INTRACELLULAR BINDING OF RUTHENIUM RED IN FROG SKELETAL MUSCLE

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During the course of an electron microscope study of the staining of skeletal muscle cell surfaces by ruthenium red (RR), it was noticed that certain staining conditions brought about a reproducible pattern of intracellular staining. The purpose of this communication is to describe that staining pattern and to discuss its implications with regard to the location of highly anionic regions within muscle cells.

The basic staining procedure was that described by Luft (1971 *a*). Bundles of 5–20 fibers dissected from frog semitendinosus muscles were exposed to RR (0.1 or 1.0 mg/ml) during a 1-h period of fixation with 3.5% glutaraldehyde in 100 mM sodium cacodylate buffer, during a rinse in cacodylate buffer containing 10% sucrose, and during a 2-h period of postfixation with 1% osmium tetroxide in 100 mM cacodylate buffer. Cross sections were examined under the light microscope to make sure there was a complete layer of RR staining around each cell, a requirement for reproducible intracellular staining. The critical parameter turned out to be the duration of exposure to RR in the rinse. Periods of time less than 1 h were not sufficient to produce intracellular staining, but when the muscles were left overnight in the rinse containing RR the complete pattern of intracellular staining did appear.

The RR used was obtained either from Sigma Chemical Co. of St. Louis, Mo., K & K of Plainview, N. J., or from Electron Microscopy Sciences of Ft. Washington, Pa., and was recrystallized once or twice by the method of Fletcher et al. (1961) and washed with ethanol and ether to bring the molar extinction coefficient at 5,300 Å in 0.1 M ammonium acetate to about 40,000. The commercial material had extinction coefficients in the range of 7,000–17,000 when corrected for absorbance at 3,600 Å and 7,340 Å by the method of Luft (1971 *a*). The pure RR is reported to have an extinction coefficient of 68,000 (Fletcher et al., 1916; Luft, 1971 *a*). Although the purity of the RR

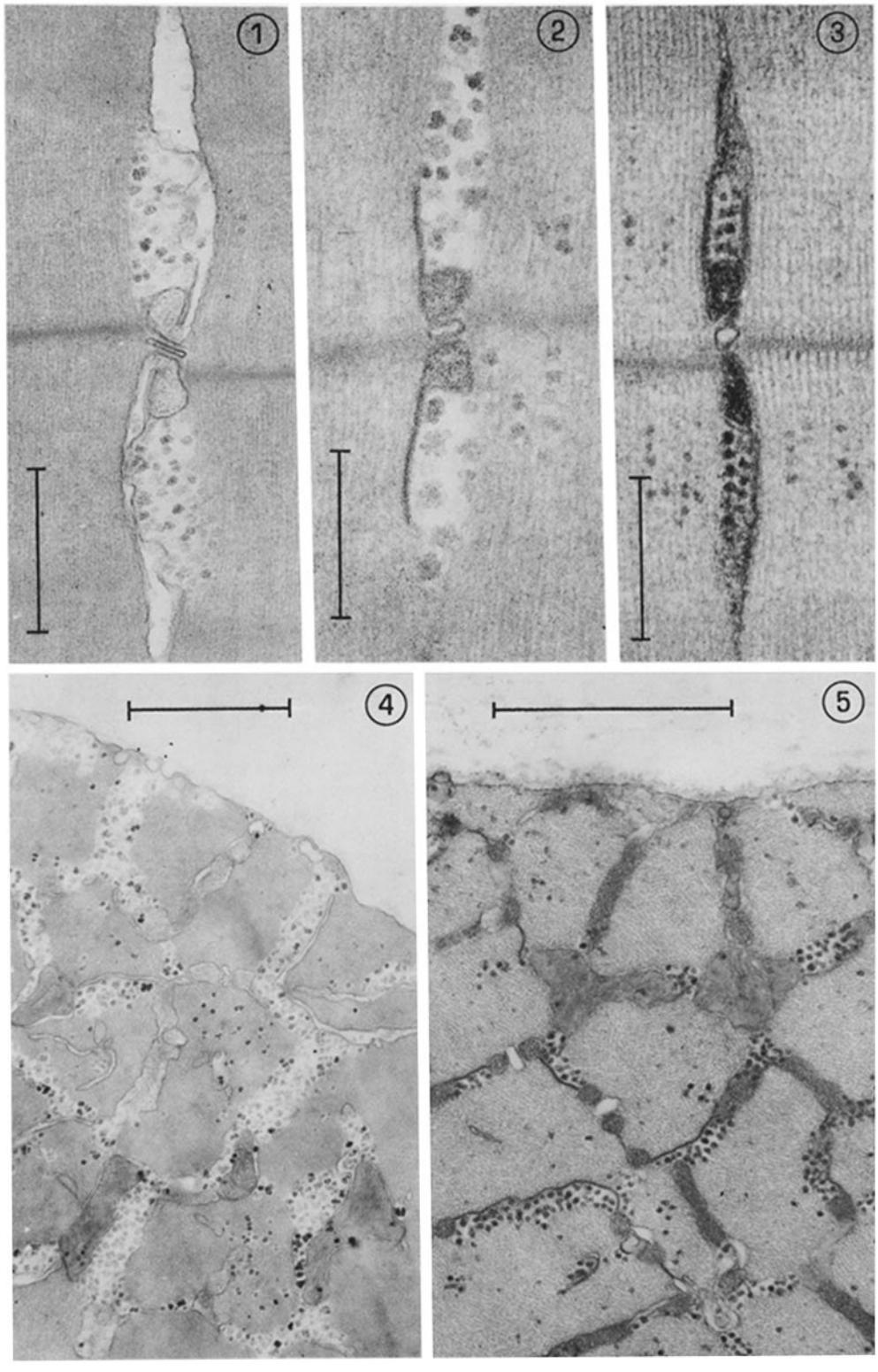
FIGURE 1 Triad from muscle fixed in the conventional way with glutaraldehyde followed by osmium postfixation. Muscle was held overnight in the cacodylate rinse between fixation and postfixation. × 47,600. Scale 0.5 μm.

