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Purification and Characterization of a Cation-stimulated Adenosine Triphosphatase from Corn Roots[1]

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ABSTRACT

A membrane-bound, monovalent cation-stimulated ATPase from *Zea mays* roots has been purified to a single band on sodium dodecyl sulfate gel electrophoresis. Microsomal preparations with K^+-stimulated ATPase activity were extracted with 1 M $NaClO_4$, and the solubilized enzyme was purified by chromatography on columns of *n*-hexyl-Sepharose, DEAE-cellulose, and Sephadex G-100 Superfine. A 500-fold purification over the activity present in the microsomes was obtained. The K^+-stimulated activity shows positive cooperativity with increasing KCl concentrations. The purified enzyme shows K^+-stimulated activity with ATP, GTP, UTP, CTP, ADP, α + β-glycerophosphate, *p*-nitrophenyl phosphate, and pyrophosphate as substrates. Under most conditions ATP is the best substrate. Although dicyclohexyl carbodiimide and Ca^{2+} inhibit and alkylguanidines stimulate the K^+-ATPase while bound to microsomes, they have no effect on the purified enzyme.

The absorption of inorganic ions by tissue of higher plants is presumed to be mediated by cation-activated ATPases (9). Of the ATPases associated with plant cell membranes the monovalent cation-stimulated ATPases associated with the plasma membranes of plant roots have received the most attention. The main reason for this attention has been the similarity between the kinetics of short term K^+ influx and the K^+ stimulation of an ATPase associated with the microsomal fraction enriched in plasma membranes (13-15).

In animal and bacterial cells, several ATPases have been shown to be directly involved in the transport of inorganic ions. The proof of the involvement of the $(Na^+ + K^+)$-ATPase of the plasma membrane (8, 25) and the Ca^{2+}-ATPase of sarcoplasmic reticulum (11) in ATP-dependent ion transport came after the enzymes had been purified and reconstitution experiments performed with the purified enzymes. Similar proof that plant ATPases are responsible for ion transport is lacking.

This paper reports the purification and characterization of a monovalent cation-stimulated ATPase from the microsomal fraction of corn roots. This work opens the way for attempts to reconstitute ion transport in lipid vesicles with purified ATPase, providing a direct test of the role of the cation-stimulated ATPase in ion transport.

A preliminary report of portions of this work has appeared (1).

MATERIALS AND METHODS

Materials. β,γ-Methyleneadenosine 5'-triphosphate and α,β-methyleneadenosine 5'-diphosphate were purchased from Miles Laboratories, Inc. P^1,P^5-di(adenosine-5'-)pentaphosphate was purchased from Boehringer Mannheim. The sample of 2-methyl-2-thiopseudourea sulfate was purchased from Eastman Kodak Company. Corn seed (*Zea mays* L., var. W64A) with N cytoplasm was obtained from Clyde Black and Son Seed Farms, Ames, Iowa.

Preparation of Hexyl-Sepharose with CNBr Activation. *n*-Hexylamine was coupled to Sepharose 4B by a combination of methods (21, 24). A 100-ml sample of Sepharose 4B, which had been allowed to settle overnight, was washed with 1 liter of deionized H_2O in a 350-ml sintered glass funnel. Ten g of CNBr dissolved in 10 ml of dioxane was added to the Sepharose, which had been suspended in 200 ml of deionized H_2O. This solution was stirred with a 7.5-cm stirring bar in a 600-ml beaker. A solution of 5 N NaOH was used to adjust the pH to 11, and the pH was maintained at 10.5 to 11 by the addition of 5 N NaOH. Crushed ice was added to maintain the temperature at 20 to 25 C. After 15 min the reaction had slowed considerably as evidenced by the slow drop in pH. The activation was terminated by the addition of crushed ice, filtration on a sintered glass funnel, and washing the gel with 1 liter of ice-cold deionized H_2O.

The *n*-hexylamine (4 mol/mol of CNBr) was dissolved in 80 ml of a solution composed of 40 ml of N,N-dimethylformamide and 40 ml of 0.1 M $NaHCO_3$ (pH adjusted to 9.5 with 3 N HCl). The activated Sepharose was suspended in 200 ml of 0.1 M $NaHCO_3$ (pH 9.5), and the *n*-hexylamine solution was added immediately with swirling. The reaction suspension was swirled on a gyrotory shaker (New Brunswick Scientific Company, New Brunswick, N.J.) overnight at room temperature. The hexyl-Sepharose was then washed according to the procedure of Shaltiel (24). The alkyl-Sepharose was stored in 0.1 M Na_2 EDTA (pH 4.8) to maintain gel stability (27).

Preparation of Alkylguanidines. The alkylguanidines were prepared by reacting *n*-alkylamines with 2-methyl-2-thio-pseudourea sulfate (23).

Preparation of Microsomal Enzyme and $NaClO_4$ Extract. The preparation of corn root microsomes was accomplished by modification of procedures described previously (10). Corn seeds were germinated in covered trays lined with paper towels moistened with a solution containing 0.1 mM $CaCl_2$ and 5 mM KCl. After 3 to 4 days in the dark at 30 C, entire primary roots were excised and immersed in ice-cold deionized H_2O. The roots were drained, blotted dry with paper towels, and weighed. All subsequent steps were carried out at 4 to 5 C. The roots were cut into approximately 1-cm lengths and transferred into a solution of 250 mM sucrose, 3 mM Na_2EDTA, and 15 mM Tris-HCl (pH 7.2) (5 ml/g of roots). The root tissue was homogenized with a Polytron PT-10ST homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.) for 1 to 2 min at a speed setting of 4. For each g of corn roots, 0.25 g of PVP was added and the solution stirred and filtered through four layers of cheese cloth. The solution was centrifuged at 1,600*g* for 10 min (1,600*g* pellet). The supernatant was removed and centrifuged at 12,000*g* for 20 min (12 K pellet). The supernatant from the 12,000*g* run was then centrifuged for 1 hr at 80,000*g*. The

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80,000*g* pellet was suspended in the homogenization buffer and recentrifuged at 80,000*g* for 1 hr.

