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PURIFICATION AND CHARACTERIZATION OF $(Na^+ + K^+)$-ATPase

IV. ESTIMATION OF THE PURITY AND OF THE MOLECULAR WEIGHT AND POLYPEPTIDE CONTENT PER ENZYME UNIT IN PREPARATIONS FROM THE OUTER MEDULLA OF RABBIT KIDNEY

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SUMMARY

1 The purpose has been to examine the purity of the highly active preparation of $(Na^+ + K^+)$-ATPase described recently (Jørgensen, P L. (1974) Biochim. Biophys. Acta, 356, 36–52) and to determine the molecular weight and the polypeptide content per enzyme unit.

2 The concentration of sites for binding of ATP and ouabain and for sodium-dependent phosphorylation is proportional to the activity of $(Na^+ + K^+)$-ATPase in a range from 15 to 37 μmoles $P_i \cdot min^{-1} \cdot mg^{-1}$ protein. The maximum content of the large phosphorylated polypeptide ($M_r = 96\,000$) is 72 % of the total protein and the content of small polypeptide ($M_r = 35\,000$–$57\,000$) is close to 1 mole per mole large chain.

3 The receptor capacities (pmoles $\cdot$ (μmoles $P_i \cdot min^{-1})^{-1}$) are 105 ± 13 for ATP, 112 ± 5 for ouabain and 221 ± 12 for sodium-dependent phosphorylation The maximum weight per site is 250 000 g protein per mole ATP bound, 278 000 g per mole ouabain bound and 132 000 g per mole phosphate incorporated

4. The results suggest that the enzyme unit binding one molecule of ATP or ouabain contains two chains of large polypeptide and that both chains are phosphorylated from ATP in the presence of Mg^{2+} and Na^+.

5. The purity of the preparation with respect to protein is close to the maximum if the enzyme contains one mole of small chain per mole of large chain. The capacities for binding of ATP or ouabain are higher than or equal to the highest values reported before. The catalytic functions of $(Na^+ + K^+)$-ATPase are well preserved since the molar activity is high and all large chains in the preparation can be phosphorylated from ATP

INTRODUCTION

Information on the molecular size and composition of the protein part of the sodium pump may be derived from studies on purified preparations of $(Na^+ + K^+)$-

ATPase if exact determinations of the number of enzyme units can be related to the content of protein in the preparations. Recently, different procedures for purification of $(Na^+ + K^+)$-ATPase have been developed [1–9] The protein (M_r = 90 000–100 000) that has been identified as an important component of the enzyme [2–11] forms a major part of the total protein in preparations with specific activities of $(Na^+ + K^+)$-ATPase varying from 10–13 [2, 3] to 25 [4–7], 32–37 [8] and 7–117 [9] μmoles $P_i \cdot min^{-1}$ mg^{-1} protein In some of the preparations the number of enzyme units has been determined as the number of sites for sodium-dependent phosphorylation [4, 9, 10] or for binding of cardiac glycosides [7, 12, 13] The data show that the molar activity per enzyme unit varies as much as the specific activity This may mean that the specific activity cannot be regarded as a valid criterion of purity, but it can also mean that the various purification steps have different effects on the catalytic properties of the enzyme In either case, the variability of the data hampers comparison of the purity of the different preparations

In the present work, the purity of the highly active preparations of $(Na^+ + K^+)$-ATPase described in a previous paper [8] has been examined by several different criteria and an attempt was made to determine the molecular weight and the polypeptide content per enzyme unit The relationship between the activity of $(Na^+ + K^+)$-ATPase and the capacities for binding of ATP [14, 15] and ouabain [16, 17] and for sodium-dependent phosphorylation [18, 19] was determined for a series of membrane preparations with specific activities ranging from 15 to 37 μmoles P_i min^{-1} mg^{-1} protein [8] Estimates of the content of polypeptides were obtained after fractionation in sodium dodecylsulphate by gel chromatography [20] or gel electrophoresis [21, 22] The value of the above parameters as criteria of purity is discussed, and the purity of the preparations is compared with that of the other preparations

EXPERIMENTAL

Preparation and assay of $(Na^+ + K^+)$-ATPase

The enzyme was isolated from the outer medulla of rabbit kidneys by incubation of microsomal fractions with sodium dodecylsulphate and ATP followed by centrifugation in a zonal rotor (25–37 units $\cdot$ mg^{-1} protein) or in an angle rotor (15–25 units mg^{-1} protein [8]) Before determination of ATP binding or phosphorylation, the enzyme preparations were washed twice by centrifugation in 25 mM imidazole–1 mM EDTA, pH 7.5 (20 °C) to remove ions and nucleotides Analyses for protein and phosphate were done as before [8]. The activity of $(Na^+ + K^+)$-ATPase was measured as the initial rate of release of P_i in the presence of 3 mM $MgCl_2$, 3 mM ATP (Tris salt), 130 mM NaCl, 20 mM KCl, 30 mM histidine, pH 7 5 (37 °C) minus the rate of release in the same medium with 1 mM ouabain added After equilibration for 5 min at 37 °C, 0 7–1.5 μg of enzyme protein were added to 1 ml and the amount of P_i released was measured after 1, 3 and 5 min of incubation at 37 °C Initial rates were calculated from linear plots of the release of P_i versus time

