

The Majority of Potassium Ions in Muscle Cells is Adsorbed on β - and γ -carboxyl Groups of Myosin: Potassium-ion-adsorbing Carboxyl Groups on Myosin Heads Engage in Cross-Bridge Formation During Contraction

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Abstract: High-molecular weight poly(ethylene glycol) (PEG-8000) in the bathing medium prolongs the survival of 2-mm-wide frog muscle segments with open ends. In a PEG-8000-containing medium Rb^+ , K^+ , and Na^+ in the muscle segments reached new diffusion equilibrium in 2–4 hours. At this new equilibrium, the cell's preference of K^+ over Na^+ was preserved but very much weakened.

Studies of the influence of pH on the equilibrium distribution of labelled Na^+ in 2-mm-wide muscle segments confirmed the prediction that β - and γ -carboxyl groups, carried respectively on aspartic and glutamic acid residues of intracellular proteins, adsorb K^+ , Na^+ and other monovalent cations. These carboxyl groups have a characteristic pKa between 3.65 and 4.25. A pKa of 3.85 was observed.

These findings, when seen in the light of other relevant information available, led to the conclusion that β - and γ -carboxyl groups on myosin molecules adsorb — in a close contact one-ion-one-site fashion — the majority (67% to 80%) of K^+ in resting muscle cells.

Other evidence suggests that in muscle contraction, the K^+ -adsorbing β - and γ -carboxyl groups on myosin heads form salt linkages with cationic sites on actin, displacing and releasing the adsorbed K^+ . Present and earlier findings together offer support for an earlier suggestion that the formation and dissociation of these salt-linkages may underlie the force-generating, cyclic formation and dissociation of cross-bridges during muscle contraction.

IN 1951–1952, A QUANTITATIVE theory for the selective accumulation of K^+ over Na^+ in living cells and model systems was presented (Ling, 1951, 1952). In this theory, stronger electro-static interaction between intracellular fixed anions and the smaller (hydrated) K^+ than that between the fixed anions and the larger (hydrated) Na^+ leads to a preferential ad-

sorption of K^+ over Na^+ on the fixed anions. Selective accumulation of K^+ over Na^+ follows in consequence. In the same 1952 presentation, it was suggested that the fixed anions selectively adsorbing K^+ participate in muscle contraction.

Subsequently, a much broader theory of the living cell called the association-induction (AI) hypothesis has evolved from, and in some aspects superseded, the 1951–1952 model (Ling, 1962). Nonetheless, the seminal idea introduced in the earlier model of selective K^+ accumulation remains valid; its key postulates summarized below, are essential for the AI hypothesis as well.

Postulate 1: Free β - and γ -carboxyl groups carried respectively on **aspartic** and glutamic acid residues of cell **protein(s)**, function as the *fixed anions* for the selective one-on-one adsorption of K^+ in living cells. (In voluntary muscle cells, the major protein, myosin alone harbors enough β - and γ -carboxyl groups to adsorb all the muscle-cell K^+);

Postulate 2: *fixation* of ions (e.g., β - and γ -carboxyl groups on proteins) strongly enhances their association with free ions bearing the opposite electric charge (e.g., K^+) (see also Ling, 1960; 1984, p. 149).

Postulate 3: the ability of *fixed β - and γ -carboxyl groups of certain proteins in living cells* to preferentially adsorb K^+ over Na^+ — an ability not shared with isolated "native" proteins — depends on the open, or what was later called *extrovert* conformation which the participating cell proteins assume (Ling, 1991, 1991a). In this conformation, the β - and γ -carboxyl groups are available for the adsorption of K^+ and other free monovalent cations. In contrast, isolated "native" proteins assume a closed or *introvert* conformation: Their β - and γ -carboxyl groups are engaged in "salt linkages" with fixed cations (e.g., ϵ -amino groups carried on lysine residues; guanidyl groups carried on arginine residue) and thus unavailable for the adsorption of K^+ and other free monovalent cations;

Postulate 4: ATP plays a key role in maintaining the extrovert conformation of certain **protein(s)** in living cells. As a *cardinal adsorbent*, it achieves its function by adsorption onto and electronically polarizing the protein molecules involved (Ling, 1962, p. 252; 1984, p. 363; 1991, Section 8.4). In serving this role, ATP does not undergo hydrolytic degradation. A quantitative relationship between the concentration of K^+ and that of ATP (per se) in the cells was implicitly predicted.

In years following, the essence of Postulates 2, 3, and 4 has already been confirmed (for review, see Ling, 1990; 1991, Section 4.4). This communication is the first of two papers, confirming the last remaining postulate (Postulate 1), i.e., β - and γ -carboxyl groups are the seats of selective adsorption and accumulation of K^+ and Na^+ in living cells. In the present report, the criterion used to identify β - and γ -carboxyl groups is their characteristic acid dissociation constant or **pKa**. In the second report soon to follow, we identified β - and γ -carboxyl groups by their distinctive susceptibility to attack by carboxyl-specific reagents (Ling and Ochsenfeld, 1992).

Materials and Methods

Chemicals All chemicals used were of C.P. grade. PEG 8000 (Molecular weights 6000 to 9000) was obtained from Fisher Scientific (Carbowax PEG 8000, Lot 724210, 733754, 745478, 857096, Fischer Scientific, Springfield, N.J.). ^{22}Na (Lot 261BA) and ^{86}Rb (Lot 185MBq) were both obtained from Amersham, Des Plaines, IL.

Preparation of open-ended narrow muscle segments All experiments were carried out on isolated sartorius muscles of leopard frogs (*Rana pipiens pipiens*, Schreber), obtained from the State of Vermont, USA. Each sartorius muscle consists of about one thousand single muscle fibers, all of which run from one end of the muscle to the other end without interruption (Ling, 1978).

The sartorius muscle was isolated with an **uninterrupted** (roughly) 2-mm-wide band of fascia attached to the anterior-lateral margin of the muscle from end to end (Figure 1). This fascia originally covers part of the dorsal surface of the immediately neighboring muscles, i.e., **adductor longus** and part of the anterior head of triceps femoris.

The isolated sartorius muscle, after a short stay in a normal Ringer phosphate solution, was placed at its natural relaxed length on a stack of filter paper (wetted with Ringer solution) and cut into narrow sections according to procedures described below. Alternatively, the muscle was first soaked for 40–60 minutes in a cold (4°C) "sedating solution" (also known as Solution 316, the composition of which is also given below). Incubation in this

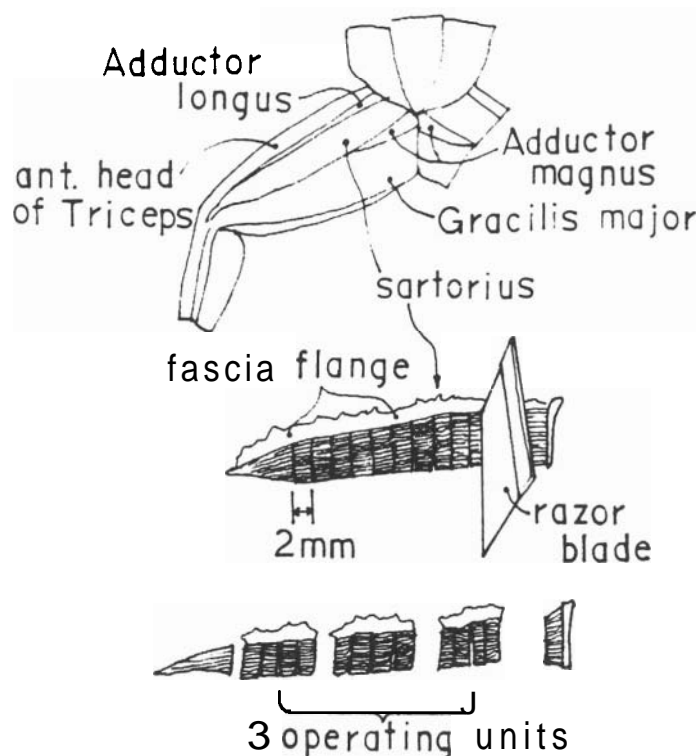


FIGURE 1. The ventral surface of the skinned thigh of a **leopard** frog, indicating the location of the sartorius and its neighboring muscles (top) and illustration of the preparation of operating units of 2 mm. open-ended muscle segments with attached fascia flange for easy handling. The **tibial** and pelvic ends of the muscle are routinely discarded.

low-sodium solution (total Na^+ concentration, 16.5 mM) renders the muscles temporarily inexcitable and thus easier to be cut into even segments.

Sartorius muscles — sedated or "awake" — were cut into 2-mm-segments with the aid of a strip of graph paper with mm-wide divisions, and a single-edged razor blade (Figure 1). Each cut went all the way across the muscle, stopping at the proximal edge of the fascia fringe. From 4 to as many as 7 consecutive segments held together by the fascia fringe were studied as an operating unit. When each unit contains 6–7 segments, the unit usually was obtained from one half of a muscle. Such a unit is accordingly referred to a "half-muscle unit." The muscle segment units were handled with jewelers' forceps by the fascia flange. Since all the muscle fibers in a sartorius muscle run all the way from one end of the muscle to the other end (Ling, 1978), these muscle segments were open at both ends in a highly uniform manner.

While the 2-mm-wide-segment assemblies were used in most experiments described in this paper, in earlier work the sartorius muscle was cut on alternate sides and these cuts did not go all the way across the muscle but stopped at about a quarter to a third of the way from the other side. As a result, the cut-muscle assemblies were a mixture of alternatingly 2-mm-wide and 4-mm-wide segments held together by the 4-mm segments instead of the carefully preserved fascia (see Ling, 1984, Figure 13.2A). This older preparation was used, for example, in the experiment described in Figure 3.

Incubation media and procedures The preset study (and the study to be described in the following paper) were made possible by the discovery of the beneficial effects of high molecular weight poly(ethylene glycol) (PEG 8000) [$\text{H}(\text{OCH}_2\text{CH}_2)_n\text{OH}$] in preserving K^+ accumulation in 2–4 mm-muscle segments with open ends (Ling, 1989). The use of open-ended-short-muscle segments, first described in 1973 (Ling, 1973, 1978), was essential. Studies depicted in this and the companion paper required externally added agents (H^+ or carboxyl reagents) to react with intracellular components rapidly. This task would be virtually impossible to achieve when an intact cell-membrane barrier intervenes between the external solution and the intracellular carboxyl groups. As muscles do not regenerate new cell membranes at the cut ends, cutting muscles into 2-mm-segments effectively removes this barrier (Ling, 1978; Cameron, 1988). However, the ability of accumulating K^+ is lost rapidly when the muscle cell segments are kept in a normal Ringer solution. PEG 8000 added to the medium arrests or at least delays this regressive process long enough to permit direct probing of ion-adsorbing groups within the open-ended muscle-cell segments.

We measured the osmotic activity of the PEG solutions on a vapor pressure osmometer (Wescor Model 5100C) (see Ling, 1983). A 16.3% to 16.7% (w/v) solution of the PEG 8000 purchased at different times (see above, for source and lot numbers) usually exhibited an osmotic activity close to that of an isotonic NaCl solution (0.18 M).

The basic incubation solution (Solution 312) contains 16.35% to 16.7% PEG 8000, 2.5 mM KCl, and 1.69 mM each of the Na^+ salts of the six organic acids: pyruvic acid, lactic acid, α -ketoglutaric acid, acetic acid, succinic acid and citric acid. The total Na^+ concentration provided by these organic Na^+ salts in Solution 312 is 16.5 mM. The pH is 7.3.

