Sodium plus Potassium-Activated, Ouabain-Inhibited Adenosine Triphosphatase from a Fraction of Rat Skeletal Muscle, and Lack of Insulin Effect on It

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ABSTRACT An ATPase, activated by Na^+ plus K^+ in the presence of Mg^{++} and inhibited by ouabain, has been obtained from rat skeletal muscle. Unlike ATPase's with similar properties obtained from other preparations, this ATPase was found only in the fraction containing fragmented sarcoplasmic reticulum. It is suggested that in rat skeletal muscle this ATPase may reside in sarcoplasmic reticulum and not in sarcolemma. This ATPase differed in its pH optimum and in its cation sensitivity from that of rat brain and from that of human muscle reported by Samaha and Gergely (1965, 1966). Because insulin accelerates Na^+ efflux from muscle, efforts were made to determine whether or not this effect of insulin could be attributed to increased $Na^+ + K^+$–activated ATPase activity. Insulin, administered either in vivo or in vitro, had no demonstrable effect on the enzyme system, nor did it protect against inhibition by ouabain.

Insulin decreases Na^+ content and increases K^+ content of muscle (Zierler, Rogus, and Hazlewood, 1966), a condition that would result if, for example, coupled active transport of Na^+ and K^+ were stimulated by insulin. This possibility grew more attractive with reports that insulin accelerates the washout of radiosodium from rat diaphragm (Creese, 1964) and from frog muscle (Moore, 1967) and with our similar observations on rat skeletal muscle (Rogus, Price, and Zierler, 1967), since Na^+ efflux occurs almost entirely by an active process.

Active transport of Na^+ and K^+ is thought to employ a membrane-associated, Mg^{++}-dependent ATPase that is synergistically activated by Na^+ and K^+ and inhibited by ouabain (for review see Skou, 1965). To study directly whether insulin exerts its effect on muscle Na^+ and K^+ movements by further

activating this enzyme system it was first necessary to separate from rat skeletal muscle a fraction having enzymic activity characteristic of the transport system. At the time these studies were begun it was uncertain that there was a $Na^+ + K^+$–activated, ouabain-inhibited ATPase in skeletal muscle. Duggan (1964) had failed to find such activity in frog skeletal muscle. However, while we were investigating the problem and had obtained active preparations, two reports appeared by Samaha and Gergely (1965, 1966) of this specific ATPase activity in human skeletal muscle. Their preparation and some of its properties differ from ours, and some characteristics of this specific ATPase system in rat skeletal muscle differ from those we obtained simultaneously from rat brain.

It is the purpose of this paper to describe some properties of a $Na^+ + K^+$–activated, ouabain-inhibited ATPase system from rat skeletal muscle, to report that it is found only in a fraction consisting mainly if not entirely of fragmented sarcoplasmic reticulum, and to present evidence that insulin has no effect on it.

METHODS

Fractionation of Tissue

An adaptation of the method of Ahmed and Judah (1964) was used. Up to 1 g of tissue, brain, or muscle trimmed from the hind limbs of 50-150 g rats, was homogenized in glass in 6 ml cold medium of pH 6.8 (measured at 25°C) and final concentration 0.25 M mannitol, 30 mM histidine, 5 mM ethylenediaminetetraacetic acid (EDTA) as tris (hydroxymethyl) aminomethane (Tris) salt, 0.1 % deoxycholic acid as Tris salt. The homogenate was centrifuged at 8,000 *g* for 20 min at 0°C. The sediment was discarded and the supernatant frozen for 18-64 hr, usually the latter. After thawing, the supernatant was centrifuged at 100,000 *g* for 35 min at 0°C in a Spinco model L preparative ultracentrifuge, using a No. 40 rotor. The pellet so formed was washed by homogenizing briefly in 6 ml cold medium containing 0.25 M mannitol, 1 mM EDTA, 0.125 M Tris-HCl buffer pH 7.2 (adjusted at 25°C) and centrifuging at 20,000 *g* for 50 min at 0°C. The washed pellet, which corresponds to the fraction designated R2 by Ahmed and Judah, was resuspended in 4-6 ml of the same medium used for washing or, after that solution was found to interfere with the protein assay, in washing medium from which mannitol had been omitted. The enzyme suspension was kept frozen until used, usually within 3 days.

Protein Assay

Protein was determined by the method of Lowry et al. (1951). Tris and EDTA, as well as mannitol, interfere with this assay but could not be diluted or omitted from the storage medium without altering properties of the enzyme system. Therefore samples of unknown protein concentration were compared with albumin standards prepared in Tris plus EDTA of the same concentrations as found in diluted enzyme suspensions.

