STUDIES OF IONIC DISTRIBUTION IN LIVING CELLS: IV. EFFECT OF OUABAIN ON THE EQUILIBRIUM CONCENTRATIONS OF Cs^+, Rb^+, K^+, Na^+, AND Li^+ IONS IN FROG MUSCLE CELLS

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SUMMARY

The equilibrium distribution of Cs^+, Rb^+, K^+, Na^+, and Li^+ was studied in normal frog sartorius muscles and in sartorius muscles treated with 3.27×10^{-7} M ouabain. The rank order of selectivity was, respectively, $Rb^+ > K^+ > Cs^+ > Li^+ > Na^+$ for normal muscles and $K^+ > Li^+ > Cs^+ > Rb^+ > Na^+$ for ouabain-treated muscles. The data are not readily predictable on the basis of the membrane theory but can be explained on the basis of the association-induction hypothesis.

INTRODUCTION

Intracellular K^+ ion in frog muscle cells can be reversibly replaced by an approximately equimolar amount of Na^+ ion, either by the withdrawal of K^+ ion from the bathing medium[1-3] or by exposure to ouabain.[4,5] In both cases, the extra Na^+ ion gained has been shown by NMR spectroscopy* to be in an adsorbed state.[5,6] These findings offer support for the association-induction hypothesis,[7,8] according to which the bulk of intracellular K^+ ion is in an adsorbed state.

These earlier studies also verified two other concepts introduced in the association-induction hypothesis: (1) that there is cooperative interaction among the adsorption sites for K^+ and Na^+ ion in living cells (for additional supportive evidence, see refs. 9, 10); (2) that the coordinated functions of living cells depend upon the control of adsorption and desorption on many (regular) sites by a small number of controlling agents, referred to as "cardinal adsorbents," which interact with key protein sites, called "cardinal sites."

In the present paper, we shall report studies on the effect of ouabain on the equilibrium distribution in frog muscles of 5 alkali-metal ions: Cs^+, Rb^+, K^+, Na^+, and Li^+. The data will provide another opportunity to test predictions of the membrane theory and those of the association-induction hypothesis.

***Note added in proof: Recent development in NMR spectroscopy by H. Berendsen *(Ann. N.Y. Acad. Sci., 1972)* has shown that this earlier interpretation of NMR data now needs revision.**

THEORY

According to the association-induction hypothesis, the ionic distribution in a resting cell represents a metastable equilibrium state. In this state, the bulk of K^+ ion and roughly half of the intracellular Na^+ ion are adsorbed on protein sites. The remaining K^+ and Na^+ ion in the cells exist as free solutes in the cell water, which, being in a physical state different from normal water—namely, the state of polarized multilayers—accommodates K^+ and Na^+ ion at equilibrium at concentrations lower than in the bathing medium.[4]

The preferential adsorption of K^+ over Na^+ ion is considered to reflect a certain electronic state of the β- and γ-carboxyl groups on cell proteins that constitute the major adsorption sites for the alkali-metal ions. This electronic state is quantitatively represented by a parameter called the "c-value." Rigorously defined elsewhere (ref. 7, p. 57), the c-value may be considered as representing the electron density of the anionic group. Differences in the c-value are by definition the underlying cause of differences in pK values. Acetic acid, a weak acid (pK = 4.7), has a high c-value; trichloracetic acid, a strong acid (pK value $<$ 1), has a low c-value.

Theoretical calculations using known physical constants led to the conclusion that at each c-value (and a specified polarizability value) of the anionic group, a specific set of quantitative relations exists among the adsorption energies for each of the 5 alkali-metal ions. Thus, at very low c-value, the sequential order of preference is $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$, while at very high c-value, the order is completely reversed: $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. At intermediate c-values, other rank orders were calculated theoretically (see Fig. 3), which exceed in number the 11 orders derived on a simpler model by Eisenman, Rubin and Casby[12] and Eisenman.[13] A more quantitative way of presenting these sequential orders is to choose K^+ ion as a reference ion and express the rank order in terms of an intrinsic equilibrium constant $K^{oo}_{K\to X}$ where X can be any other ion in question. Thus, the rank order $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ is expressed in terms of different values of $K^{oo}_{K\to Cs}$, $K^{oo}_{K\to Rb}$, $K^{oo}_{K\to Na}$, $K^{oo}_{K\to Li}$.

