

## A Study on the Property of LDH Isoenzymes of Rabbits

by

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It has been reported that many enzymes exist in more than one form within one species. A number of molecular forms of lactic dehydrogenase (LDH, L-lactate:NAD<sup>+</sup> oxidoreductase, EC. 1.1.27) have been found in the various organs of one individual (1-3). The five isoenzymes of LDH can be separated by various electrophoretic procedures. As Apella and Markert (2) reported, these isoenzymes are hybrid tetramers of two subunit polypeptides which have been designated as H and M respectively.

The H-LDH isoenzyme predominates in cardiac muscle, while, the M-LDH isoenzyme in skeletal muscle (4). These two isoenzymes are known to be separate entities as judged by physical, enzymatic, and immunochemical criteria (1,2,5). As Chan *et al.* discussed (4), the LDH of chicken breast muscle shifts from the enzymes related to the H-LDH during embryonic development, through several intermediate enzyme types, and appear in the adult as the pure M-LDH. The intermediate LDH enzyme types which appears during embryonic development are also known "hybrid" LDH's, consisting of both H- and M-LDH in different proportions. Furthermore, these different proportions are highly specific among the tissues as to acknowledge their diagnostic

significances very much in many reports (6,7).

It is, therefore, quite clear at present that the H-and M-LDH isoenzymes are under the control of separate genes, and that the hybrids are formed at random when both genes are operating in one cell, in the same manner as genetic recombinants (8). Hence, what appears to be of importance is the relative activities of the two genes responsible for the synthesis of the two different types of subunits in a given tissue. This is clearly illustrated in studies on differentiation. In the early chick embryo, the breast muscle contains almost exclusively the H-LDH as mentioned before (4). The shift of it to the M-LDH, however, reflects, no doubt, that the relative distribution of the LDH isoenzyme in a given tissue is genetically determined.

In the present paper, the author intended to study a certain aspect of kinetic behavior of these two LDH isoenzymes such as pyruvate inhibition and in order to obtain a better resolution of the electroenzymogram, a modification of electrophoretic analysis was made as to foresee a possibility of determining the kinetic properties directly on the gel by means of formazan reaction, instead of UV spectrophotometry, which require a certain knowledge on the fundamental kinetic behavior

of the enzyme at large and skilful technique as well.

## MATERIALS AND METHODS

### 1. Source of Enzymes:

Adult rabbits were sacrificed by cervical dislocation, and the heart and psoas muscles were surgically removed. They were frozen unless treated immediately.

The muscles were homogenized in a mortar, frozen previously in a deep freezer, with ice-cold 0.25 M sucrose solution to obtain 20% (W/V) tissue homogenate. Homogenization was performed by grinding it thoroughly with the addition of sea sand. They were then centrifuged at 600 x g for 20 min. in order to eliminate the nuclear fraction and cell debris. The resulting supernatant was used as the source of the H-LDH (heart muscle) and M-LDH (psoas muscle) respectively. The whole experimental procedure was performed at 4°C. unless otherwise specified.

### 2. Preparation of the Crude M-LDH

#### Isoenzyme:

The supernatant solution obtained from rabbit skeletal muscle as described previously was saturated with ammonium sulfate, salting out the 35%~65% saturated fraction.

The resulting precipitate was dissolved and dialyzed 0.02 M phosphate buffer, pH 6.0, for 7 hrs. at 4°C. It was then applied to a DEAE-cellulose column followed by washing the column with the same buffer. The M-LDH isoenzyme passes through the column while the H-LDH isoenzyme and most of the other protein are adsorbed (9).

### 3. Preparation of the Crude H-LDH

#### Isoenzyme:

The same procedure was applied as in M-

LDH isoenzyme preparation that the DEAE-cellulose column was eluted with 0.2 M phosphate buffer containing 0.15 M NaCl, since the DEAE-cellulose is known to adsorb selectively H-LDH isoenzyme (10).

