The Effects of Alkali Metal Cations and Common Anions on the Frog Skin Potential

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ABSTRACT The effects on the potential difference across isolated frog skin (*R. catesbeiana*, *R. pipiens*) of changing the ionic composition of the bathing solutions have been examined. Estimates of mean values and precision are presented for the potential changes produced by substituting other alkali metal cations for Na at the outside border and for K at the inside border. In terms of ability to mimic Na at the outside border of bullfrog skin, the selectivity order is Li > Rb, K, Cs; at the outside border of leopard frog skin, Li > Cs, K, Rb. In terms of ability to mimic K at the inside border of bullfrog and leopard frog skin: Rb > Cs > Li > Na. Orders of anion selectivity in terms of sensitivity of the potential for the outside border of bullfrog skin are Br > Cl > NO_3 > I > SO_4, isethionate and of leopard frog skin are Br, Cl > I, NO_3, SO_4. An effect of the solution composition (ionic strength?) on the apparent Na-K selectivity of the outside border is described. The results of the investigation have been interpreted and discussed in terms of the application of the constant field equation to the Koefoed-Johnsen-Ussing frog skin model. These observations may be useful in constructing and testing models ot biological ionic selectivity.

INTRODUCTION

In the recent literature, models of selectivity for alkali metal cations have appeared (1, 2). Inasmuch as such suggestions appear to offer pathways toward a more fundamental understanding of the basis of bioelectric phenomena, an attempt to furnish data for testing the validity of the hypotheses is in order. Experimental work with the alkali metal cations has a long history (3, 4), but there is a scarcity of studies in which members of the series have been quantitatively compared under conditions formulated in the light of modern theories of bioelectric phenomena (but see Sjodin (5, 6) on frog skeletal muscle).

The suitability of frog skin for such a study lies in two facts. It maintains a

stable and easily measured potential difference, and it presents two surfaces whose apparent selectivities differ. The tissue is considered to "select" that ion to which the measured potential is most sensitive.

It has been known for some time that the composition of the bathing fluids determines, at least in part, the frog skin potential (*e.g.* see Motokawa (7, 8)). Thus, it was mentioned by Dean and Gatty (9) that Rb and Cs depolarized frog skin. Huf (10) investigated the relative abilities of K, Rb, and Cs to sustain NaCl uptake by the skin when present in low concentrations in the solution bathing the inside (corium) of the skin. Zerahn (11) has studied the effect of Li on skin transport and potential. Koefoed-Johnsen and Ussing (12) demonstrated that the outside (epidermis) of the skin behaved much like a Na electrode (see also Steinbach (13)) and the inside, like a K electrode. Teorell (14) studied the effects of Li, Rb, and Cs. However, in none of these studies were all members of the alkali metal series compared at constant osmolality and ionic strength in the same animal.

The object of the present study is to extend the work of Koefoed-Johnsen and Ussing (12) on the relationship of the potential difference across the isolated frog skin to the ionic composition of the solutions bathing the inside and outside borders. The five stable alkali metals (Li, Na, K, Rb, and Cs) have been compared at both borders, and certain anions (I, Br, Cl, NO_3, isethionate, SO_4) at the outside border. In addition, skins from the two common American frogs *R. pipiens* (leopard frog) and *R. catesbeiana* (bullfrog) have been compared.

METHODS

Late spring and summer frogs of both sexes (obtained from Lemberger's of Wisconsin) were used. Bullfrogs were stored at 4°C and leopard frogs at room temperature. Pieces of belly, thigh, and calf skin (no systematic difference was noticed) were removed from the frogs and mounted in chambers of the type described by Ussing and Zerahn (15) with 2 cm^2 cross-sectional area. Usually 5 ml of solution were used to bathe each surface. The potential difference was monitored through fine-tipped 3 M KCl-agar bridges and calomel half-cells. A Philbrick P-2 differential operational amplifier was used as an electrometer-type isolation amplifier. The output was fed into a Leeds and Northrup speedomax G recorder which recorded the potential of each skin once every 30 seconds. Bridge asymmetries were checked in Na_2SO_4 solution and no bridges were used with asymmetries greater than 1 mv. However, no attempt was made to correct for possible junction potentials at the bridges when dissimilar solutions were used. A Radiometer pH meter 3 was used from time to time to check the accuracy of the recording system.

The resistance of the tissue was estimated by passing a small constant current pulse (5 to 8 $\mu a/cm^2$) and recording the potential deflection produced. Current was passed in both directions in all cases. Experiments were performed at room temperature (22 to 27°C).

All solutions were free of divalent cations, although no attempt was made to deplete the endogenous skin Ca and Mg by chelating agents. We noticed no difference in the skin potential in Ca-free solutions as opposed to that in solutions containing 1 to 2 mEq/liter Ca. When no interfering cations were present, Na and K concentrations were checked by flame photometry. A Beckman sodium-sensitive glass electrode (78178V) was used as an additional check on the Na activities of solutions. This electrode was relatively insensitive to K, Rb, and Cs at the concentration ratios used. The reference solution for most experiments was "regular" sulfate solution containing 115 mEq/liter Na, 5 mEq/liter K, with sulfate as the anion (henceforth the expression "mEq/liter" will be omitted in statements of composition). Most of the solutions were buffered with tris(hydroxymethyl)aminomethane (Sigma 7-9, 5 mM) (see 16) to pH 8, although some experiments (as noted in the Results section) were performed in the presence of a phosphate buffer at the same pH. The solutions were bubbled with with compressed air. Further details of the ionic composition are given in Results. *M* and *A* are used as chemical symbols for arbitrary cations and anions, respectively. The solutions bathing the epidermal side of the skin are referred to as "outside solutions," those bathing the corium side as "inside solutions." Similarly, reference is made to "inside cation," "outside replacement," etc.

Li_2SO_4, K_2SO_4, $Na_2SO_4 \cdot 10H_2O$, NaCl, and $NaNO_3$ were "Baker analyzed" reagents. Cs_2SO_4 and RbCl were Fisher certified reagents. NaI and KI were Baker and Adamson A.C.S. grade. Sodium isethionate was Eastman 8541. Rb_2SO_4 was prepared from RbCl by exchange on a Dowex 1-X4 column. The product did not give a precipitate with $AgNO_3$, but did give a precipitate with $BaCl_2$.

Some outside experiments were performed by serial dilution of the solution in the chambers. In these cases, a volume of solution containing *M* equal to the volume already present in the chamber was added. In this fashion, the Na concentration was progressively reduced by a factor of 2. This type of experiment does not give results which are independent of time or order. Therefore, most experiments were performed by complete replacement, twice in succession, with a given solution. Experimental periods were preceded and followed by control or recovery periods. The test solution was allowed to remain in contact with the skin until an apparent steady state was reached. The duration of the experimental and of the recovery periods for the outside was 10 minutes each; for the inside, 15 minutes each.

