equally well be interpreted as due to weak bonding of the incoming group which thus has a relatively small effect on the free energy of the transition state. The latter interpretation is more in accord with eq. 4 and the experiments of Doering and Zeiss.[3]

These results on *t*-butyl alcohol make it probable that the oxygen exchanges on 2-butanol are simple displacements on the protonated alcohol by water. The result that each exchange proceeds with inversion[14] is thus a most natural result. The author's conclusion that water was not covalently bonded to the transition state was based on the Hammett-Zucker hypothesis which is now suspect.[6]

It is of interest that substitution reactions of even triphenylmethyl derivatives become displacement reactions in non-ionizing media.[15] In these circumstances, the reactions are like those of the *t*-butyl derivatives discussed above in that the rate shows little variation with the nature of the incoming group.[16] It is hard to see how these reactions can proceed in any other way than with inversion of configuration and we are currently studying this problem.

Although we have interpreted the reactions of *t*-butyl derivatives as an extreme type of displacement reaction (SN2), some of the characteristics closely resemble those of an ideal SN1 reaction. Thus the rates depend primarily on the structure of the *t*-alkyl group and may parallel the cation stability as has been shown for solvolysis of diarylmethyl derivatives.[17] Also the large charge separation in the transition state will cause the variation of rate with media to be similar to that found for an ideal SN1 reaction. This intermediate type of nucleophilic displacement reaction as exemplified by the substitution reactions on *t*-butyl alcohol have a unique character of their own and it may be convenient to term them SN(int.) reactions.

Hydrolysis of Acrylonitrile to Acrylamide.—The data in Table V show that to within 10–30% $d \log k = -dH_0$. The transition state contains acrylonitrile and a proton. The presence or absence of water is undecided.

Acknowledgment.—We wish to acknowledge discussions with Dr. R. W. Taft, Jr.

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[CONTRIBUTION FROM THE GEORGETOWN UNIVERSITY AND NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH]

The Binding of Ions to the Muscle Proteins. Measurements on the Binding of Potassium and Sodium Ions to Myosin A, Myosin B and Actin[1,2]

BY M. S. LEWIS AND H. A. SAROFF

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The binding of sodium and potassium ions to myosin A, myosin B, actin and bovine and human serum albumin was studied using electrodes made of collodion membranes which were permselective to cations. Data showing *p*H dependence, concentration dependence and temperature dependence were obtained. It was found that myosin A and myosin B bound sodium and potassium, but that the other proteins did not. The imidazol and amino groups appear to control the binding of sodium and potassium to myosin A and B. The values of the equilibrium constants for the interaction of sodium and potassium with the myosins were determined, and values for ΔF° and ΔH°, were calculated.

Introduction

The interaction of potassium and sodium ions with the proteins of muscle is undoubtedly of considerable importance in the contractile processes in muscle. The extent of these interactions has been studied by several investigators by measuring the amount of the ion adsorbed by threads of myosin.[3–6] Their data present certain difficulties in interpretation because the threads represent a phase different from that of the solution in which they were equilibrated. This study was carried out on the viscous solutions and suspensions of the muscle proteins rather than on the precipitated thread. Other evidence for sodium and potassium ion binding to myosin can be found in Mihalyi's[7] hydrogen ion titration data at different salt concentrations.

When a suitable electrode exists for a certain ion, it is possible to determine the extent of the interaction of that ion with a protein by determining the activity of the ion in the presence of the protein. Hydrogen ion interactions with proteins have been studied using the hydrogen and glass electrodes. Scatchard, Scheinberg and Armstrong[8] studied the binding of chloride to serum albumin using silver–silver chloride electrodes. Carr has used permselective membranes as membrane electrodes to study the binding of cations and anions to a wide variety of proteins.[9]

(1) Taken in part from a doctoral thesis submitted by M. S. Lewis to the faculty of Georgetown University in partial fulfillment of requirements for the degree of Doctor of Philosophy, June, 1955.

(2) Presented before the American Physiological Society at the 40th Annual Meeting of the Federation of American Societies for Experimental Biology, April 17, 1956, Atlantic City, N. J.

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Because permselective membranes selectively permit the transfer of only a single ion species from one solution to another solution of the same electrolyte, they act as a reversible electrode for that ion species.[10,11] This makes it possible to determine the activities of ions for which no specific reversible electrodes exist or where the use of such conventional electrodes offers considerable difficulty. This method was used in the studies described here to determine the activities of potassium and sodium in the presence of muscle proteins, and thus to determine the extent of their interaction.

