CYTOCHEMICAL LOCALIZATION OF TWO GLYCOLYTIC DEHYDROGENASES IN WHITE SKELETAL MUSCLE

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ABSTRACT

The cytochemical localization, by conventional methods, of lactate and glyceraldehyde-3-phosphate dehydrogenases is limited, firstly, by the solubility of these enzymes in aqueous media and, secondly, by the dependence of the final electron flow from reduced nicotinamide-adenine dinucleotide (NADH) to the tetrazolium on tissue diaphorase activity: localization is therefore that of the diaphorase, which in rabbit adductor magnus is mitochondrial. NADH has been found to have great affinity to bind in the sarcoplasmic reticulum, and, therefore, if it is generated freely in the incubation media containing 2,2′,5,5′-tetra-*p*-nitrophenyl-3,3′-(3,3′-dimethoxy-4,4′-phenylene)-ditetrazolium chloride (TNBT) and *N*-methyl phenazonium methyl sulfate (PMS), it can bind there and cause a false staining. Since such a production of NADH can readily occur in the incubation media for glycolytic dehydrogenases due to diffusion of these soluble enzymes from tissue sections, the prevention of enzyme solubilization is extremely important. Fixation in formaldehyde prevented such enzyme diffusion, while at the same time sufficient activity persisted to allow for adequate staining. The incubation media contained PMS, so that the staining system was largely independent of tissue diaphorase activity. Application of these methods to adductor magnus of rabbit revealed by light microscopy, for both enzymes, a fine network which was shown by electron microscopy to represent staining of the sarcoplasmic reticulum. Mitochondria also reacted. These findings add further support for the notion that the sarcoplasmic reticulum is probably involved in glycolytic activity.

INTRODUCTION

The accurate cytochemical localization of the glycolytic enzymes, lactate dehydrogenase (LDH)[1] and glyceraldehyde-3-phosphate dehydrogenase (GAPD), is complicated by two factors. Firstly, both these enzymes are soluble in aqueous media

[1] The following abbreviations will be used in this paper: EDTA, ethylenediaminetetracetic acid, disodium salt; GAPD, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; NAD, oxidized nicotinamide-adenine dinucleotide; NADH, reduced nicotinamide-adenine dinucleotide; Nitro-BT, 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride; PMS, *N*-methyl phenazonium methosulfate (phenazine methosulfate); TNBT, 2,2′, 5,5′-tetra-*p*-nitrophenyl-3,3′-(3,3′-dimethoxy-4,4′-phenylene)-ditetrazolium chloride; Tris, tris(hydroxymethyl)-aminomethane.

(1–6). This may lead to loss of enzyme from tissue sections by diffusion from intracellular sites (4–6), with consequent falsely weak cytochemical reaction and possible false localization. Secondly, in the cytochemical methods, the final electron flow from the reduced nicotinamide-adenine dinucleotide (NADH) to the tetrazolium is dependent on NADH-diaphorase activity (4–8). The intracellular localization of the final reaction product (formazan) is, therefore, necessarily that of the NADH-diaphorase, which may not necessarily be that of LDH or GAPD activity. Furthermore, if the NADH-diaphorase is low in activity and therefore possibly rate-limiting, falsely low cytochemical reaction may be obtained, which may be contrary to biochemical data (6, 9).

Recently, in regard to the localization of LDH in white muscle of rabbit (adductor magnus), Fahimi and Amarasingham partly prevented enzyme diffusion by applying all the ingredients of the histochemical reaction in the form of a gelatin film to tissue sections (4). Phenazine methosulfate was incorporated in the gelatin film as an electron carrier to bypass the NADH-diaphorase, which in this muscle is confined only to mitochondria (4). By this technique, LDH in high activity was localized to a network throughout the sarcoplasm of muscle fibers, as well as to sparsely scattered dots and rods, presumably mitochondria. It was suggested, pending electron microscope confirmation, that the network represented the sarcoplasmic reticulum. In the present study, for the prevention of the enzyme diffusion and for the preservation of the fine structure we have used prefixation in formaldehyde. The relationship of the fine network which stains for LDH to the ultrastructure of the muscle has been investigated, and it is confirmed that the formazan deposits are located on segments of the sarcoplasmic reticulum.

Previously Himmelhoch and Karnovsky (10) applied their method for GAPD to skeletal muscle and reported weak reaction in white muscle fibers, which is contrary to what would be expected from biochemical data (11). Localization was presumably in mitochondria, and it was fully realized (10) that the localization was probably that of the NADH-diaphorase, and that the GAPD was solubilized in part.

Utilizing the "incubation mixture film method" of Fahimi and Amarasingham (4), as well as formaldehyde-fixed tissues, and by using PMS as an electron carrier, we have now evidence (12) suggesting that in white muscle (adductor magnus) of rabbit the localization of GAPD is similar to that of LDH at the light and electron microscope level, i.e., the activity is largely localized in the sarcoplasmic reticulum and mitochondria.

Further, in view of the possibility that the sarcoplasmic reticulum localization of glycolytic dehydrogenases could be an artifact due to substantivity, and binding of tetrazolium salts (13) and other components of the incubation medium to the sarcoplasmic reticulum, we have investigated the problems of nonenzymatic reduction and nonspecific binding of these components to white muscle. These experiments suggest that, when enzyme diffusion is prevented, the localizations obtained are most probably reliable.

MATERIALS AND METHODS

Light Microscopy

Fresh frozen sections of adductor magnus of albino rabbits were cut in a cryostat at a thickness of 2 to 4 μ. The technique of incubation used was the incubation mixture film method, which has previously been described in detail (4). In brief, the ingredients of the medium are incorporated into a 5% gelatin film, on which the fresh frozen sections, mounted on cover slips, are placed.

Formaldehyde-fixed tissues were sectioned in a cryostat, affixed to gelatinized cover slips, and incubated in an aqueous medium.

Formaldehyde-Fixation

Pieces of adductor magnus, not more than 2 mm thick, were fixed at 4°C in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.6, containing 5% sucrose, for 2½ to 3 hr. The formaldehyde was freshly prepared from paraformaldehyde (45). After fixation, the tissues were washed overnight in 0.1 M phosphate buffer, pH 7.6, containing 5% sucrose. Before freezing and incubation the pieces of muscle were rinsed several times in the same buffer solution without sucrose.

Electron Microscopy

Tissues fixed in formaldehyde were cut on a freezing microtome at 40 μ and were then transferred to the incubation medium held at 25°C. Incubation time ranged from 10 to 40 min in different experiments. After completion of the incubation, the sections were washed thoroughly in several changes of buffer and were then postfixed for 1 hr at 4°C in 1% osmium tetroxide in 0.06 M phosphate buffer,

pH 7.6, containing 5% sucrose. Dehydration was done in an ascending series of alcohols, and the sections were embedded either in Vestopal W, according to the method of Kurtz (14), or in Epon, according to the method of Luft (15).

For solely ultrastructural studies, tissues were fixed in 2.5% glutaraldehyde for 3 hr, washed in 0.1 M phosphate buffer with 5% sucrose, postfixed in osmium tetroxide, and embedded in Epon (15).

Thin sections were cut on an LKB ultratome and examined in an RCA-3F electron microscope, either not counterstained, or counterstained for added contrast with uranyl acetate (16) and/or alkaline lead (17). Counterstaining did not affect the final reaction product of the enzymatic reaction.

