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THE SEPARATION AND PARTIAL PURIFICATION OF MEMBRANE-BOUND ($Na^+ + K^+$)-DEPENDENT Mg^{2+}-ATPase AND ($Na^+ + K^+$)-INDEPENDENT Mg^{2+}-ATPase FROM FROG SKELETAL MUSCLE

R. J. BOEGMAN, J. F. MANERY AND L. PINTERIC

Department of Biochemistry, University of Toronto, Toronto (Canada)

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SUMMARY

1. Two distinct, membrane-bound, ATP-hydrolysing enzymes have been isolated from the same homogenate of frog skeletal muscle. Both are stimulated by Mg^{2+}; one shows virtually no activity without Na^+ and K^+ and the other is unresponsive to the monovalent cations. We have tentatively concluded that the ($Na^+ + K^+$)-dependent ATPase is bound to fragments of plasma membrane and the ($Na^+ + K^+$)-independent ATPase to fragments of sarcoplasmic reticulum.

2. The ($Na^+ + K^+$)-stimulated Mg^{2+}-ATPase (($Na^+ + K^+$)-Mg^{2+}-ATPase) was obtained by exposing a low-speed pellet (900 $\times$ *g*) from a coarse homogenate to high salt concentrations, followed by extensive washing and by differential centrifugation procedures. The enzyme found in the final pellet (105000 $\times$ *g*) was purified many-fold by density gradient centrifugation.

3. The highest specific activity of the purified ($Na^+ + K^+$)-Mg^{2+}-ATPase was 600 μmoles of P_i liberated per mg nitrogen per h. Ouabain inhibited the activity of this preparation by 94%. The ATPase activity was synergistically stimulated by $Na^+ + K^+$, the optimum concentrations being 105 mM Na^+ and 45 mM K^+. A broad optimum pH range of 7.3–7.7 was found. Ca^{2+} at an added concentration of 2 mM inhibited the total ATPase activity by about 50%.

4. The ($Na^+ + K^+$)-independent Mg^{2+}-ATPase was found in a microsomal fraction (44000 $\times$ *g*). After purification on a density gradient the enzyme had a specific activity of 300–400 μmoles of P_i liberated per mg nitrogen per h. Ca^{2+} activated the ATPase to an even greater degree than Mg^{2+} did.

5. There was no inhibition by ouabain and little or no stimulation by Na^+ and/or K^+ of this Mg^{2+} (or Ca^{2+}) stimulated ATPase (Mg^{2+} (or Ca^{2+})-ATPase). Attempts were made to decrease the activity of the Mg^{2+}-ATPase and thereby accentuate any ($Na^+ + K^+$)-Mg^{2+}-ATPase activity which might be present. The Mg^{2+}-ATPase was unaffected by deoxycholate but was markedly reduced by urea, NaN_3 and high salt concentrations; there was no evidence whatsoever of the ($Na^+ + K^+$)-Mg^{2+}-ATPase.

6. Examination with the electron microscope showed that the ($Na^+ + K^+$)-Mg^{2+}-ATPase and the Mg^{2+} (or Ca^{2+})-ATPase preparations were entirely membranous

Abbreviations: ($Na^+ + K^+$)-Mg^{2+}-ATPase, ($Na^+ + K^+$)-stimulated Mg^{2+}-ATPase; Mg^{2+} (or Ca^{2+})-ATPase, Mg^{2+} (or Ca^{2+})-stimulated ATPase.

and were indistinguishable. There was little evidence in either preparation of mitochondria, inner or outer mitochondrial membranes, rough endoplasmic reticulum, collagen, actin or myosin fibrils.

7. When treated with saponin both preparations showed the hexagonal pattern characteristic of artificial and natural membranes which have an appreciable cholesterol content. The cholesterol and phospholipid concentrations were determined.

8. Cytochrome *c* oxidase was not present in the purified $(Na^+ + K^+)$-Mg^{2+}-ATPase preparation but might have been a minor contaminant of two of the three bands containing the purified Mg^{2+} (or Ca^{2+})-ATPase.

9. Both preparations showed some NADH–diaphorase activity and 5'-nucleotidase activity. Hence these enzymes did not distinguish between the two types of membranes.

INTRODUCTION

From a variety of studies the suggestion has been made that in skeletal muscle as in other tissues, the maintenance of high intracellular K^+ and low Na^+ concentrations depends on the operation of an active transport process which is inhibited by ouabain[1–3]. If active cation transport in skeletal muscle is linked to the 'cation-transport ATPase" characterized by SKOU[4], then it is imperative that a highly active $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase ($(Na^+ + K^+)$-Mg^{2+}-ATPase) be found in muscle.

Difficulty has been encountered in demonstrating a $(Na^+ + K^+)$-Mg^{2+}-ATPase in muscle preparations because this enzyme is not easily separated from the highly active Mg^{2+}-ATPases in muscle which show variable sensitivities to Na^+ or K^+. In 1962 SKOU[5] reported the presence of low and inconsistent $(Na^+ + K^+)$-Mg^{2+}-ATPase activities in rabbit striated and cardiac muscle. Others[6–9] reported stimulation of a Mg^{2+}-ATPase by monovalent cations, amounting to about 30 % of the total ATPase activity, in microsomes from deoxycholate-treated homogenates of cardiac tissue.

A low $(Na^+ + K^+)$-Mg^{2+}-ATPase activity in aqueous homogenates of frog skeletal muscle of about 15 % of the total ATPase was obtained by BONTING *et al.*[10] in 1962. Subsequently they reported a 28 % stimulation by $Na^+ + K^+$ in an aged microsomal fraction from deoxycholate-treated human skeletal muscle[11]. Their earlier results[10] could not be repeated by DUGGAN[12] or by ourselves. On the other hand SAMAHA AND GERGELY[13] obtained a greater stimulation (90 %) of the Mg^{2+}-ATPase by $Na^+ + K^+$ of deoxycholate-treated human muscle but the total specific activity (*i.e.* per mg nitrogen or protein) was quite low.

Several investigators[12,14,15] have reported the presence of a Mg^{2+}-ATPase in microsomal preparations of frog and rabbit skeletal muscle which was stimulated independently by Na^+ and by K^+ and not inhibited by ouabain. It seems likely that these preparations contained a mixture of membranes arising from a variety of sites in the muscle (sarcoplasmic reticulum, plasma membranes and mitochondria) each with its characteristic ATPase activity. The most active muscle $(Na^+ + K^+)$-Mg^{2+}-ATPase (94 % stimulation by $Na^+ + K^+$) was obtained by MATSUI AND SCHWARTZ[16] from calf heart treated with 6 M NaI. The pretreatment of muscle homogenates with concentrated salt solutions appears to be the most effective method of revealing the $(Na^+ + K^+)$-Mg^{2+}-ATPase.

Applying modification of the high salt treatment used by KONO AND COLOWICK[17], KOKETSU *et al.*[18] and H. MATSUI AND L. G. ABOOD (personal communication) to fractions from a muscle homogenate, followed by a final density gradient purification, a highly active ($Na^+ + K^+$)-Mg^{2+}-ATPase has been prepared from frog skeletal muscle. The morphological purity and membranous nature of the preparation was assessed using the electron microscope.

A second membrane-bound Mg^{2+}-ATPase, not stimulated by $Na^+ + K^+$, was also isolated from the same muscle homogenate. These two morphologically similar, but biochemically different, Mg^{2+}-ATPases are compared in this paper with respect to method of isolation, influence of cations on the ATPase activity, the cholesterol and phospholipid content, the presence of other identifying enzymes and the morphological characteristics.

METHODS

Materials

For all procedures reagent-grade chemicals were used, also distilled water which had been re-distilled in an all-Pyrex distilling apparatus; frequently the water was again deionized. All water was stored in nalgene bottles. Sorvall RC2B and Spinco L2 and L2-65 preparative ultracentrifuges were used, also model B-60 from the International Equipment Co.; the rotars used are indicated below. Tris ATP and Na_2 ATP were supplied by Sigma Chemical Co.

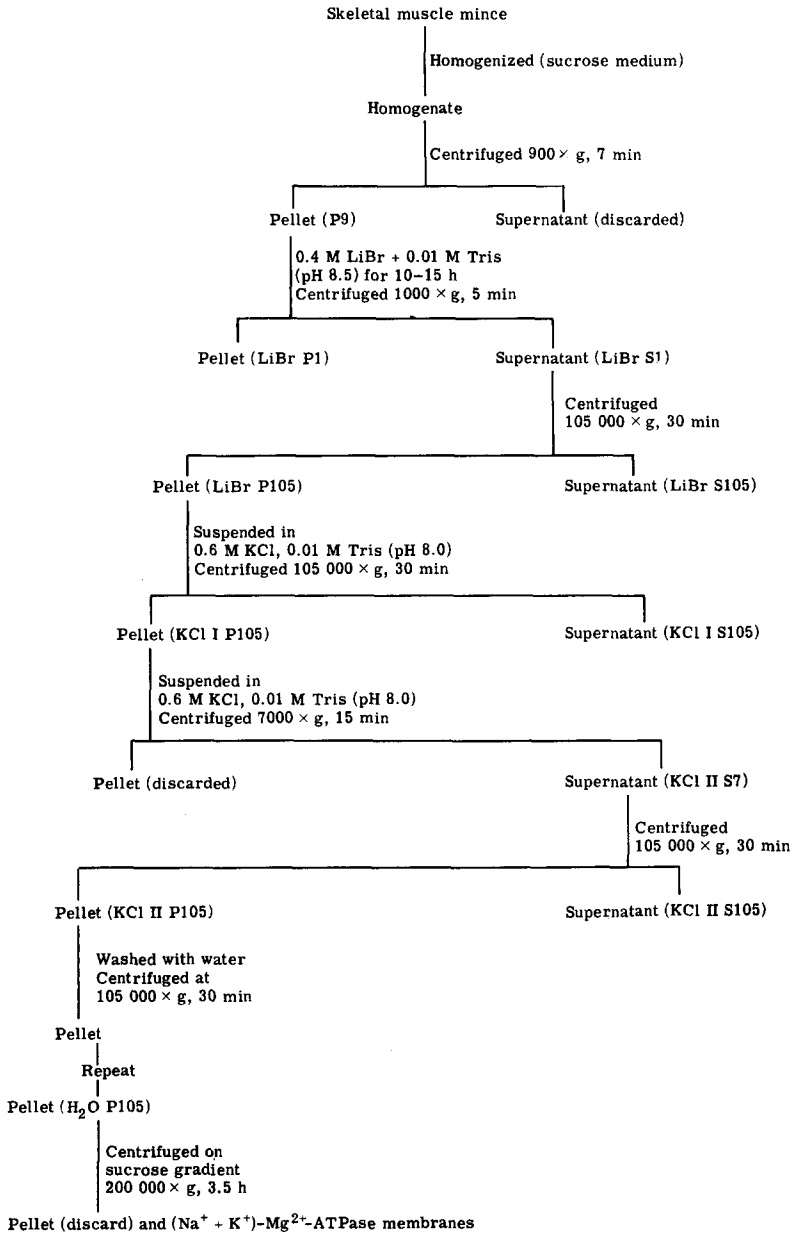
Preparation of ($Na^+ + K^+$)-Mg^{2+}-ATPase