In the association-induction hypothesis, drugs, hormones, and other biological controlling agents referred to collectively as "cardinal adsorbents" have the function of interacting with a controlling "cardinal site" on cell proteins and thus either maintaining or causing an all-or-none shift of the c-values of the many regular sites cooperatively linked to one another, and together called a "gang."[7]

If the theory and experimental facts show agreement, the theoretically calculated relations between the c-value and adsorption energies of the alkali-metal ions would permit us to estimate the c-value of the sites normally adsorbing K^+ ion, by matching the theoretical rank order of preferential adsorption of the 5 alkali-metal ions, Cs^+, Rb^+, Na^+, and Li^+, with the experimentally observed rank order found in normal resting muscles.

In the preceding paper, we have shown that each concentration of ouabain brings about a different equilibrium ratio of K^+ and Na^+ ion in muscle cells, approaching a maximum effect when the ouabain concentration reached 3.27 x 10^{-7} M, at which point nearly all cardinal sites must be occupied by ouabain. A comparison of the $K^{oo}_{K\rightarrow Na}$ value at this ouabain concentration with the theoretically calculated values of $K^{oo}_{Na\rightarrow K}$ indicates a c-value range which is compatible with the data. With the new c-value thus defined, the theoretical data will predict a new specific sequential order among the 5 alkali-metal ions. This theoretical order can then be compared with the experimentally observed sequential order of the 5 alkali-metal ions in ouabain-treated muscles, thereby offering another opportunity to test the predictions of the association-induction hypothesis.

MATERIALS AND METHODS

All experiments were pertormed on the isolated sartorius muscles of Wisconsin and Vermont leopard frogs ***(Rana pipiens pipiens***, Schreber). which were kept in running water and force-fed hamburger meat once or twice a week.

RbCl and CsCl were obtained 99.9% pure from Penn Rare Metals (Revere, Pa.). Other chemicals were of c.p. grade.

Ion equilibrium studied followed previously described procedures.[3,14] Hot HCI extraction for tissue ions described in the 1969 Ling and Bohr article were used. Rb, Cs, K, Na, and Li were determined by flame photometry using a Beckman DU spectrophotometer (with a flame attachment) and radiation buffers. The phototube was replaced by a Hamamatsu R-136 wide-range multiplier tube to increase the sensitivity for K^+, Rb^+, and Li^+ determinations.

Data of ith ion concentration in the cell $[p_i]_{in}$ and ith adsorbed ion in the cell, $[p_i]_{ad}$, both in units of μmoles per gram of fresh cells, were obtained using the following equations:

$$[p_i]_{in} = 1.09\left\{[p_i]_{tis} - (0.035 + 0.05\,\beta)\,[p_i]_{ex}\right\}, \tag{1}$$

and

$$[p_i]_{ad} = [p_i]_{in} - \alpha q_i\,[p_i]_{ex}. \tag{2}$$

which are essentially those given earlier.[3,5] $[p_i]_{tis}$ is the concentration of ith ion in μmoles per gram of fresh whole muscle tissue. β is the distribution coefficient of the ith ion between 1 gram of fresh connective tissue isolated from the vicinity of the sartorius muscle and 1 ml of the external solution. $[p_i]_{ex}$ is the concentration of the ith ion in the bathing solution. *a* is the percentage of water in 1 gram of fresh cells, q_i is the equilibrium distribution coefficient of the ith ion between the cell water and the external solutions. In the preceding paper, the value of aq_i chosen was 0.1. In this paper, a lower value (0.05) was used. The reason for this change will be made clear in the following section.

