Muscle: A Three Phase System

The partition of divalent ions across the membrane

R. FRATER, SHIRLEY E. SIMON, and F. H. SHAW

ABSTRACT The partition of sulfate, Ca++, and Mg++ across the membrane of the sartorius muscle has been studied, and the effect of various concentrations of these ions in the Ringer solution on the cellular level of Na+, K+, and Cl− has been determined.

The level of the three divalent ions in toad plasma and muscle in vivo has been assayed. Muscle was found to contain an almost undetectable amount of inorganic sulfate.

Increases in the external level of these ions brought about increases in intracellular content, calculated from the found extracellular space as determined with radioiodinated serum albumin or inulin. Less of the cell water is available to sulfate than to Cl−, and the Mg++ space is less than the Na+ space. An amount of muscle water similar to that found for Li+ and I− appears to be available to these divalent ions. Sulfate efflux from the cell was extremely rapid, and it was not found possible to differentiate kinetically between intracellular and extracellular material.

These results are consistent with the theory of a three phase system, assuming the muscle to consist of an extracellular phase and two intracellular phases. Mg++ and Ca++ are adsorbed onto the ordered phase, and increments in cellular content found on raising the external level are assumed to occur in the free intracellular phase.

This paper is devoted to a study of the partition of sulfate, Ca++, and Mg++ between the extracellular and intracellular phases. There would appear to exist in the literature a certain confusion as to the ability of these ions to...
penetrate the cell membrane. For example the sulfate ion is known to penetrate the red cell membrane (for bibliography see reference 16), and E. coli has been shown (10) to be freely permeable to it. This is in contrast to the generally held opinion that it cannot penetrate the muscle cell (18, 20). Similarly with Mg ++ one finds reports varying from complete permeation (8) to almost complete exclusion from the muscle cell (3, 13). It has generally been conceded that Ca ++ and Mg ++ are bound in the muscle (2, 8, 9) although Gilbert and Fenn (15) have invoked a transport mechanism to carry the ions out of the cell. Ussing (30), and Keynes and Lewis (21) have also suggested a transport mechanism for the nerve cell.

We have found it possible to reconcile these apparently conflicting views in the light of the three phase system. It is assumed that the normal Ca ++ and Mg ++ content of the cell is adsorbed onto the ordered phase, and that increases in the external level of these ions will result in increases in their level largely or exclusively in the free intracellular phase. This may be compared with the results obtained for phosphate distribution (4). Normal muscle contains very little inorganic sulfate, and consequently a high external sulfate level should lead to a distribution in the free intracellular phase similar to that found for Cl− (28). The experiments set out below have been found to confirm these predictions.

Methods

The sartorius muscle of the toad Bufo marinus was used throughout this study. The treatment of muscles, and the general form of the experiments were similar to that reported previously (27, 28). Eight to twelve pairs of muscles were used in each experiment, and as routine one of each pair was soaked in normal Ringer and the companion muscle in the test solution. The time of immersion was usually 4 hours.

Blood was drawn by cardiac puncture into a heparinized syringe, and analyses were performed on the plasma obtained after centrifugation.

Sulfate in plasma was determined by the method of Kleeman et al. (22). A modification of this method was also used to estimate sulfate in the muscle. Initially we attempted to analyze trichloracetic acid extracts of the muscle tissue, but found that these extracts contained an interfering substance (possibly glycogen) which produced high and erratic results. Sulfate recoveries by this method were also unsatisfactory. These difficulties were overcome by the following procedure. After soaking in the sulfate-containing Ringer the muscles were blotted, weighed, and transferred to a known volume of normal Ringer, and were left in the ice box overnight. A sample of this Ringer was withdrawn in the morning, any protein was precipitated with trichloracetic acid, and an aliquot of the supernatant taken for the sulfate analysis.

Total divalent cation was determined by the versene titration method of Friedman and Rubin (14). Interference from iron was overcome by chelating this metal with cyanide (6). Mg ++ was assayed by the Titan yellow method of Orange and Rhein (23), and Ca ++ levels were obtained by subtracting the Mg ++ content from the total
divalent cation figure. All methods were checked by the recovery of added material. Na\(^+\) and K\(^+\) were determined with the Beckman flame spectrophotometer, and Cl\(^-\) was determined potentiometrically (28). The methods were adapted so that in each experiment all the relevant ions could be determined on the one muscle. In the sulfate experiments this was achieved by cutting the muscle in two at the end of the experiment, and taking one-half for the sulfate analysis. The other half was cut up and extracted with \(\pi/50\) nitric acid and analyzed for Na\(^+\), K\(^+\), and Cl\(^-\) in the usual way. It was shown previously (4) that there is no difference in the ionic content of the two halves of a muscle.

