

# HISTOCHEMISTRY OF LACTIC DEHYDROGENASE IN HEART AND PECTORALIS MUSCLES OF RAT

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## ABSTRACT

Left-ventricular heart muscle and pectoralis major muscle of the rat were studied to determine the intracellular localization of lactic dehydrogenase (LDH) isoenzymes. Fixation of tissue for 2 hr in 2% buffered formaldehyde provided the best preservation of the ultrastructure and enzyme activity. Total LDH activity was found diffusely in the ground substance of the sarcoplasm and in the mitochondria of the heart muscle. In skeletal muscle a strong reaction was noted in the sarcoplasmic reticulum, and moderate activity was seen in the ground substance of the sarcoplasm and in the mitochondria. Differentiation of the isoenzymes of LDH was accomplished by addition of 4 M urea or application of heat. Heart-type isoenzymes were mainly localized in the mitochondria and sarcoplasm, whereas muscle-type isoenzymes were localized mainly in the sarcoplasmic reticulum of the skeletal muscle. It is speculated that the sarcoplasmic reticulum of the skeletal muscle is the site of anaerobic glycolysis and that the sarcoplasm and mitochondria are involved primarily in aerobic metabolism of pyruvate.

## INTRODUCTION

Light microscope histochemical studies of the localization of lactic dehydrogenase<sup>1</sup> (LDH) activity have suggested that the enzyme in myocardium and red skeletal muscle is localized in mitochondria (30, 46), and that in white skeletal muscle it is localized in sarcoplasmic reticulum (11). Fahimi and Karnovsky (12) reported the ultrastructural localization of LDH activity in white skeletal muscle to be in the sarcoplasmic reticulum.

Since heart and skeletal muscle differ in the

pattern of LDH isoenzymes and subcellular architecture, it is conceivable that isoenzymes of LDH have specific subcellular localization. The present study reports the localization of heart- and muscle-type LDH isoenzymes (H-LDH and M-LDH, respectively) in rat myocardium and skeletal muscle.

## MATERIALS AND METHODS

### *Animals*

The experiments were carried out on male rats of the Sprague-Dawley strain, weighing 200–300 g.

### *Fixatives*

The following fixatives were examined for their preservation of total LDH activity: 0.3 M (2%) glyoxal in 0.2 M phosphate buffer, pH 6.5 for 2 hr;

<sup>1</sup>*Abbreviations:* DMF, dimethylformamide; DMSO, dimethyl sulfoxide; H-LDH, heart-type lactic dehydrogenase; LDH, lactic dehydrogenase; M-LDH, muscle-type lactic dehydrogenase; NAD, nicotinamide adenine dinucleotide; NBT, nitro-blue tetrazolium salt; PMS, phenazine methosulfate; TC-NBT, thiocarbonyl nitro-blue tetrazolium salt.

0.2 M (2%) glutaraldehyde (41) in 0.1 M phosphate buffer, pH 7.4 for 30 min; a mixture of 0.6 M (2%) formaldehyde and 0.2 M (2%) glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4 for 30 min; and 0.65 M (2%) formaldehyde with 90 mM (1%)  $\text{CaCl}_2$  in 0.05 M cacodylate buffer, pH 7.4 for 2 or 24 hr. 0.2 M sucrose was added to all of the above fixatives. Formaldehyde was freshly prepared by heat depolymerization of paraformaldehyde (32).

### *Preparation of the Tissue*

The rats were killed by decapitation for examination of fresh tissue. For examination of fixed tissue, the upper half of the body was perfused with cold (4°C) fixative through the aorta in a retrograde direction for 10 min. Tissue blocks measuring 1–3 ml were taken from the left-ventricular myocardium and deep and superficial layers of the pectoralis major muscle and were transferred to fresh cold fixative. After 2 hr of fixation in the cold, tissues were washed for 20 hr in 0.05 M cacodylate buffer (pH 7.2) containing 0.3 M sucrose solution and 1%  $\text{CaCl}_2$  at 4°C. The tissue blocks were placed on a metal chuck, deep frozen in liquid nitrogen, and placed in a cryostat. Frozen sections for light microscope histochemistry were cut at the thickness of 10  $\mu$ , mounted on albumin-coated glass slides, incubated in the reaction medium, washed in 0.05 M cacodylate buffer (pH 7.2) containing 0.3 M sucrose, and mounted in glycerin jelly.

For electron microscope histochemistry, tissue was transferred to 7% aqueous solution of dimethyl sulfoxide (DMSO) for 20 min at 4°C, rapidly frozen in liquid nitrogen, and frozen sections were cut at a thickness of 40  $\mu$ . Sections were thawed in 7% DMSO and transferred to a preincubation medium. In some experiments 50  $\mu$  sections were obtained with a Smith-Farquhar TC-2 tissue sectioner.

### *Reaction Medium*

**TOTAL LDH ACTIVITY:** The reaction medium consisted of 100 mM L-lactate, 25% (3 mM) dextran (Sigma Chemical Co., St. Louis, Mo.), 18 mM nicotinamide adenine dinucleotide (NAD) in 0.05 M sodium cacodylate buffer, pH 7.2. 0.61 mM nitro-blue tetrazolium salt (NBT) and 0.10 mM phenazine methosulfate (PMS) were added for light microscope histochemistry. 0.50 mM thiocarbonyl nitro blue tetrazolium salt (Polysciences, Inc., Warrington, Pa.) (TC-NBT) (42) with or without 0.10 mM PMS was used for electron microscope histochemistry. The reaction was carried out in the dark at room temperature with constant agitation for 10–40 min. Preincubation medium was the incubation medium less L-lactate and NAD.

### *Selective Stains for Isoenzymes*

Selective staining of H- and M-LDH was based upon the urea sensitivity of M-LDH (41), heat lability of M-LDH (34, 37), and inhibition of H-LDH with pyruvate (36). In practice we adapted a histochemical method of McMillan (28); 4 M urea was added to the reaction medium for inhibition of M-LDH, and a combination of 8 mM pyruvate and 80 mM lactate was used to inhibit H-LDH. In order to inhibit heat-labile M-LDH, some sections were preheated in 0.05 M cacodylate buffer (pH 7.2) containing 0.3 M sucrose solution at 60°C for 30 min before incubation (48).

### *Electron Microscopy*

After incubation in the reaction medium, the sections were washed briefly in distilled water and then treated with 10%  $\text{OsO}_4$  in distilled water at 60°C for 40 min in darkness. The sections were washed quickly in distilled water, dehydrated in increasing concentrations of ethanol, and embedded in DER mixture (26). Silver to light gold-colored sections were cut with glass knives, mounted on uncoated 300-mesh copper grids, and stained with saturated ethanolic (70%) solution of uranyl acetate for 10 min and the stock solution of lead citrate (39) for 3 min. Electron micrographs were obtained in a Hitachi HU 11 B electron microscope at an acceleration voltage of 75 kv.

