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I am submitting herewith a dissertation written by Lewis R. Pollack entitled "Turnover and Regulation of Na,K-ATPase in HeLa Cells." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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TURNOVER AND REGULATION OF Na,K-ATPase
IN HeLa CELLS

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ABSTRACT

I have investigated the turnover and regulation of the catalytic subunit of Na,K-ATPase in plasma membrane ghosts isolated from logarithmically growing HeLa cells. The Na,K-ATPase was identified by specifically phosphorylating plasma membranes with radioactively labeled ATP, the principal criteria for specificity being Na^+ -dependence and K^+ -sensitivity.

When the plasma membranes were isolated from cells which had been grown in ^{13}C -substituted amino acids, the Na,K-ATPase, specifically labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, sedimented in the sodium dodecyl sulfate (SDS)-containing density gradients more rapidly than control enzyme labeled with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$. Turnover was measured by resuspending ^{13}C -substituted cells in control medium and following the decrease in density of ^{13}C -substituted enzyme as it was replaced by enzyme of control density. A lag period of about 4 to 4.5 h, or 0.16 to 0.2 of the generation time (T_G) was observed, followed by the first-order disappearance of the ^{13}C -substituted enzyme. I attribute the lag to the transit time from the cellular locus of synthesis to the surface membrane. Turnover, corrected for dilution of the ^{13}C -enriched enzyme by net growth, had a half-time of 5.5 h, or $0.24 T_G$, corresponding to a rate of constant of $3.1/T_G$.

In parallel experiments I have measured the turnover of $[^{14}\text{C}]\text{leucine}$ -labeled proteins in both total cell protein and cell-surface protein. Total protein turnover showed no lag and was approximately first-order, with a mean half-time of about 60 h. Plasma membrane

protein showed a pronounced lag, followed by a complex turnover of at least two components. The data support a model of plasma membrane turnover in which the individual proteins are turned over at different rates.

Significant isotope effects were observed when HeLa cells were cultured in ^2H substituted amino acid-containing medium. Rates of growth and of protein synthesis were significantly lower than controls; eventually, the deuterated cells ceased growing and lysed. These effects could be reduced by labeling cells for less than 24 hours, but could not be eliminated. In contrast, cells grew well in medium containing ^{13}C -substituted amino acids; such cells incorporated [^{14}C]leucine into protein at the control rate.

Density shifts obtained for specifically phosphorylated, ^{13}C -labeled Na,K-ATPase were equivalent to or larger than those obtained for ^2H -labeled enzyme, indicating that greater incorporation of ^{13}C -amino acids compensated for the theoretically smaller density shifts expected with ^{13}C -amino acids. The first-order rate constant for removal from the surface ($1.5/T_G$) obtained using ^2H -substituted amino acids gave a half-time for turnover of the Na,K-ATPase ($0.45 T_G$ or 21 h), substantially longer than obtained using ^{13}C -substituted amino acids.

Effects of long-term, subtotal inhibition of Na^+, K^+ transport, by growth of cells in low- $[\text{K}^+]$ medium, are also described for HeLa cells. After long-term growth in low- $[\text{K}^+]$ there was two-fold or greater increase in ouabain binding per cell. The increase was reversible.

To assess the corresponding transport changes for cells grown in low- $[K^+]$ I have separately estimated the contributions of increased intracellular $[Na^+]$ and of transport capacity (number of transport sites) to transport regulation. During induction as well as its reversal, short-term regulation was achieved primarily by changes in $[Na^+]_i$. More slowly, long-term regulation was achieved by changes in the number of functional transporters in the plasma membrane.

Parallel exposure of cryptic Na^+,K^+ -ATPase activity with SDS in the plasma membranes of both induced and control cells, as assayed by specific phosphorylation, showed that the long-term response cannot be accounted for by an exposure of pre-existing Na^+,K^+ -ATPase in the plasma membrane. Analyses of the kinetics of the induction and its reversal and the steady state turnover of the enzyme following induction show that it is caused by a decrease in the rate of degradation of the enzyme.

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CHAPTER I

INTRODUCTION

Turnover, or the simultaneous synthesis and degradation of cellular proteins, is important in the repair of abnormal proteins, in cellular adaption to a changed environment, and in providing metabolic fuel during starvation (for review, see Ballard, 1977). Turnover is particularly relevant to cell surface proteins since such proteins are constantly interacting with the extracellular environment which may inactivate or otherwise perturb them. At present, considerable controversy exists as to whether individual plasma membrane proteins turnover at similar (Tweto and Doyle, 1976; Hubbard and Cohn, 1975) or dissimilar rates (Dehlinger and Schimke, 1971; Kaplan and Moskowitz, 1975; Hudson and Johnson, 1977; Horst and Roberts, 1979).

Turnover studies of the individual plasma membrane proteins are complicated by the difficulty usually associated with their purification. Therefore, indirect methods have frequently been applied. The half-time for disappearance of enzyme activity of several plasma membrane proteins in the presence of inhibitors of translation have been interpreted as measurements of their turnover rates (Edelson and Cohn, 1976). However, these inhibitors may have also decreased degradation (Amenta et al., 1977; Brostrom and Jeffay, 1970; Kenney, 1967; Simpson, 1953). Down regulation or the decrease in concentration of a specific hormone receptor following cellular exposure to the hormone has been observed for many cases (for review, see Tell et al., 1978)

and implies that the receptor has been degraded. However, the receptor's binding activity may have been masked or inactivated rather than the receptor's being removed from the cell membrane and degraded. The rate of ouabain internalization has been used to measure the turnover of the Na,K-ATPase (Will et al., 1977), but it was not clear if the ligand-receptor complex or only the ligand had been removed from the membrane. Similarly, the degradation rate of α -bungarotoxin has been utilized as a measure of the turnover rate of the acetylcholine receptor (Drachman et al., 1978; Merlie et al., 1979). This method is open to criticism since recently α -bungarotoxin itself was shown to alter the degradation rate of the acetylcholine receptor (Gardner and Fambrough, 1979). However, direct measurements have been made of the turnover rates of the asialoglycoprotein receptor (Tanabe et al., 1979), fibronectin (Olden et al., 1978), H-2 alloantigens (Vitetto and Uhr, 1974) and the Na,K-ATPase in rat kidney (Lo and Edelman, 1976).

A novel technique for directly measuring turnover rates, which circumvents the complete purification of the receptor, is density labeling with heavy isotopes followed by density gradient centrifugation (Filner and Varner, 1967; Hütterman and Wendlberger, 1976). Turnover is measured by suspending density-labeled cells in control medium and following the decrease in density of the receptor. This reduces the problem of complete purification of the enzyme or receptor to identifying it with specific labels in density gradients. Since the labels are radioactive, only small amounts of material are required, making this method suitable for tissue culture cells. Density labeling was first applied

to cell surface receptors by Fambrough's laboratory (Devreotes and Fambrough, 1976; Devreotes et al., 1977). They observed a half-time of 17 hours for turnover of the acetylcholine receptor in cultured chick myotubules (Gardner and Fambrough, 1979). Recently, the half-time for turnover of the insulin receptor has been determined to be 6.7 hours by this method (Reed and Lane, 1980).

Similar to the difficulty in determining turnover rates for individual plasma membrane proteins is the problem of distinguishing an increased amount of a plasma membrane transport protein from an increase in transport activity with a fixed number of sites. If anything this is harder to demonstrate because neither specific quantitative labels nor adequate biochemical assays exist for most transport proteins. Altered V_{max} for transport has been used as an indication that the amount of a transport protein has changed (Christopher et al., 1976a; Olden et al., 1979; Tramacer et al., 1977; Salter and Cook, 1976; Heaton and Gelehrter, 1977). However, changes in transport activity can also be caused by the exposure of cryptic transporters in the membrane (Quastel et al., 1970) or by changes in phosphorylation (Nilsen-Hamilton and Hamilton, 1979). Inhibitors of transcription or translation are frequently used to detect alterations in the turnover of transporters (Christopher et al., 1976b; Kletzien and Perdue, 1975; Quastel et al., 1970; Martineau et al., 1972). However as described above for plasma membrane proteins in general, possible effects on degradation permit criticism of this approach.

Despite difficulties in studying the regulation of transport proteins, such studies can provide worthwhile basic information and are also significant by virtue of their relationship to cellular proliferation and transformation. Transport of glucose and its non-metabolizable analogs, 2-deoxyglucose and 3-O-methylglucose as well as mannose, galactose and glucosamine increase with transformation (for review, see Hatanaka, 1974). Transformed cells have higher transport activities for phosphate (Cunningham and Pardee, 1969) and for certain amino acids and amino acid analogs such as glutamine (Foster and Pardee, 1969), α -aminoisobutyric acid and cycloleucine (Isselbacher, 1972; Foster and Pardee, 1969).

The subject of this dissertation is the turnover and regulation of a specific plasma membrane transport protein, the Na,K-ATPase. This transport enzyme maintains the cell with high intracellular concentrations of potassium and low intracellular concentrations of sodium, the reverse of the relative concentrations of these ions in the extracellular fluids (for reviews on Na,K-ATPase, see Schwartz et al., 1975; Robinson and Flashner, 1979; Sweadner and Goldin, 1980). The pumping of sodium out of the cell and potassium into the cell requires metabolic energy, which is supplied by ATP (Glynn, 1968).

In 1957 Skou reported an ATPase activity in crab nerves that was stimulated simultaneously by sodium and potassium. Subsequent studies in red blood cells revealed that the concentration dependencies for activation of this enzyme activity by sodium and potassium agreed with their concentration dependencies for stimulation of active cation transport (for review see Post, 1974). Furthermore cardiac glycosides

inhibited Na,K-ATPase activity and active cation transport at similar concentrations (Post et al., 1960). These properties of the Na,K-ATPase strongly suggested that it was the cation transporter.

Several tissues contain very large amounts of Na,K-ATPase, in contrast with most of the other transporters in the plasma membrane. This has facilitated its purification from kidney (Jørgensen et al., 1971), brain (Nakao et al., 1974), electric organ (Dixon and Hokin, 1974), duck salt gland (Hopkins et al., 1976), dogfish rectal gland (Hokin et al., 1973), and brine shrimp (Peterson et al., 1978), inter alia. In all cases, the enzyme has been found to contain two subunits. The larger subunit, α , has a molecular weight of approximately 95,000 daltons. It contains the active site for ATP hydrolysis. The smaller subunit, β , has a molecular weight of approximately 40,000 daltons and is a glycoprotein. The biochemical function of the β subunit is not known. Current evidence indicates that the structure of the enzyme is probably $\beta\alpha\alpha\beta$ (Glynn and Karlish, 1975; Wallick et al., 1979), however other models are under consideration (Hastings and Reynolds, 1979; Hanson et al., 1979). When the purified enzyme was inserted into liposomes, it actively transported cations (Hilden and Hokin, 1975). This provided convincing evidence that the Na,K-ATPase is the cation transporter.

The Na,K-ATPase is an essential transport enzyme. A relationship between membrane cation transport and the control of proliferation of mammalian cells has been established (for review, see Kaplan, 1978). A great deal of evidence has accumulated that is consistent with the hypothesis that the action of cardiac glycosides on the heart is due to

inactivation of its Na,K-ATPase (see Akera, 1977 for review). The enzyme plays a role in the control of cell volume and in the generation of electrical activity in excitable cells (for review, see Baker, 1972). Cation transport is also necessary for protein synthesis (Ledbetter and Lubin, 1977).

Adaptive changes in Na,K-ATPase have been observed in response to hormones (Jørgensen, 1972; Lin and Akera, 1978; Lo et al., 1976; Lo and Edelman, 1976; Ismail-Beigi and Edelman, 1973), cardiac glycosides (Cuff and Lichtman, 1975), changes in osmolarity (Stewart et al., 1976), and potassium starvation (Chapter IV). In addition, altered Na,K-ATPase activity has been observed in a variety of disease states including cystic fibrosis (Breslow et al., 1977; Epstein and Breslow, 1977), uremia (Welt et al., 1964, 1967), hyperthyroidism (Smith and Samuel, 1970), chronic alcoholism (Lindsay, 1974a,b; Knox et al., 1972), hereditary spherocytosis (Wiley, 1969), nonspherocytic hemolytic anemia (Shohet, 1973) and malaria (Dunn, 1968).

That there are two independent quantitative estimations of the amount of Na,K-ATPase provide it with a distinct experimental advantage over other transport proteins. The α subunit can be phosphorylated in the presence of Na^+ , Mg^{+2} , and ATP; K^+ dephosphorylates it (Post et al., 1965). This Na^+ -dependent, K^+ -sensitive phosphorylation is diagnostic for the Na,K-ATPase and can be used as a specific covalent quantitative label. Cardiac glycosides, such as ouabain, also bind to the α subunit (Hegyvary, 1975), although not covalently. They inhibit cation transport activity and Na,K-ATPase activity. Ouabain binding

provides a second quantitative measure of the amount of the enzyme both in membrane preparations (Jørgensen and Skou, 1971) and in whole cells (Baker and Willis, 1970).

The chapters below describe experiments I have performed on the turnover and regulation of Na,K-ATPase in HeLa S3 cells. The purpose of these studies was to measure directly the turnover of the Na,K-ATPase and to determine whether the increased transport activity observed when these cells were cultured in low K^+ medium (see Chapter IV) represented an increase in the amount of this membrane protein.

HeLa S3 cells were used in these experiments. As a tissue culture line these cells can be manipulated in ways that are impossible in whole animal experiments. Since they have been cloned, their use decreases the heterogeneity of responses that can occur among the mixed cell types of a more complex tissue. HeLa cells are a human cell line and bind ouabain tightly. In contrast, in cultured rodent cells, bound ouabain rapidly dissociates from the membrane (Baker, 1976). Plasma membrane ghosts enriched approximately 10-fold in Na,K-ATPase (Chapter II; Constantino-Ceccarini, 1978) are readily prepared from HeLa cells (Atkinson, 1973). Using these preparations, the rate of removal of the Na,K-ATPase from the plasma membrane can be measured (Chapter II).

Chapter II describes experiments in which I have directly measured the rate of removal of the Na,K-ATPase from the plasma membrane (turnover) by the technique of density labeling followed by density gradient centrifugation. The catalytic subunit of the enzyme was

identified in the density gradients by specific phosphorylation of plasma membrane preparations with radioactive ATP. In that chapter I also compare the turnover of the Na,K-ATPase to that of the plasma membrane as a whole. The data show that the Na,K-ATPase turns over rapidly, with a half-time of 5.5 hours and that the individual HeLa cell plasma membrane proteins turn over heterogeneously.

In initial density labeling experiments, medium containing ^2H -substituted amino acids was used because of the large number of deuteriums per amino acid, their high degree of substitution, and their relative low cost. However, HeLa cells grew poorly in this medium. As described in Chapter III, cells cultured in ^2H -substituted amino acids exhibited decreased rates of growth, of protein synthesis, and of Na,K-ATPase turnover. In contrast, ^{13}C -substituted amino acids produced comparable density shifts without these deleterious effects. Deuterium-labeled amino acids have previously been used for density shift experiments (Devreotes et al., 1977; Reed and Lane, 1980). Chapter III indicates that they should be used with caution and support my use of ^{13}C -substituted amino acids in Chapter II.

Chapter IV describes the changes in Na,K-ATPase that occur when HeLa cells are grown in low K^+ medium. The goal of these studies was to differentiate an increase in the amount of Na,K-ATPase from an increase in its activity. Since the activity of the enzyme is stimulated by monovalent cations (for review, see Schwartz et al., 1975), it was essential to differentiate changes in activity caused by alterations in intracellular cation concentrations from changes in the

amount of enzyme. The results indicate that the initial cellular response is an increase in the enzyme's activity and that the long-term response is an increase in the amount of Na,K-ATPase itself. The increase appears to be due to a decrease in its degradation rate.

CHAPTER II

TURNOVER OF Na,K-ATPase IN HeLa CELLS

Introduction

The (Na^+ , K^+)-activated adenosinetriphosphatase (ATP phosphohydrolase, EC 3.6.1.3), or Na,K-ATPase, is located in the plasma membrane of most animal cells, and transports K^+ into the cell while extruding Na^+ . Many studies have described the kinetics of transport and the activity of the Na,K-ATPase and its partial reactions under various conditions (for review see Schwartz et al., 1975). Much less is known about the synthesis and assembly of the enzyme into the plasma membrane, its subsequent removal from the plasma membrane, and its ultimate degradation. A full understanding of the regulation of cation transport will require an examination of these processes and their controls.

HeLa cells grown in suspension have 10^6 Na,K-ATPase molecules per cell, as measured by [^3H]ouabain binding to intact cells (Will et al., 1977). Methods exist for the rapid preparation of clean membrane ghosts from these cells (Atkinson, 1973). Such ghosts are 10-fold enriched in Na,K-ATPase compared with the starting homogenate (Constantino-Ceccarini et al., 1978). The rate of Na,K-ATPase assembly into or removal from the plasma membrane can be explored in these preparations without the interference of contamination by other organelles.

The internalization of [^3H]ouabain bound to HeLa cells has previously been used to measure the rate of removal of the Na,K-ATPase

from the plasma membrane (Will et al., 1977). Ouabain binds tightly but not covalently to the enzyme (Schwartz et al., 1975). It was therefore not clear if the internalization represented that of the drug-receptor complex or only that of the ligand. It was also possible that ouabain, which inhibits the Na,K-ATPase, altered its rate of removal from the plasma membrane.

This chapter describes the direct measurement of the rate of removal (turnover) of the Na,K-ATPase from the plasma membrane of growing HeLa cells. I have determined this by labeling cells with ^{13}C -amino acids and measuring the rate of removal of ^{13}C -labeled Na,K-ATPase from the plasma membrane by a density gradient analysis. The catalytic subunit of the enzyme in the plasma membrane preparation can be specifically and covalently phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{33}\text{P}]\text{ATP}$. This has allowed me to locate the catalytic subunit among the other plasma membrane proteins in the density gradients. The data show that there is a transit time for the Na,K-ATPase, i.e., a delay before newly synthesized enzyme is inserted into the plasma membrane, presumably reflecting intracellular processing. In addition, by comparing the rate of removal of the Na,K-ATPase with the turnover of the plasma membrane proteins as a whole, I show heterogeneity in the turnover of the individual HeLa cell plasma membrane proteins.

Materials and Methods

Materials

Eagle's minimal essential medium with Spinner salts (S-EMEM), fetal calf serum, dialyzed fetal calf serum, and glutamine were obtained

from Grand Island Biological Co., and gentamicin (Schering) from Microbiological Associates. Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and ATP were from Calbiochem. [^3H] ouabain and [^{14}C]leucine were from Schwarz/Mann. $^{32}\text{P}_i$, $^{33}\text{P}_i$, and [$\gamma\text{-}^{32}\text{P}$]ATP were from New England Nuclear. D_2O (98 atom %) was from ICN. ^{13}C -algal amino acid mixtures (80 atom %) were obtained from Merck, Sharp & Dohme. Metrizamide, glyceraldehydephosphate dehydrogenase and 3-phosphoglycerate phosphokinase (both from yeast), glyceraldehyde-3-phosphate, molecular weight standards, NAD, and ADP were from Sigma. Acrylamide and N,N' -methylenebisacrylamide were purchased from Eastman. All other chemicals were reagent grade.

Cell Culture and Media

HeLa S3 cells (Puck et al., 1956) were maintained in suspension in S-EMEM supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 $\mu\text{g/ml}$ gentamicin, 20 mM Hepes (pH 7.4), and 2.5 mM sodium bicarbonate, as previously described (Brake et al., 1978). Cell growth was monitored with a Coulter counter (Model B). In the density-shift experiments, cells were grown in the complete medium, except that the amino acids were replaced by 600 mg/l of ^{13}C -algal amino acid mixture, 10 mg/l tryptophan, 21.8 mg/l cystine, and 35 mg/l isoleucine, and that 4% dialyzed plus 1% whole heat-inactivated fetal bovine serum was used instead of the usual 5% whole serum. The algal amino acid mixtures were dissolved in amino-acid-free S-EMEM (1 g/50 ml) by lowering the pH to 2.8 and heating at 50°C for 5 min.

Plasma Membrane Preparation

HeLa cell plasma membrane ghosts were prepared by the step-gradient method of Atkinson (1973) with the exclusion of iodoacetate from the gradients (Atkinson, 1975). Centrifugation of the step-gradients at $8000 \times g$ was strictly limited to 15 min to prevent the accumulation of contaminating mitochondria on the lower shelves of the gradients. Details of the final preparation are further described in "Results."