In the Ca\(^{++}\) and Mg\(^{++}\) experiments all the ions were assayed on the nitric acid extract used for the monovalent ion determination. As it has been reported (2) that Ca\(^{++}\) is adsorbed on the residue left after ultrafiltration of muscle this method was checked for complete extraction by comparing the results obtained by ashing the muscles in porcelain crucibles with this extraction procedure.

Unless otherwise stated all intracellular ionic contents were calculated from the extracellular space found in each individual muscle. This space was estimated either with radioiodinated serum albumin (RISA), or with inulin. It has been shown previously (29) that these two methods yield comparable results.

**Solutions** Ringer solutions used were modifications of those described previously (26), and will be described in the text.

\(\text{Na}^{+}\text{SO}_4\) was obtained as the carrier-free salt from A.E.R.E., Harwell. Radiations were counted with a conventional end window G. M. tube, and a Philips scaler. Efflux was carried out by serially transferring the \(^{35}\text{SO}_4\)-containing muscle through inactive Ringer. Samples of the Ringer were then dried on planchette under an infrared lamp. Standards were made up in Ringer under the same conditions as the efflux samples, so as to compensate for self-absorption in the source. In order to be able to follow the efflux for 6 hours, levels of approximately 50 microcuries per ml. were used in the influx solution. This meant that at early times the efflux samples had high levels of radioactivity. It was found possible to avoid errors due to the dead time of the tube by counting these samples at a lower level in the G. M. tube castle than was convenient for later samples. The relation between the two levels was accurately determined using a series of standards.

**RESULTS**

The \(\text{SO}_4^{2-}\), Mg\(^{++}\), and Ca\(^{++}\) Content of Toad Plasma and Muscle

These results may be obtained from the Documentation Service, Library of Congress.

The Effect of High Sulfate Ringer on Ionic Content

A Ringer solution was made up to contain 200 m.eq. per liter Na\(^+\), 80 m.eq. per liter Cl\(^-\), and 55 mM per liter sulfate. This was a hyperosmotic solution,
but the relatively large amount of NaCl was necessary to enable the accurate estimation of Cl⁻. The estimation of this ion, and consequently of the ratio Cl⁻_{out}/Cl⁻_{in}, was carried out in order to compare it with the ratio of external to internal sulfate in the same muscle. Groups of eight to twelve pairs of muscles were used in each experiment, with one muscle of each pair being held in normal Ringer for the stated time, and the companion being held in the high sulfate solution. The muscles were soaked in these solutions for 4 and 16 hours, and the results of two experiments are set out in Table I. The figures shown in this table are intracellular levels calculated with the use of a group mean for the extracellular space of 15 per cent. Since the muscles used were all over 300 mg. in weight this is probably a reasonable figure (29). The absolute magnitude of ionic levels in this table may be held in doubt, but it is valid to use them for comparative purposes in conjunction with the sulfate analyses obtained in muscles with a known extracellular space. How-

### Table I

<table>
<thead>
<tr>
<th>Time in Ringer</th>
<th>4 hrs.</th>
<th>Ratio</th>
<th>16 hrs.</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na in Ringer, m. eq. per liter</td>
<td>200</td>
<td></td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>SO₄ in Ringer, mM per liter</td>
<td>55</td>
<td></td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Cl in Ringer, m. eq. per liter</td>
<td>80</td>
<td></td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Volume change, per cent</td>
<td>-10.5</td>
<td></td>
<td>-6.8</td>
<td></td>
</tr>
<tr>
<td>Na, m. eq. per kg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>43.2</td>
<td>3.0</td>
<td>37.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Treated</td>
<td>70.1*</td>
<td>2.9</td>
<td>76.2*</td>
<td>2.6*</td>
</tr>
<tr>
<td>Cl, m. eq. per kg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>32.8</td>
<td>3.3</td>
<td>32.5</td>
<td>3.3</td>
</tr>
<tr>
<td>Treated</td>
<td>26.0*</td>
<td>3.1</td>
<td>32.0</td>
<td>2.5*</td>
</tr>
<tr>
<td>SO₄, mM per kg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Treated</td>
<td>8.8</td>
<td>6.3</td>
<td>13.2</td>
<td>4.2</td>
</tr>
<tr>
<td>K, m. eq. per kg.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>74.0</td>
<td></td>
<td>66.2</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>79.8*</td>
<td></td>
<td>64.2</td>
<td></td>
</tr>
</tbody>
</table>