ATP binding

Aliquots of the enzyme preparation each containing 0.1–0 17 mg protein were mixed in centrifuge tubes (1 cm × 6 cm) at 0 °C with 0.06 μCi [^{14}C]ATP

(purified Tris salt [14]) and unlabelled ATP in a total volume of 1 ml 30 mM Tris–EDTA, pH 7 5 (20 °C). In six tubes the concentrations of ATP were varied from 0.1 to 1 2 μM To estimate unbound [^{14}C]ATP in the sediments, 3 mM unlabelled ATP was added to two control tubes. After centrifugation at 50 000 rev./min for 30 min at 0 °C the supernatant fluid was removed and 25 μl were transferred to counting vials Remaining droplets were wiped off and the sediments were dissolved in 0 25 ml 1 M NaOH with heating for 30 min at 60 °C 0 1 ml of the dissolved pellets were transferred to vials containing 10 ml of a Triton–toluene scintillation solution [23] and counted in a Packard scintillation counter equipped with an internal standard for correction of quenching. 25–50 μl of the remaining dissolved pellet were used for protein determination in duplicate by the Lowry method [24] The recovery of protein in the sediment was 96$\pm$8 % (S E , n = 12) of the amount of protein added to the medium

Ouabain binding

Storage and determination of the purity of [^{3}H]ouabain were done as described by Hansen [17, 25] Portions of the enzyme preparation each containing 0.1–0.16 mg protein were added to eight centrifuge tubes (1 cm$\times$6 cm) Six of the tubes contained 1 ml of 3 mM ATP (Tris salt), 3 mM $MgCl_2$, 120 mM NaCl, 25 mM Tris, pH 7.5 (37 °C), 0 1 μCi [^{3}H]ouabain and unlabelled ouabain to final concentrations in a range from 0 08 to 2 5 μM In two tubes the concentration of unlabelled ouabain was brought to 1 mM to determine unbound [^{3}H]ouabain in the sediment After incubation for 30 min at 37 °C, the tubes were cooled in an ice bath, centrifuged at 50 000 rev /min for 30 min, and further processed as described under ATP binding The recovery of protein in the sediments was 94$\pm$6 % (S E , n = 12).

Phosphorylation

Portions of the enzyme preparation containing 0 1–0 3 mg protein were incubated for 4–6 s at 37 °C with 30 μM [γ-^{32}P]ATP (0 3 μC ml^{-1}, purified Tris salt [14]) in 3 ml of 3 mM $MgCl_2$, 30 mM Tris–HCl, pH 7 4 (37 °C) and 100 mM NaCl or 100 mM KCl. The reaction was stopped with 3 ml 8 % ice-cold perchloric acid and the protein was separated by centrifugation and washed four times with 0 1 % trichloroacetic acid, 10 mM P_i, 0.1 mM ATP [19] The precipitate was dissolved in 0 25 ml 1 M NaOH with heating for 30 min at 60 °C Portions of 25–50 μl were used for protein analysis in duplicate by the Lowry method [24], and 100 μl were transferred to 10 ml of scintillator solution [23] and counted with 100 % efficiency in a Packard scintillation counter Alternatively, a filtration [4, 26] procedure was used to control the results obtained by the procedure above. Aliquots containing 10–50 μg of protein were incubated for 5 s at 37 °C in 0 5 ml of the medium used above. The reaction was stopped with ice-cold 5 % trichloroacetic acid containing 0 6 mM ATP and 0 6 mM orthophosphate. The protein was filtered by suction on a 0 45 μM Sartorius filter and washed six times with 5-ml portions of the stopping solution. The filter was dissolved in 10 ml of Bray's solution and counted as above

Gel chromatography

Sepharose 6B columns (2 5 cm$\times$180 cm) equilibrated with 0 2 % sodium dodecylsulphate were prepared in principle as described by Rosenberg and Gui-

dotti [20] Aliquots of the preparations containing 3–10 mg protein were suspended in 2–5 ml 2 % sodium dodecylsulphate, 1 % mercaptoethanol, 20 mM sodium phosphate, pH 7 0 (20 °C) After heating for 3 min at 100 °C, 1 g sucrose was added and the sample was applied with a syringe Fractions of 5–8 ml were eluted with 0 2 % sodium dodecylsulphate, 20 mM sodium phosphate, pH 7.0 (20 °C) at a rate of 6–8 ml/h maintained by a peristaltic pump Protein in the fractions was determined by the Lowry method [24] The partition coefficient was determined from $K_{av} = V_e - V_0 / V_t - V_0$ [27], where V_e = elution volume for the fractions, V_0 = void volume of the column determined with Blue Dextran and V_t = the total volume of the gel bed