The isolation procedure has been outlined in Scheme 1. Bullfrogs (*Rana catesbiana*) were most frequently used although sometimes muscle was obtained from *Rana pipiens*. The animals were pithed and the hind legs removed. All of the leg muscle was quickly placed in a solution referred to as sucrose medium which contained 0.25 M sucrose, 0.2 mM Na_2 EDTA, 0.1 M Tris buffer (pH 7.6); the solution was cooled in ice. After removal of much of the connective tissue, blood vessels and large nerves, the muscle was sliced with scalpel blades on a perspex petri dish into pieces less than 5 mm^2. The pieces were immediately placed in a volume of sucrose medium (in ice) equal to the muscle weight. All subsequent operations were carried out at 0–4°.

The sliced muscle was homogenized in 3–5 vol. (3–5 times the original muscle weight) of sucrose medium in a loose fitting Potter–Elvehjem homogenizer (radial clearance 0.2 mm) for five complete strokes. The homogenate was rehomogenized with 2 vol. (2 times muscle weight) of sucrose medium in a tight fitting homogenizer (radial clearance 0.1 mm) for two complete strokes.

The homogenate was centrifuged at 900 × *g* for 7 min. The pellet (P9) containing cell segments was suspended by swirling in an equal volume of cold water. To this was added a volume of 0.8 M LiBr in 0.02 M Tris buffer (pH 8.5) equal to the volume of the water. The suspension was slowly stirred at 0–1° for 10–15 h. A viscous suspension resulted which was centrifuged at 1000 × *g* for 5 min. The pellet (LiBr P1) was discarded and the supernatant (LiBr S1) centrifuged at 105000 × *g* for 30 min. A small pellet (LiBr P105) and a clear viscous supernatant were obtained (LiBr S105).

The pellet (LiBr P105) was suspended in a volume of 0.6 M KCl in 0.01 M Tris



Scheme 1. Procedure for the isolation and purification of ($Na^+ + K^+$)-Mg^{2+}-ATPase (0–4°). Sucrose medium: 0.25 M sucrose, 0.2 mM Na_2EDTA, 0.01 M Tris (pH 7.6) at 0°. Sucrose gradient: 15–35% sucrose, 0.5 mM H_4EDTA, 4 mM Tris (pH 8.4).

buffer (pH 8.0) equal to that of the original muscle weight. The homogenate was centrifuged at 105000 × *g* for 30 min. The light brown pellet (KCl I P105) was rehomogenized in the same KCl–Tris medium using half the previous volume. Centrifugation at 7000 × *g* for 15 min provided a supernatant (KCl II S7) which was

centrifuged at 105000 × *g* for 30 min. The pellet (KCl II P105) was washed twice with cold water by homogenizing it and centrifuging at 105000 × *g* for 30 min. A light brown pellet (H_2O P105) was obtained which was homogenized in a small volume of water. This aqueous suspension, called the input, was layered on a continuous sucrose gradient containing 15–35 % sucrose (d = 1.06–1.132) with 0.5 mM H_4EDTA and 4 mM Tris buffer (pH 8.4). The sucrose gradient was made using a Buchler gradient mixer. The gradient tubes were centrifuged at 200000 × *g* for 3.5 h at 1° (Spinco SW-41 rotor). After centrifugation the tubes were placed in a Buchler tube-piercing device connected to the flow-cell of a recording spectrophotometer. The gradient was pumped out with a polystatic pump. The absorbance at 280 nm was recorded and appropriate fractions collected. The fractions were diluted to 3 vol. with cold water, mixed by inversion and centrifuged at 165000 × *g* for 45 min. The light brown pellets were suspended by homogenization in a small volume of cold water. The preparation was divided among a number of vials frozen rapidly, and stored at −20 or −80° as the final purified enzyme preparation.

Preparation of ($Na^+ + K^+$)-independent (Mg^{2+}-ATPase)

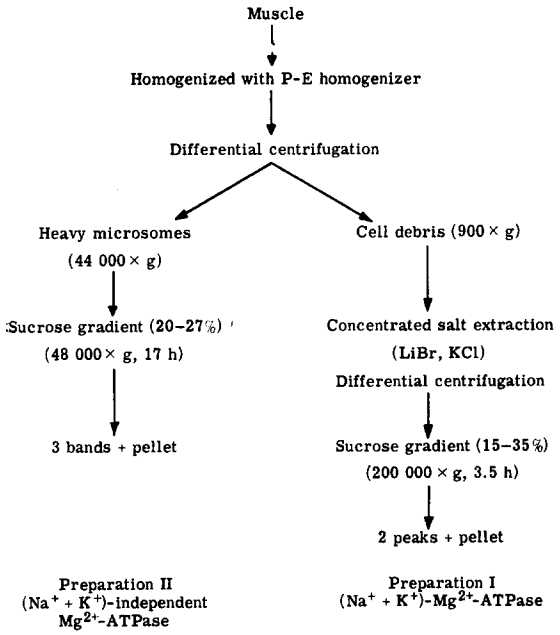
For the final purified preparation frog muscle homogenates prepared as in Scheme 1 were centrifuged at 900 × *g* for 7 min and the pellet discarded or used to prepare the ($Na^+ + K^+$)-Mg^{2+}-ATPase. The supernatant was centrifuged at 9000 × *g* for 10 min to bring down mitochondria, and the supernatant obtained was centrifuged at 44000 × *g* for 60 min which provided a heavy microsomal pellet. The pellet was washed twice (0.115 M Tris buffer (pH 8.2) containing 0.2 mM H_4EDTA) by homogenization followed by centrifugation at 105000 × *g* for 30 min. The pellet was washed once in 0.25 M sucrose containing 1 mM $MgCl_2$ and 1 mM Tris buffer (pH 8.5) and, after being suspended in this medium was layered on a discontinuous sucrose gradient 20–23.5–27 % (d = 1.082–1.095–1.106) and centrifuged at 5000 × *g* for 30 min followed by 48000 × *g* for 17 h at 3° (Spinco SW-25.2 rotor). After centrifugation the bands were located by vertical illumination with a microscope light and were removed with a U-tipped syringe. The fractions were diluted to 3 vol. with cold water, mixed by inversion and centrifuged at 105000 × *g* for 30 min. The pellets were suspended in sucrose medium by homogenization, divided into a number of vials and stored at −20°.

A comparison of the procedures for obtaining the two enzymes is shown in Scheme 2.

Enzyme assays

ATPase (ATP-phosphohydrolase EC 3.6.1.3 and EC 3.6.1.4) assays were carried out at 37° in a total volume of 2.0 ml containing 3 or 5 mM $MgCl_2$, 2 or 3 mM Na_2ATP or Tris ATP, 100 mM NaCl, 20 mM KCl and 20–50 mM Tris buffer (pH 7.5), measured at room temperature. Usually 1 mM H_4EDTA was added to the assay medium. Ouabain (0.25 mM) was used where indicated. The reaction was initiated by adding the membrane suspension to the assay tubes after equilibration at 37°. After 30 min the reaction was stopped by adding 1.0 ml of 15 % trichloroacetic acid at 0°. The P_i released did not exceed 8 % of the terminal phosphate group of ATP.

Cytochrome *c* oxidase (EC 1.9.3.1) was assayed according to COOPERSTEIN AND LAZAROW[19] and NADH-diaphorase (EC 1.6.99.3) according to KAMAT AND WALLACH[20].



Scheme 2. Summary of preparative procedures.

5'-AMP nucleotidase (EC 3.1.3.5) activity was determined qualitatively by paper chromatography and quantitatively by measuring the P_i liberated from AMP using the assay medium described for the ATPase measurement.

Chemical analyses

Cholesterol and phospholipid were measured using the methods of STADTMAN[21] and FOLCH *et al.*[22]. P_i from the ATPase assay was determined as described by SWANSON *et al.*[23], phospholipid phosphate according to BARTLETT[24] and protein according to LOWRY *et al.*[25] using a standard solution of crystalline bovine albumin from Armour Pharmaceutical Co.

Electron microscopy

For morphological studies the different preparations were routinely examined in a Philips EM 200 by negative staining procedures[26] with 1 % phosphotungstic acid (pH 6.0–7.0). Thin sections of some of the pellets were also examined. Saponin (0.025 %) in distilled water was allowed to react with membrane fragments for 2 min at room temperature before phosphotungstic acid was added and the sample was removed for examination in the electron microscope.

RESULTS

$(Na^+ + K^+)$-Mg^{2+}-ATPase

Many attempts were made initially to obtain tubules of pure sarcolemma. In the methods used the muscle fibres were cut into segments which were then exposed to various treatments designed to remove the cytoplasm. All attempts based on

suggestions in the literature[17,27,18], failed despite the use of LiBr, KCl, trypsin and NaOH to aid in the emptying of the tubular fragments. Examination of the preparations using phase-contrast microscopy and the electron microscope showed many segments containing mitochondria and actin and myosin myofibrils. In addition the cell segments were always aggregated; removal of nucleic acid or of Ca^{2+} failed to prevent aggregation. Because of the aggregation and incomplete emptying of the sarcolemmal tubules this approach was abandoned in favour of isolation of membrane fractions from a muscle homogenate as described in Schemes 1 and 2.