ATPase Assay

For measurement of Mg^{++}-dependent ATPase activity in the absence of added monovalent cations (Mg^{++}-ATPase), enzyme suspension containing 40-100 μg muscle protein or 4-10 μg brain protein was added to the reaction mixture to yield a final volume of 1 ml and final concentrations of 3 mM $MgCl_2$, 3 mM Tris-ATP (Sigma Chemical Corp.), 0.1-0.2 mM EDTA, 125 mM Tris-HCl buffer, pH 6.9 (measured at 37°C). For measurement of total ATPase activity, 100 mM NaCl and 10 mM KCl were included. The $Na^+ + K^+$–stimulated increment in activity ($Na^+ + K^+$–ATPase) was obtained by difference. Each condition was tested in triplicate. Blanks were prepared by omission of enzyme and by omission of Mg^{++}. The reaction tubes were kept in an ice bath until all additions were made, then incubated in a gently shaking water bath at 37°C for 1 hr. The reaction was stopped by returning the tubes to the ice bath and adding 1 ml cold 20% trichloroacetic acid. Reaction tubes were kept cold until analysis for inorganic phosphate (Pi) was performed.

Liberation of Pi proved to be linear with incubation time and with enzyme added until 60% of the terminal phosphate of ATP initially present in the reaction mixture had been hydrolyzed. Under the routine conditions here described, the amount of ATP split rarely exceeded 20% of that initially present. However, with higher enzyme concentrations or lower ATP concentrations it was demonstrated that more than 1 μmole of Pi could be liberated per μmole ATP added, presumably because myokinase was present.

Inorganic Phosphate Assay

2 ml color reagent containing 4% ferrous sulfate freshly dissolved in 1% ammonium molybdate in 1.15 N sulfuric acid (Bonting et al., 1961) was added directly to each reaction tube. Color was allowed to develop for 10-20 min at room temperature and optical density read at 700 mμ.

RESULTS

Activity of Fractions

The preparative procedure yielded a fraction of skeletal muscle exhibiting ATPase activity of the type associated with active transport of Na^+ and K^+.

First, the final preparation contained Mg^{++}-dependent ATPase activity that was stimulated by Na^+ and K^+ added together, but not separately (Table I). The slight but consistent inhibition produced by Na^+ alone could also be achieved by high concentrations of K^+ alone. Under no circumstance did separate addition of 100 mM NaCl to the final muscle fraction result in activation as reported by Skou (1960) and by Matsui and Schwartz (1966) in preparations from crab nerve and calf heart, respectively, and as observed in our brain fraction, also shown in Table I. Insulin, 0.1 U/ml, in the reaction mixture did not alter the activity ratio.

Second, the $Na^+ + K^+$–stimulated increment, but not Mg^{++}-ATPase activity, was sensitive to ouabain. Dose-response curves (Fig. 1) for muscle and brain fractions were similar with 50 % inhibition occurring near 10^{-6} M ouabain. A third curve in Fig. 1 shows that the presence of insulin, 0.1 U/ml, during the enzyme reaction did not affect inhibition by ouabain.

TABLE I

CATION DEPENDENCE OF ATPase ACTIVITY

Additions	Activity ratio	
	Muscle	Brain
—	0	0
Mg^{++}	1.00	1.00
$Mg^{++}+Na^+$	0.82±0.01 (36)	1.18±0.08 (6)
$Mg^{++}+K^+$	1.00±0.01 (34)	0.94±0.02 (6)
$Mg^{++}+Na^++K^+$	2.59±0.08 (87)	5.81±0.32 (22)
$Mg^{++}+Na^++K^++$insulin	2.53±0.07 (87)	

Cation concentrations (mEq/liter) were: Mg^{++}, 6; Na^+, 100; K^+, 10. Activity ratio is the ratio of ATPase activity with given cation additions to that in the presence of Mg^{++} alone. Data are mean ± standard error of the mean (SEM) for the number of observations shown in parentheses. Measurements of total activity without and with insulin, 0.1 U/ml, in the reaction mixture were made using the same 87 muscle preparations.