RESULTS

Binding of ATP and ouabain

Fig 1 shows that the data of ATP binding to preparations with specific activities of 20, 33 and 36 units · mg^{-1} protein fell on straight and parallel lines in a Scatchard plot with dissociation constants $0.14 \pm 0\ 02\ \mu M$ (S E , $n = 5$), that are close to the values reported before [14, 15] The intercepts at the ordinate calculated by the least square method yields the concentrations of binding sites for ATP that are plotted against the $(Na^+ + K^+)$-ATPase activities in Fig 3 for comparison with the ouabain binding data

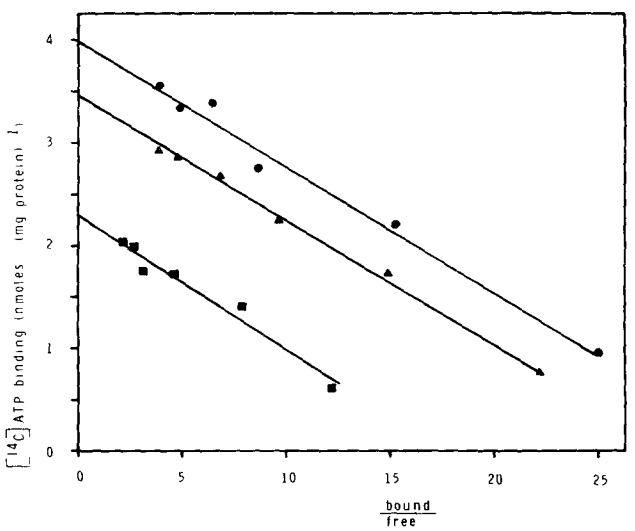

Fig 1 Scatchard plot of ATP binding to three preparations of $(Na^+ + K^+)$-ATPase with specific activities of 19 2 (■), 33 2 (▲), and 36 3 (●) units mg^{-1} protein Determination of ATP binding and enzyme assay were done simultaneously as described under Experimental

The concentration of ouabain-binding sites was nearly the same whether binding was stimulated by Mg^{2+} and P_i or by Mg^{2+}, Na^+ and ATP (Fig 2) The apparent dissociation constant was 2-fold higher in the presence of Mg^{2+} and P_i than in the presence of Mg^{2+}, Na^+ and ATP (cf refs 17 and 25). It has been shown

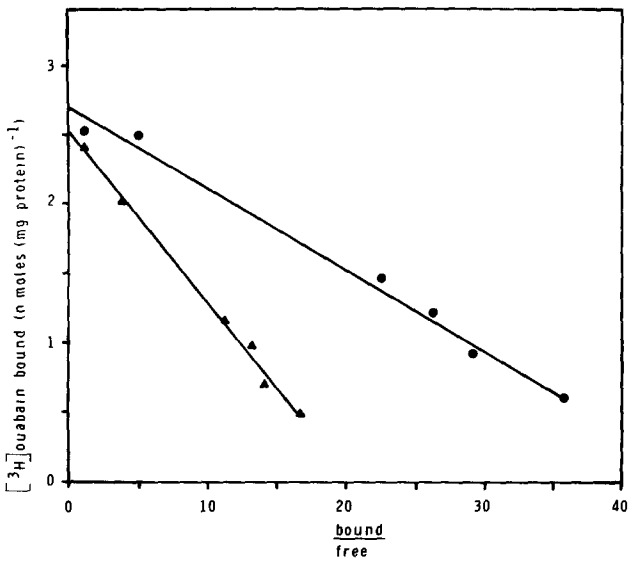

Fig 2. Scatchard plot of ouabain binding in the presence of 3 mM $MgCl_2$, 120 mM NaCl and 3 mM ATP (●) or 3 mM $MgCl_2$, 3 mM Tris phosphate and 120 mM choline chloride (▲) to a preparation with a specific activity of 27 7 units mg^{-1} protein Binding was determined as described under Experimental

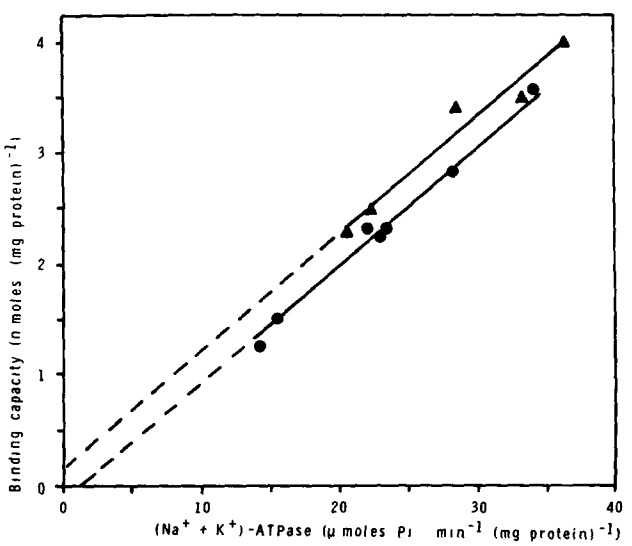

Fig. 3. Relationship between the concentration of sites for binding of ATP (▲) and ouabain (●) and the activity of $(Na^+ + K^+)$-ATPase. Determination of binding and enzyme assay were done simultaneously as described under Experimental. The equations for the regression lines calculated by the method of least squares are $y = (0\ 105 \pm 0.013)\ x + (0\ 16 \pm 0\ 39)$, $r = 0\ 98$ (▲) and $y = (0.112 \pm 0\ 005)\ x - (0\ 28 \pm 0\ 12)$, $r = 0\ 99$ (●)

