ABSTRACT

A microsomal fraction was isolated from rabbit psoas muscle by a modification of Muscatello's method. The fraction contained a Mg-dependent ATPase which had a pH optimum of 7.5. Activity was further stimulated by addition of Na or K or other monovalent cations to the reaction mixture, but synergistic activation by Na and K, and ouabain inhibition, could not be demonstrated. The enzyme hydrolyzed only ATP (adenosine triphosphate) and ITP (inosine triphosphate) at appreciable rates, but Na or K stimulated activity only when ATP was used as substrate. Activity was inhibited by Ca and by low concentrations of Na deoxycholate, and was sensitive to inhibition by thiol group reagents. The enzyme could be distinguished from another enzyme, also present in the fraction, which was Ca-activated, and which exhibited a wider substrate specificity, different pH activation characteristics, lower specific activity, lack of stimulation by Na or K, and less sensitivity to inhibition by deoxycholate and by thiol group reagents. These findings formed the basis for demonstration of the Mg-dependent ATPase in situ.

Numerous physiological studies have suggested that in skeletal muscle, as in other tissues, the maintenance of internal high K and low Na concentrations depends on the operation of active transport processes (1-3). The transport system fails when the energy stores of the muscle become low after prolonged incubation in vitro (4), and may be inhibited by metabolic poisons (5). In addition, the extrusion of Na and the influx of K are inhibited by ouabain (6, 7). This glycoside also inhibits stimulation of Na influx by external K (8), or by lactate and insulin (5), but does not affect the concentration of ATP in the muscle fiber (9).

Although the sarcolemma is both an obvious and likely site for active transport to occur, the sarcotubular system (sarcoplasmic reticulum and T system) may also be involved. A growing body of evidence suggests that ionic gradients are maintained within the muscle fiber as well as at its surface. In some muscles, membranes of the

Abbreviations used: ATP, adenosine triphosphate; ITP, inosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; ADP, adenosine diphosphate; IDP, inosine diphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; NEM, N-ethyl maleimide; PCMB, p-hydroxymercuribenzoate; DOC, deoxycholate; EDTA, ethylene glycol bis (β-aminoethylether) -N,N'-tetraacetic acid; EGTA, ethylene diaminetetraacetic acid.

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T system have been shown to be in direct morphological continuity with the sarcolemma (10), and the ingress of ferritin from externally applied solutions (11) suggests that the T system may be regarded as an inward extension of the extracellular space. Physiologically, the T system appears to be the site of transverse impulse conduction (12). This, in turn, implies the existence of a potential gradient across its membranes during the inactive state, and the restoration of this potential during recovery.

It seems increasingly likely that, following transverse impulse conduction, Ca is released from the interior of the sarcoplasmic reticulum into the sarcoplasm to initiate contraction (13, 14). However, nothing is known about the distribution of cations other than Ca between the sarcoplasm and sarcoplasmic reticulum during the resting state. Nor is it known what the electrical position of the sarcoplasmic reticulum is with respect to the T system and the sarcoplasm. The extent to which the sarcoplasmic reticulum may be involved in the electrical events of activation and the way in which depolarization of the T system effects Ca release are not yet clear.

A differential distribution of monovalent cations between the T system, the sarcoplasmic reticulum, and the sarcolemma might be due simply to the effects of the Donnan equilibrium or also to the operation of active transport processes. If the sarcotubular system were a site of active transport, then a cation-activated ATPase might also be associated with it. Cation-activated ATPases were first characterized by Skou (15, 16) in microsomes from crab nerve, as Mg-dependent ATPases exhibiting synergistic, ouabain-inhibitable stimulation by Na or K of Mg-dependent activity, and were subsequently demonstrated in whole homogenates of a number of tissues (17), as well as in microsomal fractions of cerebral cortex (17–20), liver (17), kidney (20–23), and cardiac muscle (24–25). Similar activity was studied extensively in the erythrocyte membrane (26–31). Because of the close parallelism between the properties of the enzyme and those of the active transport system in the erythrocyte, it was suggested that the enzyme constituted part of the enzymatic basis for transport activity (26–33).

In 1962, Skou briefly noted that Na-K-stimulated ATPase activity was present in rat skeletal muscle microsomes, but stated that only low Na-K-activation ratios could be obtained (20). The presence of such activity was also noted by Bonting in frog skeletal muscle homogenates (17). Järnefelt reported that, in rat skeletal muscle microsomes isolated from a medium containing 50 mM KCl, some activation of the Mg-dependent enzyme could be obtained with Na alone (34).

Recently, several reports have appeared concerning ATPases in muscle sensitive to stimulation by monovalent cations. Samaha and Gergely reported the presence of a synergistically activated ATPase in microsomal fractions of human cadaver muscle isolated in the presence of DOC, histidine, and EDTA (35). Maximal activation ratios were seen in the presence of Na azide, although this agent also reduced the total activity of the fraction.

Other investigators have described, in microsomal fractions of rabbit and frog skeletal muscle, a Mg-dependent ATPase which was stimulated independently by Na and K (36, 37). No synergistic activation by these cations was noted, and ouabain did not inhibit the Na- or K-stimulated activity. In other respects the enzymes studied resembled the Mg-dependent ATPase described by Muscatello et al. (38).

