Improved purification of brine-shrimp (*Artemia salina*) ($\text{Na}^+ + \text{K}^+$)-activated adenosine triphosphatase and amino-acid and carbohydrate analyses of the isolated subunits

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(Received 4 March 1980/Accepted 19 May 1980)

Purification of the ($\text{Na}^+ + \text{K}^+$)-activated ATPase has been improved 2-fold with respect to both purity and yield over the previous method [Peterson, Ewing, Hootman & Conte (1978) *J. Biol. Chem.* **253**, 4762–4770] by using Lubrol WX and non-denaturing concentrations of sodium dodecyl sulphate (SDS). The enzyme was purified 200-fold over the homogenate. The preparation had a specific activity of about $600 \mu\text{mol}$ of P_i/h per mg of protein, and was about 60% pure according to quantification of Coomassie Blue-stained SDS/polyacrylamide gels. The yield of purified enzyme was about 10 mg of protein per 100 g of dry brine-shrimp (*Artemia salina*) cysts. The method is highly suitable for purification either on a small scale (10–25 g of dry cysts) or on a large scale (900 g of dry cysts) and methods are described for both. The large ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunit (α -subunit) was isolated in pure form by SDS-gel filtration on Bio-Gel A 1.5 m. The small subunit (β -subunit) was eluted with other contaminating proteins on the Bio-Gel column, but was isolated in pure form by extraction from SDS/polyacrylamide gels. The amino acid and carbohydrate compositions of both subunits are reported. The α -subunit contained 5.2% carbohydrate by weight, and the β -subunit 9.2%. Sialic acid was absent from both subunits.

The ($\text{Na}^+ + \text{K}^+$)-activated ATPase of the brine shrimp (*Artemia salina*) has been used in this laboratory for studies related to the biosynthesis and structure of the enzyme. The ($\text{Na}^+ + \text{K}^+$)-activated ATPase in the developing brine-shrimp nauplius appears to be one of the most promising model systems for the study of the biogenesis of this important plasma-membrane protein (Peterson *et al.*, 1978a; Peterson & Hokin, 1979). A good deal is now known about the basic biochemistry of brine-shrimp development (Finamore & Clegg, 1969; Bagshaw & Warner, 1979; Warner *et al.*, 1979). Certain basic structural studies of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase have been undertaken to permit a more complete analysis and interpretation of the biogenesis experiments. These include unequivocal establishment of the stoichiometry of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits, clarification of the nature of the isoenzymes of the ($\text{Na}^+ + \text{K}^+$)-activated ATPase

that have been observed in the brine shrimp (Peterson *et al.*, 1978a,b; Peterson & Hokin, 1979) and the complete amino-acid and carbohydrate compositional analysis of the subunits. The last of these structural studies is described in the present paper. These biogenesis and related structural studies all require a degree of purity of the enzyme that will permit identification and isolation of pure subunits.

Purification of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase has represented a formidable challenge. Because of its small size (about 0.5 mm), it is not practical to attempt to isolate tissues within the animal that are particularly rich in the ($\text{Na}^+ + \text{K}^+$)-activated ATPase. We estimate that the ($\text{Na}^+ + \text{K}^+$)-activated ATPase in brine-shrimp homogenates represents about 0.3% of the total protein. Complete purification of the ($\text{Na}^+ + \text{K}^+$)-activated ATPase has only been achieved from substantially richer sources of the enzyme usually requiring an overall purification of 100-fold or less (Kyte, 1971; Hokin *et al.*, 1973; Lane *et al.*, 1973; Jørgensen, 1974; Hopkins *et al.*, 1976). In a previous report by one of us (Peterson *et al.*, 1978b), a purification method was presented that routinely

Abbreviations used: SDS, sodium dodecyl sulphate; Temed, *NNN'*-tetramethylethylenediamine.

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achieved a 100-fold purification of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase to 30% purity, as determined by SDS/polyacrylamide-gel electrophoresis. In the present paper, we report on an improved purification procedure for the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase that represents a 2-fold increase in both purity and yield over the previous method. The yield of this preparation is 10 mg of protein per 100 g of starting material, which is comparable with the purification procedures cited above. Techniques are described for both small-scale (10–25 g of dry cysts) and large-scale (900 g of dry cysts) preparations of the partially purified brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase. In addition, the present paper describes techniques for the isolation of pure subunits from the partially purified brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase and reports the complete amino-acid and carbohydrate composition of the subunits. A preliminary report of part of this work has been published (Peterson & Hokin, 1979).

Experimental

Materials

Acrylamide, *NN'*-methylenebisacrylamide and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A., as electrophoresis-purity-grade reagents. SDS (Sequanal grade) and methanesulphonic acid [prepared as a 4 M solution in 0.2% 3-(2-aminoethyl)-indole] were obtained from Pierce Chemical Co., Rockford, IL, U.S.A. Lubrol WX was obtained from Supelco Inc., Bellefonte, PA, U.S.A., and from Sigma Chemical Co., St. Louis, MO, U.S.A. Instant Ocean Sea Salts were obtained from Aquarium Systems Inc., Eastlake, OH, U.S.A. All other chemicals were obtained from various sources as analytical-grade reagents. Miracloth was obtained from Van Water and Rogers Scientific. Dried brine-shrimp eggs (cysts) were obtained from San Francisco Bay Brand, Newark, CA, U.S.A., and stored at -20°C .

