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The Influence of Temperature on the Thick Filaments of Vertebrate Smooth Muscle

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The influence of temperature on the thick filaments of vertebrate smooth muscle

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[Plates 17 to 19]

When fixation of taenia coli from adult guinea-pigs is initiated at 37 °C only thin filaments and 10 nm filaments are preserved. At 37 °C (i.e. as *in vivo*) thick filaments are very labile; to preserve them during fixation much thinner muscles must be used such as taenia coli from very young animals. The thick filaments from taenia coli of adult guinea-pigs can however be stabilized by pre-cooling the living muscles before fixation at 37 °C.

An ion analysis of these muscles *in vivo*, and during fixation at 37 and 4 °C, showed that there is a K and Na ion exchange in the tissue both on cooling and during fixation; the exchange is most rapid on fixation particularly when it takes place at 37 °C. The Mg^{2+} content appears to be unaffected by these conditions, but the Ca^{2+} content rises both on cooling and during fixation (when the uptake is unexpectedly large). The selective destruction of the cell membrane is greatest when fixation is carried out at 37 °C. It is suggested that pre-cooling may alter thick filaments.

Introduction

Laszt & Hamoir in 1961 showed that vertebrate smooth muscle actomyosin is soluble at low ionic strength in the presence of ATP. Subsequently Hanson & Lowy (1964), Kaminer (1969) and also myself (Shoenberg 1969*a*, *b*) examined negatively stained homogenates of vertebrate smooth muscle and were only able to observe thick filaments after special pretreatment. These findings suggested strongly that if thick filaments were present *in vivo* these must be extremely labile and it was therefore not surprising that it was so difficult to find them in sections of intact muscle or that the filaments which were observed varied so much in size, shape and total numbers, from one laboratory to another and were indeed not seen at all by one group of workers. Even when they were successfully observed, after the use of a particular technique, the results were not always reproducible.

It seemed therefore worth while to see how the speed of fixation and the temperature at which fixation takes place might affect the presence and type of thick filament seen in sections. It also seemed important to know what changes take place in the ion content of the tissue during fixation.

Methods

To eliminate at least one variable all the experiments were carried out on taenia coli of guinea-pig. Since the shock of excision causes ion disturbances in the muscles these were equilibrated for at least 1 h in phosphate free bicarbonate buffered Ringer solution kept at 37 °C. This solution was originally introduced by Goodford (1962) when, as here, Ca^{2+} content is of interest. 1 g weights were attached at one end of each piece of muscle and these usually showed continuous rhythmic contraction during equilibration.

The muscles were fixed in either $2\frac{1}{2}$ or 5% buffered glutaraldehyde, followed by several rinses in an appropriate buffer, postfixation in 1 or 2% buffered osmic acid, and staining in a

saturated aqueous solution of uranyl acetate, then dehydration in graded ethanols and embedding in araldite. The glutaraldehyde was made up in the Goodford Ringer solution except where otherwise stated. Most muscles were fixed for 10 min at 37 °C followed by 1 h and 50 min at 4 °C. These will be referred to as muscles fixed in the warm to distinguish them from muscles fixed entirely in the cold. These latter were also fixed for 2 h.

Much of the work was carried out on muscles fixed for an initial 10 min at 37 °C in glutaraldehyde diluted with Ringer solution because it seemed desirable to reproduce as nearly as possible conditions *in vivo*.

Electron microscope observations

Measurements of extracellular space and ion analysis of muscles in vivo *and during fixation*

To understand what happens during such a fixation an experiment was carried out (see Shoenberg, Goodford, Wolowyk & Wootton 1973) in which muscle sections were examined in the electron microscope, the extracellular space was measured before and at the onset of fixation and an ion analysis was carried out in the living muscle as well as during fixation. The extracellular space was measured by the [^{14}C]sorbitol technique and the ion analysis was made by atomic absorption spectrometry. Electron microscope examination of sections of taenia coli from adult guinea-pigs indicated that whether the muscle was fixed in $2\frac{1}{2}$ % or 5 % glutaraldehyde the thin filaments were always well preserved, (figure 1*a*) but that organelles such as mitochondria were not always well preserved. Thick filaments were only rarely seen and then usually in only a few of the sections of a preparation. When they were seen they appeared to have a circular profile in cross-section.