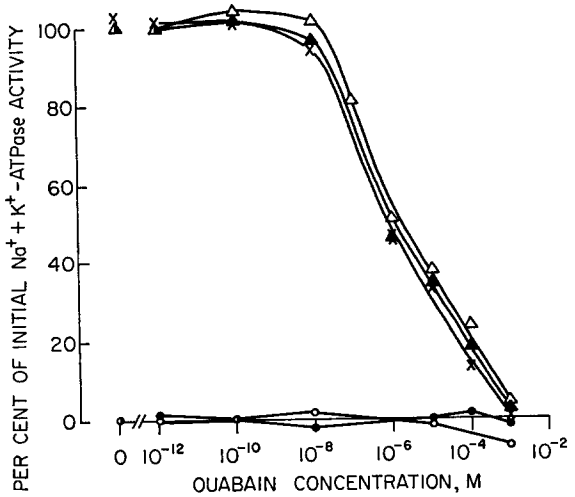

FIGURE 1. Effect of ouabain on ATPase activity of muscle (filled symbols) and brain (open symbols) fractions. Circles are Mg^{++}-ATPase activity and triangles $Na^+ + K^+$–ATPase activity, both expressed as per cent of $Na^+ + K^+$–ATPase activity in the absence of ouabain. Crosses are $Na^+ + K^+$–ATPase activity of muscle fractions with insulin, 0.1 U/ml, added to reaction mixture. Each point is a mean observation from two muscle or five brain fractions.

Third, ouabain-sensitive $Na^+ + K^+$ stimulation of ATPase activity was found in muscle only in the subcellular fraction where fragments of sarcoplasmic reticulum are presumed to occur. Specifically, it was found only in the 8,000-100,000 *g* pellet obtained by differential centrifugation of deoxycholate-treated muscle homogenate. It was not found when prolonged exposure to deoxycholate was omitted from the procedure or when sucrose replaced mannitol in the homogenizing medium. In contrast, it was found in some measure in all fractions of brain in all homogenizing media tested.

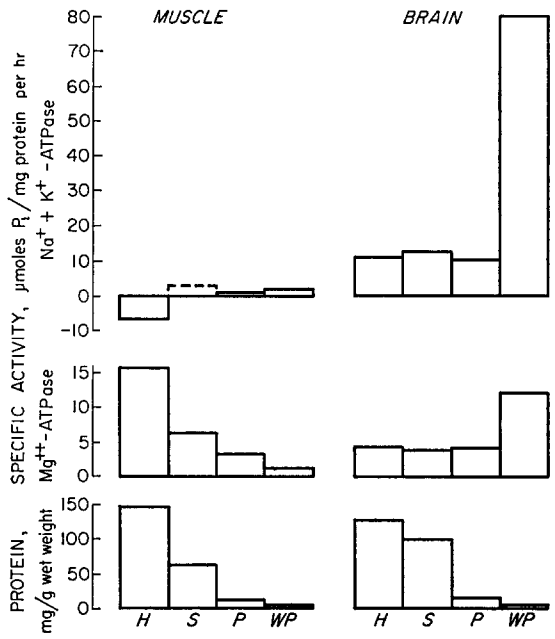

FIGURE 2. Mean specific activities of Mg^{++}-ATPase and $Na^+ + K^+$–ATPase and protein content of successive fractions of two muscle and three brain homogenates. Fractions are *H*, crude homogenate; *S*, 8,000 *g* supernatant; *P*, 8,000-100,000 *g* pellet; *WP*, washed 8,000-100,000 *g* pellet. All fractions of a given tissue sample were assayed on the same day. Dashed bar indicates ouabain-insensitive activation by Na^+ or K^+ alone.

The distributions of Mg^{++}-ATPase and $Na^+ + K^+$–ATPase activities and protein in muscle and brain fractions obtained at successive stages of preparation are compared in Fig. 2. Specific activity of the final washed pellet was 40 times as great in brain as in muscle. Na^+, alone or in combination with K^+, inhibited Mg^{++}-ATPase activity of the aged muscle homogenate while Na^+ and K^+ caused nonsynergistic, ouabain-insensitive activation in the aged 8,000 *g* supernatant. Other fractions, those that were discarded rather than sequentially treated further, are not shown. Each fraction displayed Mg^{++}-dependent ATPase activity. Ouabain-sensitive stimulation by $Na^+ + K^+$ was

observed in each discarded brain fraction, but ouabain-insensitive inhibition by either Na^+ or K^+ was observed in each discarded muscle fraction. Na^+ + K^+ stimulation was not detected in any discarded muscle fraction.

Optimal pH and Monovalent Cation Concentrations

The final fractions derived from muscle and brain responded differently to variations in pH and in Na^+ and K^+ concentrations.