The sedating solution mentioned above is a modified 312 Solution, in which the PEG 8000 is replaced by 210 mM of sorbitol. The pH of the sedation solution remains at 7.3. Since the composition of the solution used sometimes deviated from the basic formula of Solution 312, the detailed compositions of the media used are described in the text and/or in the table and figure legends when needed.

The main effort of this investigation was to determine the effects of different pH's on the ionic uptake of open-ended muscle segments. For this purpose, the incubation solutions contained beside 16.3% PEG 8000 and 2.5 mM KCl, two kinds of pH buffers. For pH above 7.5, glycine-NaOH buffers were used. In these buffers, a constant Na⁺ concentration of 18.9 mM was maintained by adjusting the concentration of Na₂SO₄ in the solutions. For pH below 7.5, H₂SO₄/Na₂SO₄ buffers were used. These bathing solutions contained unvarying concentrations of PEG 8000 (16.3%), of KCl (2.5 mM), and of Na₂SO₄ (9.4 mM) in addition to varying concentrations of H₂SO₄, ranging from 0 to 116 mM. The pH's of all incubation solutions and the osmotic activity of most of the solutions were measured after the muscle segments had been taken out at the conclusion of the experiments. Due to the large ratio of the bathing-solution volume to the muscle-segment weights (see below), the pH's held well in all incubation solutions, despite the low buffering capacity of the sulfate buffers. At the end of the incubation, the osmotic activity of the bathing solutions varied between isotonic (236 mOsM.) to as high as 357 mOsM. at the lowest pH (1.31). This higher osmotic activity at low pH's is due to the high concentration of H₂SO₄ that had to be added to bring the pH down to this level.

In this group of experiments, six half-muscle units weighing about 0.3 gram together were incubated at 1°C in 30 ml of incubation solution with gentle shaking. At the conclusion of the experiment, two of the half-muscle units were blotted dry on moist filter paper, weighed on a torsion balance and dried. To avoid the loss of PEG in the samples, this drying was carried out at room temperature for from 5 to 8 days in *vacuo* in the presence of phosphorus pentoxide and the (dried) samples weighed again. From the weight losses, the water contents of the segment units were calculated.

Each of the remaining four half-muscle units was blotted on filter paper which was wetted with the same incubation solution in which the muscle segments were incubated, and extracted individually for 15 minutes in 1.5 ml portions of a 5.1% trichloroacetic acid (TCA) solution in a 15 ml-Nalgene centrifuge tube heated in a boiling water bath. Aliquots of the TCA extracts were diluted with Universal-(Radiation)-Buffer-Extractive (UBE) solution to provide in all final sample extracts, standards and blanks, constant concentrations of LiCl (97 mM) and NH₄HPO₄ (3 mM). Analysis of the ionic contents of the extracts was by atomic absorption spectroscopy on an AA spectrophotometer from Instrument Laboratory Inc., Model IL 357. Radioactivity of ²²Na and ⁸⁶Rb was assayed on a Nuclear-Chicago Automatic y-counting System, Model 4216.

Labelled Na⁺-efflux studies The efflux of radioactive Na⁺ (1) from intact sartorius muscles and (2) from assemblies of 2 and 4-mm-wide cut muscle-segments (of whole muscles) were incubated in PEG-Ringer solution containing ²²Na at 2°C for 3 hours. After gentle blotting to remove adhering fluid, the muscle or muscle-segment-assembly was washed in successive portions of a nonradioactive solution containing 8.17% PEG 8000, 4.89 mM KCl, and 16.4 mM of the Na-organic acid mixture used in the low-sodium, sedating solution (Solution 316) described above. (The lower PEG 8000 concentration used in these efflux studies was to reduce the extent of shrinkage which occurred in the presence of higher concentrations of PEG 8000) (see Table I and text below for explanation). The radioactivity of the washing solutions and that remaining in the muscle at the conclusion of the experiment were assayed. The data obtained were used to reconstruct the efflux curves (for a more detailed description of the procedure used for the efflux curve reconstruction, see Ling et al., 1981).

Calculations The cut muscle (segments) like their intact counterparts contain two major "contaminants": fluid found in the space between the muscle fibers and the connective-tissue complex including loose connective tissues, fascia, small blood vessels, nerves, etc. Past work has established that the extracellular-space fluid of a sartorius muscle — after having been blotted according to a standardized procedure (Ling and Bohr, 1969) — is 9% (w/w) (Ling and Kromash, 1967; Ling and Walton, 1975). Other studies showed that the connective-tissue complex makes up 9.1% (w/w) of the sartorius muscle fresh weight (Ling, 1962, p. 210, Table 8.8; Ling et al., 1969). To determine the **labelled-Na⁺** content of muscle cells, it is necessary to correct for the labelled Na⁺ in both the extracellular space and in the connective tissue complex.

In equilibrium studies like the present one, the **labelled-Na⁺** content in the extracellular space is equal to that of the bathing solution. With the percentage of the extracellular space volume in the **muscle-already** known, correction for this contaminant is simple. To correct for the contamination due to the connective tissue complex, we used the method earlier described (Ling and Bohr, 1969): Loose connective tissues were isolated from the surface of the skinned thighs (and legs) from which the sartorius muscles were isolated, and exposed to the same incubation solution and for the same length of time as the muscle segments were. The **labelled-Na⁺** contents and water content of the connective tissue complex were assayed in the same way the muscle segments were.

The **labelled-Na⁺** concentration in whole sartorius muscles was represented as [Na*]_{mu}, that in the extracellular space fluid (which equals the labelled-Na* concentration in the final bathing solution) as [Na*]_{ex}, and that in the connective tissue complex as [Na*]_{ct}. With the exception of [Na*]_{ex}, which was in units of **millimolarity**, all concentrations **including** [Na*]_{cell} in equation 1 below, were in units of **μmoles/gram** of fresh tissue or cells. With all the parameters defined, we calculated the **labelled-Na⁺** concentration in (pure) muscle cells ([Na*]_{cell}) with the aid of the following equation:

$$\begin{aligned}
 [\text{Na}^*]_{\text{cell}} &= \{ [\text{Na}^*]_{\text{mu}} - (0.09 - 0.6 \times 0.091)[\text{Na}^*]_{\text{ex}} - 0.091 [\text{Na}^*]_{\text{ct}} \} / \\
 &\quad \{ 1 - 0.09 + 0.6 \times 0.091 - 0.091 \}, \\
 &= 1.145 \{ [\text{Na}^*]_{\text{mu}} - 0.035 [\text{Na}^*]_{\text{ex}} - 0.091 [\text{Na}^*]_{\text{ct}} \}. \quad (1)
 \end{aligned}$$

Note that for the correction of labelled Na* in the extracellular space, one cannot subtract a full 9% of [Na*]_{ex} from [Na*]_{mu}, but must reduce it by the factor 0.06 x 0.091 for the following reason: The isolated connective-tissues complex contains water equal to 60.3% x 1.09% of its total fresh weight. Previous studies have shown that this water is fully accessible to extracellular space probes including sucrose, D-mannitol, etc. (Ling et al., 1969), and is therefore not different from an equivalent portion of the 9% extracellular-space fluid. In making a 9.1% correction for the connective tissue complex, 9.1% x 60% = 5.46% of extracellular space fluid had in fact already been applied. Therefore, only a part of the 9%, equal to (0.09 - 0.0546) = 3.54% remains to be subtracted from the total muscle-tissue Na* concentration, ([Na*]_{mu}) to achieve the full and accurate correction.

Results

Time course of ionic equilibrium in **2-mm-muscle** segments at neutral pH Figure 2 (and Table I) shows the time courses of changes in the concentrations of Rb⁺, K⁺ and Na⁺

K⁺ ADSORPTION ON CARBOXYL GROUPS OF MYOSIN

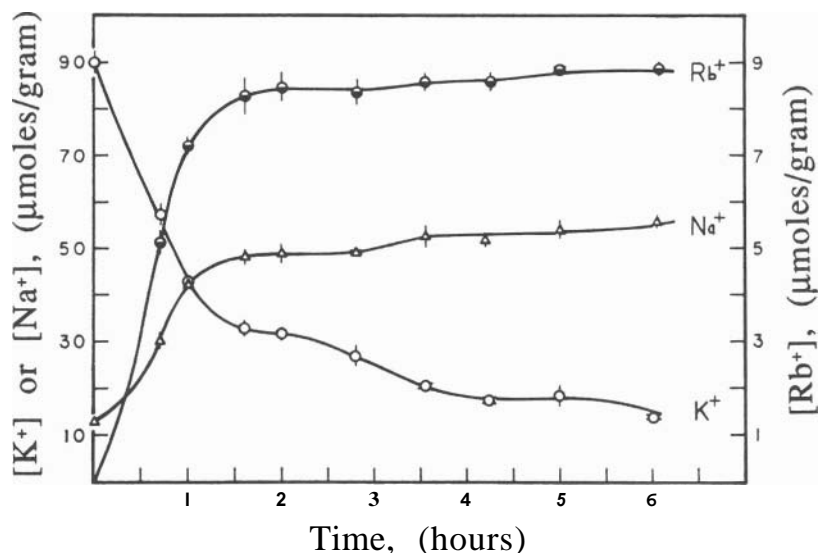


FIGURE 2. Time course of uptake of Na⁺ and (labelled) Rb⁺ by, and release of K⁺ from 2-mm-muscle segments at neutral pH (7.2) (0°C). Isolated frog muscles were preincubated for from 40 to 60 minutes at 4°C in sedating solution (Solution 316) and cut into 2-mm segments. The half-muscle units were then transferred to a standard 312 solution containing K⁺ (2.5 mM), ⁸⁶Rb⁺ labelled Rb⁺ (2.5 mM), and Na⁺ (18.5 mM) in addition to 16.5% PEG-8000 at 0°C. To each tube containing 30 ml of Solution 312, 6 to 7 half-muscle units of muscle segments were introduced. No two units in any one tube came from the same muscle or its pair. Muscles were removed at time intervals indicated. The final concentration in the incubation solution at the conclusion of the experiments were for Rb⁺, 2.4 mM, for K⁺, 3.4 mM and for Na⁺, 17.5 mM. Detailed numerical data are given in Table I.

in 2-mm-muscle-segment assemblies at neutral pH (7.3). The incubation solutions for these muscle segments contained 16.3% PEG 8000, 2.5 mM each of KCl and RbCl, and 16.5 mM of Na⁺ (as the salt of 1.69 mM each of lactate, pyruvate, a-ketoglutarate, acetate, succinate and citrates).

Both Rb⁺ and Na⁺ concentrations in the muscle-segment assemblies increased rapidly, until at 2 hours they reached a temporary equilibrium, only to resume their increases an hour later to reach another temporary equilibrium at about 3.5 hours. The K⁺ concentration, on the other hand, decreased rapidly first and then more slowly until it too approached an unsteady equilibrium at 1.5 hours, and after that declined further until another transient equilibrium was reached at 4 hours and maintained there for another hour.

