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THE EFFECT OF CATHEPTIC ENZYMES ON CHILLED BOVINE MUSCLE,(U)
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The Effect of Catheptic Enzymes on Chilled Bovine Muscle

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The toughening of meat which has been caused by cold shortening prior to the onset of rigor is of significant commercial importance. Various studies have shown that catheptic enzymes produce degradative changes to meat which are very similar to those which occur during the natural aging process and which lead to a more tender meat product. Because of the tenderizing action of cathepsins, this study was undertaken to determine whether these enzymes could hasten the reversal of the cold shortening process. Samples which were cold shortened for 24 or 72 hrs were soaked in either a control solution or one containing catheptic enzymes. The sarcomere lengths of all samples were measured by laser diffraction, transmission electron microscopy and scanning electron microscopy, while the ultrastructural appearance was assessed by both forms of electron microscopy. Results showed that the microstructural appearance and significantly longer sarcomere lengths of the enzyme treated samples were consistent with a reversal of the cold shortening process.

INTRODUCTION

The chilling of bovine skeletal muscle prior to the onset of rigor mortis produces a reversible cold shortening effect (Locker and Hagyard, 1963) which causes toughening in cooked meat when there is a shortening of between 20 to 50% (Marsh and Leet, 1966). However, when shortening exceeds 50%, the severe contraction of the myofibrillar ultrastructure causes fiber fracture leading to a decline in toughness (Marsh, Leet and Dixon, 1974).

As a result of postmortem aging meat becomes tender, but this tenderness is caused by factors unlike the supraphysiological shortening mentioned above. Aged meat is tender because of the degradation of Z discs (Davey and Gilbert, 1967) and disruption of the sarcolemma (Varriano-Marston et al, 1976) as well as certain other factors including loss of Ca^{+2} accumulating ability (West et al, 1974) and modification of collagen (Marsh, 1977). Eino and Stanley (1973 a,b) and Robbins and Cohen (1976) found that catheptic enzymes produce degradative changes to the bovine myofibrillar ultrastructure which were quite similar to those which occur during postmortem aging.

Since there are similarities between morphological changes within the myofibrills caused by physiological contraction and cold shortening (Davey and Gilbert, 1974), the addition of catheptic enzymes to cold shortened muscle might act to reverse some of the effects induced by cold shortening. The purpose of the present paper is to investigate this possibility.

MATERIAL AND METHODS

Within 15 min of slaughter, a 5 cm^2 strip of sternomandibularis muscle was dissected from a cow of undetermined age. The muscle was divided into 3 smaller strips, one to be used for laser diffraction (LD) one for transmission electron microscopy (TEM) and one for scanning electron microscopy (SEM). Sarcomere length measurements were determined by all three methods (LD, SEM, TEM).

Sampling periods were as follows: at death control (C); 24 hr cold shortened control (C_1); 24 hr cold shortened enzyme treated (E_1); 72 hr cold shortened control (C_2); and 72 hr cold shortened enzyme treated (E_2).

The strips were refrigerated at $2^{\circ}C$ in an unrestrained condition within freezer wrapping paper for 24 or 72 hrs. At these times the samples were soaked overnight (16 hrs) in a 1% KCl control solution or in a catheptic enzyme solution (1.2 activity units/ml) (Robbins and Cohen, 1976).

1. Laser Diffraction-Sarcomere Length Measurements

Muscle fiber bundles from each sampling period were teased from the larger portion of muscle and fixed for 1 hr in 2.5% glutaraldehyde in KCl-borate buffer (pH 7.1) and then washed twice in the buffer solution (10 min each).

Next, individual fibers were placed into a drop of the buffer on a glass slide and a coverslip placed over the drop. The slide was mounted on a modified microscope stage and positioned so that the muscle fiber was in the path of a laser beam (Spectra Physics HeNe 632.8 nm) so that a diffraction pattern was formed on a ground glass screen 10 cm from the sample. The sarcomere length was determined by measuring the distance between the 0th and 1st order diffraction bands and using the formula $d \sin\Theta = n$ where d = sacromere length,

θ = angular separation between the 0th and 1st order bands, λ = the wavelength of laser light (632.8 nm) and n = the order of particular diffraction band (Cleworth and Edman, 1969). A total of 50 sarcomere length measurements were made for each sampling period.

