Donnan Potential of Rabbit Skeletal Muscle Myofibrils I:
Electrofluorochromometric Detection of Potential

(fluorescent dye/glycerol extraction)

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ABSTRACT  The fluorescence of the dye CC-6 [(3-hexyl-2-(3-hexyl-2-benzoxazolinylidyne)-1-propenyl)-benzoxazolium iodide] has been shown to indicate Donnan potentials in rabbit skeletal muscle myofibrils. These results are in agreement with previously published work in which the potentials were measured with microelectrodes on glycerol-extracted muscle fibers. The magnitude of the Donnan potential of the myofibrils has been shown to be dependent on the state (rigor or relaxed) of the system.

Boyle and Conway (1) suggested that the resting potential across the membrane of skeletal muscle is determined largely by the potassium equilibrium potential, which is established by a Donnan equilibrium; that is, an electrolyte-excluding fixed charge system determines the distribution of the ionic species which is largely responsible for the resting potential. This conclusion was based on studies of the movement of potassium and chloride ions across the cell membrane. Since then others have supported this view (2-5). Other aspects of the ionic basis of the potential across the muscle membrane at rest have been discussed by Hodgkin and Horowicz (6). More recently it was demonstrated that microelectrode imbalances of glycerol-extracted cardiac muscle fibers, which presumably lack a functional membrane, gave changes in potential which were similar in sign to the resting membrane potential (7, 8). This potential has been shown by Collins and Edwards (9) to behave as if it were originated from a Donnan equilibrium, since: (i) the magnitude of the potential was the same when potassium ion was exchanged for sodium ion, (ii) the magnitude of the potential decreased when the concentration of ions in the bathing medium increased, and (iii) the potential reversed sign when the pH was changed from 7 to 4 as might be expected from the fact that the proteins of muscle have isoelectric points at about pH 5.5. It was concluded from these results that the contractile proteins constitute a region of fixed negative charge and effectively exclude anions, thereby establishing a potential. More recent studies by Pemrick and Edwards (10) have shown changes in the potential of glycerol-extracted skeletal muscle and, therefore, changes in the charge distribution of the muscle fibers, on going from rigor to the relaxed state.

The work to be reported here was initiated to test a system which potentially could be used to localize microscopically and to quantify the contributions of the various portions of the sarcomere to the Donnan equilibrium and secondly to rule out any possible interference by the microelectrodes caused by the factors such as possible changes in the tip potential (11). The studies use the electrofluorochrome CC-6, which apparently binds to the membrane of the giant axon of the squid and which shows changes in the magnitude of its fluorescence during an action potential (12). Similar changes in fluorescence related to changes in membrane potential have also been shown in erythrocytes (13), synaptosomes (14), and mitochondria (15). The mechanism of action of this effect is unclear, however.

MATERIALS AND METHODS
Rabbit skeletal back and leg muscle was either glycerinated by standard procedures or frozen immediately. The entire myofibrillar isolation was carried out at 4°. Ten grams of either type of muscle preparation were chopped into 0.5 cm segments. The minced muscle was added to 100 ml of isolation medium [40 mM KCl, 1 mM MgCl₂, and 10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (Pipes) buffer at pH 6.8] in a Waring blender and homogenized for 2.5 min in 0.25 min pulses with 1 min cooling between the pulses. The resulting slurry was centrifuged for 5 min at a force of approximately 1000 × g. The supernatant was discarded and the myofibrils were resuspended in 60 ml of the isolation medium and recentrifuged. The resuspension and centrifugation were repeated. The final resuspension was in a rigor-producing solution [RPS: 5 mM KCl, 5 mM MgCl₂, 5 mM potassium ethyleneglycol bis(β-aminomethyl ether)-N,N'-tetraacetate (K₂EGTA), and 5 mM Pipes buffer at pH 6.8]. This suspension was then homogenized with 10 strokes of a Dounce homogenizer fitted with a size A pestle (Kontes). The myofibrillar suspension was checked by sodium dodecyl sulfate gel electrophoresis (10%) to insure that none of the major contractile proteins was removed by the isolation procedures.

