Water Loss during Contracture of Muscle

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ABSTRACT The relationship of contracture and exudation of water in frozen-thawed frog muscle was studied. With maximum shortening, there was a water loss of 35 per cent of the weight of muscle. By restricting the contraction, it was demonstrated that the amount of water loss was proportional to the degree of shortening, there being no significant loss with isometric contraction. Muscle already shortened by tetanic stimulation also exuded water on subsequent freezing and thawing. The force of contraction could be reduced by depleting the muscle of calcium and it was shown that the amount of water exuded was also proportional to the tensile ability of the muscle. In a smooth muscle (anterior byssus retractor of Mytilus) which did not contract vigorously only a little water exuded. Contracture produced by caffeine was similarly associated with a loss of water. Microscopic studies revealed a disruption of the sarcomeres of the frozen-thawed muscle which contracted; glycerol-extracted and calcium-depleted muscles, which did not contract on freeze-thawing, did not show such disruption. Freezing and thawing of actomyosin caused a reversible syneresis of the protein. It is concluded that the exudation of the water is not merely due to the freezing and thawing but is also dependent on the contractile events.

INTRODUCTION

It is well known that syneresis of actomyosin occurs when it is “superprecipitated” with ATP (1). Likewise, water is lost during contraction of both freshly homogenized (2) and glycerol-extracted muscle fibers (3). In the light of these particular findings and on other general grounds, Szent-Györgyi suggests that water is an integral part of the contractile system (4). Nevertheless, no further direct evidence relating to this idea has been forthcoming. It is well, however, to recall and reconsider the early observations reported by Hermann on frozen-thawed muscle (5). In this preparation contracture follows on the thawing and is associated with exudation of water. Whether this exudation is only a result of disruption of tissue barriers due to freezing and thawing, or whether it may have some relationship to the contractile events, forms the subject of study in the present investigation.
METHODS

Experiments were carried out on the ileofibularis muscle of the frog (*Rana pipiens*) during winter and summer months. The tendons were tied with ligatures between which the resting length was measured. The excised muscle was blotted with filter paper in a standardized fashion and weighed on a torsion balance. The muscle, attached at rest length to a special holder, was frozen in hexane at $-74^\circ$C for 3 minutes and then thawed at room temperature in a flask saturated with water vapor. The muscle contracted (isometrically or isotonically when one end was released) and after 12 minutes of thawing was reblotted and reweighed. A number of muscles were electrically stimulated to contract tetanically and while being thus activated were frozen (in this case with dry ice powder) and then thawed. Another group of muscles was preliminarily soaked in Ca-free Ringer’s solution containing ethylenediaminetetraacetic acid disodium salt (EDTA) $4 \times 10^{-3}$ M (pH adjusted to 7.0–7.1) for periods of 1 to 4 hours. Controls were soaked for comparable periods of time in Ringer’s solution (NaCl 115 mm, KCl 4.0 mm, CaCl$_2$ 2.1 mm, NaH$_2$PO$_4$ 0.5 mm, Na$_2$HPO$_4$ 1.25 mm, pH 7.1).

Shortening was measured with a ruler and tension was recorded with a Grass transducer (F.T.10) and an ink writing oscillograph. The degree of shortening was varied by attaching the muscles to a special holder by means of loops with varying lengths.

The sodium and potassium concentrations in the exudate were determined by flame photometry (Advanced Instruments, Inc.). Determinations were made on four samples of pooled exudate, each obtained from three to five frogs.

Phase contrast microscopic observations were made on dissected fibers and frozen sections; and electron microscopic preparations were made according to described techniques (6).

In another series of experiments, irreversible contracture was produced by the application of caffeine $5 \times 10^{-4}$ M for about 2 minutes. The muscle was then likewise placed in a flask saturated with water vapor for 12 minutes and thereafter the weight loss was determined.

The effect of freezing and thawing was also studied on a smooth muscle, the anterior byssus retractor of *Mytilus*.

Actomyosin (myosin B) was prepared from rabbit muscle (1) and purified by reprecipitation with distilled water three times. Aliquots of the protein solution (in 0.6 M KCl) were diluted in a concentration series of KCl (0.1 M to 0.4 M) and frozen to $-74^\circ$C for 5 minutes. After thawing, the preparations were centrifuged in graduated Kolmer type tubes and the degree of syneresis was determined by measuring the volume of precipitated actomyosin (7). Corresponding non-frozen samples served as controls and for comparison syneresis was also produced with 0.004 M ATP.

