We have examined freeze-fracture replicas and conventional thin-section images of rat myofibers prepared by perfusion and by conventional immersion fixation protocols, and myofibers of normal and dystrophic human myofibers prepared by similar immersion fixation methods. In both rat and human myofibers, the size and distribution of caveolae was found to differ substantially according to (1) the method of glutaraldehyde exposure, (2) the depth of the myofiber from the surface exposed to the fixative, and (3) if surgically bisected, the distance from the cut end of the myofiber. Conventional immersion fixation resulted in unavoidable but predictable alterations in sarcolemmal caveolae. These reproducible artifacts of fixation technique substantially complicate the use of caveolae as reliable markers for the characterization of human neuromuscular disease.

CAVEOLAE PRESERVATION IN THE CHARACTERIZATION OF HUMAN NEUROMUSCULAR DISEASE

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Caveolae ("little caves") are small, cup-like deformations of the plasma membrane. In conventional thin-section images of glutaraldehyde-fixed skeletal myofibers, caveolae are seen as a continuum of shallow depressions, Ω-shaped indentations, and occasionally, as strings of small spherical or flattened vesicles that are directly continuous with the plasma membrane. In freeze-fracture replicas of glutaraldehyde-fixed muscle, caveolae are resolved either as small P-face indentations having well-defined circular borders, as volcano-like or mesa-like E-face projections, or as a continuum of cup-like and saucer-like deformations of the plasma membrane.

In vertebrates, caveolae occur in a variety of cells, but they are most common in transporting epithelia (vascular endothelia), skeletal, cardiac, and smooth muscle. Although several models have been proposed to account for the presence of caveolae in these tissues, it is not the intent of this investigation to identify the mode of formation or the function of caveolae. Rather, we have investigated previous proposals that the number, size, and distribution of caveolae (plus T-tubule openings) serve as reliable markers for differentiating dystrophic from normal human muscle fibers. In addition to caveolae, a few of the openings in the sarcolemma represent openings of T-tubules, but it is not possible to differentiate these two distinct structures using current freeze-fracture techniques. However, previous freeze-fracture investigations reporting differences in normal and dystrophic sarcolemmas based their conclusions on differences in the sum of these two structures. To facilitate comparison, we use the same convention.) Normal myofibers were reported to have large (75 nm), uniform diameter caveolae distributed in distinctive "bands" running parallel and perpendicular to the fiber axis, whereas dystrophic myofibers were reported to have equally large to slightly larger (60–85 nm), variable diameter, "randomly" (irregularly) distributed caveolae. Moreover, caveolae in Duchenne dystrophy were reported to have an overall density greater than that seen in normal myofibers.
In contrast, in our earlier freeze-fracture studies of sarcolemmas in both rat and human myofibers, we also observed numerous caveolae, but of substantially smaller size than those reported by others in human myofibers. Moreover, we had observed primarily an irregular (non-banded) distribution in perfusion-fixed rat myofibers and in most human myofibers. However, those studies did not include quantitative analyses of caveola size or distribution, but instead were directed toward devising techniques to minimize artifactual alterations of membranes and intramembrane particles (IMPs), such as those caused by hypoxia, circulatory interruption, cutting of fibers, and variable depth of fixative penetration. In this investigation, we have compared conventional and improved fixation techniques using a rat model system in an attempt to determine if the reported alterations in caveolar morphology and distribution in normal versus dystrophic myofibers occur as a result of variations in common tissue preparation procedures. We then analyzed human myofibers to ascertain which, if any, of the observed fixation artifacts also occurred in normal and/or dystrophic human tissues.

**MATERIALS AND METHODS**

For each preservation protocol, we have examined from five to eight replicas from each of two rats and from two human patients with each of the following neuromuscular diseases [Duchenne, facioscapulo-humeral (FSH), and limb-girdle (LG) muscular dystrophies; and myasthenia gravis (MG)].

**Rat Myofibers.** Sprague-Dawley rats were anesthetized with 5–12 mg of sodium pentobarbital (50 mg/kg body weight). All rats were approximately 6 months old and weighed 175–250 g. The rats were treated according to the National Research Council's guide for the care and use of laboratory animals.