Experimental

Myosin A was prepared by a modification of the procedure of Kessler and Spicer.[12] Five hundred grams of chilled, ground rabbit muscle was extracted for 10 minutes with 1500 cc. of ice-cold solution of 0.3 *M* KCl and 0.15 *M* *p*H 6.5 phosphate buffer. After 10 minutes, 6 liters of ice-cold water was added and the suspension was strained through gauze. The residue was used for the preparation of actin. The filtrate then was diluted slowly with 10 liters of cold water and the precipitated myosin allowed to settle. The supernatant was removed by aspiration and the precipitate was centrifuged. The separated precipitate was dissolved in 0.6 *M* KCl. This solution was diluted to 0.12 *M* by the addition of 4 volumes of cold water and clarified by centrifugation. Myosin was precipitated from the supernatant by dilution of the KCl to 0.03 *M* by the addition of 3 volumes of cold water. The myosin was collected by centrifugation and then re-dissolved in 0.6 *M* KCl. The myosin was stored at 0°.

At the time it was used the myosin solution was diluted to 0.12 *M* KCl and clarified by centrifugation. It was noted that the amount of insoluble material increased with the age of the preparation until after about seven days, no myosin would remain in solution. Whether this was essentially denatured myosin or some aggregate of myosin is not known. After centrifugation, the supernatant was diluted with 20 volumes of cold water, the *p*H adjusted to 5.4, and the myosin allowed to settle. The supernatant was removed by aspiration and the myosin concentrated by centrifugation. This procedure gave a protein concentration of 0.8 to 1.0% with a potassium content of about 0.006 *M*. Myosin A prepared in this manner was found to be homogeneous electrophoretically and in the ultracentrifuge. Measurements were made on the myosin within a period of 5 hours after the completion of the preparation.

Myosin B (natural actomyosin) was prepared[13] by extracting the ground muscle for 5 hours with an ice-cold solution of 0.75 *M* KCl, 0.01 *M* Na_2CO_3 and 0.04 *M* $NaHCO_3$. After extraction, the mixture was strained through gauze and then clarified by centrifugation. The binding activity of this protein was found to remain constant for at least three weeks when stored at 0°. The myosin was precipitated by diluting the solution with 30–40 volumes of cold water, and adjusting the *p*H to 6.8. After the myosin settled, the supernatant was removed and the precipitate concentrated by centrifugation. It was then redispersed in 30–40 volumes of cold water, the *p*H adjusted to 5.3, and the myosin allowed to settle again. The supernatant was removed and the precipitate was again concentrated by centrifugation. This gave a myosin concentration of 1.5 to 2.5% and with a potassium ion content of 0.001 *M* or less. This preparation was homogeneous neither electrophoretically nor in the ultracentrifuge.

Actin was prepared by the method of Tsao and Bailey.[14] The G-actin thus prepared was then clarified prior to use by centrifugation for 30 minutes at 75,000 × gravity. The amount of active polymerizable actin in the solutions thus produced was determined by measuring the protein content of the sample, polymerizing the actin, centrifuging down the gel, and determining the protein content of the supernatant which should be free of polymerized actin. Actin solutions prepared by this procedure and tested as described above had a protein concentration of about 2%, of which about one-half was active polymerizable actin.

The bovine serum albumin was the Armour crystalline product. The human serum albumin was fraction V obtained from the American Red Cross. These were dialyzed before use.

Membranes.—The strong acid type (negatively charged) collodion-base membranes used as the membrane electrodes were prepared by the method described by Neihof.[15] The solution used to prepare the membranes contained 0.15% sulfonated polystrene (mol. wt. ~70,000) and 3% collodion in a 50–50 alcohol–ether solution. The membranes were prepared by pouring this solution over 25 × 100 mm. test-tubes rotated in a horizontal position. Three layers were cast with an interval of five minutes between each layer. After the membranes were dried on the forms overnight, they were soaked in water for 30 minutes and water was forced between the membrane and the glass to facilitate removal of the membrane from the form. Glass rings were fitted to the membranes at their open end. After being dried for one hour in an oven at 60–70°, the membranes were stored in 0.1 *N* KCl or NaCl for at least three days before use.

These membranes were clear, colorless and free of wrinkles. They were approximately 50 μ thick and their resistance was approximately 200 ohms/sq. cm. in 0.1 *M* KCl. These membranes gave an average potential of 15.55 mv. (corrected for difference in liquid junction potential) with 0.2 *M* KCl on one side of the membrane and 0.4 *M* KCl on the other. This compares well with the theoretical maximum of 16.00 mv. at 27°, and indicates a high degree of selectivity.

