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Functional Heterogeneity of the Sarcoplasmic Reticulum within Sarcomeres of Skinned Muscle Fibers

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Summary. Precipitation of Ca oxalate in the sarcoplasmic reticulum of chemically skinned rabbit psoas fibers caused an increase in light scattering which was proportional to the amount of Ca accumulated per unit fiber volume. The increase in scattering was used to measure net accumulation rates and steady-state Ca capacities of the sarcoplasmic reticulum in single fibers. The data obtained were qualitatively and quantitatively similar to those reported for isolated vesicle preparations.

Under conditions in which Ca was not depleted from the medium, Ca accumulation was linear with time over much of its course. Steady-state capacities were independent of the Ca concentration; uptake rates were half-maximal at 0.5 μM Ca^{++} and saturated above about 1.0 μM. Both rate and capacity varied with the oxalate concentration, being maximal at oxalate concentrations $\geqq 5$ mM and decreasing in proportion to one another at lower concentrations, with a threshold near 0.25 mM. At the lower loads, electron micrographs showed many sarcoplasmic reticulum elements empty of precipitate alongside others that were full, whereas virtually all were filled in maximally loaded fibers. These data indicate that the Ca oxalate capacity of each fiber varies with the number and volume of elements in which Ca oxalate crystals can form at a given oxalate concentration, and that individual regions of the sarcoplasmic reticulum within each sarcomere differ in their ability to support Ca oxalate precipitation. Our working hypothesis is that this range in ability to form Ca oxalate crystals involves differences in ability to accumulate and retain ionized Ca inside the sarcoplasmic reticulum.

Vesicles of the sarcoplasmic reticulum (SR)[1] isolated from homogenates of skeletal muscle retain the ability to transport Ca against a large concentration gradient. Calcium is accumulated in the lumen of SR vesicles by an energy-dependent process which is mediated by a $Ca^{++}+Mg^{++}$-dependent ATPase in the SR membranes [22, 66]. It is widely supposed that the SR *in vivo* accumulates Ca by a similar mechanism, and that in the intact state it functions with rates and capacities for Ca uptake that are equal to or greater than those observed *in vitro* [14, 41, 70]. One goal of the present study is to compare data reported for isolated SR preparations[2] with SR function measured under similar conditions in a more intact preparation.

In fibers that have been skinned either chemically or mechanically, the sarcolemma is reduced or eliminated as a barrier to the passage of ions [8, 11, 15, 28, 31, 47, 54, 77] and the composition of the medium bathing the SR and the myofilaments can be controlled by the experimenter. In chemically skinned mammalian fibers, the SR retains most of the architectural features seen in electron micrographs of intact muscle [11], and the preparation is a favorable one for testing the capabilities of these intracellular membranes in a relatively intact state. Skinned-fiber preparations have been valuable in studies of Ca release mechanisms [14, 16, 64]. However, kinetic data on Ca accumulation by skinned fibers cannot be obtained easily with the techniques currently being used to estimate their Ca content, and the range of experimental conditions which it is practical to study is somewhat limited [14, 19, 64]. A second goal of this study is to describe a new technique which makes it possible to monitor kinetic as well as steady-state characteristics of Ca accumulation by the SR in single skinned fibers.

In a medium containing Ca and MgATP, the addition of oxalate causes skinned fibers to become visibly darker [50]. The lumen of the SR becomes electron-

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[1] *Abbreviations used:* SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis (β-aminoethyl ether) N,N′-tetraacetic acid.

[2] The term *preparation* is used in this manuscript to refer to all fibers isolated from the same rabbit.

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opaque [7, 9, 17, 51; *see also* 52], and the amount of light scattered by the fibers increases. We use the increase in light scattering to determine rates and steady-state capacities for Ca accumulation in the presence of different Ca and oxalate concentrations. From these data and from electron micrographs of fibers loaded to different steady-state levels with Ca oxalate, we conclude that within each sarcomere of a chemically skinned fiber there are elements of the SR which differ in their ability to form Ca oxalate precipitates. We propose that differences in ability to accumulate and retain ionized Ca may be involved. A preliminary report of some of these data has appeared [63].

Materials and Methods

Preparation of Chemically Skinned Fibers

Bundles containing several hundred fibers were removed from rabbit psoas muscles after being tied at rest length. Each bundle was chemically skinned by exposure to a solution containing 5 mM EGTA, 5 mM imidazole, 2.5 mM Mg, 2.5 mM ATP, and 170 mM K propionate, at pH 7.0 [11, 55, 77]. After 24 hr in the skinning solution at 0°, the bundle was transferred to skinning solution made up in 50% glycerol, and stored at −20° until used [56]. For most experiments, single fiber segments were used. Pieces 3–8 mm long were dissected from the bundle. Fiber diameters measured at 120–130% of slack length in relaxing solution (*see* below) ranged from 25 to 100 μm, with a mean of 50 μm. Histochemical tests based on myosin ATPase activities have shown that the psoas contains primarily type II fibers, which are characteristic of fast-twitch muscles [74].

Ultrastructural changes which characterize the chemically skinned and stored fibers are consistent with increased permeability of the sarcolemma and have been described elsewhere [11]. For most experiments, preparations had been stored for two to 40 days. During this period, rates of accumulation of Ca oxalate from the standard loading medium (*see* below) remain stable (Table 1). Data are also included from one preparation which was first tested under standard conditions after five months of storage (last line of Table 1).

It should be pointed out that our use of a glycerol-containing relaxing solution for storing chemically skinned fibers [56] differs in several respects from both the classical glycerinated fiber preparation [65] and the brief glycerol treatment used by others to disrupt the sarcolemma [13, 21, 28, 51, 52] or the transverse tubular system [26]. For our preparation, major osmotic pressure changes are avoided because the sarcolemma is first rendered freely permeable by the isosmotic skinning solution before exposure to the hyperosmotic glycerol solution.

Solutions

With a few exceptions noted in the text, concentrations of MgATP, EGTA, and H^+ in the experimental solutions were kept constant. The solution used for maintaining skinned fibers in a relaxed state ("relaxing" solution) in the absence of Ca contained 5 mM K_2Na_2ATP, 2.5 mM $MgCl_2$, 5 mM K_2 EGTA, 170 mM K propionate, and 5 mM imidazole, pH 7.0. For measurements of Ca accumulation, the loading medium used in most experiments was identical except for the concentration of K_2EGTA, which was replaced by a mixture of 2.15 mM CaEGTA and 2.85 mM K_2EGTA. With the addition of 5 mM K oxalate, this solution served as the "standard loading medium". Ionized Ca of this solution is 0.39 μM (pCa 6.4), calculated using apparent association constants of 1.919×10^6, 40, 5×10^3, and 10^4 M^{-1} for CaEGTA, MgEGTA, CaATP, and MgATP, respectively (for references, *see* 49). When other ionized Ca concentrations were used, the ratio of CaEGTA to EGTA was altered, keeping total EGTA at 5 mM. Control experiments were performed using buffered Ca solutions containing a total of 15 mM EGTA in order to ensure that diffusion of Ca into the fibers was not rate-limiting in our experiments, even at the lowest Ca concentration used.

Caffeine, ATP, ITP, EGTA, and imidazole were obtained from Sigma Chemical Company (St. Louis, Mo.). CaEGTA was prepared from $CaCO_3$, H_4EGTA, and KOH. Brij-58 was purchased from Ruger Chemical Company (Irvington, N.J.). A23187 was the gift of Dr. R.L. Hamill of Eli Lilly Co. (Indianapolis, Ind.). A stock solution of 7 mM in ethanol was stored in a dark bottle in the cold, and a fresh dilution to 0.01 mM in water was prepared each day. For each experiment, aliquots of 0.5–20 μl were added to the bath. X-537A (Na salt) was a gift from Hoffman-LaRoche (Nutley, N.J.). A fresh stock solution was prepared each week (13 mM in ethanol), and aliquots of 0.1–1.25 μl were added to the bath.

Apparatus

The experimental setup used to monitor light scattering in isolated fibers has been described previously in connection with a study of changes in light scattering induced by actin-myosin interactions [30]. In the present study, changes in light scattering due to actin-myosin interactions were prevented by the use of a high concentration of MgATP, and either by using low concentrations of ionized Ca (pCa 7–6.4) or, for experiments at pCa 6.2–5, by stretching the fibers to 180% of slack length before introducing Ca into the medium (*see* Fig. 6). Control experiments were performed at pCa 6.4 on fibers stretched to 130% and 180% of slack length. No significant differences were observed.

Fibers were mounted between two clamps in a chamber (0.5 ml) with a quartz bottom. Rapid stirring of the bathing medium was provided by a small probe attached to a vibrating motor. Fibers were clamped at 120–130% of slack length (measured to within 5%) and positioned in the light beam at the region of maximum sensitivity [30]. Experiments were performed either with white light or with a monochromatic beam (490 nm). Part of the incident light directed vertically into the chamber from below was scattered by the fiber. The component that was scattered at 90° was collected by a fiber-optics light guide mounted at right angles to both the fiber and the incident beam, and directed into a photomultiplier. The voltage output from the photomultiplier was amplified, filtered and displayed on a linear strip chart recorder. Scattering due to chamber and solutions was measured prior to each experiment and subtracted from all readings (*see* Fig. 1*a*). This background scatter was virtually independent of the composition of the experimental solutions and, as indicated in the legend of each figure, it accounted for 10 to 20% of the total scattering signal from a single fiber in relaxing solution.

