Lactate Dehydrogenase in the Rat Mammary Gland (34590)

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Lactate dehydrogenase (LD) consists of five isoenzymes (1–3), each a tetramer made up of two protein molecules "M" and "H" in the following combinations: HHHH (LD_1), MHHH (LD_2), MMHH (LD_3), MMMH (LD_4), MMMM (LD_5). The isoenzymes have different kinetic properties, hence each might play a distinct metabolic role (4–6). Differences in molecular weight, electrical charge, and spatial configuration are reflected by differences in electrophoretic mobility.

Supporting evidence has been presented by Karlsson (7) for his earlier hypothesis that the changes in the relative isoenzyme activities of mammary gland might be used as naturally occurring biochemical indicators of the hormonal induction of differentiated cell lines. Mammary glands of virgin and early pregnant rats reportedly lack LD_5 activity (7). From midpregnancy to peak lactation, LD_5 shows the greatest increases; LD_4, LD_3, and LD_2 increase in progressively smaller amounts while LD_1 changes very little.

Limited information is available on the LD isoenzyme forms in rat mammary gland. The hypothesis by Karlsson requires further validation, and the relative proportions of type "M" and "H" proteins should be investigated. No direct evidence for the location of LD in rat mammary tissue is available.

Methods and Materials. Enzyme preparation. Virgin and primiparous Holtzman rats were used. Those 5–10 weeks old with their vagina closed were listed as prepubertal, those with the vagina open were listed as pubertal. The abdominal–inguinal mammary glands were removed and carefully trimmed to remove lymph nodes and as much of the fat as possible. The glands were weighed, minced, and placed immediately in ground glass homogenizers containing cold 0.05 *M* Tris chloride buffer at pH 7.5. The buffer was approximately 10 times the volume of the tissue. An aliquot of the homogenate was centrifuged at 4° for 1 hr at approximately 31,000*g* in an IEC model PR-2 centrifuge with angle head No. 295. The supernatant fluid was carefully aspirated with a Pasteur pipette to avoid contamination by milk fat, which is present in the parturient and lactating glands.

Lactic acid dehydrogenase activity. Total activity in 0.1 ml of supernatant fluid was estimated by the rate of oxidation of NADH to NAD, as pyruvate is reduced to lactate by NADH catalyzed by LD. The reaction was carried out in a quartz cuvette in 3 ml of phosphate buffer at pH 7.4 at 25°. The disappearance of NADH per unit time was measured by decreased absorption at 340 mμ (4). A unit of activity is defined as the amount of LD that changes the optical density of NADH by 0.001 at 340 mμ in 1 min (4).

Isoenzyme determination. Aliquots of the supernatant fluid were diluted to equal specific activities by the addition of 20 % sucrose solution in 0.05 *M* Tris chloride buffer at pH 7.5. One drop of 0.1 % bromphenol blue was then added to each sample so the accuracy of placing an aliquot in the slot of the gel could be assessed. Aliquots of 10 or 20 μl were placed in the respective 1.0-cm slots in the acrylamide gel. The E-C Vertical Gel Electrophoresis specifications (8) were employed with the following minor alterations: (1) the electrophoresis cell used was described by Raymond (9) and modified by Anderson (10); (2) 7 % Cyanogum-41

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gel (Fisher) was used, but the Tris–Na_2 EDTA-boric acid buffer was at pH 9.3 instead of pH 9.6, (3) the gel slab was incubated in at least 100 ml of medium which was 0.3 *M* Tris chloride buffer at pH 7.4 with the following additions per 100 ml, NAD, 40 mg; DL-lactate-480 mg; 3 (4.5 dimethyl thiazolyl 1-2) 2, 5 diphenyl tetrazolium bromide (MTT), 45 mg; and phenazine methasulfate, 3.5 mg. The gel was then washed with 5 % acetic acid until clear, and photographed.

A densitogram for each sample was recorded in an E-C densitometer (E-C Apparatus Co. Ltd.). The total area under the five peaks was summed. The area under a particular peak, expressed as a percentage of the total area, was used as an index of the proportion of the total activity attributable to that particular isoenzyme. The percentage of the total activity attributable to each isoenzyme was compared to the theoretical binomial distribution, and estimates of the relative proportions of "M" and "H" present were made. It is recognized that this procedure for evaluating isoenzyme activity has limited accuracy (5), and correction factors are recommended for registering absolute activities (7). In this investigation the relative percentages fit the binomial distribution well, hence use of correction factors is not necessary for the interpretation of the results. For mammary gland tissue, aliquots containing approximately equal amounts of activity were loaded onto the gel for each tissue to avoid bias from this source.