Most experiments were of the split plot design (17); four pieces of skin from the same frog were followed simultaneously. A skin was rejected if the initial potential in sulfate solution was below 80 mv. Potentials of 100 to 120 mv were quite regularly obtained with frogs which showed no signs of molting, infection, or skin disease and which had not been exposed to vapors of organic solvents. The data presented in this paper are unselected. The sole criteria for acceptance of an experiment were a suitable initial potential and proper following of the protocol. Experiments involving leopard frogs (body lengths up to 4 inches) were interleaved with those involving bullfrogs. Identical experimental designs involving the same solutions were employed for both species of frogs.

Sample protocol: outside replacements, low total cation.

1. Four pieces of skin were mounted in regular (115 Na, 5 K, SO_4) solution.

2. Outside solution was replaced twice with 20 Na, the control solution. The inside solution was always 115 Na, 5 K.

3. When a steady potential was reached, the resistance was measured.

4. The control solution was replaced twice in succession by the first of the experimental solutions.

5. When a steady potential was again reached, the resistance was measured.

6. At approximately 10 minutes, the experimental solution was replaced twice with the control solution. Resistance and potential measurements were made in a similar fashion for each experimental solution.

The experimental solutions used are shown in the following sample solution asignment schedule.

Solution assignments:

	CHAMBER			
Experimental period	A	B	C	D
		Frog 1		
	10 Na 10 M	*5 Na 15 M*	*2.5 Na 17.5 M**	*1.25 Na 18.75 M*
1st	Cs	K	Cs	Rb
2nd	Rb	Cs	K	K
3rd	K	Rb	Rb	Cs
		Frog 2		
	2.5 Na 17.5 M	*5 Na 15 M*	*1.25 Na 18.75 M*	*10 Na 10 M*
1st	K	Rb	K	Rb
2nd	Rb	K	Rb	Cs
3rd	Cs	Cs	Cs	K
		etc.		

* An experiment such as this is illustrated in Fig. 1.

The experimental design and statistical analysis are further discussed in the Appendix.

RESULTS

I. *Outside Replacements*

1. SERIAL DILUTION OF REGULAR SULFATE SOLUTION WITH Li, Kb, R, Cs (MAY AND JUNE)

In this set of experiments, regular sulfate solution was chosen as the base solution (115 Na, 5 K, SO_4 as anion, phosphate-buffered to pH 8). The inside of the skin was bathed in this solution at all times. After a stable control level was established, the outside solution was serially diluted by a solution containing 115 K, Rb, Cs, or Li, plus 5 K (SO_4 as anion, phosphate-buffered). This experiment was done only with bullfrogs.

The recovery was reasonably good with all four cations, but poorest with Li. This factor is important in the case of Li because the difference between initial and recovery potentials is close in magnitude to the difference betwen initial and experimental potentials. Fig. 2 shows a graph of the change in potential from the initial level (ΔV) *versus* the logarithm of the ratio of the sodium concentration in the test case to the control sodium level (Na_2/Na_1).

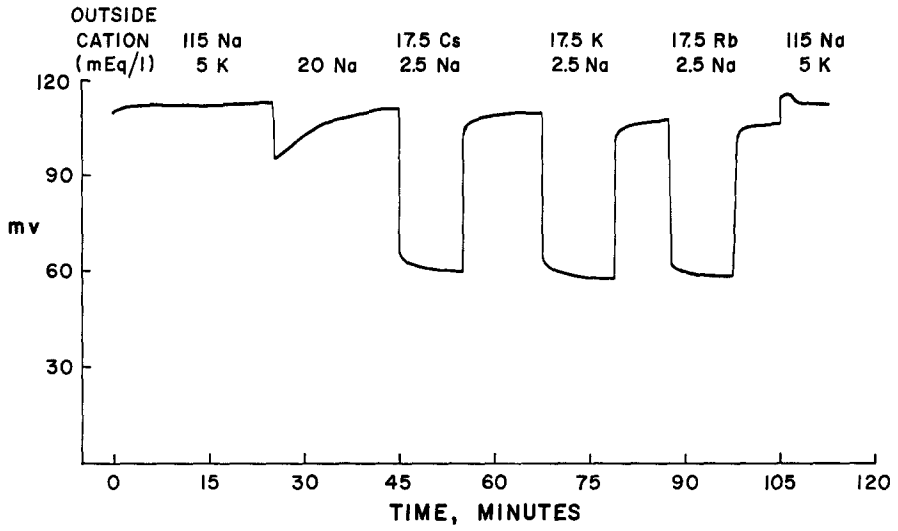

FIGURE 1. Bullfrog skin potential with hypotonic sulfate solution as outside bathing solution. This graph (redrawn from the original strip chart recording) shows the time course of a representative experiment (see sample protocol in text). Concentrations of cations are indicated above the line for the period during which those concentrations were present. The outside control solution was 20 Na, tris. The inside bathing solution was always 115 Na, 5 K. The times of solution changes can be seen from the fast changes in potential.

This method of relating potential changes to sodium concentration is justified in the Discussion. Notice that the curves depart from the 59 mv slope by a considerable amount. The curve for Rb is significantly ($p < 0.05$) higher than that for K in the range $\frac{1}{8}$ to $\frac{1}{32}$. The curve for Li is suspect because a skin with Li in the outside bathing solution sometimes deteriorates progressively with time. We saw no evidence of "oscillations" (14) in these experiments. The lack of independent controls for each test solution makes the method of obtaining ΔV somewhat arbitrary.

2. Na-K REPLACEMENTS USING HIGH, REGULAR, AND LOW TOTAL CATION CONCENTRATIONS (MAY)

Other conditions were tried in order to approach more closely the 59 mv slope. K was used as the replacement for Na in serial dilution. The four con-

ditions chosen were (*a*) low total cation (20 Na, 5 K, phosphate buffer, SO_4); (*b*) high total cation (200 Na, 5 K, phosphate buffer, SO_4); (*c*) regular (115 Na, 5 K, phosphate buffer); and (*d*) low total cation plus mannitol (20 Na, 5 K, phosphate buffer, 150 mannitol). The inside remained in regular sulfate solution (phosphate buffer). The means for these experiments are

