The Relation Between the Late After-Potential and the Size of the Transverse Tubular System of Frog Muscle

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ABSTRACT This is an investigation of the effects on the late after-potential of immersing frog sartorius muscles in three kinds of modified Ringer's fluid; hypertonic, low chloride, and potassium-free. The late after-potential has been attributed to the depolarizing effect of an accumulation of potassium, during a preceding train of impulses, in the intermediary space of the model of a muscle fiber proposed by Adrian and Freygang. Both the hypertonic and low chloride solutions prolonged the late after-potential reversibly and the potassium-free solution shortened it. The effect of the low potassium solution fitted those data calculated from the model, but the effect of the hypertonic and low chloride solutions required an increase in size of the intermediary space of the model in order to fit the calculated data. An electron microscopic study of the muscles showed that the size of the transverse tubular system changed reversibly in the hypertonic and low chloride solutions in almost the amount necessary to fit the experimental data to the calculated data. This agreement between the change in size of the transverse tubular system and that of the intermediary space indicates that the intermediary space may be the transverse tubular system.

INTRODUCTION

A negative (depolarizing) after-potential that follows a train of impulses in frog muscle fibers was the subject of an earlier communication (Freygang, Goldstein, and Hellam, 1964). This after-potential was called the *late after-potential* in order to distinguish it from the after-potential that follows a single impulse. Freygang *et al.* explained the presence of the late after-potential by postulating that the potassium concentration in the intermediary space of the model of Adrian and Freygang (1962 *a*, *b*) was raised by the train of impulses and had a depolarizing effect. In the experiments to be described here, the

time course of the late after-potential was changed by certain alterations of the fluid in which the muscles were immersed. The empirical equations of Adrian and Freygang (1962 *a*), which describe their model, did not predict all the changes in the late after-potential that were found, unless it was assumed that there was a change in the size of the intermediary space. A change in size of the sort required was found in the transverse tubular system (T system). These changes in size were reversed when the muscles were reimmersed in Ringer's fluid, as were the changes in the late after-potentials.

We suggest that the reader refer to our earlier communication on the late after-potential before proceeding here because our notation and assumptions are defined there. In brief, the model of Adrian and Freygang consists of a three compartment system. The compartments are (1) the external fluid, (2) an intermediary space located between the sarcoplasm and the external fluid, and (3) the sarcoplasm. Ionic currents may flow between any two of the three compartments. Chloride can flow between compartments 1 and 3, potassium between 2 and 3, and both sodium and potassium between 1 and 2. The relation between the ionic currents that flow between the compartments and the potential difference between the compartments are described by constant field equations with fixed permeability coefficients, except for transfer between compartments 2 and 3 where the permeability to potassium varies with the potential difference in such a way as to have the properties generally referred to as anomalous rectification. The barrier between compartments 1 and 2 is twenty times more permeable to potassium than it is to sodium. Movement of anions between compartments 1 and 2 and between 2 and 3 is slight in comparison to the passage of cations during the time course of the transients that are calculated from the model. Therefore, the volume of compartment 2 is assumed not to change during the calculated transients. The membrane potential which is measured experimentally corresponds to the potential difference between compartments 1 and 3, or V_{13}. Transients in this potential difference, except for action potentials which are generated across the sarcolemma, reflect primarily the change in the potential difference between compartments 2 and 3, which in turn is dependent upon the potassium concentration in compartment 2. Therefore the effectiveness in changing V_{13} of a net flow of potassium in or out of compartment 2 will depend directly upon the volume of compartment 2.

A preliminary account of part of this work has appeared (Hellam, Goldstein, Peachey, and Freygang, 1963).

METHODS

These experiments were performed on frog sartorius muscles (*Rana pipiens*) by the same methods as those described previously by Freygang *et al.* (1964). The composition of

the solutions in which the muscles were immersed is listed in Table I. All the solutions had the same ionic strength as Ringer's fluid.

The calculations were done in the same way as those of Freygang *et al.* (1964). The parameters that were varied to simulate the conditions of muscle fibers in the various solutions are listed in Table II. The changes of v (the volume of the intermediary space per unit area of surface membrane) that occur in the calculations which relate to some

TABLE I

COMPOSITION OF SOLUTIONS*

Solution	K^+	Na^+	Ca^{2+}	Cl^-	SO_4^{2-}	HPO_4^{2-}	$H_2PO_4^-$	Sucrose	Relative tonicity
Ringer's, *mM*	2.5	120	1.8	121	—	2.15	0.85	—	1
Hypertonic, *mM*	2.5	120	1.8	121	—	2.15	0.85	232	2
K-free, *mM*	—	122.5	1.8	121	—	2.15	0.85	—	1
Low Cl, *mM*	2.5	90	6.5	30	36	1.32	0.53	85	1

* All solutions contained 10^{-5} M *d*-tubocurarine chloride.

TABLE II

CONCENTRATIONS USED FOR CALCULATIONS

Solution	$[K_1^+]$	$[Na_1^+]$	$[Cl_1^-]$	$[K_3^+]$	$[Cl_3^-]$	$[A_2^-]$
Ringer's, *mM*	2.5	117.5	120	137	2.9	120
Hypertonic, *mM*	2.5	117.5	120	218	4.0	120
K-free, *mM*	—	120	120	137	2.9	120
Low Cl, *mM*	2.5	90.5	30	137	0.75	93*

* With respect to the calculation of the late after-potential in the low chloride solution, it should be noted that the concentration of anions in the intermediary space was reduced from 120 to 93 mM. This implies that the junction between the intermediary space and the external solution is sufficiently permeable to anions to allow their redistribution in a period of one-half hour or less. It is still assumed in the calculation, however, that any redistribution of anions during the time of the late after-potential is negligible.

of the solutions are described later. All the other parameters had the same values that they had in the previous calculations. In the calculations related to this work P_K was a function of V_{23} alone, except in the Appendix where P_K was taken as a function of the driving force acting on potassium.

For electron microscopy, muscles were removed from the experimental solutions while held at the length used in the experiments and quickly immersed in cold (0 to 4°C) fixative consisting of 1 per cent (w/v) osmium tetroxide in 0.1 M sodium phosphate buffer at pH 7.1 to 7.3 (osmolality = 250 to 260 mOsM/kg water). In some cases the muscles used for the electrical measurements were fixed for electron microscopy; in other cases the experimental conditions were duplicated but without inserting any electrodes, and then the muscles were fixed. After approximately 15 minutes of fixation

a piece about half as wide and half as long as the whole muscle was cut from the proximal end of the muscle and placed in fresh, cold fixative for an additional hour. These pieces were taken from regions of the muscle not used for electrical recording, when records had been taken, in order to avoid any fibers that had been injured by electrode penetration.

Dehydration was in graded ethanol/water mixtures, followed by two changes of propylene oxide, after which small pieces of muscle were put into a 1:1 mixture of propylene oxide and an epoxy mixture consisting of epon 812, 50 parts, dodecenyl succinic anhydride 10 parts, nadic methyl anhydride 38 parts, and DMP-30 (Rohm and Haas) 2 parts. Jars containing the tissue samples in small amounts of this mixture were left open overnight at room temperature to evaporate the propylene oxide, and the muscle pieces were embedded the next morning in fresh epon mixture in open gelatin capsules at 60°C.

Longitudinal sections were cut on Huxley/Cambridge or Porter-Blum/Sorvall microtomes using glass or diamond knives. The cutting direction was kept perpendicular to the muscle fiber axis to prevent compression distortion in the longitudinal direction. Sections were stained by immersion for 3 minutes in Reynold's lead citrate (1963), and electron micrographs were taken at a magnification of 11,300 in an RCA EMU-3F electron microscope with a 25 micron lower focal plane objective aperture and an accelerating voltage of 58 kv. Selection of fields for recording was made on the basis of visibility of triadic elements.

RESULTS

The late after-potential, postulated by Freygang *et al.* (1964) to result from the accumulation of potassium in an intermediary space of the muscle fiber, is considered to decay as a consequence of a replacement of potassium by sodium in that intermediary space. The expected time course of the decay can be calculated for the muscles in each solution from the equations which describe the model, and the results of such calculations are illustrated in Fig. 1. In terms of the model, the primary effect of the hypertonic solution is to raise the intracellular potassium concentration to 218 mM. The calculated late after-potential for fibers in the hypertonic solution is not very different from that calculated for those in Ringer's fluid. More striking are the effects of low external chloride ($[Cl_1] = 30$ mM) and removal of the external potassium ($[K_1] = 0$), both of which increased the expected rate of decay of the late after-potential.

Effect of the Hypertonic Solution

Fig. 2 illustrates the effect on the late after-potential of immersing a muscle in the hypertonic solution. The lower traces were recorded at a faster sweep speed and lower gain than were the upper traces. All the lower traces were recorded with the same gain and sweep speed. The late after-potential appears in the upper traces. Part B of this figure illustrates the almost fivefold

prolongation of the late after-potential that occurred in the hypertonic solution in comparison with its duration in Ringer's fluid (part A). The upper trace of part B is at five times slower sweep speed than the upper traces of parts A and C. Such an effect is not predicted by the calculated result illus-

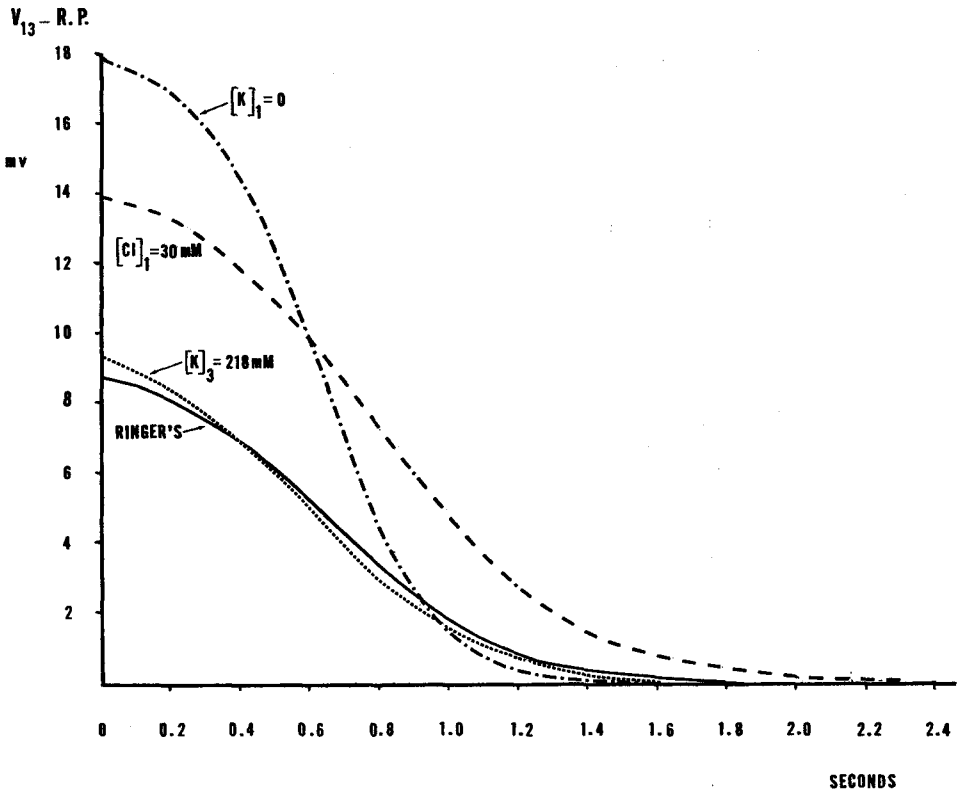


FIGURE 1. The late after-potentials simulated by the theoretical model of Adrian and Freygang (1962 *a*, *b*) and calculated in the manner which has been described by Freygang *et al.* (1964). The ordinate is the change in membrane potential, V_{13}, from the resting potential. In all these calculations the volume of the intermediary space per unit surface area, v, was 3.5×10^{-6} cm^3/cm^2. The curve that is labeled $[K_3] = 218$ mM refers to conditions in the hypertonic solution, the one labeled $[Cl_1] = 30$ mM refers to conditions in the low chloride solution, and the one labeled $[K_1] = 0$ refers to conditions in the potassium-free solution.