### 4. Assay of LDH Activity:

With the use of the Calbiometer (Calbiochem Co.), a UV spectrophotometer, LDH activity was assayed after the Neiland's procedure (11). The reaction mixture in a cuvette contained 180  $\mu$  moles of glycine-NaOH buffer, 2  $\mu$  moles of NAD<sup>+</sup>, and 50  $\mu$  moles of sodium lactate in a final volume of 2.0 ml. The reaction was started by the addition of 0.02 ml. of the enzyme sample. Throughout the procedure, the reagents used in the assay were maintained at 25°C. in a water bath, and the Calbiometer was thermostatically regulated as well. A unit of LDH activity is defined as a  $\mu$  mole of NAD reduced during a minute incubation.

### 5. Protein Determination:

The protein content was monitored by the method of Lowry *et al.* (12) in order to obtain specific activities of the enzyme preparations. Bovine serum albumin of the Nutritional Biochemicals Corp. was used as a standard, determining the N content of it by Kjeldahlometry.

### 6. Electrophoresis:

Agarose gel electrophoresis was carried out according to Wieme (13), with 0.05 M Veronal buffer, pH 8.6, of ionic strength 0.04. Electrophoresis was run at 15 V/cm. and 3.5 mA/cm at 4°C.

However, visualization of enzyme activity was carried out in a modified fashion as follows by means of formazan reaction. Cellulose acetate (Separaphore III) strip was

**Table I.** Summary of crude preparation of the M-LDH from rabbit psoas muscle.

Preparation steps	Total activity (mole/min.)	Total protein (mg.)	Specific activity (unit/mg. prot.)	Yield (%)	Purification (fold)
Supernatant of 20% homogenate	316	367.6	0.82	100.0	1.00
35% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	300	278.0	1.08	95.0	1.31
65% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	296	113.0	2.62	93.7	3.20
Dialyzate	290	111.5	2.60	91.4	3.17
DEAE-cellulose eluate	114	27.0	4.22	36.0	5.15

soaked in the substrate mixture containing  $10^{-2}$  M Na-L-lactate,  $2 \times 10^{-3}$  M NAD<sup>+</sup> (nicotinamide adenine dinucleotide),  $2 \times 10^{-4}$  M PMS (phenazine methosulphate) and  $1.5 \times 10^{-3}$  M NBT (nitroblue tetrazolium). The soaked strip was then overlapped on the agarose gel bed after electrophoresis followed by incubation. The incubation with this method was carried out at 37°C for 30 min. The agarose gel bed underneath and the overlapped cellulose acetate

strip were altogether taken into 3% acetic acid for decoloration of background for about three hours.

Densitometric evaluations of both were scanned with the Gelman Densitometer (Gelman Instrument Co.) and interpreted by means of a planimeter. The sum of densitometric areas of both gels were proportional to the amounts of nitroformazan formed.

## RESULTS

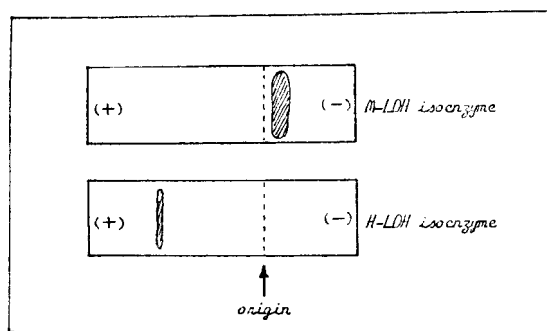
### 1. Electrophoresis of the H- and M-LDH

#### Isoenzyme:

As shown in Fig. 1, clearly separated single band of formazan was appeared at cathode side in case of the M-LDH isoenzymes and at anode side in case of the H-LDH, which illustrated the fact that the preparation procedure was satisfactory.

Densitometrical analysis of both isoenzymes are shown Fig. 2, which is the sum of formazan areas on both gels, the agarose gel bed (A) and overlapped cellulose acetate gel (C).

As shown in Fig. 3, the enzyme activities



**Figure 1.** Typical electroenzymogram of H-LDH and M-LDH isoenzyme prepared from rabbit cardiac and psoas muscle respectively.

