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°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) 4×10^{-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_2PO_4 0.5 mM, Na_2HPO_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 5×10^{-3} 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°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

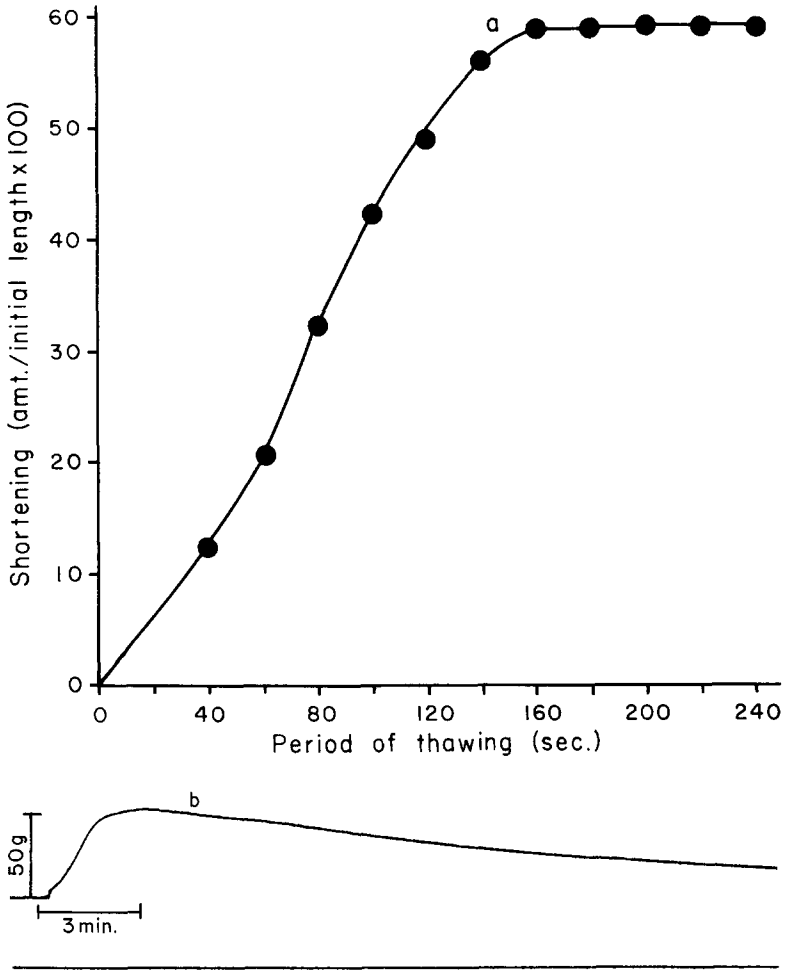

FIGURE 1. Examples of graphs depicting shortening (*a*) and isometric tension (*b*). The first small vertical rise in (*b*) is the applied initial tension; after a maximum is reached there is a decline in the isometric tension which may be due to tearing of fibers.

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.

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

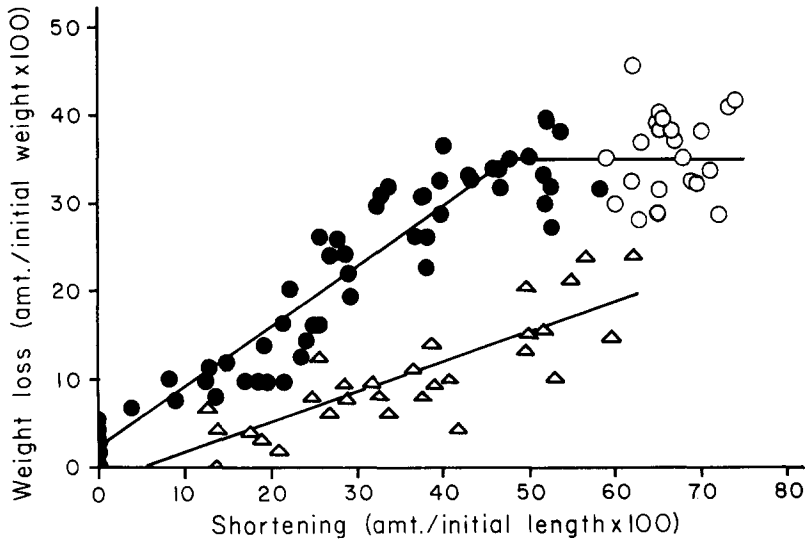

FIGURE 2. The relationship between percentage shortening and percentage loss of weight of frozen-thawed muscles. Filled circles, values for muscles which were restricted in their degree of shortening by means of restraining ligatures. Muscles soaked in Ringer's solution (1 to 4 hours) are included; soaking did not influence the results. Open circles, unrestricted preparations. Open triangles, values for muscles soaked in a Ca-free–EDTA Ringer's solution (1 to 4 hours). None of these muscles was restricted and all contracted to the maximum of their ability.

