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ATPase ACTIVITIES OF RAT EPIDIDYMAL ADIPOSE TISSUE

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SUMMARY

1. A Mg^{2+}-activated ATPase activity (ATP phosphohydrolase, EC 3.6.1.4) has been observed in rat epididymal adipose tissue. The activity was concentrated in the microsomal subcellular fraction. Evidence indicated that a large fraction of this microsomal Mg^{2+}-ATPase activity was located in the cytoplasmic membrane in the intact cell.

2. The incubation of microsomes with [γ-^{32}P]ATP resulted in incorporation of ^{32}P into trichloroacetic acid-precipitable material. No appreciable fraction (larger than 5 $\mu\mu$moles/mg protein) of this phosphorylated material possessed a rate of turnover compatible with its being a possible intermediate in the Mg^{2+}-ATPase-catalyzed reaction.

3. The influence of several membrane-active agents (detergents, vitamin A, gramicidin, histones, phospholipase C and lysolecithin) suggested a very intimate relationship between the integrity of the membrane and the activity of the ATPase.

4. Evidence is presented establishing the existence of a Mg^{2+}-requiring (Na^+ + K^+)-activated ATPase activity in adipose tissue.

INTRODUCTION

Considerable effort has been dedicated in the past few years to the clarification of the role of particulate ATPase (ATP phosphohydrolase, EC 3.6.1.4) activities which appear to be present in most tissues[1,2]. ATPase activities requiring Mg^{2+} or Ca^{2+} or Mg^{2+} + Na^+ + K^+ are most commonly found[2-6] in subcellular fractions resembling membranes and microsomes. There is strong evidence to attribute the role of providing energy for the active transport of Na^+ and K^+ across the cellular membranes, to the (Mg^{2+} + Na^+ + K^+)-requiring ATPase activity[6-9]. On the other hand, the significance of the bivalent ion-requiring ATPase activities located in the cytoplasmic membranes or membranous portions of microsomes[10] is not well understood. Several hypotheses have been advanced[11-15]. The most interesting seem to be those attribut-

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ing to this type of activity a role in controlling the permeability or other properties of the membranes in which they are located[13–15].

ATPase activities have not been well characterized in adipose tissue. BONTING, SIMON AND HAWKINS[1] found in homogenates of cat adipose tissue a relatively low Mg^{2+}-requiring ATPase (Mg^{2+}-ATPase) activity. A (Mg^{2+} + Na^+ + K^+)-requiring ATPase (Na^+ + K^+-ATPase) activity was not detected. The present paper reports the properties of a Mg^{2+}-ATPase activity and describes the detection of a (Na^+ + K^+)-ATPase in the adipose cells.

EXPERIMENTAL

Preparation of the tissues, isolated adipose cells and subcellular fractions

Male Sprague–Dawley albino rats fed Purina Chow Pellets *ad libitum* and weighing between 180 and 250 g when sacrificed, were used throughout this work. The tissues to be incubated were quickly removed, weighed and immersed in the appropriate buffer or 0.154 M NaCl at 30–37°. Tissues to be homogenized were chilled at 0° in the homogenation medium.

Isolated fat cells were prepared by treatment with bacterial collagenase, as described by RODBELL[16], in Krebs–Ringer bicarbonate buffer containing 4% bovine serum albumin or 0.05% gelatin[17].

The subcellular fractions were prepared at 0 to 4° according to the following procedure. Several epididymal fat pads were homogenized in 10 to 15 volumes of 0.25 M sucrose containing 5 mM Tris–HCl (pH 7.5), 1 mM EDTA–Tris and 1 mM mercaptoethanol (medium H) in an all-glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 16 000 × *g* for 15 min. The supernatant fluid was decanted and saved; the pellet and floating fat were homogenized again with the same volume of fresh medium. After centrifugation at 16 000 × *g* for 15 min the supernatant fluid was decanted and pooled with the previous supernatant fraction. The floating fat and the pellet were suspended separately by homogenation in medium H (fractions F and P16, respectively). Fraction P16, which contained most of the mitochondria, nuclei and debris, was sometimes fractionated in a continuous exponential sucrose density gradient prepared by passing 10 ml of a solution of density 1.32 through 10 ml of a solution of density 1.05. Both sucrose solutions were 5 mM in Tris–HCl (pH 7.5) and 1 mM in EDTA–Tris. 5 ml of fraction P16 were layered on top of the gradient and centrifuged at 16 000 × *g* in a Servall HB-4 swinging bucket rotor for 2 h. After centrifugation, the fluid at the top of the tube appeared clear. Towards the center of the tube, there was a whitish opalescent band identified as relatively pure mitochondria by electron microscopy. A pinkish pellet was observed at the bottom of the tubes. The mitochondrial band and top clear fraction were individually separated by careful suction. The three resulting fractions (SG, clear supernatant fraction; M, mitochondria; and R, residue) were then made up to convenient volumes (usually 5 or 10 ml) with medium H. No structures resembling the cytoplasmic membrane of adipose cells were seen, under phase-contrast microscopy, in any fraction from the gradients.

