Water and Electrolyte Content of the Myofilament Phase in the Chemically Skinned Barnacle Fiber

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ABSTRACT  Muscle fibers from the giant barnacle, Balanus nubilus, were placed inside the lumen of a porous glass capillary and equilibrated for 48 h in an electrolyte solution containing 2% Tween. The glass capillary prevented the chemically "skinned" fiber from swelling with a water content beyond 80%. Isotope exchange studies using \textsuperscript{23}Na, \textsuperscript{40}K, and \textsuperscript{36}Cl indicated the existence of an intermediate rate constant and compartment which varied with pH. This intermediate rate was attributed to counter-ions and co-ions in the myofilament phase. Analysis of the electrolyte composition of the fiber at pH 8 predicts that the myofilaments contain about 0.3 of the fiber water, and that a -15 mV Donnan potential exists at the myofilament surface. An open-tipped (1-\textmu m) microelectrode in the skinned fiber measured a potential (similar in magnitude to the Donnan potential), which decreased and reversed sign as the pH was lowered. The measured cation contents of the fiber between pH 5 and 8 were found to be similar to the cation contents predicted from the measured Donnan potentials. The net negative charge of the myofilaments at pH 7.5 and at ionic strength 0.56 is estimated to be 41 eq per 10^6 g of dry weight.

INTRODUCTION

Considerable evidence suggests that the water and monovalent ionic content of the muscle fiber from the giant barnacle is separable into two phases, neither of which can be attributed to the extracellular space nor to an internal membranous organelle (see Hinke et al., 1973, for review). Briefly, Hinke (1970) found that only 75% of the intrafiber water is osmotically active and functions as solvent for the free monovalent ions in the myoplasm. In addition, Hinke and his co-workers have shown that the intrafiber monovalent ions can be divided into free and "bound" fractions (McLaughlin and Hinke, 1966; Hinke and McLaughlin, 1967; Allen and Hinke, 1970) and that the diffusion of the ions and water in the myoplasm can be described as occurring in two distinct fluid phases (Caillé and Hinke, 1972, 1973, 1974). Caillé (1975, 1977) measured the electrical conductivity of the myoplasm (along the long axis) and showed that it could be described as occurring along two electrical pathways. Sachs and Latorre (1974) examined the electron spin resonance of...
an appropriate probe molecule in the myoplasm and obtained evidence of two fluid environments, presumably with differing viscosities. Pézolet et al. (1978) examined the laser Raman vibration spectrum of the myoplasmic water proton at varying temperatures and concluded that a significant water fraction (>20%) fails to freeze.

The two water phases must be located inside the myofibril since in the barnacle muscle the myofibrils occupy ~90% of the intrafiber volume (Brigden et al., 1971; Hoyle et al., 1973). Morphologically, the myofibril is separable into a myofilament and an internyofilament phase, both of which contain water and electrolytes. The intramyofilament water content can be roughly estimated to be ~15–20% of the total fiber water if one assumes that the myofilaments occupy more than 35% of the myofibrillar volume when the total fiber water content is 80%.

Some recent proton nuclear magnetic resonance studies on the barnacle fiber (Foster et al., 1976)\(^1\),\(^2\) indicate that only about 5% of the fiber water lacks rotational mobility. Therefore, most of the water molecules within the myofilament domain are probably quite mobile, although perhaps less mobile than the water molecules outside the myofilament phase. Within the myofilament domain, the water molecules must to some extent interact with and be influenced by the macromolecules (myosin, actin, etc.), certainly more so than the water molecules outside the myofilament domain. However, it is unlikely that the interactions between macromolecules and water are sufficiently strong and long-lasting to cause all the myofilament water to be highly ordered and in a nonfluid state. More probably, the myofilament water is fluid-like, but with an increased viscosity (Sachs and Latorre, 1974) and with a type of ordering which resists ice formation at low temperatures (Pézolet et al., 1978).

It is well known that the thick and thin myofilaments carry net negative charges at physiological pH. If one assumes that the myofilament phase must be electrically neutral, it follows that the ionic content of the myofilament phase will differ from the ionic content of the internyofilament phase, that a Donnan equilibrium must exist between the two phases, and that a Donnan potential must exist somewhere at the boundary between the two phases. Needless to say, this Donnan equilibrium does not require a membrane because the confinement of the macromolecular charge to the myofilament phase is not dependent on a membrane.

This two-phase model for the myoplasm of the barnacle fiber gains additional support from the experiments to be described in this paper. All experiments were conducted on the chemically “skinned” fiber, a preparation

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1 Burnell, E. E., M. E. Clark, W. R. Chapman, and J. A. M. Hinke. Studies on water in barnacle muscle fibers. III. An NMR study of motions of water molecules in fresh fibers and membrane-damaged fibers equilibrated with selected solutes. Manuscript submitted for publication.

2 Clark, M. E., E. E. Burnell, N. R. Chapman, and J. A. M. Hinke. Studies on water in barnacle muscle fibers. IV. Water self-diffusion coefficients and macromolecular hydration in fresh fibers and in membrane-damaged fibers equilibrated with selected solutes. Manuscript submitted for publication.
free of diffusional restrictions from the plasma membrane and free of possible complications from membrane-enclosed organelles, much like the glycerinated muscle preparation. One group of experiments (using $^{22}$Na, $^{42}$K, and $^{36}$Cl isotopes) demonstrates the existence of an intermediate isotope exchange rate constant for an ionic compartment which varies in size with pH. A second group of experiments provides electrolyte concentration data which are used to calculate the myofilament phase water fraction and the Donnan potential on the surface of the myofilaments. In a third group of experiments, a Donnan-like potential was measured (using 3 M KCl micropipettes) between the chemically skinned fiber and the bathing solution at a number of pH conditions. These measured potentials were similar in magnitude and sign to the calculated Donnan potentials at the myofilament surface, and accurately predicted the electrolyte composition of the chemically skinned fiber at four of the five pH conditions.

**METHODS**

Giant barnacles, *Balanus nubilus*, were collected from fast tidal narrows in the Strait of Georgia (British Columbia, Canada) and stored in aerated seawater at 10°C for 1 wk before an experiment. Barnacles were transferred to the laboratory aquarium containing artificial seawater (Instant Ocean, Aquarium Systems Inc.) with a salinity similar to the barnacle Ringer’s solution (see below).