**Table II.** Summary of crude preparation of the H-LDH from rabbit cardiac muscle.

Preparation steps	Total activity (mole/min.)	Total protein (mg.)	Specific activity (unit/mg. prot.)	Yield (%)	Purification (fold)
Supernatant of 20% homogenate	64.6	87.8	0.73	100.0	1.00
35% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	60.5	77.1	0.78	93.6	1.06
65% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	45.5	36.3	1.25	70.4	1.69
Dialyzate	43.2	36.1	1.20	67.0	1.62
DEAE-cellulose eluate	4.60	28.5	3.36	7.1	4.54

**Table III.** The activity of M-LDH and H-LDH in various pyruvate concentrations.

Pyruvate concentration	0	$5 \times 10^{-3} M$	$10 \times 10^{-3} M$	$15 \times 10^{-3} M$	$20 \times 10^{-3} M$
M-LDH activity	3.87*	2.97	2.07	1.68	1.29
H-LDH activity	1.55	1.08	0.75	0.38	0.34

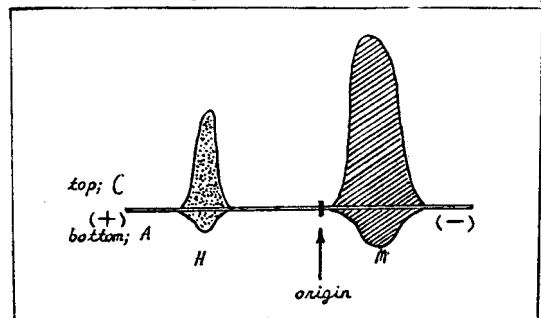
\* Figures denote unit/ml of LDH activity.

determined spectrophotometrically are plotted against amounts of nitroformazan formed on both gels. Total activities higher than 275 mU/ml. was used as a starting point since an activity lower than 50 mU/ml. could not be detected densitometrically.

The first part of the curve, up to an activity of about 1,000 mU/ml. showed a linear relationship between the activities and densitometric areas. At higher activities, however, the Lambert-Beer's law seemed to lose its validity and the change in activity did not correspond to the change in optical density.

## 2. Purification of LDH Isoenzymes:

As can be seen from Table I and II, 70% of the H-LDH activity was recovered upon fractional salting out with ammonium sulfate,



**Figure 2.** A schematic representation of the densitometric analyses performed on both gels simultaneously.

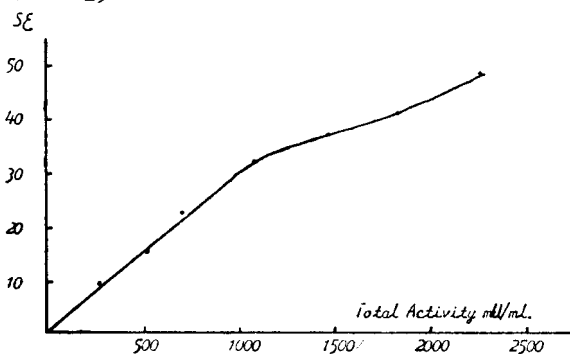
C: Cellulose acetate gel  
A: Agarose gel  
H: H-LDH isoenzyme  
M: M-LDH isoenzyme

while 93.7% of the M-LDH recovered upon the same salting out step.

On dialysis, little amount of protein was precipitated out, not affecting, though, the total activity in both preparations.

Applying the dialyzates to DEAE-cellulose, a large amount of protein was eliminated. In case of M-LDH the protein content was depressed down from 111.5 mg. to 27.0 mg. but the specific activity did not show the inversely proportional increase.

Though crude the preparations were, both enzymes were reasonably purified (4.54~5.15 folds) to carry out the present investigation. Furthermore, the two preparations showed distinctly pure isoenzyme bands on gels. (Fig. 1 and 2).



**Figure 3.** Calibration curve for formazane formation. The total activities determined spectrally are plotted against  $S_x$ , the amounts of nitroformazan produced, expressed as the sum total of extinction.

**Table IV.** The activity of M-LDH and H-LDH with 1 M urea in various pyruvate concentrations.

Pyruvate concentration	0	$5 \times 10^{-3} M$	$10 \times 10^{-3} M$	$15 \times 10^{-3} M$	$20 \times 10^{-3} M$
M-LDH activity	3.87*	0.30	0.39	0.41	0.43
H-LDH activity	1.55	1.21	0.99	0.59	0.48

\* Figures denote unit/ml LDH activity

### 3. Effects of Pyruvate on LDH Isoenzymes:

When pyruvate was present in the incubation mixtures both LDH isoenzymes showed remarkable depression of their activities as tabulated in Table III and Fig. 4.