Let us assume that 9% of the muscle-segment weight is due to the extracellular-space fluid, that 60% is the water content of the muscle segments, and that the equilibrium distribution coefficients, or q-values of Rb⁺, K⁺ and Na⁺ in the water of the muscle segments are all equal to 0.79 (for definition of the q-value, see Ling, 1984, p. 170; 1969, p. 18; see footnote on p. 143 for the source of the 0.79 figure). With these assumptions and the water- and ion-content data given in Table I, one estimates on the basis of the simplified equation 1 as applied to Rb⁺: $[Rb^+]_{ad} = \{[Rb^+]_{mu} - 0.09[Rb^+]_{ex}\} / (1 - 0.09) - 0.6q[Rb^+]_{ex}$, that at

TABLE I

Incubation (hrs)	H ₂ O (%)	[Rb*] _{in} (μmoles/g.)	[K ⁺] _{in} (moles/g.)	[Na ⁺] _{in} (μmoles/g.)
0	77.9	0 ± 0	90.7 ± 2.02	12.2 ± 1.38
0.6	70.3	5.13 ± 0.28	57.2 ± 1.78	30.4 ± 1.41
1	69.8	7.19 ± 0.20	41.8 ± 2.57	42.1 ± 2.16
1.6	67.5	8.29 ± 0.38	32.9 ± 1.33	48.1 ± 1.64
2	66.8	8.45 ± 0.30	31.4 ± 1.37	48.8 ± 1.53
2.8	63.9	8.32 ± 0.36	27.0 ± 2.14	48.5 ± 0.88
3.55	62.8	8.62 ± 0.15	20.7 ± 0.67	52.4 ± 2.55
4.28	61.7	8.61 ± 0.10	17.2 ± 0.53	52.0 ± 0.99
5	61.3	8.88 ± 0.36	18.6 ± 1.77	54.2 ± 1.65
6.05	60.5	8.79 ± 0.14	13.7 ± 0.29	55.8 ± 1.26

The time course of uptake of Na⁺ and of ⁸⁶Rb labelled Rb⁺ by and release of K⁺ from 2-mm-muscle segments at 0°C and pH 7.3. This table presents detailed numerical data graphically represented in Figure 2. For details see legend under Figure 2.

the 2-hour equilibrium, the ratios of the intracellular adsorbed ions (in units of μmoles per gram of fresh weight) over their respective extracellular concentrations (in units of molarity) are for Rb⁺, K⁺ and Na⁺ respectively 3.14; 9.31; and 2.34 in units of ml./g. At 4.28 hour, these ratios became respectively 3.21; 7.00; and 2.70. Throughout the entire 6-hour duration, a significantly higher preference for K⁺ over Na⁺ persisted, even though at magnitudes far lower than in normal intact muscles, in which case the ratios for K⁺ and Na⁺ are respectively 20.00 and 0.045 ml./g. (Ling and Bohr, 1971).

To be noted among the data given in Table I are the steadily declining water contents of the muscle segments, approaching a more or less constant level between 60% and 61% after approximately four hours from an original 80% in normal intact frog muscle. This shrinkage of the muscle segments will be the subject matter of another publication. Suffice it to say here, that the primary cause of this shrinkage is not the slight hypertonicity of the majority of the bathing solutions used (i.e., 5% above an isotonic 0.118 M NaCl solution) but is more complex, interesting and apparently related to the protective functions of PEG 8000.

Time course of ionic equilibrium in muscle segments at acid pH Figure 3 shows the time course of equilibration of labelled Na⁺ in muscle segments at an acid pH of 3.4. In contrast to the more complex one at neutral pH shown in Figure 2, the time course here is simple. Equilibrium was reached in 2 hours and maintained essentially unchanged for at least another 4 hours. The concentration of labelled Na⁺ after it has reached the plateau was

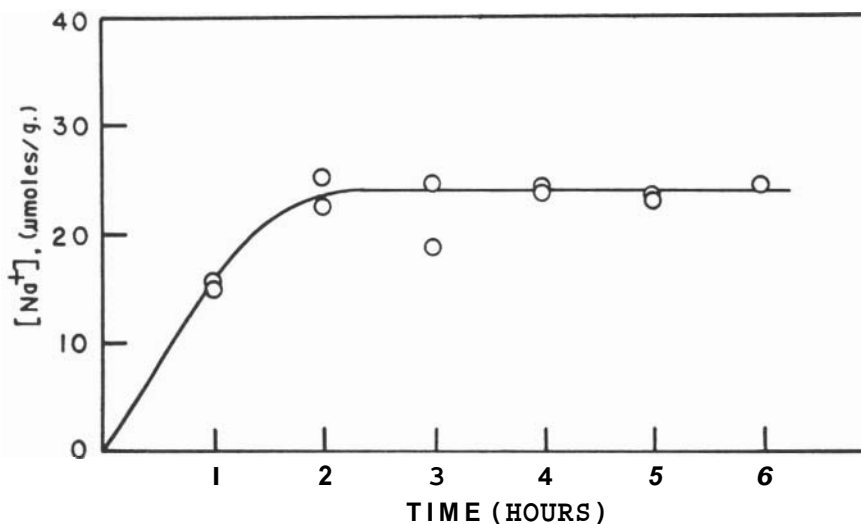


FIGURE 3. Time course of uptake of ²²Na labelled Na⁺ by 2 mm - 4 mm muscle segments at an acid pH (3.4) (0°C). Twelve half-muscle-units of 2mm- 4-mm muscle segments (from different frogs) were incubated in 30 ml of incubation solution, containing 12.5% PEG 8000, 1.9 mM KCl, 7.6 mM citrate buffer, 5.2 mM Na₂SO₄ at pH of 3.4. At each time interval indicated, 2 half-muscle-units were removed and analyzed. Each point in the graph represents a single determination.

barely 24 μmoles/g, and thus much lower than the level reached at 2 hours at neutral pH, suggesting that many adsorption sites were taken over by H⁺.

The data presented in Figures 2 and 3 show that a 4-to-5-hour incubation is adequate for achieving (metastable) equilibrium at both acidic and higher pH's, and was chosen for the study of ionic uptake at different pH's to be described after the section immediately following.

Labelled-Na⁺ efflux of intact and muscle segments in PEG-containing solution 312

To find out if the high viscosity of the PEG 8000-containing incubation solution (Solution 312) affects the rates of ionic traffic in and out of muscle cells, we carried out a labelled-Na⁺-efflux study of intact frog sartorius muscles at 1°C in such a solution. The results showed that the high viscosity of the bathing medium did not materially retard the Na⁺-efflux rate. Thus the half-time of exchange, or t_{1/2}'s of both the fast fraction (3.5 min.; 4 min.) and the slow fraction (90 min.; 127 min.) are either close to (fast fraction) or even faster (slow fraction) than the t_{1/2}'s from similar muscles washed in normal Ringer solution at a slightly lower temperature of 0°C (fast fraction, 4 min.; slow fraction, 170 min.) (Ling, 1962, Figure 11.25A).

We also carried out a labelled Na⁺-efflux study of 2- and 4-mm-segment assemblies of (whole) sartorius muscles. In this case, the main (slow) fraction of Na⁺ efflux has become much faster than from intact muscles. From 3 sets of data, the mean and S.E. of the half-time of exchange, t_{1/2}'s are 21.2 ± 1.77 min. The corresponding time for 99% exchange

($t_{0.99}$) are 149 ± 12.2 min. The $t_{0.99}$ obtained agrees with the time (2 hours) it took for diffusion equilibrium to be reached in 2-mm muscle segments from influx studies (Figures 2 and 3). This agreement between results of efflux and influx data is significant for at least two reasons: (1) it shows that the shift of ionic specificity among Rb^+ , K^+ and Na^+ ions from the 2-hour levels to the 4-hour levels reflect *intracellular* events; (2) it left no doubt that in studying ion distribution at different pH's after a 4-hour incubation (see the preceding section), we deal with (metastable) ionic equilibria in the cytoplasm, and not with rates of ion permeation, which too have been shown to be pH-dependent (Ling and Ochsenfeld, 1965).

The effect of pH variation upon the uptake of labelled Na^+ in PEG-protected 2-mm-muscle segments Figure 4 shows the uptake of labelled Na^+ by 2-mm-muscle segments at different pH's after incubation for from 4 hours to 4 hours and 50 minutes. We chose to study the uptake of labelled Na^+ due to the availability of the less expensive long-lived Na^+ isotope, ^{22}Na . In work to be reported elsewhere, we also studied the pH-dependency of the uptake of ^{86}Rb -labelled Rb^+ with essentially similar results. At pH above 6 to the highest pH reached (9.7), the uptake of labelled Na^+ remained essentially constant at approximately $70 \mu\text{moles/gram}$ of fresh-muscle-segment weight. Not shown in this figure and Figure 5

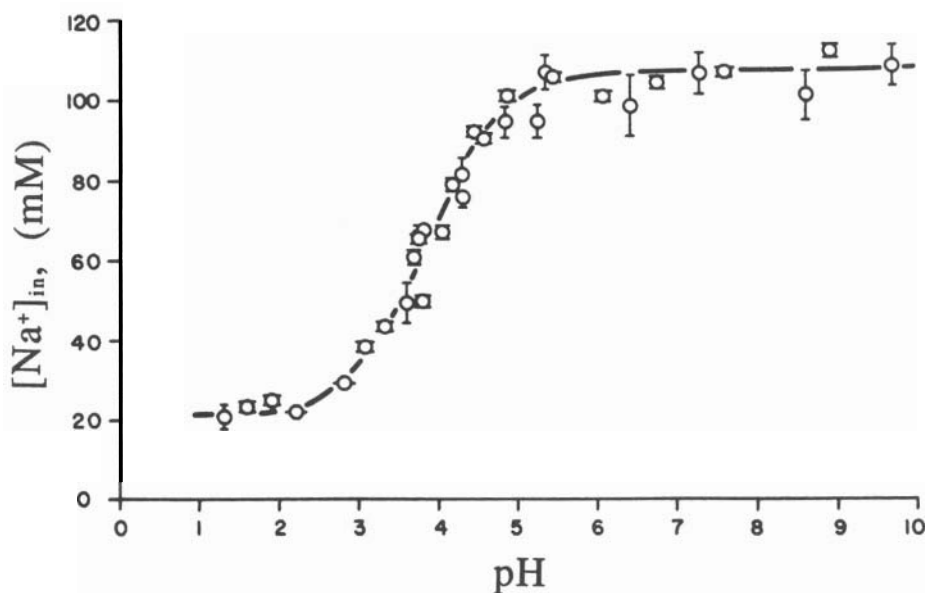


FIGURE 4. Uptake of labelled Na^+ by 2-mm-muscle segments at different pH's (0°C). Six half-muscle units from different frogs were included in a tube containing 30 ml of modified Solution 312 labelled with ^{22}Na . From the lowest pH to pH 7.5, $\text{H}_2\text{SO}_4 - \text{Na}_2\text{SO}_4$ buffers were used; for pH above 7.5, glycine- NaOH buffers were used as described under Methods. Each point represents the mean \pm S.E. for 4 half-muscle-unit samples. pH's of the incubation solution at the conclusion of the experiment were determined and shown in the graph and Table II. Ordinate represents uncorrected labelled Na^+ concentration in total tissue water given in molarity. The symbol $[\text{Na}^+]_{\text{in}}$ used here has the same meaning as $[\text{Na}^+]_{\text{mu}}$ in equation 1.

following is the retention respectively of 20 and 30 pmoles of K⁺ per gram of fresh muscle segment weight. A more complete presentation of the experimental data of this series of experiments is given in Table II.