Incubation Media

LACTATE DEHYDROGENASE: The medium for electron microscopy was slightly modified from that used previously (4), to give the maximum contrast in the shortest possible incubation time.

	Stock solution *mg/ml*	Aliquot *ml*
TNBT	2	4
NAD	7	1
PMS	2.7	1
Lithium lactate	192	1

The final volume was made up to 10 ml with 0.2 M Tris buffer, pH 7.2.

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE: The substrate was prepared as described previously (10).

	Stock solution *mg/ml*	Aliquot *ml*
TNBT	2	2
NAD	7	0.5
GAPD substrate as prepared in (10).		0.3–0.4
PMS	2.7	0.5
EDTA	6	0.3

The final volume was made to 5 ml with 0.2 M phosphate buffer, pH 7.2.

NADH-DIAPHORASE: The incubation medium contained:

	Stock solution	Aliquot *ml*
TNBT	2 *mg/ml*	2
NADH	7 *mg/ml*	0.5
Tris buffer, pH 7.2	0.2 M	2.5

All incubations with media containing PMS were carried out in complete darkness, to avoid the photosensitive reduction of this compound. The stock solutions were made up in water. The TNBT was dissolved with warming, and filtered. For light microscopy, the final concentration of TNBT was 0.4 mg/ml and of PMS 0.05 mg/ml of the incubation medium. In the case of the GAPD experiments, where the incubation media were unstable and the tetrazolium started to be reduced, sections were immediately removed, washed, and transferred to freshly prepared incubation media.

Controls

I. Substrate-Free Media

Sections were incubated in media lacking the substrate.

II. Inhibitors

Sections were treated with 10^{-3} N solution of iodine, 10^{-4} M copper acetate, 10^{-2} M silver nitrate, and 10^{-2} M mercury acetate for 5 min, washed thoroughly with distilled water, and incubated for LDH or GAPD activity up to 60 min. In other experiments, 1 ml of a 1 M solution of potassium oxalate or 1 ml of a 0.1 M solution of sodium oxamate was added to the incubation medium for LDH and the sections were reacted for 30 min.

III. Binding Experiments

BINDING OF OXIDIZED TNBT: Sections were treated with a solution of TNBT in water (2 mg/ml) for 5 min, washed, and then placed in a solution of 1% ascorbic acid, or 0.1 N sodium hydroxide.

BINDING OF PMS: Sections were treated with a solution of oxidized PMS (2.7 mg/ml) for 5 min, washed, and the PMS was reduced by exposing the sections to a solution of 1% ascorbic acid; then the sections were washed and finally exposed to TNBT (2 mg/ml).

In another experiment, PMS (2.7 mg/ml) was first reduced with an equal volume of 1% ascorbic acid and then applied to sections for 5 min. Sections were then washed and exposed to TNBT (2 mg/ml).

BINDING OF NADH: Sections were treated with a solution of NADH (7 mg/ml) for 5 min, washed, and exposed to a solution containing PMS, TNBT, and Tris buffer at the same concentrations as in the incubation medium for LDH. In another experiment, sections were added to a solution containing equal parts of PMS (2.7 mg/ml) and NADH (7 mg/ml) for 5 min, washed, and then exposed to TNBT (2 mg/ml).

IV. Influence of Addition of External LDH

Sections were treated for 5 min with an inhibitor such as 10^{-3} N solution of iodine, washed, and in-

cubated in the LDH medium for 30 min. Since no staining was noted, the incubation medium was reduced by addition of 1 ml of a solution containing LDH extracted from rabbit muscle. In other experiments the medium was reduced by addition of ascorbic acid (1%), or phenylhydrazine (1%).

All control experiments were carried out on formaldehyde-fixed sections, since the treatment of sections in aqueous media would have solubilized the enzymes. Control I was also carried out on fresh frozen sections. Except for controls I and IV, which were processed for both light and electron microscopy, all other controls were examined only by light microscopy. It should be noted that control experiments are better evaluated at the light microscope level because of the sampling error in electron microscopy. The purpose of controls I and II was to show that the reactions were substrate dependent (I) and enzymatic in nature (II). The purpose of controls III and IV was to investigate the substantivity (binding) of different ingredients of the incubation medium for particular components of the muscle cells, such as the sarcoplasmic reticulum.

RESULTS

Light Microscopy

LDH AND GAPD: The localization by light microscopy of LDH activity, utilizing PMS and the incubation mixture film method, has previously been given in detail (4). Because the results with GAPD are similar, only these will be presented briefly here.

In cross-sections of the muscle, enzymatic activity was localized in a prominent, fine network throughout the sarcoplasm (Fig. 1). In addition, small dots observed at the interstices of the network also stained. In longitudinal sections a pattern of cross-striation and a pattern of longitudinal lines, parallel to the longitudinal axis, were both seen. The cross-striation staining pattern for LDH activity has previously been localized at the A–I junction (4). If PMS was omitted from the incubation medium, the reaction product was mainly localized in dots, rods, and so called stellate formations (18) as described here for NADH-diaphorase.

When formaldehyde-fixed sections were incubated in an aqueous medium, localizations similar to those seen with the incubation mixture film method were obtained for both LDH and GAPD activities. Although enzymatic activities were considerably inhibited by the fixation, sufficient activity was preserved to give satisfactory preparations. Diffusion of enzyme after fixation appeared to be minimal, despite incubation in aqueous media.

