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Lactate Dehydrogenase Activity in Dog Adipose Tissue.* (31812)

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In the course of our studies in fatty acid metabolism it became desirable to investigate the enzyme systems responsible for channeling metabolites to alternate metabolic pathways. An approach of this sort can provide information that is not necessarily obtained by following turnover rates of specific metabolites, the synthesis and degradation of important intermediates or by assaying end products of various routes.

Some 30 years ago fat stores were believed to be metabolically entirely passive. Experimental evidence of the past few years (1-4) has changed this concept radically, revealing adipose tissue as the site of very active metabolic processes of great diversity.

Lactic dehydrogenase of dog adipose tissue was one of the enzymes whose activity was of interest to us. Although lactate dehydrogenase catalyzes the reversible oxidation of lactate irrespective of its origin, each tissue enzyme possesses characteristic physicochemical properties. Therefore, it was important first to provide some of the necessary chemical and physiological background for subsequent interpretation of metabolic data in a meaningful manner.

This paper is concerned with some of the functional and kinetic characteristics of dog adipose tissue lactate dehydrogenase (LDH).

Materials and methods. Subcutaneous inguinal and pericardial adipose tissue were used as the source of enzyme. A 10% homogenate in isotonic KCl solution was prepared in a ground glass close-fitting motor driven homogenizer. The homogenate was centrifuged for 30 minutes at a speed of 100,000 $\times$ *g* in a Spinco preparative ultra-centrifuge. The supernatant fluid was diluted 240-fold as a rule for an activity level suitable for measurement.

Measurement of enzyme activity was usually made in 0.1 M phosphate buffer at physiological pH (7.4) at 5.0×10^{-4} M pyruvate concentration in the presence of 1.3×10^{-4} M final concentration of DPNH. Decrease of optical density at 340 mμ due

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to oxidation of DPNH was measured in a Beckman DU spectrophotometer under the following conditions: light path: 1 cm, final volume: 3.0 ml, temperature 24-27°C, blank cuvette containing distilled water. The fall in optical density was followed at 60-second intervals for 4 or 5 minutes starting at 45 seconds after addition of the enzyme. There was no disappearance of DPNH in the absence of substrate. Later, by combining a Gilford Model 220 absorbance indicator and a Model 210 automatic cuvette positioner with a Beckman DU monochromator and a Beckman potentiometric strip-chart recorder, an automatic recording system was provided for the measurement of LDH activity. Occasionally enzyme activity was also measured from the lactate side according to the method of Neilands(5), in 0.1 M glycine-NaOH buffer at pH 10.0, at 0.5 M Na-dl lactate concentration in the presence of 1×10^{-3} M final concentration of DPN.

Enzyme activities were calculated from ΔOD values and expressed in terms of μmoles of substrate utilized/hour/gram wet weight of tissue(6) or μmoles of substrate utilized/hour/mg protein N. Protein nitrogen was determined by the micro-Kjeldahl method as modified by Markham(7). Under the conditions of the assay, proportionality of enzyme activity to enzyme concentration prevailed. Reaction rate was linear during the observed time periods. Flavoprotein content was measured in the homogenate supernatant by measuring the difference in absorbancies at 465 and 510 mμ respectively (8).

LDH isozymes were investigated by a combination of thin gel electrophoresis and fluorometry(9). A Turner Model 310 Thin Gel Electrophoresis System was used in conjunction with a Turner Model 111 Fluorometer. Electrophoretic separation was effected in barbital buffer pH 8.8, $\mu = 0.05$, during one hour runs at 5°C with 24 ma current.