**Perfusion fixation.** Vascular perfusion fixation minimizes the distance required for diffusion of the fixative within the tissue and exposes the myofibers to the fixative before they are cut or damaged. Two anesthetized rats were perfused at 125–175 mm Hg pressure through the left ventricle with 150 ml of oxygenated (95% O₂, 5% CO₂) rat Ringer's buffer (37°C, pH 7.2) containing 10 U/ml of heparin ("clearing solution"). After 10 minutes, the muscles were chilled, and fixation was continued at 4°C for 12–15 hours. The myofibers were dissected into two groups of samples (Fig. 1a and b). The first group consisted of those fibers at or within 0.25 mm of the surface exposed to the fixative, whereas prolonged secondary fixation was at 4°C to minimize lipid "leaching" and "blebbing." The myofibers were dissected into two groups of samples (Fig. 1a and b). The first group consisted of those fibers at or within 0.25 mm of the surface of the muscle (designated surface fibers). The second group consisted of fibers obtained about 1 mm from the surface (deep fibers). The fibers in both groups were cut into 1-mm strips and were prepared for freeze fracture as described above.

**Thin section analysis of caveolae diameter vs. depth of fixation.** To document the effects on caveola size and morphology as a function of myofiber distance from the surface exposed to the fixative, two rat EDL muscles were excised, immersed for 1 hour in 2.5% glutaraldehyde in oxygenated rat
Ringer’s solution, and bisected with a sharp razor blade. A thin wedge of muscle containing cross-sections of fibers from the surface to a depth of 1 mm (Fig. 1c) was then placed in Ringer’s buffered 1% OsO₄ for 1 hour and subsequently prepared for conventional thin-section analysis.²⁶

Immersion fixation of cut myofibers, surface fibers at selected distances from the cut end. To approximate the conditions often employed in the preparation of human biopsy samples, both EDL muscles were removed from two anesthetized rats and cut transversely with sharp razor blades. The muscle halves were immediately immersed in 37°C 2.5% glutaraldehyde in oxygenated rat Ringer’s solution. After 10 minutes, the samples were chilled to 4°C and stored for 12 hours. Surface fibers of the bisected muscles were dissected free and sliced transversely into the following samples (Fig. 2): (a) surface fibers within 1 mm of the cut edge, (b) surface fibers 2.5–3.5 mm from the cut edge, and (c) surface fibers 5–6 mm from the cut edge. All samples were prepared for freeze–fracture as described above.

Human Myofibers. Duchenne and LG dystrophic human muscle biopsies were obtained at the University of Maryland School of Medicine, Baltimore, MD. Normal (nonweak), Duchenne, and FSH dystrophic human muscle biopsies were obtained at the University of Colorado School of Medicine, Denver, CO. (The procedures followed the standards of the Human Volunteers Committee of the respective institutions.) Five to eight samples each of normal human quadriceps muscle were obtained from one man and from one woman (45 and 20 years old). Similarly, from three Duchenne patients (two 5-year-old boys and one 10-year-old boy), several small samples (5–8) were obtained from the quadriceps, vastus lateralis, and deltoid, respectively. These samples were classified as normal based on histologic examinations or as Duchenne based on family history, creatine phosphokinase (CPK) levels, and histologic evaluations.

For the other neuromuscular diseases, 5–8 samples each were obtained from: (1) the biceps brachii of one man (28 years old) and the external intercostal of one woman patient (20 years old) with FSH dystrophy, (2) the external intercostal from one man (33 years old) and the quadriceps from another man (26 years old) with LG dystrophy, and (3) the full thickness biopsies of intercostal muscles obtained at the time of thyroctomy from two MG women (32 and 42 years old).

Human muscle biopsies were immediately placed in 30–37°C oxygenated 2.5% glutaraldehyde in phosphate buffer (pH 7.2–7.5). After 5–15 minutes, the samples were cooled to 4°C and stored in fixative for 1–7 days. Bundles of surface fibers were cut in 1-mm strips and prepared for freeze–fracture. In two instances, human myofibers from a fixation depth of 1 mm were also obtained.

Freeze–Fracture and Electron Microscopy. Rat and human muscle fibers were fractured and replicated in Balzers 301 or 360M freeze–etch devices (Balzers, Hudson, NH) according to our published methods.¹⁸,²⁸ Replicas were examined in a Philips 400T (Philips Electronic Instruments, Mahwah, NJ) or a Siemens 101 (Siemens Corporation, Cherry Hill, NJ) transmission electron microscope operated at 80 kV. The freeze–etch nomenclature of Branton et al.⁴ is used in this report.