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

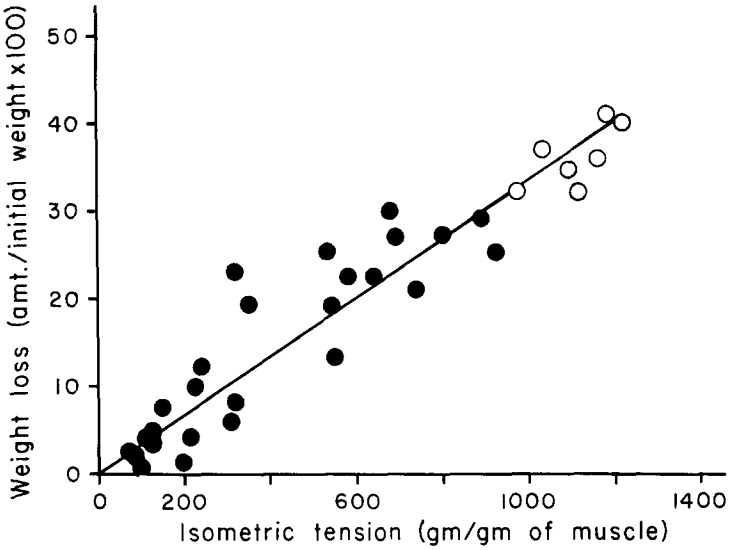

FIGURE 3. The relationship of isometric tension and per cent weight loss during shortening in paired muscles after freeze-thawing. Filled circles, values for paired muscles soaked in Ca-free–EDTA Ringer's solution (1 to 4 hours). Open circles, values for normal muscles.