Results and discussion. The level of LDH activity as assayed from either side of the reaction (pyruvate $\xrightarrow{\text{DPNH}}$ lactate or lactate $\xrightarrow{\text{DPN}}$ pyruvate) is presented in

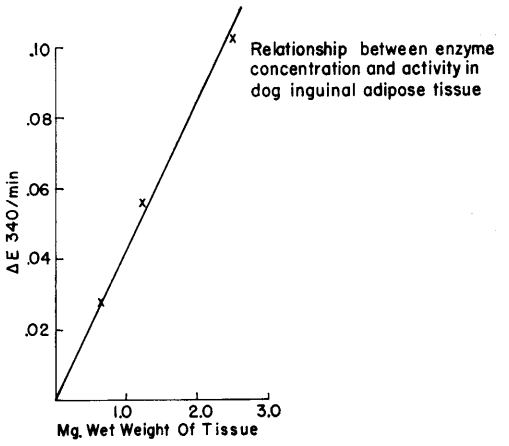

FIG. 1.

Table I. Good proportionality of activity with amounts of tissue added was found over a four-fold range from .062 to 2.5 mg wet weight of adipose tissue (Fig. 1).

Within the same animal LDH activity is significantly higher in the pericardial than in the inguinal adipose tissue. This difference is statistically highly significant, Table I. When activity is expressed in terms of per mg nitrogen of the tissues, a mean value of 281 for inguinal and 447 for pericardial tissue LDH is obtained. Due to individual variations the difference between these two values is statistically not significant. Nitrogen content of inguinal adipose tissue ranged between 3.7 and 5.8; in pericardial adipose tissue between 2.6 and 6.1 mg per gram wet weight. Weber *et al* reported 257 μmoles of substrate metabolized per hour per mg nitrogen at 37°C by LDH of rat adipose tissue(10). Several investigators pointed out

TABLE I. Adipose Tissue LDH Activity.

	Inguinal	Pericardial
Pyruvate $\xrightarrow{\text{DPNH}}$ lactate	1733	3280
Lactate $\xrightarrow{\text{DPN}}$ pyruvate	924	1200

Activities expressed as μmoles of substrate utilized per hour per g wet weight of tissue.

Mean of differences between inguinal and pericardial activity	514
Standard error of mean of differences	±139
No. of experiments	12
P	<.005

that differences in nitrogen content of adipose tissue exist, even between the proximal and distal parts of the epididymal fat pad(11).

In a few experiments LDH activity was also determined in abdominal (omental) and in perirenal adipose tissue. Abdominal adipose tissue activity is of similar order of magnitude as that of subcutaneous inguinal adipose tissue. Perirenal LDH is similar in activity to the pericardial enzyme.

There is a marked difference in the degree to which the activity of the "M" and "H" type subunits of LDH is inhibited by pyruvate. As an additional means of characterizing dog adipose tissue LDH, enzyme activities were recorded at "low" (3.33×10^{-4} M) and at "high" (1×10^{-2} M) pyruvate levels and the results expressed as a ratio of $\frac{\text{activity at "low" pyruvate}}{\text{activity at "high" pyruvate}}$. These ratios obtained with enzyme from inguinal and pericardial adipose tissue have an average value of 1.8 and 2.0 respectively, indicating that LDH from either source seems to be inhibited to the same extent by high pyruvate concentrations.

K_M (pyruvate) values were calculated from each experiment according to the method of Lineweaver and Burk(12). The mean value for inguinal adipose tissue LDH was found to be 1.9×10^{-4} M, that for pericardial adipose tissue LDH 2.0×10^{-4} M. It appears, therefore, that the significant difference observed in activity between LDH of inguinal and pericardial adipose tissue is not due to a difference in affinity of one enzyme or the other for its substrate.

In an effort to clarify the nature of the difference in activity between LDH of inguinal and pericardial adipose tissue, the possibility was explored that the difference may be due to a relative deficiency in the member next to the pyridine nucleotides in the electron transport chain of inguinal adipose tissue.

In 2 experiments when flavoproteins were measured, elevated flavoprotein content was found in the pericardial adipose tissue (18.2 mμ moles/g wet weight *vs.* 5.45 in the inguinal adipose tissue in one instance, and 12.7 mμ moles/g wet weight *vs.* 9.1 in the other) simultaneously with much greater LDH activity (about double) than in the inguinal adipose tissue. In one dog, one of the very few and unusual cases in the entire series, when LDH activities of the 2 adipose tissues were the same, the flavoprotein content was also identical.

