*RELATION BETWEEN ADENOSINETRIPHOSPHATE ACTIVITY AND SARCOMERE LENGTH IN STRETCHED GLYCEROL-EXTRACTED FROG SKELETAL MUSCLE**

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A now familiar feature of myofibrillar structure is the interdigitating array of thick and thin filaments first described by Hanson and Huxley.[1] Based on this structural feature, a model of muscular contraction has been proposed[2] which is briefly as follows. Contraction takes place by sliding of thin filaments (I filaments) toward the center of each sarcomere through interaction with thick filaments (A filaments) at projections on the latter termed "bridges." Adenosinetriphosphate (ATP) is hydrolyzed at the bridges, and the interaction of thin filaments at these sites accelerates the rate of hydrolysis. The thick filaments are composed of myosin and the thin filaments, at least partially, of actin. Huxley[3, 4] has summarized a number of lines of evidence which support this model of the contractile mechanism.

Experiments on isolated contractile proteins of skeletal muscle indicate that actin greatly enhances the magnesium-activated adenosine triphosphatase (ATPase) activity of myosin which has a pH optimum at pH 7.3.[5] We thought it would be of interest, in relation to the model of contraction proposed by Huxley, to examine the relationship between extent of overlap of thick and thin myofilaments and the rate of Mg^{++} activated ATP hydrolysis at pH 7.3. Glycerol-extracted skeletal muscle bundles were used, following the method of preparation introduced by Szent-Györgyi.[6] These bundles contract in the presence of ATP, and the amount of tension developed is approximately that developed by the intact muscle on excitation.[6] ATPase activity was assayed in bundles of approximately rest length and in ones stretched to various lengths beyond rest length. Sarcomere lengths in unfixed and uncontracted bundles were estimated by phase microscopy. The amount of myofilament overlap was gauged by electron microscopic examination of sections taken at random points along the length of representative bundles included in the study.

Materials and Methods.—Paired semitendinosus muscles of adult frogs (*Rana pipiens*) were dissected free of surrounding tissue in Tris Ringer's solution (Tris (base) 127 meq/1; HCl 114; KCl 2.5; Ca gluconate 1.8). Tris was used in this solution to serve as a buffer; furthermore, since Tris replaced sodium in the Ringer's solution, action potentials were eliminated during dissection, thereby avoiding contractions. The *in situ* length of each muscle while the limb was in the mid-extension, mid-flexion position was used as the "reference length." Both muscles were excised at their tendinous ends and tied onto hooked glass rods (see Fig. 1): one muscle was tied at 85–110% of reference length and its partner at 110–130% of reference length. By careful dissection, the diameter of each muscle was then reduced to 200 μ or less. The paired muscles were placed in 50% glycerol in water at 0° for 72 hr, then in a fresh sample of 50% glycerol in water for 2–5 weeks at −20°.[6]

Just prior to use, the length of each bundle was remeasured. After soaking for 1 hr at 0° in 15% glycerol, the bundle was mounted, still attached to the glass rod, in a 2.3-ml bath containing 100 mM Tris, 50 mM KCl, 5 mM $Mg\,Cl_2$, and 5 mM ATP at pH 7.3 and 25° (see Fig. 1). Mixing of the bath was achieved by magnetic stirrers. ATPase activity was estimated by measuring the rate of increase in the concentration of inorganic phosphate in the bath. One hundred μl aliquots

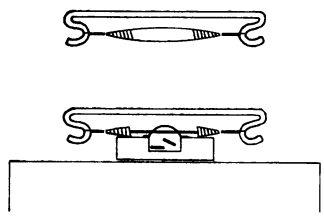


FIG. 1.—At top, a frog semitendinosus muscle tied on glass rod (shaded areas are tendonous ends). Below, muscle bundle, its diameter reduced to less than 200 μ by dissection, mounted in convex meniscus of 2.3 ml muscle bath in well of Teflon block. The bath contains two magnetic "fleas" and Teflon block stands on magnetic stirrer.

of the bath were taken before and 10 and 20 min after immersing the muscle bundle in the bath, and inorganic phosphate concentration was estimated using the phase separation method described by Martin and Doty.[9]

The bundles were assumed to be ellipsoidal in cross section, and the major and minor diameters were estimated using a microscopic eyepiece micrometer. From these measurements and that of the length of the portion of the bundle exposed to the bath, the volume of each bundle was derived. In 10 bundles, protein content of the segment exposed to the bath was assayed by dissolving it in 0.1 N NaOH at room temperature and measuring optical density at 295 mμ. A conversion factor was thus derived by which volume measurements obtained on all bundles were converted to milligrams of protein.