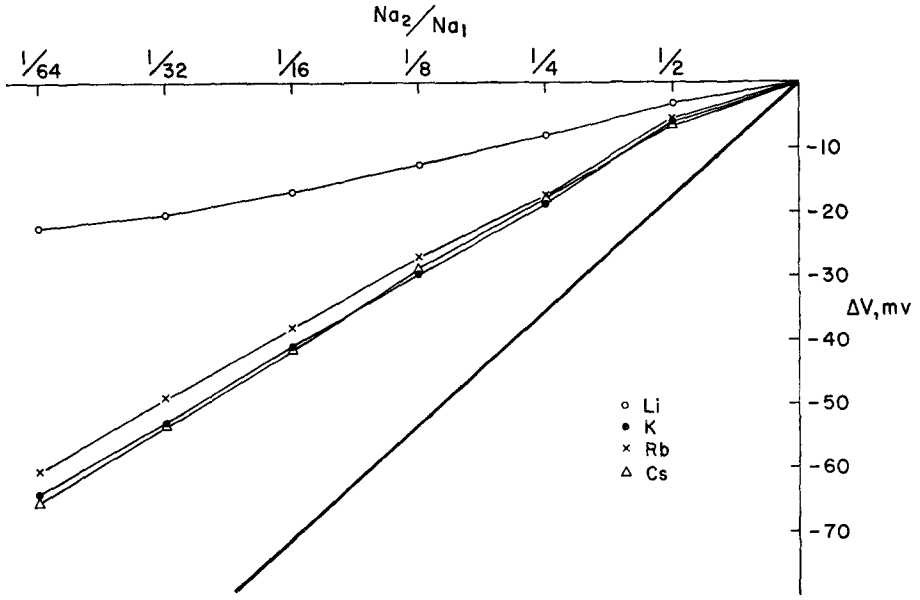

FIGURE 2. Relation between bullfrog skin potential and outside Na concentration in sulfate solution (total cation 120). The values plotted represent changes from the initial control level as the outside Na concentration was reduced by serial dilution with "*M* solution". The lines have been drawn to connect the experimental points, which are the means of ten experiments. The inside solution was always 115 Na, 5 K. The heavy straight line gives 59 mv per tenfold change in concentration. The standard error of the mean for each point is about 10 mv. However, the balance of the experimental design allows comparisons to be made within frogs; thus the standard error of the difference between two mean potentials is on the order of 1 mv. The mean initial potentials for Li, Rb, Cs, and K were 105.4, 108.1, 110.4, and 101.8 mv; the mean recovery potentials were 94.9, 105.1, 104.9, and 95.7 mv.

given in Table I. Observation of a plot of the means, combined with the following experiment, suggested that a different choice of conditions might be advisable.

3. Na-K REPLACEMENTS IN REGULAR AND LOW CATION CONDITIONS BY SERIAL DILUTION AND TOTAL REPLACEMENT (JULY)

The outside solutions in this case were (*a*) regular (115 Na, 5 K, SO_4, 5 tris) and (*b*) low total cation (20 Na, SO_4, 5 tris). The inside was bathed in 115

Na, 5 K, SO_4, 5 tris for all experiments. Each of the two types of solutions was used with the serial dilution procedure described in Methods and with a total replacement procedure. Total replacement means complete removal of the control solution and addition of the test solution (containing K), per-

TABLE I
CHANGES IN POTENTIAL OF BULLFROG SKIN FROM CONTROL LEVEL AT DIFFERENT OUTSIDE TOTAL CATION CONCENTRATIONS

Sodium concentration (Na_2/Na_1)	Regular	Low total cation	High total cation	Low total cation + mannitol
	mv	*mv*	*mv*	*mv*
1/2	−9.00	−12.50	−12.75	−11.00
1/4	−19.25	−25.25	−26.75	−24.00
1/8	−30.00	−39.75	−38.75	−37.75
1/16	−41.00	−54.00	−50.75	−51.50
1/32	−51.50	−67.25	−60.00	−66.00

Each value is the mean of four experiments. K was used as the replacement for Na.

TABLE II
CHANGES IN POTENTIAL OF BULLFROG SKIN FROM CONTROL LEVEL WITH DIFFERENT SOLUTION-CHANGING PROCEDURES AND DIFFERENT OVER-ALL OUTSIDE COMPOSITIONS

Sodium concentration (Na_2/Na_1)	Regular		Low total cation	
	Total replacement	Serial dilution	Total replacement	Serial dilution
	mv	*mv*	*mv*	*mv*
1/2	−11.50	−10.50	−14.00	−13.00
1/4	−21.25	−22.25	−31.00	−26.25
1/8	−37.00	−36.00	−42.50	−39.75
1/16	−44.50	−50.50	−51.00	−57.00

Each value is the mean of four experiments. K was used as the replacement for Na.

formed twice (a third replacement did not alter the potential). Four bullfrogs were used. Means are given in Table II. The means indicate that the curve approaches a 59 mv slope more closely when the total cation concentration is low. Furthermore, the frog skin seems to be a remarkably stable and vigorous preparation when the outside surface is bathed in low ionic strength solution, a condition which resembles the frog's usual environment. Therefore, succeeding experiments on outside ion replacements were performed

with a base solution of 20 Na, SO_4, 5 tris. The inside solution was 115 Na, 5 K, S_4O, 5 tris for the remaining outside replacements.

4. TOTAL REPLACEMENTS AT LOW TOTAL CATION WITH Li, K, Rb, Cs (JULY)

In one experiment, the cation was assigned as the factor common to all experimental periods on a given piece of skin, and replacements were done on

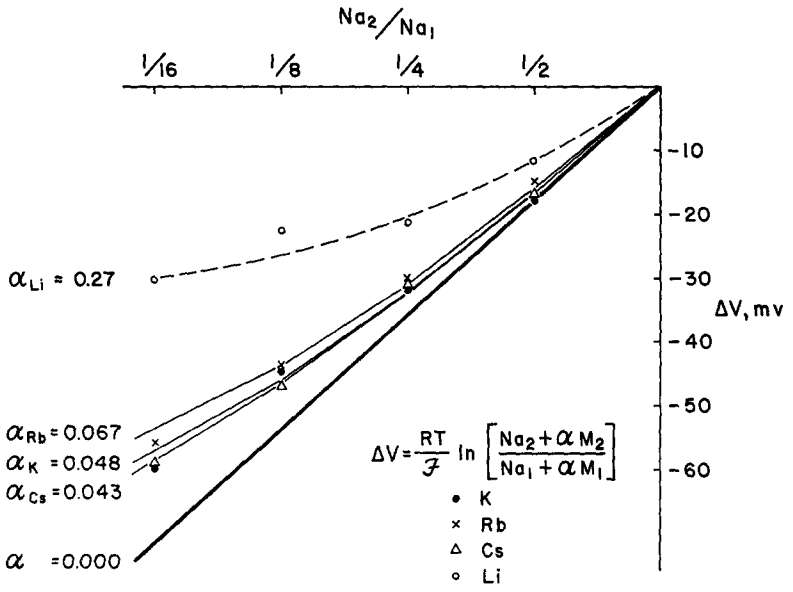

FIGURE 3. Relation between bullfrog skin potential and outside Na concentration in hypotonic sulfate solution. The points for K, Rb, and Cs represent nine experiments and those for Li, five experiments. The estimation of α for K, Rb, and Cs is discussed in the text. The heavy straight line gives 59 mv per tenfold change in concentration.

