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Title: A STUDY OF THE SODIUM + POTASSIUM-ACTIVATED ADENOSINETRIPHOSPHATASE IN BRINE SHRIMP, ARTEMIA SALINA, NAUPLII

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Abstract approved: Frank P. Conte

Studies were made on the kinetic characteristics, anatomical and biochemical localization, and the molecular and kinetic properties of partially purified Na+K-activated ATPase in the nauplius larvae of the brine shrimp, Artemia salina. The amount of ATPase that is stimulated maximally in the presence of Na^+, K^+, and Mg^{++}, and inhibited by ouabain compares with levels found in specialized transport tissues of vertebrates (about 500 μmoles P_i/hr/g fresh weight tissue). The kinetic characteristics are similar for various membrane and partially purified membrane preparations. Maximum activity is observed at pH 7.2 (imidazole buffer) with 5 mM ATP at a Mg^{++}/ATP ratio of 2/1. Monovalent cation stimulation is optimum at a Na/K ratio of 4/1 with two total ionic strength maxima near 200 mM and 400 mM ($Na^+ + K^+$). Linearity of P_i release is observed

for 30 minutes at 42° C, but is lost in less than 20 minutes at 45° C. Localization of the enzyme, anatomically by microdisection techniques, and subcellularly by marker enzyme biochemical studies, suggest that it is widely distributed in the plasma membranes of various cells of the animal. The larval salt gland or the intestine are not conspicuously enriched in the Na+K-activated ATPase. Alternatives for the function of this enzyme in the brine shrimp nauplii are discussed. Membranes isolated from nauplii after 40-48 hrs incubation at 26° C, have a specific activity of 60 μmoles P_i/hr/mg protein and represent a yield of about one third of the enzyme in the animal. Treatment of the membranes with the detergent sodium deoxycholate (DOC) produces a particulate enzyme (DOC-membrane preparation) which is resuspended in DOC-free homogenizing media (buffered sucrose-EDTA) and frozen at -12° C. DOC treatment gives a 2.5 fold purification of the membrane and an additional 1.5 fold activation, however; activation is observed only after resuspension in DOC-free media. A further 2 to 3 fold purification is obtained by gentle thawing and isolation by differential or discontinuous sucrose gradient centrifugation of a Freeze-Thaw (FT) vesicular preparation. This final partially purified preparation consists of small and frequently open vesicles or membrane fragments. Attempts to solubilize this preparation or other membrane preparation were made with several detergents under a variety of conditions.

None of these produced any significant solubilization of the brine shrimp enzyme, whereas they do solubilize this enzyme obtained from several vertebrate sources reported in the literature. Under maximum assay conditions the partially purified FT enzyme preparation has a specific activity of 300-600 μmoles P_i/hr/mg protein with a yield of about 40% of the isolated membranes. The highest specific activity preparation obtained was 712 μmoles P_i/hr/mg protein, and is estimated to be 48% pure by estimate of contamination of acrylamide gels and γ^{32}P-ATP binding. The turnover member from γ^{32}P-ATP binding studies is 6460 min^{-1}. Two protein regions enriched during partial purification and both appear as double bands on SDS polyacrylamide gels. In the large molecular weight region the bands have apparent molecular weights of 101,000 and 95,200. Both contain the catalytic subunit(s) and stain positive with PAS. In the small molecular weight region the bands have apparent molecular weights of 40,000 and 37,800 and are considered the glycoprotein subunit(s). The mass ratio for large to small subunit is 2:1 and corresponds to a molar ratio of 1 large:2 small. Evidence is presented and discussed for the possible existence of two isozymes of the Na+K-activated ATPase in the brine shrimp nauplii, with different kinetic and molecular properties.

A Study of the Sodium + Potassium-activated Adenosinetriphosphatase in Brine Shrimp *Artemia salina*, Nauplii

by

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Typed by Opal Grossnicklaus for Gary L. Peterson

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I. BACKGROUND

The translocation of ions across biological membranes is one of the general physiological phenomena of underscored importance in both the plant and animal kingdoms. The most thoroughly investigated and understood ion transport system is the cation transport enzyme, the ouabain-sensitive sodium + potassium-activated adenosinetriphosphatase (abbreviated Na+K-activated ATPase or simply Na-K ATPase). Since the discovery of this enzyme by Skou in 1957, it has been the subject of numerous reviews attempting to keep abreast of the rapidly expanding literature concerning its related biochemistry and physiology (e. g., Skou, 1965, 1971; Albers, 1967; Katz and Epstein, 1968; Bonting, 1970; Hokin and Dahl, 1972). Despite this intense investigation, the molecular mode of action, the molecular organization, and the biogenesis of this important enzyme are incompletely understood. These interrelated problems are currently very active areas of research.

Nearly all of what is known about the mechanism of action of this enzyme in its oriented membrane arrangement derives from studies of red blood cells or ghosts, particularly the low K-high K model system found in sheep. Blood cells, however, are not richly

supplied with the Na+K-activated ATPase, and other studies of a biochemical approach not requiring the orientation of the enzyme have exploited the use of tissues that are. These have principally involved vertebrate nervous, renal or extrarenal excretory tissues. The demand in studies probing the molecular organization of the enzyme is for continuously available material in large quantities. Beef brain, dog and rabbit kidneys and dogfish rectal glands are examples of the type of systems used for these studies. Model systems suitable for investigating the biogenesis of the Na+K-activated ATPase are slow in developing. They have generally employed vertebrate renal or extrarenal excretory organs challenged to induction of the Na+K-activated ATPase by salt loading and/or hormone injection. The cumbersome attributes of these systems and in particular their complexity, relatively low and/or slow induction of new enzyme, and frequent lack of complimentary adaptability to enzyme purification, has thwarted progress in this area, and stimulated the search for new model systems.

This thesis is addressed to the development of a new model system for the study of the biogenesis of the Na+K-activated ATPase. Such a system would ideally incorporate many of the convenient features associated with the use of organisms such as bacteria. (Bacteria themselves are unsuitable as they apparently contain little or none of this enzyme (Bonting, 1970).) Among the desirable features,

the organism of choice would include the following: (1) consistently available and convenient to store, (2) easy and inexpensive to rapidly rear in large quantities, (3) adaptable to sterile procedures, (4) of simple biological organization, (5) synthesize a rich supply of the enzyme in a relatively short period of time, and (6) adaptable to purification of the enzyme. One organism which is sufficiently promising to pursue as a possible organism of choice is the nauplius larva of the brine shrimp, Artemia salina.

The brine shrimp, A. salina, is a cosmopolitan species that inhabits brine pools, salterns and salt lakes (Weldon, 1909; Martin and Wilbur, 1921; Lochhead, 1941; Dempster, 1953; Carpelan, 1957). It is exposed in its natural environment to extremes of salinity ranging from that approaching freshwater during heavy rains to saturating solutions during prolonged evaporation. These salinity tolerances have been reproduced in the laboratory (Evans, 1913; Martin and Wilbur, 1921; Boone and Baas-Becking, 1931; Jennings and Whitaker, 1941), and were suggested very early to be accompanied by an internally regulated salt content under one half that of sea water (Martin and Wilbur, 1921; Medwedewa, 1927). The ionic and osmoregulatory physiology of the adult brine shrimp has been extensively examined by Croghen (1958a-d) Thuet, Motais and Maetz (1968), and Smith (1969a, b). The salt secretory epithelium of the adult lies in the metepipodite segments of the paired branchiae and their fine

structure has been examined by Copeland (1966, 1967). From the study of Augenfeld (1969) the adults are known to contain a Na+K-activated ATPase that increases in activity with high salt stress.

A more intriguing situation is present in the nauplius stage of the brine shrimp. The encysted cryptobiotic eggs (gastrulae) are abundant, commercially available, and can be stored frozen for years (Dempster, 1953). The nauplius hatches in about 24 hours from cysts placed in sea water (for life history refer to Lochhead (1941) and Dempster (1953)). The cysts can be sterilized (Finamore and Clegg, 1968), and hatched in sterile sea water in virtually any desirable quantity. Morphologically, the nauplius (see Anderson, 1967) is relatively simple, consisting of a single layer of cells about the outside and along the length of the gut enclosing a haemocoelic cavity with a few scattered mesodermal cells and musculature for the swimming antennae. According to Croghen (1958b) the nauplii regulate their internal salt levels, apparently through the neck organ (Dejdar, 1930; Croghen, 1958c), which is the larval equivalent of the adult branchial medepipodites.

Recently, evidence has been obtained in this laboratory confirming and extending previous work that the brine shrimp nauplius can survive and regulate its internal salts over a wide range of external salinities (Conte, Hootman and Harris, 1972), and that this regulation is apparently achieved by the salt secretory epithelium

of the neck organ (Conte, Hootman and Harris, 1972; Hootman, Harris and Conte, 1972). Furthermore, evidence of a Na+K-activated ATPase in the nauplius is implicated in the ouabain-sensitive survival in high salinities (Ewing, Peterson and Conte, 1972). The cysts are virtually impermeable to anything other than H_2O, O_2, CO_2 and perhaps some other gases, which solves the osmotic problems of the early embryo. After hydration, macromolecular synthesis (Clegg, 1966; Clegg and Golub, 1969) and polysome formation begins (Hultin and Morris, 1968; Golub and Clegg, 1968), but cell number and DNA content remains constant till emergence (Nakanishi et al., 1962, 1963). This and other evidence suggested to Morris (1971) that differentiation does not begin till about 12 hours after hydration. The possibility for the de novo synthesis of the Na+K-activated ATPase in a convenient study organism is thus considered very promising.

Little information is available on the Na+K-activated ATPase from invertebrate sources, although interestingly enough it was first described and characterized from preparations of leg nerves of the shore crab, Carcinus maenas (Skou, 1957, 1960). It is the intention of this thesis to develop a well characterized model system with the embryonic brine shrimp tissue, for studies on the molecular organization and formation of the Na+K-activated ATPase. Thus, the kinetic properties and subcellular localization of this enzyme in the

brine shrimp nauplius are investigated in addition to the purification and biogenesis studies. Each aspect of study is introduced in more detail in its appropriate section.

II. CHARACTERIZATION OF THE Na+K-ACTIVATED ATPase

Introduction

The implication of a Na+K-activated ATPase being found in nauplius larvae of Artemia salina has been previously introduced. Augenfeld (1969) has assayed this enzyme in the adults of Artemia, but has not provided a careful characterization of its various kinetic parameters. The kinetic properties of this enzyme have been carefully examined in two other crustacea; from the pleopods of the isopod Sphaeroma serratum (Philpott, Thuet and Thuet, 1972), and from the leg nerves of the shore crab Carcinus maenas (Skou, 1957, 1960). These preparations have similar pH, temperature, Mg and ATP (Mg/ATP ratio) optima, and compare closely with such properties of the enzyme from vertebrate sources (Skou, 1965; Bonting, 1970). However, they differ strikingly from one another and other enzyme sources in the activation characteristics of Na and K. S. serratum appears to have two K dependent Na activation optima at 30 and 100-120 mM Na, both with Na/K ratios near 6/1. C. maenas on the other hand has only one, as observed with other enzyme sources, but Na activation is optimum at 40 mM Na and a Na/K ratio of 1/1, which is quite unique. This section describes the kinetic characteristics of the Na+K-activated ATPase in the brine shrimp nauplius.

Methods

Membrane Preparations

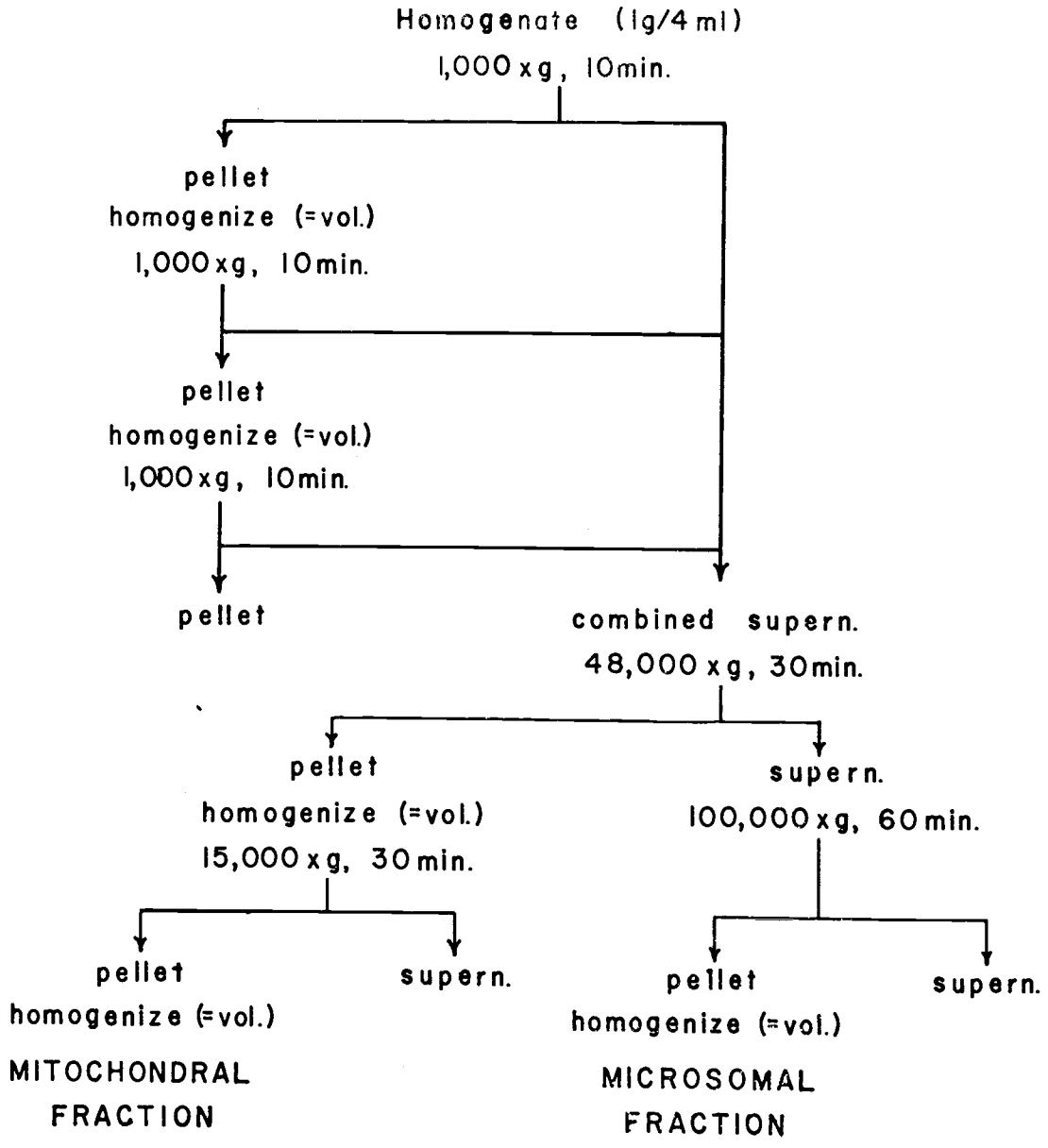
Brine shrimp nauplii were harvested after 36 hours incubation as previously described by Conte, Peterson and Ewing (1973). The nauplii were homogenized in a Potter-Elvehjem glass-teflon homogenizer at a ratio of one gram fresh weight nauplii per four ml of homogenizing media (H. M.). The homogenizing medium contained 250mM sucrose, 100mM Tris-HCl buffer (pH 7. 4), and 2mM Na_2EDTA. The homogenate was fractionated by differential centrifugation according to the flow diagram shown in Figure 1. All pellets were resuspended in 250mM sucrose. The 15, 000xg and 100, 000xg pellets were termed the mitochondrial and microsomal fractions, respectively.

Kinetic Characterizations

The Na+K-activated ATPase (ouabain sensitive, $Mg^{++}+Na^{+}+K^{+}$-activated adenosine triphosphate phosphohydrolase, EC 3. 6. 1. 3) was assayed in the following reaction mixture: 320mM NaCl, 80mM KCl, 10mM $MgCl_2$, 5mM Na_2ATP, 100mM imidazole buffer (pH 7. 2), with and without 0. 5mM ouabain. The reactants in a total volume of 0. 4 ml were premixed with 0. 1 ml of enzyme at 0° C (ice bath) and the reaction initiated by placing the reaction tubes in a 37° C

Figure 1. Schematic for subcellular fractionation of naupliar homogenates.

FRACTIONATION FLOW DIAGRAM



water bath. The reaction tubes were shaken at this temperature for 20 minutes in a Gilson metabolic incubator. The reactions were then stopped by plunging the tubes in an ice bath, then adding 3.0 ml of 1.66N H_2SO_4. By this procedure as many as 100 separate reactions can be run simultaneously. Reaction mixtures which were varied for the different parameters are individually described in the appropriate figure legends. Each reaction tube was analyzed for total inorganic phosphate by the method of Ernster, Zvetterstrom, and Lindberg (1950). Duplicate or triplicate reactions with and without ouabain were averaged for amount of inorganic phosphate and the difference was calculated to represent the activity due to the Na+K-activated ATPase. The results reported for the various substrate and/or cofactor requirements are in terms of concentrations yielding half-maximal activity rather than Km's because of the complex nature of the enzyme system. Protein was estimated after the method of Lowry et al. (1951), using crystalline bovine serum albumin as the standard.

Results

Ion Requirements

The ions tested for stimulation of ATPase activity are shown in Table 1. A ouabain sensitive component of this activity is shown

Table 1. Ion requirements for ATPase activity from nauplii

Reaction Conditions[1]	Specific Activity of ATPase (μmoles P_i/hr/mg protein)		
	Without ouabain	With 0.5mM ouabain	Difference
0.1 ml enzyme + 100mM imidazole, pH 7.2, + 5mM Tris-ATP	1.6	2.1	-0.5
+ $MgCl_2$	5.2	5.1	0.1
+ $MgCl_2$ + KCl	5.1	5.3	-0.2
+ $MgCl_2$ + NaCl	4.8	5.0	-0.2
+ $MgCl_2$ + NaCl + KCl	13.1	6.9	6.2
+ $MgCl_2$ + NaCl + NH_4Cl	12.4	6.2	6.2
+ $MgCl_2$ + NH_4Cl + KCl	3.8	3.8	0.0
+ $CoCl_2$ + NaCl + KCl	5.7	4.1	1.6
+ $MnCl_2$ + NaCl + KCl	5.1	4.4	0.7
+ $CaCl_2$ + NaCl + KCl	3.0	3.0	0.0

[1]All ionic additions were 1mM in the reaction.

to depend upon the presence of Mg^{++}, Na^{+} and K^{+}. Co^{++} and Mn^{++} replace the Mg^{++} requirements for the ouabain sensitive ATPase activity, but are less efficient than Mg^{++}. NH_4^{+} could be substituted for K^{+} but not for Na^{+}.

Distribution of Activity in the Subcellular Fractions

The activity of the Na+K-activated ATPase isolated by differential centrifugation principally resides in the mitochondrial fraction (15,000xg pellet) as shown in Table 2. Most of the remainder appeared in the microsomal fraction (100,000xg pellet). Slight variations in the relative distribution between these two fractions occasionally occurred and appears to be dependent upon the extent of mechanical disruption brought about by prolonged homogenization.

Kinetic Characterization

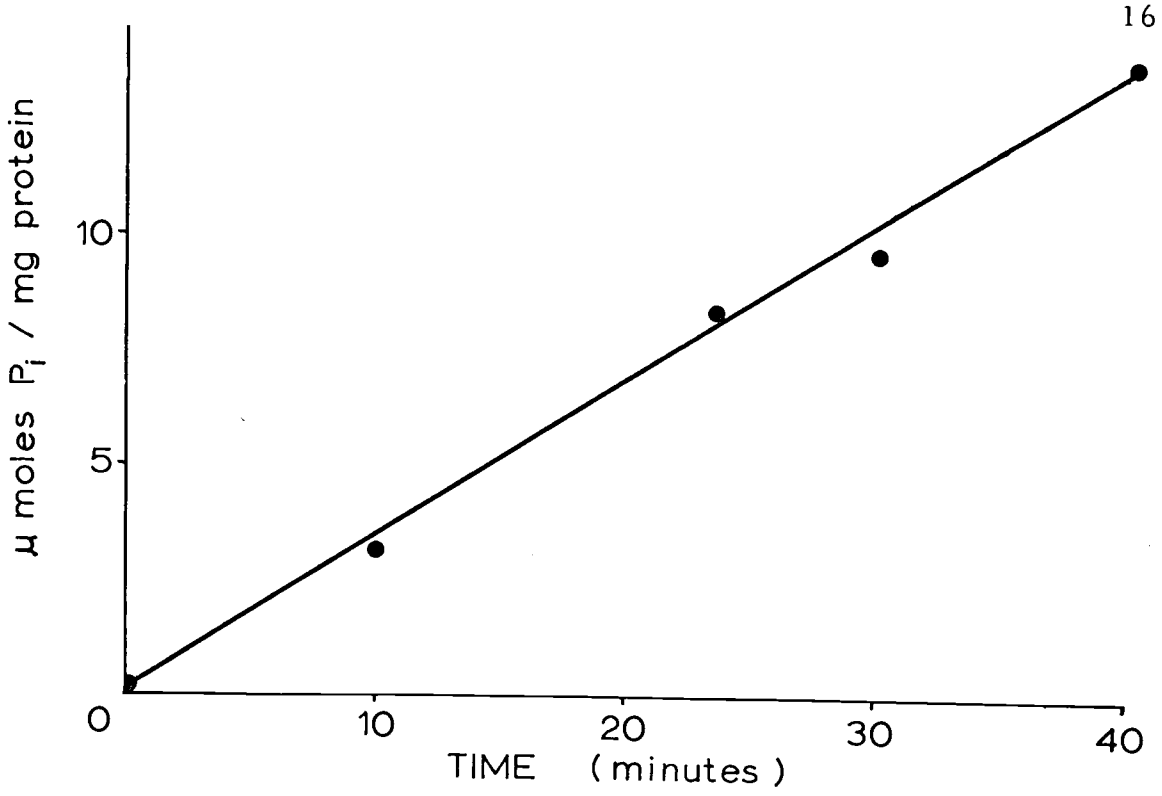
Characterization of the Na+K-activated ATPase was performed simultaneously on both the mitochondrial and microsomal preparations in the event these represented populations of kinetically different ATPases. Figure 2 shows that the hydrolyisis of ATP as measured by the release of inorganic phosphate is linear for at least 40 minutes when incubated at 37° C. Optimal reaction rates were obtained in a temperature range between 40-45° C (Figure 3), but the stability of the enzyme system declined rapidly at the higher

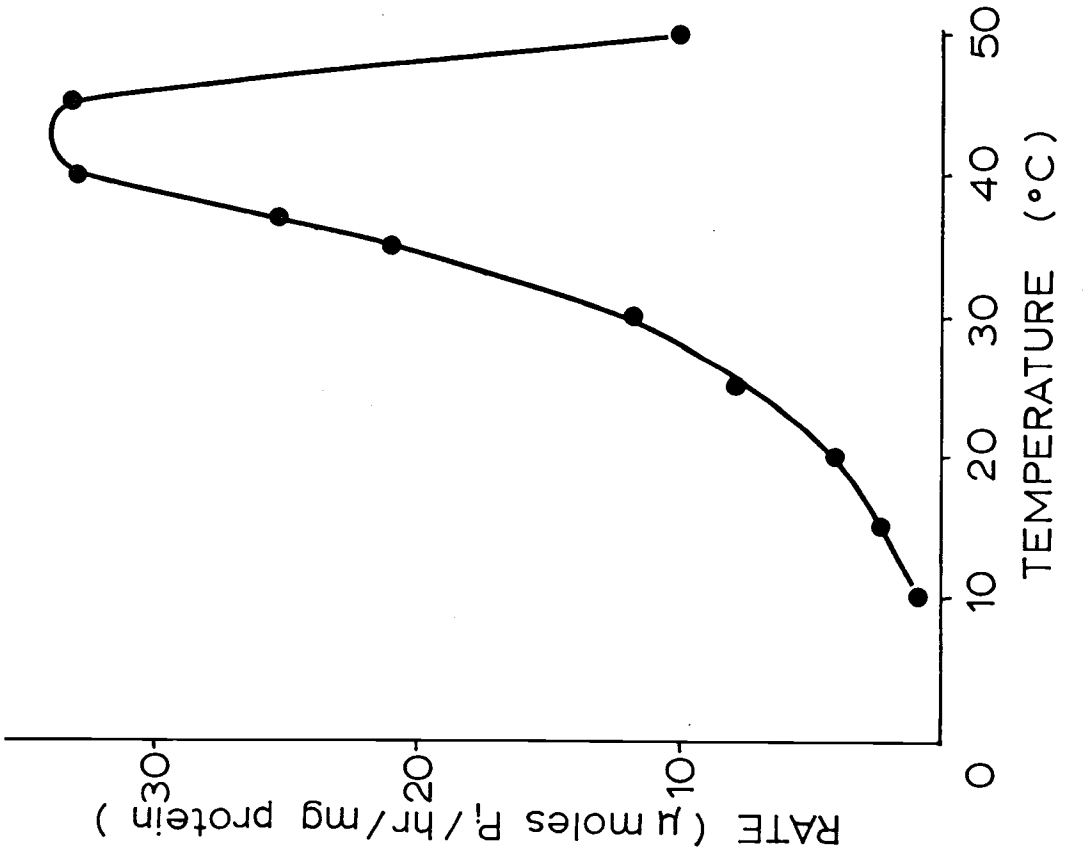
Table 2. Subcellular fractionation of Na+K-activated ATPase from nauplii

Fraction	Total Activity		Specific Activity
	µmoles P_i/hr/gram	Percent	µmoles P_i/hr/mg protein
1000xg pellet	15.4	7	2.4
1000xg supern.			
48,000xg pellet			
15,000xg pellet	135.0	60	21.4
15,000xg supern.	38.9	17	18.4
48,000xg supern.			
100,000xg pellet	35.4	16	11.9
100,000xg supern.	1.6	1	0.1

Figure 2. Ouabain-sensitive release of P_i from ATP as a function of time. Assay conditions as described in Methods.

Figure 3. Effect of temperature on Na+K-activated ATPase activity. Reaction conditions as described in Methods using a mitochondrial preparation.


μ moles P_i / mg protein
0
5
10
0
10
20
30
40
TIME (minutes)


RATE (μ moles P_i / hr / mg protein)
0
10
20
30
0
10
20
30
40
50
TEMPERATURE (°C)

temperatures. Inorganic phosphate released after the first 20 minutes at 45° C became non-linear; therefore, all subsequent reactions were carried out at the lower temperature (37° C).

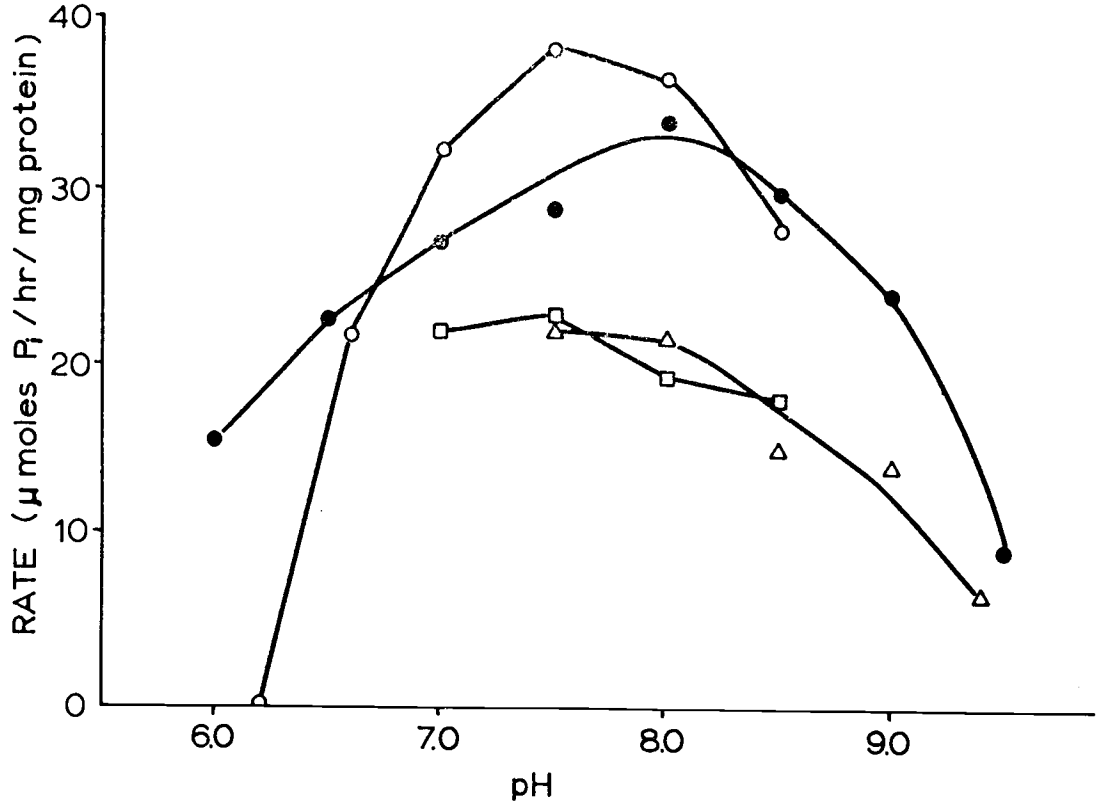
The effect of pH and buffering agent is shown in Figure 4. The optimum pH for the reaction lies in the vicinity of pH 7. 5. Histidine buffer showed a shift toward a higher pH optimum of 8. 0, while HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), not shown in the figure, shifted maximal activity nearer to pH 7. 0. Imidazole had a slightly greater activity than the other buffers, and was adopted for subsequent use.

Ouabain inhibition of ATPase activity is shown in Figure 5, and the concentration that gives half-maximal inhibition is 8×10^{-6}M for the mitochondrial fraction and 5×10^{-6}M for the microsomes. A concentration of 5×10^{-4}M was equally effective for maximal inhibition of ATPase in both fractions, and was thereafter used in all the assays.

The ATP substrate curve for Na+K-activated ATPase in the presence of 10mM Mg^{++} is shown in Figure 6. When ATP concentrations were held constant at 1, 5 and 10mM, the half maximal concentrations of Mg^{++} were 0. 7mM, 1. 1mM and 2. 0mM respectively (Figure 7). This interdependence of Mg^{++} and ATP is shown in Figure 7 to have a ratio of approximately 2/1 (Mg^{++}/ATP) in each experiment for optimal rate of ATP hydrolysis by the Na+K-activated

Figure 4. Effect of pH and different buffers on Na+K-activated ATPase activity. All buffers were present at a concentration of 100 mM. Other reaction conditions were as described in Mthods. Buffers shown are: Histidine, ●----●; imidazole, 0----0; Tris, △----△; TES, □----□.

