Table 1. Effects of ethacrynic acid (EA) on urine flow and chloride excretion. Data listed are mean values of output from the right kidney during 10-minute intervals before injection (control) and after injection (treated) of the drug.

Group	Urine flow (ml/min)	Chloride excretion (meq/min)
Control	6.6	0.43
Treated		
(0.5 to 3.0 mg EA/kg)	11.6	1.23
S.E. mean difference	1.4	0.15
p	<.01	<.01

which ethacrynic acid acts, we estimated the concentration of protein-bound sulfhydryl groups in renal cells and found it reduced in dogs that had been treated previously with ethacrynic acid.

Experiments were performed on seven mongrel dogs anesthetized with pentobarbital. Catheters were placed surgically into each ureter for separate collection of urine. The left kidney was removed and immediately afterward ethacrynic acid was administered intravenously. Twenty minutes later, when diuresis was maximal or increasing, the remaining kidney was removed. Thin slices of kidney tissue were fixed for 18 to 24 hours in 10 percent trichloracetic acid. Histological sections, 10 μ in thickness, were prepared and stained for protein-bound sulfhydryl groups by the method of Barrnett and Seligman (*4*).

Sections were then used for estimation of cytoplasmic protein-bound sulfhydryl groups of proximal and distal tubular cells, and medullary collecting-duct cells, by the method of Cafruny *et al.* (*5*).

In pilot experiments we discovered that the renal tubules of the diuresing right kidney were markedly dilated. For this reason, all animals included in this study were infused continuously with 10 percent mannitol at the rate of 1 ml/kg per minute. The ensuing osmotic diuresis caused maximum dilation of the tubules of both kidneys, thus insuring against spurious changes in the concentration of protein-bound sulfhydryl groups.

Table 2. Effects of ethacrynic acid (EA) on protein-bound sulfhydryl groups of renal cells. The results are expressed as the mean extinction value ± the standard error of the mean; kidneys from seven dogs were used.

Kidney region	Left kidney (control)	Right kidney (0.5 to 3.0 mg EA/kg)	p
Proximal convoluted tubules	0.641±.015	0.474±.007	<.001
Distal convoluted tubules	.591±.012	.494±.020	<.01
Medullary collecting ducts	.591±.026	.509±.015	<.02

Results are listed in Tables 1 and 2. The control group in Table 1 shows the average rates of urine and chloride excretion from the right kidney before administration of ethacrynic acid; the results obtained with the treated group shows that there was a significant increase in output of the right kidney following administration of ethacrynic acid.

Concentrations of protein-bound sulfhydryl groups, estimated spectrophotometrically as a colored complex with maximum absorption at 530 mμ, are given in Table 2. Values represent extinction [log(1/transmission)] determined through cylinders of cytoplasm (diameter, 2 μ; height, 10 μ). A total of 20 measurements in proximal and distal tubular cells were utilized for deriving a mean extinction value for each kidney; for the collecting ducts, 10 measurements were used. The standard deviation of these measurements did not exceed 12 percent of any mean value so derived. Extinction values listed in the table are averages of seven kidneys, 12 degrees of freedom being considered permissible in applying Fischer's "t" test for comparing two populations. The left kidney, removed before treatment with ethacrynic acid, served as a control.

A decrease in protein-bound sulfhydryl groups occurred in cells of the right kidney which had been exposed to a single injection of ethacrynic acid. Data are grouped even though the dose of ethacrynic acid ranged from 0.5 to 3.0 mg/kg, because the changes in renal sulfhydryl groups were of the same magnitude, as were changes in urine and chloride excretion (see Table 1), at each dose level. Cafruny *et al.* (*6*) have shown previously that concentrations of protein-bound sulfhydryl groups in the right and left kidneys of normal dogs do not differ.

These data support the premise that the mechanism of action of ethacrynic acid may be similar to that of mercurials. However, the drugs may differ with respect to site of action. Ethacrynic acid affects the protein-bound sulfhydryl groups of distal tubular cells; mercurials do not.

