THE RELATIONSHIP BETWEEN SODIUM, POTASSIUM, AND CHLORIDE IN AMPHIBIAN MUSCLE*

By SHIRLEY E. SIMON, F. H. SHAW, SUSANNE BENNETT, AND MARGARET MULLER

(From the Department of Pharmacology, University of Melbourne, Melbourne, Australia)

(Received for publication, December 19, 1956)

In a previous publication (28) it was shown that the cells of the sartorius muscle of the toad *Bufo marinus* were able to maintain their internal K^+ at an absolute level despite great fluctuations (0 to 25 m.eq. per liter) of the external K^+. When the external level was raised to 50 m.eq. per liter there was an intracellular increase amounting to about 17 per cent of the control level in normal Ringer. This is in contrast to the capacity of the cell to maintain an absolute ratio Na^+_{out}/Na^+_{in} of approximately 3:1 in the face of large variations in the external Na^+ (45 to 650 m.eq. per liter).

Boyle and Conway (2) had shown that K^+ entered the cell in proportion to the increase in external K^+, from which they drew the conclusion that the intracellular K^+ content would result from the electrochemical gradient, the product $K^+_{in} \times Cl^-_{in}$, being equal to the product $K^+_{out} \times Cl^-_{out}$.

In this paper it is our intention to correlate the changes in the intracellular Na^+ and K^+ content brought about by alterations in the external ionic environment with the concomitant movements in chloride ion.

A bibliography of the earlier literature relating to the estimation of chloride in muscle has been given by Fenn (9). We shall refer later to the work of Carey and Conway (3), Levi and Ussing (18), and Harris and Martins-Ferreira (10).

The method of estimation of chloride employed by some of the earlier workers (the Van Slyke acid digest), has been shown by Heilbrunn and Hamilton (11) and Wilde (34) to yield results up to 30 per cent too low. The methods used by us for the estimation of Na^+, K^+, and Cl^- were such that all three ions could be estimated on the same muscle. This has not been possible with previous methods.

The movements of Na^+ and Cl^- are so closely linked that we have formed a tentative hypothesis that these ions exist together in an intracellular phase. The amounts of the two ions in this phase are usually equivalent, and depend on the concentration of the same ion in the external medium. The amount of

* This work was undertaken with the aid of a grant from the National Health and Medical Research Council of Australia.

intracellular chloride is little, if at all, influenced by the K^+ content of the cell or by movements of this ion. The results will be employed (*a*) to show that the accumulation of intracellular K^+ is not associated with a Donnan mechanism, and (*b*) to suggest that the existence of a sodium pump in the resting muscle cell is an unnecessary postulate.

Methods

Treatment of Muscles.—Muscles which were analysed *in vivo* were carefully dissected from the animal, blotted, weighed, and immediately subjected to the analytical procedure outlined below. Isolated muscles were removed from the animal, blotted and weighed, soaked in the appropriate solution for the stated time, blotted, weighed again, and then analysed. It will be noted that we did not obtain a dry weight for our muscles, as in previous experiments. This was due to the fact that the analysis of chloride required a wet extraction. The results of our analyses over the preceding 4 years had shown that the water content of muscles *in vivo* was constant (27). By means of the above two weighings any volume change could be detected, and hence any change in dry weight.

Usually 10 pairs of muscles were used in each experiment. The companion muscle always served as a control for the treated, and unless otherwise stated was soaked in normal Ringer for a comparable time. The results were then subjected to the appropriate statistical analysis.

Collection of Blood.—Blood was taken by cardiac puncture into a heparinized syringe. It was placed in a centrifuge tube under an oil seal, and spun immediately.

The Estimation of Na,$^+$ K,$^+$ and Cl.$^-$—Preliminary experiments had shown that there was an almost complete loss of Cl^- on ashing at 560°C. Eventually it was found that the following technique was satisfactory. The muscle was very finely sliced in a centrifuge tube with scissors, the scissors were then thoroughly washed down with N/50 nitric acid, and the volume made up to 10 ml. in a centrifuge tube. The tubes were allowed to stand at room temperature for 48 hours. They were then stirred thoroughly and centrifuged; 3 ml. of the supernatant was accurately removed for the estimation of Na^+ and K^+ by means of a Beckman flame spectrophotometer. The proteins in the remainder of the supernatant were precipitated by the method of Somogyi (31), and the filtrate was used for the estimation of $Cl.^-$ The efficiency of the extraction method was compared with ashing techniques on companion muscles, and the results showed that the removal of Na^+ and K^+ was complete.

The chloride ion was estimated by a potentiometric technique similar to that of Sanderson (22). We are indebted to Mr. Gordon Bennett for the following modification of the above method.

The titration apparatus consisted of an inert reference electrode of platinum mounted in the tip of a microburette containing 0.005 N silver nitrate. The burette tip was just immersed in the chloride solution, which was contained in a small glass bucket. A pure silver wire electrode was placed in the bucket, and the contents were stirred by a constant speed electric motor.

The main functional modifications of Sanderson's circuit were such as to achieve the greater sensitivity and higher stability necessary when working with chloride

solutions of the order of 1,000-fold more dilute than this author used. Stability was achieved by operating all electrical apparatus from a voltage regulator transformer, and the use of voltage regulator tubes in the rectifier circuit. These tubes also supplied reference voltages which were used to ensure reproducible zero and full scale deflections of the indicating meter. The system had a high input resistance, and a linear response to input voltage, consequently a microammeter with an arbitrary linear scale was used as indicator of the electromotive force across the titration cell.

The standard $AgNO_3$ made up for the titrimeter was checked daily with the standard NaCl solution made up for the flame spectrophotometer, and the titration agreed within 1 per cent of the calculated normality. The NaCl solution was regarded as the absolute standard, so that the error in determining Na^+ and Cl^- in a muscle should be confined to the error inherent in the methods.

Extracellular Space.—The extracellular space was determined by the method of Wilde (34). The mean value of 8 determinations was (±S.E.), 15.8 ± 0.6. Some values must be as low as about 10.8 per cent, assuming there is no intracellular Na^+ (see reference 28 for discussion). These values are lower than those found by Harris and Martins-Ferreira (10) in frog muscle soaked in Ringer. These latter findings are based on the assumption that there is no intracellular chloride, an assumption with which we cannot agree (see below).

Solutions.—Ringer solutions used have been described (24), and were buffered with phosphate or bicarbonate. In low Na^+ Ringer the osmotic pressure was made up with sucrose.

RESULTS

In Vivo Muscles.—In order to obtain the intracellular ionic content of the muscles in the living animal it was necessary to determine the level of these ions in the plasma. The plasma was taken from each animal, and analysed separately. The mean ionic content ±S.E. of the plasma was Na^+ 122 ± 1.7 m.eq. per liter, K^+ 2.7 ± 0.02 m.eq. per liter, Cl^- 91.7 ± 2.4 m.eq. per liter (31 observations).

The correlation between Na^+ and Cl^- in the sartorius muscle is shown in Fig. 1. The complete lack of any relationship between K^+ and Cl^-, and Na^+ and K^+, is shown in Figs. 2 and 3. In an earlier publication (27) we noted a marked variation in the Na^+ content of sartorius *in vivo*. In the particular series analysed for this paper the variation was not so definite, most muscles having a low Na^+ (and Cl^-) content. We were, however, able, following the technique of Fenn *et al.* (8), to "create" high Na^+ toads by permitting the animal to bathe in 0.8 per cent NaCl solution for 2 to 3 days. To prevent contamination, the solution was changed twice daily. The increase in plasma Na^+ and Cl^- brought about by this procedure is small compared with the large increment in the muscle.

The correlation between Na^+ and Cl^- is highly significant, and the relationship has been analysed as follows:—

A multiple regression of Na^+ and K^+ as determinants of Cl^- was computed.