Figure 5. Ouabain inhibition of Na+K-activated ATPase from mitochondrial (●----●) and microsomal (0----0) preparations. Assay conditions were as described in Methods, except for variable ouabain concentrations.


RATE (μ moles P_i / hr / mg protein)
40
30
20
10
0
6.0
7.0
8.0
9.0
pH

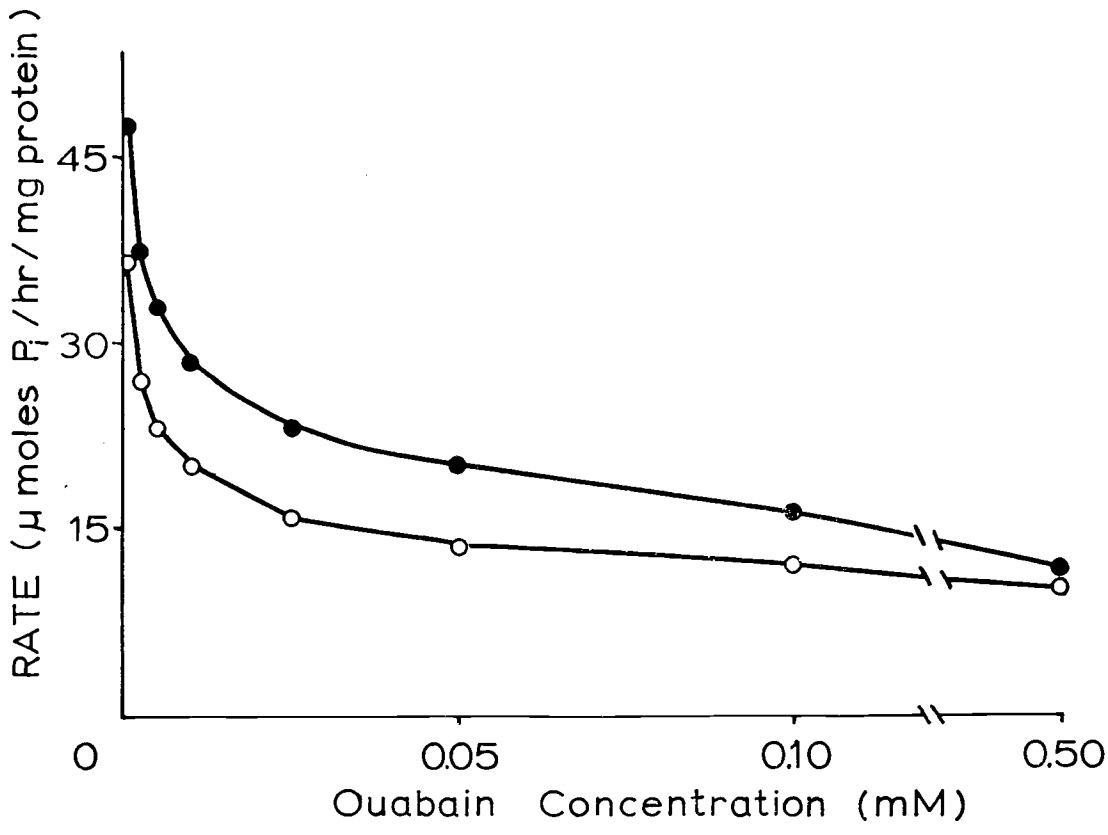
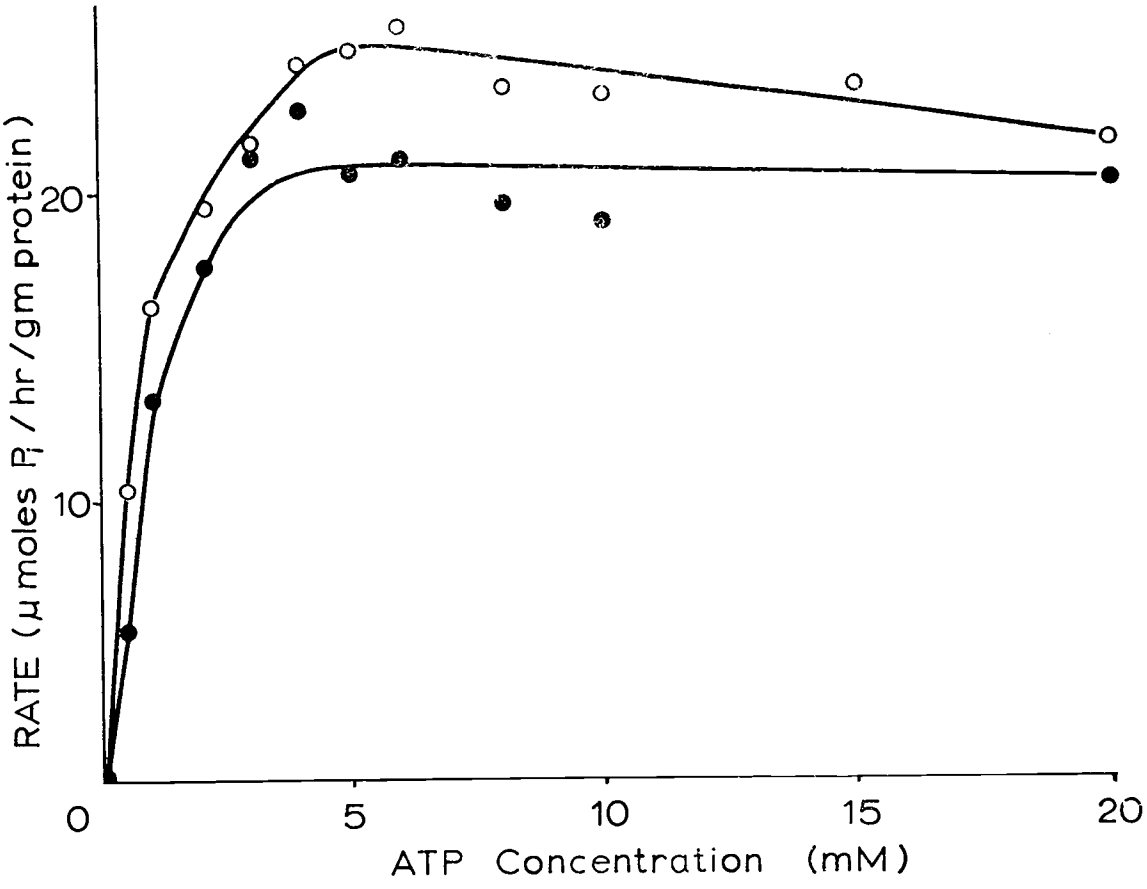
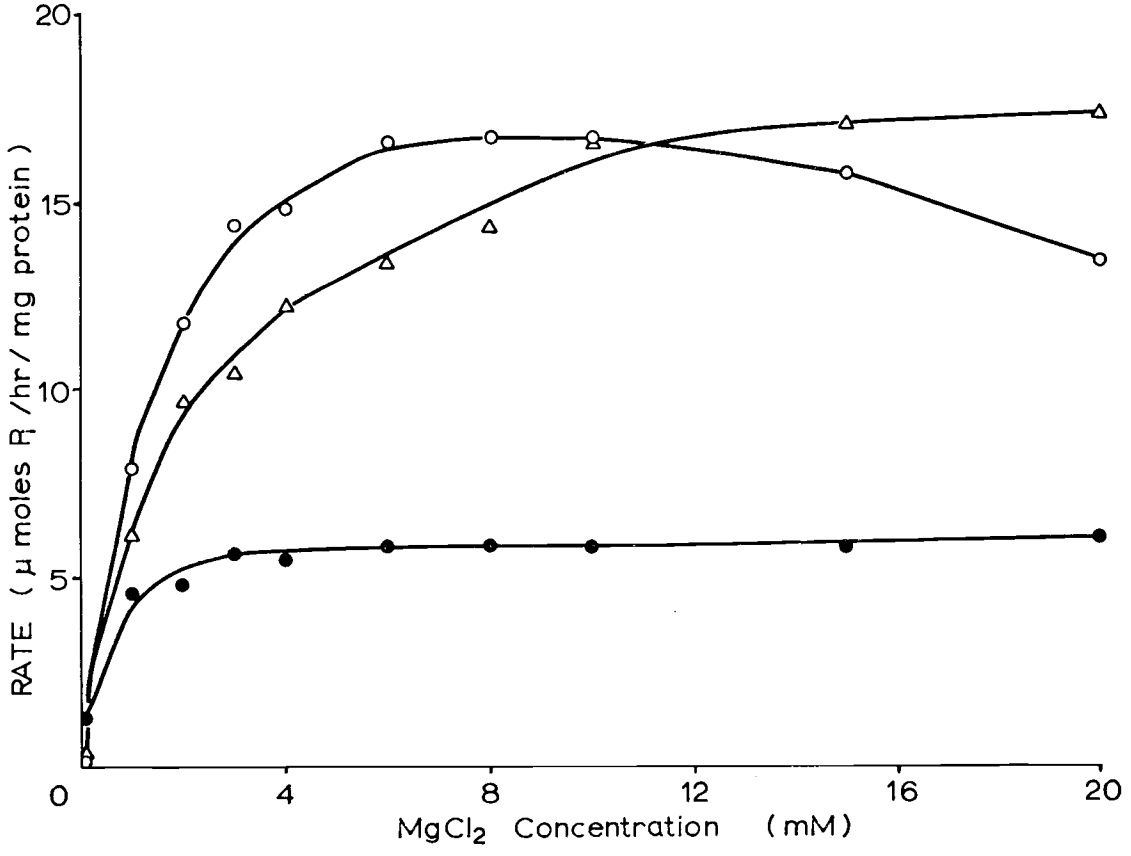

RATE (μ moles P_i / hr / mg protein)
45
30
15
0
0.05
0.10
0.50
Ouabain Concentration (mM)

Figure 6. Effect of ATP concentration on Na+K-activated ATPase activity in mitochondrial (●----●) and microsomal (0----0) preparations. Reaction conditions except for ATP concentrations were as described in Methods.

Figure 7. Effect of $MgCl_2$ concentration on Na+K-activated ATPase activity at varying concentrations of ATP. Except for Mg^{++} and ATP, assay conditions were as described in Methods. $MgCl_2$ was varied under conditions of constant Na_2ATP concentrations of: 1 mM, ●----●; 5 mM, 0----0; 10 mM, △----△.


RATE (μ moles P_i / hr / gm protein)
20
10
0
5
10
15
20
ATP Concentration (mM)


RATE (μ moles P_i /hr/ mg protein)
20
15
10
5
0
4
8
12
16
20
$MgCl_2$ Concentration (mM)

ATPase.

Since Na+K-activated ATPase has been shown to be dependent upon concentrations of monovalent cations, the ratios of these cations, and the total ionic strength of the incubation medium, it was necessary systematically to examine these relationships. At a constant KCl concentration of 50mM, NaCl was varied from 0 to 300mM. The half maximal activities for both microsomal and mitochondrial preparations under these conditions was $1.0\text{-}1.2 \times 10^{-2}$M NaCl as seen in Figure 8. However, if the NaCl concentration was held constant at either 200mM or 300mM respectively, and the KCl was varied from 0 to 100mM, the half maximal activity for both preparations was obtained at 8.5×10^{-3}M KCl (Figure 9a, c). Substituting NH_4Cl for KCl and varying its concentration from 0 to 100mM, the half maximal concentrations for both preparations was $1.9\text{-}2.1 \times 10^{-2}$M NH_4Cl (Figure 9b, d). The latter experiments did not maintain a constant total ionic strength, but varied between total ionic strengths of 50mM to 420mM. In order to determine optimum Na^+/K^+ ratios, reaction conditions were maintained at constant ionic strengths. As shown in Figures 10 and 11, the enzyme is active over a wide range of ratios for Na^+/K^+ and Na^+/NH_4^+, respectively, but the optimal ratios appear to be 4/1 for Na^+/K^+, and 4/3 for Na^+/NH_4^+. No significant differences appear to exist between the mitochondrial and microsomal preparations. When total ionic

Figure 8. Effect of NaCl concentration on Na+K-activated ATPase activity from mitochondrial (●----●) and microsomal (0----0) preparations. Reaction conditions were as described in Methods, except that NaCl concentration was varied and KCl was held constant at 50 mM.

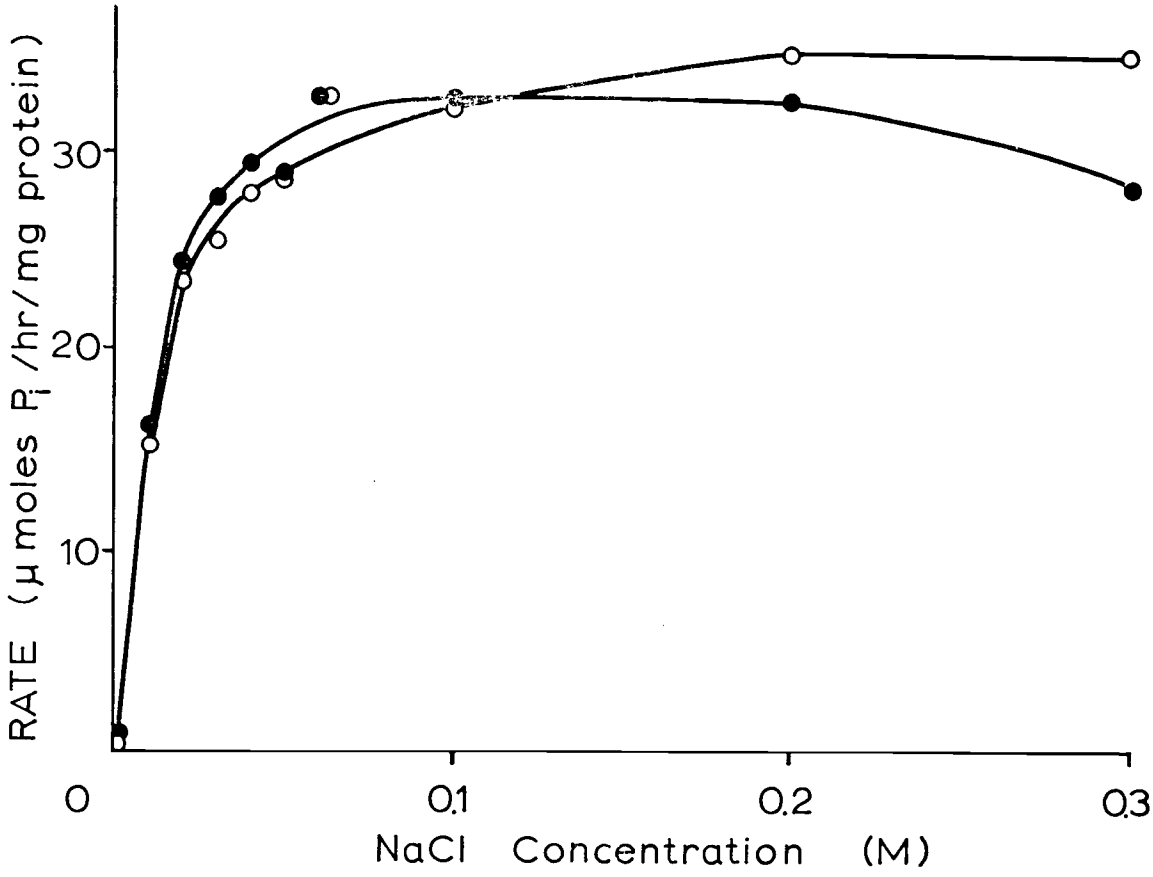

RATE (μ moles P_i /hr/mg protein)
30
20
10
0
0.1
0.2
0.3
NaCl Concentration (M)

Figure 9. Effect of KCl (●----●) and NH_4Cl (0----0) concentration on Na+K-activated ATPase activity in mitochondrial (A and B) and microsomal (C and D) preparations. The concentrations of KCl and NH_4Cl were varied in the reaction mixture in the presence of constant NaCl concentrations of 0.2 M (A and C) and 0.32 M (B and D). All other reaction conditions were as described in Methods.

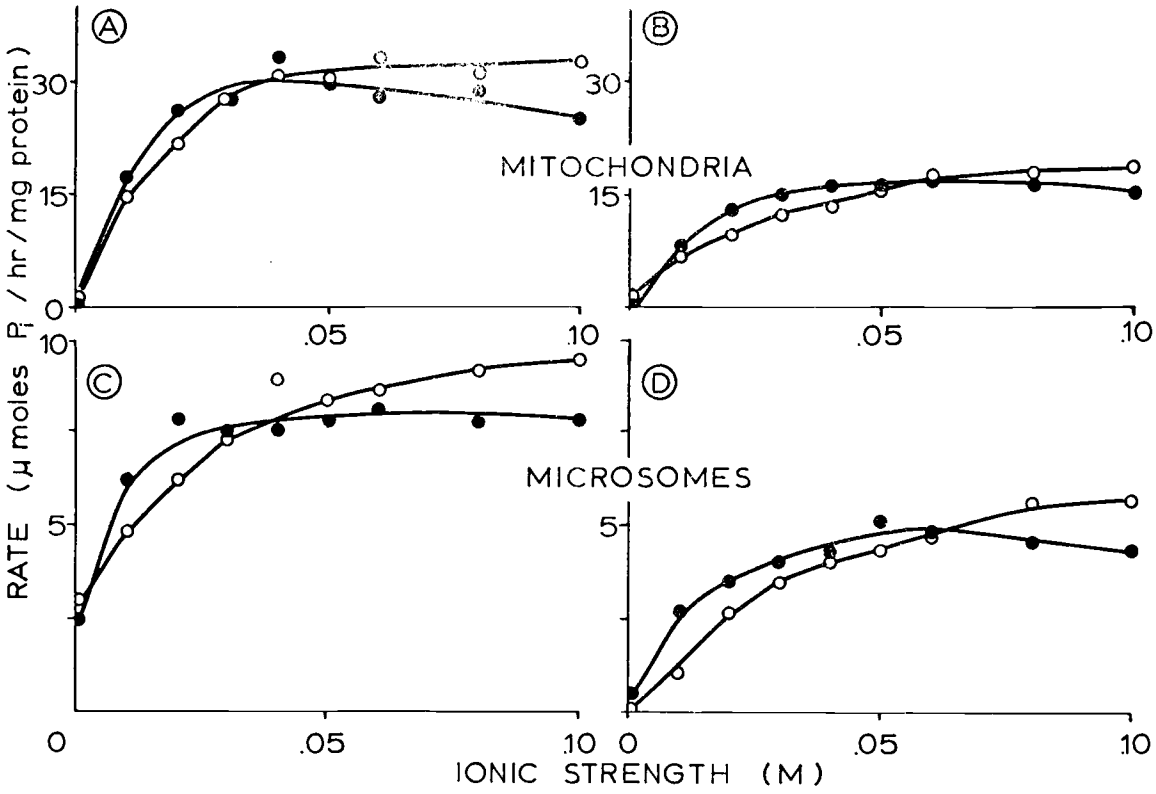

A
B
C
D
MITOCHONDRIA
MICROSOMES
RATE (μ moles P_i / hr / mg protein)
IONIC STRENGTH (M)
0
15
30
5
10
.05
.10

Figure 10. Effect of varying Na^+/K^+ ratios at constant ionic strength on Na+K-activated ATPase activity from mitochondrial (●----●) and microsomal (0----0) preparations.

A. Ionic strength held constant at 0.25 M

B. Ionic strength held constant at 0.4 M

All other reaction conditions were as described in Methods.

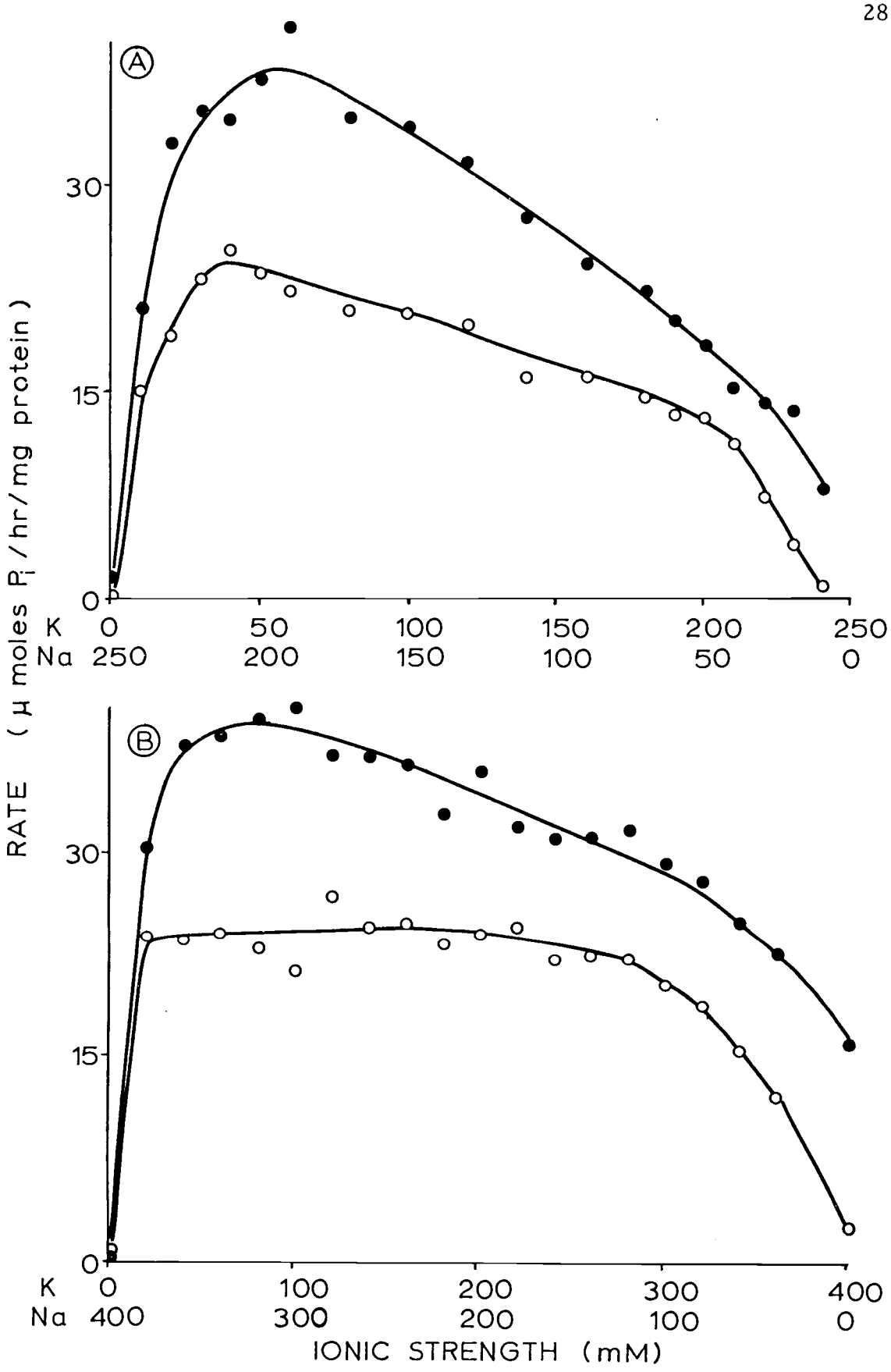
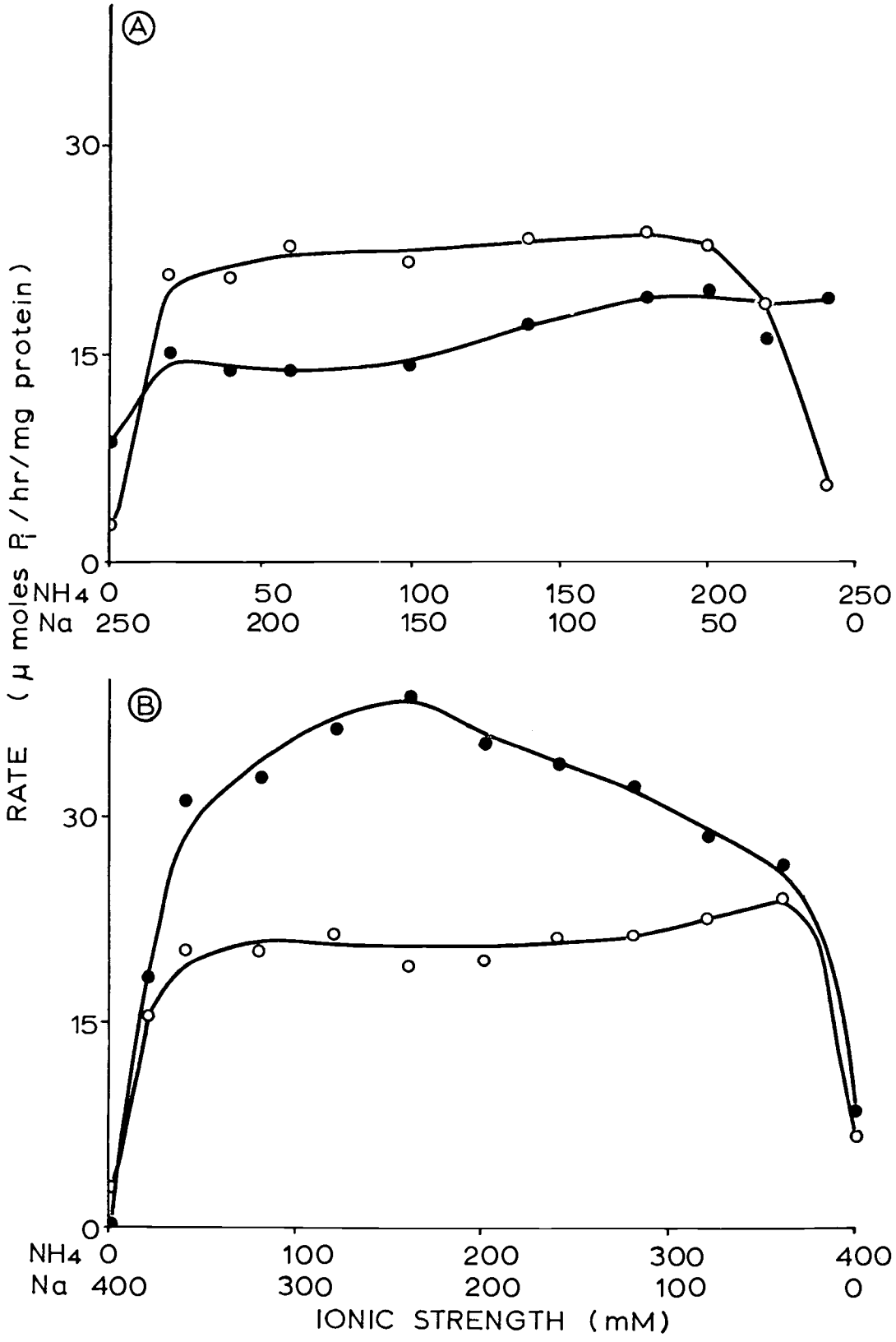

A
B
30
15
0
RATE (μ moles P_i / hr / mg protein)
K 0 50 100 150 200 250
Na 250 200 150 100 50 0
K 0 100 200 300 400
Na 400 300 200 100 0
IONIC STRENGTH (mM)

Figure 11. Effect of varying Na^+/NH_4^+ ratios at constant ionic strength on Na+K-activated ATPase activity from mitochondrial (●----●) and microsomal (0----0) preparations.

A. Ionic strength held constant at 0.25 M

B. Ionic strength held constant at 0.4 M

All other reaction conditions were as described in Methods.


A
30
15
0
RATE (μ moles P_i / hr / mg protein)
NH4 0 50 100 150 200 250
Na 250 200 150 100 50 0
B
30
15
0
NH4 0 100 200 300 400
Na 400 300 200 100 0
IONIC STRENGTH (mM)

strengths of the reaction mixture were varied from 0 to 1000mM (Figure 12) and the Na^+/K^+ ratios held constant, the mitochondrial preparation had maximal enzymatic activity when the Na/K ratio was 4.0 and the total ionic strength was either 250mM or 400mM. The bimodal peak of activity was repeatable and not due to experimental variability. The reduction of activity at the higher ionic strengths for this ratio was not observed to occur at lower Na/K ratios, suggesting that sodium may be inhibitory at very high concentrations. Na^+/K^+ ratios lower than 4.0 showed decreased activity and no evidence of a bimodal peak. Similar results were obtained for Na^+/NH_4^+ ratios (Figure 11), except that the optimum activity was obtained at 250mM total ionic strength. No significant differences were observed between mitochondrial and microsomal preparations.

Discussion

The discovery of the larval salt gland in the free-swimming nauplis of Artemia has led to the belief that this larval stage should be rich in the cation transport enzyme, Na+K-activated ATPase. The results confirm this prediction. The levels of enzyme per gram of nauplii are among the highest found in animal tissues (Table 3). The larval Na+K-activated ATPase enzyme system is found to have kinetic properties similar to a variety of Na+K-activated ATPase found in other animal tissues (Skou, 1965; Bonting, 1970). However,

Figure 12. Activity of Na+K-activated ATPase as a function of ionic strength at various ion ratios. (A) Ratios are: 4/1 Na^+/K^+, ●----●; 1/1 Na^+/K^+, 0----0; 1/4 Na^+/K^+, △----△; (B) Ratios are: 4/1 Na^+/NH_4^+; ●----●; 1/1 Na^+/NH_4^+, 0----0. All other reaction conditions were as described in Methods.