In this combined biochemical and histochemical study, we have attempted to establish whether or not cation-activated ATPase activity could be demonstrated in association with the relatively intact isolated membranes of a sarcotubular fraction, and with the sarcotubular system of muscle in situ. A vesicular fraction of rabbit psoas muscle was prepared which contained a Mg-dependent, Ca-inhibited ATPase, further stimulated by monovalent cations. This enzyme was found to differ in its properties from a Ca-activated ATPase also present in the fraction. In the following paper (39) the histochemical localization of the Mg-dependent enzyme is reported.

**METHODS**

A sarcotubular fraction of rabbit psoas muscle was prepared using a slight modification of Muscatello's procedure (38). Adult rabbits were killed by a blow on the head or by a lethal dose of Nembutal. The psoas muscles were quickly excised, blotted with filter paper, weighed, and then thoroughly minced with razor blades on a glass plate with the presence of a small volume of 0.88 M sucrose. This and all subsequent steps were carried out at 0°–4°C. The mince was further diluted with 0.88 M sucrose and homogenized in a Potter-Elvehjem homogenizer chilled with ice. The volume of the homogenate was adjusted to
approximately 10 times the weight of the muscle with 0.88 m sucrose. This was centrifuged twice at 70,000 g for 40 min in a Spinco Model L centrifuge and the sediments (fractions 1 and 2) were usually discarded. The supernatant was then centrifuged for 60 min at 105,000 g. The pellet thus obtained (fraction 3) was suspended in 1.5 ml of ice cold 0.25 m sucrose per gram of muscle, and was gently rehomogenized in a Potter-Elvehjem homogenizer to give a protein content, as determined by the Lowry method (40), of 0.2 to 0.3 mg/ml. Enzyme assays were carried out on aliquots of this suspension either immediately or after storage at -20°C for periods not exceeding 10 days. However, only fresh suspensions were used when the effects of Ca were studied. Since occasional batches of sucrose contained trace amounts of Ca, deionized sucrose free from Ca was used in the experiments reported here.

For morphological studies, pellets of the three fractions were doubly fixed in 6.25% glutaraldehyde (41) and osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon. The blocks were oriented in such a way that the depth of the pellet was included in each section. Thin sections of the embedded material were stained with lead hydroxide (42) and examined in a Siemens Elmiskop I. In addition, negative staining procedures with phosphotungstic acid were carried out on fractions 2 and 3, usually in the cold (43, 44).

ATPase activity was assayed at 37°C in a 1 ml test system containing 25 mM Tris-maleate buffer (pH 7.5 or as stated), 5 mM Tris-ATP (Sigma Chemical Corporation, St. Louis, Mo.), 4 mM MgCl₂ (or as stated), with NaCl, KCl, or other chloride salts added as stated. 0.9 ml of the reaction mixture without the enzyme and the enzyme suspension was incubated separately in a metabolic shaker. After thermal equilibration for 10 min, the reaction was started by addition of 0.1 ml of the enzyme suspension to the reaction mixture. The reaction was terminated, usually after 20 min, by addition of 0.1 ml of 1 m perchloric acid, and the inorganic phosphate content determined by the Fiske-SubbaRow method (45). When lead nitrate was also present in the final reaction mixture, the concentration of Tris-maleate buffer was increased to 125 mM and the inorganic phosphate released was assayed by the method of Martin and Doty (46).

RESULTS

Morphological Evaluation of the Fractions

Sections of fraction 1 revealed the presence of masses of myofibrils, intact and fragmented mitochondria, glycogen particles, nuclei, and cellular debris, as well as scattered vesicular structures. Fraction 2 also contained a few myofibrils, mitochondria, and recognizable mitochondrial fragments, but consisted in large part of smooth-surfaced vesicles (600 to 800 A in diameter). Fraction 3 consisted almost exclusively of vesicular material of similar dimensions (Fig. 1). These vesicles appeared as spherical or flattened structures bounded by a single continuous membrane, and often contained material of variable, low electron opacity. At times, these were closely apposed, forming small stacks or 'rouleaux' of two to six vesicles. Other vesicles consisted of several (2 to 4) irregular or circular concentric membranes containing amorphous material of somewhat greater electron opacity. Although it was possible that sections perpendicular to the long axis of the vesicles, or parallel to flattened vesicular stacks, could have accounted for some of the double-contoured vesicles, this seemed unlikely in view of the relative frequency with which they were observed.

Negative staining of the two lighter fractions (2 and 3) revealed morphological detail not apparent in sectioned material. A large proportion of the vesicles seen in fraction 2 were associated with characteristic mitochondrial elementary particles (44, 48-50), and hence could be presumed to represent fragmented mitochondria. In contrast, virtually none of the vesicles in fraction 3 had the substructures associated with mitochondria or their derivatives. Some of the vesicles and tubules in fraction 3 had a finely granular center with a periphery of similar granularity. Other vesicles or tubules had a darker interior of nearly the same density as the surrounding phosphotungstic acid (Fig. 2). The outer side of both types of tubules and vesicles bore innumerable small projections about 20 A wide and 40 A long (Fig. 3). Some, but not all, vesicles of both types appeared to arise as dilated ends of a narrower tubule (Fig. 2).