The inhibition of activity brought by pyruvate was more pronounced in the H-LDH isoenzyme as compared to the M-LDH isoenzyme in its magnitude; that is, H-LDH activity was inhibited from 1.55 units ml. to 0.34 unit ml. with  $20 \times 10^{-3} M$  pyruvate, but the M-LDH activity was inhibited with the same concentration of pyruvate to about 1/3 of original activity (from 3.87 units to 1.29 units), which appeared to be a good revelation of the fact that the latter LDH isoenzyme is more resistant than the former. But on the other hand, under the presence of urea plus pyruvate in the reaction mixture, the inhibition profile, as can be seen in Table IV and Figure 5 and 6, disclosed an inverse effect of urea as compared to that obtained by pyruvate alone. In other words, the pyruvate-sensitive H-LDH turned out to be a little more resistant to pyruvate when urea was added, as is apparent from Figure 5, showing depressed increment of  $V/V_i$  ratio as a function of pyruvate concentration (where  $V$  denote activity in the absence of urea, while  $V_i$  the activity in the presence of urea).

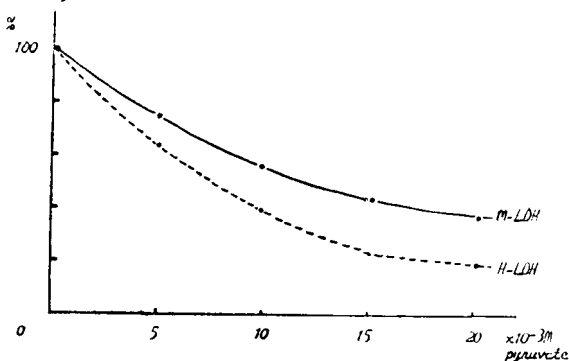


Figure 4. Pyruvate inhibition of LDH isoenzymes at its various concentrations.

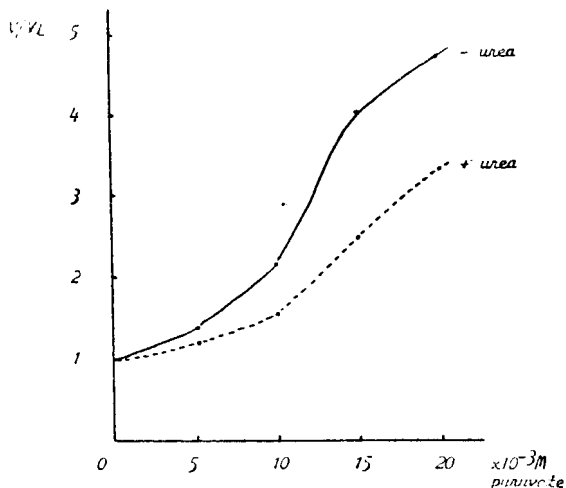


Figure 5. Effect of 1.5M urea on pyruvate inhibition of H-LDH in various concentrations.

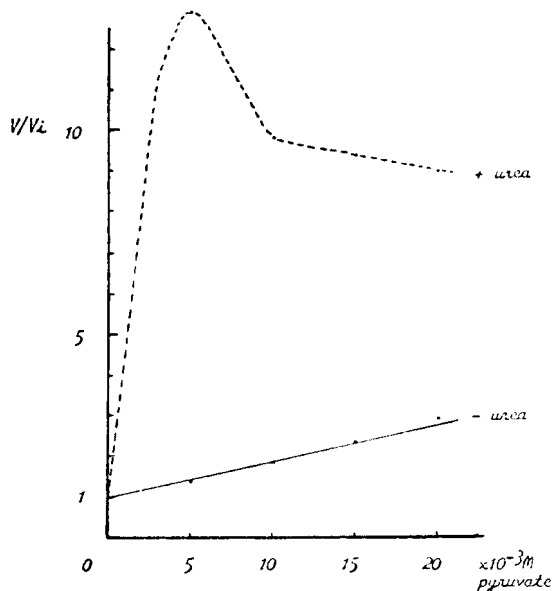


Figure 6. Effect of 1.5M urea on pyruvate inhibition of M-LDH in various concentrations.