ATPase activities (Mg^{++}-ATPase, Na^+ + K^+–ATPase, and their sum) as functions of pH are shown in Fig. 3. When Na^+ and K^+ were present, ATPase

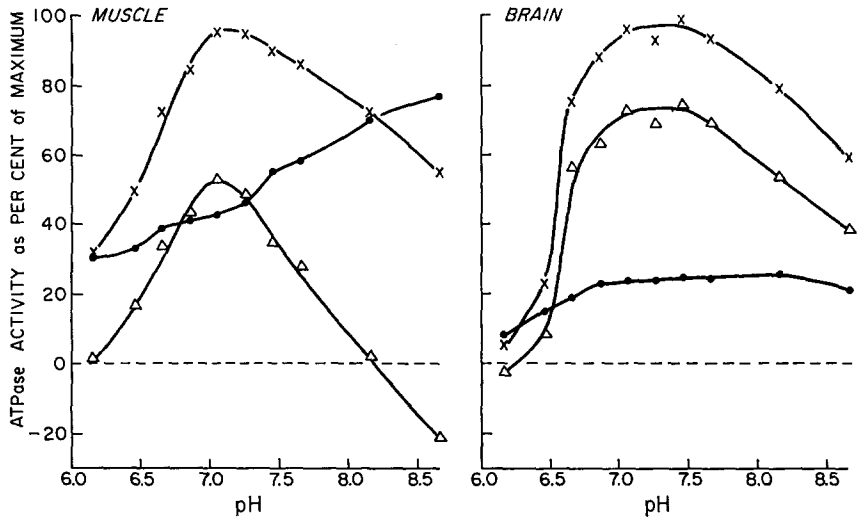

FIGURE 3. Effect of pH of reaction mixture on ATPase activities of muscle and brain preparations. Ordinate is per cent of maximum total activity. Points are means from four muscle or three brain fractions. Crosses are total activity; filled circles, Mg^{++}-ATPase activity; open triangles, Na^+ + K^+–ATPase activity. pH was measured at 37°C.

activity of the muscle preparation reached a sharp peak in the region of pH 7.0 while that of the brain preparation maintained a broader maximum over the range pH 7.0 to 7.6. Mg^{++}-ATPase activity of the brain fraction was relatively insensitive to variations between pH 6.6 and 8.6, but that of the muscle fraction increased with pH. The latter curve could be flattened by reducing ATP concentration to 1.5 mM.

Na^+ + K^+–ATPase activity as a function of both Na^+ and K^+ concentrations is shown in Fig. 4 as isoactivity plots, a method of presentation used by Samaha and Gergely (1965). Each contour is designated by the per cent of maximum stimulation that may be induced by any combination of the Na^+ and K^+ concentrations it encloses. Peak activity of the muscle preparation occurred at 50 mEq Na^+ and 5–20 mEq K^+ per liter, but in the brain prepara-

