CYTOCHEMICAL LOCALIZATION OF LACTIC DEHYDROGENASE IN WHITE SKELETAL MUSCLE

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ABSTRACT

The limitations of the conventional histochemical methods for localization of lactic dehydrogenase (LDH) in white skeletal muscle have been analyzed quantitatively. It is demonstrated that more than 80 per cent of LDH diffuses into the incubation medium within the first 10 minutes of incubation. Furthermore, it is confirmed that the addition of phenazine methosulfate (PMS) to the ingredients of the histochemical reaction for LDH increases substantially the capacity of the white muscle extract to reduce Nitro-BT. Based on these observations, a modified method of cytochemical localization of LDH has been developed. This method prevents the leakage of LDH from tissue sections by the application of all the ingredients of the histochemical reaction to tissue sections in a thin gelatin film. The incubation mixture contains PMS so that the staining system is independent of tissue diaphorase. The application of this method to the adductor magnus muscle of the rabbit revealed a fine reticulum in the sarcoplasm of all muscle fibers, in addition to the staining of mitochondria. The distribution of the staining suggests that LDH is localized in the sarcoplasmic reticulum.

INTRODUCTION

Lactic dehydrogenase (LDH)[1] plays a key role in the metabolism of a wide variety of cells. It is particularly significant in cells which depend upon anaerobic glycolysis for their energy production since it provides a mechanism for oxidizing reduced coenzyme I without requiring the presence of oxygen (reaction I).

$$H^+ + \text{pyruvate} + NADH \xrightleftharpoons{LDH} \text{Lactate} + NAD^+$$

The significance of this enzyme in the intermediary metabolism of skeletal muscle is reflected in the high content of LDH in this tissue (2–5). Histochemically, LDH has been localized in or around the muscle mitochondria (6–11). In biochemical studies, however, using the method of differential centrifugation of homogenized tissues, this enzyme is extracted with the soluble cytoplasmic fraction, which is devoid of mitochondria (12–15).

The applicability of the standard histochemical

[1] The following abbreviations have been used throughout this report: LDH, lactic dehydrogenase; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; PMS, phenazine methosulfate; Nitro-BT, 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium-chloride; tris, tris (hydroxymethyl)-aminomethane.

methods for LDH (16, 17) is limited by (*a*) dependence of the staining results on the presence in tissue of a diaphorase which must be associated with the dehydrogenase to transfer the electrons from the reduced coenzyme I(NADH) to the tetrazolium salt (18–22) and (*b*) by the leakage of LDH from tissue sections into the incubation medium (22–24, 88).

In this study, (*a*) the limitations of the standard histochemical methods for LDH are analyzed quantitatively; (*b*) evidence is presented that the standard methods are inadequate to reflect the sites of enzymatic activity in white skeletal muscle; (*c*) a modified method for cytochemical localization of LDH is presented which prevents the diffusion of LDH into the incubation medium and, at the same time, makes the staining system independent of tissue diaphorase; and (*d*) it is demonstrated that this modified method when applied to the adductor magnus muscle of the rabbit reveals a fine reticulum in the sarcoplasm of muscle fibers in addition to the usual staining of the mitochondria.

MATERIALS AND METHODS

Domestic albino rabbits, weighing 2 to 3 kilograms, were killed by exsanguination, and the white adductor magnus muscle was dissected away from the adjacent red semitendinosus and placed on ice. Transverse and longitudinal blocks, 1 to 2 mm in diameter, were immersed for 20 seconds in a mixture of 3 volumes liquid propane and one volume isopentane cooled to −180°C with liquid nitrogen. These blocks were wrapped in aluminum foil, stored at −70°C and used within 2 weeks.

1. To estimate the amount of LDH which leaks out of frozen sections of white muscle into the aqueous incubation media, serial sections were cut at 4 and 16 μ thickness in a cryostat at −23°C (Harris Refrigeration Co., Cambridge, Massachusetts). Care was taken to make the sections of uniform thickness and of equal size. Every third section was placed in 5 ml 0.2 M tris buffer pH 7.4 and then frozen and thawed twice at −20°C. An additional 15 ml of buffer was added and the tissues were further disrupted by sonic oscillation in a Raytheon model DF 101 sonic oscillator until no gross evidence of particulate material was noticed. The LDH activity of this extract was taken as the reference standard in calculating the extent of leakage of LDH from the two other sections cut serially. The two other sections were picked up on coverslips, finger thawed, and dried for 3 minutes under a blower. One of these sections was incubated directly in 10 ml 0.2 M tris buffer pH 7.4 at room temperature, whereas the other section was coated with a thin film of 2.5 per cent purified gelatin (Fisher Scientific Co., Fair Lawn, New Jersey) prior to incubation in the buffer solution; for each experiment, 24 serial sections were used. At time intervals of 3, 5, 10, 15, 30, and 60 minutes, 1 ml of the buffer medium was removed for LDH determinations and the volume was replaced with fresh buffer. LDH activity was determined in duplicate by following the rate of oxidation of NADH in the presence of pyruvate in a Gilford model 2000 multiple absorbance recorder at 340 mμ. Each reaction mixture contained 2.7 ml 0.2 M phosphate buffer pH 7.5, 10 μmoles sodium pyruvate (Nutritional Biochemical Co., Cleveland), and 0.2 mg NADH (Pabst Laboratories, Milwaukee). The final volume was 3 ml and the reaction was carried out at 25°C. NADH oxidase activity was assayed by omitting the pyruvate from the above reaction mixture. Each experiment was repeated three times.

2. To assess the importance of the intermediate electron carrier, phenazine methosulfate (PMS), in transfer of electrons from the substrate to Nitro-BT, by the white skeletal muscle, a tissue extract was prepared, according to the method described above, from 4-μ-thick cryostat-cut sections by means of freezing and thawing and sonic oscillation. Varying concentrations of PMS were added to a reaction mixture consisting of 0.5 ml of muscle extract, 1 μmole Nitro-BT (Dajac Laboratory, The Borden Co., Philadelphia), 2.5 μmoles NAD^+ (Pabst Laboratories), 500 μmoles lithium lactate (pH 7.4), and 80 μmoles tris buffer pH 7.4. The final volume was 5 ml. The reaction was carried out at 25°C and was stopped after 5 minutes by addition of 1 ml of 1 N HCl. To disperse the formazan, 10 mg of purified gelatin was added to this mixture (25, 26) and the optical density read at 540 mμ in a Beckman model B spectrophotometer. All experiments were performed in triplicate.

3. For the histochemical studies, sections 2 μ thick were cut in a cryostat maintained at −23°C. The sharpness of the blade, the knife-tissue angle and the angle of the anti-roll device, as well as the size of the blocks, had great influence in obtaining 2-μ-thick ribbon sections. Sections were picked up on coverslips, finger thawed, and air dried under a blower at room temperature for 3 minutes. These sections were used either directly without any further treatment, or were fixed in cold acetone (27, 28) or in cold calcium-formol (29, 30) in order to evaluate the relative merits of these different fixatives. A few control sections were placed in a tris-buffer solution (pH 7.4, 0.2 M) for 10 minutes. All sections were then dried and stained according to the method described below.

Preparation of Incubation Mixture and Staining

The following mixture was prepared in a test tube: 0.2 μmoles Nitro-BT; 0.5 μmoles NAD^+; 100 μmoles lithium lactate; and 0.2 μmoles PMS. This mixture was mixed with an equal volume of 0.2 M tris buffer containing 5 per cent purified gelatin and the pH was adjusted to 7.4. The total volume was 1 ml, and 0.25 ml of this final mixture was spread evenly on the surface of 75 × 25 mm slides and allowed to form a gel on a level surface. Gelatination occurred usually within a few minutes, and such slides could be stored in a light-tight container for 2 hours at 4°C without any evident loss of activity. If the incubation mixture was left at room temperature and under the usual lighting conditions of the laboratory, a blue formazan precipitate was noted within a few hours. The addition of crystalline LDH (Mann Research Laboratories, New York) to slides coated with the incubation mixture film resulted in formation of the formazan within a few seconds.

The air-dried sections mounted on coverslips were applied gently to the surface of slides which were coated with a thin film of incubation mixture. As soon as the section came into contact with the surface of the incubation mixture film, the preparation was placed under a microscope and the progressive development of formazan precipitate was observed at room temperature. When adequate staining was achieved, sections which were still attached to coverslips were detached from the gelatin film layer, washed in distilled water, dehydrated, and mounted on slides. Although a faint staining was detectable at 30 seconds after the incubation, 3 to 5 minutes were required for adequate staining of the white adductor magnus muscle of rabbit. Serial sections from the same blocks were stained for NADH-diaphorase and for LDH according to the method of Nachlas *et al.* (16). Control sections were stained in the same manner as described above, but the specific substrate, lactate, was omitted from the incubation mixture. In another set of controls, sections were stained without the addition of PMS.