Quantitative Analysis. Freeze–fracture greatly simplifies the analysis of membrane components because this technique regularly exposes large expanses of sarcolemma. Thus, 100 sq µm of sarcolemma was examined for each rat myofiber and for each normal and dystrophic human myofiber fixation protocol. Caveolae in rat myofibers were counted and their diameters measured on 10,000× prints using a 10× Peak scale lupe (Ted Pella, Inc., Tustin, CA) containing a micrometer eyepiece. In normal and dystrophic human myofibers, caveolae were counted and measured on 10,000–60,000× prints. Based on these standard areas, 700–2700 caveolae were counted for each fixation protocol. (For comparison, we note that in conventional thin sections, the examination of an equivalent membrane area would have required photographing at high magnification 1000–2000 µm of sarcolemmal profiles and the analysis of 400–800 high magnification 8 × 10

Caveolae in Skeletal Muscle

FIGURE 2. Method for obtaining surface fibers at selected distances from the cut end of the fiber: (a) fibers within 1 mm of the cut edge, (b) fibers 2.5–3.5 mm from the cut edge, (c) fibers 5–6 mm from the cut edge.
inch micrographs for each fixation protocol. Freeze-fracture thus eliminated the need to examine the several thousand 8 × 10 inch photographic prints that would have been required in a comparable analysis of thin-section images.) Nevertheless, conventional thin sections of similarly prepared muscles were examined from "surface" to "deep" fibers and were compared with the freeze-fracture images.

RESULTS
Rat Myofibers

Perfusion-fixed myofibers. Freeze-fracture replicas of normal rat EDL myofibers from perfusion-fixed animals had irregularly distributed caveolae at a density of 6.9 sq μm of sarcolemma surface (Fig. 3A). These caveolae had an average diameter of 28 nm (Table 1 and Fig. 4). Only very rarely were caveolae seen in bands.

Immersion fixation, uncut fibers. Surface Myofibers: Freeze-fracture replicas of intact myofibers obtained at or near the surface of immersion-fixed muscle showed numerous small caveolae (Fig. 3B), often arranged in bands oriented perpendicular to the muscle fiber axis (see also, Fig. 5). In these fibers, there were 9.5 caveolae/sq μm. The average diameter of these caveolae was 37 nm (Table 1 and Fig. 4).

Deep Myofibers: Freeze-fracture replicas of intact immersion-fixed deep fibers had numerous large caveolae, which were almost always arranged in bands oriented perpendicular and parallel to the myofiber axis (Fig. 3C). The bands in both directions had center-to-center spacings of about 2 μm, with the longitudinal bands containing fewer caveolae than the transverse bands. The caveolae were present at a density of 8.3 sq μm and had an average diameter of 83 nm (Table 1 and Fig. 4), an increase in diameter of 290% from those in perfusion-fixed myofibers.

Thin sections of rat myofibers from fixation depths of 0–1000 μm (Fig. 6) confirmed the data obtained from freeze-fracture replicas that an increase in diameter of caveolae occurs with increasing fixation depth. These thin-section images and the companion freeze-fracture images show

FIGURE 3. (A) Sarcolemmal P-face of perfusion-fixed EDL muscle. The sarcolemma contains irregularly arranged caveolae, 25–30 nm in diameter. (B) Sarcolemmal P-face from a surface fiber of an immersion-fixed EDL muscle. Caveolae are 30–45 nm in diameter. (C) Sarcolemmal P-face of a fiber approximately 1 mm deep within an immersion-fixed EDL muscle. Caveolae are 80–90 nm in diameter. (Bar = 0.1 μm.) (Prefracture contamination in A and C.)
that the preponderance of the larger invaginations
seen in the deeper fibers represent closed-ended
or Ω-shaped caveolae, rather than transverse in-
foldings of the plasma membrane or the expanded
opening of T-tubules. [However, a small fraction
(<10%) represents “coated pits” (see Fig. 6C),
which are not easily differentiated from “true”
caveolae in freeze-fracture replicas.]

**Immersion fixation of cut myofibers; surface fibers at
selected distances from the cut end.** To identify the type
and magnitude of the alterations induced follow-
ing surgical dissection (or following tissue mincing
before or during the initial fixation), whole rat
muscles were transected by a single cut and im-
mediately immersed in the fixative solution. Only
surface fibers were prepared for subsequent exam-
ination. Sarcolemmas within 1 mm of the cut end
of the myofiber possessed an extremely large
range of caveolar sizes (Fig. 7A). Many of the

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**Table 1.** Numerical data (number and mean diameter) of caveolae in rat EDL muscles after
different fixation methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>No./100 sq μm</th>
<th>No./sq μm</th>
<th>Mean diameter and standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfusion</td>
<td>690</td>
<td>6.9</td>
<td>28 nm (±17)</td>
</tr>
<tr>
<td>Immersion (surface fibers)</td>
<td>954</td>
<td>9.5</td>
<td>37 nm (±11)</td>
</tr>
<tr>
<td>Immersion (center fibers)</td>
<td>834</td>
<td>8.3</td>
<td>83 nm (±17)</td>
</tr>
</tbody>
</table>