five bullfrogs and two leopard frogs at four levels of Na. In another experiment, only K, Rb, and Cs were used (to avoid possible toxic effects of Li on the outside), and the Na concentration was assigned as the common factor. Four bullfrogs and four leopard frogs were used. The protocol for this experiment is described in Methods and a typical experiment is shown in Fig. 1. Means for bullfrogs are plotted in Fig. 3. The results for leopard frogs were almost identical to those for bullfrogs. There was a shift in the order of the mean values for K, Rb, and Cs, but this was not significant. The estimates of the constant field parameter are given in the Discussion. The curves were fitted from a least squares model based on the constant field equation (see Discussion). The constant field parameter, α, is the ratio of permeability coefficients, in this case, of each alkali metal to that of sodium. The curve for

Li was fitted by eye and the estimated constant field parameter cannot be tested for significance. However, in terms of effects on the potential, Li was significantly different from the other cations. The differences among the constant field parameters for Rb, K, and Cs are not statistically significant. Since the graphs in Fig. 3 give only the change in potential from the control level, a question might arise as to the condition of the skins throughout these experiments. Table III gives the mean values during control and recovery periods (cation as common factor, Na concentration as common factor—bullfrogs); the entries give the initial values of the potential and the level to

TABLE III
CONTROL AND RECOVERY POTENTIALS FOR BULLFROG SKINS WITH LOW NA SOLUTION OUTSIDE

Ion	Initial potential	Recovery potential after experimental period			
		1st	2nd	3rd	4th
	mv	*mv*	*mv*	*mv*	*mv*
Li (5 experiments)	106.0	100.8	99.6	98.4	97.6
K (5 experiments)	110.8	107.6	103.0	99.0	97.6
Rb (5 experiments)	103.0	103.0	104.0	102.8	101.4
Cs (5 experiments)	110.2	111.8	112.2	111.6	111.4
Na_2, Na_1 (Na replaced by K, Rb, and Cs)					
1/2 (4 experiments)	106.0	99.5	97.5	96.5	
1/4 (4 experiments)	105.7	104.7	102.5	100.0	
1/8 (4 experiments)	113.0	108.7	106.2	104.2	
1/16 (4 experiments)	104.7	97.0	92.7	88.7	

which the potential returned after each treatment period. The values for leopard frogs are essentially the same as those for bullfrogs; *e.g.*, the over-all mean initial control (Na concentration as common factor) for bullfrogs was 107.5 mv (sixteen pieces of skin), for leopard frogs 105.2 mv (twenty pieces of skin). The corresponding final recovery figures were 97.3 and 97.7 mv, respectively. At no time in any of these experiments did the potential reverse in sign (become positive on the outside).

5. EFFECT OF THE ANION ON OUTSIDE Na-K REPLACEMENTS (AUGUST)

Experiments were performed as above (section 4) for the replacement of Na by K (low total cation, tris), but in the presence of I, Br, Cl, or NO_3 as the anion. The inside solution was always 115 Na, 5 K, SO_4, tris. Five bullfrogs and five leopard frogs were used. Figs. 4 and 5 show plots of the means. In these figures data from a large number of SO_4 experiments have been included for comparison. Table IV summarizes the initial control and final

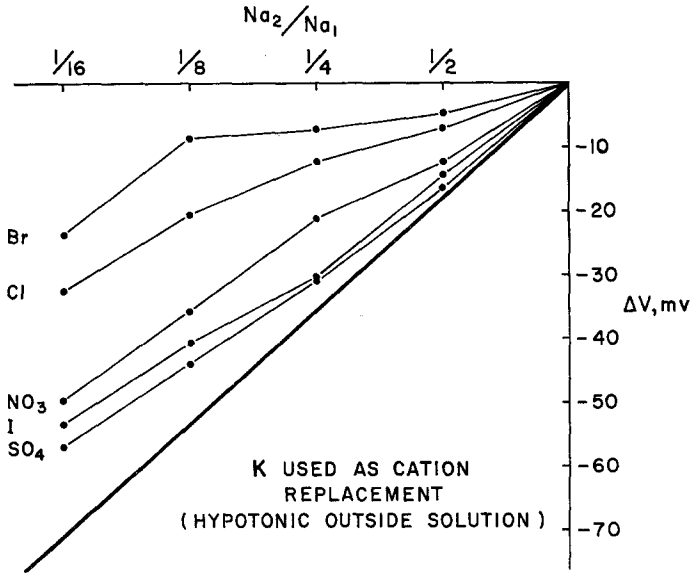

FIGURE 4. Relation between bullfrog skin potential and outside Na concentration with different anions. The sulfate points represent the means of forty-four experiments, and the other points, five experiments. Lines connect the experimental points. The heavy straight line gives 59 mv per tenfold change in concentration.

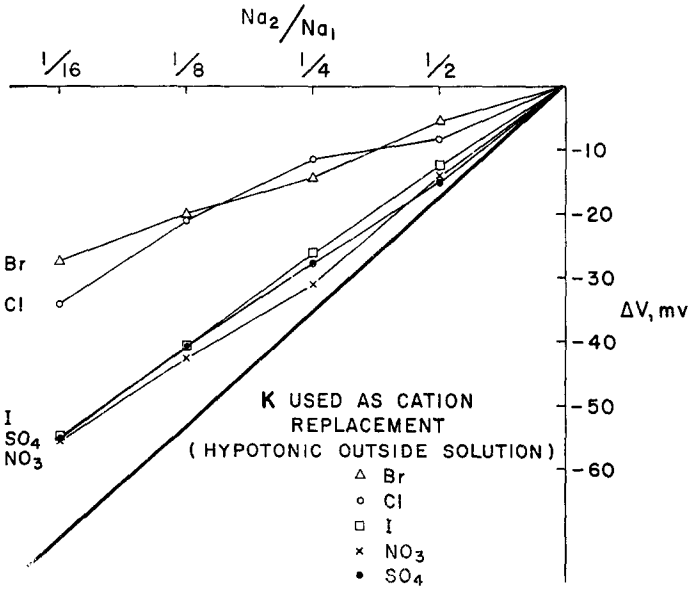

FIGURE 5. Relation between leopard frog skin potential and outside Na concentration with different anions. The sulfate points represent the means of six experiments and the other points, four experiments. Lines connect the experimental points. The heavy straight line gives 59 mv per tenfold change in concentration.

TABLE IV
FROG SKIN POTENTIALS IN VARIOUS ANIONS

	Frog skin potential		
	Preliminary value in 115 Na 5K sulfate	Initial control 20 NaA outside	Final recovery 20 NaA outside
	mv	*mv*	*mv*
Bullfrogs (5)			
A = NO_3	125.8	86.6	80.0
I	110.6	91.4	78.4
Br	121.8	55.8	45.6
Cl	111.6	60.8	62.8
Leopard frogs (5)			
NO_3	109.4	101.0	96.0
I	112.4	102.0	102.0
Br	113.0	70.2	68.8
Cl	114.0	75.4	73.4

recovery values for the skins used in these experiments. Skin potentials were lowest when bromide was the anion.