RESULTS

1. QUANTITATIVE DETERMINATIONS OF ENZYME LEAKAGE: Fig. 1 demonstrates the rapidity and the extent of diffusion of LDH from 4-μ-thick frozen sections of the white muscle into the incubation medium. On the basis of several experiments, it was estimated that the reproducibility of the method used for determination of enzyme diffusion ranged about ±15 per cent.

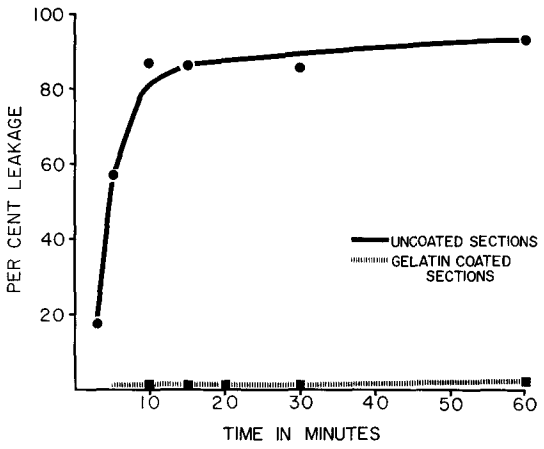


FIGURE 1 Leakage of LDH from 4-μ-thick sections of white adductor magnus muscle of the rabbit (see Methods).

Furthermore, it was noted that increasing the thickness of tissue sections decreased the leakage of the enzyme only slightly (Table I). The coating of tissue sections with a thin film of gelatin, however, reduced to negligible proportions the diffusion of the enzyme from sections into the medium (Fig. 1). The enzyme NADH-oxidase did not diffuse in any detectable amounts into the incubation medium.

2. EFFECT OF PMS: Fig. 2 demonstrates the effect of varying concentrations of PMS on tetrazolium reduction by extracts of the white adductor magnus muscle of the rabbit, in the presence of the ingredients of the histochemical reaction for LDH. It was clearly demonstrated that, although the white muscle extract could reduce Nitro-BT in the absence of PMS, the addition of the electron carrier PMS increased by many fold the capacity

TABLE I

Leakage of LDH from Sections of White Skeletal Muscle of Rabbit

Time of incubation	Thickness of sections (μ)	
	4	16
min.	*per cent*	*per cent*
3	29*	59
5	59	67
10	89	76
15	89	74
30	86	75
60	94	84

* Enzymatic activity in the incubation medium, using the activity of extract of serially cut sections as 100 per cent (see text)

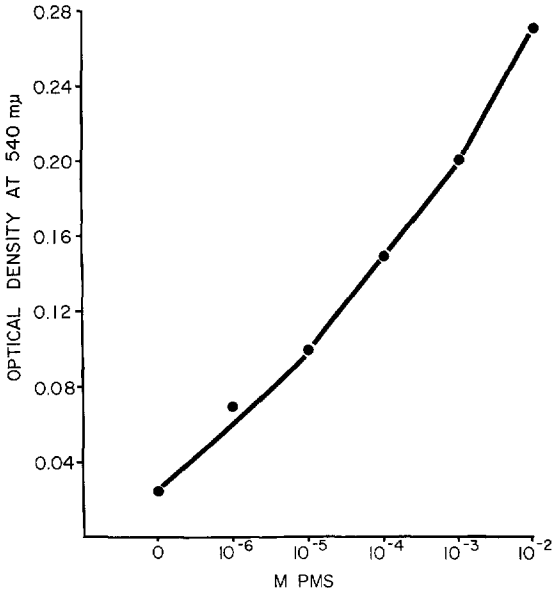


FIGURE 2 The effect of PMS on reduction of Nitro-BT by extracts of white skeletal muscle in the presence of lactate and NAD^+ (see Methods).

of the tissue extracts to reduce Nitro-BT. The deviation from linear kinetics in our results is, at least in part, due to the insoluble nature of formazan (25).

3. HISTOCHEMICAL RESULTS: A staining period of 3 to 5 minutes was sufficient to give adequate staining in sections stained by the method described in this study. A similar intensity of staining was reached by the sections stained by the conventional methods for LDH (16) only after 30 minutes of incubation.

At low magnification, a marked difference was noted in the staining of individual muscle fibers in sections stained by the LDH method of Nachlas *et al.* (16). The smaller red muscle fibers stained more prominently, whereas the white fibers, which have a larger diameter, stained only faintly (Fig. 3), a finding which is in agreement with previous reports on localization of LDH in the skeletal muscle (6–8, 10, 11). In contrast, sections which were stained with our method showed a rather uniform staining pattern as viewed under low magnification (Fig. 4). This uniformity of the staining was apparently due to the increased staining of the white muscle fibers which now could not be distinguished from the red muscle fibers simply on the basis of intensity of staining under low power magnification. Figs. 5 and 6 and 7 and 8 show serial sections of the same individual muscle fibers stained with the conventional method for LDH (16) (Figs. 5 and 7) and with the method described above (Figs. 6 and 8). Each individual muscle fiber has been labeled alphabetically so that all fibers labeled in Figs. 5 and 7 correspond to those labeled similarly in Figs. 6 and 8, respectively. At this magnification, it is evident that sections which were stained with conventional methods for LDH (Figs. 5 and 7) showed small round or rod-shaped foci of staining which were presumed to be the sites of mitochondrial activity (31) and are referred to as such. The red muscle fibers (*B* and *D* in Fig. 5; *A*, *B*, and *E* in Fig. 7) appeared rich in their mitochondrial content, but white fibers (*A* and *C* in Fig. 5) contained only a

few mitochondria. In addition, there were some "intermediate" type muscle fibers (*C*, *D*, and *F* in Fig. 7) which contained more mitochondria than the white fibers, but less than the red fibers. Serial sections of these same individual muscle fibers, stained with the modified staining method for LDH herein described (Figs. 6 and 8), showed that there was a prominent staining of a fine and distinct network in the sarcoplasm of all muscle fibers in addition to the staining of mitochondria. The network was present throughout the entire sarcoplasm and in all types of muscle fibers irrespective of their mitochondrial content. Mitochondria were identified at the intersections of the reticular network. Furthermore, there was some evidence suggesting that, depending upon the level of the sarcomere at which each individual fiber was cut, a somewhat different reticular pattern was obtained. For example, in Fig. 8 the reticular pattern in fiber *E* is apparently different from that of all other fibers in the section.

Figs. 9 to 12 demonstrate further, at higher magnification, the predominantly mitochondrial staining which is obtained with the conventional method for LDH (16) (Fig. 9) and, in contrast to that, the reticular pattern demonstrated by the method for LDH described in this communication (Figs. 10 to 12). The uniformity of the reticular staining pattern in small, medium-sized, and large fibers is demonstrated in Figs. 10 to 12, respectively.

Furthermore, the capillaries that are located between the muscle fibers, and which were stained only faintly by the conventional methods for LDH, became more prominently stained with the technic described in this communication (Figs. 10 to 12) Figs. 10 to 12 show further that the nuclei of muscle fibers remained unstained for LDH, as areas of negative staining in the subsarcolemmal spaces. This fact was confirmed by counterstaining the nuclei in a few sections with the Feulgen reaction (32).

In longitudinal sections stained by the incubation mixture film method described above, all muscle fibers stained uniformly throughout their entire length and two distinct patterns of staining were noted. Whereas a pattern of cross-striation was noted in parts of some fibers (Fig. 13), other muscle fibers showed a pattern of longitudinal lines parallel to their longitudinal axis, either along their entire length or in certain parts (Figs. 14 and 15). Fig. 14 demonstrates, in a single muscle

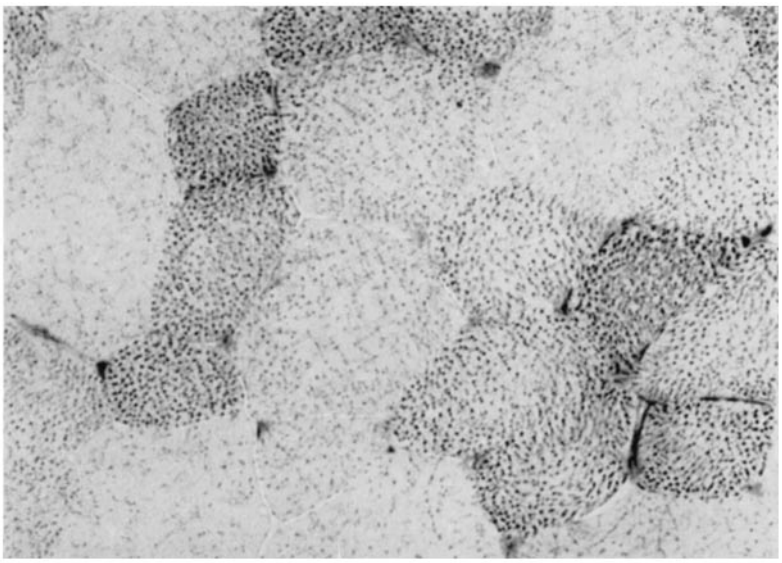


FIGURE 3 Cross-section of the white adductor magnus muscle of the rabbit stained for 30 minutes with the conventional method for LDH (16). Red muscle fibers have a smaller diameter and stain more prominently than the larger white fibers. There is also slight staining of the capillaries between the muscle fibers. × 400.