Based on the data given in Table II and equation 1, we obtained the labelled-Na⁺ concentrations in (pure) muscle-cell segments at different pH's shown in Figure 5. The unit of labelled Na⁺ is $\mu\text{moles/gram}$ of fresh-muscle-segment weight. The standard errors of the means, not shown, are of course quite similar to those shown in Figure 4. The concentration of labelled Na⁺ is high and essentially unchanging at pH 6 to 9.69 pH; it decreases with decreasing pH from pH 6 on down. At pH between 2.3 and 1.3 — the lowest pH reached in this series — the decrease of labelled-Na⁺ uptake levelled off. By extrapolation, one estimates that as the pH approaches 0, the labelled-Na⁺ concentration approaches 9 $\mu\text{moles/gram}$ of fresh-muscle-segment weight. This sharp reduction in the level of labelled-Na⁺ concentration at low pH's indicates that when the concentration of H⁺ reaches a high enough level, it effectively displaces the labelled Na⁺ adsorbed*. The water content of the muscle segments was $61.9\% \pm 0.24\%$ (mean \pm S.E.M.) (Table II). It seems reasonable to assume that the 9 $\mu\text{moles/gram}$ of labelled Na⁺ found at near 0 pH is mostly dissolved in this water (see Ling, 1991, Sect. 8.1) and at a concentration of approximately $910.619 = 14.5 \text{ mM}$, to be compared with the labelled Na⁺ concentration in the bathing solution at the same low pH range (pH 1.31 to pH 2.32): $18.4 \pm 0.12 \text{ mM}^{**}$.

Between pH 1.3 and 6, the uptake of labelled Na⁺ follows a sigmoid-shaped curve, typical of acid titration data. To obtain the true inflection point of this part of the curve, the value of 9 $\mu\text{mole/g}$. must be subtracted from all the figures (since this amount of labelled Na⁺ found in the segment water, was not adsorbed and thus not directly influenced by the pH). After making this adjustment, we obtained from the data given in Figure 5 (and Table II), an inflection point at pH 3.85. This pKa falls well within the range of pK's of β - and γ -carboxyl groups. Thus the pK₂ of the β -carboxyl groups of aspartic acid is 3.65; the pK₂ of the γ -carboxyl groups of glutamic acid is 4.25 (Stecker et al., 1968, p. 107 and p. 497). There are no other known acidic groups in frog muscle totalling more than 70 $\mu\text{mole/gram}$ of fresh muscle that has a pKa of, or near 3.85.

Discussion and Conclusion

The main conclusion: K⁺ and its surrogates are adsorbed on β - and γ -carboxyl groups

Prior investigations have established that selective accumulation of K⁺ in living cells results

* The equilibrium distribution coefficient or q-value of labelled Na⁺ in the segment water equals $14.5/18.4 = 0.79$. It is much higher than the q-value of Na⁺ in normal intact muscle cells (0.18) (Ling, 1969, p. 24, Figure 15). In theory at least, this rise of the q-value of Na⁺ can be attributed to the reduced q-value of Na⁺ in the PEG-8000-containing-bathing solution itself. Thus from the published data of Ling and Ochsenfeld (Figure 6, Ling and Ochsenfeld, 1983), the q-value of Na⁺ (as sodium citrate) of a 16.7% PEG solution is below 0.4, when compared with that in a normal aqueous solution. A rough estimate of $0.79 \times 0.4 = 0.32$ for the q-value of Na⁺ in the water of the cut-muscle segment is obtained, when compared to a normal aqueous solution like the Ringer solution. While this estimate at least reassures us that the water in muscle segments under the protective actions of PEG 8000 has not completely deteriorated to the physical state of normal liquid water (with a q-value of 1.0), too much emphasis should not be placed on the specific figures obtained due to the complicated route by which they are derived.

** Edelmann (1991) showed recently that the *in vitro* Cs⁺ uptake by thin EM muscle sections in the presence of LiCl was greatly reduced when the pH of the staining solution was reduced to 3, supporting the conclusion reached here.

TABLE II

Expt. No.	pH	[Na*] _{mu} (μ moles/g.)	[Na*] _{ct} (μ moles/g.)	[Na*] _{cell} (μ moles/g.)
6H8I	1.31	11.31 \pm 1.70 (61.0;18.2)	12.6 (64.7)	10.9
6H8II	1.60	13.21 \pm 0.54 (62.2;18.2)	13.1 (58.7)	13.0
6H8III	1.93	13.98 \pm 0.71 (62.1;18.8)	10.3 (52.6)	14.2
6H8IV	2.32	13.25 \pm 0.19 (61.3;18.5)	11.2 (55.4)	13.2
6H8V	2.82	17.87 \pm 0.26 (61.1;18.9)	9.62 (45.7)	18.7
6H8VA	3.07	23.72 \pm 0.94 (63.2;18.3)	12.0 (51.0)	25.2
6H15a	3.32	27.11 \pm 0.79 (62.6;18.6)	11.7 (58.3)	29.1
6H15b	3.60	30.54 \pm 2.92 (61.5;18.2)	13.0 (55.7)	32.9
6H22f	3.69	37.55 \pm 1.22 (61.5;18.4)	14.4 (61.0)	40.8
6H22g	3.76	38.78 \pm 1.46 (59.0;18.2)	14.3 (56.7)	42.2
6H8VI	3.80	30.08 \pm 0.56 (60.8;18.3)	11.9 (58.8)	32.5
6H22h	3.81	41.01 \pm 0.73 (58.2;18.2)	12.9 (58.3)	44.9
6H22n	4.05	41.87 \pm 1.29 (62.6;17.5)	15.0 (57.5)	45.6
6H22i	4.18	47.16 \pm 1.23 (59.6;18.2)	14.9 (57.7)	51.7
6H22j	4.27	48.20 \pm 2.40 (59.0;18.3)	15.3 (58.8)	52.9
6H15c	4.30	45.38 \pm 1.21 (60.1;18.1)	14.3 (50.8)	49.7
6H22k	4.45	55.64 \pm 0.72 (60.5;18.7)	16.1 (64.7)	61.3
6H8VII	4.57	54.99 \pm 0.57 (61.0;17.4)	13.4 (59.2)	60.9
6H8VIIA	4.84	61.49 \pm 0.66 (61.0;17.9)	12.3 (62.2)	68.4
6H15d	4.86	58.77 \pm 2.20 (61.9;18.1)	18.5 (64.7)	64.6
6H8VIIIB	5.25	60.21 \pm 2.41 (63.2;17.8)	19.9 (65.7)	66.2
6H22m	5.35	65.81 \pm 3.19 (61.9;18.1)	18.5 (64.7)	72.7
6H22l	5.38	64.78 \pm 0.49 (61.3;18.3)	17.0 (62.3)	71.7
6H22e	6.05	68.98 \pm 0.38 (62.2;18.0)	19.1	76.3
6H8VIII	6.42	61.06 \pm 5.31 (62.2;17.7)	20.0 (66.0)	67.1
6H8IX	6.76	63.23 \pm 1.11 (61.0;18.2)	20.5 (67.1)	69.5
6H8XI	7.28	68.58 \pm 3.18 (62.1;16.1)	20.3 (71.0)	75.8
6H8XVI	7.63	67.57 \pm 0.75 (63.1;18.2)	19.7 (63.3)	74.6
6H8XVIA	8.60	65.03 \pm 3.91 (63.9;18.2)		71.7
6H8XVII	8.89	68.75 \pm 1.11 (61.5;18.3)	19.7 (67.9)	75.9
6H8XVIII	9.69	67.31 \pm 3.02 (61.8;18.2)	33.6 (70.0)	72.8

Detailed data on the effect of pH on the total labelled Na⁺ concentrations in whole muscle tissue (third column) in the connective tissue complex (fourth column) and in (pure) muscle segments (fifth column). The average labelled Na⁺ concentration in the third column is given in units of μ moles of labelled Na⁺ per gram of fresh whole muscle tissue, plus/minus the standard error of the mean from four assays. The first number in the parenthesis under this column is the percentage (w/w) of water content of the muscle sections; the second number is the final labelled Na⁺ concentration in the bathing medium in millimolarity. The labelled Na⁺ concentration of connective tissue complex — isolated from the same frogs providing the sartorius muscles and treated in the same way as the muscles — is also given in units of pmoles per gram of fresh total connective tissue complex without corrections. The number in the parenthesis under this column is the percentage (w/w) water content of the wet connective tissue. The labelled Na⁺ concentration in (pure) muscle segments given in the fifth column is in units of pmoles of labelled Na⁺ per gram of fresh muscle cell segments. The SEM's are not shown but are similar to the corresponding total labelled Na⁺ concentration shown in the third column.

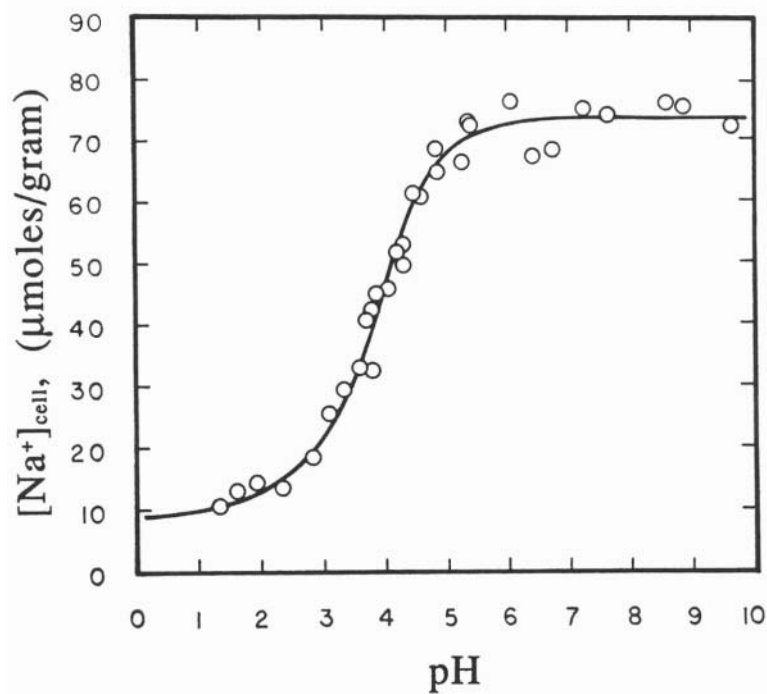


FIGURE 5. The effect of pH on the labelled Na⁺ concentration in 2-mm wide, open-ended frog sartorius muscle segments. Labelled Na⁺ concentration is that exclusively in the cell segments, calculated from the data given in Table II with the aid of equation 1 and given in μmoles per gram of fresh muscle cell (segments). Standard error of the means, not shown, are not very different from those shown in Figure 4.

from close-contact, one-on-one adsorption of the ion onto cytoplasmic sites (for recent review, see Ling, 1990; 1991, Section 4.4). In addition, these investigations showed that Tl⁺, Li⁺, Na⁺, Rb⁺, Cs⁺ as well as K⁺ compete for the same sites (Ling and Bohr, 1971; Ling, 1977a).