All bundles used were less than 200 μ in diameter, a diameter selected on the basis of considerations of the diffusion properties of ATP in glycerol-extracted skeletal muscle.[7] Calculations based on the Meyerhof-Schultz equation[8] also indicated that diffusion of ATP would be a limiting factor in the hydrolysis of ATP in bundles of diameter greater than 200 μ.

Ten bundles, at a variety of lengths from 90 to 130% of reference length, were examined under a phase microscope before exposure to ATP to obtain estimates of sarcomere length. Photomicrographs of at least 2 areas in each bundle were examined. The lengths of 10 sarcomeres in each micrograph were measured and mean values of length were obtained. Sarcomeres of a given bundle were quite uniform in length. These measurements showed a linear relation between length as per cent of reference length and sarcomere length, and this relation was used to derive mean sarcomere length of each of the bundles studied from reference length.

Eight pairs of muscle bundles were prepared for electron microscopic examination. While still tied on the glass rod, each was first fixed in 10% acrolein, at pH 7.4, for 45 min and then washed three times with 0.1 M phosphate buffer, pH 7.4. The middle portion of the bundle which had been exposed to the bath was cut into small blocks and fixed in 1% buffered osmic acid, pH 7.3, for 2 hr. The blocks were then washed with water, dehydrated in ethyl alcohol, and embedded in Epon. Sections obtained at random points along the length of each bundle were examined in an RCA electron microscope, model EMU 3G.

Results.—Sarcomere lengths measured by use of the electron microscope were quite variable in the shorter bundles (85–110% of reference length). In the longer bundles (110–130% of reference length), sarcomere lengths were more uniform and agreed closely with sarcomere lengths estimated by phase microscopy. In all instances, however, sarcomere lengths were less uniform in electron photomicrographs than in phase micrographs. This was true both in preparations exposed to ATP and in those which had not been so treated. Apparently, the process of fixation caused contraction and, as a result, in these bundles, in which over-all length was fixed, some sarcomeres shortened while others became stretched. Because of this heterogeneity following fixation, all estimates of sarcomere length, for comparison with enzymatic activity, were made on phase photomicrographs of uncontracted bundles.

In general, the rate of hydrolysis of ATP was considerably greater in unstretched bundles than in stretched ones. From data obtained on all bundles, a plot of ATPase activity (rate of liberation of inorganic phosphate (iP) per mg protein per minute) as a function of sarcomere length was constructed and this is shown in Figure 2. As the sarcomere length increased from 2.2 μ to about 4.2 μ, the ATPase activity decreased. At sarcomere lengths greater than 4.2 μ, the enzymatic activity may

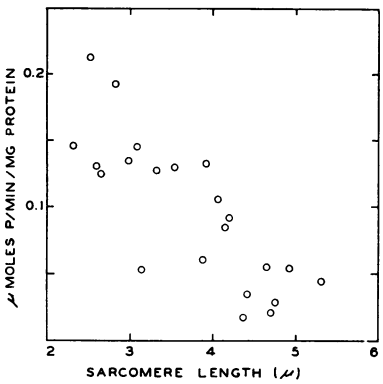


FIG. 2.—Adenosinetriphosphatase (ATPase) activity plotted as a function of mean sarcomere length in muscle bundles examined in these experiments.