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

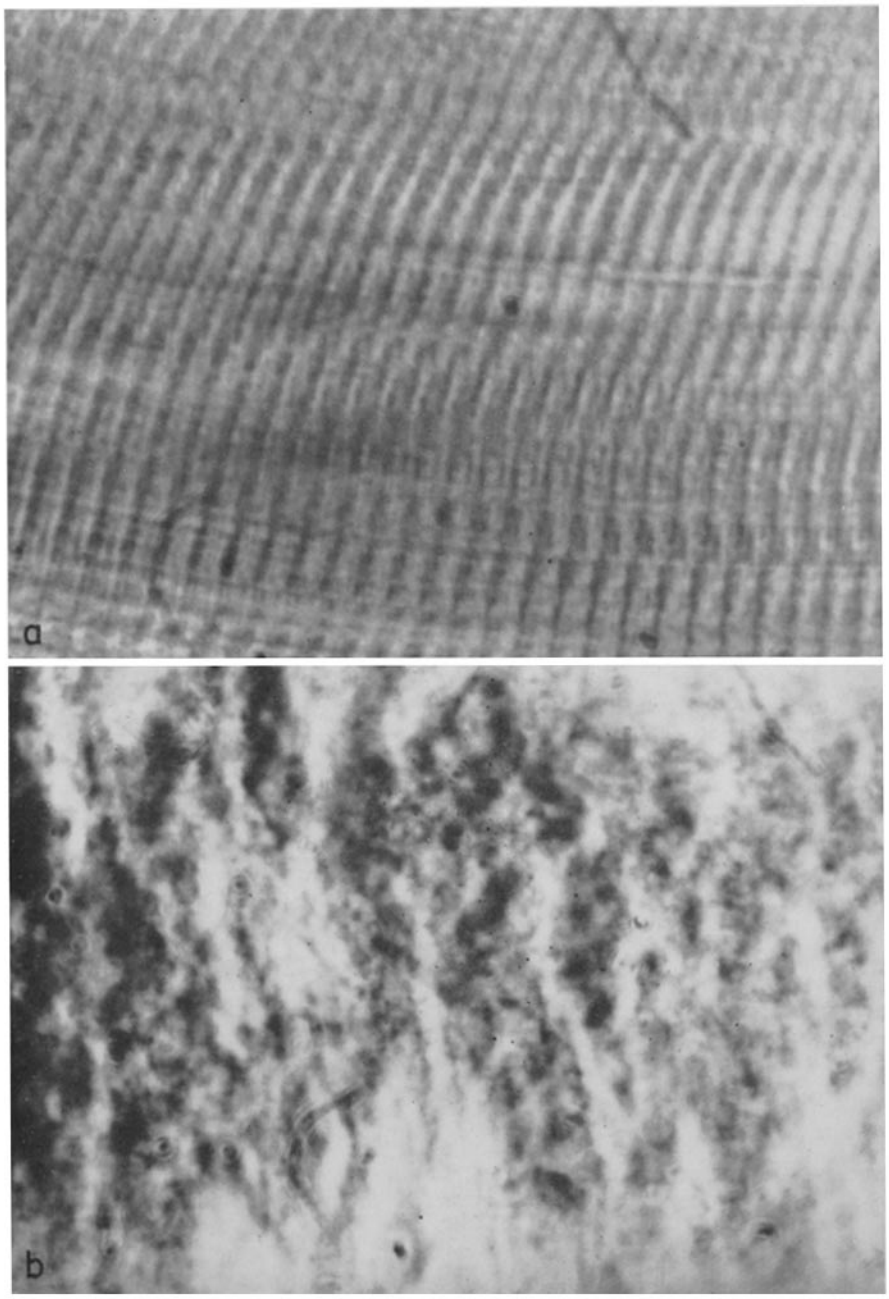

FIGURE 4. *a*. Phase contrast micrograph of normal frog muscle. × 1850.
FIGURE 4 *b*. Phase contrast micrograph of frozen-thawed muscle shortened maximally. The sarcomeres are completely disorganized. × 1750.