When microsomes were to be prepared for assay, the final 80,000*g* pellet was resuspended in 250 mM sucrose, 3 mM $MgCl_2$, 3 mM Na_2EDTA, and 15 mM Tris-HCl (pH 7.8), giving a final protein concentration of about 4 mg/ml. This preparation is referred to as the microsomal enzyme. If the extract of the 80,000*g* pellet was to be prepared, the pellet was resuspended in a solution containing equal portions of the microsomal buffer and a solution of 1 M $NaClO_4$, 5 mM $MgCl_2$, 5 mM Na_2EDTA, and Tris-HCl (pH 7.8), giving a final protein concentration of about 4 mg/ml. The mixture was incubated at 30 C for 45 min and centrifuged at 80,000*g* for 1 hr. The supernatant was collected and is referred to as the $NaClO_4$ extract. The $NaClO_4$ extract was stable for several months when stored at −20 C.

A modification of this procedure can eliminate two of the high speed centrifugation runs. The preparation of the $NaClO_4$ extract in some of the later experiments employed a $CaCl_2$ precipitation of microsomes from the 12,000*g* supernatant. The supernatant from the 12,000*g* centrifugation was made 50 mM in $CaCl_2$ by addition of 3 M $CaCl_2$ solution. This solution was allowed to stand for 45 min in an ice bath during which time the solution became turbid. The solution was centrifuged at 12,000*g* for 20 min, and the pellet was taken up in 20 mM Na_2EDTA, 250 mM sucrose, 15 mM Tris-HCl (pH 7.2) (1 ml/g of roots). The suspension was recentrifuged at 13,000*g* for 20 min. This pellet was used to prepare the $NaClO_4$ extract as the second 80,000*g* pellet had been before.

To prepare microsomes from cortex and stele, the cortex tissue was separated by pinching the primary root near the kernel and pulling away from the kernel. The stele was obtained by slicing off the remaining strand of tissue with a razor blade. Microsomes from both cortex and stele were prepared by the centrifugation procedure just described.

To prepare extracts from 1,600*g* and 12,000*g* pellets, the pellets were resuspended in the homogenization buffer and repelleted at 1,600*g* and 12,000*g*, respectively. These pellets were then treated exactly as the second 80,000*g* pellet and extracted with $NaClO_4$ solution at 30 C for 45 min.

Purification of the 80,000*g* pelleted microsomes was accomplished by the discontinuous sucrose density gradient method of Leonard and Hotchkiss (15). This procedure produced microsomes that banded at the interface between 34 and 45% (w/w) sucrose and are reported to be about 70% plasma membrane (17). Another method of preparing microsomes by discontinuous sucrose density gradient centrifugation was used to obtain a spectrum of microsomes banding at the interfaces between 20 and 30%, 30 and 38%, 38 and 45%, and 45 and 50% sucrose (w/w) (10).

Chromatography. The hexyl-Sepharose column (1.6 × 34 cm) was prewashed with 100 ml of 2 M $NaClO_4$, 1.25 mM Na_2EDTA, 1.25 mM $MgCl_2$ (pH 7.8) with Tris followed by a similar solution containing 0.25 M $NaClO_4$ instead of 2 M $NaClO_4$. The $NaClO_4$ extract (20–30 ml) was applied and followed by 20 to 30 ml of 0.25 M $NaClO_4$ solution. This wash was followed by a 100-ml linear gradient of 0.25 M to 2 M $NaClO_4$ solution. A flow rate of 6 to 8 ml/hr was used to collect 3-ml fractions Immediately after use the column was washed and stored in 0.1 M Na_2EDTA, pH adjusted to 4.8 with NaOH. The enzyme which had been purified by chromatography on the hexyl-Sepharose column was concentrated to 10 ml either in a dialysis bag covered with dry Aquacide II-A or by the use of a Millipore immersible molecular separator kit (Millipore Corp., Bedford, Mass.). After dialysis into 0.1 M NaCl, 1.25 mM Na_2EDTA, 1.25 mM $MgCl_2$, pH adjusted to 7.8 with Tris, the enzyme was applied to a DEAE-cellulose column (0.9 × 9 cm) and eluted by gravity flow. The enzyme solution was followed by a 10-ml wash of 0.1 M NaCl solution and finally at a 50-ml wash with 1 M NaCl, 1.25 mM Na_2EDTA, 1.25 mM $MgCl_2$, pH adjusted to 7.8 with Tris. A column of Sephadex G-100 Superfine (2.6 × 36 cm) was used as the final purification step. This column was calibrated for determination of mol wt (22). The enzyme solution from the DEAE-cellulose column was dialyzed into 1 M $NaClO_4$, 1.25 mM $MgCl_2$, 1.25 mM Na_2EDTA, pH adjusted to 7.8 with Tris. The enzyme solution was concentrated to a final volume of 2 to 3 ml and made 10% with respect to sucrose before application to the column of Sephadex. The flow rate was maintained at 6 ml/hr, and 2-ml fractions were collected.

Enzyme Assays. Assays for K^+-stimulated ATPase activity were carried out as described by Tipton *et al.* (26). The reaction mixture routinely contained 0.75 μmol Bis-Tris buffer (pH 6.6), 50 μl or 100 μl of enzyme solution in a total volume of 1 ml. The reaction was initiated by addition of the enzyme (unless otherwise noted) and was carried out at 37 C for times up to 1 hr. The K^+-stimulated ATPase activity is determined by subtracting the activity determined in the absence of KCl from that determined in the presence of KCl. Substrate blanks were subtracted to calculate all enzyme activities.

One unit of enzyme is defined here as the amount of enzyme that will produce 1 μmol of Pi/hr.

The protein in microsomes was determined by the method of Lowry *et al.* (20). The protein determinations for $NaClO_4$ extract and more purified fractions were performed by the 1 to 10-μg range biuret-phenol method (4) and a dye-binding method (3).

SDS Gel Electrophoresis. SDS gel electrophoresis, staining of gels, and mol wt determinations were performed by the Weber and Osborn procedure (28).