Additional studies were carried out to assess the degree of mitochondrial contamination of fraction 3. When this fraction was assayed manometrically for cytochrome oxidase and succinic dehydrogenase activities with Potter's method (47), it was found to be free from succinic dehydrogenase activity and to possess only negligible cytochrome oxidase activity (0 to 14 µl of oxygen per milligram of protein per hour). Oligomycin inhibited ATP splitting less than 5% at a concen-
Liberation of Inorganic Phosphate

Incubation of fraction 3 in the presence of ATP but in the absence of added inorganic cations, at either pH 6.5 or 7.5, resulted in release of 5 to 20 μmoles of inorganic phosphate per milligram of protein per hour. This activity varied considerably from preparation to preparation, but could be abolished by dialysis of the enzyme suspension against 0.25 M sucrose for 16 to 24 hr, at 4°C. However, suspensions of fraction 3 were not usually subjected to dialysis, since this procedure rendered the enzyme suspension unstable and resulted in subsequent rapid loss of the cation-activated ATPase activity.

When the enzyme suspension was incubated in the presence of Mg at either pH 6.5 or 7.5, considerably more inorganic phosphate was released, and this activity was still further increased when either Na or K was added to the medium. Under all these conditions the rate of phosphate liberation over a 20 min period was proportional to the amount of enzyme protein added, between 0.015 and 0.15 mg of protein per milliliter of final reaction mixture, and was linear with time over a 30 min incubation period in a reaction mixture containing 0.055 mg of protein per milliliter.

Influence of pH on ATPase Activity

In the presence of 4 mM Mg the enzyme appeared optimally active between pH 7.5 and 8 (Fig. 4). ATP splitting was further enhanced by the addition of either Na or K, or both, without appreciable change in the pH optimum, although the relative stimulatory effects of Na or K were slightly greater at pH 6.5 than at 7.5.

Effects of Mg

At pH 7.5 (Table I), half maximal stimulation of ATPase activity occurred at Mg concentrations of less than 1 mM, maximal stimulation at 4 to 6 mM (Mg/ATP = 0.8 to 1.2), while Mg levels between 8 and 16 mM slightly inhibited activity. At pH 6.5 (Table II), half maximal stimulation of activity occurred at Mg concentrations of 1 mM and maximal stimulation at 16 mM (Mg/ATP = 3.2).

Effects of Added Na and K on the Mg-Dependent Enzyme at pH 7.5

In the absence of Mg, neither Na nor K, added together or separately, enhanced the liberation of inorganic phosphate. The effects of added monovalent cations were markedly dependent on the Mg concentration (Table I, Figs. 5 and 6), and in general the relative stimulatory effects of Na and/or K were highest at Mg concentrations of 2 to 4 mM (Mg/ATP = 0.4 to 0.8). In the presence of 16 mM Mg there was little or no further stimulation of enzyme activity by added Na or K.

The addition of 1 mM K, at Mg concentrations of 1 to 10 mM, resulted in a significant increase in ATPase activity (Fig. 5). Half maximal stimulation occurred at K concentrations between 1 and 5 mM. When more than 10 mM K was present,
stimulation of ATPase activity by this cation gradually diminished, and little further stimulation by K occurred when more than 100 mM had been added.

In the presence of 1 to 8 mM Mg, the addition of 10 mM Na stimulated activity only slightly (Fig. 6). Half maximal stimulation occurred at Na concentrations between 10 and 100 mM. Comparison of the effects of Na and K shows that, with 8 mM Mg or less, Na was much less effective than K in stimulating ATPase activity.

When varying amounts of Na and K were added together through a wide range of concentrations, at Mg levels of 1 to 16 mM, the combined stimulation produced by the two cations was always less than the sum of their separate effects. In the presence of 50 mM K, addition of more than 50 mM Na caused either no further stimulation or inhibition of enzyme activity (Table I). This effect was found at all Mg levels, but was more marked at lower Mg concentrations.

**Effects of Na and K on the Mg-Dependent Enzyme at pH 6.5**

At pH 6.5, again, addition of either Na or K enhanced activity of the Mg-dependent ATPase (Table II). Although Na was relatively less effective than K in stimulating enzyme activity, maximal stimulation by either ion was attained at concentrations between 50 and 100 mM. The relative stimulation produced by Na or K was highest at Mg concentrations of 1 to 2 mM (Mg/ATP = 0.2 to 0.4), and declined progressively at higher Mg concentrations, though to a lesser extent than at pH 7.5. Again, no concentration of Na or K could be found at which the combined stimulation by the two ions was greater than the sum of their separate effects. In the presence of 50 mM K (Table II), addition of more than 20 mM Na resulted in either no further stimulation, or in inhibition, of ATPase activity. This inhibitory effect was more marked at lower Mg concentrations and was greater at pH 6.5 than at pH 7.5.

**Effects of other Monovalent Cations on the Mg-dependent ATPase**

In the presence of 4 mM Mg, increased enzyme activity was observed when up to 100 mM NH₄⁺ was added.