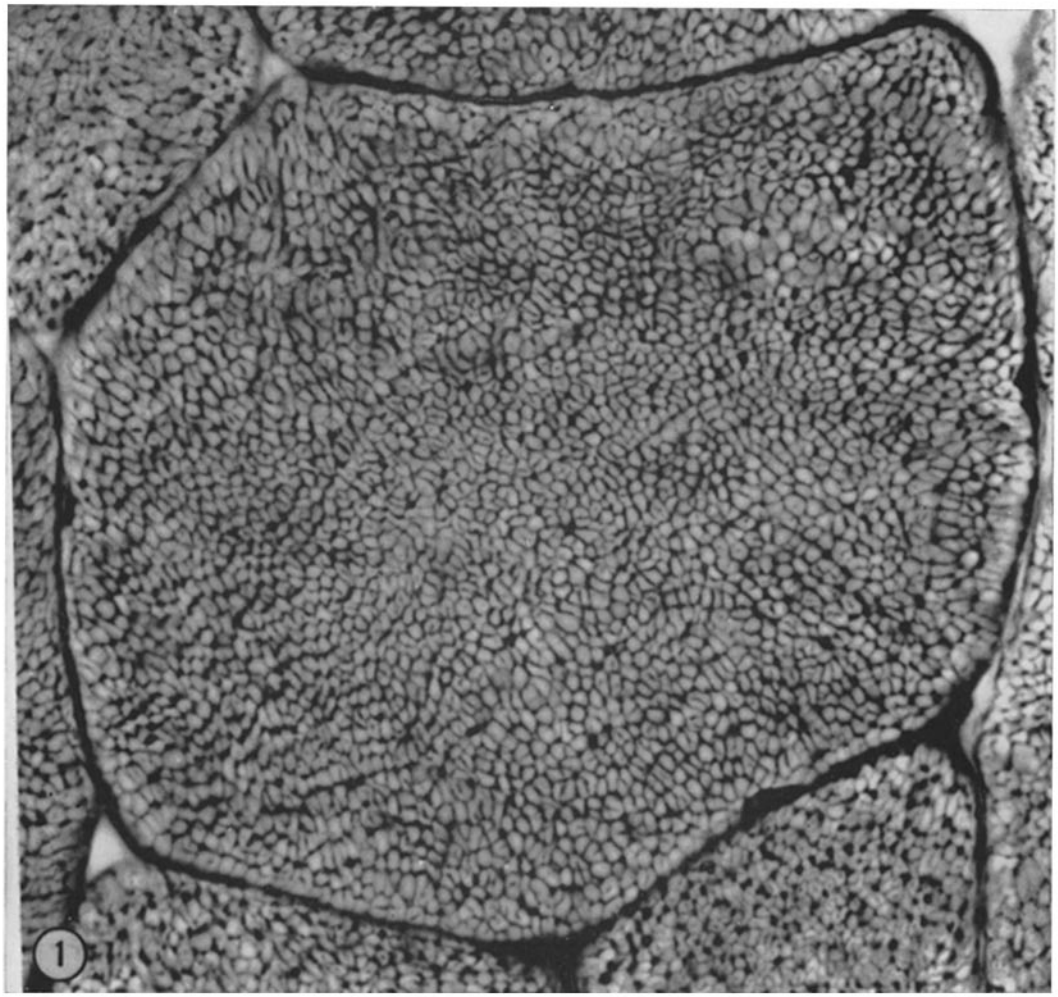
NADH-DIAPHORASE: This enzyme also survived the formaldehyde fixation quite well, and no difference was noted in the localization in sections of fixed tissue and in fresh frozen sections. Histochemically, "red" muscle fibers stained more prominently than the "white" fibers. Cytochemically, the staining was confined to dots, rods, and so called stellate formations (18) (Fig. 2). After prolonged incubation (60 min), or occasionally with thick sections (15 μ), or in sections with physical damage due to slow freezing and thawing, a generalized faint staining of a fine network in the sarcoplasm was also noted. The intensity and the extent of this staining, however, were much less in comparison to the prominent staining obtained after incubation for LDH or GAPD activity. The tissues reacted for NADH-diaphorase were not examined by electron microscopy.

Electron Microscopy

ULTRASTRUCTURE OF THE WHITE MUSCLE ADDUCTOR MAGNUS OF RABBIT: Here,

FIGURE 1 Cross-section of the white adductor magnus muscle of rabbit, reacted for GAPD activity for 10 min, using the incubation mixture film method (4). There is a prominent staining of a fine network in the sarcoplasm of the large white fiber. In addition, in the interstices of the network, darkly staining bodies, presumably mitochondria, are also noted. The capillaries situated between the muscle fibers also stain prominently. Light micrograph. × 2100.

FIGURE 2 Cross-section of the same muscle, reacted for NADH-diaphorase activity for 30 min. The staining is confined to dots, rods, and the so called stellate formations (18), which all represent mitochondria. The reticular staining is only minimal and not at all comparable with the results obtained for glycolytic dehydrogenases, LDH and GAPD. Light micrograph. × 1300.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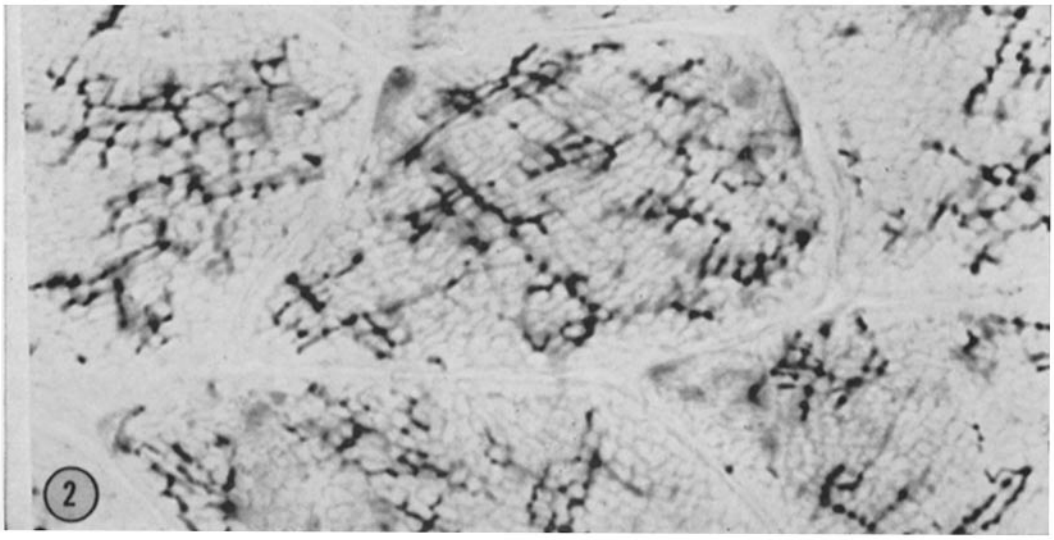
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only the sarcoplasmic reticulum and the mitochondria are described. Fig. 3 is a low-power electron micrograph of a longitudinal section of this muscle. The interfibrillar sarcoplasm appears markedly well developed, in contrast to the sparseness of the mitochondria. At higher magnification (Fig. 4) it is evident that the mitochondria are small, sparse, and contain only a few cristae, while, in contrast, the segments of the sarcoplasmic reticulum are very prominent and appear in close association with particles of glycogen. There are two sets of "triads", one on either side of the Z line at the junction of the A–I bands. The mitochondria are usually located at the I band level on either side of the Z line.

ULTRASTRUCTURAL LOCALIZATON OF LDH AND GAPD: In sections reacted for LDH and GAPD, an electron-opaque material was deposited in close association with the elements of the sarcoplasmic reticulum (Figs. 5 and 6). The reactions obtained with the GAPD method were, in general, less intense than those obtained with the LDH method, but the distribution of the reaction product was the same. In lightly reacted preparations the reaction product was seen as a grayish deposit apparently filling the lumen of the sarcoplasmic reticulum or alternatively deposited on the surface of the reticulum (Fig. 7). In heavily reacted preparations, structural detail was somewhat obscured, and the material apparently spilled out into the perireticular sarcoplasm (Fig. 5). It should be emphasized that the reaction product had low electron opacity, and, therefore, fairly heavily reacted preparations were required to visualize clearly the sites of activity. The longitudinal elements of the sarcoplasmic reticulum reacted well and the regions of triads were strongly reactive (Figs. 5 to 7), but it was difficult to resolve whether or not the intermediate element (the T system (21)) was also participating in the reaction. Mitochondria reacted positively (Fig. 7). However, the formazan obscured the ultrastructural details in these small and sparse organelles, a fact which made certain identifications sometimes difficult.

It may be noted here that adductor magnus of rabbit is essentially a white muscle, but red fibers are also admixed with the white fibers (4). The mitochondria are much more numerous, larger, and more prominent in the red fibers than in the white fibers. Cytochemically prominent reactions for LDH and GAPD were noted in the sarcoplasmic reticulum and the mitochondria of these red fibers. The reaction product in mitochondria filled the whole organelle and obscured the cristae. Extensive observations on red muscle fibers and mitochondria were not made in this study.