Measurements of the extracellular space was only possible in the living muscle as, on addition of the fixative, the extracellular space became almost indistinguishable from the intracellular space and the semi-permeability of the cell membranes appeared to be selectively destroyed. There was an immediate uptake of Na^+ in the tissue and a loss of K^+. The exchange was most

Description of plate 17

The muscles in figures 1*a*, *b* and 2*a*, *b* were fixed in 5 % glutaraldehyde made up in phosphate-free bicarbonate-buffered Ringer solution (Goodford–Ringer). The pH of the buffer was maintained between 6.9 and 7.2 by bubbling a CO_2 and O_2 mixture (5 % CO_2, 95 % O_2) into the fixative throughout the period of fixation. The tissues were then rinsed in 0.1 mol/l phosphate buffer at pH 7.1 and postfixed in 1 % osmic acid made up in Palade's buffer at pH 7.1.

The fixation in glutaraldehyde took place for a preliminary 10 min at 37 °C followed by a further 1 h and 50 min at 4 °C. Rinsing was also at 4 °C. Postfixation in osmic acid took place at room temperature.

The muscles were then briefly washed in distilled water and stained for 30 min to 1 h in a saturated aqueous solution of uranyl acetate and were then dehydrated in graded ethanols and embedded in araldite. Sections were stained at 45 °C in a saturated solution of uranyl acetate in 70 % alcohol and counter-stained with Reynold's lead citrate. The sections were examined in an A.E.I. 6B electron microscope.

Figure 1*a*. Cross-section of taenia coli from adult guinea-pig equilibrated for 4 h in Goodford–Ringer solution at 37 °C before fixation. Note well-preserved thin filaments and absence of thick filaments.

Figure 1*b*. Cross-section of taenia coli from an adult guinea-pig equilibrated for 1 h in Goodford–Ringer solution at 37 °C followed by equilibration for 3 h at 4 °C in the same Ringer, before fixation. Note the well-preserved thin and thick filaments and the occasional 10 nm filaments. The thick filaments have both the narrow elongated appearance of ribbon-shaped filaments and the irregular more or less circular appearance of the more conventional thick filaments. The filaments with a 10 nm diameter can occasionally be seen to be hollow. Thin filaments ↓; thick filaments (*a*) ribbon shaped: ↕; (*b*) cylindrical: ⇕. 10 nm diameter filaments: ↓.

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FIGURE 1. For legend see facing page.

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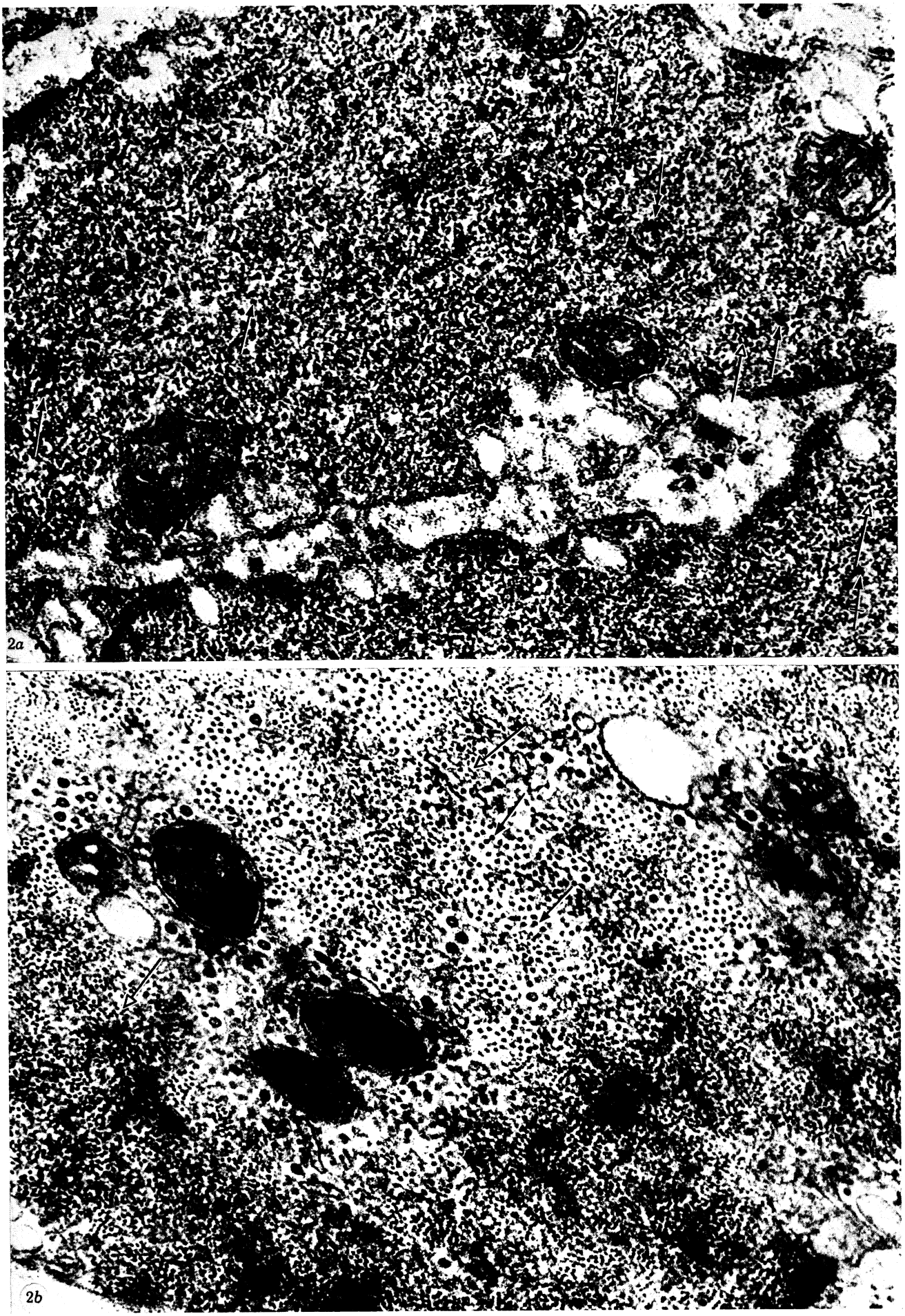