In two preliminary experiments each fraction of Scheme 1 was assayed for $(Na^+ + K^+)$-Mg^{2+}-ATPase activity and protein nitrogen to indicate their distribution during the isolation procedure (Table I). Although there is variation between the preparations and the specific activities are low compared with later preparations, it is obvious that there is no ouabain inhibition until after the second KCl extraction

TABLE I

DISTRIBUTION OF $(Na^+ + K^+)$-Mg^{2+}-ATPase IN FRACTIONS OBTAINED FROM MUSCLE AFTER SALT TREATMENT

The assay media contained the following: 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 1.0 mM H_4EDTA, 2 mM Na_2ATP and 50 mM Tris buffer (pH 7.5) with ouabain (0.25 mM) where indicated. The activity values are given as the mean of three individual incubations; the difference from the mean of any individual value was not greater than 4%. The results are from two individual preparations (1, 2) of three frogs per preparation.

Fraction (Scheme 1)	*Total nitrogen (mg)*		*Total ATPase (μmoles P_i per mg nitrogen per h)*		*ATPase + ouabain (μmoles P_i per mg nitrogen per h)*		*Ouabain inhibition (%)*	
Prep.:	*1*	*2*	*1*	*2*	*1*	*2*	*1*	*2*
LiBr P1	79.8	198	6.00	13.2	6.00	13.2	0	0
LiBr P105	10.0	72.2	22.8	46.8	21.7	45.6	0	0
LiBr S105	235	48.7	0.0	0.0	0.0	0.0	0	0
KCl I S105	3.4	23.7	0.0	0.0	0.0	0.0	0	0
KCl II S7	3.9	8.7	13.2	43.3	14.4	10.9	0	75
KCl II P105	0.6	1.2	69.6	128	15.7	60.0	77.8	53.3
KCl II S105	3.4	6.0	18.7	0.0	10.8	0.0	40.6	0

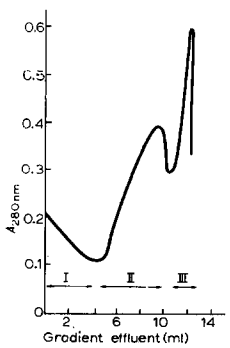


Fig. 1. A representative absorbance profile of the $(Na^+ + K^+)$-Mg^{2+}-ATPase preparation (H_2O P105, Scheme 1) after sucrose gradient (15–35%) centrifugation at 200000 × *g* for 3.5 h.

(KCl II S7). The highest total ATPase activity (78 and 53 % of which was ouabain-sensitive) was found in the high-speed fraction (105000 × *g*) after the second KCl treatment (KCl II P105) suggesting that the enzyme was associated with small membrane fragments.

The effectiveness with which LiBr and KCl removed most of the muscle protein is also shown in Table I (Column 2). Pellet P9 contained the sum of the amounts of nitrogen in the first three fractions (LiBr P1, LiBr P105, and LiBr S105); this sum amounted to 305 and 319 mg in Preparations 1 and 2, respectively. The insoluble fraction LiBr P105 which is carried forward represented about 3 % of the total protein in P9 in one preparation and 23 % in the other. Thus LiBr "solubilized" more than 75 % of the protein in P9. The protein content of the $(Na^+ + K^+)$-Mg^{2+}-ATPase fraction (KCl II P105) obtained after KCl extraction was about 0.3 % of that in Pellet P9.

Three fractions were obtained by centrifuging Pellet H_2O P105 (Scheme 1) on a linear sucrose gradient (15–35 %) at 200000 × *g*. Fig. 1 shows the absorbance profile most frequently obtained. The first fraction which contained approx. 50 % of the protein applied to the gradient, was part of the small pellet present in the tubes after centrifugation. The protein content of Fractions II and III varied between 15 and 25 % of that applied to the gradient.

The results of ATPase assays carried out on the fractions before (*i.e.* the inputs) and after (*i.e.* I, II and III) the sucrose gradient purification are shown in Table II.

TABLE II

PURIFICATION OF THE $(Na^+ + K^+)$-Mg^{2+}-ATPase PREPARATION

The final pellet, H_2O P105 of Scheme 1, which is called the input, was placed on the top of a continuous sucrose gradient (15–35 %) and centrifuged for 3.5 h at 200000 × *g*. Fractions I, II and III obtained in the gradient tube are shown in Fig. 1. The ATPase activities of the input and each fraction are given. The assay media contained 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM Na_2ATP and 50 mM Tris buffer (pH 7.5) with ouabain (0.25 mM) as indicated. The results from 3 representative individual preparations of three frogs per preparation are presented. Each figure is the mean of at least three incubations; the difference from the mean for an individual ATPase value was not greater than 5 %. Preparation 3 showed a somewhat different protein profile in the gradient tubes; the lightest fraction (III in other preparations) was not tested.

Gradient fractions	*Total ATPase (μmoles P_i per mg nitrogen per h)*	*ATPase + ouabain (μmoles P_i per mg nitrogen per h)*	*Ouabain inhibition (%)*
Prep. 1			
Input	128	13.2	90
I	42	24.1	43
II	300	43.3	86
Prep. 2			
Input	128	60.0	53
I	35	35.3	0
II	383	49.2	87
III	50	21.5	57
Prep. 3			
Input	61	11.8	81
II	484	27.5	94
III	599	34.7	94

Representative types of preparations are shown. The highest specific activities (Column 1), ranging from 300–600 μmoles P_i per mg nitrogen per h, were found in Fraction II or III of each preparation (Fig. 1). The total ATPase activities listed in the table show that purification on the gradient was achieved by separating the $(Na^+ + K^+)$-Mg^{2+}-ATPase from enzymically inactive protein. Fraction I must have contained much inactive protein since its specific activity was low; but some ouabain-inhibited ATPase was also present (Column 4). The low specific activity of the input in every preparation can be attributed to this inert protein. In Preparations 1, 2 and 3 nearly all of the ATPase in the input was inhibited by ouabain (Column 4) showing that there was little or no contamination with an Mg^{2+}-ATPase. When the ouabain inhibition of the activity in the input was only 50 % (Preparation 2) a marked increase to 87 % (Fraction II) occurred during the sucrose gradient centrifugation. Either of these two types of preparations were usually obtained. In these preparations the $(Na^+ + K^+)$-independent Mg^{2+}-ATPase was obviously removed leaving a relatively pure $(Na^+ + K^+)$-Mg^{2+}-ATPase. A 10-fold purification of the $(Na^+ + K^+)$-Mg^{2+}-ATPase was achieved in Preparation 3 by the removal of inactive protein (Column 1). The activity of this purified enzyme was 3–4 times as high as that obtained by MATSUI AND SCHWARTZ[16] (176 μmoles P_i per mg nitrogen per h) from a preparation of beef heart after treatment with NaI and deoxycholate.

The activity of the most highly purified enzyme described in Table II (Preparation 3) was tested over a pH range of 6.7–8.0 (measured at room temperature). There was no detectable difference over the range from 7.3 to 7.7; at 6.7 and 8.0 a decrease of 14.4 and 13.0 %, respectively, was found.

The synergistic stimulation by Na^+ and K^+ of the purified ATPase (Fraction II from the sucrose gradient) is shown in Fig. 2; the Na^+ and K^+ concentrations were

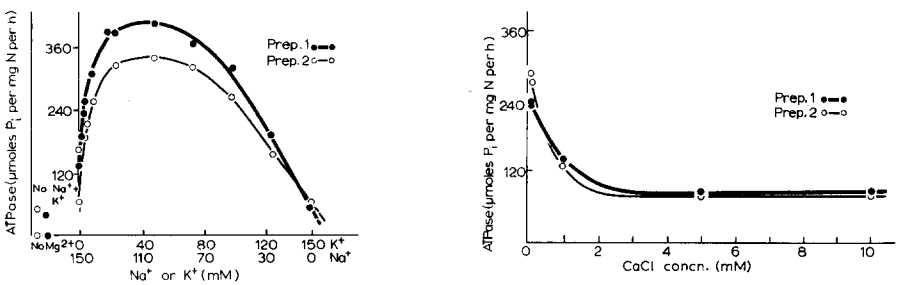


Fig. 2. The effect of different Na^+ and K^+ concentrations on the purified $(Na^+ + K^+)$-Mg^{2+}-ATPase. The assay media contained different concentrations of Na^+ and K^+ as indicated, 5 mM $MgCl_2$, 1 mM H_4EDTA, 2 mM Tris ATP and 50 mM Tris buffer (pH 7.5). The results from two individual preparations of three frogs per preparation are presented. Each point is the mean of six individual incubations for Prep. 1 and three incubations for Prep. 2. The difference of any individual value from the mean for both preparations was not greater than 3 %. The lower two points on the left which show no activity were obtained in the presence of Na^+ (100 mM) and K^+ (20 mM) but without Mg^{2+}; the upper two points show the low activity with Mg^{2+} and neither Na^+ not K^+.

Fig. 3. The effect of Ca^{2+} on the purified $(Na^+ + K^+)$-Mg^{2+}-ATPase. The assays were carried out in the presence of 100 mM Na^+, 20 mM K^+, 5 mM $MgCl_2$, 1 mM Na_2EDTA, 2 mM Na_2ATP and 25 mM Tris buffer (pH 7.5). Different amounts of $CaCl_2$ were added to give the concentrations indicated. The results from two preparations of three frogs per preparation are presented. Each point represents the mean of three individual incubations. The difference of any individual value from the mean for both preparations was not greater than 3 %.

varied inversely in the assay medium with the sum always being 150 mM. There was no ATPase activity in the absence of Mg^{2+} even when Na^+ and K^+ were present. A low Mg^{2+}-ATPase activity (42 and 54 μmoles P_i per mg nitrogen per h) was obtained in the presence of MgATP but without Na^+ and K^+. With only Na^+ (150 mM) present, a slight (1.3–3-fold) increase in the basic ATPase (Mg^{2+}-ATPase) was obtained. If, however, only K^+ (150 mM) was present the ATPase activity was the same as that with Mg^{2+} alone. With both Na^+ and K^+ in addition to Mg^{2+} the enzyme activity increased and reached a maximum value of 406 and 341 μmoles P_i per mg nitrogen per h in the two preparations, respectively, at 105 mM Na^+ and 45 mM K^+.