2. TEM Processing

Small muscle strips taken at each sampling period were macerated and then fixed in 2.5% glutaraldehyde in 0.10 M cacodylate - 0.11 M sucrose buffer (pH 7.3) for 24 hrs. This was followed by three (30 min ea) rinses in cacodylate sucrose buffer, post fixation in 1% OsO_4 in 0.04 M cacodylate - 0.14 M sucrose buffer for 1 hr at 4°C, three buffer rinses (10 min ea) and dehydration in a graded series of ETOH (70, 95, 100 and 100%). After dehydration the strips were transferred to propylene oxide for 30 min and then into a mixture of propylene oxide and Epon-Araldite (1:1) [Epon 812 (25 ml), Araldite 6005 (15 ml), DDSA (55 ml), DMP-30 (2 ml), DBP (3 ml)] for 1 hr. This was followed by Epon-Araldite (1:3) for an additional 1 hr before final infiltration in Epon-Araldite. The resin mixture was then polymerized for 48 hrs at 60°C.

A trapezoid shaped block face was hand trimmed, smoothed by a glass knife with final ultrathin sectioning by diamond knife. Sections were picked up with 300 mesh copper grids and stained with 5% uranyl acetate in 50% methanol for 15 min followed by 2% lead citrate for 7 min, and then viewed using a JEOL Model 100B (JEOL, Medford, MA) at an accelerating voltage of 60-80 kV. At least 5 sections from each sample were examined and measurements of 40 sarcomeres were made.

3. SEM Processing

Following overnight washing in the KCl-borate buffer solution, mentioned in the laser diffraction preparation, samples were dehydrated in 70, 90, 95, and

100% ETOH for 1 hr each. Then the fibers were critical point dried from liquid CO_2. Following this step the fibers were mounted on SEM stubs with double sided sticky tape and sputter coated (Commonwealth Sci., Alexandria, VA) for 10 min with gold-palladium.

After sputtering the samples were placed in a Coates and Welter Model 100-2 field emission SEM (Sunnyvale, CA) for observation. Photographs were taken using an emission current of 10 μ A and an accelerating voltage of 15kV. Twenty-five sarcomere length measurements were made for each treatment group.

4. Statistical Analysis

Sarcomere length measurements obtained by laser diffraction, transmission and scanning EM were subjected to a two way analysis of variance with repetitions followed by the Tukey test for multiple comparison of means. Significance was computed at the 95% confidence level ($p < 0.05$) for all mean comparisons.

RESULTS AND DISCUSSION

The SEM micrograph (Fig. 1) of a pre-rigor control (C) shows the even register of sarcomeres with transverse (T) tubules overlying the Z-bands. In Fig. 2, the A, I and Z-bands as well as the mitochondria can be clearly distinguished in the corresponding TEM micrograph. The average sarcomere lengths of pre-rigor, pre-cold shortened muscle, as seen in Table 1, ranged from 2.05 μm (LD), to 2.15 μm (SEM) to 2.29 μm (TEM) which is consistent with the at death sarcomere lengths of 2.4 μm (TEM) determined by Henderson, Goll and Stromer (1970).

After cold shortening for 24 hrs (C_1), the values decreased to 1.03 μm

(LD), 1.21 µm (SEM) and 1.28 µm (TEM). Once again, the mean sarcomere length as determined by TEM (1.28 µm) was almost identical to the value published by Henderson, Goll and Stromer (1970) who obtained 1.3 µm (TEM) after cold shortening for 24 hrs.

According to Locker and Hagyard (1963), the degree of muscle shortening was approximately the same (47.7%) at $0^{\circ}C$ and $2^{\circ}C$. Our results show that sarcomere lengths of muscle cold shortened at $2^{\circ}C$ for 24 hrs and then soaked in a control solution ($2^{\circ}C$) overnight, shortened between 43.7 and 48.8% (Table 1).

Chilling the samples for 24 hrs resulted in severe contraction of the myofibrils and altered the surface morphology considerably (Fig. 3). The degree of contraction was such that it was difficult to distinguish the untreated (C_1) sample from the treated sample (E_1) (Fig. 4).

As seen in Fig. 5, chilling induced sarcomere contraction producing an overlapping of myofibrillar filaments resulting in the disappearance of the I band causing the sarcomere to take on a concave appearance. Separation and distortion of the individual myofilaments also occurred. The overlapping of actin and myosin filaments by sliding across one another substantiates the sliding-filament hypothesis of Marsh and Carse (1974) who explained that filament overlapping is a significant stage in the cold-shortening process.

When the 24 hr cold shortened muscle was soaked in a catheptic enzyme solution (E_1), there was a significant increase ($p < 0.05$) in sarcomere length (Table 1) in the LD and SEM samples but not the TEM sample. The degree of chilling induced contraction made it difficult to differentiate the untreated sample (Fig. 5) from the enzyme treated one (Fig. 6) in the TEM micrographs.