### Table I

**Effects at pH 7.5 of Varying Mg Concentrations on ATPase Activity Alone or in the Presence of 50 mM Na or K, and 50 mM K plus Varying Concentrations of Na**

<table>
<thead>
<tr>
<th>Mg, mM</th>
<th>Na, 50 mM</th>
<th>K, 50 mM</th>
<th>Na, 10 mM</th>
<th>Na, 20 mM</th>
<th>Na, 50 mM</th>
<th>Na, 100 mM</th>
<th>Na, 150 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>141 (1.42)</td>
<td>170 (1.71)</td>
<td>186 (1.88)</td>
<td>170 (1.71)</td>
<td>170 (1.71)</td>
<td>136 (1.37)</td>
<td>144 (1.45)</td>
</tr>
<tr>
<td>2</td>
<td>106 (1.64)</td>
<td>186 (1.73)</td>
<td>193 (1.82)</td>
<td>193 (1.82)</td>
<td>192 (1.81)</td>
<td>174 (1.64)</td>
<td>174 (1.64)</td>
</tr>
<tr>
<td>4</td>
<td>106 (1.70)</td>
<td>189 (1.78)</td>
<td>192 (1.81)</td>
<td>188 (1.77)</td>
<td>192 (1.81)</td>
<td>183 (1.73)</td>
<td>193 (1.83)</td>
</tr>
<tr>
<td>5</td>
<td>118 (1.42)</td>
<td>170 (1.44)</td>
<td>173 (1.47)</td>
<td>181 (1.53)</td>
<td>193 (1.63)</td>
<td>188 (1.59)</td>
<td>181 (1.53)</td>
</tr>
<tr>
<td>16</td>
<td>101 (1.21)</td>
<td>94 (0.92)</td>
<td>99 (0.98)</td>
<td>97 (0.96)</td>
<td>96 (0.95)</td>
<td>96 (0.95)</td>
<td></td>
</tr>
</tbody>
</table>

Unbracketed figures indicate μM of inorganic phosphate released per milligram of protein per hour. Bracketed figures represent activity ratios obtained by dividing each activity by that observed in the presence of magnesium only. Other experimental conditions are indicated in the text.

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TABLE II
Effects at pH 6.5 of Varying Mg Concentrations on ATPase Activity Alone or in the Presence of 50 mM Na or K, and 50 mM K plus Varying Concentrations of Na

<table>
<thead>
<tr>
<th>Mg, mM</th>
<th>Na, 50 mM</th>
<th>K, 50 mM</th>
<th>Na, 10 mM</th>
<th>Na, 20 mM</th>
<th>Na, 50 mM</th>
<th>Na, 100 mM</th>
<th>Na, 150 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69</td>
<td>143 (2.08)</td>
<td>155 (2.25)</td>
<td>183 (2.66)</td>
<td>178 (2.58)</td>
<td>133 (1.93)</td>
<td>115 (1.67)</td>
</tr>
<tr>
<td>2</td>
<td>88</td>
<td>172 (1.95)</td>
<td>176 (2.00)</td>
<td>175 (1.99)</td>
<td>174 (1.98)</td>
<td>169 (1.92)</td>
<td>153 (1.74)</td>
</tr>
<tr>
<td>4</td>
<td>103</td>
<td>177 (1.72)</td>
<td>188 (1.83)</td>
<td>189 (1.84)</td>
<td>185 (1.80)</td>
<td>182 (1.77)</td>
<td>174 (1.69)</td>
</tr>
<tr>
<td>10</td>
<td>102</td>
<td>171 (1.68)</td>
<td>189 (1.85)</td>
<td>191 (1.87)</td>
<td>190 (1.86)</td>
<td>188 (1.84)</td>
<td>170 (1.67)</td>
</tr>
<tr>
<td>16</td>
<td>120</td>
<td>179 (1.49)</td>
<td>167 (1.41)</td>
<td>169 (1.41)</td>
<td>165 (1.38)</td>
<td>168 (1.40)</td>
<td>162 (1.35)</td>
</tr>
</tbody>
</table>

Unbracketed and bracketed figures have the same meaning as in Table I. Other experimental conditions are indicated in the text.

Figure 5: Effects of varying concentrations of Mg and K on ATPase activity at pH 7.5. Values relative to cations indicate their concentrations (mM/liter) in the final reaction mixture.

Cs, Rb, or Li (as chloride salts) was present in the reaction mixture (Table III). Progressive activation of enzyme activity was observed which was maximal with 100 mM NH₄, 50 mM Cs, and 10 mM of either Rb or Li. The relative stimulating effect of these cations depended on the concentration added: if 10 mM were added, the order was Rb > NH₄ > Cs = Li; if 100 mM were added, it was NH₄ > Cs = Rb > Li. If more than 10 mM Li were added, enzyme activity was progressively reduced to the level seen with Mg alone. In the absence of Mg, the rate of inorganic phosphate release was not significantly stimulated by addition of these cations.

Enzyme Activity in the Presence of Different Substrates

In the presence of 4 mM Mg, considerably less enzyme activity was seen if ITP, CTP, or UTP replaced ATP as substrate, with activity decreasing in that order (Table IV). In contrast to the effects seen with ATP, further addition of 50 mM Na and/or K did not enhance enzyme activity with other nucleoside triphosphate substrates. With ADP as substrate in the presence of Mg, a rela-
TABLE III
Effect of Other Monovalent Cations on Mg-Dependent ATPase

<table>
<thead>
<tr>
<th>Monovalent cation</th>
<th>Mg, 4 mM</th>
<th>NH₄⁺</th>
<th>Cs⁺</th>
<th>Rb⁺</th>
<th>Li⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>124</td>
<td>—</td>
<td>184 (1.49)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>179 (1.44)</td>
<td>—</td>
<td>205 (1.65)</td>
<td>158 (1.27)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>193 (1.55)</td>
<td>158 (1.27)</td>
<td>207 (1.67)</td>
<td>148 (1.19)</td>
<td></td>
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<tr>
<td>50</td>
<td>216 (1.74)</td>
<td>169 (1.36)</td>
<td>197 (1.59)</td>
<td>147 (1.18)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>247 (1.99)</td>
<td>207 (1.67)</td>
<td>200 (1.67)</td>
<td>128 (1.03)</td>
<td></td>
</tr>
</tbody>
</table>

Unbracketed and bracketed figures have the same meaning as in Table I. Other experimental conditions are indicated in the text.

