

To the Graduate Council:

I am submitting herewith a dissertation written by Norman J. Karin entitled "Biosynthesis and Turnover of Na,K-ATPase in Cultured Rat Hepatoma Cells." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

John S. Cook

John S. Cook, Major Professor

We have read this dissertation
and recommend its acceptance:

Kai-Lin Lee

Leaf Huang

Darrell Doyle

Stephen J. Kennel

Accepted for the Council:

LE Anne Cook

Vice Chancellor
Graduate Studies and Research

BIOSYNTHESIS AND TURNOVER OF Na,K-ATPase IN
CULTURED RAT HEPATOMA CELLS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Norman J. Karin

June 1983

1182503

This work is dedicated to the memory of Norman Karin, Sr., whose curiosity and love of all living things are the true essence of biology. He inspired me more than I realized.

ACKNOWLEDGEMENTS

I am grateful to Dr. John S. Cook for the support and faith in me he has expressed over the past four years. My committee members, Drs. Steven Kennel, Kai-Lin Lee, Leaf Huang (The University of Tennessee, Knoxville, Tennessee) and Darrell Doyle (Roswell Park Memorial Institute, Buffalo, New York), were instrumental in keeping this research along a productive path. In addition, Dr. Kennel and his assistant Patricia Lankford provided patient instruction in many aspects of immunology.

I am indebted to Dr. Dorothy Skinner and her co-workers, who were very generous with their equipment and supplies. I also thank Drs. Michael Weber (University of Illinois) and Chu Shek Lo (Uniformed Services University of the Health Sciences, Bethesda, Maryland), whose helpful discussions led directly to the solution of several technical problems.

In the many years that were spent approaching this point, my family never ceased to give me encouragement. I am happy that they now see this goal attained.

Finally, I am very pleased to acknowledge the unyielding support of my wife Charlotte. She was my psychiatrist when, like every graduate student, I was convinced that my project was the most difficult ever attempted; she was my typist and editor during the later stages of this work; and always, she was my companion, seeking to lighten my burden at every chance.

This work was supported by the Carcinogenesis Training Grant NIH-CA-09104 from the National Institutes of Health, awarded to The University of Tennessee Graduate School of Biomedical Sciences, at the Biology Division of the Oak Ridge National Laboratory (under contract with Union Carbide Corporation).

ABSTRACT

Na,K-ATPase is an integral protein of the plasma membrane of cells in all vertebrates. The details of its biosynthesis and, to a lesser degree, its degradation (turnover) remain unclear after more than 25 years of investigation on this enzyme. I have used antibodies prepared to the catalytic (α) subunit of rat kidney Na,K-ATPase to investigate the biosynthesis and turnover of the α subunit in HTC cells, a cultured rat hepatoma line.

Na,K-ATPase was isolated in a form approximately 50% pure, as indicated by its activity and SDS-PAGE profile, by the detergent extraction of rat kidney microsomes, followed by ultracentrifugation through 50% glycerol. The pellet of purified enzyme was denatured in SDS and the α subunit collected by gel filtration. Anti- α IgG, isolated from the serum of rabbits immunized with this preparation, precipitated a major protein of 93,000 daltons from HTC cells labeled with [^{35}S]methionine. The evolutionarily conserved structure of this subunit was confirmed by the cross-reactivity of anti-rat α subunit with the enzyme from hamster, dog and human cells.

No ^{35}S -labeled glycoprotein (β) subunit was detected in the immunoprecipitates from cells lysed under a variety of nondenaturing conditions, or when chemical cross-linking preceded the detergent solubilization. A protein corresponding in size to the β subunit was observed in immunoprecipitates from cells labeled with either [^3H]leucine or a mixture of [^3H]fucose and [^3H]mannose, suggesting

that the β peptide of Na,K-ATPase in HTC cells contains little or no methionine.

The α subunit was turned over, when corrected for cell generation time (T_G), with a half-life of $.45 T_G$ and a turnover coefficient of $1.5 T_G^{-1}$. A 5 hour lag period was observed prior to the onset of turnover which was interpreted as the transit time of the newly-synthesized α proteins from intracellular membrane compartments to the plasma membrane. This is inconsistent with theories that α is translated as a soluble protein and inserts directly into the plasma membrane. Furthermore, immunoprecipitable (93,000 daltons) α subunit was found, after brief pulses of [^{35}S]methionine, only in cell membranes, not in the cytosol. I conclude that the α subunit of Na,K-ATPase follows the classic pathway for membrane protein biogenesis, namely translation on bound ribosomes and transport through intracellular (Golgi apparatus) membranes to the plasma membrane.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
II. ISOLATION OF Na,K-ATPase FROM RAT KIDNEY	28
Introduction	28
Materials and Methods	29
Results	32
Discussion	50
III. PRODUCTION AND CHARACTERIZATION OF ANTIBODIES AGAINST RAT KIDNEY Na,K-ATPase	57
Introduction	57
Materials and Methods	57
Results	63
Discussion	81
IV. BIOSYNTHESIS AND TURNOVER OF THE HTC CELL Na,K-ATPase α SUBUNIT	85
Introduction	85
Materials and Methods	87
Results	87
Discussion	96
LITERATURE CITED	106
APPENDIX	116
VITA	134

LIST OF TABLES

TABLE	PAGE
I. Recovery and Purification of Rat Renal Na,K-ATPase	44
II. The Effect of Anti- α IgG on Na,K-ATPase Activity . . .	72
III. α Subunit Turnover Parameters in HTC Cells	97
A-I. 5'-AMP Hydrolysis by HTC Cell Homogenates	121
A-II. HTC 5'-AMPase Activity $\pm$ Calcium	128
A-III. 5'-AMPase Activity $\pm$ Cytosol in Various Cell Types . .	129

LIST OF FIGURES

FIGURE	PAGE
1. Na,K-ATPase Activity During the Extraction of Rat Kidney Microsomes with SDS	34
2. Influence on Na,K-ATPase Activity of Protein Concentration During the Detergent Extraction of Microsomes	36
3. The Effect of SDS Concentration During the Extraction of Rat Kidney Microsomes on the Subsequent Recovery and Purification of Na,K-ATPase	38
4. SDS-PAGE Analysis of Na,K-ATPase Isolated from Rat Kidney Microsomes Treated with Various Concentrations of SDS	41
5. Identification of the Subunits of Na,K-ATPase	45
6. Ferguson Analysis of the Na,K-ATPase Subunits by SDS-PAGE	48
7. The Effect of Heating Na,K-ATPase in Sample Solvent Prior to SDS-PAGE	51
8. Gel Filtration of SDS-Denatured Na,K-ATPase	64
9. Recovery of the Na,K-ATPase α Subunit from HTC Cells vs. Anti- α IgG Concentration	66
10. Immunoprecipitation of Na,K-ATPase from HTC Cells Lysed Under Different Conditions	69
11. Immunoprecipitation of Na,K-ATPase from Radiolabeled HTC Cell or Kidney Membranes that Had Been Chemically Cross-Linked	74
12. Immunoprecipitation of Na,K-ATPase from HTC Cells Labeled with [^{3} H]leucine or [^{3} H]mannose and [^{3} H]fucose	77
13. Cross-Reactivity of Anti-Rat α Subunit IgG with Na,K-ATPase of Other Species	79
14. Time Course of [^{35} S]methionine Incorporation into HTC Cell Protein	88

FIGURE

PAGE

15. Turnover of Total Triton-Soluble Protein and the Na,K-ATPase α Subunit in HTC Cells Labeled for Three Days with [^{35}S]methionine	91
16. Turnover of Total Triton-Soluble Protein and the Na,K-ATPase Subunit in HTC Cells Pulse-Labeled with [^{35}S]methionine	94
17. Immunoprecipitation with Anti- IgG from the Cytosol and Total Membrane Fractions of ^{35}S -Labeled HTC Cells . .	98
A-1. Heat- and Proteinase-Sensitivity of the Cytosolic Inhibitors of 5'-AMPase	123
A-2. Eadie-Hofstee Analysis of the Cytosolic Inhibition of 5'-AMPase in HTC Cells	126

CHAPTER I

INTRODUCTION

Cells consume a large portion of their metabolic energy in the maintenance of monovalent cation balance. This energy was found by Skou in 1957 to be utilized in the form of ATP by an enzyme, sodium + potassium-dependent-adenosine triphosphatase (Na,K-ATPase, EC 3.6.1.3), to concentrate K intracellularly while extruding Na. Both of these processes occur against concentration gradients. Since 1957, the enzyme has been the object of extensive enzymological analysis (for review, see Robinson and Flashner, 1979). Briefly, Na,K-ATPase (or "sodium pump") in human erythrocytes has been characterized by a "3-2-1" stoichiometry, i.e., 3Na are extruded and 2K are internalized per 1ATP hydrolyzed (Post and Jolly, 1957), although in other tissues the stoichiometry may not be fixed. Na and ATP bind to saturable sites on the cytoplasmic domain of the enzyme, while the K-binding site is extracellular. Enzyme activity is inhibited by the binding of cardioactive glycosides, also to external sites.

The exact structure of Na,K-ATPase is not yet certain. A 90-100,000 dalton peptide, termed α , and a smaller (40-60,000 daltons) heavily-glycosylated peptide (β) are found without exception in purified enzyme preparation (for review, see Jorgensen, 1975). The α subunit contains the binding sites for both Na and K. In addition, the ATP (cytoplasmic) and glycoside (external) binding sites are both on the larger peptides, indicating a transmembrane architecture.

No specific functions have been ascribed to the β subunit, which also spans the membrane bilayer entirely (Girardet et al., 1981). Removal of 50% of the enzyme's carbohydrate (largely or entirely accounted for by the β subunit) with neuraminidase fails to alter Na,K-ATPase activity in a purified preparation from the dog fish salt gland (Marshall, 1976). Plant lectins (Churchill and Hokin, 1976) and antibodies prepared against the β subunit (Rhee and Hokin, 1975; Jean et al., 1975), however, have been found to inhibit Na,K-ATPase, suggesting a functional role for this component.

Further insight into the structure of Na,K-ATPase has been gained from reconstitution and chemical cross-linking studies. Goldin (1977) reconstituted purified kidney enzyme into unilamellar liposomes and recovered ATP-dependent Na/K countertransport with a stoichiometry resembling that of the pump in vivo. The α and β subunits comprised 98% of the total protein (greater than 12,000 daltons) in this system. To date, neither subunit alone has exhibited any enzyme activity. A small (12,000 daltons) proteolipid may also be associated with the enzyme, as evidenced by its ability to be labeled with a photoaffinity derivative of ouabain (Forbush et al., 1978).

The cross-linking of Na,K-ATPase preparations has yielded information regarding the stoichiometry and interactions of its subunits. When the enzyme is treated with bifunctionally-reactive imidoesters $\alpha\beta$ complexes result (Kyte, 1972; Sweadner, 1977). Cupric phenanthroline, however, leads to $\alpha\alpha$ dimers (Giotta, 1976). $\beta\beta$ dimers are not observed with either of these reagents. These studies indicate

a functional unit of $\beta\alpha\alpha\beta$, although other combinations may be possible (see Sweadner and Goldin, 1980).

Purified Na,K-ATPase has been solubilized in active form in nonionic detergents and analyzed by gel filtration or sedimentation in an analytical ultracentrifuge. These studies have indicated a molecular weight of between 280,000 and 500,000 daltons (Nakao et al., 1973; Hastings and Reynolds, 1979; Esmann et al., 1980), consistent with a multimeric arrangement of $\alpha\beta$ units. Recently, however, Brothier et al. (1981) have found an active enzyme form of 170,000 daltons, suggestive of an $\alpha\beta$ or $\alpha\beta_2$ functional unit. They cite the enzyme's propensity to aggregate as a reason for the higher estimates. The activities measured in this preparation, Na and K-dependent ATP hydrolysis and K-dependent nitrophenylphosphatase (a partial reaction of Na,K-ATPase), do not indicate whether or not the 170,000 dalton complex is capable of the full complement of enzyme functions. Vectorial ion transport may require multimers of the $\alpha\beta$ unit.

Based on the above observations several models of the enzymes transport mechanism have been proposed (cf. Kyte, 1981; Wiggins, 1982), which invoke conformational changes in the peptide subunits (resulting from the transitory phosphorylation by ATP) to translocate the ions. The Na,K-ATPase subunits clearly do not rotate through the lipid bilayer since the binding of a hydrophilic, 150,000 dalton antibody molecule to the pump fails to alter ion translocation (Kyte, 1974). A common feature of many of the models is the extrusion of Na through a transient pore between two α subunits while sequentially transporting K through the same or a different channel. To my knowledge, no

model for a functional (transporting) unit of $\alpha\beta$ has been published. Some models have suggested that only half of the Na,K-ATPase sites are functional during one pump cycle (i.e., fueled by one ATP molecule). These have been criticized on the basis that too often the molecular weight (grams protein per mole enzyme) of the enzyme is overestimated (Kyte, 1981).

Na,K-ATPase is ubiquitous to all animal cells, both vertebrate and invertebrate, and is deemed similar or identical by the following criteria. The enzyme always cleaves only the γ -phosphate of ATP, a process that requires both Na and K, and is inhibited by cardioactive glycosides, although the sensitivity to these compounds varies greatly with both species and tissue type (Tobin and Brody, 1972). In addition to the functional similarities structural homology exists over a great evolutionary distance, shown by the immunologic cross-reactivity of antibodies to canine kidney Na,K-ATPase with the enzyme of the insect Manduca sexta (Schellenberg et al., 1981).

Na,K-ATPase is localized in the plasma membrane of animal cells and is commonly monitored as a marker enzyme for that membrane compartment during fractionation studies (DePierre and Karnovsky, 1973). In specialized cells the enzyme may be restricted to specialized domains of the cell surface. In polarized epithelia, for example, Na,K-ATPase is generally found exclusively in the basolateral membrane (Stirling, 1972; Ernst and Mills, 1980), a characteristic which is retained in culture by certain cell lines (Lamb et al., 1981). In retinal epithelia this appears reversed, with Na,K-ATPase occupying only the apical surface (Steinberg and Miller, 1973).

Many metabolic processes are coupled to the action of Na,K-ATPase at the cell surface. These processes include the accumulation of sugars and amino acids by absorptive epithelia, reuptake of neurotransmitters and the regulation of cell volume (for review, see Albers, 1976).

As might be expected from the importance of Na,K-ATPase, the enzyme's activity is modulated in a variety of ways, categorized as "short-term" and "long-term" regulation by Cook and his colleagues (Pollack et al., 1981b, 1982; Karin and Cook, 1983). Short-term regulation refers to the rapid (in either onset or reversal, or both) stimulation or inhibition of enzyme activity by substrates, substrate analogs or other compounds such as glycosides. The best example of this is the stimulation of Na,K-ATPase activity by cytoplasmic [Na]. The enzyme has a high affinity for K and ATP and the binding sites for these molecules are always at or near saturation. The binding of Na occurs with a low affinity ($K_m = 10-15\text{mM}$) relative to the internal Na concentration (10-30mM in HeLa cells; Pollack et al., 1982) and therefore small changes in cellular [Na] lead to proportional changes in Na,K-ATPase activity. The modulation of Na,K-ATPase activity by a variety of hormones, neurostimulatory agents, mitogenic lectins and tumor promoters appears to be via changes in Na influx (for review, see Rozengurt and Mendoza, 1980).

The primary interest of this laboratory is in the long-term regulatory mechanisms, i.e., those that govern the number of enzyme copies through the modification of biosynthesis or degradation (turnover). A clear example of this type of regulation is the

modulation of Na,K-ATPase by chronic ion stress. Lamb and his co-workers (Boardman et al., 1972, 1974) reported increased Na,K-ATPase activity in cultured cells that had been maintained in medium containing low K or sublethal levels of ouabain. Pollack et al. (1981a) have confirmed and expanded these observations in HeLa cells. They found low K-stress (0.5mM K) to increase ouabain binding, K-sensitive phosphorylation, and the $V_{\max}$ for Rb transport (an analog of K), all pointing to an increase in the number of enzyme units. This response was specific in that another plasma membrane enzyme, 5'-nucleotidase, was unaffected by the stressed growth.

The mechanism of this regulation was determined (Pollack et al., 1981b) by analyzing the enzyme's synthesis and turnover in stressed vs. control cells (grown in 5.5mM K). The α subunit (the β peptide was not analyzed) exhibited similar rates of synthesis and processing in both cell groups. The low [K]-grown cells, however, displayed a significantly reduced degradation rate, the turnover coefficient decreasing to 1.3/cell generation from the control value of 3.1/cell generation. The magnitude of this change can account fully for the observed differences in enzyme concentration. This differs from the classical concept of induction in that synthesis is not altered, rather degradation is decreased. The authors speculate that this mode of regulation allows a more rapid response to a changing ionic environment (Pollack et al., 1981b). Whether the cells are responding directly to the low [K] or to the subsequent alteration in cytosolic [Na] (Boardman et al., 1974) remains unanswered.

Long term Na,K-ATPase regulation has also been documented in whole animals subjected to ion stress. Among the most dramatic examples is the response to salt loading and deprivation by certain animals, notably fish and marine birds, which ingest large quantities of salt in their diet. A common adaptation in these organisms is the presence of extrarenal glands which function to maintain plasma osmolarity. Salt stress is often accompanied by changes in the amount of Na,K-ATPase in these organs (for review, see Karin and Cook, 1983).

One of the most widely studied of these systems is the nasal salt gland of the duck. Salt loading of these animals leads to extensive cytological changes in this gland, a large increase in the surface area of the basolateral membrane being consistently observed (Ernst and Ellis, 1969). Cytochemical analysis revealed increases in Na,K-ATPase-related activities, such as [^3H]ouabain binding and K-dependent phosphatase activity (Ernst, 1972; Hossler et al., 1978). These activities were restricted to the basolateral membrane; no such activities were associated with apical membranes. Na,K-dependent ATP hydrolysis was also increased 4 to 5 fold over about 9 days in membranes from these glands without concomitant large increases in the activities of other membrane enzymes, such as Mg-ATPase and 5'-nucleotidase (Ernst et al., 1967; Hossler, 1978). These responses were reversible, declining slowly to control levels over several days as would be expected for a long term regulatory event.

The mechanism for the observed elevation in salt gland Na,K-ATPase has been explored by Stewart et al. (1976) and

Lingham et al. (1980). Both of these groups employed polyacrylamide gel electrophoresis to quantitate the levels of the Na,K-ATPase subunits during salt stress. The conclusion of these reports is that salt loading leads to elevated de novo synthesis of the enzyme peptides.

Several hormones have been implicated in the long term regulation of Na,K-ATPase. The thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) have long been known to regulate oxygen consumption (Q_{O_2}) and, correspondingly thermogenesis in homoiothermic vertebrates. Treatment of either euthyroid or hypothyroid rats with T_3 increases Q_{O_2} in a variety of tissues, namely liver (Ismail-Beigi et al., 1979; Ismail-Beigi and Edelman, 1970, 1971, 1974; Somjen et al., 1981) kidney (Ismail-Beigi and Edelman, 1971; Katz and Genant, 1971) and skeletal muscle (Asano et al., 1976; Ismail-Beigi and Edelman, 1970), while having no effect on brain respiration (Ismail-Beigi and Edelman, 1971).

Na,K-ATPase meets the criteria for being the active cellular component of heat generation, being ubiquitous in target cells for thyroid hormones and able to hydrolyze sufficient ATP to affect respiration (Ismail-Beigi and Edelman, 1970). Largely due to the efforts of Edelman and his colleagues, the enzyme has been identified as a major instrument in thyroid hormone action. A significant portion of the hormone-induced increase in Q_{O_2} was found to be sensitive to inhibition by ouabain (Asano et al., 1976; Ismail-Beigi et al., 1979; Ismail-Beigi and Edelman, 1970, 1971, 1974; Somjen et al., 1981). This effect is especially apparent in the liver of both euthyroid and

hypothyroid rats where ouabain prevents greater than 90% of the T_3 -specific increase in QO_2 (Ismail-Beigi and Edelman, 1970, 1971, 1974). The Na,K-ATPase-dependent increments of QO_2 in rat skeletal muscle and kidney are less evident.

Analysis of ouabain-sensitive ATP hydrolysis in these tissues has revealed a pattern similar to that of oxygen consumption. Na,K-ATPase activity, following thyroidectomy, is decreased in kidney (Ismail-Beigi and Edelman, 1971; Katz and Lindheimer, 1973; Somjen et al., 1981), liver (Somjen et al., 1981), and skeletal muscle (Asano et al., 1976). The brain enzyme is unaffected (Ismail-Beigi and Edelman, 1971). Exogenous T_3 , when administered to either hypothyroid or euthyroid rats, increases Na,K-ATPase activity in the target tissues.

The nature of the change in Na,K-ATPase remained to be elucidated, i.e., increased activity of the existing enzyme vis-à-vis increased enzyme concentration. Asano et al. (1976) analyzed skeletal muscle from hypothyroid rats and found that T_3 treatment elicited an increase in V_{max} (ATP) with no change in K_m (ATP). Lo et al. (1976) characterized a variety of parameters of Na,K-ATPase in renal tissue from hypothyroid rats, reporting that a pulse of T_3 increased substantially the specific activity of microsomal Na,K-ATPase from the cortex but only slightly in microsomes from the medulla; papillary tissue was unresponsive. With chronic T_3 treatment only the cortical enzyme responded with increased activity. In an analysis of the kinetics of renal cortical Na,K-ATPase in hypothyroid animals they found that T_3

increased the $V_{\max}$ for ATP, Na and K, without a concomitant alteration in the K_m for ATP or the $K_{1/2}$ for Na and K. Two other indicators of enzyme number, [^3H]ouabain binding and K-sensitive phosphorylation, were increased by a magnitude similar to that of the Na,K-ATPase specific activity after T_3 treatment. T_3 -induced increases in [^3H]ouabain binding were also reported by Lin and Akera (1978) in liver and muscle, as well as kidney. As with activity measurements, binding levels were unchanged in brain tissue.

Although these data imply an increase in the number of enzyme molecules, hormone-induced exposure of latent Na,K-ATPase is also possible. Evidence that this is not occurring comes from observations that T_3 -induced increases in [^3H]ouabain binding (Lin and Akera, 1978) and ouabain-sensitive ATP hydrolysis (Lo and Lo, 1979) are the same when assayed in the presence of detergents so as to provide maximum accessibility of ligand or substrate to cryptic sites. Another possibility is that T_3 is effecting an increase in all enzymes of the plasma membrane (i.e., inducing a generalized membrane synthesis). This is ruled out by the reported lack of T_3 effects on the other marker enzymes by the plasma membrane, such as Mg-ATPase (Ismail-Beigi et al., 1979; Ismail-Beigi and Edelman, 1971; Katz and Lindheimer, 1973; Lo et al., 1976; Lo and Lo, 1981), 5'-nucleotidase (Ismail-Beigi and Edelman, 1971; Katz and Lindheimer, 1973) and adenylate cyclase (Levey et al., 1969).

To determine the basis for increased enzyme concentration, the synthesis and turnover rates of renal cortical Na,K-ATPase were determined. To this end rats were placed on a T_3 regimen which

increased Na,K-ATPase concentration to a new steady-state level (Lo and Edelman, 1976). Metabolically double-labeled Na,K-ATPase was partially purified and analyzed by polyacrylamide gel electrophoresis (Lo and Edelman, 1976; Lo and Lo, 1980). These experiments allowed determination of degradation rate constants for each subunit and revealed that the degradation of neither subunit was affected by T_3 treatment, indicating increased synthesis of both subunits (Lo and Lo, 1980). These data were systematically shown to be independent of artifacts due to possible hormonal influence on enzyme purification, recovery or latency (Lo and Lo, 1981). The observed effects of T_3 on both Q_{O_2} and Na,K-ATPase were initially attributed to direct hormonal action on target cells (Ismail-Beigi and Edelman, 1970, 1971). This view was challenged in light of evidence by Katz and his co-workers (Katz and Genant, 1971; Katz and Lindheimer, 1973) that renal Na,K-ATPase activity could be increased in hypothyroid animals by treatments which increase Na absorption. Enzyme activity, decreased by about 25% in both the cortex and medulla by thyroidectomy, was restored to control levels by partial nephrectomy or the administration of methylprednisolone (Katz and Lindheimer, 1973). Infusion of isotonic saline into rats increased both Na,K-ATPase and Mg-ATPase activities in microsomes from renal medulla but not the cortex (Katz and Genant, 1971). Furthermore, T_3 added directly to renal microsomes in concentrations up to $10^{-5}M$ had no effect on Na,K-ATPase activity (Katz and Lindheimer, 1973). Taken together, these data indicated to the authors that renal Na,K-ATPase was altered in

hypothyroid rats primarily by tubular sodium reabsorption and only secondarily by T_3 .

Many reports have subsequently shown that, while not acting directly on the protein itself, T_3 increases Na,K-ATPase activity through direct interaction with target cells. That the activity is altered by thyroidectomy and T_3 treatment in nonrenal tissues, i.e., liver (Ismail-Beigi et al., 1970; Ismail-Beigi and Edelman, 1970, 1971, 1974; Somjen et al., 1981) and skeletal muscle (Asano et al., 1976; Ismail-Beigi and Edelman, 1970), implies a mechanism independent of renal sodium absorption.

Possibly the most convincing evidence for direct T_3 action comes from Ismail-Beigi et al. (1979) who found that T_3 stimulates both Na,K-ATPase and respiration in primary cultures of hepatocytes from hypothyroid rats. As with liver in vivo, about 90% of the Q_{O_2} increase was sensitive to ouabain. These effects were seen under serum-free culture conditions and were independent of insulin and corticosterone. While T_3 at $8 \times 10^{-11} M$ elicited a half-maximal effect on both Na,K-ATPase activity and Q_{O_2} , nearly 90% of the hormone was catabolized by 6 hr. Further, a brief (4 hr) exposure to T_3 is sufficient to elicit a significant increase in Na,K-ATPase activity (75% was seen after a 48 hr exposure). These data imply that T_3 may be necessary only briefly and in minute amounts to initiate a response by the target cells. These hormonal effects in hepatocyte cultures have not been observed in established cell lines, such as rat hepatoma cells (Ismail-Beigi et al., 1979; Karin, unpublished observations) or fibroblastic and epithelioid cells

derived from normal kat kidney cells (Karin, unpublished observations). It is conceivable that among the alterations in growth properties necessary for a cell to proliferate in vitro is the loss of the sodium pump's responsiveness to thyroid hormones.

These results clearly indicate a direct action of T_3 on target cells, resulting in increased net Na,K-ATPase synthesis and subsequently higher enzyme activity. This is in accordance with the current model of T_3 action, i.e., that the hormone binds to membrane receptors and eventually reaches specific sites in the nucleus, leading to increased synthesis of mRNA and protein (Tata, 1968; Oppenheimer et al., 1979; Somjen et al., 1981). Indeed, the dose dependence for saturable nuclear binding and increased Na,K-ATPase activity were similar in magnitude in both liver and kidney, implying a quantitative relationship between the two events (Somjen et al., 1981).

It is well established that sodium uptake through many transporting epithelia is decreased in adrenalectomized animals and that the administration of either mineralocorticoids or glucocorticoids can partially or totally restore normal transport. Na,K-ATPase activity declines following adrenalectomy and is restored by adrenal steroids (Chignell and Titus, 1966), and has therefore been implicated in the hormonal action on sodium uptake. But despite years of research by many laboratories a clear mechanism remains elusive. Complicating the resolution of a role for Na,K-ATPase are the many types and sources of tissue studied, as well as variations in protocol regarding types of fractionation, the presence or

absence of detergent during fractionation and assay, and hormone dosage—especially in light of the possible occupancy of glucocorticoid receptors by aldosterone (Marver, 1980). The following reviews work relating to the action of these hormones to Na,K-ATPase biosynthesis.

The synthesis of both RNA and protein is required for aldosterone action on sodium uptake (Edelman et al., 1963; Law and Edelman, 1978a). In addition, certain of these proteins, those destined for the cell surface, require fatty acid production for their expression (Scott et al., 1979). Rossier and his co-workers (Rossier et al., 1977; Rossier, 1978), studying the effects of various RNA synthesis inhibitors on toad bladder, reported that aldosterone induces the synthesis of mRNA [(both poly(A)(+) and poly(A)(-)] during the 30-60 minute latent period between the administration of hormone and the observed physiological effect. Prevention of their synthesis abolishes the response to aldosterone. 3'-Deoxycytidine, an inhibitor of rRNA synthesis, does not alter hormone action during the first three hours, with later effects attributed to general toxicity.