FIGURE 2. For legend see facing page.

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rapid during the 10 min of fixation at 35 °C. When the divalent ions were examined it was found that the Mg^{2+} content of the tissue did not change significantly throughout fixation, but that the Ca^{2+} content rose from an initial 4 μmol/g in the living muscle to 12 μmol/g after 4 h of fixation, when it appeared to be still rising. This concentration far exceeds that present in either the Ringer solution of the glutaraldehyde.

The ion analysis results were in good agreement with those of Krames & Page (1968) on heart muscle fixed in either formaldehyde or osmic acid. Attempts to stabilize the thick filaments by fixing in the cold with glutaraldehyde made up in Ringer solution were unsuccessful. The preservation of both the thin filaments and the cell organelles was appalling whether $2\frac{1}{2}$ or 5% glutaraldehyde was used. Unfortunately the extracellular space changes during fixation were not measured in these experiments. The K^+ and Na^+ exchange of muscles fixed entirely at 4 °C was considerably slowed down, but the Mg^{2+} content remained virtually unchanged and only the Ca^{2+} content rose as rapidly as when fixation was initiated at 35 °C. It seems reasonable to assume that since the K^+ and Na^+ exchange was slowed down the semi-permeability of the cell membranes was not as effectively destroyed when fixation took place in the cold and that the high osmotic pressure of the Ringer-buffered glutaraldehyde took effect before fixation occurred.

Since the myosin was so labile at 35 °C attempts were made to increase the speed of penetration of the fixative in the hope that thick filaments if present might be fixed before disintegration could take place.

Preservation of thick filaments at 35 °C

In the first place monomer and polymer glutaraldehyde were compared as fixatives; they were tried in concentrations of $2\frac{1}{2}$ and 5%; the results were not well defined but it seemed that when monomer glutaraldehyde was used thick filaments were more frequently found, though they were still usually only present in small numbers, but quite a number of the 10 nm diameter filaments described by Lowy & Small (1970) were observed. This only occurred when 5% glutaraldehyde was used. The 10 nm filaments can often be seen to be hollow and clearly separated from one another; the individual filaments do not appear to be surrounded by thin filaments. Sometimes these 10 nm filaments cover quite large areas (figure 2*a*).

Next taenia from very young guinea-pigs were fixed in 5% glutaraldehyde. A number of thick filaments were now found in the preparations (figure 2*b*). In cross-sections most of these appeared to have a more or less circular outline and to be 15 to 20 nm in diameter. Some of the 10 nm filaments were also seen in these sections. Presumably the thick filaments were preserved, in these muscles fixed in the warm, because the muscles of young guinea-pigs were thinner and the cells were surrounded by less collagen so that penetration of the fixative was more rapid and fixation could take place before the disintegration of the thick filaments had occurred.