The 16 000 × *g* pooled supernatant fluids were filtered through cheese-cloth to separate some remaining congealed fat. The filtrate was again centrifuged at 16 000 × *g* for 15 min and the supernatant fluid decanted and saved. The small pellet was suspended in 5 ml of medium H (fraction HM). The supernatant fraction was centri-

fuged at 86 000 × *g* for 1 h. The supernatant liquid was decanted (fraction S, soluble material) and the pellet washed by suspension in medium H. After centrifugation at 86 000 × *g* for 1 h, the washed pellet was suspended in 10 ml of medium H (fraction T, microsomes). All fractions were used immediately or stored frozen at −20°.

ATPase activity assays

ATPase activity was measured by the release of P_i from ATP. For subcellular fractions and homogenates, unless otherwise specified, the reaction mixtures had the following composition: 3.6 mM $MgCl_2$, 3.3 mM ATP (Na^+ or Tris salt), 12 or 23 mM Tris–HCl (pH 7.5), 0.3 mM EDTA–Tris, 105 mM sucrose (if present), 107 mM NaCl (if present), 21 mM KCl (if present), enzyme fractions and other additions in a total volume of 1.5 ml. Reagents were adjusted to pH 7.5 by addition of Tris base or HCl. For isolated adipose cells the above medium with the total volume at 3 ml, or one containing 3.6 mM $MgCl_2$ and 3.3 mM ATP (pH 7.5) in 3 ml of Krebs–Ringer bicarbonate buffer (5% CO_2–95% air as the gas phase) with or without 4% bovine serum albumin and other additions, were used.

The reactions were started by the addition of either the enzyme preparation or ATP. They were conducted at 37° in a Dubnoff shaking incubator for 30 to 60 min. The conditions were chosen so that, in most cases, the total P_i released did not exceed 10% of the total phosphate of ATP in the reaction mixture. The reactions were terminated by the addition of 1 ml (subcellular fractions) or 3 to 6 ml (isolated cell suspensions) of 10% trichloroacetic acid. P_i was assayed by the SUMNER method[18].

ATPase activity in short-time incubation experiments was measured by radiochemical methodology. The $^{32}P_i$ released from [γ-^{32}P]ATP was separated from the unhydrolyzed [γ-^{32}P]ATP by electrophoresis[19] on paper strips. Radioactivity in the $^{32}P_i$ and [γ-^{32}P]ATP bands was measured in an automatic Vanguard strip scanner. The method proved reproducible and the results were equivalent to those obtained by chemical assays.

Other assays

Incorporation of ^{32}P from [γ-^{32}P]ATP into trichloroacetic acid-precipitable microsomal material was accomplished by the method of POST, SEN AND ROSENTHAL[20] with modifications.

Extraction of lipid from isolated cells and the determination of ^{14}C incorporated from uniformly labeled [^{14}C]glucose into total lipid was according to RODBELL[16].

Protein assays were performed by the LOWRY method[21] using bovine serum albumin fraction V as a standard.

Whenever possible the standard deviations of a single observation have been included with the data.

Materials

ATP–Tris was prepared by stirring solid ATP (barium salt) with Dowex 50-H^+ resin, until complete dissolution of the salt occurred. The ATP acid was separated by filtration and neutralized to pH 7.5 with Tris base. [γ-^{32}P]ATP was prepared by the method of GLYNN AND CHAPPELL[22]. The labeled ATP was purified by absorption on a Dowex 1-Cl^- resin column. Tris–azide was prepared by neutralization of Tris base with gaseous hydrazoic acid.

ADP, ATP (Na^+ or Ba^{2+} salt), bovine serum albumin fraction V, gramicidin (potency 980 μg/mg), calf-thymus histone (type II), lysolecithin, ouabain, phospholipase C and vitamin A alcohol were from Sigma Chemical Company; deoxycholic acid (enzyme grade) from Mann Research Laboratories; beef zinc insulin crystalline (26 U/mg) from Lilly; bacterial collagenase from Calbiochem.

RESULTS

Distribution of Mg^{2+}-ATPase activities in subcellular fractions of adipose tissue

Table I shows that more than 70% of the Mg^{2+}-ATPase activity was recovered in the non-sedimentable material at 16 000 $\times$ g. Approx. 3/4 of this activity was sedimented in the microsomal fraction. Very likely these figures should be somewhat higher since we have observed, by comparing the activities of the 16 000 $\times$ g supernatant fraction and those of the resulting microsomes and 86 000 $\times$ g supernatant fluid, that only about 65% of the enzymatic activity was recovered after the centrifugation at 86 000 $\times$ g. The lost activity could not be recovered by mixing and assaying together the microsomes and the supernatant fluid.

Attempts to further fractionate the microsomal preparations through sucrose density gradients showed that the enzymatic activity was concentrated in a single slowly sedimenting band relatively poor in 260-mμ-absorbing material. Towards the bottom of the gradient, fractions poorer in the enzymatic activity but with higher ratios of 260-mμ-absorbing material to protein were encountered.