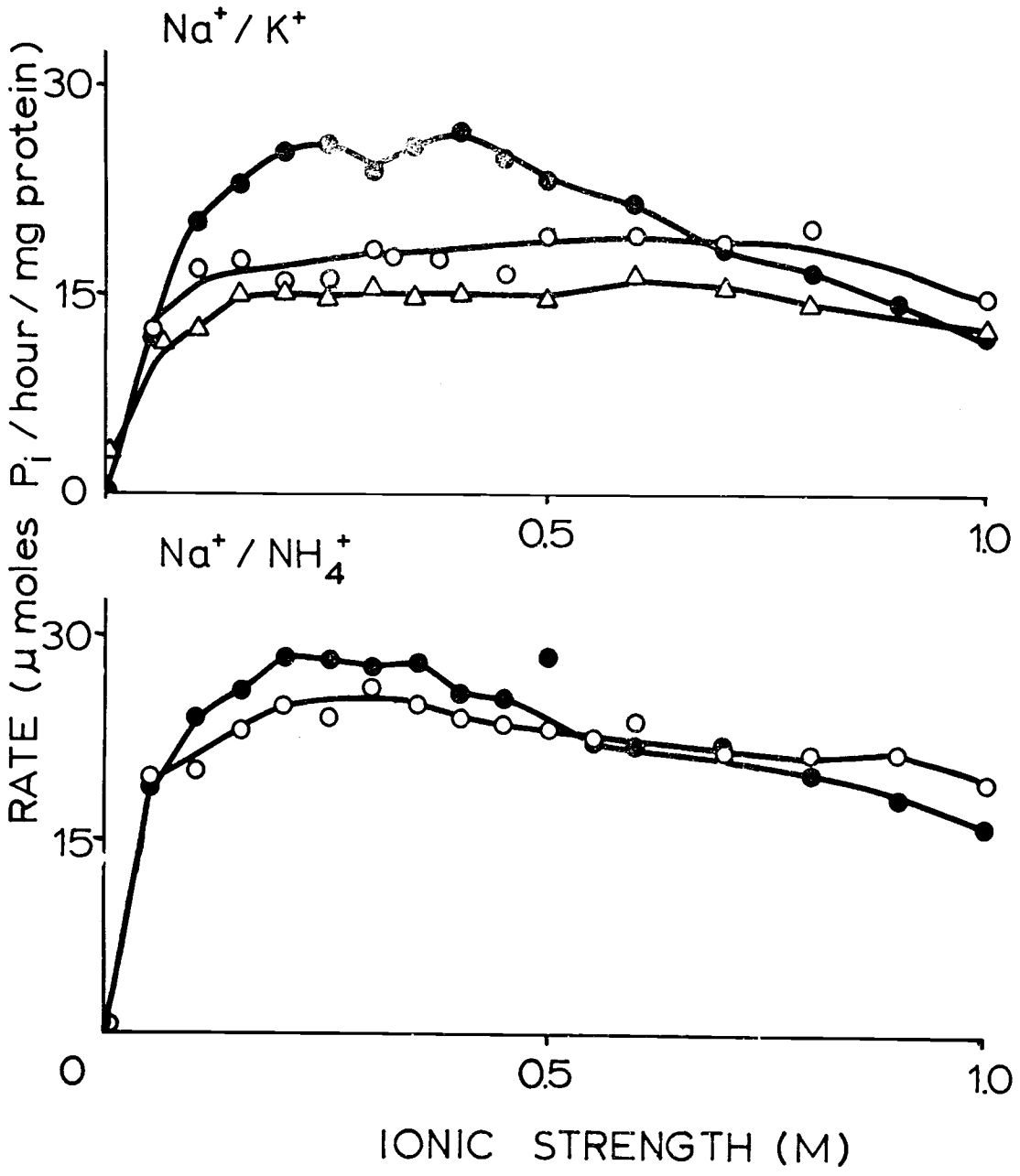

Na+ / K+
Na+ / NH4+
RATE (μ moles Pi / hour / mg protein)
30
15
0
0.5
1.0
IONIC STRENGTH (M)

Table 3. Comparison of Na+K-activated ATPase activities from homogenates of tissues from various organisms

Organism	Tissue	Specific Activity[1] (units/mg protein)	Yield[1] (units/ g tissue)	Reference
Duckling	salt gland			
	fresh water	1.1	170	Ernst et al. (1967)
	salt water	9.1	1440	Ernst et al. (1967)
Bovine	brain cortex	6.4	768	Uesugi et al. (1971)
Squalus	rectal gland	38	1800	Hokin et al. (1973)
Rabbit	kidney medulla	53	6200	Jorgensen et al. (1971)
Artemia	whole nauplius	5.8	500	Present study

[1]Unit = μmoles P_i released/hour

a few differences do exist, such as the evidence that the majority of the enzyme activity resides in a very heavy membrane fraction, and that the enzymatic activity is maintained throughout a wide range of Na^+/K^+ ratios and ionic strengths. The optimum Na/K ratio for the brine shrimp enzyme (4/1) is at the lower extreme of the range (up to 20/1) observed in other animals and compares favorably with the isopod, S. serratum, enzyme Na/K ratio of 6/1 (Philippot, Thuet and Thuet, 1972). The unique 1/1 optimum Na/K ratio found in crab (C. maenas) leg nerve preparations (Skou, 1957, 1960) is suboptimal in most of the brine shrimp preparations, with the exception of the high ionic strength (400mM) curve for the microsomal preparations shown in Figure 10B. Most peculiar of the brine shrimp enzyme kinetic properties is the very high ionic strengths for the monovalent cation activations. Maximal activity occurs at 250 and 400mM Na+K, whereas most other sources of this enzyme show maximal activation with ionic strengths much less than 200mM Na+K. The two ionic strength peaks for the K dependent Na activation are especially interesting in view of similar observations by Philippot, Theut and Theut (1972) on the isopod. These authors have suggested the possibility of two enzymes present in the pleopods of S. serratum.

III. LOCALIZATION OF THE Na+K-ACTIVATED ATPase

Introduction

In previous studies on the export of salt from the adult brine shrimp, Artemia salina, it was found that the structure responsible for providing the organism with its extreme capability of secreting large quantities of NaCl was located in the epithelial lining of the metepipodite segment of the branchiae (Copeland, 1967; Croghan, 1958b). Ion regulation in the adults has been shown to be dependent upon the active transport of ions, either sodium ion (Thuet, Motais and Maetz, 1968) or chloride ion (Smith, 1969a, 1969b) and is apparently linked with the Na+K-activated ATPase (Augenfeld, 1969).

In Artemia nauplii, there is a lack of metepipodites because at this stage of embryonic development the anlage of the legs segments have not formed their definitive structures. Nevertheless, the nauplii are capable of regulating the ionic composition of the hemolymph against wide ranges of salinities (Croghan, 1958a; Conte, Hootman and Harris, 1972; Hootman, Harris and Conte, 1972). Ewing, Peterson and Conte (1972) have shown that mortality of the nauplii exposed to 10^{-4}M ouabain is enhanced at higher salinities. The previous section reported that the nauplius stage of the brine shrimp is rich in the Na+K-activated ATPase. These data, together with the strong correlate of Na+K-activated ATPase being found in

mitochondrial rich epithelia (Ernst, 1972), suggest that this enzyme might be concentrated in larval salt gland. The quantitative anatomical distribution of the Na+K-activated ATPase is examined in the nauplii and adults in order to test this hypothesis.

As reported in section II, preliminary subcellular fractionation by differential centrifugation had shown a predominantly mitochondrial distribution of the Na+K-activated ATPase. From other tissue preparations, this enzyme has been variously reported to be in the nuclear, mitochondrial, heavy microsomal, or microsomal fractions (see reviews: Skou, 1965; Albers, 1967; Katz and Epstein, 1968). This type of distribution characterizes the fractionation behavior of a particular tissue preparation under specified homogenation and centrifugation conditions, but is only very crudely suggestive of subcellular localization.

The Na+K-activated ATPase is found in the mammalian erythrocyte cell membranes and squid axon sheath (Katz and Epstein, 1968). A microsomal preparation from Ehrlich ascites tumor cells was shown by biochemical and immunological techniques to contain plasma membrane fragments which possessed most of the Na+K-activated ATPase activity (Wallach and Ullrey, 1964; Wallach and Kamat, 1964; Kamat and Wallach, 1965). Liver cells also show plasma membrane distribution of Na+K-activated ATPase activity (Barclay et al., 1967; Emmelot and Bos., 1966). More recently,

Philpott and co-workers (Dendy, Deter and Philpott, 1973; Dendy, Philpott and Deter, 1973) have combined biochemical and morphological characteristics of the teleost pseudobranch in suggestion of the plasma mambrane localization of Na+K-activated ATPase.

In view of the interest in studying the biogenesis of the Na+K-activated ATPase in the brine shrimp, confirmation of the ultimate organellar site for the enzyme would be of great value. Histochemical localization techniques have to date been thwarted by fixation problems. Biochemical localization through the use of marker enzymes is, therefore, employed to indirectly examine subcellular localization of Na+K-activated ATPase in the brine shrimp. Quantitative assays of cytochrome oxidase, NADH oxidase, glucose-6-phosphatase and 5'-nucleotidase are made in order to characterize the subcellular fractions resulting from differential centrifugation. Each marker enzyme is sufficiently characterized kinetically to permit comparative quantification of the enzyme activities. Sucrose density gradients and electron micrographs of the various fractions are used to distinguish between the membrane fragments that possess the Na+K-activated ATPase activity.

Methods

Anatomical Localization

Microdissection and Enzyme Preparation. Salt glands and sections of bodies of whole nauplii were dissected from animals frozen in liquid nitrogen. Comparison of frozen and nonfrozen whole larvae showed that this treatment had no deleterious effects on enzymatic activity of Na+K-activated ATPase. Microdissected portions from 10 larvae were transferred frozen to microhomogenizers and homogenized in 0. 1 ml of 0. 1M imidazole buffer, pH 7. 2. The homogenizer was washed twice with 0. 1 ml of a solution containing 0. 8M NaCl and 0. 2M KCl and the washings combined in enzyme reaction tubes. Reactions were initiated by addition of 0. 2 ml solution containing 0. 125M imidazole buffer, pH 7. 2, 0. 025M $MgCl_2$, and 0. 0125M Na_2ATP (with and without 0. 00125M ouabain). Reaction mixtures were incubated for four hours at 37° C and assayed for inorganic phosphate by the method of Ernster, Zvetterstrom and Lindberg (1950). Intestines with intact midgut regions were extracted from fresh nauplii by anchoring the animal over the neck organ region with a probe and pulling on the telson with a second probe. Homogenization and ATPase assay was performed as described above, using 10 micro-dissected portions for each reaction tube.

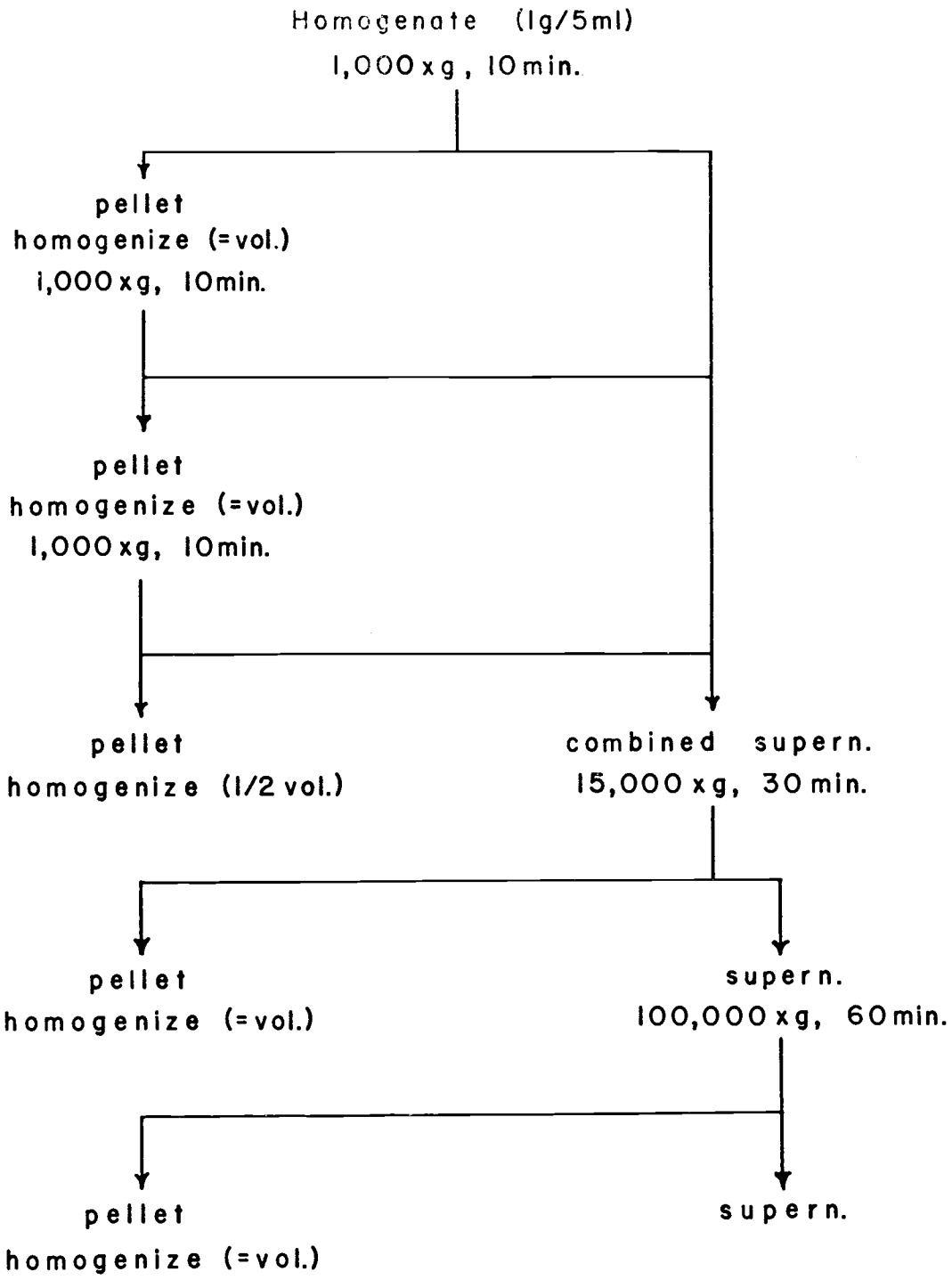
Biochemical Localization

Membrane Preparations. Nauplii were harvested after 36-40 hours incubation of prehydrated cysts. They were homogenized at 1g fresh weight per 5 ml homogenizing media, H. M. (250mM sucrose, 100mM imidazole buffer, pH 7. 2, and 2mM Na_2 EDTA), and fractionated by differential centrifugation as shown in the flow diagram of Figure 13. All pellets were resuspended by homogenation in H. M.

Preparation of membranes rich in Na+K-activated ATPase was accomplished with a slight modification of this fractionation procedure. Nauplii were homogenized in 55 ml glass-teflon homogenizers at 1g fresh weight per 2. 5 ml H. M. The heavy pellet resulting from a 5 minute centrifugation at 700xg in a SS-34 Sorvall rotor was homogenized in one-half volume H. M. and recentrifuged at 700xg for 5 minutes. The combined supernatants were centrifuged at 1000xg for 10 minutes, and the resulting pellet similarly washed with one-third volume H. M. The combined supernatants from the 1000xg spins were then centrifuged at 15, 000 xg and the supernatant carefully decanted leaving behind the loose layer over the pellet. The light colored loose layer of this 15, 000 xg pellet was swirled off with additional washing of H. M. leaving the dense darker colored lower pellet as undisturbed as possible. The 15, 000 xg, 30 minute centrifugation was repeated on the extracted upper pellet layer and

Figure 13. Schematic for subcellular fractionation of naupliar homogenates for characterization of marker enzymes and Na+K-activated ATPase distribution.

FRACTIONATION FLOW DIAGRAM



again extracted as above in a final volume of 1/20 to 1/40 of the original pre-15,000 xg material.

Marker Enzymes and Assays. Cytochrome oxidase (ferrocytochrome C: oxygen oxidoreductase, EC 1. 9. 3. 1) was used as the marker enzyme for mitochondria. Distribution of this enzyme is exclusively mitochondrial in a wide variety of organisms and tissues (de Duve, Wattiaux and Baudhuin, 1962) and is found in the inner membrane (Schnaitman and Greenawalt, 1968; Shnitka and Seligman, 1971). The enzyme activity was measured after the method of Wharton and Tzagoloff (1967). The brine shrimp mitochondrial enzyme preparation was characterized for optimum pH and substrate (cytochrome C reduced by addition of $Na_2S_2O_4$). The pH optimum in phosphate buffer was broad and centered near pH 6. 7. The reaction conditions were the following: 2. 7 ml of 10 mM phosphate buffer, pH 6. 7 and 0. 2 ml 1% reduced cytochrome C. The reaction was initiated with addition of 0. 1 ml of enzyme preparation (the blank was made with 0. 1 ml of 10 mM $K_3Fe(CN)_6$ in place of the enzyme preparation). The loss in absorbance at 550 nm was monitored continuously at 23-24° C using a Gilford Recorder attached to a Beckman DU Spectrophotometer. Activity was reported either as ΔA_{550}/min/ml of enzyme (gradient assays) or calculated to μmoles

of cytochrome C oxidized from the mM extinction coefficient E(550, 1 cm) = 28 $cm^2/\mu mole$.

NADH oxidase (rotenone-sensitive, reduced NAD:potassium ferricyanide oxidoreductase, EC 1. 6. 99. 3) is found in the endoplasmic reticulum and outer mitochondrial membranes (Kamat and Wallach, 1965; Sottocasa et al., 1967; Emmelot and Benedetti, 1968; Schnaitman and Greenawalt, 1968). Kinetic properties examined for this enzyme in a mitochondrial preparation from the brine shrimp included pH and substrate (NADH). The reaction mixture (after Avruch and Wallach, 1971) consistent with optimal assay conditions for brine shrimp was prepared as follows: 2. 5 ml of 10mM phosphate buffer pH 6. 7, 0. 2 ml of 37. 5 μM rotenone, 0. 1 ml of 10mM $K_3Fe(CN)_6$, 0. 1 ml of 3mM NADH, and 0. 1 ml of enzyme preparation to initiate the reaction. As with the cytochrome oxidase assay, the reaction was monitored continuously by recording the loss of absorbance at 340 nm at 23-24° C. Enzyme activity was calculated from the mM extinction coefficient E(340, 1 cm) = 6. 22 $cm^2/\mu mole$.

5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3. 1. 3. 5) is a well known marker for plasma membranes (e. g., Golfischer, Essner and Novikoff, 1964; Coleman and Finean, 1966; Song and Bodowsky, 1967; Bosman et al., 1968; Avruch and Wallach, 1971; Blomberg and Perlmann, 1971). Substrate specificity showed that following relative activities: UMP, 1. 00; IMP, 0. 99; GMP, 0. 89;

CMP, 0. 60; and AMP, 0. 39. Substrate concentration and pH optimum were examined and the following reaction conditions adopted: 0. 2 ml of 0. 3M Tris-HCl buffer, pH 7. 8 and 12. 5mM $MgCl_2$, 0. 2 ml of 12. 5 mM 5' UMP, and 0. 1 ml enzyme preparation. All reactants were added in the cold, 0° C, and the reaction initiated by immersing the tubes in a 37° C water bath. The difference in the amount of inorganic phosphate released between t_i-t_{30} minutes (where i=2-4 hours) was used to calculate the enzyme activity. Inorganic phosphate was measured by the method of Ernster, Zvetterstrom and Lindberg (1950).

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1. 3. 9) is localized to the endoplasmic reticulum and nuclear envelope (Rosen, Kelley and Peters, 1966; Pollak and Shorey, 1968; Goldfischer, Essner and Novikoff, 1964; Leskes, Siekevitz and Palade, 1971). The optimum reaction mixture includes the following: 0. 2 ml of 0. 3M histidine buffer, pH 6. 4 and 25mM Na_2 EDTA, 0. 2 ml of 100mM D-glucose-6-phosphate, and 0. 1 ml of enzyme preparation. The enzyme activity was determined in the same manner as the activity of 5'-UMPase above.

Protein was estimated by the method of Lowry et al. (1951), using crystalline bovine albumin as the standard.

Sucrose Density Gradient Centrifugation. The mitochondrial and microsomal fractions were subjected to sucrose density gradient

centrifugation using linear gradients of 0. 25M to 2. 0M sucrose. The high density Na+K-activated ATPase membrane preparations were examined on linear gradients of 15-45% sucrose with a 51% sucrose cushion. The gradients were centrifuged in a Beckman L-2 preparatory ultracentrifuge in a SW-25. 1 rotor at 22, 000 RPM for 90 minutes. The gradients were either fractionated from the top using an ISCO density gradient fractionator, or from the bottom after the method of Tan (1971). The density of each fraction was determined from refractometer measurements.

Electron Microscopy of Mitochondrial Pellets. Pellets representing the mitochondrial preparation were fixed in 5% glutaraldehyde in the presence of 0. 25M sucrose and 0. 15M cacodylate buffer (pH 7. 5), and post fixed with 1% OsO_4 in 0. 1M phosphate buffer (pH 7. 2). Specimens were embedded in Araldite, thin sectioned, and stained with uranyl magnesium acetate and lead citrate for examination with an RCA EMU 3H electron microscope.

Results

Anatomical Localization

The results shown in Table 4 reveal the distribution of the Na+K-activated ATPase in the various anatomical regions of the nauplius larvae and the adult. In the nauplius, the abdominal region

Table 4. Anatomical localization of Na+K-activated ATPase activity in *Artemia* nauplii and adults

	Activity nmoles P_i/hr/animal	Percent Total Activity
Nauplii, Frozen		
whole	2.9 ± 0.4 (4)	100
cephalothorax (including salt gland)	1.5 ± 0.5 (3)	54
abdominal region	1.3 ± 0.3 (3)	44
salt gland	0.2 ± 0.0 (5)	7
Nauplii, Fresh		
whole	3.3 ± 0.3 (3)	100
intestine	1.1 ± 0.1 (3)	33
body less intestine	1.8 ± 0.5 (3)	55
Adults, Fresh		
whole	455 ± 48 (5)	100
phyllopodia	252 ± 36 (5)	55
body less phyllopodia	223 ± 59 (5)	49

Values are given as mean ± standard deviations. Number of replicates is given in parentheses.

had 40-45 percent of the Na+K-activated ATPase activity, the cephalothoracic region had 50-55 percent, the isolated salt gland contained less than 10 percent, and the intestine about 33 percent. By comparison, the leg segments of the adult containing the salt organelles contained about 50 percent of the total Na+K-activated ATPase activity.

Biochemical Localization

The subcellular fractionation of the brine shrimp homogenate by differential centrifugation according to the flow diagram in Figure 13, gives the distribution of protein and enzyme activities as shown in Table 5. The recoveries are based on the sum of the amount of protein or enzyme activity present in the 1,000 xg pellet, 15,000 xg pellet, and 100,000 xg pellet and supernatant, relative to that assayed in the crude homogenate.

Cytochrome oxidase activity was quite variable in the homogenate and 1,000 xg supernatant fractions, but repeatably of constant high activity in the mitochondrial preparation, and of very low or no activity in the postmitochondrial fractions.

Most of the NADH oxidase activity was present in the mitochondrial fraction, but a significant portion was also distributed in the postmitochondrial fractions. Both 5'-UMPase and glucose-6-phosphatase showed greater amounts of activity in the mitochondrial

Table 5. Distribution of protein and enzyme activity from subcellular fractionation by differential centrifugation

Fraction	Protein	Na+K-ATPase		5'-UMPase		Glu-6-Pase		NADH oxidase		Cytochrome oxidase	
	%	%	S.A.[1]	%	S.A.[1]	%	S.A.[1]	%	S.A.[2]	%	S.A.[3]
Homogenate	100	100	7.1	100	0.15	100	0.17	100	20.3	100	4.5
1000xg pellet	30	10	2.3	0	0.00	13	0.07	11	7.6	11	1.7
1000xg supern.	85	104	8.7	96	0.17	110	0.22	104	24.8	365	19.5
15,000xg pellet	25	74	21.3	42	0.23	40	0.27	49	39.9	143	26.2
15,000xg supern.	60	48	6.1	68	0.18	58	0.17	30	10.8	15	1.2
100,000xg pellet	15	33	16.3	29	0.30	26	0.30	5	7.0	3	0.9
100,000xg supern.	42	11	2.1	40	0.15	19	0.08	12	6.4	0	0.0
Recovery[4]	109	128		111		98		78		158	

[1]S.A. = µmoles Pi/hr/mg protein

[2]S.A. = µmoles NADH oxidized/hr/mg protein

[3]S.A. = µmoles cytochrome c oxidized/hr/mg protein

[4]Sum of all pellets and final 100,000xg supern. divided by homogenate

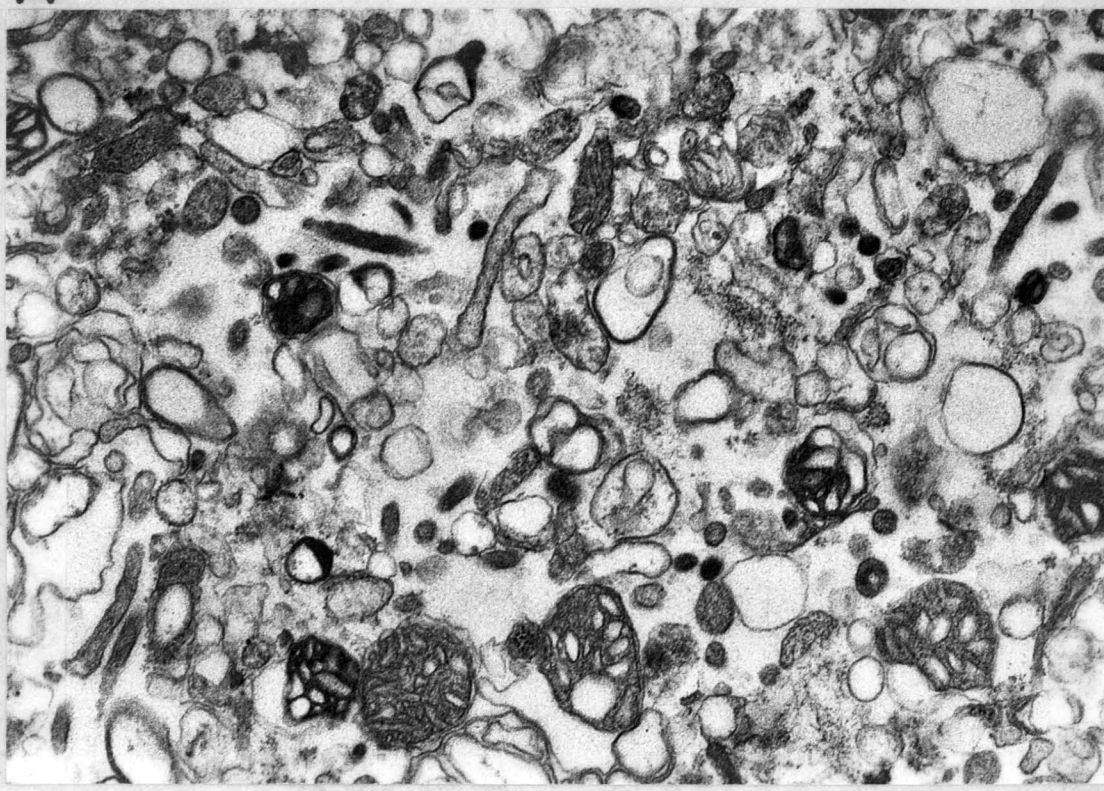
than the microsomal fractions. As much as two-thirds to three quarters of the Na+K-activated ATPase was found in the mitochondrial fraction, and most of the remainder sedimented in the microsomal fraction, as was shown previously in section II. All enzymes, except for cytochrome oxidase showed activity in the 100,000 xg supernatant fraction.

Electron photomicrographs of the mitochondrial fraction demonstrated that the pellet is stratified (Figure 14), containing an upper layer of membranous fragments (Figure 14A) and a lower layer of intact mitochondria (Figure 14B). Sucrose density gradient fractionation of the mitochondrial preparation on 0.25M to 2.0M linear gradients showed the characteristics demonstrated in Figure 15. The Na+K-activated ATPase activity was present in two distinct peaks at densities of 1.10 g/ml and 1.14 g/ml. The mitochondrial marker, cytochrome oxidase banded separately (density = 1.17 g/ml) from these two peaks. Similar sucrose density gradient fractionation of the microsomal fraction also gave two comparable Na+K-activated ATPase peaks.

The relative distribution of protein and the various enzymes from the separated top and bottom layers of the mitochondrial pellet is shown in Table 6. Cytochrome oxidase and NADH oxidase remained principally with the bottom portion of the pellet, while 80% of the Na+K-activated ATPase was found in the upper layer. Protein was

Figure 14. Electron micrographs of the mitochondrial pellet.
A. Upper portion of the pellet (4,100 X)
B. Lower portion of the pellet (24,000 X).

A



B

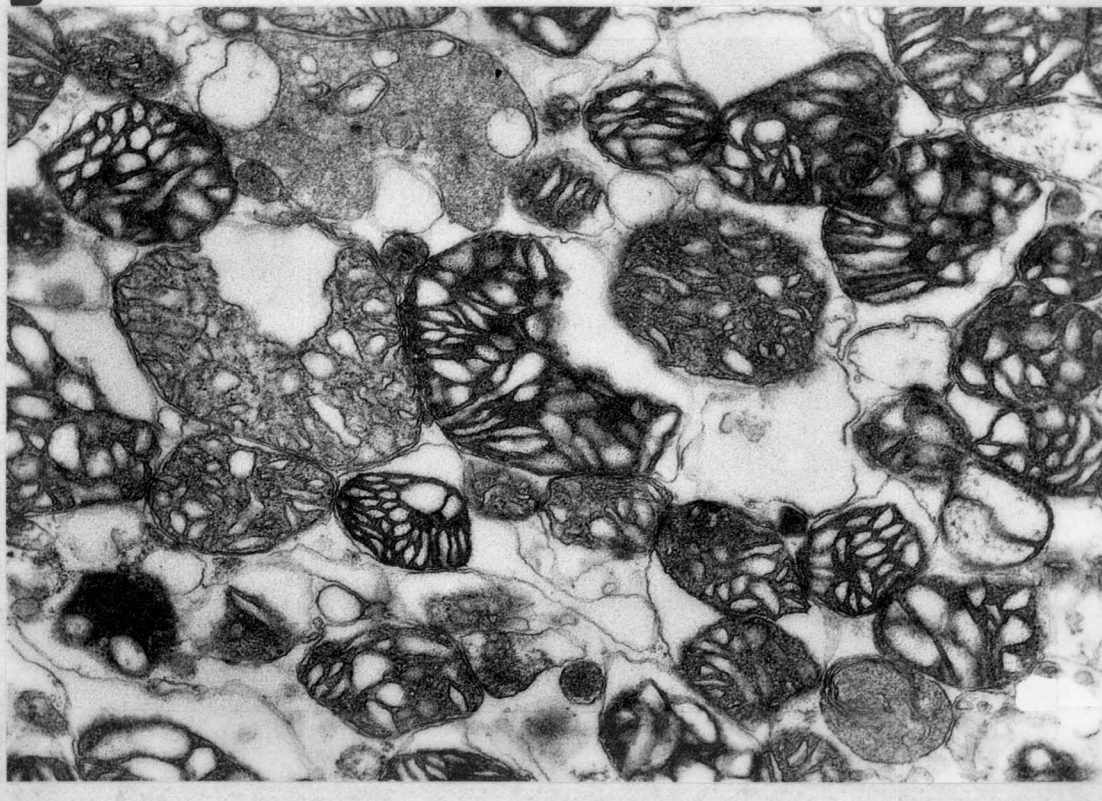


Figure 15. Sucrose density gradient profile of the mitochondrial fraction. The top illustration represents the light scattering visible in the gradient tube when mounted in the Isco density gradient fractionator.