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7 October 1963

Contraction-Band Formation in Barnacle Myofibrils

Abstract. *Contractions induced by adenosine triphosphate, in myofibrils isolated from the barnacle,* Balanus aquilia, *were observed with a phase microscope. The formation of contraction bands was well under way before the A-band came into contact with the Z-membrane. This is in disagreement with the hypothesis that contraction bands are formed when the A-band pushes against the Z-membrane.*

In a study of the changes which take place in myofibrils during contraction induced by adenosine triphosphate (ATP), we used the barnacle, *Balanus aquilia.* This species is similar to *B. nubilius*, which was described by Hoyle and Smyth (*1*) and shown by them to have especially large single fibers. In *B. aquilia*, both the single fibers and the individual myofibrils are large. The average length of the sarcomeres in the myofibrils is 9.2 μ, which is approximately four times the length of those in rabbit psoas muscle. The large sarcomeres in *B. aquilia* make certain features of the contraction of these myofibrils resolvable with the phase microscope.

A recent study by Hoyle and McAlear (*2*) resulted in the contention that the A-filaments of the barnacle

myofibril do not change in length during supercontraction. These authors considered that contraction bands result from the overlapping of A-filaments which have penetrated the Z-disks. A key point in this argument was that the distance between exterior edges of adjoining contraction bands could not be less than the width of the A-band when the myofibril was at reference length. The studies which are presented in this report show a further consideration of this question.

Myofibrils were prepared from whole barnacle muscle fibers by the method of Bendall (*3*). The scutal-tergal adductor and depressor muscles were used, after being carefully removed from the animal with a piece of the shell intact. A thread was attached to the tendon at the scutal-tergal end of the muscle which was then mounted on a glass rod at the desired length, and placed in a glycerol buffer solution at 2°C for several days (*4*). The length the fibers assumed with the plates closed, but not pulled down, was taken as the reference length; this is the same length that the undamaged fiber assumes under zero tension. In fresh, healthy animals the muscles could easily be stretched to 200 percent of the reference length.

In order to view the myofibrils under a phase microscope they were washed in the buffer solution (*4*) minus glycerol and homogenized in a high-speed homogenizer, the tissue being maintained at 2°C. Isolated myofibrils were viewed under a phase microscope and changes in sarcomere length were induced by the addition of various amounts of an ATP solution placed under the cover slip. This enabled us to view the myofibrils continuously during the process of contraction. Photomicrographs were taken at various stages during the contraction process. Stages of partial contraction were obtained by the use of very low concentrations of ATP.

The appearance of barnacle myofibrils during ATP-induced contraction is shown in Figs. 1 to 6. Figure 1 shows two myofibrils lying side by side at reference length. In the center of the A-zone there is a faintly appearing H-band. Three distinct bands have appeared within the A-band in Fig. 2. In Fig. 3, it is particularly interesting that the center of the A-band appears to be splitting into two separate segments, and that the A-band is not resting directly against the Z-membrane; a space between the A-band and the Z-membrane can be observed. In Fig. 4 the "separation" of the A-band is complete and the "two segments" are moving toward the Z-membrane. In Fig. 5, the myofibril is essentially fully contracted and shows clear contraction bands. A faint line exists down the center of the dark band, this being the Z-membrane. Figure 6 shows the appearance of a myofibril in which only one part, which has approximately doubled in width, has contracted.

Since these photographs are not all of the same myofibril, and since some variability is to be expected between different myofibrils, comparative values

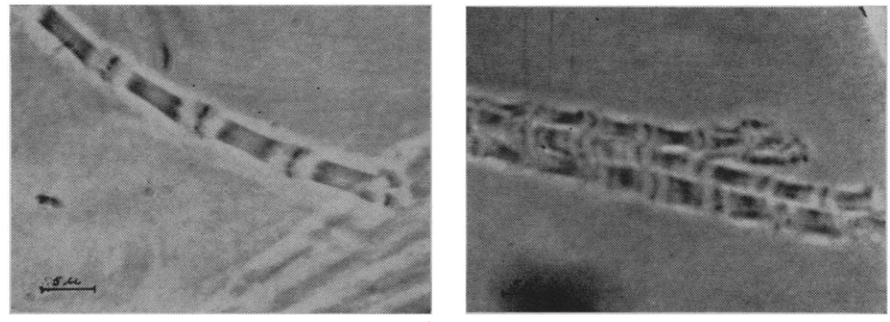

Fig. 1 (left). Two myofibrils lying side by side at reference length. The length of the sarcomere is approximately 9.2 μ and the width of the A-band is approximately 5.0 μ. Fig. 2 (right). The appearance of myofibrils which have contracted to approximately 60 percent of reference length. Three distinct bands appear within the A-band.