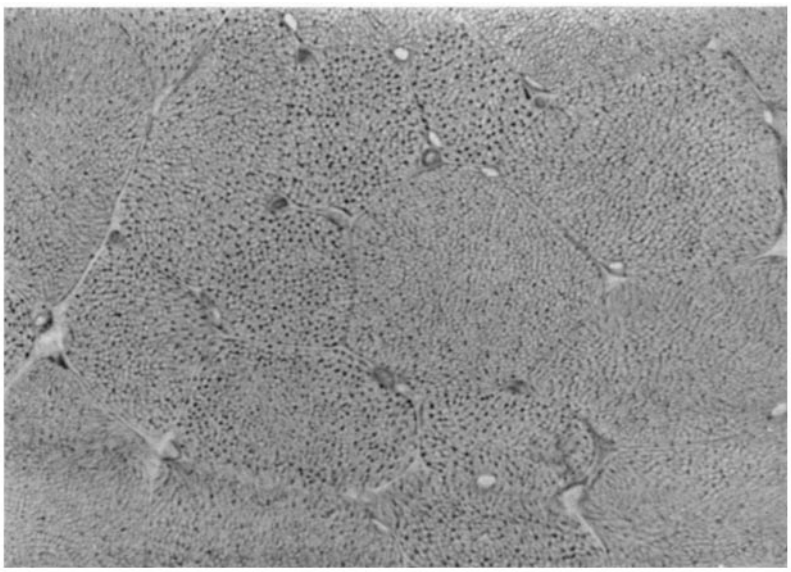


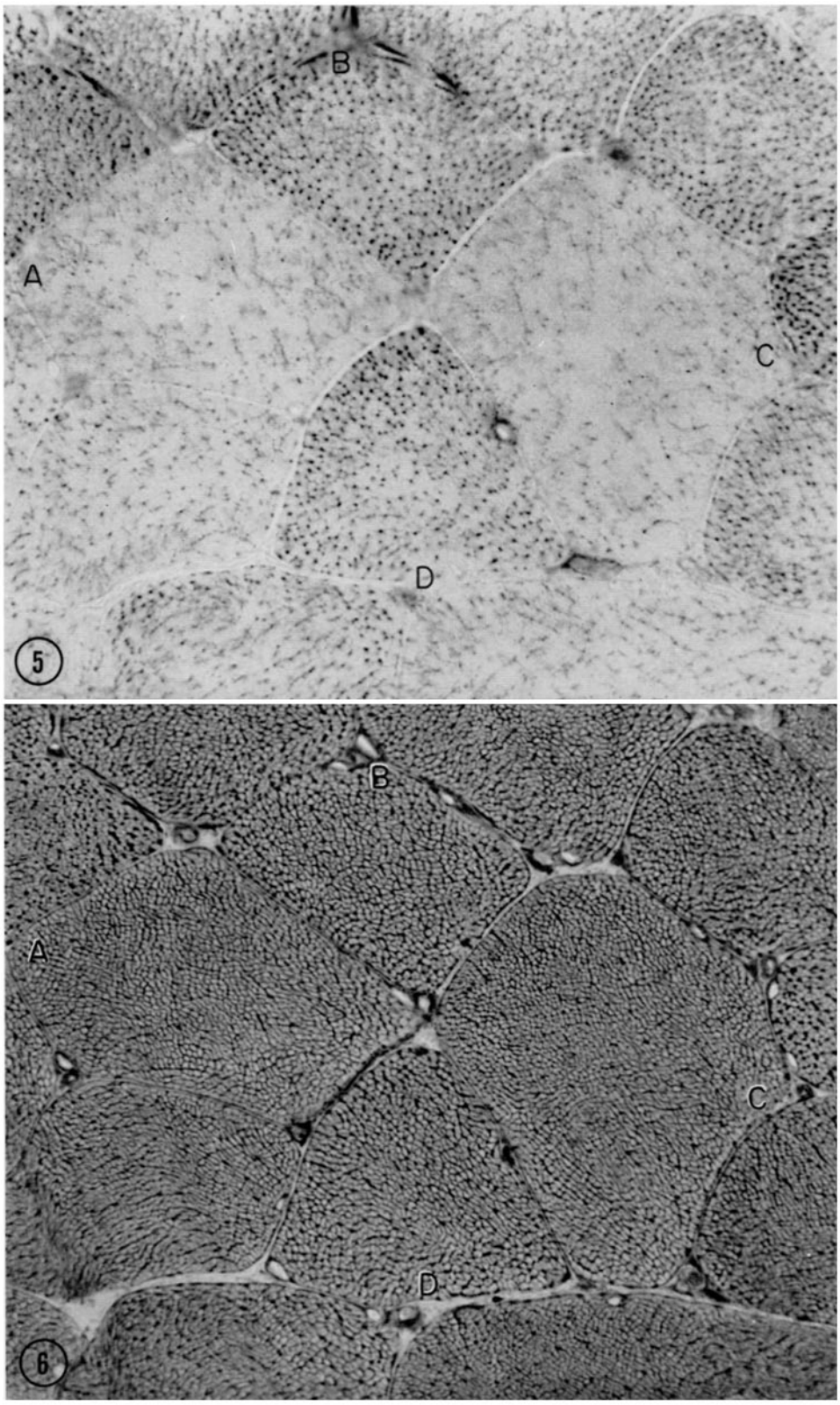
FIGURE 4 Cross-section of the white adductor magnus muscle of the rabbit stained for 3 minutes with the method for LDH described in this report. Note the equal intensity of staining of red and white fibers. The capillaries between the muscle fibers have stained more prominently than in the Fig. 3. Nuclei have remained unstained. × 400.

fiber, a fine reticular network at both ends where the fiber was cut transversally, and a pattern of parallel longitudinal lines in the middle portion of the fiber where it was cut longitudinally. Fig. 15, at higher magnification, shows that the reticular network of the cross-section is closely related to the pattern of parallel lines observed in longitudinal sections and that both are apparently different views of the same intracellular organelle. In fibers which showed the pattern of cross-striation, it was possible to identify the exact site of LDH staining relative to the A and I bands by examining the same individual muscle fibers under ordinary light (Fig. 16 *a*) and under the polarized light (crossed prisms) (Fig. 16 *b*). The LDH staining was localized at the level of the isotropic (I) bands, whereas the anisotropic (A) bands remained unstained. Furthermore, in Fig. 17, at high magnification, it is demonstrated that the I-band staining consisted of two parallel transverse lines on both sides of the I band, at the junction of the A and I bands. The Z disc which occupies the center of the I bands remained unstained. Furthermore, as Fig. 17 shows, pairs of mitochondria were located on either side of the Z discs along the I bands.

Control sections stained with the method for LDH described above, in which the substrate (lactate) was omitted from the incubation mixture, showed only a trace of staining after 10 to 30 minutes. Another set of controls in which the electron carrier PMS was omitted from the incubation mixture, as well as those sections which were stained for NADH-diaphorase according

FIGURES 5 and 6 represent serial cryostat sections of the same individual muscle fibers from the adductor magnus muscle of the rabbit. The four fibers designated as *A*, *B*, *C*, and *D* in Fig. 5 correspond to the fibers designated similarly in Fig. 6.

The section in Fig. 5 was stained for 30 minutes by the conventional method for LDH (16), while in Fig. 6 the adjoining section has been stained for only 3 minutes by the method described in this communication. Note the prominent staining of the mitochondria in Fig. 5 as contrasted with the staining of mitochondria and the "reticulum" in Fig. 6. Fibers *A* and *C* represent white muscle fibers, while *B* and *D* are red fibers. × 760.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to the method of Nachlas *et al.* (16), showed only staining of mitochondria. The latter pattern of staining was identical to the pattern obtained by the staining of muscle fibers with the conventional method for LDH (16) (Fig. 9). In unfixed sections, occasionally, a non-specific deposition of formazan was noted on lipid granules and other adipose tissues (28, 33, 89) (Fig. 15). This problem was best avoided by a short fixation of sections in cold acetone, as suggested previously by several authors (27, 28). The short fixation in cold acetone did not appear to alter significantly the enzymatic activity. Other control sections, which were placed for 10 minutes in aqueous media such as tris buffer or calcium-formol before staining by the method described in this communication, showed a weak, irregular, and patchy staining pattern which was probably due to the leakage of LDH into the aqueous media. The diffusion from the larger "white" fibers appeared to be more prominent. The method of fixation of tissue blocks in formalin as suggested by Walker and Seligman (30) was not tried because of supposed distortion and shrinkage of muscle fibers which results from the fixation in formalin (90).