trated in Fig. 1. Part C illustrates that the prolongation was not maintained after the muscle was returned to Ringer's fluid. The prolongation of the late after-potential did not appear in its full amount immediately after the muscle was immersed in the hypertonic solution, nor was the shortening of the late after-potential completed immediately after the muscle was returned to Ringer's fluid. About a half-hour was required for these changes to approach completion. Except for the change in duration, the shape of the late after-

potential was much the same in the hypertonic solution as it was in Ringer's fluid.

Table III is a summary of the experiments on muscles in hypertonic solutions. The significant change in late after-potentials with change of solution occurs in the half-times, the time to fall from 6 to 3 mv measured from the resting potential at the end of the late after-potential. It might be expected that the initial resting potentials in Table III would be somewhat higher in muscle

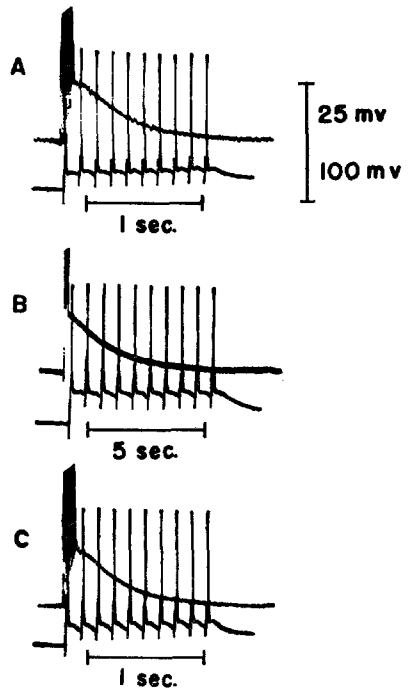


FIGURE 2. The change in the late after-potential in the hypertonic solution. The recordings are from different fibers of the same muscle (fibers 4, 7, and 9 of muscle h, Table III). The lower traces show the train of ten impulses that were separated by 10 msec. between each impulse. The lower traces were recorded simultaneously with the upper traces but at a lower gain. The time scale refers to the upper trace of each part of this figure. Part A, in Ringer's fluid. Part B, in the hypertonic solution. Part C, returned to Ringer's fluid.

fibers in the hypertonic solution because of their higher internal potassium concentration. These are the membrane potentials before the stimuli were applied, however, and not those measured immediately after impalement. It is possible that resting potentials were higher generally, but we chose fibers that twitched alone (without excitation of neighboring fibers) and that therefore would be expected to have resting potentials nearer to the threshold for contraction than the neighboring fibers. Fibers with higher resting potentials did not twitch alone and recordings from these fibers were distorted by movement artifacts.

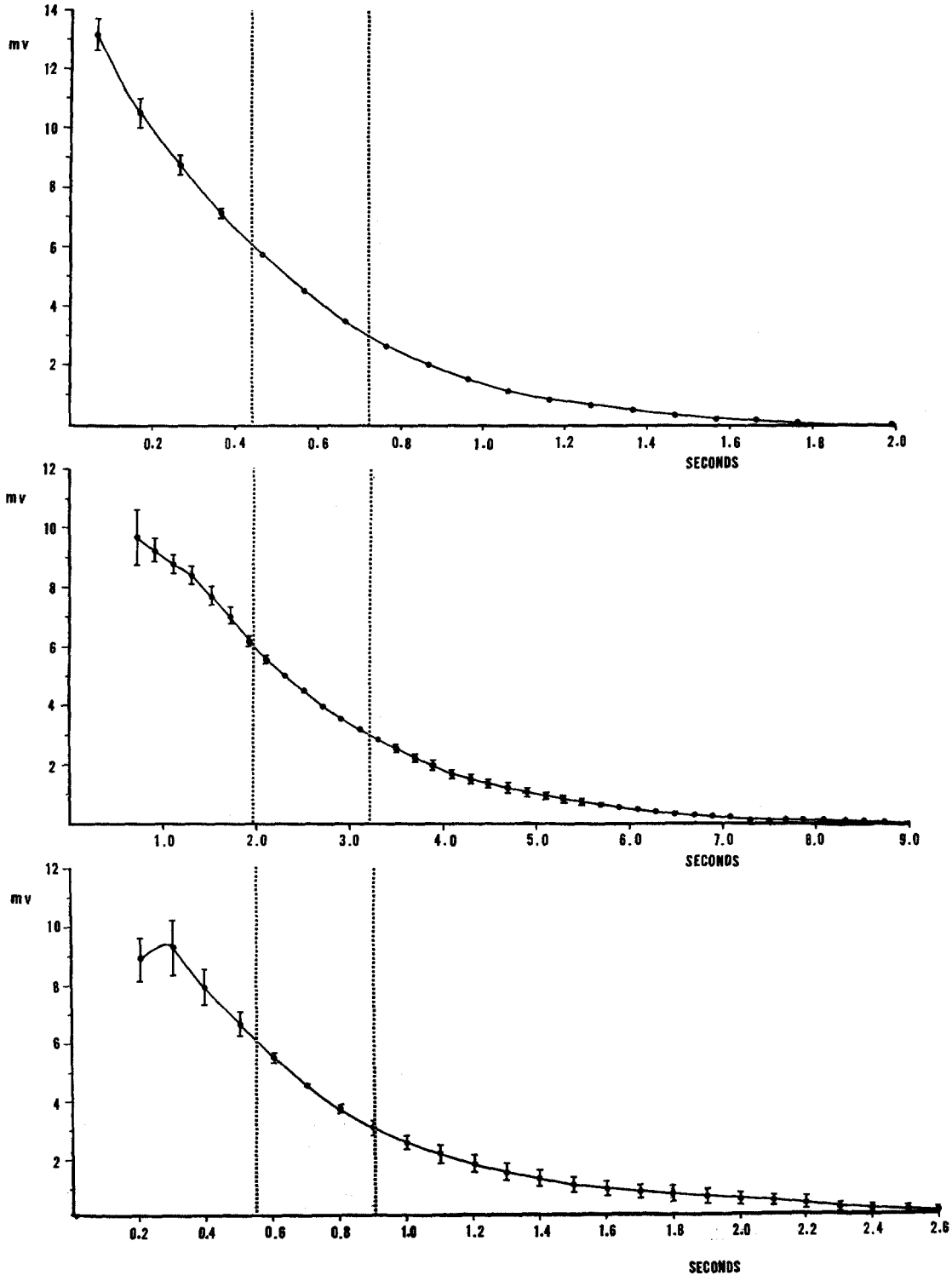
Plotted in Fig. 3 are the means, and standard errors of the means, of measurements of the late after-potentials that were recorded from muscles in Ringer's fluid, in the hypertonic solution, and after being returned to Ringer's fluid. In each graph the time scale has been adjusted so that the half-time of all the

TABLE III

LATE AFTER-POTENTIALS OF FIBERS IN HYPERTONIC SOLUTION

Muscle reference	Fiber No.	Initial resting potential	Loss of resting potential during train	No. of impulses	Peak amplitude*	Time to fall from 6 to 3 mv	Solution
		mv	*mv*		*mv*	*msec.*	
g	1	−82	0.47	11	15.9	280	Ringer's
	2	−87	0.33	10	14.7	275	
	3	−90	0.37	10	15.0	240	
	4	−86	0.51	10	18.2	280	
h	1	−83	0.30	11	11.7	260	
	2	−86	0.50	10	12.3	260	
	3	−82	0.71	9	9.8	270	
	4	−88	0.01	10	13.0	240	
i	1	−82	0.69	10	8.3	325	
	2	−82	0.57	9	11.9	400	
	3	−83	0.84	10	8.6	295	
				Mean ±SE =	12.7±0.9	284±14	
g	5	−86	0.60	10	8.3	1820	Hypertonic
	6	−85	0.86	10	8.2	1460	
	7	−84	0.47	9	10.1	1150	
	8	−82	0.85	9	7.6	2080	
h	5	−87	0.43	11	11.0	880	
	6	−80	0.69	10	8.6	1500	
	7	−97	0.87	11	14.2	1260	
i	4	−83	0.65	10	9.8	1240	
	5	−89	0.16	9	9.5	1160	
	6	−85	0.30	10	10.7	1120	
	7	−84	0.99	11	11.5	960	
	8	−84	0.53	10	9.2	1400	
	9	−90	0.42	9	10.6	1400	
				Mean ±SE =	10.0±0.5	1341±90	
h	8	−83	0.60	10	11.5	385	Returned to Ringer's
	9	−87	0.46	10	12.5	235	
	10	−92	1.00	10	15.7	275	
i	10	−85	0.50	10	10.0	625	
	11	−81	1.00	10	10.0	540	
				Mean ±SE =	11.9±1.1	412±75	

* Measurements of the peak amplitude of the late after-potential were made at the inflection point at which one might suppose that the early after-potential had decayed sufficiently to reveal the late after-potential. Since this point is not clearly defined because it depends in large part on the membrane time constant, the significance of changes in this measurement is doubtful. If significance is attached to the failure to find the decrease in peak amplitude that might be anticipated if v were to increase in muscles immersed in one of the experimental solutions, the failure would be difficult to interpret because an increase in v implies an increase in A_{23} and therefore an increase in P_K. As can be seen in Fig. 11 of Freygang *et al.* (1964), a concentration $[K_2]$ will have a greater depolarizing effect as P_K is raised.


mv
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SECONDS

curves occupies the same distance on the abscissa. It may be noted that the mean curves are very similar in shape when they are plotted in this fashion.