Preparation of $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

$[\gamma\text{-}^{33}\text{P}]\text{ATP}$ was prepared by the procedure of Wallace (1964, 1972), with modifications. The reaction mixture contained (per ml): 0.25 to $3.8 \mu\text{mol}$ DL-glyceraldehyde-3-phosphate; $13 \mu\text{mol}$ NAD; $2 \mu\text{mol}$ ADP; 1.0 mCi carrier-free $^{33}\text{P}_i$, $1.6 \mu\text{mol}$ MgSO_4 , $100 \mu\text{mol}$ Hepes (pH 7.3); $10 \mu\text{g}$ glycine; $100 \mu\text{g}$ glyceraldehydephosphate dehydrogenase (EC 1.2.1.12), and $100 \mu\text{g}$ 3-phosphoglycerate phosphokinase (EC 2.7.2.3). After 1 h at 25°C essentially all the $^{33}\text{P}_i$ was converted to ATP under these conditions. The reaction mixture (2 ml) was placed on a DEAE-cellulose column ($0.7 \times 17.5 \text{ cm}$) previously equilibrated with 0.01 M NH_4HCO_3 (pH 8.6). A 110-ml linear gradient ranging from 0.01 to 0.25 M NH_4HCO_3 (pH 8.6) was then passed through the column. $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ was eluted at a concentration of 0.12 to 0.15 M NH_4HCO_3 . The solvent was eliminated by repeated flash evaporation, which was carried out exhaustively since ammonium ion inhibits the specific phosphorylation of the Na,K-ATPase (data not shown, and Post et al., 1965). Using luciferin/luciferase to determine the concentration of ATP (Strehler, 1974), the specific activity of the

product was found to be 0.7 to 7.3 Ci/mmol. The higher specific activities were obtained with the lower concentrations of glyceraldehyde-3-phosphate, which at a pH of 6 or above breaks down to liberate free phosphate and thereby dilute out the $^{33}\text{P}_i$. In some experiments $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was similarly prepared from $^{32}\text{P}_i$; in others $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was obtained commercially from either New England Nuclear (2 to 10 Ci/mmol or 10 to 40 Ci/mmol) or from Amersham (3000 Ci/mmol).

Specific Phosphorylation of the Na,K-ATPase

The reaction mixture contained (final concentrations) 30 mM Tris/HCl, 5 mM MgCl_2 , 0.5 mM EGTA, 100 mM NaCl or 25 mM KCl, $2.5\text{ }\mu\text{M}[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (initially 0.5 to 30 Ci/mmol) or $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (initially 0.7 to 7.3 Ci/mmol); 20 to 3800 μg of purified HeLa cell plasma membranes were added. The reaction was started by addition of the radioactive ATP, and continued for 20 s at 0°C . It was stopped by addition of 10% sodium dodecyl sulfate (SDS) to a final concentration of 2%, and the solution was then warmed to room temperature for 1 to 2 min to facilitate solubilization of the membranes. The phosphorylated membranes were assayed by precipitation with trichloroacetic acid onto filter discs (Mans and Novelli, 1961).

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

I stopped the phosphorylation reaction and solubilized the membranes with 0.25 parts (v/v) of a solution containing 12.5% SDS, 20% glycerol, 10% B-mercaptoethanol (added immediately before use), 30 mM Tris/HCl, and 0.005% bromophenol blue (as the tracking dye) at

pH 7.7. The final concentration of SDS was 2.5%. Proteins were analyzed by SDS-PAGE essentially by the method of Laemmli (1970), except for my use of 0.22 M Tris/borate buffer (pH 8.3). The gels were run for 3 to 4 h at 4°C. Some gels were immediately frozen and divided into 2-mm slices with a Brinkman gel slicer. Each gel slice was incubated overnight with 0.7 ml of 30% H_2O_2 at 55°C. The dissolved gels were allowed to cool to room temperature, mixed with 0.5 ml of water and 10 ml of aqueous counting scintillant (Amersham), and assayed for ^{32}P content. The other gels were stained for protein with Coomassie blue by the method of Fairbanks et al. (1971). Gel densitometry was done with a Gilford spectrophotometer. Cross-linked albumin ($M_r = 66,000$ to 264,000) and a cross-linked hemoglobin ($M_r = 16,000$ to 64,000), both from Sigma, were used as molecular weight standards.

Velocity Sedimentation Analysis of Phosphorylated Intermediate

The phosphorylation reaction was stopped by addition of SDS to 2%, and the solubilized membranes were layered upon continuous 3.7-ml density gradients which contained Tris/citrate (pH 3.0), 0.1% SDS, and either metrizamide or sucrose. Either D_2O or H_2O was used as the solvent. Details of the individual gradients are described in the figure legends. For density-shift experiments, I specifically phosphorylated ^{13}C -substituted membranes with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, mixed them with controls specifically phosphorylated with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$, and applied the mixture to the gradients. The phosphorylated membranes were sedimented at 50,000 rpm (4°C) in an SW 56 rotor by use of a Beckman L-2

$$\text{percent density shift} = \frac{(\underline{M}_D - \underline{M}_C)}{\underline{M}_C} \times 100, \quad (2)$$

where $\underline{M}_D$ = mean migration of ^{13}C -substituted enzyme and $\underline{M}_C$ = mean migration of control enzyme.

Since I measured the acid-precipitable radioactivity only, any radioactive phosphate which dissociated from the enzyme during the centrifugation did not interfere with my sedimentation measurements. Using this method, I obtained low backgrounds even at the top of the gradients, although the entire reaction mixture was layered onto the gradients. Since each peak of phosphorylated enzyme contained 10 to 20 fractions (see "Results"), each of which contributed to the analysis, small changes in density were readily measurable. The method relies only on the mean position of the peaks in the gradient and not on either peak height or peak symmetry. The ^{32}P and ^{33}P are radioactive markers for the density labeled enzymes, and the extent of phosphorylation in the control versus the experimental sample is not a critical factor, nor is the extent of dephosphorylation during centrifugation.

Na,K-ATPase Assay

Na,K-ATPase activity was assayed in tubes containing 2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (25 $\mu\text{Ci}/\text{mmol}$), 100 mM NaCl, 20 mM KCl, 2.1 mM MgCl_2 , 0.1 mM EGTA, 50 mM Tris/HCl (pH 7.5), 50 μg of purified plasma membranes, and with or without 10^{-4} M ouabain. The total volume of the assay mixture was 0.5 ml. Following a 10-min, 37°C preincubation, the reaction was started by addition of the radioactive ATP, and after 20 min at 37°C it was stopped by the addition of 3 ml of 1.67 N H_2SO_4 .

Free $^{32}\text{P}_i$ was separated from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by the method of Ernster et al., (1950). Na,K-ATPase activity was calculated as the difference in the rates of phosphate release in the presence and absence of ouabain, the reaction was linear with time for at least 45 min. Greater than 50% of the activity was ouabain sensitive.

Turnover of Plasma Membrane Proteins and Total Protein

Turnover of plasma membrane proteins and of total cell protein was measured isotopically. Cells were labeled in medium containing 0.2 $\mu\text{Ci/ml}$ [^{14}C] leucine for 48 h and then resuspended in growth medium containing only nonradioactive leucine. At various times after the resuspension, plasma membranes were isolated and the amount of radioactivity per μg plasma membrane protein was determined. At the outset of each membrane isolation procedure, a portion of the homogenate was removed, precipitated on filter discs, and analyzed for radioactivity as described by Mans and Novelli (1961). The rest of the sample was used for protein determination. Data for both the plasma membrane and the whole homogenate were expressed as a percentage of the initial specific radioactivity.

Other Methods

[^3H]ouabain was bound to whole cells as described previously (Cook et al., 1976). 5'-Nucleotidase was assayed as 5'-AMPase by the method of Avruch and Wallach (1971) as modified by Brake et al. (1978). p-Nitrophenyl-N-acetyl- β -D-hexosaminidase was measured by the procedure

of Barrett (1972). Cytochrome c oxidase was assayed by the method of Smith (1955) as modified by Brake et al. (1978). Protein was estimated by solubilization in 4 ml of 0.2% SDS and by determination of the intrinsic fluorescence of tryptophan residues at excitation and emission wavelengths of 286 and 338 nm, respectively (Avruch and Wallach, 1971). Bovine serum albumin dissolved in 0.2% SDS was used as a standard. Refractive indices of solutions were determined with a Bausch & Lomb refractometer.

Results

Identification of the Na,K-ATPase in HeLa Cell Plasma Membrane Preparations

The HeLa cell plasma membrane ghost preparations were enriched approximately 10-fold in both ouabain binding, which is specific for the Na,K-ATPase, and 5'-nucleotidase activity. Of the total activities applied to the gradients, less than 5% of the lysosomal enzyme β -hexosaminidase and the mitochondrial marker cytochrome c oxidase was found in the plasma membrane fraction of the discontinuous gradients used to isolate the ghosts. Morphologically, under phase-contrast microscopy, the final preparation consisted almost entirely of large plasma membrane ghosts, in agreement with previously published micrographs of plasma membranes prepared by this method (Atkinson, 1973; Constantino-Ceccarini et al., 1978). Few contaminating mitochondria or small vesicles were seen.

To identify the Na,K-ATPase in purified HeLa cell plasma membrane preparations, I covalently labeled the enzyme by specifically

phosphorylating it with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ in the presence of Na^+ (100 mM) and Mg^{2+} (5.0 mM) for 20 s at 0°C . When K^+ (25 mM) was included instead of Na^+ , the extent of phosphorylation was much less. This Na^+ -dependent, K^+ -sensitive phosphorylated intermediate is diagnostic of the catalytic subunit of Na,K-ATPase (Post et al., 1965). K^+ -sensitive phosphorylation was more than half the total achieved in the presence of Na^+ : $57.9 \pm 3.4\%$ (S.D., $n = 5$) assayed with a centrifuge method (Post and Sen, 1967), or $52.2 \pm 8.6\%$ (S.D., $n = 12$) with the filter disc method. At saturating concentrations of ATP (2.5 μM), K^+ -sensitive phosphorylation was 4.4 ± 1.4 (S.D., $n = 10$) pmol phosphate bound per mg plasma membrane protein. Compared with purified Na,K-ATPase preparations (Jorgensen, 1974), this amount of K^+ -sensitive phosphorylation is approximately that expected for a preparation with this Na,K-ATPase activity (1 to 3 $\mu\text{mol}/\text{mg}/\text{h}$). Other evidence indicated that I was specifically labeling the Na,K-ATPase. When HeLa cells were grown in low K^+ ($<K_m$ for transport) medium, the increase observed in the amount of phosphorylated intermediate quantitatively corresponded to the increase in $^{86}\text{Rb}^+$ transport and in $[^3\text{H}]$ ouabain binding, other measures of the amount of enzyme (Pollack and Cook, 1978; and Chapter IV).

The phosphorylated intermediate in SDS-PAGE migrated as a single K^+ -sensitive radioactive band with an apparent molecular weight of 93,000 (Figure 1), in good agreement with the molecular weight obtained for the catalytic subunit of the purified enzyme from other sources (Schwartz et al., 1975). Other phosphorylated proteins are spread

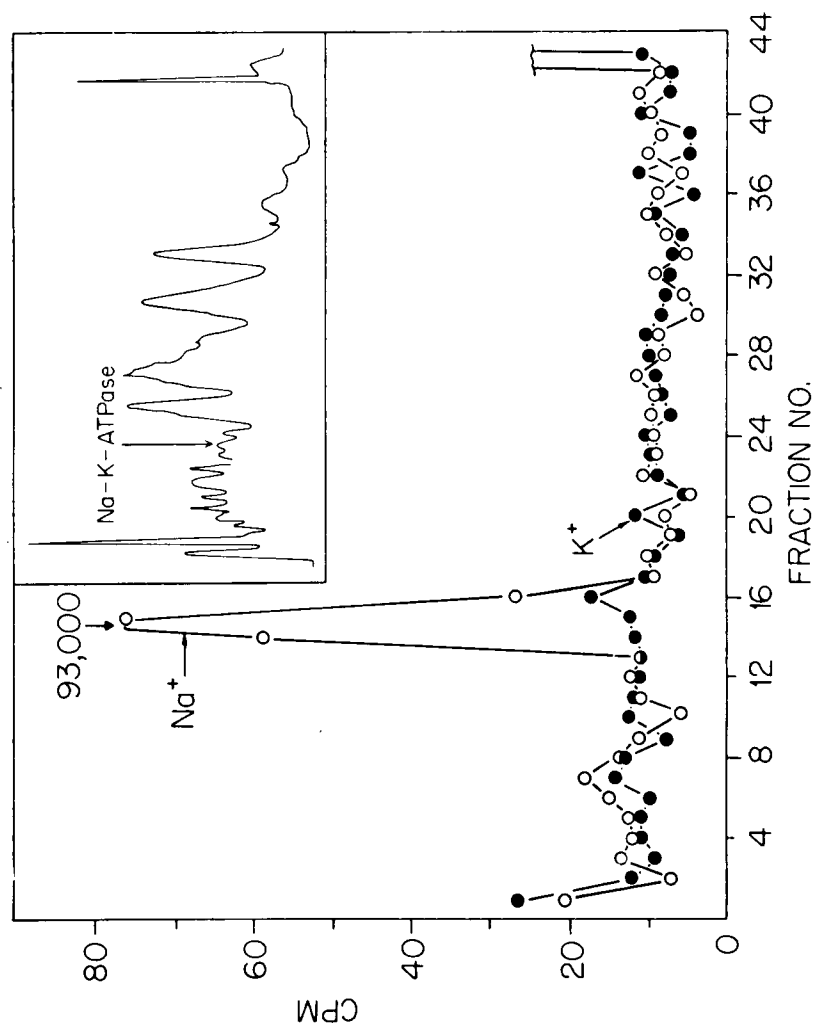


Figure 1. SDS-PAGE of phosphorylated HeLa cell plasma membranes. Purified HeLa cell plasma membranes were phosphorylated with [γ - ^{32} P]ATP (0.5 Ci/mmol), and the membrane proteins were separated by electrophoresis as described in "Experimental Procedures." Na⁺-dependent, K⁺-sensitive phosphorylation of the Na,K-ATPase. The radioactivity is plotted for each slice of the gels containing membranes labeled in the presence of either 100 mM NaCl (O) or 25 mM KCl (●). The Na⁺-dependent, K⁺-sensitive peak is indicated by the arrow. Scan (inset) of a Coomassie blue stained gel of the phosphorylated plasma membrane proteins. The band that corresponds in molecular weight to the Na⁺-dependent, K⁺-sensitive radioactive peak is indicated by the arrow.

throughout the gel at very low activity. The K^+ -sensitive phosphorylation specifically labeled the catalytic subunit of the Na,K-ATPase from among the many other plasma membrane proteins (Figure 1, inset). The Coomassie-blue-stained polypeptide that comigrated with the K^+ -sensitive radioactive peak contained approximately 2.5% of the total membrane protein (determined by SDS-PAGE of membranes from [^{14}C]leucine labeled cells).

In sucrose gradients, the phosphorylated intermediate appeared as a single K^+ -sensitive peak of radioactivity. After centrifugation for 17 h, the catalytic subunit of the enzyme sedimented into the middle of the gradient (Figure 2). Gradients were run at low pH (3.0) and low temperature (4°C) to limit hydrolysis of the aspartylphosphate bond (Forbush and Hoffman, 1979). The decreased height of the K^+ -sensitive peak observed at longer centrifugation times was partly due to this hydrolysis and partly due to diffusion of the enzyme during the run. Even after the long centrifugation times, I could still easily identify the phosphorylated enzyme in the gradients, and therefore I could estimate the sedimentation velocity of the enzyme.

Density Shift and Turnover Experiments

When control membranes were phosphorylated with [γ - ^{32}P]ATP and mixed with identical control membranes which had been specifically phosphorylated with [γ - ^{33}P]ATP, the peaks of the two isotopes sedimented at the same velocity (Figure 3), i.e., there was no detectable differential effect of the radioactive labels.

Labeling cells with ^{13}C -amino acids for 69 h shifted the ^{13}C -substituted enzyme to a higher sedimentation velocity, as shown in

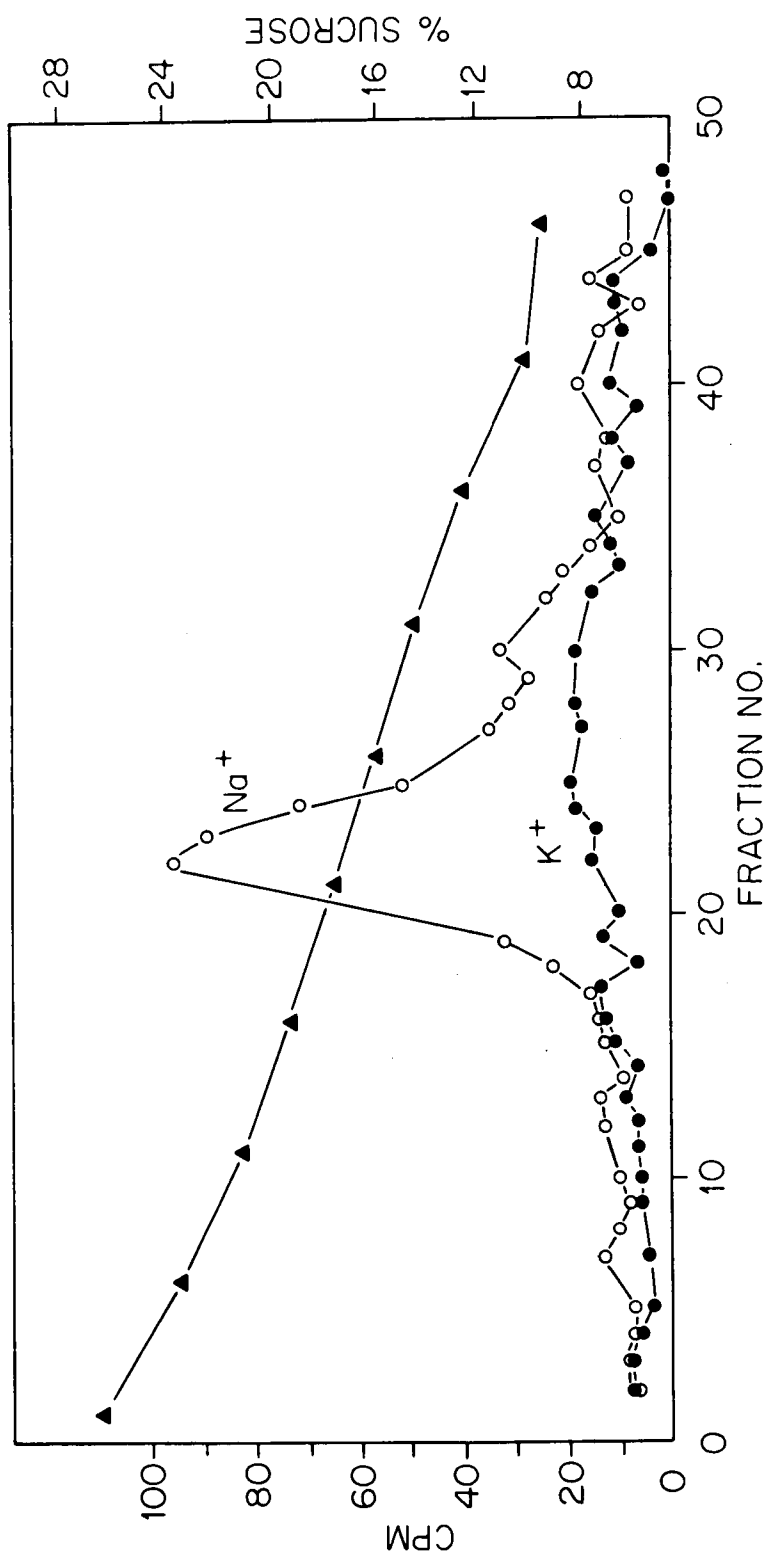


Figure 2. Density gradient centrifugation of phosphorylated HeLa cell plasma membranes. Purified HeLa cell plasma membranes were phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.225 Ci/mmol) and sedimented in linear 10 to 25% sucrose- H_2O -SDS (pH 3.0) gradients for 17 h at 50,000 rpm (SW 56 rotor). The amount of acid-insoluble radioactivity present in each fraction is shown for the gradients containing membranes labeled in either 100 mM NaCl (○) or 25 mM KCl (●). Also shown is the % sucrose in every sixth fraction (▲).