In this table, ionic contents are calculated as intracellular levels, assuming 15 per cent extracellular space. Values given are the mean of twelve observations and are significantly different from controls in normal Ringer only if marked with an asterisk, $P \geq 0.05$. "Ratio" refers to extracellular concentration/intracellular concentration.
ever, the absolute level of intracellular $SO_4^{2-}$ was also determined in seven muscles in which the extracellular space was estimated with RISA.

1. The amount of muscle water available to Na$^+$ at the shorter soaking time was not altered by sulfate. The rise in internal Na$^+$ was proportional to the external increase and consequently the ratio $Na^{+\,\text{out}}/Na^{+\,\text{in}}$ was unchanged. At the longer soaking time there was a disproportionate increase of Na$^+$ into the cell compared with the controls in normal Ringer.

2. Similar results were obtained for the partition of Cl$^-$ across the membrane. The space available to Cl$^-$ was unchanged at the shorter time of soaking but was increased at the longer time. Since the Cl$^-$ and Na$^+$ ratios were altered to the same extent it seems likely that this is a toxic effect of the foreign anion.

3. There was a slight increase in the K$^+$ level of the muscle at the short soaking time, and no change at the longer period. However, the muscles shrank somewhat in the hyperosmotic solution, and when the K$^+$ levels were calculated on the initial muscle weights it was found that there was no change in level at 4 hours, and a slight loss at 16 hours. This finding is in line with the increase in the Na$^+$ and Cl$^-$ space noted above.

4. The space available to sulfate was compared with the Cl$^-$ space in the same muscle, and it was found that the Cl$^-$ space significantly exceeded the sulfate space. Both Cl$^-$ and sulfate spaces increased with increased time of soaking.

The absolute magnitude of the sulfate space was determined in a second set of muscles in which the extracellular space was determined with radioiodinated serum albumin. The normal ionic constituents were not determined in these muscles since we wished to avoid any possibility of variation in the extracellular space due to cutting the muscle in two. The mean ratio found for seven muscles after 4 hours' soaking was $sulfate_{\text{ext}}/sulfate_{\text{in}} = 7.25$. The range was from 4.7 to 12.0. It will be recalled that this mean ratio, and range of observations are the same as those found for Li$^+$ and I$^-$ (27). This figure is also close to that shown in Table I (ratio = 6.3) and may serve as a justification for the mean extracellular space that was used in calculating this data.

It would appear from these experiments that sulfate can substitute for part of the cell Cl$^-$. It causes slight movements of Na$^+$ and K$^+$ down the concentration gradient over a prolonged soaking time. This effect was, however, found to be reversible. Muscles were equilibrated in the usual sulfate-containing Ringer for 4 hours, and then one of each pair was transferred to normal Ringer for a further 2 hours. The muscles contained a barely detectable amount of sulfate after soaking in normal Ringer (see kinetic section) and there was a significant decrease in the Na$^+$ and Cl$^-$ space compared to the level in the sulfate-treated muscles. That is the loss of Na$^+$ was more than proportional to the external decrease and the gain of Cl$^-$ was less than proportional to the external increase.
The Effect of Alteration of the External Cation on the Sulfate Distribution

It was shown previously (28) that there is a disproportionate entry of Cl− into the cell when high levels of KCl are added to normal Ringer. It has been assumed that this results from the penetration of Cl− into the ordered phase. In the experiment reported here the K+ level of the Ringer was raised by adding 50 mM/liter K2SO4 to a solution containing 80 m.eq./liter NaCl. It was noted that muscles soaked in this solution showed a small but significant volume increase, compared to companion muscles soaked in normal Ringer. The pattern of Na+, K+, and Cl− distribution was similar to that found previously for high KCl and high potassium phosphate–Ringer (4). The relationship between the Cl− and SO42− spaces in the same muscle was similar to that shown in Table I; i.e., the Cl− space exceeded the SO42− space. It was also observed that the muscles in K2SO4-Ringer showed a significant decrease in Mg2+ content, and increase in Ca2+ content compared to controls in normal Ringer.