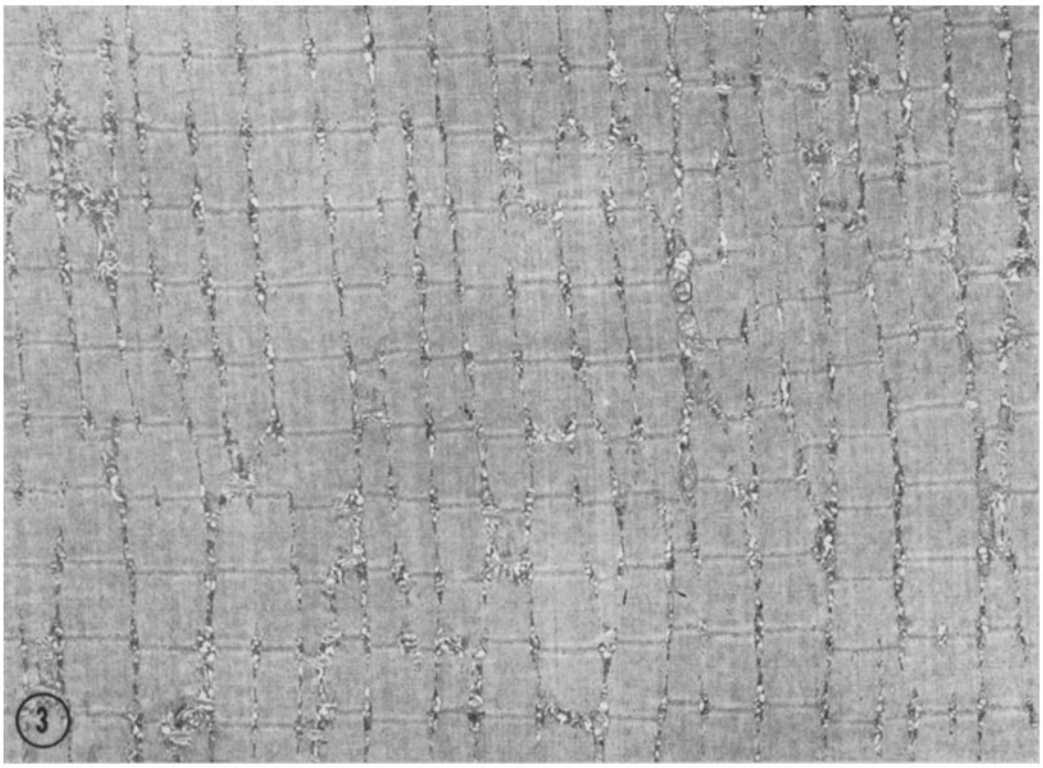
Controls

SUBSTRATE-FREE MEDIA

No electron-opaque reaction product was noted in sections incubated in the absence of the substrate (Fig. 8), using the high concentrations of PMS and TNBT as suggested for electron microscopy (see Methods). These same sections, however, showed a slight degree of staining when examined in the light microscope. With the lower concentrations of PMS and TNBT, as suggested for light microscopy (see Methods), there was a faint staining of the sarcoplasmic reticulum in the absence of the substrate. This staining occurred shortly after the incubation and did not increase in intensity even after prolonged incubation. It was less pronounced in formaldehyde-fixed sections than in fresh frozen sections.

FIGURE 3 A low-power electron micrograph of the white adductor magnus muscle of rabbit. The interfibrillar sarcoplasm is quite prominent and is surrounded by large amounts of prominently staining particulate glycogen. The mitochondria are small and sparse. Fixation, 2.5% glutaraldehyde, osmium tetroxide; stained with alkaline lead (17). × 6000.

FIGURE 4 A higher magnification of the same muscle as in the previous figure. The cisternae of the sarcoplasmic reticulum appear well developed and are in close association with particles of glycogen (*G*). There are two sets of triads (*T*), one on either side of the Z line (*Z*) at the junction of the A and I bands. The mitochondria (*M*) are small, have only a few cristae, and are mainly located on both sides of the Z lines. Same conditions as in Fig. 3. × 18,000.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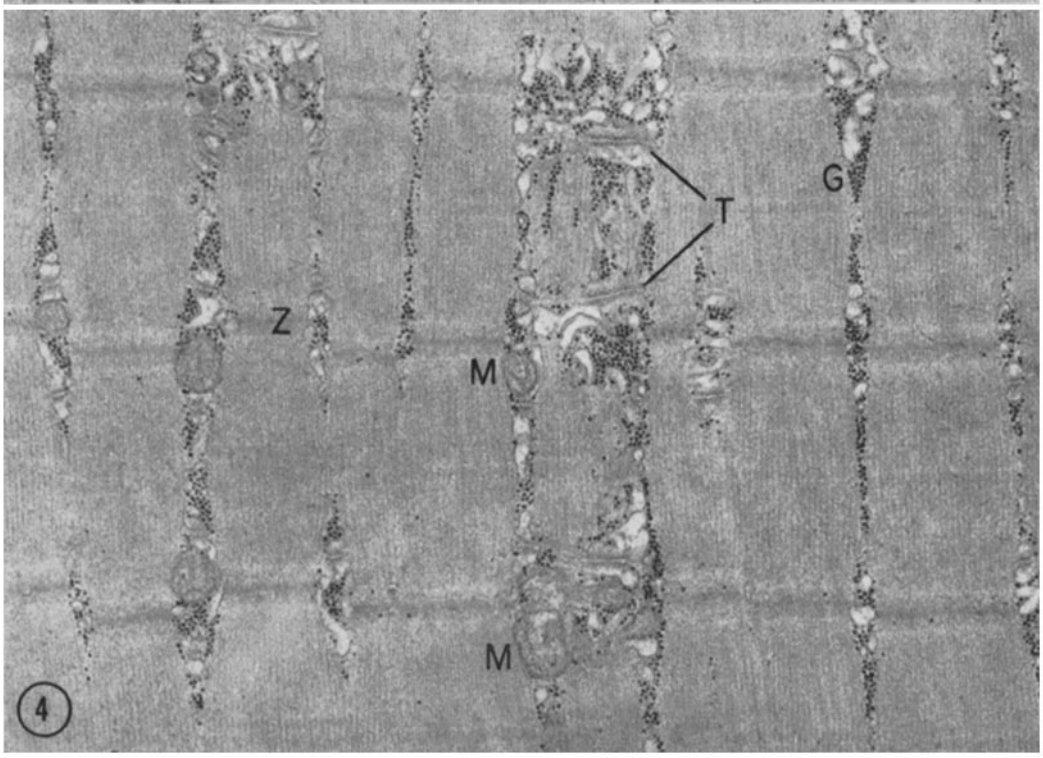
T
G
Z
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M
4

Inhibitors

There was complete absence of staining in sections which were treated with different enzyme inhibitors. The addition of oxalate and oxamate, however, did not inhibit the cytochemical reaction for LDH.

Binding Experiments

BINDING OF TNBT: Sections treated with TNBT, washed, and then exposed to an alkaline solution, such as sodium hydroxide, revealed immediately a prominent staining. The formazan was deposited not only on the mitochondria and the sarcoplasmic reticulum but also on the myofibrils and even occasionally on nuclei (Fig. 9). This suggests that TNBT in oxidized form is bound by all components of the muscle cell and that a special affinity for a particular organelle does not exist. However, if the TNBT was reduced with ascorbic acid, a prominent staining of the sarcoplasmic reticulum and mitochondria was noted which resembled the results obtained for LDH and GAPD activities.