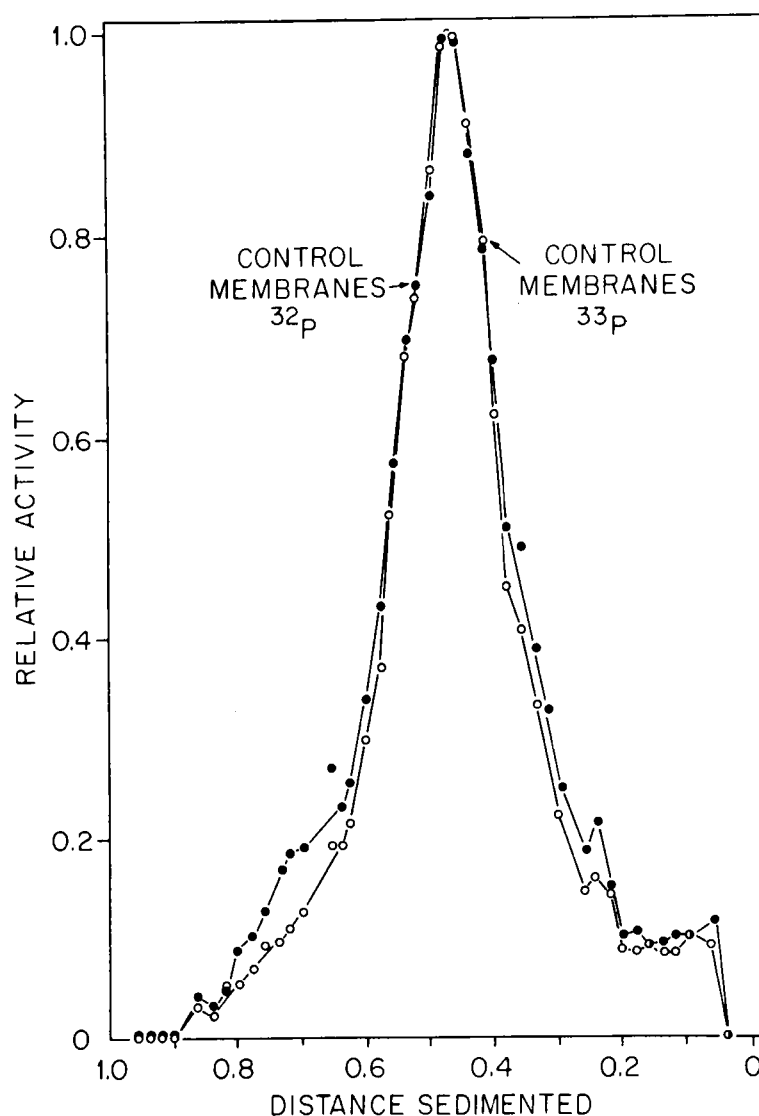


Figure 3. Density gradient centrifugation of control membranes independently labeled with ^{32}P or ^{33}P . 180 μg of purified plasma membranes from control cells were phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3.7 Ci/mmol), mixed with 500 μg of control plasma membranes which had been separately phosphorylated with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (2.5 Ci/mmol), and sedimented in a linear 8 to 23% metrizamide- D_2O -SDS gradient (pH 3.0) for 16 h at 50,000 rpm (SW 56 rotor). Both the ^{32}P - and ^{33}P -labeled membranes were phosphorylated in the presence of 100 mM NaCl (without KCl). The normalized acid-insoluble ^{32}P (●) and ^{33}P (○) radioactivity present in each fraction of the gradient is plotted.

Figure 4. ^{13}C substitution did not alter cellular rates of growth or of protein synthesis as assayed by [^{14}C]leucine incorporation into acid-insoluble material (see Chapter III). Control membranes phosphorylated with [γ - ^{33}P]ATP were included as a marker in the same gradient with the ^{13}C -substituted membranes specifically phosphorylated with [γ - ^{32}P]ATP, since the density shifts produced were small (~5%) and this procedure facilitated their demonstration. I calculated the mean density shift, which I assumed to be proportional to the concentration of ^{13}C -substituted Na,K-ATPase in the plasma membrane, as follows:

$$\text{Mean density shift} = \frac{\underline{S}_D - \underline{S}_C}{\underline{S}_C} \propto \frac{[\underline{E}_D]}{[\underline{E}_{\text{tot}}]}, \quad (3)$$

where $\underline{S}_D$ and $\underline{S}_C$ = sedimentation velocities for density-labeled and control Na,K-ATPase, respectively, and $[\underline{E}_D]$ and $[\underline{E}_{\text{tot}}]$ = concentrations of ^{13}C and total Na,K-ATPase. Metrizamide- D_2O -SDS gradients (Munthe-Kaas and Seglen, 1974) used in this analysis yielded larger separations of density-labeled and control enzyme than did sucrose- D_2O -SDS or sucrose- H_2O -SDS gradients after comparable centrifugation times (Chapter III). As before, specific phosphorylation was K^+ -sensitive.

After resuspension of ^{13}C -substituted cells in growth medium containing normal ^{12}C -amino acids, the mean density observed on the gradients gradually diminished as new membrane was synthesized and as the ^{13}C -substituted enzyme was removed from the membrane (Figure 5). The ^{32}P -labeled peaks were broad and appeared to be composed of two components, heavy and control density Na,K-ATPase molecules, which were

Figure 4. Na^+ -dependent, K^+ -sensitive phosphorylation of ^{13}C -substituted membranes (^{32}P) and control membranes (^{33}P) sedimented in metrizamide- D_2O -SDS. 85 μg of purified plasma membranes from cells labeled for 69 h with ^{13}C -amino acids were specifically phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10.2 Ci/mmol), mixed with 685 μg of controls which had been specifically phosphorylated with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (1.2 Ci/mmol), and centrifuged in a linear 11 to 26% metrizamide- D_2O -SDS gradient for 20 h at 50,000 rpm (SW 56 rotor). Shown are the amounts of acid-insoluble ^{32}P (O, Δ) and ^{33}P ($\bullet$, $\blacktriangle$) radioactivity present in each fraction of the gradient phosphorylated in the presence of either 100 mM NaCl (O, $\bullet$) or 25 mM KCl (Δ , $\blacktriangle$).

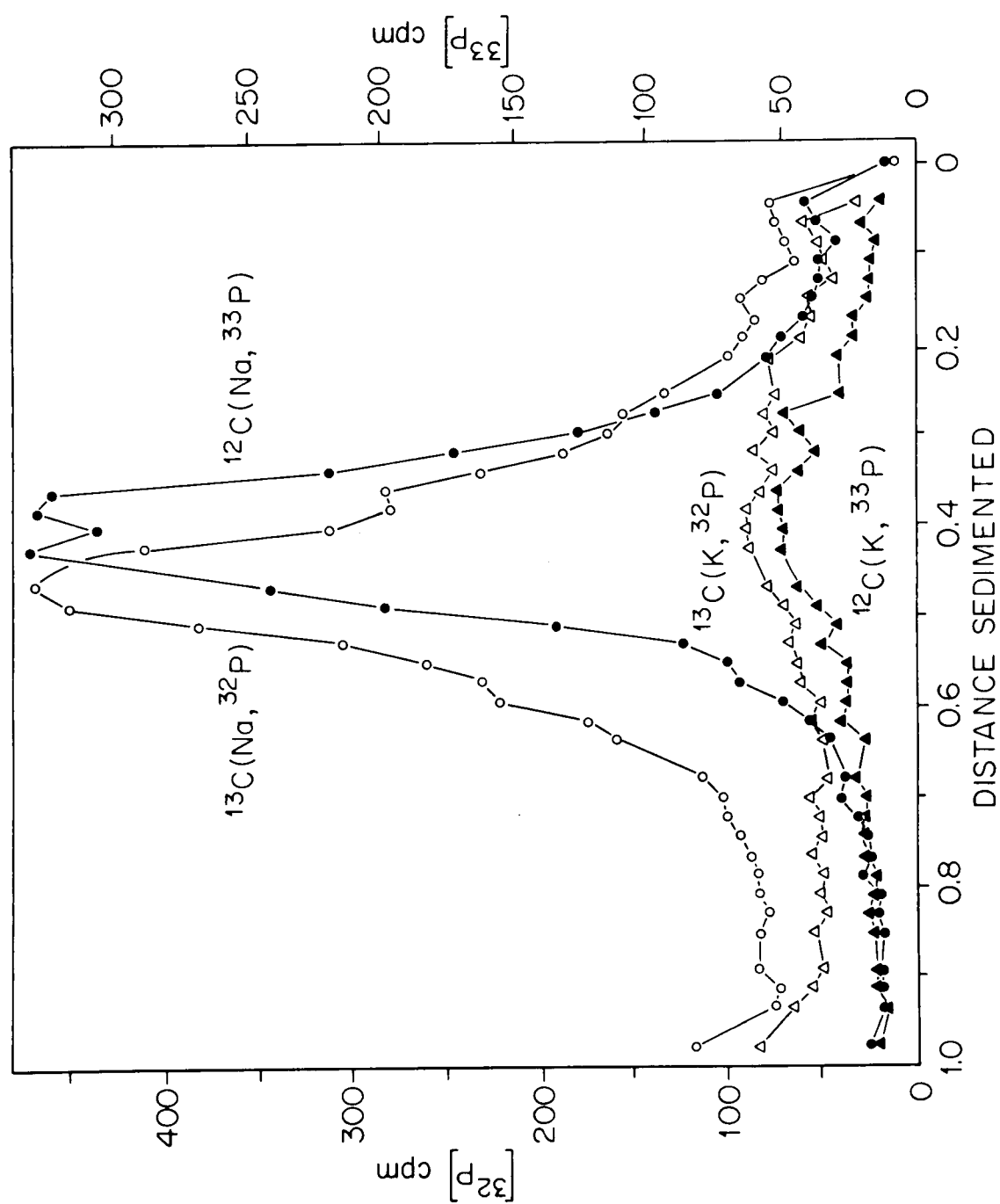


Figure 4.

Figure 5. Density shifts after resuspension of ^{13}C -substituted cells in normal growth medium. Cells labeled for 44 h with ^{13}C -amino acids were resuspended in growth medium containing normal ^{12}C -amino acids. At various times after resuspension, plasma membranes were isolated, phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5.5 Ci/mmol), mixed with control membranes that had been specifically phosphorylated with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (0.9 Ci/mmol), and sedimented in linear 11 to 26% metrizamide- D_2O -SDS gradients for 20 h at 50,000 rpm (SW 56 rotor). Both ^{13}C -substituted and control membranes were phosphorylated in the presence of 100 mM NaCl. About 240 μg of ^{13}C -substituted membranes (^{32}P) and 575 μg of control membranes (^{33}P) were applied to each gradient. Shown are the amounts of acid-insoluble ^{32}P (○) and ^{33}P (●) radioactivity present in each fraction of the gradients containing the ^{13}C -substituted membranes that were isolated at 0 (a), 6 (b), 11 (c), and 23 (d) h after resuspension in ^{12}C -medium.

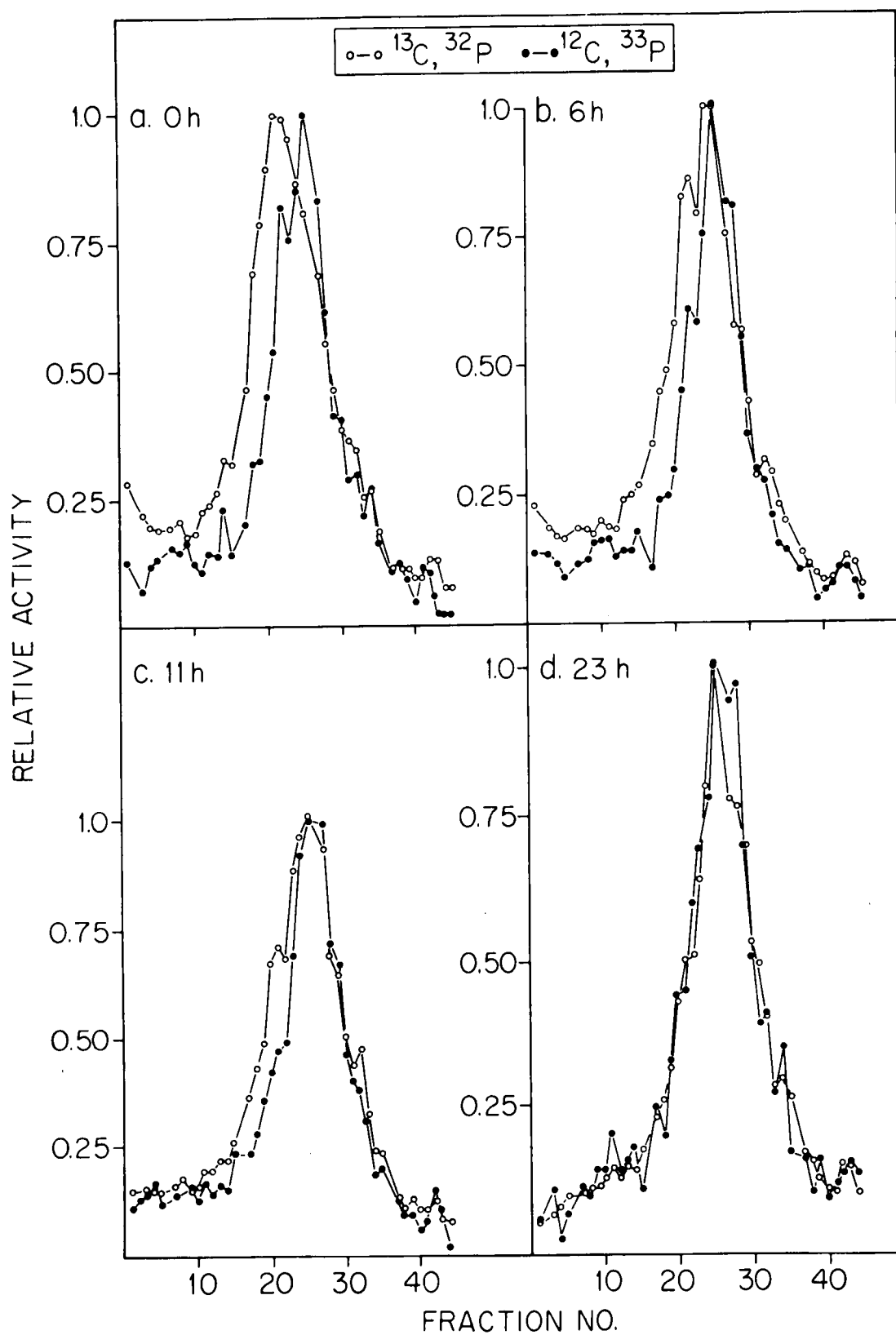


Figure 5.

incompletely separated in the density gradients. At the longer chase times, as the concentration of ^{13}C -substituted Na,K-ATPase molecules in the plasma membrane decreased due to cell growth and turnover of the enzyme, the denser component of the ^{32}P peak decreased in size. I plotted the density shifts observed during the chase as a function of time expressed as a fraction of the generation time (Figure 6). In these experiments, the generation time during the chase was 24 h, identical to that of controls.

There was an initial lag of 0.16 to 0.2 of the generation time (T_G), or 4 to 4.5 h, before the mean density of the enzyme began to decrease. Following this, its density decreased as an apparent first-order process. It is evident from Figure 6 that the density shift decreased at a greater rate than could be accounted for by the 50% dilution of ^{13}C -substituted enzyme per generation due to growth alone. Therefore, ^{13}C -substituted enzyme was being removed from the plasma membrane and presumably was being degraded. I used this linear portion of the curve to calculate the half-time for removal of the enzyme from the plasma membrane ($t_{1/2}$ for turnover) as follows:

$$\frac{[E_D]}{[E_{\text{tot}}]_t} = \frac{[E_D]_0 e^{-kt}}{[E_{\text{tot}}]_0 2^{t/T_G}}, \quad (4)$$

where k is the first-order rate constant for turnover, t is the time in h, and t/T_G is the fraction of the generation time. The numerator expresses the loss of E_D due to turnover, and the denominator the dilution of ^{13}C -substituted enzyme due to growth alone.

Figure 6. Rate of removal of ^{13}C -substituted Na,K-ATPase from the plasma membrane. Shown are the data derived from the experiment presented in Figure 5 (■) and from a separate experiment (●) in which cells were labeled with ^{13}C -amino acids for 66 h before resuspension in growth medium containing normal amino acids (chase). In this latter experiment, at various times during the chase plasma membranes were isolated, phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10.2 Ci/mmol), mixed with control membranes that had been specifically phosphorylated with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (7.0 Ci/mmol), and sedimented in linear 11 to 26% metrizamide- D_2O -SDS gradients for 20 h at 50,000 rpm (SW 56 rotor). As before, both ^{13}C -substituted and control membranes were phosphorylated in the presence of 100 mM NaCl. About 125 μg of ^{13}C -substituted membranes and 330 μg of control membranes were applied to each gradient. The percentage density shift for each gradient was determined for both experiments as described in "Experimental Procedures" and Equation 3. The density shifts for the experiment presented in Figure 5 were normalized to the initial density shift in the latter experiment. The logs of the observed density shifts and a line with the slope of the change in the density shift expected due to growth alone (-----) are plotted as fractions of the generation time (t/T_G) during the chase, which was 24 h in both experiments.

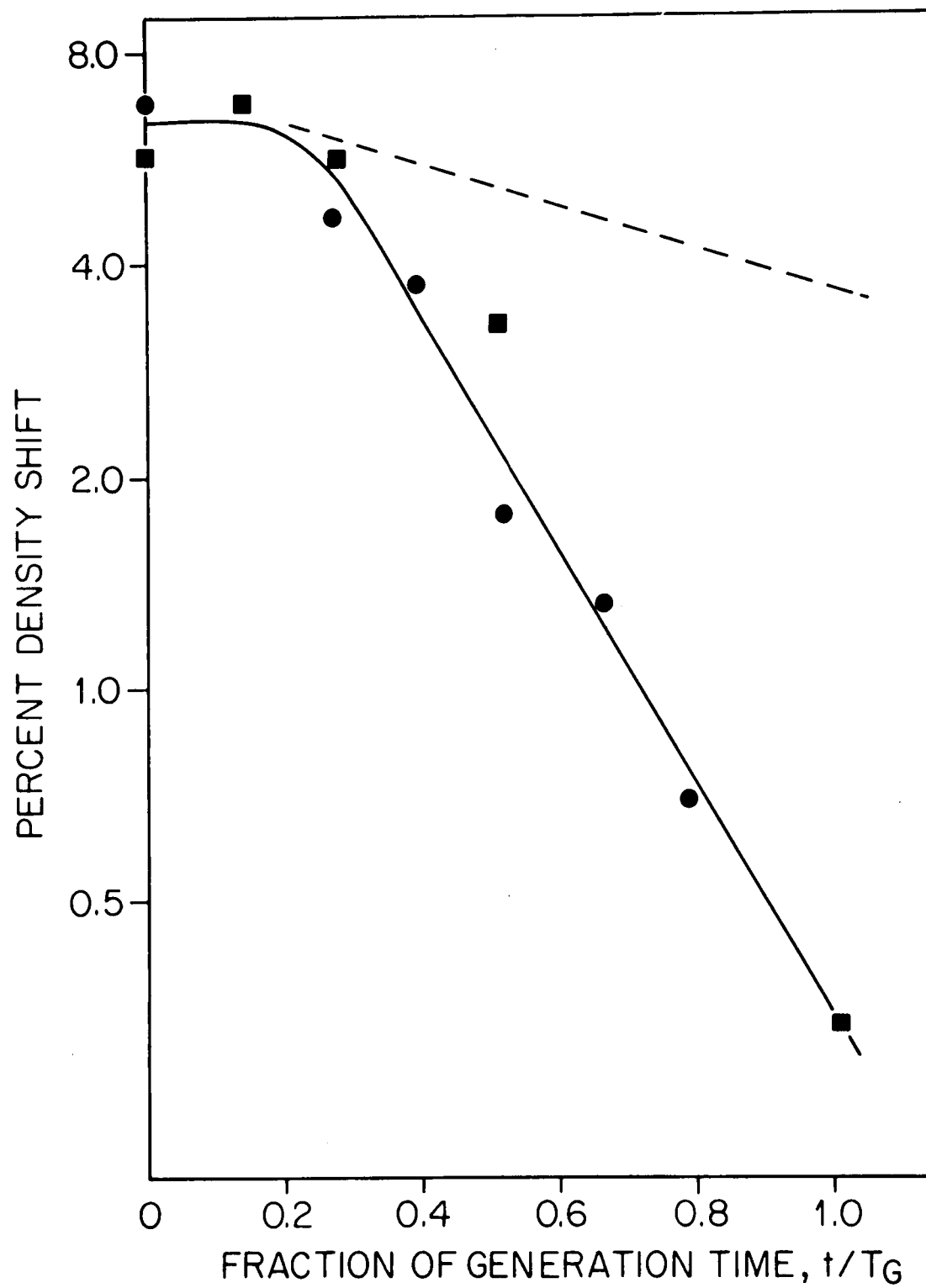


Figure 6.