### Table II

<table>
<thead>
<tr>
<th></th>
<th>Total concentration</th>
<th>Intracellular concentration</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na, m. eq. per kg.</td>
<td>Control</td>
<td>66.2</td>
<td>41.9</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>72.0</td>
<td>49.0</td>
</tr>
<tr>
<td>Mg, mM per kg.</td>
<td>Control</td>
<td>7.6</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>25.8*</td>
<td>16.1</td>
</tr>
<tr>
<td>Cl, m. eq. per kg.</td>
<td>Control</td>
<td>43.9</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>38.8*</td>
<td>10.7*</td>
</tr>
<tr>
<td>Sulfate mM per kg.</td>
<td>Control</td>
<td>19.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>K, m. eq. per kg.</td>
<td>Control</td>
<td>61.1</td>
<td>83.4</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>63.3</td>
<td>87.6</td>
</tr>
<tr>
<td>Inulin space, per cent</td>
<td>Control</td>
<td>27.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>28.4</td>
<td></td>
</tr>
</tbody>
</table>

In this and subsequent tables figures in parentheses refer to difference between control and treated level of the ion in question.
The effect of high external levels of MgSO₄ on the ionic pattern of muscle was tested by comparing the intracellular ionic levels of muscles soaked in Ringer to which had been added 50 mM/liter MgSO₄ with those of companion muscles soaked in normal Ringer. Muscles were soaked for a total of 4 hours, 2 hours in MgSO₄-Ringer and in normal Ringer, both of which contained inulin, then subsequently in similar solutions without inulin for a further 2 hours. The quantity of inulin released into the Ringer was estimated, and the ionic content of the muscle was determined in the usual way at the end of the experiment.

The results of this experiment are set out in Table II.
1. There was no significant change in the Na⁺ content of the muscle.
2. Mg²⁺ entered the muscle, so that the increase in Mg²⁺ content in high MgSO₄-Ringer compared with the control in normal Ringer, when divided into the external level (50 mM per liter), gave a ratio of 7.9. (See section on the effect of Mg²⁺-Ringer on ionic content.)
3. There was a decrease in the Cl⁻ content, and an increase in the Cl⁻ gradient in the MgSO₄-Ringer. Thus the space available to Cl⁻ is less in MgSO₄-Ringer than in normal Ringer, although the Na⁺ space is unaltered.
4. Sulfate entered the cell in the usual fashion, yielding a ratio of external to internal sulfate of 7.0. This ratio was not significantly different from the ratio of external Mg²⁺ to increase in internal Mg²⁺.
5. There was no significant change in the K⁺ level of the cell.

Thus there is a similar amount of muscle water available to sulfate and magnesium, and this space appears to be virtually identical with that available to Li⁺ and I⁻. The high, and most unphysiological level of MgSO₄ used in this experiment has had little effect on the normal ionic pattern; further it was found that the muscles all responded to electrical stimulation.

The Rate of Loss of Sulfate from the Cell
The rate of loss of SO₄ from the cell was determined by loading the muscle in Ringer containing ³⁵SO₄ for 18 hours, and then effluxing into normal Ringer as described in the Methods section. The efflux was carried on for 6 hours, and then the muscles were transferred to Ringer containing RISA, and after a further 3 hours' equilibration the RISA space was determined.

The necessity for carrying on the efflux for this period of time has been commented on previously (27). Shorter efflux times can only be justified if it can be shown that the efflux curve has indeed straightened during the period of observation. Thus it was seen that the Li⁺ efflux curve straightened after 50 minutes, but the Na⁺ efflux was still slightly curved after 6 hours had elapsed. I⁻ efflux was found to be curved throughout the total period of observation. Curves obtained over briefer periods of observation can fre-
quently be analyzed as the sum of two or three exponentials, although these may give a false impression of the actual process of diffusion.

The shape of the $^{35}$SO$_4$ efflux curve may be seen in Fig. 1. It is seen to be the sum of at least three exponentials. The amount of material in the fast component was compared with that which would be expected if this represented

![Graph showing the efflux of $^{35}$SO$_4$ from muscle](image)

**Figure 1.** The efflux of $^{35}$SO$_4$ from muscle. The lower curve was obtained from a muscle equilibrated for 80 minutes in Ringer containing tracer amounts of $^{35}$SO$_4$. The upper curve was obtained from a muscle equilibrated for 18 hours in a similar solution.