As described in the introductory section of this paper, the *main objective of this investigation was to test the postulate that β- and γ-carboxyl groups of cell proteins provide the adsorption sites for K⁺, Na⁺, and other monovalent cations. The data presented in Figure 5 and Table II has fully confirmed this postulate.* This then is our main conclusion. However, this is not the only conclusion. Other available data to be reviewed below furnish evidence (1) that *myosin* provides the majority of the K⁺-adsorbing β- and γ-carboxyl groups; and (2) that the K⁺-adsorbing anionic β- and γ-carboxyl groups on *myosin heads* and cationic groups on *actin* form cross bridges during muscle contraction.

Most muscle-cell K^+ and its surrogate monovalent cations are adsorbed on β - and γ -carboxyl groups in the A bands Based on the available data obtained in the late 1950's, Ling and Ochsenfeld estimated that more than sixty percent of the β - and γ -carboxyl groups belong to myosin (Ling and Ochsenfeld, 1966, see footnote*). In striated muscle, myosin is found only in the A bands (Engelmann, 1873; Huxley, 1880; Hanson and Huxley, 1953). When combined, these two sets of facts led to the prediction that much of the selectively accumulated K^+ (or its surrogates, Rb^+ , Cs^+ , Tl^+) in striated muscle should be found in the A bands (Ling, 1977). This prediction has been extensively tested and confirmed in more recent studies (Ling, 1977; Edelmann, 1977; for reviews, see Edelmann, 1984, 1988; Ling, 1991, Section 4.4), as well as in studies published earlier (Nesterov and Tigyi-Sebes, 1965; see also footnote on p. 320 in Ling, 1977). In this undertaking and its continued pursuit thereafter, Dr. Ludwig Edelmann has made extensive important contributions.

In the earlier efforts both Ling and Edelmann took advantage of the fact that Cs^+ and Tl^+ ions can stoichiometrically and physiologically replace the K^+ in living frog muscle cells (Ling and Ochsenfeld, 1966; Ling and Bohr, 1969, 1971; Ling, 1977a). The availability of inexpensive, long-lived radioactive isotopes of these two ions, then made it possible to use autoradiography to test and eventually to confirm the predicted localization of Cs^+ and Tl^+ (and indirectly K^+) at the A band (Ling, 1977). Edelmann utilized another attribute of this pair of ions, namely, their high electron density. Because of this high electron density, Cs^+ and Tl^+ can be visualized directly in EM sections of muscle (whereas the electron-light K^+ normally present in the cell cannot), after these ions were introduced into the muscle cells either **physiologically** followed by freezing and imbedding (Edelmann, 1977), or, as in Edelmann's later work with Cs^+ , after the muscle cells were frozen, imbedded and cut into thin sections (Edelmann, 1984, p. 884; 1991).

Both the early autoradiographic study of Ling and the early **transmission-electron** microscopy study of Edelmann had clearly confirmed the predicted concentration of these surrogate ions for K^+ in the A band of frog muscle. In varying degree of clarity, these studies also revealed that Cs^+ and Tl^+ existed at higher density in the two marginal zones of the A band than in the more central portion of the A band. A much smaller amount of these ions, though at high density, was also taken up by the Z line. Uptake of these ions in the I band (not including the Z line) was less intense (see below; Edelmann, 1977, 1986).

Even in the early days, it became obvious that the autoradiographs and in particular the transmission electron-microscopic (EM) pictures of muscle sections physiologically loaded with Cs^+ or Tl^+ show remarkable resemblance to pictures of EM sections prepared by the conventional procedures and stained with uranyl and lead ions. This resemblance suggests that much of the uranyl and lead ions in conventional EM pictures adsorb on similar β - and γ -carboxyl groups that adsorb Cs^+ and Tl^+ (Ling, 1977). One recalls that in 1960, Hodge and Schmidt (1960) suggested that (cationic) uranium (also) binds onto β - and γ -carboxyl groups in the fixed and stained protein, tropocollagen. (For reference to even earlier work

* With progress in the techniques of isolating and **identifying** proteins, and with the discoveries of new proteins, the percentage composition of muscle proteins has seen significant changes since the time we **first** made the estimate of the myosin carboxyl groups. The latest estimates provided by Murayama (1986) and Ohtsuki *et al.* (1986) are as follows: myosin (43%); **actin** (22%); tropomyosin (5%); troponin (5%); **titin (i.e., connectin)** (10%); nebulin (5%); M-protein (2%); C-protein (2%); α -actinin (2%). Based on this set of figures, and the amino-acid-residue analyses available and certain simplifying assumptions, the percentage of β - and γ -carboxyl groups in myosin is now estimated to be about 47%.

on this important subject, see Kühn *et al.*, 1959; Home *et al.*, 1956). Ling and Edelmann's findings described above lend support to the validity and broader applicability of this view.

Why are the two edges of the A bands darker? The two edges of the A band is where the thin and thick filaments overlap in the resting muscle. As a result, there is a higher electron density at the edges of the A bands due to the higher density of atoms belonging to both kinds of filaments in these overlapping regions.

However, from EM photographs of various muscle-cell preparations including those which are naturally "loaded with K⁺" (Edelmann, 1977, Figure 1, frame 5; 1984, Figure 7a); or having been loaded with electron-dense ions which were subsequently removed by leaching (Edelmann, 1977, Figure 1, frame 4) or during wet cutting (Edelmann, 1984, Figure 2c), and especially from frozen hydrated normal unstained muscle sections (Edelmann, 1986, Figure 5b; 1988 Figure 4a), we estimated that the contribution of higher protein density *per se* to the higher electron density of the two marginal zones in EM sections is minimal.

Rather, it is the adsorbed electron-dense ions Cs⁺, Tl⁺ as well as uranyl and lead ions that contribute the lion's share of the electron density of the marginal zones of the A band and the Z line. The following experimental fact provides support for this view:

In autoradiographic studies of air-dried (Ling, 1977) and frozen-hydrated muscles (Edelmann, 1980), the silver granules are found at much higher density at the two margins of the A band than at the center of the A band and at the I band (excluding the Z line). Clearly, the density of silver granules reflect *only* the location of the radioactive Cs⁺ or Tl⁺ and not the electron density of the proteins present.

While higher protein density *per se* at the marginal zones makes but a very minor contribution to the high electron density of the marginal zones of the A band, the higher protein density at the marginal zones of the A band may, in theory at least, play an *indirect* role in enhancing the electron density at the marginal zones of the A band. Since the K⁺-adsorbing carboxyl groups may be present on both the thick and the thin filaments, more K⁺, its surrogate ions, as well as uranyl and lead may be adsorbed in the overlapping zones than in the region of A band where only the thick filaments are found. In support of this interpretation, Trombitas and Pollack (1991) — confirming early work of H. E. Huxley (1953) and of H. E. Huxley and Hanson (1954) — showed that with increasing degree of stretching, the darker-staining marginal zones of the A band (in uranium- and lead-stained frog-muscle sections) became narrower and narrower until they vanished when the thin filaments were entirely pulled out of the A band. (Nonetheless, some dark-staining material projecting from the thick filaments remained, specially in lateral regions that are stained the darkest in normal muscle at resting length.)

To what proteins in muscle belong the β- and γ-carboxyl groups adsorbing K⁺ and its surrogates? As Edelmann's new techniques improved, he was able to demonstrate more details in the Tl⁺ and Cs⁺ "stained" EM sections. Thus Cs⁺ (introduced by Edelmann's *in vitro* section-staining procedures, see Edelmann, 1984, 1991) and Tl⁺ (loaded physiologically) in the A bands, are not uniformly spread over the A bands. Rather, they are concentrated at the *thick filaments* in the A band, and at the less well-defined materials between the thick filaments in the two marginal zones of the A bands (for example, see Figure 14d in Edelmann, 1984; Figures 16a and 16b in Edelmann, 1986a). *The incorporation of these K⁺ surrogate ions into the whole lengths of the thick filaments in the A bands offers direct*

evidence that a substantial amount of K^+ (and its surrogates) is indeed adsorbed on myosin, which makes up virtually all the proteins of the thick filaments.

Evidence to be presented toward the end of this Discussion led us to believe that, beside overlapping thin filaments, the dark-staining materials between the thick filaments may include *myosin heads* projecting away from the thick filament proper.

An estimation of the percentage and absolute concentration of muscle-cell K^+ adsorbed on the β - and γ -carboxyl groups of myosin. While autoradiography and transmission electron microscopy have revealed that the K^+ -surrogate ions, Cs^+ and Tl^+ are adsorbed in the A band (and the Z line), *dispersive X-ray microanalysis* provides the means of demonstrating the localized distribution of K^+ itself in the A band (and Z line) (Edelmann, 1978, 1983, 1984; Trombitas and Tigyi-Sebes, 1979).

Dispersive X-ray microanalysis cannot only detect that presence of K^+ itself, it can also yield data on the concentration of K^+ in small loci of ultrathin (EM) sections. In early studies using this new technique, estimates had been made of the *relative concentration of K^+* in different regions of the sarcomere: more K^+ was found in the A band than in the I band (without the Z line) and that higher concentration of K^+ was found in the Z line than in the A band (Edelmann, 1978, 1983). However, it was in the recent studies of von Zglinicki (1988) that the latest improvements of the technique were further exploited. As a result, *the absolute concentrations of K^+ , Na^+ , Cl^- and phosphorus as well as percentage dry weights in very small loci of ultra-thin, heart-muscle sections* were obtained for the first time.

von Zglinicki determined the ionic concentrations and dry mass of two small circular loci (each approximately $0.1 \mu m^2$ in area) in ultrathin sections (100–200 nm thick) of frozen-dried rat heart muscle. These loci were respectively in the I band including the Z line, and in the A band at the region where the thick and thin filaments overlap. The K^+ concentrations determined are respectively 128 ± 13 ($n = 20$) $\mu moles/g.$ of compartment water in the A band; and 47 ± 7 ($n = 20$) $\mu mole/g.$ of compartment water in the I band. The corresponding dry masses are $24\% \pm 1\%$ ($n = 16$) of the wet weight in the A band and $15 \pm 1\%$ ($n = 16$) of wet weight in the I band. In all cases, n represents the number of assays performed.

Based on these figures, we obtained the K^+ concentration of $97.3 \mu moles/g.$ of fresh weight in the overlapping region of the A band, and $40 \mu moles/g.$ of fresh weight in the I band containing the Z line. This unit conversion is necessary because our objective was to determine ion concentrations in different parts of the *whole* muscle substance, including both water and "dry matter."

From von Zglinicki's Figure 1 and the measured average width of Z lines obtained from his figure as well as other EM pictures (0.055 μm , see below), we estimated that roughly 20%* of the circular area in the I band he chose for study is occupied by the Z line. Assum-

* We are aware that Figure 1 represents only one of many similar sections from which von Zglinicki derived his average ionic concentrations and compartment dry weights. Since von Zglinicki made no special mention of arbitrarily assigning a special location of the Z line in the circular area he examined, we figure that in most of his sections examined, the location of the Z line in the loci should be not too different from that shown in the illustrative figure, i.e., not exactly at, but not too far away from the center. It was on the basis of this assumption that we felt justified in using the 20% figure. However, if under the highly unlikely situation, that the average position of the Z line in the circular locus examined is strongly displaced from the center of the circular locus, and as a result, the length of the Z line enclosed reduced to one half the diameter of the circle, the calculated K^+ concentration in the I band (without the Z line) and in the A band (without the thin filaments and connectin, see below) would become respectively 34 and 63 $\mu moles/g.$ of fresh weight (instead of 25.7 and 71.6 $\mu moles/g.$). This supplementary calculation shows that even under the extremely unlikely scenario described, the predominant presence of K^+ in the A band would stay unchanged.

ing that the K⁺ concentration in the Z line is equal to that of the A band in the overlapping area (i.e., 97.3 $\mu\text{mole/g.}$), we obtained a concentration of K⁺ in the I-band area away from the Z line of $(40 - 97.3 \times 0.2)/0.8 = 25.7 \mu\text{mole/g.}$ of fresh weight.

