Developmental Changes in Ruminant Lactate Dehydrogenase

MARY HINKS AND C. J. MASTERS

From the Department of Biochemistry, University of Queensland, Brisbane, Australia

Received June 18, 1964

Developmental progressions of ruminant lactate dehydrogenase have been described in terms of electrophoretic patterns, substrate-activity ratios, and the percentage of subunit types present. This evidence demonstrates that the early embryonic form in bovine and ovine tissues is predominantly "B" type, in contrast to previous suggestions in the literature. Inaccuracies inherent in the prediction of the embryonic form of lactate dehydrogenase from late fetal analyses are examined, and evidence is presented in support of the suggestion that the two genes controlling the synthesis of lactate dehydrogenase may both be active in the newly fertilized ovum.

In recent years, the heterogeneity of mammalian lactate dehydrogenase (E.C. 1.1.1.27) has been extensively investigated. 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 (Markert and Møller, 1959; Plagemann *et al.*, 1960; Fine *et al.*, 1963; Lindsay, 1963). Furthermore the individual isoenzymes have been shown to be tetramers, formed by the combination of two types of polypeptide subunit, each of which is apparently under the control of separate genetic loci (Markert and Apella, 1961; Cahn *et al.*, 1962; Markert, 1963a; Zinkham *et al.*, 1964). These component monomers have been termed "A" and "B" (Markert and Ursprung, 1962), or "M" and "H" (Cahn *et al.*, 1962), in reference to their predominance in adult skeletal muscle and heart, respectively.

During fetal development and maturation after birth, the multiple forms of lactate dehydrogenase undergo redistribution in a manner which is characteristic of a particular tissue source (Cahn *et al.*, 1962; Markert and Ursprung, 1962; Vesell *et al.*, 1962; Wiggert and Villee, 1962; Fine *et al.*, 1963; Smith and Kissane, 1963). This paper describes the developmental progression of lactate dehydrogenase in two species of the *Ruminantia*. It was initiated in view of the considerable biological significance of these developmental changes (Cahn *et al.*, 1962; Markert, 1963b), the need for comparative biochemical studies in his field (Fine *et al.*, 1963), and as an extension of previous studies into the heterogeneity of ovine lactate dehydrogenase (Masters 1963, 1964; Hinks and Masters, 1964).

METHODS

Tissue Extracts.—A number of sheep and cattle were selected to cover a representative range of intervals during gestation, infancy, and adulthood. The hearts and livers were excised from these freshly slaughtered animals along with samples of rump skeletal muscle. These tissues were then frozen and stored at −10° until required for analysis.

Electrophoresis.—Homogenates (approximately 10%) were prepared in phosphate buffer (*p*H 7.4, *I* 0.1), centrifuged (2000 × *g*, 20 minutes), and zone electrophoresis of the supernatant carried out on horizontal gels made from hydrolyzed starch (Connaught medical Research Laboratories, Toronto, Canada) and barbital buffer (*p*H 8.6, *I* 0.07). The tissue extracts were suitably diluted so that they possessed equivalent activity, then absorbed onto strips of filter paper (Whatman 3 MM, 1.0 × 0.5 cm) and inserted into slits in the gel. The gels were 27 × 14 × 0.8 cm in size and were connected to bridge solutions by barbital buffer (*p*H 8.6, *I* 0.1). Separations were carried out at 4° with a voltage drop of 6–7 v/cm, and a run of 16 hours' duration.

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

These zymograms were scanned in a Chromoscan densitometer, and the percentage of "B"-type subunits was calculated from the peak areas on the basis of an assumed tetramer structure (Markert and Apella, 1961; Cahn *et al.*, 1962).

Enzyme Assay.—Lactate dehydrogenase activity was determined by measuring the rate of optical density decrease at 340 mμ resulting from the oxidation of reduced NAD (Sigma Chemical Co., St. Louis, Mo.) in the presence of 0.00084 M sodium pyruvate and a suitable dilution of enzyme (Wroblewski and La Due, 1955). Samples were also assayed against α-oxobutyrate (0.0033 M) as substrate (Rosalki and Wilkinson, 1960). These measurements were made with a Beckman Model B spectrophotometer at *p*H 7.4 and 30°. Enzyme activity was measured as international units/liter.

RESULTS

Typical zymograms of a ruminant tissue are shown in Figure 1 with the corresponding percentage distributions of the isoenzymes in Table I. These results are intended to illustrate the methodology of these investigations.