BINDING OF PMS: Sections treated with PMS and washed showed a slight, greenish staining of the entire cut surface of the muscle fibers. If these sections were then treated with ascorbic acid to reduce the PMS, washed, and then exposed to TNBT, no reduction of the TNBT occurred. Similarly, the sections treated with PMS reduced by ascorbic acid either did not show any staining at all, or only showed a slight, patchy staining over parts of the sections.

BINDING OF NADH: If sections were treated with NADH, washed with distilled water, and then exposed to a solution containing PMS and TNBT, a prominent staining of the sarcoplasmic reticulum and mitochondria occurred (Fig. 10). This finding suggests that NADH has a great affinity for the same sites where the enzymes LDH and GAPD are apparently localized. It further indicates that, if NADH is generated freely in the incubation medium in which PMS and TNBT are present, a prominent staining of the sarcoplasmic reticulum may occur. Furthermore, if sections were exposed to a solution of PMS reduced by NADH, washed, and then exposed to TNBT, a prominent staining of the sarcoplasmic reticulum and mitochondria also occurred.

Influence of External LDH

If the endogenous LDH activity in tissue sections is inhibited by a short treatment with iodine and if these sections are placed in the incubation medium for LDH and this medium is reduced artificially by addition of the exogenous enzyme, only a slight, patchy, and irregular binding of formazan to tissue sections occurs. While the staining in parts of the sections is confined to sarcoplasmic reticulum and mitochondria, in other parts it appears only on myofibrils, leaving the reticulum unstained (Fig. 11). If the incubation medium, however, is reduced by ascorbic acid or by phenylhydrazine, the staining is more intense and occurs mainly in the sarcoplasmic reticulum and mitochondria.

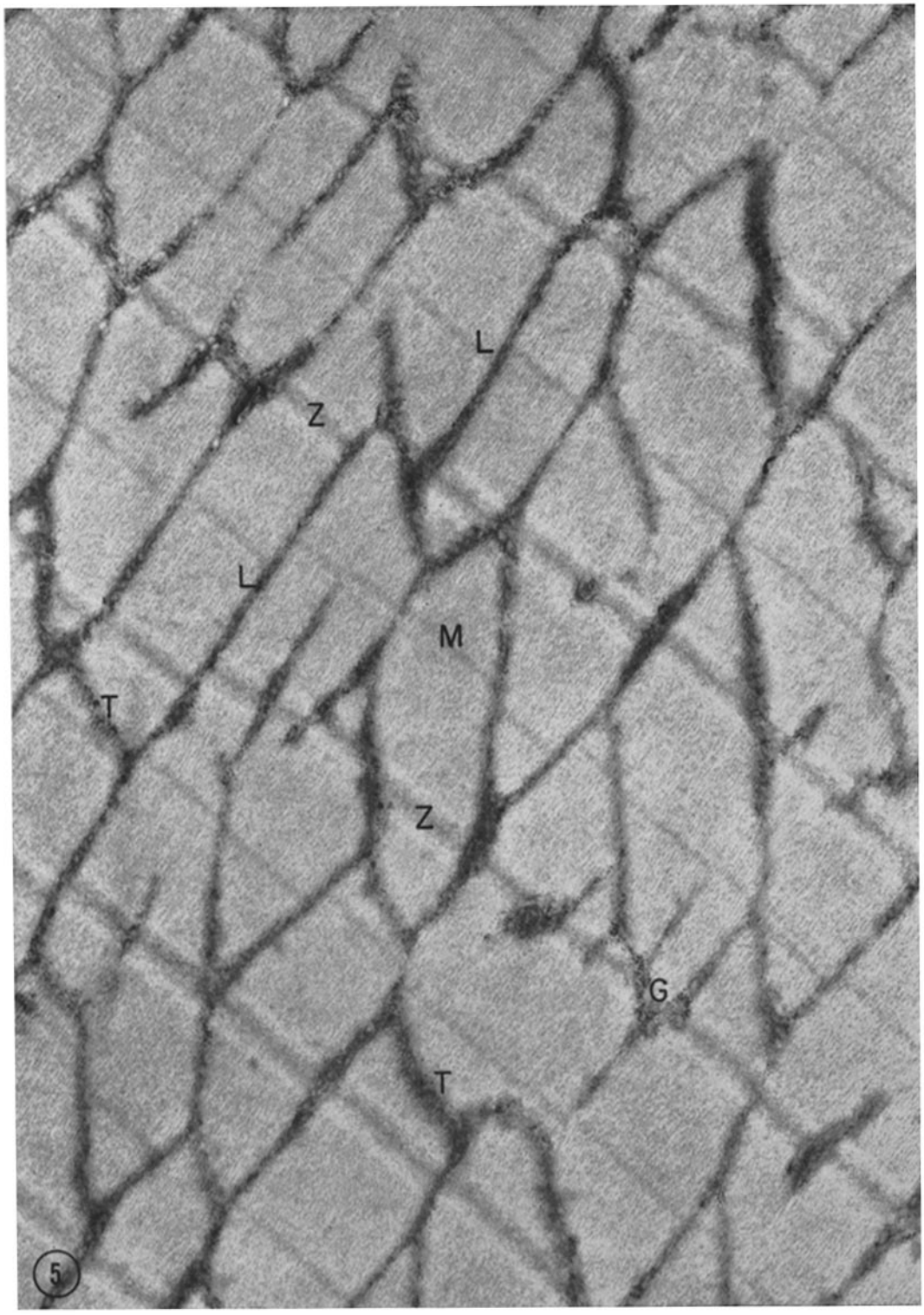
DISCUSSION

The electron cytochemical localization of LDH in the sarcoplasmic reticulum as presented here confirms and extends previous observations made with the light microscope (4). This work also extends such localization to another glycolytic dehydrogenase, GAPD.

Correlation of Biochemical Findings with Ultrastructure

Le Page and Schneider in 1948, by using the method of differential centrifugation of homogenized liver and tumor tissues, found the glycolytic enzymes mainly in the supernatant or soluble fraction (1). Subsequent biochemical studies have essentially confirmed their original findings (2, 3). In skeletal muscle the ultrastructural basis for compartmentalization is given by the membranes of the sarcoplasmic reticulum and the mitochondria. The contractile myofibrillar component is located in an environment which is separated

FIGURE 5 Oblique section of a white muscle fiber, reacted for LDH activity for 15 min. A strongly reacting network is delineated, representing staining in, and somewhat obscuring, the elements of the sarcoplasmic reticulum. In some areas components of the reticulum are, however, discernible. Both longitudinal (*L*) and transverse (*T*) elements of the reticulum react. *M*, M band; *Z*, Z line; *G*, glycogen. × 25,000.