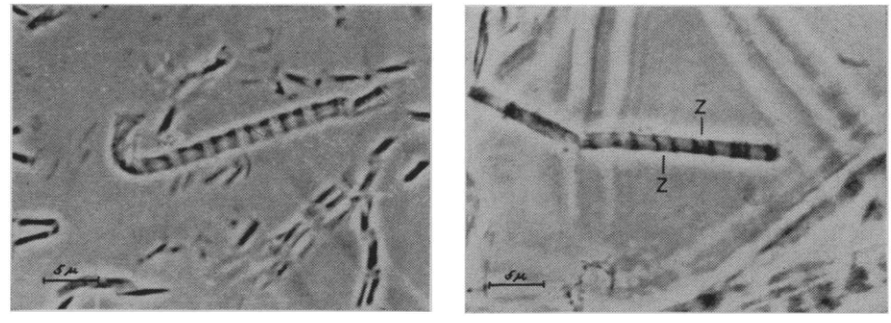

Fig. 3 (left). The length of the sarcomere has decreased to 3.3 μ, the width of the A-band has decreased to approximately 2.2 μ, and a thickening of the myofibril has occurred. Gross changes have occurred in the A-band, which is not in contact with the Z-disk. Fig. 4 (right). A further stage in the "separation" of the A-band and the movement of the two segments toward the Z-membrane. The length of the sarcomere is 3.3 μ, and the A-band width is 2 μ.

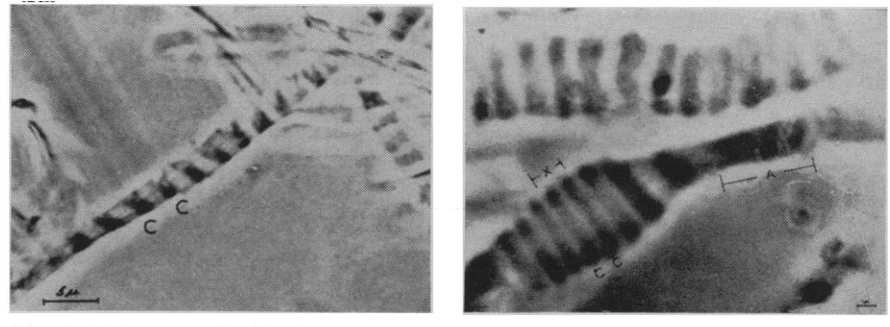

Fig. 5 (left). A myofibril is shown which is almost fully contracted. The dark area (*C*) appears to have been formed by "migration" of material from a portion of the A-band on one side of the Z-disk and a portion of the A-band on the other side of the same Z-disk. The length of the sarcomere is 3 μ. Fig. 6 (right). A single myofibril is shown with only one portion contracted. The approximate doubling in width of the myofibril which accompanies supercontraction is clearly seen. The distance between the edges of two contraction bands (*C*) is represented by (*X*). It is considerably smaller than the resting A-band width (*A*).

of A-band width are only approximate. The relative change in A-band width compared to changes in the sarcomere width provide better measurements for comparison.

A plot of sarcomere width versus A-band width (see Fig. 7) shows that during the initial period of shortening (down to 60 percent reference length) relatively little change takes place in the width of the A-band. It is only after this stage that changes in the A-band width become exceptionally pronounced. These changes have been observed in rabbit psoas myofibrils and are considered to be the first stages in the formation of contraction bands. It was previously implied (*5*, *6*), however, that the formation of contraction bands was due to a thickening of the A-bands as they pushed up against the Z-membrane. Figures 3 and 4 indicate that there is an apparent separation of the A-band before it is actually pressed up against the Z-membranes. This is in disagreement with the hypothesis that contraction-band formation is due to a pushing of the A-band material against the Z-membrane. It seems probable from these photomicrographs that an actual separation of the material in the A-band does occur.