Aldosterone stimulates the synthesis of a discrete set of proteins in toad bladder, as determined by polyacrylamide gel analysis of single or double isotope labeled tissue. Scott et al. (1978, 1981) have separated mitochondria-rich (MR) cells from mitochondria-poor granular (G) cells of the bladder mucosa and found that the aldosterone response involves MR cells exclusively. Analysis of subcellular fractions from hormone-treated and control MR cells indicated the existence of distinct sets of aldosterone-induced proteins (AIP's)

in the plasma membranes and cytosol compartments. The functional identity of these proteins cannot be ascertained by electrophoretic mobility alone and consequently, these studies neither rule out nor verify Na, K-ATPase as an AIP. It is possible that a membrane-associated AIP of 110,000 daltons is the α subunit of Na,K-ATPase (Scott et al., 1981).

Ambiguity has characterized the results of experiments designed to assign a role for Na,K-ATPase in aldosterone action (Marver, 1980). Knox and Sen reported in 1974 that synthesis of the α subunit (and possibly the β subunit) was increased in the kidney following administration of high doses of aldosterone to adrenalectomized rats. Geering et al. (1982a) have observed increases in immunoprecipitable Na,K-ATPase subunits following aldosterone treatment of toad bladder. It is interesting to note that they also reported a synergistic effect of T_3 .

Many investigators have attributed the stimulation of Na,K-ATPase to the increased sodium loading which aldosterone elicits in target tissues (Jorgensen, 1969; Will et al., 1980, 1981; Doucet and Katz, 1981; Handler et al., 1981). The evidence which favors this secondary effect of adrenal steroids is manifold. The hormone-induced alteration in sodium excretion is observed by 1-2 hours and may involve amiloride-sensitive channels (Will et al., 1981), whereas renal Na,K-ATPase does not increase until 4-5 hours later (Jorgensen, 1969). Studies with dissected nephron segments have similarly failed to reveal any immediate (3 hours) effects of aldosterone on Na,K-ATPase

which coincide with the observed changes in cation transport (Doucet and Katz, 1981). Also, Na,K-ATPase is induced by feeding rats a low-sodium diet (leading to elevated endogenous aldosterone levels); the rapid reversion to control levels following dietary sodium replacement implies a secondary effect (Will et al., 1980). Handler et al. (1981) failed to observe an increase in sodium-dependent ATP hydrolysis in aldosterone-treated toad bladder epithelia in culture. They did report elevated ouabain binding to homogenates, but that amiloride abolished this, indicative of a sodium effect on expression of Na,K-ATPase activity.

Although it is not known whether the work of Knox and Sen (1974) reflects a direct or secondary action of aldosterone, the increases in Na,K-ATPase protein observed by Geering et al. (1982a) were insensitive to the presence of amiloride, implying a direct effect of the hormone. A recent report by Garg et al. (1981) presented evidence for a mineralocorticoid-induced increase in renal Na,K-ATPase concentration. They compared the rates of Na-dependent ATP hydrolysis and Na transport in isolated nephron segments from rats which were either fed a low-Na diet or treated with deoxycorticosterone (DOCA), a potent mineralocorticoid. They found that DOCA increased Na,K-ATPase activity 6 fold in the cortical collecting duct while low-Na stress caused only a 2 fold enhancement. It is important here to note that the ATP hydrolysis assays were performed under conditions which expose all cryptic activity. This may be critical in that enzyme latency has been cited as a significant factor in the present confusion regarding the mode of adrenal steroid action on epithelial

transport (Marver, 1980). A high correlation between Na transport and Na,K-ATPase was observed in both treatment groups. The authors also monitored cell number by quantitating nuclei after staining the segments with acridine orange. They concluded that the membrane density of Na,K-ATPase molecules per cell was increased by both treatments, but to different degrees. That DOCA augments the basolateral surface area of the renal collecting tubules only 2 to 3 fold (Wade et al., 1979) indicates that the 6 fold enhancement of Na,K-ATPase cannot be attributed only to general membrane synthesis.

Other possible mechanisms of steroid action on cation transport have been proposed. The stimulation of Na,K-ATPase has been attributed to increased intracellular ATP levels—a consequence of the induction of citrate synthase (Kirsten et al., 1968; Law and Edelman, 1978b; Suzuki, 1981; Voute et al., 1975), although this outcome is not universally observed (Handler et al., 1981; Will et al., 1981). Aldosterone has also been reported to interact directly with the enzyme proteins themselves in erythrocyte ghosts, leading to a 450% increase in activity (Hamlyn and Duffy, 1978).

Several studies have indicated that the aldosterone effects on Na,K-ATPase are implemented through glucocorticoid receptors. Aldosterone stimulated Na,K-ATPase activity in the proximal tubules of the rat despite a lack of mineralocorticoid receptors and the presence of canrenone, an effective blocker of mineralocorticoid receptors (Aperia et al., 1981). In the intestinal epithelium, Na,K-ATPase stimulation by aldosterone lags behind the increase in sodium resorption and is likely to be a secondary effect of the hormone,

whereas enzyme induction by dexamethasone, a potent synthetic glucocorticoid, parallels the sodium uptake and is postulated to be a direct response to hormone induction (Will et al., 1981). Possibly the most convincing evidence for receptor cross-occupancy is that of Rodriguez et al. (1981) who determined doses of aldosterone and dexamethasone which stimulated gluconeogenesis and altered urinary electrolyte excretion, respectively, without cross-reacting. Renal Na,K-ATPase was only affected by dexamethasone; stimulation by aldosterone was achieved only at doses which increased gluconeogenesis, indicating occupancy of glucocorticoid receptors.

These results correlate with observations from 1967 by Katz and Epstein of stimulation by the glucocorticoid methylprednisolone of sodium-dependent ATP hydrolysis in parallel with elevations in glomerular filtration and renal sodium reabsorption. Similarly, Will et al. (1981) reported increased Na,K-ATPase in the intestinal mucosa following dexamethasone treatment. In agreement with the direct hormonal induction theories, the dexamethasone stimulation of Na,K-ATPase can be entirely accounted for by an increase in the number of enzyme molecules. Hormone treatment restores renal Na,K-ATPase to normal levels in adrenalectomized rats and results in increased K-sensitive phosphorylation, a measure of enzyme amount¹

¹It is interesting to note that dexamethasone was reported to increase Na,K-ATPase within 2 hours of addition (Sinha et al., 1981). Pollack et al. (1981b) and Churchill and Hokin (1979) have observed a transit time of more than 2 hours for the newly synthesized α subunit to insert into plasma membranes. This may imply hormonal regulation by an immediate change in rate of degradation, involving no lag, as opposed to an increased synthetic rate, as was elicited in response to T_3 (Lo and Lo, 1980).

(Rodriguez and Klahr, 1980; Sinha et al., 1981). The $V_{\max}$ values for Na, K and ATP are also increased, without changes in substrate affinities (Sinha et al., 1981). The elevation of specific enzyme activity, when normalized to DNA content, also indicates the presence of more enzyme per cell (Sinha et al., 1981). These data correlate with the stimulation by dexamethasone of the incorporation of [^{35} S]methionine into protein in the renal medulla (Law and Edelman, 1978a).

In polarized epithelia, Na,K-ATPase is generally thought to be located in the basolateral plasma membrane. It is relevant here to mention observations by Kashgarian (1980) that adrenal steroids elicit increases in the surface area of this membrane and may have a role in cation transport. Support for these observations comes from biochemical evidence that glucocorticoids alter Na,K-ATPase without affecting enzymes localized in the brush border, namely sucrase and alkaline phosphatase (Will et al., 1981). On the other hand, it is worth noting that adenylate cyclase, another basolateral membrane marker, is unchanged by adrenal steroids (Hendler et al., 1972; Handler et al., 1981).

In comparison to enzymological and physiological information on Na,K-ATPase, relatively little is known about the enzyme's biosynthesis. Membrane and secretory proteins are generally believed to be synthesized in the rough endoplasmic reticulum (RER) and cotranslationally inserted partially (membrane proteins) or completely (secretory proteins) into the lumen of that organelle (Palade, 1975). Here, the small hydrophobic signal sequence (Blobel and Dobberstein,

1975) is removed and, where appropriate, common oligosaccharide precursors are transferred to the peptide backbone. RER-derived vesicles containing these proteins are then shuttled to the Golgi apparatus where further processing occurs, such as oligosaccharide trimming and terminal glycosylation, and selective proteolysis from "pro" to mature forms (Rothman, 1981). These proteins are sorted via some unknown process and transferred to specific organelles or, in the case of secretory products, exported out of the cell by exocytosis (for review, see Sabatini et al., 1982). The translocation and covalent modification of the proteins accounts for a several hour lag or "transit time" between translation and the arrival at their functional destination (Devreotes and Fambrough, 1976).

Churchill and Hokin (1979) analyzed the biosynthesis and insertion of Na,K-ATPase subunits into eel electroplax membranes in vitro. Sachs organs were incubated with [³H]valine, Na,K-ATPase was isolated and the subunits electrophoretically analyzed. The authors observed a 2.5 to 3 hour lag period before radioactivity was detected in the isolated enzyme, with subsequent linear incorporation into both the α and β subunits. The ratio of specific radioactivity for the two subunits was nearly unity at all sampling times. These results were interpreted to mean that holoenzyme assembly occurs 2 to 3 hours after de novo synthesis of the individual subunits. The congruency of the lag period for the incorporation of radioactive label into the nonglycosylated (or poorly glycosylated) α and heavily glycosylated β subunits indicated to the authors either

assembly of the enzyme prior to glycosylation or that the availability of the small subunit is rate-limiting. Similarly, when protein synthesis is inhibited by cycloheximide, a 1 1/2 hour delay is seen before the specific radioactivity decreased in either subunit, pointing to concerted assembly. That the rate of incorporation of label is equal in both subunits implies a similar rate of turnover.

Sabatini and his co-workers have reported some unusual properties of Na,K-ATPase biosynthesis (Sherman et al., 1980; Sabatini et al., 1982). Using a cell-free translation system they found that while the β subunit is synthesized on membrane-bound polysomes, its insertion does not appear to involve the cleavage of a signal peptide. The glycosylation kinetics indicate that the β protein is covalently modified in the Golgi apparatus, as is true for other membrane and secretory glycoproteins. More surprising yet were their findings regarding the biosynthesis and insertion of the α subunit. They found it to be synthesized in vitro only by free, not bound, polysomes and that in MDCK cells (a cultured epithelial line) it manifests itself in the plasma membrane without appearing in the RER. Accordingly, no change in the electrophoretic mobility of the α subunit was apparent when translation was carried out in the presence of tunicamycin, a specific inhibitor of protein glycosylation. That this subunit thus appears to be without asparagine-linked oligosaccharides is in agreement with a biosynthetic pathway which bypasses the classic sites of glycosylation. McDonough et al. (1982) have also observed α subunit synthesis in the cell-free translation of guinea pig kidney mRNA.

Since only free ribosomes were present (no β subunit was synthesized under these conditions) this concurs with the findings of Sabatini's group, indicating cytosolic biosynthesis of this peptide. Recently, Geering et al. (1982b) were able to immunoprecipitate radiolabeled Na,K-ATPase α subunit from both crude microsomes and the cytosol from toad bladder epithelial cells that had been pulsed for 10 minutes with [^{35}S]methionine. After a 2 hour chase with cycloheximide all of the labeled α subunit was associated with the plasma membrane. The authors feel that these observations are consistent with the synthesis of α peptides on free ribosomes. However, after the pulse they find nascent α chains in the microsomes but none in the cytosol. This suggests instead a microsomal site of α synthesis.

Pollack et al. (1981b), in analyzing the turnover of HeLa cell Na,K-ATPase, found that newly-synthesized α subunits required a period (transit time) of several hours to reach the plasma membrane. This was interpreted as the result of transfer from an intracellular (membrane) site of synthesis and processing to the cell surface. This is in clear contradiction to the concept of direct insertion into the plasma membrane (Sabatini, 1982). In the few cases where the cytosolic translation of integral membrane proteins have been studied, for example certain myelin proteins (Colman et al., 1982) and subunit 9 of the mitochondrial F_1 -ATPase (Schmidt et al., 1983), no detectable transit time was observed.

It can be concluded from these results that the pathway of synthesis of Na,K-ATPase may deviate in several ways from most other membrane proteins. Cytoplasmic synthesis of membrane components is

not, however, without precedent, particularly for some membrane proteins of mitochondria and chloroplasts. Theories are presently being forwarded involving insertion of membrane proteins via transient hydrophobic conformations, allowing spontaneous intercalation into lipid domains (Wickner, 1980; Engelman and Steitz, 1981). Also, like the Na,K-ATPase β subunit, the insertion of a number of other membrane proteins appears not to involve proteolytic processing (Sabatini et al., 1982).

Another aspect of Na,K-ATPase which requires elucidation in terms of biosynthesis is the presence of two tissue-specific forms of the α subunit (Sweadner, 1979). Sweadner described a form of catalytic subunit ($\alpha(+)$) found specifically in myelinated axons from brain which is about 20 amino acids larger than that found in unmyelinated neurons or kidney tissue. A functional significance has been suggested in that these proteins differ in their affinities for cardiac glycosides and may be involved in the enzyme's regulation by putative endogenous compounds with glycoside-like activity. Schellenberg et al. (1981) have found that the α and $\alpha(+)$ proteins are immunologically cross-reactive and present in a wide range of animal species. It is conceivable that both forms arise from a common mRNA with posttranslational modification accounting for the observed size difference. The complete pathway of synthesis for this enzyme is not yet well-defined. An understanding of the determinants which guide the individual subunits to their functional location represents one of the more important concepts in present-day cell biology.

Na,K-ATPase, with its varied structural and synthetic properties, represents an interesting system with which to study the assembly of membrane components.

The periodic degradation (turnover) of cellular proteins performs both a repair function, allowing replacement of faulty or damaged proteins, and a regulatory role, as was seen for Na,K-ATPase during ion stress (Pollack et al., 1981b). Steady state turnover appears to be a random process, although the accelerated degradation of proteins containing abnormal amino acids has been clearly demonstrated (Goldberg and Dice, 1974).

The turnover of Na,K-ATPase in HeLa cells has been examined by Cook and his colleagues (Pollack et al., 1981b and references therein). They have analyzed its turnover by two methods. In their early experiments they took advantage of the high binding affinity and specificity of ouabain for HeLa (human) cell Na,K-ATPase. Using [^3H]ouabain they found that the recovery from glycoside intoxication occurred by two modes, namely, dissociation of the ligand at the cell surface and internalization of the ouabain-receptor (Na,K-ATPase) complex, this latter mechanism being predominant (Cook et al., 1976).

The internalization kinetics in sublethal levels of [^3H]ouabain were interpreted to represent the turnover of Na,K-ATPase. A necessary provision for this conclusion is that the ouabain is not perturbing the normal turnover of the enzyme. Two observations supported this hypothesis. First, the internalization rate was independent of the extent of ouabain binding to available receptors.

Second, SDS-polyacrylamide gel electrophoresis of metabolically-labeled plasma membranes revealed turnover rates of proteins in the molecular weight range of 90-100,000 daltons (near that of the Na,K-ATPase α subunit) that closely approximate the kinetics of glycoside internalization, which occurs with a half-time of approximately 5 hours (Cook et al., 1976).

This value, corresponding to a turnover coefficient of 3.1/cell generation, has been confirmed by a second method of analysis. Pollack et al. (1981b) employed a density shift technique which has been extensively used by Fambrough and his co-workers to study the acetylcholine receptor (cf. Gardner and Fambrough, 1979). Briefly, HeLa cells were grown in the presence of ^{13}C -substituted amino acids then placed into normal medium. Density gradient centrifugation of SDS-solubilized plasma membranes allowed the analysis of Na,K-ATPase α subunit (labeled by K-sensitive phosphorylation from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$) turnover by determining the kinetics of its return to a control density. This method is independent of possible glycoside-induced alternations in Na,K-ATPase turnover.

An interesting ramification of these experiments is that they indicate that the α subunit of Na,K-ATPase is turned over faster than the bulk of the proteins which make up the HeLa cell plasma membrane, suggesting heterogeneous turnover of plasma membrane proteins (Pollack et al., 1982). The question of homogeneous (cf. Doyle and Baumann, 1979) vs. heterogeneous (cf. Horst and Roberts, 1979; Tauber and Reutter, 1981) plasma membrane turnover is currently a hotly

contested issue, and one that studies such as that of Pollack et al. (1982) on individual membrane components have the potential to elucidate.

The pathway of Na,K-ATPase turnover has been inferred by isolating subcellular compartments from HeLa cells at various times after [^{3} H]ouabain addition (Cook and Brake, 1978; Cook et al., 1982). At early times, the bound glycoside co-fractionates with the plasma membrane marker 5'-nucleotidase. After 46 hours most of the [^{3} H]ouabain was found associated with β -hexosaminidase, a lysosomal component. The ouabain and β -hexosaminidase also were equally sensitive to shear forces at this time, determined by carefully controlled homogenization. These data indicate the transfer of the ligand from the cell surface to lysosomes during steady state turnover. No intermediate compartments could be detected, suggesting a rapid transfer step.

In these studies it was assumed that the ouabain remained bound to Na,K-ATPase until the enzyme was degraded, at which time free glycoside was released (HeLa cells cannot metabolize ouabain). The authors concluded that Na,K-ATPase is degraded in lysosomes. Caution may be advised in assuming that the ligand and receptor are always co-transported into the same compartment in view of work by Krupp and Lane (1982) who presented evidence for lysosomal degradation of insulin but non-lysosomal turnover of its receptor.

In summary, Na,K-ATPase is clearly an extremely important enzyme, both at the cellular and physiological levels. Given the

role of the biosynthesis and degradation of this and other membrane proteins in maintenance, repair and regulation it is evident that a better understanding of the pathways for these processes is necessary. In this dissertation, I present the results of my efforts to elucidate these aspects of Na,K-ATPase. For this purpose I have isolated the enzyme from kidney tissue where it is plentiful and prepared polyclonal antibodies to the large (α) subunit. These antibodies were utilized to analyze the dynamics of this protein in cultured cells, where I took advantage of their clonal homogeneity and the ease with which radiolabeled precursors can be introduced.

CHAPTER II

ISOLATION OF Na,K-ATPase FROM RAT KIDNEY

Introduction

Na,K-ATPase has been isolated from many species and tissue types, both in detergent solubilized and membrane-bound form (for review, see Jorgensen, 1975). One of the most often cited isolation methods involves the mild detergent treatment of kidney microsomes, followed by the collection of enzyme as a membrane-bound particle by centrifugation (Jorgensen, 1974). This has been useful for the rapid preparation of very pure enzyme from the kidneys of rabbit, sheep, dog and pig. Its use with rat kidney has met with somewhat less success (Lo and Lo, 1980).

For the purpose of this study, the characterization of the Na,K-ATPase from cultured rat hepatoma (HTC) cells, I have chosen to raise antibodies using purified enzyme as immunogen. Kidney tissue was selected as a source of Na,K-ATPase because it is readily available in relatively large quantities and exhibits approximately fifty times the enzyme activity (and presumably fifty times the amount of enzyme) of HTC cells (data not shown).

The use of rat Na,K-ATPase as immunogen ensures optimum antibody reactivity with the enzyme from rat HTC cells. This chapter reports the modification of Jorgensen's (1974) isolation procedure to produce

Na,K-ATPase of suitable purity and yield for subsequent use (Chapter III) as immunogen.

Materials and Methods

Materials

Rat kidneys (saline-perfused, frozen) were purchased from Pel-Freez Biologicals. Sodium dodecylsulfate (specially pure) was from BDH Biochemicals. Vanadate-free ATP (disodium salt) was from either Sigma or Boehringer. NaB^3H_4 (116.6 mCi/mmol) was obtained from New England Nuclear. All reagents and molecular weight standards (myosin, 200K; β -galactosidase 116.5K; phosphorylase B, 94K; bovine serum albumin, 68K; ovalbumin, 43K) for polyacrylamide gel electrophoresis were from Bio-Rad. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol) and aqueous counting scintillant (ACS) were from Amersham.

Na,K-ATPase Preparation

Rat kidneys were thawed at 4°C for several hours in homogenization buffer containing 0.25M sucrose, 5mM EDTA and 30mM DL-histidine, pH 7.4, then cut in half longitudinally and dissected free of papilla and connective tissue. Microsomes ("L fraction") were prepared according to the method of Lo et al. (1976) and extracted with sodium dodecylsulfate (SDS) in 50mM imidazole, pH 7.5, containing 3mM ATP and 2mM EDTA (Jorgensen, 1974). SDS was added dropwise from a stock solution of 2 mg/ml with constant stirring and the mixture was incubated at room temperature for 30 minutes. Optimum conditions for detergent extraction, determined experimentally (see "Results"),

were 0.9 mg SDS/ml at 4.0 mg microsome protein/ml. The membranes were layered over 50% glycerol (w/w) in 1mM EDTA, 25mM imidazole, pH 7.5 (Liang and Winter, 1976), and centrifuged at 50,000 RPM (Spinco 60Ti rotor) for 110 minutes. The resulting pellet containing the Na,K-ATPase was suspended in 1mM EDTA, 25mM imidazole, pH 7.5, and stored at -20°C.

Assays

Total ATPase activity was measured in a 0.5 ml volume containing 100mM NaCl, 20mM KCl, 2.1mM MgCl₂, 0.1 mM EGTA and 50mM Tris-HCl, pH 7.5. Reactions were initiated by the addition of ATP to a final concentration of 2mM and incubated at 37°C. An equal volume of 10% trichloroacetic acid was added, precipitate was removed by centrifugation and the amount of released P_i was determined (Fiske and Subbarow, 1925). For the experiments shown in the figure on p. 36 ATPase activity was assayed as the release of ³²P_i from [γ-³²P]ATP (final specific activity 12-17 μCi/mmol). The reaction was stopped with 3 ml 1.67N H₂SO₄ and released ³²P_i was separated from unreacted [γ-³²P]ATP by the method of Ernster et al. (1950). Na,K-ATPase activity was determined as that fraction of the total which could be inhibited by 1mM ouabain in the absence of K.

Protein was estimated by the fluorometric method of Avruch and Wallach (1971), or, where indicated, with the technique of Lowry et al. (1951), both of which were calibrated with bovine serum albumin.

Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was performed using the buffer system of Laemmli (1970). Slabs (1.5 mm) were cast with a 4.5% stacking gel and either a 7.5% or 9% resolving gel, as indicated in the figure legends; the cross-linker to monomer ratio (%C) was 2.7% in both gel sections. Samples were solubilized in solvent buffer (2% SDS, 5% β -mercaptoethanol, 62.5mM Tris, pH 6.8) at room temperature since heating caused agglomeration of the Na,K-ATPase α subunit (see "Results"). Electrophoresis was performed at 20mA until the tracking dye entered the resolving gel, at which time the current was increased to 40mA. For some experiments, cylindrical gels were cast in 6 mm (i.d.) x 90 mm glass tubes and electrophoresis was carried out at 1.5mA/gel, then increased to 3mA/gel when the tracking dye entered the resolving gel.

Gels were stained in 0.01% Coomassie blue in 10% acetic acid, 50% methanol for several hours, then destained in several changes of 7.5% acetic acid. For fluorography, unstained slab gels were fixed in 10% trichloroacetic acid, dried, overlaid with preflashed Kodak X-ARO film (Laskey and Mills, 1975) and exposed at -70°C with a Dupont Cronex intensifying screen. The films were scanned on a Joyce-Loebl automatic recording microdensitometer, model MK III C. Alternatively, cylindrical gels containing radioactive samples were frozen in 95% ethanol/dry ice and sliced manually into 2.5 mm sections. These were placed in scintillation vials with 0.6 ml 30% H_2O_2 , capped tightly and incubated at 60°C overnight. The vials were cooled and counted with 10 ml aqueous scintillation cocktail.

Identification of the Enzyme Subunits

Specific phosphorylation of purified Na,K-ATPase by ATP to label the α subunit was performed in a 60 μ l volume containing 10 μ Ci [γ - ^{32} P]ATP (3000 Ci/mmol) and either 100mM Na or 25mM K, as described by Pollack (1980). The reaction was terminated with an equal volume of SDS-PAGE solvent buffer and immediately loaded onto a slab gel, prepared as described above, and electrophoresed at 4°C for 2.5 hours at 40mA. Performing the electrophoresis at this temperature improves the recovery of the phosphorylated species (Sweadner, 1979). The gel was then fixed in 10% trichloroacetic acid for 1 hour, dried and fluorographed.

The β subunit was identified by its sequential oxidation with periodate and reduction with NaB^3H_4 , a label specific for sialoglycoproteins. I used the technique of Van Lenten and Ashwell (1971) as modified by Lo and Lo (1980). Labeled enzyme was electrophoresed and the gel was sliced and counted, as previously described.

Results

Purification of Rat Renal Na,K-ATPase

Renal Na,K-ATPase has been successfully obtained in a highly purified form by the selective extraction of other proteins from microsomal membranes by detergents (Jorgensen, 1974). As a prelude to optimizing the conditions for the isolation of Na,K-ATPase the sensitivity to SDS of the membrane-bound enzyme was analyzed. When rat kidney microsomes were incubated with SDS in the presence of ATP a biphasic response of Na,K-ATPase activity to detergent was observed

(Figure 1). At the lower concentrations (0.2-0.6 mg/ml) SDS stimulated activity to 1.7 fold over that of untreated membranes, presumably due to the exposure of latent enzyme sites to substrate. At higher detergent levels inhibition occurred, with complete inactivation evident at 1.3-1.6 mg SDS/ml. As was reported for kidney microsomes from other species (Jorgensen, 1974), the detergent sensitivity of Na,K-ATPase is a function of the protein concentration (Figure 2). A positive correlation was observed between the protein concentration and the concentration of SDS necessary to inactivate the enzyme. This relationship is not linear and the optimum ratio of detergent to protein must be determined for each concentration of microsome protein. Therefore, all detergent extractions were performed with 4.0 mg microsome protein/ml.

Fractions enriched in Na,K-ATPase were isolated as pellets after centrifuging SDS-treated microsomes through buffered 50% glycerol. The recoveries of Na,K-ATPase activity and protein in these fractions as a function of the detergent concentration used during the treatment of the microsomes are shown in Figure 3. An inverse relationship is apparent between the purification (specific activity) and recovery (total activity) of Na,K-ATPase with increasing SDS in the extraction step. The amount of protein recovered decreased in parallel to total activity. Ouabain-insensitive (Mg-ATPase) activity was not detected in the pellets from microsomes that were treated with SDS at 0.6 mg/ml or higher (results not shown). Mg-ATPase was sometimes detected in subsequent Na,K-ATPase preparations isolated under optimum conditions

Figure 1. Na,K-ATPase activity during the extraction of rat kidney microsomes with SDS. Microsomes were suspended at a final concentration of 4.0 mg protein/ml in 50mM imidazole, pH 7.4, 2mM EDTA, 3mM ATP and SDS at the concentrations indicated on the abscissa. After 30 minutes at room temperature 10 μ l aliquots were removed and assayed for Na,K-ATPase activity. The results of three experiments (O, $\bullet$, $\blacktriangle$) are shown. The data are expressed as percent of the maximum activity in each experiment, which ranged from 34-49 μ mol P_i $\cdot$ mg protein $^{-1}$ $\cdot$ h $^{-1}$.