Rearing brine-shrimp nauplii

The dried brine-shrimp cysts were mixed and prehydrated in 3–4 vol. of tap water for a minimum of 4 h at 4°C . At the end of the prehydration period, the cysts were thoroughly mixed and allowed to settle. Those cysts that floated at this stage were largely non-viable and were discarded. The remaining cysts were emptied into wire-mesh containers lined with Miracloth and washed under continuous running cold tap water for 5–10 min. The cysts were allowed to drain, and then placed into half-strength sea water (Instant Ocean Sea Salts) and incubated with aeration at 28 – 30°C for up to 48 h. For small-scale preparations the rearing flasks

consisted of 2-litre separatory funnels containing 1900 ml of half-strength sea water. Heating was provided by circulating water from Haake Constant Temperature Circulators through Tygon tubing wrapped around the funnels. Aeration was provided by one Silent Giant Air Pump per two funnels operating at full capacity through MistAir (A-501) air stones fitted into the apex of the funnel. Hydrated cysts from up to 25 g of dry cysts could be incubated in each funnel. For large-scale preparations, hydrated cysts from 200 g of dry cysts were incubated in 20-litre Nalgene polyethylene bottles fitted with spigots. The tops of the bottles were cut off and the opening covered with flat Plexiglas plates fitted to permit six equally spaced MistAir air stones set within the periphery of the bottom of the bottles. Three Silent Giant Air Pumps operating at full capacity provided air to the six air stones and the bottle was heated with a Haake circulator through Tygon tubing coiled around the sides of the bottles and insulated with aluminium foil. At the end of the incubation period, the air stones were removed and the hatched nauplius larvae were allowed to settle to the bottom and collected free of the floating cyst shells through the spigots at the bottom of the funnels or 20-litre bottles. The unhatched cysts could not be separated from the nauplii and the harvest from short incubation periods (12–14 h) contained a large amount of unhatched cysts, whereas the harvest after longer incubation periods contained few cysts. The harvested nauplii and cysts were washed through a Miracloth filter and rinsed three times with water.

Small-scale purification of brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase

The small-scale purification procedure was designed for experiments involving brine-shrimp nauplii, which were reared from 10–100 g of dry cysts. The procedures described below are those for nauplii originating from 25 g of dry cysts, which is the maximum incubated in each 2-litre rearing funnel. The harvested nauplii were homogenized for 10 s at low speed in a Waring blender containing 150 ml of homogenizing medium (250 mM-sucrose, 50 mM-imidazole/HCl, 2 mM-disodium EDTA, 2 mM- β -mercaptoethanol, pH 7.2) per 25 g of original dry cysts. Purification of the ($\text{Na}^+ + \text{K}^+$)-activated ATPase from the homogenate involved four steps: (a) crude membranes; (b) gradient membranes; (c) Lubrol pellet; (d) SDS-treated enzyme.

(a) *Crude membranes.* The homogenate was centrifuged at 12000 rev./min for 2 h at 4°C in a Sorvall GSA rotor ($r_{av.} = 14.5$ cm). The supernatant was decanted except for the last few millilitres to avoid loss of any pellet. The top membrane layer of

the pellet, which appears orange-tan in colour (more tan in young animals) and is easily removable, was recovered with the aid of a glass rod and saved. Care was taken not to remove the soft but fibrous textured underlying pellet layer. The remaining pellet was re-homogenized in 150 ml of homogenizing medium per 25 g of original dry cysts and centrifuged as above. The combined membranes from the two centrifugations were re-suspended in homogenizing medium with a glass/Teflon homogenizer to give a final volume of 60 ml per 25 g of original dry cysts and stored at -80°C unless used directly.

(b) *Gradient membranes.* The crude membranes were centrifuged through a step gradient consisting of 9 ml of 40% (w/v) sucrose, 9 ml of 20% (w/v) sucrose and 20 ml of crude membranes in a Beckman SW27 cellulose nitrate tube. The sucrose solutions were prepared in imidazole/EDTA buffer (25 mM-imidazole/HCl, 1 mM-disodium EDTA, pH 7.2). The gradients were placed in a Beckman SW27 rotor ($r_{\text{av.}} = 11.83$ cm) and centrifuged at 27000 rev./min for 1 h at 4°C in a Beckman model L2-65B or model L3-40 ultracentrifuge. The membranes banding at the 20–40% sucrose interface were collected by aspiration and diluted with at least 1 vol. of imidazole/EDTA buffer. The diluted membranes were then centrifuged at 30000 rev./min in a Beckman 30 rotor ($r_{\text{av.}} = 7.8$ cm) for 1 h at 4°C to isolate the membranes free from the high concentrations of sucrose. The supernatant was decanted carefully to avoid loss of the top fluffy part of the pellet. The pellet was then re-suspended in imidazole/EDTA buffer with a glass/Teflon homogenizer to a final protein concentration of 6 mg/ml.

(c) *Lubrol pellet.* The gradient membranes (6 mg of protein/ml) were treated with 0.01 vol. of 15 mM-ATP mix (15 mM-disodium ATP, 250 mM-imidazole/HCl, 10 mM-disodium EDTA, pH 7.2) followed by 0.12 vol. of 16% (w/v) Lubrol WX while stirring. (The 16% Lubrol solution was prepared from Sigma Lubrol WX by first completely dissolving the detergent in water at room temperature or slightly warmer, then cooling overnight to 4°C , and centrifuging for 30 min at 4°C and 100000 g to remove the cold precipitated material. The clear supernatant from this centrifugation was stored at 4°C until used. Lubrol WX obtained from Supelco, Inc., or solutions from which the cold precipitable material was not removed resulted in poorer enzyme purifications.) The Lubrol-treated membranes were centrifuged at 40000 rev./min for 2 h at 4°C in a Beckman 40 rotor ($r_{\text{av.}} = 5.9$ cm), and the supernatant was completely decanted together with any light fluffy pellet material. The pellet was re-suspended in homogenizing medium with a glass/Teflon homogenizer to a final protein concentration of 4.0 mg/ml for animals less than 24 h old, and 3.0 mg/ml for older animals. If the final step was not

completed directly, the Lubrol pellet was stored frozen at -80°C .