The percentages of B-type activity and the pyruvate-

TABLE I

THE PROGRESSION OF LACTATE DEHYDROGENASE IN BOVINE LIVER

Age[a]	Percentage of Total Activity				
	A_0B_4	A_1B_3	A_2B_2	A_3B_1	A_4B_0
Prenatal					
5	6	30	36	24	4
10	9	28	36	20	7
18	14	26	32	21	7
27	20	38	28	12	2
39	20	32	32	16	0
65	27	43	27	3	0
74	33	46	19	2	0
Postnatal					
3 years	56	38	6	0	0

[a] Prenatal age is expressed in terms of crown-rump length (cm).

TABLE II
CHANGE IN SUBSTRATE-ACTIVITY RATIOS DURING DEVELOPMENT[a]

Bovine				Ovine			
Age[b]	Muscle	Liver	Heart	Age[b]	Muscle	Liver	Heart
5	2.0	2.4	2.2	2.0	1.9		
10	2.3	2.3	2.1	3.5	2.0	2.1	
19	2.7	2.3	2.1	5.0	2.2	2.2	2.0
28	3.1	2.1	2.0	6.5	2.7	2.2	2.0
39	3.7	2.2	2.1	9.0	2.9	2.2	2.0
50	4.0	2.1	2.0	15	3.0	2.0	2.0
65	4.0	2.0	1.9	24	3.5	1.8	1.9
74	4.2	1.9	1.9	33	3.8	1.8	1.8

[a] Relative activities against pyruvate and α-oxobutyrate (Plummer *et al.*, 1963). [b] Prenatal age is expressed in terms of crown-rump length (cm).

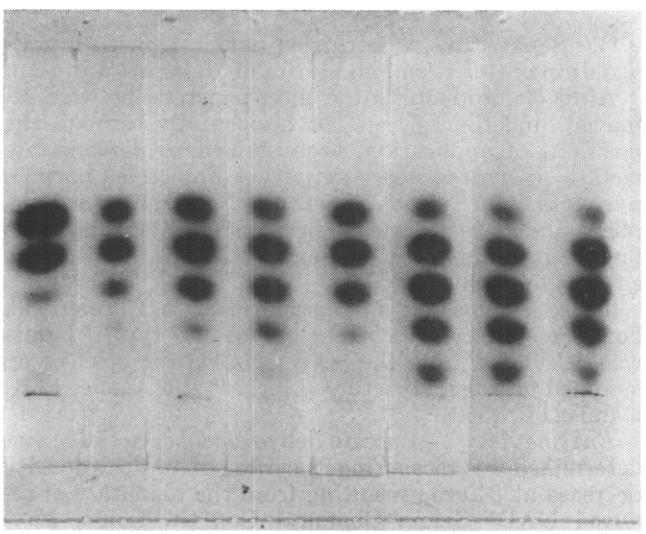

FIG. 1.—Starch-gel electrophoresis zymograms, stained to reveal the multiple forms of lactate dehydrogenase in bovine liver at different developmental stages. Tissue sources are identical with and in the same order as those listed in Table I. Youngest fetal tissue is on the right, adult tissue on the left. Maximum migration distance approximately 4 cm.

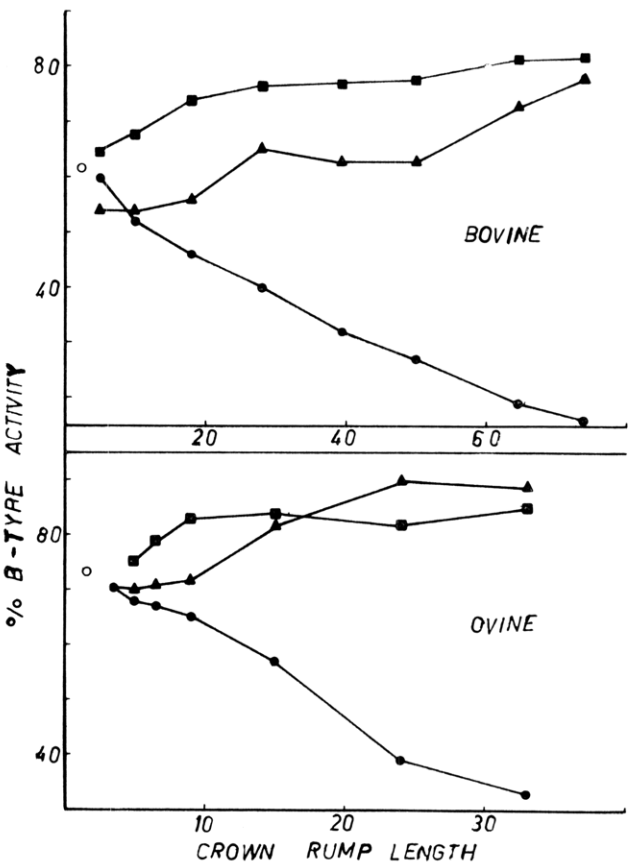

FIG. 2.—Change in ruminant lactate dehydrogenase during development. ■, heart; ▲, liver; ●, muscle; O, whole embryo.