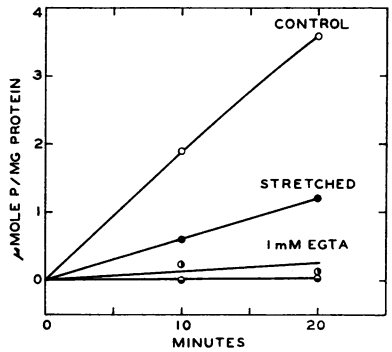


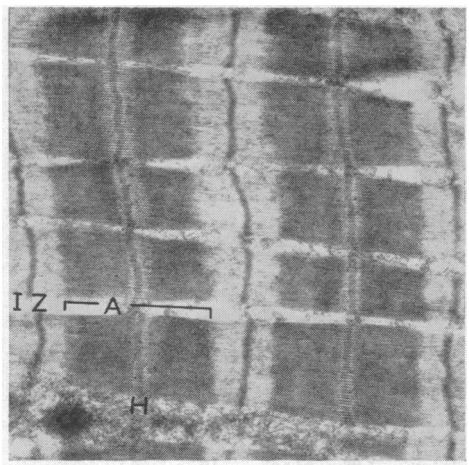
FIG. 3.—Effect of EGTA on ATPase activity of muscle bundles. Control bundle had mean sarcomere length of about 2.5 μ, and stretched one, mean length of about 5.0 μ. After addition of EGTA to bath to obtain a concentration of 2 mM: control bundle: ◑—◑; stretched bundle: ◒—◒.

have been independent of length, though the numbers of observations made were not enough to make this suggestion very secure.

In an additional experiment, the effect of addition of ethyleneglycol-bis-(amino-ethylether)-tetra-acetic acid (EGTA) to the bath was assessed. The rate of hydrolysis by a stretched and unstretched pair of muscle bundles was compared with that of a similar pair in which EGTA was included in the bath at a concentration of 2mM. The stretched bundles in this experiment were tied at 127 per cent of reference length (mean sarcomere length, 4.65 μ). Results of this experiment are illustrated in Figure 3. In the unstretched bundle (control) the rate of hydrolysis was reduced by EGTA from 0.19 μM to about 0.01 μM/min/mg protein. In the stretched bundles, the rate was reduced from approximately 0.06 μM/min/mg protein to about 0.

Electron micrographs of representative sections in bundles having mean sarcomere lengths of 2.5–5.5 μ (90–130% reference length) are shown in Figures 4, 5, 6, and 7. At a mean sarcomere length of about 2.5–3.0 μ (Fig. 4), considerable overlap of thick and thin filaments was noted. Overlap was almost gone at mean sarcomere lengths of 4.0–4.5 μ (Fig. 5), while at sarcomere lengths above 4.5 μ, scarcely any overlap was seen in all sections examined (see Figs. 6 and 7). A pronounced gap between thick and thin filaments was seen at these extreme lengths: a few scattered thin filaments bridged the gap (Fig. 7). It is perhaps worthy of note that in stretched myofibrils in which a distinct gap between thick and thin filaments is seen, the thin filaments maintain their linear orientation, as if some structure were holding them in place, side by side (Figs. 6 and 7).

Measurements of the lengths of filaments in our electron micrographs at sarcomere lengths of 2–3 μ gave values of 1.5–1.6 μ for thick filaments and 1.0 μ for thin filaments, measuring the latter from *Z*-line to edge of the *H*-zone. These lengths agree with those obtained by Page and Huxley.[10] At these filament lengths, overlap should be lost at sarcomere lengths above 3.6 μ. Our data, however, suggest that, in the bundles we studied, overlap was lost at sarcomere lengths greater than



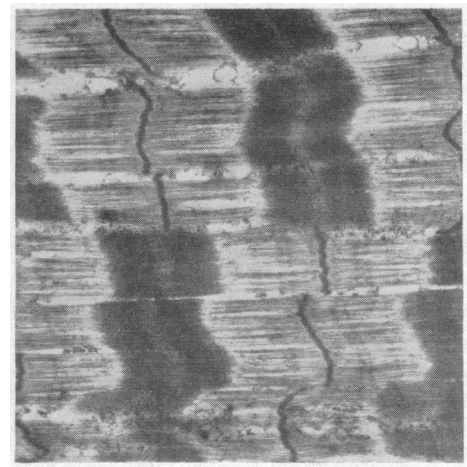
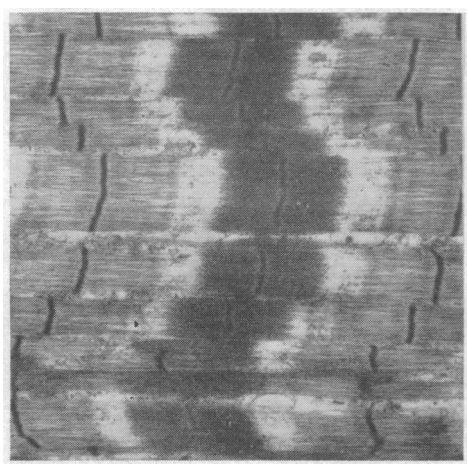


FIG. 4. (*left*).—Electron photomicrograph of glycerol-extracted frog semitendinosus muscle bundle, mean sarcomere length about 2.5 μ. Narrow *H* zone is seen and extensive overlap between thick and thin filaments. (Magnification: 13,500.)