(d) *SDS-treated enzyme.* The Lubrol pellet was treated with 0.4 vol. of the 15 mM-ATP mix and allowed to sit on ice for 15 min. To this was added 0.6 vol. of SDS (1.667 mg/ml) at a rate of 1 ml/min while stirring at 0°C , and the mixture was incubated for 30 min at 30°C . This material was then centrifuged through a step gradient consisting of 15 and 35% (w/v) sucrose solutions prepared in imidazole/EDTA buffer. The gradients were prepared in cellulose nitrate tubes using one of the following Beckman swinging-bucket rotors and corresponding protocols: SW 27 ($r_{\text{av.}} = 11.83$ cm), 6 ml of 35% sucrose, 10 ml of 15% sucrose, up to 22 ml of sample; SW 27.1 ($r_{\text{av.}} = 11.25$ cm), 3 ml of 35% sucrose, 4 ml of 15% sucrose, up to 11 ml of sample; SW 40 ($r_{\text{av.}} = 11.27$ cm), 3 ml of 35% sucrose, 3 ml of 15% sucrose, up to 7 ml of sample. Samples not occupying the full volume of the gradient tube were overlaid with imidazole/EDTA buffer to fill the tube. Regardless of the rotor type, the samples were centrifuged at 27000 rev./min ($r_{\text{av.}} = 11.25$ – 11.83 cm) for 4 h at 4°C in a Beckman model L2-65B ultracentrifuge. Material banding at the 15–35% sucrose interface was collected by aspiration through small-bore tubing connected to an Auto Analyzer proportioning pump. To concentrate the SDS-treated enzyme and remove the high concentrations of sucrose, the SDS-treated enzyme fraction collected from the gradient was diluted at least 2-fold with imidazole/EDTA buffer and centrifuged at 40000 rev./min in a Beckman 40 rotor ($r_{\text{av.}} = 5.9$ cm) for 4 h at 4°C . The final pellet was re-suspended in homogenizing medium to a final protein concentration of 1–10 mg/ml and stored frozen at -80°C .

Large-scale purification of brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase

The large-scale purification procedure was designed for experiments involving brine-shrimp nauplii, which were reared from 300–900 g of dry cysts. The purification steps are the same as for the small-scale purification procedure, except for the modifications introduced to cope with the larger volume of material. The homogenate was prepared as described for the small-scale preparation except that a larger blender flask was used with 300 ml of homogenizing medium and nauplii originating from 50 g of dry cysts. The large-scale purification procedure described below is for nauplii originating from 900 g of dry cysts.

(a) *Crude membranes.* This step was identical with that given for the small-scale purification procedure with the exception that 125 ml of homogenizing medium per 25 g of original dry cysts was used on the second centrifugation instead of 150 ml.

The crude membranes were accumulated from 900 g of original dry cysts.

(b) *Gradient membranes.* The crude membranes were run through the same 20–40% sucrose step gradient as in the small-scale preparation except that the gradient was run using a Beckman Ti-15 zonal rotor in a Beckman model L4 ultracentrifuge. Crude membranes from 300 g of original dry cysts (720 ml) were thawed and added to the zonal rotor before loading the rotor into the ultracentrifuge. After bringing the rotor to loading speed, 350 ml of 20% sucrose was pumped in from the wall at about 25 ml/min with a Gilson Minipuls 2 peristaltic pump. This was followed by 500 ml of 40% sucrose and 100–150 ml of 60% sucrose until the rotor was full. The gradient was centrifuged at 29 000–33 000 rev./min ($r_{av.} = 4.45$ cm) for 1.5 h at 4°C, and the rotor was allowed to come down to loading speed without the brake. Fractions (about 25 ml) were collected from the wall by pumping 2% sucrose into the centre of the rotor, initially at about 15 ml/min, then at about 25 ml/min after the heavy sucrose had been pumped out of the rotor. The fractions containing the band of membranes in the 20–40% sucrose junction were pooled and concentrated by ultrafiltration through Amicon XM-300 Diaflo membranes. The pooled fractions totalled 300–350 ml and were concentrated to 50 ml or less. Concentration was achieved overnight with the Amicon Thin-Channel model TCF10 ultrafiltration system consisting of a 550 ml chamber and a 90 mm-diameter filter. Similar concentration through the 400 ml Amicon Standard ultrafiltration cell (76 mm diameter) required about 36 h. The larger chamber volume and faster filtration rate of the thin-channel system allowed for adequate dilution of the high sucrose concentration with buffer during ultrafiltration, which circumvented the need for an additional centrifugation step. Three such zonal runs were performed to accumulate gradient membranes from 900 g of original dry cysts.

(c) *Lubrol pellet.* This step was performed exactly as described for the small-scale purification method except that the larger volume Beckman 30 rotor was used in place of the 40 rotor for centrifugation.

(d) *SDS-treated enzyme.* SDS treatment of the Lubrol pellet was performed in the same manner as for the small-scale purification method, except that the SDS solution was added at a rate of 3 ml/min instead of 1 ml/min. The accumulated Lubrol pellets from 900 g of original dry cysts were treated together and centrifuged over a 15 and 35% (w/v) sucrose step gradient prepared in a Beckman Ti-15 zonal rotor. The SDS-treated Lubrol pellet consisted of 650–750 ml and was added to the zonal rotor before loading in the ultracentrifuge (Beckman model L4). The gradient was prepared and fractionated in a similar manner to the methods

described for the large-scale preparation of the gradient membranes. The gradient consisted of 500 ml of 15% sucrose and 450–550 ml of 35% sucrose as needed to fill the rotor. The gradient was centrifuged at 29 000–33 000 rev./min ($r_{av.} = 4.45$ cm) overnight, or a minimum of 4 h at 4°C. The fractions (25 ml) obtained were analysed for ($\text{Na}^+ + \text{K}^+$)-activated ATPase activity and protein, and those containing high-specific-activity ($\text{Na}^+ + \text{K}^+$)-activated ATPase were pooled and concentrated overnight by ultrafiltration through an Amicon XM-300 Diaflo membrane by using a 400 ml (76 mm diameter) Amicon Standard ultrafiltration cell. The concentrated material was then diluted at least 2-fold with imidazole/EDTA buffer and centrifuged at 30 000 rev./min in a Beckman 30 rotor ($r_{av.} = 7.8$ cm) for 4 h at 4°C. The final pellet was re-suspended in homogenizing medium to a final protein concentration of 5–10 mg/ml and stored frozen at –80°C.