that equilibrium levels of binding are attained for each concentration of ouabain [17, 25] under the conditions of the experiment in Fig 2 Addition of ions to the medium containing Mg^{2+} and P_i alter the dissociation constant but has no effect on the concentration of binding sites [25]. It is, therefore, inferred that the total concentration of ouabain binding sites is determined by extrapolation to infinite ligand concentrations from equilibrium levels of binding supported by Mg^{2+}, Na^+ and ATP

Fig 3 shows that the concentrations of binding sites for ATP and ouabain were linearly related to the activity of $(Na^+ + K^+)$-ATPase in the preparations (cf refs 12, 14 and 17). The intercepts of the regression lines at the ordinate were not significantly different from zero, indicating proportionality between the parameters The results support the conclusion from studies on ox brain preparations [28] that the molar ratio of ATP to ouabain bound is close to 1 The receptor capacity for ATP (Table II) was slightly lower than reported before [14, 15] Ouabain-binding capacities close to 100 pmoles · (μmoles $P_i \cdot min^{-1})^{-1}$ were found before for a variety of preparations of $(Na^+ + K^+)$-ATPase with specific activities varying from 0 004 to 4.4 units · mg^{-1} protein [16, 28–32], but binding capacities of 400 [32] and 800 [13] pmoles per unit of enzyme activity have also been reported

TABLE I

SODIUM-DEPENDENT PHOSPHORYLATION DETERMINED BY A CENTRIFUGATION TECHNIQUE AND BY A FILTRATION TECHNIQUE

A batch of enzyme prepared by centrifugation in an angle rotor [8] was used in a series of determinations of phosphorylation by the two methods described under Experimental Mean values ±S E are given with the number of determinations in brackets Ouabain binding was calculated from a Scatchard plot as in Fig 2 Assay of $(Na^+ + K^+)$-ATPase as described under Experimental

$(Na^+ + K^+)$-ATPase (μmoles P_i min^{-1} mg^{-1} protein)	Ouabain binding (nmoles mg^{-1} protein)	Sodium-dependent phosphorylation	
		Centrifugation (nmoles mg^{-1} protein)	Filtration (nmoles mg^{-1} protein)
26 2	2 7	5 6±0 2 (8)	6 1±0 2 (7)

Phosphorylation

To determine the maximum level of the sodium-dependent phosphorylation from ATP the time course of phosphorylation was followed A stable level was reached within 1 s and maintained for more than 10 s (not shown, cf refs 19 and 26) The data in Tables I and II are average values of determinations made after 4 and 6 s of incubation. There was a linear relation between the incorporation of ^{32}P and protein content The phosphorylation level in presence of Mg^{2+} and K^+ was equal to the level with Mg^{2+} alone and formed about 4 % of the level in presence of Mg^{2+} and Na^+ The recovery of protein after washing of the precipitate by centrifugation was 70±8 % (S E., $n = 23$) of the protein added to the medium As a control, phosphorylation was therefore also determined by a procedure [4, 26] in which the phosphoprotein was washed on a filter that does not allow passage of the precipitated protein A comparison of the data in Table I shows that the phosphorylation capacity

TABLE II

COMPARISON OF DATA OF ATP BINDING, OUABAIN BINDING AND SODIUM-DEPENDENT PHOSPHORYLATION

Receptor capacities and molar activities were calculated after analysis by the least-square method of the data in Fig 5 and of the data of phosphorylation determined by the centrifugation technique The maximum molecular weights are reciprocal values of the highest concentrations of sites for binding and phosphorylation Mean values ±S E are given with the number of determinations in brackets

	Receptor capacity (pmoles (μmoles P_i $min^{-1})^{-1}$)	Apparent molar activity (P_i min^{-1})	Maximum molecular weight (g protein $mole^{-1}$)
ATP binding	105±13 (5)	9100±1100 (5)	250 000
Ouabain binding	112±5 (7)	8800±400 (7)	278 000
Sodium-dependent phosphorylation	221±12 (9)	4400±250 (9)	137 000

determined by the filter technique was 9 % higher than that obtained by the centrifugation technique It is, therefore, unlikely that the loss of protein during wash of the precipitate by centrifugation leads to an overestimation of the phosphorylation when it is calculated from the concentrations of phosphate and protein in the dissolved precipitate.

The level of sodium-dependent phosphorylation was linearly related to the specific activity of $(Na^+ + K^+)$-ATPase and there was proportionality between the parameters (not shown, cf. refs 9, 18, and 26) The phosphorylation capacity (Table II) was within the range of some of the values reported before [10, 13], but was higher than the values of 85–160 pmoles per unit of enzyme activity found by most authors [4, 9, 18, 26, 33].