Therefore:

$$\ln \left(\frac{[E_D]}{[E_{tot}]_t} \right) = \ln \left(\frac{[E_D]}{[E_{tot}]_0} \right) - (kT_G + \ln 2) \underline{t}/T_G, \quad (5)$$

and

$$\underline{m} = -(kT_G + \ln 2), \quad (6)$$

where $\underline{m}$ is the slope of the plot of log density shift versus time as a fraction of T_G (Figure 6). From Equation 6 $\underline{k}$ is obtained; finally $\underline{t}_{1/2}$ is obtained from the relationship:

$$\underline{t}_{1/2} = \frac{0.693}{\underline{k}}. \quad (7)$$

From the linear decrease in Figure 6, I calculated a $\underline{k}$ of $3.1/T_G$ and $\underline{t}_{1/2}$ of 5.5 h for turnover. Since the generation time may be shorter than the doubling time, which was used to calculate the $\underline{t}_{1/2}$, this value may be a slight overestimation.

Turnover of HeLa Cell Plasma Membrane Protein and Total Cell Protein

I compared the turnover of the Na,K-ATPase with that of the plasma membrane proteins as a whole and that of total cell protein by labeling cells with [^{14} C] leucine for 48 h and then, at various times after they were resuspended in normal nonradioactive growth medium, measuring the radioactivity remaining in plasma protein and in acid-insoluble material from the homogenate. As shown in Figure 7, the radioactivity in the homogenate began to decrease immediately. For total protein the decrease in radioactivity was essentially linear, with a half-time of 19 h. Since the cells were dividing with a generation time of 27.5 h, from Equation 6, this corresponded to a mean half-time for turnover of

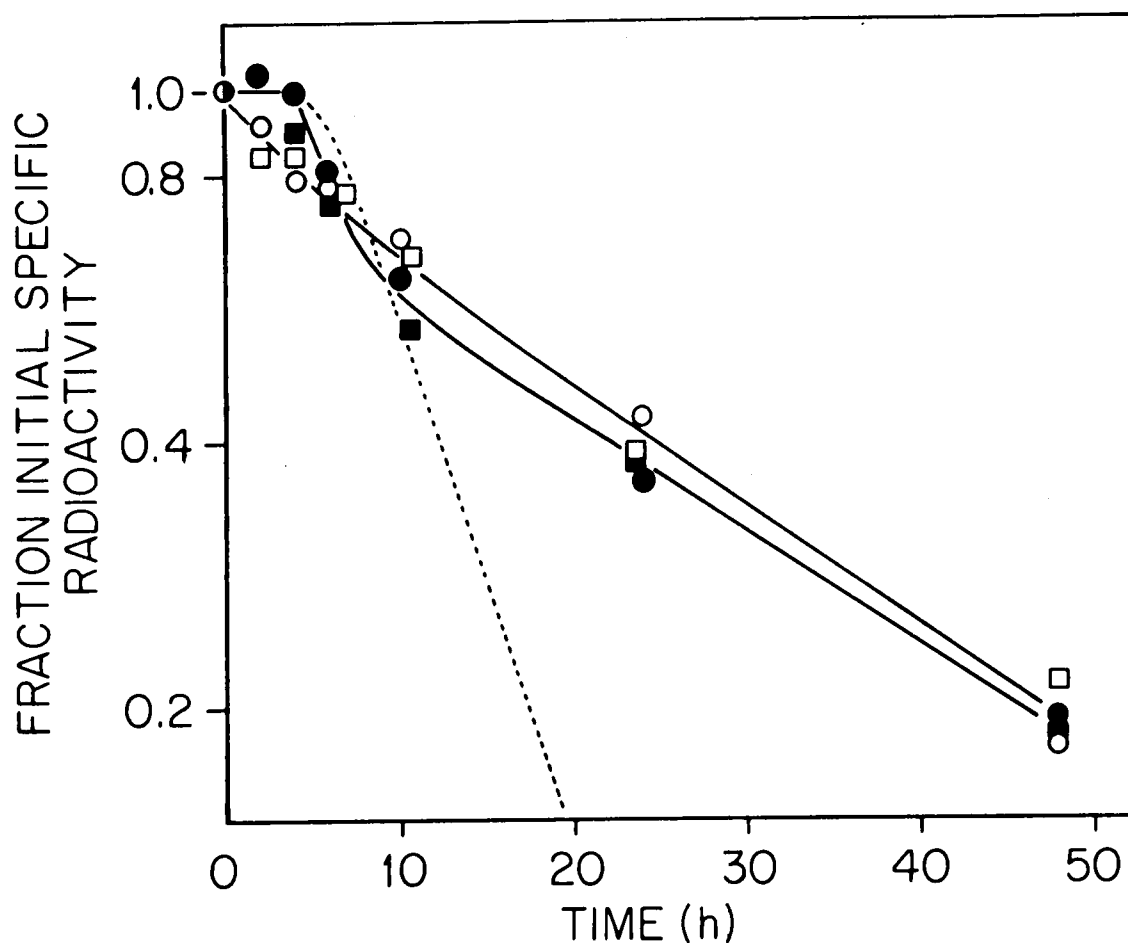


Figure 7. Turnover and dilution by growth of total HeLa cell proteins and plasma membrane proteins. Cells labeled for 48 h in growth medium containing 0.2 $\mu\text{Ci/ml}$ [^{14}C] leucine were resuspended in growth medium containing only nonradioactive leucine. At various times after resuspension, plasma membranes were isolated and the specific radioactivities of the total homogenate (○, □) and of the plasma membranes (●, ■) were determined as described in "Experimental Procedure." The data from two separate experiments (circles and squares) are shown. The dotted line shows the corresponding rate of removal of ^{13}C -substituted Na,K-ATPase from the plasma membrane taken from Figure 6.

about 60 h for total proteins. In contrast, there was a 3- to 4-h delay before the radioactivity in the plasma membranes decreased, indicative of a transit time for many of the plasma membrane proteins. Following the transit time, the radioactivity in the plasma membrane appeared to decrease in two phases. After subtracting out the slowly turning over phase, one-quarter to one-third of the proteins turned over very rapidly, with a mean half-time of 2 to 3 h. The rest turned over very slowly, with a calculated half-time of greater than 70 h after allowance was made for dilution of radioactivity by cell growth (Equation 6).

Discussion

The principal conclusions from this study are that (a) catalytic subunits of the Na,K-ATPase are removed from the membrane of logarithmically growing HeLa cells with a rate constant of $3.1/T_G$ and a $t_{1/2}$ of 5.5 h; (b) after cells are transferred from labeling to nonlabeling medium there is a lag of about 0.2 generation before the label starts to decrease in the Na,K-ATPase and in the plasma membrane, but not in the total homogenate; (c) there is heterogeneity in the turnover rate of the individual HeLa cell plasma membrane proteins.

The numerous small vesicles that were present in the low-speed supernatant or on the upper shelf of the discontinuous gradients used in the preparation of the ghosts were essentially absent in the plasma membrane preparation I used. This observation, along with the biochemical data on distribution of various organelle marker enzyme activities, indicated that my plasma membrane preparations were suitable for measuring the removal of the Na,K-ATPase from the plasma membrane

only, independently of any internal compartments containing Na,K-ATPase molecules "in transit" to the plasma membrane or internalized prior to their degradation.

From the quantity of protein in the band which contained Na,K-ATPase on SDS-PAGE, from the amount of K^+ -sensitive phosphorylation observed, and with the assumption that the functional enzyme contains two catalytic subunits (Hastings and Reynolds, 1979) of which only one is phosphorylated (Forbush and Hoffman, 1979), I calculate that this band is at least 25% Na,K-ATPase and that Na,K-ATPase is only about 1% of the total membrane proteins. Using the density gradient analysis of specifically phosphorylated, density-labeled Na,K-ATPase, I have circumvented the complete purification of the enzyme. I solubilized the phosphorylated enzyme in 2% SDS to dissociate it from lipids and other proteins in the plasma membrane. Metrizamide- D_2O -SDS gradients were used because their high density and low viscosity enabled me to obtain a separation of heavy and control Na,K-ATPase molecules with relatively short centrifugation times, thereby limiting hydrolysis of the protein-phosphate bond, presumably an aspartylphosphate (Post and Kume, 1973). The results show that in addition to their use in equilibrium sedimentation (Devreotes & Fambrough, 1976; Hüttermann & Guntermann, 1975), metrizamide- D_2O gradients can be a valuable tool for velocity sedimentation analyses.

The lag before the ^{13}C -substituted enzyme's mean density decreases (Figure 6, p. 31), after resuspension of density-labeled cells in control growth medium, occurs while normal growth and presumably normal turnover are continuing; there is no growth lag on resuspension. I interpret this

as indicating that there is a significant delay before a newly synthesized Na,K-ATPase is inserted into the plasma membrane. The existence of a transit time for the catalytic subunit of the enzyme, which was previously thought to be nonglycosylated (Kyte, 1972; Giotta, 1976), was not expected since Atkinson (1975) has noted that only the glycosylated HeLa cell plasma membrane proteins exhibit transit times. However, a low level of carbohydrate has very recently been observed in the catalytic subunit of the Na,K-ATPase isolated from the electroplax of Electrophorus electricus (Churchill et al., 1979). More study will be needed to determine if the catalytic subunit is synthesized on membrane-bound ribosomes like the glycosylated vesicular stomatitis virus G protein, which also exhibits a transit time before it is assembled into the HeLa cell plasma membrane (Atkinson, 1978).

Churchill and Hokin (1979) have recently investigated the incorporation of $\text{L-[3,4-}^3\text{H] valine}$ into the large and small subunits of the Na,K-ATPase purified from Electrophorus. They observed a 2.5- to 3-h delay in incorporation into both subunits in vitro. These results are in agreement with my data. However, it was not clear if the delay they observed represented a delay in the assembly of the enzyme into the plasma membrane, since they purified the enzyme from an $18,000 \times g$, 35-min pellet of their homogenate, and the pellet was not further characterized with respect to possible contamination by endoplasmic reticulum, Golgi, or other organelles. Whether or not the intracellular Na,K-ATPase molecules in transit are active is in any case unclear. Addis et al. (1979) have recently reported that the Na,K-ATPase is less

active in the Golgi than in the plasma membrane. Peterson et al. (1978) observed a doublet in SDS-PAGE for the brine shrimp Na,K-ATPase and suggested that this may represent a product-precursor relationship. Both bands underwent Na^+ -dependent, K^+ -sensitive phosphorylation from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, which suggests that the intracellular enzyme may be active.

The rate of removal of the enzyme from the HeLa plasma membrane is very similar to the rate at which ouabain is internalized ($t_{1/2} = 5$ to 8 h) (Will et al., 1977) and sequestered in lysosomes (Cook and Brake, 1978; Tate and Cook, submitted). The quantitative agreement between the two measurements of the rate of Na,K-ATPase removal from the plasma membrane is an independent confirmation of each, and also indicates that inhibition of the Na,K-ATPase by ouabain does not markedly alter the turnover of the enzyme. Similarly, α -bungarotoxin, the potent, essentially irreversible cholinergic blocking agent, has only minor effects on the median lifetime of the acetylcholine receptor, increasing it 1.3-fold in chick myogenic cells (Gardner and Fambrough, 1979).

With respect to turnover of other individual surface proteins, the rate of removal of the Na,K-ATPase from the HeLa cell surface is somewhat similar to the 11-h half-time measured for the α -bungarotoxin receptor in chick sympathetic neurons (Carbonetto and Fambrough, 1979), the 17-h half-time for turnover of the acetylcholine receptor in chick myotubes (Gardner and Fambrough, 1979) or in calf myotubes (Merlie et al., 1978), and the 14-h half-time for 5'-nucleotidase activity in macrophages (Edelson and Cohn, 1976). These half-times are much less than the 120-h

half-time for turnover of the kidney Na,K-ATPase (Lo and Edelman, 1976), the 88-h half-time for the rat liver asialoglycoprotein receptor (Tanabe et al., 1979), or the turnover of the H-2 alloantigens in murine splenocytes (Vitetto and Uhr, 1974).

There was a significant transit time for the plasma membrane proteins in general as compared with total protein, in which the specific radioactivity decayed without a lag. One-quarter to one-third of the [^{14}C]leucine radioactivity in plasma membrane proteins turned over very rapidly, with a half-time of 2 to 3 h. The rest turned over very slowly, with a half-time greater than 70 h. Since reutilization of [^{14}C]leucine is possible, these half-times are maximum values. However, the multiphasic pattern of plasma membrane turnover I have observed (Figure 7) cannot be explained by reutilization of isotope. My results agree with turnover studies in which iodination was used to label plasma membrane proteins in L-cells (Hubbard and Cohn, 1975), and HeLa cells (Huang et al., 1973). Biphasic kinetics can be explained by the turnover of all the plasma membrane proteins as a unit with biphasic kinetics or by the rapid turnover of some of the proteins and the slower turnover of the rest. The radioisotopic turnover data alone do not distinguish between these alternatives or a combination of them. However, there was no evidence of biphasic turnover for the Na,K-ATPase even after a 24-h chase, at which time greater than 90% of the density-labeled enzyme had been removed from the plasma membrane. Therefore, the former alternative, that the plasma membrane proteins are turned over as a unit, appears unlikely. These results differ from the half-time

for turnover of rapidly labeled HeLa cell plasma membrane proteins ($0.5 T_G$) measured isotopically by a double-label SDS-PAGE analysis (Cook et al., 1976). The measurement did not include the turnover of membrane proteins with significant transit times, such as I have now shown for the Na,K-ATPase and a significant fraction of HeLa cell plasma membrane proteins, and others have shown for various plasma membrane and viral glycoproteins (Atkinson, 1978; Eisenman and Vogt, 1978; and Kaplan et al., 1979).

The half-time for the removal of the Na,K-ATPase from the plasma membrane was similar to the half-time for the decay of the specific radioactivity of the rapidly turning over plasma membrane proteins. However, this group of proteins may be composed of a number of proteins which are turning over with somewhat different rates. The Na,K-ATPase is only about 1% of the membrane protein, and it is difficult to determine whether such a minor ^{14}C -labeled component of the plasma membrane is turning over with a half-time identical to that which was measured for the Na,K-ATPase by density gradient analysis. However, it is certain that the Na,K-ATPase is removed from the plasma membrane more rapidly than the slowly turning over majority of the plasma membrane proteins.

When the proteins from the ^{13}C -substituted plasma membrane preparations isolated for the Na,K-ATPase turnover experiments were analyzed for ^{13}C content by mass spectroscopy, it was again observed that the majority of the plasma membrane protein was turning over slowly, with a half-life of at least 60 h (data not shown). These results, plus the

controls showing an absence of ^{13}C -isotope effects, indicate that ^{13}C -substitution does not affect Na,K-ATPase turnover; they also independently support the radioisotopic turnover measurements.

Some laboratories have observed that the plasma membrane proteins turn over at different rates (Kaplan and Moskowitz, 1975; Schmidt-Ullrich et al., 1974; Horst and Roberts, 1979; and Hudson and Johnson, 1977); however, others have measured similar degradation rates for the individual proteins (Tweto and Doyle, 1976). By comparing the turnover rate of the Na,K-ATPase with that of the plasma membrane as a whole, I have obtained strong support for heterogeneous turnover rates for the individual plasma membrane proteins. In addition, the techniques described here are applicable to studies of the turnover of the Na,K-ATPase under various physiological conditions (see Chapter IV).

CHAPTER III

COMPARISON OF THE USE OF ^2H -SUBSTITUTED AMINO ACIDS WITH ^{13}C -SUBSTITUTED AMINO ACIDS FOR DENSITY LABELING OF HeLa CELL Na,K-ATPase

Introduction

With the use of density labels, the synthesis and degradation of individual proteins can be studied without their prior purification (for review, see Hutterman and Wendlberger, 1976). This advantage can be particularly valuable in studies of specific plasma membrane proteins (Devreotes et al., 1977; Gardner and Fambrough; 1979; Reed and Lane, 1980; Chapter II), which are commonly difficult to purify. Using a density gradient analysis of specifically phosphorylated, density-labeled Na,K-ATPase, I have investigated the turnover of the catalytic subunit of Na,K-ATPase in logarithmically growing HeLa cells (Chapter II). Initially I labeled the enzyme with deuterated amino acids because of the large number of hydrogen atoms per amino acid, high degree of substitution, and relatively low cost. However, it became apparent that HeLa cells grew poorly in ^2H -substituted amino acids; therefore, ^{13}C -substituted amino acids, in which the cells grew well, were used in the analysis. This chapter describes the detrimental effects of culturing HeLa cells in deuterated amino acids on their growth rate, incorporation of amino acids into total protein and into Na,K-ATPase, and on Na,K-ATPase turnover. In contrast, cells labeled with ^{13}C -amino

acids exhibit satisfactory rates of growth and protein synthesis and contain more density label in their Na,K-ATPase than do cells labeled with deuterated amino acids.

Materials and Methods

Materials

Eagle's minimal essential medium with Spinner salts (S-EMEM), fetal calf serum, dialyzed fetal calf serum, and glutamine were obtained from Grand Island Biological Co., and gentamicin (Schering) from Microbiological Associates. Hepes and ATP were from Calbiochem. [^{14}C]leucine was from Schwartz/Mann. ^{32}P and ^{33}P were from New England Nuclear. D_2O (98 atom %) was from ICN. ^2H and ^{13}C -algal amino acid mixtures (98 and 80 atom % respectively) were obtained from Merck, Sharp and Dohme. Metrizamide was from Sigma. All other chemicals were reagent grade.

Cell Culture and Media

HeLa S3 cells (Puck et al., 1956) were maintained in suspension in S-EMEM supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 $\mu\text{g}/\text{ml}$ gentamicin, 20 mM Hepes (pH 7.4), and 2.5 mM sodium bicarbonate, as previously described (Brake et al., 1978). Cell growth was monitored with a Coulter Counter (Model B). In the density labeling experiments, cells were grown in the complete medium except that the amino acids were replaced by 600 mg/l of ^2H - or ^{13}C -algal amino acid mixture, 24 mg/l cystine, 52 mg/l isoleucine and 10 mg/l

tryptophan; also 4% dialyzed plus 1% whole heat-inactivated fetal bovine serum was used instead of the usual 5% whole serum. The algal amino acid mixtures were dissolved as previously described in Chapter II.

Protein Synthesis

Cells were suspended in growth medium containing 1.5 Ci/ml [^{14}C] leucine for 20 minutes at 37°. Equilibration of the acid-soluble pools occurs in less than one minute in these cells (Proctor, 1974). The labeled cells were pelleted by centrifugation at 2500 rpm for 2 minutes and the supernatant was removed. After the excess fluid was eliminated by wiping the walls of the tubes, the pellet was solubilized in 2% SDS and transferred onto Whatman 3 MM filter circles. Precipitation and washing the discs were by the method of Mans and Novelli (1961).

Other Methods

HeLa cell plasma membrane ghosts were prepared by the method of Atkinson (1973). [γ - ^{32}P]ATP and [γ - ^{33}P]ATP were prepared using a modification (see Chapter II) of the procedure of Wallace (Tenner and Wallace, 1972; Wallace, 1964). In some experiments, [γ - ^{32}P]ATP was obtained commercially from either New England Nuclear (2-10 Ci/mmol or 10-40 Ci/mmol) or from Amersham (3000 Ci/mmol). The specific phosphorylation and velocity sedimentation analysis of the Na,K-ATPase, and the calculations of the mean density shift for the density labeled enzyme and the half-time for its removal from the plasma membrane ($t_{1/2}$ for turnover) were done as described in Chapter II. Protein was measured by the fluorescent method of Avruch and Wallach (1971).