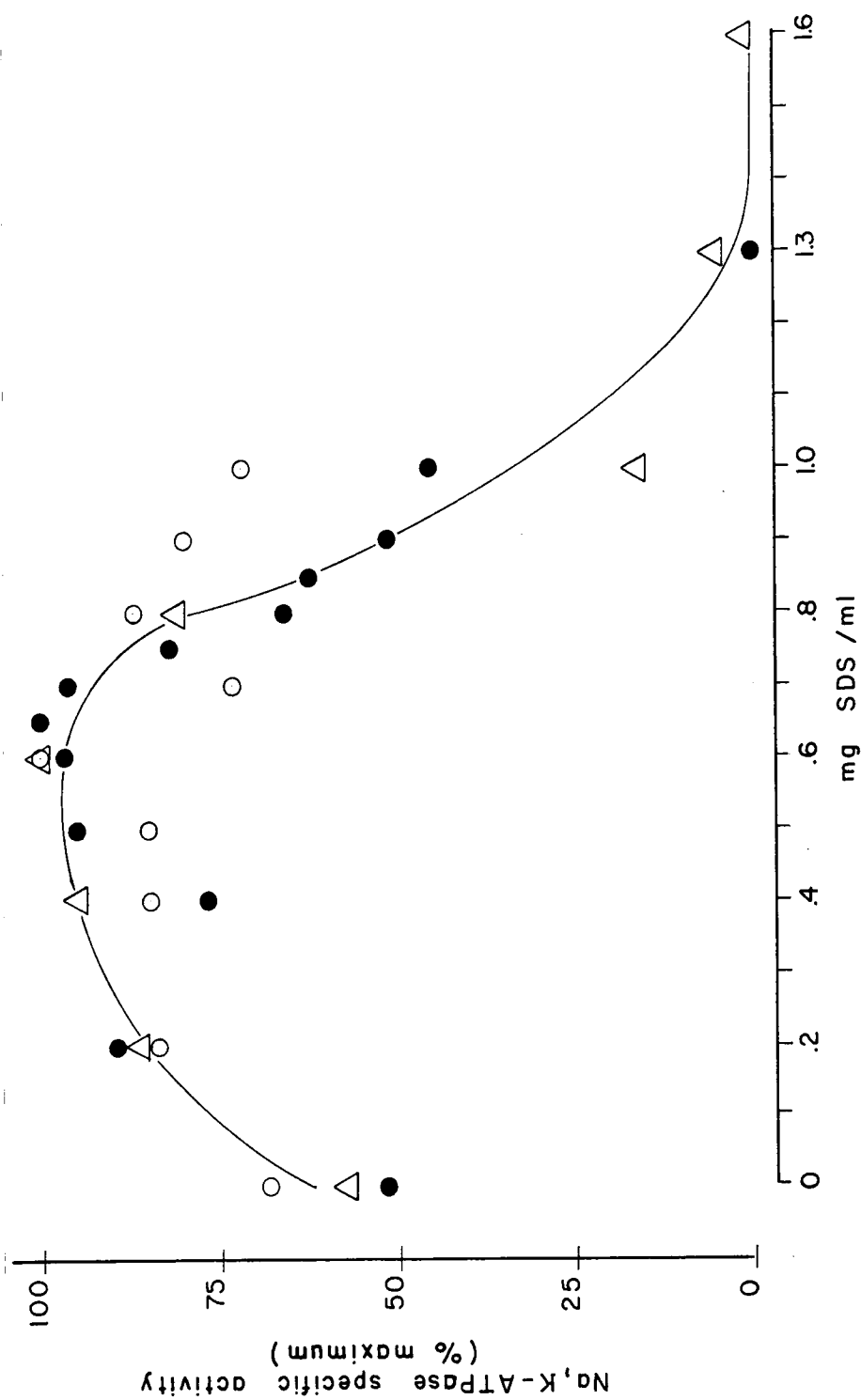


Figure 1

Figure 2. Influence on Na,K-ATPase activity of protein concentration during the detergent extraction of microsomes. Microsomes were suspended as in Figure 1 at 1.2 (○), 2.0 (●), or 4.0 (Δ) mg protein/ml and SDS at the concentrations indicated on the abscissa. After 30 minutes at room temperature aliquots were transferred to tubes for assay of Na,K-ATPase, determined in this experiment by the release of $^{32}\text{P}_i$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

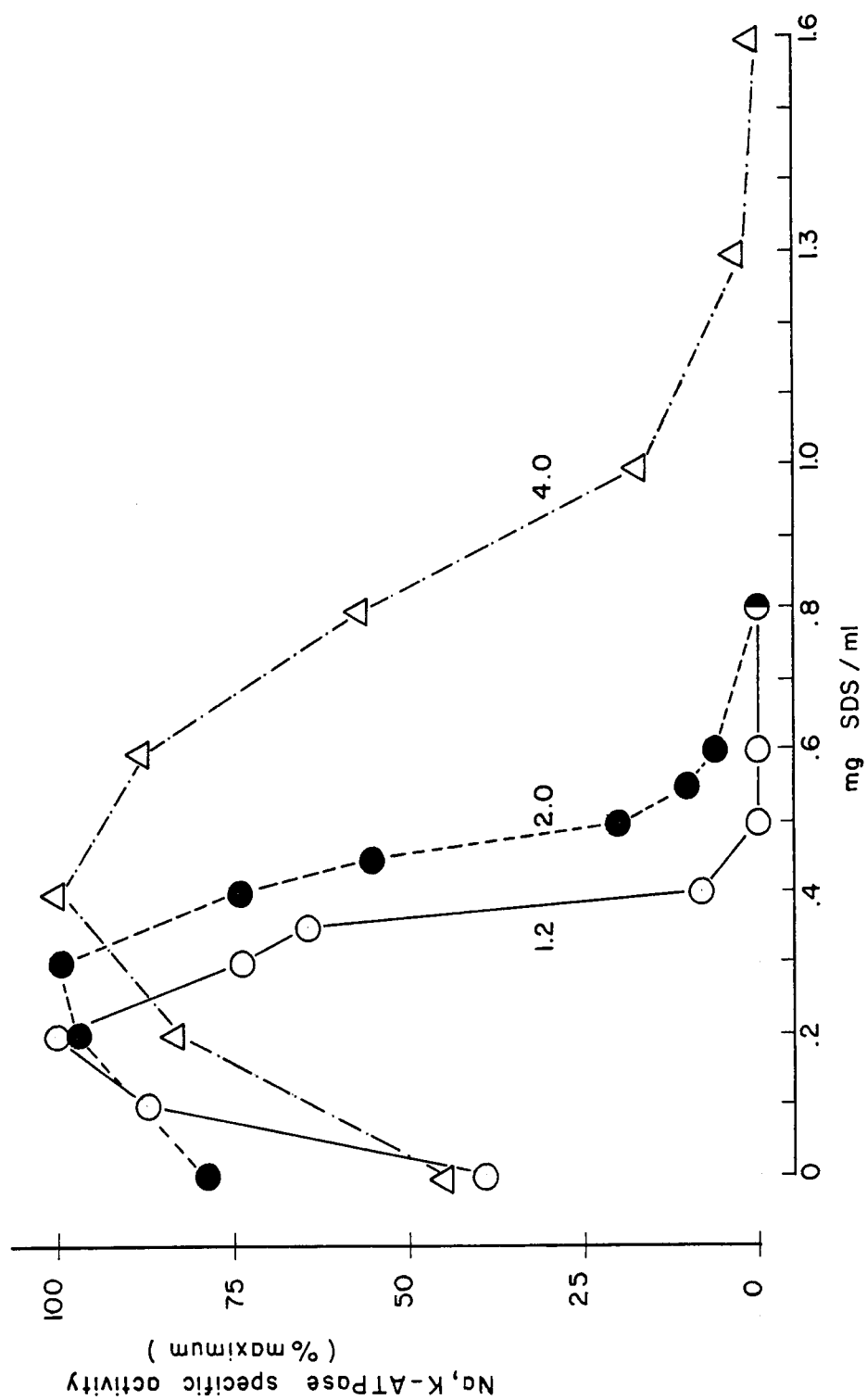


Figure 2

Figure 3. The effect of SDS concentration during the extraction of rat kidney microsomes on the subsequent recovery and purification of Na,K-ATPase. Microsomes, 32 mg protein at 4.0 mg/ml, were treated with SDS at the concentrations shown on the abscissa and pelleted through buffered 50% glycerol, as described in "Materials and Methods." Enzyme activity was determined in the pellets from each detergent concentration. The specific activity (○), total activity (●) and total protein recovery (Δ) are shown.

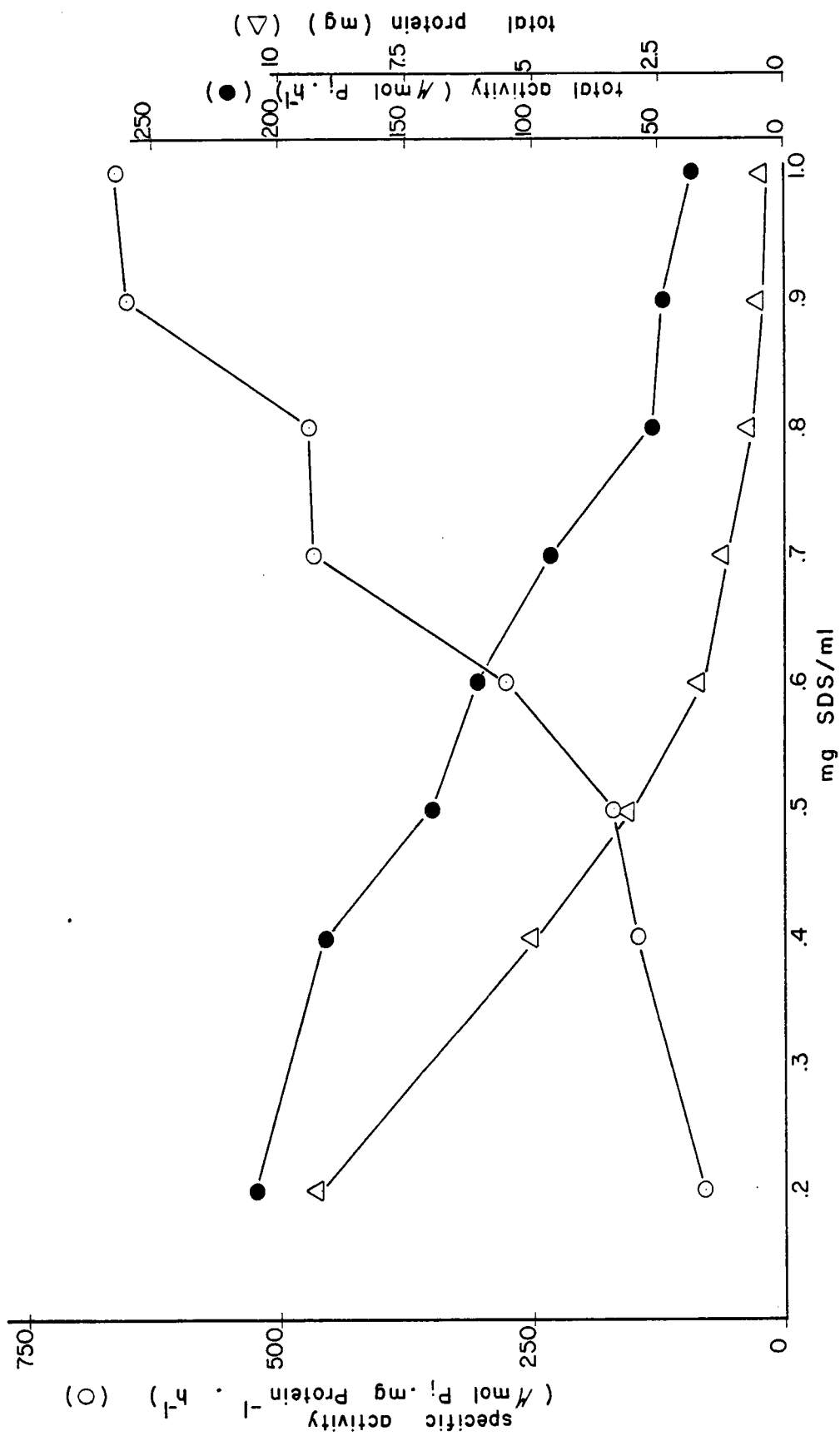


Figure 3

(see below) but never accounted for more than 3% of the total ATPase activity.

The relative purification of the Na,K-ATPase preparations shown in Figure 3 may be underestimates because of the detergent inactivation superimposed on the extraction procedure (Figure 1, p. 34). Also, while the specific activity ($>600 \mu\text{mol P}_i \text{ released} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$) compares favorably with that obtained from rat kidney by Lo and Lo (1980), our method of protein determination (intrinsic fluorescence; Avruch and Wallach, 1971) gave consistently higher values for the purified enzyme than did the Lowry et al. (1951) method used by Lo and Lo. In one such experiment, a preparation of Na,K-ATPase was found by the Lowry method to contain 1.56 mg protein/ml while simultaneously estimated to be 6.20 mg protein/ml by intrinsic fluorescence, a difference of 4 fold.

To further characterize the purity of the Na,K-ATPase preparation described in Figure 3, SDS-PAGE analysis was performed (Figure 4). As the purity of the Na,K-ATPase (i.e., specific activity) increased, the protein composition of the preparation, as indicated by Coomassie blue staining, simplified to three major bands of molecular weights 93,000, 53,000, and 32,000 daltons. Several minor bands were also present. The molecular weights of the larger two major bands correspond to published values (determined by PAGE) for the α and β subunits of Na,K-ATPase (Kyte, 1972; Lo and Lo, 1980, and references therein). The diffuse staining of the 53,000 dalton component is consistent with it being the highly glycosylated β subunit (Kyte, 1972). From the above PAGE data, in conjunction with the estimation of enzyme

Figure 4. SDS-PAGE analysis of Na,K-ATPase isolated from rat kidney microsomes treated with various concentrations of SDS. Untreated microsomes (M) and Na,K-ATPase preparations isolated from microsomes treated with SDS at the concentrations shown above each lane were from the experiment described in Figure 3. The samples (35 μ g/lane) were separated on a 9% slab gel and stained with Coomassie blue. The last lane (Stds) contains molecular weight standards of 200K, 116.5K, 94K, 68K and 43K daltons.

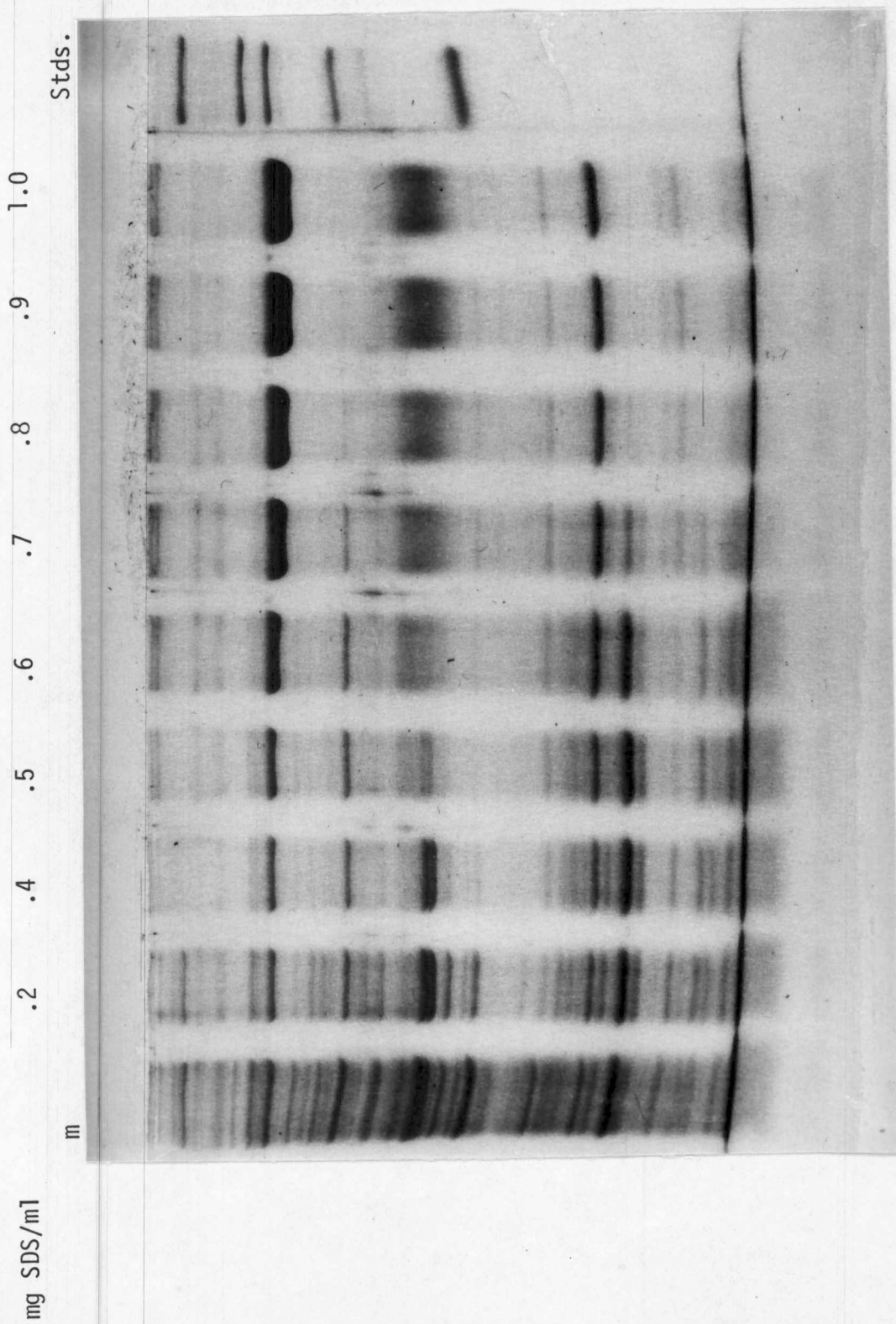


Figure 4

purification and yield based on Na,K-ATPase activity (Figure 3), the optimum detergent concentration for the extraction of microsomes was 0.9 mg SDS/ml at 4.0 mg protein/ml. Enzyme isolated under these conditions is henceforth referred to as "purified Na,K-ATPase." In Table I are shown results of a typical Na,K-ATPase isolation. The specific activity of the microsome fraction shown in this table is that expressed in the absence of detergent. This value ranged from 18 to 33 $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$ over an eighteen-month period. After the final step (centrifugation of the detergent-treated membranes through glycerol) 10% of the total activity is recovered with a concomitant removal of greater than 99% of the starting (homogenate) protein. The 58 fold purification, as indicated by the specific activity, may not be an accurate estimate because of detergent effects on the activity. In this preparation 97% of the ATPase activity in the purified Na,K-ATPase was ouabain-inhibitable.

To confirm the identity of the subunit proteins specific labeling techniques were employed. The large (α) subunit of Na,K-ATPase includes the site of Na-dependent, K-sensitive phosphorylation by ATP (Kyte, 1971). Purified enzyme was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of either Na or K and subjected to SDS-PAGE. The ^{32}P -containing band was located by autoradiography, shown in Figure 5A. When the labeling was performed in the presence of Na a single band of molecular weight approximately 94,000 daltons was labeled ^{32}P . No bands were detected when K was substituted for Na, thus establishing the 93,000 dalton band as the α subunit of Na,K-ATPase.

Table I
Recovery and Purification of Rat Renal Na,K-ATPase

Fraction	Total protein mg (%)	Total activity $\mu\text{mol P}_i \cdot \text{h}^{-1}$ (%)	Specific activity $\mu\text{mol P}_i \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$ (purif. ²)	Na,K-ATPase as fraction of Total ATPase ¹
Homogenate	832 (100)	9300 (100)	11.2 (1.0)	.53
Microsomes ³	152 (18)	3250 (35)	21.4 (1.9)	.68
Purified enzyme	1.50 (0.2)	975 (10)	650 (58)	.97

¹Ouabain-sensitive ATPase specific activity/total ATPase specific activity (not shown)

²Specific activity of the fraction/specific activity of the homogenate.

³Not detergent-treated.

Figure 5. Identification of the subunits of Na,K-ATPase. (A) Specific phosphorylation of the Na,K-ATPase α subunit. α Purified Na,K-ATPase (200 μ g) was phosphorylated with [γ - ^{32} P]ATP in the presence of either 100mM Na or 25mM K, when electrophoresed on a 9% slab gel and fluorographed. Presented are densitometric tracing of the developed film image. The large film density at the dye front is due to unreacted ATP and P_i released from the enzyme during electrophoresis. (B) Specific labeling of the Na,K-ATPase β subunit with ^{3} H. Purified Na,K-ATPase (200 μ g) was incubated with 4 μ M NaB ^{3} H $_4$ (116.6 mCi/mmol) with (●) or without (○) periodate pretreatment and electrophoresed on cylindrical 9% gels which were then sliced and counted. Shown at the top of the figures are the mobilities of molecular weight markers (same as in Figure 4) from (A) a lane of the same gel, or (B) a duplicate gel which was stained with Coomassie blue.

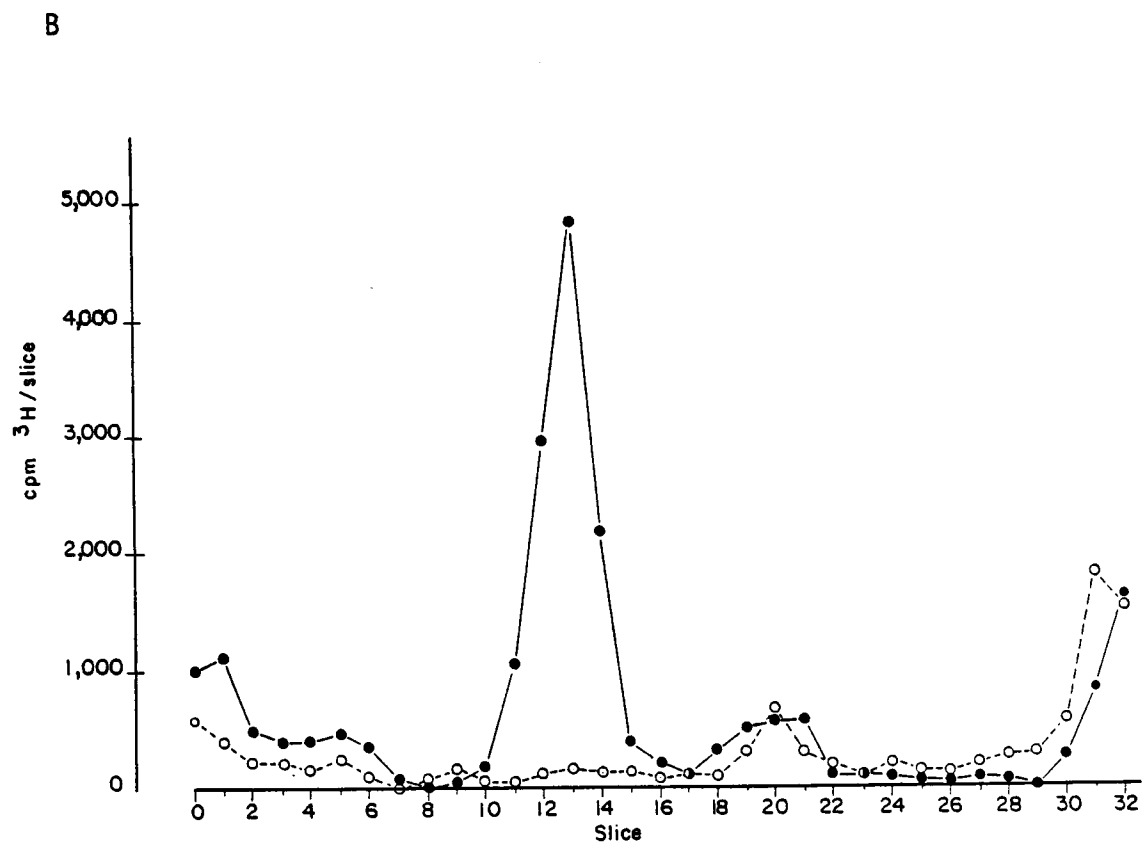
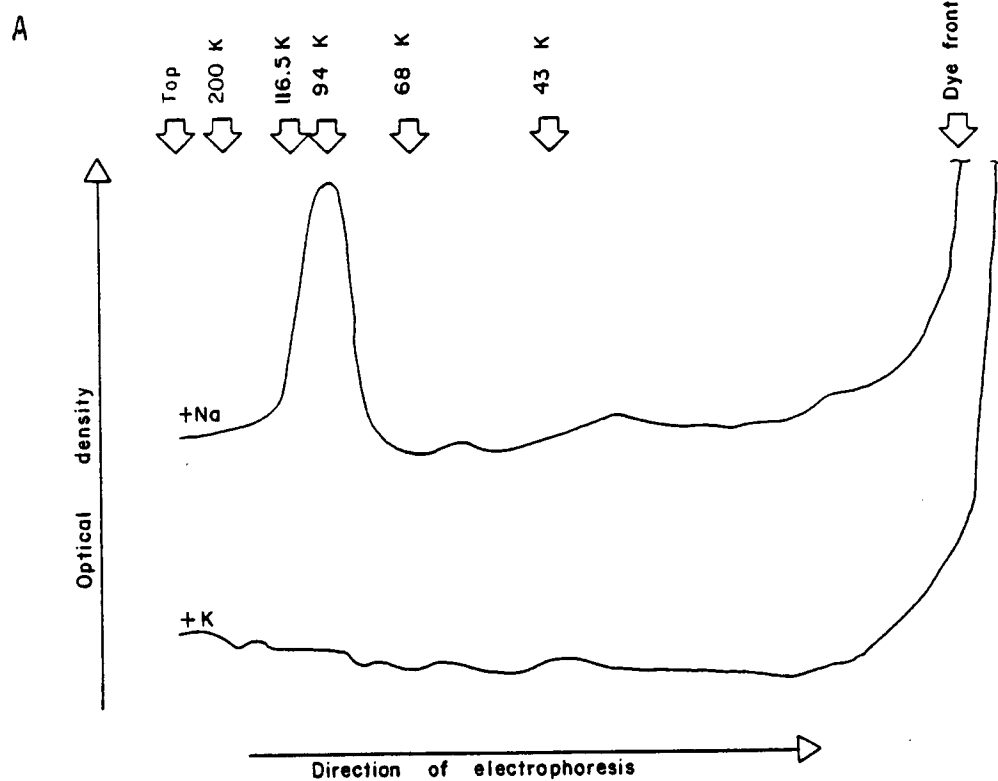


Figure 5

To identify the β subunit I employed the technique of Van Lenten and Ashwell (1971) as modified by Lo and Lo (1980) to label sialic acid-containing glycoproteins by the sequential oxidation with periodate and reduction with NaB^3H_4 . Purified Na,K-ATPase, labeled in this way, was solubilized in SDS and electrophoresed and the gel sliced. Nonspecific labeling was determined in the absence of periodate pretreatment. The radioactivity profile of these gels is shown in Figure 5B. A single peak of specific label was observed at a position corresponding to a molecular weight of approximately 56,000 daltons. This correlates well with the protein band of 53,000 daltons observed on stained gels. The results of ^3H labeling and the staining pattern, taken together, are strong evidence for the identity of the β subunit. The 32,000 dalton protein consistently observed in the preparation is a contaminant which may be a proteolytic fragment of one of the subunits.

The homogeneity of the bands containing the α and β subunits was estimated by analyzing their mobility on SDS gels of various acrylamide concentrations (Ferguson, 1964). Single bands corresponding to the α and β subunits were observed at each acrylamide concentration tested. When the relative mobility of each band was plotted versus the acrylamide concentration (Figure 6) a series of points were obtained which closely fit a straight line, as indicated by the correlation coefficients ($\alpha = -.999$, $\beta = -.995$). From this, it can be concluded that the Na,K-ATPase subunit-containing bands seen after SDS-PAGE are homogeneous with respect to molecular weight.

Figure 6. Ferguson analysis of the Na,K-ATPase subunits by SDS-PAGE. Purified Na,K-ATPase was subjected to electrophoresis on duplicate SDS gels containing the acrylamide concentration indicated on the abscissa. The cross-linker concentration (%C) was 2.7% of the free monomer in each case. The gels were stained with Coomassie blue and the mobility of the α (●) and β (○) bands was measured relative to the tracking dye (bromphenol blue). The correlation coefficients (r) are shown next to their respective lines.