RESULTS

Equilibrium Distribution of Cs^+, Rb^+, K^+, Na^+, and Li^+ in Normal Muscles

Isolated frog sartorius muscles were introduced sterilely into Ringer-GIB medium at 25°C in flasks shaken at the rate of 60 excursions per minute. The medium contained approximately 1 mM each of K^+, Rb^+, and Cs^+ ion; 5 or 10 mM Li^+ ion; and 100 mM Na^+ ion. As shown in Figure I, the muscles, when introduced into these media, promptly began to accumulate Rb^+, Cs^+, and Li^+ ion and to lose not only K^+ ion but often Na^+ ion as well. Equilibrium was reached in about 3 days and maintained for at least an equal length of time.

The final equilibrium concentration of each ion from the data shown in Figure 1, as well as two similar sets of data, are tabulated in Table 1. To arrive at the values shown, it was necessary to know the equilibrium contents of the 5 alkali-metal ions in the connec-

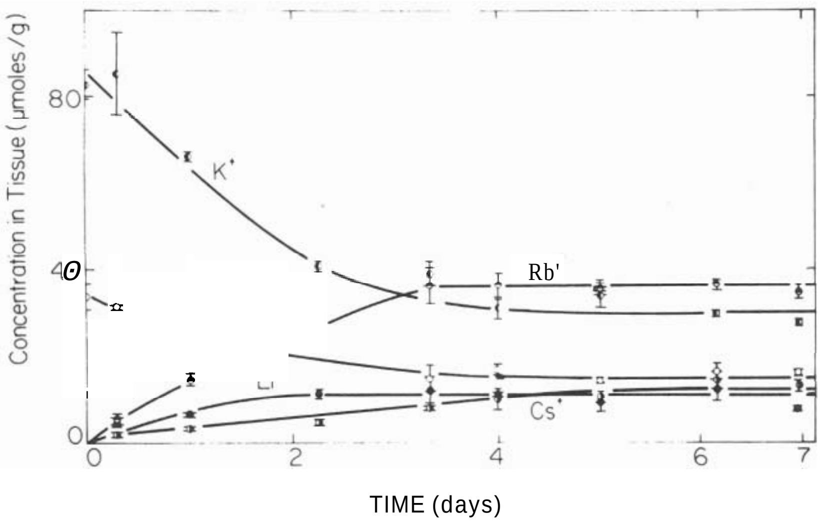

Figure 1. Time course of the uptake of Rb^+, Cs^+, and Li^+ ion and loss of K^+ and Na^+ in normal frog muscles at 25°C.
Each point represents the average (±S.E.) of 4 determinations. The final concentrations of the external solutes were 0.83 (Cs^+), 0.88 (Rb^+), 1.35 (K^+), 8.20 (Li^+), and 99.9 mM (Na^+). Experiments of 1-17, 1969. Other data given in Table 1. Solutes were changed once after 48 hours of incubation.

tive tissues. These data, given in Table 2, were obtained from normal connective tissues and from connective tissues treated with ouabain in exactly the same manner as the muscles. The ion concentrations in the connective tissues, divided by those in the external medium, referred to as β, were then used for all data calculated according to Equation 1.

The data presented in **Table** 1 show that if we use a value of aq_i equal to 0.1, the Na^+-ion concentration in the cells often becomes significantly negative. For this reason, we have decided to use the lower value of 0.05.

Table 1. The Equilibrium Distribution of Cs^+, Rb^+, K^+, Na^+ and Li^+ in Normal Frog Muscles at 25°C. Data are the averages and S.E. of 3 sets of data exactly the same as those shown in Figure 1. Each individual value is the average of the last 4 points of the time course after the curves have completely flattened.