Before calculating the late after-potential that would be predicted by the model for the conditions of a muscle fiber in the hypertonic solution, it was necessary to estimate the internal potassium concentration, $[K_3]$, of muscle fibers in this solution. This was done in two ways. The first method was by direct chemical analysis. The assumption was made here that the percentage of extracellular space was the same in the hypertonic solution as in Ringer's fluid (Dydyńska and Wilkie, 1963). The second method was to photograph a single fiber while it was immersed in the solutions. The change in volume was calculated from the change in diameter by assuming a circular cross-section. This method showed that the reduction in diameter observed in fibers in the hypertonic solution was reversible when they were returned to Ringer's fluid. It was also assumed that the amount of potassium in a fiber was not changed significantly by immersing it in the hypertonic solution. Both methods indicated that $[K_3]$ is about 1.6 times greater in fibers in the hypertonic solution than in those in Ringer's fluid. Therefore a value of 218 mM (1.6 × 137 mM, Table II) was chosen for $[K_3]$ for fibers in the hypertonic solution. An internal chloride concentration $[Cl_3]$ of 4.0 mM was chosen so that the resting potential would be −96 mv in the "steady state" (Conway, 1957).

The solid line in Fig. 4 is the calculated change in membrane potential, V_{13}, and the points are the mean experimental data that also appear in the middle graph in Fig. 3. The major difference between the experimental and calculated results is the time scale, the experimental one being five times slower than the calculated one. The deviation between experimental and calculated data for depolarizations greater than 7 mv has been discussed by Freygang *et al.* (1964).

The resting potentials which preceded the trains of impulses were usually lower than the calculated resting potentials for all the conditions listed in Table II. The calculated resting potential is determined primarily by the concentration of chloride in the sarcoplasm $[Cl_3]$. We chose values that gave resting potentials closer to those of fibers that would have been difficult to

FIGURE 3. The mean late after-potentials of the muscle fibers in Ringer's fluid before being immersed in the hypertonic solution (top part), of fibers in the hypertonic solution (middle part), and after being returned to Ringer's fluid (bottom part). These data are from the fibers listed in Table III. The amplitude of each late after-potential was measured from the resting potential that remained after the late after-potential had subsided. The graphs are arranged so that each mean depolarization of 4.5 mv is at the same distance from the origin along the abscissa. The dotted vertical lines indicate the half-times, the time to fall from 6 to 3 mv. The time scale of each graph has been adjusted so that each half-time occupies the same distance along the abscissa. Each late after-potential was measured at 100 msec. intervals before and after the time at which it had an amplitude of 4.5 mv. Twice the standard error of the means (*i.e.*, ±) of these measurements is indicated by the vertical bars. Standard errors less than 0.1 mv are not indicated.

stimulate without excitation of neighboring fibers, as discussed above. It can be seen in Fig. 9 of the earlier paper on the late after-potential (Freygang *et al.*, 1964) that the form of the calculated late after-potential is not particularly sensitive to changes in $[Cl_3]$. Also, in Tables III to V of this paper the half-time of the late after-potential does not appear to be related to the membrane po-

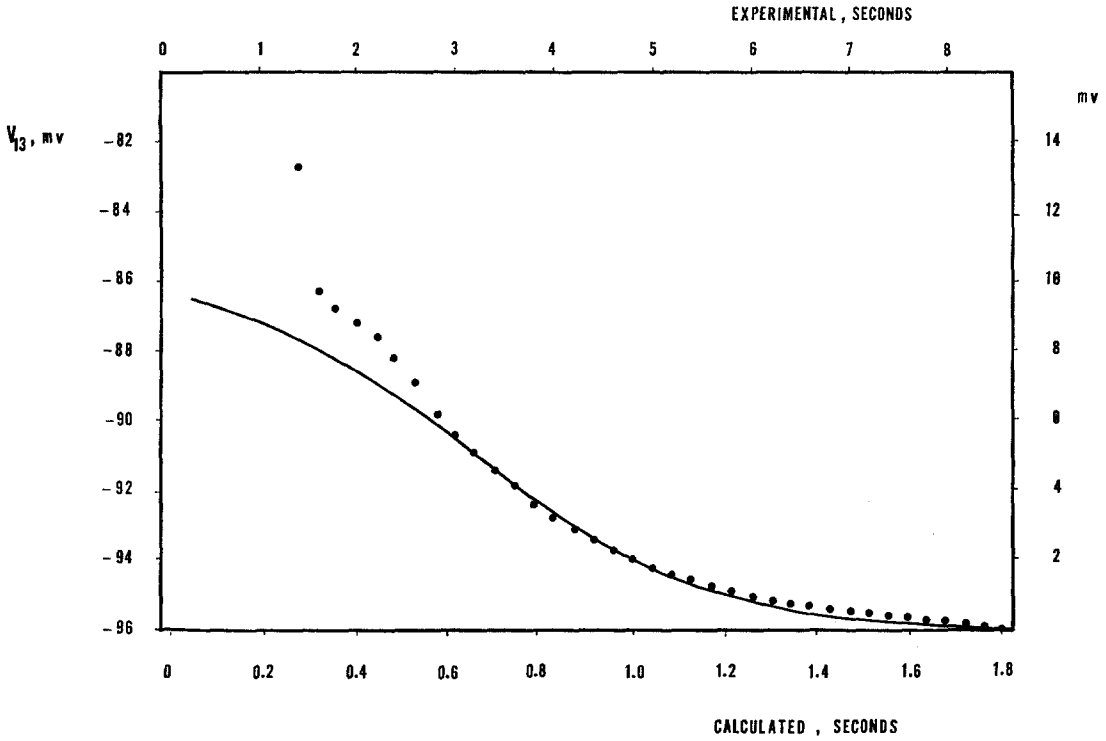


FIGURE 4. Comparison between mean experimental data (points) and the calculated late after-potential for fibers in the hypertonic solution. Each set of data has its own time scale. V_{13} is the calculated membrane potential. The experimental data are the same as those in the middle part of Fig. 3 and the scale for these data is marked on the ordinate on the right. The calculated data are the same as those in Fig. 1.

tential that preceded the train of impulses. This was true for fibers with resting potentials greater than −80 mv.

When the volume per unit surface area of the intermediary space of the model is enlarged, the effect on the calculated result is to expand the time scale of the calculated late after-potential (equation 7 of Freygang *et al.*, 1964) because the value of v is directly proportional to time. Therefore, using electron microscopy, a search for a reversibly expanding intracellular compartment was made. Figs. 5 and 6 show the normal appearance in the electron microscope of longitudinal sections of the frog's sartorius. The sarcoplasmic

TABLE IV

LATE AFTER-POTENTIALS OF FIBERS IN LOW CHLORIDE SOLUTION

Muscle reference	Fiber No.	Initial resting potential	Loss of resting potential during train	No. of impulses	Peak amplitude	Time to fall from 6 to 3 mv	Solution
		mv	*mv*		*mv*	*msec.*	
d	1	−84	0.30	10	10.4	315	Ringer's
	2	−85	1.00	11	11.9	295	
	3	−104	0.90	11	12.7	350	
	4	−81	0.60	10	12.5	235	
l	1	−83	1.00	10	8.2	310	
m	1	−83	0	9	7.8	390	
	2	−82	0	11	12.8	285	
	3	−80	0.50	10	9.4	305	
	4	−86	0.35	9	11.8	265	
	5	−88	1.00	10	13.6	310	
				Mean ± SE	11.1±0.7	306±14	
d	5	−86	0.45	9	13.7	345	$Low[Cl]_i$
	6	−90	0.80	8	10.7	565	
	7	−83	0.55	10	13.1	390	
	8	−82	0.10	10	9.4	410	
	9	−88	1.00	10	14.1	550	
m	2	−80	0	9	10.8	600	
	3	−80	−0.50	10	10.3	400	
	4	−84	1.00	9	9.0	600	
	5	−86	0.43	11	6.6	550	
	6	−86	0.20	10	9.2	320	
	7	−81	0.50	9	10.5	480	
	8	−85	−0.50	9	10.2	520	
				Mean ± SE	10.6±0.6	478±29	
l	2	−86	0.35	10	10.2	285	Returned to Ringer's
	3	−87	0.05	10	10.1	265	
				Mean	10.2	275	

reticulum occupies the spaces between the myofibrils, along with clusters of large, densely stained glycogen granules (*gly*). Smaller, less dense granules (*gr*) are found within the I bands of the myofibrils. In searching for the morphological identity of the intermediary space of the model, we concentrated our attention on the sarcoplasmic reticulum and especially on the triads. These structures lie in the region of the Z lines of the myofibrils and consist of pairs of terminal cisternae (*tc*) and central transverse tubules (*tt*); see Figs. 5 and 6.

Figs. 7 to 9 are typical electron micrographs from muscles that were fixed following immersion, respectively, in Ringer's fluid, in the hypertonic solution,

TABLE V

LATE AFTER-POTENTIALS OF FIBERS IN POTASSIUM-FREE SOLUTION

Muscle reference	Fiber No.	Initial resting potential	Loss of resting potential during train	No. of impulses	Peak amplitude	Time to fall from 6 to 3 mv	Solution
		mv	*mv*		*mv*	*msec.*	
j	1	−92	0.85	9	10.5	365	Ringer's
	2	−92	0.60	10	10.5	470	
	3	−88	0.85	9	9.0	470	
	4	−84	0.80	9	8.5	335	
k	1	−83	0.95	10	13.2	420	
	2	−84	0.75	8	12.0	400	
	3	−82	0	10	12.3	340	
	4	−87	0.40	10	10.7	400	
				Mean ± SE	10.8±0.6	400±19	
j	5	−104	0	10	6.8	245	K-free
	6	−94	0.50	10	6.8	245	
k	5	−82	0.20	10	9.4	200	
	6	−97	0.57	10	7.9	105	
	7	−88	0	9	6.4	90	
				Mean ± SE	7.5±0.5	177±33	
k	8	−85	0	9	9.6	360	Returned to Ringer's
	9	−86	0.70	10	7.9	230	
	10	−83	0	9	9.3	225	
	11	−87	0.05	10	7.2	330	
	12	−94	0.45	10	11.5	345	
	13	−93	0.15	10	10.5	305	
				Mean ± SE	9.3±0.7	299±24	

and in Ringer's fluid after having been immersed in the hypertonic solution. It can be seen in these micrographs that the T system swelled in the hypertonic solution, and returned to its usual size when the muscle was reimmersed in Ringer's fluid. An irregular swelling of the terminal cisternae in the hypertonic solution was also observed in some muscles, and this swelling was not always reversed when the muscles were returned to Ringer's fluid. Thus in these experiments it was the T system that changed size in the way predicted from the model.

