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DISTRIBUTION AND MOVEMENT OF MUSCLE CHLORIDE

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This study was started with the aim of finding the concentration dependence of the movement of frog muscle chloride, and of discovering whether evidence of an adsorption step as a rate-determining factor might appear. As, however, the work inevitably entailed a large number of analyses, figures became available for the distribution of chloride, potassium and sodium between the tissue and solution under a variety of conditions. This aspect will be mentioned first because it has a bearing on the interpretation of the kinetic results.

Boyle & Conway (1941) showed that in media having raised potassium and high chloride concentrations the internal chloride of the muscle cell, expressed as concentration in the total fibre water, approximates to the amount expected for electro-chemical equilibrium of potassium chloride. The sodium distribution was not brought into account, and even the potassium chloride relation failed when lower and more physiological potassium concentrations were used. Later, Carey & Conway (1954) described the increase of sodium and chloride which took place during storage of isolated muscle; they were led to propose that part of the muscle sodium, which is readily replaced by potassium, is situated in a 'special region'. They did not divide up the chloride. Steinbach (1947) and later Simon, Shaw, Bennett & Muller (1957) proposed that the muscle cell comprises two regions in addition to the extracellular space, with one admitting all ions and the other specific for potassium. The size of the non-specific region was considered by the latter authors to grow at the expense of the potassium region when the cell deteriorated. However, these authors state that 'the results cannot be reconciled with either a Donnan concept for the accumulation of potassium, or a linked carrier system'. A three-compartment hypothesis has been put forward at various times to explain the peculiarities of the kinetics of ion movements (e.g. Harris, 1950; Edwards & Harris, 1957; Harris, 1958) and it is desirable to apply the hypothesis to all three major ions. The hypothesis leads to an explanation for the inequality found between external and internal KCl activities when the latter activity is calculated in the total cellular water; it also provides a region for the accommodation of the cell sodium.

The analytical data can be fitted to an ion distribution such that there is electro-chemical equilibrium of potassium chloride throughout the system and of sodium chloride between the solution and one cellular compartment, with sodium excluded from the other. Although sodium impermeability was once an accepted explanation of the ionic composition of cells, it now meets objection even when applied to a part of the cell, and the idea that potassium selectivity though high is not absolute may be preferred. In this event the sodium would be distributed according to some selectivity ratio between the two cellular compartments, according to the appropriate values of the ratio in the various conditions of the experiments. Failing this knowledge it has appeared best to proceed with the simplest model to show the advantage of apportioning the cell chloride between two regions for interpretation of the kinetic data. Furthermore, the presence within the cell of an unspecific region provides a ready explanation of the increase of 'extracellular' space with the time used for its measurement.

According to the three-compartment model, substances in the unspecific compartment will be free to diffuse into the medium and a clear distinction between this compartment and the true extracellular space may not be possible. Electroneutrality requires that such chloride as is in the potassium-specific compartment can only move either along with potassium or when chloride or other penetrating anions are offered in exchange. Movement with potassium is a non-steady-state condition, whereas if the KCl activity is held constant the chloride can be exchanged for labelled chloride without net movement. It is this latter process which is of major interest in the kinetic study.

METHODS

Analytical procedures. The potassium, sodium and chloride were measured in the combined extracts made by leaving the muscles in (first) 2 ml. of 0·5 % nitric acid overnight and (secondly) washing in 1 ml. of the same mixture. The chloride in the combined extracts was titrated electrometrically against 10 mM-$AgNO_3$ (cf. Adrian, 1960). The titration fluid was then made up to a volume suitable for flame photometry for sodium and potassium; the trace of suspended silver chloride did not interfere. The accuracy of chloride titration was better than 0·1 μequiv; this represents about 2 % of the total chloride when portions of solution were being analysed, and 5–10 % of the chloride in muscle analyses. The accuracy of the flame photometry is judged to be about 2 %.

Materials. The experiments were made on the sartorius and semitendinosus muscles of *Rana temporaria*; the two muscles have similar ion contents. The frogs used have usually been about 1 week in captivity and well nourished specimens were selected. It was difficult to obtain good specimens in periods of hot weather, perhaps because of raised metabolism. Few experiments were made in the months of June and August and none in July. Chloride labelled with ^{36}Cl was obtained from the Radiochemical Centre, Amersham.

The tissue weight was always taken after a light blotting.

Kinetic measurements. The exchange of chloride for labelled chloride was measured by two methods. Muscles which were first loaded with labelled chloride were suspended in

successive portions of inactive washing-out solution for measured times, so that a time curve of the loss could be reconstructed; this procedure is similar to that described previously (Harris, 1958). Alternatively, the muscle was pre-treated in an inactive solution and then immersed for a measured time in the active solution so as to find a single point for 'uptake'.

For the output method it was usually convenient to use a sartorius plus a semitendinosus muscle for each run. The weighed tissue was loaded in a mixture containing potassium and sodium salts (Table 1). All the mixtures had 20 m-equiv bicarbonate/l. and 1 mM calcium acetate. During immersion a mixture of 95 % O_2 + 5 % CO_2 was bubbled through. Measured concentrations of the major ions were used in making the final calculations. The most usual load condition used was an overnight exposure at 0–4° C. If shorter times at 20° C were used the chloride content obtained from a given solution was lower than that obtained by the longer period in the cold; this may be attributable to a biochemical change in the tissue (see Results). At the end of loading the tissue was blotted and then passed through a series of portions of fluid of which all but the first and last had compositions approximating to

TABLE 1. Compositions of solutions

All mixtures had 20 m-equiv bicarbonate and 2 m-equiv Ca/l.

Reference	Cl	K	Na	Methylsulphate anion
	m-equiv/l.			
A	100	100	20	0
B	100	100	115	95
C	100	50	115	45
D	50	100	65	95
E	100	25	115	20
F	50	50	115	95
G	25	100	40	95
H ('Ringer')	93 to 95	3 to 5	115	0
J	10	100	25	95
K (washing soln.)	0	20	95	95
L	0	20	0	0 (6 % sucrose)

that of the load solution. Times of immersion were chosen so far as possible to equalize the quantities of ^{36}Cl collected in each portion; this minimizes error due to carry-over. The first portion of fluid was always 5 ml. of Cl-free mixture (*K*) at 0° C, in which a 1 min wash was given. The final immersion was also for 1 min at 0° in Cl-free solution (*K*) or (*L*). In the first minute, in absence of external chloride, it was considered that most of the extracellular load solution would be removed. This contention is supported by consideration of the quantity of chloride collected (see Results). The final wash in Cl-free solution removes much of the extracellular immersion fluid, so that the analysis is of a tissue having mainly Cl-free washing solution in its extracellular space. Solutions (*K*) and (*L*) furnished similar results after correction for extracellular ions had been made, but (*L*) had the advantage that the relatively large correction for trapped sodium was obviated. Also, the sodium collected in a portion of (*L*) could be measured. The volumes of the portion other than the first used for the output was 1 ml. By limiting the times of soaking the ratio of labelled chloride collected to total chloride in the portion was kept below 1 %, so that no correction was deemed necessary for the back-flow of labelled ion.

The radioactivity in the portions or in a suitable fraction was measured after evaporation of salt solution to dryness in a planchette containing a lens-paper disk to assist even spreading. When mixtures containing sucrose were in use the chloride was precipitated out after addition of sufficient additional chloride as carrier to provide a final weight of silver chloride approximating to that of the salts usually present. It was found that the precipitated silver chloride could readily be transferred to the planchette by using a drop of concentrated ammonia solution as solvent.