Properties of the Mg^{2+}-ATPase activity

The ATPase activity of microsomal preparations and isolated cells showed an absolute requirement for bivalent cations. Maximal activity was obtained at a Mg^{2+} concentration equal to the ATP concentration. Ca^{2+} and Mg^{2+} were equally effective in promoting the ATPase activity, but Mn^{2+}, at the same concentration, was only 66%

TABLE I

DISTRIBUTION OF Mg^{2+}-ATPASE ACTIVITY AND PROTEIN IN SUBCELLULAR FRACTIONS OF RAT ADIPOSE TISSUE

The means and standard deviations of a single observation have been calculated from individual data for the three columns. The specific activity of the Mg^{2+}-ATPase in the unfractionated homogenate was 14.1 $\pm$ 1.3 μmoles P_i per mg protein per h. The summation of all the Mg^{2+}-ATPase activities recovered in the different fractions amounted to 75 $\pm$ 15% of the total activity in the unfractionated homogenate. See text for fractionation procedure and designations for fractions.

Fraction	*Number of fractions assayed*	*Protein (% total recovery)*	*Mg^{2+}-ATPase (μmoles P_i per mg protein per h)*	*Mg^{2+}-ATPase (% total recovered activity)*
Residue (R)	6	22.1 $\pm$ 1.1	6.2 $\pm$ 1.2	7.4 $\pm$ 1.0
Mitochondrial (M)	6	3.0 $\pm$ 0.7	27.7 $\pm$ 3.9	5.7 $\pm$ 1.2
Supernatant gradient (SG)	6	2.1 $\pm$ 0.4	15.0 $\pm$ 5.3	2.3 $\pm$ 0.9
Fat (F)	6	4.0 $\pm$ 2.6	10.3 $\pm$ 2.3	4.3 $\pm$ 0.9
Small pellet (HM)	7	2.1 $\pm$ 0.1	37.9 $\pm$ 3.8	7.6 $\pm$ 0.8
Supernatant 86 000 $\times$ g (S)	7	60.6 $\pm$ 4.4	2.7 $\pm$ 0.3	15.4 $\pm$ 1.3
Microsomes (T)	7	7.1 $\pm$ 0.6	82.3 $\pm$ 4.2	55.7 $\pm$ 2.5

as effective. The addition of Ca^{2+} when Mg^{2+} was maintained at the optimal concentration resulted in a definite inhibition.

The microsomal Mg^{2+}-ATPase activity showed a slight inhibition (10%) in the presence of 100 mM Na^+, K^+, Li^+ and NH_4^+ (added as the chlorides). The activity displayed a broad pH maximum near pH 8.2.

Iodoacetic acid and *N*-ethylmaleimide, tested at concentrations ranging from 0.001 mM to 1 mM, were without effect. *p*-Hydroxymercurobenzoate caused only slight inhibitions—10% at 0.03 mM and 23% at 1 mM.

The hyperthyroid condition in the animals, induced by administration of 3,3',5-triiodothyronine, did not change the specific activity of the Mg^{2+}-ATPase or its distribution in the subcellular fractions. The lipolytic hormones glucagon, epinephrine, norepinephrine, oxytocin, TSH, and ACTH, were without effect on the ATPase activity of isolated cells or their resulting homogenates.

Mg^{2+}-ATPase activity in isolated cells and cell homogenates

When Mg^{2+}-ATPase activity was measured in isolated adipose cells and in their homogenates, it was consistently found that both preparations presented quantitatively the same ATPase activity (percent Mg^{2+}-ATPase activity: cells 100.0 ± 5.7; homogenates 99.2 ± 9.5). Since the available evidence indicates that the isolated adipose cells are impermeable to ATP (ref. 23), it was surprising that disruption of the cells did not result in a change of the activity of the ATPase. Breakage of the cells during incubation did not seem a likely explanation of these results since the cells were perfectly responsive to insulin 2 h after isolation (Table II). RODBELL[16,23] has shown the intimate correlation between the integrity of the isolated cells and their response to the hormone. The possibility that the response to insulin was due to only a small fraction of unbroken cells in the preparation was thought not to be the case since approximately the same levels of ^{14}C incorporation into lipid were observed

TABLE II

INCORPORATION OF [^{14}C]GLUCOSE INTO TOTAL LIPID AND ATPASE ACTIVITY OF ISOLATED CELLS AND THEIR HOMOGENATES

Isolated cells were incubated for 2 h at 37° in Krebs–Ringer bicarbonate buffer with 4% bovine serum albumin and 3 mM glucose. At the end of this period part of the cell suspension was homogenized in a motor-driven homogenizer fitted with a Teflon pestle. 1-ml aliquots of the cells and homogenates were diluted up to 3 ml with buffer, 4.5 μmoles [^{14}C]glucose (0.5 μC per flask) and other additions, incubated for 30 min, and assayed for P_i and incorporation of ^{14}C into total lipid. Reactions were performed in duplicate.

Preparation assayed	*Additions*	*Lipid ^{14}C, (counts/min)*	*ATPase (μmoles P_i per flask)*
Cells	—	856	—
Cells	ATP	654	0.79
Cells	Insulin (3 mU)	1727	—
Cells	ATP + Insulin (3 mU)	1462	0.77
Homogenate	—	441	—
Homogenate	Insulin (3 mU)	398	—
Homogenate*	ATP	—	0.69

* No [^{14}C]glucose was added to these flasks.

when the incorporation was initiated immediately after the isolation of the cells. This would imply that little cell breakage occurred during the 2-h preincubation. The isolated cells immediately after preparation should be mostly whole cells[23].

