THE ELECTROPHORETIC HETEROGENEITY OF OVINE LACTATE DEHYDROGENASE

By C. J. Masters*

[*Manuscript received December* 10, 1962]

Summary

The lactate dehydrogenase activity of ovine tissues has been measured and the existence of electrophoretically distinct forms of this enzyme confirmed. Distribution patterns of these isoenzymes have been charted for the main tissues, and the plasma pattern has been utilized as a clinical parameter of tissue function in prolonged protein depletion.

As a result, previous findings have been corroborated and extended. Liver is indicated as the tissue most affected by the protein loss, with skeletal muscle exhibiting a smaller degree of terminal tissue necrosis; the cellular integrity of the other major protein stores appears to be well preserved.

I. Introduction

In recent years, several enzymes have been shown to exist in multiple molecular forms, not only within a single organism, but even within a single tissue (Markert and Møller 1959). These protein forms may possess the same enzymatic specificity, and yet be capable of differentiation by electrophoretic, immunological, chromatographic, and kinetic criteria (Nisselbaum and Bodansky 1959; Kaplan *et al.* 1960; Plagemann, Gregory, and Wroblewski 1960*b*). Perhaps the most widely investigated instance of enzyme heterogeneity is mammalian L-lactate nicotinamide–adenosine diphosphate (NAD) oxidoreductase (i.e. lactate dehydrogenase, E.C. 1.1.1.27) which has been resolved into several distinct components by starch-gel electrophoresis.

The clinical usefulness of this phenomenon is associated with the characteristic distribution of these multiple forms or isoenzymes within the various tissues. Whereas elevations in human serum lactate dehydrogenase have diagnostic implications (Wroblewski 1959; Abderhalden 1961; Wilkinson 1962) it is difficult to specify the tissue source precisely because of the almost ubiquitous distribution of this enzyme. In this respect, the highly individual characteristics of tissue isoenzyme composition provide a significant refinement. Alterations of the proportions of isoenzymes in the serum tend to reflect the isoenzyme constitution of the contributing tissues, and in this way provide an organ specific diagnostic parameter in many instances (Wroblewski 1961, 1962).

With these facts in mind, and as an extension of previous investigations of ovine tissue chemistry (Masters and Horgan 1962*a*, 1962*b*; Masters 1963), the lactate dehydrogenase isoenzyme distribution of the major tissues has been charted, along with the plasma response to prolonged protein deprivation.

* Department of Biochemistry, University of Queensland, St. Lucia, Brisbane.

II. Methods

(a) *Treatment of Experimental Animals*

The four animals used in the protein-depletion experiments were well-matched Merino ewes, 3–4 years of age. After feeding for 3 weeks on lucerne chaff *ad libitum*, with free access to water, the animals were converted to a ration of 3 kg per week of chaffed wheat straw (digestible crude protein 0·5%) with water, *ad libitum*, for a period of 8 weeks. During the period of restricted intake, weekly blood samples were collected by venipuncture, and the plasma separated from the heparinized blood by centrifugation.

(b) *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 barbital buffer of ionic strength 0·07, pH 8·6. The extracts were clarified by centrifugation.

(c) *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 inserted into slits in the gel with a supporting medium of starch granules (Smithies 1955). The gels were 27 by 14 by 0·8 cm in size, and were connected to bridge solutions of barbital buffer, ionic strength 0·1, pH 8·6. Separations were carried out at 4°C, with a voltage drop of 6–7 volts per centimetre, and a run of 20 hr duration.

After electrophoresis, the gel was cut into 0·3-cm strips at right angles to the direction of migration and frozen. The enzyme activity was recovered by thawing and macerating the individual strips in 2 ml of a solution of 1·0 mg of α-amylase† per millilitre in 0·067M phosphate buffer, pH 7·0, which was 10% saturated with ammonium sulphate. After 30 min incubation at room temperature, the starch debris was removed by centrifugation. The lactate dehydrogenase activity of the clear supernatant fluid in each tube was determined as described below. Overall recovery of enzyme was approximately 50%. Duplicate isoenzyme determinations showed a variation within 15%.

The identity of the peaks of activity obtained from individual tissues were confirmed by the electrophoresis of mixed tissue extracts.

(d) *Lactate Dehydrogenase Assay*

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). All measurements were made with a Beckmann model B spectrophotometer at pH 7·4

* Connaught Medical Research Laboratories, University of Toronto, Canada.

† British Drug Houses Ltd., Poole, England.

‡ Sigma Chemical Co., St. Louis, Missouri, U.S.A.

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.