Description of plate 18

Figure 2*a*. Cross-section of taenia coli from a very young guinea-pig fixed after equilibration for 4 h in Goodford–Ringer solution at 35 °C. Note good preservation of both thick and thin filaments and the 10 nm filaments. The thick filaments have mostly the appearance of conventional thick filaments; a few elongated profiles of ribbon shaped filaments can be seen on rare occasion as in this section.

Figure 2*b*. Cross-section of taenia coli from an adult guinea-pig fixed after equilibration for 4 h at 35 °C. Note thin filaments and an exceptionally large number of 10 nm filaments. Thin filaments: ↓; thick filaments (*a*) conventional: ↓; (*b*) ribbon-shaped: ↓; 10 nm diameter filaments: ↓.

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It would seem that when fixation is carried out at 37 °C the speed at which fixation occurs if of paramount importance, since the thick filaments are then very labile.

Changes in taenia coli during cooling of living muscles

Lowy and Small (1970) have shown that pre-cooling stabilizes the thick filaments in taenia coli. They fixed their muscles in the cold after a pre-equilibration in Ringer solution for 2 to 4 h at 4 °C and observed numerous ribbon-shaped thick filaments in their preparations.

It seemed important to know if these stabilized thick filaments were the same as those occurring in the living muscle at 37 °C. At 37 °C the K^+, Na^+, Mg^{2+} and Ca^{2+} tissue content of taenia coli remains unchanged for several hours when the muscle is kept in Goodford–Ringer solution. Freeman-Narrod & Goodford (1962), however, showed that when living taenia coli from guinea-pig pre-equilibrated for 1 h in Ringer solution at 37 °C is then transferred to Ringer kept at 4 °C and is kept at this temperature for several hours, there is a slow exchange of K^+ and Na^+ in the tissue. The Mg^{2+} content remains virtually unchanged, but as Bauer, Goodford & Hüter (1965) showed the Ca^{2+} content rises as it does in other conditions of shock. The changes in ion content taking place on cooling indicate a distinct difference between muscles equilibrated at 37 °C and those equilibrated at 4 °C.

An important observation of Wootton's in the course of some of her work on vesicles in muscles pre-cooled for 3 h and then fixed in the warm, made it possible to show also a distinct difference between thick filaments from pre-cooled muscles and those from muscles pre-equilibrated at 37 °C. Wootton observed numerous thick filaments in sections of pre-cooled taenia coli fixed in the warm. I repeated her experiments and confirmed her observations. When taenia coli from adult guinea-pigs were pre-equilibrated at 37 and at 4 °C and these muscles were fixed as already described in the warm there were no thick filaments in sections from the muscles pre-equilibrated in the warm (figure 1*a*) and there were numerous thick filaments in sections from muscles pre-equilibrated in the cold. (figure 1*b*). The same results were also obtained when 0.1 mol/l cacodylate buffer was used instead of Ringer to make up the fixative; it was furthermore possible to show in this instance that the thick filaments were as numerous in pre-cooled muscles fixed at 37 °C as in those fixed at 4 °C. Usually both cylindrical and ribbon-shaped thick filaments were present in the cooled muscles fixed in the warm, though occasionally only cylindrical filaments could be seen. There were always a number of 10 nm elements. It was found that stabilization of the thick filaments could take place after as little as 10 min at 4 °C, but that it might also take a longer time and conversely that if pre-cooled muscles were then again incubated at 37 °C for varying numbers of minutes before fixation, the acquired stability rapidly disappeared. After 5 min of post-incubation at 37 °C few if any thick filaments were present in the preparations. Thus pre-cooling altered the thick filaments so that even when muscles from adult animals were fixed at 37 °C, thick filaments were present in the preparations.

Further experiments are planned to see in what other respects thick filament formation and stability in taenia coli equilibrated at 37 °C differ from these properties in taenia kept at 4 °C.