FIG. 5. (*right*).—Mean sarcomere length of this bundle is 4.2 μ. Ends of *I* filaments appear to be at the *A–I* junction. Note region of decreased density in center of *A* bands. This is probably the portion of the thick filaments which is devoid of bridges and which does not vary with sarcomere length. It has been termed by some the *L* line. (Magnification: 9,500.)



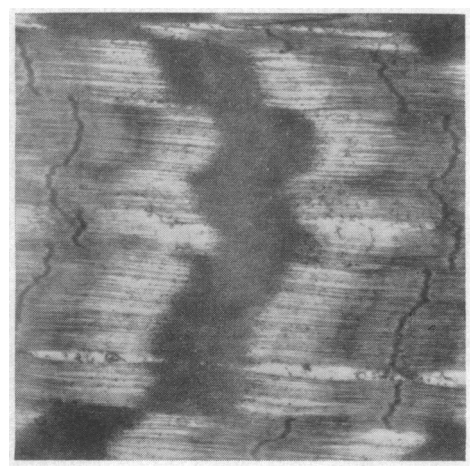


FIG. 6. (*left*).—Mean sarcomere length of this bundle is 4.7 μ. A distinct gap has appeared between thick and thin filaments and there is no overlap. This feature was seen in all sections from this bundle. (Magnification: 10,000.)

FIG. 7. (*right*).—Mean sarcomere length is 5.9 μ. The gap between thick and thin filaments is even more prominent than in Fig. 6. Some distortion of the bundle is apparent: *A* bands and *Z* lines are no longer neatly in register. Some scattered thin filaments, greatly stretched, appear to bridge the gap. (Magnification: 9,400.)

4.2 μ. Stretching of the *I* filaments when the length of the muscle bundle is extended may explain this discrepancy. Some of our electron micrographs suggest this explanation. For example, in Figure 4, the *A* filaments measure 1.6 μ in length, and the thin filaments 1.05 μ. In Figures 6 and 7, where a large gap between thick and thin filaments is apparent and the sarcomere lengths are about 4.7 and 5.9 μ, respectively, the thick filaments measure 1.5–1.6 μ, and the thin filaments, 1 μ in

length. At an intermediate length, however, a length at which the gap between *A* bands and *I* filaments is just beginning to appear (sarcomere length: 4.2 μ, Fig. 5), the thin filaments measure about 1.4 μ in length. It is possible that there is some stretching of thin filaments just before the overlap is gone and that at sarcomere lengths beyond this they retract again to their usual length of 1 μ. Page and Huxley,[10] on the other hand, found no change in length of *I* filaments up to sarcomere lengths of 3.8 μ.

Discussion.—These experiments indicate that in glycerol-extracted frog skeletal muscle ATPase activity decreases as the sarcomere length is increased beyond that at which maximum tension develops on contraction. Gordon, Huxley, and Julian[11] found that, in isolated fibers from frog semitendinosus muscle, maximum tension occurred at a sarcomere length of about 2.1–2.2 μ, and tension fell off steadily beyond this length.

The observed relation between length and ATPase activity of glycerol-extracted muscle bundles suggests that ATPase activity correlates with the amount of overlap between thick and thin myofilaments, as proposed by the sliding filament hypothesis.[2] The electron micrographs we have obtained at different lengths of bundle, representative examples of which are seen in Figures 4, 5, 6, and 7, support this contention. If ATP is hydrolyzed at cross-bridges between thick and thin filaments in the area of overlap and the number of such bridges is a function of the amount of overlap, then reducing the latter from a maximum value can be expected to result in a diminished rate of hydrolysis of ATP which is the result we have obtained.

It may be pertinent to point out that in these experiments stretch was applied to the fresh muscle bundles *before* glycerol-extraction. This was necessary because we were not able to stretch glycerol-extracted bundles without tearing them, even in the presence of ATP and EGTA. This circumstance may be due to increased cross-linking between thin and thick filaments in the glycerol-extracted bundles. Because streching was done on fresh muscle and essentially no further change in length took place during and after glycerol-extraction, our measurements of length and enzymatic activity may be compared with length-tension measurements in fresh muscle preparations.