### *Biochemical Analysis of Fixed and Unfixed Muscle Homogenate*

Both unfixed and fixed heart and pectoralis muscles were studied. Fixation was performed with formaldehyde by the same technique used for histochemistry. Samples of left-ventricular cardiac muscle and pectoralis major muscle measuring approximately 1 mm in diameter were washed for 20 hr in cacodylate buffer 0.3 M sucrose at 4°C. In the cold, tissue was homogenized and centrifuged at 10,000 *g* for 10 min. One-half of the supernatant from unfixed and fixed muscle homogenates was frozen in liquid nitrogen and quickly thawed before biochemical assay. The other half of the supernatant was assayed without freezing. Total tissue LDH activity was determined by the method of Wroblewski and LaDue (49). Electrophoretic separation of tissue isoenzymes was performed in the polyacrylamide (9) and agarose gels (35). LDH enzyme activities in the agarose gel were quantitated fluorometrically in a Turner fluorometer, (G. K. Turner Associates, Palo Alto, Calif.) measuring  $\text{NADH}_2$  produced by conversion of L-lactate to pyruvate (35). Polyacrylamide gel discs (9) were visually stained in the histochemical staining medium as previously described, and LDH activities were quantitated in a Gilford 240 spectrophotometer

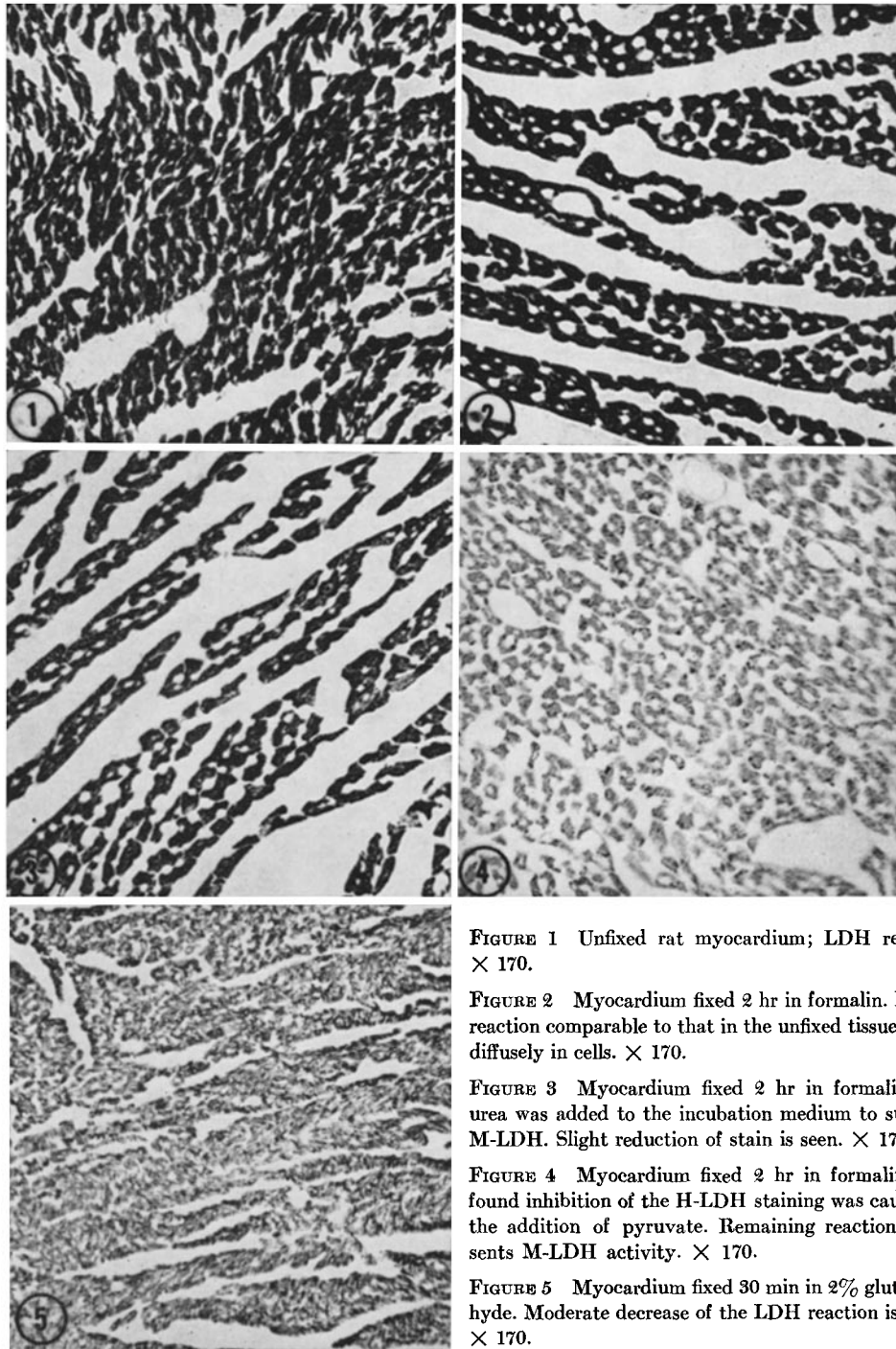


FIGURE 1 Unfixed rat myocardium; LDH reaction.  $\times 170$ .

FIGURE 2 Myocardium fixed 2 hr in formalin. Intense reaction comparable to that in the unfixed tissue is seen diffusely in cells.  $\times 170$ .

FIGURE 3 Myocardium fixed 2 hr in formalin. 4 M urea was added to the incubation medium to suppress M-LDH. Slight reduction of stain is seen.  $\times 170$ .

FIGURE 4 Myocardium fixed 2 hr in formalin. Profound inhibition of the H-LDH staining was caused by the addition of pyruvate. Remaining reaction represents M-LDH activity.  $\times 170$ .

FIGURE 5 Myocardium fixed 30 min in 2% glutaraldehyde. Moderate decrease of the LDH reaction is noted.  $\times 170$ .

(Gilford Instruments Labs., Inc., Oberlin, Ohio) with a linear transport attachment at a wavelength of 570 m $\mu$ .

## RESULTS

### *Light Microscope Observation*

**HEART MUSCLE:** The effect of fixation on total LDH activity was studied in the light microscope. Frozen sections of unfixed myocardium showed deep staining of the muscle fibers (Fig. 1). After fixation in glyoxal, glutaraldehyde, and glutaraldehyde-formaldehyde mixture, LDH activity was markedly inhibited (Fig. 5). 2 hr fixation in 2% formaldehyde provided the best preservation of LDH activity, and the intensity of staining was comparable to that seen in unfixed myocardium (Fig. 2).