At the end of the series of immersions the tissue was extracted with dilute acid, as described above. The only additional step was that of evaporating the combined extract to dryness on a platinum planchette after the chloride titration. A drop of strong ammonia solution was used to transfer the deposited silver chloride from titration vessel to planchette. The chloride activity in the dried extract was measured and finally the residue was extracted with water for analysis of sodium and potassium. Attention was paid to the greater back-scatter from platinum, which resulted in a count rate 1·16 times higher than when nickel planchettes were used.

The uptake of ^{36}Cl by weighed muscles was measured in terminal experiments. The tissues were first treated for some hours or overnight in a solution chemically similar to the ^{36}Cl mixture to be used. After blotting they were kept for measured times in the ^{36}Cl mixture, through which O_2-CO_2 was passed. Finally, the tissue was washed for 1 min at 0° C in Cl-free solution (*K*) or (*L*), and after re-weighing was put in 2 ml. of dilute acid for extraction of the chloride as before. The ^{36}Cl total, chloride, and the extracted cations were measured. It is to be noted that in both procedures for measuring the exchange the muscles had been washed for 1 min in Cl-free solution before analysis and estimation of ^{36}Cl.

Some points common to both procedures need to be mentioned. The radioactivity on the planchettes was measured by means of a scintillation counter with 0·5 mm thick anthracene crystal and a pulse analyser. With the selected pulse-height range a background of 2 c/min was obtained, the ratio: (count rate):(background) was twice that of a thin-window G–M counter, and the interference from the natural radioactivity of ^{40}K was reduced to negligible amount. The use of the pulse spectrum analyser has also the advantage that the measurements are practically insensitive to small changes of sample weight per unit area. The count rate/m-equiv labelled Cl/kg tissue was about 100/min in 'uptake' experiments (for muscle), and double this in 'output' experiments in which two muscles were used together. It may be useful to note that the chloride can be recovered from used solutions by precipitation as silver chloride followed by treatment of the silver salt with hydrogen sulphide to give hydrochloric acid and silver sulphide.

RESULTS

Extracellular space

The experiments made with labelled chloride provided a number of values for the amount of chloride which left the tissue in 1 min in Cl-free solution at 0° C. The net loss of chloride along with cation can take place both from the true extracellular space and from the cells. However, loss from the cells appears to proceed more slowly; so, at least when in the tissue the ratio of external chloride to internal chloride is high, one may obtain some measure of the extracellular space from the chloride collected in the first minute. Muscles loaded in the low-K solution (*H*) had a 1 min chloride space equal to $0·129 \pm 0·02$ ml./g (S.D. 13 results). In another group of nineteen muscles the 1 min chloride space came to $0·118 \pm 0·02$ ml./g, and with the same tissues the 1 min space found from the sodium given up to a Na-free washing solution was $0·117 \pm 0·025$ ml./g. These figures are close to those measured by inulin (Boyle, Conway, Kane & O'Reilly, 1941; Desmedt, 1953) and napthol green in short exposures (Bolingbroke, Harris & Sjodin, 1961). The 1 min chloride space of muscles loaded in media with 25 m-equiv K/l. or more was $0·169 \pm 0·039$ ml./g

(S.D. 31 results) which is significantly (t = 3·5, P 0·001) higher than the previous value. This increased chloride loss in 1 min is probably due to some loss of the cellular chloride rather than to a bigger extracellular space. Results obtained with 3 min washing were still higher; 3 min space = 0·12–0·29, (mean 0·19 ml./g) which is explicable by the still greater contribution from cellular chloride.

It is valuable to obtain a measure of the rapidly equilibrated space in swollen tissue. The 1 min chloride space referred to the *original* weight, in tissue swollen to between 1·5 and 2 times original weight, came to 0·142 ± 0·031 ml./g (S.D. 10 results). From this one may conclude that the absolute space is little changed by swelling, although of course when expressed as a fraction of the swollen weight the figure is lower. The conclusion agrees with that drawn from inulin space measurements made by Adrian (1961) in respect of shrunken tissue; some values reported by Tasker, Simon, Johnstone, Shankley & Shaw (1959) for both swollen and shrunken tissue show the same qualitative behaviour. It is not claimed that the 1 min space is identical with the extracellular space, but it does afford a basis for the deduction from the analytical figures of the quantities of extracellular ions introduced in a 1 min washing.

Chloride and potassium distribution

The analysis obtained after 1 min washing in solution (*K*) or (*L*) were corrected for the extracellular ions assumed to be present in 0·13 ml. extracellular fluid/g original weight, at the concentrations holding in the wash fluid. The amounts of potassium and sodium so corrected and the amount of chloride found by the titration were converted to concentrations $[K]_t$, $[Na]_t$ and $[Cl]_t$/g water in the cell at the time of analysis; values are given in Table 2, cols. 11–13. For the calculation of intracellular water the absolute space was taken, from the last section, to be invariably 0·13 ml./g original weight and the solid matter was taken as 0·19 g/g fresh tissue. The latter value is a mean of twelve determinations made on six sartorius and six semitendinosus muscles.

The ion contents were usually obtained from pooled sets of four analyses. When calculated as concentrations in the total cell water the $[K]_t \times [Cl]_t$ product found did in some conditions approximate to the product: $[K]_e \times [Cl]_e$ (the external concentrations), but in other solutions, particularly those of lower potassium concentration, the value for the muscle ions exceeded by several times the external value (Table 2, col. 14). The single internal compartment hypothesis has the additional failing that it ignores the cell sodium. For these reasons it seemed desirable to re-examine the partition of the three ions between two separate *intra*-cellular compartments and the solution. In one cellular compartment (subscript 1)

TABLE 2. Distribution of ions between solution and two cellular compartments such that NaCl and KCl are in electrochemical equilibrium but Na is excluded from compartment (2). These results were calculated from analytical results according to the equations in the Appendix, using sets of four pooled analyses. The analytical contents, corrected for extracellular ions in 0·13 ml./g tissue, were divided by the final 'cell water' to obtain $[K]_t$, $[Na]_t$ and $[Cl]_t$. The 'final cell water' was taken as equal to the final weight − 0·32 times original weight. Col. 14 shows that the product $[K]_t \times [Cl]_t$ exceeds $[K]_e \times [Cl]_e$ particularly when the external K concentration is low