In order to estimate the change in size of the T system, we measured the longitudinal dimension of the tubules; *i.e.*, the dimension along the axis of the muscle fiber (see arrows, Fig. 6). Assuming that the width of the tubules, *i.e.*, the dimension perpendicular to the axis of the fiber, remains constant, the

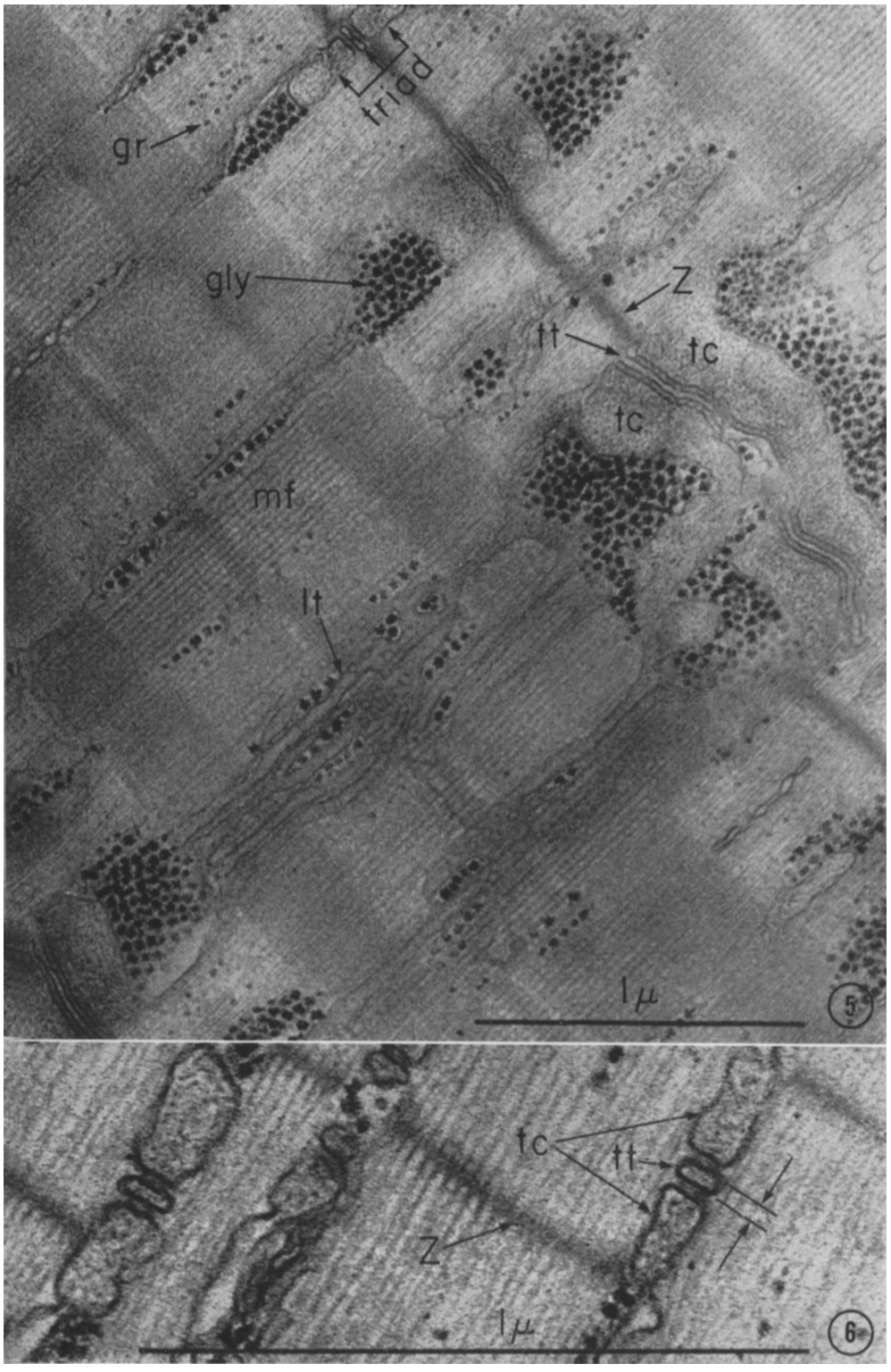
dimension we measure is directly proportional to tubular volume. This assumption seems to be reasonable since we observed little lateral swelling of the tubules, presumably because they are restrained from swelling laterally by the myofibrils.

The results of the measurements are summarized in Table VI. It can be seen in this table that the size of the T system in the hypertonic solution was 3.8 times larger than it was in Ringer's fluid or after return from the hypertonic solution to Ringer's fluid, while the latter two were not different from each other. Another finding was a large increase in the variance of the measurements on muscles in the hypertonic solution, reflecting an irregularity of the swelling. This irregularity, which may be seen in Fig. 8, did not represent differences between fibers but rather was an irregularity of swelling within each element of the T system of each fiber.

If the changes in fiber volume that occurred during fixation and dehydration were appreciably different for fibers fixed after being in Ringer's fluid in contrast to those fixed after immersion in the hypertonic solution, comparison of data for the two cases would be meaningless. To examine this possibility the diameters of isolated fibers that had been in either Ringer's fluid or the hypertonic solution were measured while they were being fixed in osmium tetroxide fixative and dehydrated in a series of ethanol solutions. Measurements of diameter were made at a fixed point along the fiber while the fiber was held at constant length in such a way as to minimize rotation of the fiber about its long axis. In four fibers fixed and dehydrated after being in Ringer's fluid, diameters decreased by 6, 19, 11, and 13 per cent (mean 12 per cent). The corresponding decreases in volume were approximately 11, 35, 21, and 25 per cent (mean 23 per cent) from the living state to the fixed-dehydrated state in absolute ethanol. Four fibers immersed in the hypertonic solution, similarly fixed and dehydrated, had decreases in diameter of 20, 9, 20, and 14 per cent (mean 16 per cent), the corresponding decreases in volume being approximately 36, 17, 35, and 26 per cent (mean 29 per cent). The mean volume changes for the two groups of fibers differ by only 6 per cent, indicating that similar size changes occur during fixation and dehydration and that measurements for fibers in the two cases were comparable. If the T system shrank laterally during preparation for electron microscopy in proportion to the decrease in fiber diameter, then the ratio of volumes of the T system as measured in fibers that had been in the hypertonic solution as compared to those that had been in Ringer's fluid would be the same in fixed, dehydrated fibers as it had been before fixation and dehydration.

Effect of the Low Chloride Solution

Fig. 10 illustrates the effect on the late after-potential of varying the duration of the train of impulses in the low chloride solution. After the longer trains the amplitude of the late after-potential was increased, but the time course of its decline was not changed. This constancy of time course was also found in muscle fibers that were immersed in Ringer's fluid, as can be seen in Fig. 1 of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Freygang *et al.* (1964). That figure was obtained from another fiber of the same muscle as the one from which Fig. 10 of this paper was obtained.

The half-time of the late after-potential was about 1.6 times longer in the low chloride solution than it was for muscles in Ringer's fluid, as may be seen in Table IV. Means of the measurements of the late after-potentials, as well as their standard errors, are plotted in Fig. 11, for the muscles first in Ringer's fluid, then in the low chloride solution, and finally on being returned to Ringer's fluid. Again the time scale of each of the graphs has been adjusted so that the half-times of all the curves occupy the same distance on the abscissa, and again the mean curves are similar when they are plotted in this way.

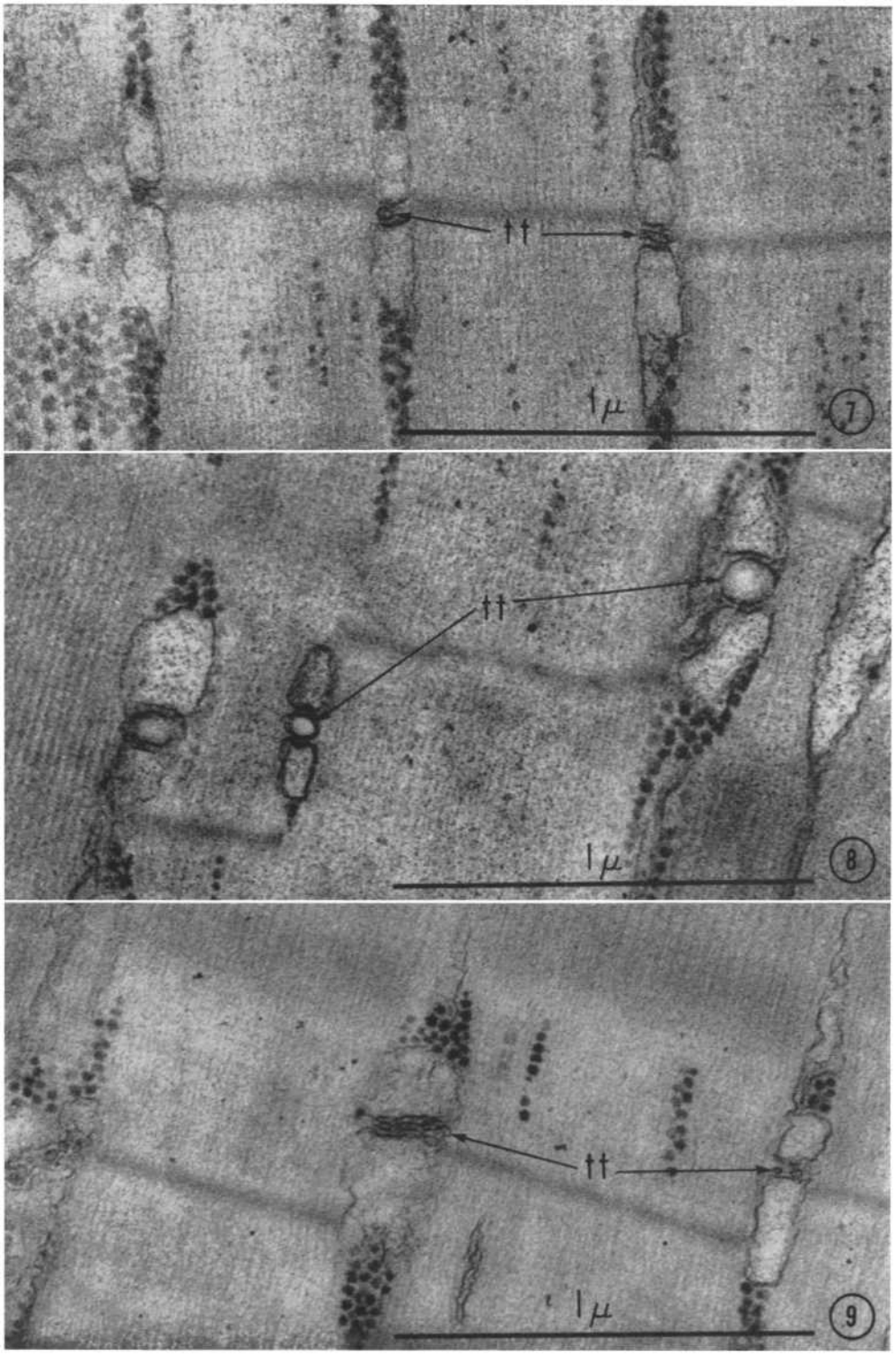
The slowing of the decay of the late after-potential that was found in the low chloride experiments was a change in the opposite direction from that which is calculated from the model (Fig. 1) unless a volume change in the intermediary space is assumed. In Fig. 12 the calculated change in membrane potential, V_{13}, and the experimental data are plotted. After adjustment of the time scale of the calculated data, the fit is quite close. These results, as did those related to the hypertonic solution, suggested that we look for an enlargement of some intracellular compartment.

