THE MULTIPLE FORMS OF OVINE LACTATE DEHYDROGENASE

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[*Manuscript received May* 4, 1964]

Summary

The extent of the multiplicity of lactate dehydrogenase in ovine tissues has been investigated by means of ion-exchange chromatography on CM- and DEAE-cellulose, starch-gel electrophoresis, and substrate activity ratios. The existence of five multiple forms of this enzyme has been demonstrated along with an unusual distribution of activity amongst the isoenzymes. These findings have been discussed in relation to methodology, ontogeny, physiological function of the parental tetramers, and ruminant metabolism.

I. Introduction

In recent years, the heterogeneity of mammalian lactate dehydrogenase (E.C. 1.1.1.27) has been extensively investigated (Nisselbaum and Bodansky 1959; Plagemann, Gregory, and Wroblewski 1960; Fine, Kaplan, and Kuftinec 1963). It is now recognized that this enzyme exists in most vertebrate sources as five separate molecular forms, which may be differentiated by electrophoretic, immunological, chromatographic, and kinetic criteria (Cahn *et al.* 1962).

In a previous investigation, however, only two electrophoretically distinct forms of ovine lactate dehydrogenase were revealed by the clinical procedure used (Masters 1963). While this result satisfied the diagnostic requirements of the situation, it was realized that it was not necessarily a complete expression of the enzyme multiplicity, and also that this apparently reduced degree of heterogeneity raised several questions of considerable biological significance (Fine, Kaplan, and Kuftinec 1963; Masters 1963).

In order to clarify the situation, therefore, the extent of multiplicity of ovine lactate dehydrogenase was studied by means of the more sensitive techniques of ion-exchange chromatography on CM- and DEAE-cellulose, starch-gel electrophoresis, and substrate activity ratios.

II. Methods

(*a*) *Tissue Extracts*

Organs and tissues were obtained from freshly slaughtered Merino ewes by excision, and frozen until required for analysis. Tissue extracts were prepared by grinding the thawed tissues in an all-glass Potter–Elvehjem homogenizer with an appropriate buffer (for chromatography on DEAE-cellulose: Tris–phosphate buffer, 0·01M, pH 8·6; for chromatography on CM-cellulose: phosphate buffer, 0·01M, pH 6·0; for starch-gel electrophoresis and enzyme activity determinations: phosphate buffer, 0·1M, pH 7·4), after which the extracts were clarified by centrifugation (2000 *g*, 20 min).

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Blood samples were collected by venipuncture and the plasma separated from the heparinized blood by centrifugation.

(*b*) *Ion-exchange Chromatography*

Columns of CM-cellulose (Whatman CM30) and DEAE-cellulose (Whatman DE50) (each 18 by 2 cm) were equilibrated with the appropriate buffers and operated under an air pressure of a few pounds per square inch.

Tissue homogenates, representing about 50 mg protein, were dialysed overnight against the starting buffer. The dialysed solutions were loaded on to the columns, and a linear concentration gradient of sodium chloride applied at a flow rate of 0·2–0·3 ml/min. Aliquots (3 ml) were collected from the column in an automatic fraction collector and analysed for lactate dehydrogenase activity (see below). The optical density at 280 mμ was also measured and used as an indication of protein concentration.

Overall recovery of enzyme was better than 95%. The identity of peaks of activity obtained from individual tissues was confirmed by the chromatography of mixed tissue extracts.

(*c*) *Starch-gel Electrophoresis*

Zone electrophoresis was carried out on horizontal gels made from hydrolysed starch* and barbital buffer (ionic strength 0·07, pH 8·6). The tissue extracts and plasma specimens were suitably diluted so that they possessed equivalent activity, then absorbed on to strips of filter paper (Whatman 3MM; 1·0 by 0·5 cm) and inserted into slits in the gel. The gels were 27 by 14 by 0·8 cm in size and were connected by bridge solutions to barbital buffer, ionic strength 0·1, pH 8·6. Separations were carried out at 4°C, with a voltage drop of 6–7 V/cm and a run of 16 hr duration.

After electrophoresis, the filter paper strips were removed, the gel was cut horizontally through the positions of application, and then stained to demonstrate lactate dehydrogenase activity. For this purpose, pyruvate was used as substrate, NAD as coenzyme, phenazine methosulphate as electron acceptor, and nitro blue tetrazolium† as the dye (van der Helm *et al.* 1962).

(*d*) *Enzyme Assay*

Lactate dehydrogenase activity was determined by measuring the rate of optical density decrease at 340 mμ resulting from the oxidation of $NADH_2$† in the presence of 0·00084M sodium pyruvate and a suitable dilution of enzyme (Wroblewski and La Due 1955). Samples were also assayed against sodium α-oxobutyrate (0·0033M) as substrate (Rosalki and Wilkinson 1960).