diffusion from the extracellular space as determined with RISA. It was found that the fast component material grossly exceeded that deriving from the RISA space in every case. Thus the true extracellular $^{35}$SO$_4$ was found to be approximately two-thirds of the total $^{35}$SO$_4$ space, whilst that in the fast component amounted to four-fifths of the total $^{35}$SO$_4$. Thus it is not possible to distinguish kinetically between intracellular and extracellular $^{35}$SO$_4$. It may be seen from Fig. 1 that 90 per cent of the $^{35}$SO$_4$ has left the muscle in the first 40 minutes. This movement is somewhat slower than that reported for iodide previously (28), but nevertheless sulfate movement across the membrane must be regarded as being very rapid. The ratio of sulfate$_{out}$/sulfate$_{in}$ was calculated for six such effluxes, and the mean figure obtained was 7.8. This is in good agreement with the figure obtained by the chemical assay.
method for 4 hours' equilibration, but is higher than that found for 18 hours' equilibration. As the $^{35}$SO$_4$ was added in tracer amounts it has presumably not caused the expansion of the free intracellular phase that was attributed to the high sulfate-Ringer.

Efflux from muscles equilibrated in $^{35}$SO$_4$ for 18 hours was compared with

<table>
<thead>
<tr>
<th>TABLE III</th>
</tr>
</thead>
<tbody>
<tr>
<td>THE EFFECT OF HIGH CALCIUM-RINGER ON THE IONIC CONTENT OF MUSCLE</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca in Ringer, m.eq. per liter</td>
<td>16</td>
</tr>
<tr>
<td>Volume change, per cent</td>
<td>±0</td>
</tr>
<tr>
<td>Na, m.eq. per kg.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>26.6</td>
</tr>
<tr>
<td>Treated</td>
<td>28.8</td>
</tr>
<tr>
<td>Ca, m.eq. per kg.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>(5.4)</td>
</tr>
<tr>
<td>Treated</td>
<td>13.1</td>
</tr>
<tr>
<td>Mg, m.eq. per kg.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>16.1</td>
</tr>
<tr>
<td>Treated</td>
<td>17.1</td>
</tr>
<tr>
<td>Cl, m.eq. per kg.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>21.2</td>
</tr>
<tr>
<td>Treated</td>
<td>23.2*</td>
</tr>
<tr>
<td>K, m.eq. per kg.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>76.0</td>
</tr>
<tr>
<td>Treated</td>
<td>75.4</td>
</tr>
</tbody>
</table>

In this table figures refer to intracellular ionic contents calculated on the assumption of an extracellular space of 15 per cent.

efflux from companion muscles which were held in normal Ringer for 18 hours, then transferred to the active Ringer for 80 minutes. The extracellular space was determined in these muscles with RISA after 6 hours' efflux, in the usual way. It may be seen from Fig. 1 that the chief difference between the two curves consists in the almost complete absence of the slowest component of efflux in the short equilibrated muscle. Since this component only appears after 2 hours' efflux it is not unreasonable that it is absent in the short equilibrated muscles. These muscles were, however, shown to contain approximately 15 per cent of the total $^{35}$SO$_4$ in the intracellular compartment.
The Effect of High Ca\textsuperscript{++}-Ringer on the Ionic Content

This Ringer was made by raising the CaCl\textsubscript{2} level of normal Ringer to 16 or 30 m.eq./liter. The usual experimental procedure was adopted, and the results are set out in Table III. The extracellular space was not determined in these muscles, and consequently the absolute magnitude of the figures listed may be questioned. Since, however, the amount of muscle water available to Ca\textsuperscript{++} exceeds the Na\textsuperscript{+} space there can be no question that increase in the external level of Ca\textsuperscript{++} leads to an internal increase.

1. There was no alteration in Na\textsuperscript{+} content at the lower level of Ca\textsuperscript{++} tested, but there was a significant increase in content at the higher level.

2. The Ca\textsuperscript{++} content of the muscles increased at both levels tested, and the ratio of external concentration to the increase in Ca\textsuperscript{++} content over the control level was in both experiments significantly less than the Na\textsuperscript{+} ratio. Thus more of the muscle water appears to be available to Ca\textsuperscript{++} than to Na\textsuperscript{+}.

3. Increasing the external Ca\textsuperscript{++} has not brought about any alteration in the Mg\textsuperscript{++} content of the tissue.

4. There was a disproportionate increase in the Cl\textsuperscript{−} content of the cells in 30 m.eq. per liter Ca-Ringer.

5. There was no change in the K\textsuperscript{+} content of the cell. The slight increase seen at the higher Ca\textsuperscript{++} level is not significant if referred to the initial volume of the cell.