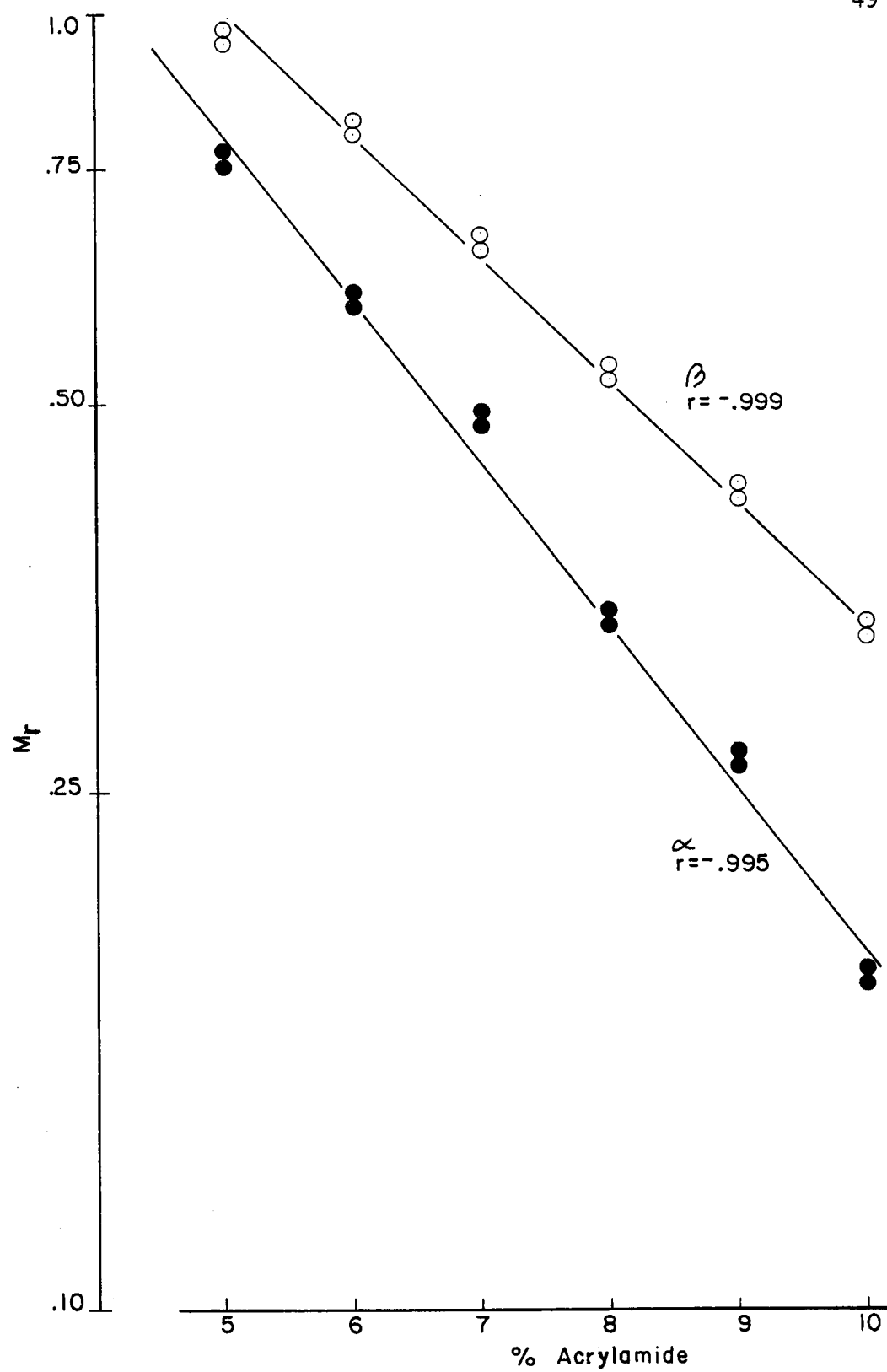


Figure 6

All SDS-PAGE analyses in this study were performed without prior heating of the samples in the solvent. I have found that Na,K-ATPase is completely solubilized at room temperature in solvent buffer containing 2% SDS and 5% β -mercaptoethanol, and that heating the enzyme in this solution results in agglomeration artifacts. Figure 7 shows SDS gels containing purified Na,K-ATPase that had been either heated or kept at room temperature prior to electrophoresis. The room temperature sample showed the normal protein pattern, while the heated sample exhibited a specific decrease in the staining density of the α subunit with the concomitant appearance of Coomassie blue-positive material that failed to enter the resolving (9%) gel. Residual stained material also appeared at the top of the stacking (4.5%) gel (not shown in this photograph) after electrophoresis of the heated enzyme. Interestingly, none of the other proteins in the Na,K-ATPase preparation seem to be affected by heating.

Discussion

This chapter reports the adaptation of methods for the isolation of Na,K-ATPase from the kidneys of large mammals (dog, rabbit, sheep) to use in rat kidney. Jorgensen (1974) has reported a rapid method for the isolation of mg quantities of pure Na,K-ATPase from the mammalian renal medulla by selectively extracting extraneous protein from microsomal membranes with detergents, maintaining the enzyme in an active particulate form. I have tailored these techniques to facilitate the isolation of Na,K-ATPase from rat kidney.

Figure 7. The effect of heating Na,K-ATPase in sample solvent prior to SDS-PAGE. Purified Na,K-ATPase (100 μ g) was mixed with sample solvent and (1) heated in a boiling water bath for 3 minutes, or (2) kept at room temperature. The samples were then electrophoresed on cylindrical 9% gels and stained with Coomassie blue.

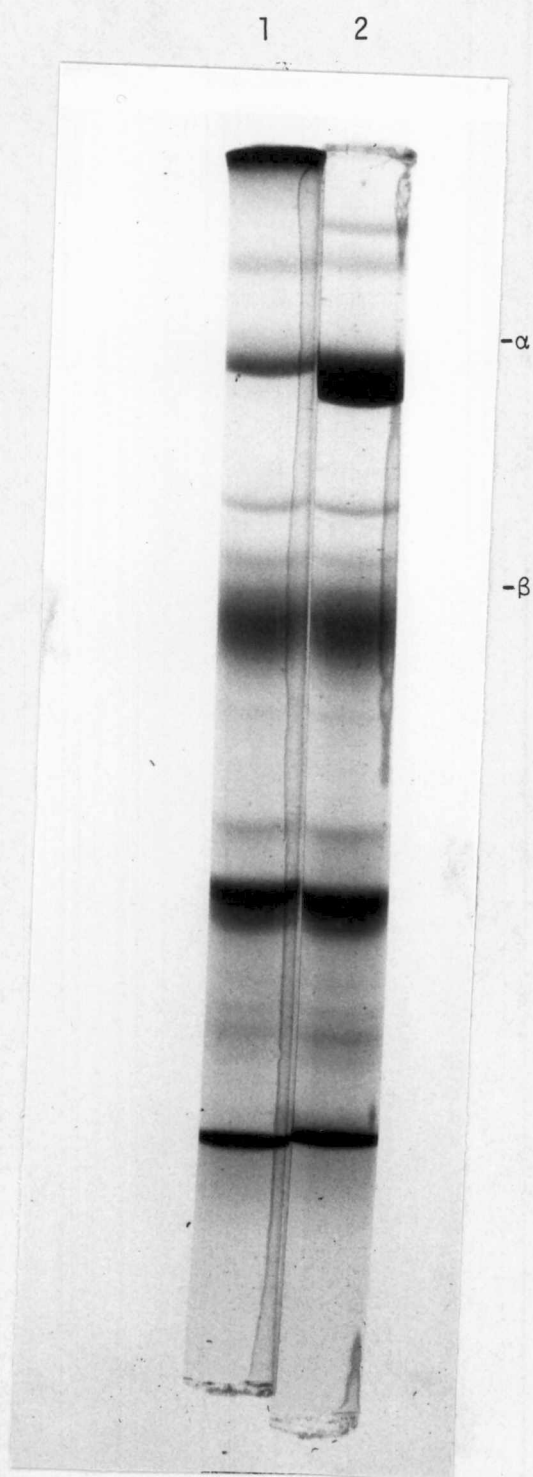


Figure 7

Microsomes were prepared by the method of Lo and Edelman (1976) and exhibited Na,K-ATPase activity ($18-33 \mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$) comparable to the value of $19.7 \mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$ in the original report. These values are, however, approximately ten fold lower than the activity ($240-300 \mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$) reported by Jorgensen (1974) for microsomes prepared from rabbit renal medulla. This probably reflects in part a species variation since the method of membrane preparation was similar. The use of only renal outer medulla may also account for part of the activity discrepancy since that region does have a higher content of Na,K-ATPase enzyme than other portions of the kidney (Jorgensen, 1980).

Upon the addition of detergent (SDS) to these membranes a biphasic response in Na,K-ATPase activity was observed (Figure 1, p. 34). The stimulation of activity at low detergent concentrations is interpreted as the exposure of latent enzyme active sites to ATP by the partial dispersal of membrane structure. In the rat kidney microsomes, detergent treatment increased activity by 1.7 fold, in contrast to the 5 fold value reported for rabbit medulla membranes (Jorgensen, 1974). My use of frozen tissue may lessen the amount of latent enzyme, i.e., ice formation may partially disrupt some membranes. While Jorgensen (1974) stored microsomes at -25°C before detergent treatment, the presence of 0.25M sucrose in the buffer can serve as a cryoprotectant (Moller, 1971). Jorgensen (1974) also used a lower concentration of microsome protein ($1.41 \text{ mg} \cdot \text{ml}^{-1}$). The degree of enzyme latency in this study was not influenced by the

protein concentration used during the detergent treatment (Figure 2, p. 36).

When the detergent-extracted microsomes are centrifuged through 50% glycerol, the resulting pellet is enriched in Na,K-ATPase (Figure 3, p. 38). The degree of purity depends upon the SDS concentration during the extraction. I observed the highest specific activity ($650\text{--}660 \mu\text{mol } P_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$) in the pellets of membranes treated with 0.9 to 1.0 mg SDS/ml. The ouabain-insensitive component of the total ATPase activity in these fractions was no more than 3%. This compares favorably the activity of preparations obtained by Lo and Lo (1980) from rat renal cortex of $430\text{--}540 \mu\text{mol } P_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$. It is, however, somewhat less active than preparations from rabbit kidney ($1900\text{--}2200 \mu\text{mol } P_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$; Jorgensen, 1974) and other sources such as dogfish rectal gland ($1200\text{--}1500 \mu\text{mol } P_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$; Hokin et al., 1973) and pig brain (up to $7000 \mu\text{mol } P_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$; Nakao et al., 1973), although the authors of the latter report cited problems in protein determination. The low activity cannot be accounted for by detergent inactivation since membranes extracted with 0.9 mg SDS/ml retain 80-85% of their maximum specific activity (Figure 1, p. 31). I encountered difficulties when comparing the specific activity of my enzyme preparation to those reports because of apparent differences in the method of protein determination. The technique used throughout this study was the measurement of intrinsic fluorescence (Avruch and Wallach, 1971) which gave a value four fold higher than that indicated by the Lowry et al. (1951) method which was (or

modifications thereof) used by the authors cited above. If I correct the specific activity of my preparation by this factor, a value of $2600 \mu\text{mol } P_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$ is obtained. This, on the other hand, is likely too high, especially after consideration of the SDS-PAGE analysis (Figure 4, p. 41). While the preparation obtained at 0.9 or 1.0 mg SDS/ml is highly enriched in peptides whose molecular weights are consistent with the Na,K-ATPase subunits, one major contaminant and several minor bands are present. No bands other than the enzyme's two subunits are seen in the high activity preparation from rabbit kidney (Jorgensen, 1974). Using the uncorrected specific activity of $650 \mu\text{mol } P_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$, the final Na,K-ATPase was purified approximately 60 fold and contained 10% of the total activity of the homogenate.

As mentioned above, the SDS-PAGE pattern (Figure 4, p. 41) corresponds well to that expected for highly-enriched Na,K-ATPase. The major bands of 93,000 and 53,000 daltons are very similar to the mobilities of the α and β subunits, respectively, found in other preparations (Jorgensen, 1975). The α subunit contains the site for ATP hydrolysis (Kyte, 1971) and can, in the presence of Na and absence of K, be specifically labeled with ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Jorgensen, 1975). I confirmed with this technique the identity of the 93,000 dalton protein as the Na,K-ATPase α subunit (Figure 5A, p. 45). Similarly, the fact that the β subunit is highly glycosylated was exploited for its identification. The 53,000 dalton protein was recognized as the β subunit by its ability to be reduced by NaB^3H_4 , a technique devised by Van Lenten and Ashwell (1971) to label sialoproteins. The two

subunit-containing bands were judged to be homogeneous by Ferguson (1964) analysis (Figure 6, p. 48). However, since SDS-PAGE separates solely by molecular weight (assuming SDS binds equally to all proteins on a molar basis) this does not rule out the co-migration of contaminants of the same size as the Na,K-ATPase subunits.

The sensitivity of the Na,K-ATPase to heating when solubilized in SDS-PAGE solvent buffer (Figure 7, p. 51) was also noted by Sweadner (1979) who reported diminished resolution of heated Na,K-ATPase samples. Heating samples for SDS-PAGE, while a common practice, has also been cited as causing proteolysis which cannot be explained by the action proteolytic enzymes (Kowit and Maloney, 1982). The specific coagulation of the α subunit after heating has recently been used as one means to identify the target of a monoclonal antibody as Na,K-ATPase (Fambrough and Bayne, submitted).

I conclude that I have prepared Na,K-ATPase from rat kidneys in a highly-enriched form that can be further processed and used as an immunogen. The production and characterization of the subsequent antibodies are described in the next chapter.

CHAPTER III

PRODUCTION AND CHARACTERIZATION OF ANTIBODIES

AGAINST RAT KIDNEY Na,K-ATPase

Introduction

Because of their specificity and binding affinities, antibodies (both polyclonal and monoclonal) have been employed in the analysis of a variety of membrane components, one of which is Na,K-ATPase. Antibodies against the holoenzyme or its component peptides have been utilized in active site structural analysis (McCans et al., 1974; Rhee and Hokin, 1975; Schellenberg et al., 1981), subunit identification (Rhee and Hokin, 1975; Jean et al., 1975), determination of subunit structural orientation and sidedness (Girardet et al., 1981) and ion transport mechanism analysis (Kyte, 1974). It is the aim of this study to characterize the biosynthesis and turnover of Na,K-ATPase in cultured rat hepatoma (HTC) cells by the use of analytical immunoprecipitation.

In this chapter I present details of antibody production against the α subunit of Na,K-ATPase prepared from the rat kidney enzyme, purified as described in Chapter II.

Materials and Methods

Materials

Methionine-free RPMI 1640 was from Flow Laboratories; all other cell culture reagents were from GIBCO. Na¹²⁵I (15.1 mCi/ μ g iodine),

L-[4,5-³H]leucine (1 Ci/mmol), D-[1-³H]mannose (5 Ci/mmol) and L-[1-³H]fucose (5.3 Ci/mmol) were from Amersham. L-[³⁵S]methionine and Enlightening were from New England Nuclear. Protein A agarose was from BRL. Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone (BSOCOES) was purchased from Pierce Chemical Co.

Gel Filtration

Purified rat kidney Na,K-ATPase (5-8 mg) was solubilized in 2% SDS and applied to a 1.5 x 90 cm Bio-Gel A1.5m column equilibrated in 0.1M Tris-HCl, pH 7.4, 0.2% SDS and merthiolate (diluted 1:10,000) (Girardet et al., 1981). Fractions (1 ml) were collected at a flow rate of 6 ml/hr. The fractions containing the α subunit (as determined by SDS-PAGE) were pooled, concentrated by vacuum dialysis and dialyzed against 10 μ M Tris-HCl, pH 7.7, in 0.1% SDS.

Immunization and Purification of Antibodies

Male New Zealand White rabbits, 3-4 months old, were immunized with 200-400 μ g Na,K-ATPase α subunit (prepared by gel filtration of the purified enzyme) according to the following schedule: week 1—intradermal injection at multiple sites on the haunches in Freund's complete adjuvant; weeks 2, 3, 5—subcutaneous (s.c.) injection in Freund's incomplete adjuvant. Booster injections were given s.c. in incomplete adjuvant every 5 weeks. Animals were bled from ear medial vein under light anesthesia with Innovar-vet (Pitman-Moore, Inc.; Stickrod and Chadwick, 1982) at week 4 and the week after each booster.

For the affinity purification of the anti- α antibodies Na,K-ATPase was purified by detergent extraction of rat kidney microsomes under

conditions (0.6 mg/ml SDS and 4.0 mg/ml protein) where purity was sacrificed for increased yield (see Chapter II, Figure 3, p. 38). The enzyme (12 mg) was solubilized in 0.5% SDS and coupled to 2 ml CNBr-activated Sepharose 4B (a gift of Dr. S. J. Kennel of this institution) by the method of Cuatrecasas (1970). A coupling efficiency of approximately 95% was determined by including a trace amount of radioiodinated protein during the reaction.

Antisera were incubated with the derivatized Sepharose at 4°C for 16 hours with gentle mixing. The beads were allowed to settle, the serum was reserved and a slurry of the beads in phosphate-buffered saline (PBS) was prepared and poured into a Bio-Rad 0.7 x 10 cm Econocolumn. The Sepharose was washed with PBS until the A_{280} of the effluent was zero. The bound antibody was eluted with 3M isothiocyanate and the fractions containing A_{280} -absorbing material were pooled. Ammonium sulfate was quickly added to the pooled fractions to 40% saturation, the precipitate was pelleted, and dissolved in PBS to a final concentration of 1-3 mg IgG/ml (based on the specific absorption coefficient of 1.5 at 280nm for a 1 mg IgG/ml solution). The reserved serum was repeatedly reprocessed until no further IgG was eluted. Normal rabbit IgG was prepared by ammonium sulphate precipitation from the serum of a nonimmunized sibling.

Cell Culture

HTC cells, a cloned rat hepatoma line kindly provided by Dr. D. Doyle, Roswell Park Memorial Institute, Buffalo, N.Y., were grown in suspension in Eagle's minimal essential medium with Spinner's

salts supplemented with 5% fetal calf serum, 2mM L-glutamine, 25 $\mu\text{g/ml}$ gentamycin and 20mM N-2-hydroxyethylpiperazine ethane sulfonic acid (HEPES). Stock cultures were maintained in log growth ($1-6 \times 10^5$ cells per ml); under these conditions the cells doubled in approximately 30 hours.

Three other cell types were used for the experiments in the figure on p. 79. HeLa(S3) cells derived from human cervical carcinoma were maintained exactly as described above for HTC cells. V-79 (Chinese hamster lung) and MDCK (canine kidney epithelium) cells were grown as monolayers in Eagle's minimal essential medium containing 10% fetal calf serum, 2mM L-glutamine and 25 $\mu\text{g/ml}$ gentamycin. The monolayers were maintained under 5% CO_2 /95% air.

Cell Labeling

For long term labeling, HTC cells were incubated for 2-3 days in complete medium containing either [^{35}S]methionine (10 $\mu\text{Ci/ml}$), [^3H]leucine (15 $\mu\text{Ci/ml}$), or a mixture of [^3H]mannose and [^3H]fucose (10 μCi each/ml). For pulse labeling, HTC cells were suspended at $6-8 \times 10^6$ cells/ml in methionine-free RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum and 2mM L-glutamine and pre-incubated for 10-30 minutes at 37°C under 5% CO_2 /95% air. Labeling was initiated by the addition of [^{35}S]methionine to a final concentration of 40-100 $\mu\text{Ci/ml}$. The tracer was chased by the addition of unlabeled methionine to a final concentration of 10mM.

Immunoprecipitation

³⁵S-labeled cells were pelleted and dissolved in 0.5-1.0 ml lysis buffer containing 1% triton X-100 and 5mM EDTA in PBS. Insoluble material, consisting of nuclear and cytoskeletal elements, was removed by centrifugation at 35,000 RPM (50Ti) for 30 minutes and the supernatant was mixed with 100 μ l 2.5% NP-40, 25 mg/ml bovine serum albumen, 5mM EDTA in PBS and either nonimmune or anti- α IgG. Following incubation at 4°C for 16-24 hours, protein A agarose (binding capacity = 8.8 mg IgG/ml of gel), in a 50% slurry, was added and incubated 60 minutes at room temperature with intermittent mixing. The agarose was pelleted for 30 seconds at 12,800 x g (Eppendorf 5412 centrifuge) and washed 4 times with lysis buffer. The pellets were suspended in SDS-PAGE sample solvent (see Chapter II, "Materials and Methods") at room temperature to dissociate the antibody-antigen complexes.

The eluates were electrophoresed and the gels were fixed, impregnated with Enlightening and dried. The dry gels were fluorographed with preflashed film and the radioactivity in the α subunit band was quantitated from densitometric scans of the film by either cutting out and weighing the peaks or by calculating their area, assuming the peaks approximate isosceles triangles.

Protein Cross-Linking

Membrane proteins were covalently cross-linked by a modification of the method of Zarling et al. (1980) using the alkali-reversible homobifunctional reagent BSOCOES. HTC cells (2×10^7) that had been

pulse labeled with [^{35}S]methionine and chased for 8 hours were lysed by 3 freeze/thaw cycles in 6 ml 10mM tris, pH 7.7. Rat kidney microsomes, prepared as described in Chapter II, were radioiodinated by a chloramine T method (McConahey and Dixon, 1966) and suspended in 6 ml 10mM tris, pH 7.7.

The labeled lysate and microsome fractions were divided as 2 ml ("mock-link") and 4 ml ("cross-link") samples. The cross-link samples were treated with 40 μl 200mM BSOCOES in DMSO while the mock-link samples received 20 μl DMSO alone. The reaction was carried out for 90 minutes at 0°C and then terminated by the addition of 0.2 ml (mock-link) or 0.4 ml (cross-link) 1M arginine-HCl in PBS, followed by 5 ml (mock-link) or 10 ml (cross-link) 0.1M arginine-HCl in PBS. The membranes were pelleted for 30 minutes at 38,500 RPM (60Ti), dissolved in immunoprecipitation lysis buffer and cleared of insoluble material by centrifugation at 35,000 RPM (50Ti) for 30 minutes.

The cross-link samples were divided into two equal aliquots (cross-link, cross-link/cleave) and all the samples were immunoprecipitated with anti- α IgG.

The antibody-antigen complexes were eluted from the protein A agarose with either SDS-PAGE sample solvent, pH 6.8 (mock-link, cross-link), or reversal solvent containing 2% SDS, 5% β -mercaptoethanol, 3M urea and 50mM tris, pH 11.6 (cross-link/cleave), and incubated for 2 hours at 37°C. The pH of the cross-link/cleave samples was adjusted to pH 6.8 prior to SDS-PAGE.

Results

Ge1 filtration has been successfully used to isolate the individual subunits of SDS-denatured Na,K-ATPase (Kyte, 1972; Hopkins et al., 1976; Girardet et al., 1981). Purified rat kidney Na,K-ATPase, prepared as described in Chapter II, was solubilized in 2% SDS and applied to a Bio-Gel A1.5m column equilibrated with 0.1% SDS buffered with Tris (pH 7.4). Several distinct peaks of protein were eluted (Figure 8A). SDS-PAGE analysis (Figure 8B) indicates that peak II is a nearly homogeneous preparation of the α subunit (lane b). While peak IIIA is highly enriched in the β subunit, the 32,000 protein component of the enzyme preparation co-elutes with it (lane d). More importantly, the diffuse β subunit region observed after SDS-PAGE also contains 1-3 discrete Coomassie blue-staining bands, seen more clearly on gels overloaded with the pooled peak IIIA fractions (not shown). I have attempted unsuccessfully to re-chromatograph these fractions on Concanavalin A-Sepharose to sequester the heavily glycosylated β subunit (not shown). The presence of the contaminants which co-migrate within the β subunit molecular weight range make the peak IIIA fractions unsuitable for use as immunogen for my purposes.

The α subunit-containing fractions of peak II were pooled, concentrated and injected into rabbits. The anti- α subunit IgG was purified from the sera of immunized rabbits by chromatography over Sepharose 4B to which highly-enriched Na,K-ATPase had been coupled. Figure 9 shows the results of immunoprecipitation from HTC cells labeled with [^{35} S]methionine. Between 10 and 15 μ g of

Figure 8. Gel filtration of SDS-denatured Na,K-ATPase. Rat kidney Na,K-ATPase (5-8 mg) was purified as described in Chapter II, solubilized in 2% SDS and applied to a Bio-Gel A1.5m column (1.5 x 90 cm) equilibrated in 0.1% SDS, merthiolate (diluted 1:10,000) and 0.1M Tris, pH 7.4. Fractions (1 ml) were collected at a flow rate of 6 ml/h. Protein (A) was assayed by the fluorometric method of Avruch and Wallach (1971), which circumvents the SDS artifacts seen when A_{280} is monitored (Kyte, 1972; Hopkins et al., 1976). Aliquots containing 10 μ g protein from selected column fractions (indicated by arrows) were electrophoresed on a 9% slab gel and stained with Coomassie blue (B). At the right are the mobilities of molecular weight standards.

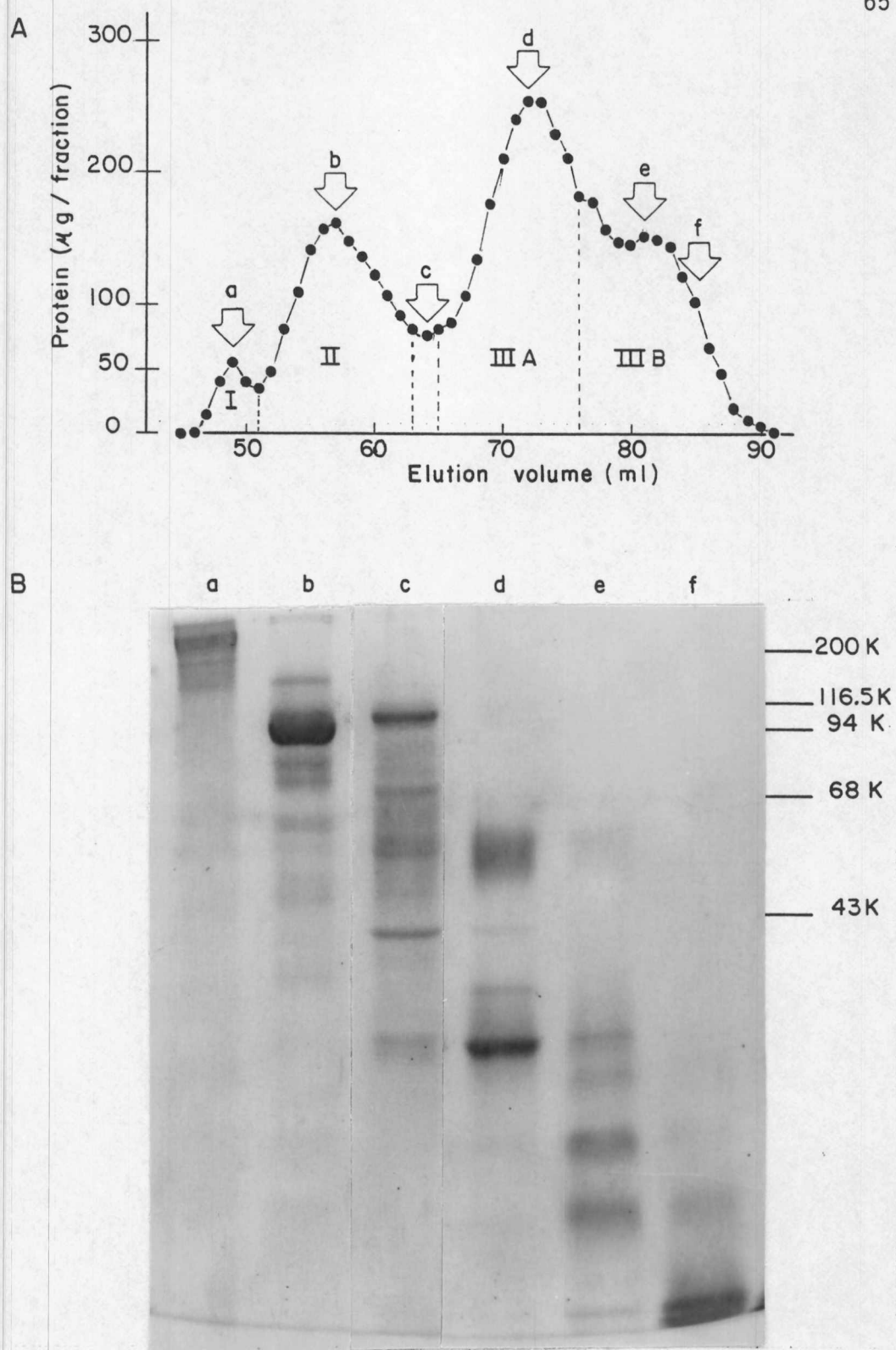


Figure 8

Figure 9. Recovery of the Na,K-ATPase α subunit from HTC cells vs. anti- α IgG concentration. HTC cells were incubated in methionine-free medium (6.4×10^6 cells/ml) for 30 minutes prior to the addition of [^{35}S]methionine (final concentration = $40 \mu\text{Ci/ml}$) for an additional 3 hours (lanes a-g). Another culture of HTC cells was labeled for 72 hours in complete medium containing $10 \mu\text{Ci}$ [^{35}S]methionine/ml (lane h). The cultures were centrifuged and the cell pellets were dissolved in 1% triton X-100, 5mM EDTA in PBS and immunoprecipitated as described in "Materials and Methods" in tubes containing 0.5 ml lysate (derived from 5×10^6 cells) and 0-20 μg anti- α IgG. All tubes contained 20 μg total IgG, the difference being made up of nonimmune IgG. The immunoprecipitates were solubilized in SDS-PAGE sample solvent and electrophoresed on 7.5% gels. The gels were fluorographed (A) and the resulting films were densitometrically scanned to quantitate the α subunit band (B). The numbers above the gel lanes in (A) indicate the amount (μg) of anti-IgG present during the immunoprecipitation. The data in (B) represent the mean from two film exposures of the same gel.