RESULTS

Purification. The microsomes after sucrose density gradient centrifugation have highest K^+-ATPase activity at the 30 to 38% and 38 to 45% sucrose boundaries (see Table I). These regions correspond to the positions on the gradient reported to have the greatest percentage of plasma membranes (17). The K^+-ADPase activity was maximal at the interface between 30 to 38%, and the activity of an enzyme that hydrolyzed AMP was maximal at the 38 to 45% interface and at the top of the gradient. There was no K^+ stimulation of the hydrolysis of AMP.

The results in Table II show that while there is no difference in the K^+-ATPase and K^+-ADPase activity between microsomes prepared from cortex and stelar tissue, there is a significant difference between the two tissue types in the amount of activity that is not KCl-stimulated. In cortex, the hydrolysis of ADP is

Table I Hydrolysis of ATP, ADP, and AMP by microsomes purified by sucrose density gradient centrifugation.

Gradient Position	Activity (units/mg protein)		
(% sucrose)	K^+-ATPase[1]	K^+-ADPase[1]	AMPase[2]
Top	0	0.55	7.2
20-30%	1.8	1.7	4.0
30-38%	4.4	3.0	6.0
38-45%	3.4	1.6	8.5
45-50%	2.0	0.3	7.4

[1] Stimulation by 50 mM KCl.

[2] Activity in absence of KCl.

Table II Assay of microsomes from cortex and stele.

			Activity (units/mg protein)		
	Protein[1]	Substrate	-K	+K[2]	K^+-Stimulation
Cortex	19.7 mg (4.7 mg/ml)	ATP	4.34	5.73	1.41
		ADP	6.04	6.66	0.62
Stele	7.1 mg (4.7 mg/ml)	ATP	2.38	3.91	1.53
		ADP	1.96	2.45	0.49

[1] Total protein isolated as microsomes.

[2] 50 mM KCl.

three times the activity of the stele enzymes. The enzymes hydrolyzing ATP also have a greater specific activity in the cortex.

Chaotropic salts have been used for the solubilization of several membrane-bound proteins (7). To compare the effectiveness of $NaClO_4$ and NaCl in the solubilization of the K^+-ATPase, a batch of microsomes was divided into two equal portions. One portion was extracted with 1 M NaCl and the other with 1 M $NaClO_4$. After incubation in the extraction medium, the suspensions were centrifuged at 80,000g, the supernatants (extracts) removed, and the pellets resuspended in microsomal buffer and assayed. The $NaClO_4$ extract removes less protein, leaves less K^+-ATPase in the pellet, and results in twice the specific activity in the extract as compared with the NaCl extract (Table III). The pellet from the $NaClO_4$ extract contains about 25% of the activity recovered while the pellet from NaCl treatment contains about 70% of the recovered activity.

The 1,600g and 12,000g centrifugations remove most of the cell wall and mitochondria (10). To determine if the $NaClO_4$-extracted K^+-ATPase originated in either of these cell fractions, the pellets from the 1,600g and 12,000g centrifugations were extracted with $NaClO_4$ solution. For comparison, results in Table IV report the protein content and activities of an extract prepared from $CaCl_2$-precipitated microsomes. It is not likely that the $NaClO_4$-extracted K^+-ATPase originates with either cell walls or mitochondria.

Chromatography of the extract over hexyl-Sepharose prepared via CNBr activation is illustrated in Figure 1. Eluting at the void volume was a large peak of 280 nm absorbing material, which contained enzyme activity that hydrolyzed ATP in the presence of Mg^{2+}, but this activity was not KCl-stimulated. As the linear elution gradient progressed from 0.25 M to 0.50 M $NaClO_4$, the K^+-ATPase activity was eluted. After several months of use, the hexyl-Sepharose had lost much of its resolving capabilities and was replaced with freshly prepared material.

The enzyme solution which had been purified by hexyl-Sepharose chromatography was dialyzed into 0.1 M NaCl solution and applied to a DEAE-cellulose column. The K^+-ATPase did not bind to the DEAE-cellulose column when the column was eluted with 0.1 M NaCl. Washing with 1 M NaCl released a large amount of 280 nm absorbing material (Fig. 2). Immediately after the enzyme had eluted from the DEAE-cellulose column, it was applied to a column (0.9 × 20 cm) of hexyl-Sepharose which was equilibrated with 0.1 M NaCl solution. This column was washed with 15 ml of 0.1 M NaCl solution and the enzyme eluted in a sharp band by washing with a short pulse of 2 M $NaClO_4$ solution. This was done to concentrate the enzyme and to get the enzyme back into a $NaClO_4$ solution.

The enzyme from DEAE-cellulose chromatography was dialyzed into 1 M $NaClO_4$ solution and applied to a column of Sephadex G-100 Superfine. The chromatographic profile in Figure 3 of Sephadex G-100 chromatography shows the presence of a single, well defined peak of K^+-ATPase. Exactly corresponding to this peak of K^+-ATPase was a peak of K^+-ADPase activity. Fractions 45 and 48 contained an activity that hydrolyzed AMP. The 280 nm absorbances in this figure are to be interpreted with care because they are very near the limits of detectability.

The results of SDS gel electrophoresis performed on protein samples at various stages of the purification are pictured in Figure 4. Only minor differences between the SDS gel electrophoresis patterns of the microsomes and extract can be seen. The separation of the four protein bands in the sample eluted from hexyl-Sepharose suggested that the proteins present differed enough in mol wt to be resolvable on a Sephadex G-100 column. However, gel electrophoresis of the protein from the Sephadex G-100 column showed three distinct bands, two at the top of the gel very close together and a single band near the middle. The bands at the top of the gel were shown to be spurious when found on a control gel to which had been applied SDS sample buffer dialyzed in a Fischer Scientific Co. dialysis bag. These spurious bands were found by Lees and Sepka (12) to be due to the growth of a microorganism on the dialysis bags. The bands were completely eliminated by the use of 0.01% sodium azide in the dialysis buffer

Table III Extraction of root microsomes with $NaClO_4$ or with NaCl.

Procedure	Total protein in Extract	Total protein in Pellet	Activity (units/mg protein) K^+-ATPase in Pellet	Activity (units/mg protein) K^+-ATPase in Extract
$NaClO_4$ Extract	5.6 mg	34 mg	0.19	3.55
NaCl Extract	8.0 mg	33 mg	0.89	1.60

[1]Starting microsomes (prepared by ultracentrifugation (10)) had specific activity of 1.89 units/mg protein.