The highest level of sodium-dependent phosphorylation found in this study, 7.3 nmoles · mg^{-1} protein, was 1.8–2 0-fold higher than the highest concentrations of binding sites for ATP and ouabain in Fig. 3. The data in Tables I and II also show that two moles of phosphate were incorporated per mole of ATP or ouabain bound to the enzyme under the experimental conditions of the present study.

The data agree with the ratio of two phosphorylation sites per molecule of ouabain bound to the preparation from eel electroplax [31]. However, Hegyvary and Post [15] found a ratio of one phosphorylation site per molecule of ATP bound and the ratio of phosphorylation to ouabain binding of other preparations varies from 1.0 to 0.25 [13, 31, 32, 34]. Apart from species differences this variability may be due to the use of different preparative procedures as it has been shown that phosphorylation, ouabain binding and ADP binding may vary independently of each other after treatment with enzymes and chemicals [34]

Content of polypeptides

Fig. 4 shows the elution profiles after gel chromatography in sodium dodecylsulphate of preparations with increasing specific activities of $(Na^+ + K^+)$-ATPase It was found by sodium dodecylsulphate gel electrophoresis that the large peak of

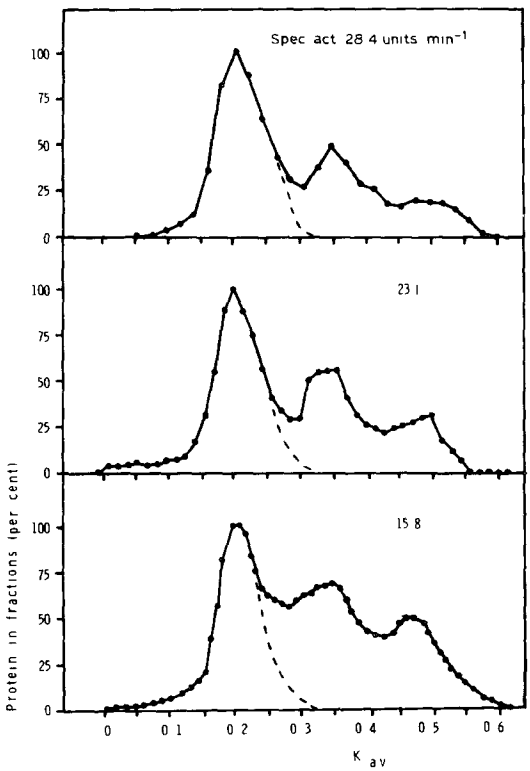

Fig 4 Elution profiles after gel filtration in sodium dodecylsulphate of three preparations of ($Na^+ + K^+$)-ATPase with specific activities of (from bottom) 15 8, 23 1 and 28 4 units mg^{-1} protein Portions containing 5 3, 3 3 and 2 8 mg protein, respectively, were solubilized in sodium dodecylsulphate and applied on Sepharose 6B columns as described under Experimental After elution, 5 1, 3 0 and 2 7 mg protein, respectively, were recovered in the fractions Assuming symmetrical elution profiles the amounts of large polypeptide eluted in the first peak formed 37 %, 50 % and 63 %, respectively, of the total protein recovered in the fractions

protein appearing at a K_{av} of 0 15–0 25 consisted of the polypeptide with a molecular weight close to 96 000 (Fig 6) Assuming that the elution profile for the protein was symmetrical, the fraction of the total protein formed by this polypeptide was determined and plotted against the specific activity in Fig. 7 for comparison with the values obtained by planimetry of gel scans

Fig 5 shows an electropherogram of a preparation with a specific activity of 37 units · mg^{-1} protein Using the optical density of the most translucent part of the gel as baseline, it was found by planimetry that the large peak of protein (M_r = 96 000) and the small peak (M_r = 57 000) formed 68 % and 17 %, respectively, of the total area of the scan The experiment in Fig 6 was done to test the quantitative relationship between the fractional area of the large peak in the scans and the content of the large polypeptide in a mixture of membrane proteins applied to the gels The large polypeptide was isolated by gel chromatography (Fig 4) and 12 or 24 μg of this protein were mixed in various proportions with proteins from the second and third peak eluted from the Sepharose column The scans in Fig 6 show the pattern for