All measurements were made with a Beckmann model B spectrophotometer at pH 7·4 and 30°C. One unit of enzyme was defined as that amount which caused a decrease in optical density of 0·001 per minute under the specified conditions.

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III. Results

Typical results of the gradient elution from ion-exchange columns of ovine liver and muscle dehydrogenases from a mixed homogenate of liver and muscle tissue are shown in Figures 1 and 2.

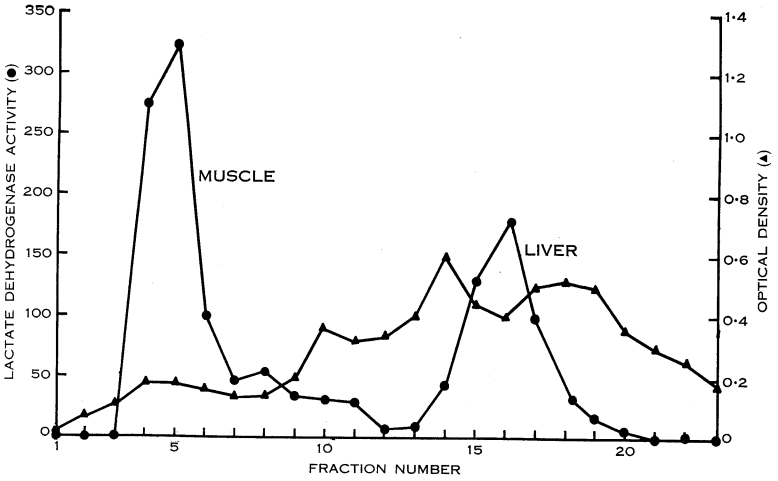

Fig. 1.—Elution of lactate deyhdrogenase from DEAE-cellulose, from a mixed homogenate of ovine liver and muscle tissue. The molarity of sodium chloride in fraction 16 was 0·07. ● Lactate dehydrogenase activity. ▲ Protein concentration measured as optical density at 280 mμ.

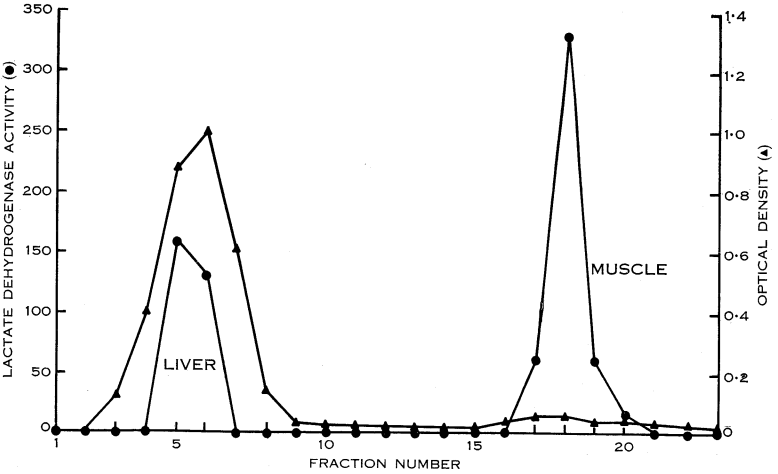

Fig. 2.—Elution of lactate dehydrogenase from CM-cellulose from a mixed homogenate of ovine liver and muscle tissue. The molarity of sodium chloride in fraction 18 was 0·08. ● Lactate dehydrogenase activity. ▲ Protein concentration measured as optical density at 280 mμ.

Liver lactate dehydrogenase was more firmly bound to DEAE-cellulose than muscle lactate dehydrogenase and was eluted as a single component after that of muscle (Fig. 1). The lactate dehydrogenases of heart, kidney, and erythrocytes also

chromatographed as single components, in positions similar to that of liver, as also did those of spleen, pancreas, and lung. The last three tissues, however, each showed an additional minor peak, intermediate in position between the muscle and liver peaks. Serum displayed two peaks of activity, the first being approximately one-quarter the area of the second.

As a control, rat kidney homogenate was also chromatographed. The lactate dehydrogenase activity of this source was known to be distributed in approximately equal proportions between five isoenzymes, and all these forms were resolved by the column.

On CM-cellulose, however, liver lactate dehydrogenase was eluted before that of muscle (Fig. 2), but the heterogeneity was the same as that on DEAE-cellulose. Furthermore, rechromatography of individual peaks from the DEAE-cellulose column on CM-cellulose provided little evidence of further multiplicity.