Table IV Extraction of pellets from low speed centrifugation with $NaClO_4$.

Sample	Protein (mg/ml)	Activity (units/mg protein) -K	+K	K+-Stimulation
1,600 g x 10 min	0.30	1.7	1.7	--
12,000 g x 20 min	1.02	7.3	7.6	0.3
Extract from $CaCl_2$ precipitated microsomes	1.70	16.7	20.5	3.8

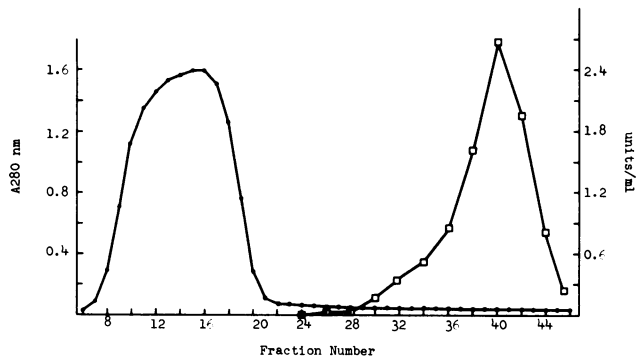

FIG. 1. Chromatography with CNBr-activated hexyl-Sepharose. *A* at 280 nm (·) and K^+-ATPase activity (□).

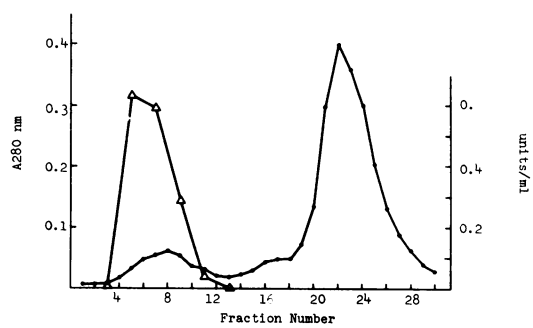

FIG. 2. Chromatography with DEAE-cellulose. *A* at 280 nm (·) and K^+-ATPase activity (Δ).

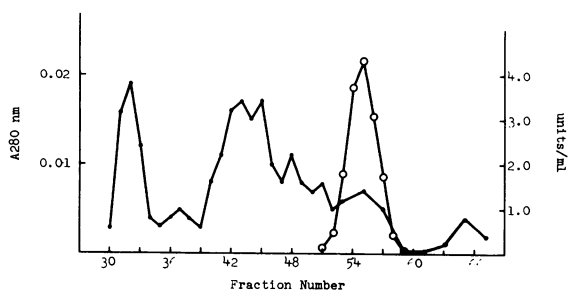

FIG. 3. Chromatography with Sephadex G-100 Superfine. *A* at 280 nm (·) and K^+-ATPase activity (○).

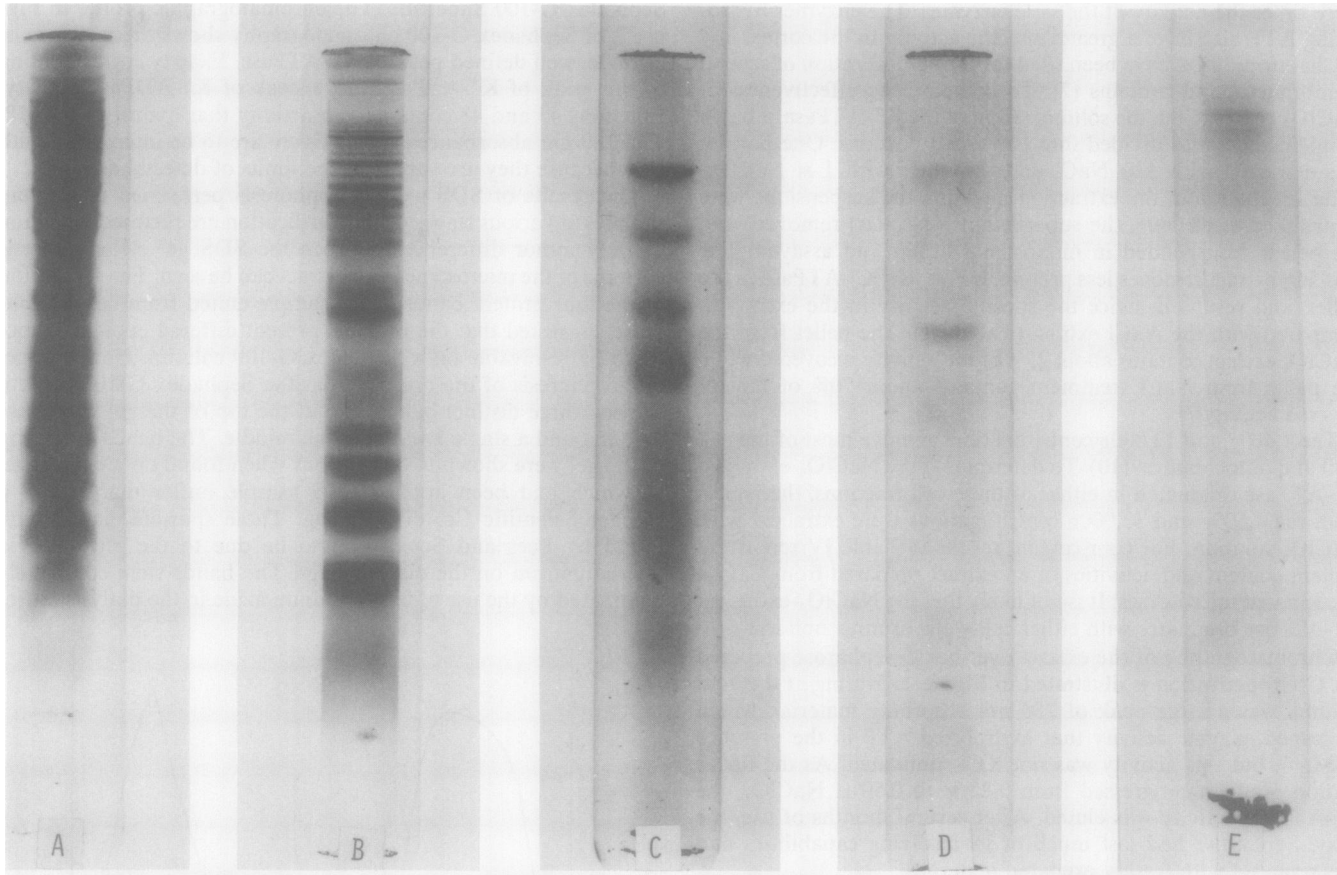

FIG. 4. SDS gels of protein at various stages of purification. *A*: microsomes, 100 μg; B: $NaClO_4$ extract, 75 μg; C: hexyl-Sepharose, 30 μg; D: G-100 purified, 10 μg; E: blank gel, see under "Results."