As part of the effort to determine the concentration and amount of K⁺ adsorbed on myosin in rat heart muscle, we introduce in the following paragraphs the data as well as simplifying assumptions used in separating the K⁺ associated with non-thick-filament proteins from the K⁺ associated with the thick-filament proteins in the marginal zones of the A band. This separation relied on the fact that the non-thick-filament proteins (thin filament proteins, connectin and desmin) exist at roughly the same concentration in the overlapping marginal zones of the A band as that in the I band in the relaxed heart muscle sarcomere.

Major proteins in the I band of voluntary muscles include those belonging to the thin filaments (actin, tropomyosin and troponin), titin (alias connectin) and nebulin (which is present only in the I band) (Wang, 1988; Maruyama, 1989). Heart muscle does not contain nebulin (Locker, 1984; Hu *et al.*, 1986); it does contain a protein absent in the skeletal muscle: desmin (Tokuyasu, 1983).

Titin exists as very long filaments starting at the M-line (0.15 μm from the center of the A band) extending all the way to the Z line (Maruyama, 1986; Nave *et al.*, 1989). Thus, roughly speaking, one may expect that in a sarcomere maintained at its resting length, the marginal zones of the A band and the I band (not including the Z line) contain a similar concentration of titin*. Accordingly, with the muscle at its resting length, the concentration of K⁺ adsorbed on titin in the marginal zone of the A band and on titin in the I band may be regarded as similar;

The overall structure and composition of the thin filament are uniform throughout its length, including the part in the A band. Similarly, desmin exists as long (intermediary) filaments extending from one Z line to the next. Again assuming that the K⁺-adsorbing properties are uniform throughout their lengths, the K⁺ associated with the thin-filament proteins and with desmin in the overlapping region of the A band and in the I band in muscle at its resting length may be assumed to be similar. Therefore, we were able to obtain the K⁺ concentration of the thick filaments in A band of rat heart muscle by simply subtracting the K⁺ concentration of the I bands (made up of thin-filament proteins, connectin and desmin) from the K⁺ concentration in the overlapping regions of the A band. Since the thick-filament proteins is to all intent and purposes myosin, we obtain the concentration of K⁺ adsorbed on myosin: $97.3 - 25.7 = 71.6 \mu\text{moles/g.}$ fresh myosin.

Our next task is to determine the relative quantities of K⁺ in the Z line, in the two half I band and on myosin. To achieve this goal, we determined next the relative volumes of these "compartments" in the fully stretched sarcomere (zero overlap) by assessing the average width of the A band, that of the fully pulled-out I band and that of the Z line.

Estimating the widths of these compartments, we began with von Zglinicki's Figure 1. From the more intact portion of the figure, we were able to estimate roughly that the sarcomere length is $2.84 \pm 0.097 \mu\text{m}$ and the A band width is $1.66 \pm 0.040 \mu\text{m}$. However, the data does not permit an estimate of the length of the fully pulled-out half I bands. Fortunately,

* There are some suggestive evidence that titin (connectin) does not adsorb a great deal of K⁺. First, connectin has been known to be a "salt-insoluble" protein (Maruyama, 1976). A common salt used for protein extraction from muscle is KCl. As a rule salt-insolubility indicates low affinity for salt ions, which in the present case may include K⁺. Second, the amino acid composition of connectin resembles that of actin (Maruyama, 1976, p. 90), which does not adsorb K⁺ *in vitro* (Lewis and Saroff, 1957).

ly, Drs. Charles Trombitas and Gerald Pollack of the University of Washington had graciously made available to us their yet-to-be-published EM plates of frog muscle with fully pulled-out I band. From their pictures and other good EM plates of frog voluntary muscle we could find in the literature, we obtained an average A band width of 1.6 μm (in agreement of the figure given by Page and H. E. Huxley, 1963), and the total width of the two fully pulled-out I band equal to $2 \times 1.0 \text{ pm}$.

At first look, these figures appear smaller than those obtained from the rat-heart muscle. However, apparently due to the complex structure of the heart muscle, it was difficult to make orthogonal cuts of the rat heart muscle preparation. As a result, the myofilaments in von Zglinicki's Figure 1 are not perpendicular to the Z line but at a $60^\circ/120^\circ$ angle. Correcting for the artificial lengthening of the sarcomere and A band, we found that in reality, the rat heart muscle and frog skeletal muscle have closely similar measurements. On this basis, and the fact that it is the *relative* length and not absolute length that matters, we feel justified in using twice the length of fully pulled-out half I band of the frog muscle, i.e., $2 \times 1.0 = 2.0 \mu\text{m}$ for the I band width of the rat heart muscle. In a similar way we derived the sum of two half Z-line width in rat heart muscle of 0.055 μm .

Assuming that the diameter of the fully stretched sarcomere is uniform along its length, we equated the width ratios of the three compartments to their respective volume ratios, i.e., A band, 43.8%; two (fully pulled-out) half I bands, 54.7%; two half Z lines, 1.5%.

Multiplying these relative-volume values by their corresponding K^+ concentrations yields the following figures for the percentage quantity of K^+ on myosin, 66.9%; on I-band proteins away from the Z line, 30.0%; on Z-line proteins, 3.1%.

The accuracy of these calculations rests upon the validity of the various assumptions made and, above all, on the accuracy of von Zglinicki's data. The large number of assays he made and the small standard errors of the means are reassuring.

While most of the figures used in the calculations are derived from the published data in the (more or less straightforward) way described, one assumption we made may appear arbitrary, i.e., that the K^+ concentration in the Z line is the same as that found in the overlapping region of the A bands. To reduce this arbitrariness, we will next make some additional **computations** based on different K^+ concentrations assumed in the Z-line. Before discussing these additional calculations, we want to reiterate that there are independent evidence that the concentration of K^+ and its surrogate ions are actually *higher* in the Z line than in the edges of the A band (Edelmann, 1978; 1984, Figure 13a). Nonetheless, the **Z-line- K^+** concentration cannot exceed 200 $\mu\text{moles/g}$. fresh weight. Otherwise, the **I-band- K^+** concentration calculated would fall below zero and become negative.

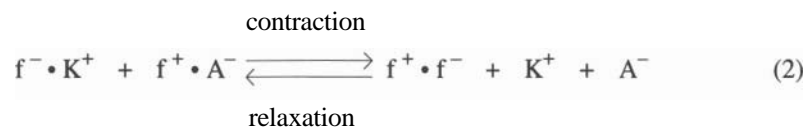
If we choose a **Z-line- K^+** concentration to have a value in between the two sets of figures discussed (97.3 and 200 $\mu\text{mole/g}$.) i.e., 150 $\mu\text{mole/g}$., the K^+ concentrations in myosin will become 84.8 $\mu\text{mole/g}$., 12.5 $\mu\text{mole/g}$. in the I band; and, of course, 150 $\mu\text{mole/g}$. in the Z line. The corresponding relative amount of the K^+ in the three compartments would become 80.3% (myosin); 14.8% (I band), and 4.86% (Z line).

From all the available data on hand, we believe that the last set of estimates may be closer to the truth. Only this estimate takes into account the evidence that the K^+ (and surrogate ion) concentration is significantly higher in the Z line than at the edges of the A bands (Edelmann, 1978, 1984). In addition, the much lower **I-band- K^+** concentration agrees with the much lower density of silver granules in the I band (excluding the Z line) than in the A band

(Ling, 1977; Edelmann, 1980), and with the fact that **actin**, the major protein of the thin filaments, does not bind K⁺ *in vitro* (Lewis and Saroff, 1956).

In conclusion, wide variation in the value assumed for the K⁺ concentration in the Z line affects primarily the estimated value of K⁺ concentration in the I bands. In all cases, myosin* remains the dominant source of β- and γ-carboxyl groups adsorbing K⁺, accounting for from 67% to as high as 80% of the total myofibrillar K⁺. We may safely conclude that *the majority of, or more K⁺ in muscle cells are adsorbed on the β- and γ-carboxyl groups of myosin.* This then is our second major conclusion.

Evidence that the K⁺ adsorbing β- and γ-carboxyl groups of muscle proteins play a critical role in muscle contraction We now review a hypothesis that links muscle *contraction and relaxation* respectively to the *desorption and adsorption* of K⁺ from the β- and γ-carboxyl groups of (some) muscle protein(s). This hypothesis was first presented with Ling's earliest model of K⁺ adsorption nearly forty years ago (Ling, 1952), and reiterated and expanded further in years following (Ling, 1962, p. 437; 1984, p. 546). Put in the simplest form, muscle contraction involves the following cyclic event:



The contraction cycle in this hypothesis involves the formation of salt-linkages (f⁺ • f⁻) followed by their dissociation. The salt linkages are formed between fixed anionic β- and γ-carboxyl groups (f⁻) — which in resting muscle cells adsorb K⁺ — and fixed cationic ε-amino or guanidyl groups (f⁺) — which in resting muscle adsorb an unspecified anion, A⁻. Relaxation involves the dissociation of the salt linkages, and the reabsorption of K⁺ and A⁻ on the once-more-liberated f⁻ and f⁺.

In the thirty years following the introduction of this hypothesis, the postulated adsorbed state of muscle K⁺ has been extensively tested and confirmed. There are strong evidence that 98% of the K⁺ in muscle cells, equal to 85.8 μmole per gram of fresh muscle cells is adsorbed (Ling, 1991, Section 8.1.3). The present study shows that the majority or even more than a simple majority of this adsorbed K⁺ is adsorbed on myosin, the major contractile protein of muscle cells.

A prediction of this hypothesis is that during muscle contraction, K⁺ is reversibly released from its adsorption sites on myosin. ~~Part~~ of the released K⁺ might be expected to leave and later to return to the muscle cells. In fact, evidence for such a reversible release of K⁺ from contracting muscle has been in the literature for a long time and will be reviewed next.

* Two proteins known to be present in the A band and at significant concentrations have not been taken into account. They are the M protein (2% of total muscle protein) present in the M line at the middle of the A band; and the C protein (also 2%) attached to myosin (Maruyama, 1986). The M line takes up more Cs⁺ and Tl⁺ than the central major portion of the A band but is flanked immediately by smaller areas (pseudo-H band) taking up less Cs⁺ and Tl⁺. Ignoring the M protein does not affect the estimated K⁺-concentration adsorbed on myosin. However, ignoring the C protein would artificially reduce the estimated K⁺ adsorbed on myosin by about 5%, if the C protein itself does not adsorb K⁺ at all. On the other hand, if the C protein adsorb K⁺ at a level comparable to that of myosin, then ignoring the C protein would also not affect the estimated concentration of K⁺ adsorbed on myosin.

Evidence that K^+ is reversibly released during normal muscle contraction. The following evidence demonstrate (reversible) release of muscle K^+ during normal contractions of voluntary, cardiac as well as smooth muscle.