Calibration

A typical experimental sequence used for converting the scattering signal to fiber Ca concentration is shown in Fig. 1*a*. Scattering (S) by the fibers in relaxing solution was recorded, and in some

Table 1. Ca accumulation rates after storage for various periods

Preparation	Storage time in days				
	1–2	9–10	21–23	28–29	>40
I	0.40 (1)	0.41 (12)	0.44 (4)	0.52 (3)	–
II	–	0.31 (2)	0.24 (5)	0.26 (2)	–
III	–	–	0.40 (5)	0.44 (2)	0.36 (3)
IV	0.56 (3)	–	–	–	0.50 (7)
V	–	–	–	–	0.28 (3); 0.37 (7)

Rates are expressed as rates of increase in light scattering, $\Delta S/S \cdot$min, measured under standard loading conditions (pCa 6.4, 5 mM oxalate; *see* text) at 23–28°. Numbers of single fibers tested at each storage period are indicated in parentheses. Each preparation remained for 24 hr at 0–4° in skinning solution before being transferred to skinning solution made up in 50% glycerol (*see* text) and stored at −20° for the times shown. Storage times in the last column were 44–48 days for preparation III, 47 days for IV, and 145 and 185 days for V. Each preparation was from a different psoas muscle except for IV, which was from another fast-twitch muscle (extensor digitorum longus).

cases diameter was measured. Then a ^{45}Ca oxalate loading solution (pCa 6.4+ox) was introduced. Loading was allowed to proceed for a different period for each bundle in order to obtain a range of increments in light scattering (ΔS). The time course of loading is described further in the Results. Loading was terminated by a single wash with a solution containing 0.25 mM EGTA, 5 mM ATP, 5 mM oxalate, and 10 mM Mg (−Ca, Fig. 1*a*). In this solution, light scattering and tension were monitored for an additional min to ensure that withdrawal of the loading solution did not cause loss of the light-scattering signal (Fig. 1*a*) nor induce contraction [57].

At the end of the wash period, the bundle was cut out from between the clamps, rinsed for 3 min in 2–4 ml of fresh wash solution, and dissolved by heating in an alkaline solution as described by Elison et al. [13]. Aliquots were taken for protein determination [36] and for counting in a liquid scintillation counter [35]. Volumes of the bundles used in these experiments were less than 0.05 µl, i.e., less than $^{1}/_{10,000}$ of the volume of the chamber used for loading. Total Ca accumulated never exceeded 0.5% of the amount present in the chamber. External ionized Ca concentration was thus essentially constant throughout these experiments, as in the study by Elison et al. [13].

Figure 1*b* demonstrates the proportionality between protein content and S in relaxing solution for 15 bundles. This finding

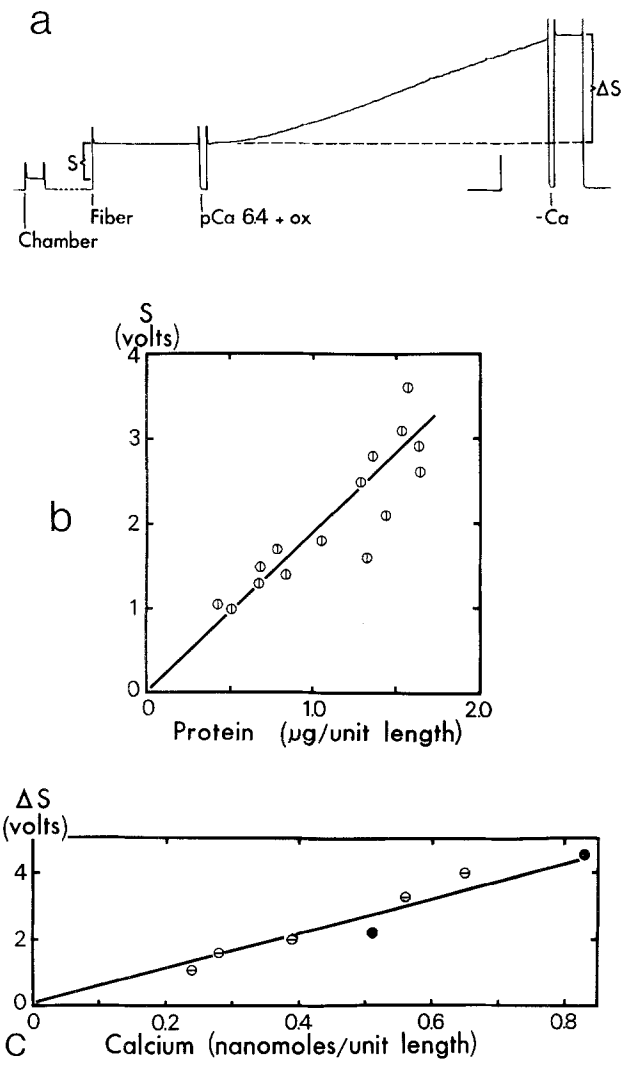


Fig. 1. Calibration of the light-scattering signal. (*a*): Record of scattering before and after exposure of a single fiber 73 µm in diameter to the standard (pCa 6.4) loading medium (2.15 mM Ca, 2.85 mM EGTA, 5 mM oxalate, 5 mM ATP, 2.5 mM Mg, 170 mM K propionate, 5 mM imidazole, pH 7.0). Fiber was exposed first to relaxing solution (*Fiber*) to obtain a reading for S, then to the loading medium containing ^{45}Ca (*pCa 6.4+ox*). After loading had proceeded for 10.5 min, the increase in scattering (ΔS) was terminated by washing the fiber with a solution containing 0.25 mM EGTA and no Ca (*−Ca*). Scattering remained steady for one min. Then the signal to the photomultiplier was blocked while the segment of fiber between the clamps was cut out and transferred to a petri dish for further washing before being digested and counted. At left, scattering signal from the chamber containing relaxing solution, before the fiber was mounted in the light path. Vertical transients are given by ambient light (upward deflection) followed by closing of a shutter (downward deflection) designed to prevent illumination of the photomultiplier during prolonged exposures of the chamber to ambient light while manipulating the fiber or changing solutions. Horizontal calibration bar, 1 min. Vertical calibration, 0.5V. Fiber was stretched to 120% of slack length. Temperature, 26 °C. Components of relaxing and washing solutions are given in Materials and Methods. (*b*): Proportionality between protein content and scattering signal (S). Bundles of 2–5 fibers stretched to 120% of slack length were exposed to relaxing solution. Protein content was determined as described in Materials and Methods. Since the diameter of the incident beam was fixed (about 2 mm), whereas the length of the bundle taken for protein and Ca determinations varied, the measured amounts of protein (Fig. 1*b*) and Ca (Fig. 1*c*) were divided by the length of the bundle in mm. (*c*): Proportionality between increase in scatter (ΔS) and Ca content. Seven of the same bundle segments shown in *b* were exposed for various periods to standard loading solution containing ^{45}Ca. *Open symbols:* loading terminated (as in *a*) before light-scattering signal had reached a plateau. *Filled symbols:* ΔS had reached a plateau (as in Fig. 2). After converting protein content to fiber volume (*see* Materials and Methods), Ca concentrations in these two bundles were calculated as 102 and 106 mM (mmol/liter fiber)

confirms an earlier observation [30]. Fig. 1*c* shows the proportionality between ΔS and the Ca taken up by seven of these same bundles. Since the increase in light scattering is proportional to the Ca content of the fiber both during net uptake and at the final plateau, we can use the increase in scattering to monitor rate of Ca accumulation, as well as capacity at the steady state.

The proportionality constant calculated from the ratio of the inverse of the slopes of the lines in Fig. 1*b* and *c* provides a means of relating the relative increase in light scattering ($\Delta S/S$) caused by loading with Ca oxalate to nanomoles Ca/μg protein in any fiber from the same animal. The Ca concentrations can be expressed in terms of fiber volume by using the relationship between volume and protein content ($x \pm SD = 136 \pm 25$ μg protein/ml fiber) obtained from length and diameter measurements on the fibers of 11 of the bundles used for the protein determinations in Fig. 1*b*.

Electron Microscopy

The primary objective of our electron microscopic observations was to determine the extent and distribution within sarcomeres of Ca oxalate precipitates formed under different loading conditions. Therefore, we chose fixation and sectioning procedures which favor preservation and visualization of Ca oxalate crystals, at some sacrifice of contrast for the membranous elements. As others have reported, the fibers could not be stained to enhance contrast without causing the crystals they contained to dissolve.

Fibers were fixed by adding 0.1% glutaraldehyde to the loading medium. All subsequent exposures to aqueous solutions were made with the precautions suggested by Pease, Jenden, and Howell [52] to retain Ca oxalate crystals formed during loading. After 2–3 min, the solution containing glutaraldehyde was replaced by one which was identical, except that it lacked ATP and that 50 mM K oxalate had been added, together with enough $CaCl_2$ to cause precipitation of Ca oxalate. The supernatant of this suspension provided a clear solution saturated with Ca oxalate in which light scattering by the loaded fiber was monitored for 5–8 min after adding fixative in order to ensure that no decrease in the scattering signal was occurring. After 30 min at room temperature in the aldehyde fixative, fibers were transferred to a solution identical except for 1% osmium tetroxide instead of glutaraldehyde, and left on ice for 30 min. To minimize loss of Ca oxalate crystals during dehydration, fibers were passed directly into four changes of 100% ethanol [52], each lasting only 1 min. After four similar changes of propylene oxide, the fibers were embedded in Epon 812.

As an additional precaution against losing crystals or translocating them along the sarcomeres during sectioning, sections were cut at right angles to the fiber axis and were 0.25–1.0 μm thick. With these dimensions, a large number of the crystals were entirely contained within the sections and never came in contact with the knife. Sections were cut with glass knives, using water saturated with Ca oxalate in the knife boat. The sections were collected on uncoated "oyster" grids and examined in a Philips EM-200 electron microscope using an acceleration voltage of 100 kV.