Inhibition of the (Na^+ + K^+)-Mg^{2+}-ATPase by ouabain and Ca^{2+} has been described as a characteristic of the enzyme[28]. The effect of ouabain (0.25 mM) on the total ATPase activity has already been shown in Table II (Column 4). The results of Ca^{2+} inhibition of the purified ATPase preparation are presented in Fig. 3. Maximal inhibition was obtained with a $CaCl_2$ concentration of 2 mM. There is still appreciable ATPase activity at this concentration of $CaCl_2$. The ouabain inhibition of the two preparations was 86 and 79 % of the total activity while the Ca^{2+} inhibition was only 55 and 56 %, respectively; this percentage inhibition by Ca^{2+} is undoubtedly the resultant of a stimulation of the (Na^+ + K^+)-independent portion of the ATPase and an inhibition of the (Na^+ + K^+)-dependent-ATPase.

A (Na^+ + K^+)-Mg^{2+}-ATPase with the characteristics just described has frequently been suggested as a marker for the plasma membrane. Hence, we tentatively conclude that the membranes containing this enzyme are fragments of the plasma membrane of muscle.

(Na^+ + K^+)-independent Mg^{2+}-ATPase

Before the (Na^+ + K^+)-Mg^{2+}-ATPase was completely isolated and purified the 44000 × *g* fraction (see METHODS and Preparation II, Scheme 2) was being further purified and examined for morphological purity (with the electron microscope) and for ATPase activity. After washing, the suspended pellet was layered on a variety of sucrose gradients—45–35–20 % (discontinuous), 35–20 % (linear) and 27–23.5–20 % (discontinuous). The bands obtained from the shallow discontinuous gradient were completely membranous and had the highest ATPase activity. Two main bands were obtained on this sucrose gradient at the 20–23.5 % and 23.5–27 % sucrose interfaces. There was, however, also a faint band at the 8–20 % sucrose interface. A heavy gelatinous pellet always formed below the 27 % sucrose layer.

To determine whether or not these membranous preparations contained (Na^+ + K^+)-Mg^{2+}-ATPase the effect of cations and ouabain on the ATPase activity present in the three bands was determined (Table III). $MgCl_2$, $CaCl_2$ and Tris ATP were added to give a series of concentrations for each compound as follows: 0.2, 0.5, 1.0, 3.0 and 10.0 mM. Since ATP chelates both of the divalent cations the concentrations of the MgATP and CaATP complexes (believed to be the enzyme substrates[29]) were calculated using $15000/M$ for the formation constant of MgATP and $6600/M$ for CaATP; these are the formation constants calculated by O'SULLIVAN AND PERRIN[30] for MgATP and CaATP in solutions in which physiological conditions are simulated. The calculated final concentrations (mM) of MgATP used were 0.11, 0.35, 0.77, 2.58 and 9.22, and of CaATP were 0.09, 0.29, 0.68, 2.4, and 8.84.

At each concentration of $MgCl_2$ and Tris ATP (and of $CaCl_2$ and Tris ATP) the

TABLE III

THE EFFECT OF CATIONS AND OUABAIN ON THE Mg^{2+}-ATPase AND Ca^{2+}-ATPase ACTIVITY OF FROG MUSCLE MEMBRANE PREPARATIONS

The assay media contained the concentrations of MgATP or CaATP indicated and 100 mM NaCl, 20 mM KCl and 25 mM Tris buffer (pH 7.5) unless otherwise stated in the table. Ouabain (0.25 mM) was present where indicated. The activities of a pooled preparation of twelve frogs and a single preparation of three frogs (asterisked) are presented as means ± S.D. where four assays were carried out. Figures in parentheses represent the number of assays. One, two or three incubations were done on Band 1 because of lack of material. For brevity similar data from bands exposed to 0.35 and 0.77 mM MgATP (part A) and 0.29 and 0.68 mM CaATP (part B) were omitted from the table; these data likewise showed no ouabain inhibition or ($Na^+ + K^+$)-stimulation of ATPase activity. The ATPase activities at these concentrations of MgATP and CaATP are shown in Fig. 4.

Incubation media	*Ouabain*	*MgATP (mM)*	*CaATP (mM)*	*μmoles P_i per mg nitrogen per h*		
				Band 1	*Band 2*	*Band 3*
A. Mg^{2+}-ATPase activity						
Na^+, K^+, Tris	—	0.11		120 (3)	201 ± 2 (4)	204 ± 9 (4)
Na^+, K^+, Tris	+	0.11		111 (1)	191 ± 12 (4)	213 (1)
Tris 50 mM	—	0.11		165 (2)	222 (2)	195 (2)
Na^+, K^+, Tris	—	2.58		182 ± 14 (4)	250 ± 19 (4)	266 ± 7 (4)
Na^+, K^+, Tris*	—	2.58		188 ± 3 (4)	375 ± 4 (4)	345 ± 11 (4)
K^+, 50 mM Tris*	—	2.58		173 ± 5 (4)	333 ± 6 (4)	336 ± 13 (4)
K^+, 50 mM Tris*	+	2.58		174 ± 4 (4)	336 ± 0 (4)	333 ± 7 (4)
Na^+, K^+, Tris	—	9.22		444 (2)	369 (3)	285 (3)
Na^+, K^+, Tris	+	9.22		459 (1)	274 ± 22 (4)	228 (1)
B. Ca^{2+}-ATPase activity						
Na^+, K^+, Tris	—		0.09	153 ± 6 (4)	222 (3)	240 (3)
Na^+, K^+, Tris	+		0.09	150 (2)	219 (1)	237 (1)
Tris	—		0.09	159 (2)	234 (2)	264 (2)
Na^+, K^+, Tris	—		2.4	311 ± 11 (4)	373 ± 6 (4)	304 ± 8 (4)
Na^+, K^+, Tris	—		8.84	347 ± 28 (4)	441 (3)	468 (3)

activity was determined (a) with $Na^+ + K^+$ in a Tris buffer (25 mM), (b) with $Na^+ + K^+ +$ ouabain in a Tris buffer (25 mM) and (c) in a Tris buffer (50 mM) without $Na^+ + K^+$. If the ATPase was ($Na^+ + K^+$)-dependent the activity should be reduced by ouabain (b) and by the omission of $Na^+ + K^+$ (c). At 0.11 mM MgATP the activity values appeared to be lower in Bands 1 and 2 with Na^+ and K^+ than without. At 0.35 and 0.77 mM MgATP this trend was reversed. Ouabain, however, had no consistent effect on the ATPase in the presence of $Na^+ + K^+$ at all MgATP concentrations studied. We concluded from these data that there was no ATPase activity stimulated by either Na^+ or K^+, no ouabain inhibition and hence no ($Na^+ + K^+$)-Mg^{2+}-ATPase in any of the three bands.

One of the characteristics of the ($Na^+ + K^+$)-Mg^{2+}-ATPase is the inhibition of its activity by Ca^{2+}. Clearly Ca^{2+} do not inhibit the ATPase in the three bands (Table III, B); CaATP can obviously substitute for MgATP as a substrate. The Ca^{2+}-stimulated ATPase (Ca^{2+}-ATPase) activity was not stimulated by $Na^+ + K^+$; in fact the trend was toward a slight inhibitory effect. Ouabain had no effect on the Ca^{2+}-ATPase.

In each one of many preparations from frog and rabbit skeletal muscle Band 1 showed a lower Mg^{2+}-ATPase activity than that of Bands 2 and 3. Frequently the highest activity was in Band 2. A comparison of the activities of the three bands in presented n Fig. 4. The maximum activity was found at about 0.6 mM MgATP and about 0.4

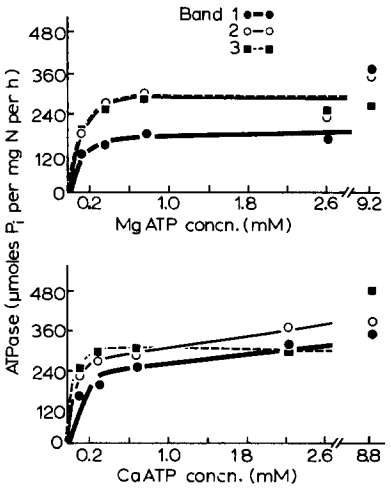


Fig. 4. The effect of varying the MgATP and CaATP concentrations on the Mg^{2+}-ATPase and Ca^{2+}-ATPase activities respectively of the three bands of Prep. II. (See description, Table III.)

TABLE IV

THE EFFECT OF DEOXYCHOLATE ON Mg^{2+}-ATPase (PREPARATION II, SCHEME 2)

Part A: Muscle was homogenized in the presence of 0.05% deoxycholate. No substitution was made when Na^+ and K^+ were omitted from the assay medium. Part B: Membrane bands from the sucrose gradient (27–23.5–20%) were preincubated for 14 h with 0.09% deoxycholate at 0°. When Na^+ and K^+ were omitted from the assay medium the Tris buffer was increased in concentration to 125 mM. If Na^+ and K^+ were omitted virtually identical ATPase activities weren obtained (with and without deoxycholate treatment) as those recorded.

Fraction	*Deoxycholate* (%)	$Na^+ + K^+$, *100 + 20 mM*	*Ouabain, 0.2 mM*	Mg^{2+}-*ATPase* (*μmoles P_i per mg nitrogen per h*)
A. Heavy microsomes				
(33000 × *g*)	0.05	−	−	260 ± 2.2 (4)
	0.05	+	−	305 ± 6.0 (4)
	0.05	+	+	309 ± 4.2 (4)
Light microsomes				
(133000 × *g*)	0.05	−	−	57.7 ± 0.7 (4)
	0.05	+	−	73.1 ± 0.0 (4)
	0.05	+	+	73.4 ± 0.2 (4)
B. Membrane bands				
1	0.09	+	−	117 (2)
2	0.09	+	−	188 (2)
3	0.09	+	−	222 (2)
1	—	+	−	153 (2)
2	—	+	−	238 (2)
3	—	+	−	260 (2)

mM CaATP. The stimulation by Ca^{2+} of this membrane-bound ATPase contrasts sharply with the inhibition of the ($Na^+ + K^+$) ATPase shown in Fig. 3.