Solution [K] (m-equiv/l.)	Solution [Na] (m-equiv/l.)	Solution [Cl] (m-equiv/l.)	Ratio: final cell water / original cell water	Compartment (1): w_1 = propn. of final cell water	Compartment (1): $\frac{[Na]_1}{[Na]_e} = \frac{[K]_1}{[K]_e} = \frac{[Cl]_e}{[Cl]_1}$	Compartment (1): Y = surplus charge (m-equiv/kg original total cell water)	Compartment (2): $\frac{[K]_2}{[K]_e} = \frac{[Cl]_e}{[Cl]_2}$	Compartment (2): X = indiffusible charge (m-equiv/kg total original cell water)	$X+Y$	$[K]_t$ (m-equiv/kg)	$[Na]_t$ (m-equiv/kg)	$[Cl]_t$ (m-equiv/kg)	Ratio: $\frac{[K]_t \times [Cl]_t}{[K]_e \times [Cl]_e}$
Equilibration for 1 hr in low-K saline mixture													
3·5	105	90	1·03	0·217	0·83	+ 9·2	44	−123·5	−114·5	121·5	18·9	25·1	9·7
3·0	118	90	0·945	0·145	1·07	− 2·2	50	−120	−122	128·5	18·3	13·8	6·6
3·2	118	98	1·03	0·20	0·99	+ 0·4	44	−115·5	−114	114	23·1	21·5	7·8
Equilibration for 3 hr minimum at 0° C or 10° C													
20*	20	20	0·985	0·315	1·10	− 2·4	10·5	−140	−142	152·5	6·9	7·0	2·7
27	105	95	1·00	0·24	1·05	− 3·2	6·2	−115·5	−118·5	134	26·7	33·6	1·7
28	125	95	1·08	0·22	0·97	+ 2·3	6·4	−137·5	−135	145	26·7	33·4	1·8
50	100	50	1·01	0·16	0·89	+ 5·7	3·4	−131	−125	149	14·4	21·4	1·3
50	118	100	1·11	0·245	0·89	+10·2	4·0	−151	−136	162	25·7	46·3	1·5
107	100	100	0·905	0·21	1·06	− 5·2	2·0	−124·5	−130	197	22·7	58·4	1·1
95	96	92	1·00	0·25	0·94	+ 8·5	2·3	−132	−123·5	184·5	22·7	55·0	1·1
100	50	48	1·00	0·19	0·90	+ 7·3	1·9	−133·5	−126	170·5	8·3	30·9	1·1
100	45	25	0·895	0·28	0·83	+13·5	2·0	−122	−108·5	169	10·6	17·3	1·2
118	17	20	0·94	0·22	0·77	+14·8	1·6	−127	−112	165	2·9	15·5	1·2
Equilibration at 20° C for 3 hr minimum													
26·5	110	104	1·00	0·22	1·00	0	6·7	−126	−126	144	24·1	34·7	1·8
25	110	96	1·00	0·28	1·25	−17·1	8·0	−135	−152	153	38·8	30·3	1·9
50	110	100	1·15	0·24	0·92	+ 6·9	3·1	−110	−103	130·5	24·1	50·0	1·4
50	100	96	0·96	0·28	1·08	−11·7	4·4	−137·5	−149	175	30·4	40·8	1·5
100	20	100	2·22†	0·21	1·25	−26·9	1·3	− 81·5	−108·5	126	5·3	79·0	1·0
100	106	100	0·85	0·27	0·89	+10·9	2·9	−159	−148	236	25·6	55·5	1·3
100	50	50	1·06	0·26	0·83	+15·5	2·1	−148	−132·5	179·5	10·8	33·0	1·2
100	45	25	0·985	0·235	1·02	− 1·6	1·9	−132·5	−134	169	10·8	15·8	1·1

* Tonicity maintained by 6% sucrose. † Grossly swollen.

sodium, potassium and chloride are supposed to be present in electrochemical equilibrium with the exterior, and associated with a proportion w_1 of the intracellular water. Then

$$[Na]_1 \times [Cl]_1 = [Na]_e \times [Cl]_e \text{ and } [K]_1 \times [Cl]_1 = [K]_e \times [Cl]_e.$$

In the second cellular compartment (subscript 2) there is a proportion $1 - w_1$ of the total cellular water and the potassium and chloride are present at equilibrium, so that $[K]_2 \times [Cl]_2 = [K]_e \times [Cl]_e$. As already mentioned, it is simplest though not necessarily correct to regard sodium as excluded from this compartment.

The equations relating w_1 and the respective ion concentrations in the compartments to the observed quantities $[K]_t$, etc., were solved (see Appendix); column 5 in Table 2 lists w_1. The first compartment appears to have a minimum of 15% of the cellular water associated with it; this corresponds to about 0·10 ml. water/g tissue. The figure found is rather variable even when similar conditions have been used. Evidently any extraneous source of sodium, such as connective tissue or damaged cells, will contribute to make w_1 too high. In the subsequent treatment of the kinetic data a fixed value for w_1 (= 0·2) has been used. The Donnan ratio in the first compartment (col. 6) is not far removed from unity, and it may be questioned whether the deviations listed do not in fact arise from errors in analyses and in the proportions of dry matter and extracellular fluid which have been assumed. A set of muscles which had been exposed to K-free solution overnight did require a high value for w_1 (0·43) and with this was associated 38 m-equiv anionic charge/kg total cell water. At the same time the anionic charge associated with compartment (2) had fallen to 93·5 m-equiv/kg total cell water, so the sum of the two (131·5) remained close to the sum found in other muscles (col. 10). This fact would be consistent with the idea that some fibres of the stored muscles had completely lost their selective permeability to potassium. In the normal tissue then, it seems likely that the Donnan ratio in compartment (1) is close to unity. The only way to preserve an electrical balance in the compartment consistent with the Donnan ratio equal or close to unity is to suppose that it admits all the anions, which include bicarbonate and methyl sulphate in these experiments. Conway (1950) has mentioned evidence for muscles containing more bicarbonate than is accommodated in the usual extracellular space and Johnson (1955) has shown that the space accessible to sulphate exceeds 13%. Part of the sulphate was found to leave the tissue more slowly than would be expected of true extracellular material.

In compartment (2) the Donnan ratio (col. 7) is high when the external potassium concentration is low. Measured potential differences (Adrian, 1956; Harris & Martins-Ferreira, 1955) in solutions of the respective

potassium concentrations approach those calculated for the Donnan ratios listed. In compartment (2) there is between 110 and 149 m-equiv anionic material associated with 1 kg total cell water. The total osmolality of the inorganic material is between one half and two thirds of the external value. Conway (1950) has shown that the osmotic contribution made by the organic substances can suffice to provide an osmotic balance.

Although there is considerable evidence, more of which is quoted in the Discussion, that the proportion of the total cell water accessible to sugar and even inulin can in sufficient time exceed 0·13 ml./g tissue, the present inference, that at least 15 % of the intracellular water (equivalent to a further 0·10 ml./g tissue) is available to such anions as methyl and bicarbonate, can profitably be reinforced. The following argument does not take the two-compartment hypothesis as starting point. The total cation (Na + K) content of a muscle, minus its chloride content, must equal the number of equivalents of other anions present. The 'other anions' here include those which have entered from the solution and those provided by the cellular material. All the following results are for tissues washed 1 min in solution *K* and are corrected for the ions present in a 0·13 ml. space/g. The sum Na + K − Cl for tissue equilibrated in Ringer's solution (*H*), having 20 m-equiv HCO_3/l. and just over 90 m-equiv Cl/l., was 79·5 ± 6·0 (S.D. 20 results) m-equiv/kg. After immersion in a mixture having 20 m-equiv each of Cl and HCO_3 with tonicity maintained with sucrose, it was insignificantly different at 83 ± 8 m-equiv/kg (S.D. 11 results). However, after immersion in mixtures *D*, *F* or *G*, having various amounts of chloride, 20 m-equiv HCO_3/l. plus 95 m-equiv methyl sulphate anion/l. the sum became significantly higher at 99·4 ± 6·6 m-equiv/kg (S.D. 116 results). Use of solution *B* led to a slightly higher value, 104·5 ± 7·0 (S.D. 36 results). That is to say, after immersion in mixtures having an additional 95 m-equiv methyl sulphate anion/l. the sum Na + K − Cl is increased by 20–25 m-equiv/kg. This would be a natural consequence if one accepted that at least 0·10 ml. of what is regarded as *intra*cellular water/g tissue admits the methyl sulphate anion at a concentration equal to or slightly more than that pertaining outside. Without this ready explanation it would be necessary either to suppose that the tissue itself provides additional anions or that the extracellular space is both larger and more slowly cleared than is indicated by the results for the 1 min Cl space. Evidently in the last resort it is difficult to distinguish between a true extracellular space and a non-specific intracellular space. If one takes the former as 0·13 ml./g tissue on the basis of short-term exposures to inulin, the latter has to be about as much again. In the next section evidence is given that the movement of chloride from the non-specific compartment (1) has little dependence upon the external solution, and in this respect differs from the chloride in compartment (2).