(e) *Serum Bilirubin Determinations*

Analyses were performed on all the plasma specimens by the methods of Malloy and Evelyn (1937).

III. Results

(a) *Lactate Dehydrogenase Levels*

The data presented in Table 1 represent the levels of total lactate dehydrogenase (LD) activity in the various tissues investigated. Considerable variation is evident amongst the tissue sources, with skeletal muscle exhibiting the greatest activity per unit wet weight. Kidney, heart, and liver also possess a high enzyme content, with spleen, pancreas, lung, and erythrocytes being minor sources by comparison.

Table 1

TOTAL LACTATE DEHYDROGENASE ACTIVITIES IN MERINO SHEEP TISSUES

Activities are given in Wroblewski–La Due spectrophotometric units per milligram wet weight of tissue

Tissue	Activity	Tissue	Activity
Skeletal muscle	400	Pancreas	55
Kidney	190	Lung	25
Cardiac muscle	150	Erythrocytes	25
Liver	70	Plasma (normal)	0·68
Spleen	60		

The determination of plasma total LD levels in 10 Merino ewes gave mean values (± S.D.) of 680±110 units/ml at 30°C.

In Figure 1, the results of typical electrophoretic separations of tissue LD isoenzymes are illustrated. Most tissues contain only one of the two electrophoretically distinct forms, with the faster-migrating component (LD_1) possessing the wider distribution. This form represents the total LD activity of liver, kidney, heart, pancreas, spleen, lung, and erythrocytes. In skeletal muscle extracts only the slower-migrating form (LD_2) is present, but both isoenzymes are evident in the serum.

Alterations in the concentrations of the serum isoenzymes during the period of restricted protein intake are outlined in Figure 2. These graphs have been plotted in terms of the arithmetic means of the results for four experimental animals, but similar responses were obtained in the individual cases. The sequence of changes shown is an initial diminution of LD_1 activity, followed by a terminal increase of both isoenzymes. LD_1 exhibited the more marked elevation in the final stages of protein depletion.

The final blood samples from two of the experimental animals exhibited elevated serum bilirubin levels. Mean value (± S.D.) was 1·0±0·7 mg/100 ml (four animals). All the other serum bilirubin values were less than 0·3 mg/100 ml.