Analysis of the purification fractions

ATPase enzyme reactions were run in the presence of 5 mM-disodium ATP, 10 mM-MgCl₂, 40 mM-imidazole/HCl, pH 7.2, and 160 mM-NaCl for 5, 10 or 20 min incubations at 37°C. ($\text{Na}^+ + \text{K}^+$)-activated ATPase activity was determined as the difference between the reaction tubes containing the above reactants plus 40 mM-KCl and reaction tubes containing the above reactants plus 1.0 mM-ouabain (no KCl). Phosphate was determined by the method of Peterson (1978). Protein was estimated by the Peterson (1977) modification of the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The protein composition of the fractions was analysed by SDS/polyacrylamide-gel electrophoresis with the Laemmli (1970) buffer system exactly as described by Hokin *et al.* (1973).

Isolation of brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits

The α -subunit was isolated from the purified brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase by gel filtration on a Bio-Gel A 1.5 m (200–400 mesh) column (2.5 cm $\times$ 90 cm), similar to that described by Marshall & Hokin (1979). The SDS-treated enzyme from 48 h-old brine shrimp was used as this contained primarily the $\alpha 1$ band. From 30 to 40 mg of the SDS-treated enzyme was solubilized and denatured in 2.5% (w/v) SDS in a final volume of 16 ml, and heated in a boiling-water bath for 3 min. The sample was then cooled and reduced by addition of 0.4 ml of 100% β -mercaptoethanol. The sample was layered on the Bio-Gel column, which was pre-equilibrated with 0.1% SDS, 0.1 M-Tris/HCl, pH 7.4, and 0.02 mg of NaN_3 /ml. The protein was eluted with the equilibration buffer at about 20 ml/h

under a 100cm hydrostatic pressure head. The eluate was monitored at 280nm with a Gilson model 261 u.v. monitor and 3ml fractions were collected with the aid of a Gilson model FC-80K Micro-fractionator. The fractions were analysed by SDS/polyacrylamide-gel electrophoresis to verify the position of the subunits. Fractions containing the subunits were pooled, freeze-dried and the residue was dissolved in 2ml of water. This was dialysed against three changes of 500ml of water at 12°C over a 48–72h period. The α -subunit, which was homogeneous, was filtered through 0.45 μm Millipore filters fitted in 13mm-diameter Sweenex holders, and stored frozen at -80°C .

The β -subunit was contaminated with other protein bands in the fractions eluted from the Bio-Gel column. It was, therefore, further purified by SDS/polyacrylamide-gel electrophoresis and eluted. The freeze-dried and dialysed β -subunit from a single Bio-Gel column run (2ml volume) was prepared for electrophoresis by addition of 0.2ml of 10% (w/v) SDS, 0.2ml of 50% (v/v) glycerol, 0.1ml of 0.05% Bromophenol Blue and 0.05ml of 100% β -mercaptoethanol. The sample was then layered on a 0.3cm $\times$ 14cm $\times$ 12cm slab gel prepared with the Bio-Rad model 220 slab-chamber apparatus. The gel consisted of 6% (w/v) acrylamide, 0.16% bis-acrylamide, 0.1% SDS, 40mM-Tris/acetic acid, pH 7.4, 0.1mM-disodium EDTA, 0.03% ammonium persulphate and 0.025% (v/v) Temed. The reservoir buffer consisted of 0.1% SDS, 40mM-Tris/acetic acid, pH 7.4, and 0.1mM-disodium EDTA. The gels were pre-electrophoresed for 30 min at 20mA per gel and run at 30mA per gel until the tracking dye reached the bottom of the gel. A 0.5cm vertical section was cut from the centre of the gel, fixed and stained with Coomassie Blue (Hokin *et al.*, 1973), and the remainder of the gel was covered with Saran wrap and stored at 4°C. The β -subunit located on the stained gel slice was cut from the unstained gel according to relative migration. The unstained gel pieces containing the β -subunit were macerated by

ejection through the bore of a syringe, and then extracted three times at 30°C over a 36h period with 5ml of 0.1% SDS/0.1% NaHCO_3 /0.1% (v/v) β -mercaptoethanol. The combined extracts were freeze-dried, dissolved in 1ml of water, centrifuged to remove any gel particles and finally dialysed and filtered as described for the final preparation step of the α -subunit.

Chemical analysis of the isolated ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits

Amino-acid analysis was done on a Beckman Spinco model 120C amino-acid analyser by the method of Spackman *et al.* (1958). The protein samples were hydrolysed *in vacuo* with 4M-methanesulphonic acid for 22, 48 and 72h at 115°C as described by Simpson *et al.* (1976). Tryptophan was determined by the spectrophotometric method of Edelhoch (1967). Total hexosamines were determined on the short column of the amino-acid analyser by the method of Spackman *et al.* (1958). For amino sugar analysis, the protein samples were hydrolysed *in vacuo* with 4M-HCl for 4h at 100°C (Spiro, 1966). Neutral sugars were determined by the orcinol/ H_2SO_4 method of Francois *et al.* (1962) with mannose as the standard. Sialic acid was determined by the thiobarbituric acid method of Warren (1959). Protein was estimated by the Peterson (1977) modification of the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Purification of brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase

Examples of the purification of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase from nauplii of different ages are shown in Table 1 for the small-scale preparations (25g of dry cysts) and in Table 2 for the large-scale preparations (900g of dry cysts). The relative yields and purification of the

Table 1. *Small-scale purification of ($\text{Na}^+ + \text{K}^+$)-activated ATPase from brine-shrimp nauplii reared from 25g of dry cysts*

Age (h)	Purification step	Total activity (μmol of P_i/h)	Total protein (mg)	Specific activity (μmol of P_i/h per mg of protein)	Enzyme yield (%)	Relative purification (fold)
24	Homogenate	8010	2960	2.70	100	1.0
	Crude membranes	5680	411	13.8	71	5.1
	Gradient membranes	3070	45.8	66.9	38	24.8
	Lubrol pellet	4230	44.3	95.3	53	35.3
	SDS-treated enzyme	1900	3.90	486	24	180
42	Homogenate	9720	3490	2.79	100	1.0
	Crude membranes	4950	380	13.1	51	4.7
	Gradient membranes	2480	51.6	48.1	26	17.2
	Lubrol pellet	3580	27.5	130	37	46.6
	SDS-treated enzyme	2370	4.44	534	24	191