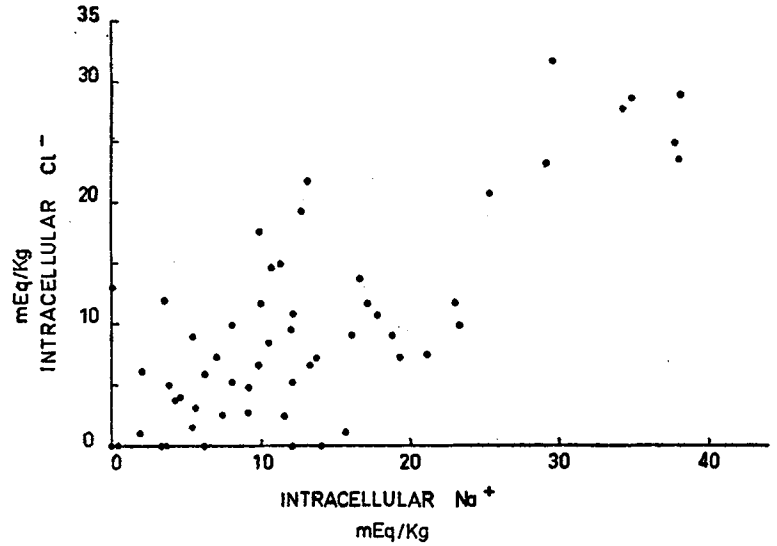


FIG. 1. The relationship between intracellular Na^+ and Cl^- in the intact animal. In this and subsequent figures each point represents the analysis of one muscle. For regression coefficient see text.

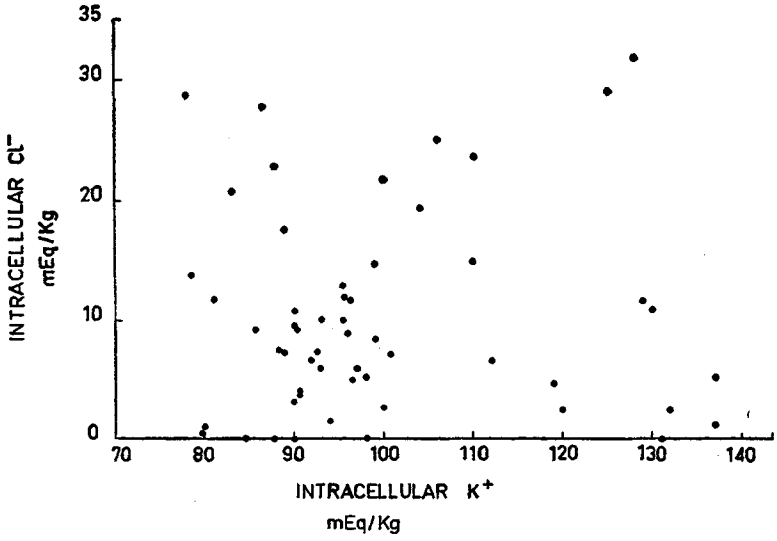


FIG. 2. The relationship between intracellular K^+ and Cl^- in the intact animal. There is no significant correlation.

Partial regression coefficients and their standard errors were obtained as follows:—

Na^+, b_1 = 0.671 S.E. = 0.072, significant at 1 per cent level.

K^+, b_2 = 0.067 S.E. = 0.045, not significant.

On recalculating the regression ignoring K^+, the coefficient was significant at the 1 per cent level, and the equation was obtained:

$$Cl^- = 1.22 + 0.658\ Na.^+$$

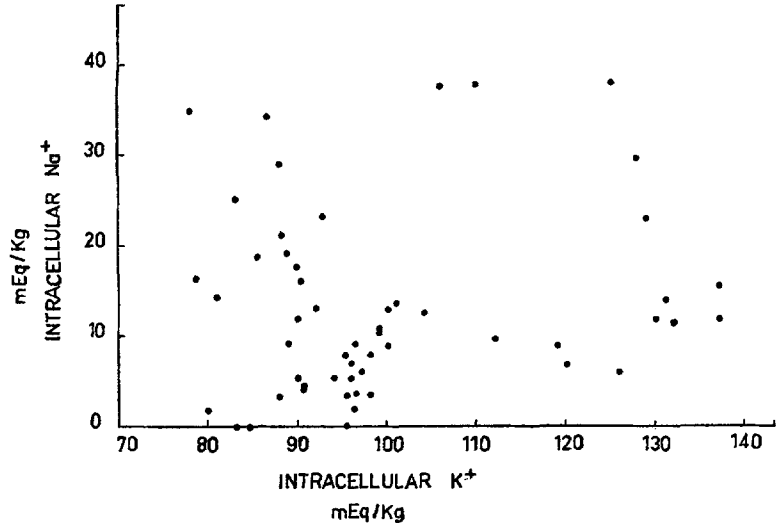


FIG. 3. The relationship between intracellular Na^+ and K^+ in the intact animal. There is no significant correlation.

This was compared with the slope 92/122 (0.754), being the ratio of Cl^- to Na^+ in plasma.

$$t = \frac{0.754 - 0.671}{0.072} = 1.16, \text{ not significant.}$$

Hence the experiment does not constitute evidence against 0.754 as slope.

The relation between Na^+ and Cl^- (Table I) within paired muscles was investigated. The regression of Cl^- on Na^+ was significant at the 5 per cent level.

Muscles Soaked in Normal Ringer (Isolated Muscles).—The ionic content of muscles *in vivo* was compared with that of the companion muscle soaked for 4 hours in normal Ringer. It is well known that when muscles are soaked in normal Ringer there is a gain of Na^+ and loss of K^+, until at the end of 2 hours a

TABLE I

Ionic Content of Paired Muscles in Vivo

Total Na, *m.eq./kg. wet weight*	Intracellular Na, *m.eq./kg. wet weight*	Total Cl, *m.eq./kg. wet weight*	Intracellular Cl, *m.eq./kg. wet weight*	Intracellular K, *m.eq./kg. wet weight*
22.2	5.4	20.9	9.0	96.0
20.7	3.5	23.6	12.0	95.6
32.3	17.0	23.2	11.8	81.0
22.2	5.4	14.7	1.6	94.0
19.1	1.9	14.3	1.0	86.0
27.9	12.0	21.4	9.6	90.0
13.4	0	12.9	0	84.6
14.5	0	13.5	0.5	79.8
36.1	21.0	19.7	7.6	88.3
20.4	3.4	12.0	0	87.8
24.4	8.0	21.8	10.0	95.4
23.4	7.0	19.5	7.4	92.6
21.6	4.5	16.9	4.0	90.6
22.7	5.6	15.8	3.1	90.0
24.3	8.0	17.8	5.2	98.0
23.1	6.2	18.1	5.9	97.0
20.8	3.8	17.5	5.0	96.5
19.7	2.0	18.3	6.0	93.0
32.4	9.8	28.3	17.6	89.0
32.4	9.8	23.2	11.7	96.3
21.2	4.2	16.4	3.8	90.6
17.6	0	24.5	13.0	95.4
20.9	3.6	9.2	0	98.0
25.4	9.0	15.5	2.6	100

In this and subsequent tables ionic contents are calculated on a wet weight basis and refer to intracellular levels, assuming 15 per cent extracellular space.

There is an apparent large variation in the intracellular Na^+ and Cl^- content of paired muscles. This variation is probably due to slight differences in the extracellular space in individual muscles. When the total content of an ion is contained mainly in the extracellular space (*e.g.*, Na^+ and Cl^-) slight variations in this space produce large differences in the estimated intracellular content. This disadvantage does not occur when the concentration of the constituent in the external fluid is small (*e.g.*, K^+). Thus in the above table the paired analyses agree better for K^+ than they do for Na^+ or Cl^-.

new steady state is established. It has also been known for some time (7, 8, 21) that the total Cl^- content of muscles increases on soaking in Ringer. Some of these authors (7, 8) were loath to admit that the Cl^- had passed into the cell. Boyle and Conway (2) followed the rate of entry of Na^+ and Cl^- into the cell and the concomitant K^+ loss. They found an approximately equivalent Na^+ gain and K^+ loss, and the anion gain could be considered equivalent to either the Na^+ gain or the K^+ loss. These authors also noted that the ratio K^+_{in}/K^+_{out} was not equal to Cl^-_{out}/Cl^-_{in} in muscles soaked in normal Ringer, and ascribed this to a drop in resting potential. It has since been shown (27) that such a fall in potential does not occur.