FIGURE 2 Triad from muscle exposed to RR (1 mg/ml) overnight in rinse between glutaraldehyde fixation and osmium postfixation. Fixation and postfixation solutions also contained RR. × 94,200. 0.3 μm.

FIGURE 3 Triad from muscle exposed overnight to Ringer's containing RR (0.2 mg/ml) before glutaraldehyde fixation. Fixation was followed by a brief rinse and osmium postfixation. × 48,400. Scale 0.5 μm.

FIGURE 4 Cross section of control muscle treated as described in the legend for Fig. 1. × 24,200. Scale 1 μm.

FIGURE 5 Cross section of RR-treated muscle as described in the legend for Fig. 2. × 35,000. Scale 1 μm.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did not seem to affect staining patterns, as was pointed out earlier by Luft (1971 *a*), the impurity of commercial preparations indicates that it is necessary to analyze RR spectrophotometrically before stating the molar concentration of a solution made from commercial material.

Figs. 2 and 5 illustrate RR staining of the sarcoplasmic reticulum (SR) in longitudinal and cross section respectively; they can be compared with similar sections of muscles not exposed to RR illustrated in Figs. 1 and 4. The most obvious feature of the SR in muscle exposed to RR is that the lumens of the intermediate cisterns appear to be totally obliterated by fusion of the membranes which normally form the opposite walls of the cisterns. The membranes appear to have fused into single pentalaminar structures which can be seen in Figs. 2 and 6. The width of the pentalaminar structures as compared to the width of single, unfused SR membranes is shown in Table I. The

TABLE I

*Widths of SR Membranes in RR-Treated Muscle Cells**

Exp. No.	Single	Fused	Ratio-fused/single
74	71 Å	132 Å	1.86
120	78 Å	130 Å	1.67
122 (L)‡		118 Å	
98		135 Å	
112	75 Å		
114 (L)‡	70 Å		
Mean widths and ratio of mean widths	73.8 Å	128.8 Å	1.75

* In cells where fusion was incomplete, membrane widths could be measured for both single and fused membranes.
‡ L means that the measurements were made on longitudinally sectioned muscle. The other measurements were made on cross sectional material.

average width of single SR membranes measured from the outer edges of the dark lines was 73.5 Å whereas the fused membranes measured 128.8 Å in total width, suggesting that an actual fusion of the two membranes does occur.

In order to observe fused SR membranes it was generally necessary for the muscles to be exposed to RR overnight. In a few cases where the exposure to RR in the rinse was much shorter, namely between 1 and 2 h, partial fusion was observed. This is illustrated in Fig. 7, where intermediate cisterns appear partially occluded with some open lumen remaining. The process of fusion appears to resemble a zipping together of the membranes of the opposite faces of the intermediate cisterns. The hexavalent cations of RR probably provide ionic bridges binding negative sites on both membranes. The reason that fusion is not seen in the terminal cisterns may simply be that the granular material contained within the terminal cisterns prevents the opposite membranes from coming close to one another.