If glycerol-extracted skeletal muscle of the frog has approximately the same composition as that of the rabbit, the myosin content may be taken as 33 per cent of total protein.[3] At a sarcomere length of roughly 2.2 μ, the rate of splitting of ATP is about 0.47 μM/min/mg myosin. The molecular weight of myosin has been found to be about 500,000.[12, 13] Using this value, we obtain a rate of 4 molecules of ATP hydrolyzed per second per molecule of myosin or per bridge, if each myosin molecule represents one completed bridge. This is a reasonable value and may be compared with that obtained by Carlson and Siger[14] of 2–3 molecules of ATP (or creatine phosphate) split per molecule of myosin in a single twitch. It must be acknowledged, however, that not all bridges may be complete: that at shorter sarcomere lengths, more bridges may be completed and a higher rate obtained. At average sarcomere lengths of 2.2 μ, on the other hand, one might reasonably expect a maximum number of bridges between thick and thin filaments on the basis of the following: the "bare" mid-portion of each thick filament is about 0.2 μ in length;[3] thus an overlap of 0.7 μ on each half of a thick filament should permit

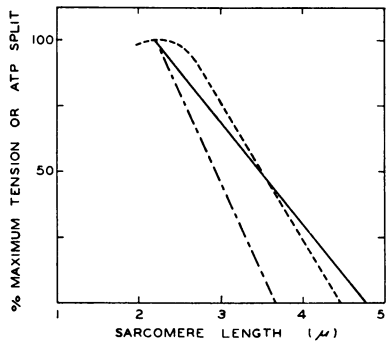


FIG. 8.—Developed tension and rate of splitting of ATP as functions of sarcomere length. Solid line is regression line of our measurements of rate of splitting of ATP; dotted line, measurements made of tension development of single frog muscle fibers by Ramsey and Street;[15] and dashed line, measurements made of tension development of single frog muscle fibers by Gordon *et al.*[11] (see text for detailed explanation).

completion of all bridges, making the *I*-bands 0.6 μ in width. The sarcomeres will average 2.2 μ in length under these circumstances.

We have attempted to compare our results with the length-tension measurements of Ramsey and Street[15] obtained on isolated frog semitendinosus muscle fibers. In doing so, we set the length for maximum tension at a sarcomere length of 2.2 μ. We arbitrarily set the maximum length for rate of ATP hydrolysis at this same value, though our data show no maximum. Furthermore, we assumed that the amount of enzymatic activity found at sarcomere lengths in excess of 4.2 μ is independent of length and thus probably not myofibrillar in origin.[16] Accordingly, we subtracted this activity (which had a mean value of 0.034 μM/min/mg protein) from the total rate of hydrolysis, in plotting a regression line of enzymatic activity versus sarcomere length. This plot is illustrated in Figure 8 and is compared with a plot of the length-tension measurements of Ramsey and Street. The length-tension curve obtained by Gordon *et al.*[11] is also included. These latter authors used an elegant technique[17] in which the tension measurements were confined to the central portion of the fiber where the sarcomere spacing is most uniform and the degree of stretch is greatest. Our measurements can be more readily compared with those of Ramsey and Street who measured tension developed by the whole length of intact single muscle fibers. It can be seen that the relationship between rate of ATP hydrolysis and length compares well with that of tension and length in the range of sarcomere length studied.

EGTA is a potent inhibitor of the pH 7.3 ATPase of actomyosin. At the concentration we used (2mM), its inhibitory effect is a consequence of its affinity for calcium ions. The suppression of ATPase by EGTA to about 5 per cent of the uninhibited rate (Fig. 3) is approximately the magnitude of effect to be expected for actomyosin ATPase.[18] Concerning the effect on stretched bundles, it should be noted that EGTA is also an inhibitor of the Mg^{++} activated ATPase of the sarcoplasmic reticulum.[19]

Infante, Klaupiks, and Davies[20] observed that in isolated fresh frog sartorius muscle treated with 1-fluoro-2,4-dinitrobenzene, which inhibits the rephosphorylation of ADP by creatine phosphate, the amount of ATP hydrolyzed in a series of contractions decreased as the length of the muscle increased beyond rest length. Our findings are in accord with these on whole muscle and indicate that the decreased rate of splitting of ATP at lengths beyond rest length can be explained by

decreased filament overlap and a linearly progressive reduction in the number of sites on the myofilaments at which ATP is hydrolyzed.