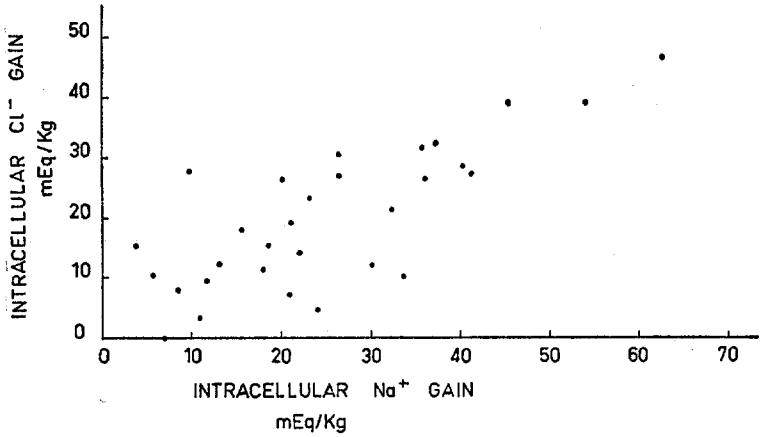


FIG. 4. The relationship between the gain of Na^+ and Cl^- on soaking in normal Ringer. For regression coefficient see text.

In Fig. 4 gain in Na^+ has been plotted against gain in Cl^-. In this particular series 7 out of 30 muscles did not lose K^+ on soaking. This is a rather unusual occurrence, but it enabled us to show that the correlated gain of Na^+ and Cl^- was independent of any K^+ movement. The correlation between the gains of Na^+ and Cl^- was highly significant ($r = 0.80$). Fig. 4 is drawn from the results obtained with 30 pairs of muscles. Figs. 5 and 6 illustrate the lack of relationship between gain of Cl^- and loss of K^+ and between loss of K^+ and gain of Na^+.

In addition to the results presented in Figs. 4, 5, and 6, which were obtained by a comparison of paired muscles, one *in vivo*, the other soaked in normal Ringer, we were able to correlate the ionic content in a very large series (124 muscles) of soaked muscles. These results have been culled from other experiments in which muscles soaked in normal Ringer served as controls. There are, of course, no *in vivo* mates for these muscles, since their companions were subjected to other experimental conditions. Hence in Figs. 7, 8, and 9 the absolute amounts of the ions are plotted, and not the gains or losses.

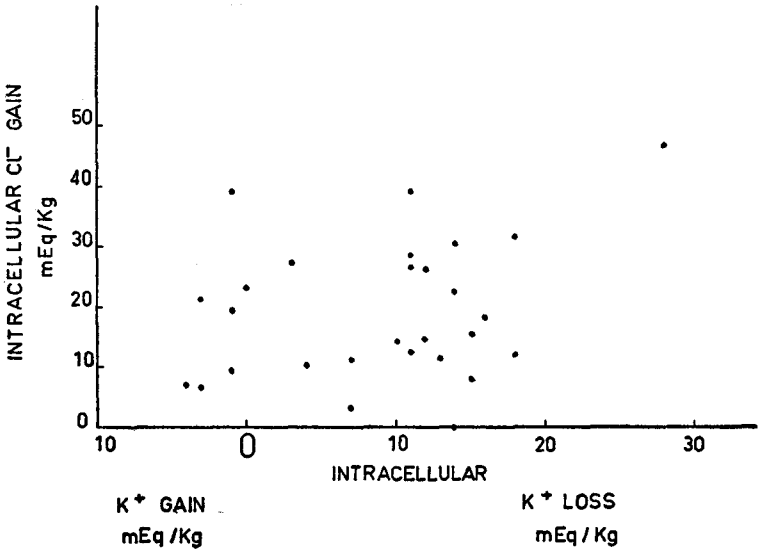


FIG. 5. The relationship between change in intracellular Cl^- and K^+ content on soaking in normal Ringer. There is no significant correlation.

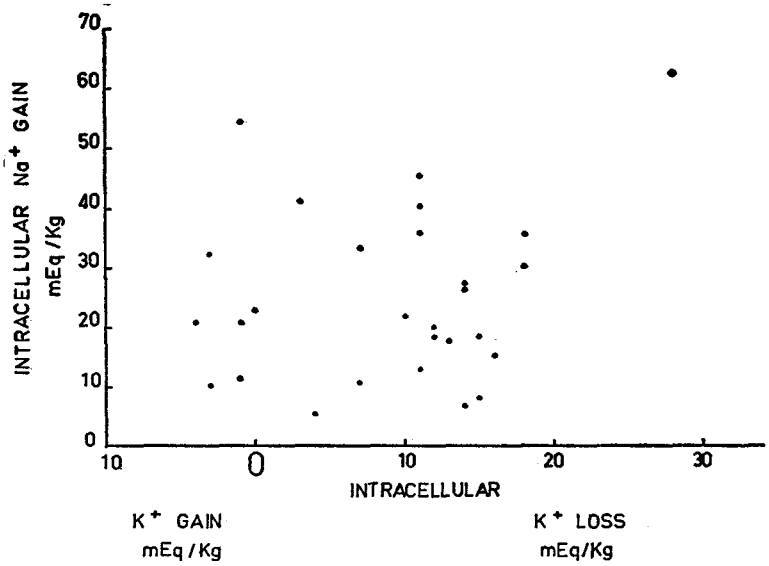


FIG. 6. The relationship between change in intracellular Na^+ and K^+ content on soaking in normal Ringer. There is no significant correlation.

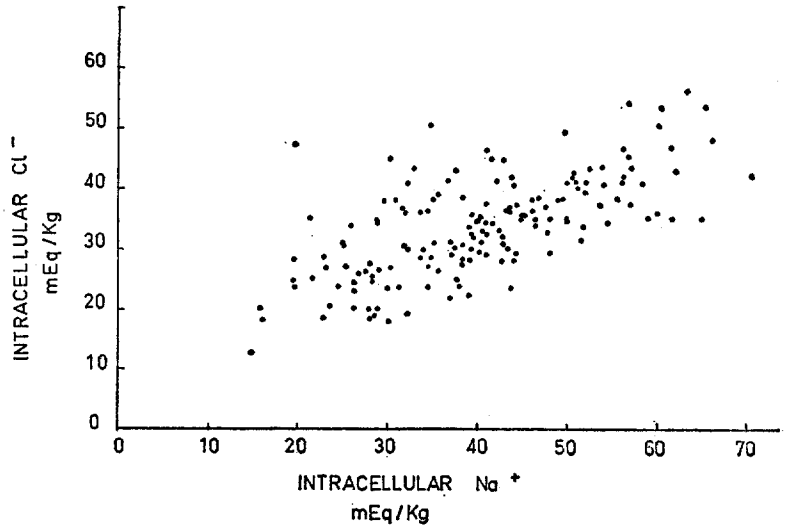


FIG. 7. The relationship between Na^+ and Cl^- in sartorii soaked in normal Ringer For regression coefficient see text.

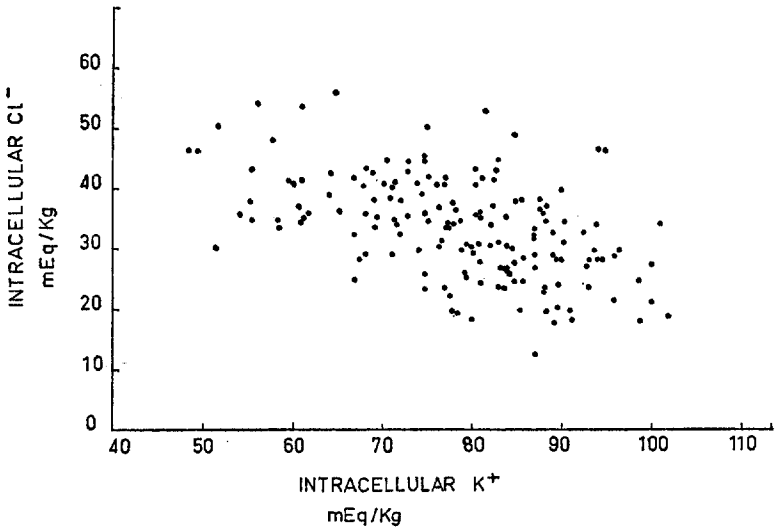


FIG. 8. The relationship between intracellular K^+ and Cl^- in sartorii soaked in normal Ringer. For discussion of correlation see text.