A

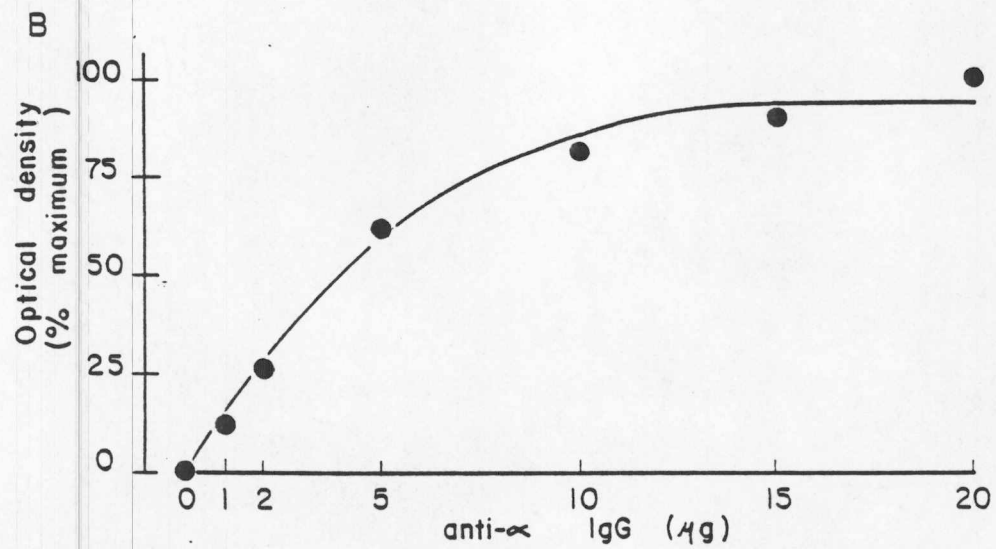
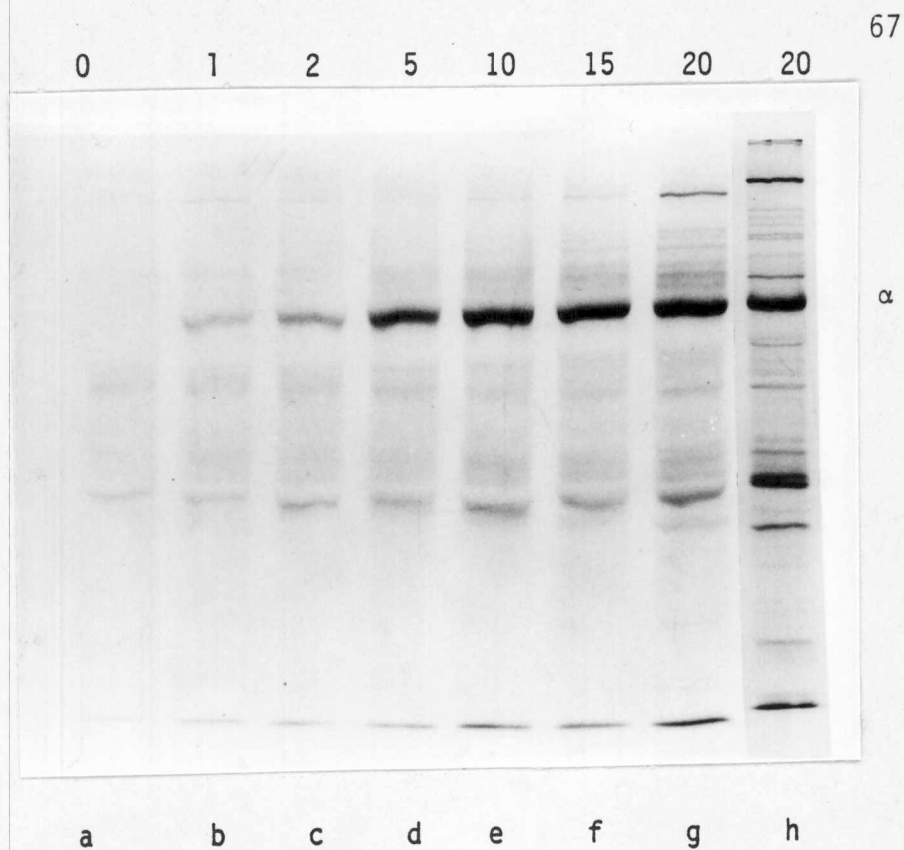


Figure 9

affinity-purified anti- α IgG was required to completely sequester the Na,K-ATPase α subunit from 5×10^6 cells (approximately 1.25 mg total cellular protein) that had been pulse-labeled with [^{35}S]methionine (Figure 9A, lanes a-g, and 9B).

It was surprising that no radioactivity was noted in the gel corresponding to the β subunit in these immunoprecipitates since the cells were lysed under nondenaturing conditions (triton X-100/EDTA/PBS). Similarly, no β subunit was observed in immunoprecipitates from cells that had been labeled for 3 days (Figure 9A, lane h), which suggests that the absence of ^{35}S -labeled material which corresponds to the β subunit is not due to either a slower synthesis rate or insufficient time for labeled holoenzyme to assemble.

Immunoprecipitates from lysates prepared with or without EDTA in either triton X-100 or Nonidet P-40 (NP-40), another nonionic detergent, all lacked ^{35}S -labeled bands corresponding in mobility to that expected of the Na,K-ATPase β subunit (Figure 10). The α subunit was present in all the extracts. An unidentified high molecular weight band appeared in immunoprecipitates from the EDTA-containing lysates and may be related to the nonspecific trapping that has previously been attributed to the presence of the chelator (Sefton et al., 1982). The other minor bands seen here and in Figure 9A are likely due to nonspecific adhesion, a malady sometimes associated with immunoprecipitation from detergent extracts of membranes (Salter et al., 1982).

The possibility remained that the antibodies were directed against a 93,000 dalton protein which was, except for its size,

Figure 10. Immunoprecipitation of Na,K-ATPase from HTC cells lysed under different conditions. Cells were suspended (8×10^6 cells/ml) in methionine-free medium for 10 minutes. [^{35}S]methionine was added to a final concentration of $50 \mu\text{Ci/ml}$ and the cells were incubated for an additional 30 minutes. The culture was divided into 4 portions of 5×10^6 cells each, pelleted and solubilized in 1% triton X-100 or 1% NP-40 containing 0 or 5mM EDTA. The lysates were immunoprecipitated with a saturating concentration of anti- α IgG and analyzed by SDS-PAGE (7.5%) and fluorography.

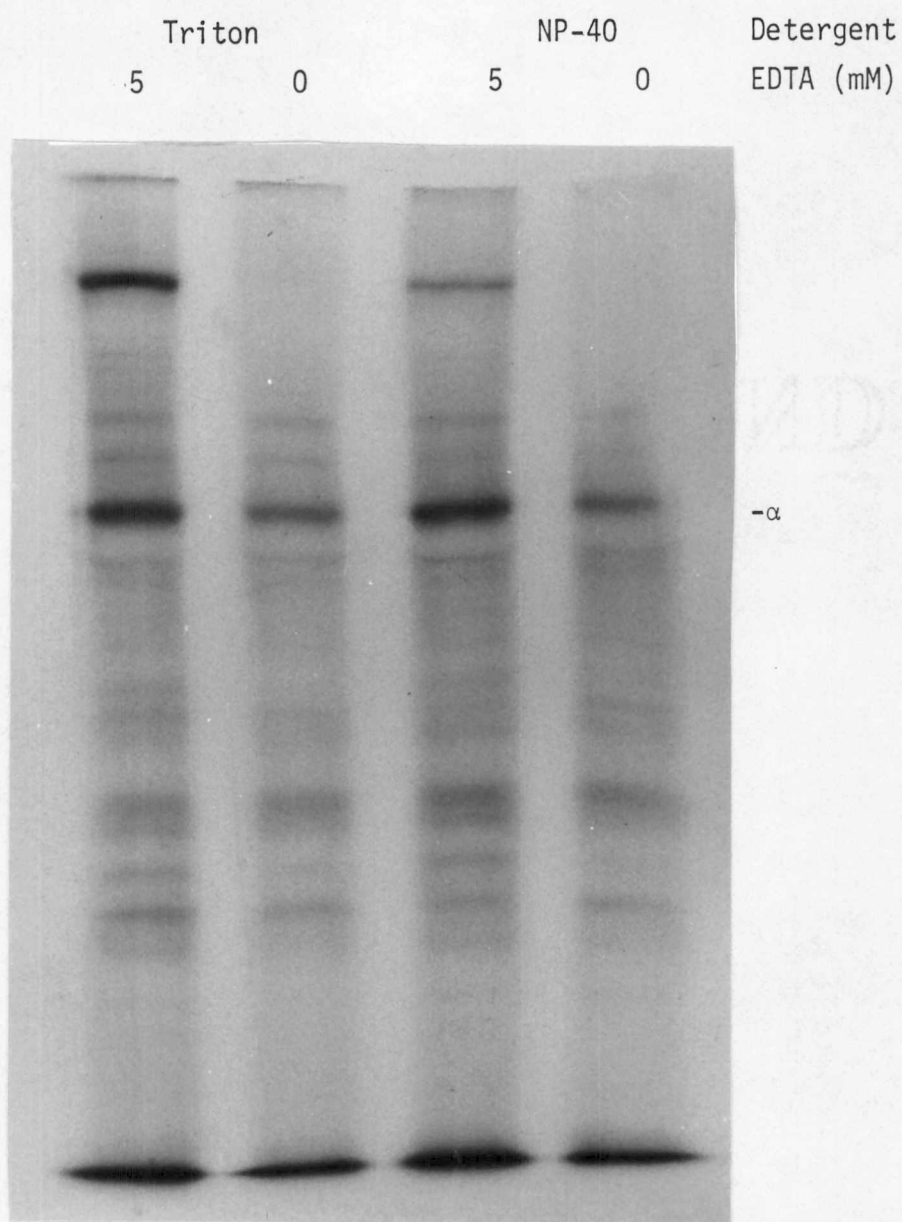


Figure 10

unrelated to the Na,K-ATPase subunits. This was tested by assaying purified Na,K-ATPase for ATP hydrolysis activity after incubation with the antibodies (Table II). The anti- α IgG was found to inhibit Na,K-ATPase activity in a dose-dependent manner while nonimmune IgG was without effect, showing that the antibodies were indeed recognizing the enzyme. It is important to emphasize that the inhibition of activity was due simply to the binding of the antibodies, not to the removal of enzyme by its precipitation. In this experiment some of the Na,K-ATPase remained active even in the presence of a 50-fold mass excess of anti- α IgG. This could mean that 3 hours at 0°C was insufficient to achieve complete binding, or that only a subpopulation of antibodies is capable of inhibiting activity.

If the nonionic detergents used here, contrary to their effect on other membrane protein systems, were denaturing the Na,K-ATPase, then covalently cross-linking the subunits prior to detergent solubilization could serve to "capture" the β subunit so as to observe it after immunoprecipitation with anti- α antibodies. BSOCOES, a homobifunctional cross-linking reagent which has been employed in the analysis of lymphocyte surface antigens (Zarling et al., 1980), was utilized to cross-link the Na,K-ATPase subunits in HTC cell membranes prior to detergent lysis.

HTC cells were pulse labeled with [^{35} S]methionine, chased for 8 hours (to ensure the complete assembly of the labeled subunits into holoenzyme) and then treated with or without the cross-linking

Table II

The Effect of Anti- α IgG on Na,K-ATPase Activity

IgG:Enzyme ($\mu\text{g}:\mu\text{g}$)	Activity (% control)	
	Anti- α	Non-immune
10	42.3 $\pm$ 0	106 $\pm$ 7.5
50	23.1 $\pm$ 8.0	102 $\pm$ 3.1

Purified Na,K-ATPase (5 μg) from rat kidney was incubated for 3 hours on ice with 50 or 250 μg of either anti- α or non-immune IgG that had been dissolved in saline buffered with Tris. The enzyme was then assayed for ATP hydrolysis activity, normalized to that of enzyme which had been incubated in the buffered saline only. Ouabain-insensitive ATPase activity was not detected in this enzyme preparation. The data are the means $\pm$ the range of duplicate assays.

agent prior to solubilization in the triton-containing lysis buffer. After immunoprecipitation with anti- α IgG half of the cross-linked sample was treated at pH 11.6 to cleave the cross-link, and all of the samples were analyzed by SDS-PAGE and fluorography (Figure 11), lanes a-c). Immunoprecipitation of the mock-linked (M) sample (lane a) revealed a single labeled band of 93,000, which corresponds to the α subunit. As in previous experiments (Figures 9 and 10), no labeled β subunit was detected. Lane b indicates that the immunoprecipitable material in the cross-linked (X) sample was in a form which was unable to penetrate significantly into the 7.5% resolving gel (some was also either insoluble or too large to enter the 4.5% stacking gel). When the cross-links in this material were cleaved (X/C) labeled protein was only detected in the α subunit (lane c).

This protocol was repeated with kidney microsomes that had been labeled at tyrosine residues with ^{125}I (Figure 11, lanes d-f). Unlike the ^{35}S -labeled HTC cell membranes, the mock-linked kidney microsomes (lane d) exhibited immunoprecipitable bands which correspond to both the α (93,000 daltons) and β (53,000 daltons) subunits of Na,K-ATPase. These were retained during cross-linking since both were visible in material which was cross-linked and then, after immunoprecipitation, cleaved at alkaline pH (lane f). Immunoprecipitates from ^{125}I -labeled membranes consistently contained a large number of nonspecific bands (see lanes d and f). The high background seen in immunoprecipitates from HTC membranes labeled with ^{125}I (not shown) prevented their analysis.

Figure 11. Immunoprecipitation of Na,K-ATPase from radiolabeled HTC cell or kidney membranes that had been chemically cross-linked. Lanes a-c. HTC cells were preincubated for 60 minutes in methionine-free medium (6.7×10^6 cells/ml) and pulsed with [^{35}S]methionine (final concentration = $100 \mu\text{Ci/ml}$) for 30 minutes. They were then placed into 25 ml chase medium containing 10mM unlabeled methionine for 8 hours. The cells were lysed by freeze/thaw, and either cross-linked (X) with BSOCOES or mock-linked (M) with solvent alone. The membranes were pelleted, solubilized and immunoprecipitated with anti- α IgG. The immunoprecipitates were solubilized in either SDS-PAGE sample solvent (M,X), pH 6.8, or in reversal solvent (X/C), pH 11.6 (which cleaves the cross-links), and analyzed by SDS-PAGE (7.5%) and fluorography. Lanes d-f. Rat kidney microsomes were ^{125}I -labeled using chloramine T and cross-linked as described above for the HTC samples. The kidney membranes were similarly analyzed with (X/C) or without (M,X) cleavage of the cross-links. The broad lanes seen with the X/C samples result from the high ionic strength of the Tris buffer required to adjust the pH to 6.8 before SDS-PAGE. The molecular weights were determined from a lane containing standards which was removed and stained prior to fluorography (not shown).

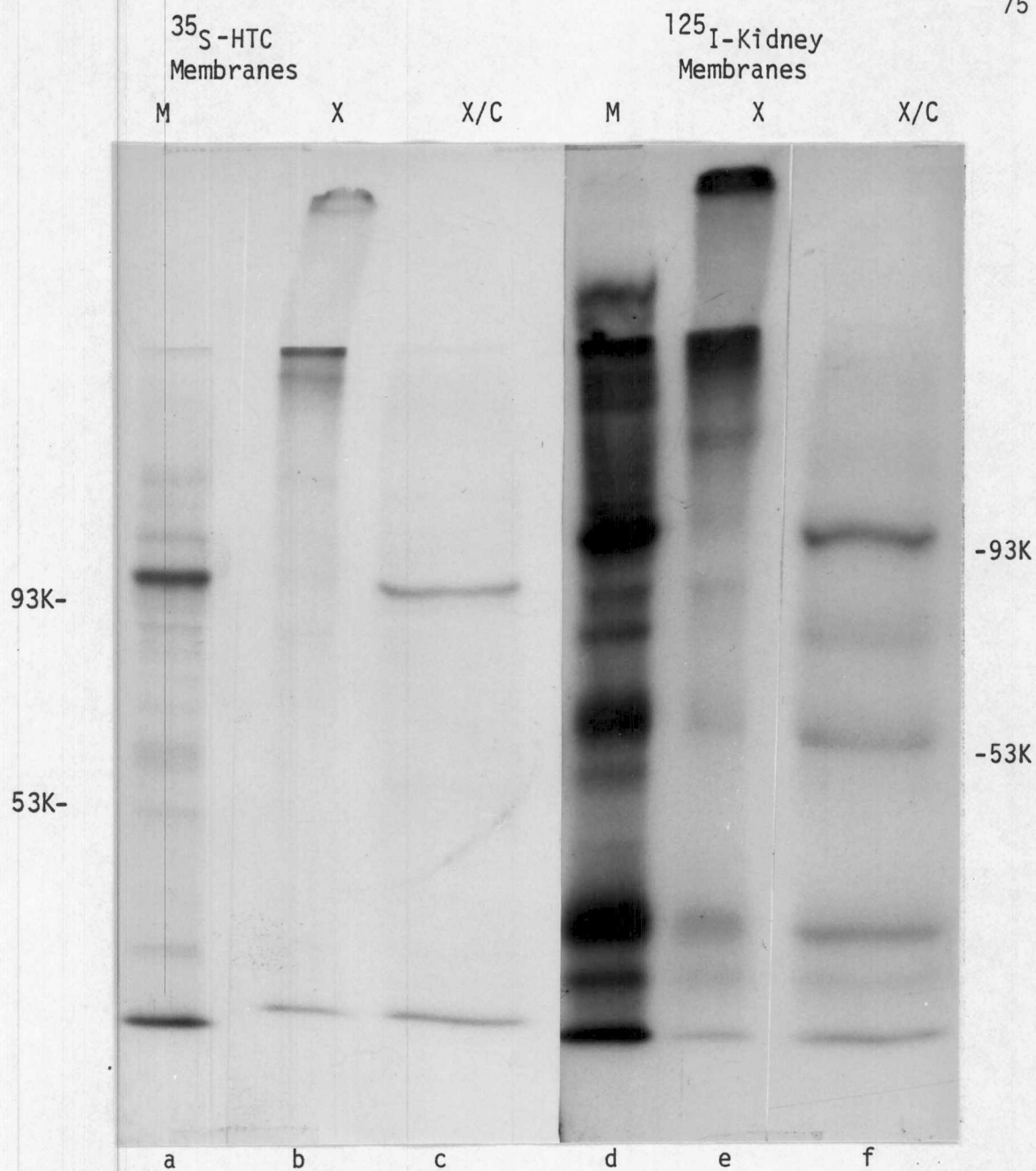


Figure 11

The results of the experiments in Figure 11 argue against holoenzyme dissociation by nonionic detergents. The alternative explanations which seemed to me most likely were that the Na,K-ATPase β subunit in HTC cells contains little or no methionine, or that the enzyme in these cells lack (recognizable) β subunits.

This problem was approached by immunoprecipitating a lysate prepared from HTC cells that had been grown with [^3H]leucine (Figure 12, open circles). Labeled proteins corresponding in electrophoretic mobility to both the α and β subunits of Na,K-ATPase were detected. The identity of the glycoprotein subunit was confirmed in a parallel experiment in which the cells were allowed to incorporate both [^3H]mannose and [^3H]fucose prior to immunoprecipitation with anti- α IgG (Figure 12, solid circles). A peak of radioactivity coincided with the putative β subunit, confirming this component to be a glycoprotein. No mannose or fucose was associated with the α subunit. A third component, also a glycoprotein, which migrated ahead of the β peptide is a contaminant that was also present when nonimmune IgG was substituted for anti- α IgG (not shown).

The anti-rat α subunit IgG cross-reacted with cells from three other species (Figure 13). Immunoprecipitation of ^{35}S -labeled protein from lysates of Chinese hamster lung cells (V-79), canine kidney epithelia (MDCK), and human cervical carcinoma cells (HeLa) revealed a major band of approximately 93,000 daltons, which represents the large catalytic peptide of Na,K-ATPase. As in HTC lysates, no labeled β subunit was evident.

Figure 12. Immunoprecipitation of Na,K-ATPase from HTC cells labeled with [^3H]leucine or [^3H]mannose and [^3H]fucose. Cells were grown for 48 hours in complete medium containing either 15 $\mu\text{Ci/ml}$ [^3H]leucine (O) or [^3H]mannose and [^3H]fucose, 10 μCi each/ml (O), solubilized and immunoprecipitated with anti- α or nonimmune IgG. The immunoprecipitates were electrophoresed on cylindrical 9% gels which were sliced and analyzed for radioactivity. The gels were aligned using the dye front as a reference and the regions which corresponded to the α and β subunits (arrows) were ascertained from a gel containing purified rat kidney Na,K-ATPase stained with Coomassie blue (not shown). The radioactivity in the gel slices containing the nonimmune IgG-incubated samples (not shown) was subtracted from each corresponding slice from gels containing the anti- α precipitates.

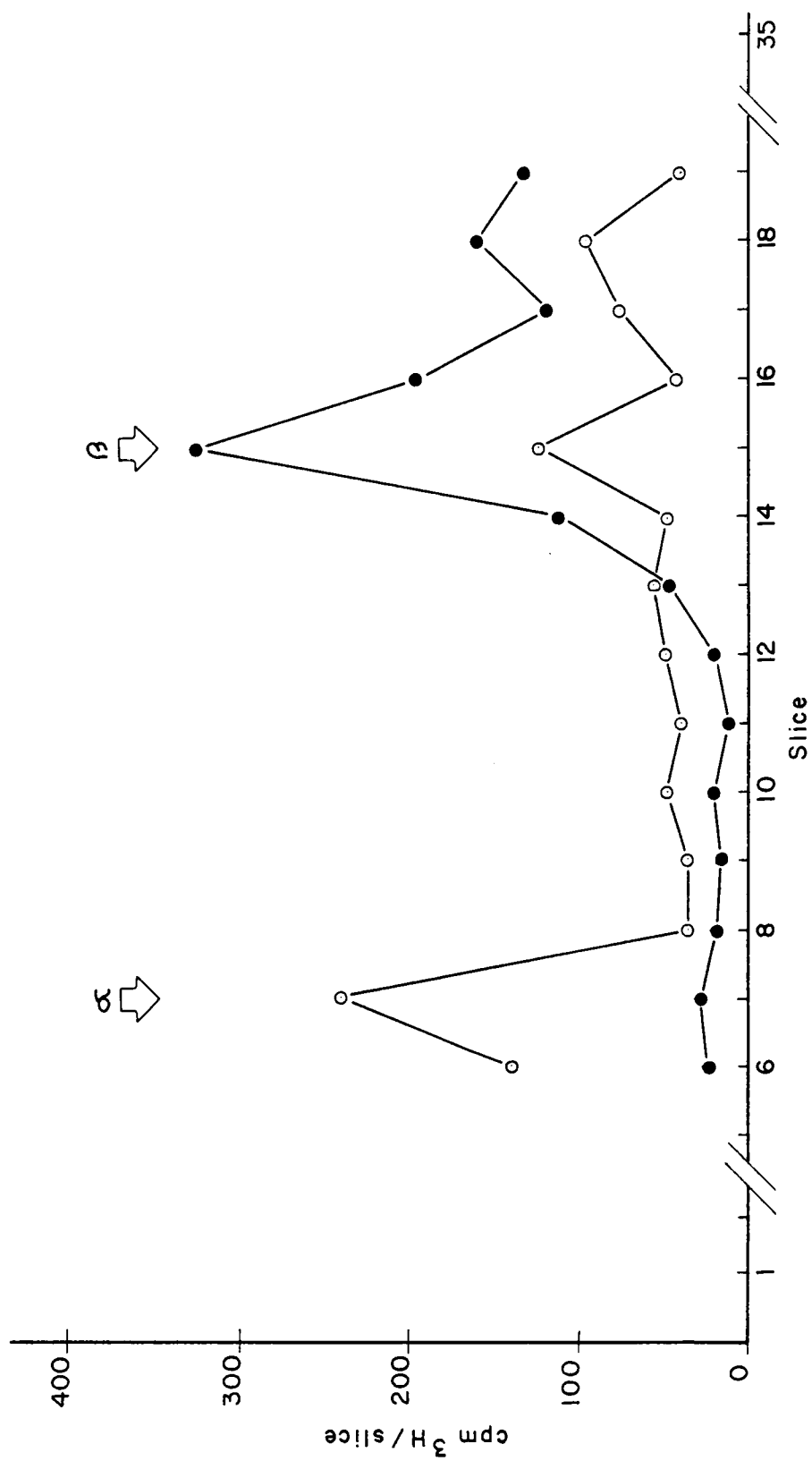


Figure 12

Figure 13. Cross-reactivity of anti-rat α subunit IgG with Na,K-ATPase of other species. Cells derived from Chinese hamster lung (V-79), canine kidney epithelium (MDCK) and human cervical carcinoma (HeLa) were grown as described in "Materials and Methods." The V-79 (lanes a,b) and MDCK (lanes c,d) monolayer cultures were preincubated for 30 minutes in methionine-free medium and labeled with [^{35} S]methionine (final concentration = 100 μ Ci/ml) for 4 hours. The cells were chased for 2 hours with unlabeled methionine (final concentration = 10mM), rinsed and scraped in PBS, and pelleted. The cell pellets were solubilized in triton X-100-containing lysis buffer and cleared of insoluble material by centrifugation at 110,000 $\times$ g for 30 minutes. HeLa (suspension) cells (lanes e,f) were incubated 30 minutes in methionine-free medium at a density of 6.7×10^6 cells/ml and labeled with 50 μ Ci [^{35} S]methionine for 4 hours. After a 2 hour chase in 10mM unlabeled methionine, the cells were pelleted, solubilized in lysis buffer and cleared by centrifugation. The lysates were incubated with either nonimmune (N) or anti- α (α) IgG, and the precipitates analyzed by SDS-PAGE and fluorography.