Saturated calomel cells were used as indifferent reference electrodes in the measurement of the potentials. Agar-saturated KCl bridges were used to connect these cells to the solutions on each side of the membrane. Both cells were maintained at the same temperature in a bath to avoid thermal asymmetry potentials. The potentials were measured with a Leeds and Northrup type K potentiometer.

In the determination of the concentration of the potassium or sodium ion in a solution,[10,11] the solution of unknown concentration was placed inside the membrane tube and the membrane was immersed to about two-thirds of its length in 100 ml. of water in a 150-ml. beaker and clamped in position. Various volumes of 1.00 *M* KCl or NaCl were added to the external solution, the external solution stirred thoroughly, and the potential measured. Slight asymmetries in the liquid junction potential were found even when the solution was the same on both sides of the membrane. This difference was measured over a range of concentrations, and used to correct the measured potentials. If the potentials obtained are plotted against the logarithm of the concentration, a straight line will result. At the zero potential obtained from their graph, the activities of the critical ion inside and outside the membrane are the same, and it has been assumed here that their concentrations are the same. In this manner it was possible to determine the concentration of an unbound ion in the presence of a protein, and since the total quantity of that ion present could be readily determined, the amount bound could be calculated.

Twenty cc. ±0.02 cc. samples of the protein solutions were used. These were weighed out because the viscosity of the protein solutions made accurate pipetting difficult. The temperature of the solution was adjusted just before use, the desired amount of 1.00 *N* KCl or NaCl added, and the *p*H adjusted with dilute KOH or NaOH of known concentration. The *p*H was adjusted to within ±0.05 *p*H unit. The salts used to adjust the K^+ or Na^+ content were recrystallized three times to remove traces of impurities which produced erratic results. The protein concentrations were determined by dry weights of samples of known volumes. The residual potassium or sodium content of the protein solutions was determined with the flame photometer.

Experimental Errors.—For myosin B at *p*H 7.7, and at a free concentration of 0.01 *M* K^+, the standard error in $\bar{\nu}_K$ is 1.5%, and at 0.1 *M* K^+ is ±7.5%. For myosin A, which was used at a lower protein concentration, the error at 0.01 *M* K^+ is ±2.5%, and at 0.1 *M* K^+ is ±18%. These errors are somewhat higher at *p*H 6.4 and lower at *p*H 9.0. These

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are the errors intrinsic in a single measurement. The errors in the apparent constants n' and k'_K were calculated from the scatter of the experimentally determined values of $\bar{\nu}_K$, after assuming that the data at pH 6.4 and pH 9.0 were essentially representative of the first and second sets of sites, respectively (see below). The errors in the apparent constants were determined by applying the least squares procedure using the equation

$$\frac{1}{\bar{\nu}_K} = \frac{1}{n'k'_K c_K} + \frac{1}{n'}$$

The values of $1/\bar{\nu}_K$ were so weighted that the variance of $1/\bar{\nu}_K$ was proportional to $1/c_K$. This was necessary to give equal weight to each value of $\bar{\nu}_K$. If this is not done, the low values of $\bar{\nu}_K$ have a much greater weight than high values of $\bar{\nu}_K$, and unrealistic variances of n' and k' result. It is shown in eq. 6 below that the apparent constant multiplied by $(1 + k_{H_1}c_H)$ gives the constant k_{K_1} when n and n' are assumed to be equal. Thus, the values of k'_{K_1} and k'_{K_2} multiplied by $(1 + k_{H_1}c_H)$ for the first set of sites (pH 6.4) and by $(1 + k_{H_2}c_H)$ for the second set of sites (pH 9.0) yield the values of k_{K_1} and k_{K_2} with their associated errors. This operation required an additional estimate of the errors in k_{H_1} and k_{H_2} and their inclusion in the calculation. The range in error of k_{H_1} and k_{H_2} was determined to be ± 0.2 pK unit from the curves shown in Fig. 1. (A change in 0.5 pK unit, the dotted curves, shifted the curve of the pH 7.7 binding by 0.3 of a pK unit too far out of a fit.) The errors shown in Tables II and III were thus calculated with the assumption that k'_K was independent of k_H.