Inhibition of M-LDH by preheating of sections or addition of 4 M urea to the reaction medium caused only slight reduction of the staining in myocardium due to the large amount of H-LDH in this tissue (Fig. 3). Addition of pyruvate caused profound inhibition of the H-LDH staining (Fig. 4). When the substrate (L-lactate) was omitted from the reaction medium, there was no reaction noted, indicating the absence of nonspecific reduction of the tetrazolium in this histochemical system.

**PECTORALIS MAJOR MUSCLE:** Since heart muscle contains little LDH-5, a skeletal muscle was selected for the study of M-LDH. The rat pectoralis major muscle consisted of two layers that were grossly recognizable: a superficial portion (approximately one-fourth) being distinctly white, and an inner part being mostly reddish. In regular electron micrographs the superficial portion consisted mostly of type II muscle fibers,<sup>2</sup> while the inner part consisted of an admixture of both type I and II fibers. A previous biochemical study showed that rat pectoralis muscle as a whole is red muscle containing all of the five LDH isoenzymes (3).

When pyruvate was added to the reaction medium, the LDH activity of the pectoralis

muscle was moderately reduced, indicating a moderate amount of H-LDH. When 4 M urea was added, the degree of inhibition was much greater. Similar inhibitory effects were noted when the sections were heated before incubation.

### *Biochemical Analysis of Fixed and Unfixed Muscle Homogenates*

The purpose of the biochemical study was to observe the effect of fixation and frozen-sectioning on LDH activity and isoenzyme distribution. The effect of 2 hr formaldehyde fixation on total LDH activity of heart and skeletal muscles is found in Table I. Changes in the electrophoretic separation pattern are shown in Figs. 6 and 7. Formalin fixation caused a moderate reduction of total LDH activity. Fixation also changed the electrophoretic pattern of LDH, and the effects were more pronounced on M-LDH. Greater degrees of formaldehyde inactivation of enzyme activity were noted in LDH-3, -4, and -5 than in LDH-1 and -2. A lack of clear separation of LDH-4 and -5 was noted in the fixed tissue; a single peak was seen in the region of LDH-4 and -5. Rapid freeze-thawing caused a small increase of the total LDH activity of the heart homogenates, and slight changes in isoenzyme distribution. Although total LDH activity was reduced by formaldehyde, the amount of enzyme activity remaining in fixed tissue was sufficient for histochemical demonstration of LDH activity. We conclude that frozen sections of formalin-fixed tissue can be used for demonstration of total LDH activity and histochemical differentiation of H- and M-LDH type isoenzymes.

We felt it necessary to exclude the possibility that formaldehyde fixation may change the chemical structure of LDH and the urea sensitivity of the isoenzymes which is the basis for the histochemical technique in this experiment. LDH isoenzymes in fresh and fixed tissue homogenates were electrophoretically separated in polyacrylamide gel, and the enzyme activities were demonstrated by the light microscope stain with or without 4 M urea (Fig. 8). In fresh heart muscle homogenate only, an extremely faint band of LDH-5 was demonstrated. Moderate urea inhibition was noted on LDH-4 and -5 in the fresh homogenate. In the fixed tissue homogenate, LDH-3, -4, and -5 were totally inhibited with urea. It may be concluded that histochemi-

<sup>2</sup>Type I fibers correspond to red muscle fibers, as described by Gauthier and Padykula (16), and type II fibers to white muscle fibers. Because the colors red and white are used for gross description of the muscle tissue, the terms type I and type II fibers are used to describe individual muscle fibers according to Dubowitz and Pearse (10).

TABLE I  
Total LDH Activity\*

	Heart muscle		% of fresh tissue	Skeletal muscle		% of fresh tissue
	Fresh tissue	Fixed tissue		Fresh tissue	Fixed tissue	
Homogenate	21,800	7600	(36)	37,000	6400	(17)
Freeze-thawed homogenate	23,700	12,400	(50)	40,000	6900	(17)

\* International units per grams of wet tissue.

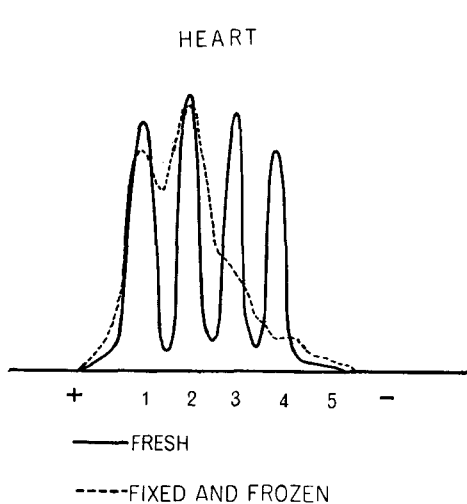


FIGURE 6 Electrophoretic pattern of rat myocardium LDH. Solid line indicates the pattern for fresh tissue homogenate. Dashed line indicates pattern for formalin-fixed and frozen-thawed tissue. Numbers indicate LDH isoenzymes. The direction of electrophoresis is shown by + (anode) and - (cathode). The height of two lines was arbitrarily set in order to compare the distributions of the isoenzymes before and after tissue manipulation. This figure does not permit comparison of absolute LDH activities in two different homogenates.

cally differentiable H- and M-LDH are LDH-1 and -2, and LDH-3, -4, and -5, respectively.

#### Electron Microscope Observations

HEART MUSCLE: Unfixed, frozen-sectioned myocardial tissue showed a considerable degree of degeneration of subcellular organelles. LDH reaction was seen only along the membranes of the mitochondrial cristae, and no cytoplasmic reaction was noted. Frozen sections of formalin-fixed (2%, 2 hr) tissue showed good preservation

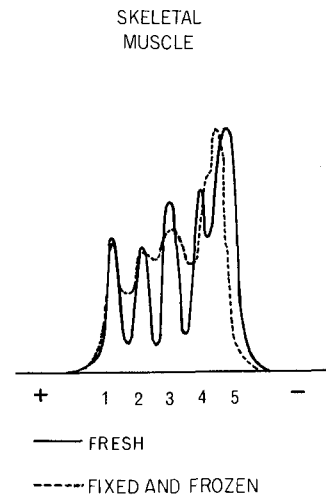


FIGURE 7 Electrophoretic pattern of rat pectoralis muscle LDH. Solid line indicates the pattern for fresh tissue homogenate. Dashed line indicates the pattern for formalin-fixed and frozen-thawed tissue. Numbers indicate LDH isoenzymes. The direction of electrophoresis is shown by + (anode) and - (cathode). The height of the lines was arbitrarily set in order to compare the distribution of isoenzymes before and after tissue manipulation. This figure does not show comparison of absolute LDH activities in two different homogenates.

of myofibrils and mitochondria (Fig. 9). The electron-opaque deposits marking the sites of total LDH activity were found in the sarcoplasmic ground substance, mitochondrial cristae (Figs. 9 and 11), and within the sarcoplasmic reticulum<sup>3</sup>

<sup>3</sup>The terms sarcoplasmic reticulum and T system are used according to Fawcett and McNutt (13). In certain areas T tubules are noted to run in the longitudinal direction (14) as shown in Fig. 12.