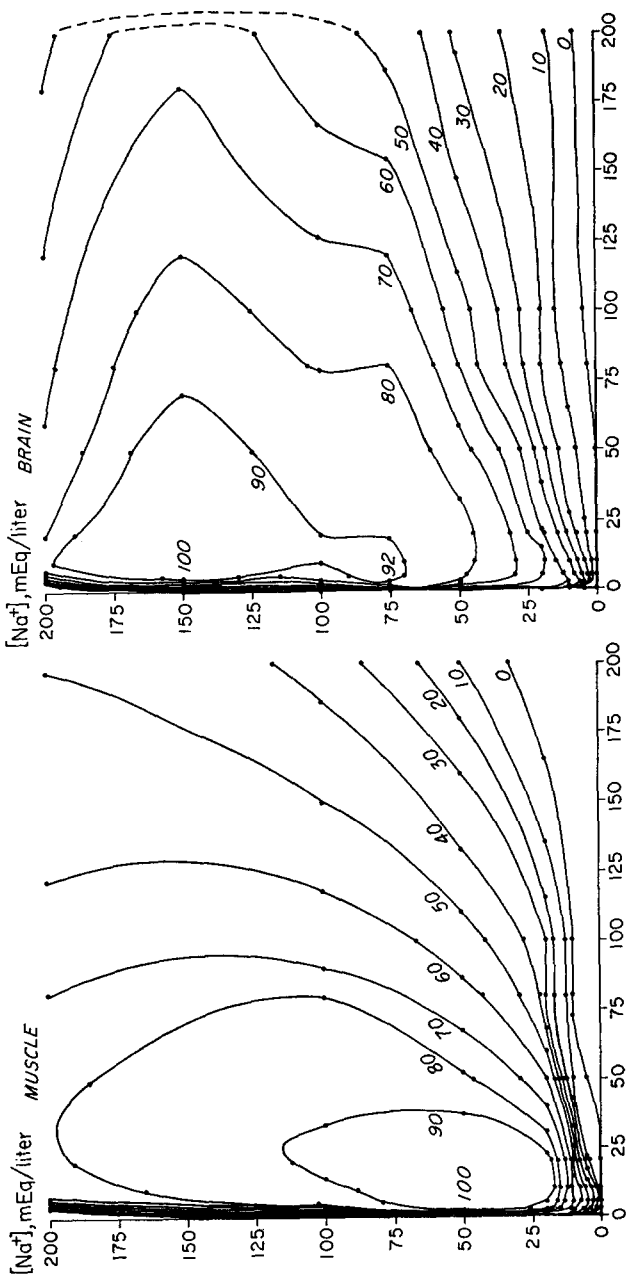

FIGURE 4. Isoactivity plots of $Na^+ + K^+$-ATPase in muscle and brain fractions. Each curve joins points of the same $Na^+ + K^+$-ATPase activity; that activity, expressed as per cent of maximum, is indicated on each line. Isoactivity contour lines at 100% are not shown. The number 100 for both muscle and brain is located in the position of 100% activity, and the number itself approximately covers the 100% contour. Activity in brain was bimodal. The minor peak, or relative maximum, was 92% of maximum, and its position and approximate size are indicated by the number 92. Inhibition is caused by combinations of Na^+ and K^+ concentrations that lie between the line marked zero and the axes. Each plot was drawn from the mean of two preparations.

tion higher cation concentrations, 150 mEq Na^+ and 10–20 mEq K^+ per liter, were required for maximal stimulation.

Close packing of isoactivity lines indicates a steep change in activity for a small change in one of the independent variables. By this parameter, both tissue preparations showed greater sensitivity to variations in K^+ than in Na^+ concentration; by the same parameter, the muscle fraction was more responsive to low concentrations of either Na^+ or K^+ than was the brain fraction. That ATPase activity of the muscle fraction was more susceptible to inhibition by high concentrations of either cation than was activity of the brain fraction is shown by the wider spacing between the line of zero activity and the axes on the muscle isoactivity plot.

In the brain fraction, at K^+ concentrations in the range of 5 to 10 mEq per liter, the plot of $Na^+ + K^+$–ATPase activity against Na^+ concentration was bimodal, with a minor peak at about 75 mEq Na^+ per liter, which was 92 % of maximum activity. This bimodality accounts for the odd, irregular shape of the isoactivity contour. The bimodality and the irregular contour lines in the isoactivity plots describing cation effects on $Na^+ + K^+$–ATPase activity of the brain fraction, as opposed to the smoother contour lines for that of muscle, suggest that there may have been two populations of $Na^+ + K^+$–ATPase in the brain fraction.

Specific Activity of Muscle $Na^+ + K^+$–ATPase Preparation

The numerical value of muscle $Na^+ + K^+$–ATPase specific activity, expressed as μmoles Pi liberated/mg protein per hr, could be increased by various treatments that affected Mg^{++}-ATPase and $Na^+ + K^+$–ATPase differentially. Addition of 5 mM NaN_3 to the enzyme reaction tube decreased Mg^{++}-dependent activity more than it decreased total activity. Preheating at 42°C of the enzyme reaction mixture, complete except for Mg^{++} and ATP, reduced Mg^{++}-ATPase activity while $Na^+ + K^+$–ATPase activity remained constant or was increased; insulin had no effect, but ATP present during preheating retarded the process. Treatment of the 8,000-100,000 *g* pellet with 2 M NaI apparently solubilized protein rich in Mg^{++}-dependent activity, for specific Mg^{++}-ATPase activity rose less than did specific $Na^+ + K^+$–ATPase activity as protein content declined. Presoaking whole tissue in Ringer-bicarbonate solution for 1 hr prior to homogenization also increased specific $Na^+ + K^+$–ATPase activity; this increase could not be attributed to altered Mg^{++}-ATPase activity or protein recovery.

Results of these treatments have been collected in Table II. Since ATPase activity and protein recovery varied appreciably among batches of preparations, only those experiments are presented in which "control" and "treated" observations were made using the same enzyme suspensions or, in the case of

NaI extraction or presoaking, when control and treated preparations were derived from the same animals, then fractionated and assayed in parallel.