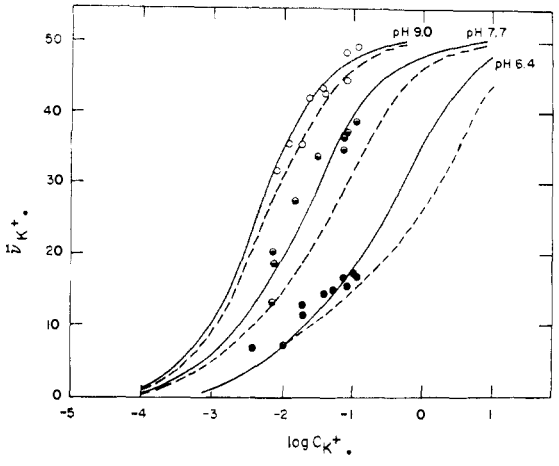

Fig. 1.—Concentration dependence data for the binding of potassium ions to myosin at 5°: ●, pH 6.4; ◒, pH 7.7; O, pH 9.0. Solid curves calculated from the constants shown in Table II using eq. 6. Dotted curves calculated from eq. 6 with the same constants except that the value of k_{H_2} has been changed to 10^9.

Myosin A—Results

Binding of potassium and sodium ions to myosin A was measured at 5°. It was important to avoid even brief exposures of myosin A to elevated temperatures. An exposure of a few hours at 27° resulted in a complete loss in binding ability of myosin A for potassium ions. Warming myosin A solutions to room temperature also brings about changes in sedimentation value.[16] The experimental points in Fig. 1 summarize our data on the concentration dependence of K^+ binding to myosin A at the pH values of 6.4, 7.7 and 9.0. Additional experiments were done at a free KCl concentration of 0.100 M at the pH values of 4.7, 5.2, 5.8, 6.4 and 7.0 to complete a pH dependence survey, the data for which are summarized in the experimental points of Fig. 2.

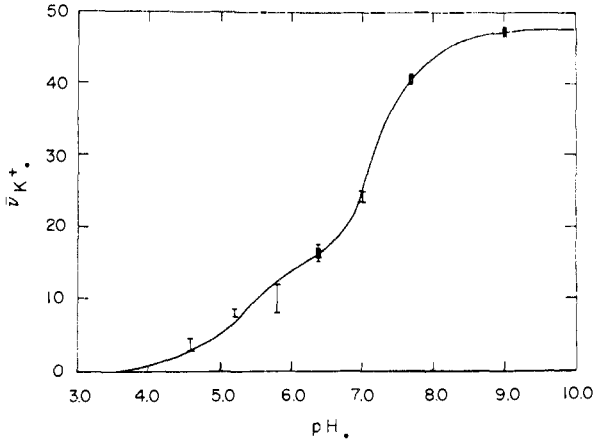

Fig. 2.—pH dependence data for the binding of potassium ions to myosin A at 5°. Concentration of free potassium ion was 0.100 M. Each symbol, I, represents the range of values from 3 to 4 measurements. The symbols, ▮, represent values found by interpolation of the concentration dependence data. The curve is calculated from eq. 6 and the constants in Table II.

Sodium ion binds to myosin A more strongly than does the potassium ion. A study of the concentration dependence of Na^+ binding to myosin A at 5° was made at pH 7.7. The data are represented in the experimental points of Fig. 3.

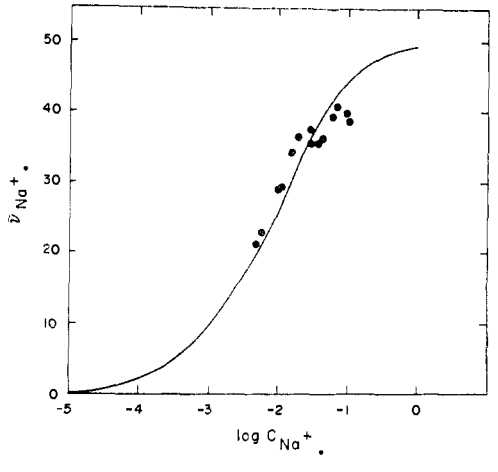

Fig. 3.—Concentration dependence data for the binding of sodium ions to myosin A at 5°, pH 7.7. The curve is that calculated from eq. 6 with the constants in Table II.