It is necessary to conclude, then, that either ATP could freely reach the majority of ATPase sites in the isolated adipose cells, or a certain fraction of ATPase activity, equal to that exposed by homogenation, was destroyed upon breakage of the cells. This last possibility cannot be ruled out, but seems unlikely.

Effects of membrane-disruptive agents

Several experiments were designed to explore the possibility of solubilizing the Mg^{2+}-ATPase activity of the microsomal preparations, by means of detergents and sonication. Solubilization of the Mg^{2+}-ATPase activity was not accomplished by these treatments; however, the experiments indicated the very intimate relationship that exists between the activity of the enzyme and the integrity of the microsomal particles. This relationship has been further explored with the help of several substances of known membrane-disruptive ability.

TABLE III

ACTION OF DEOXYCHOLATE ON MICROSOMAL ATPASE ACTIVITY

5-ml aliquots of a microsomal suspension (1.5 mg protein) were made 0.1 and 0.3% in deoxycholate, stirred and 2-ml aliquots freed of the detergent by filtration on Sephadex G-50 columns. 3-ml aliquots of the same mixtures were centrifuged at 105 000 × *g* for 1 h. The pellets were rinsed with medium and resuspended (deoxycholate pellet). 2-ml aliquots of the supernatant fluid were freed of detergent by Sephadex filtration (deoxycholate supernatant). Aliquots of all fractions were assayed for protein and ATPase activity.

Fraction assayed	*Deoxycholate treatment (%)*					
	0.1	*0.3*	*0.1*	*0.3*	*0.1*	*0.3*
	Specific activity μmoles P_i per mg protein per h		*Protein recovered (%)*		*ATPase units (%)*	
Untreated microsomes	60	71	—	—	100	100
Deoxycholate-treated microsomes	56	20	88	89	80	24
Deoxycholate pellet	56	5.4	42	22	38	1.7
Deoxycholate Supernatant	15	11	29	63	7.2	9.4

Table III shows that 0.1% deoxycholate solubilized only a small part of the microsomal protein and an even smaller portion of the total ATPase. The detergent at 0.3% was more effective in solubilizing the protein but inactivated most of the ATPase activity. No reactivation of the lost ATPase occurred when the deoxycholate-free supernatant fluid was preincubated with either untreated microsomes, or 20 μg/ml of rat-brain phospholipids containing 2 μg/ml of lysolecithin or mixtures of microsomes and phospholipids. Also, the deoxycholate-treated pellets did not respond to the addition of phospholipids. Preliminary experiments on the treatment of microsomes with Triton X-100, Triton WR 1339, Duponol C, and Brij 35 indicated that no substantial solubilization of the activity, after the elimination of the detergents, occurred in any case.

Phospholipase C, an enzyme capable of inducing lysis of the isolated adipose

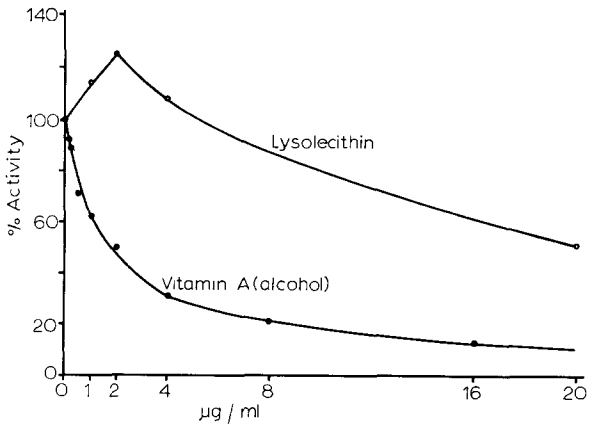


Fig. 1. Effect of lysolecithin and vitamin A alcohol on the Mg^{2+}-ATPase of microsomal fractions. Vitamin A was placed in tubes in 10 μl of ethanol solutions (10 μl of ethanol were added to control reactions). The complete mixtures were preincubated for 10 or 15 min at 37° (vitamin A) or 0° (lysolecithin) before starting the reaction by addition of ATP.

cells by hydrolyzing the phospholipids of the cytoplasmic membrane[17,23], was without effect on the ATPase activity of adipose cell suspensions, when these were incubated for 2 h in the presence of 10 μg/ml of the enzyme. This concentration of phospholipase C diminish the incorporation of ^{14}C from [^{14}C]glucose into total lipid to 12% of the incorporation in the controls without the enzyme. At the end of the incubation of the cells in the flasks containing the enzyme were almost totally lysed.