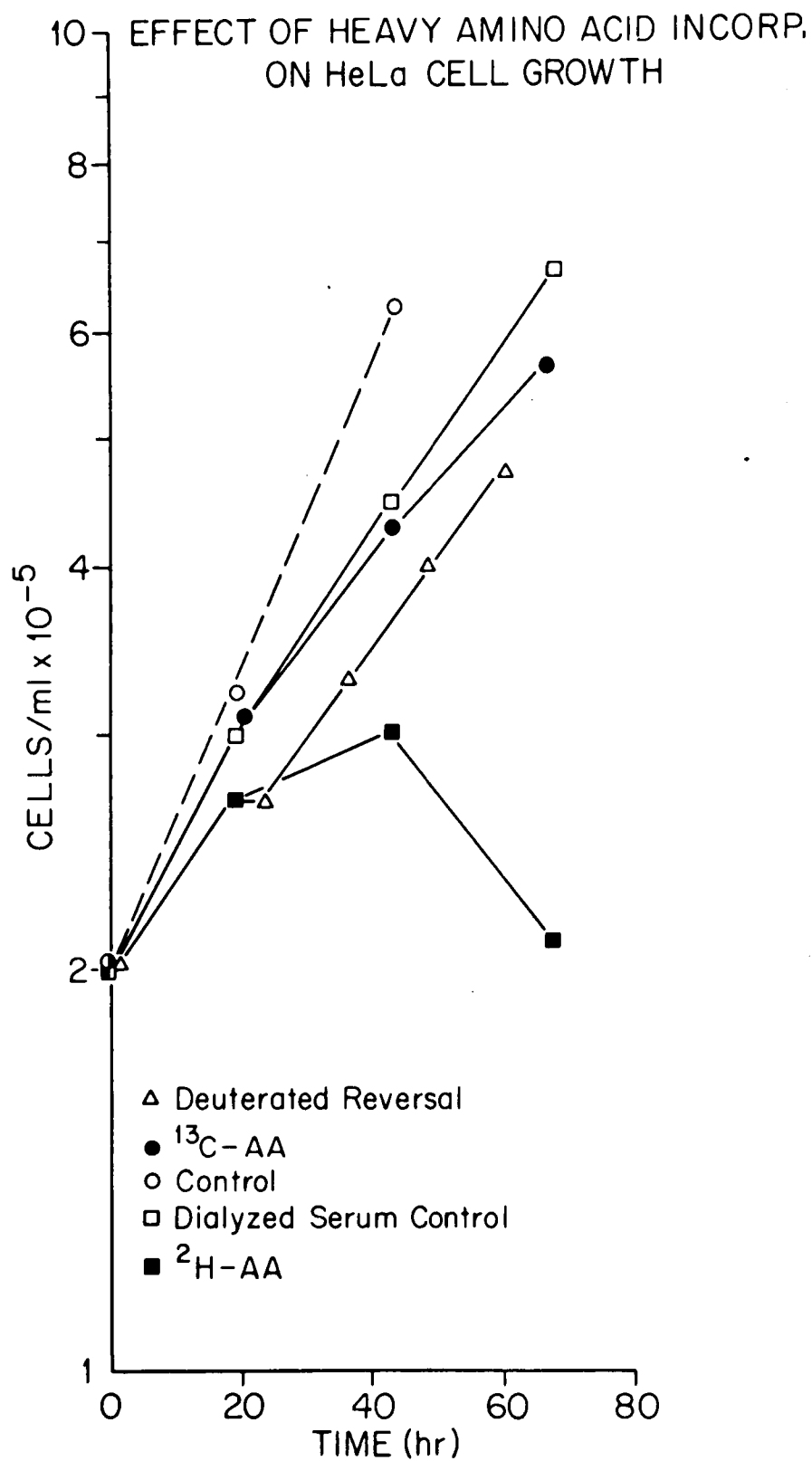
Results

Effects of Heavy Isotopes on Growth and on Protein Synthesis

Prolonged labeling with deuterated amino acids progressively decreased the rates of cell growth and of protein synthesis, as shown in Figure 8. After 19 hours labeling, cells resuspended in normal growth medium grew in log phase with a generation time of 45 hours, substantially longer than the normal generation time of 24-30 hours. Protein synthesis was stabilized at 75% of the control rate. When cells labeled for longer than 24 hours were resuspended for two days in normal growth medium, their rates of growth and of protein synthesis recovered only slowly (data not shown). Eventually, cells labeled with deuterated amino acids not resuspended in normal growth medium ceased growing and lysed. Growth was not improved by increasing the amounts of unsubstituted amino acids that were in relatively low concentration in the deuterated algal amino acid mixture and/or doubling the deuterated algal amino acid mixture in the medium. In contrast, cells in medium containing ^{13}C -amino acids grew well and incorporated [^{14}C]leucine into protein at the control rate.

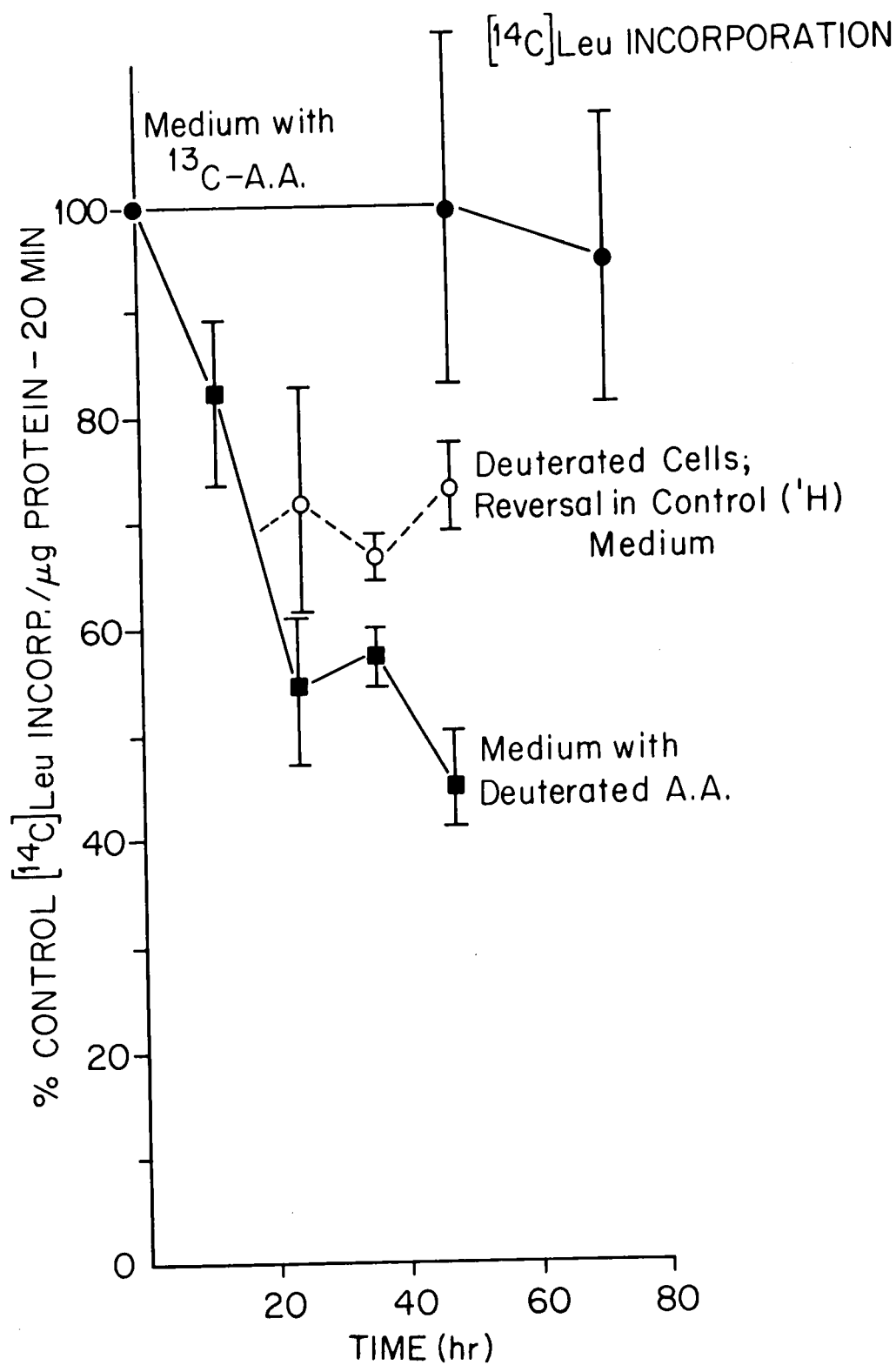
The slightly slower growth rate observed for cells cultured in ^{13}C -amino acid-containing medium can be accounted for by the substitution of dialyzed serum for whole serum in these cultures in order to prevent dilution of ^{13}C -amino acids. I observed similar growth rates for cells cultured in medium containing normal ^{12}C -amino acids when I substituted dialyzed serum for whole serum (Figure 8).

Figure 8. Effects of "heavy" amino acid incorporation on cell growth and on the rate of protein synthesis ($[^{14}\text{C}]$ leucine incorporation into acid-insoluble material). Cells were suspended in medium in which the normal amino acids were replaced by deuterated amino acids (■ — ■) or ^{13}C -amino acids (● — ●), and at various times, the cell number (a) and the rate of protein synthesis as compared with cells resuspended in control ($^1\text{H}, ^{12}\text{C}$) medium (b) were determined as described in Materials and Methods. Also shown is the growth (Δ) and rate of protein synthesis (O) of cells that had been labeled with deuterated amino acids for 19 hours and then resuspended in control (^1H) medium for the Na,K-ATPase turnover experiment presented in Figure 11, page 57. The growth of cells in control ($^1\text{H}, ^{12}\text{C}$) growth medium with 5% whole serum (O — O) or with 5% dialyzed serum (□ — □) is also shown.



(a)

Figure 8



(b)

Figure 8 (continued)

The reduced incorporation of [^{14}C]leucine into ^2H -labeled cells could conceivably have been due to an expanded intracellular pool of endogenous leucine which might have reduced the specific activity of the tracer. To resolve this question I examined the pools of trichloroacetic acid-soluble amino acids in control and deuterated cells. The amount of leucine was found to be the same or slightly reduced in the deuterated population (data not shown). Other amino acids showed more drastic quantitative changes, usually less than two-fold, but four-fold decreases were observed for lysine, arginine and histidine. These quantitative changes reflected the concentrations of these amino acids in the algal hydrolysate. They cannot, however, be rate-limiting for growth or protein synthesis because all amino acids were present in concentrations above those shown by Eagle (1955) to be required for HeLa cell growth and since the same changes in ^{13}C -medium were without effect.

Incorporation of Heavy Amino Acids into Na,K-ATPase

In purified HeLa cell plasma membrane preparations, the Na,K-ATPase was specifically phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{33}\text{P}]\text{ATP}$. In control experiments in which $[\text{}^{32}\text{P}]$ and $[\text{}^{33}\text{P}]$ membranes were mixed and sedimented in metrizamide- D_2O -SDS density gradients there was no detectable effect of the radioactive ATP labels on the sedimentation velocity of the phosphorylated enzyme (Chapter II). Labeling cells with ^2H -amino acids (24 hours) or with ^{13}C -amino acids (69 hours) shifted the ^2H -substituted enzyme or ^{13}C -substituted enzyme to higher

sedimentation velocities as shown in Figure 9. When K^+ (25 mM) was included in the reaction mixture instead of Na^+ , the extent of phosphorylation was greatly reduced (Figure 9a). The mean density shift for the ^{13}C -labeled enzyme was equivalent to if not larger than that obtained for the ^2H -labeled enzyme. Labeling with deuterated amino acids for longer times did not markedly increase the extent of the density shift, a result possibly related to the decreased viability.

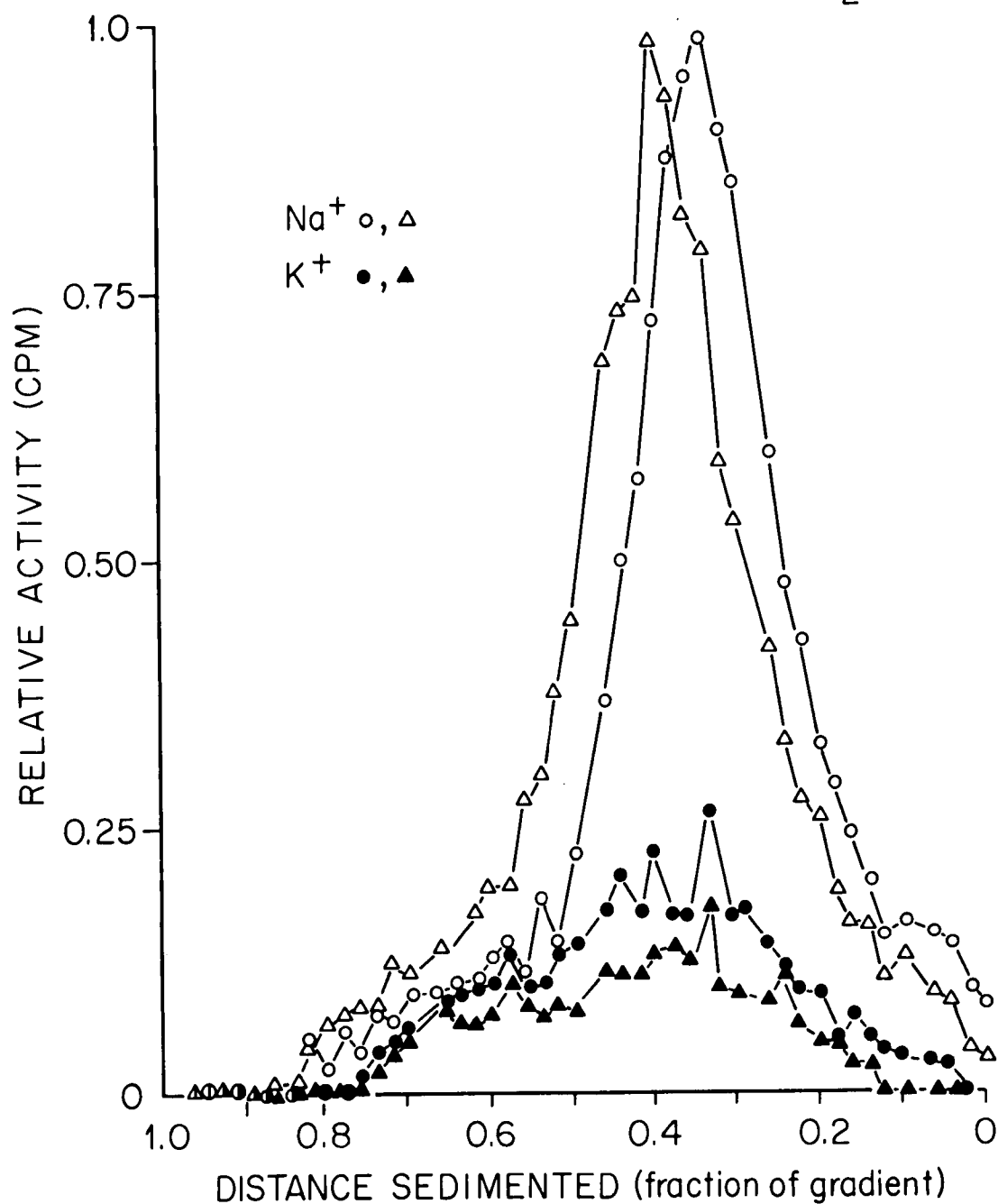
The percentage of deuterated amino acids that had been incorporated into the deuterium-labeled Na,K-ATPase was determined from the densities of the deuterated and control enzymes. To measure the densities of the enzymes, I sedimented them in gradients of different average densities, plotted the distances they had been sedimented to in the gradients as functions of the average densities of the gradients and extrapolated to 0 sedimentation (Figure 10). I obtained a density of 1.256 g/ml for the control enzyme, and 1.266 g/ml for the deuterated enzyme. From these densities, and from the published amino acid composition of the purified Na,K-ATPase from other sources (Hokin, 1974), I calculate that the deuterated enzyme is at least 15% substituted with deuterated amino acids. Direct measurements by mass spectroscopy indicate that at least 30% of the amino acids in plasma membrane preparations from ^{13}C -labeled cells are ^{13}C -substituted (data not shown).

Effects of Heavy Isotopes on Na,K-ATPase Turnover

In turnover studies, I labeled cells with ^2H -amino acids 19 hours, long enough to observe a readily measurable density shift, but not so

Figure 9. Na^+ -dependent, K^+ -sensitive phosphorylation of ^2H or ^{13}C (both $[\text{32P}]$) and control $[\text{33P}]$ membranes cosedimented in metrizamide- D_2O -SDS gradients (pH 3.0) for 20 hours at 50,000 rpm (SW 56 rotor). (a) 150 μg of purified plasma membranes from cells labeled for 24 hours with deuterated amino acids were specifically phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4.7 Ci/mmol), mixed with 900 μg of controls which had been specifically phosphorylated with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (0.86 Ci/mmol), and cosedimented in a linear 15-30% gradient. The amounts of acid-insoluble ^{32}P ($\Delta - \Delta$ or $\blacktriangle - \blacktriangle$) and ^{33}P ($\bigcirc - \bigcirc$ or $\bullet - \bullet$) radioactivity present in each fraction of the gradients containing membranes phosphorylated in the presence of either 100 mM NaCl ($\bigcirc - \bigcirc$ and $\Delta - \Delta$) or 25 mM KCl ($\bullet - \bullet$ and $\blacktriangle - \blacktriangle$) are plotted. (b) 125 μg of purified plasma membranes from cells labeled for 66 hours with $[\text{13C}]$ -amino acids were specifically phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10.2 Ci/mmol), mixed with 330 μg of controls which had been specifically phosphorylated with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (7.0 Ci/mmol); the mixture was sedimented in a linear 11-26% gradient. Both the ^{32}P and ^{33}P membranes were phosphorylated in the presence of 100 mM NaCl (without KCl). The normalized acid-insoluble ^{32}P ($\bullet - \bullet$) and ^{33}P ($\blacktriangle - \blacktriangle$) radioactivity present in each fraction of the gradient are plotted.

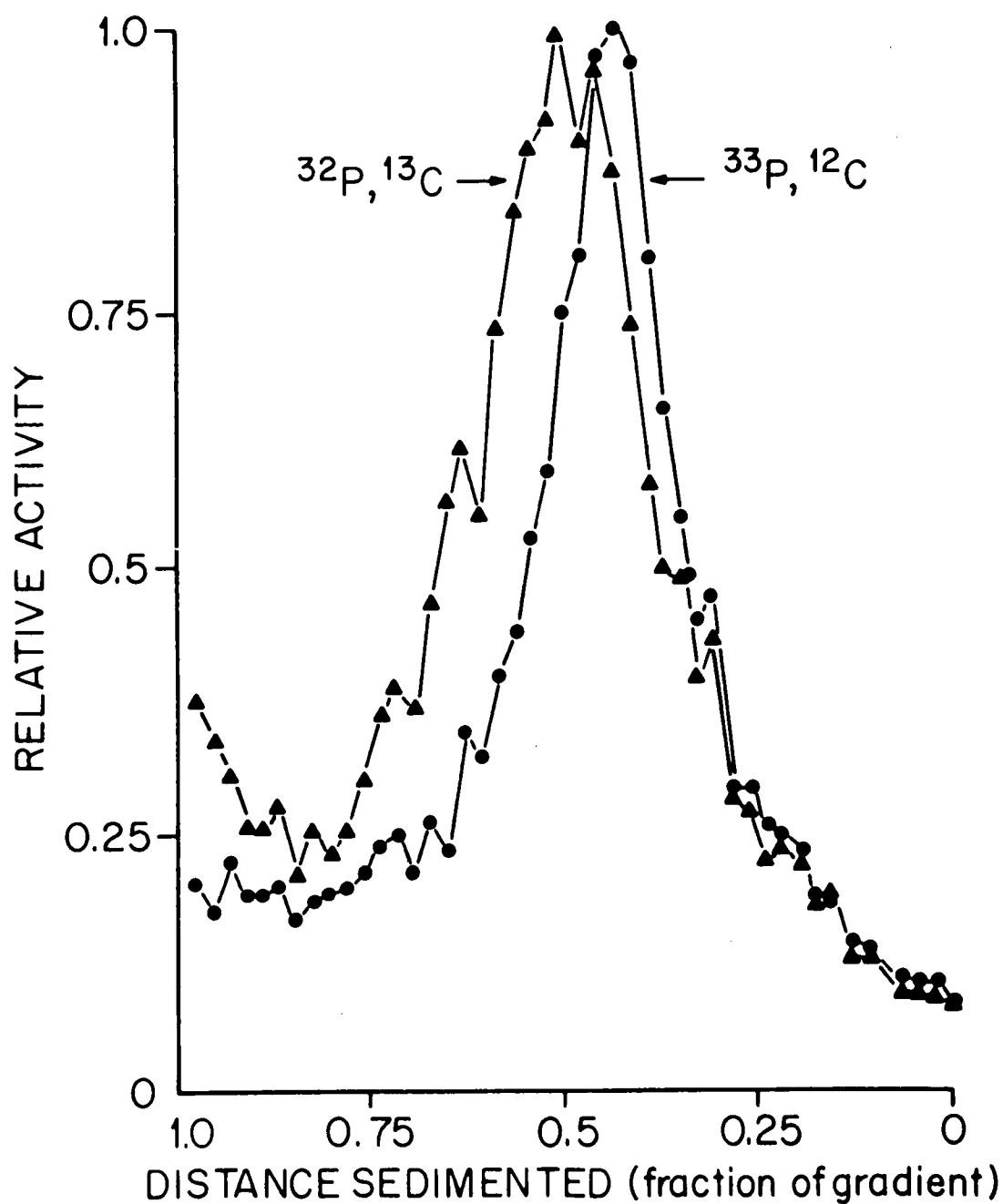
Na^+ -DEPENDENT K^+ -SENSITIVE PHOSPHORYLATION
OF DEUTERATED (^{32}P) $\Delta, \blacktriangle$ AND CONTROL (^{33}P) $\circ, \bullet$
MEMBRANES COSEDIMENTED IN Met- D_2O -SDS



(a)

Figure 9

Na^+ -DEPENDENT, K^+ -SENSITIVE
PHOSPHORYLATION OF ^{13}C -SUBSTITUTED
(^{32}P) Δ AND CONTROL (^{33}P) $\circ$ MEMBRANES
COSEDIMENTED IN Met- D_2O - SDS



(b)

Figure 9 (continued)

Figure 10. Density estimation of deuterated and control Na,K-ATPase. Purified plasma membranes from cells labeled with deuterated amino acids were specifically phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, mixed with controls specifically phosphorylated with $[\gamma\text{-}^{33}\text{P}]\text{ATP}$, and cosedimented in linear gradients of different average densities. Sucrose gradients (10-25%, 0.1% SDS) used either 100% H_2O or 100% D_2O as solvent. Metrizamide- D_2O -SDS gradients contained either 5-20%, 15-30%, or 25-40% metrizamide. The means of the migrations into the gradients of the deuterated enzyme ($\blacktriangle - \blacktriangle$) and the control enzyme ($\circ - \circ$) were determined as described in Materials and Methods, and these distances were plotted as functions of the average densities of the gradients. The data obtained using sucrose and metrizamide as solutes were separately extrapolated to zero sedimentation in order to estimate the densities of the deuterated and control enzymes. For details, see text.