DISCUSSION

The data presented here indicate that the diffusion of LDH from tissue sections into the incubation medium and the low content of NADH-diaphorase in the white skeletal muscle are serious limiting factors in the cytochemical localization of LDH with the conventional histochemical methods.

1. ENZYME DIFFUSION: Gomori (34) in 1952 pointed out that most histochemically demonstrable hydrolases tended to diffuse into the medium during the incubation, and much subsequent attention has been focused on hydrolytic enzymes (35–37); dehydrogenases, on the other hand, have been considered to be fixed or undiffusible. Novikoff and Arase (23), Novikoff (88), and recently Farber (24), however, pointed out that LDH leaks out of fresh frozen tissue sections into the incubation medium, but these authors reported no quantitative estimates of enzyme diffusion. Recently, Friede *et al.* (38) reported quantitative data on the leakage of NADH-diaphorase and glucose-6-phosphate dehydrogenase from fresh frozen sections of brain.

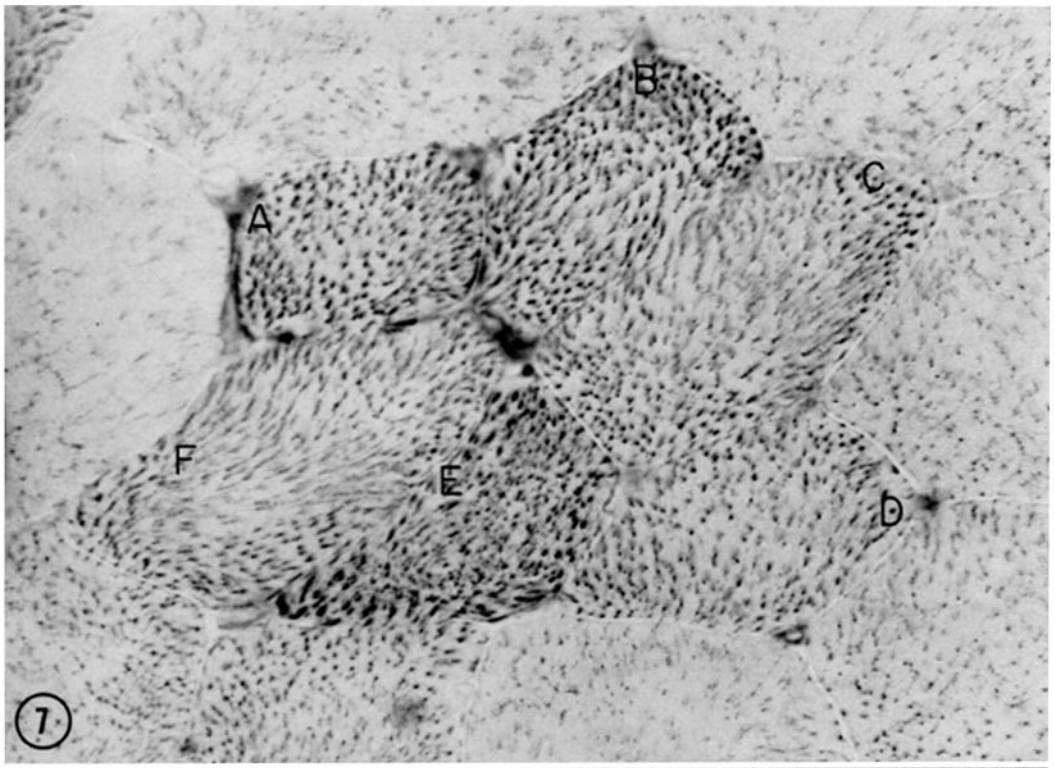
The method of estimation of enzyme diffusion reported here for LDH is somewhat similar to the method used by Nachlas *et al.* (35) for several hydrolytic enzymes, and the limitations of it have been discussed by the same authors (35, 36). Our findings demonstrate, despite the shortcomings of the method, that most of the LDH activity leaked out of fresh frozen sections of the white muscle within the first 10 minutes of incubation. The conventional methods for localization of LDH on the other hand necessitate incubation times as long as 30 minutes. In contrast to the report of Friede *et al.* (38) who found leakage of NADH-diaphorase from brain sections, we could not detect any evidence of diffusion of this enzyme from sections of white skeletal muscle. This, however, could be explained by the very low content of NADH-cytochrome *c* reductase activity in white skeletal muscle (39). Furthermore, we found no great difference in the leakage of LDH with different thicknesses of tissue sections after the first 10 minutes of incubation. This was in contrast with the findings of Friede *et al.* (38), who reported lesser enzyme diffusion in thicker sections. This discrepancy is probably due to the structure of muscle fiber, as contrasted to other tissues. In our study, only cross-sections of the white skeletal muscle were used; and probably once the cylinder of the muscle fiber has been cut across and opened,

FIGURES 7 and 8 represent serial cryostat sections of the same individual muscle fibers from the adductor magnus muscle of the rabbit. The fibers designated *A*, *B*, *C*, *D*, *E*, and *F* in Fig. 7 correspond to those designated similarly in Fig. 8.

The section in Fig. 7 was stained for 30 minutes by the conventional method for LDH, while in Fig. 8 the section was stained for 3 minutes by the method for LDH described in this report.

Note in Fig. 7 the prominent staining of the mitochondria, which appear as rod-shaped or round structures. In Fig. 8 there is, in addition to the staining of the mitochondria, evidence of staining of a fine reticulum in the sarcoplasm of all muscle fibers. The capillaries located between the muscle fibers are stained prominently in both Fig. 7 and Fig. 8.

× 800, both figures.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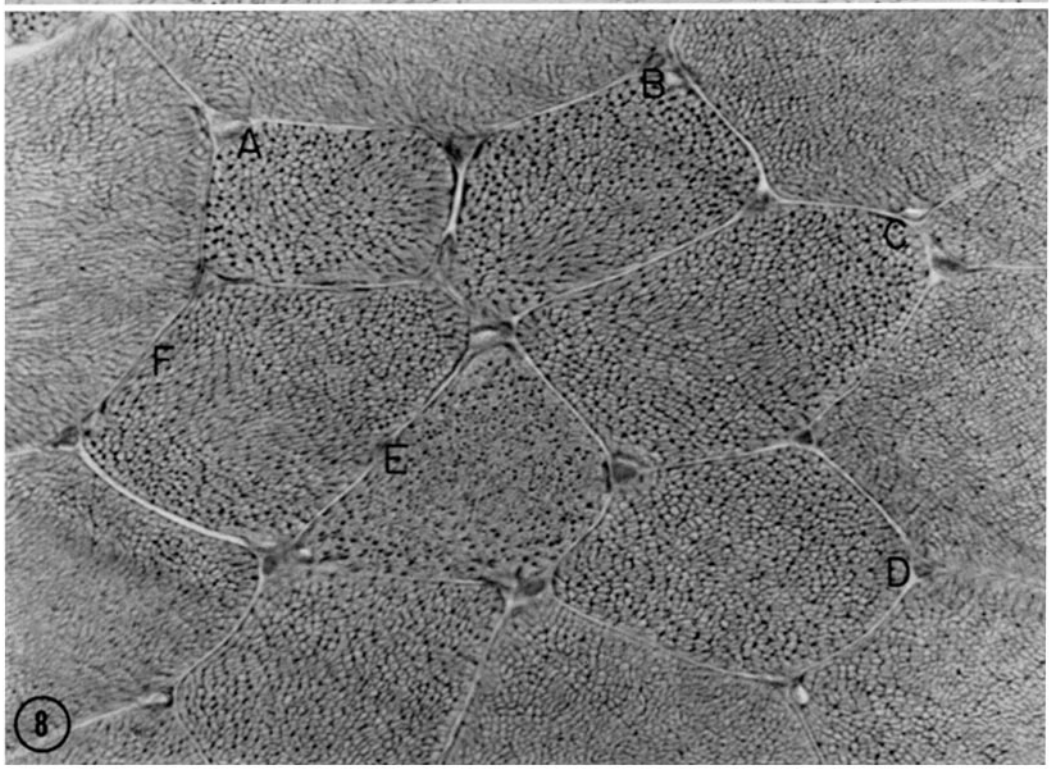
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irrespective of thickness of sections, the entire enzymatic activity may leak out. We have demonstrated further that the coating of tissue sections with a thin film of gelatin prevented the diffusion of LDH into the incubation medium.