Table 2. Large-scale purification of $(\text{Na}^+ + \text{K}^+)$ -activated ATPase from brine-shrimp nauplii reared from 900 g of dry cysts

Age (h)	Purification step	Total activity (μmol of P_i/h)	Total protein (mg)	Specific activity (μmol of P_i/h per mg of protein)	Enzyme yield (%)	Relative purification* (fold)
20	Crude membranes	245 000	19 500	12.5	100	4.2
	Gradient membranes	155 000	3740	42.5	63	14.2
	Lubrol pellet	135 000	1450	93.2	55	31.1
	SDS-treated enzyme	49 800	132	377	20	126
48	Crude membranes	168 000	11 100	15.2	100	5.1
	Gradient membranes	106 000	1800	58.6	63	19.5
	Lubrol pellet	153 000	968	158	91	30.3
	SDS-treated enzyme	57 200	96.2	594	34	198

* Calculated assuming a homogenate specific activity of $3 \mu\text{mol}$ of P_i/h per mg of protein.

enzyme at the various steps was comparable between the small- and large-scale preparations. The specific activity of the homogenate, which was not assayed in the large-scale preparations, was usually less than $3 \mu\text{mol}$ of P_i/h per mg of protein for the 20h and 48h animals represented in Table 2. Thus, the overall purification was about 200-fold. The crude membrane fraction represented about a 5-fold enrichment of $(\text{Na}^+ + \text{K}^+)$ -activated ATPase with a recovery of about 50–70% of the enzyme in the animal. The gradient membrane fraction was enriched about 4-fold more in $(\text{Na}^+ + \text{K}^+)$ -activated ATPase with about a 60% recovery. The increase in specific activity of the Lubrol pellet fraction was due in part to activation (about one-third) and in part to loss of non-enzyme protein (about two-thirds). Recovery of the $(\text{Na}^+ + \text{K}^+)$ -activated ATPase at this step was usually near 100%. In cases where the specific activity of the gradient membrane fraction was much higher than the usual $50 \mu\text{mol}$ of P_i/h per mg of protein, the Lubrol step often resulted in little purification. However, the Lubrol treatment was necessary in preparation for the final SDS step. Without prior Lubrol treatment, the SDS step resulted in little purification. The SDS treatment step resulted in a 4–5-fold purification with about a 40% recovery of the amount of enzyme found in the Lubrol pellet fraction. The specific activity of the final SDS-treated enzyme fraction was usually $350\text{--}450 \mu\text{mol}$ of P_i/h per mg of protein for the younger animals near 20h old, up to near 600 for the older animals near 48h old, and often above 600 for animals near 32h old, at which time $(\text{Na}^+ + \text{K}^+)$ -activated ATPase activity is developmentally at a maximum (Peterson & Hokin, 1979). ATPase activity not associated with the $(\text{Na}^+ + \text{K}^+)$ -activated ATPase accounts for less than 0.5% of the total ATPase activity of the SDS-treated enzyme fraction.

Fig. 1 shows the $(\text{Na}^+ + \text{K}^+)$ -activated ATPase activity and protein distribution of the crude membrane fraction from 48h-old brine shrimp after zonal centrifugation through the 20–40% sucrose

Fig. 1. Zonal centrifugation of the crude membrane fraction of brine-shrimp $(\text{Na}^+ + \text{K}^+)$ -activated ATPase

The crude membranes were prepared from 48h brine-shrimp nauplii reared from 300g of dry cysts and consisted of 3700mg of protein with an $(\text{Na}^+ + \text{K}^+)$ -activated ATPase specific activity of $13.8 \mu\text{mol}$ of P_i/h per mg of protein. The gradient, a 20 and 40% sucrose step gradient, was prepared and fractionated as described in the Experimental section. The distribution of $(\text{Na}^+ + \text{K}^+)$ -activated ATPase activity (●) and protein (○) is shown. Only the first 40 fractions collected from the wall of the zonal rotor are shown in the Figure, accounting for about 1000ml of the 1675ml gradient. The remaining gradient contains about one-half the protein, but very little $(\text{Na}^+ + \text{K}^+)$ -activated ATPase activity. Enzyme recovery in the first 40 fractions was 88% and protein recovery was 49%. The broken line indicates the concentration of sucrose in the gradient as determined by refractometry.

step gradient. The bulk of the $(\text{Na}^+ + \text{K}^+)$ -activated ATPase banded at a zone between 20 and 40% sucrose and was clearly separated from the bulk of the protein, which sedimented either slower or faster. Because of the clean separation of $(\text{Na}^+ + \text{K}^+)$ -activated ATPase-containing membranes, it was possible to make the proper cuts to obtain the gradient membrane fraction simply by visual examination of the zonal-gradient fractions. Banding in the

small-scale swinging-bucket preparations was even more clear-cut.

The effect of SDS treatment at the low concentrations effecting purification produced no evidence of additional activation of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase Lubrol pellet. The extent of inactivation was dependent on protein and SDS concentration and on age of the animals at the time of preparation. At final concentrations of 1.5 mg of protein/ml and 0.5 mg of SDS/ml, there was a 44% inhibition of the Lubrol pellet enzyme from 32-h-old animals and 76% inhibition of the Lubrol pellet enzyme from 20-h-old animals. At 2.0 mg of protein/ml and 0.5 mg of SDS/ml, the inhibition was 35% for 32-h-old animals and 28% for 20-h-old animals. About 20% of the SDS inhibition was found to be reversible by addition of bovine serum albumin to a final concentration of 1 mg/ml (Mizushima, 1976), or by removal of the SDS by the sucrose-gradient centrifugation.

The conditions worked out for the SDS purification step were arrived at by using the lowest concentration of SDS compatible with maximum yield and purification. Above 0.5 mg of SDS/ml the yield of purified enzyme decreased markedly. Protein concentrations greater than 1.5 mg/ml resulted in similar yields, but lower specific activities. Protein concentrations much lower than 1.5 mg/ml showed much lower yields as a result of greater inactivation. Protein concentrations slightly lower or higher than 1.5 mg/ml resulted in similar yields and purity, which allowed for some experimental variability in the adjustments of protein concentration. In younger brine shrimp (near 20 h old) the protein concentration of the Lubrol pellet could be increased to offset the greater SDS sensitivity without apparent loss in the specific activity of the final SDS-treated enzyme. Thus, for the younger animals, 2 mg of protein/ml was used with 0.5 mg of SDS/ml.