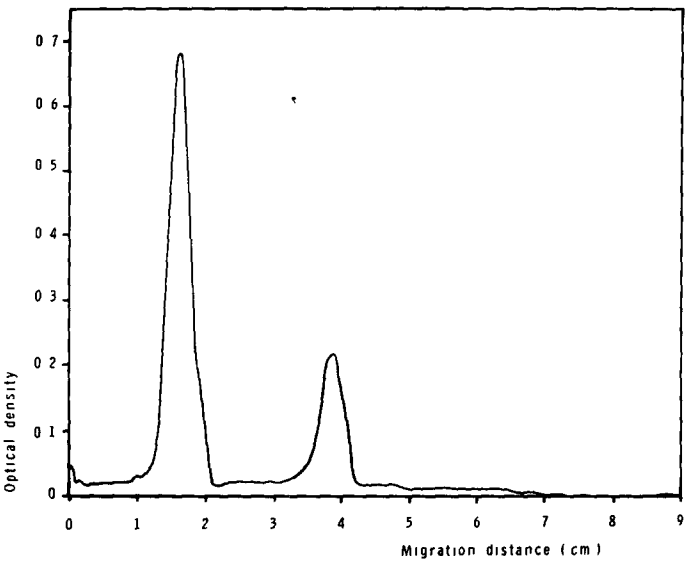

Fig 5. Scan of a separation by sodium dodecylsulphate gel electrophoresis [8] of a preparation with a specific activity of 37 units · mg^{-1} protein. The gel was scanned at 600 nm in a Zeiss spectrophorometer with a Gilford linear transport attachment and an aperture of 0 1 mm The transport speed was 1 cm min^{-1} and the chart speed was 2 5 cm min^{-1}.

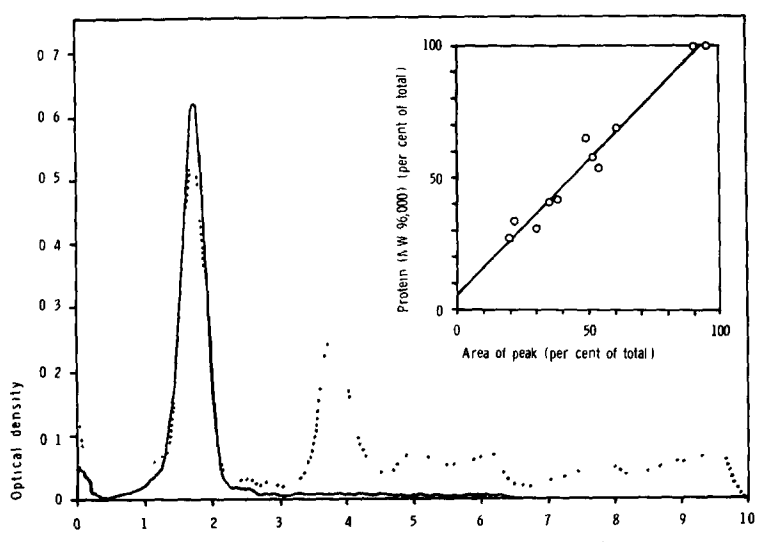

Fig 6 Relationship between the percentage area of the peak of large polypeptide (M_r = 96 000 [8]) in electropherograms and the content of this protein in a mixture of membrane proteins. A portion of a preparation of $(Na^+ + K^+)$-ATPase with a specific activity of 21 units · mg^{-1} protein containing 9 g protein was solubilized in sodium dodecylsulphate and separated on a Sepharose 6B column as described under Experimental. After elution, protein was determined by the Lowry method and fractions of 8 ml from the top of each of the three peaks (Fig. 4) were lyophilized and resuspended in 0 3 ml water. Aliquots of fractions from the first peak (K_{av} = 0.2, Fig. 4) containing 12 μg or 24 μg protein were mixed in various proportions with aliquots of fractions from the two other peaks and samples containing 12–45 μg protein were prepared for polyacrylamide gel electrophoresis in sodium dodecylsulphate [8] The gels were scanned at 600–660 nm as described in Fig 5. ——, protein from the first peak (Fig 6) and , a mixture of proteins from the three peaks. The area under the large peak in per cent of the total area of the scan was determined by a Haff planimeter and was plotted against the fractional content of protein from the first peak

the isolated large polypeptide and for a mixture in which the large polypeptide formed 28 % of the total protein It is seen from the inset in Fig. 6 that the percentage area of the large peak was linearly related to the fractional amount of large polypeptide added to the mixtures The standard error in measurements of the content of large polypeptide by this method was ±10 %

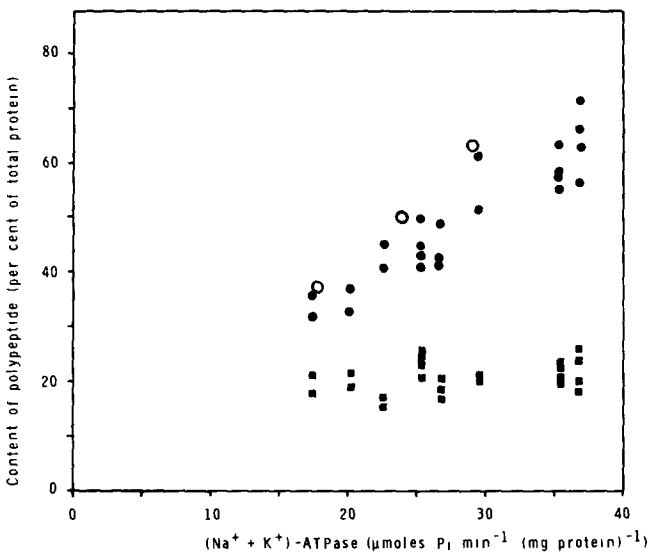

Fig. 7 Relationship between the activity of $(Na^+ + K^+)$-ATPase and the content of large polypeptide (M_r = 96 000) determined by gel filtration (○) (Fig 6) or by planimetry of polyacrylamide gels (●) (Figs 7 and 8) and the content of small polypeptide (M_r = 57 000) determined by planimetry (■) (Fig 7)

