THE CYTOCHEMICAL LOCALIZATION OF MYOGLOBIN IN STRIATED MUSCLE OF MAN AND WALRUS

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Myoglobin, the heme-protein responsible for the characteristic color of striated muscle (Kendrew, 1954), was the first protein to have its three-dimensional structure unravelled (Kendrew, 1961). However, virtually nothing is known concerning its localization within the muscle cell.

Myoglobin oxidizes benzidine in the presence of of hydrogen peroxide. This reaction is well known and is used in the biochemical identification of myoglobin, but there have been few applications to the demonstration of the heme-protein in tissue sections. Drews and Engel (1961) first described peroxidatic activity in frozen sections of skeletal muscle and attributed it to myoglobin. Subsequently, Wachstein and Meisel (1964) suggested that this activity was associated with mitochondria and not myoglobin.

The introduction by Karnovsky (1965) of a benzidine derivative, 3,3′ diaminobenzidine (3,3′ DAB) yielding a discrete, stable, and osmiophilic stain, rather than the microcrystalline product described in previous reports (Drews and Engel, 1961; Wachstein and Meisel, 1964), prompted this light and electron microscopic study of peroxidatic activity in striated muscle.

The highest concentrations of myoglobin are found in the muscles of mammals that spend a great deal of time underwater. The muscle of whale and seal is reported to be almost black in

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color because of its high myoglobin content (Kendrew, 1954). Although we were unable to obtain a specimen of whale or seal, the death of Olaf, the walrus at the New York Aquarium, made it possible to compare the benzidine-peroxidase reaction in striated muscles of man and walrus. Our observations are consistent with the view that myoglobin may be visualized in formaldehyde-fixed tissues by virtue of its peroxidatic activity.

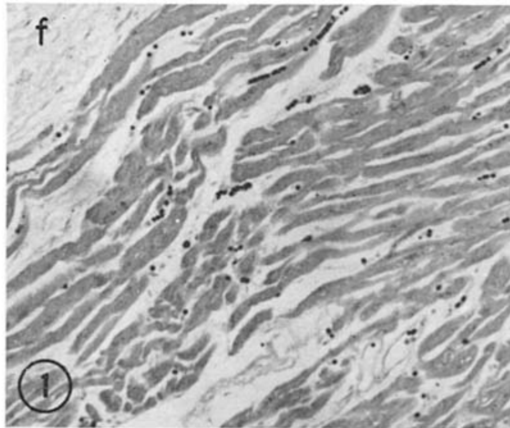

FIGURE 1 Human heart muscle. 10-μ frozen section incubated for peroxidatic activity (Graham and Karnovsky, 1966) for 30 min. This section is from an area with an old scar. The muscle fibers are stained; the adjacent fibrous tissue (*f*) is negative. Red blood cells are darkly stained. × 110.

MATERIALS AND METHODS

The myocardium, skeletal muscle (psoas, gastrocnemius, pectoral), and smooth muscle (stomach, uterus) of man were fixed at room temperature in 4% formaldehyde (phosphate buffered, pH 7.4, 0.1 M) or or formol-saline (Drews and Engel, 1961) and stored (in fixative) in the refrigerator for 48 hr–3 wk. Several specimens were fixed in 2.5–6% glutaraldehyde-phosphate (Sabatini, Bensch, and Barrnett, 1963) for periods from 2 to 18 hr. Specimens were obtained at autopsy, 4–12 hr postmortem, or at surgery.

Walrus pectoral muscle, obtained through the courtesy of Dr. Ross Nigrelli, Director of the New York Aquarium, was fixed 1 day after death and stored in unbuffered formaldehyde for 3 days.

Cytochemistry

Peroxidatic activity was visualized with the 3,3′ diaminobenzidine (3,3′ DAB) medium of Graham and Karnovsky (1966) containing three to five times the usual amount of substrate. The medium was freshly prepared by dissolving 15–25 mg of 3,3′ DAB in 10 ml of 0.05 M Tris-HCl buffer (pH 7.6) and adding H_2O_2 (0.4–0.6 ml of 1% solution). The higher concentrations of 3,3′ DAB and H_2O_2 yielded darker staining with briefer incubations.