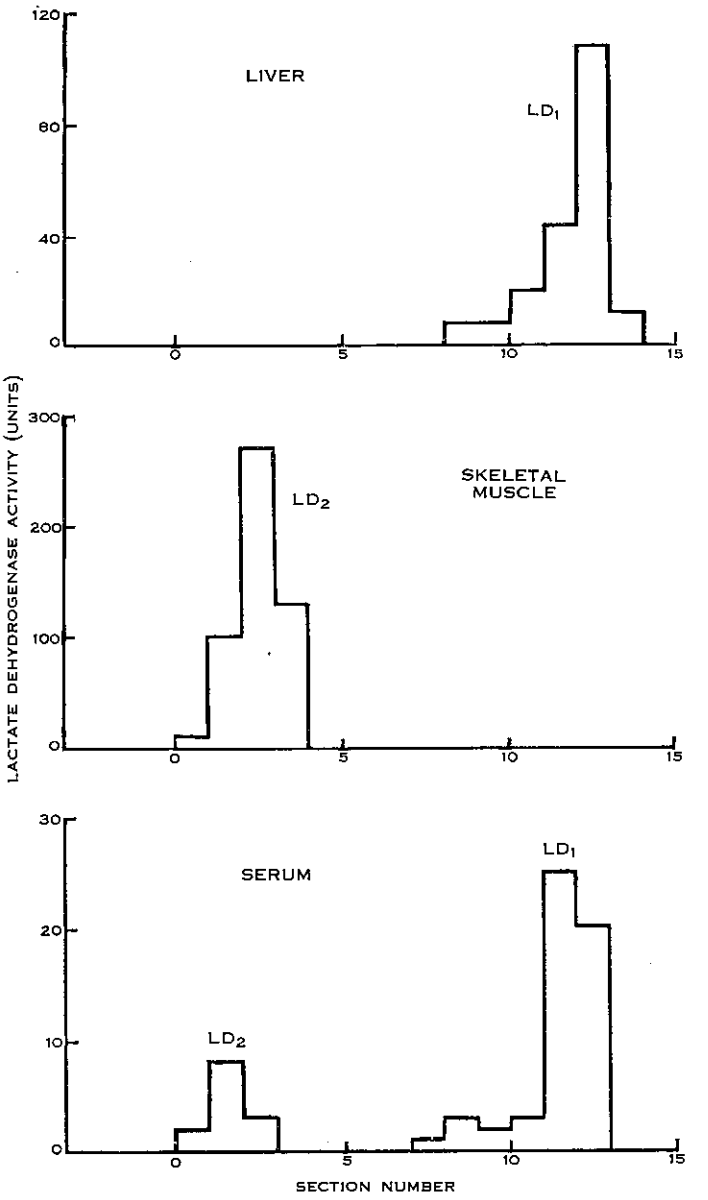

Fig. 1.—Patterns of lactate dehydrogenase (LD) activity obtained by the starch-gel electrophoresis of ovine tissue extracts at pH 8·6. The starch-gel sections are numbered from the origin towards the anode. Activity expressed as Wroblewski–La Due units per millilitre of starch-gel supernatant.

(b) Histopathology

Livers from sheep slaughtered after 8 weeks on the experimental diet exhibited a moderate degree of centrilobular necrosis and fat accumulation. Skeletal muscle evinced occasional small areas of necrotic degeneration, with scattered foci of lipid accumulation.

There was no histological evidence of renal or myocardial necrosis. A small degree of fat accumulation was evident in the kidney, and there were occasional localized fatty changes in the myocardium.

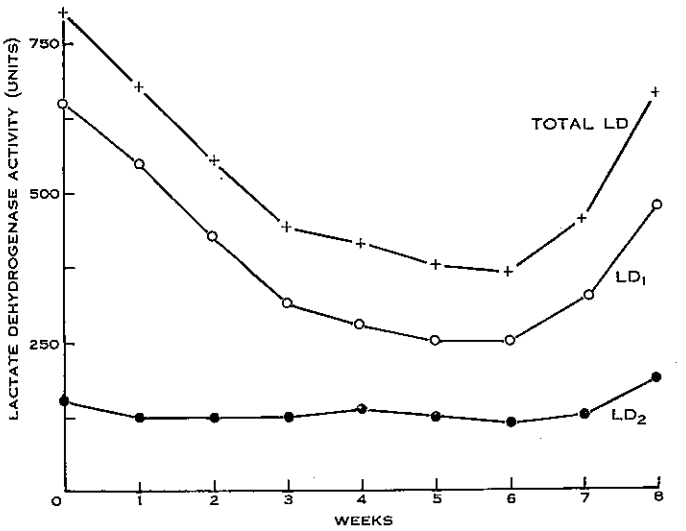

Fig. 2.—Changes in serum lactate dehydrogenase (LD) activity during the period of dietary restriction. Activity expressed as Wroblewski–La Due units per millilitre of serum.

IV. Discussion

Because of the wide diversity of methods reported in the literature for the extraction and estimation of tissue LD activity, it is difficult to correlate the results for ovine tissues (Table 1) with those for other mammalian sources. A valid comparative basis is available with rabbit tissues (Plagemann, Gregory, and Wroblewski 1960*a*), however, and in this case, activities per unit wet weight of ovine tissue appear to be of the order of three times those in the rabbit. Exceptions to this general rule are ovine muscle and liver which exhibit about one-half and one-fifth, respectively, of the activities reported for the rabbit.

The relative magnitudes of LD activities in tissues of other species have been tabulated (Green and Brosteaux 1936; Meister 1950; Wenner, Spirtes, and Weinhouse 1952; Plagemann, Gregory, and Wroblewski 1960*a*). In monogastric animals, the main sites of tissue LD activity (on a units per unit wet weight basis) are skeletal muscle, liver, heart, and kidney; with the spleen, pancreas, lung, and erythrocytes being minor sources by comparison. It is interesting to note the lower position in this tissue sequence occupied by ovine liver (Table 1). Possibly this lower status is a

reflection of ruminant metabolism, characterized as it is by a dependence on fatty acids, rather than glycolysis, as an energy source (Gallagher and Buttery 1959).

Although these studies have confirmed that electrophoretically distinct forms of ovine LD exist, it is realized that the extent of heterogeneity detected may be dependent upon the system used for resolution of the protein fractions. In fact, it has previously been reported (Markert and Møller 1959) that electrophoresis of LD from sheep's heart on borate-buffered starch gel reveals two isoenzyme bands, whereas the method used in these experiments indicated the presence of only one form. It is not a necessary corollary, however, that a higher resolution results in isoenzyme patterns of greater tissue specificity (Blanchaer 1962).

There are several points of difference between the distribution of electrophoretically distinct forms of LD in ovine tissues (Fig. 1), and the distributions obtained in other species by similar methods (Plagemann, Gregory, and Wroblewski 1960*a*).