Results

Time Course

The time course of the increase in light scattering that accompanied Ca accumulation in the presence of oxalate typically showed a small initial step, a lag, a long linear region, and a plateau (Fig. 2). The

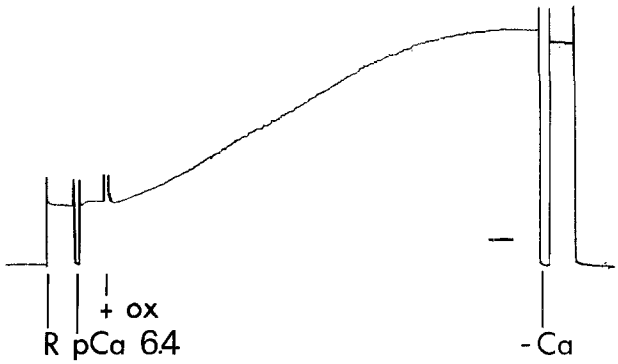


Fig. 2. Time course of the increase in scattering in the standard loading medium. Fiber was exposed first to relaxing solution (*R*) to obtain a reading for *S*. A small initial increase in scattering occurred when relaxing solution was replaced with one containing Ca but no oxalate (*pCa 6.4*). Addition of 5 mM oxalate to the bath (*+ox*) was followed by an increase in scattering which reached its maximum rate (27% of *S* per min) within about 2 min, and continued at this rate for 8–9 more minutes before approaching a plateau. At the plateau, the fiber was washed with a solution lacking Ca, as in Fig. 1*a*, and the signal remained steady (*−Ca*). Single fiber, 130% of slack length. Background scatter 5.4% of signal in *R*. Calibration, 1 min. Temperature, 25 °C

initial step occurred upon addition of Ca alone (pCa 6.4, Fig. 2); it was variable in amplitude and was absent in some fibers (*compare* Figs. 2 and 5*b*). In the absence of oxalate it reached a steady level in less than a minute. Measurements with ^{45}Ca showed that when this steady level had been reached, the fibers contained 1–3 mM Ca. The initial step was not characterized further in this study.

Following the addition of oxalate, scattering increased over a period of about 2 min until it reached its maximum rate. The rate then remained constant until a large fraction of the final signal had been attained (79% of the final ΔS value in Fig. 2). In 27 fibers from two different preparations allowed to take up Ca under the conditions of Fig. 2, scattering increased linearly with time until from 45 to 80% of the final plateau had been reached (mean 54 and 70% in the two preparations).

Measurements using ^{45}Ca have shown that in briefly glycerinated fibers [52] and in isolated SR vesicles [24], Ca accumulation in the presence of a precipitating anion is also linear with time until most of the final load has been accumulated. In these studies, as in ours, conditions were chosen so that the ionized Ca concentration remained essentially constant throughout the experiment.

The steady rate of increase in light scattering and the amplitude of the plateau attained as Ca was accumulated in the presence of oxalate varied from fiber to fiber. In one series, 46 fibers or small bundles from four different preparations were exposed to the

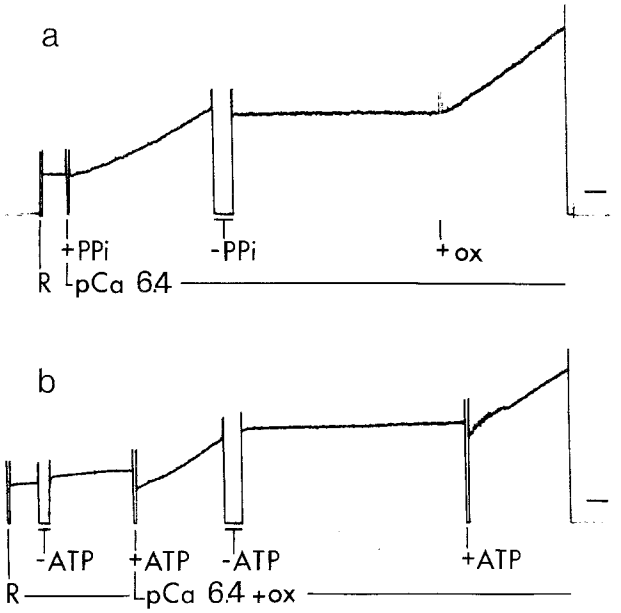


Fig. 3. (*a*): Requirement for a Ca-precipitating anion. After scattering signal was recorded in *R*, fiber was exposed to standard loading medium in which oxalate had been replaced by 5 mM sodium pyrophosphate (*pCa 6.4, +PPi*). Scattering increased to a maximum steady rate (40% of *S* per min). After 5 washes with a pCa 6.4 solution containing no precipitating anion (*−PPi*), scattering remained essentially unchanged for 9 min, until 5 mM oxalate was added (*+ox*). The maximum rate of increase in scattering was 48% of *S* per min in the presence of oxalate. Single fiber, 130% of slack length. Background scatter 11% of signal in *R*. Calibration, 1 min. Temperature, 25 °C. (*b*): Requirement for substrate. Three washes with a solution identical to *R* but lacking ATP caused a 30% increase in *S* (*−ATP*), due to formation of rigor bonds between actin and myosin [30]. Replacement of the rigor solution with standard loading medium containing ATP (*pCa 6.4+ox*) restored the original signal, followed immediately by an increase in scattering which reached a maximum rate of 42% of *S* per min. Five washes with loading solution lacking ATP (*−ATP*) reduced the rate of increase in scattering to less than 2% of *S* per min, following a small rigor step like that seen when ATP was removed in the absence of Ca. When the complete loading medium was again present (*+ATP*), scattering continued to increase at 43% of *S* per min, after an initial irregularity. Bundle of 4 fibers, 130% of slack length. Background scatter 4% of signal in *R*. Calibration and temperature as in *a*

standard loading medium as in Fig. 2, and the plateau ΔS values attained ranged from 1.2 to 8.5 times the signal from the unloaded fibers. Calibration curves relating fiber Ca concentration to the relative increase in light scattering ($\Delta S/S$) were determined for each preparation (*see* Materials and Methods). The maximum steady-state loads attained in 5 mM oxalate ranged from 20 to 170 mM (mmol/liter fiber), with a mean of 91 mM for the four preparations. Average values were ($\pm$SD, in mM) 60 ± 12 (8), 67 ± 35 (15), 89 ± 33 (18), and 147 ± 16 (5), where the numbers in parentheses indicate the number of fibers or small bundles for each preparation. These averages include direct determinations of ^{45}Ca content in 18 fibers or bundles; the rest were calculated from the calibration factors.

In the same series of experiments, maximum rates of increase in light scattering in the standard medium ranged from 0.07 to 0.74 $\Delta S/S \cdot$min at 25–29 °C. From the calibration curves, these values represented Ca accumulation rates from 1.2 to 17 mM/min. As discussed in a later section, lower rates were associated with lower plateau values.

Requirements for Increased Light Scattering

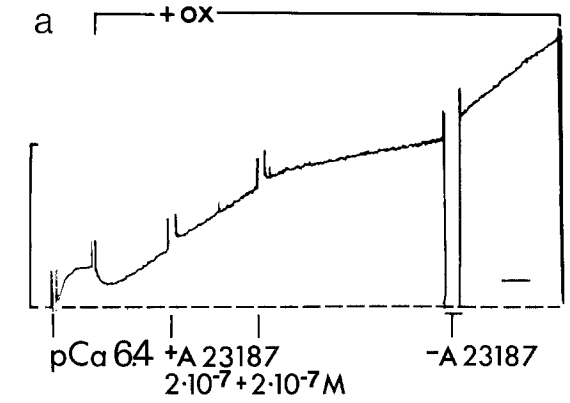
1) Calcium. Fig. 1*a* shows that Ca was essential. Removing Ca (−Ca) from the loading medium prevented a further increase in light scattering, despite the continued presence of oxalate and MgATP in the medium.

2) Anion. The data of Figs. 2 and 3*a* illustrate the requirement for a precipitating anion. In the presence of Ca, only a small increase in light scattering was observed until oxalate was added (+ox, Fig. 2; *see also* Fig. 5*b*).

The concentration dependencies for the effects of oxalate and Ca are described in a later section.

Other anions were less effective than oxalate in promoting an increase in light scattering at pCa 6.4. An experiment with pyrophosphate is shown in Fig. 3*a*. In nine bundles or single fibers from three different preparations, the rate in 5 mM pyrophosphate ranged from 43 to 78% of the rate in 5 mM oxalate, with a mean of 57% for the three preparations. Fluoride was much less effective than either oxalate or pyrophosphate (data not shown). The order of effectiveness of these three anions was thus identical to that reported for ^{45}Ca accumulation in isolated vesicles, and presumably reflects the relative insolubility of their Ca salts [22, 42]. Phosphate (up to 50 mM) caused no increment in light scattering. However, 5 mM phosphate completely abolished the increase in light scattering promoted by 1 mM oxalate. Competition between phosphate and oxalate in this concentration range has been reported for Ca and anion accumulation by isolated vesicles [2].

3) Substrate. A source of energy was required for the increase in light scattering shown in Figs. 1 and 2. Removal of ATP from the medium (−ATP, Fig. 3*b*) in the presence of Ca and oxalate virtually abolished the rise in scatter. Data from isolated SR vesicles indicate that the transport ATPase is saturated in the presence of 0.1–0.5 mM MgATP [69, 73,



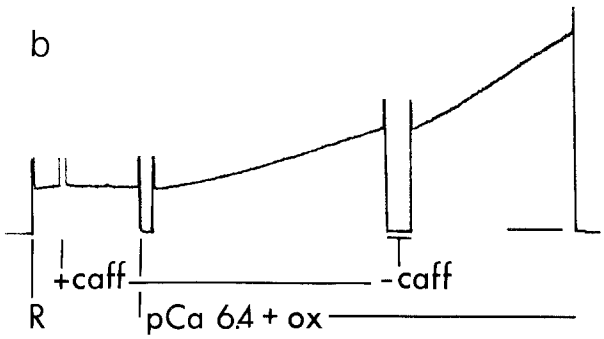
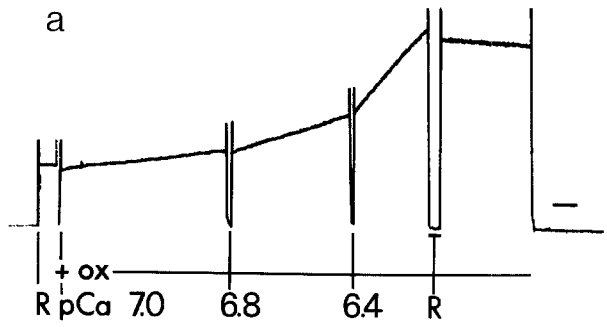