Kinetics of chloride movement

The existence of a space holding the external ions at concentrations not far removed from those in the external solution has particular importance when chloride and sodium movements are studied, because both ions are usually present at high external concentration. It does not matter particularly whether one chooses to regard compartment (1) as part of the extracellular space or not, it remains true that the chloride in it depends on the external concentration. In this respect compartment (2) differs because the chloride in it depends on $[K]_e \times [Cl]_e$. When chloride movement occurs one can think of three possible processes, namely: (*a*) Cl^--for-Cl^- exchange, measurable only by tracer methods (*b*) interchanges of

chloride with other anions which can enter the particular compartment, and (*c*) movement of the dissociated ions of a salt such as KCl. In the steady state presumably all three occur between compartment (1) and the external solution, whereas only (*a*) and (*c*) can take place between compartment (2) and elsewhere when no other penetrating anions are used, unless appreciable exchange of Cl^- for OH^- can occur. If a tissue is first loaded in a Cl^--containing mixture and then put in a Cl^--free solution, only (*b*) and (*c*) can operate for compartment (1) and only (*c*) for compartment (2).

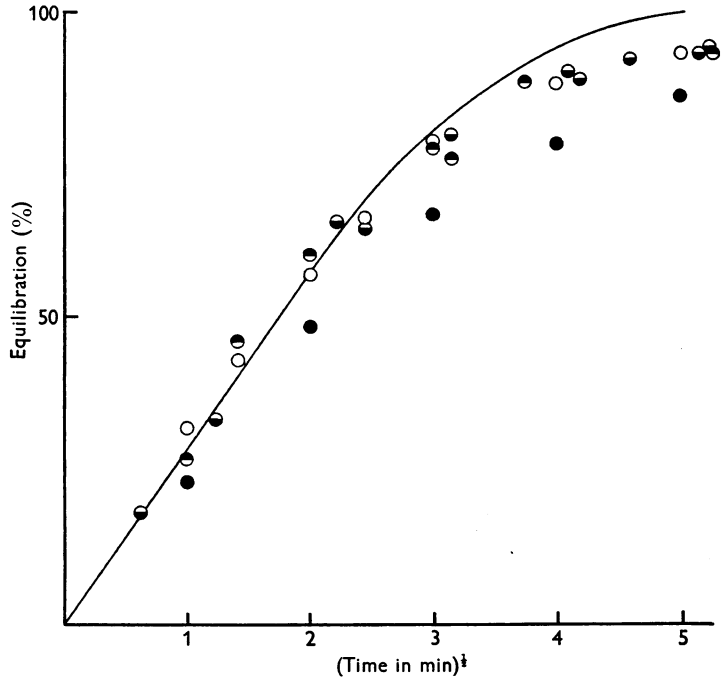

Fig. 1. The diffusion of chloride and of sodium from frog sartorius muscles. ◒ Cl at 20° C, ○ Cl at 10° C, ◓ Cl at 0° C, ● Na at 20° C. The line drawn is a theoretical diffusion curve (from a flat sheet) scaled to pass through the points for chloride movement obtained in the first 9 min of the washing-out process. Note that the muscles had been loaded with chloride in a solution having a low potassium concentration.

The time course of the exchange in the steady state of the chloride of muscles kept in media having low (3–5 m-equiv/l.) potassium concentration is shown in Fig. 1 for three temperatures. There appears to be little effect of temperature, but this may be because the curve obtained depends critically on the amount of slow-moving material present and this is not readily measured. The tissues loaded in the 'Ringer's' solution (*H*) had

between 12 and 18 m-equiv Cl/kg after 1 min washing in Cl^--free solution. According to the partition made in similar experiments (lines 1–3 in Table 2) all but about 2 m-equiv of the chloride will be situated in compartment (1). A residual amount is less mobile than the rest, as is shown by a divergence from the theoretical curve (the line in Fig. 1) for diffusion from a sheet. The apparent diffusivity of chloride from compartment (1) through the thickness of the sheet is about 5×10^{-7} cm^2/sec, but according to the present model this low value (about $\frac{1}{30}$ of that in water) arises because a still lower value holds in part or all of compartment (1). At this point it is relevant to compare the movement of cell sodium, which is supposed to reside in compartment (1), with the chloride which in the present conditions of low external potassium is mainly in the same situation. The curve obtained for sodium movement at 20° C from a muscle loaded in a high-potassium solution (to obviate replacement of cell K by Na) is shown in Fig. 1. For a given fractional movement the sodium requires 1·5–1·7 times as long as chloride. This factor is close to the ratio of the diffusivities in water, to which the observed difference may reasonably be attributed. Sodium, like chloride, does not all move according to the simple diffusion law; a small part is more firmly held than the rest and this may be associated with negative sites.

Returning to the chloride results, it was found that the output from muscles loaded in low-potassium solutions was little affected if the washing-out solution had a raised potassium concentration. With this change in conditions the tissue gained KCl during the loss of the labelled chloride, but the counter stream had no perceptible effect on chloride coming from compartment (1).

A more drastic change of the conditions, namely omission of chloride from the washing-out solution, serves to emphasize the dependence upon external chloride concentration of the movement of a small part of the total chloride present in muscles loaded in low-potassium media. In Fig. 2 the lower sets of points compare on a logarithmic scale the results obtained in chloride (*A*) and Cl-free media (*B*). That it is correct to ascribe the difference to sluggish movement of KCl from compartment (2) is confirmed by examination of the output from muscles which have been loaded in solutions with raised potassium concentration (*C*). According to the hypothesis of a uniform activity of KCl, the high external product will lead to a raised content in the compartment; since it already contains a high potassium concentration equivalent to the indiffusible anions present, the main fractional change is in the chloride content. It can be seen that the consequences of raising the chloride content of the tissue is to reduce the fraction which moves in a given interval of time. Other experiments, not illustrated, provided curves falling between *C* and *B* in Fig. 2,

according to the chloride content. The points *C* in Fig. 2 were obtained by using tissues having initially about 100 m-equiv Cl/kg, and set *B* are for tissues having initial contents of 15–18 m-equiv/kg. Consideration of the absolute amounts of chloride lost to Cl-free solutions in a short interval early in the washing-out shows that similar quantities move from tissues which have been subjected to either high- or low-potassium loading, provided the chloride concentrations in the load solutions were equal. A constant CO_2 tension must also be provided, since net KCl loss is slower in

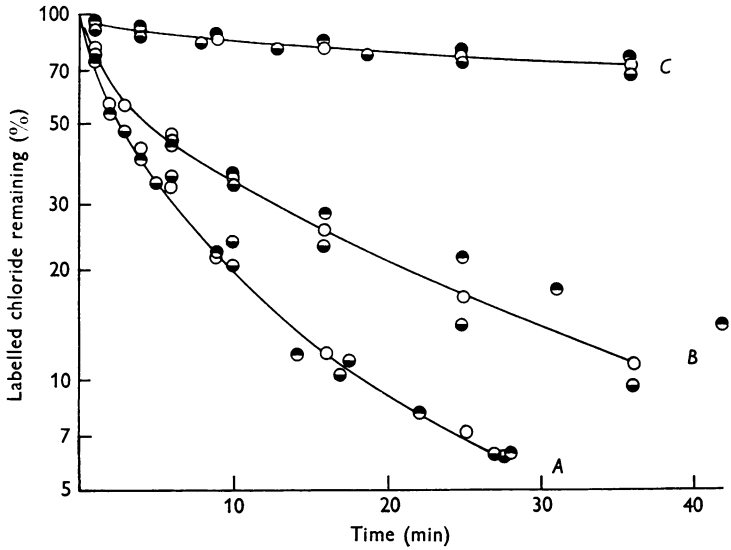

Fig. 2. The effects of removing external chloride and of increasing the chloride content of the tissue upon chloride output. *A*, the points obtained for low potassium loading and output (by exchange) to a Cl-containing solution; the points are the same as those plotted for chloride in Fig. 1. The sets of points in group *B* were obtained from tissues also loaded in low potassium solution but with output (net loss of KCl) taking place to a Cl-free solution at ◒ 20°, ○ 10°, ◓ 0° C. The three sets of points for the three temperatures in group *C* were obtained from tissues loaded in a solution with a high potassium concentration; the output (net loss of KCl) is to a Cl-free solution. These points show that the fractional movement of chloride in a given time is reduced by omission of external chloride and still more by increase of total tissue chloride. The method of plotting is unsuitable for demonstrating the independence of the behaviour of the roughly equal amounts of chloride in compartment (1) upon the conditions.