RESULTS

Response to Freezing and Thawing. Contraction commenced with thawing of the frozen ileofibularis muscle, maximum shortening or tension occurring
about 3 minutes after removal of the muscle from the hexane (Fig. 1). The contracture was irreversible and the muscle lost its plasticity.

Soon after commencement of shortening, a pinkish exudate appeared on the surface, gradually increasing in amount. To insure that all the contractile activity of the deep fibers ceased and that the maximum exudation had occurred, 12 minutes of thawing was allowed. With maximum shortening an average of 35 per cent loss in muscle weight occurred due, presumably, mainly to the water loss. The K concentration of the exudate was, on the average, 100 meq/liter and the Na 27 meq/liter.

![Figure 1](image-url)
By restricting the degree of shortening less water was lost and isometrically contracted muscle lost no water at all, or less than 5 per cent of the weight of the muscle. Fig. 2 illustrates a proportional relationship between the percentage loss of weight and the degree of shortening up to about 50 per cent of its initial length; any further apparent shortening was not associated with any further water loss. Thompson and Marsh reported (8) a weight loss of 35 per cent with maximum shortening in thaw-rigor of lamb muscle. But in their experiment a significant water loss occurred only after 50 per cent of shortening; with further contraction the water loss was proportional to the degree of shortening.

*Tetanically Contracted Muscle* The muscle shortened by about 45 per cent of its initial length during tetanic stimulation. On freeze-thawing during this contracted state there was an additional small degree of shortening of about 10 per cent of its length and a weight loss of about 35 per cent. Hence maximal water loss occurred in muscle already shortened to a major degree under more physiological conditions prior to the freezing. Tetanic isometric contraction was over 1 kg/gm muscle weight with no significant water loss occurring on freeze-thawing.
Calcium-Depleted Muscle  The contractile response to freezing and thawing was diminished in muscles previously soaked for 1 to 4 hours in Ca-free Ringer’s solution containing EDTA as has been previously reported (9, 10); the degree of shortening varied inversely with the duration of soaking. While in these preparations there was likewise an associated water loss proportional to the degree of shortening, the amount was less than that obtained in controls with a comparable degree of shortening (Fig. 2). It should be emphasized, however, that a reduction of shortening in the controls was achieved by mechanical restriction, whereas the Ca-depleted muscles were unrestricted and shortened to their maximum ability. Thus it seemed likely that the smaller amount of water loss in the Ca-depleted muscles was in actual fact associated with a reduced contractile activity which was not reflected accurately by the degree of shortening; shortening of the whole muscle often being deceptively exaggerated as an index of active contraction of the myofibrils. This likelihood was borne out in the next series of experiments. Paired ileofibularis muscles were soaked in Ca-free–EDTA Ringer’s solution for similar periods of time and after freezing, isotonic shortening and water loss were determined in the one muscle while isometric tension was recorded in the other; the isometric tension serving by extrapolation as an index of the contractile activity of the isotonically contracted muscle. The tension varied inversely with the
Figure 4. a. Phase contrast micrograph of normal frog muscle. × 1850.

Figure 4 b. Phase contrast micrograph of frozen-thawed muscle shortened maximally. The sarcomeres are completely disorganized. × 1750.
Figure 4 c. Electron micrograph of normal frog muscle. $\times 30,000$.

Figure 4 d. Electron micrograph of frozen-thawed muscle shortened maximally. Sarcomeres are disorganized but distinct filaments are seen. In some areas the filaments are oriented in a parallel fashion, whereas in other areas they are randomly oriented. The presence and orientation of the filaments varied and in some micrographs they were not discernible. $\times 52,000$. 
duration of soaking and Fig. 3 illustrates that, as anticipated, the water loss is proportional to the contractile activity.

Caffeine Contracture  Caffeine (5 × 10⁻³ M) caused irreversible contracture. In the isotonically contracted muscle water also exuded (about 25 per cent of the weight), whereas in isometric contracture there was no significant water loss. Thus, irreversible contracture produced by another method produced results similar to those obtained by freezing and thawing.

Mytilus Muscle  Freezing and thawing of the anterior byssus retractor produced shortening of about 40 per cent of its length and a water loss of only 4 to 9 per cent of the weight. Isometrically, the tension was, however, only 135 to 254 gm/gm. Thus, a small contractile response was, once again, associated with little water loss. Why this particular muscle contracted...
minimally remains to be elucidated. Consideration should be given to the possibility that the state of the paramyosin (11) prevented the contraction of the actomyosin filaments.