Effect of Actin on Binding of Potassium Ions to Myosin A.—To study the effect of actin upon the potassium binding of myosin A, various quantities of G-actin were added to samples of myosin A, the desired quantity of KCl was added, the pH adjusted to the desired value with KOH, and the free K^+ ion measured as previously described. The result of such an experiment is given in Fig. 4. If molecular weights of approximately 70,000 and 420,000 are taken for the actin and myosin A, respectively, then the potassium binding to the resulting actomyosin is at the same level as potas-

(16) K. Laki and W. R. Carroll, *Nature*, **175**, 389 (1955).

sium binding to myosin B (see below) when the molar ratio of actin to myosin A is 0.5:1 or greater. The binding of potassium to actin alone was studied at *p*H's 7.7 and 9.0 over the concentration range of 0.004 to 0.100 *M* K^+. Under these conditions, no binding of potassium to actin could be detected.

Interpretation of Myosin A Data

Apparent association constants, k'_K, and apparent maximum number of sites available n', may be evaluated from the concentration dependence data of Fig. 1 and the use of the mass law expression[17]

$$\bar{\nu}_K = \frac{k'_K c_K n'}{1 + k'_K c_K} \qquad (1)$$

where $\bar{\nu}_K$ is the average number of potassium ions found per mole of myosin, c_K is the concentration of the free potassium ions in solution, n' is the apparent maximum number of binding sites, and k'_K is the apparent intrinsic association constant. The usual methods of evaluating these constants are to plot $1/\bar{\nu}$ as a function of $1/c$,[17] or to plot $\bar{\nu}/c$ as a function of $\bar{\nu}$.[18] The values of k'_K and n' are determined from the extrapolated intercepts and slopes. Table I lists the values of k'_K and n' found in this manner from the concentration dependence curves at the three *p*H values. In Table I are listed, also, the values of k'_K calculated[8] from the k_{K_1} and k_{K_2} values of eq. 6 below. Both the variation of n' and k' with *p*H and the *p*H dependence data in Fig. 2 indicate the possibility of competition with hydrogen ion for the site to which the potassium ion is bound.

TABLE I

APPARENT BINDING CONSTANTS FOR MYOSIN A AT 5°

Bound ion	*p*H	n'	k'_K	$\frac{k_{K_1}}{1 + k_{H_1}c_H}$	$\frac{k_{K_2}}{1 + k_{H_2}c_H}$
K^+	6.4	18 ± 0.8	124 ± 24	89	..
K^+	7.7	43 ± 1.1	98 ± 11	..	..
K^+	9.0	50 ± 1.2	200 ± 33	..	150
Na^+	7.7	42 ± 0.6	225 ± 22	..	..

The following treatment involves the assumption of direct competition between hydrogen and potassium ion for the same site.[19,20]

Equations describing the competition at a single site are

$$\text{Site} + H^+ = (\text{Site } H^+)$$
$$\text{Site} + K^+ = (\text{Site } K^+) \qquad (2)$$

For a group of n sites per mole of protein, neglecting electrostatic corrections, the expression for the number of hydrogen ions bound per mole of protein, $\bar{\nu}_H$, becomes

$$\bar{\nu}_H = \frac{k_H c_H (n - \bar{\nu}_K)}{1 + k_H c_H} \qquad (3)$$

where k_H is the intrinsic association constant of the specific site ($k_H = 10^{pK}$) for hydrogen ion, c_H represents the concentration of the free hydrogen ion in solution, and $\bar{\nu}_K$ represents the number of potassium ions bound per mole of protein. The analogous expression for potassium ions is

$$\bar{\nu}_K = \frac{k_K c_K (n - \bar{\nu}_H)}{1 + k_K c_K} \qquad (4)$$

where k_K is the intrinsic association constant for the potassium ion, and c_K is the concentration of the free potassium ion. The solution of eq. 3 and 4 for $\bar{\nu}_K$ gives the expression

$$\bar{\nu}_K = \frac{k_K c_K n}{1 + k_K c_K + k_H c_H} \qquad (5)$$

If more than one set of sites is involved, equation 5 may be written

$$\bar{\nu}_K \text{ (total)} = \sum_i \bar{\nu}_{K_i} = \sum_i \frac{k_{K_i} c_{K_i} n_i}{1 + k_{K_i} c_K + k_{H_i} c_H} \qquad (6)$$

Thus, for this competitive concept, $k_{K_i} = k'_{K_i}(1 + k_{H_i}c_H)$ when n and n' are assumed to be equal.