Many preparations of the "cation-transport enzyme" from tissues other than muscle have been treated with a variety of reagents which selectively depress the ($Na^+ + K^+$)-independent ATPase and thereby show more clearly the ($Na^+ + K^+$)-dependent activity. The possibility existed that the membranes of Preparation II (Scheme 2) might contain a "masked" ($Na^+ + K^+$)-Mg^{2+}-ATPase activity. It should be noted that in all cases half of the membrane preparation was treated as described and the other half used as a control. Muscle was homogenized in the presence of 0.05 % deoxycholate[5] and various fractions were tested for ($Na^+ + K^+$)-Mg^{2+}-ATPase. Representative data for the heavy microsomal fraction (33000 × *g*) and the light microsomal fraction (133000 × *g*) from this homogenate are presented in Table IV, A. The addition of $Na^+ + K^+$ stimulated the ATPase activities of these fractions by 15–20 %. This increased activity was completely insensitive to ouabain. In other experiments the bands purified on sucrose gradients were exposed to deoxycholate (0.09 %) for 30 min to 14 h at 0°. Deoxycholate treatment for 14 h (Expt. B, Table IV) caused a reduction in activity in Bands 1, 2 and 3. However, there was no evidence of any stimulation by $Na^+ + K^+$ in either the deoxycholate-treated bands or in the untreated controls. Deoxycholate, therefore, did not reveal any "hidden" ($Na^+ + K^+$)-Mg^{2+}-ATPase activity in the microsomal fractions or in the purified membrane bands.

NaN_3 has been used to inhibit ATPase activity believed to be associated with mitochondria which, the authors[13] thought, contaminated their muscle fraction. NaN_3 (0.5 mM) added to the assay medium (Table V) caused a drop of about 50 % in the enzyme activity of both types of microsomes. Again there was no evidence of any ($Na^+ + K^+$)-sensitive activity either in the presence or absence of NaN_3.

Perhaps the most common devise used to decrease the Mg^{2+}-ATPase so that the ($Na^+ + K^+$)-Mg^{2+}-ATPase can be more readily demonstrated is to treat the membranes with urea. The heavy and light microsomal fractions were incubated with 2.0 M urea at 0° for 12 h, centrifuged and washed according to the procedure of POST AND SEN[31]. Urea caused a marked reduction in activity in the heavy microsomes compared with the untreated control, and completely abolished the ATPase activity of the light microsomes (Table V, B). Ouabain had no effect on either the treated or untreated membranes. If ($Na^+ + K^+$)-Mg^{2+}-ATPase had been present urea-treatment would have produced an activity which was inhibited by ouabain.

Finally the data in Table V, C, show the effect of exposing the three purified membrane bands to high salt concentrations. The procedure was similar to that used to prepare the membrane-bound ($Na^+ + K^+$)-Mg^{2+}-ATPase (Scheme 1). After purification on the gradient the pellets from the bands were stirred for 20 h at 0° in LiBr (0.4 M) buffered with Tris buffer (0.01 M) at pH 8.5 followed by centrifugation at 105000 × *g* for 30 min. After washing, the membranes were treated sequentially with 0.6 M KCl in Tris buffer (0.01 M, pH 8.0) and NaI (0.6 M)with H_4EDTA (1 mM). In the representative experiment described in Table V, C, the effectiveness of the salt treatment in depressing the Mg^{2+}-ATPase is clearly demonstrated. The ATPase activity remaining is completely unresponsive to ouabain.

The experiments represented by the data in Tables III, IV and V proved beyond any reasonable doubt that the ($Na^+ + K^+$)-Mg^{2+}-ATPase was not present in any

form in the membranous fraction derived from 44000 × *g* microsomes. The membranes containing this highly purified Mg^{2+}-ATPase (or Ca^{2+}-ATPase) have been tentatively identified as fragments of the sarcoplasmic reticulum.

Enzyme markers

Cytochrome *c* oxidase was shown to be located in the inner mitochondrial membrane[31]. The activity of this enzyme was used to assess the degree of contamination of the two preparations by mitochondria and inner mitochondrial membranes

TABLE V

THE EFFECT OF NaN_3, UREA AND HIGH SALT CONCENTRATIONS ON Mg^{2+}-ATPase (PREPARATION II, SCHEME 2)

Standard deviations are included with the means. Figures in parentheses show the number of enzyme assays. Expt. A: The microsomes were prepared as described in METHODS. NaN_3 was added to the assay medium in the presence or absence of $Na^+ + K^+$; no ouabain was present. Expt. B: Both microsomal fractions were treated with 2.0 M urea for 12 h. $Na^+ + K^+$ were in all assay media with or without ouabain. Expt. C: The salt used for treating the purified bands was 0.4 M LiBr, 0.6 M KCl and 0.6 M NaI (see text).

Fraction	*Treatment*	*$Na^+ + K^+$, 100 + 20 mM*	*Ouabain, 0.25 mM*	*Mg^{2+}-ATPase (μmoles P_i per mg nitrogen per h)*
A. Heavy microsomes	NaN_3, 0.5 mM			
	−	−	−	583 ± 2.9 (4)
	−	+	−	583 (3)
	+	−	−	227 ± 5.1 (4)
	+	+	−	230 ± 5.1 (4)
Light microsomes	NaN_3, 0.5 mM			
	−	−	−	75.6 (2)
	−	+	−	82.8 (2)
	+	−	−	41.4 ± 7.5 (4)
	+	+	−	28.8 (3)
B. Heavy microsomes	Urea, 2 M			
	−	+	−	280 ± 19 (4)
	−	+	+	302 ± 23 (4)
	+	+	−	147 ± 6.1 (4)
	+	+	+	125 ± 12 (4)
Light microsomes	Urea, 2 M			
	−	+	−	77.4 ± 15 (4)
	−	+	+	68.4 ± 12 (4)
	+	+	−	0 ± 0 (4)
	+	+	+	0 ± 0 (4)
C. Membrane bands	Salt			
Band 1	−	+	−	159.0 (2)
	−	+	+	157.0 (2)
	+	+	−	26.5 (3)
	+	+	+	23.5 (3)
Band 2	−	+	−	262 (3)
	−	+	+	260 (3)
	+	+	−	51.3 (3)
	+	+	+	45.6 (3)
Band 3	−	+	−	242 (3)
	−	+	+	240 (3)
	+	+	−	41.9 (3)
	+	+	+	40.5 (3)

(Table VI). Although there is some cytochrome oxidase activity in the fraction put on the gradient (input) in both ATPase preparations the purification procedure removed it almost completely. Fraction II of the first preparation and Band 1 of the second had no activity showing no contamination by inner mitochondrial membranes. Bands 2 and 3 (Expt. B) might have been slightly contaminated but most of the oxidase activity of the input was found in the pellet; this is to be expected since the buoyant density of the inner mitochondrial membranes of liver cells is approx. 1.21 (ref. 32) while a sucrose solution of 27 % has a density of 1.106.

The absence of both inner and outer mitochondrial membranes is confirmed in the section on morphology.

TABLE VI

ACTIVITIES OF CYTOCHROME *c* OXIDASE AND NADH–DIAPHORASE IN THE PURIFIED $(Na^+ + K^+)$-DEPENDENT AND $(Na^+ + K^+)$-INDEPENDENT Mg^{2+}-ATPase PREPARATIONS

The means are given with standard deviations. Figures in parentheses show the number of assays. Expt. A: The activities of two individual preparations (1 and 2) of three frogs per preparation are given. Expt. B: Each figure for oxidase activity is the mean from two individual preparations of three frogs per preparation. Each figure for diaphorase activity is the mean of three individual preparations of four frogs per preparation.

Gradient fractions	*Cytochrome c oxidase (k/mg nitrogen)* *		*NADH–diaphorase (μmoles NADH oxidized per min per mg nitrogen)*	
A. $(Na^+ + K^+)$-Mg^{2+}-ATPase (i.e. plasma membrane)				
	(1)	(2)	(1)	(2)
Input	40.8 (3)	24.0 (3)	3.6 (3)	5.8 (3)
II	0.0 (3)	0.0 (2)	8.6 (3)	10.4 (3)
B. $(Na^+ + K^+)$-independent Mg^{2+}-ATPase (i.e. sarcoplasmic reticulum)				
Input	142 ± 8 (4)		0.36 ± 0.04 (4)	
Band 1	0.0 ± 0 (4)		2.16 ± 0.04 (6)	
Band 2	13.4 ± 1.6 (4)		6.4 ± 1.7 (6)	
Band 3	16.4 ± 3.1 (4)		5.4 ± 2.0 (7)	
Pellet	84.0 ± 1.6 (4)		0.6 ± 0.24 (6)	

* k = rate constant (min^{-1}).

The distribution of the NADH–diaphorase activity has also been presented in Table VI although it was not helpful in distinguishing between the two membranes. This enzyme had been used as a marker for the endoplasmic reticulum of carcinoma cells[20] and, although not a very specific marker, it appeared to be generally associated with intracellular membranes[33]. The specific activity was definitely higher in the fractions of both preparations after purification on the sucrose gradient (*i.e.* Fraction II and Bands 1, 2 and 3 of Table VI) than in the input and heavy pellet suggesting the removal during centrifugation of an inert protein. The non-specific distribution may reflect a similarity between the two membrane fractions or it may result from adsorption of the enzyme to the fragments during the preparative procedure.

Data for 5'-AMP nucleotidase are not presented in detail because it, too, did not distinguish between the plasma membrane and the sarcoplasmic reticulum. The mean activities from three sarcoplasmic reticulum preparations in Bands 1, 2 and

3 were 112, 77 and 57 μmoles P_i per mg nitrogen per h respectively. The input from the plasma membrane preparation also had nucleotidase activity. This enzyme has been used as a marker for the plasma membrane of liver cells, but there is no reason *a priori* to believe that its distribution will be the same in muscle fibres as in liver cells.

Chemical composition

Because of the marked response of the membrane fragments of both ATPase preparations to saponin (see *Morphology of the ATPase preparations*) the cholesterol and phospholipid contents were determined (Table VII). The cholesterol concentration of the $(Na^+ + K^+)$-Mg^{2+}-ATPase preparation was approximately the same as that reported for "plasma membranes" of rat liver[34] but the phospholipid content was more than twice as high. Because of the high phospholipid content the cholesterol to phospholipid molar ratio was lower (0.12–0.26) than that (0.5–1.3) reported[34–36] for plasma membranes of liver, kidney, erythrocytes and intestinal microvilli. In the Mg^{2+}-ATPase ($(Na^+ + K^+)$-independent) preparation the cholesterol and phospholipid content decreased from Band 1 to 3 reflecting a gradual increase in the buoyant density from Bands 1 to 3. It is interesting to note that the cholesterol/phospholipid ratio of the inner mitochondrial membranes of liver (guinea pig) was much lower (0.015–0.045)[37] than that of frog muscle preparations.