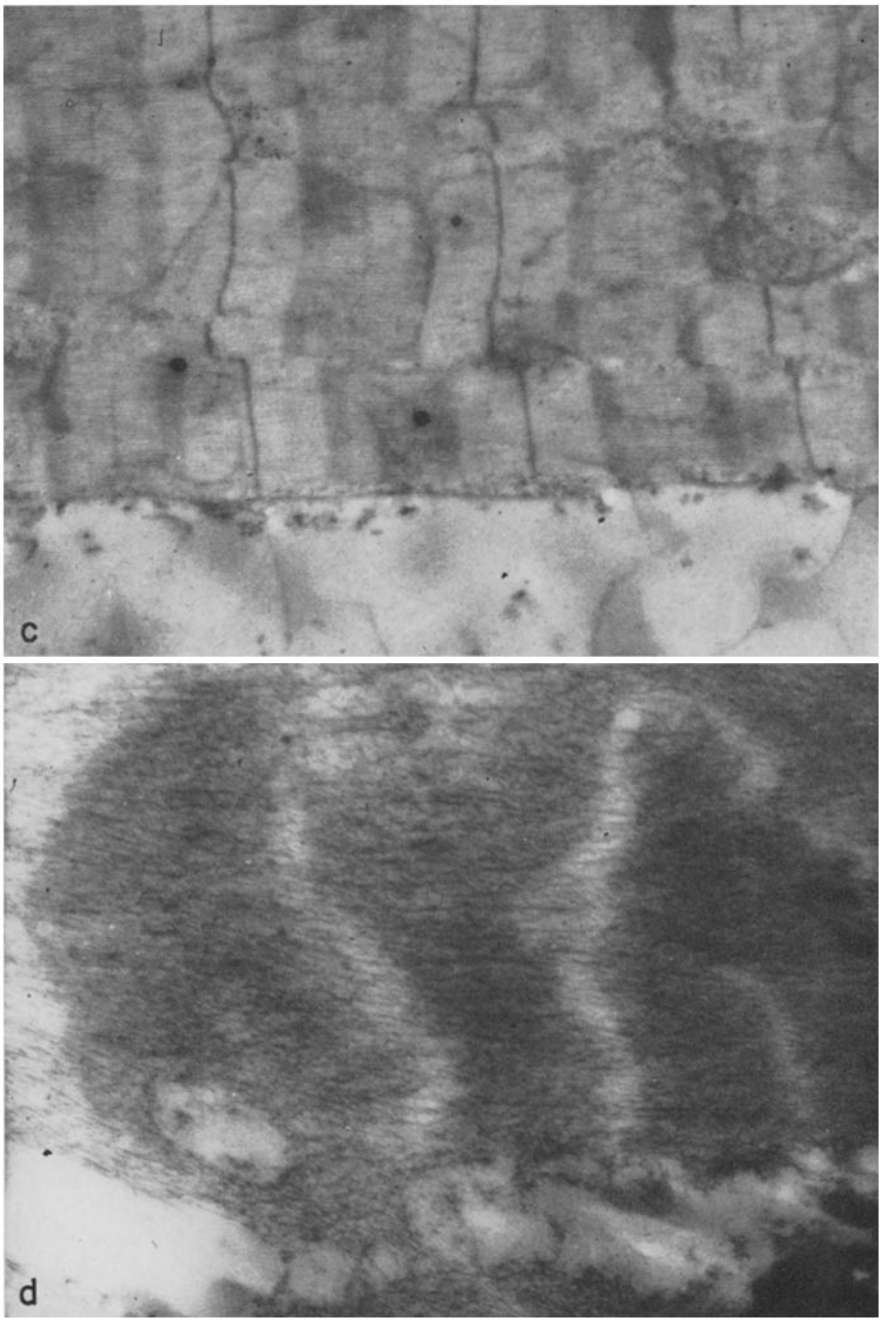

FIGURE 4 *c*. Electron micrograph of normal frog muscle. × 30,000.

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. × 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

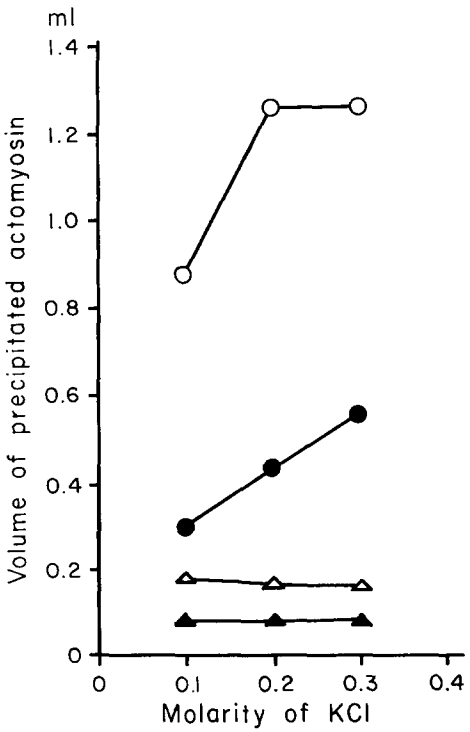

FIGURE 5. Volume of precipitated actomyosin in a concentration series of KCl after spinning in a Kolmer type tube for 3 minutes at about 2,700 RPM. The smaller the volume the greater is the syneresis. Open circles, control; filled circles, frozen-thawed actomyosin; open triangles, actomyosin treated with ATP; filled triangles, frozen-thawed actomyosin treated with ATP. There was no protein in the supernatant fluid. In 0.4 M KCl there was a very small thin precipitate only in the frozen-thawed preparations; this may have been due to some denaturation.

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, *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-

volved 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 displacement 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 membrane 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 disorganization 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.

I wish to thank Dr. Albert Szent-Györgyi for suggesting this investigation and for helpful discussions. I am also grateful to Dr. Andrew G. Szent-Györgyi for helpful discussions. Thanks are due to Delbert E. Philpott for the electron microscopy.

This research was supported by a grant from the Muscular Dystrophy Associations of America, Inc., a grant from the Commonwealth Fund, Grant No. 60 G 54 from the American Heart Association, Grant No. H-2042(C5, 6, 7,)BBC of the National Institutes of Health, and Grant No. G-5836 of the National Science Foundation. This work was done during the tenure of a Rockefeller Foundation Fellowship by the author.

Received for publication, March 8, 1962.