The M-LDH, however, showed an opposite effect of urea, on the contrary, to be manifested by more inhibition under the presence of urea as compared to that observed without urea (Table IV). It is again apparent, in Fig. 6, that  $V/V_i$  ratio was increased abruptly in the presence of urea, with only  $5 \times 10^{-3} M$  pyruvate.

This interesting distinction may serve as a

new parameter differentiating the two isoenzymes from one another.

## DISCUSSION

### 1. on the electrophoretic procedure:

As to the conventional electrophoresis, the author was interested from the start in minor technicalities involved in staining difficulty when agarose gel was used. The gel resolved satisfactorily any isoenzyme or protein as reported by Wieme (13), but the formazan stain diffused through the gel bed, not yielding distinctly separative band, which is no doubt a handicap in quantitative aspect of isoenzymes.

The author, therefore, adopted a new procedure, overlapping on top of it with a cellulose acetate strip (Separaphone III, Gelman Co.) which was pre-soaked in the staining mixture, instead of soaking agarose gel in staining mixture or instead of dropping a minute quantity of the staining mixture on top of the agarose gel bed. This modification brought an unique resolution of the isoenzymograms on cellulose acetate and agarose gel, when combined together into a sum of densitometric areas, which enabled the author to study kinetic properties of the enzyme only with its densitometric analysis, because Lambert-Beer's law was apparently valid when the activity of LDH was less than 1000 mU/ml. (Fig. 3). The reason for this probably lies in the fact that the enzymogram resulted as a sharply resolved band after the modified procedure as in the present report and in so doing quantitative approach would be possible.

### 2. Effect of Pyruvate and Urea:

As is reported by many authors (4, 14, 15) the variations in substrate inhibition of LDH isoenzymes may be related to their metabolic

functions. For one thing, as Dawson *et al* (15) pointed out, there is a marked difference between the H-LDH and M-LDH isoenzymes in substrate inhibition by pyruvate, when the purified enzymes are assayed at 25°C. In other words, the H-LDH isoenzyme is maximally active at low concentration of pyruvate and inhibited by excess pyruvate. The H-LDH isoenzyme is known to be predominating, for example, in heart, an aerobic tissue where high levels of pyruvate would not develop, because pyruvate would be oxidized by mitochondria. Inhibition of the H-LDH as in the present study, by pyruvate would, therefore, favor the oxidative pathway, but on the other hand, the M-LDH which is predominating in relatively anaerobic tissues such as skeletal muscle, is resistant to inhibition by excess pyruvate as elucidated in the present study.

Since the reduction of pyruvate to lactate by the H-LDH is strongly inhibited by quite low concentrations of pyruvate, as in the present results, it has been suggested that rapid accumulation of lactate could not occur in a tissue rich in this isoenzyme, such as the heart, and complete oxidation of glucose *via* the citric acid cycle is therefore to be expected. But the M-LDH isoenzyme, on the other hand, functions more efficiently when exposed to concentrations of pyruvate inhibitory to H-LDH and is inhibited only by much higher concentrations. It appears, therefore, that tissue rich in the M-LDH, such as skeletal muscle, would allow the rapid conversion of pyruvate into lactate, and hence the establishment of an oxygen debt under anaerobic conditions (4).

On the basis of this theoretical ground, the present results with regard to pyruvate inhibition on both isoenzymes could be justified and from the present result it is quite evident that

that pyruvate counteracts the urea denaturation in the H-LDH isoenzyme and that it accelerates the urea denaturation in M-LDH isoenzyme within certain high range of pyruvate concentration.

## CONCLUSION

The present paper reported on the nature of pyruvate inhibition of LDH isoenzymes with and without urea. The crude LDH isoenzymes were prepared from rabbit heart muscle for the H-LDH and from rabbit psoas muscle for the M-LDH, with the following conclusions.

1. It is possible to obtain 4~5 fold purification of the LDH isoenzymes from rabbit heart and psoas muscles only with ammonium sulfate fractionation and DEAE-cellulose treatment.