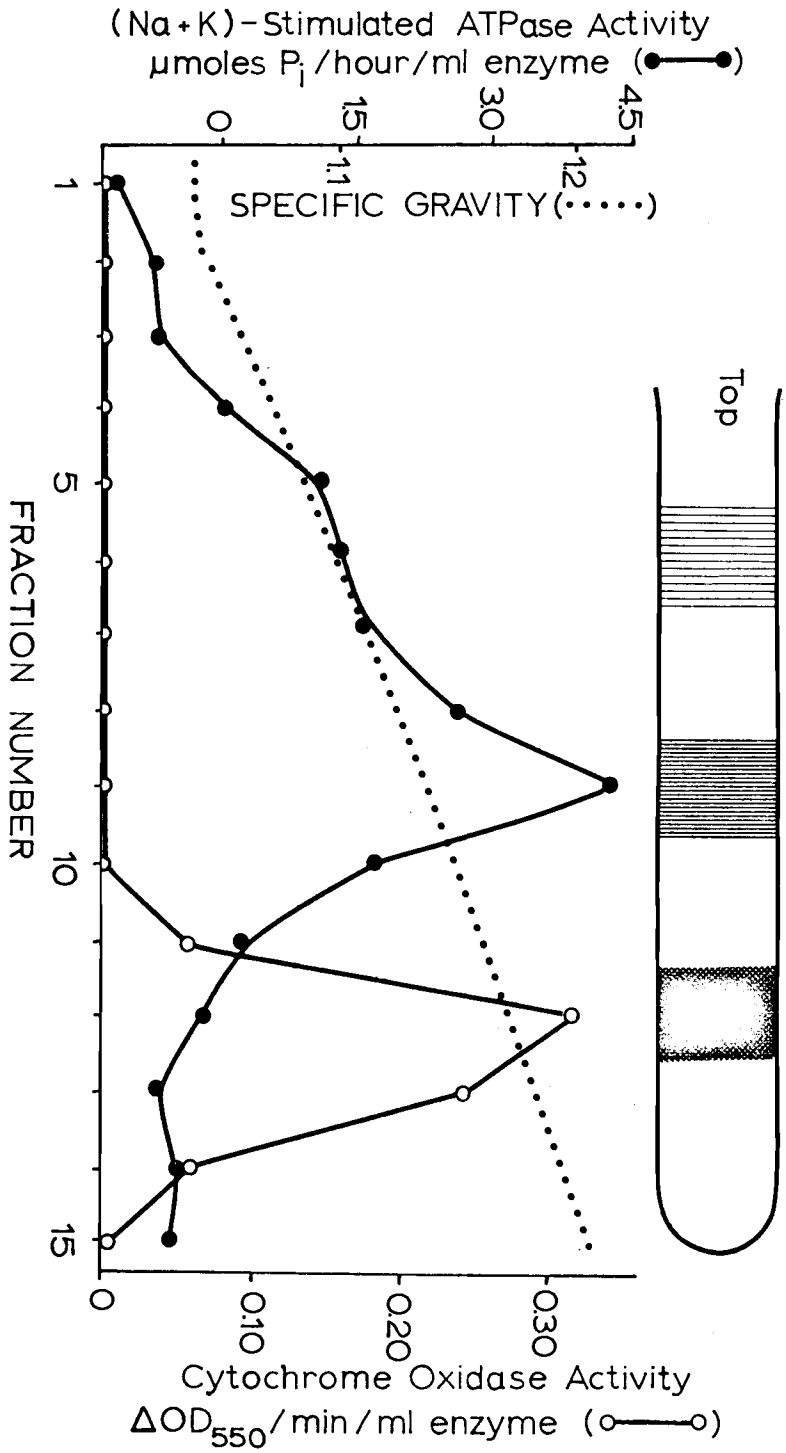

(Na + K) - Stimulated ATPase Activity
μmoles P_i / hour / ml enzyme (●—●)
0
1.5
3.0
4.5
1.1
1.2
SPECIFIC GRAVITY (·····)
Top
FRACTION NUMBER
1
5
10
15
0
0.10
0.20
0.30
Cytochrome Oxidase Activity
ΔOD_{550} / min / ml enzyme (○—○)

Table 6. Distribution of protein and enzyme activity in the separated layers of the stratified mitochondrial pellet

	Top		Bottom	
	%	S.A.[1]	%	S.A.
Protein	51.3		49.7	
Na+K-ATPase	80.8	32.7	19.2	7.9
5' UMPase	32.2	1.07	67.8	2.27
Glu-6-Pase	27.2	6.21	72.8	16.9
NADH oxidase	4.0	17.2	96.0	414.0
Cytochrome oxidase	1.5	2.25	98.5	145.0

[1] S.A. See notations for the specific activity for each enzyme at the bottom of Table 5.

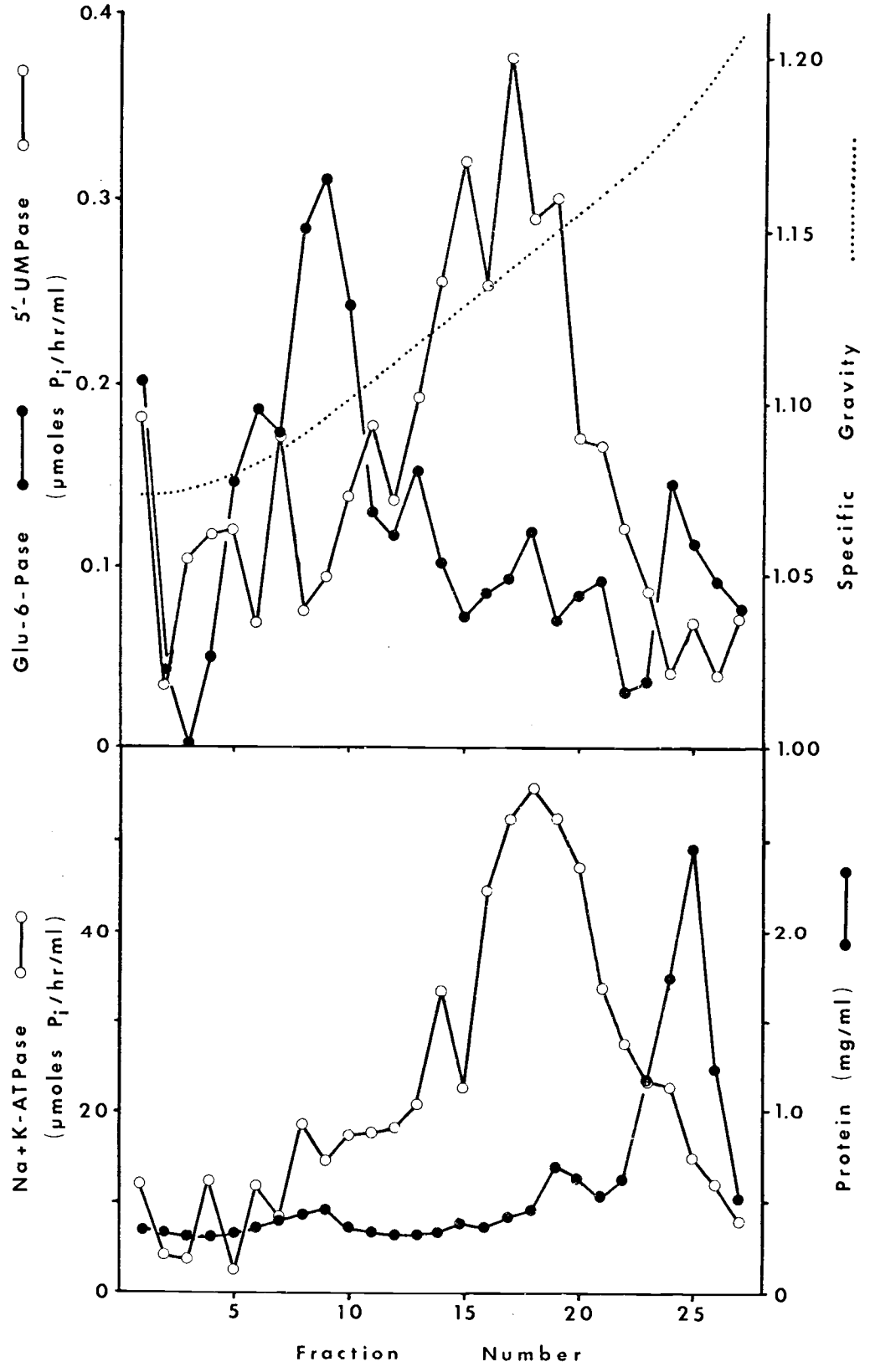
split in half and approximately one third of the 5'-UMPase and Glu-6-Pase activities was present in the top layer.

Sucrose density gradient centrifugation of this membrane fraction rich in Na+K-activated ATPase is shown in Figure 16. 5'-UMPase banded further down the gradient tube in a peak separate from glucose-6-phosphatase. Na+K-activated ATPase was distributed in a fashion similar to 5'-UMPase.

Discussion

The Na+K-activated ATPase in the nauplius larvae of the brine shrimp is found principally distributed down the long axis of the animal. There is no exceptional accumulation in the isolated salt gland or intestine, even though as pointed out in section II, the whole nauplius contains large amounts of this enzyme. The phyllopodia of the adults, which contain the metepipodite salt glands, possess about half the Na+K-activated ATPase. However, these are complex gill structures and anatomical localization to the salt gland is thereby incomplete. This is in an apparent opposition to the strongly implicated salt secretory function of the salt gland (Dedjar, 1930; Conte, Hootman and Harris, 1972), and would pose two basic questions: 1) how does the salt gland function to secrete salts? and 2) what role is the Na+K-activated ATPase playing if it is not directly responsible for secretion of excess salts out of the organism?

Figure 16. Sucrose density gradient centrifugation of the Na+K-activated ATPase rich membranes. The bottom of the gradient tube is to the right.


Glu-6-Pase
5'-UMPase
(μmoles Pi/hr/ml)
Specific Gravity
Na+K-ATPase
(μmoles Pi/hr/ml)
Protein (mg/ml)
Fraction Number
0.4
0.3
0.2
0.1
0
1.20
1.15
1.10
1.05
1.00
40
20
0
2.0
1.0
0
5
10
15
20
25

An answer to the first question lies in the possible alternative of an active transport of Cl^- taking place in the larval salt gland. The independence of hemolymph ion regulation against the external media from the Na+K-activated ATPase transport system in Artemia has been previously suggested by Smith (1969a, b). Smith found from electrochemical and flux studies of adult Artemia that salt secretion is mediated through active transport of Cl^- to the external medium, and K^+ is taken up actively while Na^+ is distributed passively. Smith further demonstrated that the principal Cl^- and Na^+ effluxes in the adults occurred across the gill epithelium, and the ultrastructural similarities of the adult and larval salt glands has been demonstrated (Conte, Hootman and Harris, 1972). Another salt secretory tissue of similar function to the Artemia salt glands, the teleost gill, has also been recently suggested to have an active component of Cl^- transport (Epstein, Maetz and de Renzis, 1973). Since Cl^- transport has been shown to use HCO_3^- as a counterion (Maetz and Garcia Romeu, 1964) and intracellular HCO_3^- is in equilibrium with CO_2, the dense accumulations of mitochondria present in these salt transport organs (Copeland, 1967; Conte, Hootman and Harris, 1972) and others (Philpott and Copeland, 1963; Ernst, 1972) may suggest a close association between the processes of ion transport, respiration and gaseous exchange.

Supporting evidence for an active transport of Cl^- in brine

shrimp nauplii has recently been obtained. Ewing, Peterson and Conte (unpublished data) have found that survival of the nauplius in the presence of an inhibitor of carbonic anhydrase, acetalzolimide, is greatly reduced in higher salinities. J. A. Mangos (University of Wisconsin Medical School, personal communication) has found by micropuncture studies of the nauplii that the hemolymph concentrations of Na and K are very similar to those of the adults, and that Na efflux is predominantly across the salt gland. The numerous parallels between adults and nauplii strongly suggest the likelihood that hemolymph regulation with respect to the external media is active for Cl^- and passive for Na^+. Other necessary ion and electrical measurements are not as yet available.

One possibility in answer to the second question, for the role of Na+K-activated ATPase in nauplii is that it may serve to regulate the intracellular composition of cells bathed by hemolymph. The hemolymph of adult Artemia contains a Na^+/K^+ ratio of nearly 20/1 (Croghan, 1958a; Smith, 1969b), which is opposite that generally found within cells (ratios near 1/10). Naupliar homogenates show a Na^+/K^+ ratio of 4/1 (Conte, Hootman and Harris, 1972). The value reported by Croghan (1958a) of 0.22M Na^+ in the hemolymph of nauplii suggests that the 4/1 ratio of homogenates probably reflects a mixing of intracellular pools containing high K^+ levels with extracellular pools containing low K^+ levels (hemolymph and/or

contaminating sea water trapped in the gut). The recent micropuncture studies by J. A. Mangos (University of Wisconsin Medical School, personal communication), have confirmed these implications (hemolymph Na=100mM and K=3. 6mM; ratio 27/1). Alternatively, or probably additionally, it may function in salt transport across the intestinal epithelium. In any event, the enzyme remains important if only indirectly to salt regulation with external media in Artemia as evidenced by: 1) salt dependent ouabain-sensitive survival (Ewing, Peterson and Conte, 1972); 2) an apparent augmentation of Na+K-activated ATPase in adults challenged to higher salinities (Augenfeld, 1969); and 3) retention of significantly greater amounts of the enzyme in nauplii exposed to 2. 5M salts than to 0. 5M salts for 40 hours (see section V). With regard to this later evidence, it is of interest to note an additional observation by Mangos, namely that naupliar hemolymph Na levels go from 100mM Na in sea water animals to 180mM Na in animals exposed to 2. 5M salts. Although the nauplius is regulating, the regulation is not absolute and cells lining the hemolymph are stressed with nearly a two fold greater hemolymph Na concentration.

The distribution of the marker enzymes in the various subcellular fractions prepared by differential centrifugation (Table 5) do not show any peculiarities that would question their reliability as markers. Measureable enzyme activity for all enzymes except cytochrome

oxidase was observable in the 100,000 xg supernatant fractions. Membranes that form very small vesicles or sheets entrapped in light lipid droplets, or that are not sufficiently heavy in themselves to completely sediment in one hour at 100,000 xg would account for this. The membranes of the microsomal fraction contain from one fourth to one third of the Na+K-activated ATPase, 5'-UMPase and glucose-6-phosphatase activities. The mitochondrial fraction contains about 40 percent of the 5'-UMPase and glucose-6-phosphatase activities as well as from two thirds to three fourths of the Na+K-activated ATPase activity. Both the microsomal and mitochondrial fractions are considered to contain mixtures of membranes derived from the endoplasmic reticulum and plasma membranes.

The electron micrographs (Figure 14) together with the sucrose density gradient experiments (Figure 15) indicate the Na+K-activated ATPase activity in the mitochondrial fraction residues in the contaminating heavy membrane fragments which are separable from mitochondria. As shown in Table 6, the majority of the Na+K-activated ATPase in the mitochondrial fraction can be isolated from the mitochondria by gently removing the upper layer of the 15,000 xg pellet. This results in a membrane preparation with a very high specific activity of the Na+K-activated ATPase. The NADH oxidase activity remains principally in the bottom portion with the cytochrome oxidase, which suggests that its location in Artemia is probably

in the outer mitochondrial membrane. Approximately 30% of the 5'-UMPase and glucose-6-phosphatase activities in the mitochondrial preparation are removed in the upper layer with the Na+K-activated ATPase. Sucrose density gradient centrifugation of the isolate upper layer of the mitochondrial fraction (shown in Figure 16), separated 5'-UMPase from glucose-6-phosphatase. The activity of Na+K-activated ATPase follows that of 5'-UMPase. This evidence is taken to support the view that the Na+K-activated ATPase from Artemia nauplii is located in the plasma membranes of the cell.

The disproportionate separation of Na+K-activated ATPase and 5'-UMPase in the top and bottom layers of the mitochondrial preparation (Table 6) suggests that some of the plasma membranes may be particularly rich in Na+K-activated ATPase. This situation could result from any one of the following enzyme distributions: 1) certain localized areas of many cells or all cells, such as the basal surface of the cells in contact with the hemolymph, 2) a few cells with more or less extensive surface areas that are richly supplied with the enzyme, such as perhaps the salt gland or intestinal epithelium; or 3) some intermediate combination of (1) and (2). From available information a situation most like (1) is currently favored. Anatomical localization of the enzyme activity has not produced evidence of any peculiar enrichment of certain cells. Furthermore, the distribution of the cells in the nauplii is, in crude outline, a single layer

enveloping the hemolymph (Anderson, 1967). Essentially only the epithelial cells of the intestine have a basement membrane (Hootman and Conte, manuscript in preparation), and thus most cells of the nauplii are in direct contact with hemolymph. Also the range of densities (1.10 to 1.14) for the membranes containing the Na+K-activated ATPase (Figures 15 and 16) is consistent with plasma membranes originating from various cell types. Although these experiments may not be isopycnic, similar preparations have been sedimented to equilibrium in sucrose gradients centrifuged in a Beckman Ti-14 Zonal rotor. At least seven density peaks for the Na+K-activated ATPase are consistently observed between specific gravities (20° C) 1.100 and 1.153. Three major peaks appear at 1.120, 1.127 and 1.138. A protein and Mg-ATPase peak, suggestive of mitochondria in the zonal experiments, bands at a density between 1.166 and 1.170.

In conclusion the above information suggests the following hypothesis: The Na+K-activated ATPase in brine shrimp nauplii is distributed in at least part of the plasma membranes of most cells, it does not show an extreme enrichment in any particular cell type, and, although it is important to the organisms' ability for regulation and survival in hypertonic media, it probably assumes some secondary role, such as ionic regulation between cell interior and exterior (hemolymph).

IV. PURIFICATION OF THE NA+K-ACTIVATED ATPase

Introduction

Studies concerning the biogenesis of the Na+K-activated ATPase and its regulation in brine shrimp would be advanced if purification could be achieved. Knowledge of the molecular properties of the enzyme are germane to such studies and require at least partial purification. The production of antibodies to this enzyme or its subunits provides a very powerful tool in both biogenesis and regulation studies, which again requires purification. In recent years purification of this enzyme from various vertebrate sources have met with reasonable success (Towle and Copenhaver, 1970; Jørgensen, Skou and Solomonson, 1971; Kyte, 1971a; Uesugi et al., 1971; Hokin et al., 1973; Lane et al., 1973). The numerous methods applied in obtaining at least partially purified preparations ($\geq$ 25%) of active vertebrate enzyme encouraged the attempt to purify the enzyme from Artemia salina.

Basically these approaches involve initial isolation of a vesicular membrane fraction (microsomes) by differential centrifugation. Detergents have been useful for further purification of either a particulate or "soluble" enzyme fraction, (supernatant from centrifugation at 100,000 xg for one hour). Hokin's group (Uesugi et al., 1971; Hokin et al., 1973) and Jørgensen's group (Jørgensen, Skou

and Solomonson, 1971) have been particularly successful with use of zonal centrifugation for purification of the enzyme in preparative quantities. Complete purification apparently necessitates solubilization of the enzyme at some later step in the purification.

This section reports on a method for large scale isolation of membrane fragments rich in the Na+K-activated ATPase from crude homogenates of Artemia salina nauplii. A method is described for the partial purification of a particulate (vesicular) enzyme preparation from these membranes. This preparation has a 100-200 fold higher specific activity than the original homogenate, with a theoretical purity estimate of 30-60% based on scans of acrylamide gels. The molecular and kinetic properties of the partially purified enzyme of A. salina are examined and compared with those of the theoretically purified enzymes from other sources.

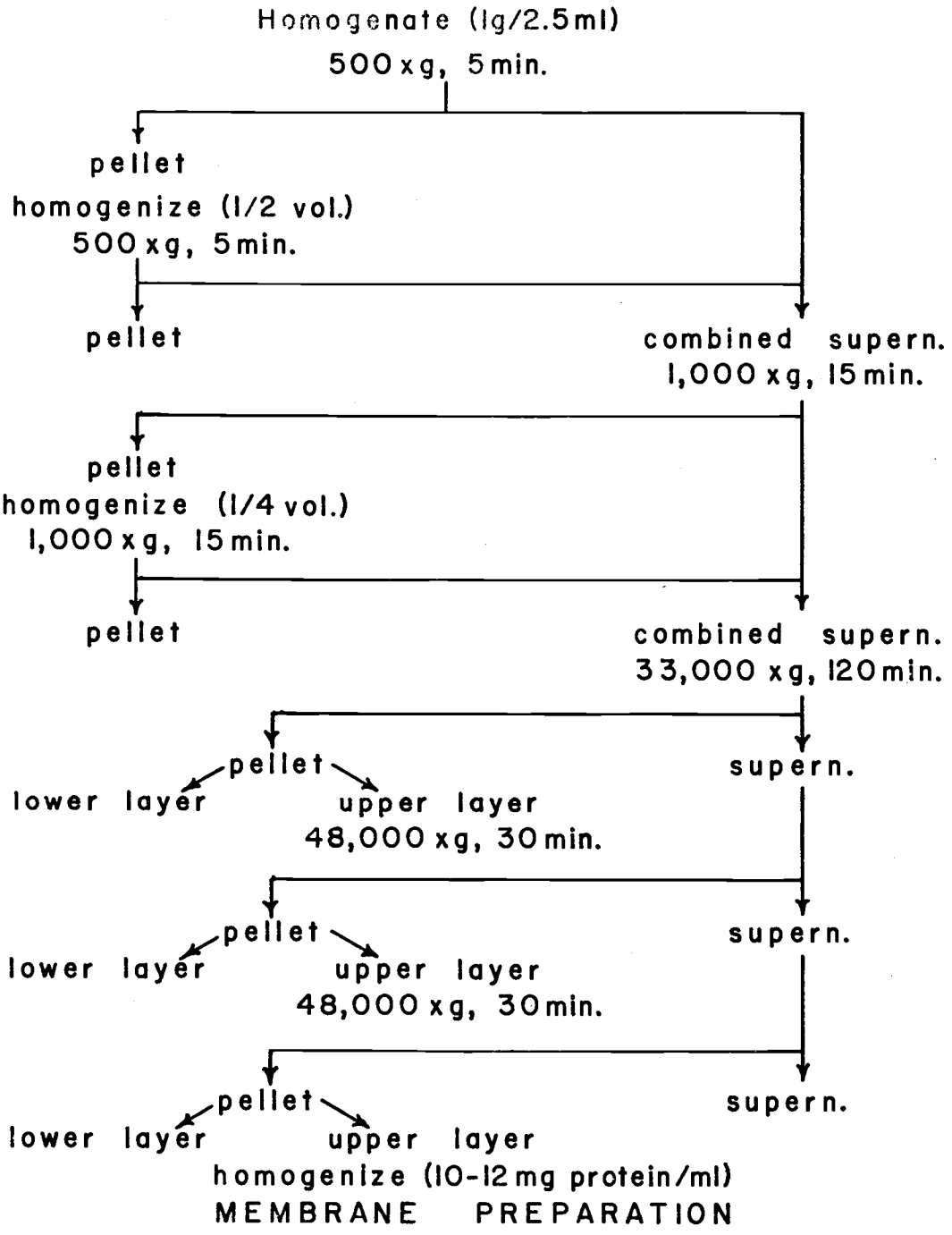
Methods

Large Scale Preparation of Na+K-activated ATPase Rich Membranes

The method for the large scale isolation of membranes containing the Na+K-activated ATPase from homogenates of the brine shrimp nauplii is outlined in the flow diagram of Figure 17. Three hundred grams of dry weight cysts are washed and prehydrated in the cold, then distributed among twelve 2200 ml Fernbach culture flasks

Figure 17. Schematic for the large scale isolation of Na+K-activated ATPase rich membrane preparations.

FRACTIONATION FLOW DIAGRAM



containing 500 ml of Instant Ocean Sea Salt solution and incubated at 27° C with shaking for 40-48 hours. This produces a harvest of about 200 g fresh weight of nauplii.

The homogenizing medium consisted of 250 mM Sucrose, 100 mM Imidazole buffer pH 7.2 and 2 mM Na_2EDTA (H. M.) or contained in addition 2 mM 2-mercaptoethanol (H. M. -S. H.). This reducing agent was found to markedly enhance the stability of the membrane preparation over that of cysteine and dithiothreitol (see Table 7).

The large scale preparation of the membranes were prepared in a Sorval RC2-B refrigerated centrifuge using the Sorval GSA rotor for the lower g forces. The 48,000 xg centrifugations were done with the smaller SS-34 rotor in-as-much as the preparation was reduced to smaller volumes by this stage and the higher g force provided more favorable layering of the pellet. The homogenate, 500 xg pellet and 1000 xg pellet were homogenized with two strokes of a 55 ml Vitro glass Teflon homogenizer. Other pellets were resuspended by single strokes of the homogenizer. The teflon pestle was rotated approximately 2500 rpm. Excessive or inadequate grinding resulted in low yield of the final membranes. The characteristic layering of the high speed pellets parallels the mitochondrial vs. membrane separations reported in similar preparations shown in Figure 13 and Table 4 of the Section III. The high speed upper layer includes a light fluffy

Table 7. Effects of reducing agents on storage of the membrane enzyme at 0°C

Reducing Agent	Days of Storage			
	0	3	6	15
Control (H_2O)	100.0[1]	69.6	64.4	36.8
1 mM Cysteine	97.8	75.0	50.0	28.8
1 mM Dithiothreitol	100.3	85.3	70.6	38.2
1.3 mM 2-mercaptoethanol	98.1	97.1	89.8	53.9

[1] Values are percent of control activity on day 0.

uppermost material down to a slightly, but detectably darker, buff-colored layer. The upper membrane layer amounts to approximately one third of the first high speed pellet.

The membrane enzyme preparation is resuspended to give a protein concentration of 10-12 mg/ml. The Na+K-activated ATPase activity was determined as outlined in Section II, unless altered for variation of the kinetic parameters examined later. Protein was estimated by the method of Lowry et al. (1951).

Partial Purification of the Na+K-activated ATPase from the Isolated Membranes

The membrane suspension is diluted one to one (v/v) with 0.70% (w/v) sodium deoxycholate (DOC), and allowed to sit at room temperature for 30 minutes. It is then centrifuged at 48,000 xg for one to two hours using the Sorvall SS-34 rotor. The supernatant is carefully aspirated off to avoid loss of the floculent pellet. The pellet is resuspended in homogenizing media to give a volume one-half of the original membrane suspension, and then frozen at -12° C. This preparation is referred to as the DOC-Membranes and can be stored frozen up to one month.

The frozen membranes when thawed slowly and only very gently mixed contain separable smaller vesicles that are two to three fold richer in Na+K-activated ATPase than the original DOC-membrane

vesicles. These vesicles, referred to as the Freeze-Thaw (FT) membrane or vesicular preparation can be recovered either by differential centrifugation or by discontinuous sucrose density centrifugation. Preparation by differential centrifugation is achieved by removal of heavy membranes low in Na+K-activated ATPase activity by centrifugation at 5,000-8,000 xg for 15 minutes, followed by a second centrifugation of the supernatant at 48,000 xg for 30 minutes. The resulting high speed pellet has a specific activity ranging from 200 to 300 μmoles P_i/hr/mg protein. The discontinuous sucrose density gradient centrifugation method, however, is more reproducible in terms of higher yield and specific activity than the differential centrifugation method for the FT enzyme preparation. In this case the thawed DOC-membranes are layered on a discontinuous gradient prepared as follows: 5 ml of 28% sucrose, 7 ml of 18% sucrose, sample (up to 20 mg protein), and distilled water overlay to give a final volume of 25 ml. The gradient is centrifuged at 22,000 rpm for 25 minutes using a Beckman SW 25.1 rotor. Part of the FT enzyme bands at the interface between the 18% and 28% sucrose layers, and is collected by aspiration. The material penetrating the 28% sucrose layer forms a loose pellet, the upper portion of which contains membranes with specific activities routinely greater than 200 μmoles P_i/hr/mg protein. These membranes, included in the FT enzyme preparation, are recovered by decanting

after aspiration of the gradient material above the 28% sucrose layer is complete. Removal of the heavy sucrose in these preparations is achieved by pelleting the membranes at 48,000 xg for one hour. The pellet obtained is once again layered, the lighter upper portion has a higher specific activity than the membranes of the lower portion, ranging from 300 to 500 μmoles P_i/hr/mg protein.

Sucrose Density Gradient Centrifugation

Linear sucrose gradients were prepared and fractionated as indicated in Section III. Conditions of the gradients are noted in the figure legends.

Electron Microscopy of Isolated Pellets

Pellets from various stages in the purification procedure were fixed, stained and sectioned for electron microscopy. The methodology is described in Section III.

Sodium Dodecyl Sulfate Polyacrylamide Disc Gel Electrophoresis

The procedure of SDS gel electrophoresis was taken from that described by Hokin et al. (1973). The gels were prepared in 0.60 mm (I.D.) x 10 cm tubes acid cleaned and coated with 2% (in carbon tetrachloride) SCI-87 Dri Film (Pierce Chemical Co.). The separating gel (2.4 mg/tube) contained 8.75% acrylamide, 0.234%

N, N'-methylenebisacrylamide (Bis), 0.025% tetramethylethylene-diamine (TEMED), 0.03% ammonium persulfate (AP), 0.375 M Tris-HCl (pH 8.8), and 0.1% SDS. The stacking gel (0.2 ml/tube) contained 3% acrylamide, 0.08% Bis, 0.1% TEMED, 0.03% AP, 0.12 M Tris-HCl (pH 6.8), and 0.1% SDS. The gels were overlayed with 0.1% SDS during polymerization at room temperature.