L
Z
L
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5

from the inner phases of the above two systems. On the basis of biochemical analysis of the fractions derived from the fragmentation of the sarcotubular system, Margreth et al. (19) suggested that this system is involved in the regulation of the glycogen cycle and the Embden-Meyerhof glycolytic pathway. Several of the glycolytic enzymes including LDH, however, were solubilized during the homogenization procedures and were recovered in the soluble fraction. As demonstrated previously (4), glycolytic dehydrogenases are easily solubilized from tissue sections and could therefore also be readily solubilized from their intracellular sites by the traumatic techniques of homogenization. The cytochemical demonstration of glycolytic dehydrogenases in formaldehyde-fixed material suggests that, if the enzyme diffusion is prevented, these enzymes can be stained in the inner phase or the lumen of the sarcoplasmic reticulum (Fig. 7). Such an intracellular localization would provide a morphological basis for the separation of the acidic products of glycolysis (e.g. lactic acid) from the sensitive myofibrillar environment and for the segregation of the anerobically produced ATP from the actomyosin ATPase and the myofilaments. Furthermore it is interesting to note that in fast-acting white skeletal muscles, which depend for their energy metabolism primarily on glycolysis (11), the elements of the sarcoplasmic reticulum are markedly well developed (20, 21), whereas in red skeletal muscle, which depends primarily on mitochondrial aerobic metabolism (11), the sarcoplasmic reticulum is small and only poorly developed (20, 21). On the basis of these purely morphological observations, Porter and Franzini-Armstrong (22) recently suggested that the sarcoplasmic reticulum is involved in synthesis of ATP via the glycolytic pathway. Our findings emphasize their views.

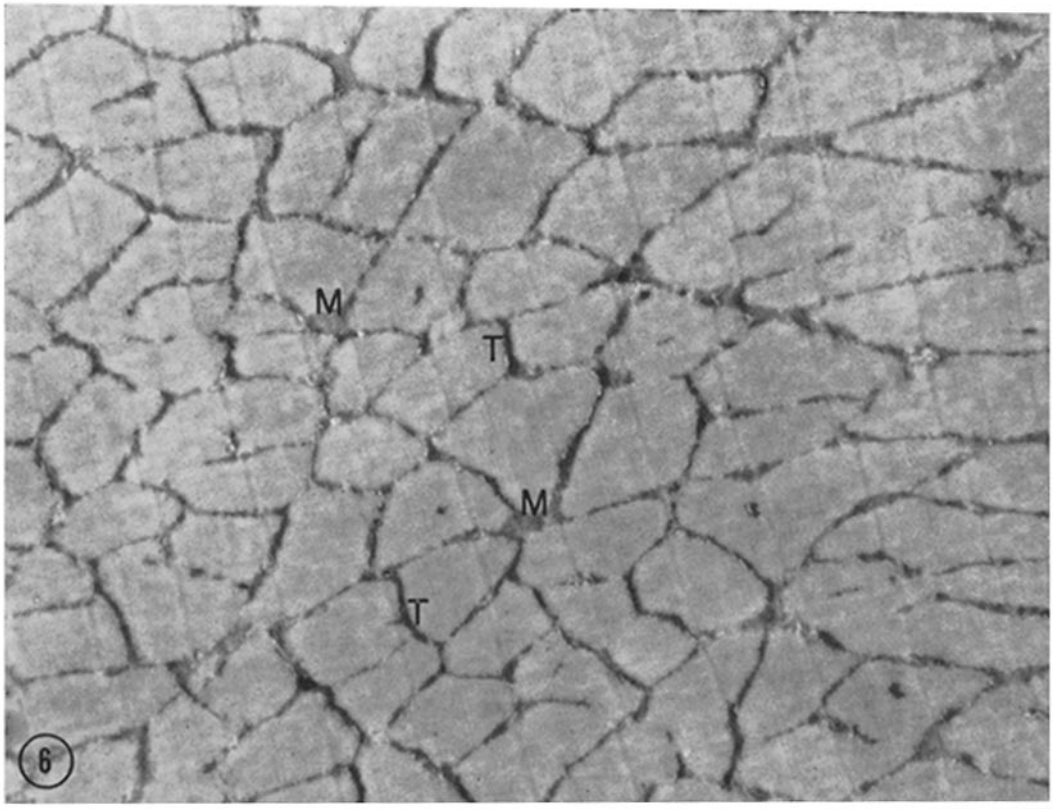
Possible Sources of Artefactual Staining of the Sarcoplasmic Reticulum

Even though there are many indications suggesting that the sarcoplasmic reticulum is actually the intracellular site of anerobic glycolysis (see previous paragraph), we must exclude the possibility that the cytochemical localization of glycolytic dehydrogenases, as presented here, is an artefact of the staining procedure. Recently Smith (13) has questioned the accuracy of the tetrazolium techniques for glycolytic dehydrogenases at the intracellular level. She argues that, if Nitro-BT is reduced in the cytoplasm, it could precipitate on the membranes of the sarcoplasmic reticulum, thus giving a false localization. It is interesting to note that Smith obtained (13) a "reticular" staining pattern for all dehydrogenases including the mitochondrial enzyme succinic dehydrogenase and that she did not prevent the diffusion of glycolytic dehydrogenases from tissue sections. Furthermore, some of the reticular pattern may represent mitochondrial stellate formations (18).

In the present study, we have investigated the substantive properties of the different components of the cytochemical staining medium for glycolytic dehydrogenases. The oxidized TNBT was found to bind not only to the sarcoplasmic reticulum and mitochondria but also to the myofibrils and even to nuclei. Oxidized PMS did not bind to tissue sections, but PMS added to NADH stained the sarcoplasmic reticulum and mitochondria. It was interesting to note that NADH

FIGURE 6 Oblique section of a white muscle fiber, reacted for GAPD activity for 20 min. A network between the myofibrils is prominently delineated (cf. Fig. 1), representing staining of the sarcoplasmic reticulum. More intensely reacting transverse elements at or near the A–I junction probably represent triads (*T*). Staining of small mitochondria (*M*) at the interstices of the reticulum is questionable. At the right of the micrograph, where the section is cut longitudinally, staining of the longitudinal elements of the reticulum is evident. × 9,500.

FIGURE 7 Longitudinal section of a white muscle fiber, lightly reacted for LDH activity (10 min). Grayish, electron-opaque reaction product is seen in the lumen of the sarcoplasmic reticulum. Longitudinal elements (*L*) and the transverse elements (*T*) (triads) both react. Glycogen particles (*G*) are located at the periphery of the deposits of formazan. Small mitochondria (*M*) situated near the Z line (*Z*) also apparently react, but their structural detail is obscured. × 25,000.