2. THE ROLE OF NADH-DIAPHORASE: The histochemical reaction for coenzyme-linked dehydrogenases is a multi-step reaction. Electrons are transferred first to coenzymes by the specific dehydrogenases and then *via* a diaphorase to the tetrazolium compounds causing precipitation of a formazan (18, 19). Nachlas *et al.* (16, 40) and Hess *et al.* (17), who described the histochemical methods which are now in wide use for coenzyme-linked dehydrogenases, presumed that the diaphorases were distributed abundantly in all tissues in association with dehydrogenases. Recently, however, Allen and Slater reported (20) that in the mouse epididymis the LDH system gave a weaker reaction histochemically than did the NADH-diaphorase system, whereas quantitative biochemical assays indicated that the activities of both enzyme systems were comparable. Similarly, in studies on white and red skeletal muscle, Blanchaer and Van Wijhe (21) suspected that the NADH-diaphorase content must be a limiting factor for the histochemical demonstration of LDH activity. These authors found that the red muscle stained much more strongly for LDH than did the white muscle, although biochemical assays of tissue homogenates indicated that white muscle had higher levels of LDH activity than did the red muscle (41). At the time of preparation of this manuscript, these authors reported also (22) that the addition of PMS to the staining system for LDH increased the staining of the white muscle fibers. Their results were, however, affected by deposits of formazan over the section, presumably due to leakage of LDH from tissue sections.

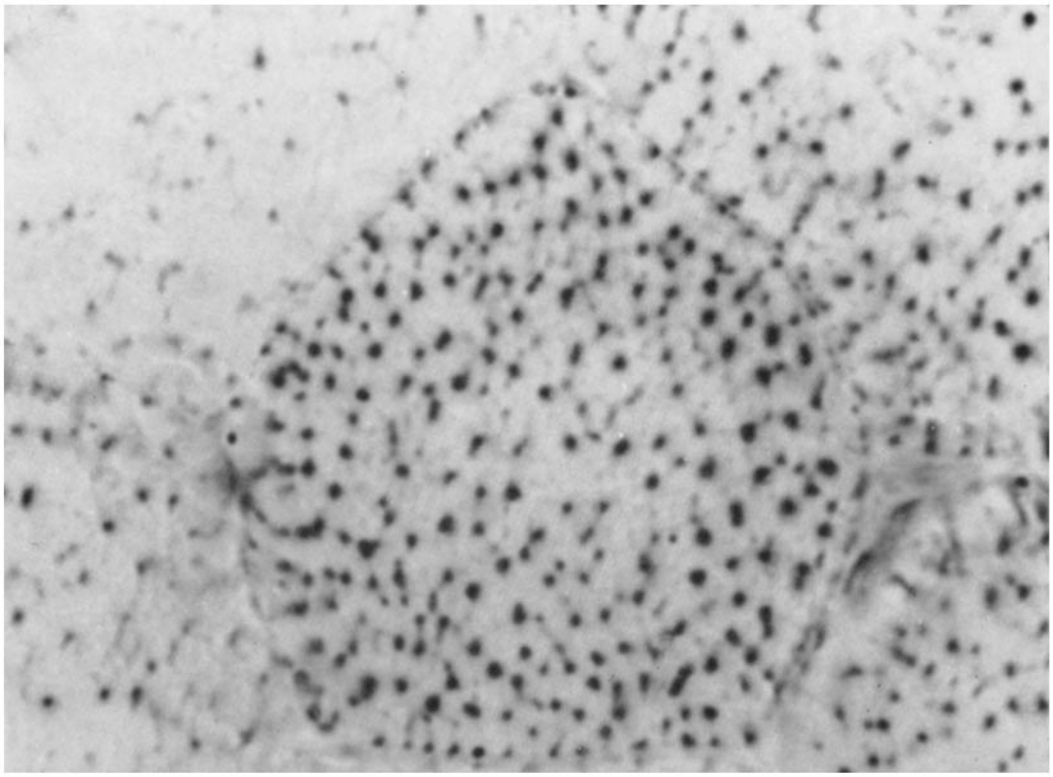


FIGURE 9 An individual red muscle fiber from the white adductor magnus muscle of the rabbit stained for 30 minutes by the conventional method for LDH.
There is evidence of staining only of the mitochondria. Some of the mitochondria appear slightly swollen, probably because of the prolonged incubation time. × 2160.

In this study, we have demonstrated that al-

though total homogenates of the white skeletal muscle had only a low capacity for reducing Nitro-BT in the presence of lactate and NAD^+ as substrates, this reducing capacity could be enhanced manyfold by the addition of the electron-carrier PMS to the system.

The basic similarity of PMS in its electron-transferring capacity to the flavin enzyme (diaphorase) was first reported by Dickens and McIlwain in 1938 (42), and subsequent studies have essentially confirmed their findings (43, 44). As Dewey and Conklin (45), and Nachlas *et al.* (26) have pointed out, PMS transfers the electrons from NADH to the Nitro-BT and this function has been the basis of the recent widespread application of this compound in the staining of LDH isozymes separated by means of electrophoresis (45).

The application of intermediate electron carriers in the histochemical staining reactions for dehydrogenases was first suggested by Farber and Louviere (46). These authors, in trying to improve the staining reaction of dehydrogenases with blue tetrazolium as electron-acceptor, tested a large number of electron carriers and noticed the increased rate of staining after addition of these compounds to the medium. Later, with the introduction of other tetrazolium salts with higher redox-potentials (47), the use of intermediate electron carriers, which were applied merely for the improvement of the staining reaction, became unnecessary (16, 40). As Nachlas *et al.* (48) have reported, the site of the electron transfer to the tetrazolium salts differs with different tetrazolium compounds. Several recent reports have indicated that intermediate electron-carriers such as coenzyme Q_{10}, menadione (vitamin K_3), and PMS can increase the intensity of staining of several dehydrogenases when Nitro-BT or MTT-cobalt are used as final electron-acceptors (9, 22, 29, 49–53). It should be emphasized that the enhancement of the staining reaction with intermediate electron carriers, if the same tetrazolium salt is used, depends primarily upon the endogenous diaphorase content of the tissues which are tested. White skeletal muscle which represents an extreme case, since it has a very low level of NADH-diaphorase activity, requires the use of high concentrations of PMS in the histochemical method for localization of LDH. Such high concentrations of PMS, however, appear to inhibit the histochemical reaction for LDH in tissues such as heart, liver, renal cortex, and red skeletal muscle with a high content of endogenous diaphorase (54). While some authors (22, 29) have reported such inhibitory activity for PMS, others have questioned its validity (44).

Cascarano and Zweifach (19) have emphasized that not only the level of diaphorase activity but also its subcellular location is important in the cytochemical localization of coenzyme-linked dehydrogenases. In skeletal muscle the entire NADH-cytochrome *c* reductase and the diaphorase activity is restricted to the mitochondria (7, 8, 55), and therefore it seems probable that the results reported by the application of the conventional methods for LDH to the skeletal muscle (6–11) reflect only the sites of NADH-diaphorase activity, namely the mitochondria. The results of the histochemical studies reported in this communication indicate that, if sufficient electron-transferring capacity is incorporated into the cytochemical staining system, a fine reticular component is stained in the sarcoplasm in addition to the mitochondria.

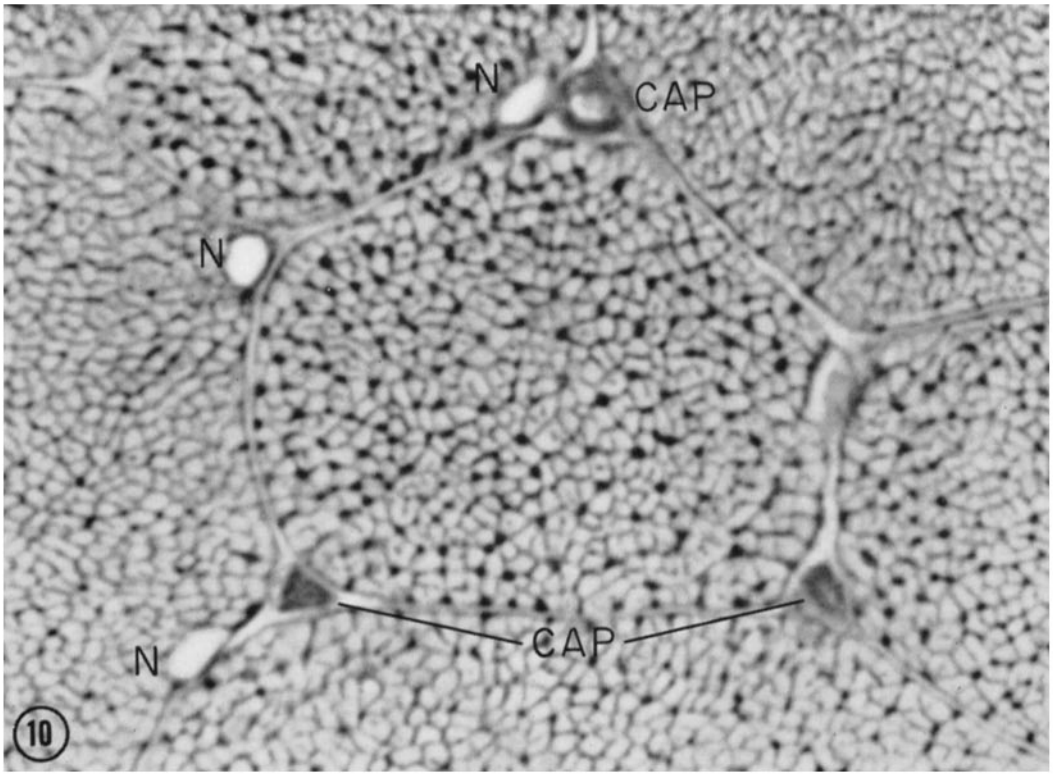
3. THE METHOD OF INCUBATION MIXTURE FILM AND POSSIBLE SOURCES OF ARTEFACTUAL STAINING: By combining the two observations reported in the first part of this communication, a modified method for the cytochemical localization of LDH (see Methods) in the white skeletal muscle was developed which is referred to as the "incubation mixture film" method. By applying all the ingredients of the histochemical reaction to the tissue sections in the form of a thin gelatin film, the diffusion of LDH into the incubation medium is prevented, while at the same time, by the incorporation of PMS, the staining system is made independent of tissue diaphorase. This method is somewhat similar to the substrate film methods for nucleases (56) and the recently described starch film method for localization of amylase (57). The major difference, however, is that in the substrate film methods only the substrate (*e.g.* DNA or starch) is contained in the film layer, and after incubation the sites of enzymatic activity are detected by comparing the stained substrate film layer with the stained tissue section. In the method described here for LDH, however, the entire incubation mixture is contained in the film layer and the formazan precipitation occurs directly on the tissue section, so that the gelatin film layer is discarded after the completion of the staining reaction. Thus, the function of the gelatin film base in our method for LDH is only to prevent the diffusion of the enzyme