The sample was solubilized at room temperature in a mixture (referred to as sample buffer) with a final concentration of 10% sucrose, 5% 2-mercaptoethanol, 3% SDS and 0.0625 M Tris HCl (pH 6.8). Solubilization is immediate. Five μl of 0.1% bromphenol blue was added to each gel as a tracking dye. The reservoir buffer was 0.025 M Tris, 0.19 M glycine and 0.1% SDS (pH 8.3). The gels were stacked at a constant current of 1 ma/tube and run at 2 ma/tube. At the end of the run (4-5 hours) the tracking dye was marked with drawing ink and the gels simultaneously fixed and stained overnight in methanol:H_2O: acetic acid (5:4:1) containing 0.05% Coomassie brilliant blue. The gels were electrically destained in 7.5% acetic acid at 4 ma/tube and stored in 7.5% acetic acid. Calibration of the gels was done with the following molecular weight markers: β-galactosidase subunit, 130,000; phosphorylase A, 92,500; bovine serum albumin, 68,000; catalase, 57,500; pyruvate kinase, 57,200; glyceraldehyde-3-phosphate dehydrogenase, 37,000; hexokinase, 27,500; ribonuclease A, 13,700; cytochrome C, 11,700;

insulin, 5,700.

Glycoproteins were detected by PAS staining according to a combination of techniques borrowed from Zacharius et al. (1969) and Fairbanks, Steck and Wallach (1971). Gels were fixed overnight as above but without the Coomassie brilliant blue stain. They were then electrically "destained" to remove excess SDS which interferes with PAS staining. The gels were then immersed in 1% periodic acid (in 3% acetic acid) for one hour and washed with 7.5% acetic acid overnight with several changes. Staining with fuchsin-sulfite prepared according to McGuckin and McKenzie (1958) was done at room temperature in the dark for one hour. The gels were washed, with several changes, in freshly prepared 0.5% sodium metabisulfite, and stored in 7.5% acetic acid. Fading required that the gels be photographed within one week after preparation.

Gel scanning was performed with a Photovolt Densicord Densitometer on Response 5 which approximates an absorption scale. Four protein standards (phosphorylase A, bovine serum albumin, glyceraldehyde-3-phosphate dehydrogenase and ribonuclease A) were tested for densitometric quantification after Fishbein (1972). The relative peak areas were determined either by planimetry or by weighing xerox copies of the peaks. Linearity of these areas to protein applied to the gel was found for all standards for 1-30 μg protein. In agreement with Fishbein (1972) for Coomassie blue

stained acrylamide slabs, Coomassie brilliant blue stained disc gels show breaks in the densitometric quantification that are more pronounced in the higher molecular weight proteins. However, beyond 60 μg protein the protein to peak relation regains the original linear slope at least up to the 100 μg protein level examined. Fishbein did not report on protein values above 55 μg, and did not note a return to the original slope. The enzyme preparations examined in this study, because of the nature of their protein profiles can be analyzed quantitatively by densitometry with up to 200 μg total protein per gel. The peculiarities of the procedure, however, are expected to place an analytical tendency toward underestimation of the relative amounts of the higher molecular weight proteins (i. e., 90, 000 and above), apparently a result stemming from the packing characteristics found with such molecular sieving gels.

Phosphorylation of Na+K-activated ATPase with γ^{32}P-ATP and SDS Gel Electrophoresis

The kinetic parameters examined for optimal binding conditions of Na+K-activated ATPase to γ^{32}P-ATP were Mg^{++} concentration (0. 05 mM), Na^{+} concentration (0. 001-400mM), K^{+} concentration (0-80mM) and time of incubation (0-60 seconds). The optimum phosphorylation reaction was run at 0° C in a total volume of 0. 25 ml containing: 12. 5 μg FT enzyme preparation, 0. 68 μM γ^{32}P-ATP (7×10^{14} cpm/mole), 100mM NaCl, 0. 1mM $MgCl_2$, 100mM Imidazole

buffer (pH 7. 2). Nonspecific phosphorylation was controlled by an identical reaction mixture except that 20mM KCl was substituted for the 100 mM NaCl. The reactions were initiated by addition of 0. 1 ml of enzyme-buffer solution and terminated (optimally at 20 seconds) by addition of 0. 2 ml of 5% trichloroacetic acid (TCA) containing 0. 6mM Na_2ATP and 0. 6mM NaH_2PO_4, and 0. 05 ml of 5 mg/ml BSA as carrier protein. After 10 minutes at 0° C the mixture was filtered on Whatman 3MM filter discs and washed three times with 2 ml of the TCA solution at 0° C. According to analysis of the filtrates, protein recovery on the filter discs was about 80%. The discs were counted with 15 ml of a BBOT (2, 5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene) toluene: methylcellosolve (5:3) scintillation fluid in a Model 3310 Packard Tri-Carb liquid scintillation spectrometer.

For processing on SDS polyacrylamide gels 100 μg of enzyme were incubated under optimal binding conditions in a total volume of 2. 5 ml and stopped with 2. 5 ml of the cold TCA sclution (above), but without carrier protein. After 10 minutes at 0° C the samples were centrifuged at 48, 000 xg for five minutes, washed with 2. 5 ml of the cold TCA solution, and recentrifuged at 48, 000 xg for five minutes. Protein recovery was about 98%. Solubilization and electrophoresis was as described above, except that all operations were carried out at 0° C. Gels were run in duplicate, one set stained for protein, the

other sliced (0. 5 mm thickness) and counted by liquid scintillation.

Comparison of the Kinetic Properties of the Membrane Enzyme and the Partially Purified Freeze-thaw Enzyme

Temperature, pH, substrate, and monovalent ion characteristics were examined in a fashion patterned after that presented in detail in Section II. The membrane enzyme preparation had a specific activity of 43 μmoles P_i/hr/mg protein, and the FT enzyme an activity of 222 μmoles P_i/hr/mg protein according to previous assay methods. In the case of the temperature profile studies a DOC-membrane enzyme preparation (specific activity, 100 μmoles P_i/hr/mg protein) was also examined. The pH profile was performed in a 25 mM histidine-25 mM Imidazole buffer system. The substrate curves were obtained using a constant Mg^{++}:ATP ratio of 2:1, and reactions were run for 10 minutes. The movalent ion characteristics investigated were variable ionic strengths at a constant Na:K ratio of 4:1, and variable Na:K ratios at a constant ionic strength of 160 mM (Na+K).

Results

Isolation of Na+K-activated ATPase Membranes

Fractionation of brine shrimp nauplii according to the flow diagram in Figure 17 results in a distribution of enzymatic activity

and protein in the various fractions for a typical experiment, as shown in Table 8. From six experiments the average total yield of Na+K-activated ATPase per unit amount of embryo was 69.1 ± 6.0 μmoles P_i/hr/gr nauplii ($\overline{X}$ ± SEM). Activity of the enzyme in the homogenate was difficult to assess due to its crude nature. Approximately one third of the total enzymatic activity in the embryo was recovered in the final membrane fraction. These membranes had about a 20 fold increase in the specific activity of the enzyme over that in the homogenate, averaging 39.0 ± 2.4 μmoles P_i/hr/mg protein ($\overline{X}$ ± SEM, n=6).

Partial Purification of the Na+K-activated ATPase from Membrane Fragments

Early approaches to the purification included screening of a number of detergents: Brij 35, Brij 36T, DOC (sodium deoxycholate), Lubrol PX, Lubrol WX, Nonidet P-40, SDS (sodium dodecyl sulfate), Triton CF-32, Triton X-100, and Tween 80. Detergent characteristics examined were optimum concentrations for enzyme activation (operationally defined as the concentration permitting maximum activity, or $\geq$ 90% activity if an activation peak was not observed), effects on enzyme stability at 0° C and 25° C, and effect on protein and enzyme redistribution after centrifugation of a detergent-treated membranes. DOC was found to be particularly promising for

Table 8. Distribution of protein and Na+K-activated ATPase from naupliar homogenates fractionated by differential centrifugation according to the flow diagram of Figure 1.

Fraction	Protein		Na+K-activated ATPase		
	Total mg protein / g nauplii	Percent (%)	Total Activity µmoles Pi / g nauplii/hr	Percent Activity (%)	Specific Activity µmoles Pi / mg protein/hr
Homogenate	72.9	100.0	141.4	100.0	1.94
500 xg pellet	38.8	53.2	79.2	56.0	2.04
1000 xg pellet	1.9	2.6	7.9	5.6	4.14
"Bottom" pellets combined	3.1	4.3	27.7	19.6	8.82
Final upper membranes	1.7	2.3	67.6	47.8	40.00
Combined high speed supern.	16.4	22.5	30.9	21.8	1.88
Recovery		84.9		150.8	

purification of the particulate membranes. A summary of several of these detergent characteristics is provided in Table 9. For a number of these detergents including DOC it was found that concentrations 10 fold in excess of their characteristic optimum could be tolerated if adequate dilutions were made before enzyme assay. Proper detergent to protein (or lipoprotein) ratio is not as important to activation properties (Jørgensen and Skou, 1971) as it is to solubilization (Banerjee et al., 1970). Salts in accompaniment with the detergents are also useful in solubilization (Banerjee et al., 1970; Kyte, 1971a). Numerous experiments covering a wide variety of these detergent complexities have not provided conditions for solubilization of the brine shrimp enzyme. The DOC treatment described earlier for the particulate enzyme appears most favorable for further purification of the enzyme in the membrane form.

The effect of various concentrations of DOC on the enzyme activity after 30 minutes exposure at room temperature is shown in Figure 18. No activation was observed with the DOC treated enzyme. All other detergents with the exception of Tween 80 produced an activation of the enzyme activity. The activation amounted to 120-130% of control levels except for Lubrol WX which was nearly 160%. The optimum DOC concentration was assigned as 0.075%. The stability of the enzyme at this concentration of DOC for temperatures of 0° C and 25° C is reported in Table 10. The relative

Table 9. Summary of various characteristics of the detergents screened for potential use in purification of the Na+K-activated ATPase from brine shrimp nauplii

Detergent	Optimum[1]		Purification-solubilization analysis[2]					
	Percent Conc.	Percent Activation	15,000 xg pellet		15,000 xg supern.		Recovery (%)	
			rel. pur.	yield (%)	rel. pur.	yield (%)	Proteins	Enzyme
Control (H_2O)	---	100	1.08	73.4	0.25	8.9	103.2	82.3
Brij 35	0.050(v/v)	127	2.25	66.1	0.27	18.4	96.6	84.5
Brij 36T	0.015(v/v)	132	1.85	60.7	0.28	18.0	98.2	78.7
DOC	0.075(w/v)	91	2.86	97.6	0.25	16.5	95.4	114.1
Lubrol PX	0.010(v/v)	133	1.92	57.0	0.25	17.1	94.6	74.1
Lubrol WX	0.050(w/v)	157	1.83	53.2	0.34	24.9	102.7	78.1
Nonidet P-40	0.025(v/v)	125	2.14	44.9	0.60	44.1	99.7	99.0
SDS (+3mM ATP)[3]	0.005(w/v)	87	1.07	90.4	--	7.3	106.3	97.7
Triton CF-32[3]	0.075(v/v)	114	0.92	67.5	0.12	6.3	97.0	73.8
Triton X-100	0.030(v/v)	120	2.02	59.4	0.38	26.5	98.2	85.9
Tween 80	0.100(v/v)	93	4.45	117.3	0.02	13.7	99.7	131.0

[1]Optimum concentration (defined in Methods) is the concentration that the enzyme is incubated in prior to direct sampling for enzyme assay.

[2]Purification-solubilization analysis was performed on a mitochondrial preparation in a final protein concentration of 0.35 mg/ml in the presence of the optimum detergent concentrations. The relative purification is the specific activity of the fraction divided by the specific activity of the incubate.

[3]These detergents examined subsequently to others. Studies were performed using membrane enzyme preparations centrifuged at 40,000 xg, 30 min. SDS requires that the enzyme be pretreated for 30 minutes with 3 mM ATP (in enzyme solution).

Table 9. Continued

Detergent	UV Absorption[4] 260 nm $E^{1\%}_{1\ cm}$	UV Absorption[4] 280 nm $E^{1\%}_{1\ cm}$	Interference[5] with Lowry protein assay PCA ppt.	Interference[5] with Lowry protein assay Direct
Control (H_2O)	0	0	1.00	1.00
Brij 35	0.072	0.054	1.02	1.00
Brij 36T	0	0.013	1.02	0.98
DOC	0.690	0.630	1.01	0.98
Lubrol PX	0.024	0.012	1.02	0.98
Lubrol WX	0.200	0.160	0.52	1.05
Nonidet P-40	8.730	22.100	0.99	1.00
SDS (+3mM ATP)[3]	0.044	0.018	0.99	0.98
Triton CF-32[3]	0.304	0.260	1.00	1.00
Triton X-100	8.270	17.300	1.04	0.99
Tween 80	0.380	0.270	1.04	1.00

[4]The 1% extinction coefficients were determined from standard curves of aqueous solutions of the detergents prepared either as w/v or v/v (refer to optimum concentration column).
[5]Determined by assay of 0.2 ml sample containing 250 µg crystalline BSA and detergent at optimum concentration. Values represented are relative to those of control.

Figure 18. Activity of Na+K-activated ATPase exposed to various concentrations of DOC. The concentrations are those to which the enzyme is exposed during the 30 minute, room temperature (22° C) preassay incubation. Enzyme activity was assayed from 0.1 ml aliquots of such solutions.

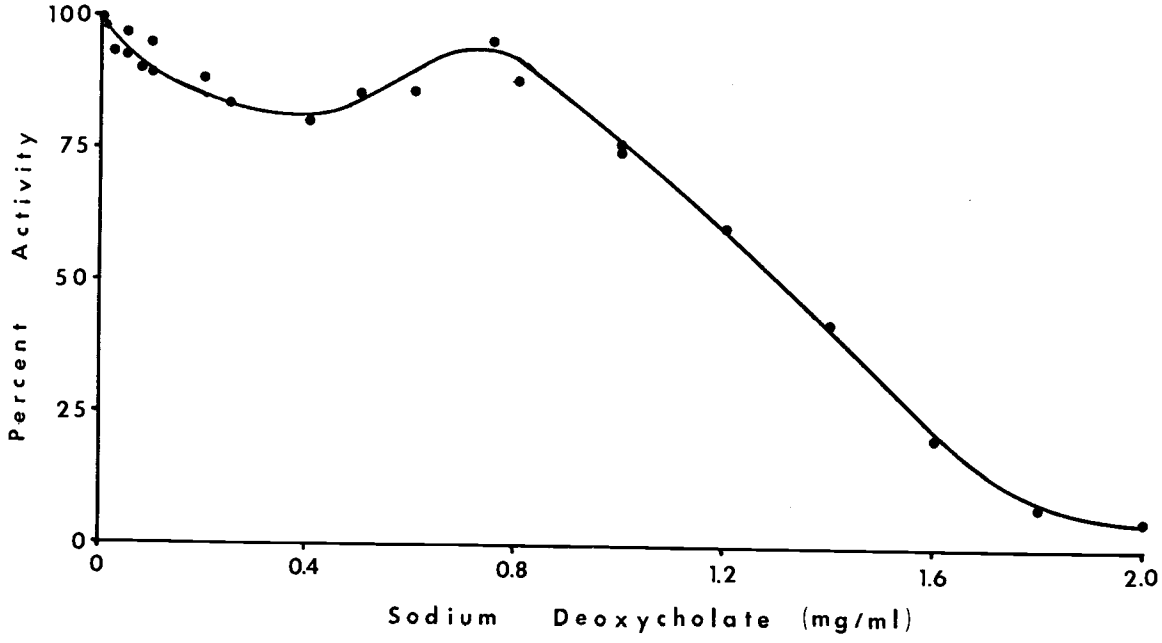

100
75
50
25
0
Percent Activity
0
0.4
0.8
1.2
1.6
2.0
Sodium Deoxycholate (mg/ml)

Table 10. Stability of the Na+K-activated ATPase exposed to 0.075% DOC at 0°C and 25°C. Activity is expressed as percent of control (H_2O) at 30 minutes.

Time (hours)	0°C		25°C	
	Control	DOC	Control	DOC
0.5	100.0	89.9	100.0	87.7
6	103.9	87.8	91.5	67.8
20	95.1	81.7	62.9	22.9
30	95.6	82.9	43.0	6.4

distribution of protein and enzyme from a mitochondrial preparation incubated with H_2O (control) or 0.075% DOC (protein concentration was 0.38 mg/ml) for 0, 2 and 6 hours and recentrifuged at 15,000 xg for 30 minutes is displayed in Table 11. The large recovery value for the DOC treated enzyme is the result of activation in the pellet after removal of the DOC supernatant. The relative purification of 3.49, 3.38 and 3.92 for the 0, 2 and 6 hour exposure is a combination of activation and purification. The relative purification corrected for activation, estimated from the recovery values, is 2.36, 2.58 and 2.57, respectively.

Efforts at purification of the Na+K-activated ATPase beyond the DOC-membrane step involved a variety of approaches. The DOC-membrane preparation was extremely sensitive to retreatment with the detergents examined. Activation was absent in all cases except for Triton X-100, in which a 110% activation was observed at a concentration (0.003%) 1/10 of the previous optimum concentration (Table 8). Inactivation of the enzyme was otherwise immediately apparent at very low concentrations of any of the detergents. Following Triton X-100 treatment a two-fold purification of the particulate enzyme was found, but no significant solubilization. NaI purification according to Uesugi et al. (1969), including several modifications of the procedure gave at best only a 1.5 fold increase in specific activity with 70% recovery. A similar situation occurred in the attempts to

Table 11. The effect of exposure time on redistribution of protein and Na+K-activated ATPase from a mitochondrial preparation treated with 0.075% DOC

Time (hours) / Fraction	Control (H_2O) Percent Protein	Control (H_2O) Percent ATPase	Control (H_2O) Specific Activity	DOC treated Percent Protein	DOC treated Percent ATPase	DOC treated Specific Activity
0						
Incubate	100	100	30.7	100	100	21.9
15,000 xg pellet	60.6	87.3	45.8	40.3	130.2	76.4
15,000 xg supern.	39.4	8.1	6.6	59.7	17.0	6.8
Recovery	96.5	95.4		92.7	147.0	
2						
Incubate	100	100	27.8	100	100	22.7
15,000 xg pellet	49.7	87.6	48.9	34.4	116.1	76.5
15,000 xg supern.	39.3	9.5	6.7	61.2	15.5	5.8
Recovery	89.0	97.1		95.6	131.6	
6						
Incubate	100	100	29.3	100	100	22.3
15,000 xg pellet	50.5	86.6	50.2	34.3	134.9	87.5
15,000 xg supern.	41.7	9.6	6.7	58.5	18.0	6.9
Recovery	92.2	96.2		92.8	152.9	

utilize the NaI purification procedure with the dogfish rectal gland enzyme (Hokin et al., 1973). These approaches were abandoned since none gave promising leads.

Freezing the DOC-membrane enzyme, followed by gentle thawing and sedimentation through a linear 15% to 45% sucrose gradient gave an enzymatic activity and protein distribution as shown in Figure 19A. Thawing followed by homogenization altered the distribution pattern of protein as illustrated in Figure 19B. A very similar method was successfully applied by Jørgensen and Skou (1971) and Jørgensen, Skou and Solomonson (1971) in purification of rabbit kidney Na+K-activated ATPase, however, in their case freezing was not a necessary step. Effective separation of enzyme and protein as in Figure 19A by the differential centrifugation method gave a yield of 10 to 30%. Similar purification was obtained with the discontinuous sucrose gradient method, however, the yield of the higher specific activity enzyme was more favorable (25-60%). With the discontinuous gradient preparation the heavy membrane vesicles of low enzyme activity pass through the 28% sucrose (D_4^{20} = 1.105 g/ml). Use of a 30% sucrose (D_4^{20} = 1.1126 g/ml) often floated the heavy membranes. The results of the method are shown by the fractionation pattern of two gradient tubes (Table 12). Following recovery of the enzyme enriched membranes, a recentrifugation at 48,000 xg for one hour to remove the heavy sucrose resulted in the

Figure 19. Linear sucrose density gradient (15-45%) centrifugation of the frozen and thawed DOC-membrane showing the profiles of protein and Na+K-activated ATPase.

A. Thawed without homogenization

B. Thawed with homogenization.

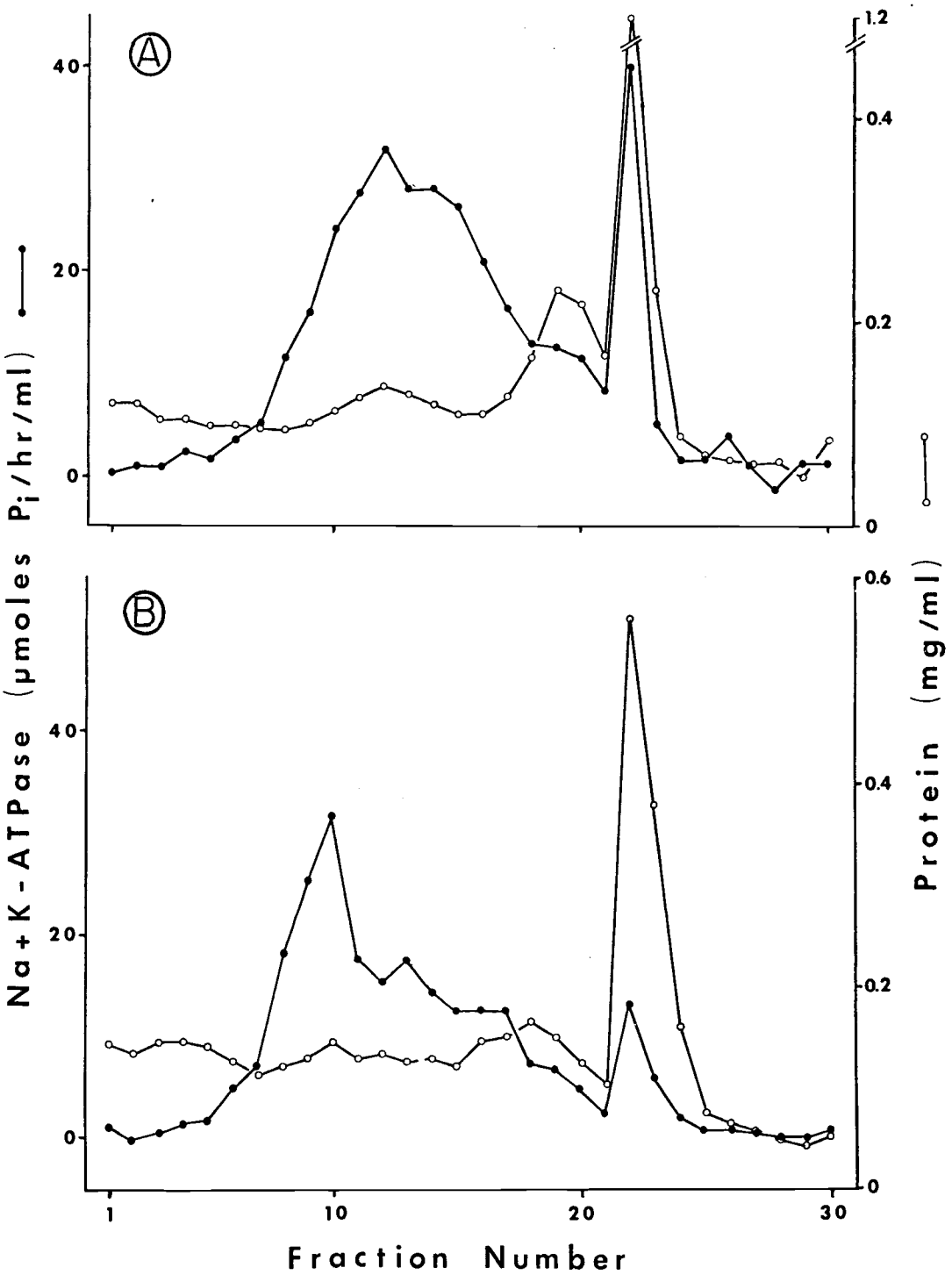

A
B
40
20
0
1.2
0.4
0.2
0
0.6
1
10
20
30
Na+K-ATPase (μmoles P_i/hr/ml)
Protein (mg/ml)
Fraction Number

Table 12. Distribution of Na+K-activated ATPase and protein in a discontinuous sucrose (18-28%) gradient preparation of the Freeze-Thaw Enzyme

Fraction	Tube #1			Tube #2		
	Total		Specific activity	Total		Specific activity
	Protein (mg)	Na-KATPase (µmoles/hr)	(µmoles/hr/mg)	Protein (mg)	Na-KATPase (µmoles/hr)	(µmoles/hr/mg)
Upper zone Sample + dist. H_2O	2.98	38	13	2.76	5	2
Sample - 18% interface	1.27	382	301	--	379	--
18 - 28% interface	1.65	765	463	0.88	536	609
Upper portion of pellet	3.80	1185	312	4.45	1177	264
Remaining pellet	6.03	977	162	6.67	1016	152

distribution of enzyme and protein shown in Table 13. The data presented in Table 13 was obtained from a combination of the appropriate fractions of tubes #1 and #2 shown in Table 12. The relatively high specific activity of the enzyme derived from the 18%-28% sucrose interface shown in Table 12, is slightly suspect because of the difficulty in assaying low amounts of protein in the presence of high concentrations of sucrose.

Table 14 demonstrates an example of the partial purification of the Na+K-activated ATPase containing membranes originally derived from 210 g of embryos. Approximately 30 percent of the total membrane enzyme activity is recovered in the high specific activity FT enzyme preparation. The membranes in turn represent approximately one third of the enzyme present in the organism (see Table 8). The FT enzyme was more stable to detergent treatments than the DOC-membrane enzyme, but resisted treatments for solubilization or further purification.

The storage characteristics of the membrane, DOC-membrane and FT enzyme preparations are shown in Tables 15 and 16. For freeze storage (-12° C) both the membrane and DOC-membrane enzyme are fully active over a period of 3-4 weeks. Activity is slowly lost when the enzyme is stored non-frozen at 0° C. The DOC-membrane enzyme appears only slightly less stable than the non-detergent treated membrane enzyme. Preparation of the FT enzyme

Table 13. Distribution of Na+K-activated ATPase and protein from fractionation by differential centrifugation at 48,000 xg for one hour of the 18-28% interface and upper portion of the pellet from the discontinuous sucrose gradient preparation of the Freeze-Thaw enzyme

Fraction	18-28% interface			upper portion of pellet		
	Protein	Na-KATPase		Protein	Na-KATPase	
	(mg)	(μmoles/hr)	(μmoles/hr/mg)	(mg)	(μmoles/hr)	(μmoles/hr/mg)
upper pellet	1.90	773	408	1.19	566	475
lower pellet	1.11	272	246	6.69	1490	222
supernatent	--	22	--	--	38	--

Table 14. Purification of the Na+K-activated ATPase from membranes prepared from 210 g fresh weight of nauplii

	Total		Specific activity	Percent yield
	Protein (mg)	Na-KATPase (µmoles/hr)	(µmoles/hr/mg)	
Membranes	370.00	12,700	34.3	100.0
DOC-membranes	47.40	8,510	179.0	67.0
FT enzyme				
18-28% Interface	2.59	1,330	514.0	10.5
Upper pellet	8.44	2,410	286.0	19.0

Table 15. Stability of the Na+K-activated ATPase in untreated membrane and DOC-membrane preparations stored frozen (-12°C) or non-frozen (0°C) in Homogenzing Media

Untreated Membrane Enzyme

frozen storage		non-frozen storage	
Days	Percent activity	Days	Percent activity
0	100.0	0	100.0
1	124.4	3	91.5
3	90.0	9	86.8
7	98.3	19	80.2
8	102.0		
31	109.1		
121	39.8		

DOC-Membrane Enzyme

frozen storage		non-frozen storage	
Days	Percent activity	Days	Percent Activity
0	100.0	0	100.0
1	105.7	7	116.8
3	100.4	9	101.9
5	106.4	11	77.3
6	92.4	19	70.0
8	107.6		
13	100.2		
19	97.8		
26	82.7		
29	86.3		

Table 16. Stability of the Freeze-Thaw Enzyme prepared at various storage times of the frozen DOC-membranes and subsequently stored non-frozen (0°C) in Homogenizing Media

Days of storage of FT Enzyme at 0°C after preparation	Days before preparation of FT Enzyme (storage time of frozen DOC-Membrane Enzyme)			
	1	6	7	25
0	100.0	100.0	100.0	100.0
4	95.3	-	-	-
5	-	-	-	72.9
6	-	-	-	55.2
10	-	-	86.6	-
13	-	73.1	-	-
21	59.6	-	-	-
37	-	51.1	-	-

from DOC-membranes frozen for 25 days produced a less stable enzyme than those prepared within 7 days. The data shown in Tables 15 and 16 are from storage experiments in the presence of homogenizing media without the reducing agent 2-mercaptoethanol, which promotes the shelf-life (Table 7). Freeze storage characteristics of the enzyme at -90° C or -196° C (liquid N_2) were not examined.

Alternative Methods for Purification of the Brine Shrimp Na+K-activated ATPase

At the time the majority of the experiments for the above described purification were completed three successful methods for the purification of enzyme from different tissues were reported (Hokin et al., 1973; Lane et al., 1973; Papers presented by L. E. Hokin, P. L. Jørgensen, and L. K. Lane, International Conference on the Properties and Functions of Na^+K^+-ATPase, N. Y. Acad. Sci., New York, N. Y., November 26-29, 1973). The procedure developed at Hokin's laboratory involved solubilization of the membranes with Lubrol WX, zonal centrifugation of the lubrol extract, and ammonium sulfate purification of the concentrated, ultrafiltrated zonal enzyme. The same procedure was attempted with the brine shrimp, but, no significant solubilization was obtained using the 1.6% Lubrol concentration as published, or the 0.12% Lubrol at protein:detergent ratio greater than 1:1 scheme currently used in their laboratory

(L. E. Hokin, personal communication). Using a Lubrol membrane preparation, a swinging bucket rotor, and concentration by differential centrifugation, resulted in the two-fold increase in specific activity. Further investigation would require a zonal rotor for adequate sample size and g force, and a high pressure ultrafiltration device for rapid concentration and removal of sucrose (pelleting the zonal enzyme results in inactivation). The ammonium sulfate purification step as described (Kahlenberg et al., 1969; Uesugi et al., 1971; Hokin et al., 1973) was also attempted on the FT enzyme without effect. L. E. Hokin (personal communication) also noted that prior treatment of his enzyme preparation with DOC precluded any subsequent attempts at purification by ammonium sulfate.