neutral than in alkaline media (Harris, 1958; Tables 1 and 3). In the first 4 min between 8 and 12 m-equiv Cl/kg tissue is lost after high K loading, and between 7 and 9 m-equiv Cl/kg tissue is lost after low K loading. The appearance of the curve on the logarithmic scale depends upon the quantity of chloride in the tissue, and it is the point of the present argument that it is the amount in compartment (2) which is controlled by the

product $[K]_e \times [Cl]_e$. The curves might be used to infer a reduced 'permeability' to KCl on the part of tissue having a high content of the salt, but this only follows the tacit assumption that the rate of loss ought to be proportional to the content.

Figure 3 shows the kind of result obtained for chloride exchange under nearly steady-state conditions by using a constant $[K] \times [Cl]$ product throughout but with the washing-out solution having either 50 or 100 m-equiv Cl/l. One pair of curves was obtained at 20° C and the other at 0° C. At each temperature, and at 10° C (not shown), the absolute exchange is more in a given time when the medium having the higher chloride concentration is used. The difference is perceptible within the first minute, showing that the exchange contributes appreciably to the total movement;

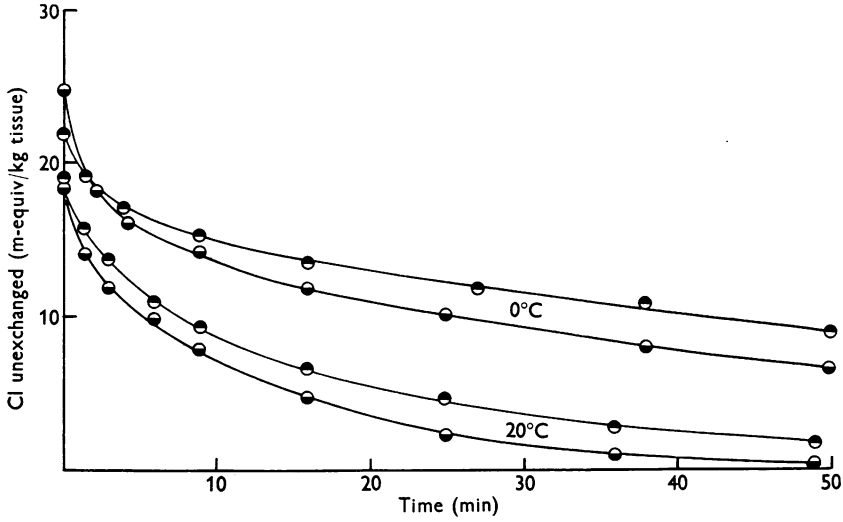

Fig. 3. The influence of external chloride concentration on the steady-state exchange of chloride. The muscles were loaded in mixture *D*. After 1 min washing in Cl-free solution at 0° C the loss of labelled chloride to media *C* ◒ and *D* ◓ (with 100 and 50 m-equiv Cl/l., respectively) was followed. The loading and washing-out media all had an equal $[K] \times [Cl]$ product. Both at 0 and 20° C there is more exchange in a given time in the higher chloride concentration.

this is the more striking in view of the loss in the preliminary washing in Cl-free solution for 1 min of nearly equal amounts of chloride, corresponding in each case to that expected for a space of about 0·15 ml./g.

In order to discover the quantitative dependence of the chloride exchange between compartment (2) and the solution, it is necessary to deduct the amount of chloride in compartment (1) from the total which is observed. The amount in compartment (1) is assumed to follow the curve

shown in Fig. 1; the deviation between the points and the curve in the figure is supposed to be due to a small amount of the ion in compartment (2). The quantity of chloride moving either from or into compartment (1), according to whether the experiment is on tracer output or uptake, has been taken as that which would be accommodated in 0·2 of the total cell water at a Donnan ratio $[Cl]_e:[Cl]_i = 0{\cdot}97$. After 100 m-equiv Cl/l. loading the amount is 14 m-equiv/kg tissue, and this is reduced in proportion to the reduction in external concentration. At each time the total chloride content of the tissue was measured the amount of unexchanged chloride in compartment (1) was calculated and deducted from the total unexchanged chloride. The differences, being the amounts unexchanged in compartment (2), were then related to time and external chloride concentration.

It appeared that at each temperature, up to about 60 % of the possible exchange was approached along a course determined by the product of time and external chloride concentration. Rather than plot the amount exchanged against the product it was preferred to use $(\text{time} \times [Cl]_e)^{\frac{1}{2}}$ as abscissa, since this made a portion of each line straight. Evidently the fact that the steady chloride content of the tissue depends upon the composition of the solution must be reflected in the ultimate content of exchanged chloride. The curves for each experiment diverge from what appears to be a common portion of the curve when more than 60 % of the chloride of the particular muscle has been exchanged; this has been indicated in Fig. 4. Use of progressively higher $[K]_e \times [Cl]_e$ products, particularly when $[Na]_e$ is low, leads to increasing chloride in compartment (2), so that more chloride moves in accordance with the common curve. At 0 and 10° C the pattern of exchange is similar (Figs. 5 and 6). In the latter figures the results plotted have been limited to those obtained for degrees of exchange less than 60 % of the possible for each experiment, so that the points are scattered about a common curve.

Figures 4–6 show compactly the information obtained in many experiments on the exchange of chloride in compartment (2) in various media. Points on Figs. 5 and 6 were obtained by both uptake and output methods. Figure 4 shows how the exchange tends to completion at different final levels, depending on both potassium and sodium concentrations. With lower sodium-salt concentration (mixture *A*, top curve) the tissue is swollen to 1·75 times its fresh weight, but the amounts of chloride exchanged, expressed per gram fresh weight, follow the common curve and appear to extend it. Raising the temperature from 0 to 10° C reduces the value of the product $([Cl]_e \times \text{time})$ required for a given exchange by a factor of 1·6 times; a further increase to 20° C reduces the product by a factor of 2·1 times.

The implication of the dependence of exchange upon the product of concentration and time has been discussed previously (Harris & Sjodin, 1961), but a more rigorous treatment of the problem of penetration through or into an exchanger can be given. Suppose that the material has N exchange sites per unit volume and that $N\delta l$ of these are exposed per unit area. The rate of replacement of adsorbed ions by labelled ions (denoted Cl^x) at the boundary is equal to the diffusion flux of labelled ions, so

$$D\,\partial Cl_s^x/\partial x = k[Cl]_e^x\,\delta l(N - Cl_s^x),$$

where D is the diffusivity within the exchanger, k is the specific self-exchange coefficient for chloride, and $[Cl]_s^x.\,\delta l$ is the amount of exchanged chloride per unit area in the exposed depth δl. The diffusion of labelled ions into the material then resembles the heat transfer case having a radiation boundary. The equilibrium level (here full exchange) is set by N, while the rate of attainment of equilibrium depends on $k[Cl]_e$. For a cylinder of radius r and values of $rk[Cl]_e\,\delta l r/D$ less than 0·3 the effect of internal diffusion is small (the error being less than 10 %) and the amount diffused depends on the rate of surface exchange and hence on ($[Cl]_e$ × time). When this condition holds the question of whether all, or only a part, of the internal chloride is adsorbed remains open for the kinetics is unaffected.