Morphology of the ATPase preparations

The electron microscope was used consistently at all stages in this work to observe the morphological components of each fraction. Mitochondria, actin, myosin, collagen, rough endoplasmic reticulum with attached ribosomes and inner mitochondrial membranes have morphological characteristics which are readily recognized. Few, if any, of these were found in the purified ATPase preparations as the electron micrographs described below will show. Unfortunately it is not possible to distinguish between the plasma membrane, sarcoplasmic reticulum, smooth endoplasmic reti-

TABLE VII

CHOLESTEROL AND PHOSPHOLIPID CONCENTRATIONS OF THE PURIFIED $(Na^+ + K^+)$-Mg^{2+}-ATPase AND $(Na^+ + K^+)$-INDEPENDENT Mg^{2+}-ATPase PREPARATIONS

Means ± S.D. are given with the numer of determinations in brackets. In Expts. A and B two individual preparations from four frogs per preparation were used.

Gradient fractions	*Cholesterol (mg/mg protein)*		*Phospholipid (mg/mg protein)*		*Cholesterol: phospholipid (molar ratio*)*	
A. $(Na^+ + K^+)$-Mg^{2+}-ATPase i.e. plasma membrane)						
	(1)	(2)	(1)	(2)	(1)	(2)
Fraction II	0.28 (3)	0.35 (3)	1.43 (2)	1.44 (2)	0.11	0.13
B. $(Na^+ + K^+)$-independent Mg^{2+}-ATPase (i.e. sarcoplasmic reticulum)						
Band 1	0.72 ± 0.19 (4)		1.68 ± 0.09 (4)		0.26	
Band 2	0.35 ± 0.04 (4)		0.96 ± 0.01 (4)		0.21	
Band 3	0.08 ± 0.01 (4)		0.36 ± 0.01 (4)		0.12	

* Molecular weight of phospholipid was assumed to be 700 and cholesterol 387.

culum, outer mitochondrial membranes and nuclear membranes with negative staining.

Thin sections of whole muscle were used to quantitate the yield of the various fractions; no rough endoplasmic reticulum was found except in connective tissue cells. Thin sections of the fraction pellets revealed no identifying markers under electron microscopic examination.

The membranous nature of the purified $(Na^+ + K^+)$-Mg^{2+}-ATPase preparation is shown in Fig. 5. The membranes vary in size, shape and surface granularity. Only occasionally were fragments of inner mitochondrial membranes observed. The irregular shape of the vesicles was probably caused by the exposure to concentrated salt solutions.

The purified $(Na^+ + K^+)$-independent Mg^{2+}-ATPase was also associated with membrane fragments (Figs. 6–8). In Bands 1–3 the membranes appeared to have smooth surfaces; in general the fragments were larger than those characteristic of the $(Na^+ + K^+)$-dependent fraction (Fig. 5) and showed less tendency to aggregate. The vesicles in Band 1 (Fig. 6) seemed to be composed of two morphologically different

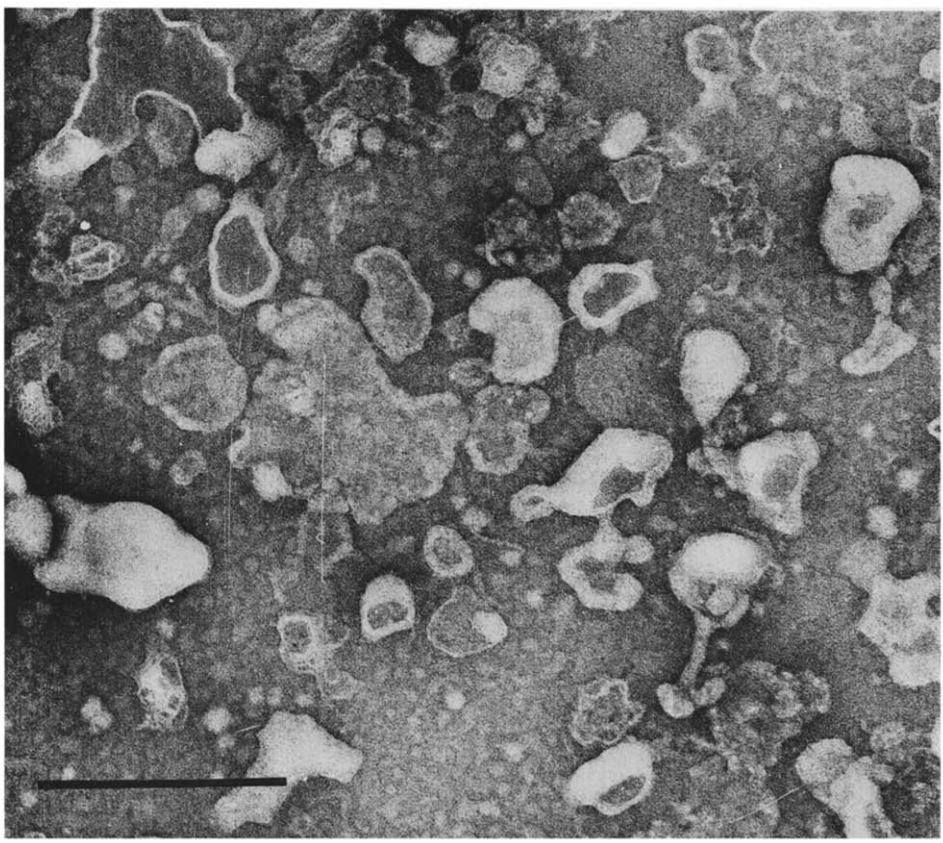


Fig. 5. A representative electron micrograph of the membrane fragments in the purified $(Na^+ + K^+)$-Mg^{2+}-ATPase preparations, *i.e.* after gradient centrifugation; they were negatively stained with phosphotungstate (1 %) at pH 7.0. The membranes were prepared by Scheme 1 and are, those found in Fraction II, Fig. 1, and Table II. The marker equals 5000 Å.

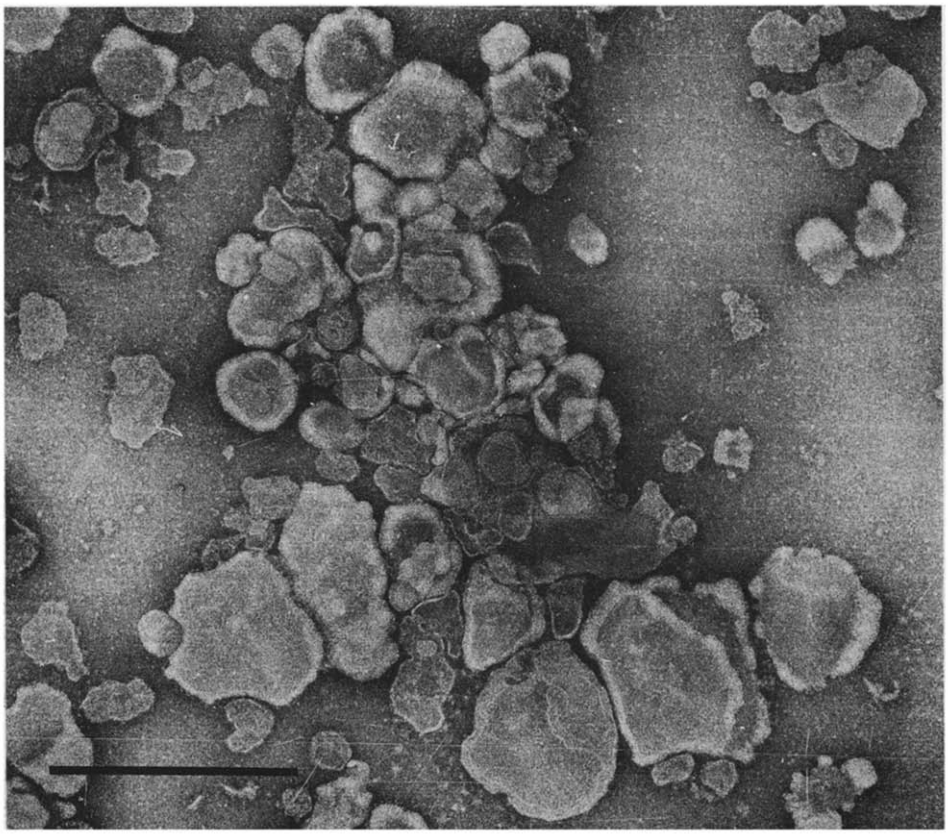


Fig. 6. Band 1. See legend Fig. 8.

types, one with a smooth thin border and the other with a raised thick border. Band 2 (Fig. 7) contained predominantly the latter type and, occasionally, strands of actin and myosin. Somewhat more actin and myosin strands were found in Band 3 (Fig. 8). This fraction was characterized by having many more vesicular extrusions than in Bands 1 and 2, caused probably by removal into water from the high sucrose concentration at the bottom of the gradient. The pellet obtained after gradient centrifugation of the $(Na^+ + K^+)$-independent Mg^{2+}-ATPase consisted of intact and partially disrupted mitochondria, inner mitochondrial membranes, actin, myosin and collagen filaments—all in a highly aggregated state.