6. ANION REPLACEMENTS (AUGUST)

Since the frog skin potential appeared to be most sensitive to bromide, experiments on anion selectivity were done using bromide as the control ion.

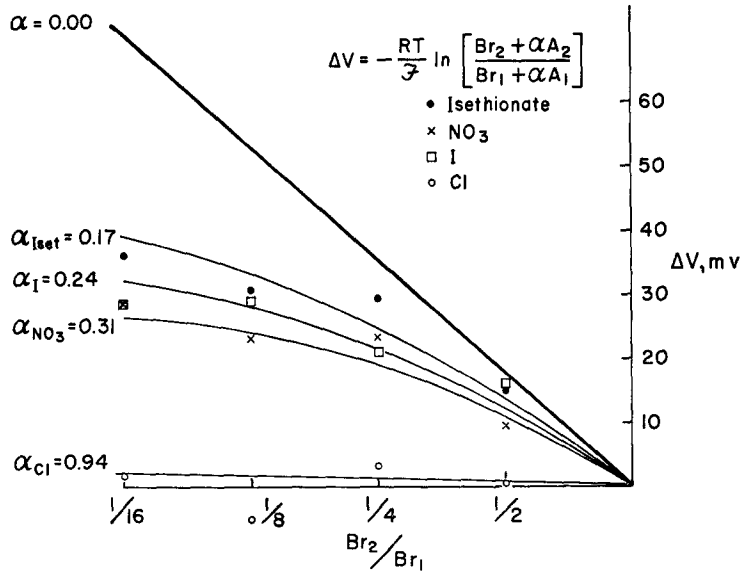

FIGURE 6. Relation between bullfrog skin potential and outside bromide concentration in hypotonic Na solution. Points represent the means of four experiments. The curves were fitted by eye. The heavy straight line gives 59 mv per tenfold change in concentration.

In these experiments, the outside anion concentration was changed, with the outside cation composition constant at 20 Na. I, Cl, NO_3, and isethionate were used to replace Br. The inside solution was always 115 Na, 5 K, SO_4, tris. The potential usually increased when the Br solution was replaced by the other solutions. Means for the four bullfrogs are shown in Fig. 6. The mean initial control potential was 65.4 mv in 20 NaBr (118.9 mv in 115 Na, 5 K, SO_4); the mean final recovery potential in NaBr was 62.5 mv.

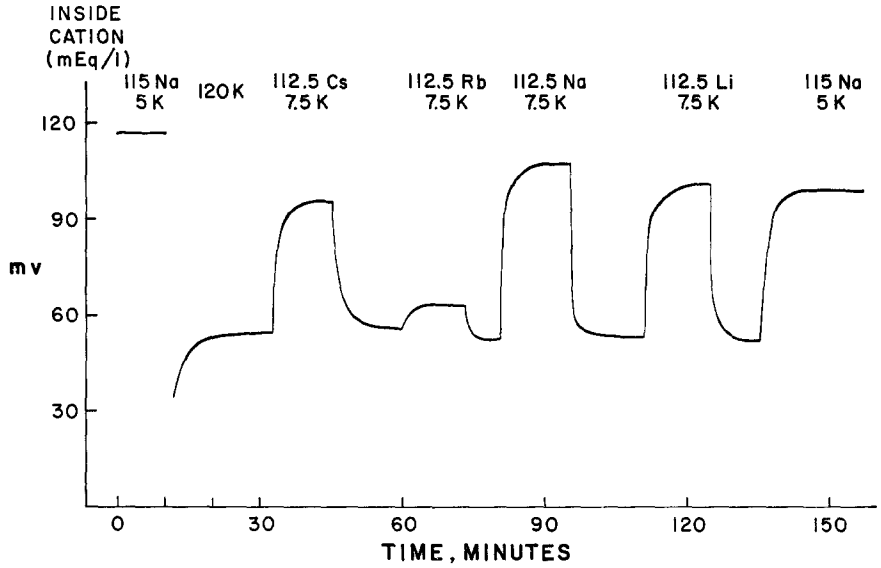

FIGURE 7. Bullfrog skin potential with changes in inside cation concentration. The time course of skin potential in a representative inside cation total replacement experiment is shown. Cation concentrations in the inside bathing solution are indicated along the top of the graph for the periods in which these concentrations were present. The inside control solution was 120 K. The outside bathing solution was always 115 Na, 5 K. All solutions were buffered with tris. The sharp changes in potential show the times at which the solution replacements were made. The graph was redrawn from the original strip chart record.

II. *Inside Replacements (June, July, and August)*

Only cation replacement experiments have been done with the inside bathing solution. The total alkali metal concentration was kept at 120 mEq/liter and two successive total replacements were made. The outside solution in these experiments was 115 Na, 5 K, SO_4, tris. Experiments were performed in both phosphate buffer (115 *M*, 5 Na) and tris buffer (120 *M*) and with both bullfrogs and leopard frogs. Fig. 7 shows a typical experiment and Fig. 8 shows the means for the bullfrog skins in the experiment using tris. Data for leopard frog are shown in Fig. 9 using a linearized plot (see Discussion). Table V gives the means. The mean initial control value with 120 K, tris inside, for

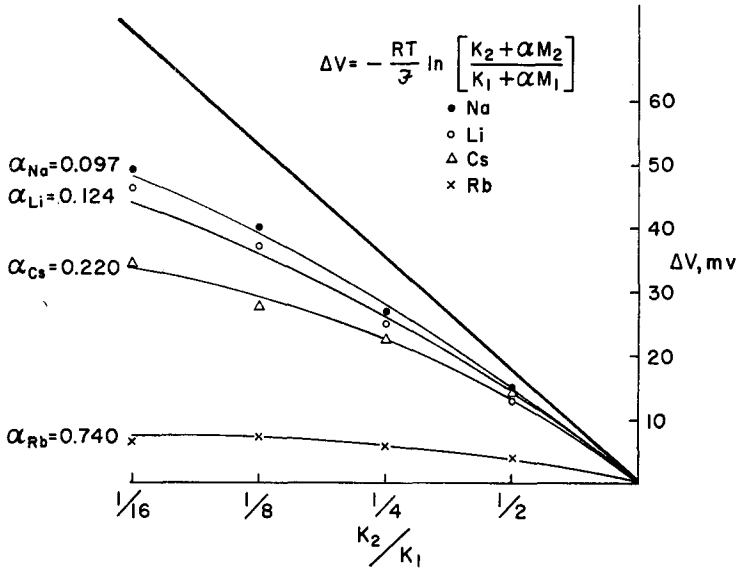

FIGURE 8. Relation between bullfrog skin potential and inside K concentration in sulfate solution. The points are the means of seven experiments with tris-buffered solutions (see Table V). The curves (which are given by the equation shown) were fitted by eye. The heavy straight line gives 59 mv per tenfold change in concentration.