TABLE IV
Inorganic Phosphate Release in the Presence of Different Substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mg, 4 mM</th>
<th>Na⁺, 50 mM</th>
<th>K⁺, 50 mM</th>
<th>K⁺, 50 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>52</td>
<td>93</td>
<td>112</td>
<td>105</td>
</tr>
<tr>
<td>ITP</td>
<td>23</td>
<td>22</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>CTP</td>
<td>12</td>
<td>7</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>UTP</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>ADP</td>
<td>59</td>
<td>71</td>
<td>73</td>
<td>71</td>
</tr>
<tr>
<td>IDP</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>AMP</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>β-glycerophosphate</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figures indicate μM inorganic phosphate released per milligram of protein per hour. The pH of the incubation media was 6.5. The concentration of each substrate in the final reaction mixture was 5 mM. ATP was added as the Tris-salt; the other substrates as the Na salts.

TABLE V
Effects of Na Deoxycholate on Mg-Dependent ATPase Activity

<table>
<thead>
<tr>
<th>Na DOC</th>
<th>Mg, 4 mM</th>
<th>Mg, 4 mM + K⁺, 150 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>R⁰</td>
<td>R¹</td>
</tr>
<tr>
<td>0.0125</td>
<td>56 (0.67)</td>
<td>57</td>
</tr>
<tr>
<td>0.0250</td>
<td>50 (0.59)</td>
<td>—</td>
</tr>
<tr>
<td>0.0500</td>
<td>43 (0.51)</td>
<td>43</td>
</tr>
<tr>
<td>0.1000</td>
<td>18 (0.21)</td>
<td>—</td>
</tr>
<tr>
<td>0.2000</td>
<td>10 (0.12)</td>
<td>6</td>
</tr>
</tbody>
</table>

Unbracketed figures indicate μM of inorganic phosphate released per milligram of protein per hour. Bracketed figures are obtained by dividing each activity by that observed in the absence of Na DOC. Other experimental conditions are indicated in the text.

Effects in inhibitors of enzyme activity

Na Deoxycholate

Addition of Na deoxycholate to the final reaction mixture in concentrations of 0.0125 to 0.2% caused progressive inhibition of activity in the presence of Mg or Mg and K (Table V). Fifty per cent inhibition was obtained with approximately 0.05% Na deoxycholate, while stimulation of activity by monovalent cations was lost when the concentration of deoxycholate exceeded 0.0125%.

Reagents for Thiol Group

N-Ethyl Maleimide (NEM): Preincubation of fraction 3 with NEM, 10⁻³ M, for 1 to 10
Effects of preincubation with NEM (1 × 10^{-3} M) on ATPase activity. (Fig. 7) caused progressive and approximately parallel inhibition of the rate of inorganic phosphate release when the fraction thus treated was added to reaction mixtures containing 4 mM Mg, 4 mM Mg and 150 mM Na, and 4 mM Mg and 150 mM K. In each case the rate of enzyme inhibition was greatest during the first minute of preincubation, and 5 min of preincubation resulted in approximately 50% inhibition of enzyme activity.

PCMB: Preincubation with PCMB, 1 × 10^{-6} M, for 1, 5, and 10 min resulted in 23, 31, and 55% inhibition of the rate of inorganic phosphate release when the fraction thus treated was added to reaction mixtures containing 4 mM Mg. The Mg-dependent enzyme activity was totally inhibited, and the stimulating effects of Na or K were lost, after a 1 min preincubation with 5 × 10^{-6} M PCMB. However, after a 10 min preincubation with cysteine, 1 × 10^{-4} M, added to the system containing 5 × 10^{-6} M PCMB, the rates of inorganic phosphate release in reaction mixtures containing 4 mM Mg and 150 mM K were 76 and 78% of those observed when the fraction was preincubated in sucrose.

Ouabain and Synergistic Activity

Fraction 3 was preincubated with 10^{-4} M ouabain for 30 min and the rate of ATP splitting measured in a reaction mixture containing 1 × 10^{-4} M ouabain and (a) 1, 2, 4, 10, or 16 mM Mg; (b) 4 mM Mg and 5, 10, 20, 50, 100, or 150 mM Na or K; and (c) 4 mM Mg, 20 mM K, and 100 mM Na. These experiments failed to demonstrate any inhibition of ATP splitting when the results were compared with those of parallel experiments in which the fraction was not exposed to ouabain.

In other experiments the effects of some variations in the isolation medium were investigated. Parallel postmitochondrial and microsomal fractions from rabbit psoas muscle and rabbit kidney were prepared in media containing 0.88 M sucrose, 30 mM histidine, and 5 mM EDTA, pH 6.8. The fractions were suspended in 0.95 M sucrose containing 30 mM histidine and 1 mM EDTA, pH 6.8, and each fraction from either muscle or kidney was then incubated in media containing (a) 4 mM Mg; (b) 4 mM Mg and 150 mM Na; (c) 4 mM Mg, 20 mM K, and 100 mM Na.