The purification procedure of Lane and co-workers involves solubilization by DOC, precipitation by glycerol, resolutilization with DOC-cholate and treatment with ammonium suflate (method different from Hokin). Their initial DOC treatment is the same as this study, however, the brine shrimp membranes vesicles completely sediment at 100,000 xg for 2.5 hours. Attempts to work with supernatants at lower g forces gave no significant purification with the glycerol step, although some purification was evident with glycerol treatment of the DOC-membrane pellets. All such enzyme preparations, however, would not tolerate retreatment with DOC, even at extremely low concentration. This procedure would require

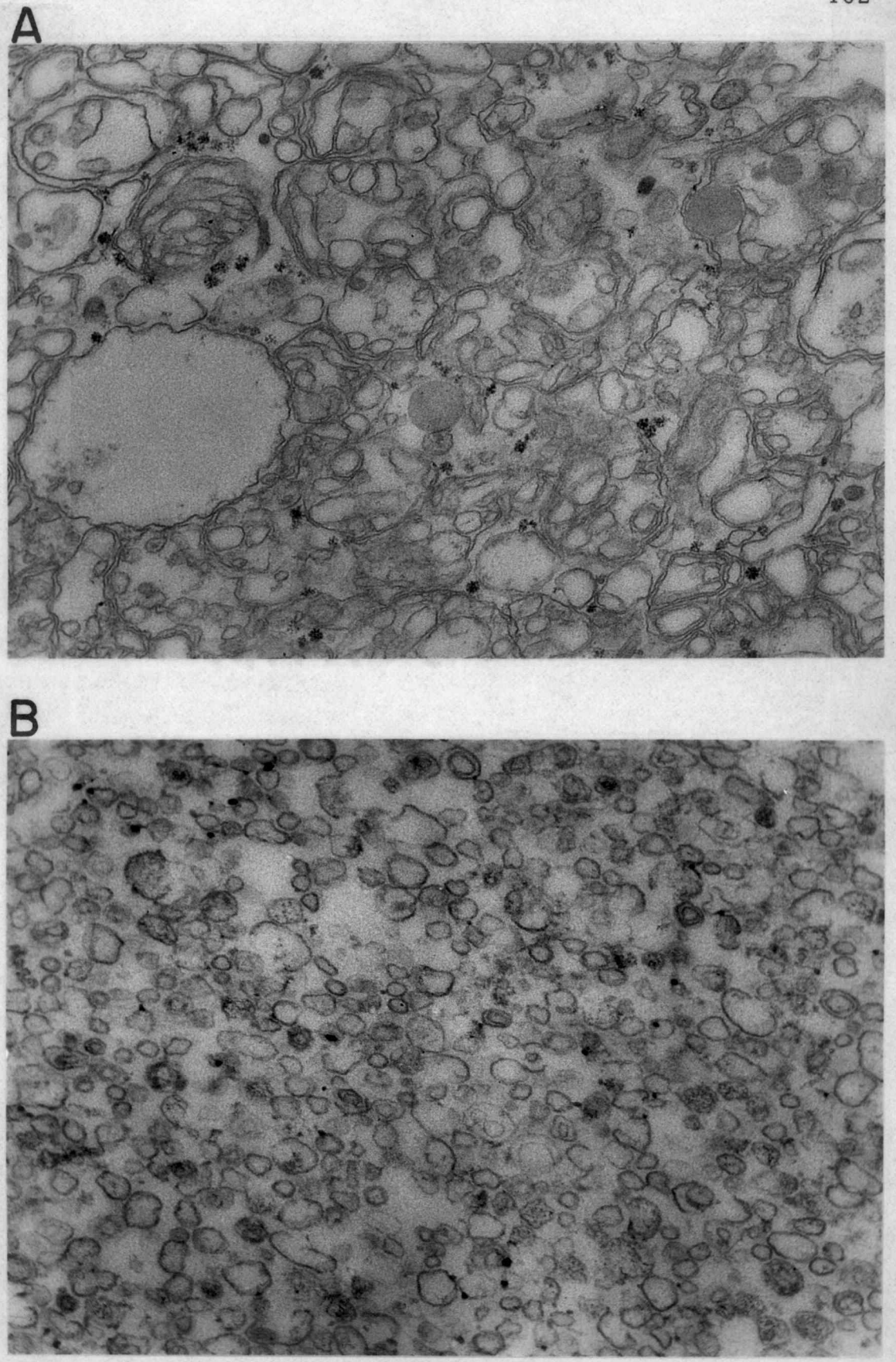
extensive modification if adaptable to purification of brine shrimp Na+K-activated ATPase, and thus does not appear immediately promising to pursue.

The technique described by Jørgensen for purification of rabbit kidney Na+K-activated ATPase required a simple zonal centrifugation of SDS treated membranes. Preliminary results from swinging bucket rotor experiments with membrane preparations indicated some protein-enzyme separation. The FT enzyme preparation is extremely sensitive to treatment with SDS. With the use of zonal rotors this procedure warrants further exploration.

Electron Microscopy

Electron microscopy of a fraction equivalent to the membrane enzyme preparation appears in Figure 15A (Section III). Thin sections of this material show a few mitochondria and a large array of heterogenous membrane vesicles. Sections of the DOC-membranes and FT enzyme preparations are shown in Figures 20A and B, respectively. The DOC-membranes appear as extensively elaborate and large membrane contours, often surrounding lipid globulules and interspersed with glycogen particles. The FT enzyme preparation (prepared by the differential centrifugation method) consists of small vesicles of relatively uniform size, frequently with broken surfaces and occasionally of double structured appearance. The

Figure 20. Electron micrographs of the DOC-membranes (A) and the FT enzyme preparation (B). (Approximately 36, 500X)


A
B

DOC-membrane preparation corresponds to a preparation with a specific activity of about 50 μmoles P_i/hr/mg protein, and the FT enzyme preparation corresponds to one at about 270 μmoles P_i/hr/mg protein.

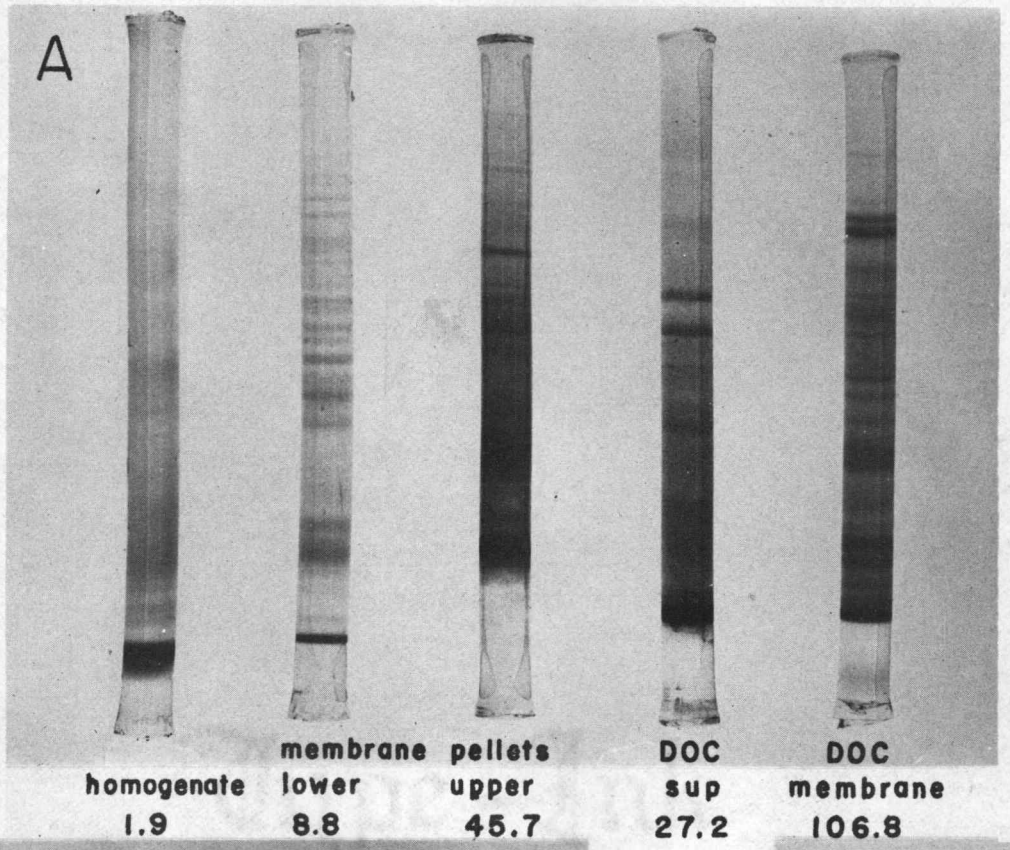
SDS Polyacrylamide Gel Electrophoresis

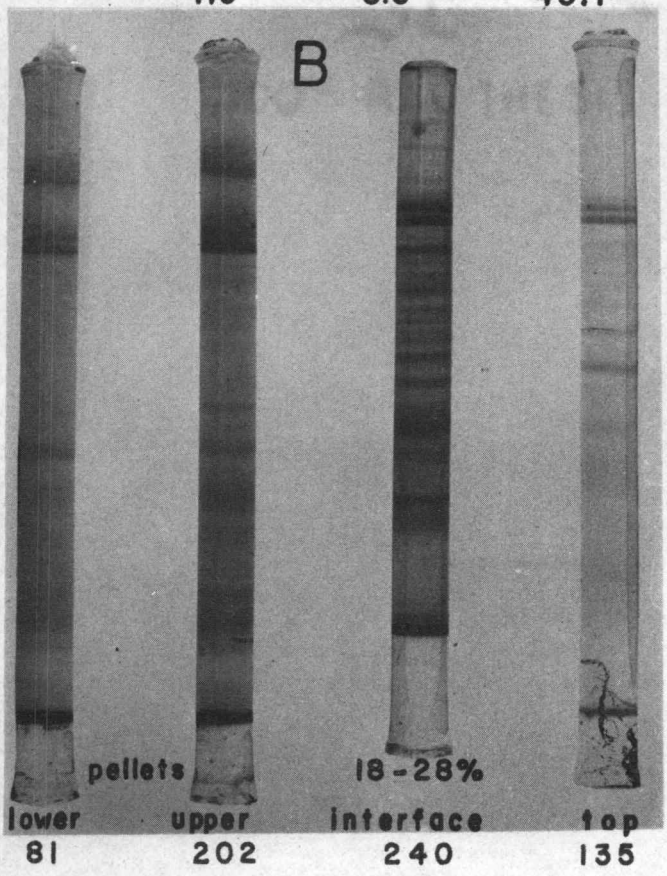
SDS polyacrylamide gel electropherograms of various fractions isolated during purification of the Na+K-activated ATPase are displayed in Figure 21. The purification up to the DOC-membrane enzyme is shown in Figure 21A. Figure 21B shows the various fractions obtained from a discontinuous gradient preparation of the FT enzyme, and Figure 21C illustrates the fractions obtained from recentrifugation of an upper pellet fraction prepared by the discontinuous gradient method. The gels shown in Figures 21A and B are representative, but derived from several preparations. Figure 21C is from one preparation. The protein in the regions marked a and b in Figure 21C are enriched during purification, and each region contains two protein bands.

Molecular weight calibration of the SDS gels is demonstrated in Figure 22. From the R_f values of regions a and b determined from several gels (indicated in Figure 22) the molecular weight estimates are as follows: a upper band 99-103,000 daltons, a lower band 93-97,500 daltons, b upper band 39-41,000 daltons, b lower

Figure 21. SDS polyacrylamide gels illustrating the protein composition of various fractions obtained during purification of the Na+K-activated ATPase. The various fractions obtained are those outlined in Methods and include the measured specific activity (μmoles P_i/hr/mg protein) of each.

A. Differential centrifugation fractions from the homogenate to the DOC-membranes.
B. Discontinuous sucrose gradient preparation of the FT enzyme.
C. Differential centrifugation of the FT enzyme (upper pellet), prepared by the discontinuous sucrose gradient method.


A
homogenate
1.9
membrane
lower
8.8
pellets
upper
45.7
DOC
sup
27.2
DOC
membrane
106.8


B
pellets
lower
81
upper
202
18-28%
interface
240
top
135

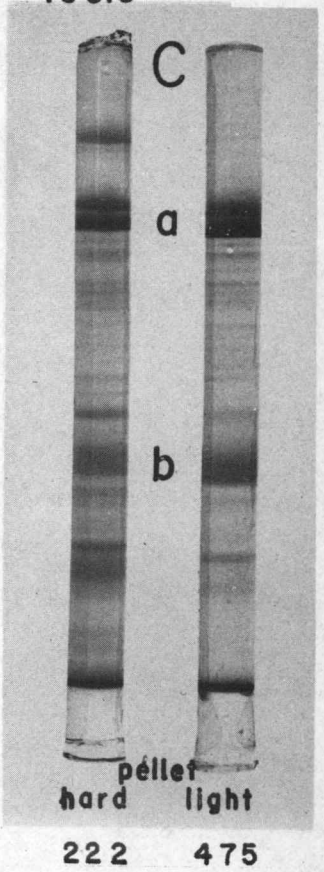
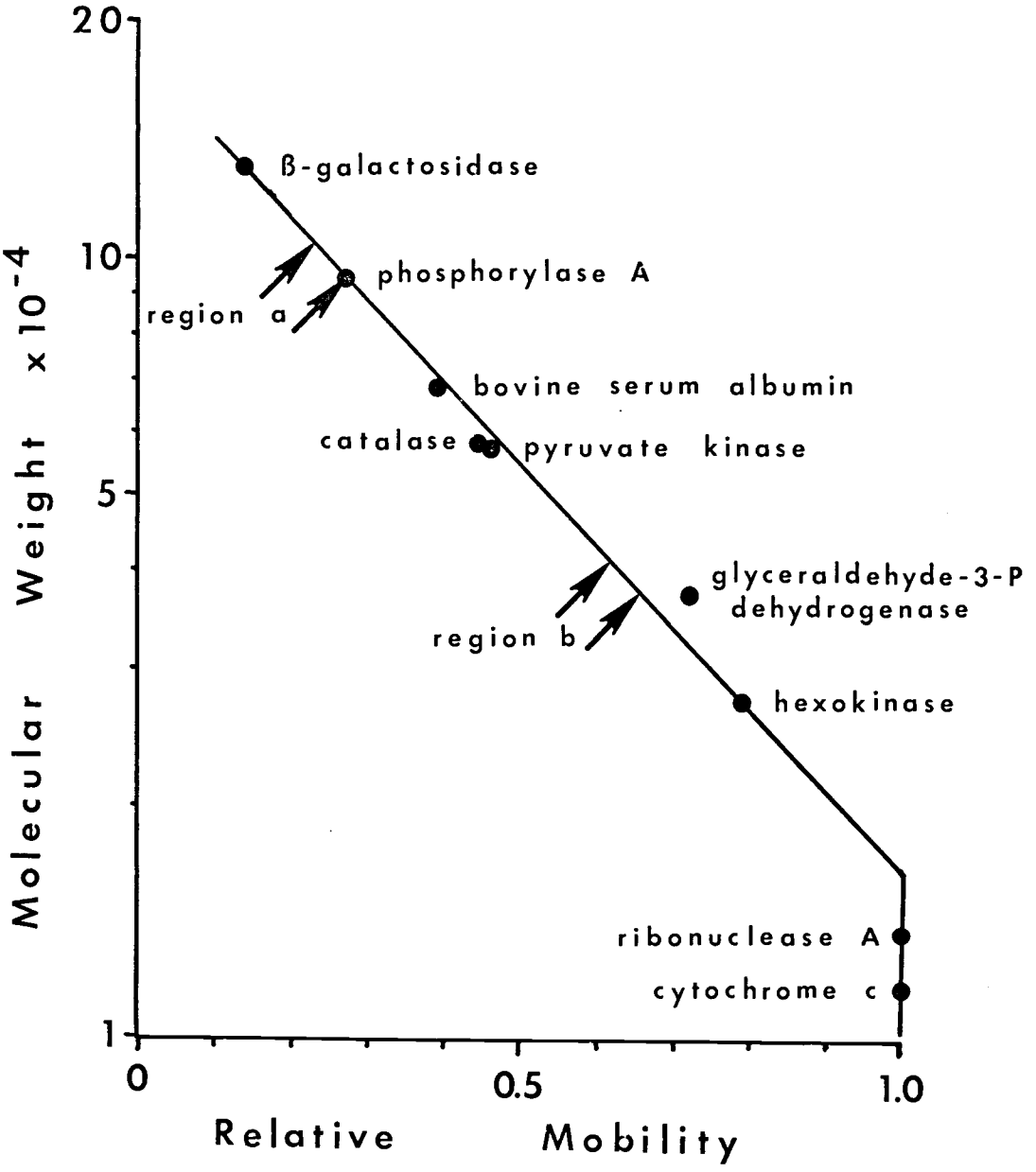

C
a
b
pellet
hard
222
light
475

Figure 22. Molecular weight calibration curve for the SDS polyacrylamide gels. Regions a and b are the R_f ranges including the doublet protein bands indicated in Figure 21 and 23 and observed to enrich during purification.


20
10
5
1
Molecular Weight x10-4
ß-galactosidase
phosphorylase A
region a
bovine serum albumin
catalase
pyruvate kinase
glyceraldehyde-3-P dehydrogenase
region b
hexokinase
ribonuclease A
cytochrome c
0
0.5
1.0
Relative Mobility

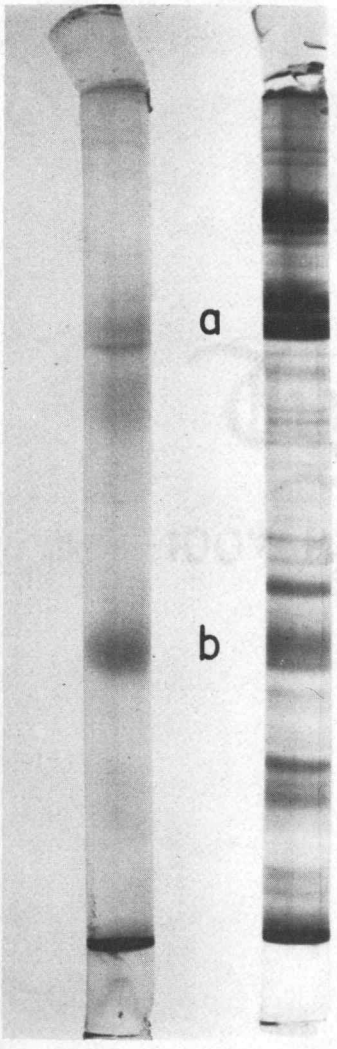
band 37-38, 500 daltons. The doublet banding is always observed, while other proteins in the same gel do not appear to follow this behavior. The doublet pattern also appears in gels at pH 7. 8 and pH 6. 8 for the Tris buffer in the running gel, or in gels where Tris is replaced with a borate buffer system. Resolution of the doublet in region b is not as good as in region a which is an expected characteristic of farther migrating components in such a system.

Preliminary examination of a SDS solubilized FT enzyme preparation (257 μmoles P_i/hr/mg protein) on the SDS-Urea gel system of Hoober (1970) was done in the laboratory of Norman Bishop (Botany Department, Oregon State University) courtesy of Jim Wong. This system demonstrated a similar protein profile on the gels and the easily recognizable equivalents of regions a and b displayed the doublet banding pattern. Molecular weight estimates from their standard curve calibrated between 17, 200 and 68, 000 daltons were as follows: region a, upper band 146, 000 daltons, lower band 127, 000 daltons; and region b, upper band 31, 300 daltons, lower band 30, 500 daltons. The molecular weight estimates for the protein bands in region a were determined by a presumed natural extension of the calibration curve.

Figure 23 shows gel stained for PAS positive material (glycoproteins). Components in both regions a and b are positively stained, while other proteins in the gel are not. The chick sera control

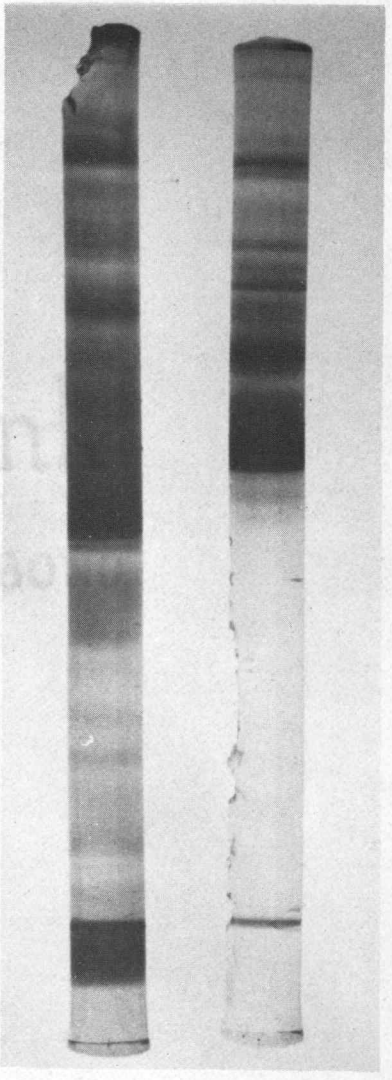
Figure 23. SDS polyacrylamide gels of the FT enzyme (222 μmoles P_i/hr/mg protein) showing gels stained for protein by comassie brilliant blue and glycoproteins by the PAS reaction. Chick sera is shown as a technique control.

FT membranes



PAS protein

chick sera



PAS protein

gels also indicate that the PAS reaction is not generally reacting with high concentrations of protein. The same characteristic staining pattern is obtained from numerous other preparations, including those at a specific activity of 408 and 475 μmoles P_i/hr/mg protein.

Quantitative densitometry was performed on 30 acrylamide gels processed with FT enzyme preparations with specific activities of 200-475 μmoles P_i/hr/mg protein. The ratio of region a to region b (mass ratio with doublets summed) was 1.99 ± 0.22 ($\overline{X}$±SEM). The specific activity of a theoretically pure enzyme preparation, assuming all of region a and region b to be the protein present in the Na+K-activated ATPase and the remainder as contaminants, was calculated as follows:

$$\text{sp. act. of pure enzyme} = \frac{\text{measured sp. act.}}{\text{(region a+b)/total}}$$

Such calculations from each of the 30 gels analyzed gave a mean of 833 ± 45 (±SEM) μmoles P_i/hr/mg protein. The mass ratio did not show a significant correlation with the measured specific activity, but did (P=0.005) with the values for the specific activity estimates for pure preparations. The analytical tendency (as noted in Methods) reduces true estimates of region a and thus minimizes the mass ratio, and at the same time maximizes the estimate of the specific activity of pure enzyme. The negative correlation observed thus contains at least a component of analytical bias in the same direction.

The amount of upper to lower band in a doublet varies from a dominance of the upper to a dominance of the lower with different enzyme preparations. The relative amounts are more clearly distinguishable in region a where resolution is greater. From 18 preparations examined quantitatively, the relative ratios of the upper and lower bands in a given region do not correlate with mass ratio (region a/region b), measured specific activity, or estimates of pure enzyme specific activity. The ratio of the upper band to the lower band in region a to the same ratio in region b doublets gave a positive and very significant correlation ($P \ll 0.001$).

Phosphorylation with γ^{32}P-ATP and SDS Gel Electrophoresis

Under the phosphorylation reaction conditions in which 12.5 μg of FT enzyme preparation is exposed to 0.135 μM γ^{32}P-ATP in an imidazole buffer system (pH 7.2) with a total volume of 0.25 ml, a peak requirement is observed for Mg^{++} at 0.20 mM and for Na^{+} at 75-100 mM. Nonspecific phosphorylation of about ten percent of maximum phosphorylation and is the same level for 10-80 mM K^{+}. Under optimum cation concentration of 0.20 mM $MgCl_2$ and 100 mM NaCl (or 20 mM KCl), maximum binding of γ^{32}P-ATP is observed between six to ten seconds and remains constant for at least 60 seconds. Addition of 0.05 ml of 100 mM K^{+} to the Na^{+} reaction mixture after 25 seconds of phosphorylation, and subsequent

incubation for ten or twenty seconds reduces binding to the nonspecific phosphorylation level.

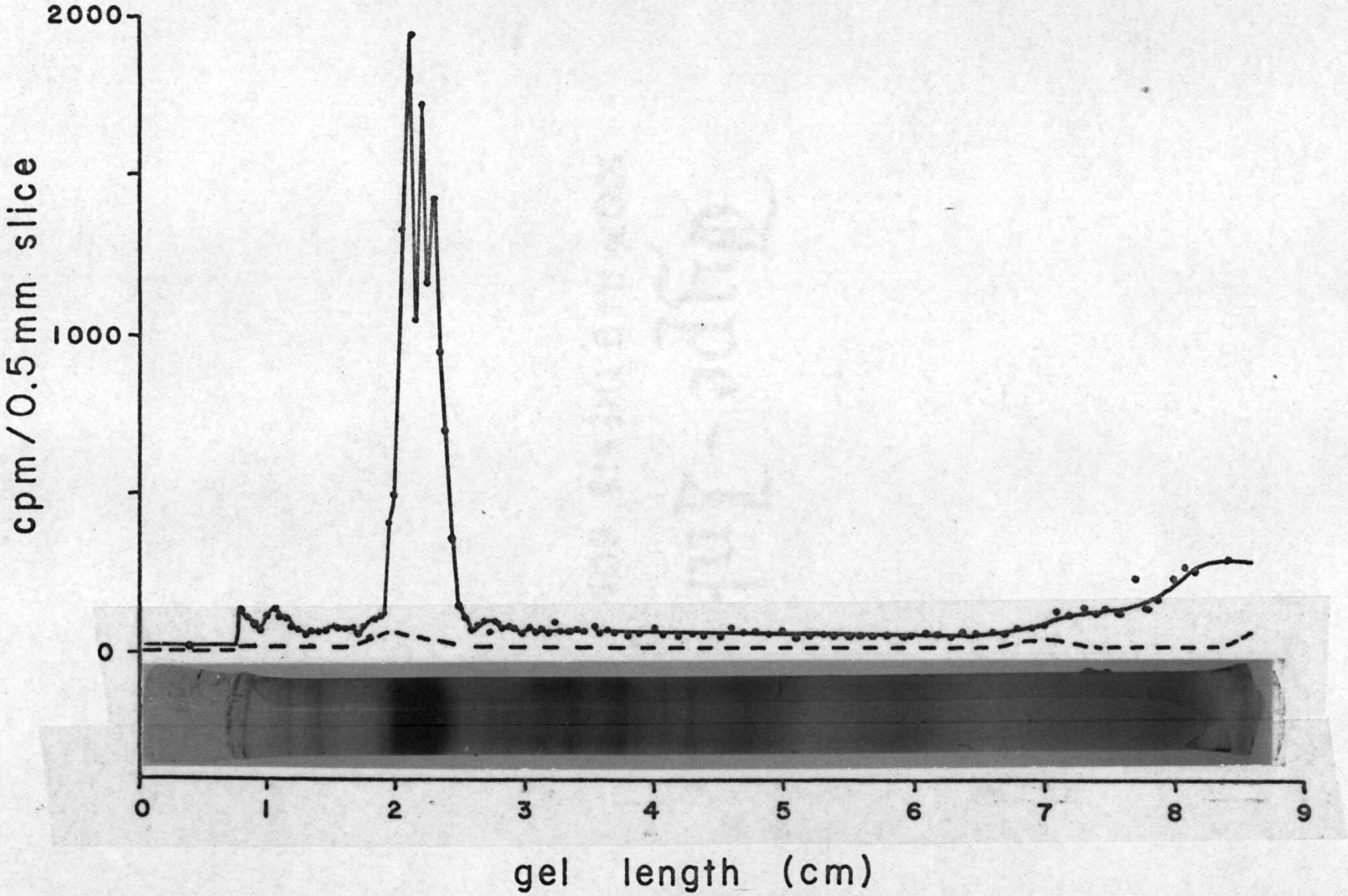
The binding data can be used to estimate a turnover number for the enzyme by the following relations:

$$\text{Turnover number } (\text{min}^{-1}) = \text{Mw } \left(\frac{\mu\text{g protein}}{\mu\text{mole ATP}}\right) \text{ X specific activity } \left(\frac{\mu\text{moles } P_i/\text{min}}{\mu\text{g protein}}\right)$$

As purity is reached the molecular weight estimate appraoches a minimum and the specific activity approaches a maximum. The relation assumes that ATP binding is complete. In practice, however, the value of 100% binding is only approached as a limit such that the calculated turonover number is actually a maximum value. Binding on one enzyme preparation was estimated by the disc method used for kinetic studies. Assuming 20% loss of protein the turnover number is 4, 300 min^{-1} for this preparation. Another enzyme preparation was examined by centrifuging the TCA precipitate (without carrier protein) as was done with the material prepared for the SDS polyacrylamide gels. The precipitate was washed once with TCA and dissolved in 0. 5 M NaOH, and the protein and radioactivity measured. This preparation gave a turnover number of 5, 050 min^{-1}.

Figure 24 shows the distribution of radioactivity from gels containing the Na dependent phosphorylated enzyme and the K dependent dephosphorylated enzyme. A protein stained Na gel is shown

Figure 24. Distribution of radioactivity in SDS polyacrylamide gels prepared from γ^{32}P-ATP labeled FT enzyme preparation in the presence of Na (solid line) or K (broken line). A protein stained Na gel is shown below.


2000
1000
0
cpm/0.5mm slice
0
1
2
3
4
5
6
7
8
9
gel length (cm)

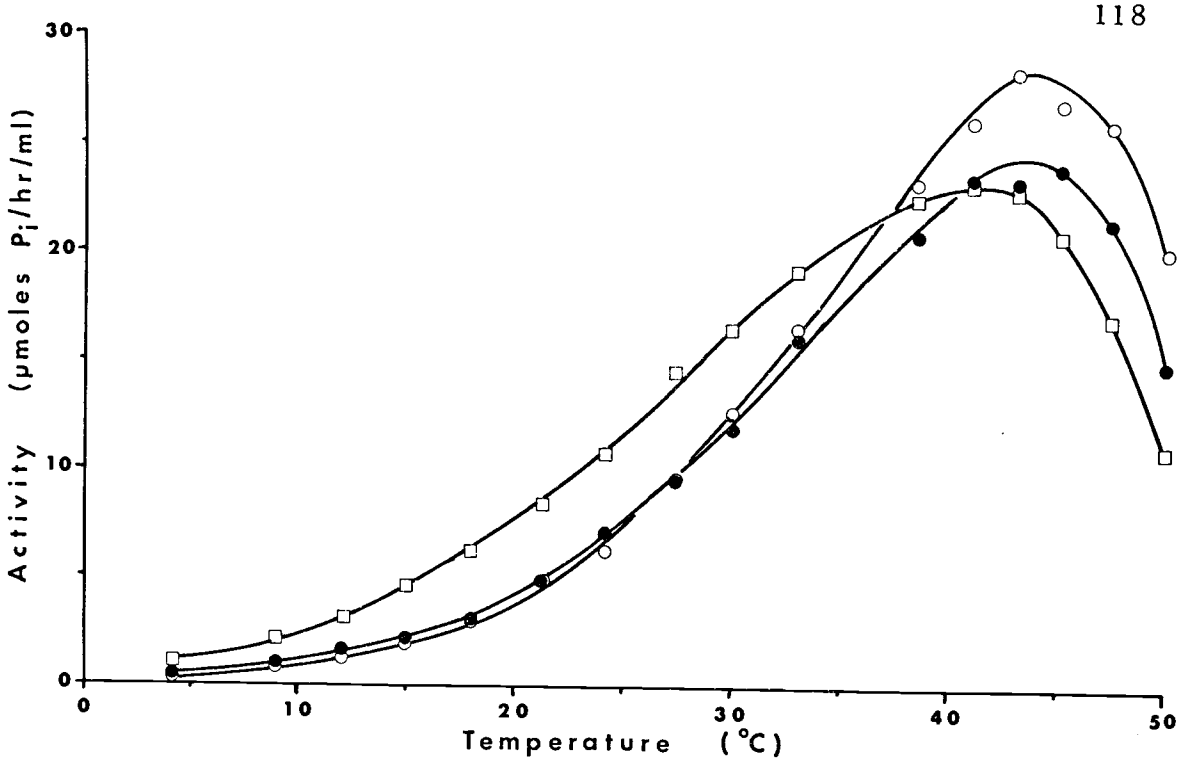
above. Na dependent phosphorylation by γ^{32}P-ATP that is labile to dephosphorylation in the presence of K appears over both of the bands in region a. The scatter of radioactivity in the lower portion of the Na gel is not associated with any protein bands. This is presumed to be an artifact of the gel system, where the high pH (8. 8) of the running gel causes breakdown of the labile phosphoenzyme intermediate (Bader, Sen and Post, 1966; Hegyvary and Post, 1971; Ratanabanangkoon and Hokin, 1973).