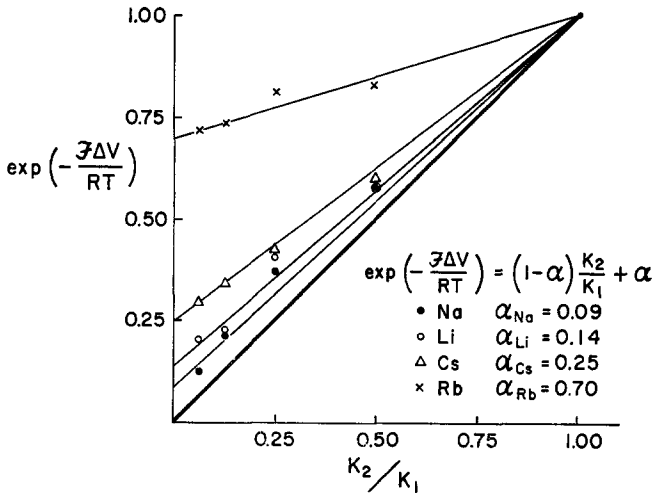

FIGURE 9. Relation between leopard frog skin potential and inside K concentration in sulfate solution. The points are the means of three experiments with tris-buffered solutions (see Table V). The lines, which were fitted by eye, are given by the equation shown. The heavy line is the curve for $\alpha = 0$, *i.e.*, when the potential slope is 59 mv per tenfold change in concentration.

28 bullfrog skins was 54.7 mv (12 leopard frog skins, 48.1 mv); the mean final recovery was 49.3 mv (leopard frogs, 41.3). In 115 K, 5 Na (phosphate buffer) initial control and final recovery potentials were slightly lower for both bullfrogs and leopard frogs.

The earliest experiments (June) on the inside were done with the serial dilution technique. On the basis of a limited number of such experiments,

TABLE V
INSIDE CATION SELECTIVITY. MEAN VALUES FOR POTENTIAL CHANGE

(K_2/K_1)	Skin potential increase (ΔV) Inside replacement cation			
	Rb	Cs	Li	Na
	mv	*mv*	*mv*	*mv*
Bullfrog (tris—7 experiments)				
1/2	3.9	14.4	13.0	14.7
1/4	5.9	22.4	25.0	27.1
1/8	7.1	27.7	37.1	39.6
1/16	6.4	34.3	45.9	49.0
Bullfrog (phosphate—4 experiments)				
1/2	3.9	11.9	10.0	12.3
1/4	4.8	24.5	20.8	23.3
1/8	3.6	27.1	30.5	35.1
1/16	6.1	28.5	38.4	44.8
Leopard frog (tris—3 experiments)				
1/2	4.7	12.7	14.0	14.0
1/4	5.3	21.7	23.0	25.3
1/8	7.7	27.0	36.3	39.7
1/16	8.3	30.3	41.3	52.7
Leopard frog (phosphate—4 experiments)				
1/2	3.5	10.5	9.9	14.9
1/4	4.5	20.6	19.9	22.4
1/8	6.9	26.6	33.3	39.9
1/16	6.0	28.1	35.9	46.6

it appeared that Cs gave increases in potential larger than those with Na at the higher K concentrations, but smaller increases than with Na at the lower K concentrations. Consequently the order of selectivity (for the inside border of the skin) given in the preliminary report (19) differed from that established by these extended studies. The differences between any two mean changes in potential shown in Fig. 8, except for Na, Li, and Cs at $K_2/K_1 = \frac{1}{2}$, are statistically significant ($p < 0.05$).

DISCUSSION

The results of this work are in substantial agreement with earlier frog skin work indicating an apparent "Na-selectivity" of the outer surface and "K-

selectivity" of the inner surface. They can be readily explained in terms of the Koefoed-Johnsen-Ussing (12) frog skin model, if one allows extension of the model from the Nernst equation to kinetic treatments of diffusion potentials. However, it is to be emphasized that these experiments by themselves do not constitute evidence uniquely in support of the Koefoed-Johnsen-Ussing model. One important assumption in the interpretation of these results is that the glands of the frog skin either do not play any significant role in determining the skin potential or else behave in a fashion identical with that of the non-glandular epithelium. A second assumption is that the observed potential difference across the frog skin is composed of two independent membrane potentials connected in series, and that the intracellular electrolyte content is relatively unaltered during the short periods in contact with the experimental solutions. Evidence for the presence of the two potential steps has been reviewed previously (20, 21). There does not appear to be any published evidence for the independence of these potentials; in fact, there may be a slight interaction of the two. Preliminary work (Edwards, Leb, Lindley, and Hoshiko, unpublished) indicates that the magnitude of the change in potential caused by altering the Na/K ratio of the outside solution may be conditioned by the composition of the inside solution; similarly, the outside Na/K ratio may affect the inside Na/K preference. The importance of such an effect would be that a change in composition of the outside solution might alter the potential at both the inner and the outer borders. These effects may be mediated indirectly by changes in "cellular" electrolyte (*i.e.*, Na/K ratio), and appear to be small under our conditions. Thus, it appears justifiable to assume essential independence of the potential steps for the present considerations. The presence of "inert" tissue at either border is ignored, except as it affects the time course of changes in the potential. It is to be noted that junction potentials could still be established before the steady state is reached, *e.g.* across the cell layer at the inside of the corium (a layer which is permeable to sucrose and sulfate ions, but perhaps not to albumin). However, the present data do not appear to warrant introduction of a more complicated version of the Ussing model, although the basic notion is susceptible to considerable extension.

The data from these experiments have been analyzed in terms of the constant field equation (22, 23). Use of this equation is not so strongly bound to a given model as it might seem, for Eisenman (24) has used a formally similar equation to express the difference in potential between a solution and a glass electrode. For the case of a system with two univalent cations ("*a*" and "*b*") as the only ions able to cross a membrane, the potential difference given by the constant field equation is (although the constant field assumption is not

necessary for this case):

$$V = \frac{RT}{F} \ln \left[\frac{P_a M_a^{\mathrm{I}} + P_b M_b^{\mathrm{I}}}{P_a M_a^{\mathrm{II}} + P_b M_b^{\mathrm{II}}} \right] \tag{1}$$

P represents the "permeability coefficient," M represents the concentration of the subscript ion, and the superscript represents the phase. V is the potential difference (phase II minus phase I). This equation as written does not include phase boundary potentials between the membrane and the adjoining phases, but it does include the possibility of a partition of salt between these phases. The permeability coefficient may be considered the product of partition and mobility terms, assumed to be constant through the thickness of the membrane.