Tissues were incubated at 37°C for peroxidatic activity as free-floating 10-μ frozen sections (15–60

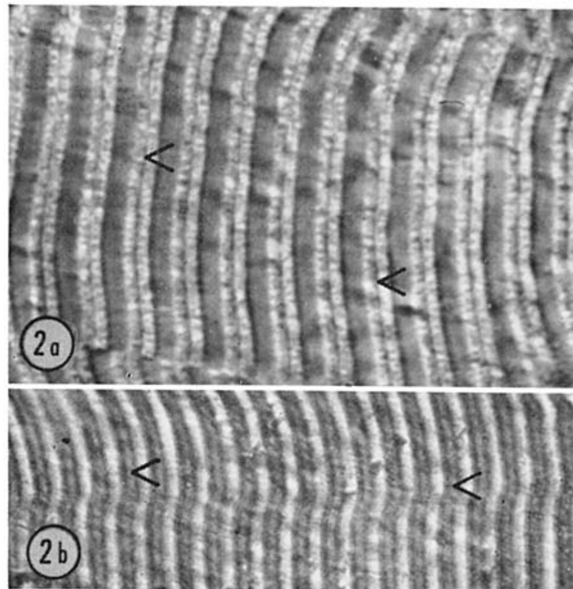

FIGURE 2 *a* Human gastrocnemius muscle. A 3-μ paraffin section incubated for peroxidatic activity for 60 min. The A bands and thin Z lines (arrow heads) are stained. × 1500.

FIGURE 2 *b* A lower power view of the same section photographed in polarized light. The A bands and Z lines (arrow heads) are anisotropic. × 1000.

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min) or as 5-μ paraffin sections (30–60 min) mounted on glass slides and brought to water.

Controls were incubated 1) in the absence of H_2O_2, 2) in the presence of KCN (4×10^2 M), and 3) after prior heating at 90°C for 5 min.

Sections were mounted in a water-soluble medium (glycerol jelly or Gurr's water soluble mounting medium) or dehydrated, cleared, and mounted in Permount.

Electron Microscopy

Frozen sections (40 μ) of skeletal muscle of human and walrus were incubated for peroxidatic activity (see above) for 20 min at room temperature, washed, postfixed for 90 min in phosphate-buffered (0.1 M, pH 7.4) 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon 812 according to Luft (1961). These sections, without lead or uranium counterstaining, were examined in an RCA-EMU3B electron microscope at 100 Kv.

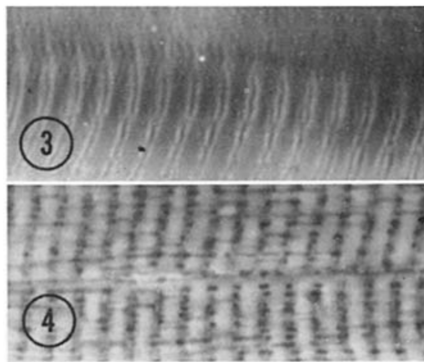

FIGURE 3 Human skeletal muscle. Frozen section incubated for peroxidatic activity for 60 min. Broad A bands and Z lines are stained. This differs from the staining pattern of mitochondria (See Fig. 4). × 1000.

FIGURE 4 A parallel section from the same specimen as that in Fig. 3. Incubated for DPNH-tetrazolium reductase activity (Novikoff, 1963) to stain mitochondria. The mitochondria are visualized as small granules at the Z line. × 1000.

OBSERVATIONS AND DISCUSSION

Light Microscopy

STRIATED MUSCLE: The cytoplasm of human cardiac and skeletal muscle (Figs. 1 and 2 *a*) was stained during incubation in the 3,3′ DAB medium. The A bands and the Z lines were visualized as brown stripes (Fig. 2 *a*) that could be identified by viewing the section in polarized light (Fig. 2 *b*). The best preparations were obtained from skeletal muscle fixed at surgery.