Examination of the *p*H dependence data (Fig. 2) reveals that two sets of sites are probably active in potassium ion binding to myosin. When, therefore, two sets of sites, the first with $n = 15$ and the second with $n = 35$, are applied to eq. 6 the solid curves shown in Figs. 1 and 2 are calculated. These curves represent a satisfactory fit to the experimental data. Table II gives the values for n_1, n_2, k_{K_1}, k_{K_2}, k_{H_1} and k_{H_2} required for these calculated curves to fit the data on the assumption that there is competition as described by the eq. 2 and 6.

It is not too difficult to assign reasonable sets of ionizing groups to the pK and n values in Table II. Amino acid analysis[21] indicates that there are 15 histidine residues per 10^5 g. of myosin A. Mihalyi's titration data[7] indicate a pK of approximately 7 for imidazole groups at 25° (7.3 at 5°). Mihalyi's data also indicate approximately 25 amino groups with a pK of about 7.6 at 25° (8.2 at 5°) as well as a larger number at a pK between 10 and 11. The minor discrepancies between these values and the values of $n_2 = 35$ and $k_{H_2} = 3.3 \times 10^8$ (pK 8.5) found here may be at least partly attributable to the differences in the protein preparations used here and in Mihalyi's experiments. Differences of even small amounts of actin in the preparations

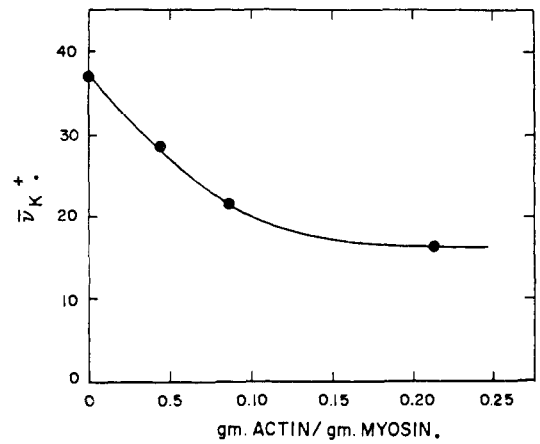

Fig. 4.—The effect of actin on the binding of potassium ions to myosin A at 5°, free K^+ concentration = 0.075 ± 0.002. (The curve is not a calculated one.)

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TABLE II

POTASSIUM, SODIUM AND HYDROGEN ION BINDING CONSTANTS FOR MYOSIN A AT 5°

	Max. no. of ions bound ($K^+ + H^+$) per 10^5 g. myosin	Association constants: Hydrogen ion	Potassium ion	Sodium ion
First set	$n_1 = 15 \pm 0.7$	$k_{H_1} = 2 \times 10^7$ ($pK_1 = 7.3$)	$k_{K_1} = 800 \pm 250$	$k_{K_1} = 1600 \pm 500$
Second set	$n_2 = 35 \pm 0.9$	$k_{H_2} = 3.3 \times 10^8$ ($pK_2 = 8.52$)	$k_{K_2} = 200 \pm 36$	$k_{K_2} = 400 \pm 72$

TABLE III

THERMODYNAMIC CONSTANTS FOR THE BINDING OF POTASSIUM IONS TO MYOSIN A AND MYOSIN B

	n_K: Myosin A	n_K: Myosin B	k_K: Myosin A 5°	k_K: Myosin B 5°	k_K: Myosin B 27°	$\Delta F°_K$, cal./mole: 5°	$\Delta F°_K$, cal./mole: 27°	$\Delta H°_K$, cal./mole av., 16°
First set of sites (imidazole groups)	15 ± 0.7	10 ± 0.5	800 ± 250	800 ± 210	800 ± 210	−3690 ± 180	−3980 ± 160	0 ± 3100
Second set of sites (amino groups)	35 ± 0.9	15 ± 1.5	200 ± 36	200 ± 52	50 ± 10	−2930 ± 150	−2330 ± 120	−10,300 ± 2500

could be responsible for these discrepancies, particularly that in the value of n. Studies on myosin B, see below, support this line of reasoning.

Two other competitive mechanisms have been considered, particularly in an attempt to explain the anomalously high association constants for the binding of Na^+ and K^+ to myosin.[22] These mechanisms involve hydrogen bonding and may be described by the reactions

$$-COO^-....H_3^+N- + K^+ = -COOK + H_3^+N- \quad (7)$$

$$-COO^-....H_3^+N- + K^- = -COO^-..K^+..H_2N- + H^+ \quad (8)$$

Equation 7 represents competition between the potassium and ammonium (or imidazolium) ions for the carboxylate ion. Equation 8 represents competition between H^+ and K^+ for the hydrogen bonded and chelated positions, respectively.