When the composition of the solution in compartment I is changed from condition "1" to condition "2" without changing the composition of the solution in compartment II, the change in potential is ΔV, the expression for which can be simplified by setting $P_b/P_a = \alpha_{ba}$. Thus, the parameter α_{ba} represents the relative permeability of b compared to a.

$$\Delta V = \frac{RT}{F} \ln \frac{(M_a^{\mathrm{I}} + \alpha_{ba} M_b^{\mathrm{I}})_2}{(M_a^{\mathrm{I}} + \alpha_{ba} M_b^{\mathrm{I}})_1} \tag{2}$$

If the initial condition, 1, is that solution I contains only a, and if the total cation concentration is constant and equal to $(M_a^{\mathrm{I}})_1$

$$\Delta V = \frac{RT}{F} \ln \left[\frac{(M_a^{\mathrm{I}})_2}{(M_a^{\mathrm{I}})_1} (1 - \alpha_{ba}) + \alpha_{ba} \right] \tag{3}$$

For the outside replacements, M_a is taken as Na, and the concentration (understood as that of the outside solution) is represented by the chemical symbol.

$$\Delta V = \frac{RT}{F} \ln \left[\frac{\mathrm{Na}_2}{\mathrm{Na}_1} (1 - \alpha_M) + \alpha_M \right] \tag{4}$$

This equation may be transformed into that of a straight line:

$$\exp (F\Delta V/RT) = (1 - \alpha_M) \frac{\mathrm{Na}_2}{\mathrm{Na}_1} + \alpha_M \tag{5}$$

A plot of the data in this form (see Fig. 9) allows the estimation of α_M from the slope or intercept. However, if the discrepancies between the observed potential changes and those predicted by equation (4) have constant variances on the scale measured, the estimation of α_M by the least squares procedure

using the transformed potential changes (equation (5)) is not satisfactory. In obtaining the least squares estimate of α_M using equation (5), the importance of random variation in the potential changes for high ratios of K_2/K_1 is exaggerated.

Such a difficulty can be avoided by an alternative method of analysis.[1] The expansion of the function

$$\Delta V = \frac{RT}{F} \ln \left[\frac{Na_2 + \alpha_M M_2}{Na_1 + \alpha_M M_1} \right]$$

in a Taylor's series about the point α_0, the initial estimate of α, is

$$\Delta V = \frac{RT}{F} \ln \left[\frac{Na_2 + \alpha_0 M_2}{Na_1 + \alpha_0 M_1} \right] + \frac{RT}{F} \left[\frac{M_2}{Na_2 + \alpha_0 M_2} - \frac{M_1}{Na_1 + \alpha_0 M_1} \right] (\alpha - \alpha_0) + \cdots \tag{6}$$

Using the first two terms as an approximation for ΔV and denoting $\Delta\alpha = \alpha - \alpha_0$, a linear equation in $\Delta\alpha$ for ΔV is obtained. $\Delta\alpha$ is the correction to be applied to α_0. The sum of squares of the deviations to be minimized with respect to $\Delta\alpha$ becomes

$$\sum [\Delta V_{obs} - \Delta V_{pred}]^2 = \sum \left[\Delta V - \frac{RT}{F} \ln \left[\frac{Na_2 + \alpha_0 M_2}{Na_1 + \alpha_0 M_1} \right] - \frac{RT}{F} \left[\frac{Na_1 M_2 - Na_2 M_1}{(Na_2 + \alpha_0 M_2)(Na_1 + \alpha_0 M_1)} \right] \Delta\alpha \right]^2 \tag{7}$$

The least squares estimate of $\Delta\alpha$ is

$$\Delta\alpha = \frac{\sum \frac{(Na_1 M_2 - Na_2 M_1)}{(Na_2 + \alpha_0 M_2)(Na_1 + \alpha_0 M_1)} \left[\Delta V - \frac{RT}{F} \ln \left[\frac{Na_2 + \alpha_0 M_2}{Na_1 + \alpha_0 M_1} \right] \right]}{\frac{RT}{F} \sum \left[\frac{(Na_1 M_2 - Na_2 M_1)^2}{(Na_2 + \alpha_0 M_2)^2 (Na_1 + \alpha_0 M_1)^2} \right]} \tag{8}$$

For the case of $M_1 = 0$,

$$\Delta\alpha = \frac{\sum \frac{M_2}{(Na_2 + \alpha_0 M_2)} \left[\Delta V - \frac{RT}{F} \ln \left[\frac{Na_2 + \alpha_0 M_2}{Na_1} \right] \right]}{\frac{RT}{F} \sum \frac{M_2^2}{(Na_2 + \alpha_0 M_2)^2}} \tag{9}$$

Through iteration an estimate of any desired degree of approximation can be obtained. The preliminary estimate α_0 is corrected by adding $\Delta\alpha$ to it, and the

[1] Suggested to us by Dr. Glenn E. Bartsch.

preliminary estimate for the second cycle is $\alpha_0 + \Delta\alpha$. This method gives a better fit in terms of the observed variable ΔV. It has been used to calculate the parameter α for some of the experiments. Because the method is tedious, we have not yet made full calculations for the other experiments, and any estimates of α other than those for the outside low total cation replacements (section 4 of Results) represent fits by eye on the ΔV *vs.* $\ln \frac{Na_2}{Na_1}$ $\left(\text{or } \ln \frac{K_2}{K_1} \text{ or } \ln \frac{Br_2}{Br_1}\right)$ plot, and on the plot in Fig. 9.

The constant field equation appears to fit reasonably well the data for the inside and for the outside alkali metal replacements at low total cation (20 mEq/liter) with SO_4 as the anion. The fact that a formally identical equation fits the anion replacements (Fig. 6) might be taken to indicate that the relative permeability of anions compared to cations is fairly high. The flux data of Mullins (25) and of Linderholm (26) indicate an order of anion relative permeabilities similar to that obtained by the present method. From the effect of anions on the Na *vs.* K curve one can draw similar conclusions.

Although the constant field equation seems a good approximation under some conditions, it is apparent from the present experiments that it does not fit at all well when the total outside cation is 120 mEq/liter. Since this is the condition one is likely to choose first in attempting to perform experiments of the type reported herein, and since this type of experiment is often used as a student exercise, the departure from the simple constant field equation is of some consequence.

We have chosen not to regard this circumstance as a failure of the Ussing model, but rather to view it in terms of a membrane potential situation which requires a more sophisticated treatment than is afforded by the simple constant field equation. Assuming a membrane mechanism, one factor would be the presence of sizable phase boundary potentials. Another possible reason for the form of the curve for outside Na replacement under the condition of 120 total cation (ignoring possible effects of the osmotic gradient, which is constant in any given experiment) is non-linearity of the rate laws governing the movement of ions across the membrane. Such non-linearity could lead to "saturation" effects, as when the capacity of the membranes for diffusing ions is not practically infinite as compared to the ionic concentration. Under any such circumstance of saturation, the apparent permeability of the membrane will depend upon the concentration of ions in the solutions. For the present, we have taken the relative permeabilities determined at the more dilute condition as an estimate of the intrinsic membrane selectivity. In no case have we found the order of permeabilities to be affected by this choice.