Lysolecithin and vitamin A alcohol, substances known to possess haemolytic properties[24,25], caused a strong inactivation of the microsomal enzymic activity (Fig. 1). However, at low concentrations, only lysolecithin activated the Mg^{2+}-ATPase activity. This activating effect was not mimicked either by low concentrations of Triton X-100, Brij 35 or deoxycholate. This implies that the activation caused by lysolecithin is a specific effect rather than the result of a non-specific loosening of the membrane structure by the detergent properties of the compound. It is of interest that MUNDER, FERBER AND FISCHER[25] have shown that concentrations of lysolecithin below 15 μg/ml protect erythrocytes from haemolysis. Minimal haemolysis occurs at 2 μg/ml of lysolecithin, the same concentration which induces maximal Mg^{2+}-ATPase activity in the adipose tissue preparation. The activating effect of lysolecithin was observed only in ATPase preparations with high specific activity. Fractions previously treated with Triton X-100, deoxycholate or cold aqueous acetone, (provided that these treatments caused a high degree of inactivation of the ATPase) were totally insensitive to the addition of lysolecithin. On the other hand, 0.1% deoxycholate treatment, which caused little inactivation of the enzyme (7%, Table III) did not impair the activating effect of lysolecithin. These results suggest that if lysolecithin is to have activating action on the ATPase activity a certain degree of membrane integrity, which is accompanied by high specific activity of the enzyme, is required.

Fig. 2 shows that the microsomal Mg^{2+}-ATPase was inhibited by calf-thymus histones and gramicidin. In both cases the inhibition appeared to level off since increasing the concentration of histones to 100 μg/ml or of gramicidin to 50 μg/ml caused a slight increase of the inhibition. Table IV shows that the inhibition caused

TABLE IV

INFLUENCE OF 20 μG/ML OF HISTONES OR GRAMICIDIN ON THE MICROSOMAL ATPASE ACTIVITY IN THE PRESENCE OF SEVERAL MONOVALENT IONS AND OUABAIN

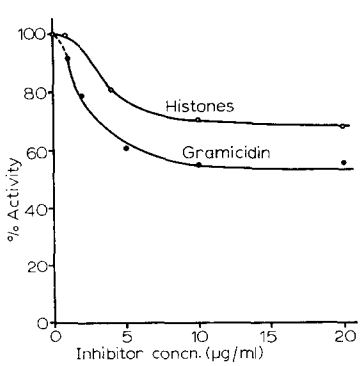
The experimental details were as in Fig. 2.

Additions	*Inhibitor*		
	Present or absent	*Histones (% activity)*	*Gramicidin (% activity)*
None	—	100	100
None	+	57	39
133 mM NaCl	+	90	58
133 mM KCl	+	92	58
133 mM LiCl	+	84	—
106 mM NaCl, 27 mM KCl	+	102	65
107 mM NaCl, 21 mM KCl, 0.1 mM ouabain	+	88	—

by these agents was partially reversed non-specifically by the presence of monovalent ions in the reaction mixture. KATCHALSKI[26] has suggested that gramicidin and some synthetic amino acid copolymers, also rich in basic and lipophylic amino acid residues can interact with biological membranes. Since histones are particularly rich in similar amino acids[27], it is possible that they can have an action on the membranes analogous to that of gramicidin and the synthetic copolymers. The analogies in the inhibition patterns of histones and gramicidin on the Mg^{2+}-ATPase activity could be the result of their similar action on the membranes. The polycation spermine at 0.1 and 1 mg/ml in the incubation mixture was without significant effect on the Mg^{2+}-ATPase.

Detection of ($Na^+ + K^+$)-ATPase activity

The simultaneous addition of Na^+ and K^+ ions to the assay mixture of micro-



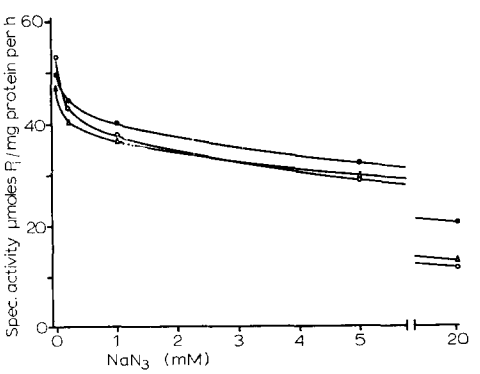


Fig. 2. Inhibition of microsomal Mg^{2+}-ATPase by calf-thymus histones and gramicidin. Gramicidin was added to the tubes in 10 μl of ethanolic solutions (10 μl of ethanol were added to the control reactions). The complete mixtures were preincubated for 10 or 15 min at 37° before starting the reaction by addition of ATP.

Fig. 3. Effect of sodium azide on the microsomal ATPase activity in the presence or absence of Na^+, K^+ and ouabain. Additions: ●—●, 103 mM Na^+, 20 mM K^+; △—△, 103 mM Na^+, 20 mM K^+, 1 mM ouabain; ○—○, without Na^+, K^+ and ouabain.

somal fractions did not result in a noticeable increase of the activity as would be expected if a Mg^{2+}-activated, ($Na^+ + K^+$)-requiring ATPase were active in these fractions. This observation could imply that either a ($Na^+ + K^+$)-ATPase was not present in the microsomal preparation, or, if it were present, its activity was low compared to the highly active Mg^{2+}-ATPase. In the latter alternative the described inhibition of the Mg^{2+}-ATPase by monovalent cations could mask completely the low activity of the ($Na^+ + K^+$)-ATPase. Since in other systems azide is a preferential inhibitor of the Mg^{2+}-ATPase activity[28-30] and ouabain is a specific inhibitor of the ($Na^+ + K^+$)-ATPase[6,7], it was considered that these inhibitors could be used to clarify the presence of a monovalent ion-requiring ATPase in the microsomal preparations.