Fig. 2 shows the ($\text{Na}^+ + \text{K}^+$)-activated ATPase and protein distribution of the SDS-treated Lubrol pellet fraction from 48-h-old brine shrimp after zonal centrifugation through the 15–35% sucrose step gradient. The bulk of the protein remained near the top of the gradient and contained little or no ($\text{Na}^+ + \text{K}^+$)-activated ATPase activity. A pellet of low ($\text{Na}^+ + \text{K}^+$)-activated ATPase specific activity was also present. The ($\text{Na}^+ + \text{K}^+$)-activated ATPase banded as a near symmetrical peak with a median density of 1.10 g/ml. Appropriate cuts were made from this gradient to obtain a pool of ($\text{Na}^+ + \text{K}^+$)-activated ATPase of high specific activity. In the gradient of Fig. 2, this pool had a peak specific activity of 705 μmol of P_i /h per mg of protein and an average specific activity of 597.

Fig. 3 shows the protein patterns of the gradient membrane, Lubrol pellet and SDS-treated-enzyme

Fig. 2. Isopycnic zonal centrifugation of the SDS-treated Lubrol-pellet fraction of brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase

The Lubrol pellet was prepared from 48-h-old brine shrimp reared from 900 g of dry cysts and consisted of 969 mg of protein with an ($\text{Na}^+ + \text{K}^+$)-activated ATPase specific activity of 94.8 μmol of P_i /h per mg of protein. SDS treatment and gradient preparation (15% and 35% sucrose step gradient) and fractionation were performed as described in the Experimental section. The distribution of ($\text{Na}^+ + \text{K}^+$)-activated ATPase activity ($\bullet$) and protein ($\circ$) is shown. The pellet and the first 40 fractions collected from the wall of the zonal rotor are shown in the Figure and account for 131% of the applied enzyme activity (after SDS treatment) and 47% of the applied protein. The remaining top part of the gradient (about 675 ml) contained 4% of the applied enzyme activity and 44% of the applied protein. The broken line indicates the concentration of sucrose in the gradient as determined by refractometry.

fractions isolated from 24-h-old brine shrimp and separated by SDS/polyacrylamide-gel electrophoresis. The two ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits, which have been previously identified (Peterson *et al.*, 1978b), are observed to enrich during purification. Also as previously observed (Peterson *et al.*, 1978b), the α -subunit consists of two bands, designated α_1 and α_2 for the upper and lower bands respectively. Preparations from older brine-shrimp nauplii contain mostly the α_1 band, with very little of the α_2 band. The developmental aspects of these changes in the α_1 and α_2 bands will be dealt with in more detail elsewhere. The β -subunit appears to consist of a single broad diffuse band. Contaminating proteins in the final SDS-treated enzyme consist of three bands between the α - and β -subunits and three bands below the β -subunit, as well as some protein at the position of the tracking dye. The contaminating protein bands are not enriched in parallel with the α - and β -subunits. They can also be detected in the gradient membrane and Lubrol-pellet-protein gel patterns and in several instances appear to be actually decreased in con-

Fig. 3. Electrophoretograms of the fractions obtained during purification of brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase

Purification of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase was by small-scale procedures (25 g of dry cysts) from 24 h-old brine shrimp as described in the Experimental section. The gels (SDS/polyacrylamide-gel electrophoresis) were stained with Coomassie Blue. The positions of the α_1 -, α_2 - and β -subunit bands are indicated. Gel (1) represents the gradient membrane fraction with a specific activity of $43 \mu\text{mol}$ of P_i/h per mg of protein. Gel (2) represents the Lubrol-pellet fraction with a specific activity of $59 \mu\text{mol}$ of P_i/h per mg of protein. Gel (3) represents the final SDS-treated enzyme fraction with a specific activity of $517 \mu\text{mol}$ of P_i/h per mg of protein.

centration with purification as the α - and β -subunits are increased. The relative amount of these contaminating bands are higher in fractions of the SDS/sucrose step gradient containing lower specific activity ($\text{Na}^+ + \text{K}^+$)-activated ATPase (results not shown). The α - and β -subunits together account for about 60% of the total protein of the final SDS-treated enzyme fraction as determined by quantitative densitometry of the Coomassie Blue-stained gels.

Isolation of brine shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits

Fig. 4 shows the separation of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits by SDS-gel filtration on Bio-Gel A 1.5 m. Peak 2 contains the α -subunit and is not contaminated by other proteins according to SDS/polyacrylamide-gel electrophoresis in the fractions indicated by the horizontal bar. Peak 3 contains the β -subunit as well as other proteins of slightly larger and smaller size. Densitometric scanning of this gel indicated that the β -subunit protein comprised about 45% of the total protein. After SDS/polyacrylamide-gel electrophoresis and extraction, the β -subunit comprised 95% of the total protein. A minor protein band of higher molecular weight and one of lower molecular weight comprised 2 and 3% respectively of the total. The thin band at the bottom of the gel was not part of the protein preparation since it was also present in blank gels.

The pooled fractions indicated by the horizontal bars in Fig. 4 after freeze-drying and dialysis produced on average 3 mg of protein for the α -subunit fractions and 4 mg of protein for the β -subunit fractions from a column run of 40 mg of holoenzyme (SDS-treated-enzyme fraction). After SDS/polyacrylamide-gel electrophoresis and extraction of the β -subunit, an average of 0.4 mg of the purified β -subunit was obtained from the original 40 mg of holoenzyme protein. It would probably be possible to obtain higher yields of the β -subunit by direct electrophoresis of the holoenzyme rather than using the fractions obtained by gel filtration. In our hands, extraction of the α -subunit from SDS/polyacrylamide gels under a wide variety of methods has always resulted in very low yields.