The other site of RR interaction with the SR was the granular material of the terminal cisterns. RR staining of this material can best be seen in sections not subsequently stained with lead citrate or in sections stained only very lightly with lead. A conspicuous example of RR staining in the terminal cisterns is shown in Fig. 8. Even in other micrographs where lead staining of the sections is more intense, RR binding to the material in the terminal cisterns can be detected by virtue of the fact that this material appears darker than any of the myofibrillar material. In the absence of RR, this material never appears more intensely stained than the myofibrillar material (Figs. 1 and 4).

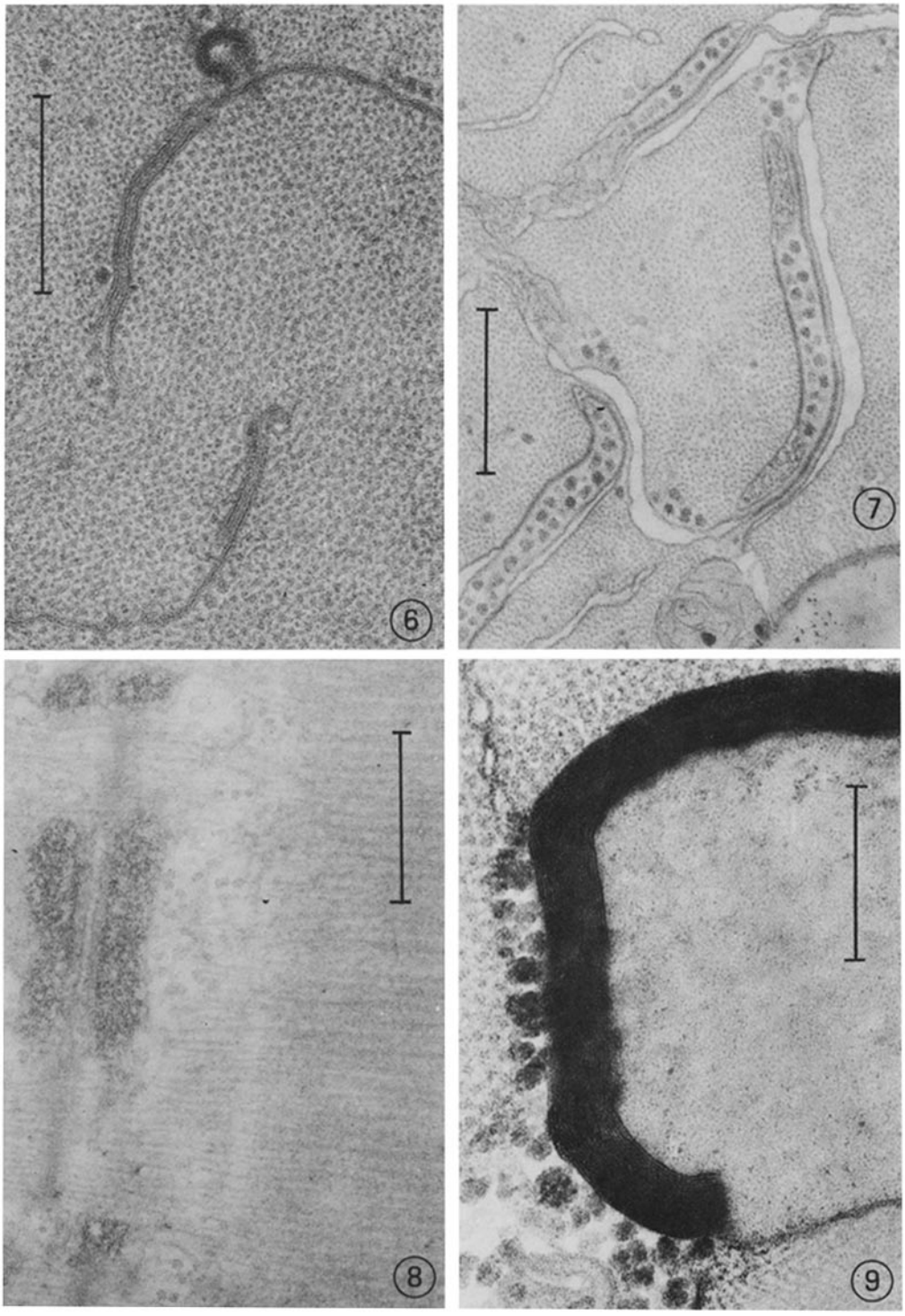
Another prominent feature of muscles exposed to RR was the occurrence of lamellar structures within the cells. These structures sometimes appear continuous with membranes of the SR, suggesting that they consist of tightly packed SR

FIGURE 6 Cross section of RR-treated muscle as described in the legend for Fig. 2. The small lamellar structure appears continuous with the fused membranes of the intermediate cisterns. × 94,200. Scale 0.3 μm.