After 72 hrs, the sarcomere lengths of the cold shortened muscles were significantly longer ($p < 0.05$) than the 24 hr group (C_1) when measured by all

three methods. The sarcomere lengths of the control samples (C_2) were 1.72 µm (LD), 1.69 µm (SEM) and 1.46 µm (TEM). However, the enzyme treated samples (E_2) had sarcomere lengths of 2.08 µm (LD), 2.06 µm (SEM) and 1.95 µm (TEM) which ranged from 21 to 22 to 34% longer than the controls, respectively. The significant differences were probably caused by the synergistic effect of soaking in the catheptic enzyme solution.

While Eino and Stanley (1973a) found that the resolution of rigor usually occured at five days post-mortem, we have found that the rigor process seems to be largely resolved by 72 hrs (plus overnight soaking). The surface morphology of the 72 hr control sample (C_2) has changed so there is no longer any difficulty in differentiating the myofibrillar features (Fig. 7). The enzyme treated sample (E_2), although similar in appearance to the untreated sample (C_2) has areas, especially in the Z-I band region, where a certain amount of degradation appears to have taken place (Fig. 8). Eino and Stanley (1973a) and Varriano-Marston et al. (1976) found similar changes in naturally aged muscle as did Eino and Stanley (1973b) and Robbins and Cohen (1976) in muscle treated with catheptic enzyme.

The greatest differences between untreated (C_2) and treated (E_2) samples are seen in the TEM micrographs (Figs. 9, 10) of the 72 hr samples. The untreated sample (Fig. 9) is similar in appearance to Fig. 5, but the sacromere has increased significantly ($p < 0.05$) in width, some degradation in the Z-band has occurred and the sarcolemma is devoid of normal appearing mitochondria.

The contraction of the untreated 72 hr samples (C_2) has been largely reversed. Because of the higher concentration of catheptic enzymes, the treated (E_2) myofibrils exhibit the typical ultrastructural morphology found in aged muscle (Davey and Dickson, 1970). Furthermore, the sarcomere lengths of the 72 hr enzyme treated (E_2) sample more closely approach those of the original pre-rigor, pre-cold shortened control (C).

Although statistical analysis clearly showed significant differences between untreated and enzyme treated samples no matter which preparative technique was used (LD, SEM, TEM), the value differences of the three methods was probably due to the techniques themselves (Varriano-Marston, 1978). If one is solely interested in measuring sarcomere lengths, the laser diffraction method is the simplest, fastest and probably most accurate method. It also involves the fewest manipulative procedures. On the other hand if information is desired concerning surface structure or ultrastructure, then SEM and TEM examination would be necessary.

Thus, there is little doubt that cathepsins both aid and speed the processes responsible for significantly increasing the sarcomere length in rigor meat by degrading the Z-I band region and sarcolemma microstructure. Our results suggest that the addition of cathepic enzymes to cold shortened muscle hastens the change from rigor to aged muscle.

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Figure Legends

Fig. 1. SEM micrograph of pre-rigor muscle showing the register of sarcomeres (S) and transverse tubules (T) overlying the Z-I band region. Bar = 5 μm

Fig. 2. TEM micrograph of pre-rigor muscle. Typical A, I and Z bands as well as mitochondria (arrows) are easily distinguished. Bar = 1 μm

Fig. 3. and 4. SEM micrographs of untreated (C_1) (Fig. 3) and enzyme treated (E_1) (Fig. 4) 24 hr chilled muscle. Degree of contraction makes differentiation of surface features of both samples difficult. Bar = 5 μm

Figs. 5. and 6. TEM micrographs of untreated (C_1) (Fig. 5) and enzyme treated (E_1) (Fig. 6) 24 hr chilled muscle. Note disappearance of I band, concavity in A band region (arrows) and apparent separation of myofilaments in both figures. Bar = 1 μm

Fig. 7. and 8. SEM micrographs of 72 hr muscle. Chilling-induced contraction is reduced in untreated sample (C_2) (Fig. 7) and surface features can be better differentiated. With enzyme-treatment (E_2) some degradation (arrows) has occurred. (Fig. 8) Bar = 5 μm

Fig. 9. and 10. TEM micrographs of 72 hr muscle. Untreated myofibrils (C_2) (Fig. 9) have undergone some aging-related changes including increased sarcomere length and some degradation of the Z-I band region. Treated sarcomeres (E_2) show extensive degradation of the Z-I band region (arrows), no separation of myofilaments and a return to convex appearing sarcomers (Fig. 10). Bar = 1 μm.

