H_4LDH (△), peafowl heart extract (□), and peafowl breast muscle extract (○). The antiserum dilutions were 1/3000, 1/2000, and 1/1000, respectively.

extract (Fig. 4*a*), but the peafowl heart extract did not react even when the antiserum concentration was raised. By contrast, antiserum to H_4 reacted strongly with the peafowl heart extract as well as with chicken H_4 LDH, but showed no reaction with the peafowl breast extract (Fig. 4*b*).

The immunological, catalytic, and thermostability experiments permit us to identify peafowl breast LDH as an M_4 isoenzyme and peafowl heart LDH, despite its low electrophoretic mobility (*24*), as an H_4 isoenzyme. Had only electrophoretic criteria been employed, the peafowl isoenzymes might have been incorrectly identified.

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References and Notes

1. Number 1.1.1.27 in *Enzyme Nomenclature*. Recommendations of the International Union of Biochemistry (Elsevier, New York, 1965).
2. Random combination of LDH subunits appears to take place in many but not all vertebrates, as shown by S. N. Salthe, O. P. Chilson, N. O. Kaplan, *Nature* **207**, 723 (1965).
3. Abbreviations used in this paper: LDH (lactate dehydrogenase); NAD (nicotinamide adenine dinucleotide); NADH, the reduced form of NAD; AcPyAD (3-acetylpyridine adenine-dinucleotide); EDTA, ethylenediaminetetraacetate; BSA, bovine serum albumin.
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8. C. L. Markert and I. Faulhaber, *J. Exp. Zool.* **159**, 319 (1965).
9. We thank C. Baldwin (San Fancisco Zoo), K. Lint (San Diego Zoo), G. Spalding (Los Angeles County Aboretum), L. Poisal (Pleasanton, California), H. Abplanalp (Univ. of California, Davis), and R. Paynter (Harvard Univ.) for assistance in obtaining specimens.
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11. Oxidation of NADH was followed by measuring decrease in absorbance at 340 mμ, with a Zeiss model PMQII spectrophotometer, in potassium phosphate buffer (0.1*M*, *p*H 7.5) at 23°C. Initial concentrations were: NADH, $1.4 \times 10^{-4}M$; sodium pyruvate, $3.3 \times 10^{-1}M$; LDH, approximately 3×10^{-2} μg per milliliter; in a final volume of 3.0 ml. As used in this paper a unit of LDH activity produces an absorbance change of 1.0 per minute in a 3-ml reaction volume at 23°C and is approximately equivalent to 1 μg of LDH or 1 international unit (at 30°C).
12. I. H. Fine and L. Costello, *Methods Enzymol.* **6**, 958 (1963).
13. I. Smith, *Chromatographic and Electrophoretic Techniques*, vol. 2, *Zone Electrophoresis* (Interscience, New York, 1960), p. 95.
14. Since no individual variation was detected in the electrophoretic mobility of the LDH's of 44 peafowl, subsequent investigations were carried out with heart and breast extracts from one or two individuals.
15. This buffer contained: 0.14*M* NaCl, 0.01*M* tris, $5 \times 10^{-4}M$ magnesium sulfate; $1.5 \times 10^{-4}M$ calcium chloride; and bovine serum albumin (1 mg/ml). The *p*H was 7.5.
16. A. C. Wilson and N. O. Kaplan, in preparation.
17. F. Kubowitz and P. Ott, *Biochem. Z.* **314**, 94 (1943).
18. The relative lack of pyruvate inhibition shown by the M_4 enzyme is thought to facilitate rapid anaerobic glycolysis, such as occurs in large skeletal muscles. The H_4 enzyme is strongly inhibited by pyruvate and has a high affinity for lactate. Since this enzyme predominates in tissues capable of sustained aerobic metabolism, such as the heart, it is though to be concerned with aerobic metabolism. See, for example, N. O. Kaplan and T. L. Goodfriend, *Advance. Enzyme Regulat.* **2**, 203 (1964); D. M. Dawson, T. L. Goodfriend, N. O. Kaplan, *Science* **143**, 929 (1964); A. C. Wilson, R. D. Cahn, N. O. Kaplan, *Nature* **197**, 331 (1963); C. L. Markert and H. Ursprung, *Develop. Biol.* **5**, 363 (1962).
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20. We have been unable to confirm a recent report that M_4 LDH becomes strongly inhibited by pyruvate at 37°C [E. S. Vesell, *Science* **150**, 1590 (1965)].
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22. D. Stollar and L. Levine, *Methods Enzymol.* **6**, 848 (1963).
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24. Two factors may account for the reversal of the relative mobilities of the peafowl H_4 and M_4 isozymes, (i) the unusually high isoelectric point (approximately 7 to 8) of this H_4 enzyme and (ii) a high histidine content in the M_4 enzyme. The M_4 enzymes of six avian species, representing three diverse orders, have been subjected to amino acid analysis. Every one contains about 60 moles of histidine per mole of LDH, whereas every avian H_4 enzyme analyzed contains about 30 moles of histidine per mole of LDH [see *6* and *10*, and T. P. Fondy and N. O. Kaplan, *Ann. N.Y. Acad. Sci.* **119**, 888 (1965)]. At *p*H 7, many histidyl residues bear a positive charge but, at *p*H 8.6 or higher, most of them are uncharged [J. Steinhardt and S. Beychok, in *The Proteins*, H. Neurath Ed. (Academic Press, New York, 1964), vol. 2, p. 223]. Because of their histidine content, avian M_4 enzymes would be expected to increase markedly in anodic mobility when the *p*H is raised from 7 to 8.6. Such an increase was observed, as is evident in Fig. 1, not only for the chicken M_4 enzyme, which has a high histidine content (63 moles per mole of LDH), but also for the peafowl M_4 enzyme, whose histidine content has not been determined.
25. Supported in part by NSF grant to A.C.W. from NSF.