In the two previous studies of peroxidase activity in muscle (Drews and Engel, 1961; Wachstein and Meisel, 1964), reaction product was reported to accumulate at the I bands. However, the crystalline nature of the final product obtained with the benzidines that were available earlier apparently precluded a precise localization. The deposition of microcrystals at the I band led to the suggestion by Wachstein and Meisel (1964) that the peroxidatic activity of muscle was associated with mitochondria rather than myoglobin. In our material skeletal muscle mitochondria were visualized, in a DPNH medium, as small and discrete granules, on either side of the Z line (Fig. 4). The staining pattern that is achieved with the 3,3′ DAB medium is clearly not mitochondrial (compare Figs. 3 and 4).

SMOOTH MUSCLE: Smooth muscle from human stomach and uterus was unstained. This finding is consistent with the chemical data of Biorck (1949) who reported that the myoglobin content of

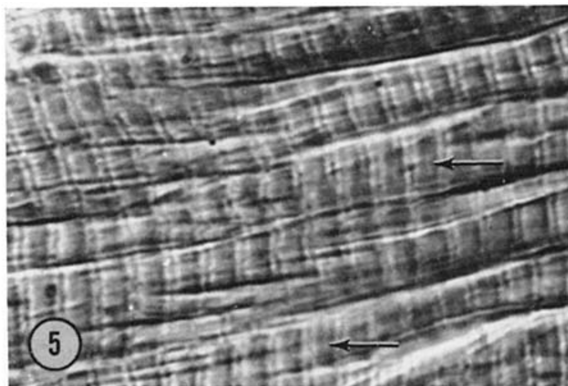

FIGURE 5 Walrus pectoral muscle. Paraffin section incubated for peroxidatic activity for 15 min. The wide A bands and thin Z lines are stained. Within the A band a central light zone, corresponding to the H zone, is evident in some areas (arrow heads). × 1650.

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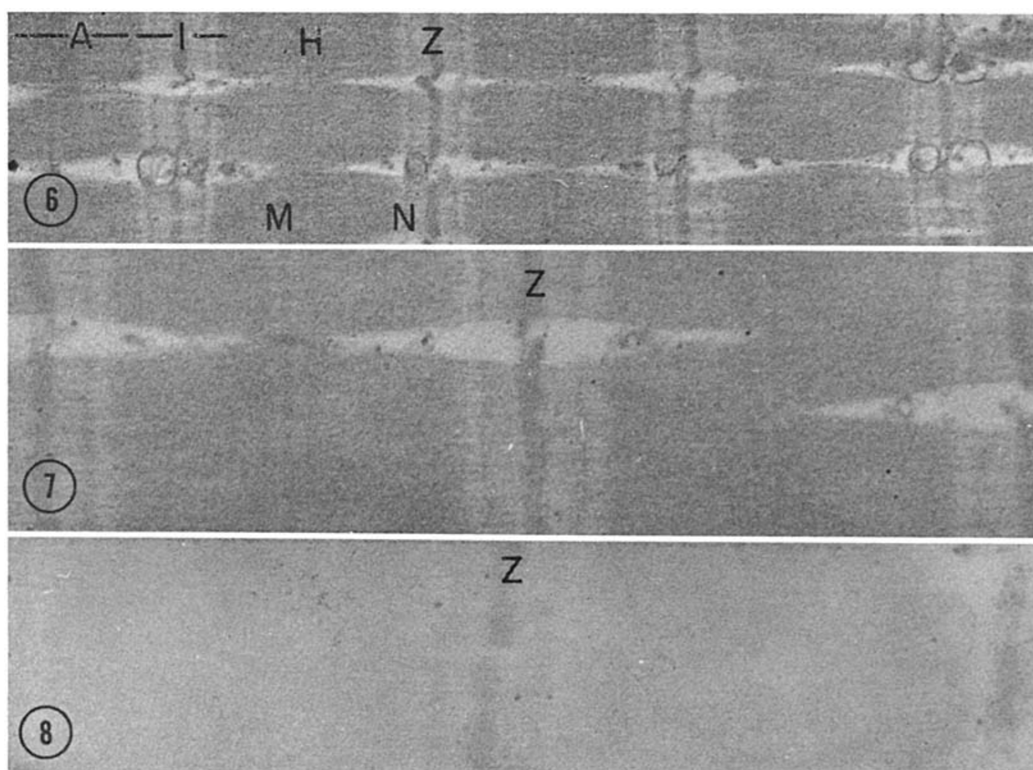
human uterus was approximately one-tenth that of striated muscle.