Fig. 4. (*a*): Reversible inhibition by ionophore. Following addition of 5 mM oxalate to complete the standard loading medium (+*ox*), the control rate of increase in scatter reached 13% of *S* per min. The first 1-μl addition of a 10^{-4} M solution of A23187 had virtually no effect (< 3%) on the rate; within 2 min after a second addition of A23187, the rate dropped to 28% of the control. Three washes with standard loading solution to remove ionophore restored the rate of increase in scattering to 120% of the control. There was some development of tension (not shown). Initial step caused by adding Ca (*pCa 6.4*) was large in this fiber (27% of *S*). Bracket at left shows magnitude of scattering signal recorded in *R*; baseline was then lowered by this same amount so that record shows only increment in scatter (*ΔS*), above *S* (dashed line). Single fiber, 130% of slack length. Background scatter 12% of signal in *R*. Calibration, 1 min. Temperature, 25 °C. (*b*): Reversible inhibition by caffeine. Signal recorded in *R* was not changed by the addition of 5 mM caffeine (+*caff*). After 3 min in the presence of caffeine, fiber was exposed to standard loading medium (*pCa 6.4*+*ox*) containing 5 mM caffeine, and a linear rate of increase in scattering (22% of *S* per min) was eventually attained. Within 1 min after fiber was washed three times with standard loading medium to remove caffeine, the rate increased to 43% of *S* per min, which is in the normal range for this preparation (compare rates in Fig. 3, from the same preparation). Single fiber, 130% of slack length. Background scatter 9.4% of signal in *R*. Calibration and temperature as in *a*



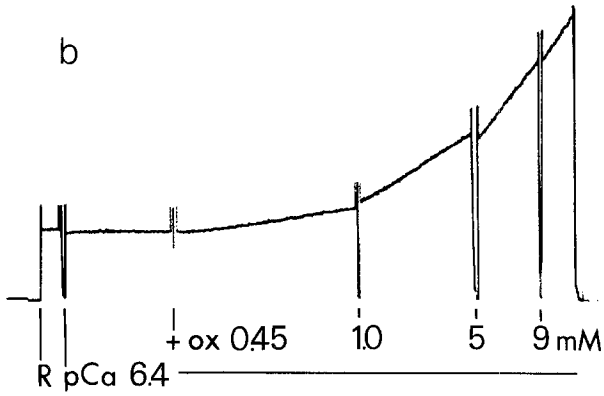


Fig. 5. (*a*): Effects of external Ca^{++} on the loading rate. Fiber was exposed first to relaxing solution (*R*), then to progressively higher concentrations of ionized Ca (*pCa 7.0, 6.8,* and *6.4*) in the presence of 5 mM oxalate. The maximum rates of increase in light scattering increased from 6 to 14% and 56% of *S* per min. When Ca was removed at the end of this sequence by washing the fiber with *R*, a very slow loss in scattering ensued. Single fiber, 180% of slack length. Background scatter 12% of signal in *R*. Calibration, 1 min. Temperature 25 °C. (*b*): Effects of oxalate on the loading rate. At a fixed external Ca concentration (*pCa 6.4*), there was no increase in light scattering until 0.45 mM oxalate was added to the bath (+*ox*); then *ΔS*/min reached 6.9% of *S*. When the oxalate concentration was increased to 1.0 and 5.0 mM, the rate of increase in scattering increased to 26% and 53% of *S* per min. Raising the oxalate concentration to 9 mM had little further effect. Note that the rate in 5 mM oxalate was nearly identical to the rate recorded in *a* for the same loading condition, although the fiber in *a* was stretched to 180% of slack length whereas this fiber was stretched to 130%. Background scatter 10% of signal in *R*. Calibration and temperature as in *a*

79]. The concentration of MgATP used in our experiments appeared to be adequate for saturating the transport ATPase throughout the cross-section of the fibers: the average rate of increase in light scattering in the medium containing 5 mM ATP and 2.5 mM Mg (7 fibers) was not significantly different from the average rate observed when 10 mM ATP and 12.5 mM Mg were present (6 fibers).

Substitution of ITP for ATP resulted in a slower rate of rise in light scattering. Ca accumulation by isolated SR vesicles is also slower with ITP than with ATP [41].

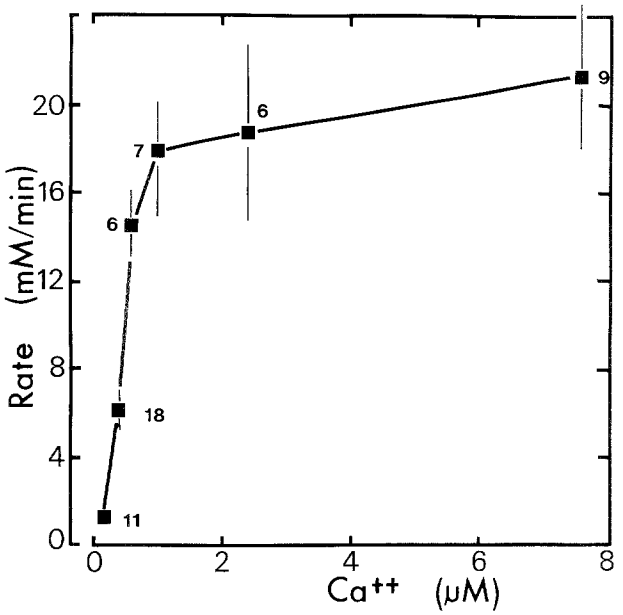


Fig. 6. Ca concentration dependence of loading rate. Forty single fibers, or bundles of 2–4 fibers stretched to 180% of slack length were each exposed to several different Ca concentrations, not necessarily in increasing order, in the presence of 5 mM oxalate. At each Ca concentration, loading was allowed to proceed until it had reached its maximum linear rate, as in Fig. 5*a*. The sequence was terminated and the final plateau value was recorded when the rate of increase in light scattering began to fall off. Not every fiber was exposed to every Ca concentration shown; points are means, and bars show standard errors, with the number of fibers exposed to each concentration beside each point. Most fibers received at least three hits, and many continued to load at a linear rate through five hits. Average maximum Ca load at the final plateau in this preparation was 67 mM; fibers had been stored in glycerol-skinning solution at −20 °C for 26–40 days. Solutions were identical to the standard loading medium except for the concentrations of EGTA and CaEGTA (*see* Materials and Methods). Temperature, 25 °C

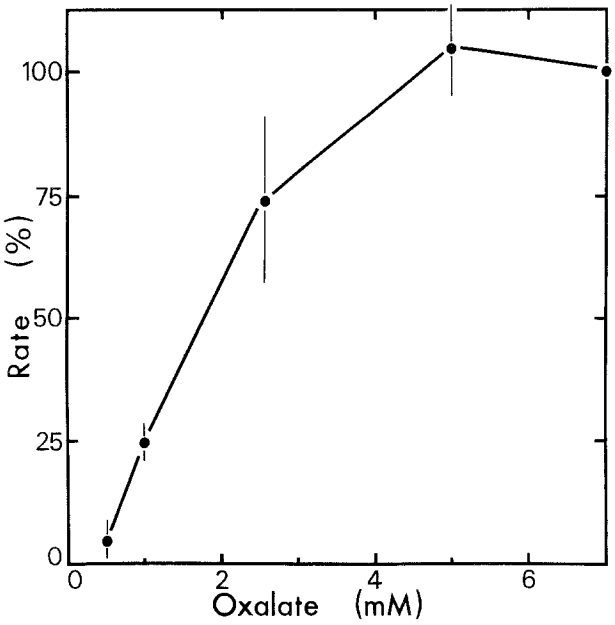


Fig. 7. Oxalate concentration dependence of loading rate. One fiber and six bundles of 2–10 fibers were each exposed to progressively increasing oxalate concentrations in the presence of a pCa 6.4 loading solution. At each oxalate concentration, loading was allowed to proceed until it had reached its maximum linear rate, as in Fig. 5*b*. The sequence was terminated when the rate of increase in light scattering began to decrease. Points show means and standard errors for 4–7 observations. For each bundle, rates at lower oxalate concentrations were normalized to the rate in 7 mM oxalate. Temperature, 24–25 °C

4) Inhibitors. Effects of several inhibitors known to act on SR or other intracellular membranes were tested. Azide has been shown to inhibit mitochondrial Ca transport [61]. Several fibers were tested for an effect of sodium azide on the rate of increase in light scattering in the standard loading medium. Rates were identical in the presence and absence of 10 mM azide.

The divalent-cation ionophores X-537A and A23187 reduce or abolish Ca accumulation in isolated SR vesicles, apparently by increasing the permeability to Ca [6]. Fibers were pre-incubated in the presence of A23187 for 4–6 min in relaxing solution, followed by 4–6 min in the standard loading medium containing the ionophore, to which 5 mM oxalate was subsequently added. With pre-incubation, the increase in light scattering normally caused by adding oxalate was reduced or blocked at ionophore concentrations of $2–3 \times 10^{-8}$ M. When the ionophore was introduced after Ca accumulation had begun, however, no effect was observed until 4×10^{-7} M had been added to the bath (Fig. 4*a*). X-537A had effects similar to those seen with A23187, but higher concentrations were required. Effects of both ionophores could be partially reversed by washing, as in Fig. 4*a* (−A23187), or by increasing the concentration of oxalate (not shown).

The presence of caffeine (0.5–20 mM) in the standard loading medium reduced the rate of increase in light scattering caused by adding 5 mM oxalate, but never completely blocked it. Inhibition caused by brief exposures to caffeine ($\leqq$10 min) was completely reversible (Fig. 4*b*).

In several preparations, fibers were pre-treated for 5–20 min in detergent (2% Brij-58 or Triton X-100 in relaxing solution) in order to disrupt the SR [44, 50, 67]. Subsequently, when Ca and oxalate were added, no increase in light scattering occurred and the accumulation of ^{45}Ca was greatly reduced in comparison with the untreated fibers. Adding detergent (0.5–2% Brij-58) to the loading medium after the light-scattering signal had increased to its maximal value caused the signal to fall rapidly to nearly the original preloading value.