The preparations were scrutinized for homogeneity and photographed. A Zeiss Universal microscope was equipped for differential interference microscopy after Nomarski (16). Photomicrographs were recorded on Kodak Panatomic-X 35 mm film.

Reagents. All the chemicals were of reagent grade. The N₄₂₅/ATP, EGTA, Pipes buffer, and ovalbumin were obtained from the Sigma Chemical Co. The electrofluorochrome CC-6 [3-(3-hexyl-2-(3-hexyl-2-benzoxazolinylidene)-1-propenyl)-benzoxazolium iodide (nomenclature of Michael M. Cone of Yale University)], also known as 3,3'-dihexyl-2,2'-oxacarbocyanine, was the gift of Alan S. Waggone of Amherst College.
Fluorescence Measurements. The fluorescence was measured in a Perkin-Elmer-Hitachi model MPF-3L spectrofluorimeter with the excitation wavelength set at 470 nm and the emission wavelength set at 505 nm. The experiments were carried out at 10–12°. The ATP solution was kept frozen until use. The pH was constant at 6.8.

The results of the experiments are expressed as the percent change in the fluorescence ($\%AF$) as defined by the equation: $\%AF = (100 \Delta F_R/\Delta F_C)$, where $\Delta F_C$ is the change in the fluorescence of the myofibrils when CC-6 is added and $\Delta F_R$ is the change when the test reagent is added. The percent change in fluorescence due to ATP was calculated with the equation: $\%AF_{ATP} = 100(DF_C - DF_A)/DF_C$, where $\Delta F_A$ is the change in the fluorescence of the myofibrils when both CC-6 and ATP are added. These equations correct for background.

To determine the binding of CC-6 to the myofibrils two types of experiments were conducted. Solutions containing CC-6 alone, CC-6 with myofibrils, and CC-6 with myofibrils and test reagents were filtered through Millipore filters (HAWP 02500) with pores 0.45 μm in diameter with Swinex 25 adapters. The fluorescence was measured both before and after the filtration and the $\%AF$ was calculated. Similarly, other preparations were centrifuged at 20,000 × g to pellet the myofibrils and the change in fluorescence was calculated as in the filtration experiments.

Potential Measurements. The potential measurements were carried out using a high input impedance amplifier with a digital voltmeter on the output and a potential-dividing circuit which was used to monitor electrode resistance. The glass microelectrodes were filled with 3 M KCl and had resistances between 12 and 25 MΩ. All the potential measurements were carried out on the glycerol-extracted muscle fibers, which apparently have no functional sarcolemma (17), before myofibrillar isolation, since the myofibrils were too small to be impaled successfully.

Membrane Integrity Studies. To insure that intact membranes were absent from the preparations, two tests were undertaken: (i) electron microscopic observations of pelleted myofibrils and (ii) the comparison of the water space of a myofibrillar pellet with the solute space of a membrane nonpenetrant of a similar pellet.

Pellets of the myofibrillar suspension were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 6.8 for 1 hr; postfixed in 2% osmium tetroxide in the same buffer for 15 min; dehydrated; embedded in Epon 812: Araldite M 6005: dibutyl phthalate (25:15:5); sectioned; stained with 1% uranyl acetate at 80° for 15 min and with Karnovsky’s lead (18) at 20° for 1–1.5 hr; and examined with a Phillips EM 300 at 60 kV.

The water space of a myofibrillar pellet was compared to the space penetrated by three radioactively-labeled solutes, two of which were considered nonpenetrants. [14C]Sucrose (4.3 Ci/mol), [carboxyl-14C]inulin (2.6 Ci/mol of molecular weight 5000–5500), and [1,3-14C]glycerol (12.4 Ci/mol) were obtained from the New England Nuclear Co. After addition of the label to a 10 ml suspension, the myofibrils were sedimented by centrifugation and extracted in 10 ml of distilled water with shaking for 3 or more hours. The radioactivity in 0.5 ml of the extract was determined after removal of the myofibrils and the total radioactivity of the sample was calculated. The volume penetrated by the radiochemical was calculated as the (cpm in the pellet/cpm per ml of the supernatant) after the radioisotope-containing suspension was pelleted. The pellet was dried at 50–80° and the weight of the water in it was calculated. The initial glycerol experiments with 10 mM glycerol showed the glycerol space to be