The effects of certain inhibitors may be used to distinguish lactate dehydrogenase isozymes. Several investigators(13-15) have found that the electrophoretically slow isozymes, LD_4 and LD_5, from a number of tissues are preferentially inhibited by urea. By contrast, Plummer and Wilkinson(16,17) found that oxalate selectively inhibits the fast isozymes LD_1 and LD_2.

Enzyme activity was measured following 15 minutes of incubation in the presence of 0.4 M, 1.0 M and 2.0 M urea, respectively. Moreover, the effect of each concentration of the inhibitor was evaluated in the presence of low (5×10^{-4} M) and high (1×10^{-2} M) pyruvate levels. At each concentration of urea investigated, the degree of inhibition was always less at the high than at the low pyruvate level, suggesting that the high substrate levels succeed in overcoming the competitive type inhibition imposed by urea. This differential inhibition was found to be statistically highly significant with a $p < .005$ (Table II). Pericardial or perirenal adipose tissue LDH activity was affected in a similar manner by varying urea and substrate (pyruvate) levels (Table III). The lower urea concentrations did not cause changes in K_M values. At the 2.0 M urea level, however, K_M (pyruvate) for both inguinal and pericardial LDH were significantly lowered (Table IV).

The effects of oxalate in final concentrations ranging from .02 to .20 mM were also investigated. Results obtained within the same animal are usually consistent at various levels of oxalate. Biological variations from one animal to the next, however, make quantitative interpretation of these results difficult.

There is less inhibition by oxalate of inguinal adipose tissue LDH, in the majority of the experiments, at the high than at the low pyruvate level. LDH of pericardial adipose tissue is less inhibited by oxalate at the low than at the high pyruvate level. This finding could

TABLE II. Differences in Inhibition of Inguinal Adipose Tissue LDH Activity by Urea at "Low" and "High" Pyruvate Concentrations.

	Urea conc M					
	.4		1.0		2.0	
	Pyruvate M					
	5×10^{-4}	10^{-2}	5×10^{-4}	10^{-2}	5×10^{-4}	10^{-2}
Mean % of uninhibited activity	64.5	105.3	62.8	113.6	41.0	119.9
Mean of differences	40.8		50.8		78.9	
Standard error of mean of differences	± 6.7		±10.7		±19.9	
No. of experiments	8		8		7	
P	<.005		<.005		<.005	

TABLE III. Differences in Inhibition of Pericardial Adipose Tissue LDH Activity by Urea at "Low" and "High" Pyruvate Concentrations.

	Urea conc M					
	.4		1.0		2.0	
	Pyruvate M					
	5×10^{-4}	10^{-2}	5×10^{-4}	10^{-2}	5×10^{-4}	10^{-2}
Mean % of uninhibited activity	81.2	94.0	76.8	115.8	46.5	102.8
Mean of differences	12.7		39.0		56.3	
Standard error of mean of differences	± 6.3		±10.7		±14.4	
No. of experiments	4		4		4	
P	<.1		<.025		<.01	

be explained by proposing a higher percentage of "H" type subunits in this enzyme protein, since excess pyruvate alone is inhibitory to the "H" type, even without the presence of oxalate. Oxalate inhibition of both substrate levels is less at pH 7.4 than at pH 6.2. An explanation for this observation may be found in the fact that the dissociation constant of oxalate from its lactic dehydrogenase-coenzyme complexes increases markedly with pH in the range between pH 6 and pH 10(18). All levels of oxalate studied affected the K_M (pyruvate) of pericardial adipose tissue LDH, but not those of the inguinal adipose tissue enzyme. The greater susceptibility of pericardial adipose tissue LDH to oxalate inhibition suggests again a greater percentage of "H" type subunits in this tissue.