and sample buffer. Approximately 10 μg of protein, eluted from the Sephadex G-100 column, was applied to the gel for SDS electrophoresis. This is the entire amount of protein purified from 400 to 600 g of fresh corn roots.

The specific activity, recovery, and purification at each stage of the purification scheme are given in Table V. Recoveries of 100% of the activity present in the extract were common after hexyl-Sepharose chromatography. This step also showed the most dramatic purification. The over-all recovery was 45% and nearly 500-fold purification was obtained by the procedure outlined in Table V.

A $NaClO_4$ extract was made using the microsomes prepared by discontinuous sucrose density gradient centrifugation (17). Microsomes prepared in this way reportedly contain approximately 70% plasma membranes (15). The $NaClO_4$ extract made from these had a K^+-ATPase activity of 6.2 units/mg of protein. The extract was further purified by hexyl-Sepharose, DEAE-cellulose, and Sephadex G-100 chromatography. The K^+-ATPase prepared by this procedure behaved the same as the material prepared without the discontinuous sucrose density gradient centrifugation.

Characterization. Estimation of mol wt was performed by measuring the mobility of the Sephadex-purified enzyme and the mobilities of proteins of known mol wt during SDS gel electrophoresis. The elution volume of the K^+-ATPase on Sephadex G-100 Superfine chromatography and the elution volume of proteins of known mol wt were also used to estimate the mol wt. SDS gel electrophoresis and Sephadex chromatography provided estimates of 30,500 and 27,000, respectively.

The enzyme purified through hexyl-Sepharose chromatography showed increasing activity as a function of increasing KCl concentration, as presented in Figure 5. The pattern of K^+-stimulation of the ATPase suggests positive cooperativity. Figure 6 shows a Hill plot of the data in Figure 5. Using K^+ concentrations from 74.6 to 205 mM, a value of n equal to 3.1 was found by linear regression.

Table V Purification and recovery of K^+-ATPase

Sample	Specific Activity[1]	% Recovery	Purification
Microsomes	1.75	--	--
$NaClO_4$ Extract	5.70	79	3.3
Hexyl-Sepharose Column	110	79	63
DEAE Column	450	65	260
G-100 Column	850	45	490

[1] Units/mg protein.

Monovalent cation stimulation at concentrations of 50 mM followed the order $NH_4^+ > K^+ > Na^+ > Li^+$, with relative activities of 104, 100, 87, and 66 for the enzyme purified through Sephadex G-100 chromatography.

Figure 7 shows a plot of 1/velocity *versus* 1/Mg·ATP concentration for the hexyl-Sepharose purified enzyme at 50 and 300 mM KCl concentrations. The substrate concentration at which maximum ATPase activity is observed is near 0.75 mM Mg·ATP. Inhibition by Mg·ATP is more pronounced at 50 mM KCl than at 300 mM KCl. Inadequate sensitivity of the assay for Pi prohibited the use of Mg·ATP concentrations less than 0.31 mM. At lower concentrations, more than one-third of the substrate would be utilized to produce enough Pi to be measured accurately. Therefore, a meaningful apparent K_m for Mg·ATP could not be determined with this assay.

The activity of the enzyme at two stages of purification on several substrates is shown in Table VI. The enzyme purified with hexyl-Sepharose shows higher specific activities with the substrates ADP and AMP than with ATP. After chromatography with Sephadex the activity with ADP is considerably lower than with ATP, and the activity that hydrolyzes AMP is nearly gone. Pyro-

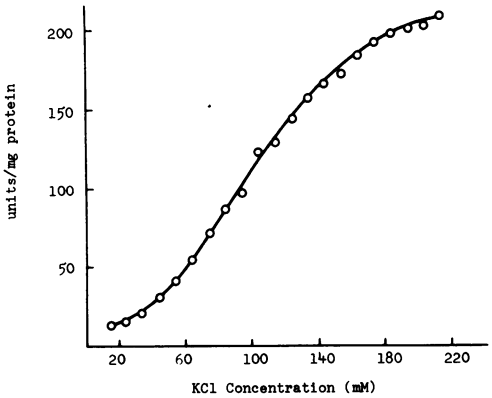

FIG. 5. Activity of enzyme at increasing KCl concentrations. Assays of hexyl-Sepharose-purified enzyme, which had been dialyzed into 20 mM HEPES, adjusted to pH 7.7 with KOH, were preformed in 20 mM HEPES, 0.75 mM ATP (potassium salt) 0.75 mM $MgCl_2$ (pH 7.7), and with 0.01 mg/ml of BSA. Potassium concentration increased from 14.6 mM (present in buffer and substrate) to 215 mM by increments of 10 mM.

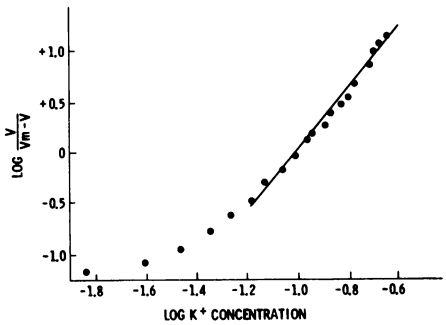

FIG. 6. Hill plot of log $V/(V_m - V)$ versus log (K^+ concentration). Using the equation, log $V/(V_m - V)$ = n log [S] − log K a value of n = 3.1 was determined by linear regression for points representing K^+ concentrations of 74.6 mM to 215 mM.