2. In electrophoresis a new method of staining is proposed as follows; after electrophoresis with agarose gel a cellulose acetate strip, presoaked in the staining mixture, is overlapped on top of the agarose gel, followed by incubation and densitometry.

In so doing, a kinetic study on the LDH isoenzymes could be possible through formazan staining, instead of UV spectrophotometry.

3. The H-LDH isoenzyme activity is more markedly inhibited than the M-LDH isoenzyme by pyruvate.

4. Under the presence of urea, pyruvate inhibition of H-LDH isoenzyme decreases, while that of M-LDH isoenzyme increases.

### —국문초록—

서울대학교 의과대학 생화학교실

### 토끼 LDH Isoenzyme의 특성에 관한 연구

鄭 德 載 · 金 昇 元

저자는 본 연구에서 토끼의 심장근육 및 대퇴 근육에서 각각 H-LDH와 M-LDH isoenzyme을 분리정제하여,

그 전기 영동상(像)의 특성을 살펴보고 아울러 pyruvate와 urea에 기인하는 이들 isoenzyme의 활성변화를 관찰하여 다음과 같은 결론을 얻었다.

1. 토끼 심장근육과 대퇴근육을 이용하여 ammonium sulfate에 의한 염석과 DEAE-cellulose에 의한 흡착만으로라도 약 4~5배의 H-LDH와 M-LDH isoenzyme의 정제를 할 수 있다.

2. 전기 영동에 있어서는 agarose gel로 각 LDH isoenzyme을 영동한 다음 gel bed 위에 cellulose acetate gel로 덮고 incubation에 함으로써 더욱 분명한 분리과 정확한 densitometry가 이루어짐으로 전기 영동만으로써 spectrophotometry에서 기대되는 kinetic study가 가능한 새로운 염색법을 시도하였다.

3. Pyruvate는 M-LDH보다 H-LDH isoenzyme의 활성을 크게 억제한다.

4. Urea 존재하에서는 상기 pyruvate 억제가 H-LDH isoenzyme에서는 감소되며 M-LDH isoenzyme에서는 증가한다.

## REFERENCES

1. Kaplan, N.O., Ciotti, M.M., Hamolsky, M., and Bieber, R.E.: *Science*, 131, 393 (1960)
2. Appella, E. and Markert, C.L.: *Biochem. Biophys. Res. Comm.*, 6, 171 (1961)
3. Markert, C.L. and Möller, F.: *Proc. Natl. Acad. sci.*, 45 753 (1959)
4. Cahn, R.D., Kaplan, N.O., Levince, L. and Zwilling, E.: *Science*, 136, 962 (1962)
5. Kaplan, N.O., and Ciotti, M.M. *Ann. N.Y. Acad. Sci.*, 94, 701 (1961)
6. Goodfriend, T.L. and Kaplan, N.O.: *Circulation*, 32, 1010 (1965)
7. Starkweather, W.H., Spencer, H.H., Schwarz, E.L. and School, H.K.: *J. Lab. and Clin. Med.*, 67, 329 (1966)
8. Kaplan, N.O., Ciotti, M.M., Hamolsky, M. and Bieber, R.: *Science*, 131, 392 (1960)
9. Boyd J.W.: *Biochem. Biophys. Acta*, 132, 221 (1967)
10. Bergmeyer, H.U.: *Enzymatic Analysis, Academic Press, N.Y.*, (1963) p. 736
11. Neiland, J.B.: *Methods in Enzymology, ed by Boyer et. al; Academic Press, N.Y. (1955) Vol. 1, p. 449*

12. Lowry, O.H., Rosenbrough, N.I., Farr, A.L.  
And Randall, R.J. : *J. Biol. Chem.*, 193, 265  
(1951)
  13. Wieme, R.J. : *Clin. Chem. Acta*, 13, 138 (1966)
  14. Wilson, A.C., Cahn, R.D. and Kaplan, N.O. :  
*Nature*, 197, 331 (1963)
  15. Dawsnn, D.M., Goodfriend, T.L. And Kaplan,  
H.O. : *Science*, 143, 929 (1964)
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