Preparation of Chemically Skinned Fibers in Glass Capillary

Single fibers in barnacle Ringer’s solution were dissected free from each other but left attached by their origin to the base plate. A silk thread was attached to the tendon of each fiber to maintain fiber length and to facilitate handling. Before exposure to detergent, the fibers were equilibrated for 30 min in Ca- and Mg-free Ringer’s solution in order to remove all extrafiber-divalent ions. The fibers were then equilibrated for another 30 min, in the same solution but with sucrose added (to 0.75 M) in order to shrink the fibers temporarily to 70% water content. One to three shrunken fibers were carefully pulled into a porous glass capillary (Vycor No. 749303, Corning Glass Works, Corning, N.Y., 1.5 mm i.d. and 1.5 cm long), which had been filled beforehand with the equilibrating solution. These capillary fibers were allowed to swell back to normal size for 3–5 h in Ca-free Ringer’s solution. If they filled the whole lumen of the capillary and did not show signs of fragmentation, the fibers were trimmed flush with both ends of the capillary, transferred to a plastic holder, immersed in one of solutions 1–4, and stored for at least 48 h in a cold room (5–8°C). The equilibrating solution (50 ml) was changed at least four times during the 48-h storage period.

The purpose of the glass capillary was to prevent unlimited swelling from occurring after exposure to the Tween 20 detergent (Atlas Chemical, Wilmington, Del.). An unrestricted fiber, following such treatment, swells continuously to a water content of ~93% in 48 hr. In contrast, the water content of a fiber in a capillary jacket can be controlled at 80% if the fibers are made to fit the capillary lumen snugly at the start of the equilibration.

After 48 h equilibration, each fiber-capillary unit was inspected with the aid of a dissecting microscope at × 20 magnification for fragmentation of myofibrils and for fiber displacement beyond the capillary glass cut ends. Only intact fiber-capillary units with the same appearance as at the beginning of equilibration (usually 85%) were selected for further experimentation and analyses.
**Equilibrating Solutions**

The composition of the barnacle Ringer's solution was: 450 mM NaCl, 8 mM KCl, 20 mM CaCl₂, 10 mM MgCl₂, and 25 mM Tris CI (tris hydroxymethyl amino methane) adjusted to pH 7.6 at 10°C. This solution was made free of divalent cations by the omission of CaCl₂ and MgCl₂ and by the addition of 0.5 mM EGTA. The solution was made hypertonic by the addition of sucrose to 0.75 M. Table I lists the compositions of a number of equilibrating solutions containing 2% Tween 20 (polyoxyethylene-20-sorbitan monolaurate). In solution 1, only the pH was changed from 7.4 to 5.4. In solutions 2, 3, and 4, the Na⁺ and K⁺ content were varied within the limits shown but their sum was always constant. In solution 4, the main anion was H₂PO₄⁻ when the pH was between 4 and 6, and the anion was propionate when the pH was between 7 and 8. Notice also that a different buffer was used in solution 4 for each pH.

| Table I | EQUILIBRATING SOLUTIONS CONTAINING 2% TWEEN-20 AND 0.5 mM EGTA |
|---------|-----------------------------------------------------------|
| Ion     | 1            | 2   | 3   | 4       |
|---------|---------------|-----|-----|---------|
| Na      | 20            | 100-0* | 100-0 | 200-0* |
| K       | 80            | 0-100 | 0-100 | 0-200   |
| Cl      | 20            | 80   | 20  | 20      |
| Propionate | 60       | 60   | 0 or 180 |       |
| H₂PO₄⁻  | 20            | 20   | 20  | 180 or 0 |
| Buffer  | -             | -    | -   | 20%     |
| pH (at 10°C) | 7.4, 5.4 | 8.0 | 8.0 | 4.5, 6, 7, 8 |

* The Na and K content were varied between the limits shown. In cols. 2 and 3, [Na] + [K] = 100 mM; In col. 4, [Na] + [K] = 200 mM.  
‡ At pH 7 and 8, 180 mM propionate was used; at pH 4, 5, and 6, 180 mM H₂PO₄⁻ was used.  
§ In col. 4 the buffers were KH tartrate at pH 4, KH phthalate at pH 5, histidine at pH 6, KH₂PO₄ at pH 7, and Tris at pH 8.

**Isotope Uptake Experiments**

The isotope uptake by the fibers while in the Vycor capillaries proved to be variable. Therefore, after the 48-h equilibration in solution 1 at either pH 7.4 or 5.4, the fibers were pushed out of the capillaries before exposure to an isotope. Curiously, these 48-h-old fibers did not swell, at least not significantly in the first 2 h after their removal from the glass capillary. Three separate uptake experiments were carried out, one for each of the following isotopes: ^22Na at 2μC/ml, ^42K at 2μC/ml, and ^36Cl at 0.2 μC/ml. At least 40 fibers were placed in a 50 ml volume containing an isotope. At time periods, 0, 2, 5, 10, 15, 25, 40, 60, 120, and 240 min, four fibers and four bath samples were removed and each was transferred either to a 10-ml volumetric flask, a γ-counting tube or a β-counting bottle. In the ^22Na uptake experiment, chemical and isotope analyses were performed on the same diluted digest. In the ^42K uptake experiment, fibers for isotope analysis were digested immediately (without drying to constant weight), diluted and counted as soon as possible. In the ^36Cl uptake
experiments, the fibers for chemical and isotope analyses were handled as described by Gayton and Hinke (1971).

In a fourth experiment, the uptake of tritiated dimethyl sulfoxide (\[^3\text{H}\]DMSO) by the fibers was measured in order to verify that the 48-h Tween-treated fibers were devoid of membrane barriers which might restrict inward diffusion. The diffusion coefficient for this compound in the barnacle muscle myoplasm is known (Caillé and Hinke, 1974). In this experiment, 32 fibers were placed in a 50-ml volume of solution 1 at pH 7.4 containing 2 \(\mu\)C/ml \[^3\text{H}\]DMSO. At time periods 1, 2, 5, 10, 20, 30, 40, and 50 min, four fibers were removed and each was analyzed separately for its \[^3\text{H}\] DMSO content.