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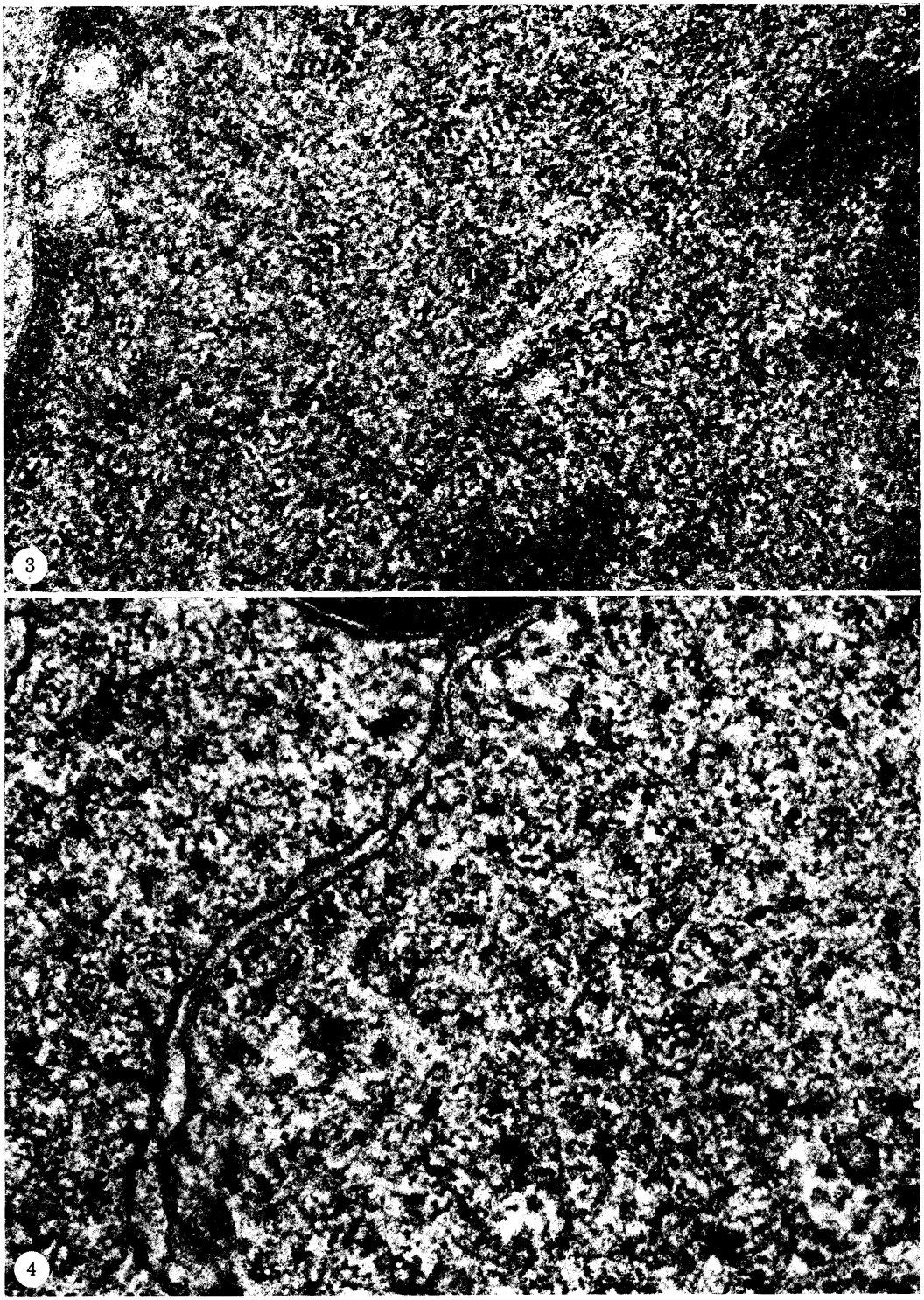

FIGURE 3. Urea-treated toad muscle. No thick filaments can be seen. (Magn. ×130 000.)

FIGURE 4. Urea-treated mouse muscle. Numerous bizarre thick filaments are present. (Magn. ×130 000.)

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Discussion

J. Rosenbluth (*Department of Physiology, New York University School of Medicine*): I would like to mention some results that bear on the question of whether thick filaments occur in smooth muscle cells normally or form only under abnormal circumstances. The following observations illustrate some special conditions under which thick filaments either do or do not appear.

Segments of the small intestine were taken from a mouse anaesthetized with chloral hydrate and from a pithed toad. Some were fixed immediately without dissection or manipulation in 3 % glutaraldehyde buffered with 0.1 mol/l cacodylate (pH 7.3). A second group was briefly glycerinated, then deglycerinated, and then fixed as in the first case, and a third group was soaked for about 1 h in Ringer's solution to which 0.6 mol/l urea had been added, then fixed as before but with urea also added to the fixative and rinsing solutions. All specimens were post-fixed in osmium tetroxide and stained *en bloc* with uranyl acetate.