oxobutyrate ratios for heart, liver, and muscle are given in Figure 2 and Table II. Percentages of B-type activity have been calculated in order to clarify the directions of the shifts in isoenzyme activity. No correction was made for differences in specific activity of the parental types because these values were not available for ruminant lactate dehydrogenase. Also, this correction would not affect the direction of the progression.

The purpose of assaying the tissue homogenates against both pyruvate and α-oxobutyrate was to provide a complementary index of isoenzyme composition based on the different relative rates of reaction against these substrates (Plummer *et al.*, 1963).

From the results of these experiments (Figs. 1 and 2; Tables I and II) it is apparent that sequential alterations occur in the pattern of lactate dehydrogenase isoenzymes during fetal and infant development of the ruminant. The direction and extent of these changes vary in different tissues, but, in general, corresponding ovine and bovine tissues exhibit very similar responses.

In the case of skeletal muscle (Fig. 2, Table II), the redistribution of multiple forms proceeds from an early fetal composition which is mainly anodal (B type) toward the predominantly cathodal distribution characteristic of the late fetus. This developmental progression in muscle is more marked than in the other tissues studied, and advances toward a different parental type. With liver and heart, increase in the age of the fetus is accompanied by an increased percentage of B-type units (Figs. 1 and 2; Tables I and II).

In two instances where the embryo was very small, the specimen was homogenized *in toto*, and analyzed An ovine fetus of 1.2 cm crown-rump length gave a pyruvate-oxobutyrate ratio of 2.0 and a calculated 74% of B-type activity; a bovine fetus of 2.0 cm length was estimated to possess 62% B-type activity and a value of 2.1 for the relative substrate-activity ratio.

A distinctive feature of the results in the convergence of the isoenzyme parameters from different tissues toward values which are common to each individual species (Fig. 2, Table II). This common range of initial values is incontiguous with either parental type of enzyme, early zymograms of bovine tissues displaying five zones of activity (Fig. 1) while the initial ovine tissues show three main bands.

Discussion

It has recently been demonstrated that the early embryonic form of lactate dehydrogenase occurs in some vertebrates as a pure parental type (Cahn *et al.*, 1962; Markert and Ursprung, 1962; Fine *et al.*, 1963; Lindsay, 1963). This was established by determining the isoenzyme composition of the young fetus, and confirmed by detailing the direction of fetal progressions in individual tissues. Such combined evidence would appear to be unequivocal.

On occasion, however, the isoenzyme constitution of lactate dehydrogenase in the germinal embryo has been predicted solely on the basis of late-fetal and infant analysis (Fine *et al.*, 1963). The data in this paper emphasize the inaccuracies inherent in this approach.

First, in both the animals studied, the lactate dehydrogenase progressions in muscle proceed with a different inclination to those in liver and heart (Fig. 2; Table II). Whereas the embryonic form of bovine lactate dehydrogenase has been predicted as the A-type tetramer from the tendency of the isoenzyme shift in extramyoskeletal tissues (Fine *et al.*, 1963), a similar extrapolation on the basis of the muscle results would imply a B-type tetramer as the initial embryonic form. These deductions are obviously incompatible, and analysis of the early embryonic enzyme has confirmed that the activity is, in fact, predominantly B type (Fig. 2; Table II; Masters, 1964).

A second relevant observation is that it is difficult to accurately establish the direction of isoenzyme redistribution when a comprehensive range of tissue specimens is not examined. There are a number of examples in the literature where consideration of only a few late-fetal specimens provides a misrepresentation of the overall trend of isoenzyme shift (Vesell, *et al.*, 1962; Wiggert and Villee, 1962).

In summary, then, there is a necessity for caution in predicting the embryonic form of the enzyme from a nonrepresentative sampling of fetal tissues.