CONTROLS: Staining did not occur in the absence of H_2O_2. The addition of KCN to the medium strongly inhibited all staining. Prior heating for 5 min at 90°C abolished white cell staining; however, the staining of muscle and red cells was not reduced. Glutaraldehyde fixation inhibited the peroxidatic activity of the muscle cell.

WALRUS: Walrus pectoral muscle was grossly brown-black and much darker than human skeletal muscle. Unlike those in human muscle, the cross-striations could be detected in unstained sections without lowering the condenser or closing the diaphragm. In such sections it was evident that the dark color of the muscle, which is attributed to its high myoglobin content, is associated mainly with the A band. The Z line was also visible in these preparations. Within the A band a central stripe, corresponding to the H zone, was colorless.

Walrus muscle displayed far more peroxidatic activity than the human specimen (Fig. 5). Sections incubated for 15 min were much darker than sections of human muscle incubated for 60 min. In these sections, the A band was stained black rather than brown. The pale H zone and a dense Z line were evident (Fig. 5).



FIGURES 6 to 8 are electron micrographs of walrus muscle. These sections were not stained with lead or uranium. Figs. 6 and 7 are of tissue incubated for peroxidatic activity in the 3,3′ DAB medium for 15 min at room temperature. Fig. 8 is a control preparation incubated for 15 min in 3,3′ DAB medium containing KCN, an inhibitor of peroxidase activity.

FIGURE 6 The A band and the Z line are stained. The H zone, save for the M line, is less dense. Note the staining of an additional line (*N*) in the I band which is also increased in density. × 17,000.

FIGURE 7 A higher power view of a portion of the tissue photographed in Fig. 6. × 34,000.

FIGURE 8 In this control preparation the characteristic bands and lines are unstained and, with the exception of the Z line, can barely be detected. This figure and Fig. 7 were photographed on the same plate and developed and printed under identical conditions. × 34,000.

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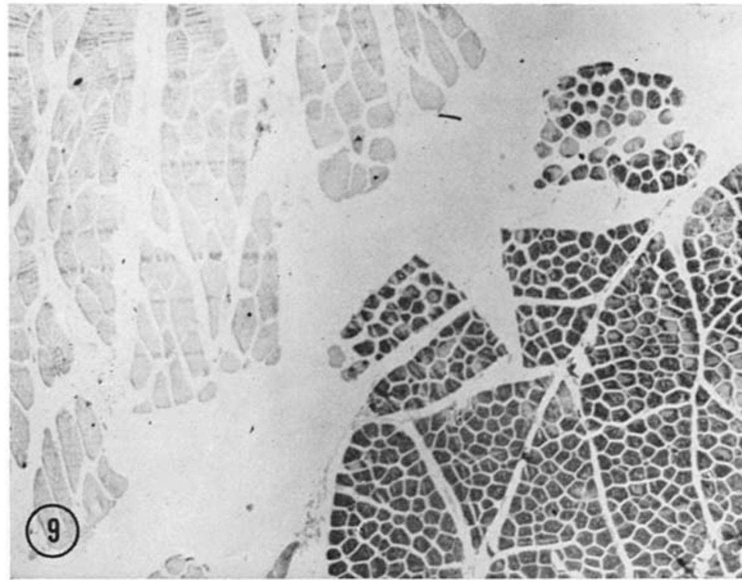

FIGURE 9 Chicken leg and pectoral muscles. The two muscles were bound together during fixation and embedding and were mounted on the same slide. Incubated for peroxidatic activity for 30 min. The myoglobin-rich leg muscle is darkly stained. The myoglobin-poor pectoral shows very little activity. A few fibers in the leg muscle are almost unstained and are presumed to be white fibers. Diffusion of stain to the white muscle is not detectable. × 25.