	$[p_i]_{ex}$	$[p_i]_{tis}$	$[p_i]_{in}$	$[p_i]_{ad}$	$[p_i]_{in}/[p_i]_{ex}$	$[p_i]_{ad}/[p_i]_{ex}$	$\frac{[p_i]_{ad}/[p_i]_{ex}}{[K]_{ad}/[K]_{ex}}$
Cs	0.93±0.04	9.70±1.96	10.4±2.17	10.4±2.17	11.4±2.79	11.4±2.79	0.55±0.11
Rb	0.90±0.04	32.7±1.96	36.3±2.84	36.3±2.84	41.0±4.54	41.0±4.54	2.0±0.21
K	1.47±0.05	27.7±0.98	29.7±0.98	29.3±0.72	20.3±1.52	20.0±1.25	1.0
Na	105±1.93	23.7±3.34	12.8±3.22	7.77±3.29	0.12±.031	0.045±0.026	0.0037±0.00017
Li	6.37±0.75	9.67±1.09	9.43±0.90	9.37±0.95	1.53±0.20	0.95±0.14	0.048±0.013

Table **2.** The Equilibrium Distribution of Cs^+, Rb^+, K^+, Na^+, and Li^+ in Connective Tissues in the Presence and Absence of 3.27 x 10^{-5} M ouabain at 25°C.

		Cs				
		Cs	Rb	K	Na	Li
CONTROL	$[p_i]_{tis}$	4.47	4.75	5.07	151	6.96
	$[p_i]_{ex}$	1.14	1.16	1.30	104	9.90
	β	(3.90)	(4.10)	(3.90)	(1.45)	(0.70)
OUABAIN	$[p_i]_{tis}$	1.81	2.65	2.62	113	6.54
	$[p_i]_{ex}$	1.14	1.16	1.30	104	9.90
	β	(1.59)	(2.28)	(2.02)	(1.08)	(0.66)

Although there are some minor flucutations among the different sets of data in Table 1, the relative intracellular-extracellular concentration ratio for the 5 ions consistently follows the same rank order: $Rb^+ > K^+ > Cs^+ > Li^+ > Na^+$. This order is in agreement with the rank order found by Cohen[15] for alkali-metal ions accumulated in *Chlorella* cells, with the previous conclusions of Ling and Ochsenfeld concerning the distribution of K^+, Rb^+, Cs^+, and Na^+ ion in frog sartorius muscle from competitive studies,[16] and with the demonstration of selective accumulation in the exposed cytoplasm of cut frog sartorius muscles.[17] The observed rank order, however, shows some difference 'from the data for K^+, Rb^+, and Cs^+ ion distribution in adult rat muscles, which have demonstrated selectivity ratio in the rank order $Cs^+ > Rb^+ > K^+$ after two weeks or longer of feeding (ref. 18; ref. 7, p. '232).

The average equilibrium content in our present data shows that Rb^+ ion is accumulated preferentially over K^+ by a factor of 2. On the other hand, K^+ ion is 20, 26, and 200 times more preferred than Cs^+, Li^+, and Na^+ ion, respectively.

The Effect of Ouabain on the Equilibrium Distribution of Cs^+, Rb^+, K^+, Na^+, and Li^+

In the preceding paper of this series, we showed that at a concentration of 3.27×10^{-7} M, ouabain has exerted virtually its maximal effect. That is, at this concentration, virtually all the involved types of cardinal sites are occupied by this drug. Figure 2 and Table 3 show that at this concentration there are not only profound alterations in the relative intracellular concentrations of K^+ and Na^+, as is well known, but also significant alterations in the distribution of Rb^+, Cs^+, and Li^+ ion as well.

Thus, in comparison with the pattern of distribution in normal muscles, there is a

Table 3. The Equilibrium Distribution of Cs^+, Rb^+, K^+, Na^+, and Li^+ in Frog Muscles in the Presence of 3.27×10^{-7} M Ouabain at 25°C.
Each value is the average (±S.E.) of a total of 24 values from 6 sets of 4 individual determinations. Muscles were equilibrated for 4 to 5 days in Ringer solution containing the five ions and ouabain.