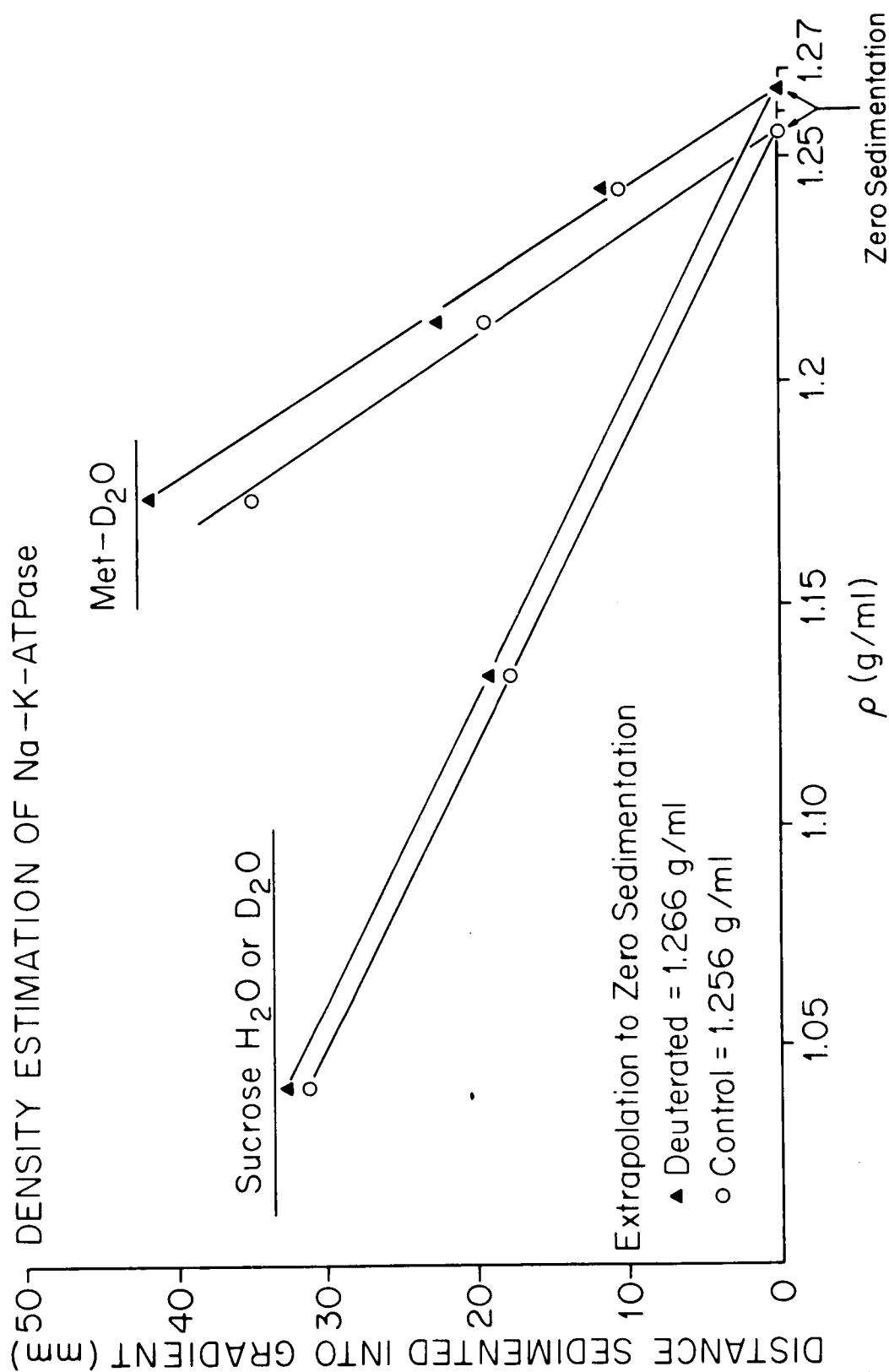


Figure 10.

long as to produce lethal isotope effects. After resuspension of deuterium-labeled cells in growth medium containing normal amino acids, the mean density of the deuterated enzyme gradually decreased as new enzyme was synthesized and as the substituted enzyme was removed from the membrane (Figure 11). There was an initial lag of 0.10-0.15 of the 45-hour generation time (T_G) or 4.5 - 7.0 hours before the mean density began to decrease. Following this, the density shift decreased as an apparent first-order process. It is evident that the density shift decreased at a greater rate than could be accounted for by the 50% dilution of ^2H -enzyme per generation due to growth alone. Therefore, ^2H -enzyme was being removed from the plasma membrane and presumably was being degraded (turnover). From the linear decrease in the deuterium density shift, I calculate a $t_{1/2}$ of $0.45 T_G$ (21 hours) for turnover of the ^2H -substituted enzyme in these slow growing cells.

When ^{13}C -substituted cells were suspended in medium containing normal amino acids, a lag of 0.16 to $0.2 T_G$ or 4 to 4.5 h was observed followed by the first-order disappearance of the ^{13}C -substituted enzyme. Turnover, corrected for dilution of the ^{13}C -enriched enzyme by net growth had a half-time of $0.24 T_G$ or 5.5 h.

Discussion

Density labeling is an important technique in studying the synthesis and degradation of specific plasma membrane proteins (Devreotes et al., 1977; Gardner and Fambrough, 1979; Reed and Lane, 1980; Chapter II). However, culturing HeLa cells in ^2H -substituted

Figure 11. Decrease in density shift after resuspension of [^2H]-substituted cells in normal growth medium. Cells labeled for 19 hours with deuterated amino acids were resuspended (chased) in growth medium containing normal (^1H) amino acids. At various times during the chase, plasma membranes were isolated, phosphorylated with [$\gamma\text{-}^{32}\text{P}$]ATP (3.0 Ci/mmol), mixed with control membranes that had been specifically phosphorylated with [$\gamma\text{-}^{33}\text{P}$]ATP (2.5 Ci/mmol), and cosedimented in linear 8-23% metrizamide- D_2O -SDS gradients for 16 hours at 50,000 rpm (SW 56 rotor). Both the deuterated and control membranes were phosphorylated in the presence of 100 mM NaCl without KCl. About 200 μg of deuterated membranes, and 500 μg of control membranes were applied to each gradient. The percentage density shift for each gradient was determined as described in Materials and Methods. The log of the observed density shifts (O — O) and the change in the density shift expected by dilution of deuterated enzyme due to growth alone (----) are plotted as functions of the generation time (TG), which during the chase was 45 hours. The broken line (----) taken from Chapter II shows the decrease in the density shift when [^{13}C]-amino acids were used as the density label.

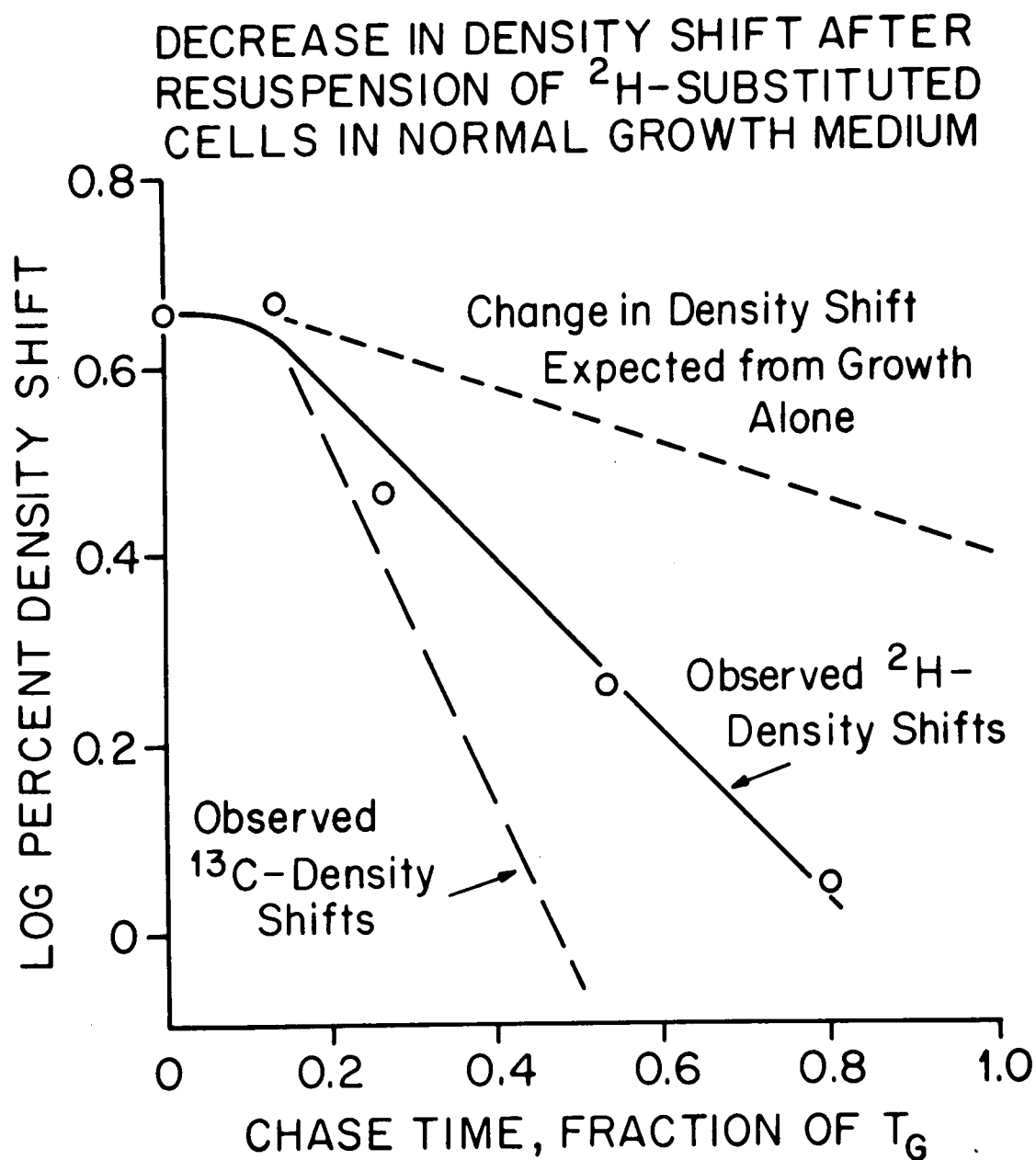


Figure 11.

amino acid-containing medium decreased their rates of growth, of incorporation of amino acids into Na,K-ATPase and into total cell protein, and of Na,K-ATPase turnover. These effects could be reduced by labeling cells for less than 24 hours, but could not be eliminated. In contrast, cells grown in ^{13}C -substituted amino acids were essentially indistinguishable from controls.

Because there are fewer carbons than hydrogens per amino acid, the density shift using ^{13}C -labeled cells was expected to be much less than using deuterium-labeled cells. From the published amino acid composition of the Na,K-ATPase from other sources (Hokin, 1974), together with the assumption that the ^2H and ^{13}C -enzymes are equally substituted with heavy amino acids, I calculate that the maximum increase in density of Na,K-ATPase obtainable with ^{13}C -amino acids is only about half of that expected with deuterated amino acids. Furthermore, the ^{13}C -amino acid mixture was only 80% ^{13}C -substituted whereas the deuterated amino acid mixture was 98% ^2H -substituted. The density shift obtained for the ^{13}C -enzyme, however, was equivalent to if not larger than that obtained for the deuterated enzyme. Therefore, increased substitution with ^{13}C -amino acids compensated for the theoretically smaller density shift that was expected.

The densities of the deuterated and control enzymes were determined by extrapolation from their velocities of sedimentation in gradients of different average densities. The relatively more rapid sedimentation of the enzymes in metrizamide- D_2O -SDS than in sucrose- D_2O -SDS or sucrose- H_2O -SDS is because at a given density, metrizamide forms less

viscous solutions than does sucrose (Rickwood and Birnie, 1975). For shallow gradients like the ones used in this analysis, the extrapolated densities at which the velocities of sedimentation equal zero are estimates of the densities of the macromolecules (Devreotes et al., 1978). These are minimal estimates because the enzymes were solubilized in SDS, which is expected to have bound to the enzymes, and thereby to have both lowered their densities and minimized the difference in density between ^2H -substituted and control enzymes. This amount of ^2H -substitution, however, is comparable to that previously reported for the acetylcholine receptor from deuterium-labeled chick myoblasts (Devreotes and Fambrough, 1976). From the extent of the ^{13}C -density shift and from the mass spectroscopy measurements, the ^{13}C -enzyme probably contains at least 30% ^{13}C -substituted amino acids.

Upon resuspension of ^{13}C -labeled cells in medium containing normal amino acids, the ^{13}C -density shift decreased in a manner similar to that observed with deuterated cells. Using either ^{13}C or ^2H -amino acids, lags of $0.1 - 0.2 T_G$ were observed prior to apparent first-order decreases in the density shifts. From the linear decreases in the density shifts, I calculate the first-order rate constants $3.1/T_G$ and $1.5/T_G$ for the removal from the plasma membrane of the ^{13}C and ^2H -substituted enzymes, respectively.

The corresponding half-times for turnover were, with ^{13}C -substituted amino acids $t_{1/2} = 0.24 T_G$, or deuterated amino acids, $t_{1/2} = 0.45 T_G$;

both half-times were much less than the generation times. The $t_{1/2}$ for the ^{13}C -enzyme, however, was considerably shorter. Deuterated amino acids appear to also interfere with the removal of the Na,K-ATPase from the plasma membrane.

When the turnover data are expressed in hours, the difference between labeling with ^2H - and ^{13}C -amino acids becomes even more apparent. The $t_{1/2}$ for turnover was about 21 hours using ^2H -amino acids, and was about 5.5 hours using ^{13}C -amino acids.

My results agree with previous reports of toxic deuterium isotope effects on cultured mammalian cells (Rothstein et al., 1960) and on intact mammals (Czajka, 1961), and with descriptions of negligible ^{13}C -effects on whole mice (Gregg et al., 1973). Toxic effects were not observed, however, when chick myoblasts were cultured in the presence of ^2H or ^3H , ^{13}C , ^{15}N -substituted amino acids (Gardner and Fambrough, 1979; Devreotes and Fambrough, 1976; Devreotes et al., 1977) or confluent 3T3-L4 cells were cultured in ^2H , ^{13}C , ^{15}N -substituted amino acids (Reed and Lane, 1980). Since one deleterious action of deuterium is its effect on cell division (for a review of deuterium isotope effects, see Finkel, 1974), perhaps part of the difference observed between cell types, if real, occurs because only the HeLa line was dividing. However, these results indicate that caution should be used in density labeling with deuterium-containing amino acids and support the use of ^{13}C -substituted amino acids in Chapter II.

CHAPTER IV

Na,K-ATPase IN HeLa CELLS AFTER PROLONGED

GROWTH IN LOW K^+

Introduction

A number of cases have been described in which mammalian cells in culture respond to deprivation of an essential metabolite by an enhanced capacity for uptake of that metabolite. Frequently this is considered an induction of the transport system. Such a response is well known for glucose deprivation (Martineau et al., 1972; Kalckar and Ullrey, 1973; Kletzien and Perdue, 1975; Salter and Cook, 1976; Christopher, 1977) and amino acid deprivation (Frengley et al., 1974; Hume and Lamb, 1974; Heaton and Gelehrter, 1977) and has been described for K^+ -starved whole animals (Chan and Sanslone, 1969). Analogously when HeLa cells were cultured for long periods in low K^+ , the total number of ouabain molecules that could be bound per cell increased with time by as much as a factor of three (Boardman et al., 1974). However, the interpretation of an increase in ouabain binding per cell as an induction of the Na^+-K^+ transport system has been unclear since the changes in transport did not appear to correlate quantitatively with the changes in numbers of bound glycosides. These changes also did not correlate temporally. HeLa cells incubated in low K^+ medium for 2.5 hours exhibited an increased transport of $^{86}Rb^+$ (a potassium analog) which was not accompanied by an increase in ouabain binding (Cook et al., 1975).

In this chapter I seek to resolve these ambiguities. I show that after long-term growth in low- $[K^+]$ medium there is a substantial increase in specific ouabain binding sites which correlates with changes in both the V_{max} of alkali-cation transport in intact cells and the specific phosphorylation of Na^+, K^+ -ATPase in isolated membranes. From this complex of responses I conclude that there is an induction of the amount of functional Na^+, K^+ -ATPase in the plasma membrane. The induction is reversible. I also show that the increase in Na,K-ATPase cannot be ascribed to the exposure of cryptic transporters in the sense that detergent treatment can expose Na^+, K^+ -ATPase. Instead, it appears to be caused by a decrease in the enzyme's degradation rate during turnover. Finally, I describe and differentiate between short-term and long-term physiological compensatory responses of HeLa cells to K^+ -deprivation.

Materials and Methods

Materials

Eagle's minimal essential medium with Spinner salts (S-EMEM), fetal calf serum, dialyzed fetal calf serum, and glutamine were obtained from Grand Island Biological Co., and gentamicin (Schering) from Microbiological Associates. N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (Hepes) and ATP were from Calbiochem. $[^3H]$ ouabain, $[^{14}C]$ sucrose, and $[^{32}P_i]$ were from New England Nuclear. $^{86}Rb^+$, used as a marker for K^+ flux measurements (Vaughan and Cook, 1972), was obtained as high specific activity $^{86}RbCl$ from ICN. $[^{14}C]$ adenosine-5'-monophosphate (5'-AMP) was purchased from Amersham Searle and was

purified as described by Brake et al. (1978), ^{13}C -algal whole hydrolysate (80 atom %) was from Merck, Sharp and Dohme. Ouabain, 5'-AMP, glyceraldehydephosphate dehydrogenase and 3-phosphoglycerate phosphokinase (both from yeast), glyceraldehyde-3-phosphate, NAD, ADP, p-nitrophenyl-N-acetyl- β -D-glucosaminide and bovine serum albumin (fraction V) were from Sigma. All other chemicals were reagent grade.

Cell Cultures

HeLa S3 cells (Puck et al., 1956) were grown in suspension in S-EMEM (Eagle, 1959) supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, 25 $\mu\text{g/ml}$ gentamicin, and 20 mM Hepes, pH 7.4, and 2.5 mM sodium bicarbonate. The cultures were swirled at 118 cycles/min at 37° in screw-cap Erlenmeyer flasks. Routine testing (Schneider et al., 1974) showed the cultures to be free of mycoplasma contamination. Cell growth was monitored with a Coulter Counter (Model B). Cell number increased exponentially from 10^5 to 8×10^5 cells/ml with a generation time of 24-30 hours. Cell volume was determined with a Coulter Channelyzer to be 1600-2000 μm^3 . For K^+ -deprivation experiments, cells were grown in K^+ -free S-EMEM supplemented as described for growth of stock cultures except that up to 2% of the 5% fetal calf serum was replaced by dialyzed fetal calf serum. In this medium, the K^+ was derived entirely from the fetal calf serum. Dialyzed serum was substituted for whole serum, which contained varying amounts of K^+ (up to 20 mM), so that the final medium K^+ -concentration was 0.55-0.80 mM. In the density labeling experiment, cells were grown in

K⁺-free S-EMEM supplemented with 3% whole serum and as described above except that the amino acids were replaced by 800 mg/l of ¹³C-whole algal hydrolysate, 24 mg/l cystine, 52 mg/l isoleucine, and 10 mg/l tryptophan.