Kinetic Properties of the Membrane and Partically Purified Freeze-thaw Enzyme Preparations

Figure 25 illustrates the temperature profile for the membrane DOC-membrane and FT enzymes. The two enzymes have similar profiles with an optimum at 43° C, as was previously obtained for a mitochondrial enzyme preparation (Figure 3, Section II). The temperature profile for a DOC-membrane preparation is uniquely different and shows a slightly lower temperature optimum of 40-41° C. Arrhenius plots of this temperature data are shown in Figure 26. Breaks in the Arrhenius plots occur at transition temperatures in the range 27-29° C for all enzyme preparations. The DOC-membranes, however, show an additional transition temperature at 15° C. Comparison of the various thermal properties of these three enzyme preparations is summarized in Table 17. Previous analysis of P_i

Figure 25. Effect of temperature on the Na+K-activated ATPase activity from membrane (0----0), DOC-membrane (□ ----□), and FT enzyme (●----●) preparations.

Figure 26. Arrhenius plot of the temperature data for the membrane (0----0), DOC-membrane (□ ----□), and FT enzyme (●----●) preparations.


Activity (μmoles P_i/hr/ml)
30
20
10
0
0
10
20
30
40
50
Temperature (°C)

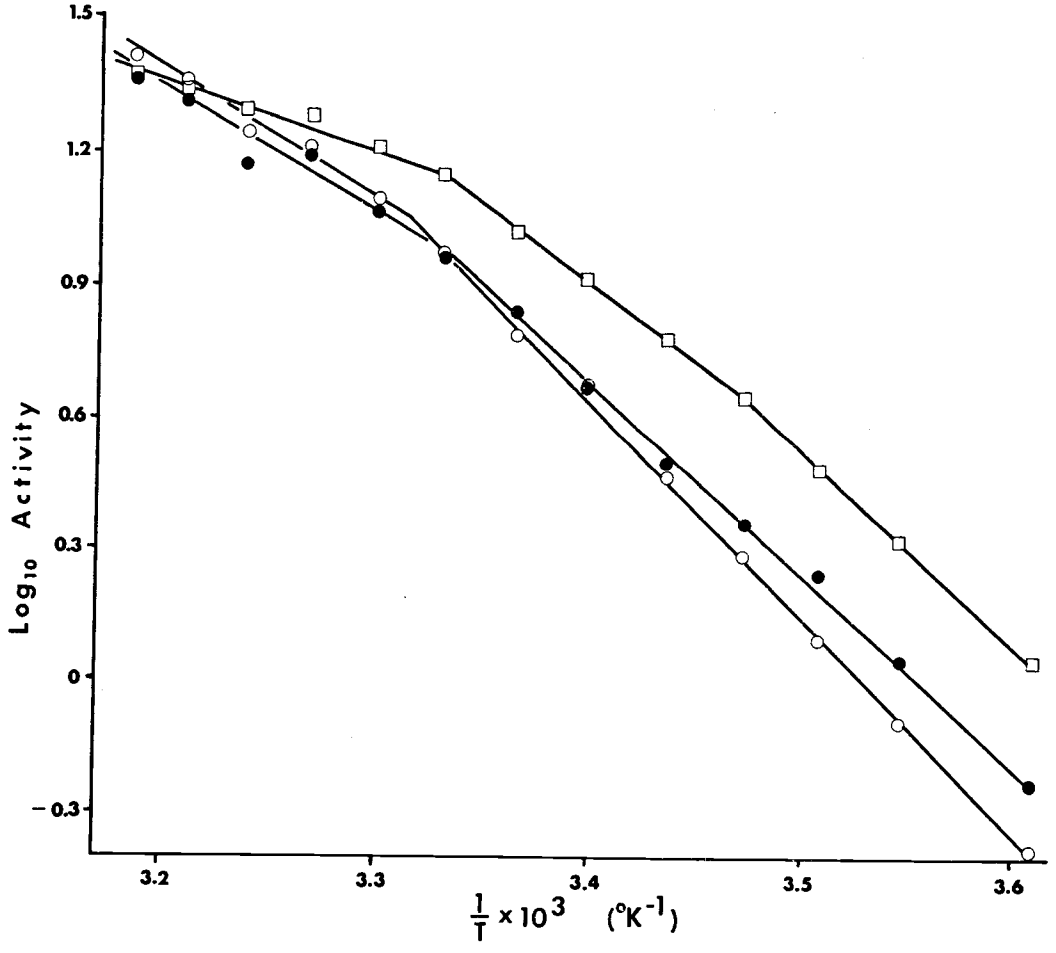

Log_{10} Activity
1.5
1.2
0.9
0.6
0.3
0
−0.3
3.2
3.3
3.4
3.5
3.6
$\frac{1}{T} \times 10^3$ (°K^{-1})

Table 17. Thermal properties of the membrane, DOC-membrane and FT enzyme preparations

Properties	Membranes	DOC-Membranes		FT Enzyme
Arrhenius plots				
Transition temp. (°C)	28.8	27.0	15.2	27.0
Energy of activation (kcal/mole)				
above transition	13.3	7.3	16.2	12.8
below transition	22.6	16.2	20.4	20.1
Q_{10} for temperatures				
10-20°C	5.0	3.1		3.2
20-30°C	3.1	2.2		2.8
30-40°C	2.0	1.4		1.9

release with time (Section II) indicated that linearity was retained beyond 40 minutes at 37° C (Figure 2), but was lost within 20 minutes at 45° C. Reexamination of the release of P_i as a function of time at 42° C is shown in Figure 27 for the membrane and FT enzyme preparations. Linearity in both preparations is retained for at least 30 minutes at 42° C.

The effect of pH on the enzyme activity from membrane and FT enzyme preparations is shown in Figure 28. The pH profile is very similar for both preparations with optimum activity at pH 7.2 and a precipitous decline below pH 7. This is in agreement with the profiles obtained previously for the mitochondrial fraction preparations in Section II (see Figure 4).

Evaluation of the effect of ionic strength at a constant Na/K ratio of 4/1 is shown in Figure 29 for the membrane and FT enzyme preparations. The two curves are nearly identical for the two preparations. The major optimum occurs between 150 and 200 mM for total $Na^+ + K^+$. A shoulder of activity is observed in both preparations at a total ionic strength of 440 mM $Na^+ + K^+$. This agrees with the similar experiment shown in Figure 12 (Section II) for a mitochondrial fraction preparation except that the second optimum peak is reduced in the preparations of Figure 29. The effect of Na/K ratios at a constant ionic strength of 160 mM $Na^+ + K^+$, is shown in Figure 30. The profile obtained is similar to that previously observed for the mitochondrial

Figure 27. Ouabain sensitive phosphate release as a function of time at 42° C for membrane (0----0) and FT enzyme (●----●) preparations.

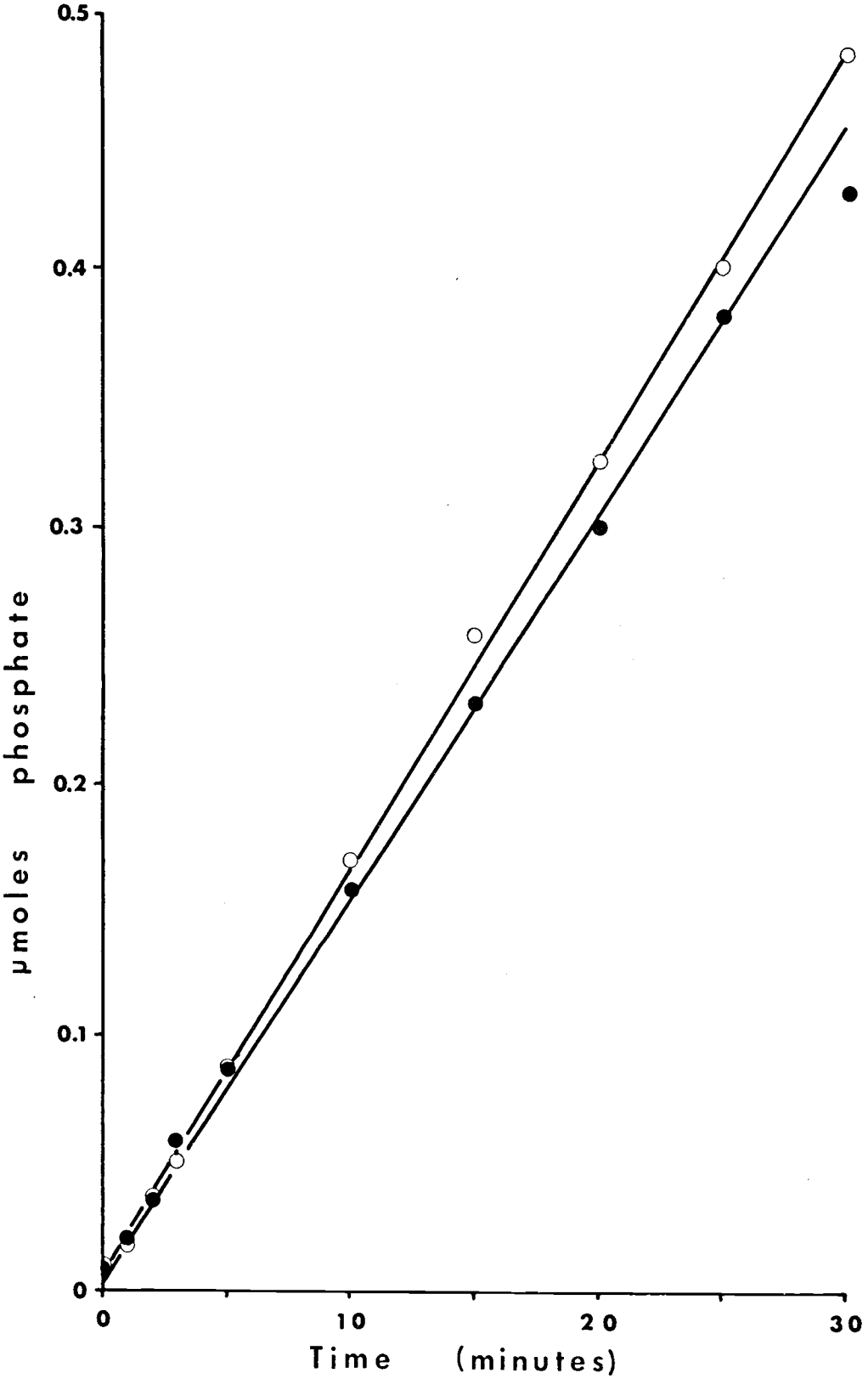

0.5
0.4
0.3
0.2
0.1
0
µmoles phosphate
0
10
20
30
Time (minutes)

Figure 28. Effect of pH using an imidazole-histidine buffer system on the activity of Na+K-activated ATPase from membrane (0----0) and FT enzyme (●----●) preparations.

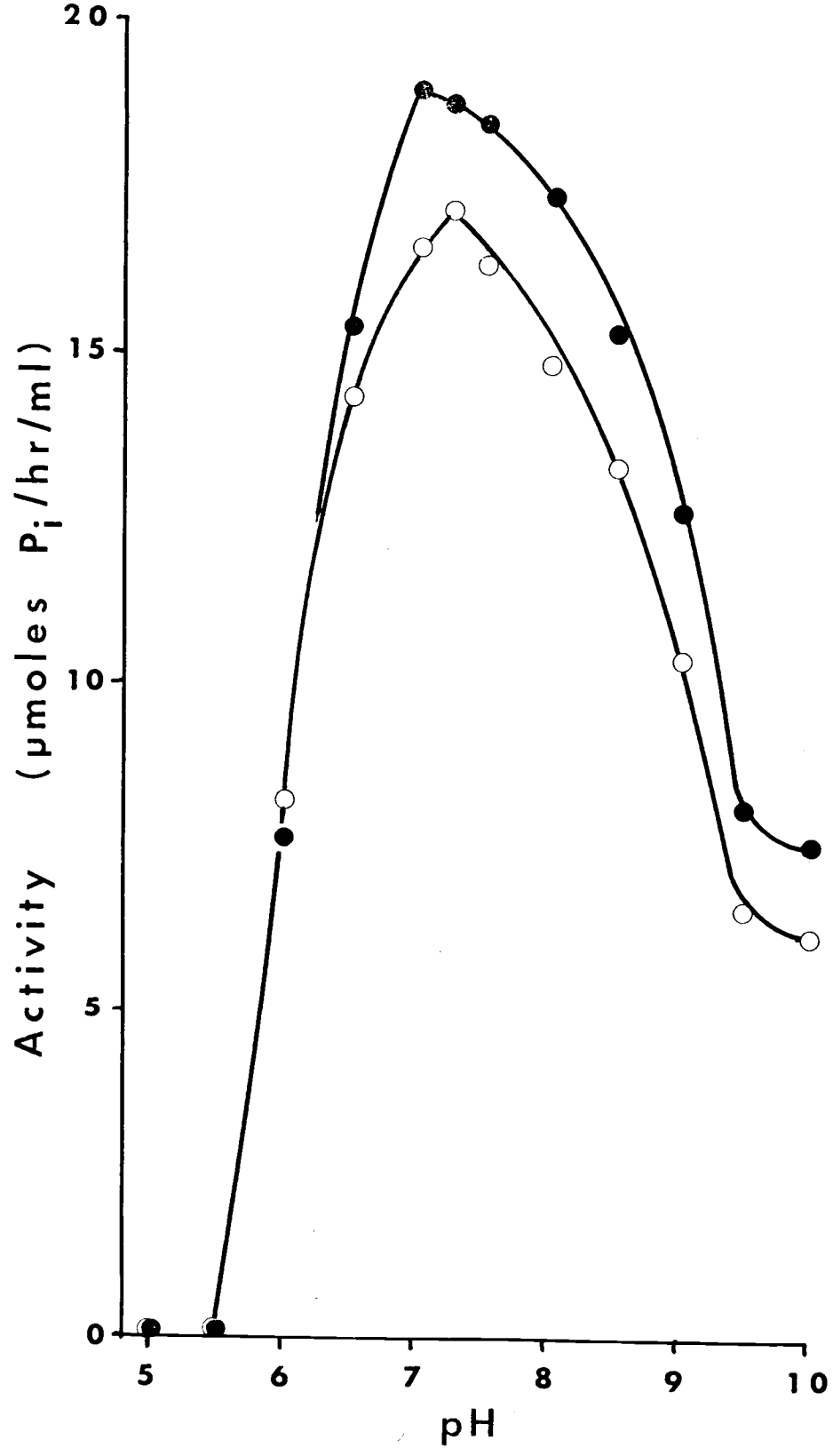

20
15
10
5
0
Activity (μmoles P_i/hr/ml)
5
6
7
8
9
10
pH

Figure 29. Effect of ionic strength, at a constant Na/K ratio of 4/1, on the activity of the membrane (0----0) and FT enzyme (●----●) preparations. The ionic strength refers to the total of Na and K.

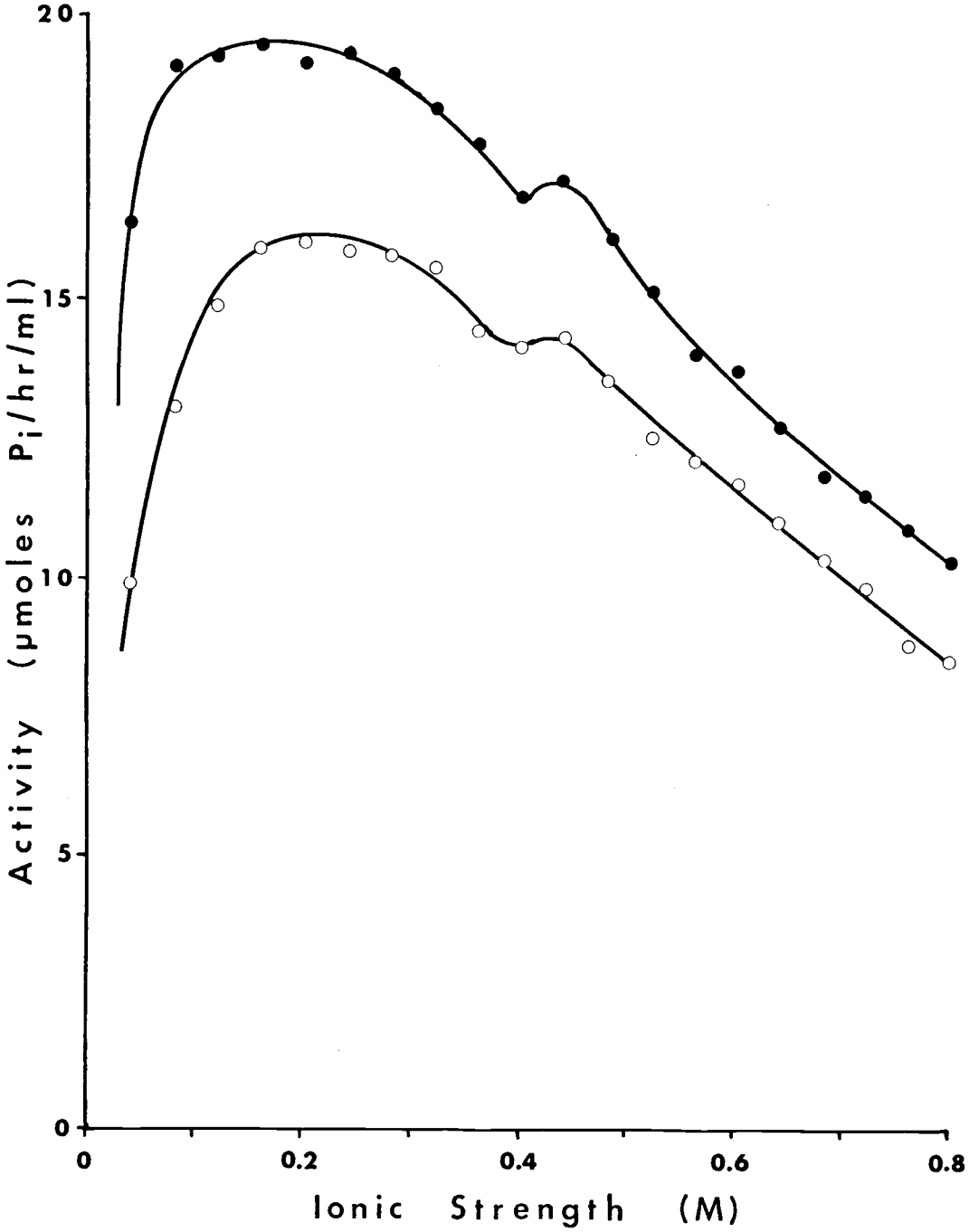
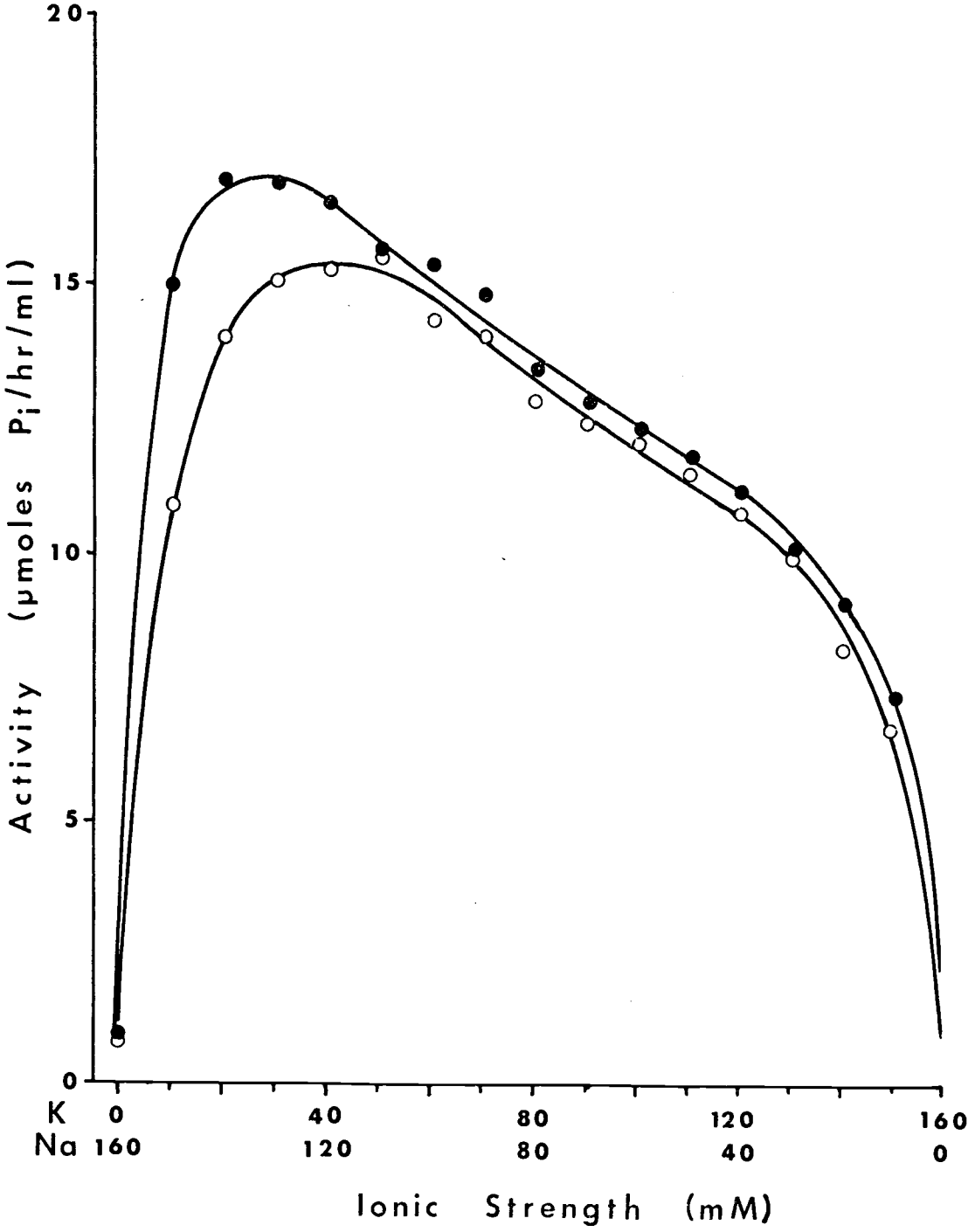

20
15
10
5
0
Activity (μmoles P_i/hr/ml)
0
0.2
0.4
0.6
0.8
Ionic Strength (M)

Figure 30. The effect of varying the Na/K ratio, at a constant ionic strength of 160 mM Na+K, on the activity of the membrane (0----0) and FT enzyme (●----●) preparations.

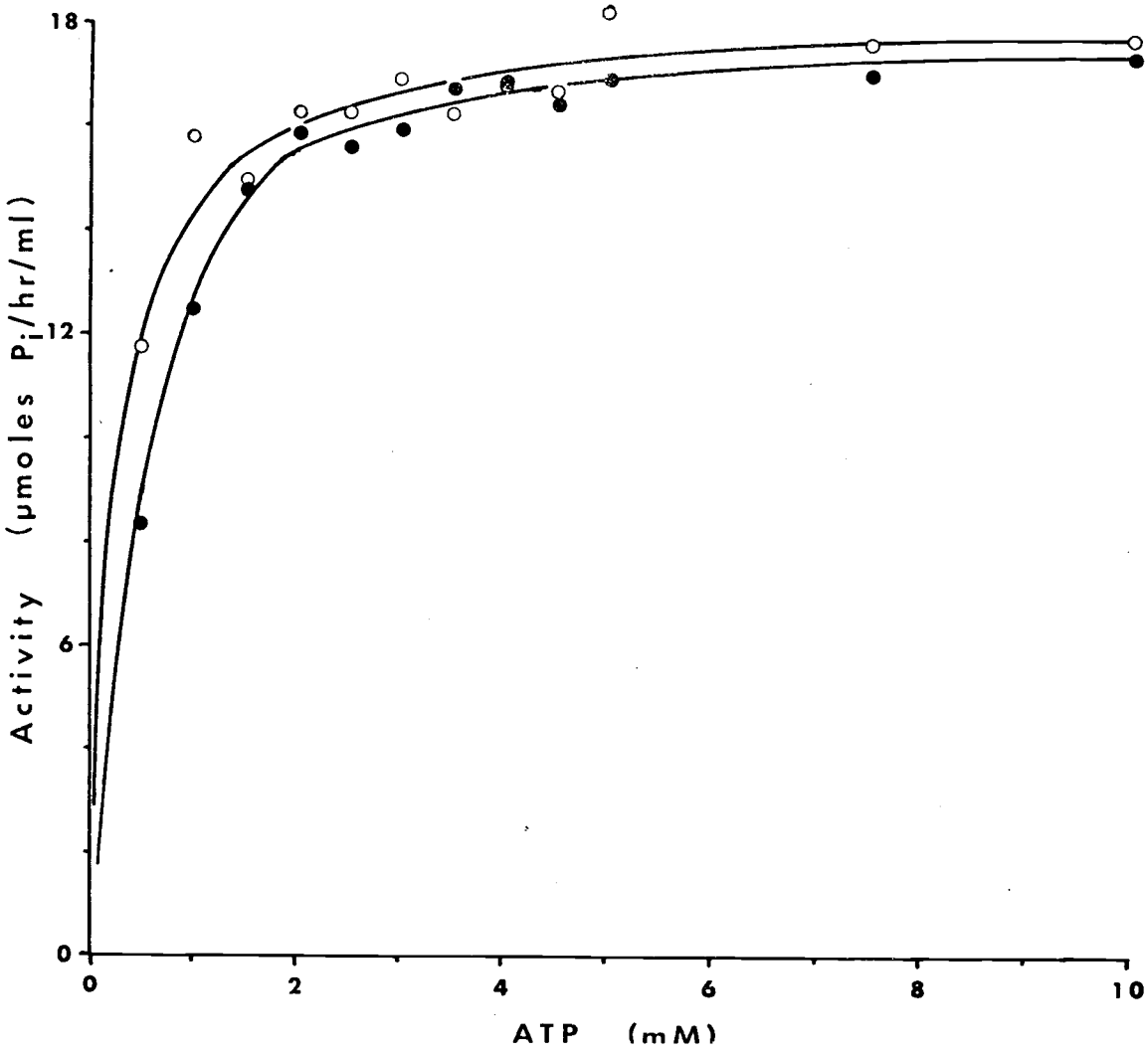

20
15
10
5
0
Activity (μmoles P_i/hr/ml)
K 0 40 80 120 160
Na 160 120 80 40 0
Ionic Strength (mM)

fraction preparation at total ionic strengths of 250 mM and 400 mM $Na^+ + K^+$ (Figures 10A and 10B, respectively). The FT enzyme shows a tendency to optimize at slightly higher Na/K ratios than the membrane enzyme. Optimum in both cases, however, includes the 4/1 ratio previously obtained (Section II).

From the previous kinetic studies in Section II, the substrate (ATP) appeared to produce optimum activity when present with $MgCl_2$ at a Mg^{++}/ATP ratio of 2/1. Maintaining a constant Mg^{++}/ ATP ratio of 2/1, the effect of ATP concentration on the activity of Na+K-activated ATPase from membrane and FT enzyme preparations gives the curves illustrated in Figure 31. Lineweaver and Burk double reciprocal plots of this data give an apparent Km for both enzyme preparations at 0.25 mM ATP.

From the above kinetic data, it is apparent that the enzyme assay mixture developed in Section II for mitochondrial fraction and microsomal fraction preparations of the brine shrimp Na+K-activated ATPase is suboptimal when applied to the membrane preparation as in this section, and subsequent partially purified enzyme preparations. Suboptimal conditions include ionic strength and temperature. Additionally there is concern that as K^+ and high temperatures antagonize the inhibitory effects of ouabain (Tobin and Sen, 1970; Akera, 1971; Hansen, 1971; Lee and Klaus, 1971; Hansen and Skou, 1973; Yoda, 1973), a true total ATPase activity that is due to the

Figure 31. The effect of substrate concentration on the activity of the membrane (0----0) and FT enzyme (●----●) preparations. The substrate, ATP, is maintained with Mg^{++} at constant molar ratio of 2/1 Mg^{++}/ATP.


18
12
6
0
Activity (μmoles P_i/hr/ml)
0
2
4
6
8
10
ATP (mM)

Na+K-activated ATPase enzyme is not being assessed. Based on these considerations a new reaction mixture was formulated as follows: 120 mM NaCl, 30 mM KCL, 10 mM $MgCl_2$, 5 mM Na_2ATP, 100 mM imidazole (pH 7.2), with ouabain (0.5 mM) controls containing 10 mM $MgCl_2$, 5 mM Na_2ATP and 100 mM imidazole (pH 7.2). Concentrations are those in the reaction, and the reaction is run at 42° C for 20 minutes. Comparison of the relative activity of the membrane, DOC-membrane and FT enzyme preparations by this New Method and the Old Method (Section II) is demonstrated in Table 18. Unless otherwise specified all enzyme activities in this thesis are determined by the Old Method.