Three other major u.v.-absorbing peaks were obtained by SDS-gel filtration of the SDS-treated enzyme fraction on Bio-Gel A 1.5 m (peaks 1, 4 and 5 of Fig. 4). Peak 1, which corresponds to the void volume of the column, contained no protein detectable on Coomassie Blue-stained SDS/polyacrylamide gels. Peak 4 contained low-molecular-weight proteins that migrated with or near the tracking dye in 8.75% acrylamide concentration SDS gels (mol.wt. approx. 10000). Peak 5 contained β -mercaptoethanol.

Fig. 4. SDS-gel filtration of partially purified brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase

The SDS-treated enzyme fraction consisting of 36.5 mg of protein (specific activity, 413 μmol of P_i/h per mg of protein) prepared from 48 h-old brine-shrimp nauplii was solubilized in SDS and fractionated by chromatography on a Bio-Gel A 1.5 m column as described in the Experimental section. The horizontal bars indicate the fractions pooled for each subunit. The electrophoretograms (SDS/polyacrylamide-gel electrophoresis) obtained from the fractions indicated by the horizontal bars are shown directly above each respective bar. Further purification of the β -subunit by SDS/polyacrylamide-gel electrophoresis and subsequent extraction produced the protein pattern shown in the third gel as indicated.

The amino-acid and carbohydrate composition of the isolated brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits is shown in Table 3. The amount of protein hydrolysed for amino-acid analysis as estimated by the Lowry protein assay (Peterson, 1977) was 250 μg for the α -subunit and 200 μg for the β -subunit. Total amino-acid analysis accounted for 215 μg of α -subunit protein and 173 μg of β -subunit protein, indicating that the Lowry protein assay overestimates the α -subunit by 12% and the β -subunit by 13%. This is in agreement with a 10% overestimate for the electric-eel ($\text{Na}^+ + \text{K}^+$)-activated ATPase by the Lowry protein assay (Peterson & Hokin, 1980). The most striking difference in the amino-acid composition of the two subunits is in the tyrosine and tryptophan content, which is much higher in the β -subunit than in the α -subunit. The β -subunit contains nearly twice the amount of carbohydrate on a weight basis as the α -subunit. Neither subunit was found to contain sialic acid, although the assay was sufficiently sensitive to detect less than 1 mol of sialic acid/mol of subunit.

From the results of careful molecular-weight analysis of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits (Peterson & Hokin, 1980), the total number of amino acid residues was estimated to be 891 for the α_1 -subunit and 357 for the

β -subunit. By using these values, the total number of each amino acid and carbohydrate residue was estimated to the nearest integer and the results are shown in Table 3. Calculated in this fashion, the α_1 -subunit contains 890 amino acid residues for a total mol.wt. of 98 100, and similarly the β -subunit contains 355 amino acid residues for a total mol.wt. of 40 000. The molecular weights of the α_1 - and β -subunits after addition of the mass contributed by carbohydrate are 103 500 and 44 100 respectively. The molecular weight of the holoenzyme, which is an $\alpha_2\beta_2$ tetramer (Peterson & Hokin, 1980) is 276 200 for the protein portion only and 295 200 for the sum of the protein and carbohydrate.

Discussion

Purification of the brine shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase by the Lubrol/SDS method described in the present paper does not result in complete purification, but represents a substantial improvement over the previous deoxycholate and freeze-thaw procedure (Peterson *et al.*, 1978b). The purity of the enzyme by the new method is about 60% as determined by SDS/polyacrylamide-gel electrophoresis. The new procedure results in a 2-fold increase in yield and purity, and furthermore

Table 3. *Amino-acid and carbohydrate composition of brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits*

The α -subunit was isolated by SDS-gel filtration on a Bio-Gel A 1.5 m column from the partially purified 48 h brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase, which consists of about 80–90% α 1-subunit band and 10–20% α 2-subunit band. The β -subunit was isolated from 20h- and 48h-brine-shrimp enzyme by SDS/polyacrylamide-gel electrophoresis and extraction. Subunit isolation and composition analyses were performed as described in the Experimental section. The molar amino-acid residue/subunit-ratio results are nearest-integer values calculated from the composition data and the total number of amino-acid residues per subunit (891 for α 1-subunit and 357 for β -subunit) estimated from extensive molecular-weight analysis by SDS/polyacrylamide-gel electrophoresis (Peterson & Hokin, 1980). The molecular-weight values shown were calculated directly from the nearest-integer residue composition.

Residue	α -Subunit		β -Subunit	
	(mol/100 mol of amino acid)	(mol/mol of subunit)	(mol/100 mol of amino acid)	(mol/mol of subunit)
Lysine	5.0	44	6.0	21
Histidine	1.0	9	0.8	3
Arginine	3.0	27	2.6	9
S-Sulphocysteine	2.7	24	3.2	12
Aspartic acid	10.4	93	11.0	39
Threonine	6.9	62	5.8	21
Serine	7.4	66	7.4	26
Glutamic acid	9.7	87	10.2	36
Proline	4.4	39	5.4	19
Glycine	7.6	68	8.2	29
Alanine	8.4	74	5.7	20
Valine	5.3	47	5.6	20
Methionine	3.3	29	2.6	9
Isoleucine	5.7	50	4.9	17
Leucine	9.0	80	7.6	27
Tyrosine	2.9	26	4.1	15
Phenylalanine	5.1	46	5.3	19
Tryptophan	2.1	19	3.6	13
Total	99.9	890	100.0	355
Hydrophobic residues (%) (Guidotti, 1972)	46.8		44.0	
Amino sugars	0.66	6	1.06	4
Neutral sugars	2.9	26	5.7	20
Sialic acid	0	0	0	0
Carbohydrate (%)*	5.20	5.25	9.19	9.21
Molecular weight				
Protein portion		98 100		40 000
Carbohydrate portion		5 400		4 100
Total		103 500		44 100

* Percentage of total by weight.

is more suitable for small-scale purification experiments than the previous method. The overall purification is consistently about 200-fold relative to the homogenate, which is higher than any published method to date (Kyte, 1971; Uesugi *et al.*, 1971; Hokin *et al.*, 1973; Lane *et al.*, 1973; Dixon & Hokin, 1974; Jørgensen, 1974; Pitts & Schwartz, 1975; Hopkins *et al.*, 1976). The yield of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase by the new purification method is about 10 mg of protein per 100 g of starting material, which is in the same range as the other preparations cited above.