Lead Nitrate

The addition of increasing concentrations of lead nitrate caused progressive inhibition of inorganic phosphate release in systems containing either 4 mM Mg, or 4 mM Mg and 150 mM K (Fig. 8). In the presence of 1 mM Pb, activity of the Mg-dependent enzyme was reduced by 85%, but further stimulation by K of inorganic phosphate release could still be detected. When the concentration of Pb was increased to 2 mM, the Mg-dependent activity was reduced by 90% and no K stimulation could be detected.
TABLE VI

Inhibition by Ca of Mg-Dependent ATPase Activity

<table>
<thead>
<tr>
<th>Ca added (M)</th>
<th>Mg, 4 mM</th>
<th>Na, 50 mM</th>
<th>K, 50 mM</th>
<th>Na, 50 mM + K, 50 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>108</td>
<td>160 (1.48)</td>
<td>168 (1.55)</td>
<td>193 (1.79)</td>
</tr>
<tr>
<td>5 × 10^{-4}</td>
<td>102</td>
<td>152 (1.49)</td>
<td>157 (1.57)</td>
<td>187 (1.83)</td>
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<td>121 (1.44)</td>
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<tr>
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<td>75 (1.10)</td>
<td>102 (1.50)</td>
<td>86 (1.26)</td>
</tr>
<tr>
<td>1 × 10^{-3}</td>
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<td>2 × 10^{-3}</td>
<td>44</td>
<td>47 (1.07)</td>
<td>54 (1.23)</td>
<td>58 (1.32)</td>
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</table>

Unbracketed figures indicated μM of inorganic phosphate released per milligram protein per hour. Bracketed figures were obtained by dividing each activity by that observed in the presence of Mg and the same concentration of Ca. Other experimental conditions are indicated in the text.

Mg; (b) 4 mM Mg and 100 mM Na; (c) 4 mM Mg and 20 mM K; and (d) 4 mM Mg, 100 mM Na, and 20 mM K; with or without the addition of 1 × 10^{-8} M ouabain. These experiments failed to demonstrate in the muscle fractions the presence of an ATPase characterized by synergistic, ouabain-inhibitable stimulation by Na and K of its Mg-dependent activity, although such an enzyme could readily be demonstrated in each of the kidney fractions.

Effects of Ca

In media containing 4 mM Mg with or without added Na or K, the addition of Ca in concentrations ranging from 5 × 10^{-4} M to 2 × 10^{-3} M caused progressive reduction of ATP splitting (Table VI). A 50% inhibition of activity in the presence of Mg was obtained with Ca levels of 5 × 10^{-4} M to 1 × 10^{-2} M. Some activation by Na or K was retained in the presence of up to 2 × 10^{-3} M, but relative activation by these cations progressively declined with increasing Ca concentration. When the free Ca of the incubation mixture was varied by addition of EGTA-Ca buffers (14) (total EGTA, 3 mM), activity in the presence of 4 mM Mg declined from 105 μM Pi per milligram of protein per hour at pCa 6 to 72 μM Pi per milligram of protein per hour at pCa 8. A parallel decrease in activity was observed in the presence of 4 mM Mg and 50 mM K (165 to 110 μM Pi per milligram of protein per hour). However, even at pCa 8, high specific activity remained, and no effect of decreasing free Ca levels on Na-K activation ratios could be observed.

When the effects of increasing concentrations of Ca alone were studied (Fig. 9), progressive stimulation of inorganic phosphate release was observed in the absence of Mg as the Ca concentration was increased from 10^{-5} to 10^{-2} M. When the effects of Ca alone were compared with its effects in the presence of 4 mM Mg, at Ca concentrations of 2 × 10^{-3} M or more, virtually all ATP splitting in the presence of Mg could be accounted for by Ca-dependent activity. No stimulation of inorganic phosphate release was noted if Na or K were added to systems containing Ca alone, and this was consistent with the loss of such stimulation in the presence of Mg and high Ca concentrations.

It was possible that the Ca-stimulated ATPase activity was due to contamination by myofibrils or myofilaments and their associated ATPase. To assess this possibility, the fraction was twice extracted for 2 hr at 4°C in 0.6 M KCl containing 1 mM Tris-HCl, pH 7, and recentrifuged for 2 hr at 105,000 g (52). The extracted material again showed a response to added Ca (Fig. 9). Stimulation was noted as the Ca concentration was increased above 1 × 10^{-5} M, with maximal activity at 1 × 10^{-2} M Ca.

Characteristics of Ca-Dependent Activity

In other experiments, activity in the presence of Ca alone was studied and compared with the Mg-dependent activity. At 37°C, pH 7.5, in the presence of 2 × 10^{-3} M Ca as the only activating cation, the inorganic phosphate released increased linearly with time over a 30 min period in a reaction mixture containing 0.029 mg of protein per milliliter and was proportional to the amount of enzyme protein added between 0.009
present as a substrate (cf. Table IV). Although 0.2% Na deoxycholate inhibited both Ca- and Mg-dependent enzymes almost completely, the Ca-stimulated activity was more resistant to low (0.1% or less) concentrations of Na deoxycholate (Table VIII). This activity was also more resistant to the effects of thiol group reagents. Preincubation of the fraction with 1 × 10^{-4} m PCMB for 10 min resulted in 18% inhibition of the rate of inorganic phosphate release when the fraction thus treated was added to reaction mixtures containing 1 × 10^{-3} Ca. Preincubation of the fraction with 1 × 10^{-3} m NEM for 1, 5, and 10 min resulted in 34, 37, and 46% inhibition of ATP splitting. Finally, the addition of 0.5, 1, and 4 mM Pb to a reaction system containing Ca resulted in much less inhibition of activity (16, 25, and 27%), compared with the effects of 0.5, 1, and 4 mM Pb added to a system containing Mg as activating ion (80, 85, and 90%).