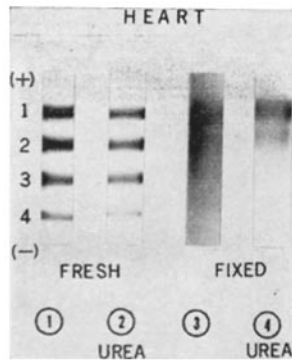


FIGURE 8 Electrophoretic pattern of rat myocardium LDH. Polyacrylamide gel was stained with NBT for LDH activity. The direction of electrophoresis is shown by + (anode) and - (cathode). LDH-5 band in the fresh homogenate (gel 1) was too faint to print. Note a distinct urea (4 M) inhibition of LDH-4 in the gel 2. Fixed tissue homogenate (gel 3) shows a predominance of LDH-1 and -2 and weak staining of LDH-3 and -4. Complete inhibition of LDH-3 and -4 is noted with 4 M urea (gel 4).

(Fig. 12). Quantitatively, the largest amount of reaction product was in the sarcoplasmic ground substance. Profiles of sarcoplasmic reticulum were sparse in the myocardium relative to skeletal muscle, and their paucity accounted for the small amount of LDH activity noticeable in the sarcoplasmic reticulum of the myocardium. Reaction product was not seen in the T system<sup>8</sup> or in the nucleus (Figs. 9 and 13). Absence of reaction in the nucleus (Fig. 13) and T system may indicate little diffusion of LDH. When 4 M urea was added to suppress M-LDH activity, a slight reduction in the amount of reaction product in the ground substance of the sarcoplasm was evident; the distribution of reaction deposits was unchanged (Fig. 10). Addition of pyruvate to the reaction medium resulted in an extremely weak histochemical reaction inadequate for electron microscope evaluation.

Light microscope observation of myocardium demonstrated that staining of total myocardial LDH activity was almost equally strong in fixed and unfixed frozen sections. This seems contradictory to the biochemical finding of a moderate reduction of enzyme activity in fixed tissue. The discrepancy probably is due to diffusion of non-mitochondrial, sarcoplasmic LDH from unfixed frozen sections. Electron microscope demonstration of a total absence of nonmitochondrial

sarcoplasmic LDH activity in the fresh preparation and presence of the enzyme activity in fixed tissue supports such an interpretation.

**PECTORALIS MUSCLE:** In contrast to myocardium, pectoralis major muscle showed more regularly oriented sarcomeres, more abundant sarcoplasm, and fewer and smaller mitochondria. In the type I muscle fibers the ground substance of the sarcoplasm showed diffusely distributed reaction product. In addition, concentration of reaction product was noted between the myofibrils, which was due to the deposition within the sarcoplasmic reticulum (Figs. 14 and 15). When 4 M urea was added to the reaction medium during incubation or when the tissue was heated before incubation, marked reduction of total LDH activity was noted. A strong reaction was noted to remain within the mitochondrial cristae (Fig. 16). In type II muscle fibers the reaction product was diffusely distributed throughout the ground substance of the sarcoplasm, and condensation of deposits was noted in the sarcoplasmic reticulum (Figs. 17 and 18). With addition of 4 M urea to the reaction medium there was a considerable reduction of LDH reaction with small but numerous deposits persisting in the sarcoplasmic ground substance and a striking reduction of reaction product in the sarcoplasmic reticulum (Fig. 19). Because of the marked reduction with urea or heat, the sarcoplasmic reticulum was considered to contain mainly LDH-4 and -5 (M-LDH) which are highly urea and heat sensitive (37, 40).

#### DISCUSSION

There are several problems in the cytochemical demonstration of LDH: diffusion of enzymes during tissue manipulation, inactivation of enzymes by fixation, and alteration of isoenzyme distribution by fixation or frozen-sectioning.

The importance of fixation in the histochemistry of LDH, which is a loosely attached, readily soluble enzyme, has been well discussed by Fahimi et al. (11, 12), who stated that a histochemical study of the localization of LDH in a fixed tissue preparation has an advantage over a biochemical cell fractionation study in which considerable diffusion and loss of loosely attached enzymes from particulate fractions can be expected. In the present study with formaldehyde-fixed tissue, no deposition of reaction product of LDH was noted in the nucleus or extracellular