Microscopic Observations  As isolated frog muscle fibers contracted while thawing, the striations disappeared. Water also exuded from them, as judged by an increase in the space between the fibers and oil in which they had been immersed. The precise time relationship between these three events, \textit{viz.} contraction, sarcomere disorganization, and water exudation, requires more detailed study.

With the disappearance of the striations, the fibers looked granular (Fig. 4 b). This was the case with isotonic as well as isometric contraction; and was also seen in frozen sections. Electron microscopy confirmed the occurrence of sarcomere disorganization but also demonstrated that distinct filaments remained in some areas (Fig. 4 d).

Ca-depleted muscles which hardly contracted had, on the other hand, an abundance of fibers with intact sarcomeres. Likewise, glycerol-extracted muscles which did not contract on freezing and thawing (or lose water) had no obvious signs of disorganization.

Syneresis of Actomyosin  Freezing and thawing of actomyosin (in 0.1 M KCl) produced marked syneresis but a little less than that obtained with ATP (Fig. 5). In higher concentration of KCl the syneresis was less marked and no precipitation occurred at 0.4 M KCl. The syneresis was reversible; for on increasing the molarity of KCl to 0.6 M the precipitated protein went back into solution and could be subsequently superprecipitated with ATP in 0.1 M KCl.

DISCUSSION

Subsequent to the original observations on the frozen-thawed muscle, investigations have included aspects of its thermodynamics (12) and the fate of its ATP during contraction (13). How the freezing and thawing induce contraction remains, nevertheless, enigmatic. It is known, however, that the frozen-thawed muscle will contract after its membrane has been depolarized (9). It also appears that ATP may be necessary for the contractile response (14) and that freeze-thawing may cause association of actin and myosin (15). In the present investigation, freezing and thawing caused syneresis of actomyosin apparently resembling that produced by ATP. Thus, by analogy, it again appears that freezing and thawing affected directly the contractile proteins in the intact muscle. That calcium is involved in this reaction is evident from the inhibition of the contraction in Ca-depleted muscles; Ca++ may well have been present in the actomyosin and also may have been in-
involved in the syneresis of the protein (16). But the point of major interest in
the present communication is the associated water loss.

From the evidence reviewed (17, 18) it is clear that a 35 per cent loss in
weight of muscle, as obtained in the present investigation, with the exudate
having concentrations of 27 meq/liter of Na and 100 meq/liter of K represents
mainly an intracellular loss. Since the amount of water lost is proportional
to both the force of contraction and the degree of shortening (with no loss
occurring in isometric contraction) it follows that freeze-thawing in itself is
not responsible for the water exudation and that shortening of the myofibrils
is a determining factor. Teleologically, one could suppose a lateral displace-
ment of water to provide space for contractile filaments to either slide (19,
20) or fold (21) during shortening. This water displacement could presumably
be caused by local pressure changes associated with the movement of the
contractile element during shortening. Whereas, in a normal muscle twitch
the water displacement would be cyclical, this would not be the case during
tetanic contraction or in irreversible contracture; the water in these instances
would remain displaced. If, at the same time, rapid stretching of membranes
did not occur to equilibrate the pressure, then exudation would ensue; this
would, of course, be facilitated by any increase in the permeability of mem-
brane barriers, a change which may have occurred with freezing (22). This
explanation could also account for the large quantity of water lost in the
muscles which had already been tetanically shortened prior to the freezing
and thawing. On the other hand, the force of the contraction may have simply
expelled the water which had already exuded into the interstitial spaces as a
result of membrane disruption. That water displacement occurs during
normal muscle contraction has been suggested to account for both the optical
changes (23) and the diminution in fibrillar volume as determined electron
microscopically (24). Also of interest in this connection is the water shift in
contracting synthetic polymers (25).

Regarding the cause of the sarcomere disorganization it is known that
freezing need not disturb the striations (26) and it also appears from the
maintained striations in the Ca-depleted and glycerol-extracted muscles that
freezing followed by thawing is in itself not responsible for the disorganization
but that the contractile process is a necessary concomitant. That the dis-
organization is causally related to the water loss is probable, particularly in
view of the similar loss in water obtained with caffeine contracture, where
structural disorganization is also known to occur (27, 28).

The explanation for the water exudation has been considered, so far,
simply in terms of water being mainly in a free state in muscle (29). But
in the light of recent evidence on the ordered structure of water in proteins
(30) and tissues (31, 32) it is worth bearing in mind that any displacement
of intracellular water, during contraction, may be preceded by changes in
the water structure. While the present observations were obtained in extreme irreversible contracture, the additional information on water movement, particularly its relation to length changes and tension, may have some bearing on normal muscle contraction.

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