*Caveolae number remains relatively constant but diameters are increased 290% in deep myofibers.*

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**FIGURE 5.** Low magnification micrograph of the P-face of the sarcolemma of a surface fiber inside an immersion-fixed EDL
muscle. The caveolae are arranged in bands perpendicular and parallel to the fiber axis. (Bar = 1 μm.)
largest caveolae (100–500 nm) had irregular profiles, perhaps indicating that they resulted from the fusion of several caveolae or, alternatively, that they represented irregular invaginations or infoldings of the sarcolemma.

At 2.5–3.5 mm from the cut edge of the surface fibers, there were fewer of the irregular large diameter (up to 200 nm) caveolae and more of the medium and small diameter caveolae (30–50 nm; Fig. 7B). The density of caveolae was essentially the same as for uncut, immersion-fixed surface fibers.

At 5.0–6.0 mm from the cut end of the surface fibers, both the size and distribution of caveolae (Fig. 7C) were similar to those in uncut immersion-fixed surface myofibers (see Fig. 3B). Thus, after immediate immersion fixation of cut rat myofibers, substantial caveolae alterations occur within 3 mm...
of the cut ends of the fibers, but at greater than 5 mm from the cut end, caveolae are similar to those in intact myofibers.

**Human Myofibers**

*Immersion fixation, cut myofibers.* Normal Myofibers, Surface Fibers Only: Immersion-fixed surface fibers from normal (nonweak) human patients (Fig. 8A) revealed irregularly (randomly) distributed caveolae at a density of 13.0 sq μm and with a mean diameter of 32 nm (Table 2 and Fig. 9). The lack of caveolae banding pattern and the presence primarily of small diameter caveolae rather than large caveolae is at variance with the data reported by others, but is consistent with the caveolae data seen in similarly prepared rat myofibers (see Fig. 3B, above).

Dystrophic Myofibers, Surface Fibers Only: Immersion-fixed surface fibers from three patients with Duchenne muscular dystrophy (Fig. 8B) also revealed irregularly (randomly) distributed caveolae with a mean diameter of 36 nm, but at an increased mean density of 26.8 sq μm (Table 2 and Fig. 9). The lack of caveolae banding pattern and the apparent increased number of caveolae are consistent with published data for Duchenne myofibers, but the much smaller diameter is inconsistent with published reports. (However, we note that these small diameter caveolae are similar to those seen in both normal human and rat myofibers prepared by our methods, see above.)

Deep Fibers (FSH Dystrophy): To compare surface versus deep fibers in human patients, we were fortunate to obtain large biopsies (> 2 mm diameter) from two patients with FSH dystrophy and one patient with MG. Immersion-fixed deep fibers (Fig. 10) revealed large and small diameter caveolae (mean diameter of 52 nm) at a density of 18 sq μm. (Similar alterations were observed in deep fibers obtained from the MG patient; not shown.) We thus confirmed that caveolar alterations (increased diameter) similar to those seen in

**FIGURE 7.** (A) E-face of an immersion-fixed EDL muscle that was cut with a sharp razor blade before fixation. This sarcolemma was less than 1 mm from the cut edge of the muscle. The sarcolemma contains extremely large caveolae and irregular membrane invaginations. (B) P-face of an EDL sarcolemma approximately 3 mm from the cut edge. Large caveolae and a few irregular membrane invaginations are present. (C) P-face of an EDL sarcolemma approximately 5 mm from the cut edge. The appearance of the sarcolemma and caveolae in this sample is virtually identical to that of the immersion-fixed intact surface fibers shown in Fig. 3B. (Bar = 0.1 μm.)
FIGURE 8. (A) Sarcolemmal P-face of normal (nonweak) human muscle. (B) Sarcolemmal P-face from a patient with Duchenne dystrophy. (Bar = 0.1 μm.)

FIGURE 9. Graph of the number and diameter of caveolae in normal versus dystrophic human myofibers. Continuous lines were drawn interconnecting datum points from the same sample.

Caveolae in Other Human Neuromuscular Diseases: Fixation-induced alterations in caveolae morphology also occur in human muscle biopsies from patients with other types of neuromuscular diseases (Figs. 10 and 11 from FSH and LG dystrophy and Fig. 12 from MG). In those micrographs, the numbers of caveolae per square micrometer were 18, 46, and 18, respectively. However, we believe that these numerical and morphological data may be of little value in the characterization of these diseases because, as we have shown, changes in caveolae size and distribution reflect various aspects of sample preparation artifact. In addition, the wide variability in caveolae number may reflect differences in age or sex of the patients, prior muscle activity, disease state, or as yet unidentified artifacts of fixation.