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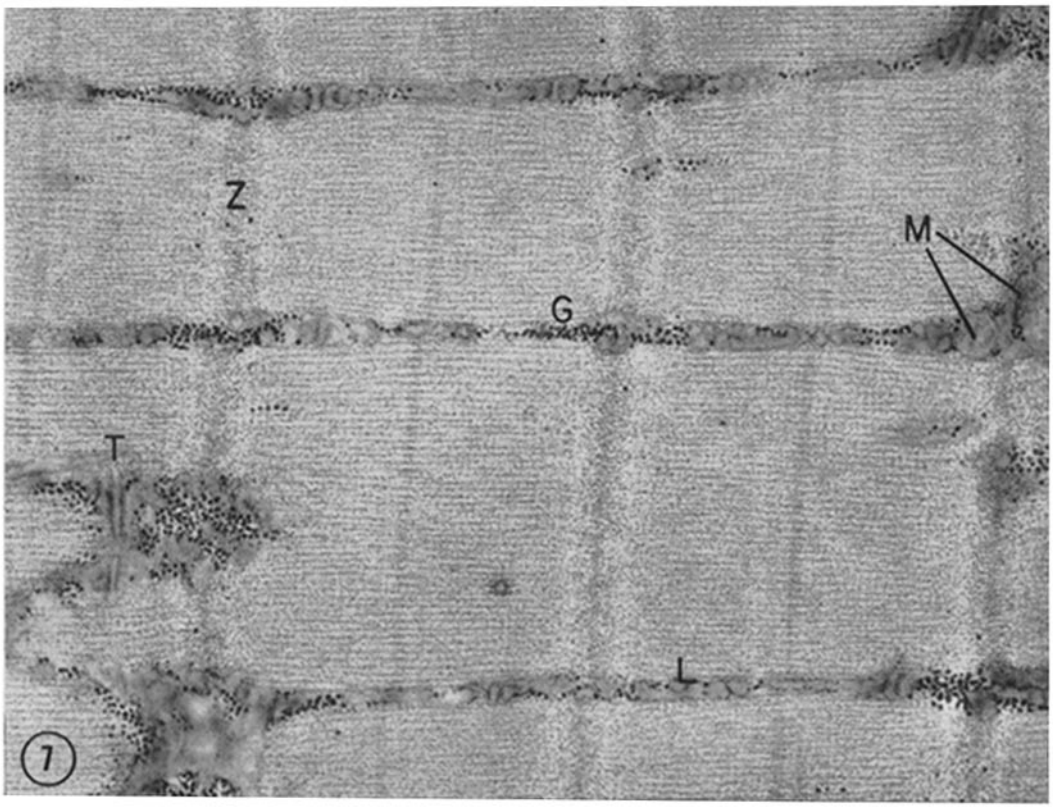

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had a great affinity to bind to the sarcoplasmic reticulum. Even though this binding could be due to affinity of NADH (coenzyme) for its respective enzymes in the sarcoplasmic reticulum, such a binding in the presence of PMS and TNBT can cause a marked staining of the sarcoplasmic reticulum, which is identical with the results obtained for glycolytic dehydrogenases. This, however, would mean that if NADH is generated freely in the incubation medium, due to leakage of the soluble dehydrogenases, an artefactual staining of the sarcoplasmic reticulum and mitochondria would be obtained. It is, therefore, imperative that the *enzyme solubilization be prevented.* The method of incubation mixture film, as suggested previously (23, 24), diminishes the enzyme leakage markedly (4), even though a small amount of diffusion into the gelatin film, which stains the gelatin, occurs. This small amount of diffusion, however, could perhaps be sufficient to cause a false *intracellular* localization. Even though for histochemical studies the incubation mixture film method appears to be most suitable, especially if fresh frozen sections are used, for fine structural cytochemistry the use of prefixed tissues is essential. The fixation in formaldehyde not only preserves the ultrastructure but also allows sufficient enzymatic activity to survive to permit adequate staining reactions. Novikoff and Masek (25) also found that fixation in formaldehyde preserved lactic dehydrogenase and NADH-diaphorase activities. Similarly Fasske et al. (44) have reported preservation of LDH and GAPD activities after fixation in Baker's formaldehyde. It should be noted that fixed sections, incubated in aqueous media, gave the same results by light microscopy as did fresh frozen sections incubated on gelatin films, but whereas the latter required 3 to 5 min for adequate reaction for LDH activity, the former required 10 to 15 min for equivalent reaction: for GAPD the incubation times were 10 to 15 min and 20 to 40 min, respectively. By electron microscopy of fixed sections, reaction product could be reproducibly visualized at the sites described. It is fully realized that failure to reveal activity at any one site is not significant because of the possibility of enzyme inhibition during the fixation and subsequent manipulations. At the present time, we do not know how to exclude the possibility of slight intracellular enzyme shifts.

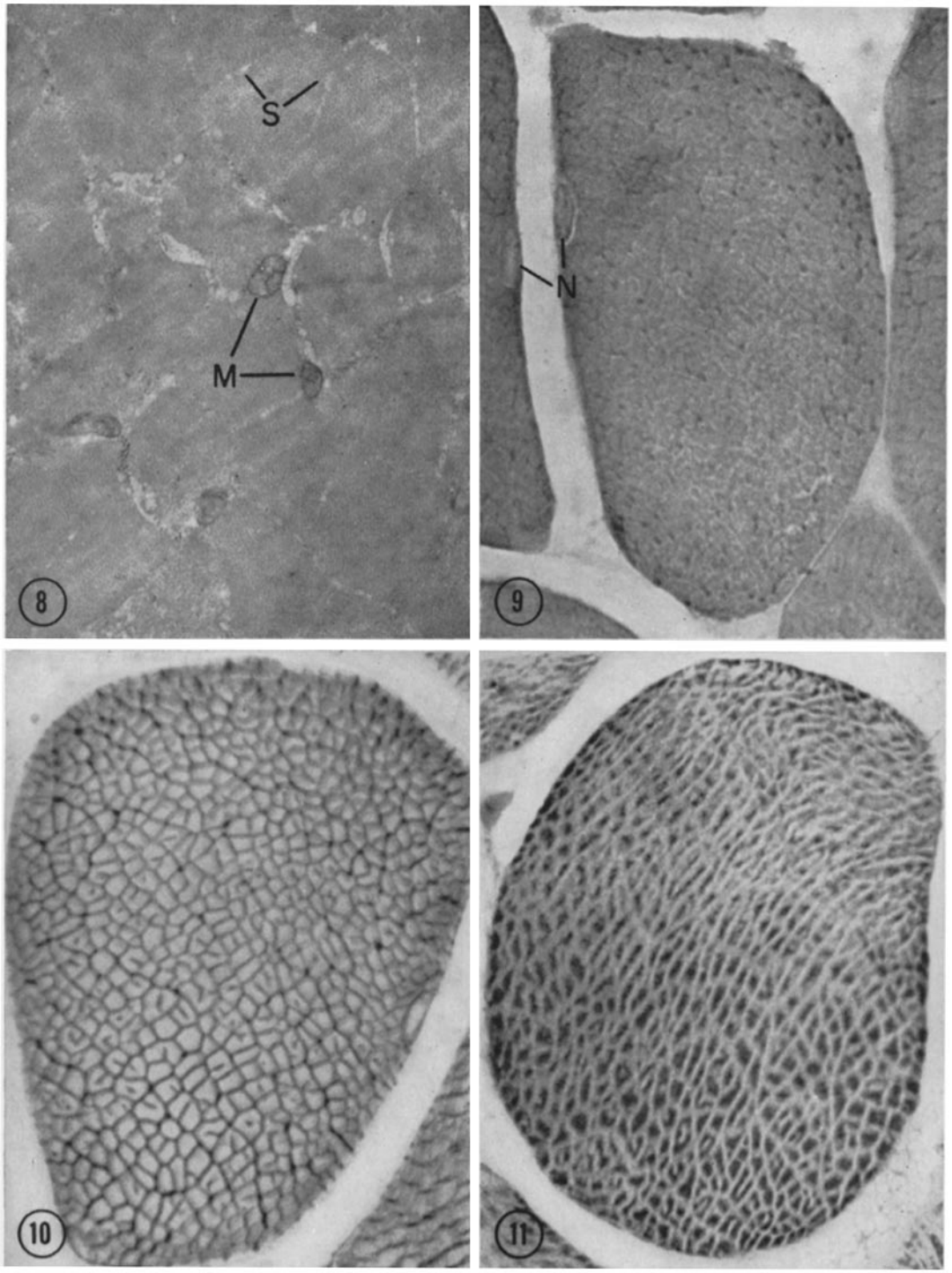
Provided the incubation times were not too prolonged, there was little or no overt discoloration of the incubation media due to formation of formazan and indicative of diffusion of enzyme from the fixed sections incubated in aqueous media. It should be mentioned that the GAPD medium was less stable than the LDH medium and that even in the absence of sections some nonenzymatic formation of formazan occurred in the medium

FIGURE 8 Cross-section of a white muscle fiber, incubated in the medium without the substrate. The sarcoplasmic reticulum (*S*) and the small mitochondria (*M*) are not stained. Compare with Fig. 6. × 19,000.