Fig 7 shows that the contents of heavy polypeptide determined by planimetry of a series of gel scans agree reasonably well with the values obtained by gel chromatography. The content in the most active preparations was 55–72 % of the total protein corresponding to 5 8–7 5 nmoles of polypeptide (M_r = 96 000) per mg protein The concentrations of heavy polypeptide calculated from the data in Fig. 4 obtained by gel filtration fell close to the regression line for the phosphorylation data and the concentrations obtained by gel scanning fell on or below this line Although the error in the analysis is appreciable, the data thus suggest that the ratio is one heavy chain per phosphorylation site. The small polypeptide did not enrich significantly on purification suggesting that proteins with the same electrophoretic mobility were removed. The mass ratio of large to small polypeptide in the most active preparations was 2.8±0.2 (S E., n = 8) and higher than the values of 2.3 [4], 1 9 [7] and 1.7 [11] reported before As the small chain is a glycoprotein [4, 7, 11], the molecular weight of 57 000 found by sodium dodecylsulphate gel electrophoresis is probably too high [35] If the true molecular weight is 35 000 [11], the mass ratio of 2.8 gives a molar ratio of one large chain per small chain.

DISCUSSION

The proportionality between the activity of $(Na^+ + K^+)$-ATPase and the concentration of sites for binding of ATP and ouabain and for phosphorylation indicates that the procedure for isolation of the enzyme leads to a true purification of the enzyme and it allows an estimation of the mutual number of these sites on the enzyme from the data.

A ratio of two phosphorylation sites per site for binding of ATP or ouabain was maintained during purification. The capacities for binding of ouabain and ATP are close to the expected values, whereas the capacity for sodium-dependent phosphorylation is higher than found by most authors. The fact that determination of the phosphorylation levels by two different methods gave nearly identical results lends validity to the data. The phosphate incorporated from ATP is bound to the large polypeptide chain [4, 7, 8, 10] and this polypeptide is homogeneous in preparations from the outer medulla of dog and rabbit kidney as judged by N-terminal analysis [11] and hydroxylapatite chromatography [36]. The present data, therefore, appear to be reasonable as they show that the phosphorylation capacity is close to one mole phosphate per mole large chain

The determinations of the maximum molecular weight per phosphorylation site (137 000) and per site for binding of ATP (250 000) and ouabain (278 000) are independent of the analysis of the protein composition. These data suggest that the maximum content of the protein with a molecular weight of 90 000–100 000 [2–10] or 139 000 [11] is one chain per phosphorylation site and two chains per site for binding of ATP or ouabain. The scheme in Fig. 8 gives a hypothetical explanation for the apparent paradox that only one molecule of ATP should be bound per two phosphorylated chains. It is assumed that binding of ATP to one chain blocks the binding site of the other chain [37]. Under the conditions of the ATP-binding experiment only one molecule of ATP is bound per two chains because further reactions are abolished In the presence of Mg^{2+} and Na^+ the acyl phosphate bond is formed, ADP is removed and the other chain can bind ATP and be phosphorylated. Dimeric [37, 38] and tetrameric [39] models for partial reactions of the system and for the active transport of cations have been proposed before.

In Table III, the present data are compared with the data of preparations purified after solubilization either by deoxycholate of membranes from the outer medulla of dog kidney [2, 7] or by Lubrol of membranes from the rectal gland of the dogfish [4]. Based on protein analysis by dye absorption [40], the preparation

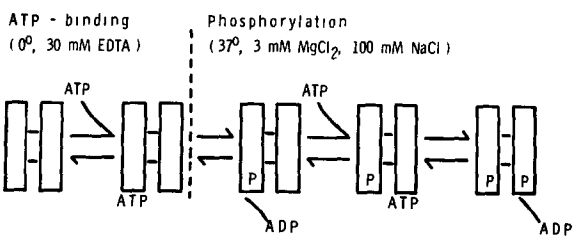

Fig 8. ATP binding and phosphorylation of a dimer consisting of two identical polypeptide chains ($M_r = 96\,000$).