FIGURE 7 Cross section of muscle exposed to RR during glutaraldehyde fixation, during a 2-h cacodylate rinse and during osmium postfixation. Note partial fusion of intermediate cistern membranes. A lipid droplet is visible at the lower right corner adjacent to a mitochondrion. × 75,300. Scale 0.3 μm.

FIGURE 8 Triads from RR-treated muscle as described in the legend for Fig. 2. RR staining of the contents of the terminal cisterns is particularly evident. × 121,000. Scale 0.2 μm.

FIGURE 9 Cross section of RR-treated muscle as described in the legend for Fig. 2. A lamellar array forms the outer edge of a portion of a lipid droplet. × 125,000. Scale 0.2 μm.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membranes (Fig. 6) whose reorganization into lamellar structures is promoted by the presence of RR. Sometimes, however, these lamellar structures can be seen within lipid droplets (Fig. 9). The repeat periodicity of the lamellar array is about 50 Å and appears to be the same as the distance between the centers of adjacent dark lines in the fused intermediate cistern membranes.

In order to be certain that the pattern of interaction of RR with muscle cells was not an artifact of glutaraldehyde fixation, unrelated to anionic sites in living cells, muscles were exposed to RR in Ringer's solution overnight before fixation. Most fibers did not survive this treatment; they were swollen and contained many retraction clots. In two experiments a few fibers in the bundles survived and were examined. They were fixed for 1 h in 3.5% glutaraldehyde, rinsed quickly in cacodylate buffer, and postfixed for 2 h with OsO_4 containing RR. The appearance of these fibers (Fig. 3) was similar to that of fibers exposed overnight to RR following glutaraldehyde fixation (Figs. 2, 5, 6, 8 and 9). The contents of the terminal cisterns were heavily stained and fusion of intermediate cistern membranes was observed.

In general the staining pattern brought about by RR in frog muscle is similar to the pattern that Luft (1971 *b*) obtained with mammalian muscle. However, Luft indicated some capriciousness of staining in the SR and tubular (T)-system. During the course of this study no staining was ever observed in the T-system except very near the cell periphery where occasionally a few T-tubules were seen to contain RR. Both staining of the terminal cisterns and fusion of the intermediate cistern membranes were observed reproducibly with the prolonged exposure to RR. The outer surfaces of lipid droplets were often stained although lamellar arrays within the lipid droplets were rare. Lamellar structures were commonly seen, particularly at the cell periphery. Some of these were quite large, of the order of a micron in length, and appeared to consist of closed, concentric lamellae with a regular spacing of about 50 Å. The only open-ended one that was seen is the one illustrated in Fig. 9. The appearance of the lamellar structure in Fig. 9 seems to suggest that RR is orienting some component of the lipid droplet itself. However, since lipid droplets are generally thought to contain primarily triglycerides (DiAugustine, 1972) and since it is difficult to imagine that RR would interact with triglycerides, it seems likely that the lamellar structure represents membrane material of SR origin organized by RR into lamellae which coalesce with the substance of the lipid droplets.

Considering the intense binding of RR to the SR observed in this study, the recent report of Vale and Carvalho (1973) to the effect that RR does not inhibit ATP-dependent calcium binding by the SR is surprising. It is true that the only binding sites demonstrable morphologically are in the interior of the SR, while the sites responsible for calcium transport must be at the outer face of the SR membranes. Nevertheless, internal binding has generally been considered to be important for the accumulation of calcium by the SR, at least in the absence of a coprecipitating anion such as oxalate (Sandow, 1970; McLennen and Wong, 1971). These observations suggest that the sites involved in RR binding are not the same as those involved in calcium binding. RR binding that is visible morphologically may be analogous to the bulk precipitation reactions which were described by Luft (1971 *a*) and which appear to require a high density of negative charges. The specific SR sites involved in calcium transport may well not bind RR.

This stands in sharp contrast to calcium-dependent processes in other tissues which are very sensitive to RR. Vasington et al. (1972) showed that RR is a potent inhibitor of calcium uptake and of calcium stimulation of respiration in rat liver mitochondria, and Watson et al. (1971) showed that the calcium-stimulated ATPase of erythrocyte membranes, but not the sodium/potassium- or magnesium-stimulated ATPases are inhibited by RR. It is interesting that no convincing RR binding to mitochondria was detectable in the present study. Although rat liver mitochondria may behave quite differently from frog muscle mitochondria, these observations on mitochondria as well as those on the SR do suggest that no simple identity exists between morphologically identifiable RR binding sites and sites where RR exerts pharmacological effects.

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