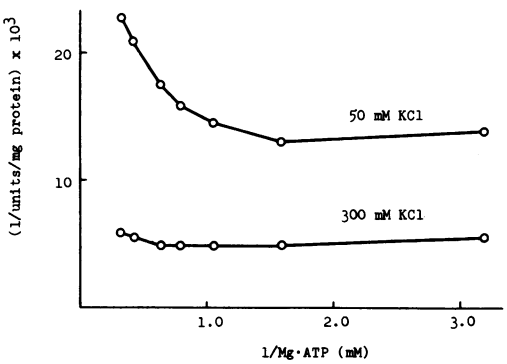

FIG. 7. Kinetics of ATPase at 50 mM KCl and 300 mM KCl. Assay buffer and enzyme as in Figure 5. Mg·ATP concentrations from 0.31 to 3.1 mM were used. The potassium concentration was 50 and 300 mM for the upper and lower curves, respectively.

phosphate and *p*-nitrophenylphosphate are substrates with approximately 10% of the K^+-stimulated activity of ATP. The inhibition by K^+ of pyrophosphate hydrolysis by the hexyl-Sepharose enzyme and stimulation after Sephadex chromatography are as yet unexplained results.

The AMP- and ADP-hydrolyzing activities in the hexyl-Sepharose-purified enzyme were initially believed to be separate enzymes, distinct from the activity that hydrolyzed ATP. After Sephadex chromatography, the AMPase activity eluted ahead of the K^+-ATPase and had a mol wt of 49,000 as calculated from its elution volume. The K^+-ADPase was found to coincide exactly

Table VI Activities of the enzyme at different stages of purification with various substrates[1].

	Purified thru hexyl-Sepharose			Purified thru Sephadex G-100		
	-K[2]	+K[3]	K+- Stimulation	-K	+K	K+- Stimulation
ATP	14.1	83.9	69.8	71	895	824
ADP	45.2	135.7	90.5	164	636	471
AMP	150	142	--	24	24	--
α+β-Glycero-P[4]	2.8	6.6	3.8	24	59	35
p-Nitrophenyl-P	13.2	21.7	8.5	106	188	82
Pyrophosphate	48.1	40.5	--	553	647	94

[1]Assay contained 0.75 mM substrate (sodium salt), 100 mM glycine, adjusted to pH 7.8 with Tris, 0.75 mM $MgCl_2$, 0.01 mg/ml BSA, and 50 mM KCl (when added).

[2]Assay without added KCl.

[3]Assay with 50 mM KCl.

[4]Mixture of 25% α and 75% β.

with the peak of K^+-ATPase activity. Because the two co-purify in this manner, it is assumed that both activities are possessed by the same enzyme. Results presented later will support this contention.

Assays of the Sephadex-purified enzyme in two different buffer systems with various nucleotides were performed (Table VII). Two portions of the enzymes were dialyzed against 1 M $NaClO_4$ solution and 0.188 M Tris-glycine solution. In the final assay mixture, the enzyme in $NaClO_4$ was assayed with 50 mM $NaClO_4$ and had a final pH of 6.7, while the enzyme in Tris-glycine was assayed with 9.4 mM Tris-glycine and had a final pH of 7.9. The rest of the assay mixture was the same as the assay mix described under "Materials and Methods," except for the different substrates. In the Tris-glycine system ATP is the best substrate of those tested, while in the $NaClO_4$ system ADP and CTP are both better substrates. The large changes in substrate specificity are probably a result of changes in pH (6.7 *versus* 7.9) and concentration of $NaClO_4$.

To test the hypothesis that the K^+-stimulated hydrolysis of both ATP and ADP are activities of the same enzyme, two inhibitors of ATP and ADP hydrolysis were used (29). The structural analog of ATP and ADP, β,γ-methyleneadenosine 5'-triphosphate (AP-POPCP) and α,β-methyleneadenosine 5'-diphosphate (AOPCP), were included in assays for K^+-ATPase and K^+-ADPase activity. Because the K^+-ATPase activity showed substrate inhibition (Fig. 7) in the range for which sensitive assays could be performed, the normal procedure of looking for competitive inhibition of both activities by both analogs could not be used. Instead an assay was performed with each analog present in the assay of each substrate. The results in Table VIII show that the ATP analog (AOPOPCP) inhibits ATP and ADP hydrolysis by 50 and 51%, respectively. The ADP analog inhibits ATP and ADP hydrolysis by 77 and 82%, respectively. The similar inhibition of hydrolysis of ATP and ADP by each of the analogs argues for the presence of both K^+-ATPase and K^+-ADPase activity on the same enzyme.

Using the same assay system as described in Table VIII, assays were run to determine the effects of an inhibitor of myokinase activity, p^1,p^5-di(adenosine-5'-)pentaphosphate [$A(P)_5A$], (19, 26). Assays with $A(P)_5A$ revealed K^+-stimulated hydrolysis of the inhibitor of 695 units/mg of protein. Assays with ADP present with the inhibitor showed 530 units/mg of protein of activity. This compound seems to be a substrate for the enzyme with activity midway between those with ATP and ADP.

Alkylguanidines have been shown to be inhibitors of K^+ uptake in barley roots (18). Of the alkylguanidines tested, the octyl and hexyl homologs were the best inhibitors. When ethyl-, butyl-, hexyl-, and octylguanidine were incubated for 15 min with microsomal preparations and the enzyme then assayed for ATPase activity, stimulation by all the alkylguanidines tested was observed (see Table IX). The stimulation of each of the alkylguanidines at 2 mM was equal to or greater than stimulation by 50 mM KCl. Of

Table VII Relative activity of Sephadex purified enzyme with various substrates.[1]

	Enzyme in $NaClO_4$[2]	Enzyme in Tris-Glycine[3]
ATP	100	100
ADP	140	45
AMP	12	1
CTP	127	84
UTP	96	85
GTP	34	64

[1]The enzyme was purified through Sephadex chromatography and dialyzed into 1.0 M $NaClO_4$, 1.25 mM Na_2EDTA, 1.25 mM $MgCl_2$, and adjusted to pH 7.8 with Tris or 0.188 M Tris-glycine pH 8.9. Assays contained 0.75 mM substrates (sodium salt), 50 µl of enzyme and the usual assay mixture as described in Materials and Methods. Activity with ATP = 100.