while bringing the reaction mixture into contact with the tissue section.

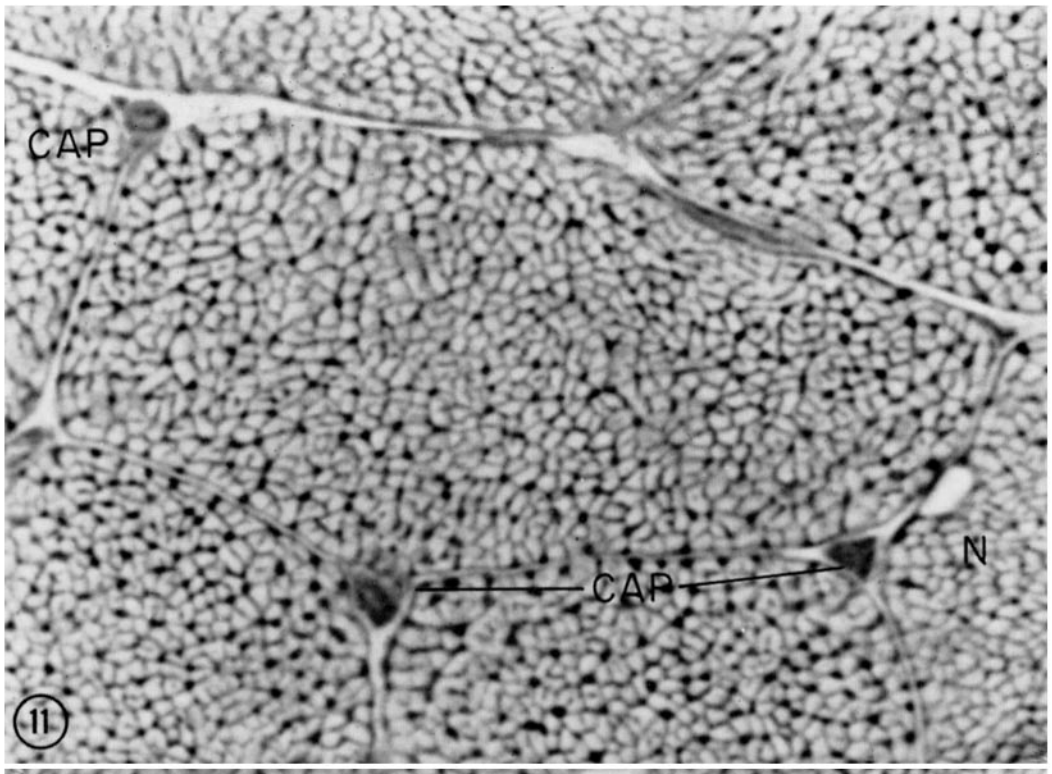
To rule out the interference of "staining artefacts" with the results of the cytochemical studies reported here, several problems were considered.

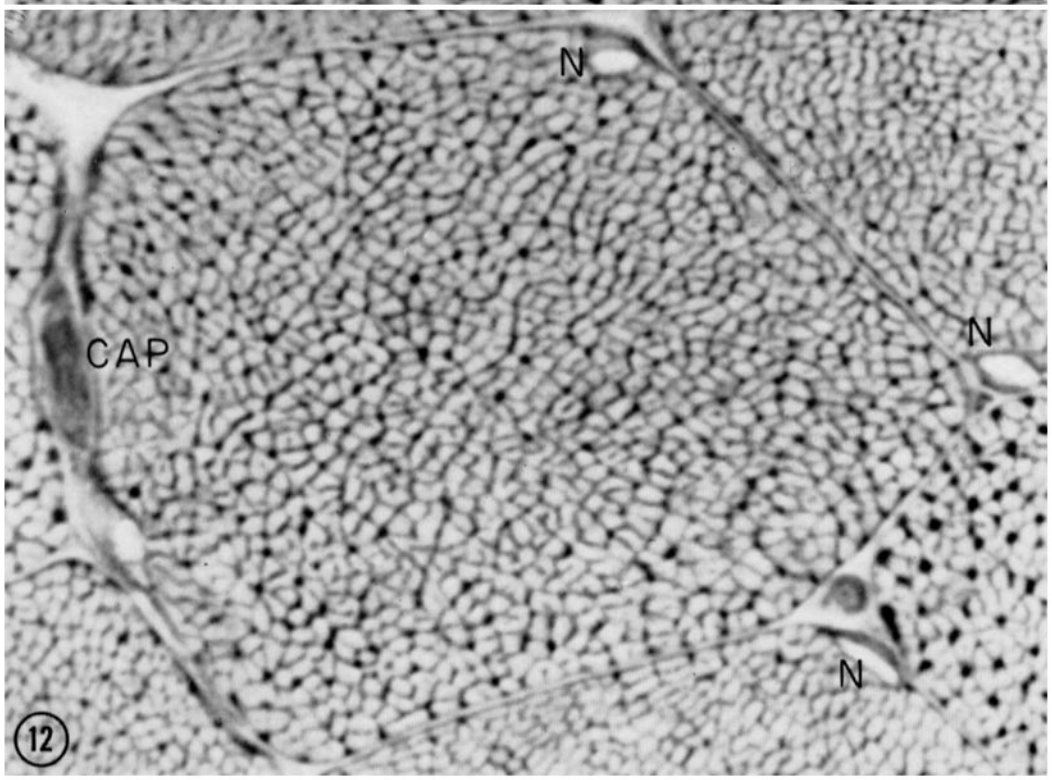
a. The affinity of Nitro-BT for lipoprotein membranes and the propensity for deposition of formazan on lipid droplets is a major source of artefactual staining (28, 33, 58, 89). In the striated muscle, lipid droplets, when stained non-specifically with formazan, can resemble mitochondria in size, shape, and location (28). Novikoff *et al.* (27) and Hitzeman (28) have reported, however, that cold acetone fixation extracts this lipid material, thus permitting a proper visualization of both mitochondrial and microsomal enzymes. All the histochemical observations reported here were made with both fixed and unfixed material and the cold acetone fixation did not alter the sites of enzymatic activity.

b. Another factor which imposes a limitation on the cytochemical demonstration of those dehydrogenases whose optimal activity lies in the alkaline range (59) is attributed to active SH-groups which cause a non-specific reduction of NAD$^+$ (60). Zimmermann and Pearse (59) have cautioned against the use of high concentrations of NAD$^+$ (2 to 3 mg per ml of incubation medium) at pH 8.0–9.0 because of interference of the so called "nothing dehydrogenase" with the specific dehydrogenase reaction. Although the optimal activity of LDH lies in the alkaline range (61), all reactions in this study were done at pH 7.4 to minimize any possible artefactual results. Further-



FIGURES 10 to 12 Three muscle fibers of different sizes from the adductor magnus muscle of the rabbit stained for 3 minutes by the method for LDH described in this report. Fig. 10 represents a small red fiber, Fig. 11 a medium-sized fiber, and Fig. 12 a large white muscle fiber.
Note the staining of the fine reticulum in the sarcoplasm in addition to the mitochondrial staining. The capillaries (*CAP*) between the muscle fibers are stained prominently, while the nuclei (*N*) of the muscle cells have remained unstained. × 2160.