\[
\begin{array}{|c|c|}
\hline
\text{Sample} & \%\Delta F \\
\hline
\text{RPS + CC-6 + 133 mM NaCl} & +1.7 \pm 8.2 \\
\text{RPS + CC-6 + 133 mM KCl} & -2.9 \pm 5.7 \\
\text{RPS + 3.3 mM ATP} & +23.9 \pm 5.5 \\
\text{RPS + CC-6 + 3.3 mM ATP} & +20.0 \pm 16.3 \\
\hline
\end{array}
\]

The fluorescence of CC-6 is unaffected by salt and ATP.

\[
\begin{array}{|c|c|}
\hline
\text{Sample} & \%\Delta F \\
\hline
\text{MF + 133 mM NaCl} & -0.3 \pm 1.2 \\
\text{MF + 133 mM KCl} & -0.1 \pm 0.9 \\
\text{MF + 3.3 mM ATP} & +0.8 \pm 3.8 \\
\hline
\end{array}
\]

Table 1: Fluorescence of CC-6 is unaffected by salt and ATP

Table 2: Background fluorescence of the myofibrils (MF) is unaffected by salt and ATP
**Table 3. Lack of significant binding of CC-6 to myofibrils (MF)**

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample</th>
<th>% Original CC-6 F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtration</td>
<td>CC-6</td>
<td>58.5 ± 12.9</td>
</tr>
<tr>
<td></td>
<td>CC-6 + MF</td>
<td>58.2 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>CC-6 + MF + 133 mM NaCl</td>
<td>53.0 ± 11.6</td>
</tr>
<tr>
<td></td>
<td>CC-6 + MF + 133 mM KCl</td>
<td>63.1 ± 8.9</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>CC-6</td>
<td>87.0 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>CC-6 + MF + 10 mM NaCl</td>
<td>89.0 ± 0</td>
</tr>
<tr>
<td></td>
<td>CC-6 + MF + 133 mM NaCl</td>
<td>87.0 ± 4.0</td>
</tr>
</tbody>
</table>

The fluorescence values are expressed as the mean (± standard deviation) for a minimum of five measurements.

**RESULTS**

**Potential Measurements.** The changes in electrical potential on impaling glyceral-extracted frog muscle fibers have the same sign as the resting potential in the presence of the sarcolemma (9). To confirm that these potentials were found in glyceral-extracted rabbit muscle, fibers were impaled with microelectrodes in rigor-producing bath solution at five different concentrations of added NaCl. All of the changes in potential were negative with respect to the reference electrode (Fig. 1). The change in potential decreased with increasing salt concentration as reported previously (9).

**Fluorescence Studies.** In these experiments, the electrofluorochrome CC-6 was used at a concentration (2.8 nM) significantly lower than those used by other investigators (12, 13). The fluorescence of CC-6 alone, at this concentration, was not affected by addition of NaCl or KCl at the highest concentration used. CC-6 fluorescence was also unaffected by ATP, although the ATP did autofluoresce at the 470 nm excitation wavelength (Table 1).

When the CC-6 was added to the myofibrillar suspension there was an increase in fluorescence of 10–30% above the background. The background output signal of the myofibrils was significant. However, it was not affected by the addition of salts or ATP, as is shown in Table 2. In all other tables only the CC-6 signal is reported as the mean of six measurements.

Since it has been proposed that in erythrocytes the change in fluorescence is due to a change in the binding of the dye to the cells, the binding of CC-6 to myofibrils was examined. No statistically significant binding of the CC-6 to the myofibrils could be demonstrated by either the centrifugation or filtration methods (Table 3). The decrease of the fluorescence of the CC-6 alone in both methods can be attributed to nonspecific binding of the dye to the filters and to the centrifuge tubes. The data for the binding following various additions to the dye fall within the range of values for the CC-6 alone.