Firstly, ovine LD (two forms) exhibits a smaller degree of multiplicity than the LD of such mammals as humans (five forms) and rabbits (five forms). In these experiments, no sheep tissue (except plasma) contained more than one LD form, whereas the tissues from other species frequently contain the full complement of that particular animal.

Secondly, amongst ovine tissues only skeletal muscle has a unique isoenzyme composition, in contrast to the distinct and characteristic LD picture displayed by the various tissues of humans and rabbits.

There are also differences in electrophoretic mobilities between the enzyme forms from different species. For example, the predominant liver isoenzyme in humans and rabbits migrates towards the cathode under the same conditions at which the sheep liver form migrates towards the anode. This possibly indicates differences in constitution of these LD forms between the species (Markert and Apella 1961).

A point of similarity between the above species is the common possession by skeletal muscle of a single isoenzyme of low mobility at pH 8·6; and a further point of interest about the ovine myoskeletal isoenzyme is the relative activity of the corresponding plasma form. Despite the fact that skeletal muscle must be the largest source of tissue lactate dehydrogenase by far (Table 1; Widdowson, McCance, and Spray 1951), serum LD_2 is the minor component of normal serum LD activity (Fig. 2).

Because of the high activity of most tissues relative to serum, the serum LD is a useful index of cell damage in many organs and has been used as diagnostic confirmation of myocardial infarction (White 1956), muscular dystrophy (Dreyfus, Schapira, and Schapira 1958) and liver disease (Wilkinson 1962).

In these experiments, the sequential pattern of serum LD activity during protein depletion is characterized in the initial stages by a diminution of activity (Fig. 2). A similar phenomenon has been observed in other mammals in response to restricted protein intake (Warter, Metris, and Keckhut 1961) and while various interpretations of this decrease are possible on the present experimental evidence

(for example, changes in the concentration of inhibitors or activators, the effect of associated enzyme systems), the diminution probably relates to a decline in the quantity of contributory tissue LD under these conditions (Weber 1960; Warter, Metris, and Keckhut 1961). Since the decrease of serum LD activity is due to the LD_1 modification, this form presumably originates in sites of high initial protein lability.

Following the initial steep decrease of serum LD activity (Fig. 2), changes in this parameter are inappreciable for some weeks. On the basis of previous investigations (Addis *et al.* 1940; Masters and Horgan 1962*b*; Masters 1963) this would seem to be associated with a slower rate of protein loss from the tissues and a developing tendency towards tissue necrosis. As regards the level of serum LD, these effects would tend to act in opposition.

In the last 2 weeks of the protein depletion a sharp increase in serum LD occurs (Fig. 2). The interpretation of this increment as an indication of extensive tissue necrosis associated with the terminal processes of protein depletion is in agreement with the histopathology, with the behaviour of serum glutamate oxalacetate transaminase in this condition (Masters and Horgan 1962*b*), and with experimental evidence in other animals (Weimer *et al.* 1959).

It is possible to improve the sensitivity and specificity of previous test procedures by following the variations of the serum LD heteromorphs. Ovine serum LD_2 provides a perceptive index of myoskeletal necrosis in the sheep and the small terminal rise occurring in response to prolonged protein depletion indicates that, in spite of the loss of more than half the normal protein content (Masters 1963), the skeletal muscle suffers only a slight degree of cellular breakdown.

Clinical experience with humans (Wilkinson 1962) and the distribution of LD_1 in sheep tissues show that the most likely sources contributing to serum LD_1 elevation would be the liver, heart, kidney, and possibly the erythrocytes. A hepatic contribution seems to be established on the basis of histopathology and other clinical findings (Masters and Horgan 1962*b*). This is not surprising when it is realized that the liver has lost nearly half its normal protein content at this stage (Masters 1963).

The protein of heart and kidney is relatively well preserved under the experimental conditions (Masters 1963) and histological examination failed to reveal extensive necrosis at these sites. Further, renal tissue is an infrequent contributor to serum LD and exhibits normal functional ability in the final stages of protein depletion (Masters and Horgan 1962*b*). Also, the experimental animals showed no symptomatology of myocardial infarction. For these reasons, a contribution to serum LD from renal or cardiac sources seems unlikely.

Although the erythrocytes are a comparatively minor source of LD_1 on a units per wet weight basis, their intimate relationship with the plasma occasionally causes significant elevations of serum LD. The possibility must be considered in this instance because of the elevation of terminal serum bilirubin values in two of the experimental animals. Since these bilirubin levels were only slightly elevated, however, and since the animals without hyperbilirubinaemia showed similar rises at this stage, the erythrocytes, also, seem an unlikely donor of increased serum LD activities.