Ouabain binding

Cells were centrifuged at 1000 times g for 2 min and resuspended in K⁺-free S-EMEM supplemented as described above (final K⁺ concentration = 0.55-0.6 mM) to which [³H]ouabain had been added to a final concentration of 2×10^{-7} M. The suspension was rotated in a 37°C water bath for 20 min. Previous experiments showed that the binding was saturated after 20 min and remained constant for at least 1 hr. Aliquots were counted for cell number during the binding assay. At the end of the incubation period, triplicate aliquots were transferred to conical, plastic 15 ml tubes and centrifuged at 1000 times g for 2 min. The medium was decanted and the tubes were wiped out, taking care to avoid the cells in the tip of the tube. After gentle resuspension the samples were centrifuged as described above, and the excess medium again wiped out. The pellets were then dissolved in 0.5 ml of 0.1 N NaOH and transferred to glass scintillation vials. Each tube was then rinsed with an additional 0.5 ml of 0.1 N NaOH which was pooled with the dissolved pellet. The samples were neutralized with 0.1 ml of 1 N HCl and were assayed for radioactivity in 10 ml of aqueous counting scintillant (Amersham).

Ouabain-Sensitive Rb^+ Transport

1. Standard assay. Cells were harvested by centrifugation and resuspended in growth medium. Aliquots were counted for cell number. The suspension was then divided into two flasks. $^{86}\text{RbCl}$ was added to flask 1 to a concentration of $0.25 \mu\text{Ci/ml}$. Flask 2 was preincubated with 10^{-3} M ouabain for one minute before the addition of $^{86}\text{RbCl}$. Following incubation at 37°C , triplicate 0.75 ml samples were removed at 0, 3, and 6 minutes, transferred into 1.5 ml tubes containing 0.75 ml of growth medium at 0°C with $0.3 \mu\text{Ci/ml}$ [^{14}C]sucrose, and were centrifuged in an Eppendorf microfuge at 15,000 times g for 20 sec. The medium was decanted and the tips of the tubes, which contained the pellets, were sliced off with a guillotine and then were placed in scintillation vials. The pellets were dissolved in 1 ml of 0.1 N NaOH and neutralized and assayed for radioactivity. Corrections were made for the amount of residual medium in the pellet based on the amount of [^{14}C]sucrose, which is extracellular only, associated with the pellet. Ouabain-sensitive Rb^+ transport was calculated by subtracting the uptake obtained in the presence of 10^{-3} M ouabain (flask 2 above) from the total Rb^+ uptake (flask 1). The ouabain-sensitive Rb^+ transport was 80-90% of the total Rb^+ uptake.

2. V_{max} measurement. To measure the V_{max} for transport, flasks were preincubated at 0°C for 4 hours, and then placed in a 37°C water bath for 1 minute before starting the assay. Further details are given in Results.

K⁺ Measurements

K⁺ was measured with a Nilab flame photometer, using LiCl as an internal standard. Previous experiments with HeLa cells showed that a decrease in [K⁺]_i is accompanied by an equivalent increase in [Na]_i (Cook et al., 1975). To measure [K⁺]_i, aliquots of cells were counted for cell number. Triplicate samples were then transferred to conical, plastic 15 ml tubes containing 4.5 ml of growth medium at 0°C, with 0.35 µCi/ml [¹⁴C]sucrose, and were centrifuged at 1000 times g for 2 minutes. The medium was decanted and the tubes were wiped out. Cells were lysed by sonication in 3 ml of 2.88 mM Li₂SO₄ and the K⁺ concentration was determined. [K⁺]_i was calculated by assuming a mean cell volume of 1800 µm³ (Cook and Brake, 1978). The extracellular fluid in the cell pellet was estimated from the trapped [¹⁴C]sucrose concentration, and appropriate corrections were made for this component.

Phosphorylation of HeLa Cell Plasma Membrane Preparations

Na,K-ATPase was specifically phosphorylated with 2.5 µM [γ-³²P]ATP for 20 seconds at 0°C, pH 7.5, in the presence of 5 mM Mg⁺², and either 100 mM Na⁺ or 25 mM K⁺, as described in Chapter II. Specific, or K⁺-sensitive phosphorylation, of the Na,K-ATPase was calculated as the amount of phosphorylation observed in the presence of Na⁺ minus that observed in the presence of K⁺. Nonspecific, or K⁺-insensitive phosphorylation, was the residual amount seen in the presence of K⁺. In some experiments, the plasma membranes were preincubated with

SDS (0.05-0.35 mg/ml) for 45 minutes at 0°C to expose cryptic phosphorylation sites. Detergent treatment at room temperature led only to inactivation of the enzyme.

Other Methods

HeLa cell plasma membranes were purified by the method of Atkinson (1973); details of the preparation are described in Chapter II. [γ - ^{32} P]ATP was prepared using the procedure of Wallace (Tenner and Wallace, 1972; Wallace, 1964) as modified (see Chapter II). For some experiments, [γ - ^{32} P]ATP was obtained commercially from Amersham (3000 Ci/mmol). Na,K-ATPase activity was assayed as described in Chapter II. The velocity sedimentation analysis of the Na,K-ATPase and the calculations of the mean density shift for the ^{13} C-enzyme and the half-time for its removal from the plasma membrane ($t_{1/2}$ for turnover) were done as described in Chapter II. 5'-Nucleotidase was assayed as 5'-AMPase by the method of Avruch and Wallach (1971) as modified by Brake et al., (1978).

Results

Increased Ouabain Binding in Cells Grown in Low- $[K^+]$ -Medium

Cells were stressed by growth in low- K^+ (~ 0.5 - 0.8 mM) medium. These concentrations are slightly below the K_m for the K^+ -activation of Na^+, K^+ transport in these cells (Vaughan and Cook, 1972). The number of ouabain molecules that could be pulse bound to the surface was assayed. Two representative experiments are shown in Figure 12.

Figure 12. Enhancement of pulse-labeled ouabain-binding sites in HeLa cells grown in low- K^+ media and reversal on restoration of normal $(K^+)_{\text{o}}$. A. Cells grown 50 h in medium with 0.5 mM $(K^+)_{\text{o}}$. At the arrows, the concentration of K^+ in the medium was restored to 5.5 mM. Open circles, numbers of ouabain binding sites as measured by the pulse label technique; open diamonds, concentration of $(K^+)_{\text{i}}$. Both sets of measurements expressed as fractions of the initial control value. B. Cells grown 44 h in medium with 0.8 mM $(K^+)_{\text{o}}$. At the arrows, a fraction of the cells grown in low- K^+ medium were resuspended in the normal (conditioned) K^+ medium in which the controls had been growing, and a fraction of the controls were resuspended in the low- K^+ medium (conditioned) in which the experimental cells had been growing. All values expressed as a fraction of the initial number of pulse-labeled ouabain-binding sites in the controls, which was 1×10^6 . Open diamonds, experimental cells growing in low- K^+ medium; closed circles, control cells growing in normal- K^+ medium; open diamonds, experimental cells resuspended at 50 h in normal- K^+ conditioned medium; open squares, control cells resuspended at 50 h in low- K^+ conditioned medium.

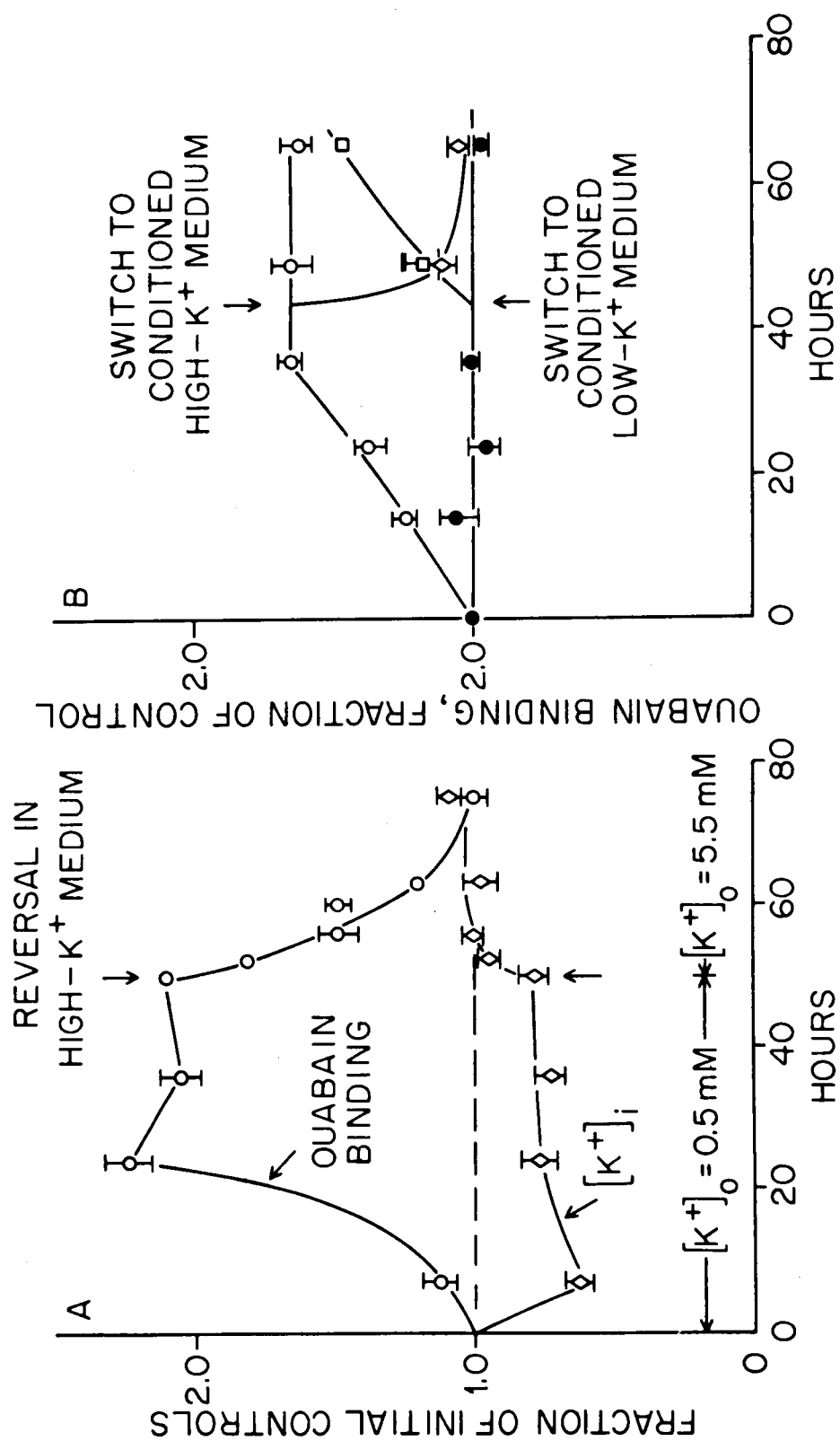


Figure 12.

In these low- $[K^+]$ media the experimental cells grew with generation times of 30-35 h, cell volume was not significantly different than controls. They gradually developed an increase in the number of ouabain-binding sites per cell. The increase was scarcely detectable after 6 h, was clear by 24 h, and had reached its maximum by 48 h. In several experiments, it appeared that the magnitude of the induction as well as the rate of its development were inversely related to the medium $[K^+]$. Nevertheless, at least 24 h were required before the induction reached its maximum and in no case was the induction more than 3-fold. A 2-fold increase was most commonly observed under the conditions described. At still lower concentrations of K^+ in the medium the generation time was greatly prolonged and the cell viability was decreased (data not shown; Gargus et al., 1978).

During growth in these low- $[K^+]$ media, the intracellular $[K^+]$ fell within a few hours to 70-85% of the control level and stabilized at these lower levels. $[K^+]_i$ seemed to increase again somewhat with time as the number of ouabain-binding sites increased (Figure 12a).

When cells grown in low $[K^+]$ were resuspended in medium containing the normal $[K^+]$, the number of ouabain-binding sites decreased promptly to control levels. The half-time for this reversal was about 5-6 h, or much less than the half-time for the original induction.

The relative slowness of the induction compared to the reversal suggested that for the former some component of the medium might be changing and that this slow change might be necessary for induction. To test this point control cells were resuspended in low- $[K^+]$ medium in which cells had already been induced. The kinetics of induction of

these fresh cells were identical to those observed with unconditioned medium (Figure 12b). There is no evidence that a progressive change in some medium component beyond the initial reduction in $[K^+]$ is required for the increase in ouabain binding.

Transport Measurements

To assess the functional properties of the ouabain binding sites two assays were utilized, one "standard assay" and one for V_{max} of $^{86}\text{Rb}^+$ transport.

The standard assay was performed in 5.5 mM K^+ medium (see Methods). At this concentration the transport system is stimulated to 90% or more of the rate attainable by extracellular K^+ -stimulation alone. This standard assay measures the ability of the cells in a given physiological state to take up K^+ from the medium; this capability is primarily a function of the number of transporters and their stimulation by internal $[Na^+]_i$. To estimate V_{max} it was necessary to raise $[Na^+]_i$ to the point where it was also saturating. This was done by preincubating the cells at 0°C in order to inhibit transport and thereby run down the cation gradients. As seen in Figure 13 (bottom left), when cells were incubated in the cold, $[K^+]_i$ slowly decreased. Since there was no significant change in volume during this period, $[Na^+]_i$ must have increased concomitantly as had observed previously with attached HeLa cells (Cook et al., 1975). When the temperature was returned to 37°C, $[K^+]_i$ recovered within 30 min (Figure 13, bottom right). This rapid net K^+ influx (and Na^+ efflux) indicated that a

Figure 13. $V_{\max}$ assay. (Top) Cells were incubated at 0°C and at various times assayed for $^{86}\text{Rb}^+$ uptake at 37°C by the microfuge assay as described in the text. The percentage of the 3-minute ouabain-sensitive uptake without incubation at 0°C is plotted. (Bottom left) At the indicated times, aliquots of the 0°C cell suspension used for the transport measurements were removed and the intracellular potassium concentrations, which are plotted, were determined as described in Materials and Methods. (Bottom right) After 3 hours at 0°C, a sample of the suspension was transferred to a separate flask, incubated at 37°C for 30 minutes, and the intracellular potassium concentration was determined.

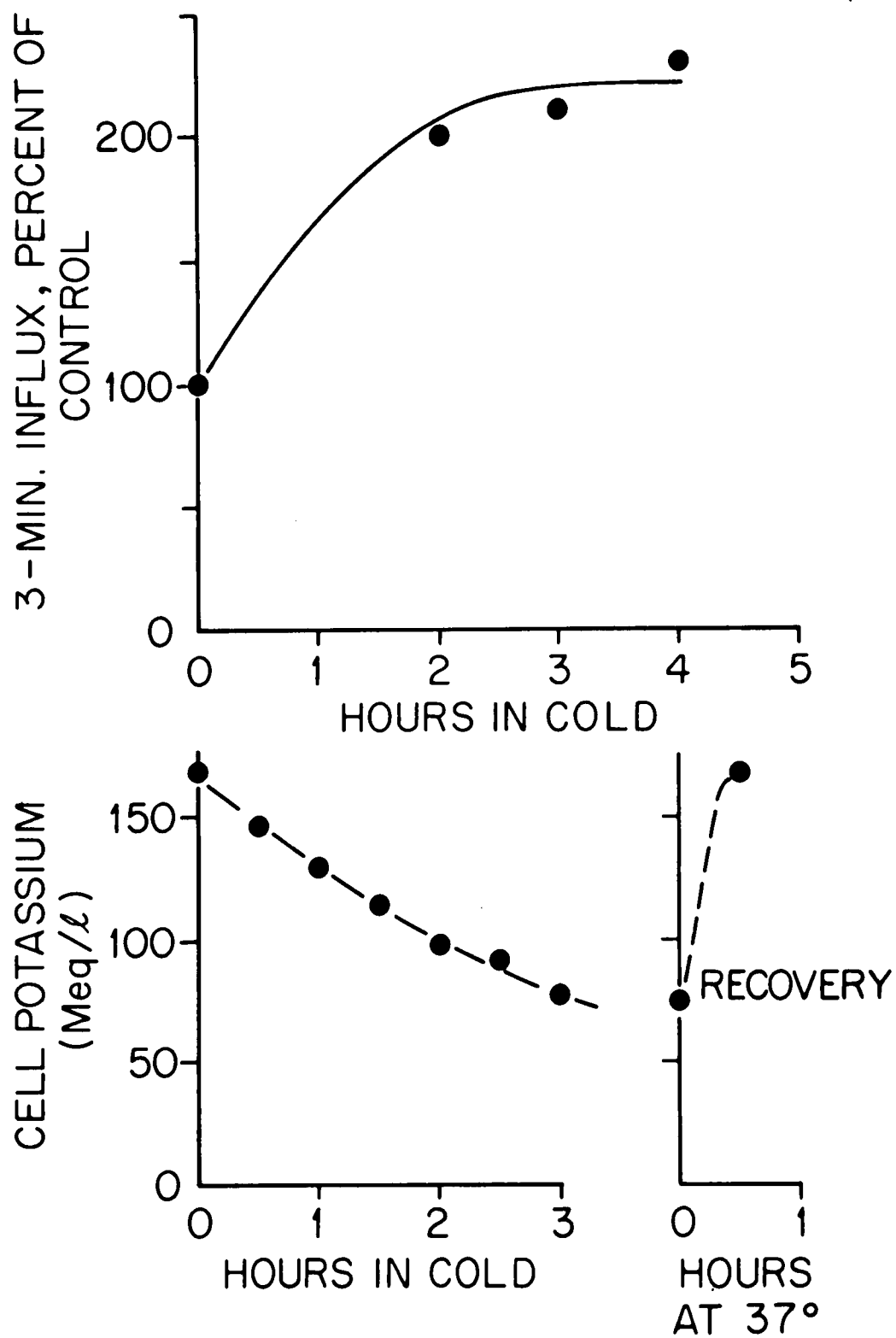


Figure 13.

usual 10 min $^{86}\text{Rb}^+$ -uptake assay (Boardman et al., 1974; Cook et al., 1975) would not be adequate for these conditions nor for those in which growth in low K^+ had increased $[\text{Na}^+]_i$. Therefore, for both the V_{max} measurement and for the standard assay, I utilized a 3 min microfuge assay which measured fluxes over a shorter time period during which they were linear (see Methods). Figure 13 (top) shows that incubating the cells in the cold doubled the $^{86}\text{Rb}^+$ influx assayed at 37°C . After about 2 hours of cold incubation, transport increased no further, indicating that the enzyme was essentially saturated with $[\text{Na}^+]_i$. In subsequent experiments V_{max} for transport was measured after a 4 hour preincubation at 0°C . The additional 2 h incubation and further depletion of $[\text{K}^+]_i$ and increase of $[\text{Na}^+]_i$ insured that during the brief microfuge assay, although Na^+ was being extruded, it still remained in high enough concentration intracellularly to keep the transporter saturated from the cytoplasmic site. Such preincubation in the cold did not affect ouabain binding (data not shown).

Quantitative Relationships between Ouabain Binding, Transport V_{max} , and Specific Phosphorylation

To investigate the quantitative relationships among the various manifestations of surface Na^+, K^+ -ATPase, I divided the observations into 4 time categories: (1) control; (2) short-term response to K^+ starvation (4-6 h) when $[\text{K}^+]_i$ has fallen substantially but the number of ouabain-binding sites has increased very little (Figure 12a, p. 70); (3) long-term regulation (24-72 h) when the number of ouabain-binding

sites has reached its peak value; and (4) mid-reversal, or 4-6 h after restoration of normal $[K^+]_o$ to fully induced cells. The data in Figure 14 show the changes in some transport parameters at these various times, expressed as fraction of the controls. After 4 h incubation in low- $[K^+]$ medium (short-term regulation), there was an increase in $^{86}\text{Rb}^+$ influx in the standard assay. Ouabain per cell and transport capacity (V_{max}) showed little change over the controls. After 48 h growth in low- $[K^+]$ medium (long-term regulation) $^{86}\text{Rb}^+$ influx in the standard assay was increased only slightly more than in short term regulation. At this time, however, both ouabain per cell and transport capacity (V_{max}) had doubled. After resuspension of these induced cells in normal (5.5 mM) $[K^+]$ medium for 4 h (reversal), the relative decrease in $^{86}\text{Rb}^+$ influx in the standard assay was much greater than the diminution in ouabain binding or transport capacity.