It is obvious from an inspection of these graphs that (*a*) there is a correlation between the Na^+ and Cl^- content of muscle in normal Ringer; (*b*) there is no connection between K^+ and Cl^- save in those muscles having an abnormally low K^+ content; (*c*) there is no connection between Na^+ and K^+. The relation-

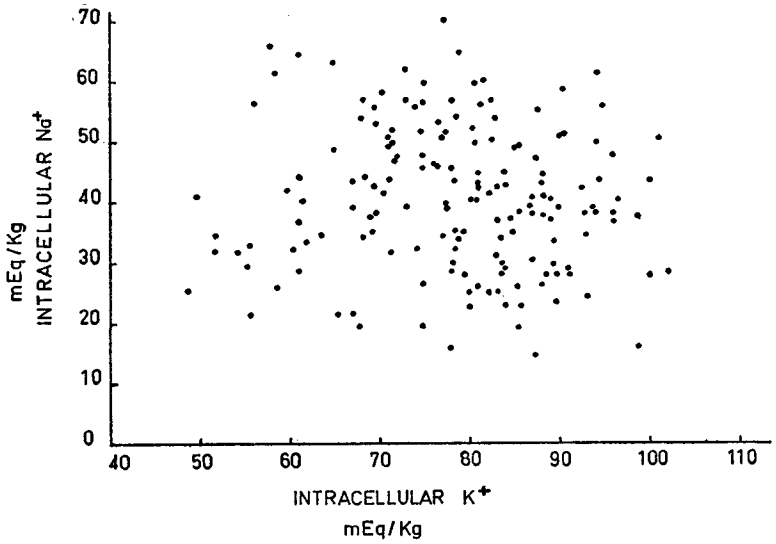


FIG. 9. The relationship between intracellular Na^+ and K^+ in sartorii soaked in normal Ringer. There is no significant correlation.

ship between Na^+ and Cl^- has been examined more closely, with the following results:

A multiple regression was performed with Na^+ and K^+ as determinants of Cl^-. The partial regression coefficients and their standard errors were as follows:—

Na^+, $b_1 = 0.582$, S.E. $= 0.050$, significant at 1 per cent level.

K^+, $b_2 = 0.196$, S.E. $= 0.065$, significant at 1 per cent level.

The experiments from which these figures were obtained were divided into two halves on a chronological basis, because in the first series there were several muscles with an abnormally low K^+ content. These abnormalities did not occur in the second series. The regression coefficient for K^+ in the second series was not significant. Thus the dependence of Cl^- on K^+ is only seen in muscles which have an abnormally low K^+ content.

When the regression coefficient for Na^+ is compared with the ratio Cl^-/Na^+ in Ringer, *i.e.* 110/130 (0.846), it is found that the difference is highly significant ($t = 5.31$, 123 d.f.). Thus the ratio of Cl^-/Na^+ in the isolated muscle differs from that of the surrounding Ringer.

The Effect of Alteration of External NaCl Level.—We have investigated the effect of alteration of the external Na^+ and Cl^- level of the Ringer on the intracellular ionic content ($Na,^+$ Cl^-, and K^+). The pattern of the experiment was the same as that described in our previous paper (28). One muscle was removed from the animal and soaked in normal Ringer solution for the appropriate time. The companion muscle was either placed in the test solution immediately (unequilibrated), or soaked in normal Ringer for 2 hours, and then placed in the appropriate test solution (equilibrated). In the equilibrated series the control muscle was analysed at the end of the equilibration period (2 hours).

TABLE II

The Effect of Reduced NaCl Ringer on Ionic Content

	Equilibrated				Not equilibrated			
Na in Ringer, *m.eq./liter*	65	Ratio	56	Ratio	65	Ratio	45	Ratio
Cl in Ringer, *m.eq./liter*	55		37		55		37	
Na, *m.eq./kg.*								
Control	40.2	3.2	31.9	4.1	39.1	3.3	32.1	4.2
Treated	29.6	2.2	23.2	2.4	17.6	3.7	20.7	2.2
Cl, *m.eq./kg.*								
Control	34.7	3.2	35.4	3.1	34.1	3.2	32.4	3.4
Treated	25.5	2.2	14.0	2.6	17.3	3.2	18.2	2.0
K, *m.eq./kg.*								
Control	72.8		67.2		83.2		85.6	
Treated	69.7		72.0		81.0		86.2	

"Ratio" means Na^+ or Cl^- (out/in). Control muscles were soaked in normal Ringer for 2 or 4 hours (see text). The changes in K^+ concentrations are not significant.

The external Na^+ level was varied between 45 and 460 m.eq. per liter, the Cl^- level between 37 and 440 m.eq. per liter. In low Na^+ solutions the osmotic pressure was made up with sucrose. High NaCl solutions were of necessity hypertonic.

Low NaCl Ringer (Unequilibrated).—As will be seen from Table II muscles placed in reduced NaCl Ringer lose intracellular Na^+ and Cl^- in proportion to the decrease in the NaCl level of the external solution. Thus, after 4 hours' soaking, when the muscle has reached a steady state the ratios Na^+_{out}/Na^+_{in} and Cl^-_{out}/Cl^-_{in} are the same in the half NaCl Ringer as in the controls. In the one-third NaCl Ringer the ratios are smaller in each case, but the Na^+ and Cl^- ratios are equal. There was no concomitant change in the K^+ level of the muscle in either case.

Low NaCl Ringer (Equilibrated).—When a muscle is soaked in Ringer it

tends to gain NaCl until a steady state is achieved, when the ratio of Na^+ and Cl^- (out/in) is approximately equal to three (see previous section). If the muscle is now removed to a NaCl-deficient solution these ions are extruded from the cell, against a concentration gradient, until the Na^+ and Cl^- ratio is almost completely reestablished. These results are set out in Table II. As was found in the unequilibrated series there was no change in K^+ levels. The relation of this Na^+ extrusion to the Steinbach (32) "pump" was discussed previously (26). This work has recently been confirmed by Van der Kloot (17).

The above results show that there is an extrusion of Cl^- against a concentration gradient. Since the levels of Na^+ and Cl^- are different in the external solution (*viz.* 130 and 110 m.eq. per liter), it is better to consider changes in

TABLE III

The Effect of High NaCl Ringer on Ionic Content

Na in Ringer, *m.eq./liter*	240	Ratio	460	Ratio
Cl in Ringer, *m.eq./liter*	220		440	
Volume change, *per cent*	−13.8		−17.2	
Na, *m.eq./kg.*				
Control	36.0	3.6	39.1	3.3
Treated	93.6	2.6	248	1.85
Cl, *m.eq.k/g.*				
Control	34.6	3.2	34.1	3.2
Treated	87.2	2.5	249	1.8
K, *m.eq./kg.*				
Control	82.0		83.2	
Treated	88.0		83.2	

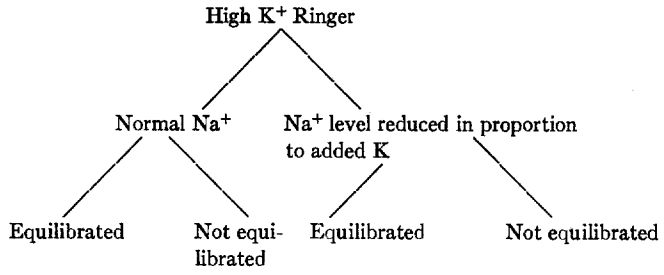
ratio rather than the absolute amount of material moved. It will be seen from Table II that the Na^+ and Cl^- ratios in the treated muscles are nearly identical, thus the extrusion of both ions is of a similar magnitude. The question whether it is necessary to postulate a Cl^- "pump", or whether an alternative mechanism must now be sought is referred to in the Discussion. These results are unique, since for the first time there has been demonstrated a gross movement of the Cl^- ion which is completely unaccompanied by a movement of K^+.

High NaCl Ringer (Unequilibrated).—The results of soaking muscle in high NaCl Ringer are set out in Table III. In agreement with our earlier results (28), it was found that as the osmotic pressure of the external solution is increased there is a progressive shrinkage of the muscle which amounts to approximately 20 per cent at 400 per cent NaCl. This shrinkage reaches a maximum after the first 30 to 45 minutes. The Na^+ and Cl^- have entered the muscle

in proportion to the external increase, but contrary to our earlier results (28) the ratio Na^+ and Cl^- (out/in) has declined at the higher level of NaCl; the Na^+ and Cl^- ratios are, however, still equal.

The Effect of Alteration of the External KCl Level

The plan of our experiments is set out in the diagram below:



The experiments were divided into two series. In one series KCl was added to normal Ringer. In the other series as the K^+ was increased an equivalent amount of Na^+ was subtracted, and thus the Cl^- level remained constant. As in the previous section (the effect of the alteration of external NaCl level) the muscles were either placed directly in the high K^+ solution (unequilibrated), or permitted to equilibrate in Ringer for 2 hours before they were placed in the high K^+ solution. Muscles immersed in normal Ringer gain Na^+ and Cl^- and frequently lose K^+ during the first 2 hours. If these changes are not reversed by raising the K^+ in the Ringer this will produce differences between the equilibrated and unequilibrated series.