The effect of NaCl concentration on the change in fluorescence of the myofibrillar preparation from the non-glycerol-extracted muscle appeared to plateau at high concentrations of NaCl and KCl (Table 4). Similar experiments conducted with the myofibrils prepared from glyceral-extracted muscle gave similar results (Fig. 2). Therefore, myofibrils prepared from the non-glycerol-extracted or the glyceral-extracted muscle showed similar changes in fluorescence as a function of added salt (Fig. 3).

For each salt concentration used, the change in potential as measured using microelectrodes (shown in Fig. 1) is plotted as a function of the change of fluorescence of the CC-6-containing myofibrillar suspension (taken from Fig. 2) in Fig. 4. The points can be fitted by a straight line.

The addition of ATP to the myofibrillar suspensions in the rigor-producing solution to a final concentration of 3.3 mM changes them from the rigor state to the relaxed state. The preparations were checked microscopically after the addition

**Table 4. Similarity of effect of NaCl and KCl on fluorescence of myofibrils + CC-6**

<table>
<thead>
<tr>
<th>Salt</th>
<th>%ΔF</th>
</tr>
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<tbody>
<tr>
<td>133 mM NaCl</td>
<td>−52.3 ± 11.1</td>
</tr>
<tr>
<td>133 mM KCl</td>
<td>−59.9 ± 11.1</td>
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</table>
of ATP to insure that no contraction occurred. Controls had shown that no interaction of the ATP with the CC-6 could be detected (Table 1), nor did the ATP change the background fluorescence of the myofibrils (see Table 2). A percent change in fluorescence of $-29.3 \pm 5.6$ was observed when ATP was added to the myofibrils.

**Morphological Studies.** That the myofibrillar isolation procedure produced fairly uniform myofibrils with little, if any, vesicular or cytoplasmic contamination is evinced by Fig. 5. Myofibrils from a non-glycerol-extracted preparation and a glycerol-extracted one are shown in Fig. 5a and b, respectively. No noticeable difference between these two myofibrillar preparations can be seen in the light microscope using Nomarski optics.

As a further check for the presence of membrane fragments, portions of several myofibrillar preparations were pelleted and prepared for electron microscopy. In many sections no membranes or membrane fragments were seen. In some sections, however, a few membrane fragments were observed, but intact vesicles were seen very rarely, if ever. In this sampling, membranes were virtually absent.

Another test for membrane-bounded space in these preparations is to compare the water space with that of molecules to which the membrane is normally considered to be impermeable. This technique will determine if any space exists in the preparation which excludes membrane nonpenetrants. If such a space were to exist it would signify a membrane-bounded volume either vesicular, reticular, mitochondrial, or sarcolemmal in nature. As can be seen from Table 5, the water space is approximately the same as the sucrose, inulin, or glycerol space. It is suggested that the aberrant inulin value may be the result of binding to the myofibrils during the extraction, as is the case for the membrane-penetrant glycerol. Therefore, it is concluded that no measurable membrane-bounded spaces exist in these preparations.

**DISCUSSION AND CONCLUSIONS**

The results suggest that the fluorescence of the CC-6 in myofibrils serves as an indicator of the potential measured in glycerol-extracted muscle with microelectrodes (Fig. 4). The dependence of the fluorescence on the salt concentration is the same regardless of whether the glycerol-extracted or the non-glycerol-extracted preparation is used (Fig. 3). These results suggest that the behavior of the fluorescence reflects events occurring in the myofibrils in the absence of a membrane. The conclusion that no membrane is involved is supported by the results of the water space measurements. These measurements showed the correspondence between the space penetrated by the probes used (one of which was of molecular weight 5000) and the water space. Further, the evidence from electron microscopic and Nomarski system observations (Fig. 5) also suggests the absence of a functional membrane. A measurable potential which reflects the Donnan equilibrium can be established at a phase boundary, such as exists at the myofibril/external medium boundary. In the intact muscle, the corresponding phase boundary exists between the myofibrils and the sarcoplasms. In these experiments the sarcoplasm has been extracted, but the medium in which the myofibrils are suspended acts as a separate phase. However, another possible boundary at which a potential change may occur is that between the thick and thin filaments within myofibrils, and evidence concerning this will be presented in a subsequent publication. It is sufficient to note here that at least one and perhaps two geometries exist which could support the establishment of a potential difference.