Table 1. Thick filaments in intestinal smooth muscle cells

	mouse	toad
fixed immediately	±	−
glycerinated	+ + +	+
0.6 mol/l urea	+ + + +	±

As table 1 indicates, thick filaments were absent from the toad muscle fixed immediately and were not clearly present in the mouse either. After glycerination, however, the mouse muscle showed large numbers of thick filaments in most of the cells and the toad showed a small number in some of the cells. These specimens also showed signs of extensive damage including swelling and interruption of cell membranes. After exposure to urea, all of the mouse muscle cells in both the circular and longitudinal layers had thick filaments in large numbers but the toad muscle did not (figure 3).

What this shows, as I see it, is that thick filaments may appear very prominently in smooth muscle cells under highly unphysiological circumstances including some in which the cells are badly damaged. An earlier report (Rosenbluth 1971) also showed large numbers of thick filaments in smooth muscle cells that had been deliberately damaged with trypsin and suggested that the myosin probably exists in a relatively disaggregated state normally but tends to aggregate under conditions that cause protein denaturation. Furthermore, not only does the demonstration of thick filaments depend on how the muscle is treated but also apparently on the species of animal selected, and the same factors that cause thick filaments to appear in a

mammal may fail to do so in an amphibian. These findings should be accounted for before contraction mechanisms requiring the presence of thick filaments are postulated.

One final point is shown in an example of urea-treated mouse muscle (figure 4). At this magnification the thick filaments present appear to be both irregular in size and bizarre in shape, some resembling composites. These aggregates look quite different from the thick filaments of striated muscles and for this reason also should not be equated with them.

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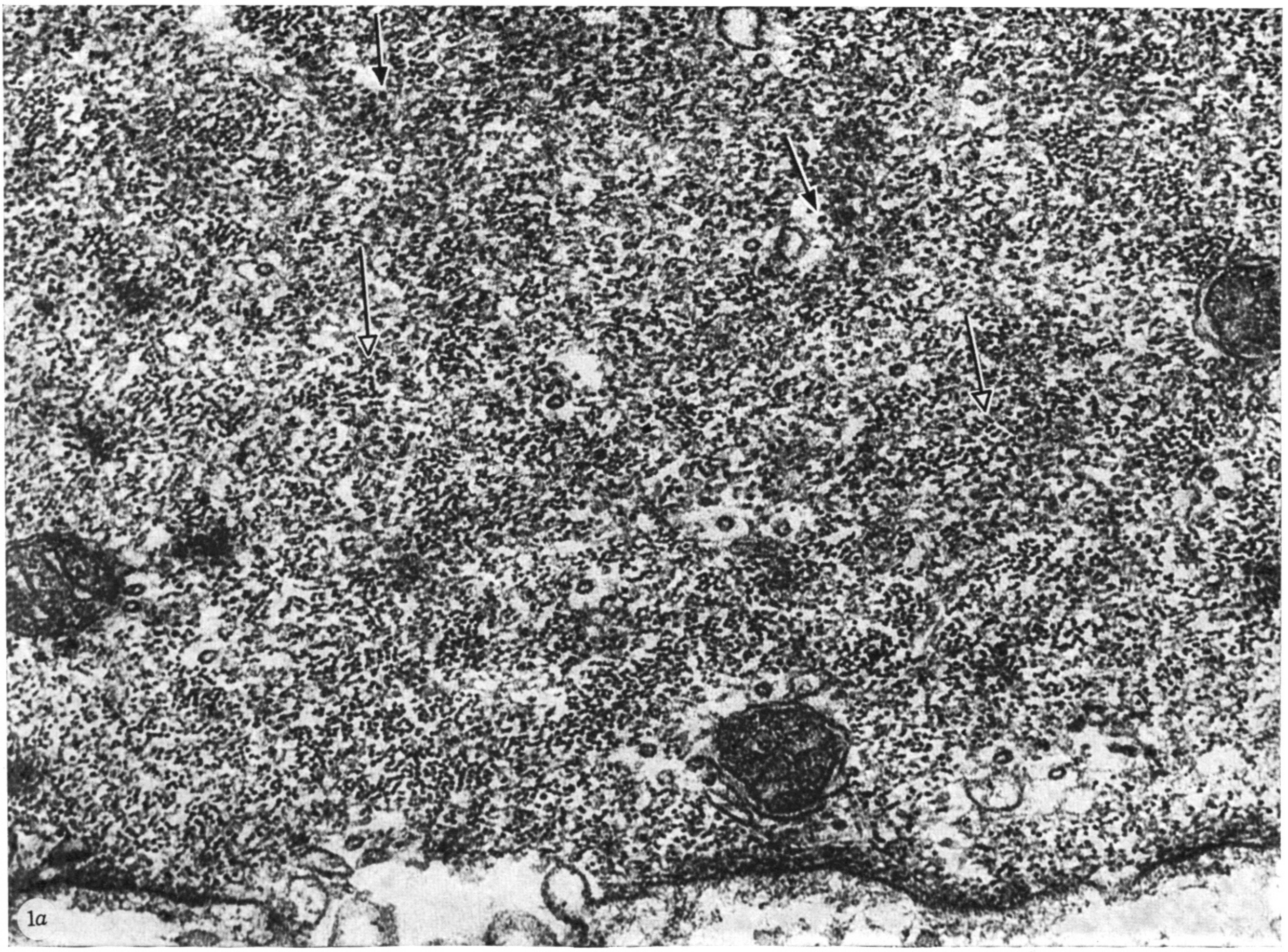