(1) *Voluntary muscle.* Figure 6 reproduces the findings of Wood *et al.* (1940). The solid curve demonstrates the loss and regain of K^+ from a **perfused** dog gastrocnemius muscle in a heart-lung-gastrocnemius-muscle preparation during and following a brief tetanus brought about by **electrical** stimulation of the sciatic nerve. The dotted curve indicates the rate of K^+ loss and regain.

(2) *Heart muscles.* Wilde *et al.* (1956) **perfused** a turtle heart which had been previously loaded with radioactive K^+ and collected continually the effluent from the coronary vessel. Figure 7 shows the simultaneous recording of the electrocardiogram (EKG), coronary flow volume and reversible release of radioactive K^+ during 6 successive contraction cycles of the heart.

(3) *Smooth muscle.* Increased release of K^+ was also found to regularly accompany the stimulation of cat small intestine (Lembeck and Strobach, 1955).

Other efforts aimed at demonstrating an expected increase of membrane permeability to K^+ in response to electrical excitation in frog muscle (Noonan *et al.*, 1941) and rat diaphragm muscles (Creese *et al.*, 1958) ended in failure. K^+ permeability of the muscle cells remained unchanged during contraction. At that time, this contrast between rapid release and regain of K^+ during muscle contraction and the lack of K^+ permeability change at the same time posed a dilemma.

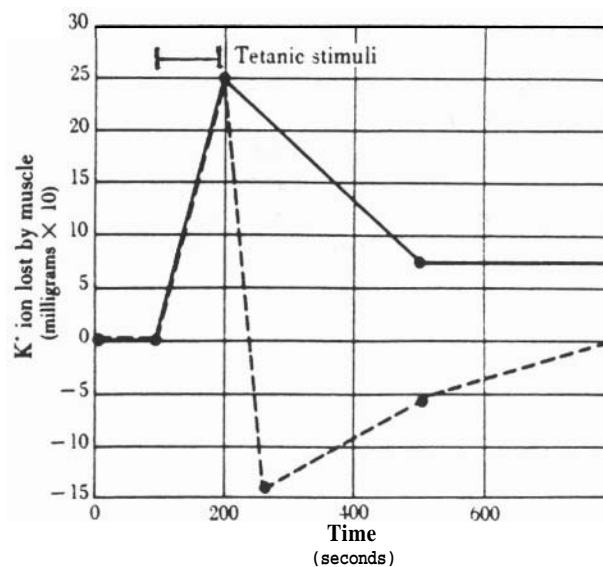


FIGURE 6. K^+ loss during a sustained **tetanic** contraction of (and its later regain in) a dog gastrocnemius muscle in a **perfused** heart-lung-gastrocnemius preparation. Electrical stimulation was applied on the sciatic nerve innervating the muscle, for a duration indicated by the horizontal line. — K^+ loss by muscle; - - rate of K^+ loss. (Data of Woods *et al.*, 1940. Picture from Ling, 1962).

Now that we know the bulk of K⁺ to be adsorbed, and that the muscle cell membrane is highly permeable to K⁺ at all times (Ling, 1984, p. 405), these findings are readily explained by a reversible desorption of the adsorbed K⁺ and its partial escape from the muscle cells followed by its return to the muscle cells and resorption on muscle proteins in agreement

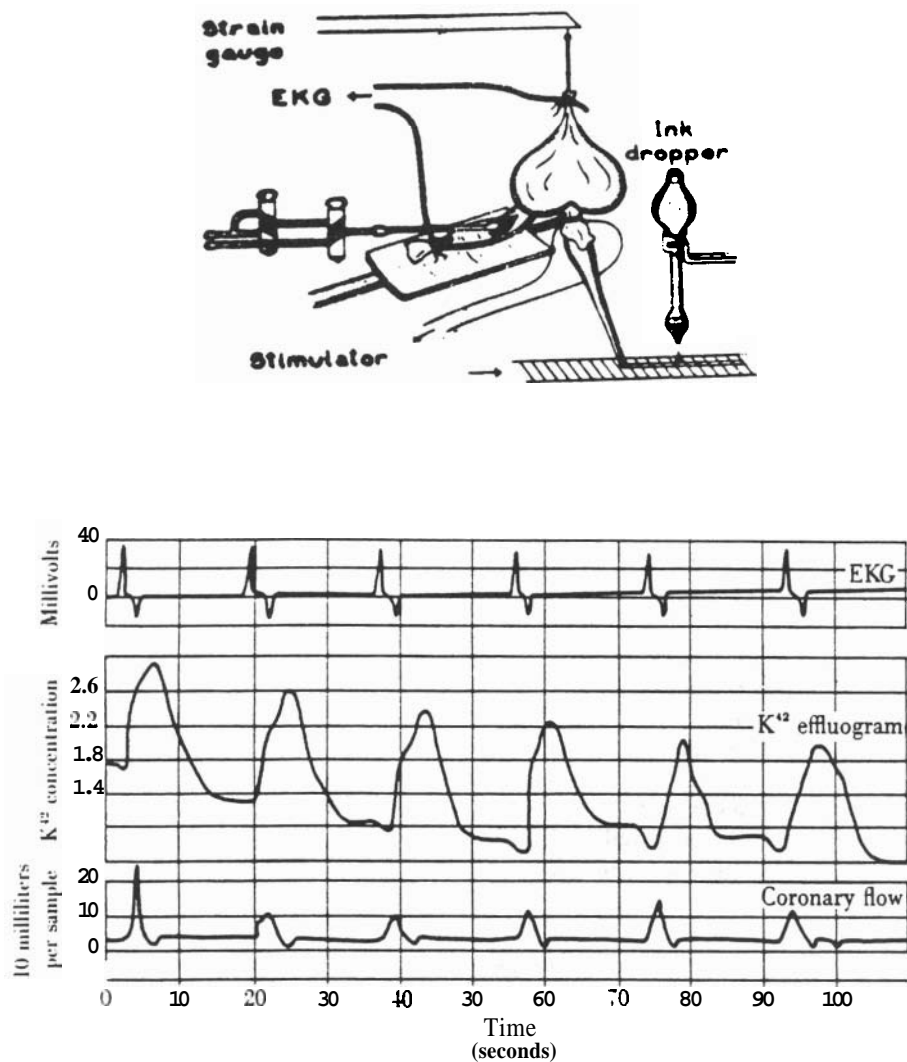


FIGURE 7. The increased K⁺-ion efflux accompanying contraction of a turtle heart. A turtle heart previously equilibrated with a high concentration of radioactive ⁴²K was perfused with a non-radioactive Ringer's solution through its coronary vessels. The effluent was continually monitored for its radioactivity. The rate of ⁴²K loss together with the electrocardiogram recording is plotted against time. Figure shows a record of six successive electrically elicited contraction cycles (Data of Wilde et al., 1956. Figure from Ling, 1962).

with theory. These findings do not tell us where in muscle cells does the K^+ released come from. Additional evidence pointing to the specific locations of these K^+ -adsorbing sites is given next.

Edelmann electrically stimulated a Tl^+ -loaded frog muscle for 1 sec at the rate of 50 stimuli per sec before cryofixation. Held in a stretched position, the tetanically contracting muscle was prevented from shortening. EM pictures of the muscle sections are reproduced in Figure 8e and 8f in comparison with an EM plate of a resting Tl^+ -loaded muscle shown in Figure 8a (Edelmann, 1989). Here, the high electron density of the A bands (and at the Z-line) so strikingly and reliably observed in the resting Tl^+ -loaded muscle (Figure 8a), is sharply reduced after the tetanic contractions.

With this reduction of the electron density of the A bands (and Z lines) of the contracting Tl^+ -loaded muscle, electron-dense granules appeared. These granules were found either within the I bands (Figure 8f) or throughout the entire sarcomere (Figure 8e). Since no new electron-dense materials had been introduced into the muscle during the contractions, these granular precipitates could only have originated from electron-dense material already present in the muscle prior to contraction but in a less conspicuous form, i.e., the Tl^+ adsorbed on the β - and γ -carboxyl groups in the A bands, especially at the two marginal zones of the A band as shown in Figure 8a.

From the literature on the solubility of various Tl^+ salts, one suspects that the electron-dense granular precipitate may be or contain TlH_2PO_4 , which has a low (but not insignificant) solubility in water*. The possibility that these granular precipitates might be the phosphate salt of Tl^+ was pointed out earlier by Edelmann (1989, p. 246).

This set of findings provided, on the one hand, additional independent evidence that the excess of Tl^+ ions adsorbed on the β - and γ -carboxyl groups account for most of the extra electron density at the two marginal zones in resting muscle; and, on the other hand, evidence for the theory of muscle contraction depicted in equation 2. The establishment of the A band as the major source of K^+ released during contraction, reaffirms our earlier conclusion that myosin carries these β - and γ -carboxyl groups that releases their adsorbed Tl^+ (or its normal counterpart, K^+) during contraction. Further evidence to the same effect are provided by other independent experimental evidence discussed next.

***In vitro* model studies suggesting that K^+ adsorbed on myosin is released during muscle contraction** It is by now well established that the key mechanism in muscle contraction involves the interaction between myosin and actin (Szent-Gyorgyi, 1947).

* The frog muscle cell is very rich in phosphates, notably creatine phosphate (30 μ moles/g) and ATP (5 μ moles/g). During a tetanic contraction, part of both compounds may become (momentarily) hydrolyzed and (in the somewhat acidic medium created by the formation of lactic acid) the products may well include enough $H_2PO_4^-$ to precipitate Tl^+ liberated from its adsorption sites as TlH_2PO_4 .

Hill has demonstrated that ATP in normal resting frog muscle is localized at the A-I boundary (Hill, 1960a, b) and CrP in the I band near the Z line (Hill, 1962). Thus the stages are set for all sorts of distribution patterns of granular TlH_2PO_4 , precipitates within the sarcomere, which in turn offers intriguing subjects for future research as they may hold valuable information connected with cell excitation and motility. With the very limited data we can gather from the publications of Edelmann, we note that in the presence of a minimal amount of water (available to the cut sections on exposure to room air), the precipitates tend to be found either at the marginal zones of the A bands or very close by (see Edelmann, 1977, Figure 1-3; 1984, Figure 9b and Figure 14b). In the abundant water of a contracting living muscle, redistributing over a much wider area becomes possible, as shown in Figures 8e and 8f.