[2]Final assay mix has 50 mM $NaClO_4$ and is pH 6.7.

[3]Final assay mix has 9.4 mM Tris-glycine and pH 7.9.

Table VIII Effect of β,γ-methyleneadenosine-5'-triphosphate and α,β-methyleneadenosine-5'-diphosphate on K^+-stimulated ATP and ADP hydrolysis[1].

	K^+-Stimulated Activity (units/mg protein)[2]
ATP	824
ADP	459
AOPOPCP	0
AOPCP	0
ATP + AOPOPCP	412
ATP + AOPCP	188
ADP + AOPOPCP	224
ADP + AOPCP	82.4

[1]Assays contained 0.75 mM ATP or ADP sodium salt), 0.75 mM analogue (if present), 0.75 mM $MgCl_2$ (1.5 mM $MgCl_2$ if analogue present), 0.01 mg BSA per ml, 10 mM glycine adjusted to pH 7.8 with Tris, and the Sephadex purified enzyme.

[2]Stimulation by 50 mM KCl.

Table IX Effect of alkylguanidines on microsomal K^+-ATPase[1].

	Activity (units/mg protein) -K	+K
Control	23.1 ± .4	25.3 ± .4
Ethylguanidine	25.3 ± .1	
Butylguanidine	25.5 ± .1	
Hexylguanidine	27.7 ± .1	
Octylguanidine	25.7 ± .1	

[1]Assays were performed by incubating the enzyme with 2 mM alkylguanidine for 15 minutes at 37°C, and starting the reaction by adding Mg·ATP (# mM final concentration).

[2]No alkylguanidine present.

the alkylguanidines tested, hexylguanidine showed the greatest stimulation of the microsomal ATPase. Figure 8 shows the stimulation by hexylguanidine at concentrations from 0.01 mM to 2 mM. The activity in 2 mM hexylguanidine is not further stimulated by the addition of 50 mM KCl, indicating that the hexylguanidine is affecting the same enzyme as is the K^+. If the hexylguanidine were stimulating another ATPase, one would expect further stimulation by 50 mM KCl. Assays of the G-100 purified enzyme preincubated with each of the alkylguanidines showed no effect on the ATPase activity.

DCCD[2] at 0.04 mM reduces activity of the microsomal enzyme to 26% of control but has no effect at 0.2 mM on the enzyme after it has been solubilized (data not shown). Treatment of microsomes or enzymes purified by hexyl-Sepharose chromatography with Nbf-Cl or with quercitin had no effect on K^+-ATPase activity (data not shown).

Calcium has been reported to inhibit the K^+-ATPase of the plasma membrane fractions from corn roots by 73% (in the presence of Mg^{2+}) at 0.5 mM Ca^{2+} (15). In our experiments, assay

[2] Abbreviations: DCCD: N,N'-dicyclohexylcarbodiimide; Nbf-cl: 4-chloro-7-nitrobenzofurazan.

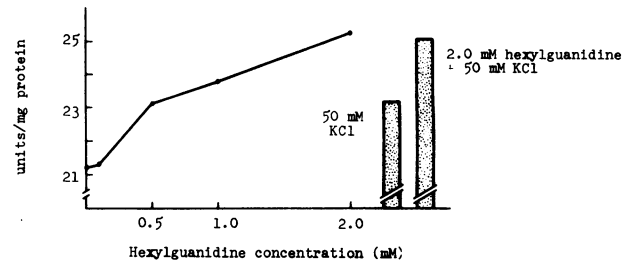

FIG. 8. Effect of hexylguanidine and 50 mM KCl on microsomal K^+-ATPase. Assays were performed using a 15-min preincubation at 37 C with hexylguanidine. Assays were started by the addition of Mg ATP (3 mM final concentration). Bar graph represents activity of the microsomes with 50 mM KCl (left bar) or with 2 mM hexylguanidine and 50 mM KCl (right bar).

Table X Effect of added $CaCl_2$ on K^+-ATPase activity[1].

$CaCl_2$ concentration (mM)	Activity (units/mg protein)
0	23.1
0.02	23.3
0.05	23.3
0.10	23.3
0.20	22.5
0.50	22.0
0.75	21.2

[1]The hexyl-Sepharose purified enzyme was assayed in 20 mM HEPES, adjusted to pH 7.7 with KOH, 0.75 mM $MgCl_2$, 0.75 mM ATP (potassium salt), 0.01 mg/ml BSA, 50 mM KCl and varying concentrations of $CaCl_2$.

of a similar membrane fraction resulted in only 22% inhibition (data not shown) and there was little or no inhibition of the purified enzyme (Table X).

DISCUSSION

The basic procedure for obtaining plasma membrane from corn roots is to prepare a root homogenate, by grinding the root tissue, and to centrifuge at 1,600*g* and 12,000*g* to remove cell walls and mitochondria. The supernatant is centrifuged at 80,000*g*, and the pellet resuspended and applied to a discontinuous sucrose density gradient. The plasma membranes band at the interface between 34 and 45% sucrose (w/w) (15). Because of low recovery of plasma membranes from discontinuous sucrose density gradient centrifugations it is difficult to get enough material to allow purification of the K^+-ATPase in quantities needed for characterization. Therefore, the pellet from the 80,000*g* centrifugation was used for further purification. The use of the 80,000*g* pellet for the extraction of the K^+-ATPase seems justifiable because when the plasma membrane fraction prepared by discontinuous sucrose density gradient centrifugation was used for preparation of the $NaClO_4$ extract, the extract prepared had a higher K^+-ATPase activity than the extract prepared from the 80,000*g* pellet (6.2 *versus* 5.7 units/mg of protein). Enzyme preparations purified from crude microsomes and from density gradient centrifugation fractions enriched in plasma membranes were identical. Extracts of pellets enriched in cell walls and mitochondria lacked significant K^+-ATPase activity (Table IV).