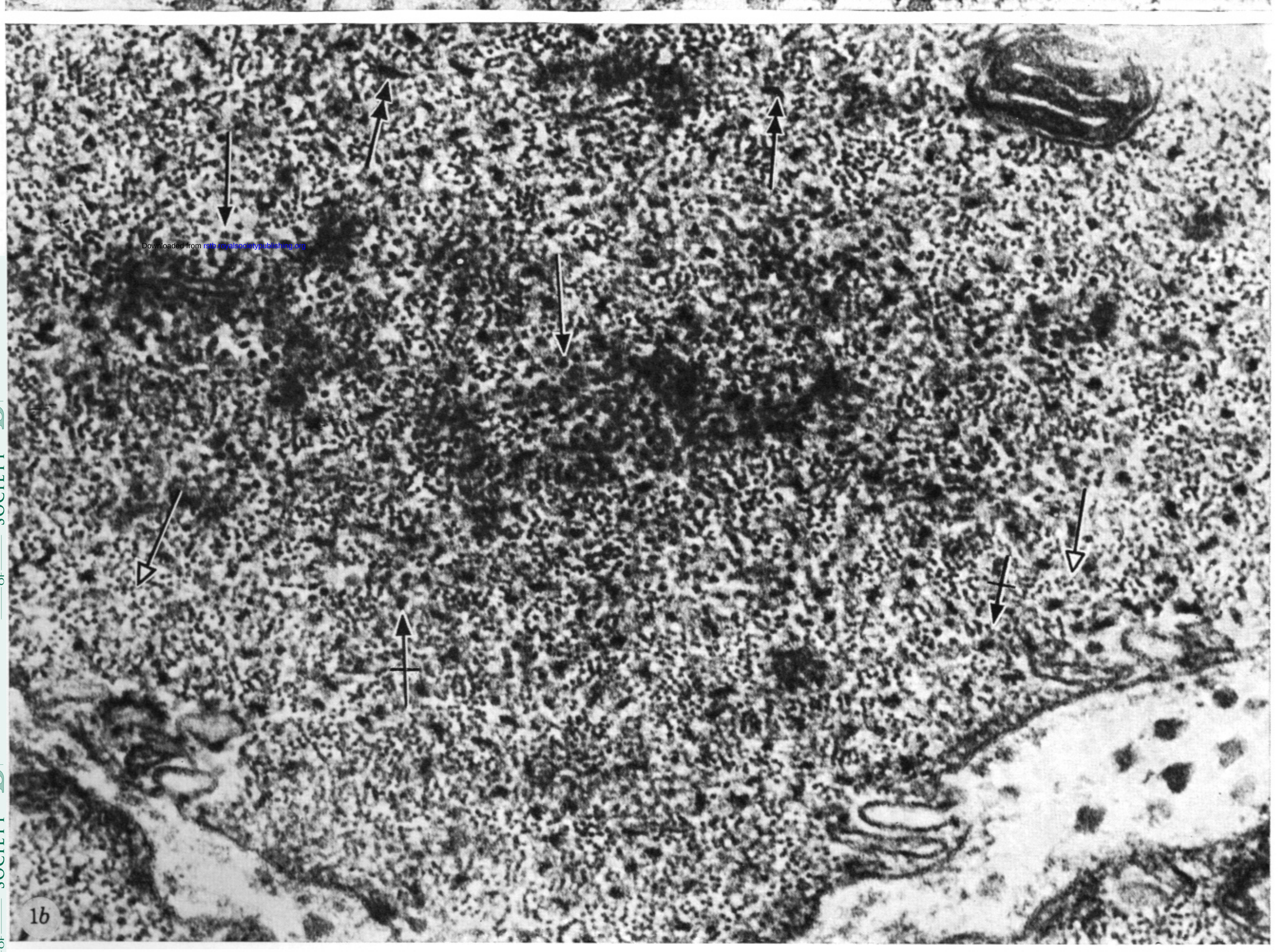

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FIGURE 2. For legend see facing page.