SARCOMERE LENGTH MEASUREMENTS (μm) OF SAMPLES

PREPARED FOR LASER DIFFRACTION (LD) SCANNING (SEM)

AND TRANSMISSION (TEM) ELECTRON MICROSCOPY

	C	C_1	E_1	C_2	E_2
LD (n=50) MEAN ± STANDARD DEVIATION	2.01 ± .02	1.03 ± .03	1.23 ± .02	1.72 ± .03	2.08 ± .01
SEM (n=25) MEAN ± STANDARD DEVIATION	2.15 ± .21	1.21 ± .09	1.39 ± .19	1.69 ± .14	2.06 ± .12
TEM (n=40) MEAN ± STANDARD DEVIATION	2.29 ± .06	1.28 ± .12	1.32 ± .15	1.46 ± .04	1.95 ± .15

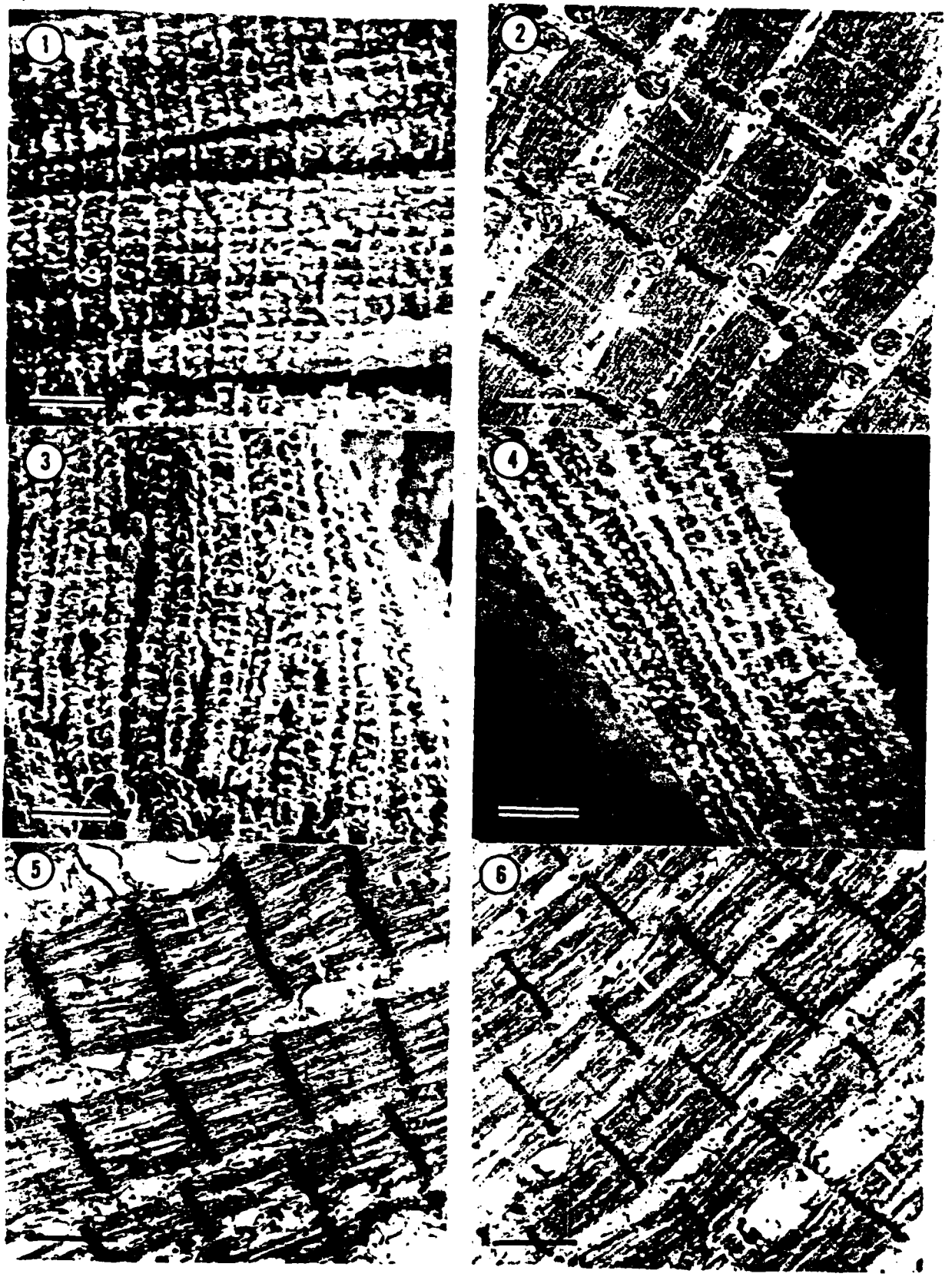