**Total Ion Analyses**

If still in its glass jacket, the fiber was pushed out of the capillary with a glass rod, blotted gently in a standard manner, and placed in a Pyrex volumetric flask. The wet and dry weights of each fiber (or group of one to three fibers) were measured in the usual way (Hinke, 1970). For cation analysis, the dried fiber was dissolved in 0.2 ml concentrated HNO\(_3\), neutralized with NH\(_4\)OH, and diluted to 10 ml all in the same flask. The cation concentrations were measured by atomic absorption spectroscopy (Varian Techtron, Palo Alto, Calif., AAS); each sample reading was bracketed by readings from two of the closest standards that contained the same amounts of HNO\(_3\) and NH\(_4\)OH as in the unknown samples. The chloride analysis was done on a Buchler-Cotlove chloridometer as described by Gayton and Hinke (1968). At least four bath samples were taken from each equilibrating solution and analyzed for \(\text{Na}^+\), \(\text{K}^+\), and \(\text{Cl}^-\) content.

**Microelectrode Measurements**

Conventional micropipettes containing 3 M KCl were prepared in the usual manner from 1.5-mm (o.d.) Pyrex capillaries. Only micropipettes with a resistance of 10–20 mΩ and with a tip potential change of \(<3\) mV were selected for use. All the fiber capillaries in this experiment were equilibrated for at least 48 h in solution 4. The fiber capillary was mounted vertically in a plastic rack (immersed in solution 4 at 5–8°C), and the top cut end was impaled with a micropipette to a depth of 0.5 cm. The micropipette was referred to a calomel reference electrode in the bath and the potential (\(E_o\)) was observed on a Keithley Instruments, Inc. (No. 616) electrometer (Cleveland, Ohio). If a consistent set of potentials was obtained after several penetrations (three to five in different locations), the fiber(s) was removed from the capillary and analyzed for its total cation content.

**RESULTS**

**Isotope Uptake Experiments**

Figs. 1–3 provide the mean isotope uptake curves for fibers which had been equilibrated for 48 h in solution 1 at pH 7.4 or pH 5.4. The isotope uptake by the fiber at each time period is shown as a specific activity (\(\text{SA}_f\)) relative to the specific activity in the bath (\(\text{SA}_B\)) at the same time period. The data points are inverted (i.e., the specific activity ratios are subtracted from unity) in order to better illustrate the difference between the two pH conditions on the logarithmic ordinate. The curves were drawn with the assistance of a
computer program designed to give the best fit to the experimental points using either the sum of two or of three exponential terms of the form, $A_i \exp k_i t$. The derived $k_i$ and $A_i$ values for each of the fitted curves are given in Tables II-IV. Also included are the results of chemical analysis of the fibers and the bath.

Note in Table II that three rate constants were required to best describe the $^{42}K$ uptake data at pH 7.4 but only two were necessary at pH 5.4. Notice also that rates I and III were identical in magnitude for the two pH conditions.

![Figure 1](image_url)

**Figure 1.** The $^{42}K$ uptake by the single fiber after 48 h equilibration in Tween Solution 1 (Table I). The ordinate is a ratio of the specific activity of the isotope in the fiber ($SA_f$) and the specific activity in the bath ($SA_b$). For graphic reasons (see text), the ratio is subtracted from unity. The fibers were removed from the Vycor glass capillaries before the uptake study. The bars on the points at $t = 40$ min are double the standard error ($n = 7$ fibers). The curves through the points are drawn according to the data in Table II (see text).

The disappearance of the rate II at pH 5.4 suggests a loss of a compartment which contained about 27 mmol/kg fiber water (FW) of fiber $K^+$ when the total fiber [K] was 108.5 mmol/kg FW. The actual measured mean loss of [K] following the pH change was 20 mmol/kg FW ($108.5 - 88.7$).

Note in Table III that three rate constants were again required to best describe the $^{22}Na$ uptake data at pH 7.4 but only two were necessary at pH 5.4. Once again it was rate II that disappeared and rates I and III that remained unchanged in magnitude. The disappearance of rate II at pH 5.4
suggests a loss of a compartment which contained about 6.4 mmol/kg FW of fiber Na⁺ when the total fiber [Na] was 31 mmol/kg FW. The actual measured mean loss of [Na] following the pH change was 5.1 mmol/kg FW (31.0 – 25.9).

Note in Table IV that only two rate constants were necessary to describe the ³⁶Cl uptake data at pH 7.4 but that three were required for the pH 5.4 data. The new rate is an intermediate one (like rate II in Tables II and III),

![Graph of pH 7.4 and pH 5.4 Na uptake](image)

**Figure 2.** The ²²Na uptake by the single fiber after 48 h equilibration in Tween solution 1. The bars on the points at t = 240 min are twice the standard error (n = 7). The curves are drawn according to the data in Table III.

and rates I and III remained identical in magnitude at the two pH conditions. The appearance of rate II at pH 5.4 suggests the gain of a new Cl⁻ compartment containing ~8.1 mmol/kg FW of [Cl] when the total fiber [Cl] was 27.7 mmol/kg FW. The actual measured mean increase in Cl⁻ following the pH change was 7.9 mmol/kg FW (27.7 – 19.8).

The mean results of the [³H]DMSO uptake is shown in Fig. 4. Also included for comparison is the first 60 min of the ²²Na uptake curve at pH 7.4 (Fig. 2).
The straight (dashed) line through the $[^3H]$DMSO curve obeys the equation,
\[
1 - \frac{C_F}{C_0} \approx \frac{4}{(2.405)^2} \exp (-kt),
\] (1)
which, according to Jost (1960, p. 45), approximately describes the radial diffusion of a substance into a cylinder with radius, $r_0$, when the concentration ($C_0$) of the substance remains constant at the surface as the cylinder content ($C_F$) increases with time ($t$). Jost (1960) defines $k$ as $(2.405)^2D/r_0^2$, where $D$ is the diffusion coefficient, but Harris and Burn (1949) found it necessary to include a tortuosity factor, $\lambda^2$, with $r_0^2$ in the denominator ($\lambda = 1.5$) when they related the $k$ and $D$ for substances diffusing into the extracellular space of whole muscle. The modified equation, $k = (2.405)^2D/\lambda^2r_0^2$, predicts a $D$ of $0.474 \times 10^{-5}$ cm$^2$s$^{-1}$ (10°C) for $[^3H]$DMSO when $\lambda = 1.5$, $k = 0.13$ min$^{-1}$ (slope of straight line, Fig. 4) and $r_0 = 0.075$ cm (mean radius of Tween-treated fibers).