In 1962 DOURMASHKIN *et al.*[38] had shown that when external cell membranes were treated with saponin an ordered structure resembling a hexagonal lattice was easily seen with the electron microscope after negative staining. Similar geometric patterns were obtained when negatively-stained preparations of mixtures of lecithin, cholesterol and saponin were investigated by electron microscopy[39–41]. It was proposed that globular saponin–cholesterol micelles of 30–35 Å in diameter aggregated and formed hexagonal rings. These were seen as light rings surrounding a dark hole which had a diameter of 80 Å. The micellular subunits between two adjacent 80-Å holes could be shared in mixtures of only saponin and cholesterol. If lecithin was present

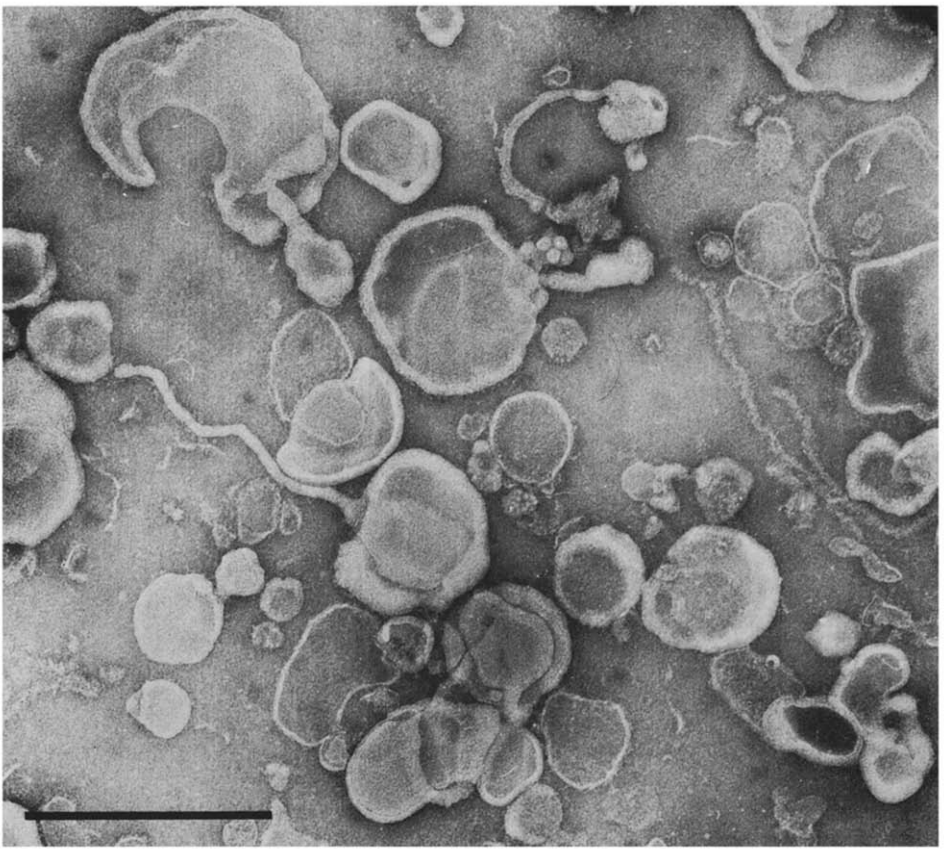


Fig. 7. Band 2. See legend Fig. 8.

this sharing was less frequent and the centre to centre spacing between holes was increased.

When the purified bands of the $(Na^+ + K^+)$-independent Mg^{2+}-ATPase were treated with saponin the hexagonal pattern just described was seen (Fig. 9). The insert shows clearly the dimensions of the micelles (29–34 Å); these can be seen forming a light ring surrounding the dark "hole" of 80 Å in diameter. The centre to centre spacing between the holes varied somewhat probably owing to the presence of lecithin–cholesterol complexes. Not all of these membrane fragments reacted with saponin. Those which showed the hexagonal pattern were counted in a large number of micrographs. A large proportion of the membranes (up to 75 %) in Bands 1 and 2 showed the saponin effect, whereas a somewhat smaller proportion of those in Band 3 responded to saponin.

When the purified $(Na^+ + K^+)$-Mg^{2+}-ATPase was treated with saponin the characteristic hexagonal pattern was seen in all fragments of the many micrographs examined (Fig. 10). Since outer mitochondrial membranes do not respond to saponin (L. PINTERIC, unpublished result) because of their low cholesterol content saponin treatment provided assurance of their absence.

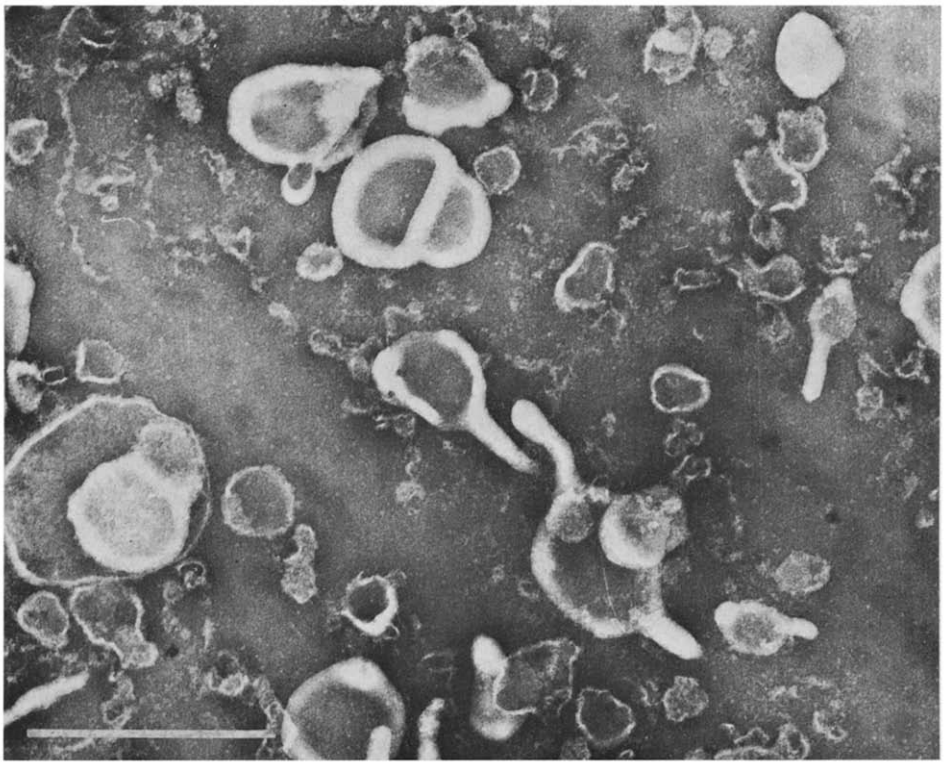


Fig. 8. Representative electron micrographs of the membrane fragments in Band 1 (Fig. 6). Band 2 (Fig. 7) and Band 3 (Fig. 8) obtained after gradient centrifugation of 44000 × *g* microsomes (Scheme 2). The membranes were negatively stained with phosphotungstate (1 %) at pH 6.0. The marker equals 5000 Å. Each band had Mg^{2+}-ATPase and Ca^{2+}-ATPase activity but no ($Na^+ + K^+$)-dependent Mg^{2+}-ATPase. (See Table III).

Thus the interaction with saponin occurred in both membranous preparations, that containing the ($Na^+ + K^+$)-dependent ATPase (PM) and that with the ($Na^+ + K^+$)-independent ATPase activity (SR). The response to saponin appears to be characteristic of membranes containing high concentrations of cholesterol and merely reflects the similarity in chemical composition (Table VII) and morphology of the plasma membrane and sarcoplasmic reticulum which have been described in this paper.

DISCUSSION

For the first time a clear-cut separation of the ($Na^+ + K^+$)-dependent Mg^{2+}-ATPase and the ($Na^+ + K^+$)-independent Mg^{2+}-ATPase from a skeletal muscle homogenate has been achieved. The enzymes are membrane-bound and each was obtained from a different fraction of the homogenate. The fact that treatment of the Mg^{2+}-ATPase with deoxycholate, urea, NaN_3 and salt solutions failed to reveal any ($Na^+ + K^+$)-dependent activity strongly suggests that the two enzymes are not located on the same membrane fragments.

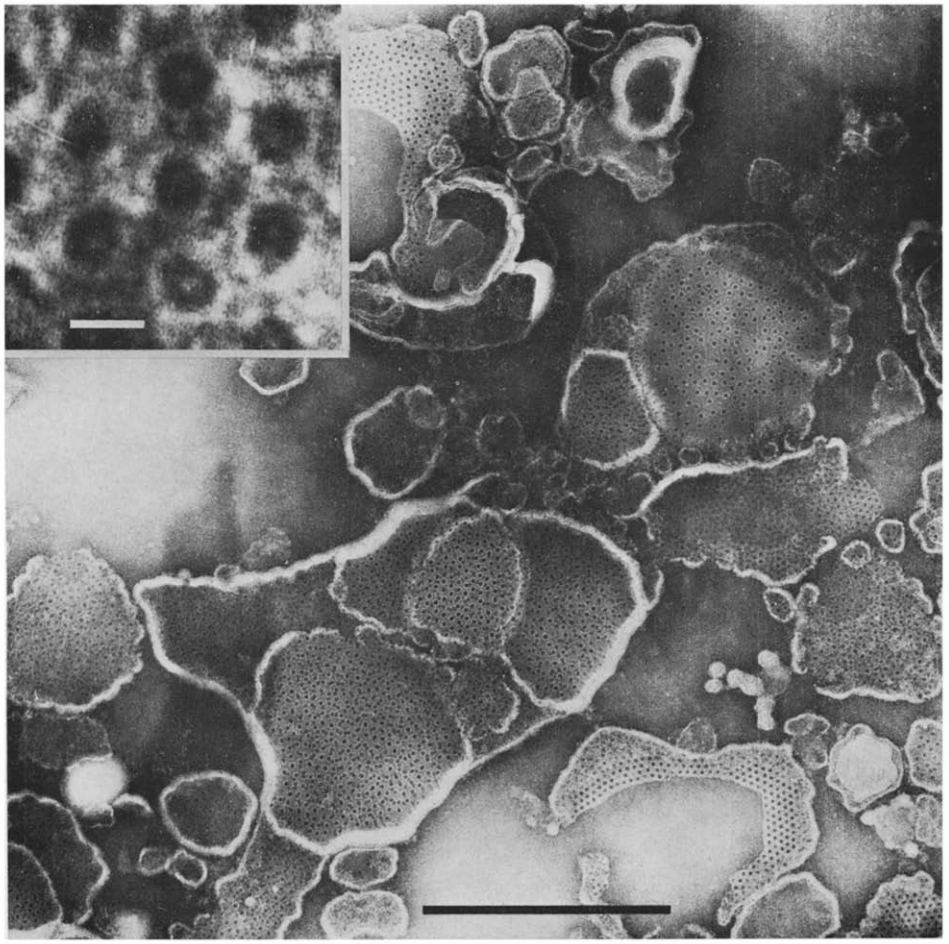


Fig. 9. A representative electron micrograph of the membrane fragments in Band 2 (Fig. 7), after treatment with saponin (0.025%). The preparation was negatively stained with phosphotungstate (1%) at pH 6.0. The marker on the large picture equals 5000 Å and on the insert 100 Å. The insert shows a magnification of the hexagonal pattern produced by saponin treatment of Band 2; the holes (80 Å in diameter) are surrounded by a light ring (29–34 Å in width) of micelles which are probably composed of a cholesterol–saponin complex.