Aging the muscle preparation did not increase specific activity of $Na^+ + K^+$–ATPase; indeed, storing the muscle enzyme suspension at −10°C caused both components of activity to decline, as shown in Fig. 5 where specific $Na^+ + K^+$–ATPase activity has been plotted upward and specific Mg^{++}-ATPase activity downward. The former component was more stable than the latter during the first few days but not throughout the aging period. Because protein

TABLE II

EFFECT OF VARIOUS TREATMENTS ON SPECIFIC ATPase ACTIVITIES

Treatment	*N*	Total ATPase	Mg^{++}-ATPase	$Na^+ + K^+$-ATPase	Effect
		μmoles Pi/mg protein per hr			
Control	7	10.9	6.5	4.4	
NaN_3, 5 mM	7	8.9	2.1	6.8	$p < 0.02$
Control	2	12.5	5.0	7.5	
Preheated at 42°C					
5–6 min	2	11.7	3.0	8.7	
10 min	1	12.0	2.2	9.8	
15 min	1	12.4	1.9	10.1	
Control	4	5.3	2.2	3.1	
NaI, 2 M	6	11.1	3.2	7.9	$p < 0.01$
Control	8	5.0	1.8	3.2	
Tissue preincubated	8	7.8	2.4	5.5	$p < 0.01$

N is the number of observations. Each observation represents either a muscle fraction from one rat or pooled fractions from up to three rats. "Effect" refers to significance of effect on $Na^+ + K^+$-ATPase activity (= total minus Mg^{++}-ATPase). *p* is probability that difference occurred by chance.

concentrations were not measured in all the muscle fractions shown, activity has been referred to the gram wet weight of fresh tissue on the left scale of Fig. 5; the scale on the right was constructed assuming uniform protein recovery of 3.98 mg protein/g wet weight, the mean recovery in 59 muscle fractionations.

Effect of Ca^{++}

Ca^{++}, when added in an effective concentration of about 9 mEq/liter (5 mM CaCl added to the mixture containing 0.2 mM EDTA) to the reaction mixture already containing 6 mEq Mg^{++} per liter, did not significantly

inhibit Mg^{++}-ATPase activity. $Na^+ + K^+$-ATPase activity was greatly inhibited, but not abolished, by Ca^{++} and remained sensitive to ouabain. Ouabain at 10^{-5} M, a concentration which caused 70 % inhibition of $Na^+ + K^+$-ATPase activity when Mg^{++} was the only divalent cation added, caused

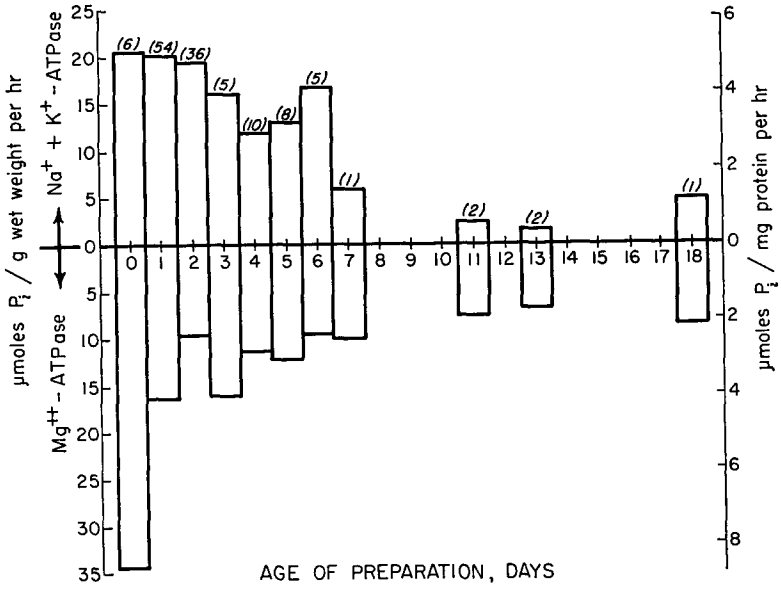

FIGURE 5. Effect of aging on muscle $Na^+ + K^+$-ATPase activity (plotted upward) and Mg^{++}-ATPase activity (plotted downward). Washed 100,000 *g* pellets were suspended in 125 mM Tris containing 1 mM EDTA, and sometimes containing 0.25 M mannitol, and stored at −10°C for the number of days indicated on the horizontal axis. The number of observations is shown in parentheses.

TABLE III

EFFECT OF Ca^{++} ON Mg^{++}-ATPase ACTIVITY AND ON TOTAL ATPase ACTIVITY WITHOUT AND WITH 10^{-5} M OUABAIN

	ATPase activity		
	No Ca^{++}	Ca^{++}	Δ Ca^{++}
	µmoles Pi/mg protein per hr		
Mg^{++}	1.19±0.23	0.92±0.11	$p < 0.10$
$Mg^{++}+Na^+ + K^+$	4.12±0.72	1.26±0.19	$p \ll 0.01$
$Mg^{++}+Na^+ + K^+$+ouabain	2.04±0.35	0.79±0.08	$p \ll 0.01$
Δ $Na^+ + K^+$	$p \ll 0.01$	$p < 0.01$	
Δ Ouabain	$p \ll 0.01$	$p \ll 0.01$	

Data are means ± SEM for eight muscle fractions. Statistical significance (based on paired observations) of inhibition by Ca^{++} (Δ Ca^{++}) appears in last column, significance of stimulation by monovalent cations (Δ $Na^+ + K^+$) and inhibition thereof by ouabain (Δ ouabain) in bottom two rows.

more than 100 % inhibition when both Mg^{++} and Ca^{++} were present ($p < 0.02$, based on analysis of differences between pairs). These results are shown in Table III.