Histochemistry. Fresh tissue was placed in fixative of 4% formaldehyde, 0.9% sodium chloride, 1% calcium chloride in 0.3 *M* Tris chloride buffer at pH 7.4 at 4° for approximately 2 hr. Sections 20–30 μ thick were cut on a freezing microtome and were floated off the microtome blade onto microscope slides with cold 0.3 *M* Tris chloride buffer at pH 7.4. The buffer was blotted off, and several drops of the medium used for gel incubation were placed directly on the tissue. The preparation was incubated for 7 – 15 min at 37° in the dark, since the medium is light-sensitive. Excess medium was blotted, and the preparation was photographed without the application of coverslips.

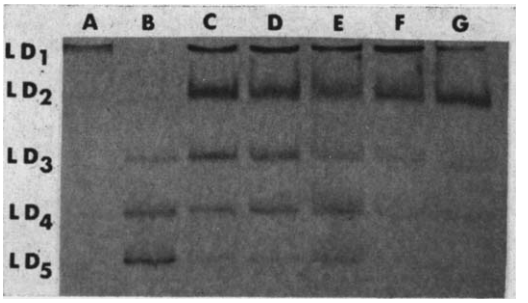

FIG. 1. Rat mammary gland lactate dehydrogenase isoenzymes. A. Thigh muscle 90 (units activity per slot), 719 (units activity per miligram wet tissue), 10 (volume in microliters of homogenate placed in the slot). B. Heart ventricle muscle 92, 619, 10. C. Prepubertal 185, 24, 20. D. Day of breeding 180, 19, 20. E., Day 6 of pregnancy 181, 22, 20. F. Parturient 186, 44, 20. G. Day 11 of lactation 188, 75, 20.

Results and Discussion. All five isoenzymes are present in the rat mammary gland at all the stages of development studied, which included prepuberty to peak of lactation (Fig. 1). This is in contrast to Karlsson's (7) conclusions, but his reported data on the virgin and pregnant animals do not fit the binomial distribution very well. They do, in fact, more closely fit a distribution that indicates LD_5 activity.

Total LD activity per milligram wet weight increased approximately 4- to 5-fold from puberty to Day 15 of lactation (Fig. 2). No correction of total LD activity per unit weight of wet tissue was made for the milk present in the lactating gland. The increase in LD activity in the gland followed a

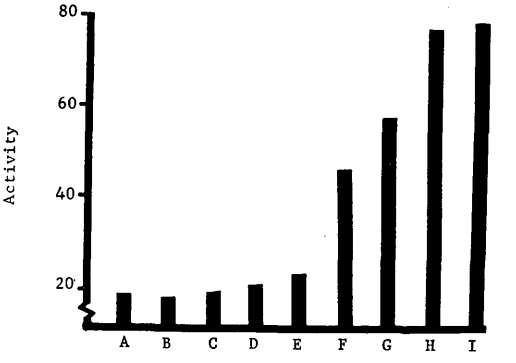

FIG. 2. Rat mammary gland lactate dehydrogenase activity. A. Weaned; B. Prepubertal; C. Pubertal; D. Breeding; E. 6th day of Pregnancy; F. Day of Parturition (P); G. P + 5; H. P + 10; I. P + 15.

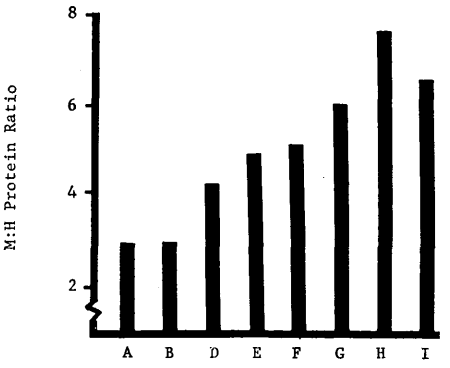

FIG. 3. Ratio of type "M" to "H" proteins at different stages of development. A. Weaned; B. Prepubertal; C. Pubertal; D. Breeding; E. Day 6 of pregnancy; F. Day of parturition (P); G. P + 5; H. P + 10; I. P + 15.

very similar pattern to other enzymes (11, 12) and increased in the same proportion as many others (11).