The experiment described in Fig. 3 shows that in the absence of azide, maximal activity was obtained in the absence of Na^+ and K^+. When azide was added a stimulation of the ATPase activity by Na^+ and K^+ was apparent and ouabain reversed this stimulation. Identical results were obtained with microsomes prepared from whole tissue or from isolated adipose cells. Table V shows that Na^+ and K^+ were simultaneously required for maximal activity in the presence of 20 mM NaN_3. The stimulation by Na^+ *plus* K^+ was half maximally inhibited at approx. 10^{-6} M ouabain. When Na^+ and K^+ were absent, and in the presence of 20 mM Tris–azide, 1 mM ouabain caused no inhibition (percent activities: without ouabain, 100.0±3.5, with 1 mM ouabain, 98.6±2.2). If Mg^{2+} were substituted by Ca^{2+} or Mn^{2+}, no stimulation either in the presence or absence of 20 mM azide was observed when Na^+ and K^+ were added to the reaction mixture. These results are consistent with the presence of a ($Na^+ + K^+$)-ATPase activity in the microsomal fractions from adipose tissue.

TABLE V

INFLUENCE OF Na^+ AND K^+ ON THE ATPase ACTIVITY OF MICROSOMAL PREPARATIONS IN THE PRESENCE OF 20 mM NaN_3

The means and standard deviations shown are the results of eight experiments performed with four different microsomal preparations from whole tissue and isolated adipose cells.

Cations added	*Percent of maximal activity**
1. 20 mM Na^+	90.9 ± 2.8
2. 127 mM Na^+	92.9 ± 4.3
3. 20 mM Na^+, 21 mM K^+	95.9 ± 1.5
4. 127 mM Na^+, 21 mM K^+	100

* Values of P, t test: (1:2), $P < 0.25$; (2:3), $P < 0.10$; (3:1), $P < 0.001$; (4:1), $P < 0.001$; (4:2), $P < 0.001$; (4:3), $P < 0.001$.

It has been reported that histones, like azide, inhibit microsomal ATPase activities[31] with preferential action on the monovalent ion-insensitive ATPases. The experiment described in Table IV shows that histones in the reaction mixture inhibited the Mg^{2+}-ATPase activity, and, under these conditions, a large stimulation of the activity occurred when Na^+ and K^+ were added to the reaction mixtures. However, since the inhibition of the Mg^{2+}-ATPase activity by histones was partially released by the presence of monovalent ions, it is likely that the large stimulation induced by the

TABLE VI

RELATIVE INHIBITION OF ATPASE ACTIVITY BY 1 mM OUABAIN IN THE PRESENCE AND ABSENCE OF INHIBITORS OF THE DIVALENT ION-ACTIVATED ATPASE ACTIVITY

Na^+ and K^+ were present in the incubation mixtures. 2 to 6 reactions were conducted with every fraction. Relative percent inhibitions by ouabain were calculated as the ratio of the absolute decrease of ATPase activity caused by ouabain to the ATPase activity of the fraction in the absence of Na^+, K^+, azide and histones.

Mg^{2+}-ATPase inhibitor added	*Divalent ion present*	*Number of fractions assayed*	*Relative percent inhibition by 1 mM ouabain*
None	Mg^{2+}	10	5.8 ± 2.1
20 mM azide	Mg^{2+}	9	8.3 ± 4.1
(0.1 or 0.02 mg/ ml histones)	Mg^{2+}	4	9.3 ± 4.2*
None	Ca^{2+}	5	0.2 ± 2.7
20 mM azide	Ca^{2+}	6	2.5 ± 2.5
None	Mn^{2+}	4	0.6 ± 4.0
20 mM azide	Mn^{2+}	4	1.7 ± 1.2

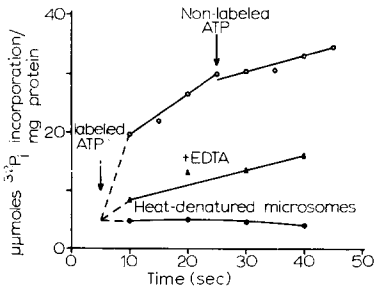
* Two experiments were performed with 0.1 mM ouabain.

presence of Na^+ and K^+ could be attributed only in part to the activity of a (Na^+ + K^+)-ATPase. This is supported by the fact that ouabain reversed only 25 to 35% of the stimulation caused by Na^+ + K^+.

Table VI shows the inhibition of ATPase activity caused by ouabain in the presence of Na^+ *plus* K^+ under different conditions. The inhibition by ouabain was significant only when Mg^{2+} was the bivalent cation present in the reaction mixture. Assuming that the suggested (Na^+ + K^+)-ATPase activity of the preparations was totally inhibited by 0.1 or 1 mM ouabain, the activity of the (Na^+ + K^+)-ATPase in the microsomal fractions would be approx. 8% of the activity of the Mg^{2+}-ATPase. The absolute values of the ouabain inhibition (suggested to be the activity of the (Na^+ + K^+)-ATPase) varied between 2 and 8 μmoles P_i per mg protein per h depending upon the microsomal preparation utilized.