	n	$[p_i]_{ex}$	$[p_i]_{tis}$	$[p_i]_{in}$	$[p_i]_{ad}$	$[p_i]_{in}/[p_i]_{ex}$	$[p_i]_{ad}/[p_i]_{ex}$	$\frac{[p_i]_{ad}/[p_i]_{ex}}{[K]_{ad}/[K]_{ex}}$
Cs	24	1.18±0.03	2.12±0.26	2.16±0.29	2.11±0.29	1.86±0.30	1.82±0.30	0.85±0.06
Rb	24	1.35±0.03	2.24±9.32	2.22±0.34	2.15±0.35	1.68±0.28	1.62±0.28	0.74±0.08
K	24	1.73±0.26	3.72±0.43	3.80±0.47	3.59±0.42	2.43±0.29	2.12±0.29	1.0
Na	24	102±0.56	96.4±1.79	94.9±2.07	89.9±2.07	0.94±0.019	0.89±0.05	0.48±0.07
Li	24	6.21±0.10	10.8±0.83	11.2±0.88	10.9±0.90	1.81±0.15	1.76±0.15	0.90±0.13

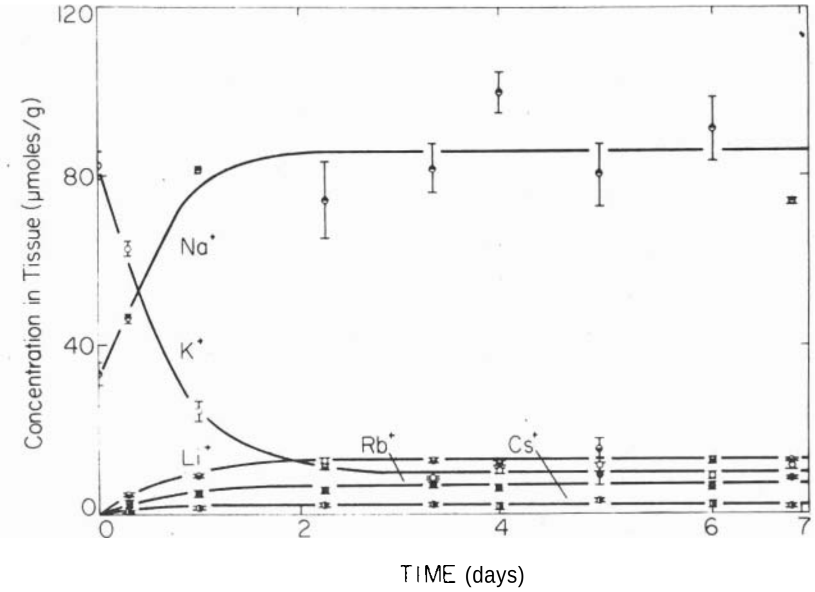

Figure **2.** Time course of uptake of Rb^+, Cs^+, Li^+, and Na^+ and loss of K^+ of frog muscle in solute containing these ions and ouabain. (3.27×10^{-7} M) at 25°C.
Final equilibrium concentrations in the external solution were 0.98 (Cs^+), 0.95 (Rb^+), 1.38 (K^+), 7.81 (Li^+), and 99.0 mM (Na^+). Experiments of 1-17, 1969. Solutions were not changed.

20-fold increase in the relative uptake of Li^+, and a more than 100-fold increase in the Na^+-ion accumulation. The relative preference for Cs^+ ion is changed the least (i.e., 1.5-fold increase). A shift also occurs in the relative preference of K^+ and Rb^+ ion. Thus, in normal muscle the selectivity is 2 to 1 in preference of Rb^+; in the ouabain muscle, it is 1 to 0.74 in preference of K^+ ion.

DISCUSSION

In. the membrane pump model, the asymmetrical distribution of alkali-metal ions is attributed to an energy-consuming pumping action. Poisoning with ouabain leads to an increase in intracellular Na^+ and intracellular K^+ ion. This is usually attributed to interference with the pump. With the pumping activity coming to a halt, the ion concentrations in the cell water approach those in the surrounding medium.

Thus, the membrane model predicts that when a new equilibrium distribution is attained in the presence of maximal concentrations of ouabain, all 5 alkali-metals should reach the same concentration as that in the external media.