The membranes were morphologically indistinguishable and were similar in their cholesterol and phospholipid concentrations. The cholesterol to phospholipid molar ratios of the two preparations are approximately the same but are about one-seventh of the ratios found in erythrocyte, liver or intestinal microvillus plasma membranes. This low molar ratio resulted from the high phospholipid content of the muscle membranes. Because of the preparative procedure the high cholesterol and phospholipid content per mg protein might not be expected to be a true reflection of the situation *in vivo*. Others[42], however, have found that the $(Na^+ + K^+)$-Mg^{2+}-ATPase is a lipoprotein with an apparent density of 1.05 and would therefore, like our membranes even after salt treatment, be found in the less dense membrane fractions.

Neither preparation showed contamination with collagen, actin, myosin, inner

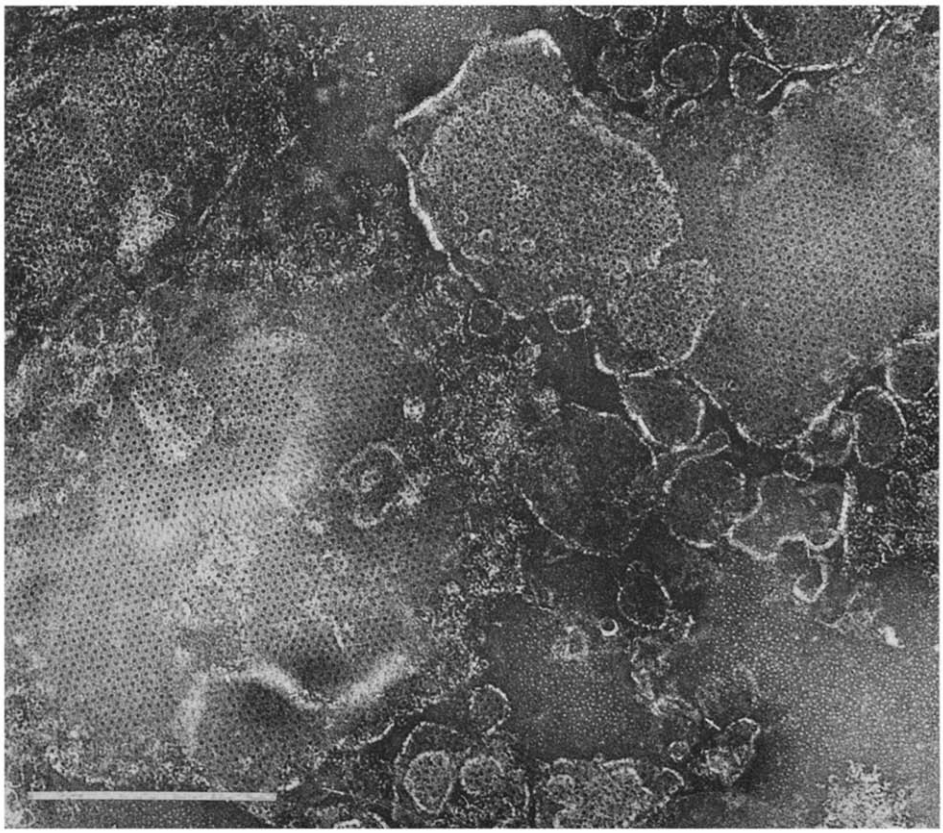


Fig. 10. A representative electron micrograph of membrane fragments in the purified ($Na^+ + K^+$)-Mg^{2+}-ATPase preparation (Fig. 5) after treatment with saponin (0.025%). The preparation was negatively stained with phosphotungstate (1%) at pH 6.0. The marker equals 5000 Å.

or outer mitochondrial membranes or rough endoplasmic reticulum. Since the two membranous fractions were highly purified, it was reasonable to suggest that the membrane with the high ($Na^+ + K^+$)-Mg^{2+}-ATPase derived from the cell fragments (900 × *g* pellet) was the plasma membrane, and that the membrane containing the Mg^{2+} (or Ca^{2+})-ATPase derived from the microsomal fraction (44000 × *g*) was the sarcoplasmic reticulum. It is obvious, however, that we have no real proof of this identity. The enzyme markers used (NADH–diaphorase and 5'-AMP-nucleotidase) failed to distinguish between the two preparations, being present in both.

The current use of ($Na^+ + K^+$)-Mg^{2+}-ATPase as a marker for the plasma membrane of cells has not yet been put to a rigorous test. If this enzyme were a part of the external membrane of a muscle fibre one might expect the enzymic activity to be manifest in the external medium surrounding the muscle. However, muscle fibres have an ATPase on the cell surface which is not inhibited by ouabain[3], is insensitive to Na^+ and K^+ (ref. 43) and appears to be stimulated, not inhibited, by Ca^{2+} (J. R. A. RIORDAN AND J. F. MANERY, unpublished). The relation between this enzyme, which resembles our sarcoplasmic reticulum ATPase, and the ($Na^+ + K^+$)-Mg^{2+}-ATPase (*i.e.* the plasma membrane ATPase) is still obscure. Two highly speculative possibilities

exist: (a) the plasma membrane ATPase might be bound into the structure of the external membrane in such a way that its $(Na^+ + K^+)$-Mg^{2+}-ATPase activity is directed only to the cell interior; (b) there might be a sarcoplasmic reticulum-like ATPase on the external cell surface which is dissociated from a buried plasma membrane ATPase by salt treatment.

Although we are in some doubt about the exact morphological source of our $(Na^+ + K^+)$-Mg^{2+}-ATPase, there is good reason to believe that the Mg^{2+} (or Ca^{2+})-ATPase membranes were derived from the intracellular sarcoplasmic reticulum network which surrounds the myofibrils[44]. It is difficult to compare our sarcoplasmic reticulum preparation directly with those described in the literature because a variety of preparative procedures have been used. An ATPase stimulated by both Ca^{2+} and Mg^{2+}, however, was found in microsomal preparations of muscle[15,45], and there was no stimulation by Na^+ and K^+ (ref. 45). Sarcoplasmic reticulum preparations showing active Ca^{2+} uptake after sucrose gradient purification also had both Ca^{2+}-ATPase and Mg^{2+}-ATPase activity[46–48].

The finding of a highly active $(Na^+ + K^+)$-Mg^{2+}-ATPase in muscle is significant in view of the reports of its absence[12,14,15]. It is not surprising that difficulty has been encountered in finding the enzyme in muscle. In contrast to liver, kidney and brain, from each of which an active $(Na^+ + K^+)$-Mg^{2+}-ATPase has been prepared, muscle is relatively resistant to cell disruption because of the well-developed network of connective tissue and the multi-layered nature of the sarcolemma[49]. Thus the $(Na^+ + K^+)$-Mg^{2+}-ATPase, if it is a part of the true plasma membrane, might not be released easily during cell disruption. Because of the abundance of the sarcoplasmic reticulum inside the cell, sarcoplasmic reticulum membranes might be expected to form the major fraction present in microsomal pellets. It seems likely that the three groups of investigators[12,14,15] who found no ouabain inhibition had predominantly an sarcoplasmic reticulum preparation. They did not use such long homogenization times or such a high deoxycholate content as SAMAHA AND GERGELY[13] did, who reported low activity in human muscle but a high percentage stimulation by $Na^+ + K^+$. The latter authors exposed the muscle to prolonged disruption (90 sec) with a high deoxycholate concentration (0.3 %) in a Waring blender followed by relatively low-speed centrifugation (10000–30000 $\times$ *g*). There is danger, however, of breaking muscle mitochondria during prolonged periods in the Waring blender and of contaminating the preparation with mitochondrial membranes. In our opinion treatment of the appropriate muscle fraction with concentrated salt solutions provides the most effective procedure for disrupting the muscle cell and for releasing fragments of the plasma membrane. Recently KAWADA *et al.*[50] have also exploited the disruptive properties of concentrated halide solutions to locate a $(Na^+ + K^+)$-Mg^{2+}-ATPase in frog skin. The inhibition of this enzyme by high K^+ at low Na^+ concentrations, as well as the monovalent cation ratio required for maximal activity agrees well with that reported by us for the $(Na^+ + K^+)$-Mg^{2+}-ATPase of frog muscle.

Since the completion of this research which has already been reported briefly[51–53], ROGUS *et al.*[54] described a relatively crude preparation of a $(Na^+ + K^+)$-Mg^{2+}-ATPase from rat skeletal muscle; the specific activity and ouabain sensitivity were appreciably lower than those reported in this communication and the optimal cation concentrations required for maximal activity differed greatly being 50 mM Na^+ and 5–20 mM K^+ instead of 105 mM Na^+ and 45 mM K^+ which is optimal for the frog muscle enzyme.

They suggest that the $(Na^+ + K^+)$-Mg^{2+}-ATPase of muscle may reside in the sarcoplasmic reticulum and not in sarcolemma, but it is likely that in this impure preparation they have failed to separate the sarcoplasmic reticulum ATPase from the plasma membrane ATPase.

The importance of isolating a highly active $(Na^+ + K^+)$-Mg^{2+}-ATPase in high yield from skeletal muscle and of identifying the plasma membrane of this tissue cannot be over-emphasized. The enzyme provides a mechanism for the marked inhibition of cation transport by ouabain found in muscle[3]; its presence in the skeletal muscle fibre, which exerts such a sensitive control over Na^+ and K^+ movements, adds final, strong support to the universality of the concept that the $(Na^+ + K^+)$-Mg^{2+}-ATPase is the cation-transport enzyme in living cells.

The isolation and unequivocal identification of the plasma membrane of muscle is also of great significance since this will permit a study at the molecular level of the many functions of this important cell constituent. At present the plasma membrane is believed to control Na^+ and K^+ translocation and thereby the osmotic integrity of the muscle cell, its irritability and contractility; if so the plasma membrane must possess binding sites for monovalent and divalent cations as well as sites for depressant and stimulating drugs. Enzymes bound to the surface of muscle cells as well as various carrier mechanisms and insulin-responsive sites are expected to reside in the plasma membrane. Thus the achievement of this isolation and identification of muscle plasma membrane with which we are now concerned will open up many important avenues of research.

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