Our estimates of the "selectivity" parameters are:

	Bullfrog	*Leopard frog*
At the outside surface:		
α_{LiNa} =	0.27	0.39
α_{RbNa} =	0.067	0.074
α_{CsNa} =	0.043	0.091
α_{KNa} =	0.048	0.074
α_{ClBr} =	0.94	
α_{NO_3Br} =	0.31	
α_{IBr} =	0.24	
α_{IsetBr} =	0.17	
At the inside surface (tris):		
α_{RbK} =	0.740	0.70
α_{CsK} =	0.220	0.25
α_{LiK} =	0.124	0.14
α_{NaK} =	0.097	0.09

For the estimates obtained by the iterative procedure discussed above, the standard error of the mean was 0.008.

The relative permeabilty to Li at the outside is somewhat difficult to evaluate. The potential in Li is not steady with time probably because of gradual accumulation of Li within the cells (see reference 11). Furthermore, the skin resistance decreases as the outside Na is replaced with Li (see reference 14), whereas it increases when other cations are used. It is thus possible that the outer border of the skin does prefer Li to Na. We have not experienced such a difficulty with Li on the inside of the skin; there is no apparent "poisoning" in this case.

At the outside surface the values for the constant field parameter are not significantly different from each other for K, Rb, and Cs within the same species of frog (Keuls' sequential range test, reference 17, p. 253). However, this should not be taken to mean that the constant field parameter is the same for these three cations. An experiment in the absence of Na would be more likely to detect any slight preference for Rb than the type of experiment reported here. Since the critical test of a model is based on quantitative prediction, we have presented the selectivity orders in terms of the mean values for potential changes and for the constant field parameter. We feel that data in this form are more suitable for model-testing than would be the qualitative results of a significance test based on a particular method of interpretation.

The models proposed by Mullins (1) and Eisenman (2) have not dealt with anion selectivity although that of Mullins might be extended to do so. The model of Eisenman does not seem to account easily for anion selectivity, although it is possible that some type of mosaic membrane postulate might do so. The values we have estimated for cation selectivity are not in complete agreement with the qualitative orders given by Mullins and by Eisenman.

Neither model predicts the order seen with the inside cation replacements, where a K-preferring membrane selects Li over Na. It is possible that modification of their models, *e.g.* allowing a complex membrane composed of parallel sites of two modes of selectivity, might fit these experimental data. It is also possible that the model of frog skin used to arrive at the selectivity values is erroneous.

It is apparent that there still exists a considerable lack both of quantitative theoretical predictions for biological systems and of precise experimental data. The present observations may be of use in constructing a quantitative theory of biological ionic selectivity.

APPENDIX

From the schedule given in Methods it can be seen that frogs are blocks, pieces of skin are the main plots, and the three experimental periods on each piece of skin are the subplots. Thus, in that experiment the dose (*i.e.*, the ratio of sodium in the test case to that in the control case) was the main plot treatment and the cation (*i.e.*, Rb, Cs, or K) was the subplot treatment.

The model adopted for statistical analysis is that described in Snedecor (reference 17, p. 369) with both main plot and subplot treatment effects fixed. We have given a summary of the analyses in terms of variance components (see also Anderson and Bancroft (18)); this information combined with the means allows the reader to make comparisons which we do not present here. Furthermore, it is possible to use such information in the design of further experiments. One can calculate the most efficient and economical design for a given number of observations to produce results with a desired degree of precision.

Since most of the experiments are of the same general design, the results of the analyses have been gathered in Table A-I. This table gives the number of frogs (blocks, b), the number of pieces of skin (main plots, m), and the number of experimental periods (subplots, t), along with the estimates of variance components. σ_B^2 is the frog variation, σ_{BM}^2 is the frog-main plot treatment interaction variance ("skins"), and σ_e^2 is the residual error term. In those experiments used to obtain least squares estimates of the constant field parameter α, the design is that of a randomized block. In these two cases we present the estimates of frog variance (σ_B^2), the number of frogs b, the residual error (σ_e^2), and the number of agents per frog m. The procedure described here explains the general lack of standard errors in this paper; an appropriate standard error, when desired, can be constructed from Table A-I.

It is possible to apply the analysis of variance to the regression problem using orthogonal polynomials. The deviations of the observations from a straight line (not constrained to pass through the origin) are often not statistically significant. Furthermore, the differences between slopes for various pairs of agents also are often not statistically significant.

In the absence of a universally accepted model for analysis, the simplest and most straightforward procedure is a direct comparison of the treatment means; *i.e.*, ordering the ions in terms of the change in potential seen in their presence. Thus we have re-

ported the qualitative orders of selectivity in terms of the ranking of the mean changes in potential. This procedure is justifiable only in the event that the lines relating potential change to concentration are roughly parallel for the ions compared in the range considered. When these conditions were met (it was sometimes necessary to

TABLE A-I

ESTIMATES OF VARIANCE COMPONENTS (OBSERVATIONS IN TERMS OF mv, ΔV; SEE TEXT FOR MEANINGS OF SYMBOLS)

Experiment*	b	m	t	σ_B^2	σ_{BM}^2	σ_e^2
Outside						
4. Bullfrogs						
(*a*) K-Rb-Cs-Li cation as main plot treatment	5	4	4	1.58	14.26	19.28
(*b*) K-Rb-Cs dose as main plot treatment	4	4	3	7.23	4.90	6.14
4. All 9 analyzed as randomized block for constant field parameter	9	3		0.00030		0.00119
4. Leopard frogs						
(*a*) K-Rb-Cs dose as main plot treatment	4	4	3	7.74	3.20	2.51
4. Randomized block for constant field parameter	6	3		0.00028		0.00066
5. Anion effect on Na-K replacements (anion as main plot treatment)						
Bullfrogs	5	4	4	44.63	53.39	50.04
Leopard frogs	5	4	4	123.50	34.62	22.18
6. Anion replacements (dose as main plot treatment)	4	4	4	30.06	27.70	40.33
Inside						
(Dose as main plot treatment)						
Bullfrogs, tris	7	4	4	5.63	7.59	15.22
phosphate	4	4	4	0.63	—	7.31
Leopard frogs, tris	3	4	4	5.97	6.20	7.21
phosphate	4	4	4	12.37	6.23	9.19

* Experiment number refers to section in the Results.

divide an experiment into two parts in order to make the parallelism approximation hold), this test was used (Keuls' sequential range test, reference 17, p. 253) to obtain the significance results reported. In cases which did not readily yield to this type of analysis, the slopes of regression lines have been compared.

Choice of a suitable regression model is essential if meaningful comparisons are to be made. For the present experiments, the usual straight line regression models are not suitable since they are not constrained to pass through the origin. Yet the physical

picture demands that the curve do so. At present we are trying to find a more suitable general regression model for such experiments. (The constant field equation suffers from some objections similar to those raised with respect to the linear model not forced to pass through the origin.)

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