In experiments specifically designed to measure the diffusion coefficient ($D$)

3 Eq. 1 is valid only when $t$ is sufficiently large (Jost, 1960). Fig. 4 indicates that $t$ must be larger than 120 s for DMSO.
of \(^{3}H\)DMSO in the myoplasm of the barnacle fiber containing varying amounts of water, Caillé and Hinke (1974) found \(D\) to be \(0.418 \times 10^{-5}\) \(\text{cm}^2\text{s}^{-1}\) (10°C) and \(0.480 \times 10^{-6}\) \(\text{cm}^2\text{s}^{-1}\) (10°C) when the myoplasmic water content was 75% and 80%, respectively. In their experiments, the fibers were not chemically skinned with Tween detergent; instead, diffusion into the myoplasm was from a cut end and along the long axis of the fiber. Since the \(D\)

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Solution} & \text{Rate constant, } k_K & \text{Coefficient, } A_K & \text{Estimated } [K] & \text{Rate constant, } k_K & \text{Coefficient, } A_K & \text{Estimated } [K] \\
\hline
\text{pH 7.4} & & & & \text{pH 5.4} & & \\
\text{I} & 1.5 \times 10^{-1} & 0.65 & 71 & 1.5 \times 10^{-1} & 0.92 & 82 \\
\text{II} & 4.0 \times 10^{-2} & 0.25 & 27 & - & - & - \\
\text{III} & 2.0 \times 10^{-3} & 0.10 & 11 & 2.0 \times 10^{-3} & 0.08 & 7 \\
\hline
\text{Fiber } [K] & \text{II} & 108.5 \pm 2.8^* & \text{II} & 88.7 \pm 1.7 \\
\text{Bath } [K] & \text{II} & 81.9 \pm 0.4 & \text{II} & 81.5 \pm 0.4 \\
\hline
\end{array}
\]

* Standard error of mean.

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Solution} & \text{Rate constant, } k_Na & \text{Coefficient, } A_Na & \text{Estimated } [Na] & \text{Rate constant, } k_Na & \text{Coefficient, } A_Na & \text{Estimated } [Na] \\
\hline
\text{pH 7.4} & & & & \text{pH 5.4} & & \\
\text{I} & 1.5 \times 10^{-1} & 0.64 & 20.3 & 1.5 \times 10^{-1} & 0.77 & 20.1 \\
\text{II} & 4.0 \times 10^{-2} & 0.20 & 6.4 & - & - & - \\
\text{III} & 2.4 \times 10^{-4} & 0.16 & 5.0 & 2.4 \times 10^{-4} & 0.23 & 6.0 \\
\hline
\text{Fiber } [Na] & \text{II} & 31.0 \pm 0.6^* & \text{II} & 25.9 \pm 0.3 \\
\text{Bath } [Na] & \text{II} & 22.3 \pm 0.2 & \text{II} & 23.2 \pm 0.2 \\
\hline
\end{array}
\]

* Standard error of mean.

obtained from the radial influx (Fig. 4) is very close to the \(D\) (80% water) obtained from longitudinal diffusion (Caillé and Hinke, 1974), one can conclude that the Tween-treated fiber lacked a membrane barrier and that \(^{3}H\)DMSO influx into the myoplasm was primarily diffusion-limited. Since the rate I values for the \(^{40}K\), \(^{25}Na\), and \(^{36}Cl\) uptake curves (Tables II–IV) are about the same magnitude as the \(k\) for \(^{3}H\)DMSO, it follows that rate
I must represent isotope diffusion in the myoplasm. For example, rate I for \(^{36}\)Cl influx at pH 7.4 (Table IV) predicts a \(D\) of \(0.620 \times 10^{-5}\) cm\(^2\) s\(^{-1}\), which compares favorably with the measured \(D\) (Caillé and Hinke, 1974) of \(0.683 \times 10^{-5}\) cm\(^2\) s\(^{-1}\) (10\(^\circ\)C) for the longitudinal diffusion of \(^{36}\)Cl in myoplasm containing 76% water.\(^4\)

In the Appendix arguments are developed which indicate (see Eq. 4 a and 5 a) that ions exist in three locations in the Tween-treated fiber: as free ions in the bulk fluid, as free ions in the myofilament phase, and as tightly bound ions to the myofilaments (or other macromolecules). Of the three rate constants in Tables II-IV, it seems reasonable to assign (a) rate I to an exchange with the free ions of the intermyofilament (bulk) space, (b) rate II to an exchange with the free ions of the intramyofilament phase, and (c) rate III to an exchange with the bound ions (B).

The assignment of rate II to the free intramyofilament ions is based on the following observations: rate II is only moderately slower than rate I; compartment II contains about the correct number of ions (20-29% of the fiber ions) for the amount of water (25%) in the myofilament phase (Hinke, 1970); and the cation content decreased and the anion content increased in compartment II when the pH was decreased to 5.4.

### Table IV
EXPONENTIAL CONSTANTS FOR CURVES IN FIG. 3 AND THE [Cl] CONTENT OF FIBERS AT END OF EXPERIMENT

| Solution | Rate constant, \(k_{\text{Cl}}\) | Coefficient, \(A_{\text{Cl}}\) | Estimated [Cl] | Rate constant, \(k_{\text{Cl}}\) | Coefficient, \(A_{\text{Cl}}\) | Estimated [Cl] |
|-----------|-------------------------------|-----------------|--------------|-------------------------------|-----------------|--------------|
| Fiber [Cl] | 19.8±0.13*                   | 27.7±0.45       | \(n=10\)     | \(n=14\)                     |
| Bath [Cl]  | 23.2±0.15                    | 22.2±0.29       | \(n=38\)     | \(n=51\)                     |

* Standard error of mean.

The assignment of rate II to the free intramyofilament ions is based on the following observations: rate II is only moderately slower than rate I; compartment II contains about the correct number of ions (20-29% of the fiber ions) for the amount of water (25%) in the myofilament phase (Hinke, 1970); and the cation content decreased and the anion content increased in compartment II when the pH was decreased to 5.4.

**Electrolyte Content and Calculations of \(a_0\) and \(E_D\) at pH 8.**

The mean electrolyte composition data at the bottom of Tables II–IV illustrate that at pH 7.4 the Tween-treated fiber accumulates K\(^+\) (Table II) and Na\(^+\) (Table III) but excludes Cl\(^-\) (Table IV) relative to the bath concentrations. At pH 5.4, the cation accumulation by the fiber is greatly reduced, but the

\(^4\) Calculation of \(D\) from a \(k\) (rate I) obtained from specific activity data is valid if the nonradioactive ionic concentrations in the fiber and bath are identical. Table IV indicates that [Cl] in the fiber was only 85% of [Cl] in the bath at pH 7.4.
anion exclusion has changed to an anion accumulation (Table IV). These results are compatible with the existence and behavior of a Donnan equilibriu between the myofilaments and the intermyofilament space (see Appendix and Eq. 7 a).