The data shown in Figure 2 and Table **3** demonstrate, however, that each ion assumed a different distribution ratio in the new equilibrium after treatment with 3.27 x 10^{-7} M ouabain. These findings therefore do not support the prediction of the membrane theory.

The association-induction hypothesis offers explanations for the effect of drugs on the pattern of ion accumulation in terms of a shift in the c-value.

From the theoretically calculated adsorption energies, the relative preference of the various alkali-metal ions in comparison with that of K^+ ion could be calculated. The

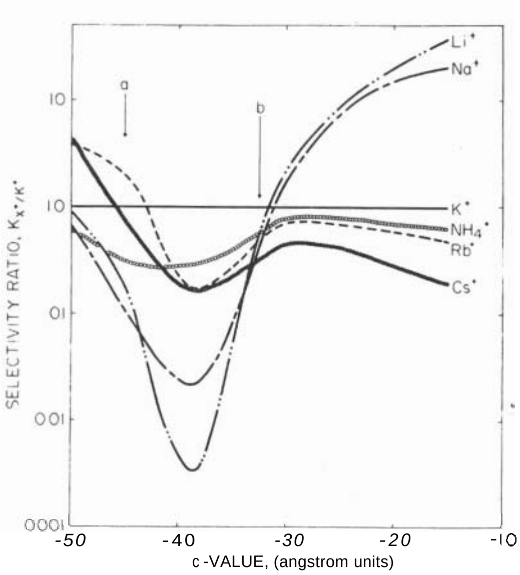

Figure 3. The relation between the selectivity ratios of various cations and the c-value. The K^+ ion is taken as unity and selectivity ratios are calculated from the association energies given in Figure **4.11** of Ref. **7,** (polarizability of anionic group: **2.0** x 10^{-24} cm^3) divided by **2** (see section **4, 4A** of Ref. 7).

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*In the theory given by Ling in **1962,** three theoretical models were calculated with a equal to **0.87** x 10^{-24}, **1.25** x 10^{-24}, and **2.0** x 10^{-24} cm^3 because at that time we had no accurate values for carboxyl polarizability. Years after the publication of the monograph, Dr. D. Reichenberg of the National Physical Laboratory, Middlesex, England, brought to our attention the fact that more reliable figures for the polarizability of the carboxyl groups place it even higher than **2.0** x 10^{-24} cm^3. For this reason, we consider the data calculated with the polarizability value of **2.0** x 10^{-24} cm^2 as the more reliable, and it is these data that are reproduced in Figure **3.**

results of such calculations based on the data* given in 1962 are reproduced as Figure **3.** Comparing this theoretical data with the experimental data of the equilibrium concentrations of the 5 alkali-metal ions in normal muscles (Table 1), one finds that the c-value at which a similar rank order is predicted is about –4.50 Å. Figures 4 and 5 give the theoretically predicted relative distribution ratios and the experimentally observed data, respectively. The general sequential orders are the same in each case: $\mathrm{Rb}^+ > \mathrm{K}^+ > \mathrm{Cs}^+ > \mathrm{Li}^+ > \mathrm{Na}^+$.*

A pronounced effect of 3.25 x 10^{-7} M ouabain was a replacement of intracellular K^+ by adsorbed Na^+ ion.[5] The theoretical c-value vs. the selectivity ratio plot reproduced in Figure **3** permits only one way to achieve this displacement of adsorbed K^+ by Na^+ ion. That is, the theory predicts an increase of the c-value to about –3.25 Å and with this a shift in the rank order of selectivity from $\mathrm{Rb}^+ > \mathrm{K}^+ > \mathrm{Cs}^+ > \mathrm{Li}^+ > \mathrm{Na}^+$ to the rank order $\mathrm{K}^+ > \mathrm{Li}^+ > \mathrm{Cs}^+ > \mathrm{Rb}^+ > \mathrm{Na}^+$.* Figures 4 and 5 show that this was what actually was observed.