Lack of Insulin Effect

Data have already been presented to show that 0.1 U/ml insulin, when exhibited to the subcellular muscle fraction during a 1 hr enzyme reaction, had

TABLE IV

EFFECT OF INSULIN ON $Na^+ + K^+$-ATPase ACTIVITY OF MUSCLE FRACTIONS

Experiment	No. of pairs	Age of preparation	Protein content		$Na^+ + K^+$-ATPase			
			Control	Insulin	Control	Insulin	Δ	
		days	*mg/g wet wt.*		*μmoles Pi/mg protein per hr*			
1. In vitro								
Insulin 10^{-3} U/ml								
A	8	1–2	5.53 ±0.17	5.80 ±0.17	2.54 ±0.17	2.89 ±0.21	+0.35 ±0.09	$p < 0.01$
A	7	3	5.49 ±0.19	5.79 ±0.20	2.35 ±0.19	2.12 ±0.19	−0.23 ±0.17	NS
B	2	1	3.13 ±0.21	3.57 ±0.34	4.13 ±0.08	4.29 ±0.02	+0.16 ±0.10	NS
Insulin 10^{-1} U/ml								
C	4	2	2.81 ±0.31	2.37 ±0.07	3.54 ±0.39	3.81 ±0.35	+0.27 ±0.47	NS
D	8	1	2.42 ±0.22	2.69 ±0.26	5.96 ±0.45	6.65 ±0.58	+0.69 ±0.71	NS
D	8	2	2.42 ±0.22	2.69 ±0.26	4.97 ±0.52	5.18 ±0.70	+0.21 ±0.88	NS
2. In vivo								
Insulin 1 U per rat								
E		1	2.81 ±0.18	2.57 ±0.18	3.49 ±0.35	3.59 ±0.45	+0.10 ±0.57	NS
E		2	2.81 ±0.18	2.57 ±0.18	2.93 ±0.51	2.84 ±0.38	−0.09 ±0.64	NS

Data are means ± SEM. For in vitro experiments Δ is the difference between pairs; for in vivo experiments Δ is the difference between means of eight control and eight insulin-treated preparations. In the last column p is the probability that Δ occurred by chance and NS indicates that Δ is not significant.

no effect on $Na^+ + K^+$-ATPase activity (Table I) or on its inhibition by ouabain (Fig. 1). Because insulin may require an intact cell membrane in order to exert its effect, two additional types of experiments were performed in which muscle was exposed to insulin prior to homogenization. Results are shown in Table IV.

1. IN VITRO PREINCUBATION WITH INSULIN Whole muscle, intact except for cut ends, was bathed in oxygenated glucose–Krebs–Ringer bicarbonate buffer at room temperature for 1 hr prior to homogenization. Contralateral muscles were bathed in the same medium with insulin, 10^{-3} U/ml or 10^{-1} U/ml, added. No insulin effect could be demonstrated with the exception of a single set of experiments in which a 14 % increase proved significant when differences between paired muscles (± insulin) were analyzed. Repeated assays on seven of the eight pairs of muscle 1 or 2 days later failed to confirm the insulin effect.

2. IN VIVO ADMINISTRATION OF INSULIN 16 rats were fasted overnight; 8 received 1 U insulin, injected intraperitoneally, 1 hr before sacrifice. Na^+ + K^+–ATPase activity in the subcellular muscle fraction derived from rats that had received insulin was not different from that of the controls.

DISCUSSION

We have obtained in a fraction of rat skeletal muscle a Mg^{++}-dependent ATPase stimulated by the presence of Na^+ and K^+ together and inhibited by ouabain. This fraction therefore has the major characteristics of the ATPase that many have associated with active transport of Na^+ from cells in exchange for K^+ (Skou, 1965, for review).