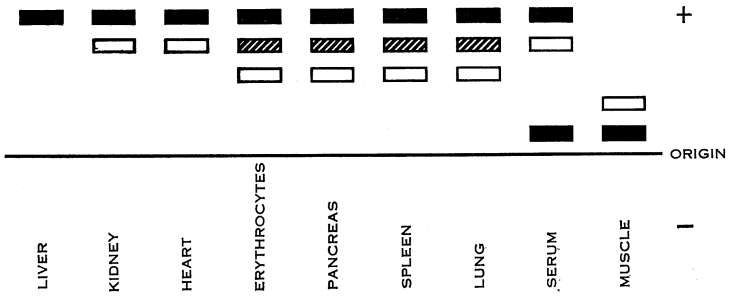

Fig. 3.—Starch-gel patterns of ovine tissue lactate dehydrogenase. Solid bands represent intense staining, shaded bands moderate staining (less than 15% of total activity), and clear bands weak staining (less than 2% of total activity).

Starch-gel patterns of ovine tissues are illustrated in Figure 3. It is evident from these results that five electrophoretically distinct forms of ovine lactate dehydrogenase exist. The activity is situated predominantly in the terminal isoenzymes but spleen, pancreas, and lung show appreciable quantities of the intermediate forms. Muscle has a unique isoenzyme composition (mainly cathodal forms), and serum is the only source containing both parental types in appreciable quantities.

Table 1 lists the ratios of activities of ovine tissue lactate dehydrogenase against pyruvate and α-oxobutyrate. It may be seen that a gradient of this ratio occurs between the extremes of liver and muscle lactate dehydrogenases. Furthermore, this ratio reflects the isoenzyme composition of the source and provides a complementary index of isoenzyme composition.

IV. Discussion

The resolution of the lactate dehydrogenase in ovine tissues obtained by chromatography on ion-exchange resins was comparable to that revealed by a previously reported procedure involving electrophoresis and elution from starch

gel (cf. Figs. 1 and 2 of Masters 1963). Studies of the substrate activity ratios in the various tissues, however, indicated the presence of more than two multiple forms. For example, lung showed no activity corresponding to muscle, yet possessed a higher pyruvate/α-oxobutyrate ratio than liver (Table 1). This may be interpreted as indicating the presence of some hybrid muscle-type activity, but gives little indication of the extent of the multiplicity.

In contrast, separation of tissue proteins by starch-gel electrophoresis, and staining of the electropherograms *in situ* clearly demonstrated the presence of five multiple forms of ovine lactate dehydrogenase (Fig. 3). This procedure then is indicated as the method of preference for demonstrating the extent of heterogeneity in tissue sources.

TABLE 1

RELATIVE ACTIVITIES OF OVINE TISSUE LACTATE DEHYDROGENASE AGAINST PYRUVATE AND α-OXOBUTYRATE

Method of assay that of Rosalki and Wilkinson (1960)

Tissue	Pyruvate/ α-Oxobutyrate Ratio	Tissue	Pyruvate/ α-Oxobutyrate Ratio
Liver	1·1	Spleen	1·6
Kidney	1·3	Lung	1·9
Heart	1·3	Serum	2·3
Erythrocytes	1·8	Skeletal muscle	6·0
Pancreas	1·6		

At the present date, no mammal has been found to possess a lactate dehydrogenase which is strikingly different from that of other mammals. Major differences have only been found between groups of animals which are very different in their evolutionary characteristics (Kaplan 1961). This fuller description of ovine lactate dehydrogenase heterogeneity, then, clarifies these particular interrelationships in respect of the sheep, and supports a general uniformity in the multiplicity of mammalian lactate dehydrogenase.

The distribution of activity amongst the isoenzymes, however, is unusual by comparison with most species in that it lies predominantly in the parental forms. The hybrid forms are present in low concentration and were not demonstrated previously (Masters 1963). The previous procedure, nevertheless, represented a close approximation to the findings by more sensitive methods and the diagnostic interpretations are unaltered by the fuller description of enzyme heterogeneity now available (Wroblewski 1961).

This unusual distribution of isoenzymes may be related to the different physiological roles of each parental type of lactate dehydrogenase, and the unusual fatty-acid-dependent metabolism of ruminants (Gallagher and Buttery 1959; Cahn *et at.* 1962). As pyruvate shows a pronounced substrate-inhibiting effect with heart-type lactate dehydrogenase compared to the muscle-type enzyme, and since the

direction of pyruvate metabolism in muscle is towards lactate and not towards oxidation, it might be expected that the lactate dehydrogenase in skeletal muscle would be of a type which would operate in the presence of relatively high levels of pyruvate. Similarly the predominance of the heart-type tetramer in the other tissues appears to be a reflection of the metabolism in these sites which takes place, in the main, through non-carbohydrate pathways involving oxidation under aerobic conditions.

In conclusion, then, these studies confirm the presence of five multiple forms of lactate dehydrogenase in ovine tissues, and add support to proposals in the literature regarding the structural and ontogenetic relationships of mammalian lactate dehydrogenase (Kaplan 1961; Fine, Kaplan, and Kuftinec 1963). Furthermore, useful information has been provided to assist in a purification of individual isoenzymes.

V. Acknowledgments

The authors wish to thank Proffessor E. C. Webb for valuable advice.

VI. References

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