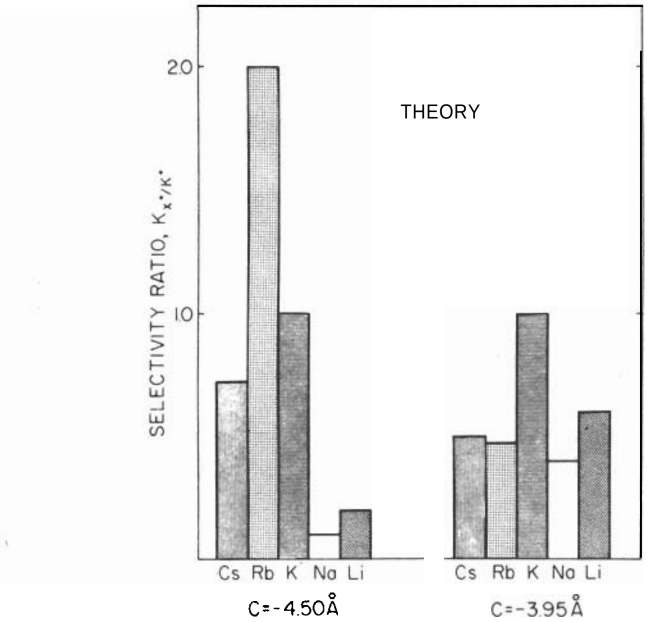

Figure 4. Theoretical selectivity ratios at a c-value of –4.50 Å of **3.24** Å. (Data from Figure 3.)

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*Neither the order of the 5 alkali-metal ion distribution in normal muscle, nor that of the ouabain-treated muscle, is predicted by the theory of Eisenman, Rudin and Casby[12] and Eisenman.[13] The eleven rank orders their theory deduced are: (I) Li>Na>K>Rb>Cs; (II) Li>Na>K>Cs>Rb; (III) Li>Na>Cs>K>Rb; (IV) Li>Na>Cs>Rb>K; (V) Li>Cs>Na>Rb>K; (VI) Li>Cs>Rb>Na>K; (VII) Cs>Li>Rb>Na>K; (VIII) Cs>Li>Rb>K>Na; (IX) Cs>Rb>Li>K>Na; (X) Cs>Rb>K>Li>Na; and (XI) Cs>Rb>K>Na>Li.

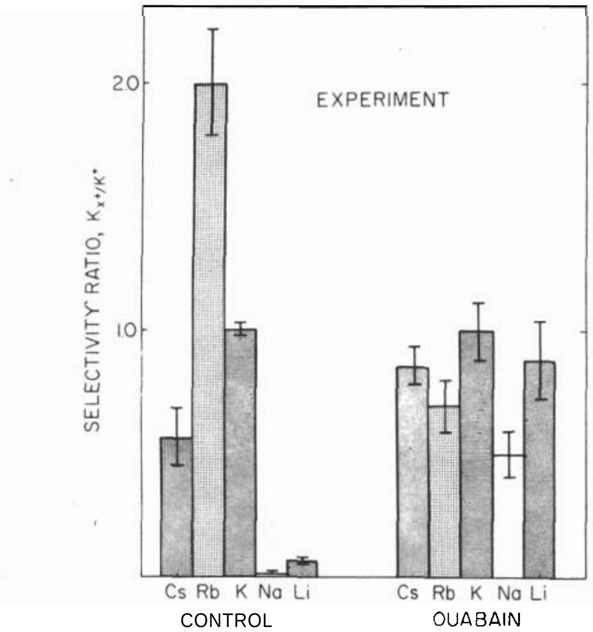

Figure 5. Experimental selectivity ratios of normal resting frog muscles and *muscles* treated with ouabain (3.27×10^{-7} M). (Data are from Tables 1 and 2.)

While this close agreement is gratifying, it is necessary to point out that the major significance of these findings does not lie in the precision of the matching but in the finding that a specific new equilibrium rank order among the 5 alkali-metal ions, different from that in the surrounding medium, was obtained in consequence of the exposure to a pharmacological concentration of a useful drug; a finding which lends weight to the c-value theory.

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