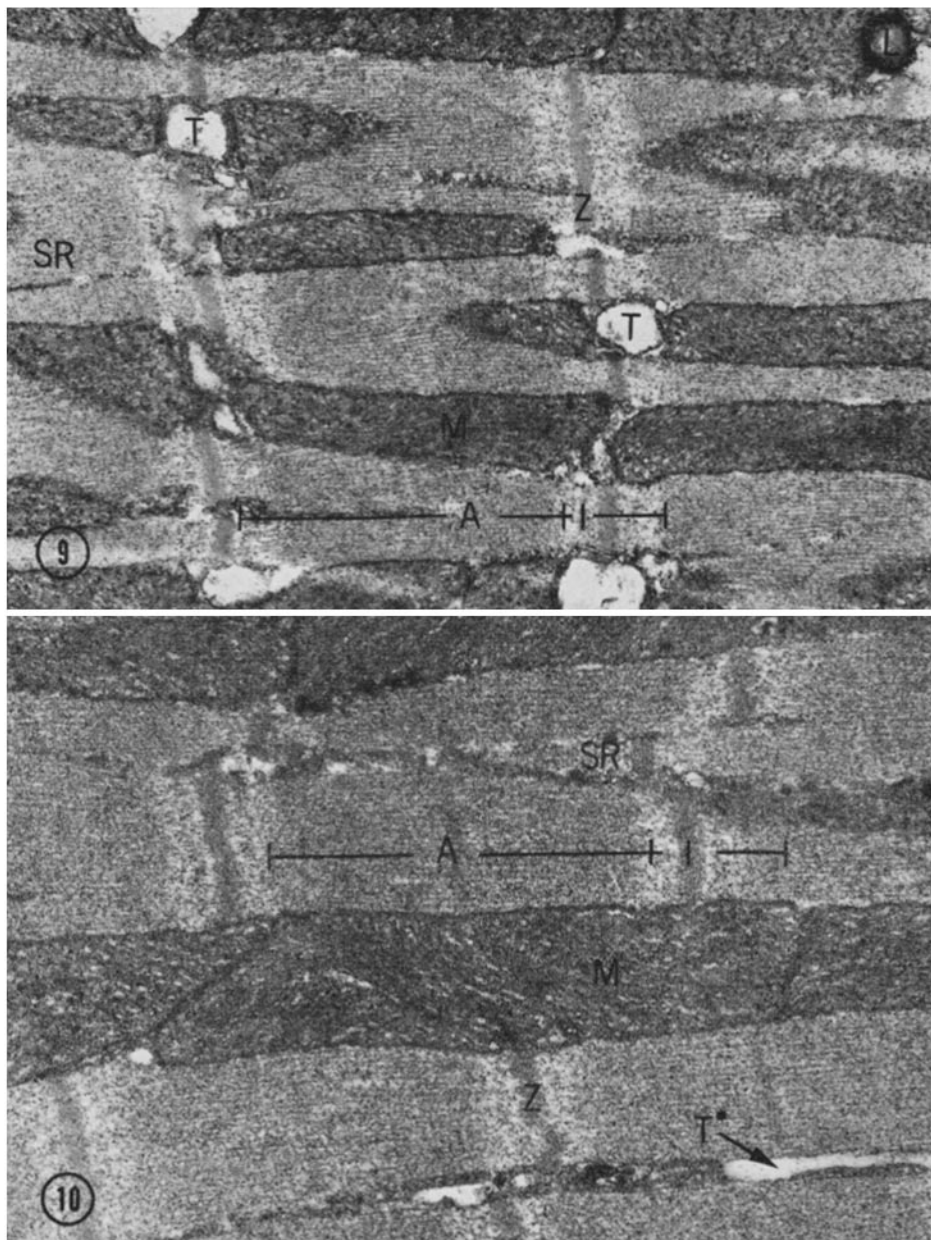
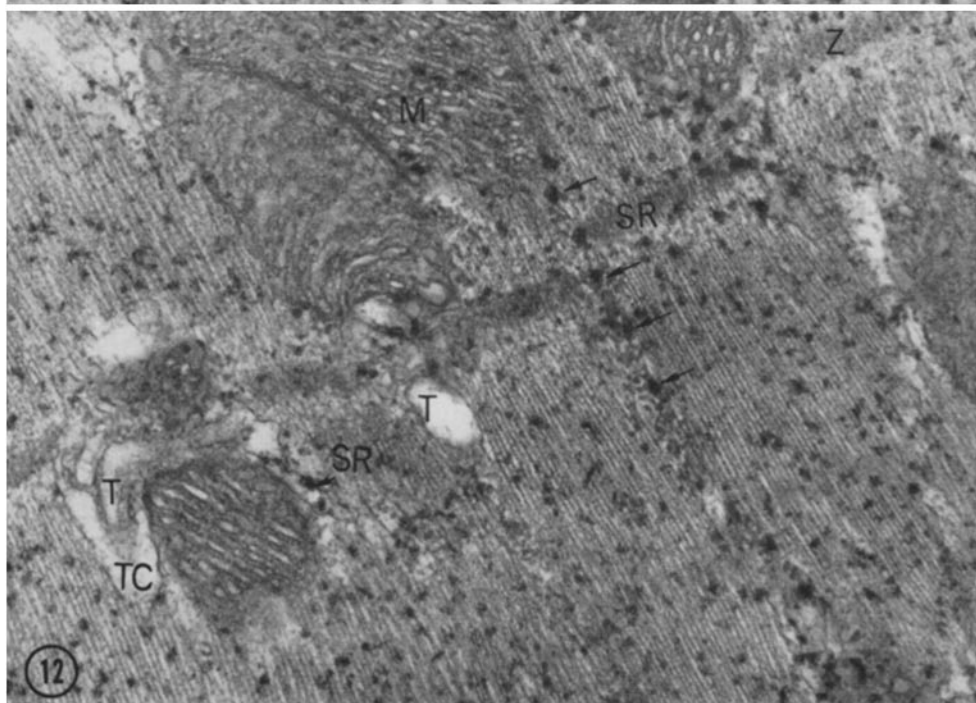


FIGURE 9 LDH reaction in formalin-fixed rat myocardium. There is a diffuse reaction in the sarcoplasm. Mitochondria (*M*) show some deposition of reaction products. Reaction products were deposited throughout the sarcoplasm. T tubules (*T*) are free of deposits. Some segments of the sarcoplasmic reticulum (*SR*) are positive for the reaction. *A*; A band; *I*; I band; *L*; lipid; *Z*; Z line.  $\times 19,500$ .

FIGURE 10 LDH reaction in formalin-fixed myocardium with 4 M urea. There is some reduction of the deposits in the ground substance of the sarcoplasm. The distribution of the reaction products was not grossly altered by heating. Faint staining is seen in the sarcoplasmic reticulum (*SR*). The reaction products present in this section indicate the activity of H-LDH. *A*, A band; *I*, I band; *M*, mitochondrion; *Z*, Z line. *T\**, longitudinal T tubules (14).  $\times 25,000$ .



**FIGURE 11** LDH reaction in the myocardium. Reaction products of LDH seen in the mitochondria are primarily located within the cristae (*c*). *m*, matrix.  $\times 93,000$ .

**FIGURE 12** LDH reaction in the myocardium. Some of the reaction products are seen in the sarcoplasmic reticulum (*SR*). The transverse tubules (*T*) and terminal cisternae (*TC*) are both negative for reaction. *M*, mitochondria; *Z*, Z line.  $\times 47,000$ .



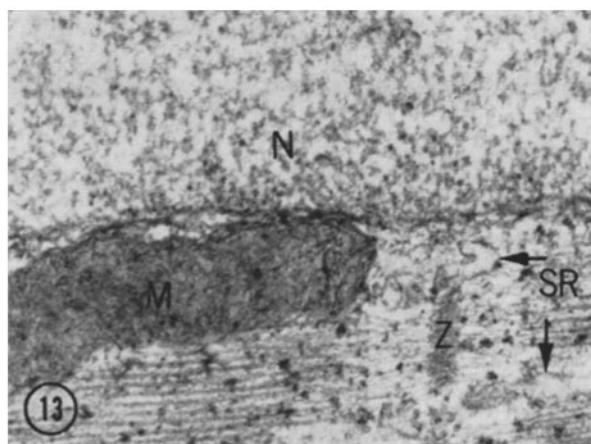


FIGURE 13 LDH reaction in the myocardium. Reaction products are present mostly in the ground substance of the sarcoplasm. Some products are in the mitochondrion (*M*). The cisternae of the sarcoplasmic reticulum (*SR*) appear to lack reaction. Note the absence of reaction in the nucleus (*N*), indicating a minimal diffusion of the enzyme or reaction products. *Z*, *Z* line.  $\times 40,000$ .