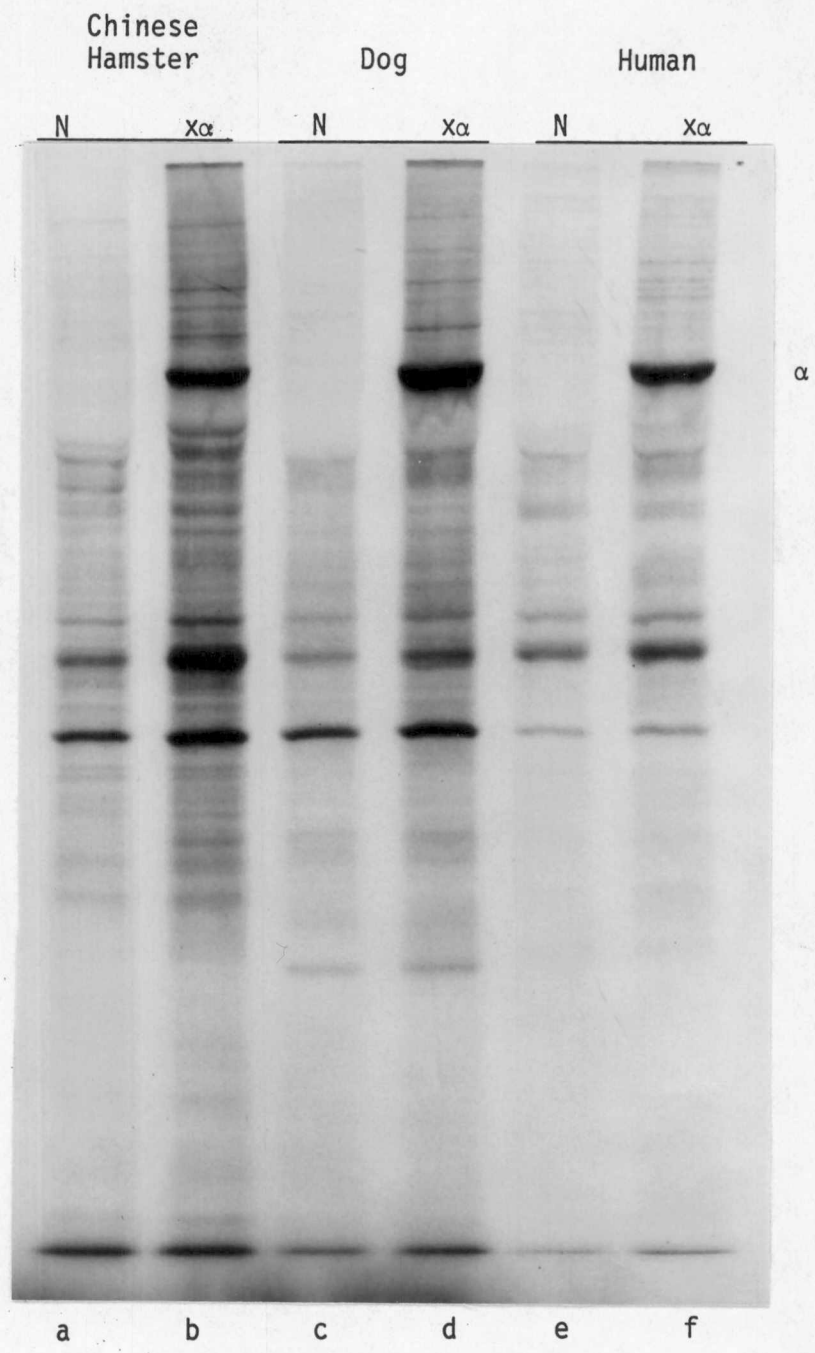


Figure 13

Discussion

The subunits of NaK-ATPase, purified from rat kidney (Chapter II), were separated by gel filtration in the presence of SDS. This approach has been used by other investigators (Kyte, 1972; Hopkins et al., 1976; Girardet et al., 1981) to obtain the enzyme's peptides in homogeneous form. Several distinct peaks of protein were observed. When analyzed by SDS-PAGE, peak II was revealed to contain nearly homogeneous α subunit. Peak IIIa consisted of both the β subunit and the 32,000 dalton contaminant of the enzyme preparation. The diffusely-staining β subunit region also contains several discrete bands, interpreted to be contaminants of similar molecular weight. One of these may be a fragment of the α subunit (Girardet et al., 1981). Regardless of their identity, their presence rendered the peak IIIa fraction unsuitable (in its present form) for use as immunogen for the preparation of monospecific antibodies to the Na,K-ATPase glycoprotein.

The antibodies produced by rabbits in response to peak II (α) precipitated a major band of 93,000 daltons (by SDS-PAGE/fluorography) from cells which had been labeled with [^{35}S]methionine. In spite of the nondenaturing conditions maintained during immunoprecipitation, no band of radioactivity was detected which corresponded to the β subunit in size or morphology, as predicted by its appearance in the kidney enzyme. This was true after both pulse and long term labeling of the cells with [^{35}S]methionine, which indicated that its absence was not a function of the rate of its synthesis or holoenzyme assembly.

Similarly, the lack of labeled glycoprotein in the immunoprecipitates was independent of whether the cells were lysed in triton X-100 or NP-40, or whether or not EDTA was present. The 93,000 dalton band, presumed to be the α subunit, was present under all of the above conditions.

The possibility existed that the 93,000 dalton peptide was not the α subunit, but rather a contaminant of similar molecular weight. That the antibody preparation inhibited the activity of purified kidney Na,K-ATPase was evidence that this was not the case. The activity was not totally inhibited under the conditions reported here. This may be due to incomplete binding of the antibodies to the enzyme in 3 hours at 0°C, or it may be indicative of both inhibitory and noninhibitory subpopulations of anti- α IgG. The latter explanation is feasible since antibodies prepared against the Na,K-ATPase α subunit of another species have been reported as both inhibitory (Jean and Albers, 1976) and noninhibitory (Kyte, 1972; McDonough et al., 1982).

Chemical cross-linking of HTC cell membranes containing Na,K-ATPase, prior to detergent solubilization, failed to elicit the appearance of ^{35}S -labeled β subunit in the immunoprecipitates. A band which corresponds to β in both size and appearance is present in immunoprecipitates from kidney microsomes labeled at tyrosine residues with ^{125}I . This band was present in both mock-linked samples and those that were cross-linked and then cleaved. BSOCOES was chosen because it is active at 0°C, diminishing the cross-linking of proteins whose proximity is only transient due to membrane fluidity. The

cross-link is also alkali-sensitive and can be cleaved by incubation at pH 11.6, a treatment which is sufficiently mild to preserve the original electrophoretic mobilities of the previously cross-linked species (Zarling et al., 1980).

These results suggest that the lack of a detectable glycoprotein subunit in HTC extracts is not due to the enzyme's dissociation prior to immunoprecipitation. A large number of nonspecific bands were observed when ^{125}I -labeled membranes were immunoprecipitated. This precluded the use of ^{125}I -labeled HTC cell membranes in those experiments.

The failure to detect labeled β subunit in the immunoprecipitates is explained as a paucity or complete absence of methionine in its peptide backbone. This was revealed by experiments which showed an immunoprecipitable protein that was labeled by either $[^3\text{H}]$ leucine or a mixture of tritiated sugars, and migrated as would be expected for a 53,000 dalton glycoprotein. Leucine, but not mannose or fucose, was incorporated into the α subunit. This agrees with the inability to detect sialic acid on the kidney enzyme's large subunit (Chapter II, Figure 5B, p. 45), and confirms its status as a nonglycosylated protein.

The lack of methionine in the β peptide was unexpected in that this subunit of Na,K-ATPase from several tissue types and species contains methionine in amounts comparable to that of the large subunit (Hopkins et al., 1976, see Tables II and III). This may reflect some evolutionary divergence in the genes for the small subunit.

The α subunit is highly conserved, based on the immunologic cross-reactivity reported by Schellenberg et al. (1981). This might

be predicted since the Na,K-ATPase is found in all animals species and is enzymologically similar or identical over a wide range of species, and that all known functional activities of this enzyme are associated with the α subunit. The anti-rat α subunit IgG precipitated a 93,000 dalton protein from cells of hamster, dog and human origin, in agreement with work cited above. No ^{35}S -labeled β subunit was observed in any of the cells tested. This was surprising in that the dog kidney cells (MDCK) have previously been used as a source of mRNA for the study of the synthesis of Na,K-ATPase β subunit glycoprotein by cell-free translation (Sherman et al., 1980) using [^{35}S]methionine.

CHAPTER IV

BIOSYNTHESIS AND TURNOVER OF THE HTC

CELL Na,K-ATPase α SUBUNIT

Introduction

A variety of techniques have been employed in the analysis of membrane protein biosynthesis and turnover. Na,K-ATPase synthesis has been characterized in rat kidney (Lo and Lo, 1980) and eel electroplax membranes (Churchill and Hokin, 1979) by administering radioactive amino acids, either to the animal or to isolated organs, and purifying the enzyme at various times afterward. This approach demands large quantities of tissue and isotope, and requires minimal variation in the recovery and purity of the isolated enzyme preparation.

Several indirect methods have been devised to analyze synthesis and turnover. Edelson and Cohn (1976) monitored the activity of 5'-nucleotidase in macrophages after treatment with translation inhibitors, the rate of activity decline considered a function of enzyme degradation. The release of bound ligands (excluding that which occurs by simple dissociation) which are specific for a single membrane component has been equated with that component's degradation in studies of acetylcholine receptors (Hartzell and Fambrough, 1973) and Na,K-ATPase (Cook et al., 1976). Both of these methods assume that the treatments do not alter the normal metabolism of the membrane

proteins being studied, although this has been shown not to be true for both cycloheximide treatment (Amenta et al., 1977) and the acetylcholine receptor-specific ligand α -bungarotoxin (Gardner and Fambrough, 1979). Also, the presence of ligand does not necessarily denote the concomitant presence of its receptor, as has been documented in an insulin receptor study (Krupp and Lane, 1982).

The direct analysis of membrane proteins by density labeling has been accomplished by Fambrough and his co-workers (cf. Gardner and Fambrough, 1979) in acetylcholine receptor investigations, by Lane and his colleagues (cf. Krupp and Lane, 1982) in the analysis of the insulin receptor, and also by Pollack et al. (1981b) to characterize the turnover of HeLa cell Na,K-ATPase. This method circumvents many of the problems associated with protein purification, but is susceptible to potential artifacts due to the toxicity of certain heavy isotopes (Pollack, 1980).

In this chapter, I utilized antibodies prepared against the α subunit of rat kidney Na,K-ATPase to characterize the biosynthesis and turnover of the enzyme in cultured rat hepatoma (HTC) cells. The plasma membrane proteins of these cells have been extensively characterized by Doyle and his colleagues (for review, see Doyle and Baumann, 1979). HTC cells provide an excellent system for this analysis because of their clonal homogeneity and the ease with which large quantities can be generated without repeated proteolysis of their surface constituents.

Materials and Methods

Cell Growth and Labeling; Immunoprecipitation

All procedures were performed as described in previous chapters. Specific details are given in the figure legends.

Results

In order to compare the degradation (turnover) rate of a single polypeptide to the mean rate of a group of proteins, it is obligatory that the radioactive tracer incorporation reach a steady-state. Figure 14 shows the results of an experiment designed to determine the time necessary to reach a steady-state of [^{35}S]methionine incorporation into total HTC cell protein. The cells were grown in complete medium containing 10 μCi [^{35}S]methionine/ml and assayed for acid-precipitable radioactivity. When expressed on a per cell basis, the incorporation into protein reached a plateau phase 50 to 70 hours after the introduction of label. The cells were growing logarithmically during the entire experiment; the decrease in the rate of incorporation per cell is not due to a lowering of the metabolic rate as cells become quiescent. The relatively high concentration of [^{35}S]methionine was necessary to produce proteins containing sufficient radiolabel for subsequent immunoprecipitation analyses. In this experiment, when corrected for counting efficiency (approximately 65%), the specific radioactivity incorporation approached 15 dpm/cell at steady state.

Figure 14. Time course of [^{35}S]methionine incorporation into HTC cell protein. Cells were incubated in 25 ml complete medium containing 10 μCi [^{35}S]methionine/ml; the cells were growing logarithmically during the entire experiment. At various times, 200 μl were removed, mixed rapidly with 1 ml unlabeled cells ($2-5 \times 10^5/\text{ml}$) and pelleted. The cells were resuspended in Tris-buffered saline, pelleted and precipitated with an equal volume of 10% trichloroacetic acid. The precipitates were solubilized in 0.1N NaOH, neutralized with HCl and counted. Cell number in the labeling culture was determined in a Coulter counter (model B).

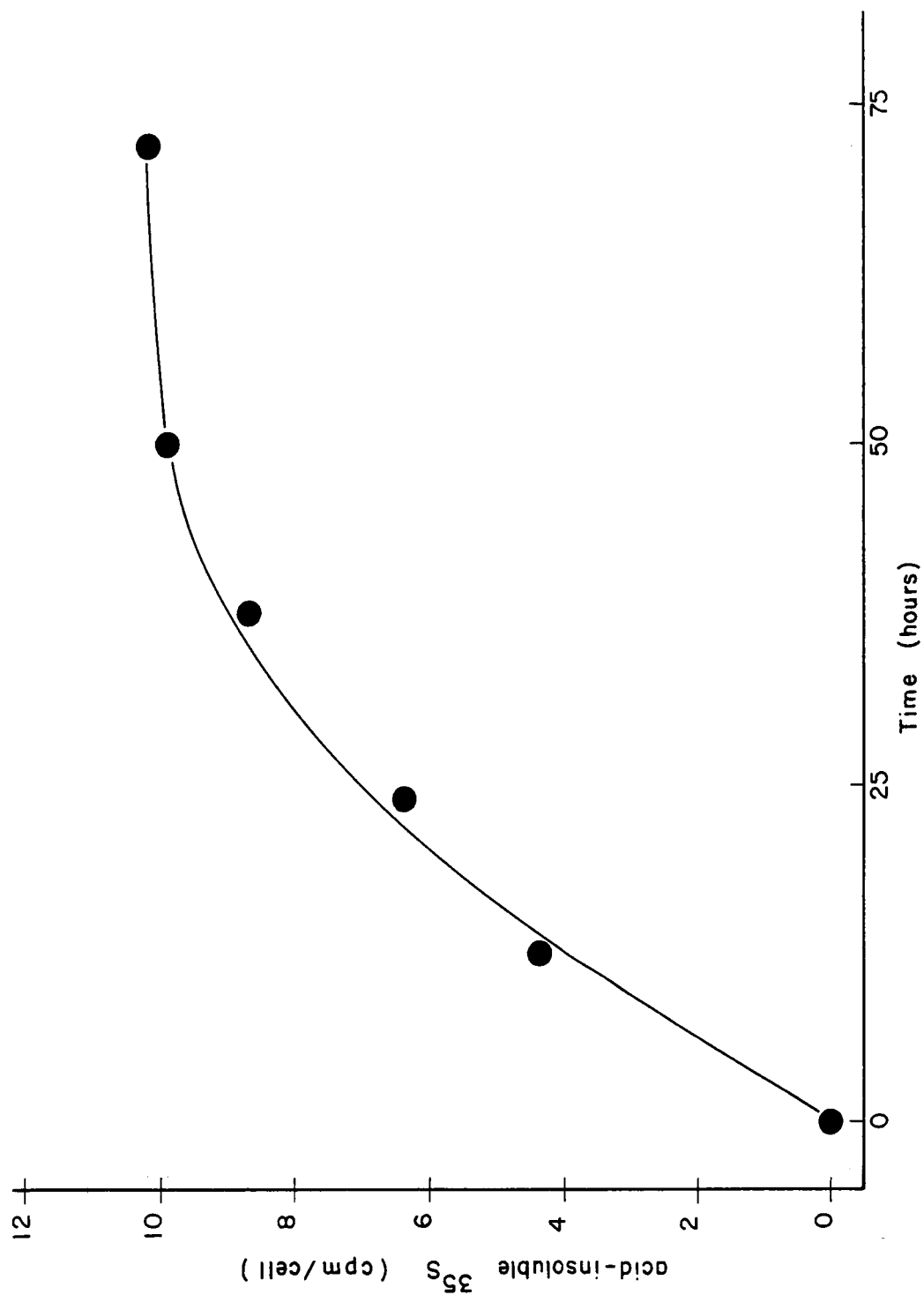


Figure 14

When cells were labeled to steady-state with [^{35}S]methionine and then placed into chase medium containing excess unlabeled methionine, the acid-precipitable radioactivity in triton-soluble material declined exponentially with a half-life of 54.5 hours (Figure 15A). The turnover in these and the following experiments was calculated based on radioactivity per volume of culture. By this method, the only source of radioactivity loss is degradation; correction for the dilution of label per cell due to cell division is avoided.

The turnover of the Na,K-ATPase α subunit, determined by immunoprecipitation, exhibited a half-life of 27 hours (Figure 15B). The line in Figure 15B was fitted only to those points 4 hours or more into chase period. This was because random (first order) turnover of radiolabeled Na,K-ATPase molecules can only take place when they are in a compartment (i.e., the plasma membrane) in which the degradation process can begin, in this case, the internalization and delivery of enzyme to the lysosomes (Cook et al., 1982). Pollack et al. (1982) reported a several hour transit time for newly-synthesized Na,K-ATPase α subunits to reach the HeLa cell plasma membrane. I assumed, and subsequently confirmed (see below), a similar transit time in HTC cells. The first order turnover of the α subunit was therefore corrected for this period during which degradation was nonrandom.

When the half-life ($t_{1/2}$) of a protein is known, the rate constant for degradation, k_d , can be ascertained from the following relationship:

Figure 15. Turnover of total triton-soluble protein and the Na,K-ATPase α subunit in HTC cells labeled for three days with [^{35}S]methionine. Cells were grown for three days in complete medium containing 10 μCi [^{35}S]methionine/ml and then placed into chase medium (final concentration = 10mM unlabeled methionine) for 0 to 60 hours. Samples ($2-4 \times 10^6$ cells) were removed and the cells were solubilized in triton-containing lysis buffer and cleared of insoluble material by centrifugation at 110,000 $\times g$ for 30 minutes. Aliquots of the cleared lysates were assayed for trichloroacetic acid-insoluble radioactivity (A). The lysates were immunoprecipitated with anti- α IgG, analyzed by SDS-PAGE and fluorography, and the films were densitometrically scanned to quantitate the radioactivity in the α band (B). The data are the results of 4 (A) and 2 (B) experiments. The correlation coefficients (r) are indicated in parentheses. The line in (B) was fitted to the points 4 hours or more into the chase period (see text for explanation).

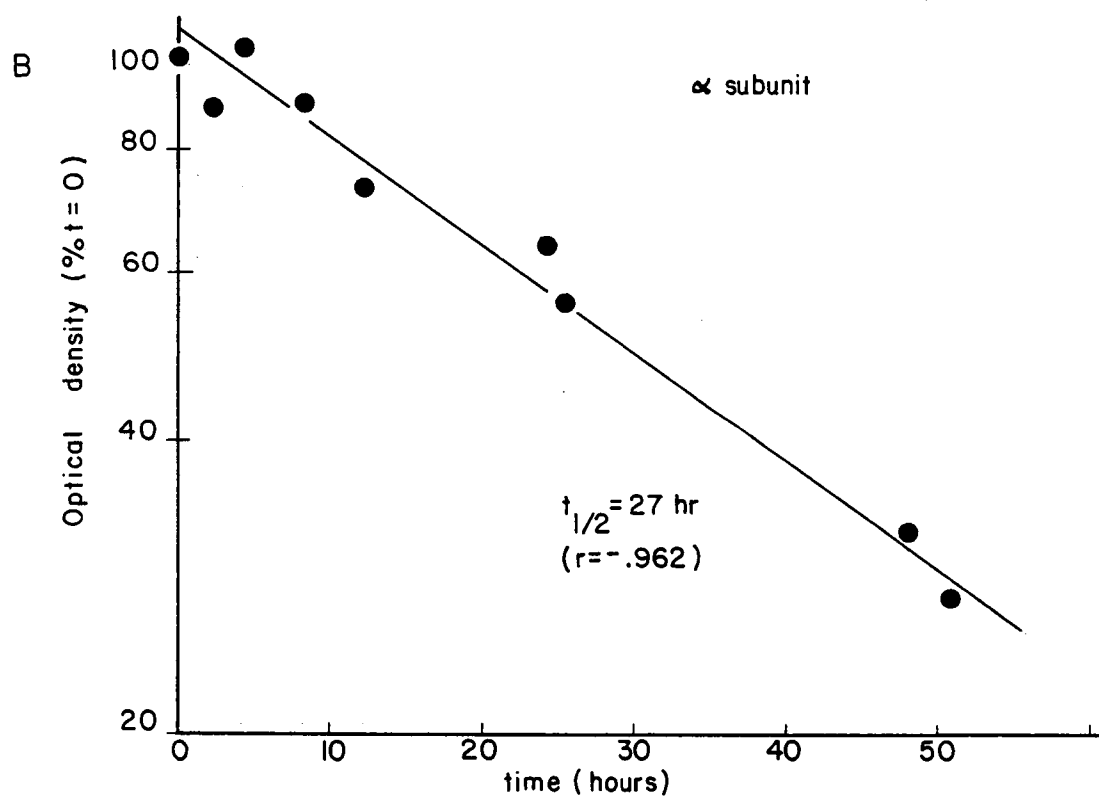
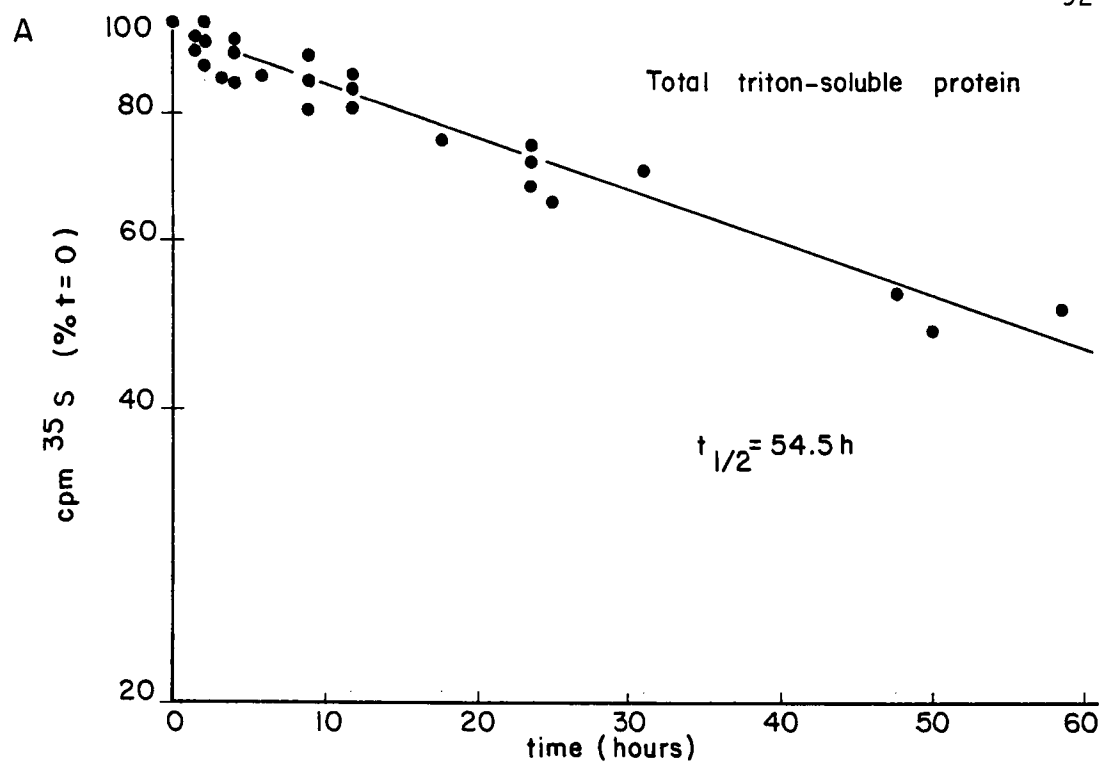


Figure 15

$$t_{1/2} = \frac{\ln 2}{k_d}$$

Pollack et al. (1981b) have found it convenient to express the k_d as a function of cell generation (T_G). The cells in the experiments shown in Figure 15 were growing with a mean doubling time of 64 hours during the chase phase (this is somewhat slower than unlabeled cells and may reflect adverse effects of the incorporated radioactivity). The $t_{1/2}$ for the Na,K-ATPase α subunit in these experiments was $.42 T_G$, which yields a k_d of $1.64 T_G^{-1}$.

HTC cell proteins can also be labeled to high specific activities by the administration of a brief pulse of [^{35} S]methionine after incubation in methionine-free medium. Experiments analogous to those in Figure 15 were performed with pulse-labeled HTC cells (Figure 16) to determine whether or not turnover of the α subunit of Na,K-ATPase begins immediately after its synthesis. In contrast to the data in Figure 15A, the total triton-soluble protein of pulse-labeled HTC cells turns over with multi-exponential kinetics (Figure 16A). The mean half-life at the linear portion of the decay (times later than 6 hours) was 70 hours. The turnover of the α subunit in these cells can be seen in Figure 16B. When a line was fitted to these data by linear regression (broken line; $r = -.988$), a lag period of approximately two hours is observed before α subunit turnover ensues. A statistically better fit occurs when only those time points later than 3 hours are considered (solid line; $r = -.996$). This line exhibits a lag time of 5 hours prior to α degradation. The $t_{1/2}$ of

Figure 16. Turnover of total triton-soluble protein and the Na,K-ATPase α subunit in HTC cells pulse-labeled with [^{35}S]methionine. Cells were incubated for 30 minutes in methionine-free medium (6.7×10^6 cells/ml) followed by a 10 minute pulse of [^{35}S]methionine (final concentration = $50 \mu\text{Ci/ml}$). The cells were then transferred to chase medium containing 10mM unlabeled methionine. At various times, samples ($4\text{--}10 \times 10^6$ cells) were removed, pelleted and lysed in triton-containing lysis buffer. Insoluble material was removed by centrifugation. (A) Aliquots of the cleared lysates were assayed for trichloroacetic acid-insoluble radioactivity. The half-time ($t_{1/2}$) and correlation coefficient (r) were determined from the linear portion of the decay curve (at times of 6 hours and longer). (B) The lysates were immunoprecipitated with anti- α IgG, and analyzed as in Figure 15B. The $t_{1/2}$ and r values were determined from the solid line which was fitted to the time points of 4 hours and longer. The dotted line depicts the best fit ($r = -.988$) of a line through all of time points. See text for explanation. The data are the result of 2 (A) and 3 (B) experiments.

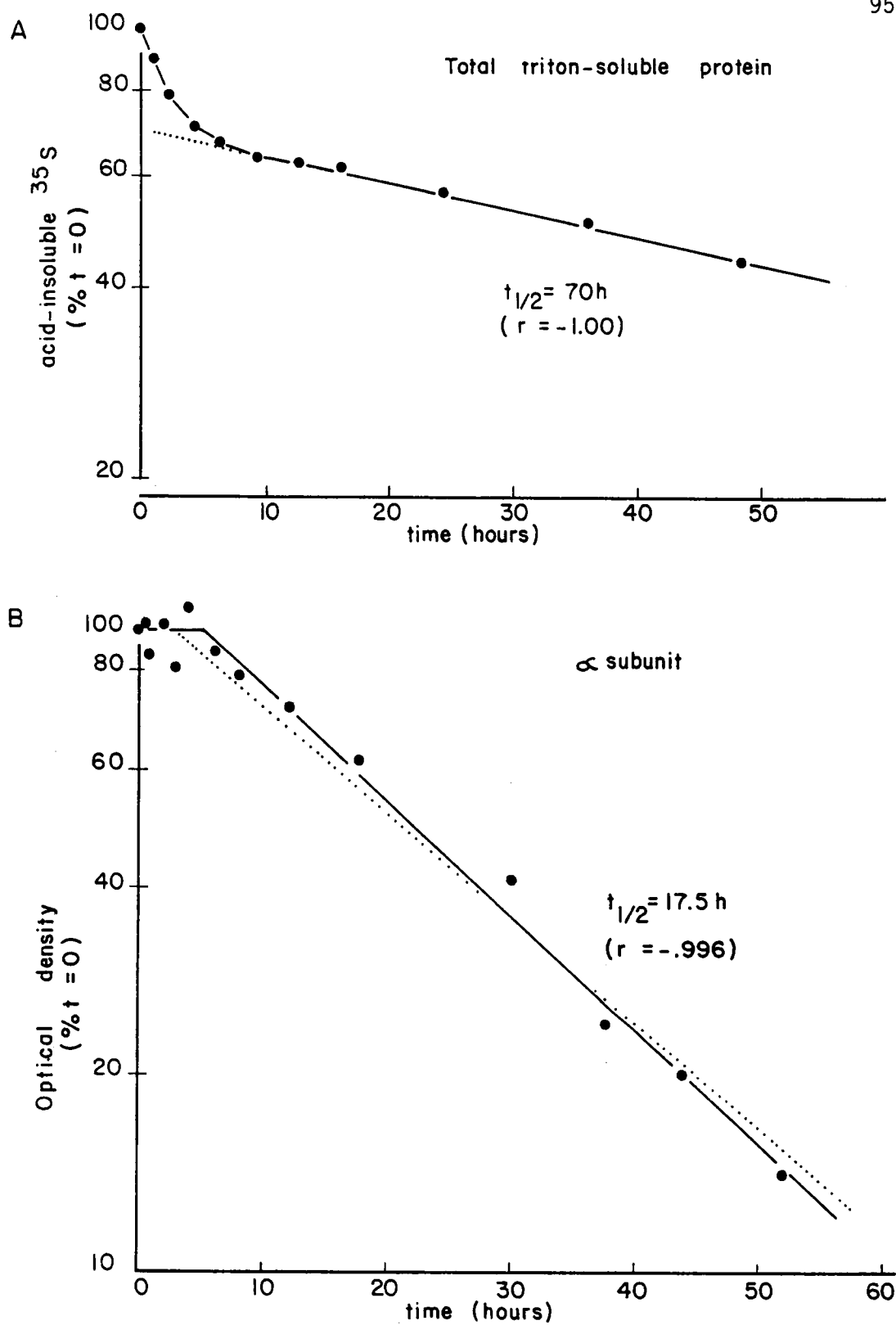


Figure 16

the Na,K-ATPase large subunit in these cells (determined from the linear portion of the decay) was 17.5 hours. These cells grew during the chase period with a T_G of 36 hours, leading to $t_{1/2}$ and k_d values of $.485 T_G$ and $1.43 T_G^{-1}$, respectively, for the α subunit. As is displayed in Table III, these data, when expressed in terms of T_G , agree well with those values obtained with steady-state-labeled cells.