DISCUSSION

In several respects, the enzyme preparation obtained from rabbit muscle resembled that obtained from frog muscle by Muscatello et al. (38). Although Muscatello’s isolation technique was the basis for that used in this study, with rabbit muscle two successive centrifugations at 70,000 g were required to obtain complete sedimentation of mitochondrial fragments from the homogenate. Fraction 3, obtained after further centrifugation at 105,000 g, was consistently free of mitochondrial and myofibrillar contamination when it was examined in the electron microscope. The virtual absence of mitochondrial contamination was further supported by biochemical studies, which showed that the preparation contained no succinic dehydrogenase activity and virtually no cytochrome oxidase activity. Lack of significant inhibition by oligomycin at concentrations of 1 μg/ml and only slight inhibition at 10 μg/ml lends further support to these findings.

The choice of preparative technique was dictated by the aims of the study: to explore the possibility that a cation-activated ATPase might be associated with chemically unaltered membranes of the sarcoplasmic reticulum. A high viscosity homogenizing medium was used to minimize contamination of the lighter fractions by fragmented mitochondria (53) or by myofilaments (38). Addition of electrolytes to the medium was avoided to prevent uptake of monovalent cations by the membranes of the fraction, which might have obscured the effects of Na and K when these were added to the final reaction mixture (54). Although some earlier studies demonstrating the presence of a “pump” enzyme of the Skou type, in microsomal fractions of rabbit and human muscle, have employed homogenizing media containing deoxycholate (20, 35), such media were avoided because of the dissolution of the sarcotubular membranes which may result from exposure to this compound (55). In two experiments carried out by us, in which 0.5% Na deoxycholate was added to the homogenizing medium, negative staining of the resulting preparation showed no recognizable sarcotubular structures. Since the study was directed ultimately toward the in situ demonstration of the enzyme in intact sarcotubules, this precluded the use of deoxycholate in subsequent experiments.

In conventionally fixed and embedded pellets, the sarcotubular fraction obtained from rabbit muscle was morphologically quite similar to analogous preparations from frog muscle (38). As noted, negative-staining techniques proved a useful adjunct to more conventional preparative procedures, and were particularly useful in the detection of mitochondrial fragments which were too small to retain a recognizable mitochondrial structure in sectioned material. The dimensions (70 × 90 Å) and characteristic, stalked shape of mitochondrial elementary particles (48-50) clearly distinguished them from the small projections (20 × 40 Å) observed on the outer surfaces of the membranes in negatively stained preparations of fraction 3.

The Mg-dependent ATPase studied in these experiments had a pH optimum between 7.5 and 8 in the presence or absence of added monovalent cations. At optimal pH, maximal stimulation by Mg was attained between 4 and 6 mM. At suboptimal pH (6.5) considerably higher Mg concentrations were required for maximal activity. Further addition of either Na or K led to further increases in ATPase activity, although the extent of the increase was affected not only by the quantity of monovalent cation added, but also by the pH and by the Mg concentration of the final reaction mixture. Either at suboptimal or at optimal pH, lower concentrations of K than of Na were required for half-maximal stimulation of the Mg-dependent ATPase activity, although the relative stimulating effect of either K or Na
and 0.029 mg/ml of final reaction mixture over a 20 min period. Activity in the presence of 2 mM Ca was found to differ from the Mg-dependent activity in several respects. Activity in the presence of Ca had a broad pH optimum between pH 6.5 and 7.5 (Table VII). Relatively more activity was obtained with substrates other than ATP; the rate of inorganic phosphate release from either ITP or UTP as substrate was 58%; with IDP as substrate, 14%; and with β-glycerophosphate, 9% of the rate observed when ATP was used.

### Table VII

<table>
<thead>
<tr>
<th>pH</th>
<th>5</th>
<th>6</th>
<th>6.5</th>
<th>7</th>
<th>7.5</th>
<th>8</th>
<th>9</th>
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</thead>
<tbody>
<tr>
<td>QPi</td>
<td>14.3</td>
<td>19.6</td>
<td>23.4</td>
<td>23.4</td>
<td>23</td>
<td>21</td>
<td>14.5</td>
</tr>
</tbody>
</table>

QPi indicates μM of inorganic phosphate released per milligram of protein per hour. The Ca concentration was 2 × 10⁻³ M. Other experimental conditions are indicated in the text.

### Table VIII

<table>
<thead>
<tr>
<th>Na DOC %</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>QPi</td>
<td>25</td>
<td>20 (0.79)</td>
<td>16.5 (0.65)</td>
</tr>
</tbody>
</table>

QPi refers to rate of inorganic phosphate release per milligram of protein per hour and is shown in the unbracketed figures. The bracketed figures are obtained by dividing each activity by that observed in the absence of Na DOC. Temperature 37°C, pH 7.5 Other experimental conditions are indicated in the text.

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was greater at suboptimal than at optimal pH, and greater at suboptimal Mg levels. Regardless of Mg concentration and pH, no concentration of the two monovalent cations could be found at which their combined stimulation was synergistic, i.e., exceeded the sum of their separate effects. In fact, in the presence of more than 50 mM K, the further addition of 50 mM Na (pH 7.5) or of 20 mM Na (pH 6.5) resulted either in no further increase, or in a decrease, in ATPase activity.