The results of our experiments are set out in Tables IV and V. It will be seen that in K-free Ringer there is no significant difference in ionic content between the treated muscle and the control muscle soaked in normal Ringer. It must be remembered that the control muscles have gained Na^+ and Cl^-, and have probably lost K^+, and so these movements have not been affected by soaking in the K-free solution. It will be observed that the cell contained 27 m.eq. per kg. Cl^-, thus it cannot be said that there was insufficient Cl^- to accompany the K^+ from the cell (see Discussion).

In our previous paper (28) we have found that increasing the KCl level to 25 and 50 m.eq. per liter produced a slight increase in the K^+ content of the cells. This increase was by no means equal to that predicted by the Donnan theory. The results set out in Tables IV and V are in agreement with this earlier work. There is no difference in K^+ increase between the series in which KCl was added to normal Ringer and that in which an equivalent amount of NaCl was subtracted. However, the muscles in the latter series increased in volume and showed an increase in Na^+ gradient in high K^+ Ringer. There is little difference between the equilibrated and unequilibrated series, the muscles in the

TABLE IV

The Effect of High KCl Ringer on Ionic Content
Na^+ Level of Ringer Not Altered

	Equilibrated						Unequilibrated							
K in Ringer, *m.eq./liter*....	25	Ratio	50	Ratio	87	Ratio	0	Ratio	25	Ratio	50	Ratio	103	Ratio
Cl in Ringer, *m.eq./liter*....	135		161		217		107		135		161		223	
Volume change, *per cent*.....	+2.3		−4.4				+0.5		+3.1		±0		−3.0	
Na, *m.eq./kg.*														
Control...	42.2	3.1	41.5	3.1	45.2	2.9	43.7	3.0	31.7	4.1	41.6	3.1	63.7	2.0
Treated...	42.8	3.0	38.7	3.4	47.9	2.7	40.6	3.2	22.9*	5.7*	38.0*	3.4*	58.1*	2.7*
Cl, *m.eq./kg.*														
Control...	35.4	3.4	31.5	3.5	40.3	3.3	32.3	3.4	33.6	3.3	40.8	2.7	38.8	2.8
Treated...	49.6*	2.9*	55.4*	2.9*	100*	2.2*	27.7	3.9	43.5*	3.1*	65.3*	2.5	92.9*	2.4*
K, *m.eq./kg.*														
Control...	69.7		63.3		80.6		80.9		64.6		60.5		87.5	
Treated...	79.7*		83.3*		142*		77.8		78.8*		94.4*		149*	

* Changes are significantly different from the control ($P < 0.05$).

TABLE V

The Effect of High K^+ Ringer on Ionic Content
Na^+ Level of Ringer Reduced Proportionately

	Equilibrated				Unequilibrated			
K in Ringer, *m.eq./liter*.....	25	Ratio	50	Ratio	25	Ratio	50	Ratio
Cl in Ringer, *m.eq./liter*.....	110		110		110		110	
Volume change, *per cent*.....	+3.1		+15.0		+7.2		+15.4	
Na, *m.eq./kg.*								
Control.....	31.9	4.1	36.5	3.5	57.9	2.2	42.9	2.8
Treated.....	16.4*	6.4*	11.4*	7.0*	43.3*	2.4*	18.1*	5.3*
Cl^-, *m.eq./kg.*								
Control.....	27.4	4.0	31.2	3.5	40.8	2.7	35.2	3.1
Treated.....	26.8	4.1	36.0	3.1*	46.2	2.4	40.9	2.7
K, *m.eq./kg.*								
Control.....	80.6		89.2		75.3		82.7	
Treated.....	89.7*		105*		89.2*		103*	

* Changes are significantly different from the control ($P < 0.05$).

latter contain more K^+ in high K Ringer than the former. Thus it would appear that the loss of K^+ found on soaking in normal Ringer has been hindered in the unequilibrated series, and that in the equilibrated series there has been a smaller return of K^+ to the cell than was found in the former experiments.

Throughout the series of experiments with high K^+ we have used as our control the K^+ content of the companion muscles soaked in normal Ringer. Alternatively one could have used the K^+ value *in vivo*, which would normally be higher than that in normal Ringer. Ideally one should compare the results of the muscles soaked in high K^+ with those *in vivo* and in normal Ringer. Unfortunately this is not possible as the toad has not three hind legs. We have endeavoured to overcome this difficulty in the following manner. Muscles from 12 toads were dissected on the one day, 6 were analysed immediately, and their companions soaked in normal Ringer to which had been added 50 m.eq. per liter KCl. Six were soaked in normal Ringer and their companions soaked in the high KCl Ringer. The results are given in Table VI. The figures show that the high KCl Ringer has caused an entry of K^+ into the cell, because the value is 15.1 m.eq. per kg. higher than that of the muscles *in vivo*. On the other hand the muscles soaked in normal Ringer lost 17.4 m.eq. per kg. K^+, compared with the *in vivo* group. When the companion muscles were soaked in high K Ringer this loss was prevented, and a further 18.5 m.eq. per kg. K^+ entered. This latter addition is in reasonable agreement with the 15.1 m.eq. per kg. K^+ which entered in the first group.

TABLE VI

The Effect of High KCl Ringer on Ionic Content Compared with Controls in Normal Ringer and in Vivo (See Text)

	Control in normal Ringer		Control *in vivo*	
		Ratio		Ratio
Na, *m.eq./kg.*				
Control	53.0	2.5	2.1	57.0
Treated	50.0	2.6	36.2*	3.6*
Cl, *m.eq./kg.*				
Control	38.2	2.9	6.2	18.0
Treated	69.1*	2.3	64.2*	2.5*
K, *m.eq./kg.*				
Control	66.5		83.9	
Treated	102*		99.0*	

Intracellular contents *in vivo* are calculated using plasma ionic levels. The test Ringer contained 50 m.eq. KCl added to normal Ringer.

* Changes are significantly different from controls ($P < 0.05$).

In the high KCl Ringer series (Na^+ level remaining constant) there has been no change in the internal Na^+ level in the equilibrated series. Muscles without equilibration showed a slight decrease in Na^+ content. When the Na^+ level of the Ringer was decreased in proportion to the added K^+ there was a marked decrease in Na^+ content, which was greater than would have been expected from the decrease in Na^+ level of the Ringer. This has resulted in an increase in the ratio Na^+_{out}/Na^+_{in}.

When we come to consider the alteration in internal Cl^- resulting from the increase in external KCl (the Na^+ level remaining constant), it is found that the Cl^- content of the cell increases, and that this increase is directly related to the outside level (*e.g.* external Cl^- 135 m.eq. per liter, increase in muscle 14 m.eq. per kg. external Cl^- 161 m.eq. per liter, increase in muscle 21 m.eq. per kg. Cl^-, equilibrated series, Table IV). However, the actual increase in the cell is greater than one would have expected from the increase in external concentration. This is shown by the fact that in all cases the ratio Cl^-_{out}/Cl^-_{in} is significantly lower in the treated muscles. This reduction in the Cl^- ratio was only seen in one out of six experiments when the Na^+ content of the Ringer was reduced in proportion to the added K^+, and consequently the Ringer Cl^- level remained the same.

The relationship between the Na^+ and Cl^- movements in high K^+ Ringer is extremely interesting. When the Na^+ level in the Ringer was not altered it was found (in the equilibrated series) that the Na^+ in the cell remained unchanged (the ratios are the same), whilst the Cl^- had more than proportionately increased, and consequently the ratio was less than in the control. In the unequilibrated series there was a significant loss of Na^+ (at 25 m.eq. per liter K^+ and higher levels), whilst the Cl^- had again entered the cell more than in proportion to the external increase. When the Na^+ level of the Ringer was reduced there was a disproportionate decrease in the Na^+ content of the treated muscles, but the Cl^- level (and ratio) did not alter in either the equilibrated or unequilibrated series (except for the one experiment mentioned above). Thus it is evident that the presence of a high external level of K^+ has altered the relationship between the movements of the sodium and chloride ions. In normal Ringer or when the NaCl level in Ringer was altered the movements of these ions were equivalent. From the results with high K^+ Ringer it would appear that the Na^+ and Cl^- ions can move independently. Thus the ratios Na^+ and Cl^- (out/in), which were previously found to be equal, have diverged under the influence of a high external K^+ level.