Incorporation of ^{32}P from [γ-^{32}P]ATP into microsomes

There is at present strong evidence indicating the existence of phosphorylated intermediates in the Mg^{2+}-activated (Na^+ + K^+)-stimulated ATPase-catalyzed reaction in membranes from several tissues[5,20]. Na^+ is required for the formation of such intermediates, and K^+ stimulates their dephosphorylation. In the absence of Na^+ phosphorylation of membrane constituents to a limited extent also occurs. In these conditions the monovalent ion-stimulated ATPase is not functional, but some ATP is hydrolyzed by the Mg^{2+}-ATPase which is present in the membrane preparations. TITUS and co-workers[5] found in beef-brain fractions, that incorporation of ^{32}P into microsomes in the absence of Na^+ rapidly leveled off. They suggested that the incorporation might represent the steady-state level of a phosphorylated intermediate in the Mg^{2+}-ATPase-catalyzed reaction. It is evident that the phosphorylated material, in order to be considered a possible intermediate in the Mg^{2+}-ATPase-catalyzed reaction, should possess a rate of turnover rapid enough to account for the Mg^{2+}-



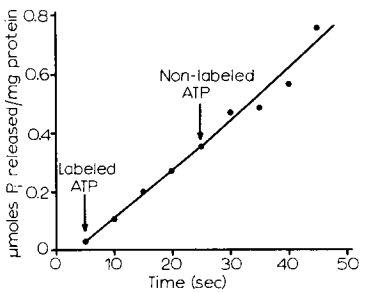


Fig. 4. Incorporation of ^{32}P into microsomal trichloroacetic acid-precipitable material from [γ-^{32}P]ATP. The reaction was carried out at 23°. It was started by addition of 0.2 ml of microsomes (0.38 mg protein) to 0.1 ml of 0.53 mM ATP–Tris, 0.69 mM $MgCl_2$ in 175 mM Tris–HCl buffer (pH 7.5). 5 sec later 0.2 ml of 0.97 mM [γ-^{32}P]ATP, 1 mM $MgCl_2$, approx. 103 mM in Tris–HCl buffer (pH 7.5), was added. The specific activity of the [γ-^{32}P]ATP in the complete reaction mixture was 80 counts/min per $\mu\mu$mole (from control experiments it was estimated that about 50% of the non-labeled ATP present at zero time was hydrolyzed at 5 sec). In the reactions containing EDTA, 0.1 ml of 100 mM EDTA–Tris, was placed in the tubes before starting the reaction. At 25 sec, 0.1 ml of 2.66 mM ATP–Tris, 3 mM $MgCl_2$ was added. Reactions were terminated by addition of 10 ml of an ice-cold solution of 5% trichloroacetic acid, 1 mM ATP, 1 mM P_i. The denatured protein was precipitated by centrifugation. Aliquots of the supernatant fluids were analyzed for $^{32}P_i$ and [γ-^{32}P]ATP. The pellets were washed once with 10 ml of the reaction-stopping mixture and three times more with 10-ml portions of cold 5% trichloroacetic acid. To the washed pellets 0.9 ml of water and 0.167 ml of 3 M NaOH were added. Dissolution was completed with gentle heating in a boiling-water bath with occasional stirring. Aliquots were analyzed, 0.3 ml for radioactivity, in gas-flow counter, and 0.5 ml for protein.

Fig. 5. Rate of hydrolysis of [γ-^{32}P]ATP in the experiment shown in Fig. 4. 61% of the initial [γ-^{32}P]ATP was hydrolyzed at 25 sec. The Mg^{2+}-ATPase activity was 60.3 μmoles P_i per mg protein per h.

ATPase activity of the preparation. Figs. 4 and 5 show the results of a representative experiment designed to test this point with the adipose tissue microsomal preparations. Fig. 5 shows that the reaction was proceeding at a linear rate within less than 5 sec after the addition of labeled ATP. This implies that the phosphate in an hypothetical intermediate should have reached isotopic equilibrium with the labeled substrate in a time interval shorter than 5 sec. Diluting the labeled ATP by the addition of non-labeled ATP should result in a rapid removal of label from the microsomes. Fig. 4 shows that the incorporation of label was dependent upon enzymic activity. However, only very small removal of incorporated radioactivity occurred upon a four-fold dilution of the [γ-^{32}P]ATP in the medium. Therefore, most of the incorporated ^{32}P could not be considered part of an intermediate of the Mg^{2+}-ATPase-catalyzed reaction. Quantitatively similar results were obtained in experiments performed at 0°.

To further substantiate the evidence indicating the presence of a ($Na^+ + K^+$)-ATPase activity in the microsomal fractions from adipose tissue the incorporation of ^{32}P in the presence and absence of monovalent cations and ATPase inhibitors was also measured. Table VII shows that a small but significant increase in ^{32}P incorporation was detected when Na^+ alone was present in the labeling reaction. 3 mM K^+ was sufficient to cancel the increment of incorporation and ouabain also interfered. Azide was without effect upon the stimulation of labeling caused by Na^+, which correlates with the lack of inhibition by azide upon the ($Na^+ + K^+$)-ATPase (Fig. 3). The

TABLE VII

EFFECTS OF Na^+, K^+, OUABAIN AND AZIDE ON THE INCORPORATION OF ^{32}P FROM [γ-^{32}P]ATP INTO MICROSOMES

The reaction mixtures and experimental details were as in Fig. 4. Total incubation time was 15 sec. The absolute incorporations observed in three experiments (between 22 and 28 $\mu\mu$moles ^{32}P per mg protein) were normalized taking as 100% the averaged incorporations in the reactions without additions or with 20 mM Tris–azide alone.