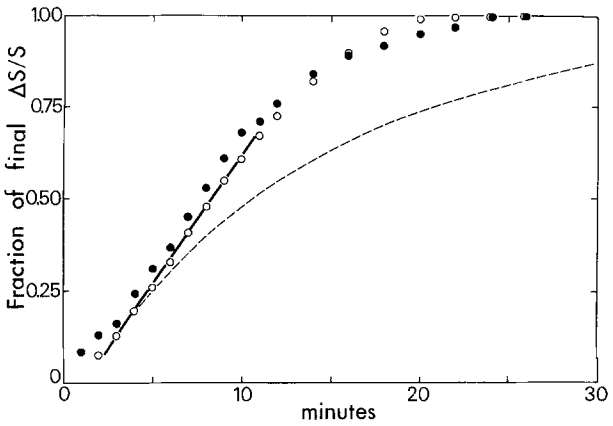


Fig. 8. Nonexponential time course of loading in the presence of saturating and nonsaturating oxalate concentrations. Two fibers from the same preparation were exposed at time 0 to a pCa 6.4 loading medium containing either 5 mM (*open circles*) or 1.25 mM (*filled circles*) oxalate. Loading was continued until the increase in light scattering reached a steady plateau. The time course of the increase in scattering is plotted as a fraction of the final plateau $\Delta S/S$ value reached in each case (in 5 mM oxalate, $\Delta S/S=4.88$; in 1.25 mM oxalate, $\Delta S/S=1.24$). From the end of the lag period (3 min) until the signal has reached 60% of its final value (10 min), both curves can be fitted by a straight line having a slope of 0.070/min. In 9 similar experiments at each oxalate concentration, loading half-times were identical ($x\pm$SD, 8.4 ± 1.7 min in 1.25 mM oxalate, 8.3 ± 1.9 min in 5 mM oxalate). Dashed line is included for comparison of the observed time course of loading with an exponential curve having the equation $y=1-(1-y_o)e^{-k(t-t_o)}$ and constructed so that the starting point (t_o, y_o) and the initial rate (k) coincide with the observed values for these quantities at the end of the lag period. Temperature, 25 °C

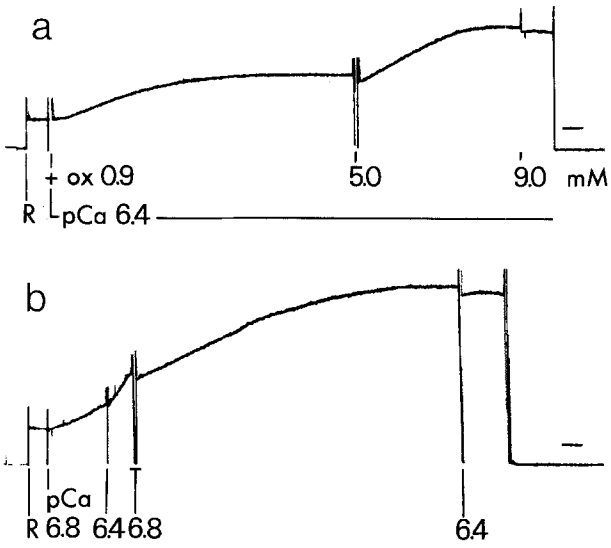


Fig. 9. (*a*): Effect of oxalate on steady-state capacity. Fiber was exposed for 29 min to a pCa 6.4 loading medium containing 0.9 mM K oxalate (*pCa 6.4, +ox 0.9*) until the scattering signal had increased to a steady plateau ($\Delta S/S=1.9$). The maximum rate of increase in light scattering (17% of S per min) was maintained until 50% of the plateau value had been reached. The standard loading medium containing 5 mM oxalate was introduced (*5.0*), and in 12 min scatter had reached a higher plateau ($\Delta S/S=3.9$). No further increase occurred when the concentration of oxalate was raised to 9 mM. Single fiber, 130% of slack length. Background scatter 12% of signal in relaxing solution (*R*). Calibration, 2 min. Temperature, 25 °C. (*b*): Effect of external Ca on rate and capacity. Loading in the presence of 5 mM oxalate (*pCa 6.8*) was briefly interrupted by exposing the fiber to a higher Ca concentration (*pCa 6.4*) long enough to determine the maximum rate at the higher concentration. Loading then continued in the original loading solution until the signal reached a plateau. Rates were 17 and 50% of S per min at pCa 6.8 and 6.4, respectively, and the final plateau ($\Delta S/S=4.46$) changed very little when the higher Ca concentration was introduced for the second time. Large differences in rate, but identical capacities, were also observed in 3 similar experiments comparing pCa 6.8 with 6.4, or pCa 6.4 with 6.0 (data not shown). Single fiber, 130% of slack length. Background scatter 7.5% of signal in *R*. Calibration, 2 min. Temperature, 25 °C

Ca Concentration Dependence of Ca Accumulation Rate

The results described so far are consistent with our initial assumption that the increase in light scattering seen in the presence of Ca and oxalate is a consequence of the active transport of Ca into the SR. The Ca concentration required for half-maximal activation of this process in psoas fibers was determined from measurements of rates of Ca accumulation during the linear portion of the loading curve. Figs. 5*a* and 6 show that in the presence of 5 mM oxalate, pCa 7.0 was close to the threshold for activation of the Ca pump; Fig. 6 shows that the pump was nearly saturated at 1 μM Ca^{++} (pCa 6.0). In several fibers from another preparation, no further increase in rate was observed when the Ca^{++} concentration was raised from 3.6 to 10 μM. A double-reciprocal plot of the data in Fig. 6 was nonlinear and so was not used to estimate V_{max}. However, Fig. 6 shows that an ionized Ca concentration of 0.5 μM (pCa 6.3) was required to attain one-half the maximum observed rate of 21.3 mM/min. K_m values reported for Ca activation of the pump in isolated vesicles range from 0.1 to 1 μM [38].

Oxalate Concentration Dependence of Ca Accumulation Rate

At pCa 6.4, threshold concentrations of oxalate for the first detectable increment in light scattering ranged from 0.2 to 0.75 mM (data not shown). Concentrations below 5 mM supported lower rates of increase in light scattering (Fig. 5*b*). The oxalate concentration dependence for the rate of increase in light scattering in seven bundles or single fibers from one preparation is shown in Fig. 7. Both Figs. 7 and 5*b* show that the effect of oxalate on rate was saturated at 5 mM for these preparations.

Enhancement of ^{45}Ca accumulation in isolated vesicles under various conditions has also been reported to exhibit oxalate thresholds of 0.1–0.5 mM [22, 32, 39, 48, 62] and saturation between 5 and 15 mM [39, 58, 61].

Despite differences in rate, loading followed a similar, nonexponential time course at both low and high oxalate concentrations, with the maximum rates remaining constant until more than 50% of the final signal had been attained (Fig. 8).

Capacity for Ca accumulation in different oxalate concentrations. In a number of experiments, fibers exposed to oxalate concentrations below 5 mM were loaded until the increase in light scattering reached a plateau. This plateau was invariably lower than that reached with saturating concentrations of oxalate present. An example is shown in Fig. 9*a*. With 0.9 mM oxalate present in the loading medium, light scattering increased until it reached a plateau at $\Delta S/S = 1.9$. This plateau was maintained for 8 min, until 5 mM oxalate was added. Light scattering then increased to a new level ($\Delta S/S = 4.0$), about twice that attained at the lower oxalate concentration. Further addition of oxalate showed that the maximum signal had been attained with 5 mM oxalate present. More than half of 45 fibers tested in another series of experiments showed no further increase in light scattering when 20 mM oxalate was added to the bath after a steady plateau ΔS value had been reached in 5 mM oxalate. The average increase in light scattering caused by adding more oxalate to the remaining fibers was only 18%.

In six fibers from two different preparations, ^{45}Ca was included in the loading medium, and the Ca concentration was measured after the light-scattering signal had reached a steady state in the presence of 1.25 mM oxalate. The average Ca concentrations were 64 and 42 mM, or 44 and 47% of the average concentrations attained at the steady state in 5 mM oxalate in other fibers from the same two preparations. These data confirm the conclusions from the light-scattering measurements and indicate that the Ca capacity of the SR was smaller at lower oxalate concentrations. Similar data have been reported for isolated vesicles [39, 61]. At a saturating oxalate concentration, on the other hand, lowering the external Ca concentration had little or no effect on capacity, although rate was reduced (Fig. 9*b*).

Morphological correlates of differences in light scattering. Two alternative explanations for the oxalate concentration dependence of the steady-state Ca capacity were considered. One possibility is that all elements of the SR had reached a steady state when they were only partially filled. Alternatively, Ca oxalate precipitates may have filled fewer SR elements at the lower oxalate concentration, leaving the rest empty. To evaluate these two possibilities, we examined electron micrographs showing the extent of Ca oxalate precipitation inside the SR of skinned fibers loaded to a steady state in a low concentration of oxalate, comparing them with controls which were loaded to their maximal steady-state levels in 25 mM oxalate.

In previous electron microscopic studies, it was noted that many SR elements did not contain Ca oxalate crystals or were only partially filled [1, 5, 7, 9, 20, 80]. Therefore, in addition to monitoring the light-scattering signal to verify the extent of loading, we included controls for the efficacy of our preparative procedures in preserving crystals (Figs. 10*a* and 11*b*), and for our ability to distinguish a partially filled element from a maximally filled element (Fig. 11*c*). These controls are considered first.

1) Maximal loading conditions. In none of the experiments (maximal or submaximal loading conditions) was there any gradation in the distribution of Ca oxalate crystals between the core and the surface of a fiber. Maximally loaded fibers from five different muscles were examined. The distribution of Ca oxalate deposits seen in Fig. 10*a* is typical. A few empty membrane profiles can be seen (*e*), as well as a few mitochondrial remnants (*M*), but most membranes are obscured by large crystals of Ca oxalate (*c*). As noted previously by Pease, Jenden and Howell [52], these are particularly abundant at the level of the I-band and over the Z-disc, but crystals can also be seen in the A-band region. A chemically skinned fiber which was block-stained with uranyl acetate, thin-sectioned, and stained with lead demonstrates the disposition of T-tubules, SR membranes, and terminal cisternae among these regions (Fig. 11*a*).