TABLE III

COMPARISON OF THE PROPERTIES OF PURIFIED PREPARATIONS OF $(Na^+ + K^+)$-ATPase

The data are the highest estimates of the parameters

	$(Na^+ + K^+)$-ATPase (μmoles P_i min^{-1} mg^{-1} protein)	Content of polypeptide (M_r = 90 000–97 000) (% total protein)	Sodium-dependent phosphorylation (nmoles mg^{-1} protein)	Ouabain binding	ATP binding
Kyte [2, 10, 11 13]	7–13	57★	1 9	3 6★★	
Hokin et al [4]	20–25	66–72	4 1	–	
Lane et al [7]	26	60–70★★★		3–4	
Present results	32–37	55–72	7 3	3 6	4 0

★ The mass ratio of large to small polypeptide is 1 7 and the two polypeptides form 90 % of the total protein

★★ From Fig 8, ref 13

★★★ From Fig 2, ref 7

by Nakao et al. [9] has a specific activity of $(Na^+ + K^+)$-ATPase varying from 7 to 117 units · mg^{-1} protein and a maximum phosphorylation level, 7.8 nmoles · mg^{-1} protein, which is close to the value found in this study; but the fractional content of the large polypeptide estimated from the scans of sodium dodecylsulphate–polyacrylamide gels [9, 41] is lower than that of the preparations in Table III.

Considering the differences between the purification procedures, it is remarkable that the content of the large polypeptide in the preparations in Table III differs by less than 10 %. This suggests that the maximum purity with respect to this protein has been reached. However, it remains an open question whether the small polypeptide is part of the enzyme. Cross-linking experiments suggest that the two chains are close to one another in the membrane [11], but the small chain has not been linked to any catalytic function. The mass ratio of small to large chain is lower in our preparation than in the three other preparations in Table III and it seems as if the small chain can be removed by ion-exchange chromatography after solubilization by Lubrol [9], but the fact that the enzyme is very unstable after this fractionation might support the suggestion that a glycoprotein serves as a fixed reference for the large chain in the membrane [11].

The highest concentration of ouabain binding sites is nearly identical for three of the four preparations in Table III, whereas the specific activity of $(Na^+ + K^+)$-ATPase and the phosphorylation capacity vary considerably. The specific activity of our preparation is 30–40 % higher than that of two of the preparations and 3-fold higher than that of Kyte's preparation. The ratio of the moles of phosphate incorporated per mole of large polypeptide varies from about 1.0 in our preparation to 0 5 and 0 3 in two of the other preparations

These differences seem not to be due to removal of protein subunits from the enzyme in the purification procedures, since the protein composition of the preparations is nearly identical

It has been suggested that the high specific activity of our preparations is due to an unspecific activation by detergent [4, 7]. This does not provide an explanation for the differences between the preparations in Table I. The three other preparations were treated with, relatively, much higher concentrations of detergent than that used in our procedure, and it has been shown that deoxycholate, Lubrol and sodium dodecylsulphate activate latent $(Na^+ + K)$—ATPase equally well [12].

The content of detergent in our preparation is low [8], whereas it is high in preparations isolated after solubilization by Lubrol [3, 41]. Kyte [13] found that deoxycholate in the concentrations necessary for solubilization depresses the rate of turnover of substrate per ouabain binding site and that this effect is partly reversed by removal of deoxycholate from the purified preparation. The decrease in activity of $(Na^+ + K^+)$-ATPase after solubilization by deoxycholate can partly be restored by addition of phospholipids [42, 43] and it may be part of the solubilization process that the detergents substitute phospholipid–protein interactions by detergent–protein associations and thus partly replace lipid as a support for the enzyme [41, 44, 45]. This may lead to alterations in the arrangement of the protein that can explain why the specific activity of $(Na^+ + K^+)$-ATPase and the phosphorylation capacity are lower for the preparations isolated after solubilization of the enzyme than for the preparation obtained by our procedure in which the enzyme is kept embedded in the membrane. It is in agreement with this hypothesis that formation of the phos-

phorylated intermediate has a specific requirement for phospholipid [46, 47] and that the $(Na^+ + K^+)$-ATPase activity is more sensitive to treatment with phospholipase than the ouabain binding capacity [34, 48, 49].

It is clear from these considerations that the fractional content of the heavy polypeptide cannot serve as the only criterion of purity for preparations of $(Na^+ + K^+)$ ATPase Other polypeptides may be parts of the enzyme molecule and the proteins must be associated with lipids to display catalytic activity To ensure that the purified preparations contain all the components of the native enzyme, it is also necessary to apply criteria for its catalytic functions The ouabain binding capacity remains in a constant ratio to the amount of heavy polypeptide even after a considerable depression of the rate of turnover of substrate (Table III) and is, therefore, an estimate of the concentration of the heavy polypeptide rather than of the catalytic functions of the protein. The molar activity of $(Na^+ + K^+)$-ATPase, the phosphorylation capacity and the activity of other partial reactions are the possible criteria for catalytic functions.

The present data show that the purity of the preparation with respect to protein is close to the maximum value if the enzyme contains one mole of small polypeptide per mole of large chain. The capacities for binding of ATP or ouabain are higher than or equal to the values reported before, but it is not known whether the maximum values have been reached The catalytic functions of $(Na^+ + K^+)$-ATPase are well preserved since the molar activity is high, 4000–5000 P_i min^{-1} per mole large chain or 8800 P_i min^{-1} per mole ouabain bound, and all large polypeptide chains in the preparation can be phosphorylated from ATP.

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