Additions	*Number of reactions*	*Incorporation (%)*	*P values (t test)*
1. None or 20 mM Tris–azide	7	100.1 ± 6.2	(2:1) $P < 0.001$
2. 100 mM Na^+*	7	112.0 ± 4.2	(2:3) $P < 0.01$
3. 3 mM K^+	4	101.5 ± 5.1	(2:4) $P < 0.005$
4. 100 mM Na^+, 3 mM K^+*	6	101.9 ± 5.0	(2:5) $P < 0.02$
5. 100 mM Na^+, 0.1 mM ouabain	4	102.0 ± 6.0	

* With and without 20 mM Tris–azide.

stimulation of labeling caused by Na^+ amounted approximately to 3 $\mu\mu$moles ^{32}P per mg protein. Presumably this would represent the amount of phosphorylated intermediate in the (Na^+ + K^+)-ATPase-catalyzed reaction. It is of interest that the approximated turnover numbers, which can be calculated using this value, are in the same order of magnitude as those found by POST, SEN AND ROSENTHAL[20] in kidney preparations.

DISCUSSION

The results presented establish that a relatively high Mg^{2+}-ATPase activity is present in rat epididymal adipose tissue. The specific activity of the Mg^{2+}-ATPase in the microsomal fractions is higher than those reported for analogous preparations from rat liver[4] and brain[32], rabbit heart[33], beef brain[5] and guinea pig[28]. It has similar or lower activity than those found in preparations from rat heart and kidney[32] and guinea-pig kidney[20].

The Mg^{2+}-ATPase activity of unbroken adipose cells was not significantly changed when the cells were homogenized or lysed by the action of phospholipase C. Since there is evidence indicating that ATP does not penetrate isolated adipose cells[23], it is likely that their Mg^{2+}-ATPase activity is mainly localized on the cell surface or in compartments of the cells readily available to the ATP in the incubation medium. The plasma membrane and its adjacent pinocytotic vesicles[34], for which an ATPase activity has been found by histochemical methods[35], are likely structures for the localization of the activity. It does not seem probable that a high percentage of the total activity would be located on the endoplasmic reticulum since this, in adipose cells, appears to be composed of round vesicles without continuity with the extracellular space. In the light of these considerations the finding that approx. 2/3 of the Mg^{2+}-ATPase activity in the homogenate from whole tissue can be attributed to the microsomal fraction suggests that the plasma membrane of adipose cells and its adjacent vesicles are broken upon homogenization in pieces sedimenting in the 16 000 to 86 000 × g fraction. It is of interest that in liver almost 50% of the Mg^{2+}-ATPase

in the homogenate sediments at 700 × *g* (refs. 3,4), in contrast to our results with adipose tissue where less than 20% of the activity is sedimentable at 15 000 × *g*. It is thought that differences in fragility of the plasma membranes of liver and adipose cells can explain the differences in distribution of the activity in the subcellular centrifugal fractions.

Contrary to the suggestion of TITUS and co-workers[5] for the beef-brain enzyme, no pool of bound ^{32}P with turnover rates high enough to be considered a possible intermediate in the Mg^{2+}-ATPase-catalyzed reaction has been detected in the adipose tissue microsomal preparations. From the experiments performed it is concluded that, if such a pool exists and the ^{32}P is bound in a state which permits its precipitation by 5% trichloroacetic acid, its steady-state level is lower than 5 $\mu\mu$moles per mg protein for microsomal preparations with specific activities about 60 μmoles P_i released per mg protein per h. The findings by SKOU[7] concerning a Mg^{2+}-dependent ATP–ADP exchange in nerve preparations devoid of adenylic kinase activity are consistent with the possible existence of a phosphorylated high-energy intermediate in the Mg^{2+}-ATPase reaction. However, it is probable that not all the ATP–ADP exchange detected in these preparations is a part of the ATPase reaction since STAHL, SATTIN AND MCILWAIN[36] have shown the solubilization of most of the ATP–ADP exchange activity of brain microsomes simultaneous with only small change in the ATPase, which was not solubilized. A small part of the exchange remained in the insoluble portion with the ATPase and could be related to the ATPase activity.

In addition to the Mg^{2+}-ATPase activity, a $(Na^+ + K^+)$-ATPase activity has been detected in the microsomal fractions from adipose tissue. Its absolute values (between 2 and 8 μmoles P_i per mg protein per h) are in the same order of magnitude as the specific activities found for $(Na^+ + K^+)$-ATPases prepared from rat liver and smooth muscle[4], rabbit heart[33], beef brain[5] and Ehrlich ascites carcinoma cells[37]. Tissues with specialized functions involving transport of monovalent cations, like brain and kidney, usually yield enzyme preparations with higher specific activities[4,6,38]. Whether this $(Na^+ + K^+)$-ATPase of adipose tissue may be involved in the transport of monovalent cations remains to be clarified.

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