Four experiments were specifically designed to document the cation-accumulating and anion-excluding ability of the Tween-treated fiber when equilibrated with solutions of differing composition. A pH of 8 was selected to ensure a near maximal net negative charge on the myofilaments, hence a near maximal Donnan distribution of the ions. The fiber-capillary units were equilibrated for 48 h in either solution 2, 3, or 4 (Table I). The Na\(^+\):K\(^+\) ratio in the equilibrating solutions was varied but the sum of the Na\(^+\) and K\(^+\) concentrations and the Cl\(^-\) concentration was always held constant in a given experiment. At the end of an equilibration, some fibers were used for cation analysis and some for chloride analysis.

The mean results of all four experiments are shown in Table V. Note that the highest water content (82\%) occurred in the first experiment when [Cl\(^-\)]\(_o\) was relatively high. In the other three experiments when propionate and H\(_2\)PO\(_4\) were the major anions, the water content of the Tween-treated fibers was normal (79–80\%). In the four experiments, [Na + K]\(_F\) was always significantly larger than [Na + K]\(_o\), and [Cl]\(_F\) was always significantly smaller than [Cl]\(_o\). In these experiments, as in earlier ones (Hinke et al., 1973), the Tween-treated fibers tended to accumulate proportionately more Na\(^+\) than K\(^+\) ions at all solution mixtures of the two cations.

Also listed in Table V are the estimations of myofilament water (\(\alpha_0\)) and...
the Donnan potential on the myofilaments ($E_D$). These have been calculated from the electrolyte data using Eqs. 8 $a$ and 9 $a$ in the Appendix. Note that the $a_2$ values are comparable in magnitude to the estimations of the compartment associated with rate II in Tables II-IV (see coefficients to rate II). The calculated $E_D$ values vary between $-13$ and $-21$ mV and are about twice the magnitude of the $E_D$ values measured on glycerinated frog sartorius and ventricle muscle (Collins and Edwards, 1971), and are slightly larger than the $E_D$ values measured on glycerinated rabbit psoas muscle (Pemrick and Edwards, 1974).

**Measured Donnan-like Potentials ($E_b$) at pH 4 to 8.**

In the Appendix it is shown that the Donnan potential ($E_D$) on the myofilaments is dependent on the hydrogen ion concentration in the bathing medium.

| Table V | ELECTROLYTE CONTENT OF SKINNED FIBERS AT pH 8 AND CALCULATIONS OF $a_2$ AND $E_D$ |
|---------|-----------------------------------------------------------------------------------|
| Equilibration solution | Bath | Fiber | Myofilament water | Donnan potential |
| | $[Cl]_o$ | $[Na+K]_o$ | $[Cl]_F$ | $H_2O$ | $[Na+K]_F$ | $a_2$ | $E_b^*$ |
| mM | mmol/kg FW | % | mmol/kg FW | mV |
| 2 | 85.8 | 103.1 | 73.7$\pm$ | 82.0 | 134.5 | 0.26 | $-18.6$ |
| ±0.3 | ±0.2 | ±1.0 | ±0.4 | ±1.2 | |
| 3 | 19.0 | 105.1 | 16.1 | 80.6 | 138.3 | 0.29 | $-17.7$ |
| ±0.1 | ±0.2 | ±0.5 | ±0.5 | ±2.0 | |
| 4 | 19.5 | 106.0 | 16.0 | 79.8 | 152.0 | 0.31 | $-21.4$ |
| ±0.1 | ±0.2 | ±0.6 | ±0.5 | ±2.2 | |
| 4 | 16.6 | 191.0 | 14.4 | 79.3 | 235.0 | 0.31 | $-13.4$ |
| ±0.1 | ±0.6 | ±0.3 | ±0.4 | ±1.8 | |

* $RT/F = 24.2$ mV.

† Mean ± standard error of mean and s.

(see Eq. 13 $a$). Collins and Edwards (1971) also demonstrated that the measured Donnan-like potential ($E_b^*$) in glycerinated frog ventricle changed from a negative to a positive value when the pH was changed from 7.5 to 4.0.

Table VI summarizes the results of an experiment in which Donnan-like potentials ($E_b^*$) were measured in the Tween-treated fiber capillary preparation when the pH of the equilibrating solution was varied from 8.0 to 4.0. The intentions of the experiment were twofold: to compare the measured $E_b^*$ with the calculated $E_D$ (Table V) at high pH; to establish whether the exponent of $E_b^*$ varied with the hydrogen ion concentration as predicted by Eq. 13 $a$.

Note first in Table VI that the $E_b^*$ values for the pH conditions from 7 to 8 are similar in sign and comparable in magnitude to the calculated $E_D$ values listed in Table V. This result was unexpected partly because Collins and Edwards (1971) obtained smaller $E_b^*$ values at comparable pH and ionic
strength conditions, and partly because it was reasoned that the 1-µm tip micropipette should only be expected to measure some fraction of the $E_D$ on the myofilaments.

To test how applicable Eq. 13 $a$ might be for the $E_D$ vs. pH data, the exp $E_D'F/RT$ and $(H)_o$ terms were calculated (Table VI) and subjected to linear regression analysis. When all points were included the regression line had an $r^2 = 0.7$, but when the pH 4 data point was omitted the regression line had an $r^2 = 0.96$. Values of the $a$ and $b$ constants for the latter regression line are given at the bottom of Table VI and will be commented on later.