Discussion

The preparation of Na+K-activated ATPase rich membranes from homogenates of brine shrimp nauplii is compared in Table 19 to similar preparations from other common sources used in the purification of this enzyme. The yield and specific activity of the brine shrimp membrane preparation compares very favorably, particularly when considering the homogenate is from whole animals and not excised tissues. Two hundred grams (fresh weight) of nauplii can be conveniently processed by one person in a day, and the resulting membrane enzyme preparation can be stored frozen for a month without loss of activity (Table 15). For partial purification of the

Table 18. Comparison of the Old and New Method for enzymatic assay of the Na+K-activated ATPase in the membrane, DOC-membrane and FT enzyme preparations. (See text for description of methods).

Enzyme Preparation	Activity (μmoles Pi/hr/ml)		Ratio of New/Old
	Old Method	New Method	
Membranes	19.28	27.54	1.428
DOC-membranes	20.68	27.00	1.305
FT enzyme	17.50	26.35	1.505

Table 19. Comparison of the yield and specific activity of Na+K-activated ATPase rich membranes from different sources

Source	Na+K-activated ATPase: Total yield (μmoles Pi/hr/g tissue)	Percent of Homogenate	Specific Activity (μmoles/hr/mg protein)	Reference
Canine renal inner medulla	160	--	39	Kyte, 1971a
Canine renal outer medulla	408	--	59	Lane et al., 1973
Bovine brain	169	45	18	Uesugi et al., 1971
Rabbit renal outer medulla	520[1]	42	53[1]	Jørgensen, Skou and Solomonson, 1971
Dogfish rectal gland (fresh)	1,790	31	400	Hokin et al., 1973
(frozen)	502	29	160	
Brine Shrimp nauplii	100[2]	30	56[2]	This study

[1]Values are corrected for 5 fold activation by DOC.
[2]Values are those corrected to the New Method of enzymatic assay (see Table 18).

enzyme from these membranes, DOC treatment is completed before freezing. Extraction of the Freeze-Thaw vesicular enzyme is completed the next morning, or as is convenient within three weeks. Isolation of the FT enzyme preparation by differential centrifugation permits 100 fold purification of this particulate enzyme to a specific activity of 300-450 μmoles P_i/hr/mg protein (New Method) without the need of expensive preparative ultracentrifuges and rotors.

The behavior of the brine shrimp Na+K-activated ATPase to detergents is unique in many respects. The optimum concentrations (Table 8) for enzyme activity compares closely with those obtained for this enzyme from mammalian kidneys (Banerjee et al., 1970; Jørgensen and Skou, 1971), yet lack the marked activation. Optimum concentrations for the detergents appear to be largely independent of protein concentration and closely parallel their intrinsic critical micelle concentration (Jørgensen and Skou, 1971). The concentration at optimum is thus expected to be principally a property of the detergent, whereas the extent of activation is considered a property of the membrane in its interaction with the detergent. The extent of DOC activation of mammalian kidney Na+K-activated ATPase is several fold, while in the brine shrimp enzyme it is antagonistic (Figure 18). The brine shrimp enzyme is stable in the presence of 0.075% DOC (optimum concentration) for several hours at 0° C, and shorter periods at room temperature (Table 10). Centrifugation to

pellet the DOC treated enzyme results in both further purification (about 2.5 fold) and activation (about 150%) after resuspension in DOC-free homogenizing media. The activation by detergents, at least for DOC, has been interpreted from binding studies (Jørgensen and Skou, 1971) and electron microscopy (Rostgaard and Møller, 1971) to be due to exposure of latent enzyme sites by opening of vesicular structures. Electron microscopy of a pellet from the DOC treated brine shrimp enzyme (Figure 20A) suggest that the effect of DOC is different in this situation. Extensive and elaborate membranes with interspersed smaller vesicles characterize this pellet. Formation of these membrane contours in the presence of DOC would presumably reduce access of some enzyme sites. Resuspension of the pellet in DOC free homogenizing media would permit dispersal of these structures apparently exposing sites previously hidden. It is interesting in this respect that the DOC-membrane enzyme displays different thermal properties from the membrane or FT enzyme preparations (Figures 25 and 26, Table 17), and is extremely sensitive to secondary detergent treatment.

Electron microscopy of a differential centrifugation preparation of the FT enzyme reveals a preponderance of small vesicular structures and membrane fragments or open vesicles (Figure 20B). It would seem that freezing promotes formation of enzyme rich aggregates (Petit and Eolidin, 1974; Wayne L. Hubbell and C. Fred Fox in

seminars at OSU, 1974) that are released as small fragments or vesicles after gentle thawing. Sucrose density gradient centrifugation of a frozen and thawed DOC-membrane preparation (Figure 19A) indicates that heavy particles or membranes of low enzyme content are present and that homogenization disturbs the desirable separation (Figure 19B). The appearance of the thin sectioned membranes of the FT enzyme preparation is very similar to the freeze-thaw activated ox kidney cortex enzyme examined by Rostgaard and Møller (1971), although no activation is observed with the brine shrimp enzyme, due to freeze-thaw alone. Activation occurs prior to freezing by resuspension of DOC treated membranes in homogenizing media free of the detergent.

During partial purification of the brine shrimp Na+K-activated ATPase two regions of protein, as detected on SDS polyacrylamide gels, are conspicuously enriched (regions a and b, see Figure 21). Other proteins are progressively reduced, although protein traveling at the position of the tracking dye is still present in substantial quantities in the most purified preparation (final gel in Figure 21C, specific activity 475 μmoles P_i/hr/mg protein). The protein in regions a and b is uniquely a doublet with average molecular weights estimated from their R_f values at 95, 250 and 101, 000 daltons for the region a and 37, 750 and 40, 000 daltons for the region b doublets. Both protein bands in region a contain the catalytic subunit(s) of

Na+K-activated ATPase as judged by its phosphorylation in the presence of Na^{+}, Mg^{++} and ATP, which is dephosphorylated in the presence of K^{+} (Figure 24). The catalytic subunit(s) is only slightly larger in molecular weight estimate by SDS polacrylamide gels than that from other organisms (Kyte, 1971b; Uesugi et al., 1971; Hokin et al., 1973; Lane et al., 1973). Protein in region b is PAS positive in agreement with the enrichment of a glycoprotein during purification of this enzyme from other organisms (Kyte, 1972a; Hokin et al., 1973; Lane et al., 1973), but is of lower molecular weight as estimated by SDS polyacrylamide gel electrophoresis.

Region a also showed a PAS positive reaction in both of the bands (Figure 23). This characteristic has not been observed in the large subunit (catalytic subunit) of the Na+K-activated ATPase from other organisms, which suggest that it is a contaminant in these preparations. If it is a contaminant, it is a large molecule with properties that permit its appearance in both of the bands of region a, or it is a small molecule which tenaciously resists dissociation in SDS from the large subunits. It is present in gels containing enzyme preparations with specific activities ranging from 200 to 475 μmoles P_i/hr/mg protein. In one gel the FT enzyme preparation was treated with periodic acid prior to SDS gel electrophoresis. In this gel protein in region a was absent, and staining at the interface of the stacking and running gel suggested that the region a protein

aggregated. The remainder of the gel showed the normal protein staining pattern including that of region b and the tracking dye position.

The double banding characteristic of both the large (region a) and small (region b) subunits are another unique feature of the brine shrimp Na+K-activated ATPase. Figure 24 demonstrates that both of the bands in region a contain the catalytic subunit(s), which strongly implicates the existence of isozymes of this enzyme in the brine shrimp. Correlation analysis on the quantity of each band supports the view that the upper bands in each doublet are enriched as a pair, as are the lower bands of each doublet. The double banding is not considered an artifact of the gel system, since it appears in gels of different pH's (7.8 and 6.8), buffers (borate), and dispersing agents (SDS-urea), and since other proteins in the gel pattern are not conspicuously double banded. However, the possibility that they are the result of some peculiar and unknown artifact unique to the preparation of this enzyme from brine shrimp, cannot be entirely eliminated.

The ratio of the large to small subunits (total amount of region a to total amount of region b) was calculated from gel scans to be $1.99:1 \pm 0.22$ ($\overline{X} \pm$ SEM, n=30). The large amount of variability is in part analytical, but may also indicate that the two subunits may not be purified strictly in parallel. The amount of variability (if any) has not been reported by other investigators for the mass ratio

estimates in their enzyme preparations. Hokin et al. (1973) mentioned that with the purification of the shark rectal gland enzyme the two subunits were purified "more or less" in parallel. Their gel scans gave a mass ratio of 3.7:1, while column chromatography on Sephadex G-150 gave a mass ratio of 2.36:1. The co-enrichment of both the large and small subunits during purification of the enzyme in a wide range of species, exists as the major line of evidence supporting the contention that the small glycoprotein is part of the Na+K-activated ATPase. Studies with dimethyl suberimidate indicate that these two subunits reside physically very close in purified preparations (Kyte, 1972a). A. E. Shamoo (N. Y. A. S. Symposia on Na-K ATPase, 1973) reported on the isolation of a Na^+ specific ionophore by limited tryptic digests of the small glycoprotein subunit. He also claimed that a molar ratio of 1:2, large to small combination of pure subunits gave a Na^+-specific voltage-independent ionophoric activity in bilayers. However, all of the enzyme ligands apparently effect sulfhydryl group reactivity on the catalytic subunit (Hart and Titus, 1973), which suggests it contains all the binding sites.

From scans of the acrylamide gels, the specific activity of a theoretically purified preparation of brine shrimp Na+K-activated ATPase is 833±45 ($\overline{X}$±SEM, n=30) μmoles P_i/hr/mg protein. Analytical bias suggests that this estimate slants towards a minimum

value. The turnover numbers estimated from two preparations were 4300 and 5040 min^{-1}. Assuming the molecular weight of a pure enzyme is 250,000 daltons, as generally accepted from the radiation inactivation studies of Kepner and Macey (1968, 1969), the maximum specific activity of a theoretically pure enzyme can be calculated from the turnover numbers at 1030 and 1210 μmoles P_i/hr/mg protein, respectively.

Table 20 summarizes and compares the molecular characteristics and purification data from several sources of the enzyme. The discrepancy in the specific activities of purified canine kidney enzyme reported by Kyte (1971a) and Lane et al. (1973) may be explained by the different assay conditions used (viz., pH 6.8 vs 7.1; Na/K ratios 5/1 vs 10/1; and Mg/ATP ratios 2/1 vs 1/1, respectively). Lane et al. (1973) were also concerned over the instability of Kyte's (1971a) preparations. The high specific activity of Jørgensen's rabbit kidney enzyme preparation seems peculiar. The activity of the brine shrimp enzyme reported in Table 20 is corrected to maximal enzyme activity using the New Method for assay (see Table 18). The specific activity for the theoretically pure enzyme based on the gel scans is 1250 μmoles P_i/hr/mg protein. Similar values obtained from the turnover numbers and the molecular weight assumption of 250,000 daltons are 1550 and 1820 μmoles P_i/hr/mg protein. The turnover numbers, likewise corrected, have the values of 6460 and 7590 min^{-1}. The

molecular characteristics of the brine shrimp enzyme appear to be in close agreement with others shown in Table 20.

Attempts to assign mole ratios for the two subunits depends not only on the troublesome mass ratio estimate, but also on the molecular weight estimates of the subunits. Radiation inactivation of the K-stimulated microsomal alkaline phosphatase gave an apparent molecular weight of about 140,000 daltons (Kepner and Macey, 1968). This enzyme is considered part of the Na+K-activated ATPase reaction, viz., the final K dependent dephosphorylation step, and presumably takes place on the large subunit. Kyte (1972a) found that his large subunit, which had a molecular weight estimate of 84,000 by SDS gel electrophoresis, was estimated as 135,000 by gel filtration techniques. Also the small subunit showed a decrease in molecular weight from 57,000 to near 40,000 by gel filtration. The SDS-urea gel system produced similar results with the brine shrimp enzyme. Kyte (1972a) favored a molar ratio of one large to two small subunits. Lane et al. (1973) favored a molar ratio of one large to one small subunit. Hokin et al., 1973) and Jørgensen (N. Y. A. S. Symposia on Na-K ATPase, 1973) favored a molar ratio of two large to one small subunit. Data on the brine shrimp enzyme is most consistent with a molar ratio of one large to two small subunits.

The kinetic properties of the FT enzyme are very similar to those of the membrane enzyme. Similar results have been obtained

Table 20. Molecular characteristics and purification data of the Na+K-activated ATPase from various sources

No.	Source	Subunit Characteristics[1] Molecular weight Large	Small	Mass ratio Large/Small	Turnover Number (min^{-1})	Method of Estimate
1.	Canine kidney medulla	84,000	57,000	1.7:1	3,000	^{32}P-ATP binding
					3,300	Ouabain inhibition
2.	Canine kidney medulla	89,000	56,000	1.88:1	6,480	^{3}H-Ouabain binding
3.	Bovine brain	94,000	53,000	--	--	--
4.	Dogfish rectal gland	97,000	55,000	3.7:1	6,300	^{32}P-ATP binding
5.	Rabbit kidney medulla	--	--	--	--	--
6.	Rabbit kidney medulla	96,000	57,000	2:1 - 4:1	9,100	^{14}C-ATP binding
					8,800	^{3}H-Ouabain binding
					4,400	^{32}P-ATP binding
7.	Electric eel	97,000	47,000	--	--	--
8.	Brine shrimp nauplii[2]	101,000	40,000	2:1	6,460	^{32}P-ATP binding
		95,200	37,800			

[1] The subunit characteristics are based on analysis of SDS polyacrylamide gels.

[2] Turnover number and specific activity (next page) values are those based on the New Method of enzymatic assay for brine shrimp (see Table 18).

Table 20. Continued

No.	Yield: mg protein per kg tissue	Yield: mg protein per preparation	Specific Activity (μmoles P_i/h/mg protein): Preparation	Specific Activity (μmoles P_i/h/mg protein): Purity	Reference
1.	3	0.46	800	890	Kyte, 1971a, 1971b, 1972a, 1972b
2.	170	22	1552	1552	Lane et al., 1973
3.	40	35	750	1500	Uesugi et al., 1971
4.	150	20	1510	1640	Hokin et al., 1973
5.	1700	18	1200	--	Jørgensen et al., 1971
6.	--	20	2200	2440	Jørgensen, NYAS symposia on Na-K ATPase, 1973
7.	--	--	1200	--	Hokin, NYAS symposia, 1973
8.	15	2.6	715	1250	This study
	50	11	450	1250	

[3]Purity is an estimate of the specific activity of a theoretically purified preparation based on the protein contamination of SDS polyacrylamide gels, assuming that the large and small subunits represent all the enzyme protein.

from studies of the kinetic characteristics of membrane bound enzyme in comparison to partially purified or purified preparations from beef brain Na+K-activated ATPase (Kline et al., 1971) and the dogfish rectal gland enzyme (Ratanabanangkoon, Dixon and Hokin, 1973). The unusual thermal profile of the DOC-membrane enzyme resembles the perturbed behavior of the lubrol extracted beef brain enzyme on Arrhenius plots (Kline et al., 1971). Discontinuities (enzyme activity thermal transitions) in Arrhenius plots reflect conformational alterations of either the enzyme or its immediate environment. Breaks in the Arrhenius plots are typically observed for membrane transport systems, and are primarily due to phase transitions or phase separations of the lipids, which occur at characteristic temperatures (transition temperatures) reflective of the type of lipid environment (Esfahani et al., 1971; Overath, Hill and Lanmek-Hirsch, 1971; Wilson and Fox, 1971; Linden and Fox, 1973; Linden, Keith and Fox, 1973; Linden et al., 1973; Tsukagoshi and Fox, 1973). Grisham and Barnett (1973) demonstrate from a combination of spin-label and lipid extraction experiments using plasma membrane and partially purified (600-840 μmoles P_i/hr/mg protein) lamb kidney Na+K-activated ATPase, that a 20° C transition temperature for enzyme activity was due to changes in the lipids surrounding the enzyme, and not due to the protein. Their work and that of Kimelberg and Papahadjopoulos (1974) suggests further that the membrane lipids

must be fluid for the Na+K-activated ATPase to function. The perturbations observed in the Arrhenius plots for the brine shrimp DOC-membrane enzyme (Figure 26) and the lubrol extracted beef brain enzyme (Kline et al., 1971) evidence the interaction of the detergent in the immediate lipid environment of the enzyme. Attempts to study the normal enzymatic properties of this enzyme in the presence of detergents would be hazardous.

The kinetic properties for the brine shrimp Na+K-activated ATPase (as pointed out in Section II) are very similar to those from other organisms (Skou, 1965; Bonting, 1970; Charnock, Cook and Opit, 1971). An interesting exception, however, appears in the ionic strength curves at constant Na/K ratio of 4/1. In Section II (Figure 12) two peaks (at 250 and 400 mM Na+K) of equal magnitude were obtained from a mitochondrial fraction preparation of the enzyme. In the membrane and FT enzyme preparations of this section, the second, high ionic strength peak is of a minor contribution (Figure 29). In the isopod Sphaeroma serratum two maxima were also observed for K dependent Na activation of ouabain sensitive ATPase (Philippot, Thuet and Thuet, 1972), although the ionic strength maxima were shifted to lower levels (30 mM Na + 5 mM K, and 120 mM Na + 10 mM K). Only a single mixima was obtained from Crab nerves (Skou, 1957, 1960). However, this later enzyme displayed the unusual behavior of being maximally stimulated by 1/1 Na/K at an

ionic strength of 80 mM Na+K. Vertebrate Na+K-activated ATPase are usually optimally activated between 5/1 and 20/1 Na/K at ionic strengths between 110 and 160 mM Na+K. These second kinetic optima in the brine shrimp and the isopod Sphaeroma for ion activation, are distinguishably different from the Site II, Na-ATPase system described by Neufeld and Levy (1970), which is inhibited by K.

The possible existence of isozymes of the brine shrimp Na+K-activated ATPase are thus suggested at the molecular level by the presence of two protein bands having the specific catalytic characteristics (phosphorylated in the presence of Na and dephosphorylated in the presence of K), and at the kinetic level by the presence of two peaks for maximal monovalent cation stimulation. It is interesting to recall from the localization studies that the density distribution of the brine shrimp membranes showed two major peaks of enzymatic activity (Figures 15 and 16). The possibility that isozymes might be distributed asymmetrically in the different membranes prompted the attempt to isolate, partially purify, and examine each peak for its kinetic and electrophoretic characteristics. The recent availability of a zonal rotor has made this experiment technically feasible.

Membranes were isolated from 70 g of nauplii using the 33,000 xg pellet (see Figure 17), with a specific activity of 16.4 μmoles P_i/hr/mg protein. The zonal experiment was done using a Beckman Ti-14 zonal rotor equipped with a B29 liner and run in a Beckman L2-65

preparative ultracentrifuge. The sample and gradient were layered from the wall while spinning at 2,000 rpm. The sample of 84 ml containing 980 mg protein was layered under 150 ml of distilled water overlay and followed by the gradient till 100 ml of overlay were displaced from the center. The gradient was prepared by pumping a reservoir of 275 ml of 2.0 M sucrose at 6.5 ml/min into a second mixing reservoir initially containing 325 ml of 0.5 M sucrose, which was pumped into the rotor at 12.5 ml/min. Material was pumped in and out of the rotor using a Technicon, Auto Analyzer Model proportioning pump. The gradient was centrifuged at 45,000 rpm for 90 minutes at 4° C, and pumped out by water displacement from the center while spinning at 2,000 rpm. One minute fractions were collected with a Gilson fraction collector at the rate of 12.5 ml/min.

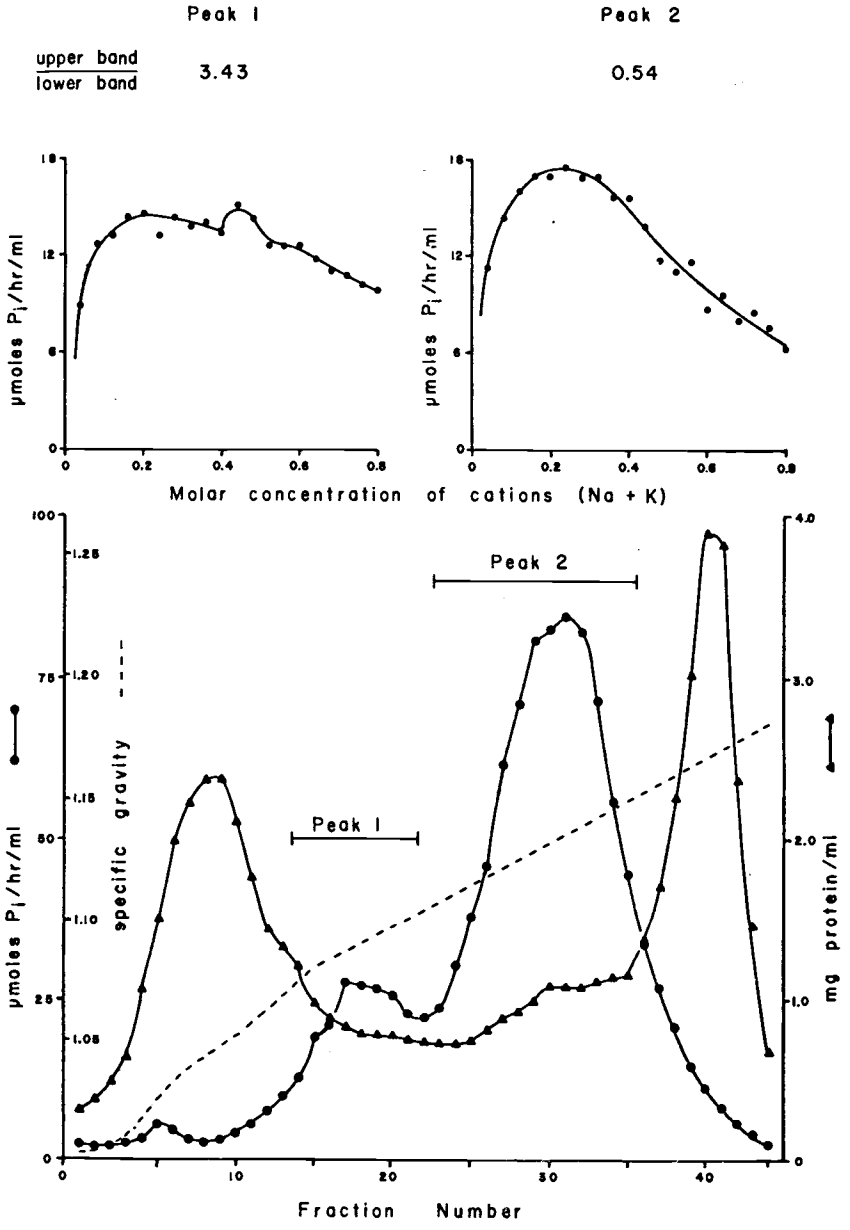
Figure 32 (bottom) shows the distribution of protein, enzyme and sucrose density (by refractometry) of the zonal experiment. The low density peak (Peak 1) was isolated from fractions 14 to 21 and the high density peak (Peak 2) from fractions 23 to 35 (note: the fractions are assayed in reverse of the collection sequence). The effect of total Na+K concentration at a constant Na/K ratio of 4/1 is shown in the middle of Figure 32 for each peak. The ratio of the upper to lower bands in the large subunit is shown at the top of Figure 32. In three other similar experiments the upper/lower band ratios were 2.19, 4.33 and 2.54 for Peak 1 and 0.70, 1.06 and 0.68 for Peak 2,

Figure 32. Asymmetric membrane density distribution of possible isozymes of the brine shrimp Na+K-activated ATPase, with their molecular and kinetic characteristics.

Bottom: Zonal experiment showing the distribution of enzyme, protein and sucrose density.

Middle: Effect of total salt (Na+K) concentration at a constant Na/K ratio of 4/1 for membrane enzymes preparations isolated from Peak 1 and Peak 2.

Top: Ratio of the amount of the upper to lower band in region a from gels of partially purified Peak 1 and Peak 2 enzyme preparations.


Peak 1
Peak 2
upper band / lower band
3.43
0.54
µmoles Pi/hr/ml
Molar concentration of cations (Na + K)
Peak 2
Peak 1
specific gravity
µmoles Pi/hr/ml
mg protein/ml
Fraction Number

respectively. The salt profiles were also very similar and distinguishable by a broad flat profile for Peak 1 and a higher maximum in the lower salt range for Peak 2. These experiments very strongly support, but do not definitively prove the existence of two Na+K-activated ATPase isozymes in the brine shrimp. It would certainly look good if the doublet banding were positively known to be of genetic origin, and only one large subunit was present in an enzyme molecule.

V. SUMMARY

It was the intended purpose of this thesis to examine the Na+K-activated ATPase in the nauplius larva of the brine shrimp, Artemia salina in an effort to develop a new model system for studies of the regulation and biogenesis of this transport enzyme. As little was originally known about this enzyme in brine shrimp, the studies presented here undertook to examine its kinetic characteristics and localization anatomically and subcellularly with the brine shrimp. With this background an effort was made to purify the enzyme from the membrane isolated from the brine shrimp, and to examine its molecular properties.

Early studies indicated that the brine shrimp contains the Na+K-activated ATPase, as determined by its stimulation by Na^{+}, K^{+}, and Mg^{++} and by its inhibition by ouabain. The amount of this enzyme in the brine shrimp was found to be comparable to levels usually only found in specialized transport tissues. Its kinetic characteristics were examined in fresh preparations of both mitochondrial and microsomal fractions. Most of the activity resided in the mitochondrial fraction, but the kinetic properties of the two preparations were nearly identical. The Na+K-activated ATPase from brine shrimp was maximally stimulated by the following concentrations of substrate and cations in the presence of 100 mM Imidazole pH 7.2, 37° C: 5 mM

ATP, 10 mM $MgCl_2$, 320 mM NaCl and 80 mM KCl (concentrations are those in the reaction). In comparison to other sources of this enzyme, the brine shrimp enzyme was very similar except that it was stimulated at a rather low Na/K ratio, and at high ionic strengths of Na+K. It was apparent that the brine shrimp enzyme was maximally stimulated by ionic strengths of either 250 or 400 mM Na+K at a Na/K ratio of 4/1. Most sources of this enzyme show maximum stimulation at 120-160 mM Na+K at ratios between 5/1 and 20/1, Na/K.

Anatomical localization studies by techniques of microdissection indicated that the naupliar salt gland does not show a particularly rich accumulation of the Na+K-activated ATPase. Evidence suggested that the enzyme is rather widely and proportionally distributed about the organism. The possibility that it may serve to regulate the hemolymph ion concentration against the internal cellular ions was discussed. Subcellular localization was investigated by biochemical techniques using marker enzymes. The large amount of activity present in the mitochondrial fraction was determined to be due to contaminating heavy membranes, which could be separated from the mitochondria. The Na+K-activated ATPase was separated from cytochromic oxidase activity by linear sucrose density gradient centrifugation. The mitochondrial fraction was found to be visibly layered by differential centrifugation. Electron microscopy of this

pellet indicated that the upper layer was largely membranes while the lower largely mitochondria. Careful removal of the upper membranes followed by linear sucrose density gradient centrifugation demonstrated that the density profile of the Na+K-activated ATPase containing membranes coincided with those of 5'-UMPase (plasma membrane marker) and was separated from glucose-6-phosphate (endoplasmic reticulum marker). The broad density range for the Na+K-activated ATPase membranes was in agreement with the broad anatomical distribution.

For purification efforts a method was developed for the large scale isolation of the membranes found to be rich in the Na+K-activated ATPase. A yield of approximately one third of the enzyme in the animal is recovered in a membrane preparation with a specific activity about 20 fold higher than the homogenate. Further purification was accomplished by treatment of the membranes with the detergent DOC (deoxychloate) and centrifugation to obtain the DOC-membrane preparation in the pellet. Freezing and thawing the DOC treated membranes was followed by isolation of a rather homogenous vesicular preparation (Freeze-Thaw enzyme preparation) either by differential centrifugation or by discontinuous sucrose density gradient centrifugation. This later preparation represents up to 15 percent of the enzyme in the animal with a purification of about 100 fold over the homogenate. Pelleting the discontinuous gradient

prepared FT enzyme to remove the heavy sucrose produced a layered pellet, the upper portion of which contained enzyme with an additional two-fold greater specific activity. This later partially purified preparation was particulate in nature and estimated by protein contamination of SDS-polyacrylamide gels and γ^{32}P-ATP binding studies to be about 50 percent pure.

Recent successful purification of this enzyme from several vertebrate sources (Kyte, 1971a; Hokin et al., 1973; Lane et al., 1973) have involved solubilization of the enzyme from membrane preparation by the use of detergents. These detergents, as well as several others have been extensively examined on the membrane, DOC-membrane and FT membrane preparations of the brine shrimp. No significant solubilization has been observed for any of these detergents under any circumstance tested. Final purification of the brine shrimp enzyme, however, may eventually depend on development of conditions which will effectively solubilize an active form of the specific Na+K-activated ATPase complex. These conditions will certainly differ from those currently used for this enzyme from other sources.

The molecular properties of the brine shrimp Na+K-activated ATPase were examined by SDS polyacrylamide gel electrophoresis and binding studies with γ^{32}P-ATP. During the partial purification of this enzyme, two regions of protein detectable on the SDS-gels

were observed to enrich. Each region contained two protein bands. From molecular weight markers, the apparent molecular weight for these protein bands are 101,000 and 95,200 for the upper region a and 40,000 and 37,800 for the lower region b. The upper region bands contain the catalytic subunit(s) and the lower contains the glycoprotein subunit(s) in parallel with similar molecular properties of the subunits of this enzyme from other sources. The large to small subunit mass ratio was found to be 2:1 from scans of 30 SDS-gels. The amount of each band in doublet for a subunit varied with different preparations, but the upper of each appeared to vary together (likewise the lower).

Unique features of the brine shrimp enzyme in comparison to those from other sources (all vertebrate) include the doublet banding appearance of the subunits, a positive PAS reaction on the large subunits, and its resistance to solubilization by detergents, and the kinetic properties were re-examined for the membrane and FT enzyme. The two preparations were similar, but differed in ionic strength (Na+K) profile from that obtained earlier from the mitochondrial fraction preparation. Both the high and low ionic strength maxima were present, however, with the new preparations the lower ionic strength maximum was more pronounced. Isolation of the membranes from two density peaks with Na+K-activated ATPase activity was done by zonal centrifugation. Analysis of the molecular and kinetic

properties of the enzyme partially purified from each peak suggested that the peculiarities of doublet subunit banding and two ionic strength optima for the brine shrimp enzyme might be due to two isozymes.

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