In light of the current controversy regarding the site of synthesis of the Na,K-ATPase α subunit (see Chapter I), it was of interest to investigate this matter in HTC cells. The problem was approached by fractionating cells into soluble (S150) and particulate (P150) components after pulses of [^{35}S]methionine. These fractions were then analyzed by immunoprecipitation with anti- α IgG (Figure 17). No radioactivity was observed in the 93,000 dalton polypeptide in either fraction after a 2 minute pulse. After 5 minutes, labeled α subunit was detected in the P150 fraction, followed by the nearly linear incorporation of [^{35}S]methionine into that protein for 30 minutes. No label was found associated with a 93,000 dalton protein in the S150 fraction at any time during this experiment.

Discussion

I chose HTC (rat hepatoma) cells for these studies because of the ease with which their metabolism can be regulated and labeled compounds are incorporated. Also, since they readily grow in suspension culture, large quantities can be generated without repeatedly proteolyzing their surface, necessary when cells are grown in a monolayer. These cells lack the complex structural domains that

Table III
 α Subunit Turnover Parameters in HTC Cells

	Steady-state label ¹	Pulse label ²
Cell generation time (T_G)	64 h	36 h
α Subunit half-life	27 h	17.5 h
	.42 T_G	.485 T_G
α Subunit k_d	1.64 T_G^{-1}	1.43 T_G^{-1}

¹Data from experiments in Figure 15B.

²Data from experiments in Figure 16B.

Figure 17. Immunoprecipitation with anti- α IgG from the cytosol and total membrane fractions of ^{35}S -labeled HTC cells. Cells were incubated for 30 minutes in methionine-free medium (3.3×10^6 cells/ml) followed by the addition of [^{35}S]methionine to a final concentration of 50 $\mu\text{Ci/ml}$. At the times indicated on the abscissa, samples (5×10^6 cells) were removed and quick-frozen in liquid nitrogen. After a total of three freeze/thaw cycles, the cells were centrifuged at $150,000 \times g$ for 60 minutes. The supernatants (S150) were reserved and the pellets (P150) were solubilized in triton-containing lysis buffer with a dounce homogenizer and cleared of insoluble material by centrifugation. Both the S150 and P150 lysate fractions were immunoprecipitated with anti- α and analyzed as in Figure 15B.

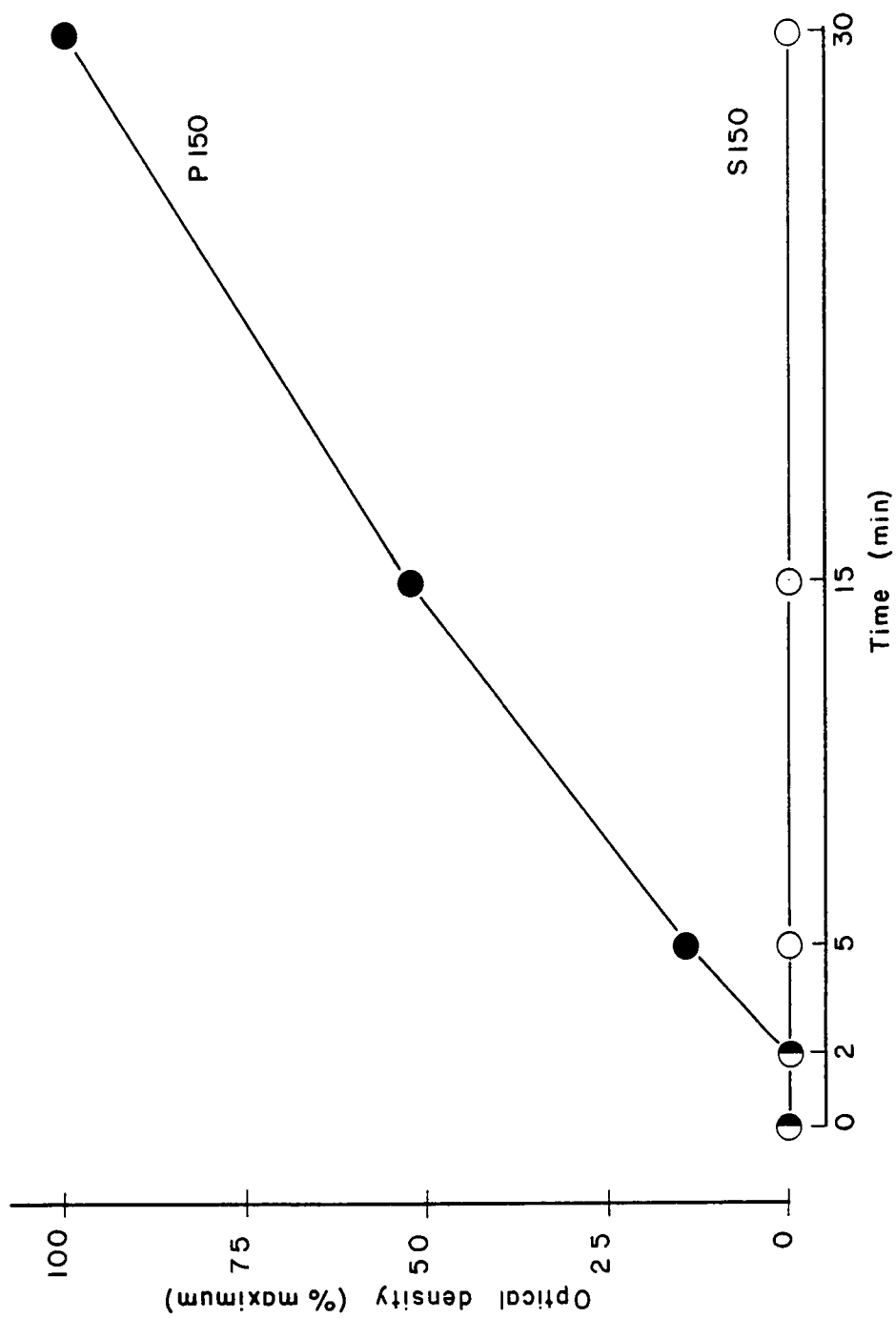


Figure 17

characterize the plasma membranes of cells of normal liver tissue and complicate the analysis of the turnover of surface constituents (see Evans and Higgins, 1978).

Doyle and his co-workers (for review, see Doyle and Baumann, 1979) have studied the plasma membrane components of these cells by monitoring the disappearance of radioactivity after labeling with a variety of macromolecule precursors and nonpenetrating reagents. In particular, they have found in experiments confirmed in this laboratory (Karin, unpublished) that HTC cell surface proteins, labeled by lactoperoxidase-catalyzed iodination, are degraded very slowly with a mean half-life of 100 hours or more (Tweto and Doyle, 1976). The results of SDS-PAGE analysis of these labeled peptides suggested to the authors that the membrane turns over as a unit, in that all the resolvable protein bands exhibited identical rates of degradation.

The Na,K-ATPase is an integral plasma membrane component and is presumably included among the peptides analyzed by Tweto and Doyle (1976). I have found that the α subunit of this enzyme is degraded somewhat faster than the bulk of the iodinated proteins of the HTC surface, exhibiting a half-life of 17.5 to 27 hours, depending on growth rates. These data imply, instead, heterogeneity in the turnover of plasma membrane proteins. Of possible relevance to Doyle's results is the observation that the α subunit of Na,K-ATPase purified from rat kidney is poorly labeled by chloramine T-catalyzed iodination (Karin, unpublished). This method, like those using lactoperoxidase, leads to iodinated tyrosine residues. If the α subunit

of HTC cells is similarly a poor substrate for iodination, its "aberrant" turnover could go unnoticed.

Heterogeneous turnover of plasma membrane proteins has been observed in both cultured cells (cf. Horst and Roberts, 1979) and in tissues in vivo (cf. Tauber and Reutter, 1981). L. R. Pollack and his colleagues in this laboratory (Pollack et al., 1982) found that the α subunit of Na,K-ATPase in HeLa cells is turned over at a much higher rate ($t_{1/2}$ = 5 to 6 hours) than the majority of the surface proteins (mean $t_{1/2}$ = 100 hours). The relatively rapid turnover of Na,K-ATPase seen in both HeLa and HTC cells may reflect the importance of this enzyme to the cells survival. That is, the turnover of Na,K-ATPase, which maintains the battery of surface enzymes in good repair through regular replacement of faulty or damaged proteins, may be independent of the routine replacement of the majority of the plasma membrane.

The turnover of the total (triton-soluble) protein complement of HTC cells is greatly influenced by the labeling regime, evident when comparing Figures 15A and 16A (pp. 91 and 94). Whereas the proteins of long-term-labeled cells turn over with a mean rate described by a single exponential, the proteins of pulse-labeled cells exhibit an early (0 to 10 hours) rapid phase of turnover, followed by a slower phase, also a single exponential. This behavior has previously been attributed to either sets of individual protein species with different rates of turnover (Poole and Wibo, 1973), or discrete degradation phases, characteristic of all proteins, i.e., the same proteins exhibit both rapid and slow turnover (Wheatley et al., 1980).

The variation in the half-life of the α subunit reported here ($t_{1/2}$ = 17.5 to 27 hours) is virtually eliminated when the turnover rate is corrected for the generation time of the cells in each experiment ($t_{1/2}$ = .42 to .485 T_G). Thus, the turnover rate of the α subunit is only secondarily affected by the duration of radiolabel incorporation prior to the chase phase. More importantly, the turnover of this protein is described by a single exponential (in pulse-labeled cells, it follows a several hour lag) with no obvious discontinuities to suggest different rate phases. This contradicts the theory of Wheatley et al. (1980) that all proteins exhibit a "metastable," or rapid turnover state, followed by a "destabilized state" of slower decay. The explanation of Poole and Wibo (1973), that the multi-exponential turnover kinetics seen after pulse labeling reflects protein heterogeneity, is more plausible.

The 17.5 to 27 hour half-life of the Na,K-ATPase α subunit observed in HTC cells is longer than that found by Pollack et al. (1982) in HeLa cells ($t_{1/2}$ = 5 to 6 hours). This difference is diminished, but not eliminated, when expressed in terms of cell generation time ($1.5 T_G^{-1}$ in HTC cells vs. $3.1 T_G^{-1}$ in HeLa cells).

While Pollack et al. (1982) measured the rate of enzyme removal from the HeLa cell plasma membrane, the HTC cell data represent the actual degradation rates of the subunit protein. It is possible in HTC cells that the Na,K-ATPase α subunit is stable for several hours after its internalization, which would account for the differences between this report and the work of Pollack et al. (1982). However, Cook et al. (1982) were unable to detect Na,K-ATPase in an

intermediate subcellular compartment (endosome) during transfer from the plasma membrane to lysosomes. They concluded that the endosome component of the membrane turnover pathway in HeLa cells is either very small or very transient, and that the degradation of membrane proteins occurs rapidly after internalization. The temporal characteristics of endosomes in HTC cells are not known.

The half-lives of the Na,K-ATPase α subunit in HTC and HeLa cells are, however, much faster than that found by Lo and Edelman (1976) in rat kidney ($t_{1/2}$ = approximately 120 hours), a system with a low mitotic index. It therefore seems likely that the turnover rate of Na,K-ATPase is both a function of the cellular metabolic rate and the tissue type.

The five hour lag time preceding α subunit turnover seen in pulse-labeled HTC cells is similar to that reported by Pollack et al. (1982) in HeLa cells and also by Devreotes and Fambrough (1976) in their analysis of acetylcholine receptor turnover. These authors attributed the lag to the "transit time" of the newly-synthesized proteins through intracellular membrane compartments (endoplasmic reticulum, Golgi apparatus) where posttranslational processing and modification can occur. This is the best explanation of the lag in these experiments as well since, after a brief pulse of [^{35} S]methionine, an essentially synchronous population of α peptides should be moving through the cells, and degradation cannot occur until these proteins have reached the plasma membrane where they can be internalized into lysosomes (Cook et al., 1982).

This interpretation is in direct opposition to the biosynthesis pathways proposed by Sherman et al. (1980) and Geering et al. (1982b),

who suggested cytoplasmic ribosomes as site of α subunit translation. I tested this directly and found immunoprecipitable (93,000 daltons) α subunits only in the membrane compartments of HTC cells. A lag of two minutes was observed prior to the appearance of radiolabeled α subunits in the membranes. This presumably is the time required for sufficient [^{35}S]methionine to enter cellular amino acid pools. No mature (93,000 daltons) α protein was found in the cytosol at any time tested.

These results, together with the observed transit time, do not support the theory that the Na,K-ATPase α subunit is translated as a soluble protein and either inserted immediately into the plasma membrane (Sherman et al., 1980), or into an intracellular membrane (Geering et al., 1982b). I cannot rule out the instantaneous association of α with (intracellular) membranes, nor could I localize nascent chains. Even though Geering et al. (1982b) propose cytoplasmic biosynthesis of α proteins, they identified nascent chains only in the microsome membranes.

While clear examples exist of integral membranes proteins being translated on free ribosomes and inserted posttranslationally (Wickner, 1980), some of the best studied examples have exhibited little or no transit time prior to reaching their functional destinations (cf. Colman et al., 1982 and Schmidt et al., 1983). This is in contrast to the observations of the Na,K-ATPase α subunit in HTC cells. The α subunit is so highly conserved among evolutionarily distant species that it is not apt to have widely divergent biosynthetic mechanisms. In view of the work presented

here, I feel the most probable path of α subunit biosynthesis involves translation on membrane-bound ribosomes.

LITERATURE CITED

LITERATURE CITED

- Albers, R. W. (1976) in Enzymes in Biological Membranes, Volume 3, A. Martinosi, ed.,
- Amenta, J. S., Sargus, M. J., and Baccino, F. M. (1977) Biochem. J. 168, 223-227.
- Aperia, A., Larsson, L., and Zetterstrom, R. (1981) Am. J. Physiol. 241, F356-F360.
- Asano, Y., Liberman, U. A., and Edelman, I. S. (1976) J. Clin. Invest. 57, 368-379.
- Askari, A., and Huang, W. (1980) Biochem. Biophys. Res. Commun. 93, 448-453.
- Avruch, J., and Wallach, D. F. H. (1971) Biochim. Biophys. Acta 233, 334-347.
- Blobel, G., and Dobberstein, B. (1975) J. Cell Biol. 67, 835-851.
- Boardman, L., Huett, M., Lamb, J. F., Newton, J. P., and Polson, J. M. (1974) J. Physiol. (Lond.) 241, 771-794.
- Boardman, L. J., Lamb, J. F., and McCall, D. (1972) J. Physiol. (Lond.) 225, 619-635.
- Brake, E. T., Will, P. C., and Cook, J. S. (1978) Memb. Biochem. 2, 17-46.
- Brotherus, J. R., Moller, J. V., and Jorgensen, P. L. (1981). Biochem. Biophys. Res. Commun. 100, 146-154.
- Chignell, C. F., and Titus, E. (1966) J. Biol. Chem. 241, 5083-5089.
- Churchill, L., and Hokin, L. E. (1976) Biochim. Biophys. Acta 434, 258-264.
- Churchill, L., and Hokin, L. E. (1979) J. Biol. Chem. 254, 7388-7392.
- Colman, D., Kreibich, G., Frey, A. B., and Sabatini, D. D. (1982) J. Cell Biol. 95, 598-608.
- Cook, J. S., and Brake, E. T. (1978) in Cell Membrane Receptors for Drugs and Hormones, R. W. Straub and L. Bolis, eds., Raven Press, New York, 343-351.

- Cook, J. S., Tate, E. H., and Shaffer, C. (1982) J. Cellul. Physiol. 110, 84-92.
- Cook, J. S., Will, P. C., Proctor, W. R., and Brake, E. T. (1976) in Biogenesis and Turnover of Membrane Macromolecules, J. S. Cook, ed., Raven Press, New York, 15-36.
- Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059-3065.
- DePierre, J. W., and Karnovsky. (1973) J. Cell Biol. 56, 275-303.
- Devreotes, P. N., and Fambrough, D. M. (1976) in The Synapse, Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, New York, 237-251.
- Doucet A., and Katz, A. I. (1981) Am. J. Physiol. 241, F273-F278.
- Doyle, D., and Baumann, H. (1979) Life Sci. 24, 951-966.
- Edelman, I., Bogoroch, R., and Porter, G. A. (1963) Proc. Natl. Acad. Sci. USA 50, 1169-1177.
- Edelson, P. J., and Cohn, Z. A. (1976) J. Exp. Med. 144, 1581-1595.
- Engelman, D. M., and Steitz, T. A. (1981) Cell 23, 411-422.
- Ernst, S. A. (1972) J. Histochem. Cytochem. 20, 23-38.
- Ernst, S. A., and Ellis, R. A. (1969) J. Cell Biol. 40, 305-321.
- Ernst, S. A., Goertemiller, C. C., Jr., and Ellis, R. A. (1967) Biochim. Biophys. Acta 135, 682-692.
- Ernst, S. A., and Mills, J. W. (1980) J. Histochem. Cytochem. 28, 72-77.
- Ernster, L., R. Zetterstrom., and Lindberg, O. (1950) Acta Chem. Scand. 4, 942-947.
- Esmann, M., Christiansen, C., Karlsson, K.-A., Hansson, G. C., and Skou, J. C. (1980) Biochim. Biophys. Acta 603, 1-12.
- Evans, W. H., and Higgins, J. A. (1978) in Federation of European Biochemical Societies 12th Meeting, S. Rapoport and T. Schewe, eds., Pergamon Press, Oxford, 169-178.
- Ferguson, K. A. (1964) Metabolism 15, 985-1002.
- Fiske, C. H., and Subbarow, Y. (1925) J. Biol. Chem. 66, 375-400.

- Forbush, B., Kaplan, J. H., and Hoffman, J. F. (1978) Biochemistry 17, 3667-3676.
- Gardner, J. M., and Fambrough, D. M. (1979) Cell 16, 661-674.
- Garg, L. C., Knepper, M. A., and Burg, M. B. (1981) Am. J. Physiol. 240, F536-F544.
- Geering, K., Girardet, M., Bron, C., Kraehenbühl, J.-P., and Rossier, B. C. (1982a) J. Biol. Chem. 257, 10338-10343.
- Geering, K., Girardet, M., Kraehenbühl, J.-P., and Rossier, B. C. (1982b) J. Cell Biol. 95, 379a (abstr.).
- Giotta, G. J. (1976) J. Biol. Chem. 251, 1247-1252.
- Girardet, M., Geering, J. M Frantes, Geser, D., Rossier, B. C., Kraehenbühl, J.-P., and Bron, C. (1981) Biochemistry 20, 6684-6691.
- Goldberg, A. L., and Dice, J. F. (1974) Ann. Rev. Biochem. 43, 835-869.
- Goldin, S. M. (1977) J. Biol. Chem. 252, 5630-5642.
- Hamlyn, J. M., and Duffy, T. (1978) Biochem. Biophys. Res. Commun. 84, 458-464.
- Handler, J. S., Preston, A. S., Perkins, F. M., Matsumura, M., Johnson, J. P., and Watlington, C. O. (1981) Ann. N.Y. Acad. Sci. 372, 442-453.
- Hartzell, H. C., and Fambrough, D. M. (1973) Develop. Biol. 30, 153-165.
- Hastings, D. F., and Reynolds, J. A. (1979) Biochemistry 18, 817-821.
- Hendler, E. D., Torrett, J., Kupor, L., and Epstein, F. (1972) Am. J. Physiol. 222, 754-760.
- Hokin, L. E., Dahl, J. L., Deupree, J. D., Dixon, J. F., Hackney, J. F., and Perdue, J. F. (1973) J. Biol. Chem. 248, 2593-2605.
- Hopkins, B. E., Wagner, H., Jr., and Smith, T. W. (1976) J. Biol. Sci. 251, 4365-4371.
- Horst, M. N., and Roberts, R. M. (1979) J. Biol. Chem. 254, 5000-5007.

- Hossler, F. E., Sarras, M. P., and Barnett, R. J. (1978) J. Cell Sci. 31, 179-197.
- Ismail-Beigi, F., Bissell, D. M., and Edelman, I. S. (1979) J. Gen. Physiol. 73, 369-383.
- Ismail-Beigi, F., and Edelman, I. S. (1970) Proc. Natl. Acad. Sci. USA 67, 1071-1078.
- Ismail-Beigi, F., and Edelman, I. S. (1971) J. Gen. Physiol. 57, 710-722.
- Ismail-Beigi, F., and Edelman, I. S. (1974) Proc. Soc. Exp. Biol. Med. 146, 983-988.
- Jean, D. H., and Albers, R. W. (1976) Biochim. Biophys. Acta 452, 219-226.
- Jean, D. H., Albers, R. W., and Koval, G. J. (1975) J. Biol. Chem. 250, 1035-1040.
- Jorgensen, P. L. (1969) Biochim. Biophys. Acta 192, 326-334.
- Jorgensen, P. L. (1974) Biochim. Biophys. Acta 356, 36-52.
- Jorgensen, P. L. (1975) Q. Rev. Biophys. 7, 239-274.
- Jorgensen, P. L. (1980) Physiol. Rev. 60, 864-917.
- Karin, N. J., and Cook, J. S. (1983) Curr. Top. Memb. Transp. 19, in press.
- Kartner, N., Alon, N., Swift, M., Buchwald, M., and Riordan, J. R. J. Memb. Biol. 36, 191-211.
- Kashgarian, M. (1980) Curr. Top. Membr. Transp. 13, 149-160.
- Katz, A. I., and Epstein, F. H. (1967) J. Clin. Invest. 46, 1999-2011.
- Katz, A. I., and Genant, H. K. (1971) Pflügers Arch. 330, 136-148.
- Katz, A. I., and Lindheimer, M. D. (1973) J. Clin. Invest. 52, 796-804.
- Kirsten, E., Kirsten, R., Leaf, A., and Sharp, G. W. G. Pflügers Arch. 300, 213-225.
- Knox, W. H., and Sen, A. K. (1974) Ann. N.Y. Acad. Sci. 242, 471-488.
- Kowit, J. D., and Maloney, J. (1982) Anal. Biochem. 123, 86-93.

- Krupp, M. N., and Lane, M. D. (1982) J. Biol. Chem. 257, 1372-1377.
- Kyte, J. (1971) Biochem. Biophys. Res. Commun. 43, 1259-1265.
- Kyte, J. (1972) J. Biol. Chem. 247, 7642-7649.
- Kyte, J. (1974) J. Biol. Chem. 249, 3652-3660.
- Kyte, J. (1981) Nature 292, 201-204.
- Laemmli, U. K. (1970) Nature 227, 680-685.
- Lamb, J. F., Ogden, P., and Simmons, N. L. (1981) Biochim. Biophys. Acta 644, 333-340.
- Laskey, R. A., and Mills, A. D. (1975) Eur. J. Biochem. 56, 335-341.
- Law, P. Y., and Edelman, I. S. (1978a) J. Membr. Biol. 41, 15-40.
- Law, P. Y., and Edelman, I. S. (1978b) J. Membr. Biol. 41, 41-64.
- Levey, G. S., Skelton, C. L., and Epstein, S. E. (1969) Endocrinology 85, 1004-1009.
- Liang, S.-L., and Winter, C. G. (1976) Biochim. Biophys. Acta 452, 552-565.
- Lin, M. H., and Akera, T. (1978) J. Biol. Chem. 253, 723-726.
- Lingham, R. B., Steward, D. J., and Sen, A. K. (1980) Biochim. Biophys. Acta 601, 229-234.
- Lo, C.-S., August, T. R., Liberman, U. A., and Edelman. (1976) J. Biol. Chem. 251, 7826-7833.
- Lo, C.-S., and Edelman, I. S. (1976) J. Biol. Chem. 251, 7834-7840.
- Lo, C.-S., and Lo, T. N. (1979) Am. J. Physiol. 236, F9-F13.
- Lo, C.-S., and Lo, T. N. (1980) J. Biol. Chem. 255, 2131-2136.
- Lo, C.-S., and Lo, T. N. (1981) Endocrinology 109, 1473-1478.
- Lopez-Saura, P., Trouet, A., and Tulkens, P. (1978) Biochim. Biophys. Acta 543, 430-449.
- Lowry, O. H., Rosebrough, N. J., Farr, N. J., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
- Marshall, M. O. (1976) Biochim. Biophys. Acta 455, 837-848.

- Marver, D. (1980) Vitam. Horm. (N.Y.) 38, 57-117.
- McCans, J. L., Lane, L. K., Lindenmayer, G. E., Butler, V. P., Jr., and Schwartz, A. (1974) Proc. Natl. Acad. Sci. USA 71, 2449-2452.
- McConahey, P. J., and Dixon, F. J. (1966) Int. Arch. Allergy 29, 185-189.
- McDonough, A. A., Hiatt, A., and Edelman, I. S. (1982) J. Memb. Biol. 69, 13-22.
- Moller, O. J. (1971) Exptl. Cell Res. 68, 347-355.
- Nakao, T., Nakao, M., Nagai, F., Kawai, K., Fujihara, Y., Hara, Y., and Fujita, M. (1973) J. Biochem. 73, 781-791.
- Oppenheimer, J. H., Dillman, W. H., Schwartz, H. L., and Towle, H. C. (1979) Federation Proc. 38, 2154-2161.
- Palade, G. (1975) Science 189, 347-358.
- Pollack, L. R. (1980) Ph.D. Thesis, University of Tennessee, Knoxville.
- Pollack, L. R., Tate, E. H., and Cook, J. S. (1981a) J. Cell. Physiol. 106, 85-97.
- Pollack, L. R., Tate, E. H., and Cook, J. S. (1981b) Amer. J. Physiol. 241, C173-C183.
- Pollack, L. R., Tate, E. H., and Cook, J. S. (1982) in Membranes in Growth and Development, J. F. Hoffman, G. Giebisch, and L. E. Bolis, eds., Alan R. Liss, New York.
- Poole, B., and Wibo, M. (1973) J. Biol. Chem. 248, 6221-6226.
- Post, R. L., and Jolly, P. C. (1957) Biochim. Biophys. Acta 25, 118-128.
- Rhee, H. M., and Hokin, L. E. (1975) Biochem. Biophys. Res. Commun. 63, 1139-1145.
- Robinson, J. D., and Flashner, M. S. (1979) Biochim. Biophys. Acta 549, 145-176.
- Rodriguez, H. J., and Klahr, S. (1980) Clin. Res. 28, 460A.
- Rodriguez, H. J., Sinha, S. K., Starling, J., and Klahr, S. (1981) Am. J. Physiol. 241, F186-F195.