FIGURE 9 *Binding of TNBT* Cross-section of a muscle fiber, treated with TNBT, washed thoroughly in distilled water, and then TNBT-reduced by exposure to an alkaline solution (0.1 N NaOH). The tetrazolium is immediately reduced and formazan deposits are noted over the entire cross-section of the muscle fiber, including the myofibrils and the nuclei (*N*). Light micrograph, × 1150.

FIGURE 10 *Binding of NADH* Cross-section of a muscle fiber, treated with NADH, washed with distilled water, and then exposed to a solution containing PMS and TNBT. The staining is confined to a fine network in the sarcoplasm and resembles the staining results obtained for glycolytic dehydrogenases. Light micrograph. × 1150.

FIGURE 11 Cross-section of a muscle fiber, treated first with 10^{-3} N iodine solution, washed, and then incubated for LDH for 30 min. Since no reaction was noted, the incubation medium was reduced by addition of exogenous LDH. The staining which follows is patchy and irregular. In some fibers, the sarcoplasmic reticulum stains; in others, as here, the reticulum remains unstained while the myofibrils stain. Light micrograph. × 1150.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used for electron microscopy after approximately 15 min: the medium was therefore changed and sections were transferred to freshly prepared incubation media every 5 to 10 min.

The concentrations of TNBT, PMS, and NAD for electron microscope cytochemistry were higher than those used previously for light microscopy (4). Also whereas Nitro-BT was used previously (4), here we used TNBT. In agreement with other authors (26–29) it was noted that the TNBT formazan has the most favorable characteristics for light and electron microscope cytochemistry, including suitable substantive properties, lack of crystal formation, good sensitivity, very fine formazan with small particle size, and some degree of electron-opaqueness. The diffuse electron-opaque image of the TNBT formazan in our results, which were obtained mostly on low-power electron micrographs, is in full agreement with the small particle size reported by others (27–29).

The absence of significant staining when substrates were lacking indicates the enzymatic nature and the substrate specificity of the reactions. Although a moiderate degree of staining was noted in the light mcroscope, when sections were incubated in media with high concentrations of TNBT, PMS, and NAD, as suggested for electron microscope cytochemistry, the same material did not show sufficient electron opacity in the electron microscope (Fig. 10). The lower concentrations of the same compounds, as used for the light microscopy, caused a generalized faint staining of the mitochondria and the sarcoplasmic reticulum in the absence of the substrates. This so called "nothing-dehydrogenase" reaction has been attributed, by different authors, to protein-bound sulfhydryl groups in tissue sections (30), the endogenous lactate (31), and alcohol dehydrogenase (32). In white muscle, which depends primarily on glycolysis for energy production, this reaction is most probably due to high content of endogenous lactate.

Another observation suggesting the enzymatic nature of the staining reaction is that a short treatment of sections with iodine, which is a potent inhibitor of LDH (33), or with several other heavy metals which also inhibit the enzyme (34), suppressed completely the staining for this enzyme. The substantivity reaction of the TNBT for the tissue sections was not affected by these inhibitory procedures. It was surprising, however, to note that oxalate and oxamate, which also inhibit the enzyme biochemically (33, 34), did not inhibit the cytochemical reaction for LDH, under our test conditions. We cannot explain this finding.

Finally, the observation that, in lightly reacted preparations, the formazan is deposited within the lumen or the inner phase of the sarcoplasmic reticulum whereas in heavily reacted preparations the formazan shows evidence of spilling over and diffusion outside the reticulum, speaks against a selective affinity of the formazan for the reticulum and suggests that the reaction product is accrued there by a gradual, time-dependent, enzymatic activity.

The Influence of NADH-diaphorase and the Role of PMS

The enzyme NADH-diaphorase has been localized in several tissues, such as liver (35), kidney, and heart (29), in the endoplasmic reticulum and the sarcoplasmic reticulum. Such a localization could interfere seriously with the localization of coenzyme-linked dehydrogenases since the NADH generated in the incubation media, owing to leakage of glycolytic dehydrogenases, could serve as a substrate for the NADH-diaphorase present in the endoplasmic reticulum or the sarcoplasmic reticulum, and thereby cause a false localization (5, 7, 8). In the white muscle adductor magnus of rabbit, studied here, NADH-diaphorase activity was confined to structures identified by light microscopy as mitochondria (Fig. 2). The sparseness of these staining sites corresponded to the low number of mitochondria found in the electron micrographs of this muscle (Fig. 2), and the mitochondrial localization correlated well with other histochemical and biochemical studies on NADH–cytochrome c reductase in skeletal muscle (11, 36–39).

In the absence of PMS, glycolytic dehydrogenases, similar to NADH-diaphorase, were also localized in mitochondria, and it was only after the addition of PMS to the staining medium that the sarcoplasmic reticulum became prominently stained. It has previously been shown quantitatively that PMS increases the rate of formazan production in the LDH reaction (4), and it is known that electrons are transferred by PMS directly from NADH to the tetrazolium, bypassing the NADH-diaphorase tetrazolium route (4–7). The high activity for LDH and GAPD observed in the presence of PMS is more in accord with

biochemical data (11) than is the weak reaction observed in the absence of PMS (10, 36, 37).

As mentioned previously (4) in a report on light microscope studies with fresh frozen sections, when high concentrations of PMS were used an inhibition of red muscle fibers was noticed. The inhibitory action of PMS upon the staining of tissues rich in cytochrome oxidase, such as red muscle fibers, can be overcome by addition of cyanide ions to the incubation medium (40, 41). Since the objective of the present study was only the localization of glycolytic dehydrogenases in the *white* muscle fibers, no cyanide ions were necessitated for the cytochemical reactions.

Mitochondrial Localization of Glycolytic Enzymes

Our observations suggesting that LDH and GAPD are probably localized in both the sarcoplasmic reticulum and the mitochondria are not necessarily in contradiction with biochemical reports indicating that the glycolytic activity is only confined to the supernatant fraction, which is essentially devoid of mitochondria (1–3). Since, at least, LDH exists in several molecular forms (isozymes) (42, 43) which are found in tissues with anerobic metabolism (M-type isozyme) as well as in tissues with aerobic metabolism (H-type isozyme), and since the glycolytic enzymes are generally associated with the supernatant fraction (1–3), while the aerobic enzymes are mainly mitochondrial (2), one might postulate that the ultrastructural localization of LDH in both mitochondria and the sarcoplasmic reticulum might be actually due to reaction of different LDH isozymes. Alternatively, the mitochondrial localization may represent incomplete bypassing of the diaphorase system by the PMS.

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