Electron Microscopy

Electron micrographs of walrus muscle incubated for peroxidatic activity were consistent with light microscopic observations (Figs. 6 and 7). Incubated sections showed an increase in density that is most evident when Fig. 7 is compared with Fig. 8, a control section incubated in the presence of KCN. Both the A and I bands were increased in contrast: however, the A band was most darkly stained. The H zone, the region of the A band free of thin filaments (Huxley, 1965), showed less staining. An additional line in the I band (Figs. 6 and 7), corresponding to the N line (Rhodin, 1963), was also stained. In human muscle the sites of staining were similar, with the exception of the N line which was not visualized.

Validity of Localization

Whether the structures stained represent the site of myoglobin within striated muscle or merely a favored site of adsorption of soluble protein or reaction product is the most critical question to be resolved. The results of several experiments indicate that the in situ localization of myoglobin is achieved by incubation in the 3,3′ DAB medium.

To test for adsorption of myoglobin, we soaked unfixed slices of rat rectus abdominal and gastrocnemius muscles overnight at room temperature in a 1% solution of myoglobin (horse heart, Pentex Inc., Kankakee, Ill.) in normal saline prior to fixation, sectioning, and incubation. These tissues were compared with specimens from the same muscles that had been soaked in saline. Light microscopy showed that myoglobin did not adsorb to the A band and Z line. Although there was no enhanced cytoplasmic staining in tissues that had been soaked in myoglobin, slight nuclear staining was present. Rat skeletal muscle has a low level of peroxidatic activity; if myoglobin had adsorbed to the cytoplasmic bands, an increase in activity would have been evident.

Evidence that we were visualizing myoglobin was also obtained by incubating a "sandwich" of chicken muscle. Dark, myoglobin-rich, leg muscle and white, myoglobin-poor, pectoral muscle were bound together and fixed in formalin and then embedded, sectioned and incubated as a "sandwich." The difference in peroxidatic activity of the two muscles was distinct (Fig. 9). After 30 min of incubation, the leg muscle was darkly stained; the pectoral was virtually unstained. No diffusion of myoglobin or reaction product from

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the dark to the light muscle was detectable by light microscopy.

To determine whether myoglobin had diffused during fixation in phosphate-buffered formaldehyde or formol-saline, chicken leg muscle and rat myocardium were prepared by freeze-substitution. Specimens were frozen in isopentane, cooled by liquid nitrogen, and substituted for 4 days at −20°C in acetone or a fixative composed of absolute ethanol (85 ml), formaldehyde (10 ml), and glacial acetic acid (5 ml), and were embedded in paraffin. The staining pattern in these tissues was similar to that in muscle fixed in aqueous fixatives in the usual manner, suggesting that significant diffusion of myoglobin did not occur. Drews and Engel (1961) have previously reported that formaldehyde fixation prevented diffusion of myoglobin from tissue sections.

Diffusion of reaction product is not significant with the 3,3′ DAB medium (Karnovsky, 1965; Graham and Karnovsky, 1966). For the purpose of minimizing diffusion of myoglobin or reaction product in electron microscopic preparations, frozen sections (no more than 40 μ in thickness) of formaldehyde-fixed muscle were incubated at room temperature for no longer than 20 min. That some diffusion may have occurred was suggested by the increased density of the membranes of the sarcoplasmic reticulum.

Perhaps the best evidence that the staining of myofibrils represents the in situ localization of myoglobin and not the diffusion of reaction product comes from light microscopic examination of *unstained* sections of walrus muscle. In such sections, it is apparent that the dark color of walrus muscle, which may be assumed to represent its high myoglobin content (Kendrew, 1954), is associated with the same sites that are stained in walrus and human muscles incubated for peroxidatic activity.

It is evident that staining results, require confirmation by other procedures. Immunological techniques in which anti-myoglobin antibodies labeled with fluorescein or ferritin are employed should prove useful in this regard. Although it has been assumed that myoglobin functions as an oxygen storage depot, Wittenberg (1965) believes that myoglobin serves to facilitate diffusion of oxygen from the capillary to the site of combustion at the mitochondrion. The physiological significance of the intimate topographic relationship of myoglobin to the contractile filaments and their sites of attachment is yet to be determined.

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