TABLE IV. Mean K_M Values (Pyruvate) $\times 10^{-4}$ M for Dog Adipose Tissue Lactic Dehydrogenase.

Inhibitor	Type of tissue	
	Inguinal	Pericardial
None	1.9 (8)	2.0 (5)
Urea .4 M	2.3 (8)	2.3 (4)
1.0 M	1.6 (8)	1.6 (4)
2.0 M	1.2*(7)	1.1*(3)
Oxalate .02 mM	2.2 (6)	3.0*(3)
.05 mM	2.3 (7)	3.7*(3)
.10 mM	2.0 (7)	3.2*(4)
.20 mM	2.1 (7)	3.2*(4)

Numbers in parentheses refer to number of experiments performed. Asterisks denote K_M values. Changed statistically significantly with "T" values of >2.0 or at least 2.0.

Using pyruvate as substrate, Plagemann *et al*(19) demonstrated that liver LDH is appreciably more thermolabile than the anionic LDH isoenzymes, characteristic of rabbit heart muscle. Inguinal and pericardial adipose tissue homogenates were pre-incubated at 59°C for 10 minutes at the same dilution as was subsequently used in the assay for LDH activity. Both enzymes were inactivated to about the same extent, losing 44 and 47% of their activity, respectively. If, however, the 10% homogenate supernatants were heated and then diluted just before the performance of the assay, 90 to 100% of the activity was lost. This observation is in line with the importance for high dilution of protein during renaturation of rabbit muscle lactate dehy-