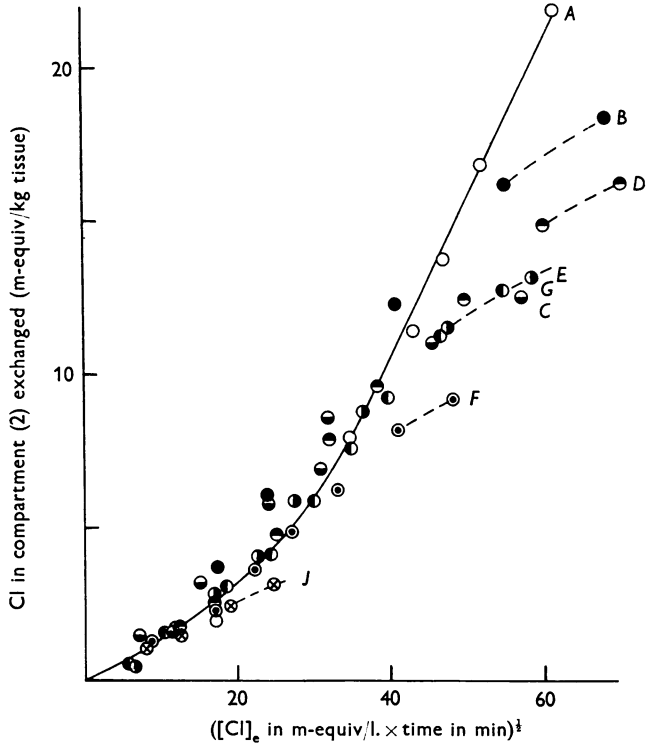

Fig. 4. The exchange at 20° C of the chloride in compartment (2) plotted against ([Cl] × time)½:$[Cl]_e$ in m-equiv/l., time in minutes. Symbols for the different solutions (see Table 1 in Methods) ○ *A*; ● *B*; ◒ *C*; ◓ *D*; ◑ *E*, ⊙ *F*; ◐ *G*; ⊗ *J*. All points shown were obtained from 'output' experiments, those for solution *C* are derived from the observations of total movement plotted as control in Fig. 7. The later parts of the curves for the separate experiments are indicated to show how they branch away from the common curve as exchange approaches completion in the respective mixtures.

Exchange inhibitors

In previous papers a slowing of the output of chloride to Cl-containing media was shown to be caused by certain other anions such as nitrate and perchlorate (Harris, 1958; Adrian, 1961). The most marked of the effects described in 1958 were obtained by using muscles loaded in mixtures having high concentrations of potassium chloride and little sodium salt (like mixture *A*). When a load solution low in potassium is used the effect

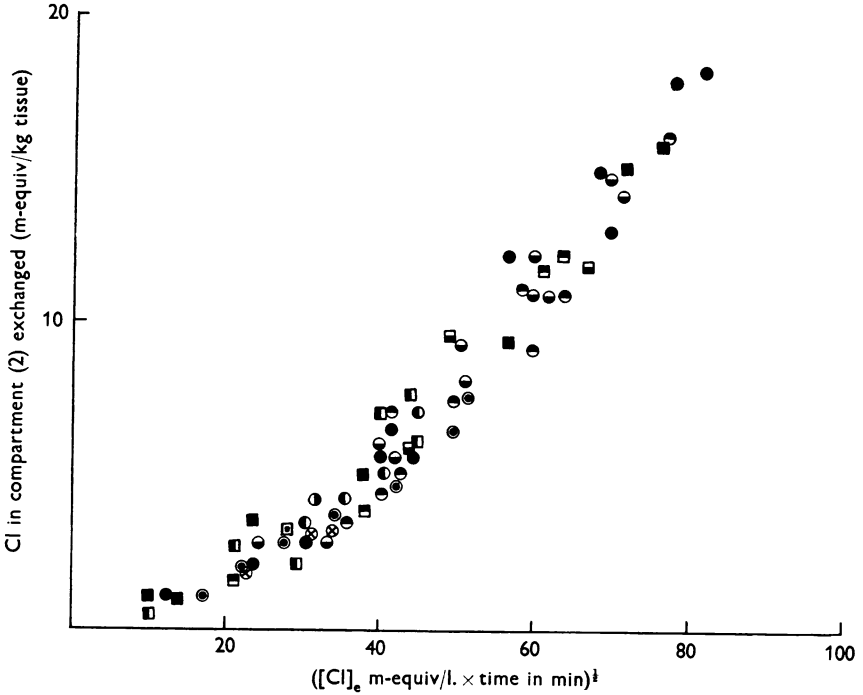

Fig. 5. The exchange at 10° C of the chloride in compartment (2) plotted against $([Cl]_e \times \text{time})^{\frac{1}{2}}$: $[Cl]_e$ in m-equiv/l., time in minutes. Symbols for 'output' experiments as in Fig. 4; uptake results are shown as □, etc. The points plotted are limited to exchanges of less than 60 % of the possible.

of these foreign anions on the movement of chloride, plotted logarithmically, is less obvious, although it appears that a small proportion of the total chloride is held up (Fig. 3 in Harris, 1958; Fig. 1 in Adrian, 1961). According to the two-internal-compartment hypothesis the use of low-potassium load will introduce little chloride into compartment (2). Inhibition of exchange, like its prevention by the use of Cl-free solution, will then affect only a small proportion of the total chloride, because movement from compartment (1) remains unimpeded. On the other hand,

tissues loaded in high-potassium load mixtures have raised chloride in compartment (2), and the output curves obtained show a large effect when inhibitor is present (Figs. 1 and 2, Harris, 1958). When the absolute movement is plotted (Fig. 7, this paper), the difference caused by the presence of inhibitor is seen to be on the less mobile part of the chloride, and there is no perceptible influence on the movement in the first few minutes.

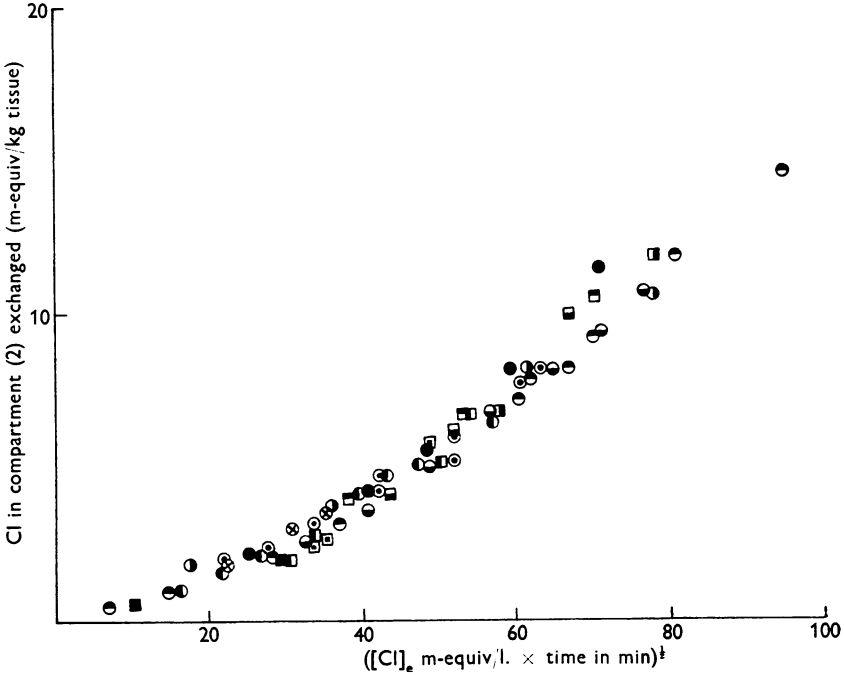

Fig. 6. The exchange at 0° C of the chloride in compartment (2) plotted against $([Cl]_e \times \text{time})^{\frac{1}{2}}$; $[Cl]_e$ in m-equiv/l. time in minutes. Symbols as Figs. 4 and 5.