The enzymatic activity remaining in preparations stretched to the extent that overlap is eliminated may be nonmyofibrillar in origin.[16] On the other hand, since part of the breakdown of ATP[20] and phosphorylcreatine[21] in a series of muscle twitches appears to be independent of length, it has been suggested that a low rate of hydrolysis of ATP occurs at the bridges even in the absence of overlap.[21, 22] The histochemical experiments of Tice and Barnett,[23] however, indicate that ATPase activity is largely confined to the bridges between thick and thin filaments in the region of overlap.

Summary and Conclusions.—The rate of hydrolysis of ATP catalyzed by glycerol-extracted frog skeletal muscle bundles was found to decrease as the length of the bundles was increased beyond rest length. The decrease in activity was approximately a linear function of increased length as the sarcomere length increased from approximately 2.2 to 4.2 μ. Above 4.2–4.5 μ, ATPase activity was low and seemingly independent of further changes in length. Electron photomicrographs revealed that at sarcomere lengths above 4.2 μ, overlap between thick and thin myofilaments was lost. These findings are consistent with the sliding-filament model of contraction proposed by Hanson and Huxley.[1] They indicate that the rate of splitting of ATP, at pH 7.3, 5 mM Mg^{++}, is dependent on the amount of overlap between thick and thin myofilaments. As the length of the bundle is increased beyond rest length, the amount of overlap is reduced in a linear progression. Sites of ATP hydrolysis are spaced at regular intervals (at "cross-bridges") in the overlap region. Thus, progressive loss of overlap is accompanied *pari passu* by decreasing rate of ATP splitting as predicted by the sliding filament hypothesis of muscle contraction.[1]

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[16] Our electron micrographs suggest that the structural integrity of the sarcoplasmic reticulum is preserved in these glycerol-extracted muscle bundles (see Fig. 5). Since these structures also have ATPase which is magnesium-activated and has a calcium dependency, it may well be that

the residual activity in overstretched bundles is that of the sarcoplasmic reticulum.

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*THEORY OF THE FLOW OF ACTION CURRENTS IN ISOLATED MYELINATED NERVE FIBERS, II**

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We continue the presentation of the theory of the isolated fiber.[1]

Dual Effect of Anesthetics upon Isolated Nerve Fibers.—In the literature, the observation, originally made by Bishop,[2] that cocaine does not alter in appreciable manner the resting membrane potential of normal nerve fibers has been repeatedly confirmed as well as extended to many other "local" anesthetics. Such anesthetics, however, when acting upon isolated fibers, exert, in addition to the primary anesthetizing action, a secondary depolarizing action.

An anesthetic applied to the central part of an internode reduces the ability of the metabolic mechanisms to supply the energy which is required to maintain sufficient flows of demarcation currents into the P and J segments (ref. 1*a*, Fig. 3). Consequently, the membrane potential of the P and J segments decreases. Occasionally this effect causes a slight, temporary decrease of the stimulation threshold of the P segment, but ordinarily cathodal depression sets in and the threshold of the P segment increases. On the other hand, if the anesthetic is applied to P or J segments of internode, interference with those electrochemical processes which underlie repolarization by the inward (anodal) flow of the electrical current[3] results in a further depolarization of the treated J and/or P segments.

If the treated J and P segments are located in the distal pool, the secondary action of the anesthetic rarely causes irreversible depolarization because, since r_e has a negligible value, the demarcation current remains large enough to keep the membrane potential of the J and P segments at values which still are sufficient to prevent the onset of irreversible deterioration. If the treated P and/or J segments are located in the central pool, the high value of r_e in the vaseline gaps, by limiting the flow of demarcation current, enhances the secondary action of the anesthetic. If only a P segment is present in the central pool, depending upon the state of the isolated fiber, the depolarization may be reversible and recovery takes place after the anesthetic has been removed (Fig. 1, *17–24*), or the depolarization may be so severe that within a few minutes the entire isolated segment becomes irreversibly depolarized. In the three experiments in which, with node N_1 and consequently also J segments in the central pool, 20 mM xylocaine was introduced in this pool,