**Changes in the Cation Content with pH**

When the $E_D$ measurements on a given fiber capillary were completed, the fiber was analyzed for its Na$^+$ and K$^+$ content. The mean cation contents $<M>_F$ of the fibers at each pH condition are listed in Table VI as $<M>_F/<M>_0 - 1$. When $<M>_F$ was greater than $<M>_0$ the $<M>_F/<M>_0 - 1$ term is positive and when $<M>_F$ was smaller than $<M>_0$ the $<M>_F/<M>_0 - 1$ term is negative. Thus, according to Table VI, the fiber accumulated cations when the pH was 6 or higher and the fiber excluded cations when the pH was 5 or lower. These results are consistent with the well-known fact that the myofilament proteins have isoelectric points somewhere between pH 5 and 6 depending on the ionic strength and divalent cation composition of the protein solution (Mihalyi, 1950; Sarkar, 1950). The $<M>_F/<M>_0 - 1$ data of Table VI are plotted as five experimental points in Fig. 5. The curve through four of the five points is drawn according to Eq. 14 $a$ when $a_2 = 0.3$ (Table V) and when $a = 0.55$ and $b = 68,000$ (Table VI). That the theoretical curve does not pass through the pH 4 point is consistent with the previous observation that the best regression line ($r^2 = 0.96$) for the $E_D$ data (Table VI) was obtained when $E_D$ at pH 4 was omitted.

It should be emphasized here that Eq. 14 $a$ depends on the assumption that $E_D' = E_D$. The only evidence in support of this assumption is found in a

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**Table VI**

**Effect of pH on the Measured Potential ($E_D'$) and on the Cation Content ($M_F$) in Chemically Skinned Fibers (5–8°C)**

| pH* | $E_D'$ | exp $E_D'F/(RT)$ | $(<M>_F/<M>_0-1)$‡ |
|-----|--------|-----------------|----------------------|
| 8.0 | -13.5±0.6§ (38) | 0.57 | 0.246±0.009 (35) |
| 7.5 | -15.7±1.3 (16) | 0.52 | - |
| 7.0 | -16.0±1.0 (39) | 0.52 | 0.235±0.012 (36) |
| 6.0 | -10.1±0.8 (43) | 0.66 | 0.185±0.010 (47) |
| 5.0 | -0.65±0.9 (43) | 0.97 | -0.053±0.006 (36) |
| 4.0 | +8.3±1.9 (34) | 1.41 | -0.110±0.010 (32) |

* Linear regression analysis of data between pH 5 and 8 (pH 4 omitted). exp $E_D'F/(RT) = a + b(H)_o$; $a = 0.55 ± 0.02$, $b = 68,000 ± 8,000$, $r = 0.98$.
‡ These data are shown in Fig. 5.
§ Mean ± standard error (number of determinations).
comparison between the \( E_D \) values of Table V and the \( E_D' \) values of Table VI. Interestingly, Eq. 14 \( a \) produces a curve passing through four of five data points when the value of \( \alpha_2 \) is as predicted in Table V and the values of \( a \) and \( b \) are as determined in Table VI. Equally noteworthy, the derived curve predicts an overall isoelectric point of about pH 5.2 for the contractile filaments, a reasonable number when one considers that the isoelectric points for pure myosin, actin, and tropomyosin are 5.4, 5.2, and 5.1, respectively (Szent-Gyorgyi, 1947; Erdos et al., 1948; Young, 1963).

As shown in the Appendix, the \( a \) and \( b \) constants of Eq. 13 \( a \) represent a number of constants in Eq. 12 \( a \) as follows:

\[
a = \frac{\alpha_2 m \gamma}{n_2 (1 - \gamma)} (M)_1, \quad b = \frac{\alpha_2 m \gamma}{n_2 (1 - \gamma)} \frac{K_H}{K_M},
\]

where \( \gamma \) is the fraction of fiber water by weight and where \( K_H \) and \( K_M \) are the apparent association constants for \( H^+ \) and \( M^+ \) at the \( n_2 \) sites. The \( m \) term is the conversion factor, kilograms of dry weight of fiber per mole of protein in the dry weight. When \( a = 0.55, \alpha_2 = 0.3, \gamma = 0.79, \) and \( (M)_1 = 0.2 \) M, then \( n_2 \) calculates as 41 charge sites per 10^5 g of dry weight in the fiber. For comparison, Collins and Edwards (1971) estimated a charge density of 33 negative sites per 10^6 g at pH 7.5 for glycerol-extracted vertebrate muscle, and
Kholodova (1972) found the maximum hydrogen ion binding capacity of myofibrils from frog muscle to be 65 eq per $10^5$g of protein. When $b = 68,000$, then the $K_H/K_M$ ratio becomes $2.5 \times 10^4$. In comparison, Lewis and Saroff (1957) found the $K_H/K_M$ ratio to be between $10^4$ and $10^5$ for pure myosin.

**Measurements with Ion-Selective Microelectrodes**

After the 48-h equilibration, the fiber-capillary preparation was occasionally impaled with a Na+, K+, or Cl− microelectrode (Hinke, 1969 a and b) to check whether a true equilibrium between the skinned fiber and the bathing solution was established and to rule out the existence of a potential generating barrier which might have been created by the Vycor porous glass. In all cases, the ion selective microelectrode recorded a potential inside the fiber capillary which did not deviate from the bath potential by more than ±1 mV. These results provided assurance that the monovalent ions in the skinned fiber were indeed in electrochemical equilibrium with the bathing solution after 48 h of equilibration, and that a reformed plasma membrane with selectively permeable properties was not present.

**DISCUSSION**

A simple two-phase model for the myoplasm of the skinned barnacle muscle fiber (see Appendix) accounts for the changes in electrolyte composition, microelectrode potential, and exchange kinetics of ions following changes in pH. The model divides the myoplasm into a myofilament and intermyofilament phase and postulates that both phases contain water and electrolytes in a relatively mobile and exchangeable form (Eqs. 4 a–6 a). Since the myofilaments are known to be negatively charged at physiological pH, the model recognizes the existence of a Donnan equilibrium and a Donnan potential between the two phases (Eq. 7 a).

In the two-phase model, the total cation concentration $(M)_F$ of the skinned fiber is best defined by the equation,

$$
\frac{(M)_F}{(M)_0} = \exp \left( \frac{-E_dF}{RT} \right) \alpha_1 \left( \frac{\exp \left( \frac{-E_dF}{RT} \right) - 1}{\exp \left( \frac{-E_dF}{RT} \right)} \right),
$$

which is obtained by rearranging Eq. 9 a and substituting $(1 - \alpha_1)$ for $\alpha_2$. In the one-phase model, $(M)_F$ is simply related to $E_d$ by the well-known relation,

$$
\frac{(M)_F}{(M)_0} = \exp \left( \frac{-E_dF}{RT} \right).
$$

It must be emphasized that the $E_d$ in Eq. 2 is the Donnan potential located on the myofilament surface whereas the $E_d$ in Eq. 3 is a Donnan-like potential between the skinned fiber and the bathing solution.