Mutual interference

It has been remarked already that the output of labelled chloride from muscles loaded in media having low potassium concentrations is not noticeably affected if the tissue is at the same time gaining chloride along with potassium. Output from muscles loaded in media having raised potassium concentration is, however, affected by counter flow, so it again appears that interference occurs at the entrance to compartment (2); only chloride in compartment (2) is held up by either inhibitors or by a stream of chloride ions moving in the opposite direction. The result is to be expected if the ions pass through a system of adsorption sites where there is competition between labelled ions and others (either unlabelled chloride or adsorbable foreign ions). On Fig. 7 the points ⊗ show that the loss of

labelled chloride to a mixture (*A*, Table 1) having a higher [K] × [Cl] product and lower Na concentration than the load solution is slower than the exchange (points ●) in a mixture having an equal [K] × [Cl] product and equal Na concentration, although the washing-out solutions had equal Cl concentrations. Exactly similar results were obtained at 0 and 10° C; in each of the three pairs of experiments the fractional rate of output at 40 min after the commencement was reduced to about half by the counter flow.

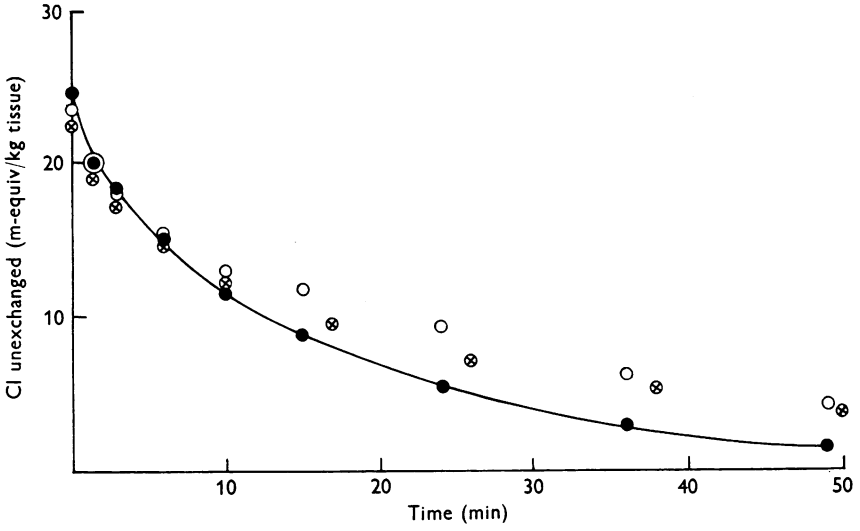

Fig. 7. The effects of an inhibitor (perchlorate) and excess counter-flow on chloride movement at 20° C. The muscles were loaded in labelled mixture *D* (Cl = 50, K = 100, Na = 65 m-equiv/l.). The control experiment (●) was run in washing-out solution *C* (Cl = 100, K = 50, Na = 115 m-equiv/l.). To inhibit chloride movement 10 m-equiv perchlorate was substituted for equivalent methyl sulphate in mixture *C*, leaving the chloride concentration unchanged (points ○). To cause excess counter-flow during the washing-out of labelled chloride the third run (points ⊗), was made in a solution having 100 mM-KCl + 20 mM each of $NaHCO_3$ and $NaCH_3SO_4$. Since the product [K] × [Cl] in this last mixture exceeds that in the load solution the muscle gains KCl during some of the time that labelled chloride is exchanging for unlabelled chloride.

DISCUSSION

The concept of the muscle cell as a three-compartment system (one being the extracellular space) has been put forward at intervals for various reasons. These include the peculiarities of ion exchange kinetics (Carey & Conway, 1954; Edwards & Harris, 1957), the ion contents found in various media (Steinbach, 1947; Shaw & Simon, 1955), dependence of duration of the after-potential on chloride exchangeability (Harris, 1958), and finally

many observations that the space accessible to large molecules (e.g. sucrose or inulin) increases slowly with time of exposure. In the last connexion Danielli & Davson (1941) found that the maltose or galactose space increased from 0·10 to 0·26 ml./g in perfused muscle. Cotlove (1954) infused sucrose or inulin into the blood stream and after 1 hr exposure found a space equal to about 80 % of the space needed to accommodate the tissue chloride at its external concentration, but after some hours the sucrose or inulin space became equal to the chloride space. This observation is particularly relevant to the present proposal that there is a non-selective region which (in media with low potassium concentrations such as plasma) holds nearly all the cell's 'internal' chloride. The fact that muscles remain at their original weight if much of the external salt is replaced by its osmotic equivalent of sucrose implies on the three-compartment model that sucrose can replace the salts in the non-specific region. One would then expect a sucrose space having an initial value corresponding to the true extracellular space (say 0·13 ml./g) and a final value about twice as large (since 15 % at least of the intracellular water is in the non-specific compartment and this is equivalent to 0·10 ml./g tissue). Other observations that both sucrose and inulin spaces increase with time have been made for toad muscle by Tasker *et al.* (1959), who state that after 2 hr inulin entered 0·126 ml./g and after 18 hr 0·193 ml./g; and by Norman, Menozzi, Reid, Lester & Hechter (1959) for rat diaphragm, in which the sucrose space increased from 0·05 ml./g at 7½ min exposure to 0·30 ml./g at 1½ or 2 hr exposure. Although these and other results, for example, those of Goodford & Lüllman (1962) who used ethane sulphonate, agree that the space depends on the time of soaking, it is of interest to know how well-differentiated the true extracellular space is from the non-specific internal space now being discussed. Two kinds of result are available to answer this. In the first place if the internal region is reached through a membrane or is a continuous gel structure it might fill more readily with smaller than with larger molecules. Tasker *et al.* (1959) (Table 4) found that sucrose penetrated a matter of 0·05 ml./g more muscle water in 3 hr than did inulin or albumin; Bozler (1961, Table 1) found an average difference in favour of sucrose in 2 hr amounting to 0·06 ml./g in frog muscle; and Norman *et al.* (1959) found differences in the same sense of 0·07–0·10 ml./g in rat diaphragm at 15 min and 2 hr, respectively. In the second place, kinetic curves showing the efflux of albumin (Tasker *et al.* 1959), sucrose (Johnson, 1955; Bozler, 1961) and sulphate (Johnson, 1955) all show composite output curves when plotted logarithmically, and there is both more slowly lost material and its rate of loss decreases as the time of loading is increased (Bozler, 1961, Fig. 1). One may conclude, then, that there is a difference in rate of penetration of the two spaces by particles of different

sizes, although even a molecule as large as egg albumin does slowly enter the unspecific space. The distribution of chloride between two cellular compartments allows the Donnan relations to be preserved over the whole range of external potassium concentrations, whereas with a single compartment the relation is only approximate at higher potassium concentrations. Evidently the use of high external potassium will make the potassium in the postulated compartment (1) approach the concentration level in compartment (2), so that the two hypotheses will tend to give similar results under these conditions.