Assuming no change in the rate of turnover of "M" and "H" and no change in selective inhibitors (13) with stages of development, the relative percentages of the total activity attributable to each isoenzyme compared to the binomial distribution allow an assessment of the relative activity of the two genes that control "M" and "H" synthesis. In contrast to Karlsson's conclusions (7), gene "M" is most active at all stages of development (Fig. 3), hence isoenzymes having a predominance of "M" protein are in the majority at all times. The activity of gene "M" increases more rapidly than that of gene "H." The relative activities shift from approximately 3 to 1 during prepuberty to approximately 7 to 1 on Days 10 and 15 of lactation. This supports previous evidence that LD_5 shows the greatest increase (7) from breeding to midlactation.

LD activity in the mammary gland is predominantly in the parenchymal tissue and fat droplets (Fig. 4). There are a few fat droplets on the connective tissue of slide A, but these may have drifted there during preparation as they can be easily disturbed by fluid currents over the tissue. Figure 4 supports recent evidence (13) that LD activity in milk is associated with the milk fat globule. Acetone washing for 1–2 min as a prefixative remove most of the large globules evident in slide B (Fig. 4) while a reduced amount of LD activity is displayed in the parenchymal cells. It is not known whether the LD is associated with fat droplets as they are being formed in the cell or whether its activity is more diffuse through the entire cell.

There is a high correlation between the predominant molecular composition of LD and the relative degree of oxygen uptake by a particular cell type throughout the biological kingdom (14). LD_1 is the tetramer that predominates in tissues that have an aerobic environment, such as heart muscle; LD_5 is the tetramer that is found where anaerobic conditions occur, but glycolysis remains important, such as in skeletal muscle. The LD_5 form is not inhibited by rapid increases in the level of pyruvate or lactate, hence allowing continued reoxidation of NADH to NAD

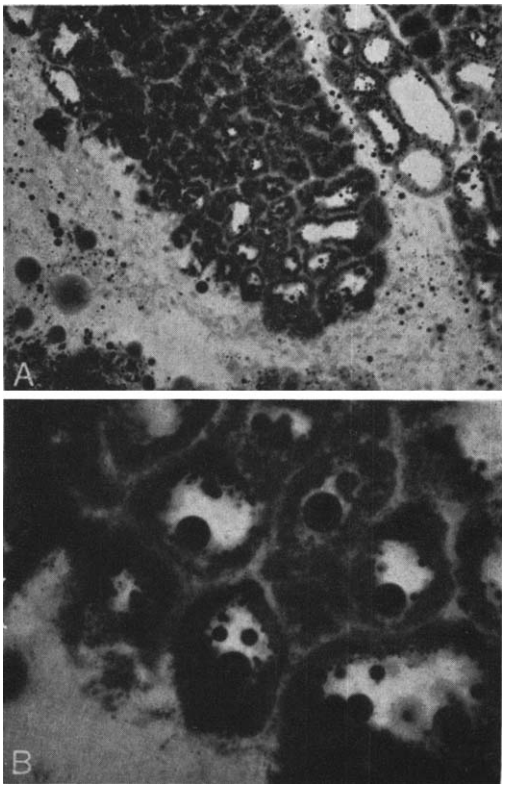

FIG. 4. Lactate dehydrogenase in rat mammary gland at Day 8 of lactation using a histochemical procedure. A, ×77; B, ×350.

to support glycolysis even under relatively anaerobic conditions (14, 15).

If the correlation between oxygen availability and type "M" protein predominance is accepted for the mammary gland, it appears that relatively anaerobic-type metabolism occurs or predominates in the rat mammary gland at the peak of lactation. At the very least, it suggests that a system that could cope with anaerobic conditions has been developed in some, if not all cells. The system is likely to be present in all cells as the isoenzyme distribution fits the expected binomial distribution very well.

Summary. The localization of lactate dehydrogenase activity in the epithelial cells and fat globules of the rat mammary gland is shown. All LD isoenzymes are present in the mammary gland at all stages of development, but their proportions change. As development progresses LD_5 shows the greatest increase in activity, LD_4, LD_3, and LD_2 increase in progressively smaller amounts, while LD_1 changes very little. The use of the relative amounts of type "M" and "H" proteins present is suggested as an index of the degree of differentiation of the mammary gland. The shift in the proportions of the isoenzymes is a further clue to controlling mechanisms of the mammary gland.

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