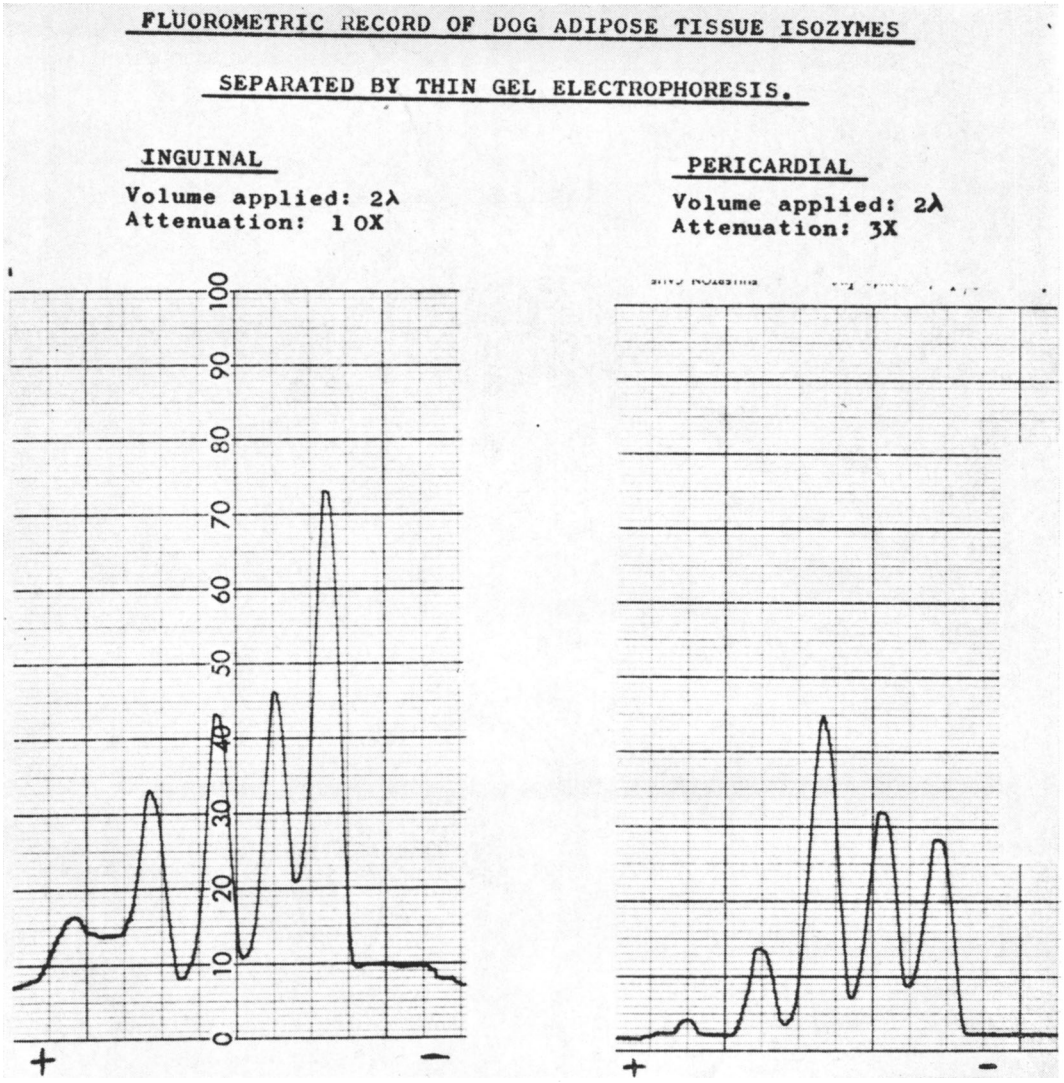

FIG. 2.

drogenase *in vitro*, in order to prevent intermolecular aggregation of unfolded polypeptide chains(20).

Five distinct isozymes of inguinal adipose tissue LDH were separated by electrophoretic migration in polyacrylamide gel and identified by formazan formation(21).

LDH of both inguinal and pericardial adipose tissues can be separated into 5 isozymes by thin gel electrophoresis and identified by fluorometry. Fig. 2 shows fluorometric records of these isozymes. Among the isozyme fractions, it is the first, slowest moving one which has the greatest fractional activity in inguinal adipose tissue, whereas in pericardial adipose tissue the third, and a faster moving (towards the anode) component displays more fractional activity than any of the others. This appears to lend further support to the observation that the behavior of pericardial adipose tissue LDH towards high substrate levels and oxalate inhibition is not inconsistent with a higher proportion of "H" subunits than in inguinal adipose tissue LDH.

Summary. Within the same animal, LDH activity is significantly higher in the pericardial than in the inguinal adipose tssue. This is related to the flavoprotein content of

the two tissues. High pyruvate concentrations inhibit LDH activity from either source to the same extent. The degree of inhibition by urea is always significantly less ($p<.005$) at the high (1×10^{-2} M) than at the low (5×10^{-4} M) pyruvate level. Oxalate inhibits inguinal adipose tissue LDH less at the high than at the low pyruvate level, while showing a reverse effect on the enzyme from pericardial tissue. Michaelis-Menten constants (K_M) for pyruvate were calculated from activities both in the absence and in the presence of urea and oxalate. K_M values were significantly decreased in the presence of 2.0 M urea. All levels of oxalate studied increased the K_M of pericardial adipose tissue LDH, but did not affect that of the inguinal adipose tissue enzyme. Incubation at 59°C for 10 minutes inactivates both enzymes to about the same extent, leading to a loss of 44 and 47% of their activity, respectively. Electrophoretic separation revealed 5 isozymes with a higher proportion of fast moving subunits in pericardial adipose tissue LDH.

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Transport of Alanine, Arginine and Glutamic Acid in the Rabbit Erythrocyte and Reticulocyte.* (31813)

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Early studies of Christensen *et al* reveal that the nucleated duck red blood cell and the human erythrocyte are able to maintain higher intracellular concentrations of apparently free amino acids than concentrations found in plasma(1). Riggs *et al* found the rabbit reticulocyte has an intracellular glycine content 3 to 5 times greater than the plasma (2). In contrast, the rabbit erythrocyte is un-

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