C - PRE-RIGOR CONTROL

C_1 - CHILLED AT 2°C FOR 24 HRS., SOAKED IN KCl SOLUTION OVERNIGHT

E_1 - CHILLED AT 2°C FOR 24 HRS., SOAKED IN ENZYME SOLUTION OVERNIGHT

C_2 - CHILLED AT 2°C FOR 72 HRS., SOAKED IN KCl SOLUTION OVERNIGHT

E_2 - CHILLED AT 2°C FOR 72 HRS., SOAKED IN ENZYME SOLUTION OVERNIGHT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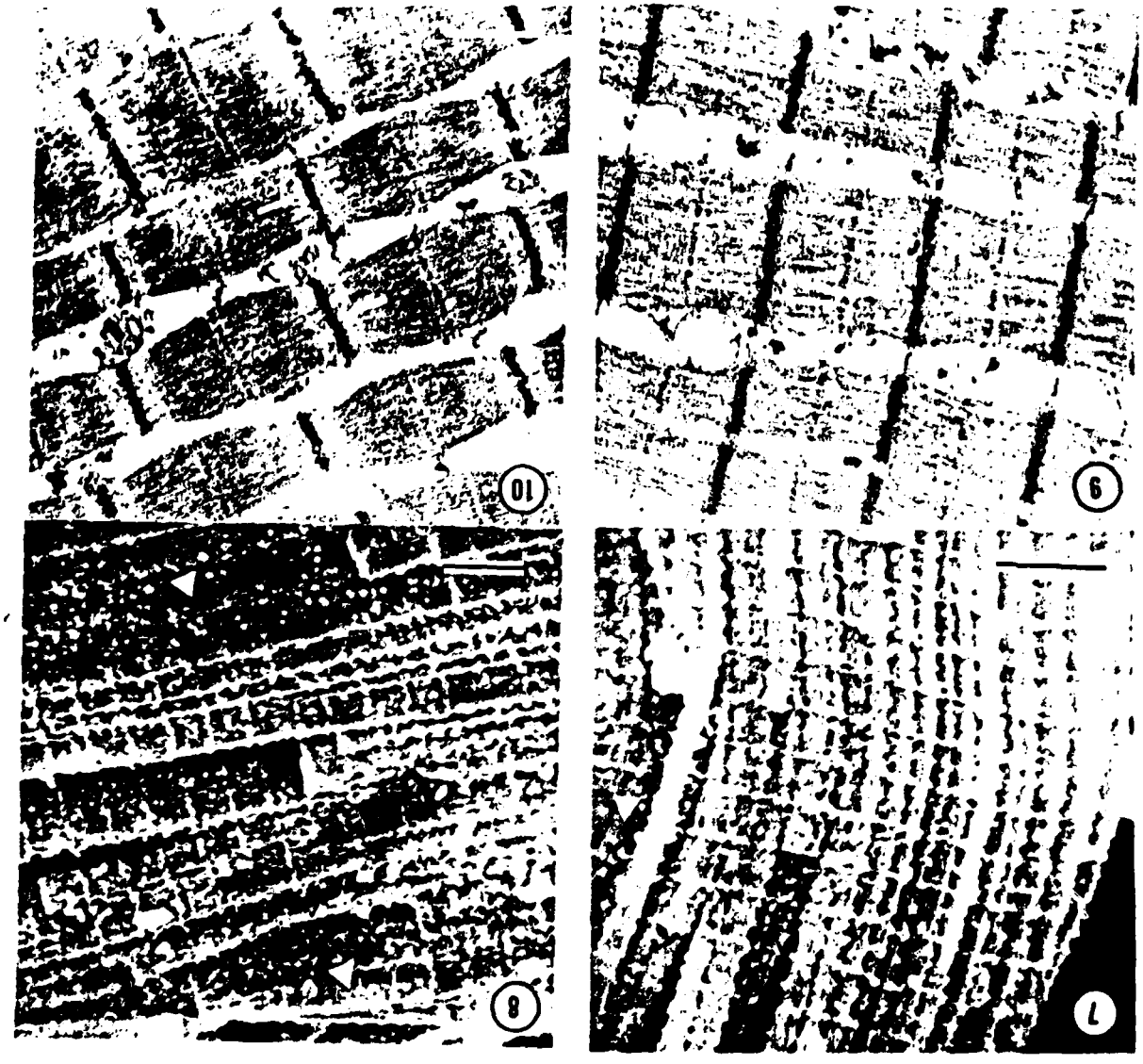
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