Although it has been clearly demonstrated that there is Na^+ + K^+–ATPase in red cell ghosts (Hoffman, 1960), in the sheath of squid giant axon (Bonting and Caravaggio, 1962), and in the membranes of neurons isolated from vestibular nuclei (Cummins and Hydén, 1962), it need not be assumed that Na^+ + K^+–ATPase is found exclusively in the plasma membrane. Indeed, Bonting and Caravaggio (1962) have shown that axoplasm contains about one-third as much so-called transport ATPase, on a dry weight basis, as does the axon sheath. The studies of Potter, Charnock, and Opit (1966), indicating that Na^+ + K^+–ATPase is probably localized in the membrane component of heart muscle cells, might implicate sarcoplasmic reticulum as well as plasma membranes as a site of transport ATPase. In general, transport ATPase activity has been found in microsomal fractions, or fractions of endoplasmic reticulum, from a variety of cell types. The assumption seems to have been made, at least tacitly, that the transport ATPase activity of this fraction is due to the presence of plasma membranes rather than to endoplasmic reticulum, and where the assumption has been explicit reference is made to the report by Hanzon and Toschi (1959) to the effect that electron microscopy of microsomal fractions from rat brain revealed the presence of cell membranes. We have not been able to recognize sarcolemma in a microsomal fraction from rat skeletal muscle. Indeed, the preparation seemed remarkably homogeneous and contained only particles recognizable as micro-

somes in electron micrographs prepared for us by Dr. Ronald Bergman, Department of Anatomy, The Johns Hopkins Medical School.

In preparations from human skeletal muscle (Samaha and Gergely, 1965) and in preparations from other tissues (for example, from brain in our own studies reported herein), so-called transport ATPase activity is distributed somewhat diffusely throughout several fractions. In contrast, we found no transport ATPase activity in any muscle fraction except the classical microsomal fraction.

These considerations at least raise the possibility that in rat skeletal muscle the so-called transport ATPase may reside exclusively in some portion of sacroplasmic reticulum and not in sarcolemma. It is difficult to prove this by electron microscopy because there is reasonable doubt concerning unequivocal identification of all structures from fragmented cells.

The $Na^+ + K^+$–activated, ouabain-inhibited ATPase system from rat skeletal muscle differs in some of its properties from those reported by Samaha and Gergely (1965, 1966) for human skeletal muscle, the only other skeletal muscle system for which there are enough reported data to permit comparison, and differs from the $Na^+ + K^+$–activated, ouabain-inhibited ATPase from rat brain treated in the same way. The major differences lie in the effects of pH and in optimal cation concentrations. In the latter, the chief difference is that maximum activity occurs in our skeletal muscle preparation at concentrations of Na^+ less than or equal to 100 mEq/liter, whereas in other preparations maximum activity occurs at concentrations of Na^+ greater than 100 mEq/liter. These observations at present say no more than that something is different. Because the system is crude there is no basis for attributing the observed differences to any particular property.

Specific activity of the transport ATPase of our muscle fraction is low. It is only about one-fifth that reported by Samaha and Gergely for an aged human skeletal muscle fraction and it is only about one-fortieth of that we observed in preparations from rat brain. In the absence of data on absolute activity of the system in human muscle observed by Samaha and Gergely we cannot compare our results for purposes of determining whether the differences are true differences between species, or due to better purification (i.e., lower protein content of the fraction for a given level of activity) by them, or due to loss of activity in our preparative procedure. That the difference is not likely to be due to some gross defect in our technique is suggested by the fact that the specific activity of the transport ATPase from rat brain is the same in our hands as in the report by Ahmed and Judah (1964). Nor is the difference likely to be due to greater contamination of our preparation by mitochondria. Samaha and Gergely (1966) have suggested that the ATPase activity of the microsomal fraction that is dependent on Mg^{++} but not stimulated by Na^+ and K^+ is due to contamination by mitochondrial fragments, morphologically

unrecognized. Evidence for this is that inhibitors of mitochondrial Mg^{++}-ATPase also inhibit the Mg^{++}-ATPase of the microsomal fraction. Our preparation had a higher activity ratio (see legend to Table I for definition) than did their fresh preparation, and aging or addition of azide was less effective in increasing it. The two preparations are simply different.

The major purpose of our study was to obtain a test system suitable for examining the possibility that insulin decreased the Na^+ content of rat skeletal muscle and increased the Na^+ efflux from it by enhancing activity of transport ATPase. Our observations fail to demonstrate an effect of insulin on this system.

Addition of insulin to the assay system, preincubation of the muscle with insulin, or injection of a large dose of insulin into the rat did not alter transport ATPase activity of the fragmented sarcoplasmic reticulum fraction nor did it protect against subsequent inhibition by ouabain.

It is reasonable to conclude that insulin exerts its effect on muscle Na^+ content and efflux either by some means not related to transport ATPase or, if by a means related to transport ATPase, that that relationship is not directly with the enzyme.

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