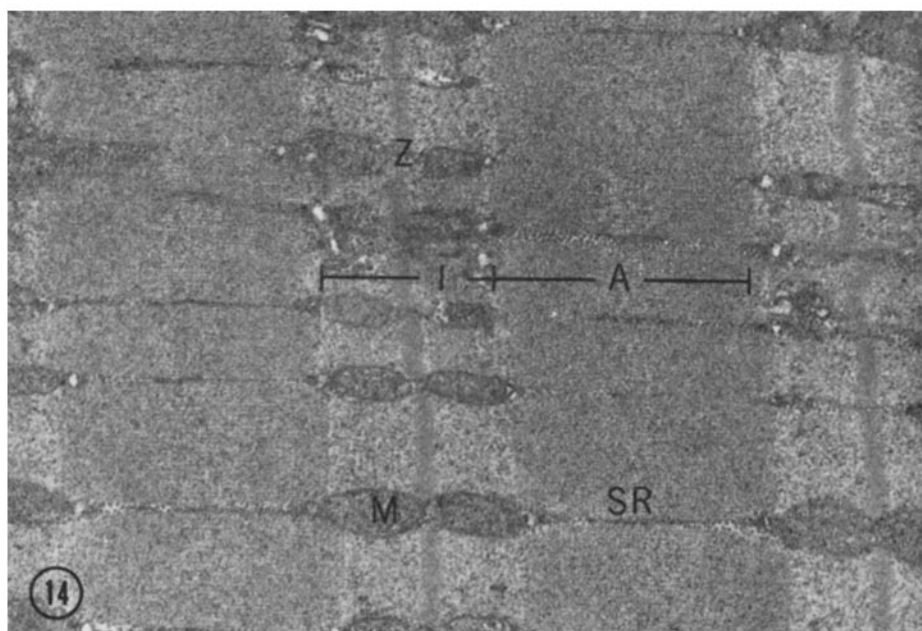
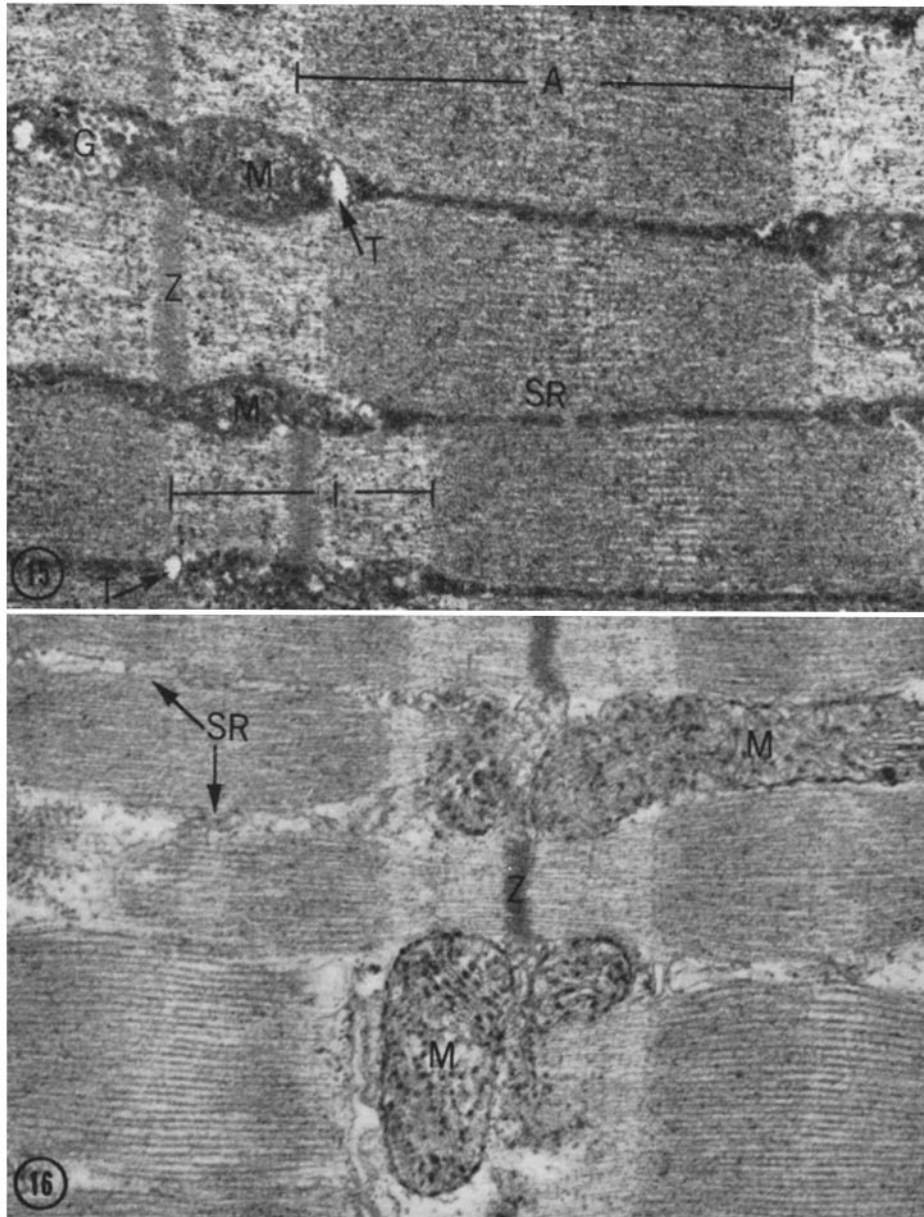


FIGURE 14 LDH reaction in formalin-fixed rat pectoralis muscle. A portion of a type I muscle fiber showing diffuse reaction in ground substance of sarcoplasm. Reaction is also seen in the mitochondria (*M*) and sarcoplasmic reticulum (*SR*). *A*, *A* band; *I*, *I* band; *Z*, *Z* line.  $\times 25,000$ .

space, indicating that little enzyme diffusion occurred.

Formaldehyde fixation resulted in a decrease in total LDH activity, with the most pronounced diminution in the M-LDH. This observation is in

agreement with Ressler's finding (38) that the LDH subunit A, which predominates in M-LDH, is more susceptible to formalin than the subunit B, which predominates in H-LDH. Histochemical data obtained by other investigators have indi-



**FIGURE 15** LDH reaction in the pectoralis muscle. A type I fiber showing reaction products in the mitochondria (*M*) and sarcoplasmic reticulum (*SR*) as well as the ground substance of sarcoplasm. Note the absence of the reaction in the lumen of the T tubules (*T*). *A*, A band; *G*, glycogen granules; *I*, I band; *Z*, Z line.  $\times 41,000$ .

**FIGURE 16** LDH reaction in the pectoralis muscle in the presence of 4 M urea. Notice a marked reduction of LDH reaction in the ground substance of the sarcoplasm. No reaction is seen in the cisternae of the sarcoplasmic reticulum (*SR*). A strong reaction remains only in the intramembranous space of the mitochondria (*M*). *Z*, Z line.  $\times 41,000$ .