In maximally-loaded fibers examined at high magnifications (Fig. 11*b*), the relationship between Ca oxalate deposits and the adjacent SR membranes could be seen only rarely, when there was some separation between them. A clearer picture of the surrounding membranes is obtained with fibers fixed before loading had reached a plateau. For Fig. 11*c*, another segment of the same fiber shown in Figs. 10*a* and 11*b* was fixed after being partially loaded in 25 mM oxalate. This was achieved by removing ATP from the medium as soon as the light-scattering signal indicated that the maximum rate of uptake had been attained. At this point, the load was only 13% of the plateau value recorded for the full load shown in Fig. 11*b*, in the other segment of the same fiber. The appearance of the crystals in the partially loaded

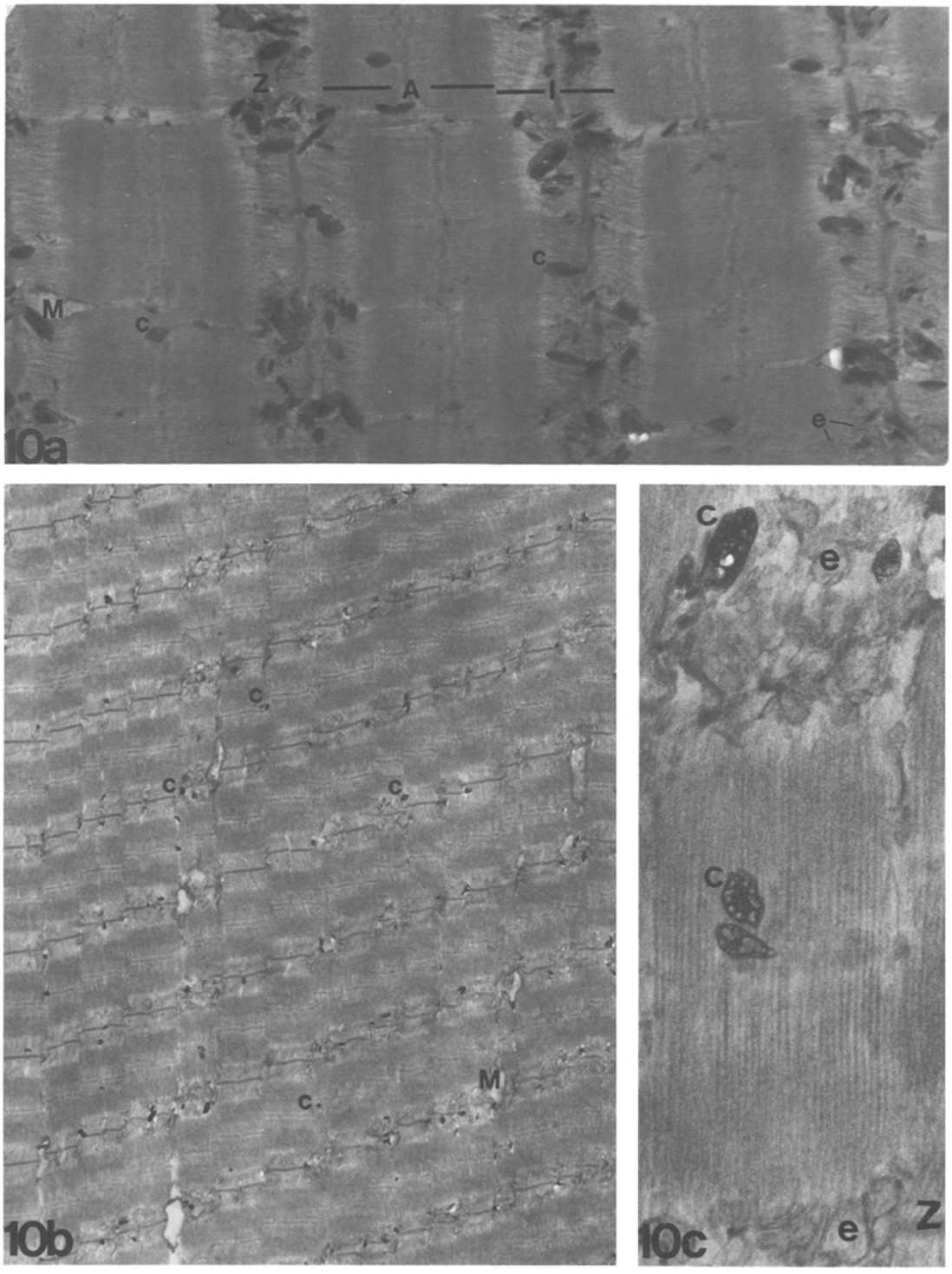


Fig. 10. (*a*): Maximally loaded fiber. A segment of a single chemically skinned psoas fiber held at 150% of slack length was exposed to a pCa 6.4 loading solution containing 25 mM oxalate. The segment was fixed (*see* Materials and Methods) after light scattering had reached a steady plateau ($\Delta S/S = 3.9$). Unstained longitudinal 0.25 μm section shows A-bands (*A*), I-bands (*I*), and Z-lines in three complete sarcomeres. Large empty membrane profile at left (*M*) is a swollen mitochondrion typical of chemically skinned fibers [11]. Large calcium oxalate crystals (*c*) are seen in both A- and I-bands; a few empty membrane profiles (*e*) are also visible. The same fiber is shown at a higher magnification in Fig. 11*b*. ×20,000. (*b*): Sub-maximally loaded fiber. This fiber was exposed to a pCa 6.4 loading solution containing only 0.75 mM oxalate, and was fixed after light scattering had reached a plateau ($\Delta S/S = 0.14$). Calcium oxalate crystals (*c*) are seen in the I-bands of most myofibrils and in the A-bands of some of them, but there are fewer in both regions than in the maximally loaded fiber of Fig. 10*a*. ×8500. (*c*): Sub-maximally loaded fiber. Higher magnification of a single sarcomere from the fiber of Fig. 10*b*, showing crystals in both A- and I-bands. Empty membrane profiles (*e*) are numerous in the I-bands, and in other fields examined could also be seen in the A-bands. Bubbles in the calcium oxalate crystals are due to beam damage [5]. Exposure to the beam at 100 kV for longer periods than those used for these photographs caused no obvious changes in number or size of the crystals in sections like these. ×60,000