Note that in Eq. 2 the two-phase model predicts that the cation content of the skinned fiber is dependent not just on the myofilament charge (through $E_d$) but also on the size of the intermyofilament space, $\alpha_1$, or the degree of hydration of the fiber. In all the experiments described in this paper, the water content of the skinned fiber was held constant (80%) and $\alpha_1$ under these
conditions turned out to be about 0.7. Both the theoretical and measured $E_D$ at physiological pH and at the same degree of hydration (80%) was about $-15 \text{ mV}$. Substitution of these numbers into Eq. 2 leads to an $(M)_F/(M)_0$ value of 1.26. The average experimental value for $(M)_F/(M)_0$ between pH 7 and 8 was 1.24 (Table VI). Using this latter value in Eq. 3, one obtains an $E_D$ of about $-5 \text{ mV}$ indicating that the one-phase model is less satisfactory in associating the measured $(M)_F/(M)_0$ with the measured $E_D$.

The two-phase model predicts the myofilament water content to be about 0.3 of the total water when the latter is held at 80%. This estimation is about the same as the nonosmotic and nonsolvent water (0.25) in the intact fiber (Hinke, 1970). However, it is larger than the estimation of water (0.2) unavailable for diffusion of ions (Caillé and Hinke, 1974), and larger than the nonfreezeable water (0.2) in the whole muscle (Pézolet et al., 1978). Only a small fraction (10%) of the myofilament water can be considered as tightly bound to proteins (Kuntz and Kauzmann, 1974; Foster et al., 1976).

The cation content of the myofilament phase at pH 7.5 calculates to be 0.44 of the total cation content of the skinned fiber when $(M)_F = 1.24 (M)_0$. In comparison, the Na$^+$ and K$^+$ isotope exchange studies (Table II and III) predict that about 0.35 of the total fiber cations are located in the myofilament phase. Earlier studies (McLaughlin and Hinke, 1966; Hinke, 1969b) with ion-selective microelectrodes estimated Na$^+$ “binding” in the intact fiber to be as high as 0.8 of the fiber Na$^+$, but these estimates were based on the assumption that K$^+$ binding was negligible, an assumption which has been shown to be invalid (Caillé and Hinke, 1973; Hinke et al., 1973). Also, this study has shown K$^+$ uptake in the skinned fiber (Fig. 1 and Table II) to be as complex as Na$^+$ uptake (Fig. 2 and Table III).

The necessity to use three exponentials to fit the isotope uptake data (Figs. 1–3) is consistent with Eqs. 4a and 5a but not completely compatible with the simplified two-phase model. The persistence of compartment III and its apparent resistance to change with pH (Tables II to IV) presents difficulties. However, few problems arise in identifying compartment I with the intermyofilament space and compartment II with the intramyofilament phase. Particularly noteworthy is the observation that K$^+$ and Na$^+$ disappeared and Cl$^-$ appeared in compartment II at pH 5.4, and the observation that the composition changes predicted to occur in compartment II were similar to those actually observed in the whole fiber (Tables II to IV).

Caillé and Hinke (1972, 1973) have shown that the diffusion of a fraction $(f)$ of the cations in the myoplasm was delayed by the charge sites on the myofilaments. At pH 7.5, the fractions were 0.21 for Na$^+$ and 0.075 for K$^+$, both smaller than the compartment II fractions (Tables II and III). However, these authors have already pointed out (Caillé and Hinke, 1973; Hinke et al., 1973) that the $f$ fraction need only represent that fraction of ions in the myofilament phase that exchanges rapidly relative to the diffusion rate of the free ion. Nevertheless, a common observation in both studies was the pH dependency of the ion exchange process, i.e., the $f$ fractions for Na$^+$ and K$^+$ changed with pH as compartment II did in Tables II and III, and the $f$ fraction for Cl$^-$ changed with pH as compartment II did in Table IV.
The two-phase model must, of necessity, include among its assumptions that the electrolyte composition of the intermyofilament space in the skinned fiber is similar to the electrolyte composition of the bathing solution. The only time this assumption gives difficulty is when one tries to imagine how a 1-μm micropipette could successfully record the $E_D$ on myofilaments having diameters in the $10^{-2} \, \mu m$ range. It would have been quite realistic to expect the micropipette to measure only a fraction of the myofilamentous $E_D$ since the measuring pipette is large enough to make contact with both the myofilament and intermyofilament phases. Yet, the magnitude of the measured $E_D$ at six pH conditions (Table VI) was compatible with the two-phase model.

In conclusion, the two-phase model for myoplasm has been reasonably successful in explaining changes in the electrolyte composition with pH, partially successful in explaining the changes in isotope exchange with pH, and partially successful in predicting the changes in Donnan potential with pH in the chemically skinned muscle fiber. In addition, the model provides acceptable estimations of the net fixed charge capacity, the water content, and the electrolyte composition of the myofilaments under physiological conditions. The model should be useful to physiologists interested in electrolyte distribution and exchange phenomena in intact muscle preparations.

**APPENDIX**

Most of the volume of a muscle fiber is occupied by myofibrils and each myofibril contains two distinct phases: the myofilament and intermyofilament phase. All other intrafiber structures (sarcoplasmic reticulum, mitochondria, nuclei, etc.) are membrane-enclosed organelles and occupy relatively little myoplasmic volume, particularly in crustacean fibers. Since the myofibrils carry a net negative charge at physiological pH, the ionic composition must be different in the myofilament and intermyofilament phases. Of course, the ionic composition may be different in each organelle. The following three equations attempt to define the heterogeneous distribution of a cation ($M$) and anion ($A$) in the muscle fiber:

\[
(M)_F = a_0(M)_0 + a_1(M)_1 + \ldots_a_n(M)_n + B_M/V_F; \quad (1a)
\]

\[
(A)_F = a_0(A)_0 + a_1(A)_1 + \ldots_a_n(A)_n + B_A/V_F; \quad (2a)
\]

\[
a_0 + a_1 + a_2 + \ldots + a_n = 1. \quad (3a)
\]

$(M)_F$ and $(A)_F$ represent the analyzed concentrations; $(M)_n$ and $(A)_n$ are the concentrations in the $n$th compartment, which contains an $a_n$ fraction of the fiber water, $V_F$; $B_M$ and $B_A$ are the total amounts of cation and anion which are truly bound out of solution. For convenience, the numbered compartments can be assigned to morphological entities as follows: extrafiber, space, 0; bulk or intermyofilament phase, 1; myofilament phase, 2; other organelles, $n$.