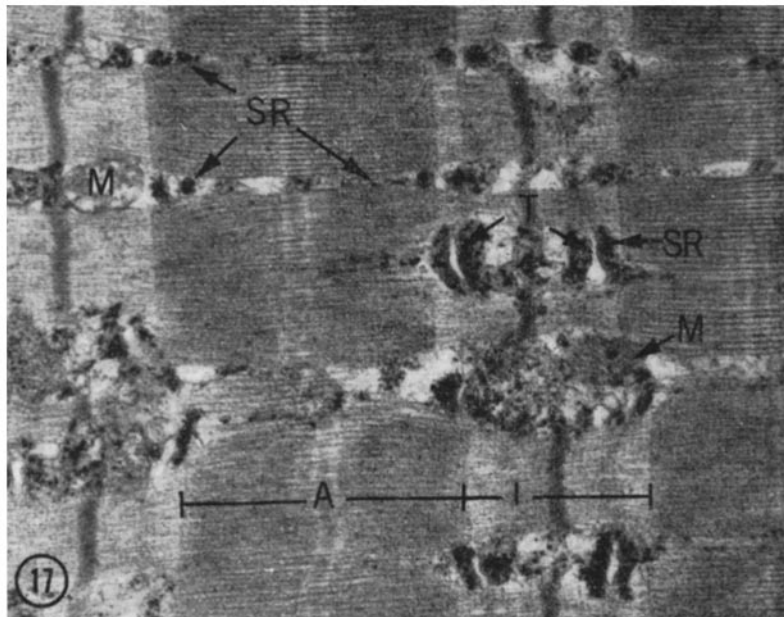


FIGURE 17 LDH reaction in the pectoralis muscle. A portion of a type II muscle is shown with strong reaction localized within the sarcoplasmic reticulum (SR), including the terminal cisternae. Note the absence of the reaction in the T tubules (T) and a faint reaction in some mitochondria (M) and in the ground substance of the sarcoplasm. A, A band; I, I band.  $\times 27,500$ .

cated that mitochondrial LDH in muscle tissue is well preserved after formalin fixation (11, 28, 44) and that extramitochondrial LDH in skeletal muscle and liver is considerably inhibited by formalin fixation (7, 11).

The preservation of tissue fine structure with formaldehyde was adequate for enzyme localization. The preservation of mitochondrial membranes was improved by adding 0.2 M sucrose to the formaldehyde. The membranes of the sarcoplasmic reticulum were noted to be quite labile, and to be particularly sensitive to freezing and thawing during frozen sectioning. This artefact was minimized by rapid freezing with DMSO in liquid nitrogen or addition of 1% calcium chloride to cacodylate-buffered formaldehyde.

Instability of LDH, particularly LDH-4 and -5, during low-temperature storage has been noted (24, 50). Dissociation and recombination of LDH subunits during freeze-thawing in salt-containing solution has been reported (27). The method in our experiment included overnight storage of the tissue fragments at 4°C, freezing of tissue for sectioning, and thawing of frozen sections before incubation. To investigate

the extent of cold inactivation and recombination of LDH subunits in our material, an electrophoretic study was performed on homogenates of formaldehyde-fixed tissue which had been freeze-thawed and then stored at 4°C. There was a change in the mobility of LDH-4 and -5 and a reduction in the activity of all isoenzymes. These changes were not sufficiently severe to preclude a cytochemical study of the localization of M- and H-LDH.

One of the common problems inherent in the histochemical demonstration of a dehydrogenase is interference by the presence of other intracellular enzymes, particularly the cytochromes (20) and diaphorase (5, 11, 12). The cytochromes which affect histochemical reactions in aerobic tissue such as heart and red skeletal muscles may be effectively blocked by cyanide in the reaction medium (20). It has been demonstrated in skeletal muscle that NADH<sub>2</sub> diaphorase (NAD-dependent tetrazolium reductase) may critically influence the intensity and intracellular distribution of reaction products of NAD-dependent dehydrogenases, and that the interference of diaphorase can be effectively avoided by the use

of PMS in the histochemical reaction medium (5, 11, 12). However, a high concentration of PMS has been shown to be quite deleterious for histochemical demonstration of loosely attached dehydrogenases such as LDH (19). In our experience this adverse effect of PMS appeared much stronger in association with TC-NBT than with NBT. Therefore, it was necessary to use a small quantity of PMS or none at all for the electron microscope study. We compared sections stained in the presence of a minimal amount of PMS and without PMS and noted that omission of PMS did not change the ultrastructural localization of LDH. Previous workers have demonstrated that NADH<sub>2</sub> diaphorase activity is concentrated in the sarcoplasmic reticulum (31) and in the mitochondrial matrix (42) of myocardium muscle cells. We observed weak LDH activity in the sarcoplasmic reticulum and no activity in the mitochondrial matrix. Therefore, we conclude that the interference of diaphorase in our experiment was negligible. Commercially available TC-NBT had an extremely low solubility in water and a high inhibitory effect from PMS, and it was necessary to dissolve it first in filtered dimethylformamide (DMF) or DMSO.

Urea inhibition of M-LDH and excess substrate (lactate and pyruvate) inhibition of H-LDH have been successfully applied in light microscope histochemistry for the identification of LDH isoenzymes in tissue sections (4, 17, 28). In our study, however, the use of excess lactate or pyruvate was not satisfactory for electron microscopy, and the use of urea or heat was a more reliable method for cytochemical isoenzyme differentiation.

Numerous histochemical studies have demonstrated the presence of LDH activity in mitochondria of various tissues (4, 20, 28-30, 44) and in sarcoplasmic reticulum of white muscle (11, 12). These studies disagree with the bio-

chemical studies which have indicated that LDH is in the soluble fraction of the cells (8); however, several investigators have recorded LDH activity in the mitochondrial and microsomal cell fractions (1, 18, 23, 33, 43, 47). The reported predominance of LDH-1 in the mitochondrial fraction (1, 18, 23, 43) and a close relationship between LDH-5 and the developing sarcoplasmic reticulum (2) are all in accord with our histochemical findings.