In muscles immersed in the low chloride solution a reversible dilatation of the T system was again found. No change in the size of other components of the sarcoplasmic reticulum could be detected. The magnitude of the swelling, as seen in Fig. 13, was not as great as in the sucrose-hypertonic solution. The ratio of mean longitudinal size of the T system in low chloride solution to that in Ringer's fluid is 1.7 (Table VI). Also there was again an increase in vari-

FIGURE 5. Normal appearance of the muscle fibers of the frog's sartorius as seen in longitudinal sections. Dense glycogen granules (*gly*) and less dense unidentified granules (*gr*) are indicated. The sarcoplasmic reticulum appears in the spaces between the myofibrils (*mf*) and, in one place, is seen in "face view" where the section is tangential to the surface of a myofibril. The large terminal cisternae (*tc*) near the Z lines and the longitudinally oriented tubules (*lt*) in the A band region form the *longitudinal reticulum*. The *transverse tubules*, or *T system* (*tt*), are confined to the regions of the Z lines where they form the central elements of the *triads*, whose outer elements are the two terminal cisternae on either side of the Z lines. The T system is believed to be continuous across the width of the fiber in vertebrate muscle (Andersson-Cedergren, 1959), but is clearly discontinuous in the longitudinal direction.

FIGURE 6. Higher magnification of a similar preparation, showing more clearly the T system and the triads. The dimension we measured is indicated by the arrows.

The micrographs in Figs. 5 and 6 are from a muscle fixed for 1 hour in 6.2 per cent glutaraldehyde, rinsed for 1 hour in 10 per cent sucrose, and then fixed for an additional hour in 1 per cent osmium tetroxide. All solutions were buffered to pH 7.3 to 7.5 with 0.1 M sodium phosphate and chilled. This method causes some swelling but otherwise gives an excellent preservation of the sarcoplasmic reticulum. Succeeding micrographs are from muscles used in the present experiments and fixed as described under Methods in order to avoid the swelling induced by the glutaraldehyde fixative,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ability of size of the elements of the T system. The size of the T system after return to Ringer's fluid (Fig. 14) was slightly smaller than normal.

Effect of the Potassium-Free Solution

Unlike the late after-potentials that were recorded from muscles immersed in the hypertonic and low chloride solutions, those recorded from muscles that were immersed in the potassium-free solution changed in the same direction as the calculated after-potential. It was not necessary to postulate an increase in the volume of the intermediary space. The calculated result drawn in Fig. 1 indicates that the rate of decline of the late after-potential should be increased in muscles in the potassium-free solution. This was the case, as can be seen in

TABLE VI

Solution	No. of muscles	No. of measurements	Mean longitudinal size (±SD)	Ratio to control
			Angstrom	
Ringer's	3	56	260±70	(1.0)
Sucrose hypertonic	3	78	980±690	3.8
Sucrose hypertonic returned to Ringer's	3	63	270±60	1.0
Low chloride	3	50	450±150	1.7
Low chloride returned to Ringer's	3	77	230±40	0.9
K-free	2	39	210±40	0.8
K-free returned to Ringer's	2	29	280±30	1.1

the experimental result presented in Fig. 15. The reversal of the change when the muscle was returned to Ringer's fluid for over a half hour was incomplete.

Fig. 16 shows that the shapes of the mean late after-potentials were the same for muscles in Ringer's fluid, in the potassium-free solution, and those returned to Ringer's fluid. The time scales have been adjusted in the same way as was described for Figs. 3 and 11. The changes in the half-times are listed in Table

FIGURE 7. Appearance of the T system in Ringer's fluid. The tubules of the T system lie between the larger terminal cisternae and measure about 260 A. Lateral continuity of the tubules is not so well preserved by osmic acid fixation alone as by the combination of glutaraldehyde and osmic acid fixation shown in Figs. 5 and 6.

FIGURE 8. Appearance in the hypertonic solution. Note the swollen T system. It is enlarged to about four times normal in the longitudinal direction. The terminal cisternae also seem to be swollen, but it is more difficult to judge this.

FIGURE 9. Appearance after return to Ringer's fluid after having been in the hypertonic solution. The T system has returned to its normal size. The terminal cisternae sometimes appear to remain swollen.

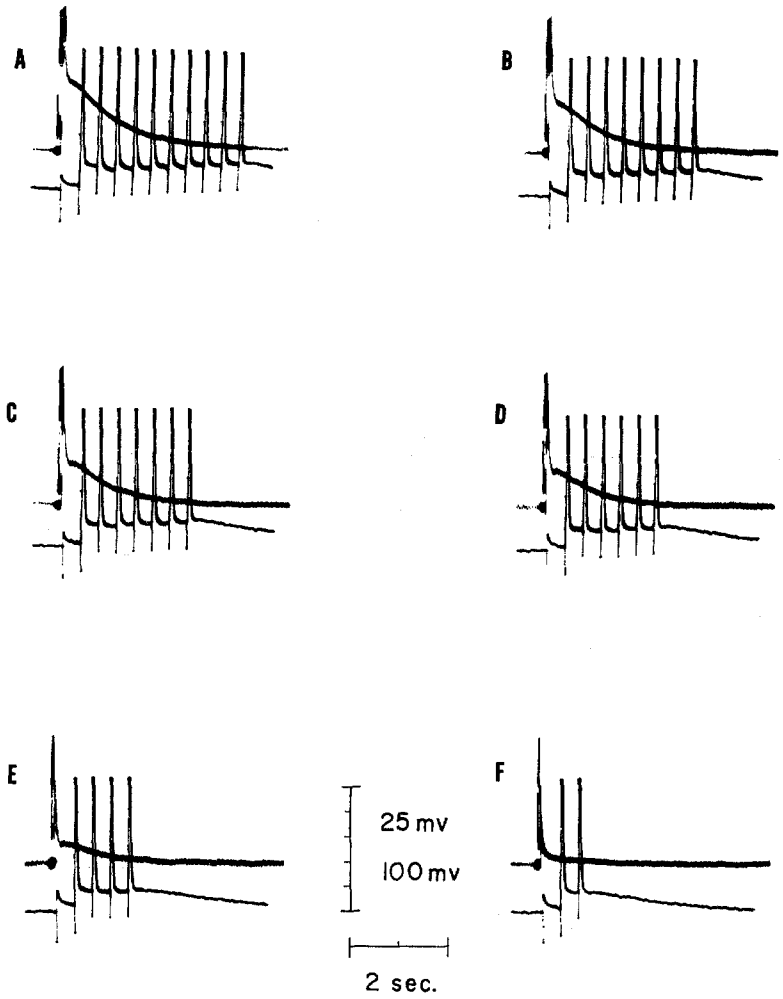

FIGURE 10. Effect of the duration of the train of impulses on the late after-potential of muscle fibers in the low chloride solution. The impulses are 10 msec. apart. The time scale refers to the upper traces which were recorded simultaneously with the lower ones. The upper traces are at the higher gain. Fiber 6 of muscle d in Table IV. This fiber did not respond to the first stimulus of the train.

V; they were partially reversible when the muscles were returned to Ringer's fluid. The initial resting potentials listed in Table V, as those in Tables III and IV, were probably biased by the method for selecting fibers.

A comparison between the mean measurements and the calculated result is illustrated in Fig. 17. Here the calculated curve has almost the same time scale as that of the experimental data.

It was of interest to examine the tubular system of muscles in the potassium-free solution because here a change in size, except for possibly a rather small