If a fiber is equilibrated in the presence of a detergent that selectively destroys the plasma membrane and the membranes of intrafiber organelles, then Eqs. 1–3 reduce to Eqs. 4–6:

\[
(M)_F = a_1(M)_1 + a_2(M)_2 + B_M/V_F; \quad (4a)
\]
(A)_F = \alpha_1(A)_1 + \alpha_2(A)_2 + B_A/V_F; \quad (5a)
\alpha_1 + \alpha_2 = 1. \quad (6a)

Since the intermyofilament phase 1 now communicates freely with the extracellular space (0) and with all membranous organelles (n), the new \( \alpha_1 \) must include the old \( \alpha_0 \) plus \( \alpha_n \), and the new \( (M)_1 \) and \( (A)_1 \) concentrations must be equal to \( (M)_0 \) and \( (A)_0 \). The intramyofilament phase (compartment 2) also communicates freely, as before, with the intermyofilament phase, but it must continue to remain as a separate compartment since its ionic content remains different.

Because of the net negative charge on the myofilaments, the cationic and anionic concentrations in the intramyofilament phase must be in accordance with a Donnan equilibrium between compartments 1 and 2. Such an equilibrium is described as follows:

\[
\frac{(M)_2}{(M)_1} = \frac{(A)_1}{(A)_2} = \exp - \frac{E_D F}{RT}, \quad (7a)
\]

where \( E_D \) is the Donnan potential at the interphase between the myofilament and its surrounding space and \( R, T, \) and \( F \) have their usual significance.

If the \( B_M/V_F \) and \( B_A/V_F \) terms are small compared to the other terms in Eqs. 4a and 5a, then one can combine these equations with Eq. 7a and obtain the following:

\[
\exp - \frac{E_D F}{RT} = \frac{((M)_F)/(M)_1 - 1)}{1 - (A)_F/(A)_1); \quad (8a)
\]
\[
\alpha_2 = \frac{(M)_F}{((M)_1 - 1)} \left/ \left( \exp - \frac{E_D F}{RT} - 1 \right) \right. \right. \quad (9a)
\]

Thus, \( E_D \) and \( \alpha_2 \) can be calculated from the cationic \( (M)_F \) and anionic \( (A)_F \) content of the detergent-treated fiber (see Table V).

The Link with pH

Lewis and Saroff (1957) showed that cation binding to myosin (and actomyosin) was dependent on the pH of the solution. By postulating competition between the cation (Na⁺ or K⁺) and the proton (H⁺) for certain charge sites (\( n_2 \)) on the protein, they defined cation binding as follows:

\[
v_M = \frac{n_2 K_M(M)_1}{1 + K_H(H)_1 + K_M(M)_1}, \quad (10a)
\]

where \( v_M \) is the average number of cations associated with a mole of contractile protein, and \( K_H \) and \( K_M \) are the apparent association constants for H⁺ and M⁺ at the \( n_2 \) sites. For \( n_M \) to be identified with \( (M)_2 \) in Eq. 7a, \( n_2 \) must be defined as the maximum net negative charge on the filaments (say at pH 8). Furthermore, one must also postulate that the \( n_2 \) charges can be neutralized either by association with protons (H) or by screening from cationic counter-
ions \((M)_1\). With these definitions, one can relate \(v_M\) to \((M)_2\) as follows:

\[
v_M = \frac{a_2m\gamma}{1 - \gamma} (M)_2, \quad (11\text{ a})
\]

where \(\gamma\) is the fraction of fiber water by weight and \(m\) is the conversion factor, kilograms of dry weight of fiber per mole of contractile protein in the dry weight. Eq. 11 a provides the link between Eqs. 10 a and 7 a which, when combined, produce the equation,

\[
\exp \frac{E_{DF}}{RT} = \frac{a_2m\gamma}{n_2(1 - \gamma)} \left[ \frac{1}{K_M} + (M)_1 + \frac{K_H}{K_M} (H)_1 \right]. \quad (12\text{ a})
\]

This equation relates the Donnan potential \((E_D)\) between the myofilament phase and the intermyofilament phase to the hydrogen concentration \((H)_1\) at a constant cation concentration \((M)_1\). If the water terms, \(a_2\) and \(\gamma\), do not change with pH, then Eq. 12 a predicts a linear relation between \(\exp \frac{E_{DF}}{RT}\) and \((H)_1\). Since Lewis and Saroff (1957) found \(K_M\) to be about 800 for \(K^+\) and 1,600 for \(Na^+\) and since \((M)_1\) in the present study was 0.2 M (solution 4, Table I), it follows that \((M)_1 > 1/K_M\) and Eq. 12 a can be simplified to

\[
\exp \frac{E_{DF}}{RT} = a + b(H)_1, \quad (13\text{ a})
\]

where

\[
a = \frac{a_2m\gamma}{n_2(1 - \gamma)} (M)_1
\]

and

\[
b = \frac{a_2m\gamma}{n_2(1 - \gamma)} \frac{K_H}{K_M}
\]

A linear relationship between the measured potential \((E_D)\) of the Tween-treated fiber and the bath pH was indeed found (Table VI).

At pH 7.5-8.0, the measured \(E'_{D}\) (Table VI) and the \(E_D\) calculated from Eq. 8 a (Table V) are similar in sign and magnitude and suggest that the microelectrode in the Tween-treated fiber may, in fact, be recording the \(E_D\) on the surface of the filaments. To test this possibility, one can assume that \(E'_{D} = E_D\) and combine Eqs. 9 a and 13 a to give

\[
\left(\frac{(M)_F}{(M)_1} - 1\right) = a_2 \left( \frac{1 - a - b(H)_1}{a + b(H)_1} \right). \quad (14\text{ a})
\]

This equation relates the cation content \((M)_F\) of the Tween-treated fiber to the proton concentration \((H)_1\) in the equilibrating solution. Support for the assumption that \(E_D = E_D\) is achieved when Eq. 14 a produces a curve. (using
appropriate $a_2$, $a$, and $b$ values), which passes through experimentally obtained $(M)_F$ vs. $(H)_1$ points (Fig. 5).

The author is grateful to Miss E. Nee, Miss J. Au-Yeung, and Dr. M. Weir for their technical assistance.
This work was supported by the Medical Research Council of Canada through grant MT 1039.

Received for publication 12 March 1979.

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