Previous reports (15) have not shown in K^+-stimulated hydrolysis of ADP that was present in the preparation of microsomal enzyme. The K^+-ADPase activity seems closely associated with the K^+-ATPase and is banded on the discontinuous sucrose gradient in fractions enriched in plasma membranes (Table I). Assays also revealed the presence of an AMP-hydrolyzing activity whose specific activity is almost twice that of the K^+-ATPase. Leonard and Hotchkiss (15) report a ratio of AMP-hydrolyzing activity to K^+-ATPase activity of about 0.5. The differences in K^+-ADPase and AMP-hydrolyzing activity may be due to the differences in the varieties of corn used in these studies. The specific activity of

the K^+-ATPase in the plasma membrane fractions is similar to that reported by Leonard and Hotchkiss (15).

Extraction of the enzyme from the microsomes in the 80,000*g* pellet was performed by using the chaotropic salt, $NaClO_4$. A 63-fold purification of the K^+-ATPase, over the activity present in the microsomes, was obtained by use of a hexyl-Sepharose column prepared via CNBr activation of the Sepharose (Table V). The chromatography with DEAE-cellulose offered significant purification (Table V), but the SDS electrophoresis gels show that this step can be eliminated and in most preparations it was omitted. Chromatography using Sephadex G-100 Superfine removed the AMP-hydrolyzing activity that had been present up to this point. A single band on SDS gels was obtained from enzyme purified through Sephadex G-100 chromatography.

Using the SDS gel as a criterion for purity, one could estimate that the protein is at least 90% pure if the Coomassie blue stain can detect 1 μg of protein as suggested by Weber and Osborn (28). The removal of the AMP-hydrolyzing activity is perhaps more important than the purity of the enzyme. The AMPase was the only activity detected by Pi assay that was not K^+-stimulated (Table VI).

The K^+-ATPase extracted from microsomes with $NaClO_4$, chromatographed sequentially over hexyl-Sepharose, DEAE-cellulose, and Sephadex G-100 Superfine was purified almost 500-fold over the activity present in the microsomes prepared from the 80,000*g* centrifugation. When stored at −20 C in 0.25 M $NaClO_4$, the enzyme was stable for several months.

The turnover number for the K^+-ATPase is 6.4/sec. Although this number is low for an enzyme-catalyzed reaction, the highest turnover number for the animal ($Na^+ + K^+$)-ATPase is only 60 (5). Most preparations of the ($Na^+ + K^+$)-ATPase are one-half this number.

Several pieces of information imply that the K^+-ATPase purified in this work is associated with membranes and that these membranes are plasma membranes. First, the K^+-ATPase activity is greatest in fractions having the greatest amount of plasma membranes (Table I and ref. 17). Second, the extraction with $NaClO_4$ of the microsomes that are enriched in plasma membranes yielded a higher specific activity for the K^+-ATPase than the extract from the crude microsomes prepared by the 80,000*g* centrifugation. The superior ability of $NaClO_4$ compared with NaCl to extract the K^+-ATPase implies that the chaotropic salt, $NaClO_4$, solubilizes a protein that is bound to the membrane by hydrophobic interactions. Since NaCl also extracts some of the K^+-ATPase, the binding to the membrane may also involve ionic interactions. Chaotropic salt treatment has not been sufficient to extract K^+-ATPase from other plant materials we have tried. Other procedures, involving detergents, are being developed for purification of the enzyme from etiolated corn shoots and other sources (unpublished experiments). The reason for the difference in behavior of the enzyme from corn roots and from other similar sources is not yet known.

The relative activities of several nucleotides as substrates for the enzyme under two different assay conditions differ markedly (Table VII). The reasons for this difference have not yet been explored, although we have preliminary evidence that the enzyme aggregates at low ionic strength.

The use of a specific inhibitor of the K^+-ATPase would certainly facilitate identification of the role this enzyme plays in the cell. Of the compounds tested, the most interesting were the alkylguanidines and DCCD. The alkylguanidines have been shown to inhibit K^+ uptake in barley roots (18). The stimulation of the K^+-ATPase of the microsomal fraction by hexylguanidine (Table IX and Fig. 8) is especially interesting when one compares the structure of hexylguanidine with the structure of the hexyl-Sepharose ligand used for purification of the K^+-ATPase. Both structures contain a positively charged group at the end of a six carbon alkyl chain. The hexyl-Sepharose chromatography may have purified the enzyme by a combination of affinity (monovalent cation) chromatography and hydrophobic (six carbon alkyl arm) chromatography. Stimulation of the membrane-bound enzyme by hexylguanidine might be explained if the alkylguanidines are concentrated in the vicinity of the enzyme by insertion of the chains into the membrane to which the enzyme is bound. The solubilized enzyme would not experience a similar stimulation at 2 mM hexylguanidine because of the lack of the membrane. Alkylguanidine may inhibit K^+ uptake by roots by uncoupling ATP hydrolysis from ion transport rather than by inhibiting the hydrolysis.

DCCD, quercitin, and Nbf-Cl have been used as inhibitors of various ion-stimulated ATPases (reviewed in ref. 2). Of these, we found quercitin and Nbf-Cl had no effect on the corn root enzyme and DCCD was an effective inhibitor only while the enzyme was membrane-bound.

The inhibition by Ca^{2+} of the microsomal K^+-ATPase (15) and the lack of an effect on solubilized enzyme (Table X) suggest that the inhibitory action of Ca^{2+} on K^+ influx in corn roots (16) may be through an indirect effect on the enzyme. Leonard and Hotchkiss (15) have made a similar suggestion.

The pattern of K^+ stimulation of the ATPase (Fig. 6) suggests that the enzyme shows positive cooperativity in K^+ binding (6). This result does not require a multisubunit enzyme (6), but it does provide a minimal estimate of the number of K^+-binding sites. Because the K^+-ATPase is a membrane-bound enzyme, the kinetics of K^+-stimulation and the kinetics of the K^+-ATPase as a function of Mg·ATP concentration (Figs. 5 and 7) are probably not directly comparable for a membrane-bound enzyme and the solubilized enzyme. Reconstitution of the K^+-ATPase with membrane lipids is being performed to compare the kinetic properties of the membrane-bound and the solubilized enzyme. The reconstituted enzyme would provide a better approximation to the native enzyme.

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