CAP
CAP
N
11


N
CAP
N
N
12

more, the concentration of NAD^+ used in our staining system is one-tenth of the critical concentration reported by Zimmermann and Pearse (59). Further caution is necessary in the method described here, because of instability of PMS. This compound is reduced easily at alkaline pH and under strong light to a leuko form (25, 44) which, in turn, reduces tetrazolium salts. At pH 7.4 and the concentration used in this study (see Methods), there was no detectable evidence of decomposition of PMS within 2 hours if the incubation mixture was protected from strong light and kept at 4°C.

of 3 to 5 minutes appears to decrease markedly the possibility of any extensive damage to tissue sections, although this problem would need further investigation with the electron microscope.

4. THE HETEROGENEITY OF SKELETAL MUSCLE FIBERS: In 1868 Krause (65) considered the white adductor magnus muscle of the rabbit, which has been used in this study, as being a white skeletal muscle in contrast to the neighboring red semitendinosus muscle. The observations of investigators in the 19th century (66, 67) that there is a marked heterogeneity of the individual fibers that compose vertebrate skeletal

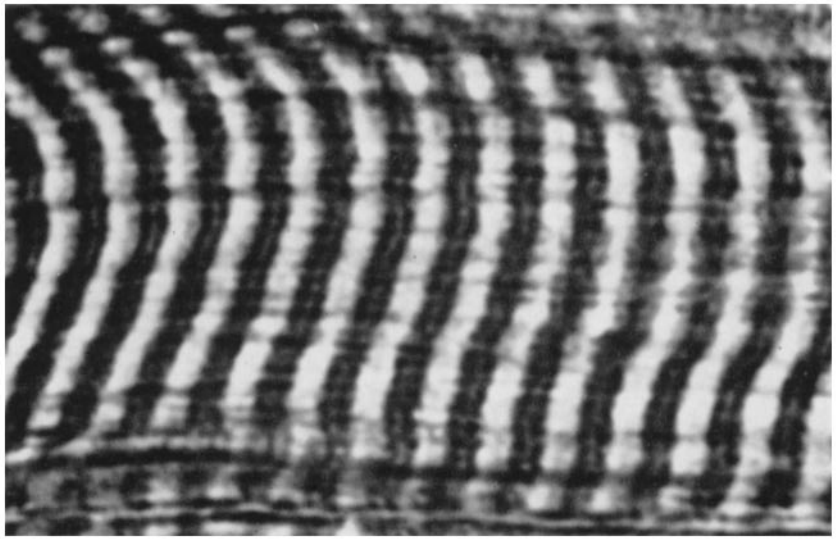


FIGURE 13 Longitudinal section of a muscle fiber stained for LDH by the method described in this report. The LDH staining has a pattern similar to the pattern of cross-striation of the muscle fibers. × 3200 (original magnification, × 1260).

c. The physico-chemical damage to mitochondria which occurs during the procedures for cytochemical localization of enzymes can distort some of the fine structural details (62). Novikoff suggested (63) the application of some "protective measures" such as polyvinyl-pyrrolidone-sucrose, and Scarpelli and Pearse (62) developed the routine use of polyvinyl-pyrrolidone at concentrations sufficient to give a mild hyperosmolarity in the histochemical procedures. Recently, Williams and Whitely (64) have questioned the necessity of such osmolar protection and recommended that it be applied only when the degree of thermal damage has been uniformly standardized. In the method reported here for LDH, the short incubation time

muscles have been confirmed by the application of modern histochemical methods (6–8, 10, 11, 68–73). Basically, there are two types of muscle fibers, a "white" and a "red" type, with some intermediate types. The "white" fibers are usually larger in diameter, have fewer mitochondria, and have low levels of myoglobin (72) and oxidative enzyme activity (6–8, 10, 11, 68–70), but a high level of phosphorylase (7, 8) and glycogen (70, 73). In contrast, the "red" fibers are usually smaller in diameter, have a high level of myoglobin (72), and are rich in mitochondria, oxidative enzymes (6–8, 10, 11, 68–70), lipids (70), and lipase (71), but have a low level of phosphorylase (7, 8) and glycogen (70, 73). Physiologically, it is

well known that the red muscle is tonic while the white muscle is phasic, *i.e.* the red muscle has a longer contraction and relaxation time than the white muscle. These basic physico-chemical and physiological differences have led to speculation that white and red muscle fibers might use different metabolic pathways for energy production (7, 8). It has been suggested that red fibers oxidize fatty acids and lipids as the source of energy, which are utilized *via* the citric acid cycle pathway, while the white fibers break down glycogen as their energy source *via* the glycolytic (Embden-Meyerhof) pathway.

The histochemical localization of high levels of LDH in both red and white muscle fibers as demonstrated in this study is not necessarily in contradiction to the basic concept of two different metabolic pathways for white and red muscle fibers. LDH is one of the several enzymes which have been reported to exist in more than one

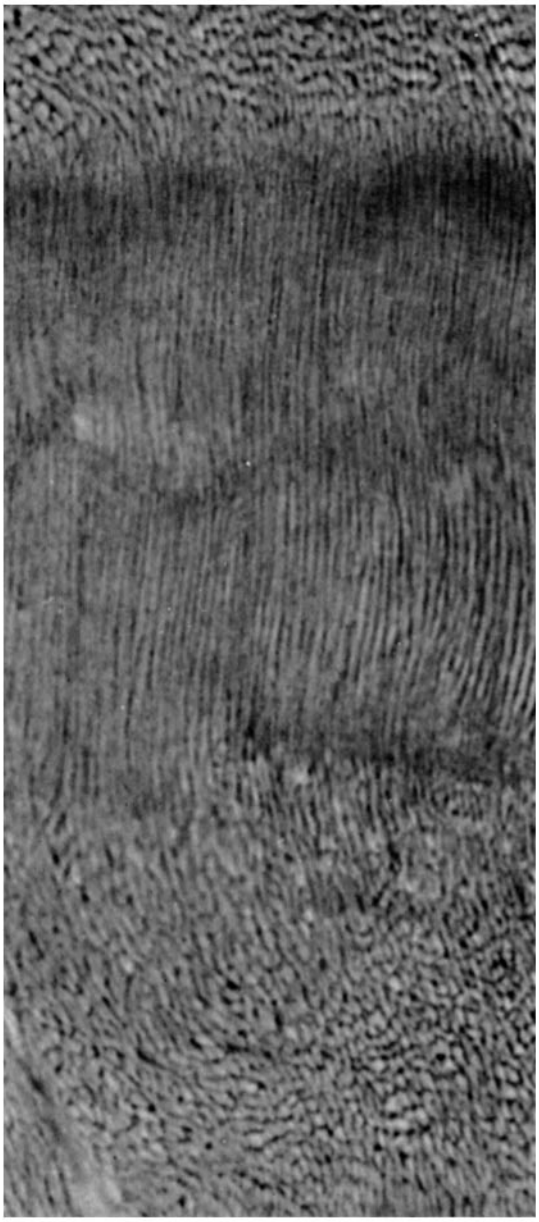


FIGURE 14 A muscle fiber sectioned longitudinally in the center portion and transversally at the ends and stained for LDH according to the method described here.

Note in the mid-portion the longitudinal lines which appear to be closely related to the reticular pattern of the cross-section. × 1200.

molecular form (45, 74). By different methods of separation a number of molecular forms (isozymes) (74) of LDH have been found in different tissues of the same animal (74–76). Recently, Blanchaer and Van Wijhe (77) have reported that the LDH-isozyme patterns of red and white skeletal muscle show some basic differences. Kaplan and Ciotti and Cahn *et al.* (75, 76) have presented evidence that there are two "pure" types of LDH, which appear to be controlled by separate genes and which are different in their catalytic, physical, and immunochemical properties. These have been designated as M (muscle) type and H (heart) type of LDH. There are, furthermore, indications that the M (muscle) type enzyme occurs in tissues with predominantly anaerobic glycolysis, while the H (heart) type enzyme is found in tissues with aerobic metabolism.