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Cell Size and Rate of Protein Synthesis in Ventral Horn Neurones

Abstract. *Autoradiographic studies show that small ventral horn neurones synthesize protein at a greater rate per unit area of cytoplasm than do large ones. It is suggested that this is related to the faster rate of firing of the smaller neurones.*

Henneman *et al.* (*1*) have shown an inverse relation between the size of motor neurones and their excitability. Small motor neurones have a low threshold to excitation and fire more often than large motor neurones.

In order to determine whether the rate of protein synthesis in neurones is related to their firing rates, I have investigated the rate of uptake of H^3-phenylalanine into large and small ventral horn neurones of the mouse. If there is a direct relation, then the smaller neurones should show a greater rate of incorporation per unit area of cytoplasm than the larger.

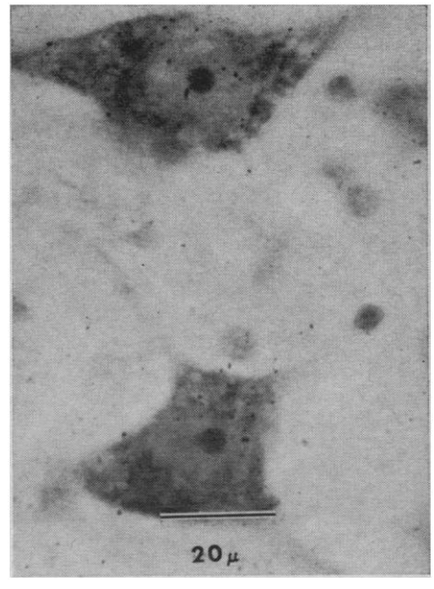

Fig. 1. An autoradiograph of two large motor neurones in the mouse lumbar spinal cord. In order to photograph cells and grains, both are slightly out of focus.

An adult mouse was injected with 100 μc of H^3-phenylalanine 4 days after the left sciatic nerve had been sectioned in the region of the midthigh. Five hours later the mouse, anesthetized with ether, was perfused through the left ventricle with 10 percent formal-saline. After several hours the lumbar enlargement was removed, fixed for 3 days, washed, dehydrated, embedded in paraffin, and serially sectioned at 10 μ. After the sections had been mounted and the paraffin removed, the slides were dipped in Kodak NTB-3 emulsion, and after a 3-week exposure were developed at 10°C in Kodak D-19.

I examined ventral horn neurones (Fig. 1) with discernible nucleoli at a magnification of 450× using a calibrated ocular micrometer. The area of the cell body was estimated by two measurements at right angles, usually the long and short axes of the cell. The nuclear diameter was also measured, and the nuclear and cytoplasmic areas calculated. Grains were counted over the cytoplasm and nucleoplasm separatedly, and the number of grains (above background) per square micron of cytoplasm was obtained. About 250 cells from several sections on two slides were examined in this manner. Unless other-