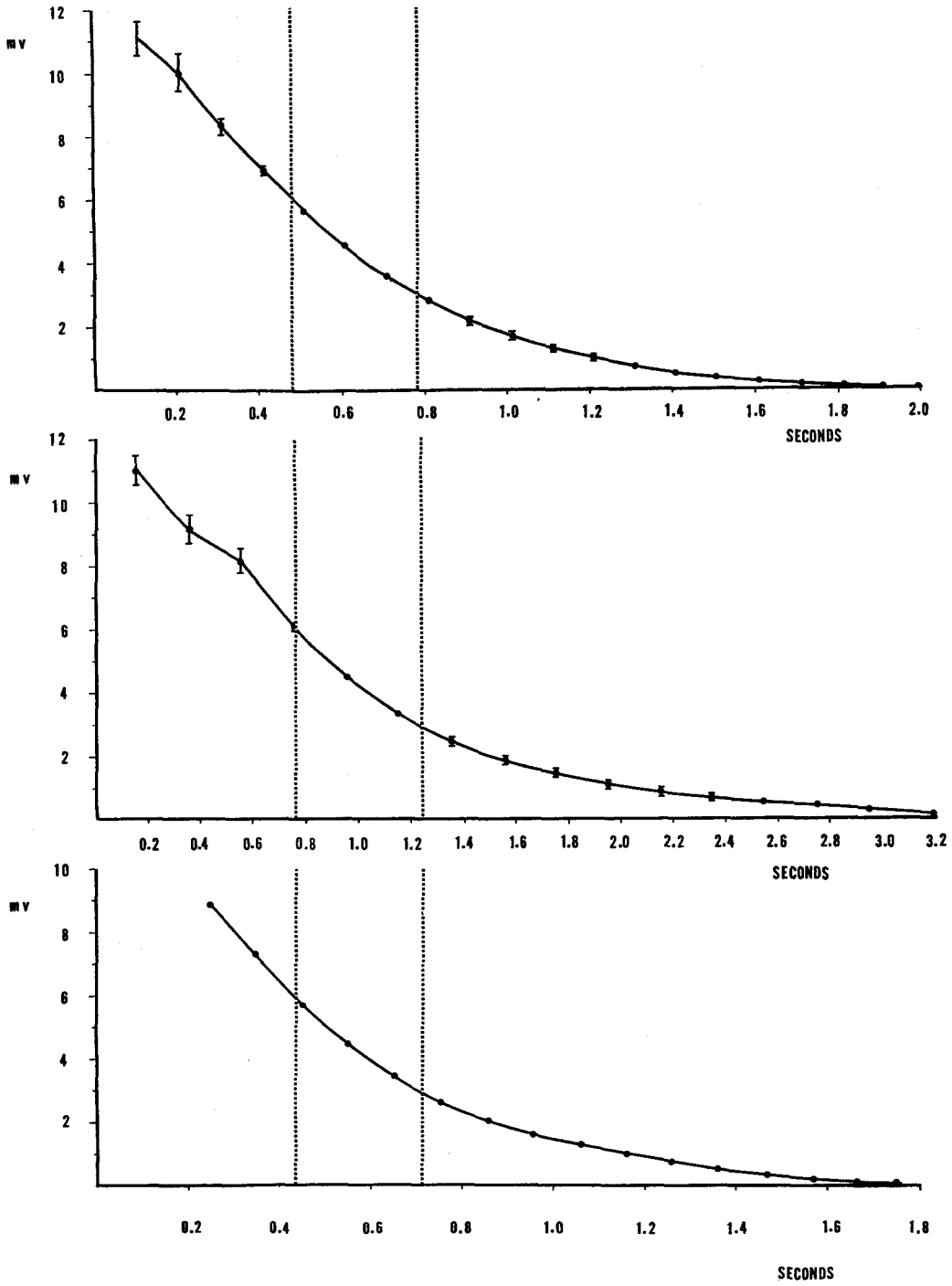

FIGURE 11. The mean late after-potentials of the muscle fibers in Ringer's fluid before being immersed in the low chloride solution (top part), of fibers in the low chloride solution (middle part), and after being returned to Ringer's fluid (bottom part). These data are from the fibers listed in Table IV. This figure was prepared in the same way as Fig. 3.

increase, was not necessary to bring the experimental and the calculated results into accord. The size change we observed microscopically was small and in the opposite direction from the two previous cases; that is, the size of the T system was slightly reduced in the potassium-free solutions to about 0.8 times its size in Ringer's fluid (Figs. 18 and 19 and Table VI). The size returned

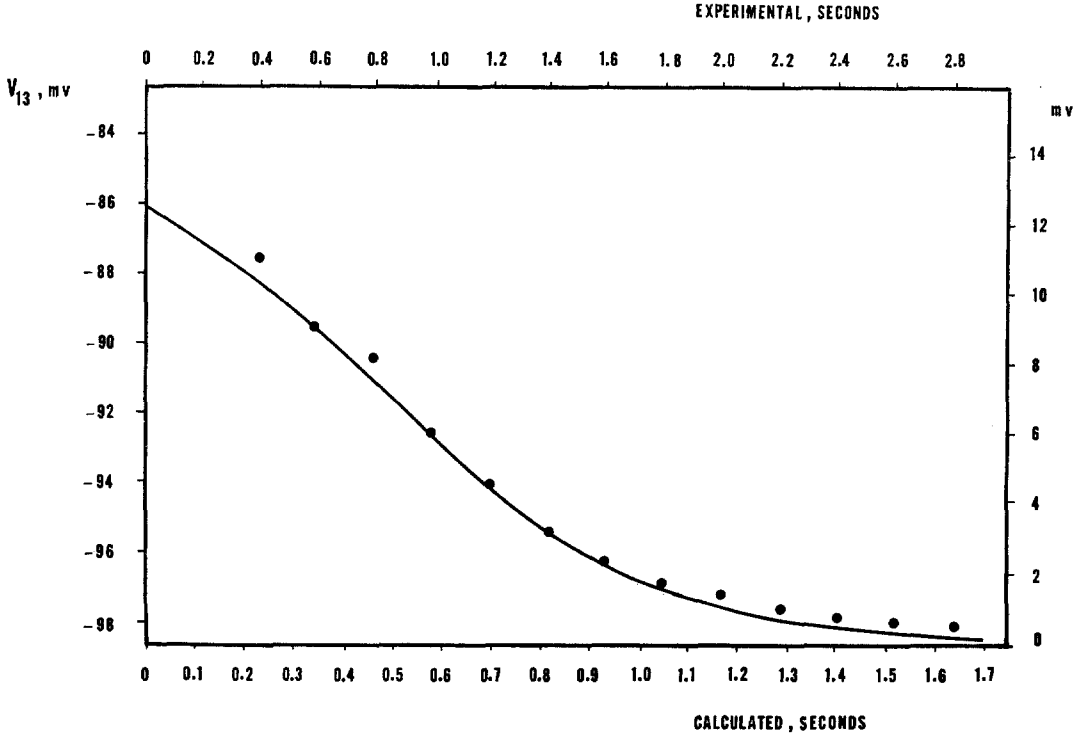

FIGURE 12. Comparison between mean experimental data (points) and the calculated late after-potential for fibers in the low chloride solution. Each set of data has its own time scale. V_{13} is the calculated membrane potential. The experimental data are the same as those in the middle part of Fig. 11 and the scale for these data is marked on the ordinate on the right. These calculated data are the same as those in Fig. 1.

almost to normal when the muscle was immersed in Ringer's fluid again. No change in variance of measurements was seen in the muscles immersed in potassium-free solution.

DISCUSSION

The primary finding that emerges from our results is that the changes in the time scale of the late after-potential correlate rather well with the changes in size of the T system. The significance of this finding is that, if the assumption of a three-compartment model is the correct explanation of our data, then the

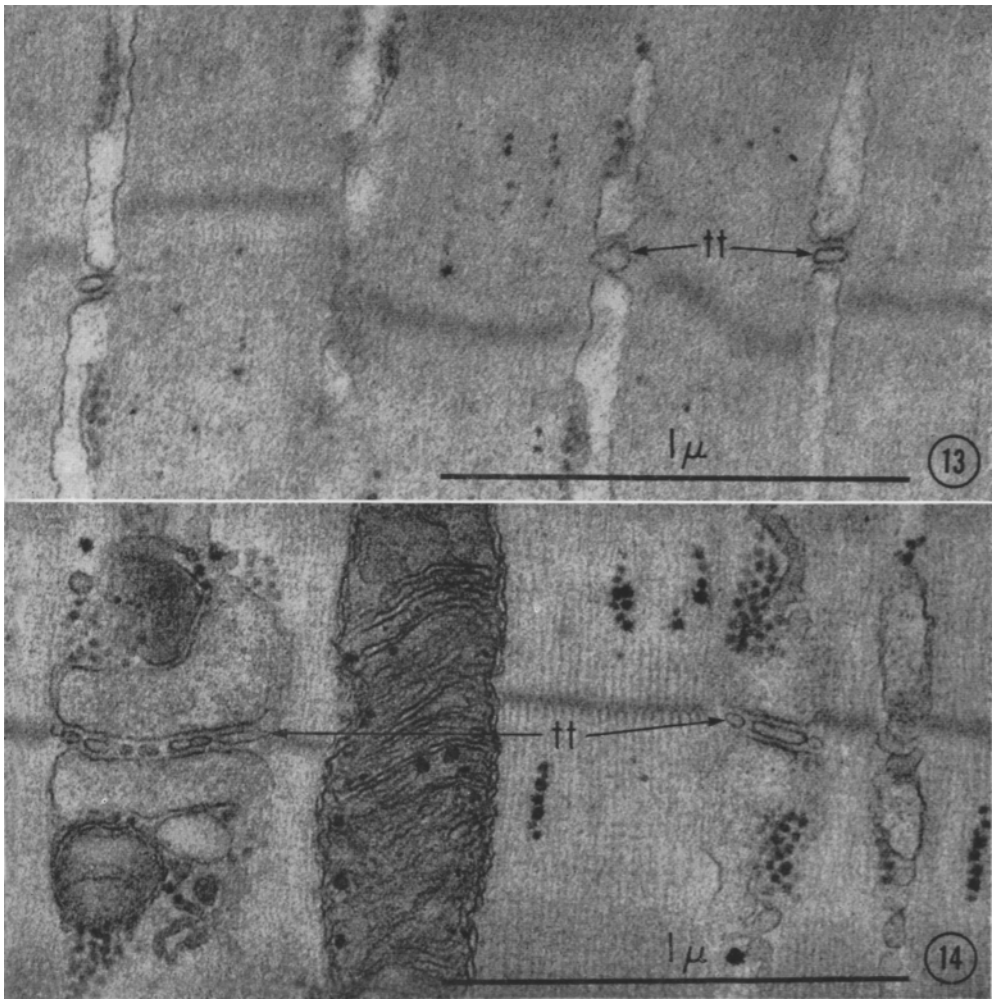

FIGURE 13. Appearance of the sarcoplasmic reticulum in the low chloride solution. Swelling of the T system can be seen. The mean longitudinal dimension of the T system is increased to about twice normal.

FIGURE 14. Appearance after return to Ringer's fluid after having been in the low chloride solution. The T system has returned to close to its normal size.

T system serves well as the required intermediary space. Also, one's estimate of worth of an intermediary space as a means of explaining these results is somewhat higher because one now has a reason to put the intermediary space in the structure of the muscle fiber in a place that is well suited to its function in the model. None of the other assumptions in the model is necessarily supported by these results.