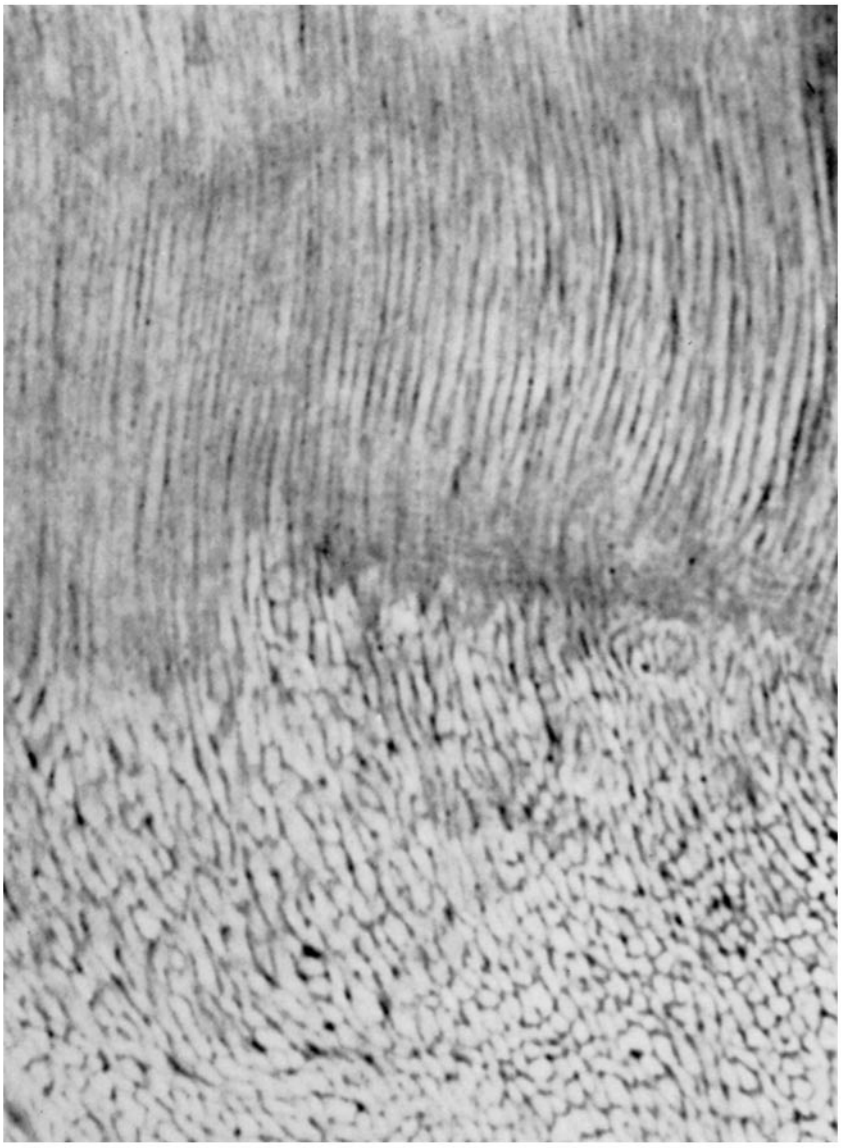


FIGURE 15 Same fiber as Fig. 14 at a higher magnification. × 2160. The close association of the longitudinal lines and the reticulum is more clearly demonstrated.

Although both white and red muscle fibers have been stained with our method for LDH, recent studies in our laboratory (54) indicate that evidently white muscle fibers contain the M (muscle) type of LDH or the hybrids (76) with dominance of the M type, while the red muscle fibers contain the H (heart) type of LDH or the hybrids with dominance of the H fraction.

5. LOCALIZATION OF LDH IN THE SARCOPLASMIC RETICULUM: The description of a fine reticular component in the sarcoplasm of skeletal muscle, which appears in a constant relation to the cross-striated pattern of the myofibrils in longitudinal sections, dates back more than half a century (78). The fine reticular staining pattern in the sarcoplasm of muscle fibers which has been obtained by the application of our LDH method to the white adductor magnus muscle of the rabbit corresponds in many of its features to the descriptions of Veratti (79) who used a modification of Golgi's silver impregnation method. Porter (80), in correlating the recent findings of electron microscopy with light microscopic observation of Veratti, pointed out that evidently Veratti's illustrations represent both the "triads" (81) and the "muscle cell equivalent of the endoplasmic reticulum."

From the striking similarity between our results and those of Veratti (79), it seems most likely that the LDH is localized in the sarcoplasmic reticulum (1); however, further confirmation of our findings must await the direct application of the staining method reported here to electron microscopy. Furthermore, our findings suggest that probably the sarcoplasmic reticulum (or the contents of its lumen) is directly engaged in intermediary metabolism of the striated muscle and, therefore, in the synthesis of energy-rich compounds such as ATP *via* the glycolytic pathway. While such a "metabolic" function for the sarcoplasmic reticulum was suggested recently by Fawcett (82) and by Fawcett and Revel (83), its validity was questioned because of lack of strong evidence (80). Further evidence in favor of such a "metabolic" function of the sarcoplasmic reticulum is presented by the localization of glycogen (84), alkaline

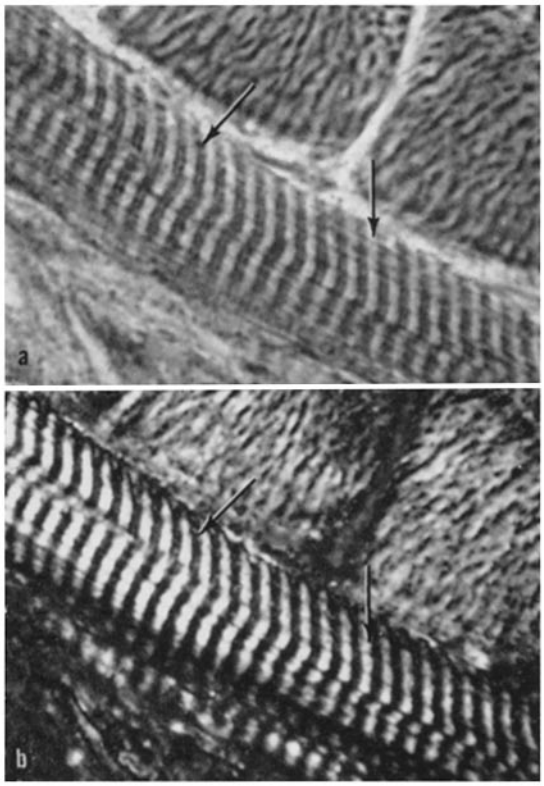


FIGURE 16 *a* and *b* Longitudinal section of a muscle fiber stained for LDH by the method described in this report.

To identify the exact site of LDH staining in relation to the A and I bands of the muscle fibers, the same field has been photographed with ordinary light (Fig. 16 *a*) and with polarized light (crossed prisms) (Fig. 16 *b*).

The LDH staining is localized at the level of the isotropic (I) bands (lefthand arrows) while the anisotropic (A) bands have remained unstained (righthand arrows). × 1600.

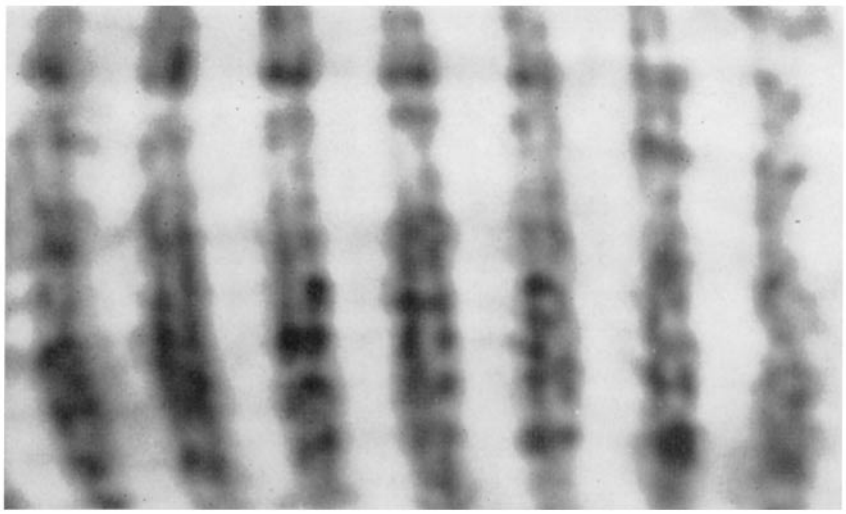


FIGURE 17 High power view of a longitudinal section of a muscle fiber from adductor magnus muscle of the rabbit stained with the method for LDH described here.

Note that the staining at the I bands consists of 2 parallel lines, each located at the A–I junction. The Z discs are unstained. The mitochondria are located on both sides of the Z discs along the I bands. × 6400 (original magnification, × 1260).

phosphatase (85), and ATPase (55, 86) in the sarcoplasmic reticulum.

While the present studies were in progress, Pette and Brandau (87) reported some preliminary observations in which they applied a method, somewhat similar to the histochemical method for LDH reported here, for the localization of two coenzyme-linked dehydrogenases, LDH and glyceraldehyde-3-phosphate dehydrogenase, in muscles of the locust, *Locusta migratoria*. Both enzymes were reported to be localized at the level of isotropic bands. This finding is essentially in agreement with our observations on the longitudinal sections of the adductor magnus muscle of the rabbit, although these authors made no mention of the localization of enzyme in the sarcoplasmic reticulum in their report.

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