DISCUSSION
This study utilized rat myofibers as a model to identify the separate changes in muscle plasma membrane ultrastructure contributed by (1) the method of glutaraldehyde exposure, (2) the depth of fixative penetration, and (3) the relative proximity of the sample to the cut end of the myofiber. We have shown that (1) the size and distribution of caveolae are substantially different in perfusion versus immersion-fixed muscle, (2) that caveolae diameter increases 3–5-fold in deep fibers as compared to surface fibers fixed by immersion, and (3)
Table 2. Numerical data (number and mean diameter) of caveolae in normal and dystrophic human muscle fibers.

<table>
<thead>
<tr>
<th>Immersion fixation</th>
<th>No./100 µm²</th>
<th>No./µm²</th>
<th>Mean diameter and standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (surface fibers)</td>
<td>1304</td>
<td>13.0</td>
<td>32 nm (± 14)</td>
</tr>
<tr>
<td>Duchenne dystrophy (surface fibers)</td>
<td>2678</td>
<td>26.8</td>
<td>36 nm (± 21)</td>
</tr>
</tbody>
</table>

*Diameters are similar in normal and dystrophic myofibers.*

that caveolae diameter in cut surface fibers is substantially increased in sarcolemmal regions close to the cut ends of the myofibers. In analogous studies of human myofibers, we have shown that similar fixation-induced changes in caveolae size and distribution occur in both normal and dystrophic myofibers and that if unaccounted for, these alterations severely complicate the characterization of caveolae in human neuromuscular disease.

From our examination of rat myofibers prepared by immersion and perfusion fixation, we have determined that: (1) rapid exposure to glutaraldehyde (as in perfusion-fixed myofibers) results in small diameter (20–30 nm) caveolae usually distributed irregularly in the sarcolemma, (2) delayed chemical fixation (as in central myofibers fixed by immersion) is characterized by large diameter (80–150 nm) caveolae that had become rearranged into bands oriented perpendicular and parallel to the fiber axis, and (3) myofiber scission (surgical biopsy) produces significant increases in caveolae diameters, with the severity of the

FIGURE 10. Sarcolemmal E face of a “deep” myofiber from a patient with facio-scapulo-humeral dystrophy (18 caveolae/sq µm). (Bar = 0.1 µm.)

FIGURE 11. Sarcolemmal P-face from a patient with limb-girdle dystrophy (46 caveolae/sq µm). (Bar = 0.1 µm.)

FIGURE 12. Sarcolemmal P-face from a patient with myasthenia gravis (18 caveolae/sq µm). (Bar = 0.1 µm.)
changes in caveolae diameter most apparent within 1–3 mm of the cut end of the myofibers.

**Relevance to Human Duchenne Dystrophy.** The distribution of caveolae in sarcolemmas has been suggested by some authors to be useful in distinguishing dystrophic from normal muscle.


Moreover, in *deep fibers*, the caveolae almost always were arranged in bands and their diameters were found to be as large as those reported by others for normal and dystrophic myofibers. Thus, our data demonstrate that the depth of the myofiber sample from the surface exposed to the fixative is of major importance in the preservation of caveolae.

A second caveat in the evaluation of biopsied muscles was traced to the induction of large irregular caveolae in all myofibers cut before fixation, and was found to be especially pronounced in areas within 3 mm of the cut ends of myofibers. To avoid the changes in caveolae size associated with muscle scission, we suggest that for ultrastructural analysis only surface fibers from the middle segments of bundles longer than 15 mm be examined or that intact myofibers be fixed by in situ infiltration before excision. (This latter procedure requires great care to insure that other tissues are not exposed to the fixative solution.)

A possible alternative to the problems inherent in immersion fixation may be provided by ultrarapid freezing of intact fibers immediately after biopsy, according to one or more of the several major variations of the technique. 2,15,21,22,34 For a recent review of the rapid freeze technique, see Rash. 24 Recent rapid freeze studies 5,20,29 suggest a substantial decrease in the number of caveolae in rapidly frozen endothelium and fibroblasts as compared with chemically fixed cells of the same type. Consequently, we are currently comparing rapidly frozen myofibers "cryofixed" following a variety of physiologic and pharmacologic manipulations (Poulos and Rash, in preparation). This instantaneous preservation technique may allow us to determine if caveolae size, number, and/or distribution may be useful in the characterization of human neuromuscular disease.