DISCUSSION

The work reported in this paper is an extension of that previously published (28) in which the intracellular changes of Na^+ and K^+ in the same muscle

were correlated with changes in the external ionic environment. These results may be summarised as follows:—

(*a*) Muscles soaked in K-free Ringer do not lose K^+ compared with companion muscles soaked in normal Ringer.

(*b*) When the external K^+ is raised stepwise to 100 m.eq. per liter a small amount of K^+ enters the cell.

(*c*) Whilst the amount of K^+ in the muscle *in vivo* is relatively constant there is great variation in the intracellular Na^+ level (0 to 70 m.eq. per kg.; *i.e.*, the ratio Na^+_{out}/Na^+_{in} varies from 1.85 to infinity). When the muscle is soaked in Ringer solution it loses or gains Na^+, until there is an approximately constant ratio of 3.

(*d*) If the external Na^+ level is raised or lowered (between 45 and 460 m.eq. per liter) Na^+ enters or leaves the cell to maintain this constant ratio.

(*e*) Rendering the Ringer solution hypertonic by the addition of KCl does not affect the volume of the cell. The addition or subtraction of NaCl to or from the Ringer solution (regardless of the K^+ concentration) results in a shrinkage or swelling of the cell. However, there is not a proportional relationship between the external NaCl level and the volume change.

In this paper we have repeated the previous work, but in addition we have analysed the same muscle for chloride (as well as Na^+ and K^+). We have confirmed the above results with respect to Na^+ and K^+. It remains to discuss the conclusions which may be drawn from the relationship between the movements of the three ions. This will be done under four headings.

1. Donnan Theory.—The accumulation of K^+ in the cell has been held in the past to be due to a Donnan distribution (2). Boyle and Conway (2) had found that an increase in external K^+ level resulted in an elevation in internal K^+ which agreed with that theoretically predicted. We are unable to confirm this (see Tables IV and V). Furthermore they stated (2) that in a K-free solution K^+ would leave the cell as long as it could be accompanied by Cl^-. We had shown (29) that under these conditions K^+ did not leave the cell, and we have now demonstrated that there is adequate Cl^- to accompany the K^+ into K-free Ringer (see Table IV). It is a fundamental requirement of the Donnan theory that the product $K^+_{in} \times Cl^-_{in} = K^+_{out} \times Cl^-_{out}$. Conway found that this relationship did not hold in normal Ringer, but only when there was an elevated K^+ level, or in the intact animal. The agreement with the Donnan ratio in muscles *in vivo* must be regarded as fortuitous, for those muscles with a low Cl^- (and Na^+) content will tend to give correct equality, but high Cl^- (and Na^+) muscles will not. This lack of correlation between K^+ and Cl^- *in vivo* is shown in Fig. 2. We were also unable to confirm this relationship in high K^+ Ringer, since there was an insufficient increase in intracellular K^+, with respect to the raised external level.

A further consequence of the Donnan theory is that there should be reciprocity between Na^+ and K^+ content. If the electrochemical gradient for K^+ is maintained by the active extrusion of Na ions, then high internal Na^+ levels should result in low K^+ levels, and conversely. It may be seen (Figs. 3 and 9) that such a correlation *in vivo* or in the isolated preparation does not exist.

We were also unable to reconcile the changes in the ionic content of muscle on soaking in normal Ringer with a Donnan mechanism. As was stated when discussing the absolute levels of intracellular ions, there should be a correlation between gain of Na^+ and loss of K^+, and a correlation between gain of Cl^- and loss of K^+. The lack of such a relationship is seen in Figs. 5 and 6.

The simple concept that the accumulation of K^+ was due to a Donnan mechanism with the Na^+ playing the role of an impermeant cation was proved untenable, because radioactive Na^+ was shown to cross the membrane freely. Since then the situation has become confused. Dean (5) introduced the concept of a Na^+ pump to account for the Na^+ permeability; following this the concept of a pump or carrier has been used to explain the movements of every cation and anion so far considered (6, 12, 20). It is difficult to ascertain at the present time to what extent the accumulation of K^+ is considered to be due to a Donnan mechanism bolstered by a Na^+ pump, with the passage of K^+ across the membrane facilitated by a carrier, and to what extent the accumulation is considered to be due to active transport alone. It would seem that the evidence cited in this paper must refute the Donnan concept.

2. Linked Carriers.—Hodgkin and Keynes (12, 13) have stated that the recovery movements of Na^+ and K^+ in squid axon after stimulation are coupled, though they do not seem to be rigidly linked. In our experiments with resting muscle we find little correlation between the movements of Na^+ and K^+.

With one exception, the movements of ions studied by us have been along concentration gradients, in the resting state. Thus our results are not strictly comparable with those obtained after the passage of an impulse in the squid axon. However, examples of non-reciprocal movement may be seen in Tables II and III and Fig. 6.

Keynes (16) found that placing a muscle in K^+-free Ringer reduced the Na^+ efflux. Hodgkin and Keynes (12) confirmed this finding in nerve. We have found that under these conditions the total Na^+ content of the muscle did not alter, and therefore if there was a decrease in efflux, there must also have been a decrease in influx. As the K^+ content of the cell after 4 hours soaking in K-free Ringer is the same as that of the companion immersed in Ringer, this suggests that K^+ efflux has remained the same as in normal Ringer, until a steady state is achieved, when efflux would appear to cease. (See also reference 29.)

When a muscle which has been soaked in Ringer is transferred to half Na^+ Ringer there is an extrusion of Na^+ against a concentration gradient (26). There is no concomitant movement of K^+. We have now shown that the Na^+

which leaves the cell is accompanied by Cl^-. Thus we have an example of the active transport of NaCl which is analogous to that performed by the frog skin (for bibliography see Ussing (33)). The analogy is not complete, because with the frog skin we are dealing with a multicellular membrane bathed on either side by a Ringer type solution. It is possible to postulate a net ionic transfer from one solution to the other taking place at a constant rate, and so fulfilling the conditions of the steady state. In this case the flux ratios will not be unity, but the assumption of a steady state justifies the calculations given by Ussing on page 9 of his paper (33). From a consideration of the flux ratios and the electrochemical gradient for the ion, he states that Na^+ is actively transported through the frog skin, and Cl^- follows passively. Unfortunately, one cannot transfer this process of reasoning to a system such as nerve or muscle where one is dealing with the flux across the membrane of a cell or group of cells, and the existence of a flux ratio which is not unity implies that the system is not in a steady state.

3. The Relationship between Na^+, K^+, and Cl^-.—As we have mentioned earlier in this discussion most workers have suggested that movements of K^+ and Cl^-, and K^+ and Na^+ are related. Our results show that there is not a first order correlation between these ions. On the other hand we have demonstrated a definite linkage between Na^+ and Cl^-.

In the intact animal there is a highly significant correlation between intracellular Na^+ and Cl^-. This is shown in Table I and Fig. 1. There is a significant correlation between Na^+ and Cl^- content in two sartorii taken from the same animal. It has also been shown that the ratio of Na^+ to Cl^- in muscle is the same as this ratio in plasma. This relationship is unaffected by alterations in the level of internal K^+.

The ion shifts found on soaking muscle in normal Ringer also reveal a highly significant correlation between Na^+ and Cl^- movements. The gains of Na^+ and of Cl^-, although closely related, are not equivalent. For each Cl ion entering the cell approximately 1.4 Na ions enter. As can be seen from Figs. 5 and 6 there is no correlation between Na^+ gain and K^+ loss, and Cl^- gain and K† loss.

There is also a highly significant correlation between Na^+ and Cl^- content of muscles soaked in normal Ringer. The ratio of Na^+ to Cl^- in muscle is, however, significantly different from the ratio of Na^+ to Cl^- in Ringer. The relationship is such that one Cl ion is associated with 1.7 Na ions. The results shown in Fig. 7 were divided chronologically into two series. In the first series the intracellular K^+ was abnormally low in several muscles (less than 55 m.eq. per kg.). A multiple correlation performed on this series showed that the Cl^- level was influenced by both the Na^+ and K^+ content. In the second series, in which these abnormal K^+ levels were not present, the intracellular Cl^- value depended only on the amount of intracellular Na^+.

When the external NaCl is raised or lowered the intracellular level of these ions follows the external change so that the ratio Na^+ or Cl^- (out/in) remains constant at approximately 2–3, and is the same in each individual muscle. There are no accompanying changes in internal K^+.