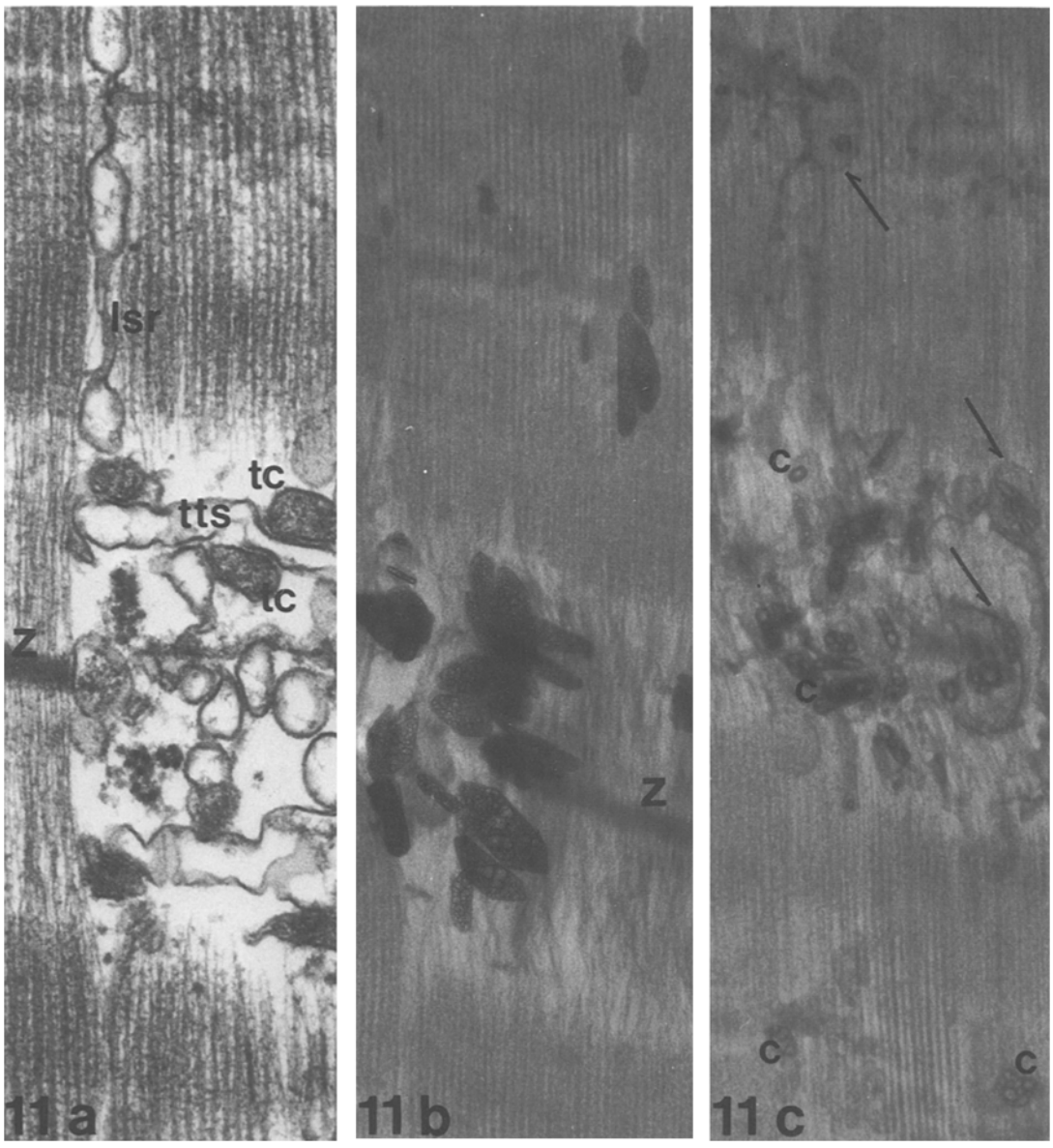


Fig. 11. (*a*): SR and T-system membranes in chemically skinned, stored fiber. Portions of two adjacent sarcomeres from a chemically skinned fiber that was fixed, stained and thin-sectioned after being stored for one week at −20 °C in 50% glycerol-skinning solution (*see* Materials and Methods). SR appears swollen, but all the normal components are present. Several terminal cisternae (*tc*) containing electron-opaque fuzz are seen in the I-band, including two that form a triad with a transverse tubular element (*tts*) between them. Triadic "feet" are not present. Other vesicular membrane profiles of SR or T-system occur over the Z-disc, and a longitudinal SR element (*lsr*) extends from the I-band across the A-band. ×60,000. (*b*): Maximally loaded fiber. Higher magnification of the fiber shown in Fig. 10*a*. Large crystals of Ca oxalate are seen throughout the sarcomere, but more prominently in the I-band. Unstained thick section. ×60,000. (*c*): Partially loaded fiber. A second segment of the fiber shown in Fig. 11*b* was exposed to the same loading solution (pCa 6.4, 25 mM oxalate), but Ca accumulation was halted by removing ATP after two min when light scattering was increasing at its maximal rate but had reached only 13% of the plateau value recorded for Fig. 11*b*. The segment was then fixed and processed as for Figs. 10 and 11*b*. Field is centered on a Z-disc, as in Fig. 11*a*. Calcium oxalate crystals (*c*) are numerous and their distribution is similar to that shown in Fig. 11*b*, but they are much smaller, and partially filled vesicular profiles can be seen (arrows). ×60,000

fiber (Fig. 11*c*) is similar to that observed by Carsten and Reedy [5] at early stages of loading in isolated vesicles. The number of precipitation sites is large, as in Figs. 10*a* and 11*b*; few of the vesicular membrane profiles are completely empty. In general, the crystals are smaller than in Fig. 11*b*, and many partially filled vesicular profiles can be found (arrows, Fig. 11*c*). A pattern that was intermediate between that shown in Fig. 11*b* and *c* was seen in four fibers from a different preparation which were loaded to about 30% of the maximum load before being fixed.

Table 2. Calcium accumulation rate and capacity in the presence of saturating and sub-saturating concentrations of oxalate

Oxalate (mM)	Rate ($\Delta S/S \cdot$ min)	Capacity (plateau $\Delta S/S$)	Ratio
5	0.22 ± 0.09 (20)	4.34 ± 1.31 (12)	0.055 ± 0.012 (12)
1.25	0.07 ± 0.03 (13)	1.24 ± 0.60 (13)	0.060 ± 0.015 (13)
$\frac{1.25}{5}$	32%	29%	109%

Single fibers were exposed to a pCa 6.4 loading medium (*see* Materials and Methods) containing either 5 or 1.25 mM oxalate. The maximum rate of increase in light scattering (column 2), the final plateau value (column 3), and the ratio of these two values (column 4) were determined for each fiber. Values shown are means and standard deviations for 12 or 13 fibers, as indicated in parentheses; rate measurements on 8 additional fibers are included for the 5 mM oxalate medium. Bottom line shows the average rates, capacities and rate/capacity ratios in 1.25 mM oxalate expressed as a percent of the average values in 5 mM oxalate. Fibers stretched to 130% of slack length; temperature, 24–26.5 °C.

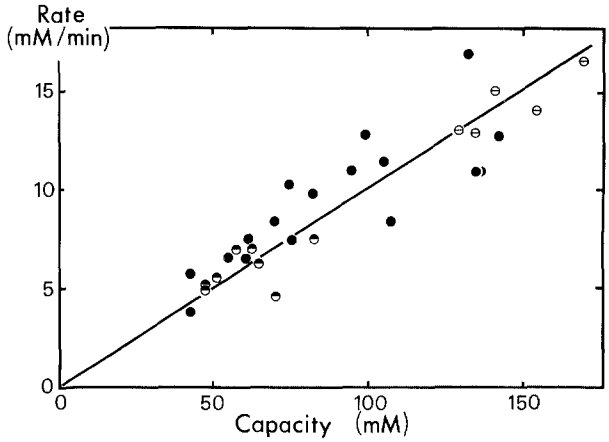


Fig. 12. Correlation between Ca accumulation rate and capacity. Each symbol represents a determination of both maximum rate and final steady-state capacity in a single fiber or small bundle exposed to the standard loading medium containing pCa 6.4 and 5 mM oxalate, as in Fig. 2. Each type of symbol represents a different animal. Temperature, 25–29 °C

2) Submaximal loading conditions. In three experiments, fibers were fixed after light scattering had reached a steady plateau in the presence of 0.75 mM oxalate. Precautions for preserving Ca oxalate crystals were identical to those employed for the experiments in 25 mM oxalate. At a low magnification (Fig. 10*b*), crystals can be seen in every sarcomere, being present in both A- and I-bands but more frequently in the latter. In contrast to the fibers that were loaded either partially or completely in 25 mM oxalate, however, the number of precipitation sites is small, and in every sarcomere there are many completely empty vesicular membrane profiles (*e*, Fig. 10*c*). From the light-scattering signal, it was apparent that no further loading would occur at this oxalate concentration; we infer that the empty elements seen in Fig. 10*c* were incapable of forming Ca oxalate deposits at this oxalate concentration. The contrast with Figs. 10*a* and 11*b* and *c* suggests that the lower steady-state capacities observed at lower oxalate concentrations are due primarily to reduction in the number of participating SR elements rather than to partial filling of the entire population.

Since fewer SR elements appeared to be involved at the steady state in lower oxalate concentrations, we reasoned that the net uptake rates (Figs. 5*b* and 7) might be lower for the same reason. A test for a correlation between rate and capacity indicated that the entire difference in loading rates could be attributed to the different volumes of SR involved (Table 2). Rate and capacity were determined for 13 fibers loaded in the presence of 1.25 mM oxalate and 12 fibers from the same preparation loaded in 5 mM oxalate. There was considerable variation in the absolute values for each condition, and the average rates and capacities differed by 3- to 3.5-fold in the two conditions. However, the ratio between rate and capacity for each fiber was quite constant, and, more importantly, was independent of the concentration of oxalate used. The last column of Table 2 indicates that net uptake filled 5.5–6% of the final capacity per minute at both oxalate concentrations. Similar fractional rates of filling at each oxalate concentration were obtained on seven of these fibers which were first loaded to a plateau with 1.25 mM oxalate and then loaded maximally in the presence of 5 mM oxalate (data not shown).

Heterogeneity Among Fibers

As already noted, Ca accumulation rates and capacities in the presence of 5 mM oxalate varied widely from fiber to fiber and in different preparations. However, lower rates were associated with lower maximal capacities for loading, in a highly predictable way. This can be seen in Table 2 for the standard loading condition, and in experiments on 30 fibers or small bundles from three other preparations in Fig. 12. Rates and capacities both varied over a fivefold range, whereas the highest and lowest ratios of rate to capacity among individual fibers differed by a factor of only two. In this series, the ratio was 0.105 ± 0.017 ($\bar{x} \pm$ SD).

Discussion

This investigation has established that measurement of the change in light scattering from chemically

skinned muscle fibers can be used as a quantitative technique for following Ca accumulation by the sarcoplasmic reticulum. While the findings with the new technique are strikingly similar to those obtained in studies on isolated SR vesicles, they provide new data on the dynamics of Ca accumulation by SR in a preparation in which the general features of the normal spatial relationship between SR and myofibrils are retained. Furthermore, the data indicate that the SR in these skinned fibers is heterogeneous in its ability to form Ca oxalate crystals.

Light-Scattering Technique

Changes in both absorption [18] and birefringence [5] have previously been shown to accompany Ca oxalate accumulation by isolated SR vesicles. Until now, changes in light scattering have been used only as indices of osmotic shifts across isolated SR membranes [34, 59].

Our experiments show an increase in light scattering which requires ATP, Ca, and a precipitating anion (Figs. 1*a*, 2 and 3) is blocked or attenuated by detergent, divalent-cation ionophores, and caffeine (Fig. 4), but not by azide; and is accompanied by formation of electron-opaque deposits inside the sarcoplasmic reticulum (Figs. 10 and 11). We infer that, under the conditions of our experiments, light scattering by a relaxed fiber increases primarily as a result of Ca oxalate precipitation in the lumen of the SR. Other Ca-precipitating anions, except inorganic phosphate, are also effective. At present we have no explanation for the observation that incubation with Ca and inorganic phosphate causes no increase in light scattering.

Since we use oxalate as a tool to provide a measurable optical change, it is desirable to know whether oxalate itself has any effect on the operation of the Ca pump or on the Ca permeability of the SR membranes. Previous studies have provided evidence that it has no effect on isolated SR vesicles [23, 42, 43, 60, 79]. In the skinned fiber, increasing oxalate above 5 mM (Figs. 5*b* and 7) had no effect on the rate of increase in light scattering, and the fractional rate of filling was exactly the same whether 1.25 mM or 5 mM oxalate was used (Table 2). Moreover, for isolated SR vesicles, the initial rates of transport measured by rapid-mixing techniques in the absence of oxalate [27, 68] are consistent with maximal rates of oxalate-supported uptake measured by slower methods [25, 45, 78]. Oxalate appears to act primarily as a sink for the entering Ca and to prevent internal ionized Ca from increasing to levels that "back-inhibit" the pump. It thus prolongs the period during which true initial rates of Ca accumulation can be measured [39, 71].

The basis for the precipitation of Ca oxalate inside the sarcoplasmic reticulum has been described [23, 42, 43, 60, 79]. Oxalate enters the SR by diffusion, and Ca is pumped in actively. The ionized Ca concentration rises until it reaches a limit set by the concentration of oxalate and the Ca oxalate solubility product, K_{sp}. Subsequently, ionized Ca remains constant and the further entry of Ca leads to formation and growth of Ca oxalate crystals.