The Lubrol WX treatment of the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase was done under

conditions similar to those previously described in this laboratory (Uesugi *et al.*, 1971; Hokin *et al.*, 1973), in an effort to obtain a Lubrol-solubilized enzyme. However, despite experimentation with a variety of conditions of temperature, protein concentration, Lubrol concentration and ionic strength, the brine-shrimp enzyme could not be solubilized with Lubrol WX. Virtually 100% of the Lubrol-treated brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase is pelleted after centrifugation at 100 000 g for 2 h. Although treatment of the brine-shrimp enzyme with Lubrol WX produces at most only a 2-fold purification, it was, nevertheless, found to be an essential preparatory step to the SDS purification

step. Previous experiments attempting to apply the SDS purification method of Jørgensen (1974) to the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase produced little or no purification of the gradient membrane fraction. After treatment with Lubrol WX, however, a 5-fold or greater purification could be accomplished by SDS.

The SDS purification step arrived at for the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase differs in several respects from that developed by Jørgensen (1974) for the mammalian kidney ($\text{Na}^+ + \text{K}^+$)-activated ATPase, and subsequently used by others for complete (Hopkins *et al.*, 1976; Hastings & Reynolds, 1979) or partial (Sweadner, 1978, 1979; Bell & Sargent, 1979) purification of ($\text{Na}^+ + \text{K}^+$)-activated ATPase from other tissue sources. The concentrations of protein and SDS were higher and lower respectively than those used by Jørgensen, and SDS treatment had to be done at 30°C rather than 20°C (Jørgensen, 1974). At temperatures less than 27°C , the purification achieved by SDS treatment decreased. The final brine-shrimp SDS-treated enzyme corresponds to a density of 1.10 g/ml at the peak of enzyme activity, as opposed to 1.125 for the mammalian kidney (Jørgensen, 1974). Differences in the density of the SDS-treated enzyme are probably a result of the particular membrane protein and SDS interactions (Sweadner, 1978), which are probably tissue specific.

SDS/polyacrylamide-gel electrophoresis of the purified brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase shows that the α - and β -subunits appear to be free of other proteins when isolated by this method. Other experiments with ten different acrylamide concentrations from 5 to 15%, or gels using acrylamide gradients, did not resolve additional protein bands (Peterson & Hokin, 1980). Precise agreement of the ratio of the α - and β -subunits determined from accurate molecular-weight measurements and from accurate mass-ratio measurements (Peterson & Hokin, 1980) lend further support to the homogeneity of these two subunits. Therefore, the brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits isolated by gel filtration or by extraction of SDS/polyacrylamide gels are assumed to be homogeneous, or nearly so, since re-examination by analytical SDS/polyacrylamide-gel electrophoresis does not indicate the presence of additional protein bands. The β -subunit preparation contained two minor components of higher and lower molecular weight, which comprised 2 and 3% of the total protein respectively.

The α -subunit of the San Francisco-Bay-variety brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase appears as two bands ($\alpha 1$ and $\alpha 2$) in agreement with the previous studies on the Great-Salt-Lake variety (Peterson *et al.*, 1978b). Both bands had been previously identified as ($\text{Na}^+ + \text{K}^+$)-activated

ATPase α -subunit by specific ($\text{Na}^+ + \text{Mg}^{2+}$)-dependent K^+ -sensitive phosphorylation for [γ - ^{32}P]ATP (Peterson *et al.*, 1978b). Evidence that both bands are nearly identical in primary structure has been recently obtained by 'mapping' partial proteolytic digests of the $\alpha 1$ - and $\alpha 2$ -bands (J. A. Fisher, G. L. Peterson & L. E. Hokin, unpublished work). The double banding is similar to that reported recently by Sweadner (1979) for mammalian brain tissue. Additional studies (results not shown) demonstrate that the two α -subunit bands of the brine shrimp represent different ($\text{Na}^+ + \text{K}^+$)-activated ATPase isoenzymes.

The amino-acid and carbohydrate analyses on the brine-shrimp α -subunit were performed on the subunit isolated from 48-h-old brine shrimp, since these animals contain principally the $\alpha 1$ band and very little of the $\alpha 2$ band (Peterson & Hokin, 1979). The compositional analyses are therefore assumed to represent that for $\alpha 1$ -subunit only. It has not been possible thus far to obtain preparations that consist exclusively of $\alpha 1$ -subunit, or those that contain more than 50% of $\alpha 2$ -subunit. Because of the low extractability of the α -subunit from SDS/polyacrylamide gels, isolation of preparative quantities of $\alpha 1$ - and $\alpha 2$ -subunits exclusive of each other by this method has also not been possible. The $\alpha 1$ -subunit of the brine shrimp enzyme has about twice the carbohydrate content of the α -subunit of the electric eel (Churchill *et al.*, 1979). The carbohydrate content of the β -subunit of the brine shrimp, however, is lower than that in the β -subunit of the other species reported to date (Kyte, 1972; Perrone *et al.*, 1975). The most striking feature about the compositional analysis of the brine-shrimp subunits is that neither one contains sialic acid, which has been found in both ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits from all other species that have been examined (Kyte, 1972; Perrone *et al.*, 1975; Churchill *et al.*, 1979). The amino-acid composition of the two brine-shrimp ($\text{Na}^+ + \text{K}^+$)-activated ATPase subunits are very similar to those of the other species and contain approximately the same percentage of hydrophobic residues.

We thank Mrs. Constance Capacio for her skilful technical assistance and Mr. Hank Ellis for the amino-acid analysis. This work was supported by Grants HL16318 and GM26000 from The U.S. National Institutes of Health, and Research Grant PCM76-20602 from The U.S. National Science Foundation. G. L. P. was supported in part by U.S. National Institutes of Health Postdoctoral Fellowship GM05934.

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