An estimation of how well the assumptions of the model agree with our

results can be obtained by calculating the ratio of the volume of the intermediary space per unit surface area of the fiber in the experimental solutions to the volume of the space per unit surface area of the fiber in Ringer's fluid that will make the half-time of the calculated late after-potential agree with those half-times that are found experimentally. This ratio then can be compared to the ratios in Table VI. For example, for the calculated data under the conditions in the low chloride solution, without allowing for a change in the volume per unit surface area, the half-time is 280 msec., while the calcu-

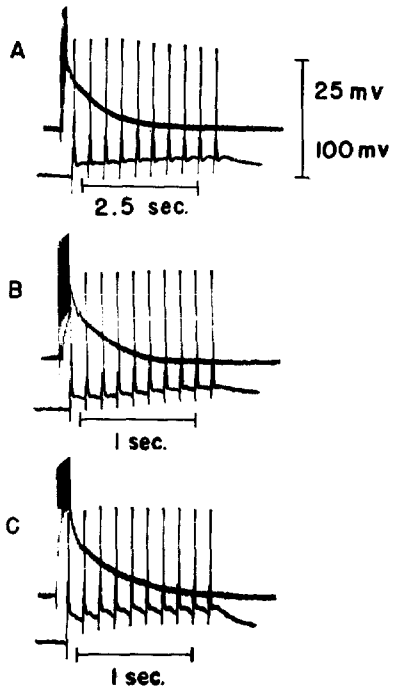

FIGURE 15. The change in the late after-potential in the potassium-free solution. The recordings are from different fibers of the same muscle (fibers 1, 6, and 13 of muscle k, Table V). The lower traces show the train of ten impulses that were separated by 10 msec. between each impulse. The lower traces were recorded simultaneously with the upper traces but at a lower gain. The time scale refers to the upper trace of each part of this figure. Part A, in Ringer's fluid. Part B, in the potassium-free solution. Part C, returned to Ringer's fluid.

lated half-time for the conditions of Ringer's fluid is 330 msec. The ratio of these half-times is 0.85. A similar ratio of the half-times that were found experimentally is $\frac{478}{306} = 1.56$ (Table IV). The ratio of these ratios, $\frac{1.56}{0.85} = 1.8$, expresses the 80 per cent increase in the volume of the intermediary space that one must postulate to make the rate of fall of the calculated late after-potential fit the rate of fall of the experimental one. Since the diameter of the fibers was the same in the low chloride solution as it was in Ringer's fluid, one can assume that the transverse dimensions of the T system do not change and can then compare the ratio 1.8 with the ratio in Table VI that expresses the observed dilatation of the T system, which is 1.7.

A similar calculation for the calculated and experimentally obtained half-

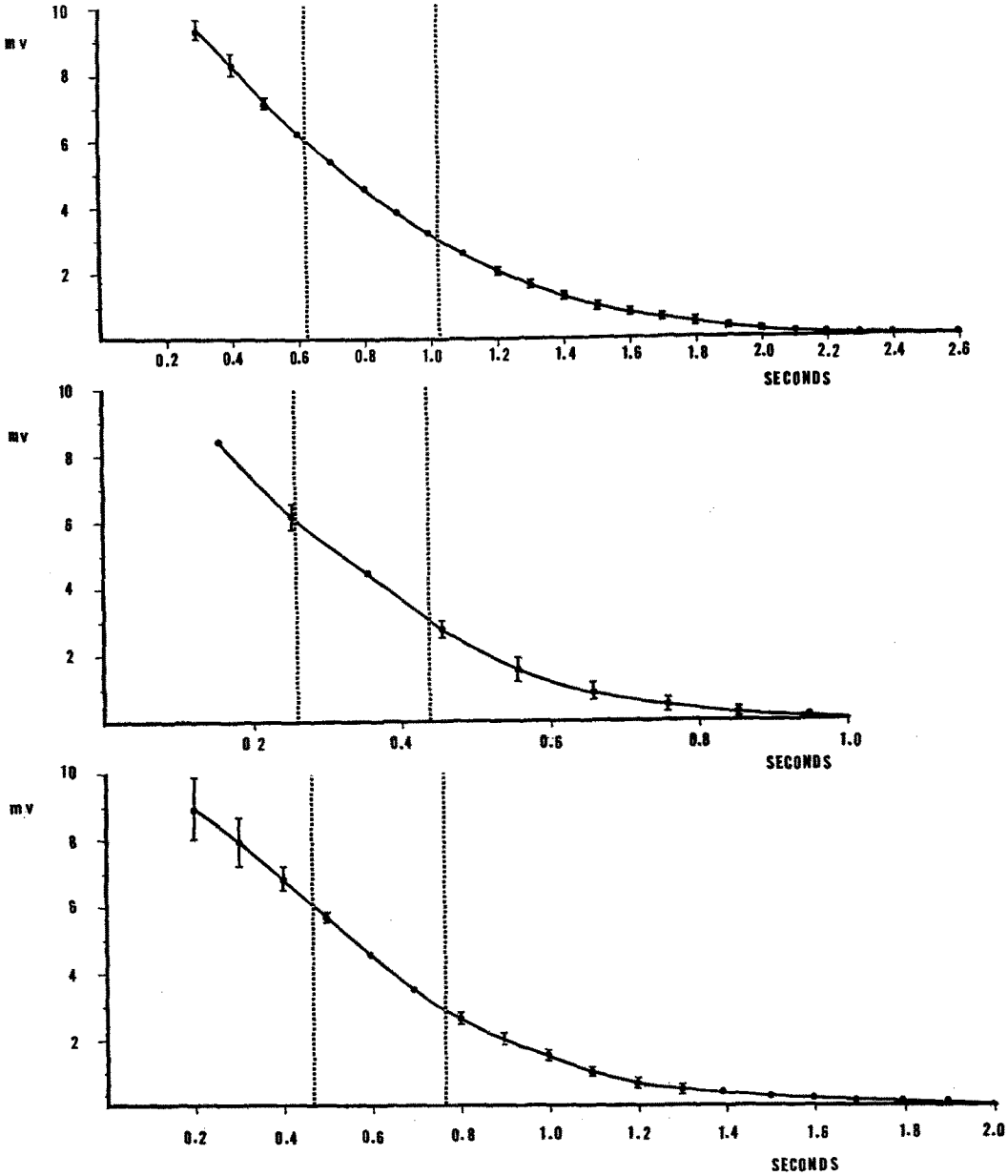

FIGURE 16. The mean late after-potentials of muscle fibers in Ringer's fluid before being immersed in the potassium-free solution (top part), of fibers in the potassium-free solution (middle part), and after being returned to Ringer's fluid (bottom part). These data are from the fibers listed in Table V. This figure was prepared in the same way as Figs. 3 and 11.

times for sucrose hypertonic solution yields a necessary increase in volume of 5.2 times. The increase in the mean longitudinal dimension of the T system tubules was 3.8 times (Table VI), but this number must be multiplied by 1.26, the ratio of fiber diameter in Ringer's fluid to that in the hypertonic solution, to adjust for the change in surface area of the fiber. In this way we estimate

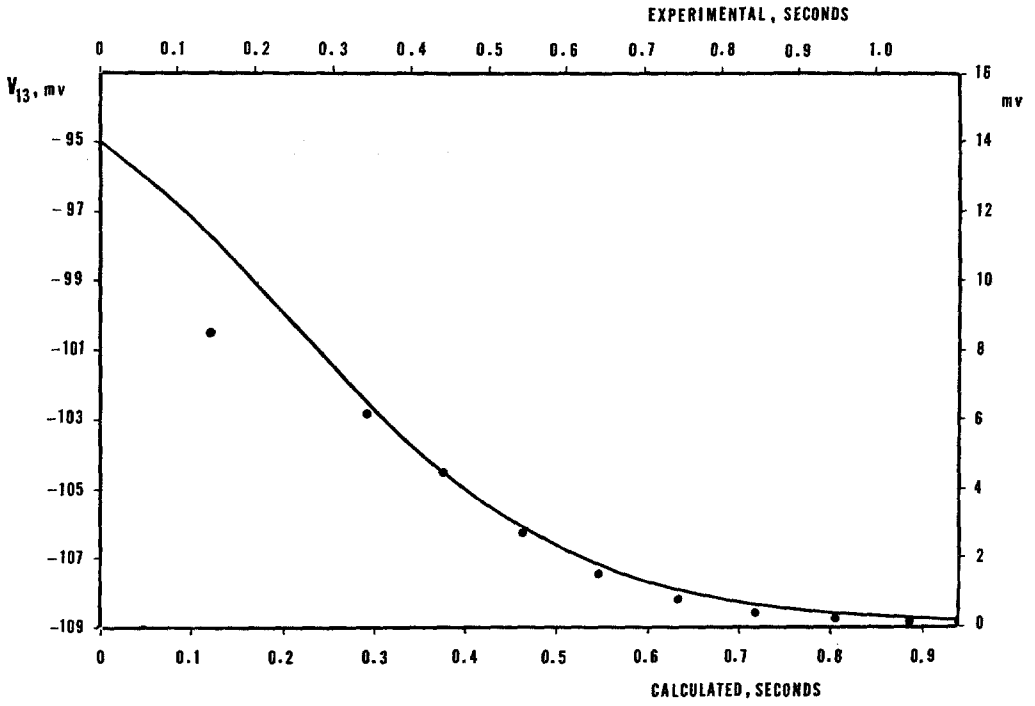

FIGURE 17. Comparison between mean experimental data (points) and the calculated late after-potential for fibers in the potassium-free solution. Each set of data has its own time scale. V_{13} is the calculated membrane potential. The experimental data are the same as those in the middle part of Fig. 16 and the scale for these data is marked on the ordinate on the right. The calculated data are the same as those in Fig. 1.

that the volume of the T system per unit surface area increased 4.8 times when the fibers were in the hypertonic solution. The agreement with the calculated ratio of 5.2 above is rather good.

A major change in the size of the intermediary space per unit area of surface membrane need not be postulated to explain the shortening of the experimentally observed half-time of fibers in the K-free solution (Fig. 1 and Table V). A small decrease in the size of the T system of about 20 per cent was found (Table VI). The model failed to predict this small decrease. It is not clear whether this failure shows the limits of accuracy in the model or that of the estimates of tubular size.