6. The muscles showed a slight shrinkage in the higher Ca-Ringer only. Thus high levels of external Ca\textsuperscript{++} would appear to increase the space available

\begin{table}
\centering
\begin{tabular}{lcccc}
\hline
 & Ratio & & & Ratio \\
\hline
Mg in Ringer, m.eq. per liter & 2.8 & & 33.0 & \\
Na, m.eq. per kg. & 37.6 & 3.5 & 38.0 & 3.4 \\
Ca, m.eq. per kg. & 5.3 & & 7.8* & \\
Mg, m.eq. per kg. & 19.4 & & 24.9* (5.5) & 6.0 \\
Cl, m.eq. per kg. & 30.5 & 3.6 & 25.9 & 5.4* \\
K, m.eq. per kg. & 80.8 & & 90.2* & \\
\hline
\end{tabular}
\caption{The Effect of High Magnesium-Ringer on Ionic Content}
\end{table}

The figures refer to the intracellular ionic contents calculated from the extracellular space determined in each instance with RISA.

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to Na\(^+\) and Cl\(^-\), without causing any alteration in the K\(^+\) level of the cell. The undue entry of Ca\(^{++}\) may reflect an increase in the amount of bound cellular Ca\(^{++}\).

**The Loss of Ca\(^{++}\) from Ca\(^{++}\) Treated Muscles**

The results obtained when muscles were soaked in a high Ca\(^{++}\) solution could be reversed when the treated muscles were transferred to normal Ringer. The ionic changes found following such a transfer are shown in Table IV, which may be obtained from the Documentation Service of the Library of Congress.

**The Effect of High Magnesium–Ringer on Ionic Content**

As was done in the Ca\(^{++}\) experiments the MgCl\(_2\) level of normal Ringer was raised to 33 m.eq. per liter. The results of these experiments are set out in Table V. The figures obtained were calculated from the observed extracellular space measured with RISA.

1. The high Mg-Ringer brought about no change in the Na\(^+\) content of the muscle.
2. There was a significant gain in the Ca\(^{++}\) content of the muscles. This Ca\(^{++}\) shift was not a constant finding in high Mg-Ringer.
3. There was an entry of Mg\(^{++}\) into the cell. The ratio of external Mg\(^{++}\) to increase in internal Mg\(^{++}\) was greater than the Na\(^+\) ratio, and was similar in magnitude to that shown in Table II.
4. The Cl\(^-\) content of the cell showed an increase which was less than proportional to the external increase, and consequently there was a significant increase in the ratio Cl\(_{eq}\)/Cl\(_{in}\). Like the Ca\(^{++}\) shift noted above this was not a constant finding in all experiments.
5. The K\(^+\) content of the cell showed a small but significant increase.

We have found in a large series of experiments that high Mg-Ringer tends to increase the K\(^+\) level of the cell and decrease the Cl\(^-\) level. This effect may be due to a prevention of the usual ion movements found on soaking the tissue. We were also able to demonstrate that high K-Ringer tends to reduce the Mg\(^{++}\) level of the cell.
6. The muscles all responded to electrical stimulation.

**The Loss of Mg\(^{++}\) from Mg\(^{++}\) treated Muscles**

The reversibility of the increase in cellular Mg\(^{++}\) in high Mg-Ringer was tested in the same way as was done in the Ca\(^{++}\) section. The results of this experiment are set out in Table VI, which may be obtained from the Documentation Service of the Library of Congress.
DISCUSSION

The concept of muscle as a three phase system as put forward in this and previous publications (4, 27, 28), rests on the postulate that the ability of the cell to maintain a concentration gradient is a function of the ability of the ordered phase to exclude or accumulate the various ions of the external medium. If the ordered phase were able to discriminate rigidly between the different ions then the “space” available to an excluded ion such as Na⁺ should be identical with the space available to all other excluded ions. If, however, the ion is also partly contained in the ordered phase or is accumulated in the free phase, then this approximation will not hold. We have no evidence that the free intracellular phase is entirely randomized, and it will no doubt contain fixed charges with a degree of order intermediate between that of the ordered phase and the external medium. We should therefore expect these charges to affect the partition of different ions between the free phase and the external phase. This effect would be expected to produce differences in distribution between ions of different charge and valency.