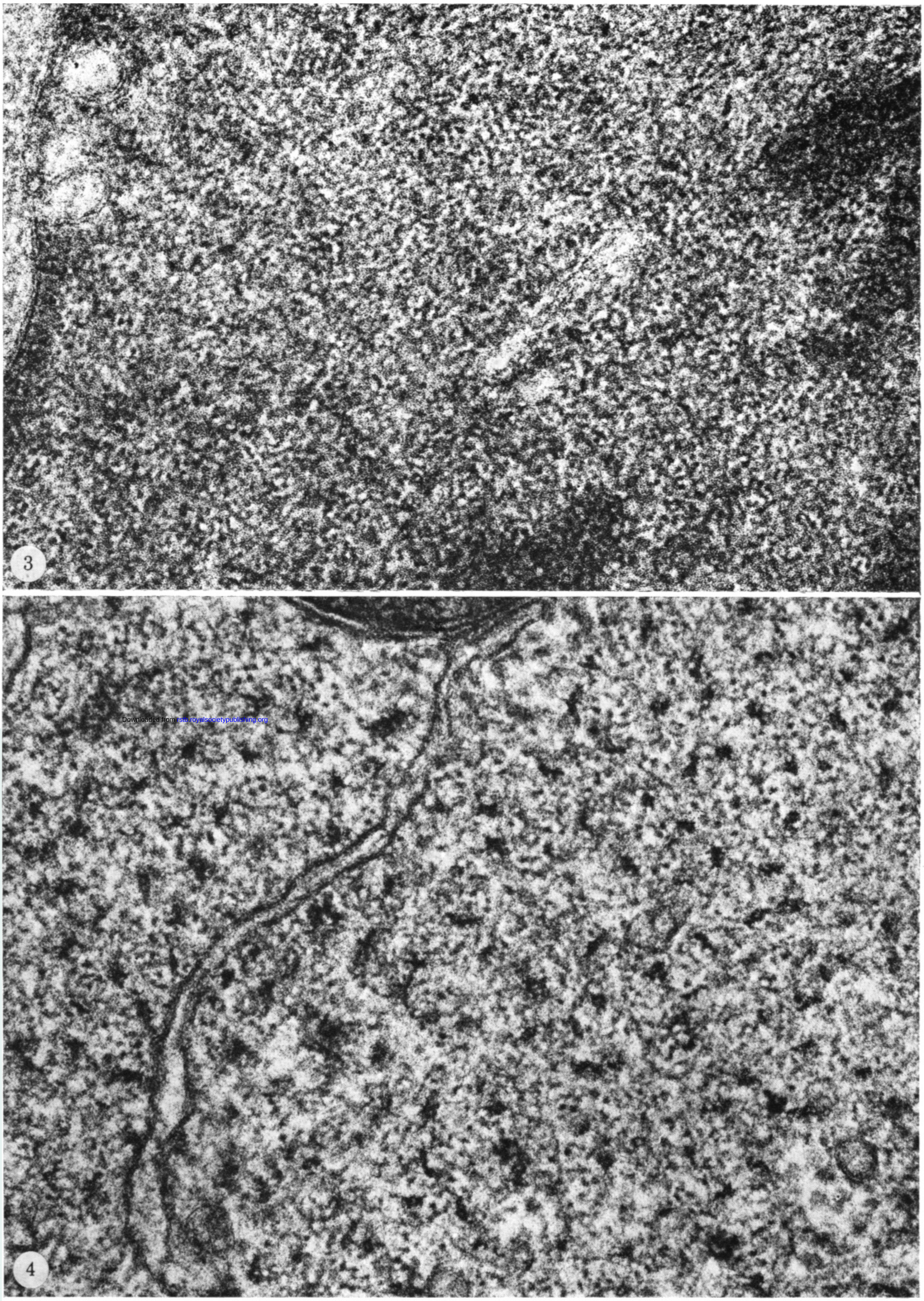

FIGURE 3. Urea-treated toad muscle. No thick filaments can be seen. (Magn. ×130000.)

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