REFERENCES

1. SZENT-GYÖRGYI, A., Chemistry of Muscular Contraction, New York, Academic Press, Inc., 1947.
2. MARSH, B. B., The effects of adenosine triphosphate on the fibre volume of a muscle homogenate, *Biochim. et Biophysica Acta*, 1952, **9**, 247.
3. STRÖBEL, G., Doppelbrechungsänderungen bei der aktiven Kontraktion des Fasermodells (aus Kaninchen-Psoas), *Z. Naturf., pt b*, 1952, **7**, 102.
4. SZENT-GYÖRGYI, A., Bioenergetics, New York, Academic Press, Inc., 1957.
5. HERMANN, L., Weitere Untersuchungen über die Ursache der electromotorischen Erscheinungen an Muskeln und Nerven, *Arch. ges. Physiol.*, 1871, **4**, 149.
6. NEWMAN, S. V., BORYSKO, E., and SWERDLOW, M., New sectioning techniques for light and electron microscopy, *Science*, 1949, **110**, 66.
7. MUELLER, H., The action of relaxing factor on actomyosin, *Biochim. et Biophysica Acta*, 1960, **39**, 93.
8. THOMPSON, J. P., and MARSH, B. B., Thaw rigor and the delta state of muscle, *Biochim. et Biophysica Acta*, 1957, **24**, 427.
9. CSAPO, A., *in* Structure and Function of Muscle, (G. A. Bourne, editor), New York, Academic Press, Inc., 1960, **1**.
10. BRIGGS, F. N., KING, R., and BARNABY, J., Freeze-thaw contracture and excitation contraction coupling, *Fed. Proc.*, 1961, **20**, 300.
11. JOHNSON, W. H., KAHN, J. S., and SZENT-GYÖRGYI, A. G., Paramyosin and contraction of "catch muscles," *Science*, 1959, **130**, 160.
12. SZENT-GYÖRGYI, A., Free energy relations and contraction of actomyosin, *Biol. Bull.*, 1949, **96**, 140.
13. SZENTKIRALYI, E. M., Changes in the nucleotides of the cross-striated muscle after freezing, *Arch. Biochem. and Biophysics*, 1957, **67**, 298.
14. PERRY, S. V., Studies on the rigor resulting from the thawing of frozen frog sartorius muscle, *J. Gen. Physiol.*, 1950, **33**, 563.
15. SZENT-GYÖRGYI, A. G., Effect of freezing and thawing of muscle, *Enzymologia*, 1950, **14**, 252.

16. WEBER, A., and WINICUR, S., The role of calcium in the superprecipitation of actomyosin, *J. Biol. Chem.*, 1961, **236,** 3198.
17. FENN, W. O., Electrolytes in muscle, *Physiol. Rev.*, 1936, **16,** 450.
18. CONWAY, E. J., Nature and significance of concentration relations of potassium and sodium ions in skeletal muscle, *Physiol. Rev.*, 1957, **37,** 84.
19. HUXLEY, H. E., and HANSON, J., Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation, *Nature*, 1954, **173,** 973.
20. HUXLEY, A. F., and NIEDERGERKE, R., Structural changes in muscle during contraction, *Nature*, 1954, **173,** 971.
21. SJÖSTRAND, F. S., and ANDERSSON-CEDEGREN, E., The ultrastructure of the skeletal muscle myofilaments at various states of shortening, *J. Ultrastructure Research*, 1957, **1,** 74.
22. LOVELOCK, J. E., The haemolysis of human red blood-cells by freezing and thawing, *Biochim. et Biophysica Acta*, 1953, **10,** 414.
23. HUXLEY, A. F., Muscle structure and theories of contraction, *Progr. Biophysics*, 1957, **7,** 255.
24. CARLSEN, F., KNAPPEIS, G. G., and BUCHTHAL, F., Ultrastructure of the resting and contracted striated muscle fiber at different degrees of stretch, *J. Biophysic. and Biochem. Cytol.*, 1961, **11,** 95.
25. KUHN, W., RAMEL, A., WALTERS, D. H., EBNER, G., and KUHN, H. J., The production of mechanical energy from different forms of chemical energy with homogeneous and cross-striated high polymer systems, *Fortschr. der Hochpolym.-Forsch.*, 1960, **1,** 540.
26. RAPATZ, G., and LUYET, B., On the mechanism of ice formation and propagation in muscle, *Biodynamica*, 1959, **8,** 121.
27. CHENEY, R. H., Microphysical changes induced in striated muscle after caffeine immersion, *Anat. Rec.*, 1939, **73,** 129.
28. CONWAY, D., and SAKAI, T., Caffeine contracture, *Proc. Nat. Acad. Sc.*, 1960, **46,** 897.
29. HILL, A. V., The state of water in muscle and blood and the osmotic behaviour of muscle, *Proc. Roy. Soc. London, Series B*, 1930, **106,** 477.
30. KLOTZ, I. M., Protein hydration and behavior, *Science*, 1958, **128,** 815.
31. ODEBLAD, E., Studies on vaginal contents and cells with proton magnetic resonance, *Ann. New York Acad. Sc.*, 1959, **83,** 189.
32. BERENDSON, H. J. C., The structure of water in tissue, as studied by nuclear magnetic resonance, *Biol. Bull.*, 1960, **119,** 287.