It was shown previously (27) that as a first approximation monovalent ions tend to be distributed either like Na⁺ or Cl⁻, according to charge. It was also shown (4) that phosphate does not penetrate as much of the cell water as does Cl⁻, and this effect was independent of pH, and therefore presumably of the valence of the ion. Our ignorance of the true value of the intracellular pH however, makes this assumption somewhat dubious. In evaluating the phosphate results the further difficulty was encountered that the cell normally contains a considerable amount of inorganic phosphate which is presumably incorporated into the ordered phase. Thus one must deduct this control level to obtain the increment in phosphate content in high phosphate-Ringer. This procedure assumes that there is an equal amount of phosphate in both paired muscles, which is known to be an approximation, and also that the test solution has not altered this control level. A similar difficulty was met with in the present series of experiments in evaluating the Ca ++ and Mg ++ results, and will be discussed more fully below. The sulfate ion is present in such small amounts in untreated muscles that this difficulty did not arise.

Taking into account these various difficulties in interpretation it would seem that the results presented for the three divalent ions fall into the same pattern as was found for the monovalent ions. The results are consistent with the concept of a three phase system and reveal interesting interrelationships between the different ionic species.

The main fact emerging from the sulfate experiments is that this ion does enter the cell. This is in distinction to the findings of other workers (18, 20), but in view of the comparison of inulin and RISA spaces and sulfate spaces
must be accepted as genuine. It would appear that less of the cell water is available to the sulfate ion than is found for Na⁺, and its partition is similar to that found for phosphate, Mg⁺⁺, Li⁺, and I⁻. The effect of high sulfate-Ringer on the partition of other ions is dependent on the cation associated with the sulfate. When it is present as the Na salt sulfate tends to increase the space available to Na⁺ and Cl⁻ without any marked effect on the K⁺ content. This effect is reversible, for when the sulfate is washed out of the cell the Na⁺ and Cl⁻ ratios increase. As was mentioned previously (27) this type of effect may be due to an increase in the dimensions of the free intracellular phase, or to an increase in the binding of these ions, or both. The possibility exists that the true dimensions of the free intracellular phase may be defined by the sulfate, magnesium, and phosphate spaces, Na⁺ being also contained in the ordered phase; or there may be an accumulation of Na⁺ in the free phase (27).

The amount of sulfate entering the cell when it is present in the Ringer as the magnesium salt is similar to that found for Na₂SO₄, but there is a marked difference in the effect on the distribution of Na⁺ and Cl⁻. The high MgSO₄-Ringer has not altered the Na⁺ content, but has brought about an increase in the Cl⁻ ratio, compared with controls in normal Ringer. It was also noted in some experiments that the K⁺ content of the cell was increased over the control level. These findings may be interpreted as indicating that under these conditions the ability of the ordered phase to exclude Cl⁻ and retain K⁺ is greater than in normal Ringer.

The effect of high K₂SO₄-Ringer (see page 86 on the muscle is similar to that found with high KCl or high potassium phosphate solutions (4, 28). We observed a similar pattern in the Na⁺, K⁺, and Cl⁻ distribution. The sulfate ratio was not altered by the presence of K⁺. It should be noted that we made up this Ringer by adding K₂SO₄ to Ringer containing 80 m.eq. per liter NaCl, not by substituting K₂SO₄ for NaCl, as has been done by other authors. It is of interest that this hyperosmotic solution did not cause any shrinkage of the cells. In this experiment the Ca⁺⁺ and Mg⁺⁺ content of the muscles was also determined, and it was found that there was an almost equivalent loss of Mg⁺⁺ and gain of Ca⁺⁺ by the muscle. We have confirmed that this Mg⁺⁺ loss is a result of the high K⁺ level, and is independent of the anion present (unpublished observations). The gain of Ca⁺⁺ was only found in SO₄-Ringer, and may be due to a precipitation of CaSO₄. Interactions between K⁺, Ca⁺⁺, and Mg⁺⁺ have been noted previously for muscle (13), and have been explored more fully for the yeast cell (7, 9, 25). We have also noted that high Mg⁺⁺, whether as the sulfate or Cl⁻, tends to increase the K⁺ content of the cell, compared to controls in normal Ringer.

The kinetic experiments confirm the thesis that part of the intracellular water is freely accessible to sulfate. The rate of movement of sulfate across the membrane is rapid, and the efflux appears to be slowed by movement through
the cytoplasm to a greater extent than it is slowed by the membrane phase. It may be said to be diffusion-limited rather than membrane-limited in the sense that it is chiefly hindered by inhomogeneities in the cytoplasmic phase such as could be envisaged as a network of fixed charges.