Szent-Györgyi and Holtzer (*7*) located specific materials within the sarcomere during immunological studies. Using antibodies specific for light Meromyosin (LMM) and heavy Meromyosin (HMM), they were able to separate the A-band material into two groups. The LMM appeared to be located at the lateral borders of the A-band whereas the HMM was concentrated in the center (M-band). They also observed A-band separation due to what they called migration of LMM toward the Z-membranes. The banding resulting from this migration formed "doublets".

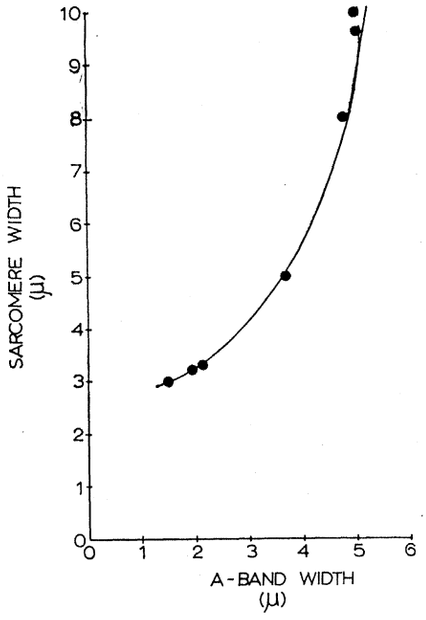

Fig. 7. The relationship between the length of the sarcomere and the width of the A-band in the barnacle myofibril. During the initial period of shortening (to approximately 60 percent of the reference length) relatively little change takes place in the A-band width.

Photographs by Hodge (*8*) support the hypothesis that A-band separation is a result of migration of the A-band material. The results reported here parallel exactly those reported by Hodge working with insect skeletal muscle.

The separation of the A-band into distinct areas of different materials provides a possible explanation for the origin of the observed contraction bands. Migration of the LMM region toward the Z-membranes would cause the observed A-band separation.

The problem of contraction in barnacle myofibrils has recently been considered by Hoyle and McAlear (*2*). They propose a situation whereby the A-filaments maintain a constant length. The observed changes, they maintain, are due to a "penetration" of the Z-disk by A-filaments. This is in disagreement with the findings presented here: Figs. 3, 4, and 5 show changes occurring in the A-band before it has come in contact with the Z-disk.

The separation of the A-band shown in Fig. 3 has been seen by a number of investigators in a variety of muscles (see *5*, *8*). The work of Hodge (*8*), in particular, shows that changes occur in the A-band when it is not in contact with the Z-disk.

If the contraction bands were formed through penetration of the Z-membranes by A-band filaments whose lengths did not change, then the minimum distance between the exterior edges of contraction bands (*X* in Fig. 6) would be equal to the A-band width (*A*). As shown by the supercontracted myofibril in Fig. 6, the distance is considerably less than the A-band width (*A*).

The results presented here support the hypothesis that muscular contraction depends upon the sliding of filaments, at least for contractions down to approximately 60 percent of the reference length. Contraction beyond 60 percent appears to be accompanied by a change in length of the A-filaments. These studies do not rule out the possibility of a shortened A-filament penetrating the Z-membrane.

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Cytochrome Content of Mitochondria Stripped of Inner Membrane Structure

Abstract. *The cytochrome composition of mitochondrial fractions which have been stripped of inner membrane subunits by exposure to high-frequency sound have been examined by low-temperature spectroscopy. The ratio of cytochrome* c *to cytochrome* a *is not changed by the treatment, but the concentration of cytochrome per milligram of protein is increased and the concentrations of cytochromes* c_1 *and* b *change slightly. These cytochromes* (c_1 *and* b) *may be at least partly located in the subunits of the inner membrane, but the idea that all the respiratory components are located in single subunits of the mitochondrial cristae may be considered to be disproved.*

Green and his co-workers propose as a plausible hypothesis that the inner membrane subunits of mitochondria (*1*, *2*) represent an integrated unit of all the components of the respiratory chain (*3*, *4*). However, some objections have been put forward, based upon what appear to be irreconcilible differences between the small size of the inner membrane subunits and the large size of the unit embracing the functions of electron transfer and oxi-