Application of the binding expression derived from the idea in equation 7 can result in a satisfactory fit of the experimental data. However, the binding constants required for the fit were such as to result in abandoning the reaction of equation 7 as an explanation for Na^+ and K^+ binding.

The chelation mechanism of equation 8 results in a binding expression which not only fits the data but does so with values of the binding constants approaching those found for the direct competitive reaction of eq. 6. This chelation mechanism, while not changing the binding constants appreciably, makes the values of these constants more acceptable in light of our knowledge of the chelation of small compounds with Li^+, Na^+ and K^+.[23]

Myosin B—Results and Interpretation

The instability of preparations of myosin A at temperatures above 5° makes difficult a study of the temperature dependence of the potassium ion binding. Myosin B may be considered to be a complex of myosin A and actin and was, therefore, chosen as the protein to study for temperature dependence of the binding. Myosin A is the protein of choice for binding studies because this protein appears monodisperse on examination in both centrifugal and electrical force fields. However, myosin B, while stable at room temperature, is polydisperse under the conditions of these potassium ion binding studies. The heterogeneity of myosin B prepared in these studies was found to increase at pH 9.0. Blum[24] has reported a study of the dissociation of myosin B occurring at pH 9 and at relatively high salt concentrations.

Measurements were made at the pH values of 6.4, 7.7 and 9.0, and at the temperatures of 5 and 27°. The experimental points of Figs. 5 and 6 represent these data. Figure 7 summarizes all the experimental data collected at pH 9.0. The data with the free potassium ion concentration above 0.06 M at pH 9.0 were not used in the interpretation of the myosin B binding since we believe that the irregularities noted were caused by the dissociation and heterogeneity phenomena. The solid curves drawn in Figs. 5, 6 and 7 for the data at 5° are calculated curves derived from eq. 6 with the same association constants as those found for myosin A but with $n_1 = 10$ and $n_2 = 15$ for myosin B. (This lower number of binding sites may be attributed to the actin content.) The data fit well the calculated curves at pH 6.4 and 7.7, while there is a discrepancy of one order of magnitude for the value of k_{K_2} at pH 9.0.

The following procedure was used in evaluating the association constants for myosin B at 27°. First, the shifts in pK values for the hydrogen ions from 5 to 27° were calculated using the van't Hoff expression

$$\Delta H°_{ion} = -4.579 \frac{T_1T_2}{T_1 - T_2}(pK_2 - pK_1) \quad (9)$$

The values of $\Delta H°_{ion}$ used were 6,000 cal. for the imidazole groups and 11,000 cal. for the amino groups. This gave association constants at 27° of $k_{H_1} = 1 \times 10^7$ (pK 7.0) and $k_{H_2} = 8.7 \times 10^7$ (pK 7.94). Then, values of n and k_K at 27° were found to fit the experimental data. These were found to be $n_1 = 10$, $k_{K_1} = 800$, and $n_2 = 15$, $k_{K_2} = 50$.

The calculated curves and experimental data show satisfactory agreement except, again, for some of the data at pH 9. With the reservation that the discrepancies at pH 9.0 must be adequately explained, it appears reasonable to assume that similar sites in both myosin A and B bind potassium ions. The difference appears to lie in the number of sites available for binding. With the assumption that eq. 1 and 6 describe the binding reaction of potassium ions to myosin and that the extrapola-

(22) A detailed treatment by H. A. S. of these competitive mechanisms will appear elsewhere.

(23) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice-Hall, Inc., New York, N. Y., 1953, Appendix I.

(24) J. J. Blum, *Arch. Biochem. Biophys.*, **43**, 176 (1953).

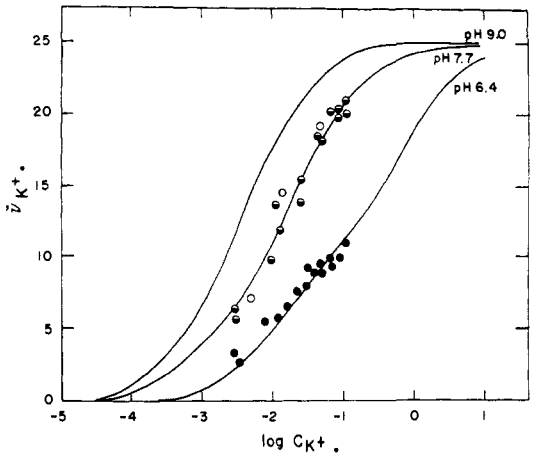

Fig. 5.—Concentration dependence data for the binding of potassium ions to myosin B at 5°: ●, *p*H 6.4; ◒, *p*H 7.7; O, *p*H 9.0. Curves calculated from eq. 6 and the constants in Table III (k_H values from Table II).