In conclusion, then, it is proposed that the clinical data provides confirmatory evidence for the view that the liver is the protein storage site which suffers most damage as a result of the prolonged protein deprivation. Skeletal muscle exhibits a smaller degree of tissue necrosis in this situation, but the cellular integrity of the other major protein stores appears to be well preserved.

V. Acknowledgments

The author wishes to thank Professor E. C. Webb for his interest and advice, and Professor O. E. Budtz-Olsen for the provision of experimental facilities. For the histopathology, he is indebted to Dr. R. Tucker, University of Queensland, and Mr. W. J. K. Hall, Animal Research Institute, Yeerongpilly. He is also grateful to Mr. R. Arentz for his assistance with the experimental animals.

VI. References

Abderhalden, R. (1961).—"Clinical Enzymology." (D. Van Nostrand Co. Inc.: London.)
Addis, T., Lee, D. D., Lew, W., and Poo, L. J. (1940).—*J. Nutrit.* **19**: 199.
Blanchaer, M. C. (1962).—In "Proceedings of the 2nd International Symposium on Enzymes in Clinical Chemistry". (Butterworths Scientific Publications: London.)
Dreyfus, J. C., Schapira, G., and Schapira, F. (1958).—*Ann. N.Y. Acad. Sci.* **75**: 235.
Gallagher, C. H., and Buttery, S. H. (1959).—*Biochem. J.* **72**: 575.
Green, D. E., and Brosteaux, J. (1936).—*Biochem. J.* **30**: 1489.
Kaplan, N. O., Ciotti, M. M., Hamolsky, M., and Bieber, R. E. (1960).—*Science* **131**: 392.
Malloy, H. T., and Evelyn, K. A. (1937).—*J. Biol. Chem.* **119**: 481.
Markert, C. L., and Apella, E. (1961).—*Ann. N.Y. Acad. Sci.* **94**: 678.
Markert, C. L., and Møller, F. (1959).—*Proc. Nat. Acad. Sci., Wash.* **45**: 753.
Masters, C. J., and Horgan, D. J. (1962*a*).—*Aust. J. Biol. Sci.* **15**: 690.
Masters, C. J., and Horgan, D. J. (1962*b*).—*Aust. J. Agric. Res.* **13**: 1082.
Masters, C. J. (1963).—*Aust. J. Biol. Sci.* **16**: 192.
Meister, A. (1950).—*J. Nat. Cancer Inst.* **10**: 1263.
Nisselbaum, J. S., and Bodansky, O. (1959).—*J. Biol. Chem.* **234**: 3276.
Plagemann, P. G. W., Gregory, K. F., and Wroblewski, F. (1960*a*).—*J. Biol. Chem.* **235**: 2282.
Plagemann, P. G. W., Gregory, K. F., and Wroblewski, F. (1960*b*).—*J. Biol. Chem.* **235**: 2288.
Smithies, O. (1955).—*Biochem. J.* **61**: 629.
Warter, J., Metris, P., and Keckhut, M. (1961).—*C. R. Soc. Biol., Paris* **155**: 108.
Weber, G. (1960).—*J. Nutrit.* **71**: 156.
Weimer, H. E., Carpenter, C. M., Naylor Foote, A. W. C., McKee, R. W., and Nishihara, H. (1959).—*Proc. Soc. Exp. Biol., N.Y.* **101**: 344.
Wenner, C. E., Spirtes, M. A., and Weinhouse, S. (1952).—*Cancer Res.* **12**: 44.
White, L. P. (1956).—*New Engl. J. Med.* **255**: 984.
Widdowson, E. M., McCance, R. A., and Spray, C. M. (1951).—*Clin. Sci.* **10**: 113.
Wilkinson, J. H. (1962).—"An Introduction to Diagnostic Enzymology." (Edward Arnold Ltd.: London.)
Wroblewski, F. (1959).—*Ann. Intern. Med.* **50**: 62.
Wroblewski, F. (1961).—In "Proceedings of the 4th International Conference on Clinical Chemistry". (Eds. C. P. Stewart, S. C. Frazer, and P. C. Jocelyn.) (E. & S. Livingstone, Ltd.: London.)
Wroblewski, F. (1962).—In "Proceedings of the Second International Symposium on Enzymes in Clinical Chemistry". (Butterworths Scientific Publications: London.)
Wroblewski, F., and La Due, J. S. (1955).—*Proc. Soc. Exp. Biol., N.Y.* **90**: 210.