In addition, the data presented in this paper bring in question the assumption that the initial embryonic form of lactate dehydrogenase is necessarily a pure parental type (Figs. 1 and 2; Tables I and II). This conclusion was based on the available evidence (which was definitive in few instances) and the analogy with fetal hemoglobin (Markert and Ursprung, 1962; Fine *et al.*, 1963). But a consideration of the isoenzyme composition of the early ruminant fetus, the rates of redistribution of the multiple enzyme forms during gestation, and the convergence of results from different tissues (Fig. 2) calls attention to the possibility that the initial enzyme form may include representation of each type of subunit. With tissues from both the species studied, for example, the attainment of an initial parental form of the enzyme would necessitate a dramatic reapportionment of the multiple forms (Fig. 2). Such a rapid initial change would appear to be inconsistent with the available literature on developmental progressions, and uncharacteristic of the initiation of derepression (Cahn *et al.*, 1962; Markert and Ursprung, 1962; Fine *et al.*, 1963).

Furthermore, the analogy with hemoglobin synthesis during gestation appears to be inapposite. There are manifest differences between the compensatory synthesis characteristic of the hemoglobins, and the independent regulation of synthesis exhibited by the lactate dehydrogenase subunits (Dawson *et al.*, 1964; Zuckerkandl, 1964).

In view of these facts, then, it is suggested that both the genes responsible for the synthesis of lactate dehydrogenase (subunits A and B) are functional in the newly fertilized ova of some species. Instead of the general occurrence of a single parental form of the embryonic enzyme, it is suggested that different species may have either of the two types of activity, or both types, during early development. Possibly the nature of the initial enzymic form is related to the environment during the gestation period (N. O. Kaplan, personal communication). While the subsequent developmental progression of lactate dehydrogenase may still be visualized as an expression of cellular differentiation correlated with enzyme repression and derepression and the different functional significance of the multiple-enzyme forms (Markert, 1963b; Dawson *et al.*, 1964), it is evident that much detailed investigation is necessary in order to ascertain the exact nature of interspecies variation with regard to these enzymic changes during gestation.

References

Cahn, R., Kaplan, N. O., Levine, L., and Zwilling, E. (1962), *Science 136*, 962.

Dawson, M. D., Goodfriend, T. L., and Kaplan, N. O. (1964), *Science 143*, 929.

Fine, I. H., Kaplan, N. O., and Kuftinec, D. (1963), *Biochemistry 2*, 116.

Helm, H. J. van der, Zondag, H. A., Hartog, H. A. Ph., and Kooi, M. W. van der (1962), *Clin. Chim. Acta 7*, 540.

Hinks, M., and Masters, C. J. (1964), *Australian J. Biol. Sci. 17* (in press).

Lindsay, D. T. (1963), *J. Exptl. Zool. 152*, 75.

Markert, C. L. (1963a), *Science 140*, 329.

Markert, C. L. (1963b), *Symp. Soc. Study Develop. Growth 21* (1962), 65.

Markert, C. L., and Apella, E. (1961), *Ann. N. Y. Acad. Sci. 94*, 678.

Markert, C. L., and Møller, F. (1959), *Proc. Natl. Acad. Sci. U. S. 45*, 753.

Markert, C. L., and Ursprung, H. (1962), *Develop. Biol. 5*, 363.

Masters, C. J. (1963), *Australian J. Biol. Sci. 16*, 709.

Masters, C. J. (1964), *Biochim. Biophys. Acta 89*, 161.

Plagemann, P. G. W., Gregory, K. F., and Wroblewski, F. (1960), *J. Biol. Chem. 235*, 2282.

Plummer, D. T., Elliott, B. A., Cooke, K. B., and Wilkinson, J. H. (1963), *Biochem. J. 87*, 416.

Rosalki, S. B., and Wilkinson, J. H. (1960), *Nature 188*, 1110.

Smith, C. H., and Kissane, J. M. (1963), *Develop. Biol. 8*, 151.

Vesell, E. S., Phillip, J., and Bearn, A. G. (1962), *J. Exptl. Med. 116*, 797.

Wiggert, B., and Villee, C. A. (1962), *Science 138*, 509.

Wroblewski, F., and La Due, J. S. (1955), *Proc. Soc. Exptl. Biol. Med. 90*, 210.

Zinkham, W. H., Blanco, A., and Kupchyk, L. (1964), *Science 144*, 1353.

Zuckerkandl, E. (1964), *J. Mol. Biol. 8*, 128.