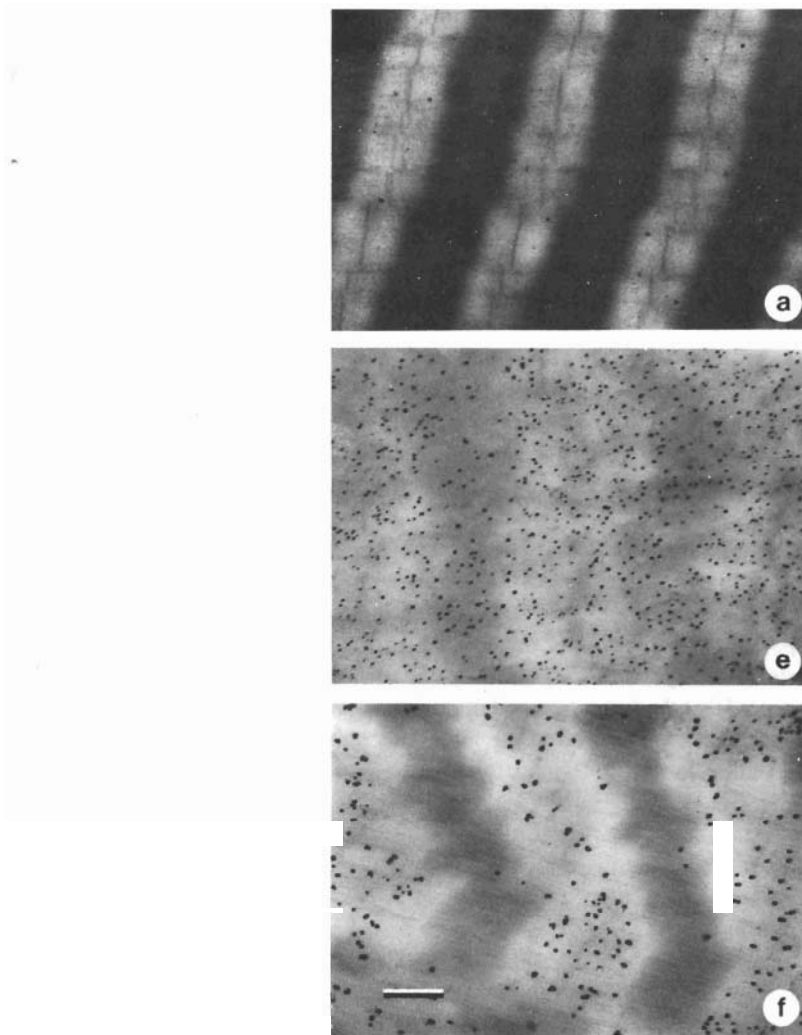


FIGURE 8. Transmission electron micrograph of a 0.3 μm thick freeze-substituted, K11M embedded, and dry-cut sections of **Tl⁺-loaded** frog semitendinosus muscle at rest (a) and **cryofixed** after having been electrically stimulated for 1 **sec** at the rate of 50 stimuli per **sec** while fixed in a stretched position (e and f). Poor contrast of A bands in e and f when compared to the control resting muscle suggests liberation and redistribution of the electron-dense **Tl⁺** as the primary component of the electron-dense granules found in the I band or throughout the entire sarcomere. Bar = 1 μm . (from Edelmann, 1989, by permission of Scanning Microscopy International).

In the publication of Lewis and Saroff mentioned earlier, these authors reported that addition of **actin** to a myosin solution reduced the amount of K^+ adsorbed on myosin. As the weight percentage of **actin** to myosin rose from zero to about 0.1, the reduction of K^+ adsorbed was linearly related to the **actin** added. Further increase of **actin** added produced lesser and lesser reduction of K^+ adsorption until a plateau was reached from whereon further addition of **actin** produced no additional reduction in K^+ binding to myosin (Figure 4 in Lewis and Saroff, 1957). This finding suggests that it is indeed myosin that releases its adsorbed K^+ during contraction and that there are two kinds of K^+ -adsorbing sites on myosin, each making up approximately half of the total number of sites, and that only one kind loses its K^+ on exposure to **actin**.

However, since whole myosin molecules were used, these studies gave no clue as to which part of the myosin molecule is involved.

Evidence that during muscle contraction cyclic salt-linkage formation (and dissociation) between K^+ -adsorbing β - and γ -carboxyl groups on myosin heads and cationic sites on **actin** molecules underlies force-generating cross-bridge formation (and dissociation). Although Ling's 1952 theoretical model of muscle contraction — a subsidiary of the association-induction (AI) hypothesis — was introduced before the sliding-filament-cross-bridge model (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954; Huxley, 1957; Huxley and Simmons, 1971), parts of the two theories can be merged together. Thus according to the combined theory, *the formation and dissociation of salt linkages between β - and γ -carboxyl groups on myosin head and actin represents the primary event in the cyclic attachment and detachment of cross bridges.*

It is by now widely accepted that the formation and dissociation of cross bridges between myosin heads and **actin** represent the key step in the force-generating process in contraction. Now, each myosin molecule can be chopped into two pieces: light meromyosin (LMM) and heavy meromyosin (HMM) (Hanson and Huxley, 1960; Bendall, 1969). Each HMM can in turn be chopped into two pieces: subfragment-1 (HMM S-1) and subfragment-2 (HMM S-2). Subfragment 1 consists of two *myosin heads* which project away from the thick filament proper and participate in the formation of the cross bridges. *Subfragment-1, and hence myosin heads, possesses all the actin-combining capacity (and ATPase activity) of the parent molecule* (Jones and Perry, 1966). This important fact, when seen in the light of what has been discussed in the preceding section, already indicates that *the one half of myosin sites, which loses its adsorbed K^+ in response to actin binding, must belong to the myosin heads*. The other half of sites which does not lose its K^+ on exposure to **actin** must be on the rod part of the thick filament (LMM + HMM S-2), as Edelman's EM pictures, mentioned earlier, have clearly revealed.

Eisenberg and Moos (1968) showed that the ATPase activity of isolated HMM is influenced quantitatively by the concentration of **actin** added to the reaction mixture. This finding was repeatedly confirmed by others. Figure 9, reproduced from Tawada and Oosawa (1969), shows double reciprocal plots of HMM ATPase activity against **actin** concentration. Different KC1 concentrations added to the system altered the slopes of the straight lines to different degrees. These varying slopes of the family of straight lines all converging on the same locus of the ordinate indicates that the reaction follows **Michaelis-Menton** kinetics. The obedience to the **Michaelis-Menton** kinetics signifies that each binding site on the myosin head binds, at any one time, only one "substrate" (i.e., **actin**) or the

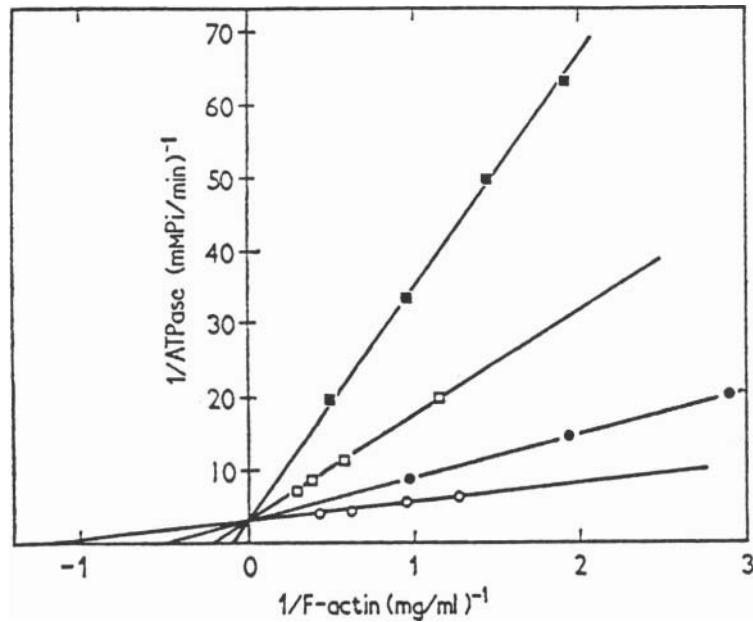


FIGURE 9. Effect of different concentration of KCl and actin concentration on the activity of acto-HMM ATPase system. Double reciprocal plots of the actin concentration (abscissa) and the ATPase activity (ordinate). —□—□—, —○—○— in the presence of F-actin at 60 and 30 mM KCl, respectively; —■—■—, —●—●— in the presence of CM-F actin at 60 and 30 mM KCl respectively. Solution contained 0.1 mg of HMM/ml, 10 mM Tris-maleate buffer (pH 7.0), 1 mM MgCl₂, and 2 mM ATP. (From Tawada and Oosawa, 1969, by permission of *Journal of Molecular Biology*).

competing species (i.e., KCl). In other words, the data reproduced in Figure 9 establishes that *actin and KCl compete for the same site on myosin heads.*

This data given in Figure 9 does not by itself tell whether it is the K⁺, the Cl⁻ or both that compete against actin for binding sites on the myosin heads. However, other available information provides the foundation for a clear answer.

According to Szent-Gyorgyi, neither actin (Szent-Gyorgyi, 1947, p. 20) nor myosin (Szent-Gyorgyi, 1947, p. 7) binds Cl⁻ *in vitro*. Szent-Gyorgyi as well as Lewis and Saroff also showed that while actin does not bind K⁺ *in vitro*, myosin does (Szent-Gyorgyi, 1947, p. 29; Lewis and Saroff, 1957). These leave no other alternative than that *the K⁺ ion and actin compete for binding sites on the myosin head, confirming at once the same conclusion reached in the preceding section and the combined theory of muscle contraction described by equation 2.*

Now the weight of evidence presented in the present paper shows that the binding sites on myosin that adsorb K⁺ are the β- and γ-carboxyl groups. We are thus led to the conclusion that the actin-binding sites on the myosin head are (or include) nothing else than the

K^+ adsorbing β - and γ -carboxyl groups, which releases its K^+ in consequence of **actin** binding.

Since it is highly unlikely that an entity which competes one-on-one for a monovalent anionic site against a bona fide monovalent cation (K^+) does not itself carry a net **cationic charge**, we are happy to discover in an old observation of Dr. Charles Trombitas, evidence that **actin** in the thin filaments does indeed carry available **cationic ϵ -amino** groups and guanidyl groups (see footnote*). Taken together, these data lend additional support for the concept of muscle contraction summarized in equation 2:

When the fired cationic site on an actin molecule successfully combines with the (actin-binding) β - or γ -carboxyl groups on a myosin head, a salt-linkage is formed. Salt-linkage formation of this kind would then represent the force-generating, cross-bridge formation step of contraction. The subsequent displacement by K^+ of the ϵ -amino or guanidyl groups on actin from the β - and γ -carboxyl groups on the myosin head then represents the relaxing, cross-bridge-dissociation step of a contraction cycle. This then is the third major conclusion of the present communication.

Hodge and Schmidt, whose work was mentioned earlier, did not just comment on the sites that bind **cationic** uranium ion, they also mentioned that phosphotungstate anion binds onto **cationic ϵ -amino** groups of lysine residues and guanidyl groups of arginine residues of collagen, citing the earlier work of Memetschek *et al.* (1955) and Kuhn *et al.* (1958).

The fact that in Trombitas' EM picture the I band (away from the Z line) is most strongly stained by phosphotungstate and that **actin** is the main component of the I band (as well as the fact that it is **actin** that binds onto myosin heads [Figure 9]) are in harmony with the notion that **cationic ϵ -amino** groups and guanidyl groups carried on **actin** of the thin filaments form salt-linkages with the β - and γ -carboxyl groups on myosin heads during muscular contraction.

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* Many years ago, Dr. Charles Trombitas of Hungary kindly sent me an (unpublished) combination EM picture of frog sartorius muscle. Side by side with a uranium stained sequence of sarcomeres is another sequence of **sarcomere** which match the **first** one, Z line to Z line, A band to **A** band. The outstanding feature of the second EM section is that it was stained with the anionic stain, phosphotungstate and that it looks very much like the "negative" of the uranium-stained one. That is, what is deeply stained in the uranium section is lightly stained in the phosphotungstate stained section and vice **versa**. The part of the **sarcomere** most strongly stained with phosphotungstate is the thin filament in the I band away from the Z line. If it is the anionic β - and γ -carboxyl groups that adsorb the **cationic** uranyl ion — now already established, it seems logical to expect that the sites that bind phosphotungstate would be the **cationic ϵ -amino** groups and guanidyl groups. In fact, there are already evidence for this idea.

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