The interpretation of the results obtained with high Ca- and Mg-Ringers is complicated, as was mentioned earlier, by the fact that the control muscles already contain appreciable amounts of these ions. The calculations shown in Tables III and IV are based on the assumptions that paired muscles contain identical amounts of the ions, and that increase in the external level of the ion does not alter the amount incorporated in the ordered phase. In the case of Ca++ in particular this latter assumption would appear to be unjustified. The apparent Ca++ space in high Ca-Ringer is greater than the Na+ space, and it would seem likely that this is due to the inclusion of part of the additional Ca++ in the ordered phase. Harris (17) has presented evidence from work with 45Ca which suggests that all muscle Ca++ is not held equally firmly, and it would seem that his results could be interpreted in the light of a system such as we propose. Gilbert and Fenn (15) have also found it necessary to assume a three compartment system to account for the influx kinetics of *SCa.

The high Ca-Ringer brought about an increase in the space available to Na+ and Cl-, but did not alter the K+ content of the cell. As was found with high sulfate-Ringer this effect was reversed when the muscles in high Ca-Ringer were transferred to normal Ringer. The high Ca-Ringer did not alter the Mg++ content of the cell. The effect may be related to the dispersing effect of Ca++ on cytoplasm, and may reflect a partial and reversible loss of order by the ordered phase. This cytoplasmic effect has been commented on by Hodgkin and Keynes (19) and Brink (5).

It has been suggested in the literature (3, 13) that Mg++ is virtually an impermeant cation. The results presented in this paper in which Mg++ spaces are compared with inulin and RISA spaces would indicate that this view is incorrect. It appears from the results listed in Tables II and V that Mg++, sulfate, and phosphate, have a similar distribution across the membrane. As was mentioned earlier in this section this partition, which is similar to that found for Li- and I- may define the true limits of the free intracellular phase. It was seen that the sulfate space is less than the Cl- space in the same muscle. It is nearer to the size of the Cl- space in the control muscle, but still somewhat greater. The Mg++ space tended to be close to the Cl- space in the same muscle, but less than that of the control. It was shown previously (27) that the I- space is not significantly different from the Cl- space. Since the distribution of Cl- may be affected by the presence of foreign ions it is not possible to state on present evidence whether the free intracellular phase, defined as the space available to these foreign ions, can be equated with the Cl- space. It is clear that the Na+ space exceeds that postulated for the free phase, but
it has also been shown (28) that the intracellular Na\(^+\) level is greater than the intracellular Cl\(^-\) level.

It is noteworthy that with the exception of the high K\(_2\)SO\(_4\)-Ringer, none of these rather unphysiological solutions prevented muscular contraction in response to stimulation. Sulfate has been shown to have relatively little effect on the resting potential, whether it was partially substituted for Cl\(^-\) in an otherwise normal Ringer (18), or whether the lowering of the resting potential in high KCl-Ringer was compared with K\(_2\)SO\(_4\) solutions (1).

Mg\(^++\) has been shown to raise the threshold of the muscle fiber to direct stimulation (11), but this effect is small compared with the great sensitivity of the neuromuscular junction to this ion (12). Ca\(^++\) partially relieves the Mg\(^++\) block under these conditions. Brink (5) has reviewed extensively the role of Ca\(^++\) in neural processes, and emphasizes the ability of this ion to alter the stability of the excitable process, and its effect on sol-gel transformations.

It has now been shown (4, 27, 28) that the distribution across the muscle membrane of Na\(^+\), Li\(^+\), Mg\(^++\), and Ca\(^++\), and Cl\(^-\), Br\(^-\), I\(^-\), PO\(_4\)\(^-\), and SO\(_4\)\(^-\) may all be described adequately within the framework of a three phase system. Such a system obviates the need for a continuous feed in of metabolic energy to maintain the ionic gradients by means of active transport. The partial exclusion of these ions is held to result from the maintenance of the structural integrity of the ordered phase. Such structural integrity will require the normal metabolic functioning of the cell, but will not require an array of pumps and carriers to keep the cell in a steady state. It will also render redundant the somewhat improbable concept of a general cation carrier such as has been proposed by Conway for the yeast cell (7, 9).

A further study of the physical chemistry of polyelectrolyte gels, ion exchange resins, and phase separation due to complex coacervation such as has been studied by Overbeck (24) will no doubt lead to a clearer understanding of the problem of electrolyte distribution in the living cell.

**BIBLIOGRAPHY**