- Rossier, B. C. (1978) J. Membr. Biol. 40, 187-198.
- Rossier, B. C., Wilce, P. A., Inciardi, J. F., Yoshimura, F. K., and Edelman, I. S. (1977) Am. J. Physiol. 232, C174-C179.
- Rothman, J. D. (1981) Science 213, 1212-1219.
- Rozengurt, E., and Mendoza, S. (1980) Ann. N.Y. Acad. Sci. 339, 175-190.
- Sabatini, D. D., Kreibich, G., Morimoto, T., and Adesnik, M. (1982) J. Cell Biol. 92, 1-22.
- Salter, D. W., Baldwin, S. A., Lienhard, G. E., and Weber, M. J. (1982) Proc. Natl. Acad. Sci. USA 79, 1540-1544.
- Salter, D. W., Custead-Jones, S., and Cook, J. S. (1978) J. Membr. Biol. 40, 67-76.
- Schellenberg, G. D., Pech, I. V., and Stahl, W. L. (1981) Biochim. Biophys. Acta 649, 691-700.
- Schmidt, B., Hennig, B., Zimmermann, R., and Neupert, W. J. Cell Biol. 96, 248-255.
- Scott, W. N., Reich, I. M., Brown, J. A., Jr., and Yang, C-P. H. (1978) J. Membr. Biol. 40, 213-220.
- Scott, W. N., Reich, I. M., and Goodman, D. B. P. (1979) J. Biol. Chem. 254, 4957-4959.
- Scott, W. N., Yang, C-P., Skipski, I. A., Cobb, M. H., Reich, I. M., and Terry, P. M. (1981) Ann. N.Y. Acad. Sci. 372, 15-29.
- Sefton, B. M., Trowbridge, I. S., Cooper, J. A., and Scolnick, E. M. (1983) Cell 31, 465-474.
- Sherman, J. M., Sabatini, D. D., and Morimoto. (1980) J. Cell Biol. 87, 307a (abstr.).
- Sinha, S. K., Rodriguez, H. J., Hogan, W. C., and Klahr, S. (1981) Biochim. Biophys. Acta 641, 20-35.
- Skou, J. C. (1957) Biochim. Biophys. Acta 23, 394-401.
- Somjen, D., Ismail-Beigi, F., and Edelman, I. S. (1981) Am. J. Physiol. 240, E146-E154.
- Stefanovic, V., Mandel, P., and Rosenburg, A. (1975) J. Biol. Chem. 250, 7081-7083.

- Steinberg, R. H., and Miller, S. (1973) Exp. Eye Res. 16, 365-372.
- Stewart, D. J., Semple, E. W., Swart, G. T., and Sen, A. K. (1976) Biochim. Biophys. Acta 419, 150-163.
- Stickrod, G., and Chadwick, C. (1982) Lab Anim. 11, 48-49.
- Stirling, C. E. (1972) J. Cell Biol. 53, 704-714.
- Suzuki, S. (1981) J. Steroid Biochem. 14, 449-456.
- Sweadner, K. J. (1977) Biochem. Biophys. Res. Commun. 78, 962-969.
- Sweadner, K. J. (1979) J. Biol. Chem. 254, 6060-6067.
- Sweadner, K. J., and Goldin, S. M. (1980) N. Engl. J. Med. 302, 777-783.
- Tata, J. R. (1968) in Regulatory Functions of Biological Membranes, Biochem. Biophys. Acta Library, Vol. 11, Elsevier, Amsterdam, 222-235.
- Tauber, R., and Reutter. (1981) Eur. J. Cell Biol. 26, 35-42.
- Tobin, T. R., and Brody, T. M. (1972) Biochem. Pharmacol. 21, 1553-1560.
- Tweto, J., and Doyle, D. (1976) J. Biol. Chem. 251, 872-882.
- Van Lenten, L., and Ashwell, G. (1971) J. Biol. Chem. 246, 1889-1894.
- Voute, C. L., Thummel, J., and Brenner, M. (1975) J. Steroid Biochem. 6, 1175-1179.
- Wade, J. B., O'Neil, R. G., Pryor, J. L., and Boulpaep, E. L. (1979) J. Cell Biol. 81, 439-445.
- Wallach, D. F. H., and Lin, P. S. (1973) Biochim. Biophys. Acta 300, 211-254.
- Wheatley, D. N., Giddings, M. R., and Inglis, M. S. (1980) Cell Biol. Int. Rep. 4, 1081-1090.
- Wickner, W. (1980) Science 210, 861-868.
- Widnell, C. C., Schneider, Y.-J., Pierre, B., Baudhuin, P., and Trouet, A. (1982) Cell 28, 61-70.
- Wiggins, P. M. (1982) J. Theor. Biol. 99, 665-679.

- Will, P. C., DeLisle, R. C., Cortright, R. N., and Hopfer, U. (1981) Ann. N.Y. Acad. Sci. 372, 64-78.
- Will, P. C., Lebowitz, J. L., and Hopfer, U. (1980) Am. J. Physiol. 238, F261-F268.
- Zarling, D. A., Watson, A., and Bach, F. H. (1980) J. Immunol. 124, 912-920.

APPENDIX

APPENDIX

ENDOGENOUS INHIBITORS OF 5'-NUCLEOTIDASE IN THE CYTOSOL OF CULTURED CELLS

Introduction

5'-Nucleotidase (5'-ribonucleotide phosphorylase, EC 3.1.3.5) activity has commonly been monitored as a marker for plasma membranes during fractionation studies of tissues and cultured cells (DePierre and Karnovsky, 1973; Wallach and Lin, 1973), although exceptions to its surface localization have been noted in certain cell types (cf. Widnell et al., 1982). The purity of a plasma membrane fraction is routinely expressed as the ratio of the specific activity of the marker enzyme in the isolated fraction to that of the total homogenate. Implicit in this calculation is the assumption that the plasma membrane enzymes are fully active in the homogenate, albeit diluted by other cell constituents. I report here that inhibitors of 5'-nucleotidase exist in the cytosol of HTC cells and two other unrelated cultured cell lines.

Materials and Methods

Materials

Cell culture reagents were all from Gibco. [^{14}C]5'-AMP was purchased from Amersham and purified by paper chromatography, as described by Brake et al. (1978). Tunicamycin and Concanavalin A

(ConA) were from Sigma. Proteinase K was from Boehringer. All other components were reagent grade.

Cells

HTC cells were grown in suspension as described in Chapter III, or where indicated, in monolayers on 15 cm dishes in Eagles minimal essential medium supplemented with 2mM glutamine and 5% fetal calf serum. In some experiments HTC cells were grown for two days prior to use in 0.1 $\mu\text{g/ml}$ tunicamycin, an inhibitor of protein glycosylation which, at this concentration, decreased the incorporation of mannose into total cell protein by 35% (data not shown). HeLa cells and HSWP cells, a human skin diploid fibroblast line, were cultured as previously described by Brake et al. (1978) and Salter et al. (1978), respectively.

Fractionation

HTC and HeLa cells were washed twice in phosphate-buffered saline, suspended at 1×10^7 cells/ml in 10mM Tris, pH 7.7 at 0°C and allowed to swell for 10 minutes. They were homogenized in a tight-fitting Dounce homogenizer to 80-90% cell breakage, as determined by phase contrast microscopy. An equal volume of 20% (w/v) sucrose was then added. HSWP cells were rinsed twice on the dish with cold phosphate-buffered saline, scraped with a rubber policeman and pelleted. The pellet was suspended in 10mM Tris, pH 7.7, at 150 μl buffer/15 cm dish-equivalent of cells and homogenized with three freeze/thaw cycles followed by sucrose addition, as above. For

some experiments cytosol and total particulate fractions from each cell type were prepared by centrifuging the homogenates at 200,000 x g for 60 minutes.

Assays

5'-Nucleotidase was assayed as 5'-AMPase and measured by the release of [^{14} C]adenosine from [^{14} C]5'-AMP, as described by Brake et al. (1978) with the addition of 0.1% triton X-100 and 5mM p-nitrophenylphosphate. In some experiments 5 μ M ConA was added to the assay mix prior to substrate addition. 5'-AMP was 100 μ M (final concentration) unless otherwise indicated. Protein was estimated by the method of Avruch and Wallach (1971).

Proteinase Digestion

The cytosol (245 μ l) was mixed with 5 μ l of a 10 mg/ml solution of proteinase K and incubated at 37°C for 3 hours. This was then added to the reaction tubes for a 20-30 minute assay. To test the susceptibility to proteolytic inactivation of the 5'-AMPase during this assay period, 5 μ l proteinase K solution was mixed with 245 μ l homogenization buffer containing 10% (w/v) sucrose. An aliquot equal in volume to that of the added proteinase-treated cytosol was included in the tubes containing the total particulate fraction and reagents for the 5'-AMPase assay as described above. Alternatively, cytosol aliquots in capped Eppendorf tubes were heated in a boiling water bath for 5 minutes prior to their addition to assay tubes.

Results

The presence of 5'-nucleotidase in HTC cells was confirmed by the results of experiments shown in Table A-I. The enzyme was assayed as the release of [^{14}C]adenosine from [^{14}C]5'-AMP in the presence of a 50 fold molar excess of p-nitrophenylphosphate, to minimize the hydrolysis of AMP by nonspecific phosphatases. When the [^{14}C]5'-AMP concentration is increased to 200 μM (double the usual assay concentration) the release of [^{14}C]adenosine is elevated by 32%, indicating that the enzyme activity is specific for 5'-nucleotides (in this case 5'-AMP) and not p-nitrophenylphosphate (see Discussion). Some p-nitrophenol is liberated during the assay conditions, as evidenced by its yellow color. This "neutral phosphatase" activity does not, however, co-fractionate with HTC 5'-AMPase; while all of the 5'-AMPase is particulate in these cells, greater than 40% of the total p-nitrophenylphosphatase activity (at pH 7.0) is soluble (data not shown).

Further evidence for the presence of 5'-AMPase is the enzyme's response to treatments which are specifically directed toward glycoproteins. The lectin Concanavalin A (ConA) is a potent inhibitor of 5'-nucleotidase in both C6 glioma cells (Stefanovic et al., 1975) and HeLa cells (Brake et al., 1978). ConA (5 μM) inhibited AMP hydrolysis by HTC homogenates by 97% (Table A-I). When I assayed homogenates which were prepared from cells grown in 0.1 $\mu\text{g/ml}$ tunicamycin, an inhibitor of protein glycosylation, AMP hydrolysis was diminished by

Table A-I
5'-AMP Hydrolysis by HTC Cell Homogenates

	$[^{14}\text{C}]\text{AMP}$ (μM)	- ConA	Specific activity + ConA	% Inhibition
Control	100	94 ± 4.4	2.4 ± 1.4	97
Control	200	124 ± 0.6	Not determined	-
Tunicamycin- treated	100	64 ± 5.6	7.6 ± 1.2	88

Homogenates from control cells or cells grown in $0.1 \mu\text{g/ml}$ tunicamycin were assayed for 5'-AMP hydrolysis as described in Materials and Methods with $[^{14}\text{C}]\text{AMP}$ at the concentrations shown above. ConA, when present, was $5 \mu\text{M}$. The data are the result of triplicate assays and are expressed as $\text{nmol adenosine} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1} \pm \text{S.D.}$

about one third. Tunicamycin treatment also led to a slight increase in ConA-insensitive AMP hydrolysis.

These data demonstrate that HTC cells contain true 5'-AMPase. In testing the validity of this activity as a plasma membrane marker, I consistently found the recovery of activity in subcellular (particulate) fractions to be several fold higher than that of the starting homogenate. In one experiment the homogenate exhibited 5'-AMPase activity of 190 nmol AMP hydrolyzed $\cdot$ mg protein⁻¹ $\cdot$ h⁻¹. After centrifugation to remove the cytosol (which contains approximately 50% of the total homogenate protein and none of the 5'-AMPase) the specific activity of the residue was expected to double, or equal approximately 380 nmol AMP hydrolyzed $\cdot$ mg protein⁻¹ $\cdot$ h⁻¹. The observed value for the total particulate fraction was 865 nmol AMP hydrolyzed $\cdot$ mg protein⁻¹ $\cdot$ h⁻¹, more than 2 fold higher than expected.

These data could be explained by inhibitory agents in the cytosol. This was confirmed by assaying a total particulate fraction from HTC cells in the presence of various amounts of the cytosol. As can be seen in Figure A-1A, 5'-AMPase was inhibited by the soluble fraction in a concentration-dependent manner. Sucrose is an inhibitor of 5'-AMPase in HeLa cells (Brake et al., 1978) but at the concentration present in the HTC homogenates was not inhibitory (data not shown). The inhibition was partially relieved by heating the cytosol prior to its addition (Figure A-1A). Figure A-1B shows that treatment of the cytosol with proteinase K also diminished its

Figure A-1. Heat- and proteinase-sensitivity of the cytosolic inhibitors of 5'-AMPase. (A) 5'-AMPase activity in the HTC total particulate (TP) fraction $\pm$ control or heat-treated cytosol. Cytosol (0.9 mg protein/ml) was prepared from an HTC homogenate by centrifugation and a portion incubated at 100°C for 5 minutes. Control (○) or heat-treated (●) cytosol was then added as indicated on the abscissa to 5'-AMPase assay tubes containing the TP fraction. (B) The effect of proteinase K digestion of the HTC total particulate and cytosol fractions on 5'-AMPase activity. HTC fractions were incubated with or without proteinase K (200 μ g/ml) at 37°C for 3 hours (cytosol) or 30 minutes (TP). 5'-AMPase activity was then measured in the proteinase-treated TP fraction and the control TP fraction $\pm$ 100 μ l of either control or proteinase-treated cytosol. The assays in both A and B contained 65 μ g TP fraction protein. The data represent the means and ranges of duplicate assays.

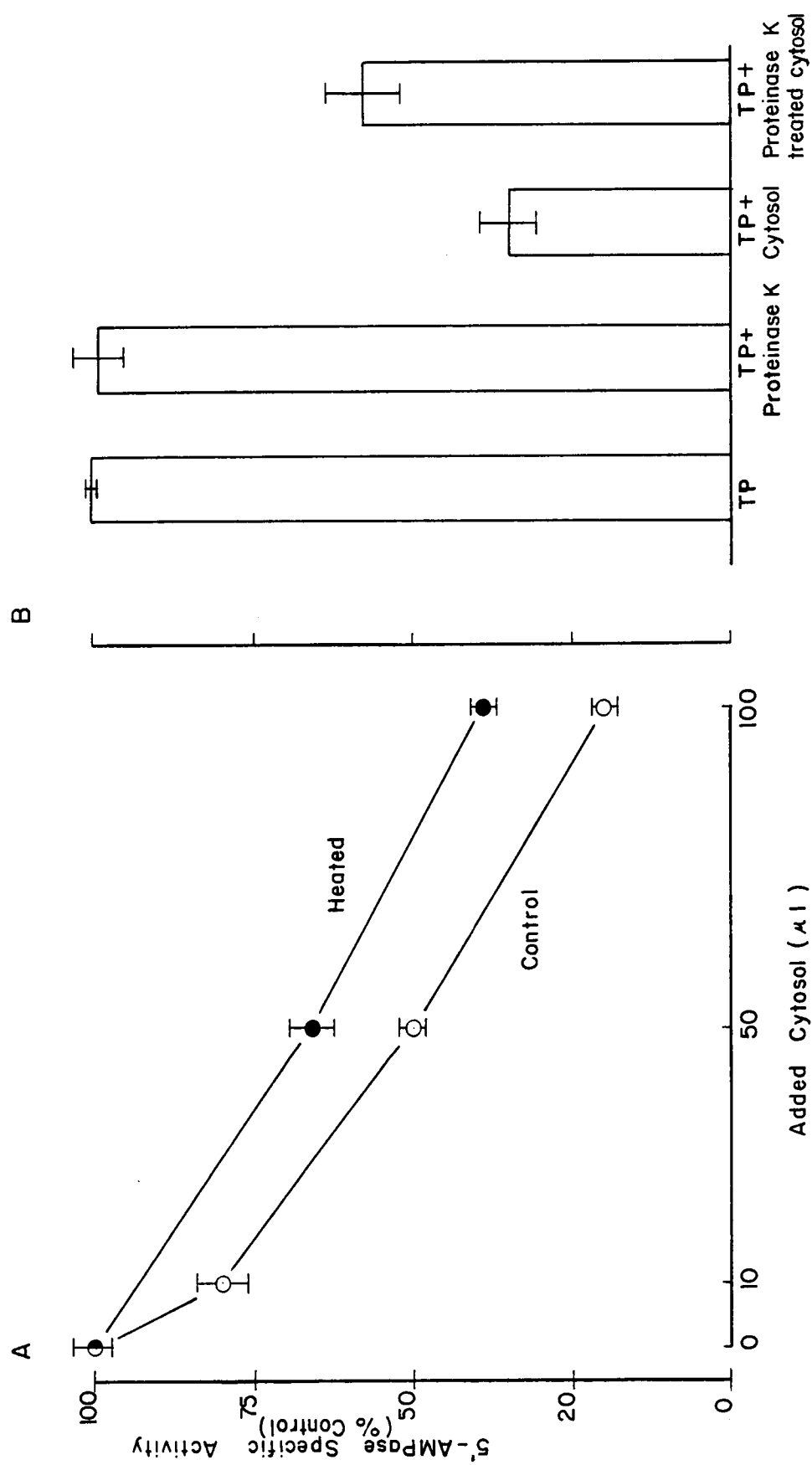


Figure A-1

inhibitory action on 5'-AMPase activity. The 5'-AMPase activity was not itself affected by the proteinase.

Figure A-2 shows the results of an experiment designed to distinguish the biochemical mode of inhibition of 5'-AMPase by the cytosol. When expressed on an Eadie-Hofstee plot, the data are best explained as the result of noncompetitive inhibition, indicated by the nearly parallel slopes of the lines.

Noncompetitive inhibition of many enzymes is observed after the chelation of required cations. 5'-AMPase requires cations since it is inhibited in HeLa cells by EDTA (Brake et al., 1978) and can be reactivated with calcium (Cook et al., 1982). The cytosolic inhibitors in HTC cells are apparently not chelators of divalent cations since 2mM Ca failed to restore the activity of 5'-AMPase (Table A-II).

The inhibitors of 5'-AMPase were studied under different growth conditions in rat HTC cells and two human lines, HSWP (diploid skin fibroblasts) and HeLa (Table A-III). 5'-AMPase activity decreased by greater than 50% in HTC cells that were grown in monolayer culture as compared to the activity in cells maintained in suspension. Their respective cytosols, however, were equally potent in inhibiting this activity. HeLa cells also contain cytosolic inhibitors of 5'-AMPase of approximately the same potency as that of HTC cells. HSWP cells exhibit 5'-AMPase activity which is 15-30 fold higher than that seen in the other two lines. This activity is decreased by about half upon reaching confluency. The cytosol of these cells also contains

Figure A-2. Eadie-Hofstee analysis of the cytosolic inhibition of 5'-AMPase in HTC cells. HTC total particulate was assayed (in duplicate) for 5'-AMP hydrolysis at various substrate ($[S]$) concentrations (10-100 μ M) $\pm$ cytosol. Initial velocity (v) is expressed as adenosine released from AMP per mg protein. The lines were fitted by linear regression and exhibited correlation coefficients (r) as indicated in parentheses.

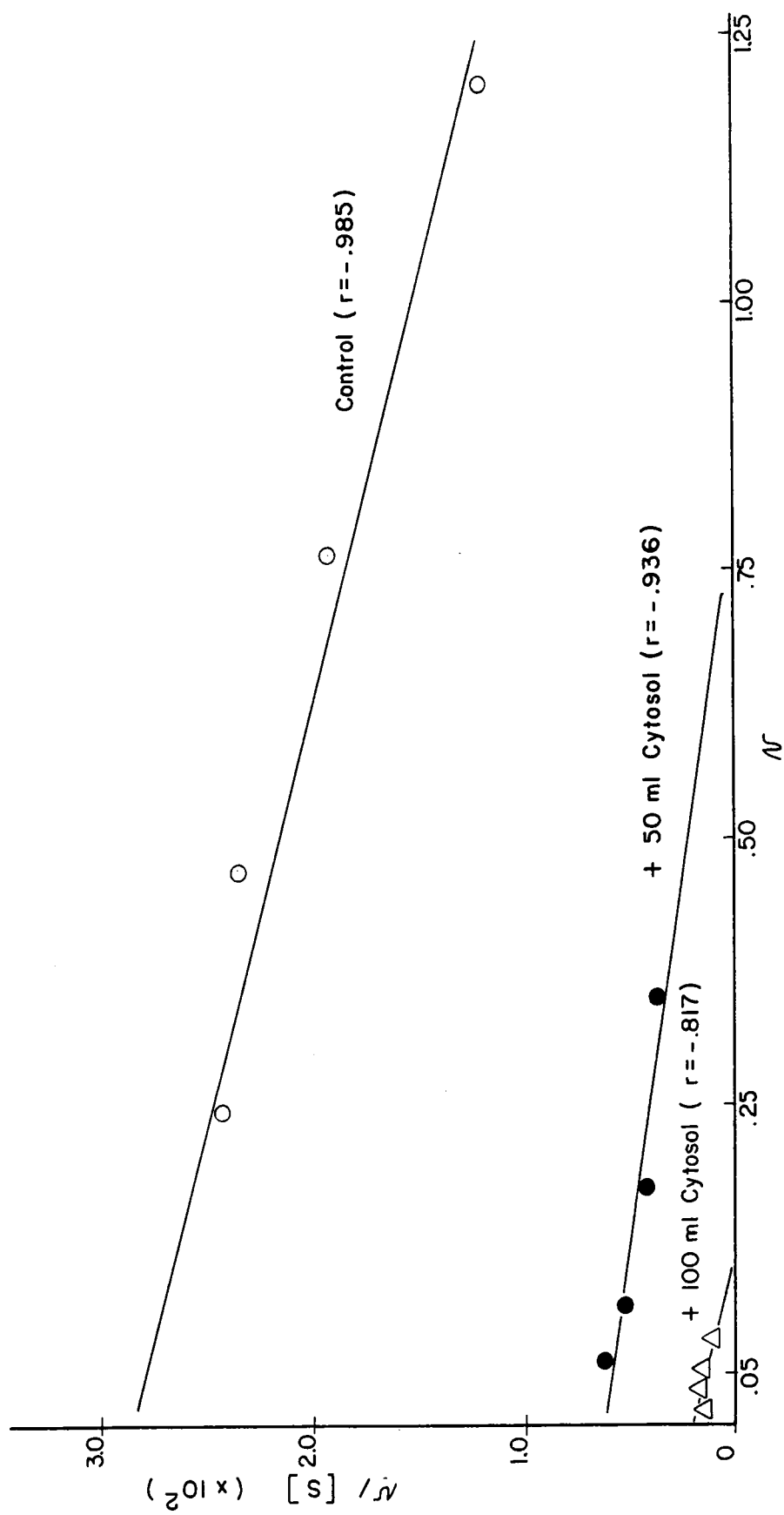


Figure A-2

Table A-II
HTC 5'-AMPase Activity $\pm$ Calcium

	% Control activity	
	-Ca ²⁺	+Ca ²⁺
Total particulate	100	108
Total particulate + 50 μ l cytosol	43	44
50 μ l cytosol	Not detected	Not detected

HTC cell fractions were assayed (in duplicate) as described in Materials and Methods in the presence or absence of 2 mM CaCl₂.

Table A-III
5'-AMPase Activity $\pm$ Cytosol in Various Cell Types

Cell	Growth condition	Cytosol in assay μ l	Specific activity μ mol adenosine $\cdot$ mg protein ⁻¹ $\cdot$ h ⁻¹	% Control
HTC	Suspension	0	1.22	100
		100	0.24	20
	Attached	0	0.53	100
		100	0.15	29
HeLa	Suspension	0	1.46	100
		100	0.57	39
HSWP	Log growth	0	17.8	100
		100	11.5	65
	Confluent	0	9.87	100
		100	7.20	73

Total particulate fractions were prepared from each of the cell lines listed above and assayed $\pm$ their respective cytosol for 5'-AMPase activity as described in Materials and Methods.

inhibitors of 5'-AMPase, although they are not as evident as in the other cell lines tested, and this inhibition is not a function (on a percentage basis) of the cells' growth state.

Discussion

Tweto and Doyle (1976) reported that HTC cells contain 5'-nucleotidase activity that co-purifies with cell surface proteins labeled by lactoperoxidase-catalyzed iodination from Na^{125}I . However, Lopez-Saura et al. (1978) have not found 5'-nucleotidase activity associated with HTC cells. I have established that HTC cells contain 5'-nucleotidase (assayed here as 5'-AMPase) by a number of criteria. First, when the AMP concentration in the assay is doubled (from $100\mu\text{M}$ to $200\mu\text{M}$), an increase in the hydrolysis of the AMP is observed. Had the activity been due to a nonspecific phosphatase this change in AMP concentration would have been negligible compared to the large excess of p-nitrophenylphosphate that is also present. In this situation, however, doubling the $[\text{}^{14}\text{C}]\text{AMP}$ concentration would have effectively doubled the specific radioactivity of the entire pool of phosphatase substrate, including both AMP and p-nitrophenylphosphate (assuming both could be hydrolyzed equally well by a nonspecific phosphatase). The expected result would be an apparent 2 fold increase in activity. The observed increase was only 32%. Using a K_m value of $76\mu\text{M}$ for AMP (determined from the control data of Figure A-2) one can calculate that increasing the substrate concentration from $100\mu\text{M}$ to $200\mu\text{M}$ (assuming a specificity

for AMP) would lead to an increase in activity of 28%. This value is in agreement with the observed 32% elevation in AMP hydrolysis, evidence for specificity of the enzyme for AMP.

Confirmation of the presence of 5'-AMPase comes from the inhibition observed with ConA or after growth in the glycosylation inhibitor tunicamycin. These data are consistent with reports that in other cells 5'-nucleotidase is a glycoprotein and inhibited by ConA (Stefanovic et al., 1975; Brake et al., 1978). The reason for the discrepancies between this report and the results of Lopez-Saura et al. (1978), who found no 5'-nucleotidase in HTC cells, is not obvious. It is possible that the cells used here differ from those of Lopez-Saura and co-workers due to the genetic instability and divergence which is a property of many transformed cell lines.

The cytosol of HTC cells contains inhibitors of 5'-AMPase. The inhibition is proportional to the amount of cytosol included in the assay and is apparently reversible to some extent since simply pelleting the membranes containing the enzyme led to activity stimulation. The inhibition could be partially relieved by either heat treatment or proteinase digestion of the cytosol prior to its inclusion in the assay. The inhibitory components may therefore be macromolecules, possibly proteins, in agreement with experiments which suggest that the inhibitor is not dialyzable (data not shown).

The noncompetitive mode of inhibition is not expected if it results from a small metabolite such as adenosine or other substrate-related compounds (Brake et al., 1978). 5'-AMPase requires divalent

cations since EDTA reversibly inhibits activity in HeLa cells (Brake et al., 1978). The cytosolic inhibitors are not likely to be simple chelators since their effects could not be reversed by the addition of calcium, a treatment which relieves the EDTA inhibition in HeLa cells. It may be important to point out that calcium acts in HeLa cells by competing for the EDTA, not by substituting for a required cation removed from the enzyme by chelation (Brake et al., 1978). If the cytosolic inhibitors sequester a necessary cofactor then the addition of another metal, such as zinc, may be effective in restoring 5'-AMPase activity.

Cytosolic inhibition of 5'-AMPase is exhibited to varying degrees in all three cell types analyzed, namely rat hepatoma (HTC), human cervical carcinoma (HeLa) and human skin fibroblasts (HSWP). The lower percentage of inhibition observed in HSWP cells may be related to their high enzyme content, as implied by the high specific activity of the homogenate.

Both the exact chemical nature and the physiological role of the soluble inhibitors of 5'-AMPase remain to be resolved. While 5'-AMPase is affected by both cell density (in HSWP) and culture conditions (attached vs. suspension growth of HTC), the percent inhibition by the respective cytosols is essentially unchanged. I am not aware of any previous reports of cytosolic inhibitors of this enzyme despite its common use as a marker for plasma membranes. However, at least one instance of a large discrepancy between the purification of 5'-nucleotidase and that of other surface markers has been reported (Kartner et al., 1977; compare Tables 1 and 2).

The 35 fold purification of 5'-nucleotidase in plasma membranes from human skin fibroblasts in that report conflicts with the modest 6 and 8 fold purification of the Na,K-ATPase and Mg-ATPase, respectively, exhibited by the same fraction. This could be explained by cytosolic inhibitors of 5'-nucleotidase, although the magnitude of such putative inhibition must, necessarily, greatly exceed that observed in any of the cells used in this study.

The presence of inhibitors of 5'-AMPase in the cells analyzed here, and conceivably in others as well, is sufficient cause for concern and care in the quantitative use of this enzyme as a plasma membrane marker.

VITA

Norman J. Karin was born on April 7, 1954, in Dunkirk, New York. He attended Dunkirk High School, graduating in 1972. Norman enrolled at the State University of New York at Fredonia and received a Bachelor of Science degree in biology in 1976. He remained at Fredonia, where he was awarded a Master of Science degree in biology prior to entering The University of Tennessee Graduate School of Biomedical Sciences in 1978.

Norman and his wife Charlotte were married in April of 1981. He is a member of the American Association for the Advancement of Science.