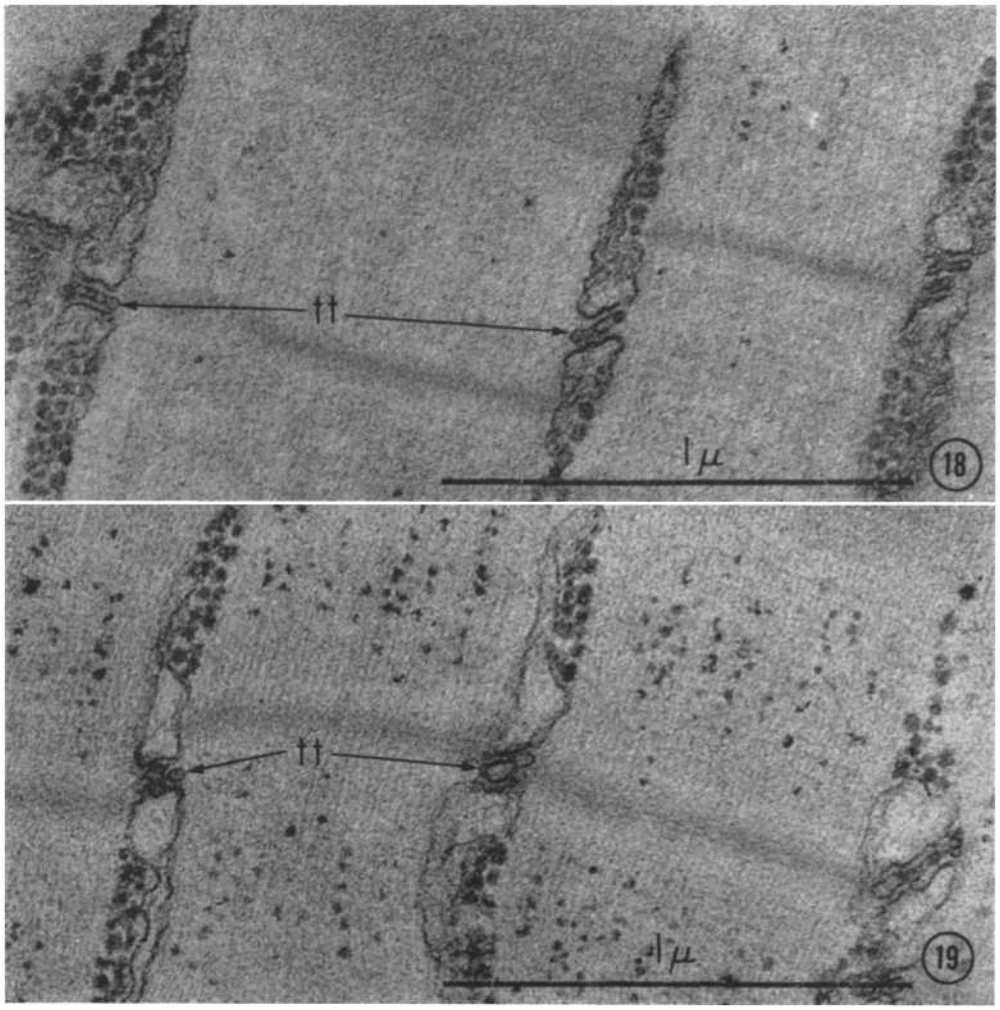

FIGURE 18. Appearance of the sarcoplasmic reticulum in K-free solutions. The T system is slightly smaller than normal.

FIGURE 19. Appearance after return to Ringer's fluid. The mean size of the T system is normal.

Swelling of sarcoplasmic vesicles in sucrose-hypertonic solutions has also been observed by Huxley, Page, and Wilkie (1963). Their most frequent finding, illustrated in their published micrographs, is a swelling of the outer elements of the triads (the terminal cisternae), in contrast to our findings in which swelling of the central element was the constant finding, swelling of the outer elements being variable and sometimes not detectable. We are unable to explain the difference in the findings of the two sets of experiments. In discussing this seemingly paradoxical finding of swollen vesicles in hypertonic solu-

tions, Huxley and coworkers mention three possible explanations, but consider them inadequate. None of their explanations accounts for the swelling of the T system in our low chloride solution, which was isotonic with Ringer's fluid. The swelling in the low chloride solution, which had sucrose in it, directs one's attention to the possibility of sucrose entry into the T system from the external fluid being involved in the mechanism of swelling, rather than hypertonicity. If sucrose does enter the T system without a simultaneous leakage of the normal osmotically active contents of the T system, water would move from the major portion of the sarcoplasm (space 3) into the T system.

Girardier, Reuben, Brandt, and Grundfest (1963) have attributed a swelling of the transverse tubular system of crayfish muscle fibers to a movement of chloride from the sarcoplasm into the tubes. Water accumulates in the tubes, in their scheme, through normal osmosis, electroosmosis, or both. In all three of our experimental solutions the fibers were exposed to a lowered external [K] [Cl] product relative to the internal [K] [Cl] product and there was an efflux of potassium chloride from the muscles in each of the solutions. If a system such as the one in crayfish muscle were responsible for the swelling of the T system that we found in frog muscle fibers in the hypertonic and low chloride solutions, there should have been a swelling of the T system in the potassium-free solution as well, but none was found.

Notwithstanding the relation between the size of the T system and the late after-potential, some of the assumptions implied in the model of Adrian and Freygang remain clearly expedient while others appear to be more in accord with reality. To us, among the more credible assumptions are the following: (*a*) The frog fast muscle fiber behaves as if it were composed of two compartments. The extra compartment is the intermediary space of the model, or compartment 2, which corresponds to the "special region" postulated by Hodgkin and Horowicz (1960) and the T system. (*b*) The potential difference across the walls of the T system, or V_{23} in the model, is determined by the difference between the potassium concentration in the lumen of the tube and the major portion of the sarcoplasm. (*c*) The walls of the T system display anomalous rectification of potassium movement but also allow potassium to accumulate in the T system during a train of impulses. (*d*) The major portion of chloride movement is located directly between the sarcoplasm and the fluid outside. This assumption follows from the finding of Hodgkin and Horowicz (1960) that changes in the external chloride affect membrane potential more rapidly than changes in external potassium.

An expedient assumption is (*e*) that there is rapid mixing of the contents of the intermediary space. This assumption is implied in the equation that relates the change in potassium concentration in the intermediary space to the net inward or outward flow of potassium (equation 7 of Freygang *et al.*, 1964). Therefore the significance of v, the volume of the intermediary space per unit

surface area, is not altogether clear. The reason this assumption was made, of course, is to avoid the enormous amount of computation required to take into consideration the gradients of concentration, potential, and permeability along the walls of the intermediary space.

The assumptions that govern the flow of ionic currents at the mouth of the tubules, the junction between the external fluid and the intermediary space, also are rather tenuous particularly in the light of some recent publications. Endo (1964) finds egress within several seconds of the dye lissamine rhodamine B200 from the I band region of an isolated frog muscle fiber. This dye carries one positive and two negative charges per molecule. The strong suggestion is that the dye can enter some part of the triad system from the external solution without entering the major portion of the sarcoplasm. Huxley (1964) finds that ferritin, which has a diameter of about 110 A, can enter the central element of the triads when sartorius muscles are immersed for a few minutes or longer in Ringer's fluid which contains the protein. Adrian and Chandler (Adrian, 1964) have evidence which they interpret as suggesting that, if there is a membrane between the intermediary space and the external fluid, it is permeable to sucrose and sulfate ions in muscles that have been soaked in solutions of low ionic strength. In fish muscle, Franzini-Armstrong and Porter (1964) find electron microscopic evidence that a direct continuity exists between the T system and the external space. All these reports lead one to consider a free communication between the intermediary space and the external fluid to be the most likely situation. On the other hand, Fatt (1964) finds it necessary to postulate a positive and a negative charge selective barrier at the mouth of the tubule in order to account for a capacity of 50 μf/cm^2 of fiber surface. His finding is derived from an analysis of the low frequency dispersion (less than 100 CPS) of transverse impedance measurements. Thus it appears that the mouths of the tubules allow slow movements of particles but more rapid movements are not always allowed. It is not clear what revisions of the model are desirable, if any are suggested by these findings, in order to improve its prediction of events on the time scale for which it is employed.

Appendix

In an attempt to explain the time course of the late after-potential, Freygang, Goldstein, and Hellam (1964) also described calculations in which P_K, the permeability of the 2–3 membrane, was taken to be a function of the driving force for potassium movement across this membrane rather than simply a function of the potential difference across it. In the calculations that are described in the major portion of this paper, P_K is a function of V_{23} alone, as in the paper of Adrian and Freygang (1962 *a*). Since the values of P_K that are employed in the calculations are derived from the data of Hodgkin and Horowicz (1959), who considered P_K to vary with the driving force on potassium, it seemed advisable to repeat the calculations with this additional complexity. The result is illustrated in Fig. 20, which should be compared with Fig. 1.

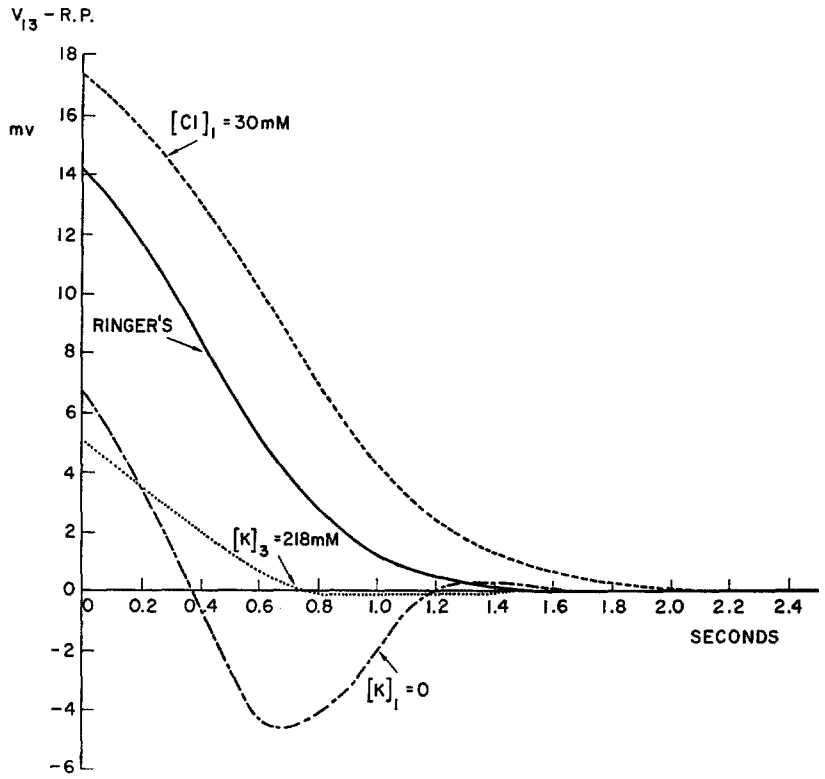

FIGURE 20. The late after-potentials simulated by the theoretical model of Adrian and Freygang (1962 *a*, *b*) and calculated in the manner which has been described by Freygang *et al.* (1964). These curves were calculated in the same way as those in Fig. 1 of this paper except that P_K is taken as a function of $[V_{23} - E_{23}^K]$ as has been described on p. 941 of Freygang *et al.* (1964). In all these calculations the volume of the intermediary space per unit surface area, v, was 3.5×10^{-6} cm³/cm². The curve that is labeled $[K_3]$ = 218 mM refers to conditions in the hypertonic solution, the one labeled $[Cl_1]$ = 30 mM refers to conditions in the low chloride solution, and the one labeled $[K_1]$ = 0 refers to conditions in the potassium-free solution.

The most obvious difference between the calculated data in Fig. 20 in comparison with the late after-potentials, or the calculated data in Fig. 1, is the result when $[K_1]$ = 0. A hyperpolarization of this magnitude was never encountered in our experiments in the K-free solution. The rate of decay of the calculated late after-potential for the conditions of the hypertonic solution in Fig. 20 is slower than that calculated for Ringer's fluid, but not enough to account for the amount of slowing that was observed experimentally without again postulating an increase in v. An increase in v is also necessary to explain the slowing of the late after-potential found experimentally in the low chloride solution if the calculated data in Fig. 20 are considered rather than those in Fig. 1.

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