Mechanism of Limiting Ca Oxalate Accumulation

With the skinned fiber, it is relatively easy to control the Ca concentration in the bath so that it does not change during loading. This has made it possible for us to obtain evidence that it is the volume of the SR rather than a progressive change in the ratio of influx to efflux that limits Ca oxalate accumulation at all Ca and oxalate concentrations. Hasselbach and Makinose [25, 39] recognized that this should be the case for isolated vesicles under maximal loading conditions. They reasoned that external and internal ionized Ca concentrations were held constant during loading by CaEGTA and Ca oxalate, respectively, and therefore that influx and efflux should remain constant until the physical limit for filling the reticulum with Ca oxalate crystals had been reached. Their evidence came from the prolonged linear time course of Ca oxalate accumulation and from the constant rate of ATP hydrolysis throughout the loading period [24]. We find a similar time course for skinned fibers (Figs. 2 and 8). Additional evidence for a primarily physical limitation at high oxalate concentrations is given in Fig. 9*b*: the capacity for Ca oxalate accumulation is independent of changes in external ionized Ca which would change influx rates and the ratio of influx to efflux but, of course, would not alter the volume of SR [*cf. also* 24 and 78].

Earlier experiments with isolated vesicles did not lead to an explanation of the mechanism for limiting the Ca oxalate capacity at submaximal oxalate concentrations [39]. In the skinned fiber, Ca oxalate accumulation also shows a nonexponential time course at low oxalate concentrations (Fig. 8). In addition, using the skinned fiber and suitable precautions in processing for electron microscopy, it is possible for the first time to show a clear difference between maximal and submaximal steady-state loads. Figures 10*b* and *c* and 11*b* indicate that participating SR elements are maximally filled at the steady state, whether low or high oxalate concentrations are used; the physical limit is smaller at a low oxalate concen-

tration because fewer SR elements are involved, not because some steady state has been established after partial filling of all the elements. Since the reduction in rates (Fig. 7) is proportional to the reduction in capacity (Table 2), it can be accounted for entirely by the loss of participating elements.

These observations on skinned fibers might explain the lower capacities found for isolated vesicles at low oxalate concentrations [24, 39, 61]. In both preparations, efflux must increase to match influx at some point near the end of the loading period, possibly because of damage from the Ca oxalate crystals as the physical limit of the membrane-enclosed space is approached [39]. Increased efflux from heavily loaded vesicles has been reported [78].

Heterogeneity within sarcomeres

The finding that different SR elements have different thresholds for Ca oxalate precipitation has led us to consider possible mechanisms for this heterogeneous response. One possibility is that of artifact. If the heterogeneity reflects surface damage or an unevenness in permeability of the sarcolemma to ions in the bathing medium, empty elements should be localized next to less permeable regions of the sarcolemma. However, empty and filled elements were distributed uniformly throughout the fibers (Fig. 10*b*). Furthermore, experiments with isolated vesicles and muscle homogenates in several different laboratories have shown a heterogeneous response to oxalate with a similar concentration dependence [39, 58, 61]. It seems unlikely that the heterogeneity we see is peculiar to the skinned-fiber preparation. We propose that it reflects a heterogeneity in the normal functional capabilities of the SR.

On this basis, possible differences of physiological interest, such as variations in internal Ca or oxalate concentrations, can be considered. Oxalate concentration differences inside the SR could exist if there were differences in permeability to oxalate. These can be ruled out on several grounds. We were repeatedly able to demonstrate a steady plateau of the light-scattering signal for low loads (e.g., Fig. 9*a*), indicating that the empty elements could not be recruited even by prolonged incubation at the lower oxalate concentrations. Nevertheless, they were not completely impermeable to oxalate since they were capable of filling when more oxalate was added (Figs. 5*b*, 7, 9*a* and 10*a*). These two observations are incompatible with the notion that some elements remain empty because they are either impermeable or less permeable to oxalate: less permeable elements should fill more slowly at the lower oxalate concentrations, but eventually all of them should fill. Finally, if oxalate permeability were a limiting factor, the Ca accumulation rates should continue to increase with increasing oxalate concentrations, rather than to show saturation (Figs. 5*b* and 7).

An alternative source of heterogeneity in precipitation might be the ability of different regions of the SR to accumulate and retain ionized Ca. Although the use of oxalate provides an artificial increase in the total capacity for Ca, it can do so only for SR elements that are inherently capable of accumulating internal ionized Ca to levels that exceed K_{sp}. If K_{sp} and oxalate concentrations are the same throughout the SR, the different thresholds for Ca oxalate precipitation in different regions of the SR must reflect different internal ionized Ca concentrations. Sarcoplasmic reticulum elements capable of generating the highest internal Ca concentrations will demonstrate the lowest threshold for oxalate (about 0.25 mM, Fig. 7). The concentration of oxalate required for complete recruitment of the SR (5 mM) gives a lower limit to the range of Ca concentrations in different regions of the SR in these skinned-fiber preparations. For a Ca oxalate solubility product of 2×10^{-8} M^2 [29], the range is 4–80 μM Ca^{++}.

The higher concentrations of internal Ca might arise from lower Ca permeabilities in some regions. This would account for the data of Table 2, where influx and net uptake were the same at both high and low oxalate concentrations even though the driving force for Ca efflux must have been fourfold higher in 1.25 mM oxalate [*cf.* 29]. However, our data do not rule out other possible explanations for the heterogeneous precipitation of Ca oxalate, such as differences in K_{sp} due to local variations in other ionic species within the SR.

Support for the concept of functional heterogeneity within sarcomeres has appeared in a number of previous studies of Ca movements in the SR. Based primarily on evidence from frog muscle, it has been suggested that storage and release of Ca occur primarily at terminal cisternae [7, 23, 53, 75]. Morphometric analyses of mammalian fibers show that 20% of the SR volume is found in the A-band, whereas 80% is located in a network of tubules over the Z-disc and in pairs of terminal cisternae forming triads with the T-tubules at each end of the I-band, near the A-I junction [12]. In our micrographs of maximally loaded fibers (Figs. 10*a* and 11*b*), as well as those of Pease, Jenden, and Howell [52], the distribution of Ca oxalate deposits between A-bands and I-bands is consistent with SR volume distribution in these regions rather than with the location of specific structures such as the terminal cisternae. However, it is the submaximal loading condition which would be expected to reveal most clearly any correlation between Ca oxalate precipitation and a particular region

of the SR in each sarcomere. Although many sarcomeres are devoid of A-band deposits in Fig. 10*b*, a detailed morphometric analysis would be required to establish whether there is a significant difference in the ratio of A-band to I-band loading sites in experiments of this type.

Comparison with Other Preparations, and Effects of Storage

For the chemically skinned rabbit psoas fibers (Fig. 12), average maximal Ca oxalate loads of 60–147 mM (mmol/liter fiber, 1.3–3.2 moles/liter SR [12]) compare favorably with values reported for Ca oxalate capacities of isolated SR vesicles (6–10 μmol/mg protein [39, 73], or 0.6–2.0 moles/liter SR [10, 72]). Capacities 2.5-fold higher were obtained for cardiac SR vesicles selected for their ability to form visible Ca oxalate crystals [5].

Maximal loads accumulated by chemically skinned psoas fibers are equal to or greater than those reported for other vertebrate skeletal muscle preparations loaded under similar conditions [4, 13, 23]. Among previous studies in which oxalate was used, maximal loading rates as large as those shown in Fig. 6 were obtained only for fresh homogenates [4].

Earlier reports have shown that various procedures for storing fibers in glycerol lead to loss of SR activity [3, 13, 33]. The comparisons cited above show no indication that this is a problem for our preparations. Furthermore, under our storage conditions (*see* Materials and Methods) there are no detectable changes in the functional properties we have measured for at least six weeks. Although continuity along a sarcomere can readily be demonstrated (Fig. 11*a*, and [11]), the SR in fibers stored for one to two weeks under our conditions shows some signs of vesiculation (Figs. 10 and 11). Nevertheless, the data of Table 1 indicate that net uptake rates in such fibers remain unchanged. It has also been determined that caffeine-induced release of Ca from the SR is unaltered during storage over this period [76].

Our data do not show whether loss of SR activity occurs during the initial exposure to skinning solution (24 hr). Experiments which will allow us to compare chemically skinned mammalian fibers before and after storage in glycerol are in progress. However, since the solution which chemically skins mammalian fibers is virtually identical to that used to bathe preparations which are skinned mechanically, our kinetic data may be compared with that reported for ^{45}Ca uptake in freshly prepared, mechanically skinned frog fibers [19; *see also* 14]. Evidence cited above indicates that the maximal rate of Ca accumulation in the presence of oxalate reflects the initial rate of Ca accumulation in the absence of oxalate. In order to compare data from frog and rabbit fibers, it is necessary to allow for the greater SR volume [12, 46] and the effect of measuring fiber diameters in silicone oil [19] in the case of the frog. On this basis, a lower limit of 14 mM/min can be estimated for frog fibers from the rate constant given by Ford and Podolsky [19] at pCa 6.0. At pCa 6.0 the preparation of Fig. 6, which had been stored for five weeks, accumulated Ca at a rate of 18 mM/min.

A different approach to the study of SR in a relatively intact state has recently been described [37]. Ten-μm cryostat sections of rabbit fast-twitch muscle (adductor magnus) were exposed to a loading solution containing 50 mM Tris oxalate and 0.8–1.0 μM ionized Ca, at pH 7 and 37 °C. It is not clear which of the differences between that study and ours could account for the lower rate, prolonged time course, and higher capacity reported for accumulation of ^{45}Ca by the cryostat sections, nor for the higher concentrations of oxalate they seem to require. Under our conditions, the effect of oxalate on both rate and capacity was saturated with 5 mM oxalate (Figs. 5*b* and 7), and at pCa 6.4 fibers were fully loaded in 10–45 min (Fig. 2).

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