In addition, unlike the enzyme described in microsomal fractions of nerve, kidney, and cerebral cortex (16–20), significant stimulation of the Mg-dependent enzyme was produced by the addition of NH₄, Cs, or Rb, as chloride salts in the absence of added Na. This stimulation appeared to be determined by the cation rather than by the accompanying chloride anion, since, depending on the cation, a wide range of activation ratios could be obtained in the presence of identical chloride concentrations. These findings could be interpreted to indicate the presence of a single site on the enzyme which can be occupied by either Na or K, with higher affinity for K than Na, or occupied by other monovalent cations.

The results might also suggest that the enzyme is not concerned with the active, linked transport of Na and K if such enzymes are invariably characterized by synergistic Na-K stimulation. The possibility that synergistic stimulation was present but was obscured by contamination of the original enzyme preparation with either Na or K seems unlikely, for addition of ouabain in high concentration did not inhibit activity in the presence of Mg, nor did it prevent stimulation of the Mg-dependent enzyme by either added Na or K. It is interesting that the Mg-dependent ATPase closely resembled the sonicated erythrocyte ATPase, in which cation activation was retained after sonication although synergistic stimulation and ouabain inhibition were lost (36). This raises the possibility that an originally ouabain-sensitive, Mg-dependent, sarcotubular ATPase might have been injured during the preparation procedure, or that the sarcotubular enzyme might be akin to that of the intact red cell but differ from it in its spatial orientation in the membrane. It is also possible that the presence or absence of synergistic stimulation by Na and K may be a function of the isolation procedure used. To date, only those authors who isolated their fractions in media containing Na deoxycholate have reported such stimulation (20, 35). Other workers, who used isolation media which did not contain this agent, have consistently found independent stimulation by Na and K, but no synergistic effects of these cations (36, 37).

The enzyme resembled other cation-activated ATPases in its dependence on free —SH groups. Activity in the presence of Mg or Mg plus monovalent cations was reduced in parallel fashion after preincubations with NEM. This supports the interpretation that a single site on the enzyme was responsible for Na or K binding, or that the —SH reagents were inhibiting allosterically.

The inhibitory effects of Pb were studied because it was to be used as the capture reagent in subsequent histochemical studies. Although this ion may combine with enzyme residues other than —SH groups, it was of interest that in concentrations of 1 mM or less, it inhibited the Mg and Mg plus monovalent cation activities in an approximately parallel manner. However, Pb in concentrations as high as 4 mM had only a slight inhibitory effect on the Ca-dependent enzyme.

Inhibition by Ca of inorganic phosphate release in the presence of Mg or Mg plus monovalent cation is in accord with the findings of Muscatello et al. (38), Fratantoni and Askari (36), and Duggan (37). In the presence of low Ca concentrations, stimulation of activity by Na or K was retained, although with increasing Ca concentrations the relative activation by monovalent cations progressively declined.

The failure of even $1.6 \times 10^{-2}$ M Ca to reduce ATPase activity in the presence of Mg to levels observed in the absence of other added cations suggested that another enzyme hydrolyzing ATP, stimulated by Ca, might also be present in the fraction. In fact, progressive stimulation of ATP hydrolysis could be demonstrated when Ca, as the only activating cation, was added to the final reaction mixture. The Ca-stimulated enzyme could be distinguished from the Mg-dependent one by its wider substrate specificity, lack of Na- or K-stimulation, and by its greater resistance to such inhibitors as Na deoxycholate, thiol group reagents, and to lead.

It was of particular interest that, in the presence of Mg, relatively high specific activity was retained at very low Ca concentrations produced by EGTA buffers, together with stimulation of activity by Na or K. Even at pCa 8, 71 μM Pi per milligram
of protein per hour was released in the presence of Mg alone, and 110 μM Pi per milligram of protein per hour in the presence of Mg + 50 mM K. Comparison of these figures with the activity, at pCa 8, of fractions active in Ca uptake (<6 μM/mg of protein per hour) (63) strongly suggests that, although the preparation contained some admixture of EGTA-inhibitable activity, most of the ATP splitting in the fraction could be attributed to an enzyme not dependent on the presence of low levels of Ca for activity, and therefore presumably not coupled to Ca transport (63). This suggestion is further supported by the relative specificity of the Mg-dependent enzyme for ATP and ITP, since Ca-accumulating activity and its accompanying phosphatase are active in the presence of CTP, GTP, and UTP as well as ATP and ITP (59, 60).

The capacity of the sarcoplasmic reticulum to accumulate calcium and the correlation of Ca uptake with “extra” ATP splitting has been intensively studied (14, 57-63). However, relatively little attention has been given to the “basic” ATPase, not inhibited by EGTA, and assumed not to be coupled to Ca transport. Its properties and possible function have not been extensively investigated. It is possible that the Mg-dependent ATPase described here, stimulated by monovalent cations, represents the basic ATPase. Its precise physiological role merits further investigation.

This work was supported by National Institutes of Health grants NB-01620-07, 5 T1-NB-5062-10, BT-722, H-5906, and by Health Research Council of the City of New York grant U-1075.

The authors are indebted to Miss Mary Rayborn for her competent assistance during part of this study. Received for publication 15 December 1965.

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