The only condition under which the equivalence of Na^+ and Cl^- ratios is not observed is that obtaining when the external K^+ is increased. Under these circumstances the Na^+ ratio tends to be increased, and the Cl^- ratio decreased; *i.e.*, there is less Na^+ than Cl^- in the cell.

4. Theoretical Considerations.—Contrary to currently held opinion there is a close correlation between Na^+ and Cl^- content, and no first order relationship between these ions and K^+. These findings have led us to consider a new concept of the distribution of ions within the cell. The nature of this concept is suggested by the results given in this paper, but must remain tentative pending the completion of confirmatory experiments.

In an earlier paper, Shaw and Simon (25) discussed the possibility that the accumulation of K^+ could be accounted for by an adsorption hypothesis. The suggestion that K^+ is adsorbed by the cell is not new (for literature see reference 25). It is no more unreasonable to assume that ionic differentiation takes place in the cell interior than that it happens within the mural structure.

The adsorption of K^+ cannot be considered to occur as the result of "classical ionic" forces; *i.e.*, it cannot be pictured as a point charge held in position under the action of electrostatic forces. If this were so then no discrimination between Na^+ and K^+ would be possible. The introduction of the concept of ionic radius raises further difficulties, for example the possibility of induced polarisation of the charge in the presence of an electric field. Indeed, it is logically inconsistent to conceive of a homogeneous sphere of charge, with an invariant shape, structure, and size, which can react as a whole with an electric field. It is clear that the radius of the ion is not invariable, but must be considered as an "interaction radius," resulting from the interaction of a particular ion with a particular environment. It is thus a quantity of secondary rather than primary significance. In this respect our views differ from those of Ling (19).

The binding of K^+ must thus be the result of a non-classical process such as electron exchange, which gives rise to a covalent type of directed binding force. This will be strong, and of very short range compared with the range of electrostatic forces. The current physical picture of the ion, with a distributed charge density whose details depend on the electrodynamics of the situation, and whose charge density will differ between equivalent ions (*i.e.*, Na^+ and K^+), gives a possible mechanism for ionic discrimination. Thus it is postulated that K^+ is adsorbed onto the ultrastructure of the cell. The K^+ sites will be highly specific, and the normal metabolic function of the cell is necessary to maintain the sites, which may be cyclically broken down and regenerated. The K^+ sites are situated in a charged, semirigid, ordered lattice structure which

occupies about two-thirds of the cellular volume. (For a general discussion of polyelectrolyte gels see Katchalsky (15).) Na^+ and other ions not associated with the structure of the lattice are excluded from the region it occupies. The other excluded ionic constituents diffuse into the remaining smaller fraction of the cell which is in physicochemical equilibrium with the Ringer. The isolation of these ions in the smaller space results in an apparent concentration gradient across the membrane when their concentration is expressed in terms of total cell volume.

The evidence for the existence and extent of this space is as follows: (*a*) Na^+ and Cl^- movements are equivalent in the majority of our experiments. (*b*) The ratio of Na^+ and Cl^- (out/in) is constant, and on the average has a value of 3. Thus the extent of this space is one third of the total cell volume. (*c*) LiCl shows the same relationships (unpublished observations). (*d*) There will also be a small amount (about 1 m.eq. per kg.) of "free" K^+ in this space in equilibrium with the Ringer. When a muscle is placed in high K Ringer there is a small gain in K^+, which is directly related to the external level. Since this increase represents diffusion into the free intracellular phase (F.I.P.) then the *increase* in K^+, compared with the controls, should be in the same ratio (K^+_{out}/K^+_{in}) as is found with the other ions. This calculation will be complicated by the fact that the increase in external K^+ is known to reduce the loss of K^+ found on soaking in normal Ringer (Table IV). Thus the increase in intracellular K^+ in the unequilibrated series will represent a reduction in this loss, plus diffusion into the free intracellular phase. If one considers the equilibrated series, in which this loss will take place during the equilibration period (Tables IV and V), then it is apparent that the increase in K^+ agrees reasonably well with that calculated. For example in Table IV (equilibrated series) the gains of K^+ at 25, 50, and 87 m.eq. per liter external level are 10 m.eq. per kg. K^+ found $\left(8 \text{ m.eq. per kg. calculated from } K^+ \text{ out} \div \frac{Cl^-_{out}}{Cl^-_{in}}\right)$, 20 m.eq. per kg. found (17 m.eq. per kg. calculated), and 62 m.eq. per kg. found (40 m.eq. per kg. calculated). In Table V the values at 25 and 50 m.eq. per liter external K^+ are 9.0 m.eq. per kg. found (6 m.eq. per kg. calculated) and 16 m.eq. per kg. found (16 m.eq. per kg. calculated.) When the increase in intracellular K^+ is compared with controls *in vivo* (Table VI) there is an actual addition of 15 m.eq. per kg. compared with a calculated increase of 17 m.eq. per kg. The undue increase found at 87 m.eq. per liter may reflect a reversal of the loss found on soaking in normal Ringer; *i.e.*, there may have been a reconstitution of adsorption sites. This would result from a decrease in the energy required to maintain a site, following a decrease in the K^+ gradient. With this one exception at 87 m.eq. per liter the differences between the found and calculated values are not statistically significant.

There are two apparent inconsistencies with the simple theory set out above.

The ratio of Na^+ and Cl^- (out/in) in the living animal is usually much greater than three, and is subject to great variation. It is now necessary to explain why the NaCl enters the muscle on soaking to give an approximate ratio of three. It is possible that the semirigid lattice structure wherein the K^+ sites are situated extends to a larger extent throughout the cell *in vivo*, and so excludes a greater amount of Na^+ and Cl^-. When the cell is removed from the body there will be a decrease in available energy to support this semirigid structure, which becomes gradually disordered. This loss of order within the cell permits the entry of a certain amount of NaCl. After about an hour a new balance is set with the available energy, and a steady state is achieved. This state is maintained for at least 8 hours, but as the cell dies a further disintegration of structure takes place, and more NaCl enters until finally the ratio approaches unity on a fiber water basis (Shaw and Simon (25)).

The ratio of Na^+ to Cl^- in the intact animal is the same as the ratio of Na^+ to Cl^- in plasma. However, when the muscle is soaked in normal Ringer there is an excess of Na^+ over Cl^- in the free intracellular phase (see Fig. 7), the additional Na^+ may preserve electroneutrality if there is an accumulation of phosphate or bicarbonate. Contrary to the above finding there is a higher ratio Na^+_{out}/Na^-_{in} than Cl^-_{out}/Cl^-_{in} when muscle is soaked in high K^+ Ringer. Both these points will be discussed in a later paper. In other words our results illustrate that the movements of Na^+ and Cl^- are passive and depend on the external concentration. To account for this we have suggested that the cell consists of two parts, first, there is an ordered semirigid gel, which specifically adsorbs K^+, and excludes other ions. Second, there is a small and relatively disordered free space within which other ions exist in equilibrium with the extracellular phase. It is quite possible that indiffusible ions exist within this free space and therefore will influence the distribution of diffusible ions according to Donnan principles. When the cell is removed from the body and the energy available to maintain the ordered structure decreases, the disordered phase will grow at the expense of the ordered phase and ions, particularly Na^+ and Cl^-, will enter from without. This free space may not necessarily be coaxial with the ordered phase, but may in parts exist within it.

This theory replaces a mural mechanism for the separation of ions with one dependent on the structural integrity of the whole cell. Cowie and Roberts (4), using an approach similar to our own, have come to the conclusion that the cell membrane plays a very minor role in the maintenance of the ionic pattern in *E. coli*. They state that it must be regarded as a morphological boundary, permitting the free access of all molecules of dimension smaller than that of proteins. The consequences of our theory for the interpretation of the bioelectric potentials will not be dealt with fully in this paper. Briefly, if the Na^+ and Cl^- are in equilibrium across the membrane it would be difficult to see how they could influence the resting or action potential as a first order. This

would be in accordance with the results presented earlier (28). The assumption of an equilibrium between the Na^+ and Cl^- in the free intracellular phase and that in the external solution obviates the necessity to postulate a sodium pump in resting muscle.