It would seem to be a logical step to accept the presence of a non-specific region which can be entered by most molecules and ions, given enough time. The total water available to what are usually regarded as non-penetrants then becomes at least 0·23 ml./g, and according to the condition of the tissue it may be more, owing to loss of specificity. Assuming an intracellular non-specific region it becomes possible to distribute cell chloride and sodium in equilibrium concentrations, so that no continuous expenditure of energy is demanded for maintenance; metabolic energy would only be required to alter the relative proportions of specific and non-specific regions and to restore the *status quo* after any deterioration such as might be associated with activity. According to this model the movement of ions in the steady state, as measured by isotopes, is an ion exchange and need have no relation to the cell's economy, a point originally made by Ussing (1949). Metabolic inhibitors slow down output of labelled sodium from Na-loaded (24 hr) muscles to a medium from which they normally gain potassium (Frazier & Keynes, 1955) in exchange for part of their sodium. An effect on sodium movement is less obvious if short-loaded muscles are used (Keynes & Maisel, 1954); this is because, one may suppose, that the smaller amount of sodium in these muscles is in a relatively steady state and there is little Na for K interchange to be poisoned. It has been established by Carey, Conway & Kernan (1959) that the net loss of sodium (in exchange for potassium) is strongly inhibited by a number of metabolic poisons. According to the present model it is the rebuilding of the K-specific region at the expense of the non-specific region which is inhibited while Na for Na exchange in the latter remains unaffected.

The idea that the exchange rate is limited by an ion exchange step is the same as that suggested to explain the observed dependence of potassium exchange on the applied concentration (Harris & Sjodin, 1961). Alternative mechanisms have to be able to explain the dependence of exchange on the external concentration of the ion in question, and its sensitivity to ionic inhibitors of the same sign and to counter-flow. It should be noted that in media having, say, 100 m-equiv K/l. the state of polarization of muscle is not greatly dependent upon presence or absence of chloride

(Harris & Martins-Ferreira, 1955), but the chloride exchange does vary with external chloride concentration.

Electron-microscopic studies of muscle reveal its composite internal structure (e.g. Porter & Palade, 1957) and it has been proposed that the endoplasmic reticulum is in relatively free communication with the external medium (Hodgkin & Horowicz, 1960). According to the figures in Table 2 a minimum of 15 % of the intracellular water is associated with the unspecific region. Although this is more than the appearance of the reticulum in unswollen tissue leads one to expect, it remains possible that the reticular protein is more strongly hydrated than is the contractile protein.

The kinetic behaviour of the chloride in compartment (2) presents strong analogies with that of potassium (Harris & Sjodin, 1961), and one is forced to suppose that both anion and cation exchangers are present. The exchanger which controls potassium entry appears to accept only potassium-like ions (Rb, Cs), since K movement is insensitive to Na concentration (Harris & Sjodin, 1961); however, part at least of the cellular sodium is associated with or passes through an ion exchanger to enforce a one-for-one exchange (Keynes & Swan, 1959) and the competition seen between sodium and calcium (Cosmos & Harris, 1961) points to the latter ions sharing a set of adsorption sites.

The relative ease of replacement of an ion by a chemically identical or similar ion should bear some relation to the contribution to the electrical conductivity made by the respective species. It has already been remarked (Bolingbroke *et al.* 1961) that rubidium and caesium replace muscle potassium less easily than potassium itself, and that use of these ions leads to a decrease of conductivity between the cell interior and the solution. Chloride ions when present provide carriers of electric charge into the cell interior and it is known that the conductance is higher in their presence than in Cl-free solution (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960). The self-exchangeability of chloride appears to be only about 0·2 times the potassium value as derived by taking the ratio of the squares of respective slopes of the uptake—(concn. × time)$^{\frac{1}{2}}$ curves at 20° C as a measure of the exchange coefficients. On this account, then, chloride would contribute only an additional 20 % to the potassium conductance. However, another factor has to be considered; in a system having fixed charges the conductivity can be increased when co-ions are added on account of the chemically equivalent number of counter ions which can then enter the system. Where potassium passes through a system of anionic sites the addition of mobile chloride ions adds not only chloride but also additional potassium ions to carry current. For this reason the additional conductance seen when chloride is added will be the sum of the true

chloride conductance and the conductance of the extra potassium chemically equivalent to the chloride, giving a sum of 1·2 times the potassium conductance. The respective self-exchange coefficients do not measure the mobilities of the ions under non-steady conditions, which may not require movement through the adsorbed condition.

SUMMARY

1. Frog skeletal muscle has been analysed for potassium, sodium and chloride after exposure to various media. The three ions can be apportioned between two cellular regions in each of which the Donnan relations hold, provided sodium is excluded from one of them.

2. According to the model, the region (1), which admits sodium also admits 'non-penetrating' ions such as bicarbonate and methyl sulphate. It has associated with it 15–35 % of the intracellular water, that is, 0·10–0·23 ml./g tissue, and is additional to 0·13 ml. water/g tissue which equilibrates with chloride in 1 min.

3. The region (2) which excludes sodium contains potassium and chloride and usually about 120 m-equiv indiffusible anionic charge/kg total intracellular water.

4. The exchange and net movement of the muscle chloride has been measured in various media at 0, 10 and 20° C, and with different conditions of loading.

5. From muscles loaded in media with low potassium concentrations most of the chloride is lost to a washing solution with a time course independent of the external chloride concentration and foreign anions or counter-flow of chloride. This insensitive fraction is supposed to be held in region (1).

6. In muscles loaded in media having high potassium concentrations the less mobile part of the chloride, presumed to be in region (2), undergoes exchange with other Cl ions with a time course dependent upon the external chloride concentration. This part of the chloride is impeded by a competing anion (perchlorate) and by counter-flow of chloride ions.

7. The existence of a non-specific cellular compartment is discussed in relation to the increase with time of exposure of the proportion of cell water penetrated by inulin and sucrose.

My thanks are due to Mrs V. Bolingbroke and Mrs J. Bennette for their careful assistance with some of these experiments.

APPENDIX

Given two regions in which equilibria for sodium and potassium chlorides pertain,

$$[Na]_e \times [Cl]_e = [Na]_1 \times [Cl]_1;$$

$$[K]_e \times [Cl]_e = [K]_1 \times [Cl]_1 = [K]_2 \times [Cl]_2 \quad \text{and} \quad [Na]_2 = 0;$$

and with w_1 parts of the total cell water in region (1) and $(1-w_1)$ parts of water in region (2), the problem is to evaluate $w_1, [Na]_1$, etc., from the known contents of total K, Na and Cl/g cell water, denoted $[K]_t$, $[Na]_t$ and $[Cl]_t$. The conservation conditions lead to the quadratic for w_1

$$w_1^2\left(\frac{[K]_t}{[Na]_t}[Na]_e \times [Cl]_e\right) - w_1(2[K]_e \times [Cl]_e) + [K]_e \times [Cl]_e + \frac{[K]_e}{[Na]_e}[Na]_t \times [Cl]_t - [K]_t \times [Cl]_t = 0.$$

From w_1 one obtains $[Na]_1 = [Na]_t/w_1$ and thence $[Cl]_1$ and $[K]_1$. The concentrations of K and Cl in (2) are found from:

$$[K]_2 = \frac{[K]_t - [K]_1 \times w_1}{1-w_1},$$

$$[Cl]_2 = \frac{[Cl]_t - [Cl]_1 \times w_1}{1-w_1}.$$

In evaluating $[K]_t$, etc. it is important to use the cell water content deduced from the final weight. The absolute extracellular space was taken as constant at 0·13 times original tissue weight (the density being taken as unity) and solid matter content was taken equal to 0·19 times original weight.

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