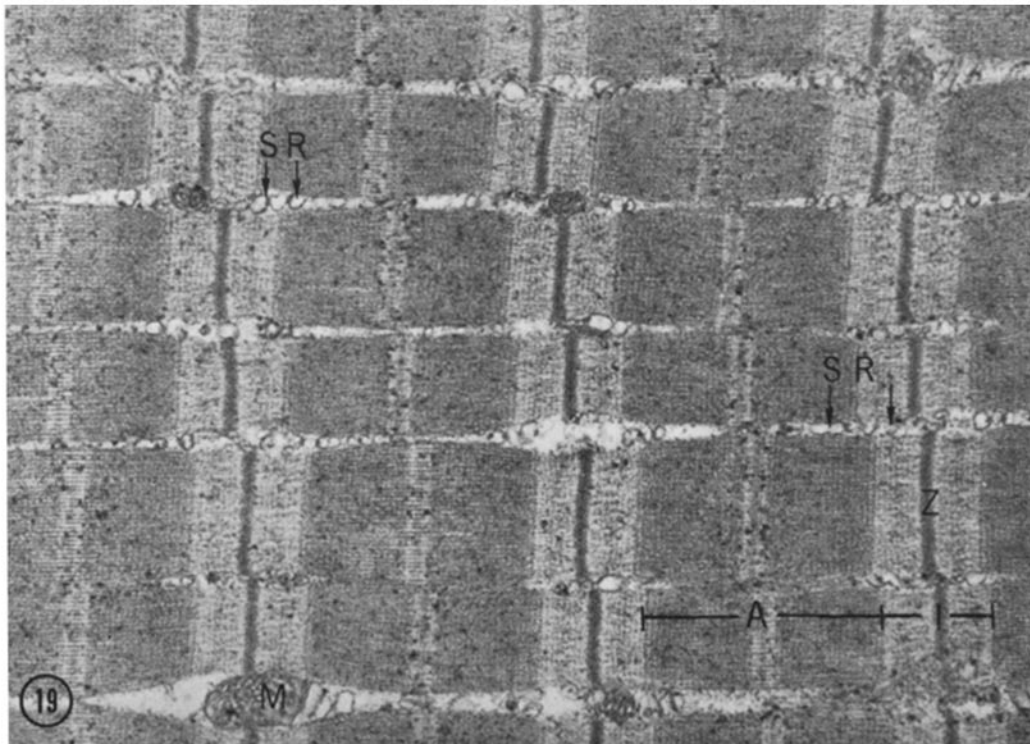
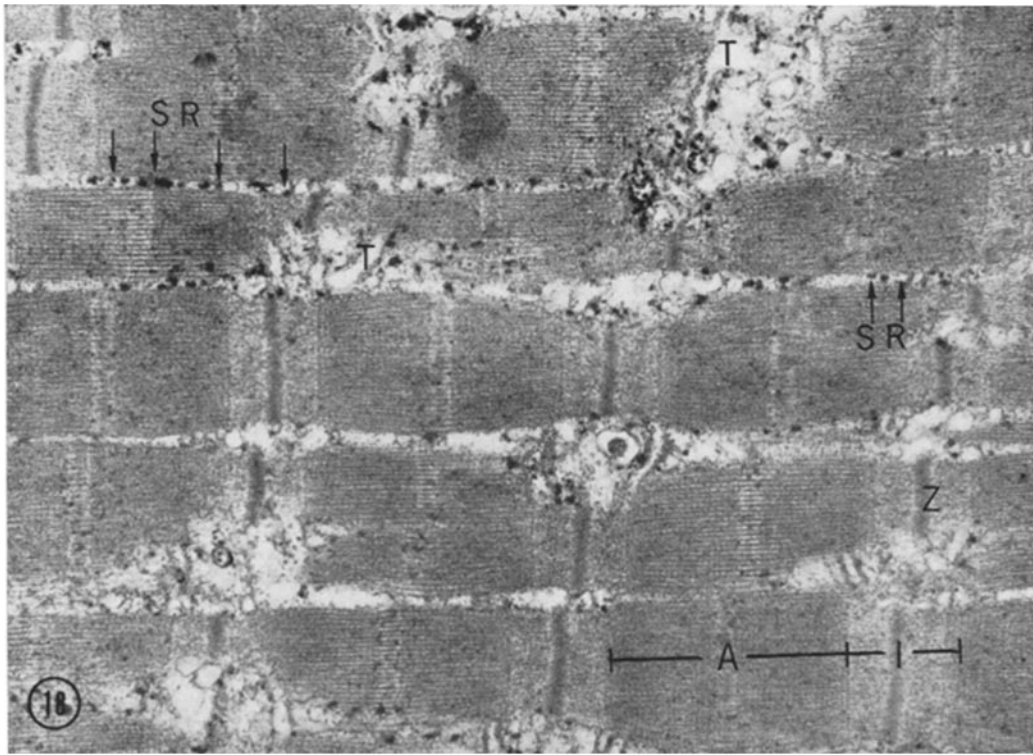
It has been shown that M-LDH is usually present in tissues that are more active in anaerobic metabolism, such as white muscle, which can actively utilize glycolytic pathways. H-LDH is found largely in tissues utilizing aerobic metabolism, such as red muscle and cardiac muscle, which utilize fatty acids as a source of energy (22, 34). An excess of substrates, lactate and pyruvate, in vitro has been shown to suppress H-LDH activity (22, 36) although such excessive concentration never seems to occur in the tissue in vivo (45). While LDH-5 is activated by the intermediates of the Krebs cycle, LDH-1 lacks such property (15). On the basis of these properties of LDH, it is speculated the H-LDH functions to insure pyruvate entry into the Krebs cycle for aerobic oxidation. H-LDH is similar to glycerophosphate dehydrogenase and NAD-dependent malate dehydrogenase in that it has both mitochondrial and extramitochondrial components (6, 21). Since both glycerophosphate and malate dehydrogenases have been known to be shuttle enzymes for reoxidation of extramitochondrial NADH<sub>2</sub> (25), a similar metabolic role (shuttle) may exist for H-LDH. Permeability of the mitochondria to lactate has not been well-demonstrated, and the lactate shuttle remains a pure speculation.

M-LDH may be associated with greater conversion of pyruvate to lactate and may allow less pyruvate for the Krebs cycle than H-LDH. In regard to the coenzyme, this would constitute

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FIGURE 18 LDH reaction in the pectoralis muscle. Reaction products are seen in the sarcoplasmic reticulum (SR). The T tubules (T) are free of reaction. Faint reaction is seen in the sarcoplasm in the areas of the myofibrils. A, A band; I, I band; Z, Z line.  $\times 23,000$ .

FIGURE 19 LDH reaction in the pectoralis muscle after heating. Marked diminution of the reaction is noted in the sarcoplasmic reticulum (SR). The sarcoplasm in the area of the myofibrils shows numerous reaction products. The disappearance of the reaction from the sarcoplasmic reticulum suggests the distribution of M-LDH, and the remaining reaction indicates that of H-LDH. A, A band; I, I band; Z, Z line; M, mitochondria.  $\times 23,000$ .



a mechanism of direct oxidation of the extra-mitochondrial NADH<sub>2</sub> (25). Coexistence of triosephosphate dehydrogenase and M-LDH in the sarcoplasmic reticulum of the muscle (12) strongly suggests that the sarcoplasmic reticulum is a site of active anaerobic glycolysis. Since the cisternae of the sarcoplasmic reticulum are closely connected with the T system by formation of triads, the presence of active glycolysis in the sarcoplasmic reticulum may facilitate disposal of the final metabolic product, lactate, into the extracellular space.

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