Other authors have suggested that there is a separate space or compartment within the cell, in which the ionic content differs from that of the remainder of the cell. Shanes and Berman (23) have postulated such an "X-phase" in the giant axon. The existence of two intracellular compartments in red blood cells has been suggested by Solomon and Gold (30). They leave the nature of the compartment unspecified, but suggest it contains K^+ at a much lower concentration than the major portion of the cell and that the concentration of K^+ in the smaller compartment is about equivalent to that in blood. Carey and Conway (3) from their results with radioactive Na^+ and K^+ suggest that most of the Na^+ of muscle is in a region with a high permeability to both Na^+ and K^+, and with concentrations of Na^+ higher, and K^+ lower than in other parts of the fiber. These authors have calculated that the amount of Na^+ in the special region is 8 m.eq. per kg., which would agree with our calculation of the Na^+ in the F.I.P., in many muscles in the intact animal. As we have said, the F.I.P. increases when the muscle is soaked in ordinary Ringer. However, we think there would be much less than 1.75 m.eq. per kg. K^+ in this space; *i.e.*, $2.5 \times \frac{8}{104} = 0.19$ m.eq. per kg. (see page 248 of original reference (3)). Carey and Conway noticed that the rate of exit of Na^+ from sartorii when immersed in isotonic glucose containing 100 mM KCl or 5 mM K_2SO_4 per liter was the same. They then say that this supports the view that the Na^+ exchanges with the K^+. Our opinion is that it is not an exchange, but a simple diffusion down a concentration gradient, and that the movements of the two ions are in no way linked. If there were a connection, the rates of Na^+ exit would be unlikely to be the same in the two different K^+ solutions.

The inhomogeneity of cells has also been stressed by Bartley *et al.* (1), who state:

"Apart from the outer boundaries (of the cell) there are many intracellular boundaries which behave like semipermeable membranes. This applies in particular to the boundaries of mitochondria. Mitochondria are compartments within a compartment, and it is very probable that mitochondria are further subdivided into smaller compartments, separated by permeability barriers. The nucleus is another subunit within the cell, separated from the rest of the cell by membrane-like barriers. These intracellular permeability barriers may be no less important than those of the outer cell wall." In muscle the highly organized structure of the contractile mechanism must also be considered in this regard. It is tempting to identify the ordered phase containing adsorbed K^+ with the "organoids" of the cell (mitochondria, etc.) and the free intra-

cellular phase with the interstices, but this would no doubt be an oversimplification. For example Itoh and Schwartz (14) have shown that nuclei contain appreciable amounts of Na^+, and it is not at present known whether this ion is in a diffusion equilibrium with the exterior (and therefore logically part of the F.I.P.) or adsorbed onto the structure of the nucleus.

SUMMARY

The $Na,^+$ Cl^-, and K^+ content of toad plasma and the sartorius muscle has been determined. Although the Na^+ and Cl^- level of the muscles in the living animal varied greatly (0 to 38.0 m.eq. per kg., and 0 to 31.8 m.eq. per kg. respectively) the K^+ level was subject to a smaller variation (76.5 to 136 m.eq. per kg.). There was a direct relationship between Na^+ and Cl^-, which was independent of the K^+ level.

There is a closely related gain of Na^+ and Cl^- when muscle is soaked in normal Ringer. These gains are not related to the K^+ loss, frequently found on soaking.

The relationship between the three ions was studied in a large series of 124 muscles in normal Ringer. As found *in vivo*, there was a correlation between Na^+ and $Cl.^-$ This correlation was independent of K^+ content, except when this was abnormally low.

Alteration of the external NaCl level produced concomitant changes in the internal levels of these ions.

Alteration of the external KCl level produced an increase in internal Cl^- similar to that found with high NaCl solutions, but the amount of K^+ entering the cell was approximately one-third of the external increase.

Removal of K^+ from the external solution did not result in a loss of K^+ from the cell, although there was an adequate amount of Cl^- present to accompany it.

The results cannot be reconciled with either a Donnan concept for the accumulation of K^+, or a linked carrier system.

A theory is proposed to account for the ionic differentiation within the cell. The K^+ is assumed to be adsorbed onto an ordered intracellular phase. The normal metabolic functioning of the cell is necessary to maintain the specificity of the adsorption sites. There is another intracellular phase, which lacks the structural specificity for K^+, and which contains Na^+, Cl^-, and K^+ in equilibrium with the external solution. The dimensions of the free intracellular phase will vary from cell to cell, but it will be smaller in the intact animal, and will increase on soaking in normal Ringer, until it is approximately one-third of the total cellular volume. The increase in this phase may be ascribed to a decrease in the energy available to maintain the ordered phase.

We are very grateful to Professor M. Belz and Mr. Rupert Leslie for carrying out the statistical analyses. We wish to acknowledge the help of Miss Alma Werner, M.Sc., with certain theoretical considerations.

REFERENCES

1. Bartley, W., Davies, R. E., and Krebs, H. A., *Proc. Roy. Soc. London, Series B*, 1954, **142,** 189.
2. Boyle, P., and Conway, E. J., *J. Physiol.*, 1941, **100,** 1.
3. Carey, M. J., and Conway, E. J., *J. Physiol.*, 1954, **125,** 232.
4. Cowie, D. B., and Roberts, R. B., Electrolytes in Biological Systems, Baltimore, The Waverly Press, 1955, 1.
5. Dean, R. B., *Biol. Symp.*, 1941, **3,** 331.
6. Eccles, J. C., The Neurophysiological Basis of Mind, Oxford University Press, 1953, 22.
7. Eggleton, M. G., Eggleton, P., and Hamilton, A. M., *J. Physiol.*, 1937, **70,** 167.
8. Fenn, W. O., Cobb, D., and Marsh, B. S., *Am. J. Physiol.*, 1934, **110,** 261.
9. Fenn, W. O., *Physiol. Rev.*, 1936, **16,** 450.
10. Harris, E. J., and Martins-Ferreira, H., *J. Exp. Biol.*, 1955, **32,** 539.
11. Heilbrunn, L. V., and Hamilton, P. G., *Physiol. Zool.*, 1942, **15,** 363.
12. Hodgkin, A. L., and Keynes, R. D., *J. Physiol.*, 1955, **128,** 28.
13. Hodgkin, A. L., and Keynes, R. D., *J. Physiol.*, 1955, **128,** 61.
14. Itoh, S., and Schwartz, I. L., *Nature*, 1956, **178,** 494.
15. Katchalsky, A., *Progr. Biophysic. and Biophys. Chem.*, 1954, **4,** 1.
16. Keynes, R. D., *Proc. Roy. Soc. London, Series B*, 1954, **142,** 359.
17. Van der Kloot, W. G., *Nature*, 1956, **178,** 366.
18. Levi, H., and Ussing, H. H., *Acta Physiol. Scand.*, 1948, **16,** 232.
19. Ling, G. N., The role of phosphate in the maintenance of the resting potential, *in* Phosphorus Metabolism. A Symposium on the Role of Phosphorus in the Metabolism of Plants and Animals, (W. D. McElroy and B. Glass, editors), Baltimore, The Johns Hopkins Press, 1952, **2,** 748.
20. Mitchell, P. J., *J. Gen. Microbiol.*, 1953, **9,** 273.
21. Mond, R., and Netter, H., *Arch. ges. Physiol.*, 1932, **42,** 230.
22. Sanderson, P. H., *Biochem. J.*, 1952, **52,** 502.
23. Shanes, A. M., and Berman, M. D., *J. Gen. Physiol.*, 1955, **39,** 279.
24. Shaw, F. H., Holman, M. E., and Mackenzie, J. D., *Australian J. Exp. Biol.*, 1955, **33,** 497.
25. Shaw, F. H., and Simon, S. E., *Australian J. Exp. Biol.*, 1955, **33,** 153.
26. Shaw, F. H., and Simon, S. E., *Nature*, 1955, **176,** 1031.
27. Shaw, F. H., Simon, S. E., and Johnstone, B. M., *J. Gen. Physiol.*, 1956, **40,** 1.
28. Shaw, F. H., Simon, S. E., Johnstone, B. M., and Holman, M. E., *J. Gen. Physiol.*, 1956, **40,** 263.
29. Simon, S. E., *Australian J. Exp. Biol.*, 1955, **33,** 178.
30. Solomon, A. K., and Gold, L. G., *J. Gen. Physiol.*, 1955, **38,** 371.
31. Somogyi, M., *J. Biol. Chem.*, 1930, **86,** 655.
32. Steinbach, H. B., *Am. J. Physiol.*, 1951, **167,** 284.
33. Ussing, H. H., Ion Transport Across Membranes, New York, Academic Press Inc., 1954, 3.
34. Wilde, W. S., *Am. J. Physiol.*, 1945, **143,** 666.