A further indication that an ordered matrix is needed to establish a potential difference of this type is that denatured egg white also functions as a Donnan system (9). To extend this observation to the electrofluorochromometric method described in the present paper, purified ovalbumin was tested for changes in CC-6-enhanced fluorescence under conditions similar to those reported above. There was no change in the fluorescence upon addition of NaCl to 10%
ovalbumin suspended in the rigor-producing solution used for the myofibril experiments. If, however, the ovalbumin were denatured by heat, thereby giving it a particulate structure, a statistically significant change in the fluorescence was observed upon addition of the salt (Table 6). By denaturation a macroscopic matrix is created in the ovalbumin system, which then contains fixed charges within a phase boundary. Only in the presence of such a matrix can the fluorescence change depend on ionic strength. This is true also for the measured potential change in glycerol-extracted muscle.

The potential dependence on the salt concentration shown in Fig. 1 is that which would be predicted for a Donnan equilibrium. This study, however, extends these findings further in that now a change in fluorescence can be correlated with the potential difference. In these studies, the potential decreases as the decrease in fluorescence increases. In contrast, in erythrocytes the relation between the sign of the change in fluorescence and the sign of the change in the potential difference is the opposite in that the potential increases as the decrease in fluorescence increases (19). However, Sims et al. (19) reported a decrease in the fluorescence of a CC-6-containing medium upon addition of a suspension of erythrocytes, whereas the addition of a myofibrillar suspension increased the fluorescence. Further, the fact that with the myofibrillar preparations no binding of the CC-6 to the myofibrils can be demonstrated, whereas with the erythrocytes a significant amount of binding is seen, implies that a different mechanism may exist in the two systems. The changes in fluorescence in erythrocytes have been attributed to dye aggregation (19). In the present study, a possible explanation for the decrease in fluorescence may be the combination of the dye with a contractile protein. However, the molar ratio of myosin to dye is greater than 200. Therefore, this model seems unlikely.

The difference probably lies at the level of the interaction of the dye with the molecular architecture of the two systems. In the case of the erythrocytes a membrane which is functional and osmotically active is known to exist and the CC-6 could lie within the lipid phase of the membrane, could permeate the membrane and distribute itself across the membrane, or could interact electrostatically with the membrane proteins. Fortes and Hoffman have demonstrated that the dye 1-anilino-8-naphthalene sulfonate both permeates the membrane and acts at the membrane/medium boundary (20). For the myofibrillar case, no functional membrane is present and the only interactions possible are with the proteins of the myofibril or at the phase boundary between the entire myofibrillar structure and the external medium.

When 3.3 mM ATP was added to a CC-6-containing myofibrillar suspension, a percentage decrease in the fluorescence of 29.3 ± 5.6 was observed. This decrease was statistically significant (compare this result with Table 1). This indicates that upon going from the rigor to the relaxed state a potential decrease of about 5 mV occurs. This implies either a masking of approximately 1/4 the negative charges in the muscle or an increase in the positive charges. These results are at variance with those of Pernick and Edwards (10), who found that the potential became more negative upon going from the rigor to the relaxed state.

In conclusion, it has been demonstrated that the electrofluorochrome CC-6 can be used to monitor changes in the Donnan potential of glycerol-extracted skeletal muscle. This method is able to detect changes in charge density which may reflect conformational changes of the thick and thin filaments. Whether the charges on the proteins within the sarcomere play a role in the contraction of skeletal muscle must remain for further investigations. However, it should be noted that several models for muscle tension generation have been proposed which are based on the charges in the contractile proteins in muscle (21-23).

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