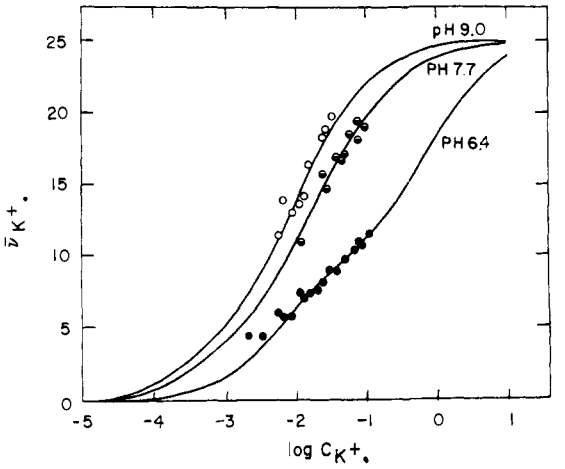

Fig. 6.—Concentration dependence data for the binding of potassium ions to myosin B at 27°, ●, *p*H 6.4; ◒, *p*H 7.7; O, *p*H 9.0. Curves calculated from eq. 6 and the constants in Table III (the k_H values were calculated as explained in the text from the van't Hoff expression).

tion from myosin A to myosin B is legitimate, the values of $\Delta F°$ and $\Delta H°$, for the binding of K^+ to the imidazole and amino groups of myosin A and B were calculated using the relations

$$\Delta F°_K = -RT \ln k_K \qquad (10)$$

$$\Delta H°_K = RT^2 \left(\frac{\partial \ln k}{\partial T}\right)_P \qquad (11)$$

Table III summarizes the values for the binding sites of myosin.

Serum albumin, both human and bovine were studied at 27° in the *p*H range from 5 to 9 and in a concentration range from 0.01 to 0.09 *M* KCl. No binding ($\bar{\nu} = 0 \pm 1$) of potassium ions was found to occur under these conditions.

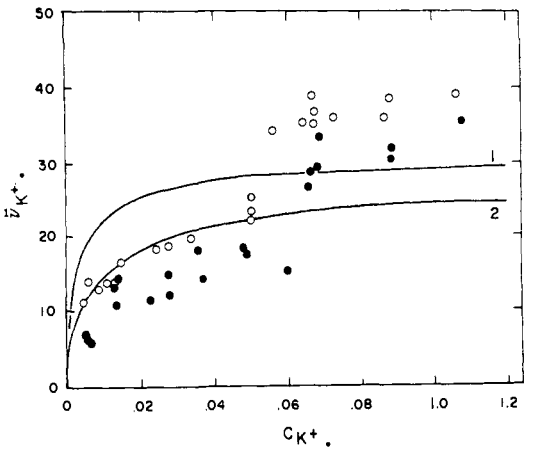

Fig. 7.—The binding of potassium ions to myosin B at *p*H 9.0: ●, 27°; O, 5°. Curve 1 calculated for 27°, curve 2 calculated for 5° from eq. 6 with the same constants as in Figs. 5 and 6.

Actin was studied at 27° and at *p*H 7.7 and 9.0. No binding of potassium ions was found in the concentration range from 0.005 to 0.09 *M* KCl.

Discussion

The validity of the assumption that the activity coefficients of the free potassium and sodium ions are not affected seriously by the myosin is supported by the following observations: (1) the binding of sodium ions is stronger than that of the potassium ions, and (2) warming the myosin A solutions to room temperature destroys the binding properties of the protein. The second supporting argument requires the qualification that large changes in what constitutes the domain of the molecule (per unit weight) in solution do not occur in the warming process. Additional study of the temperature effect will be required to describe completely the change in myosin, particularly in light of the proposed chelation mechanism.

The temperature dependence data are subject to the limitations already described. The $\Delta H°_K$ for the first set of sites (imidazole groups) is similar to that found for association reactions between ions where it is assumed that oriented water of hydration is lost to give an increase in entropy. It is difficult to speculate on the reasons for the positive $\Delta H°_K$ value for the second set of sites (amino groups) in light of the uncertainties in the *p*H 9.0 data for myosin B. The difficulties in obtaining good temperature dependence data for binding to a protein such as myosin are intrinsic in the nature of this protein.

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