From the data used to construct Figure 14 one may calculate the transport per ouabain binding site in terms of the $^{86}\text{Rb}^+$ influx in both the standard assay and the V_{max} (Figure 15). During short term regulation the $^{86}\text{Rb}^+$ transport per ouabain binding site increased. During long-term regulation, when the number of binding sites had increased substantially, transport per binding site fell somewhat but was still elevated over the controls. During the reversal, transport per binding site undershot the control value. During all of these changes, which are of regulatory significance (see Discussion), the V_{max} per binding site was unaltered.

A parallel property of the Na^+, K^+ -ATPase in membranes is the Na^+ -dependent, K^+ -sensitive phosphorylation which quantitatively labels

Figure 14. Cation transport after growth in 0.5 mM K^+ . Following suspension in low K^+ medium for either 4 hours (short-term regulation), 48 hours (long-term regulation), or for 48 hours and then resuspension in control 5.5 mM K^+ medium for 4 hours (reversal), cells were assayed for V_{max} , $^{86}Rb^+$ influx, and ouabain binding as described in the text. The average of three determinations are plotted.

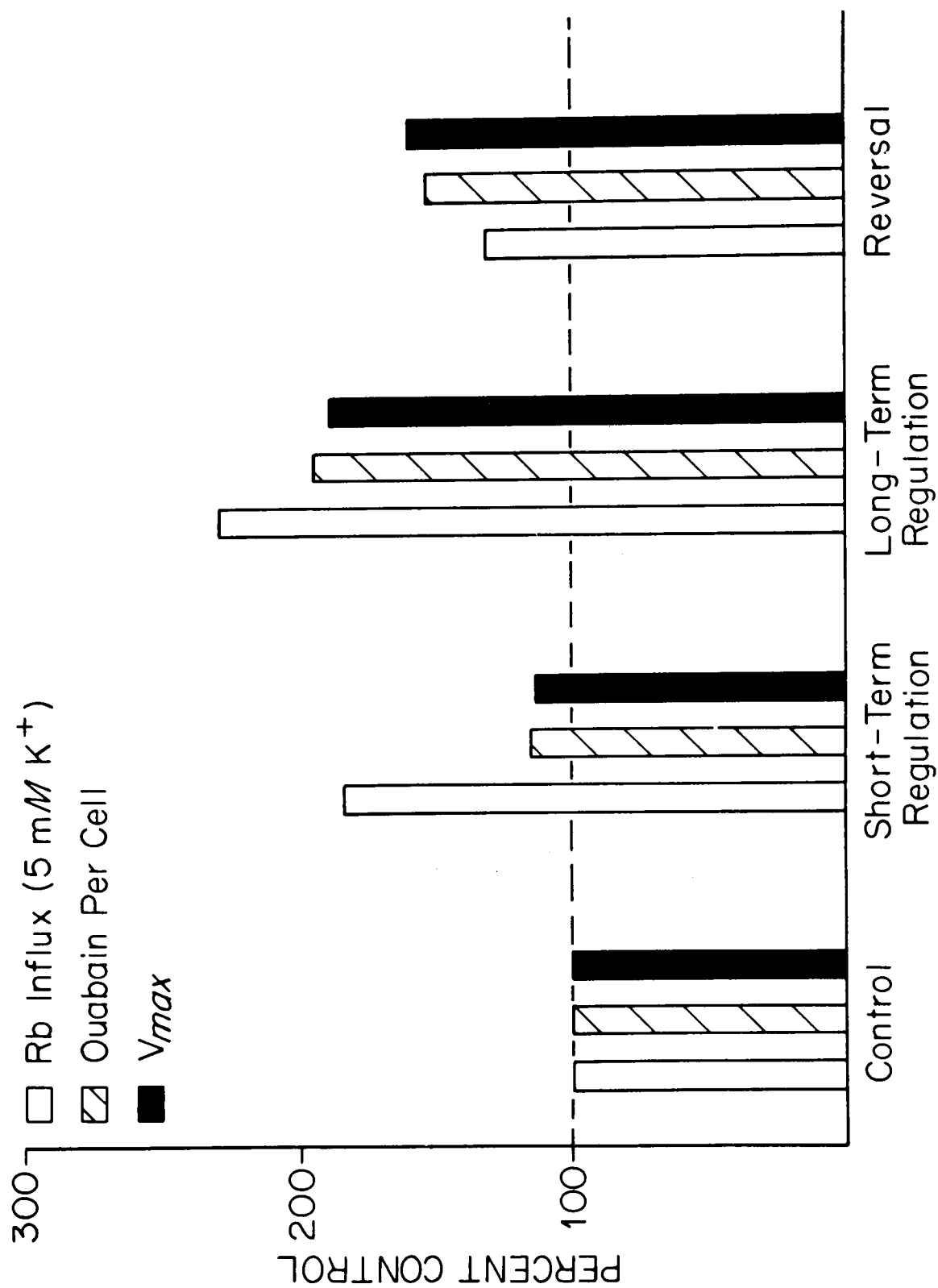


Figure 14.

Figure 15. $^{86}\text{Rb}^+$ transport per ouabain binding site after growth in 0.5 mM K^+ . The data from Figure 14, page 77, along with the $^{86}\text{Rb}^+$ radioactivity (0.25 $\mu\text{Ci/ml}$) and potassium concentration (5.5 mM) in the assay medium was used to calculate the molecules of $^{86}\text{Rb}^+$ transported per minute per ouabain binding site for both the $^{86}\text{Rb}^+$ influx and transport capacity (V_{max}) measurements.

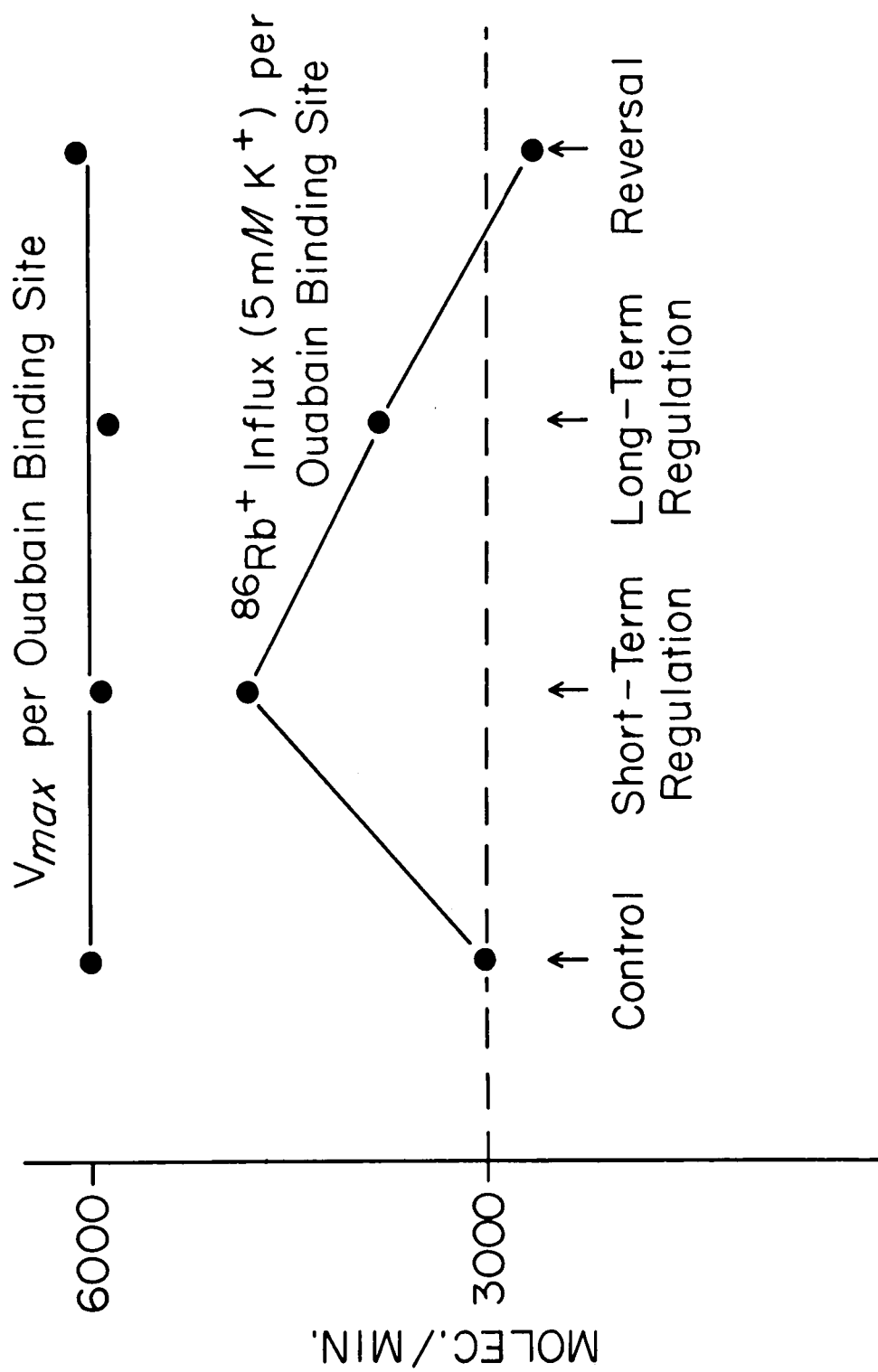


Figure 15.

the enzyme on the cytoplasmic side of the membrane (Post et al., 1965). In isolated plasma membranes both from controls and from cells which had been grown in low $[K^+]$ the Na^+, K^+ -ATPase was specifically phosphorylated as described in Chapter II. Under these conditions, when compared with purified Na,K-ATPase preparations (Jørgensen, 1974), the control amount of phosphorylation approximated that expected for a preparation with its Na,K-ATPase activity. Therefore, these conditions quantitatively label the enzyme. As seen in Figure 16, the induced cells showed an increased K^+ -sensitive phosphorylation that was very similar in relative magnitude to the increase in ouabain binding per cell.

The specificity of the increase in ouabain binding and K^+ -sensitive phosphorylation on prolonged growth of the cells in low- $[K^+]$ medium is also evident in Figure 16 in that neither K^+ -insensitive phosphorylation of the membranes nor the specific activity of 5'-nucleotidase (5'-AMPase) was significantly altered.

Detergent Activation of Specific Phosphorylation in Control and Induced Cells

Cryptic sites of Na^+, K^+ -ATPase in membrane preparations can be exposed by low concentrations of detergent (Jørgensen and Skou, 1971). The next question to be addressed was whether the enhanced amount of Na,K-ATPase in induced cells was due to the physiological exposure of such cryptic sites. A representative experiment is shown in Figure 17.

Plasma membranes were treated with SDS for 45 min at $0^\circ C$ and the amount of Na^+, K^+ -ATPase present was quantitated by specific

Figure 16. Plasma membrane activities after prolonged growth in low K^+ . Cells were suspended in 0.5 mM K^+ for 88, 135, or 208 hours or in 5.5 mM K^+ for 66, 142, or 190 hours, and then ouabain binding per cell was determined and plasma membranes were isolated. Each preparation was individually assayed for 5'-AMPase, K^+ -insensitive phosphorylation, and K^+ -sensitive phosphorylation as described in Materials and Methods. The average values for the low K^+ cells $\pm$ 1 S.D. are plotted as percentages of their respective average control values.

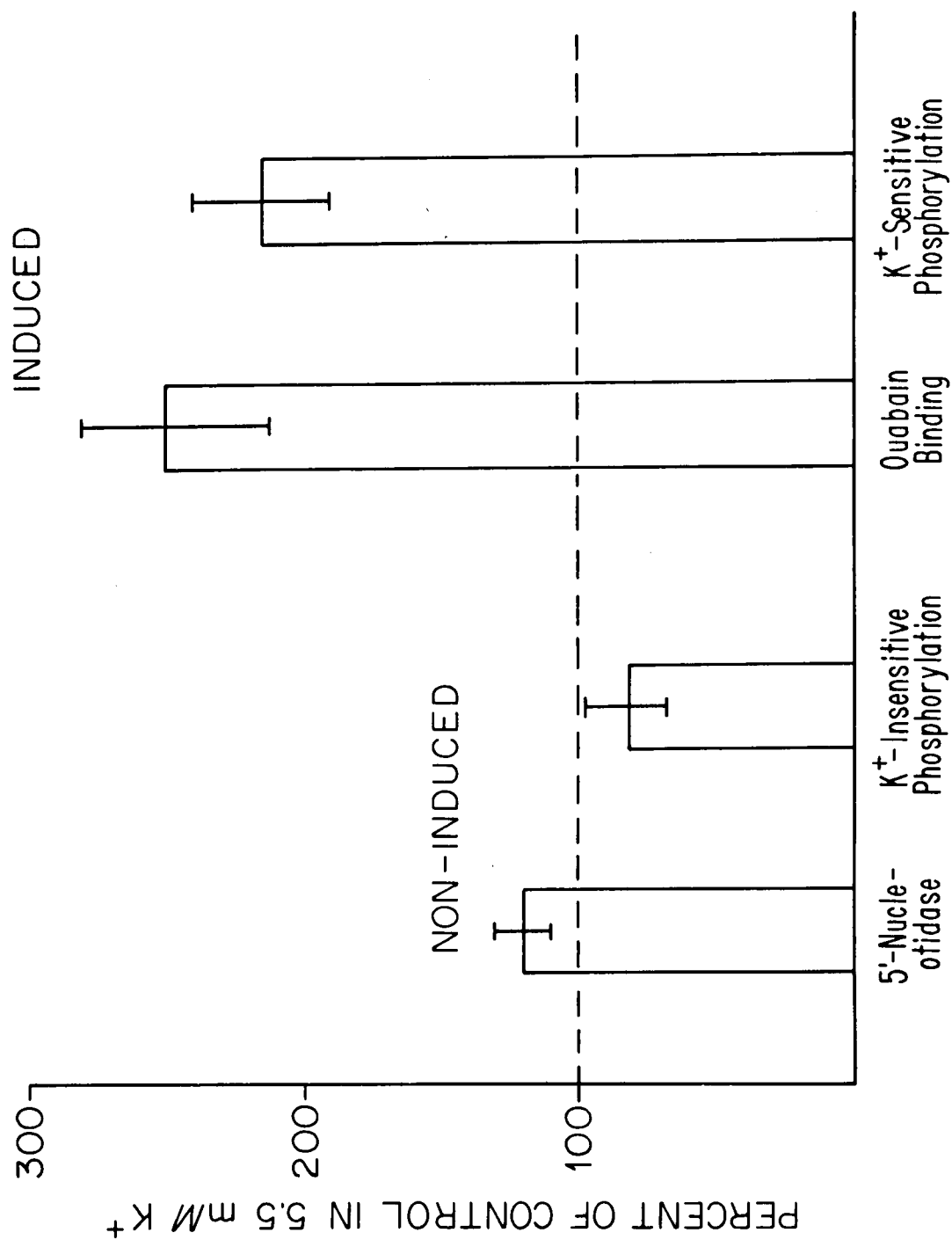


Figure 16.

Figure 17. Effect of SDS on phosphorylation of plasma membranes from cells grown in low K^+ . Cells were suspended in 0.5 mM K^+ (low K^+) for 48 or 122 hours or 5.5 mM K^+ (controls) for 25 or 125 hours and then plasma membranes were isolated. Membranes from low K^+ cells and controls were separately pooled and 48 μ g of each were pre-incubated with various concentrations of SDS for 45 minutes at 0°C and then phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in Materials and Methods. Phosphorylation was carried out in the presence of either 100 mM Na^+ (O — O or ● — ●) or 25 mM K^+ (Δ — Δ or $\blacktriangle$ — $\blacktriangle$). The acid insoluble material incorporated into membranes from cells grown in low K^+ (O — O or Δ — Δ) or controls (● — ● or $\blacktriangle$ — $\blacktriangle$) is plotted. Each point is the average of two determinations. The error bars are the ranges.

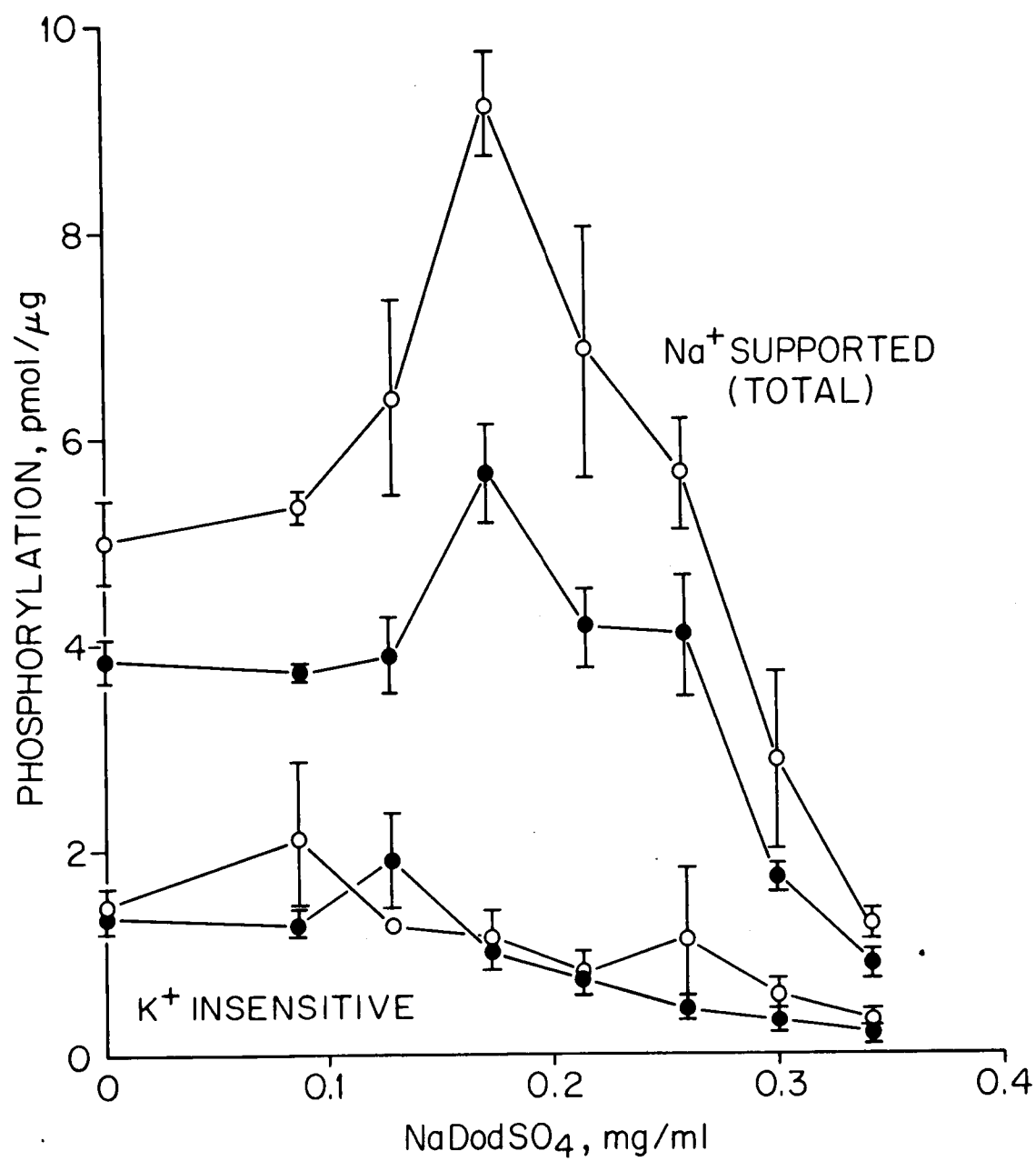


Figure 17.

K⁺-sensitive phosphorylation with [γ -³²P]ATP. In the range of 0.15-0.2 mg/ml, SDS increased the specific phosphorylation (difference between total and K⁺-insensitive) about 2-fold in the control cells, giving an amount about equal to that observed in membranes from the induced cells in the absence of detergent. However, the same detergent concentrations also increased the specific phosphorylation about 2-fold in membranes from the induced cells. These parallel increases in detergent activation suggest that the same proportion of cryptic sites is present in both control and induced cells and that physiological exposure of cryptic sites does not account for the induction.

In contrast to the K⁺-sensitive phosphorylation, the K⁺-insensitive phosphorylation was about the same in both control and induced cells and showed only inactivation with increasing concentrations of detergent (Figure 17).

At concentrations higher than 0.2 mg/ml, SDS also inactivated the K⁺-sensitive phosphorylation capacity of both control and induced cell membranes, again in parallel. The concentration of detergent that resulted in inactivation depended upon the concentration of protein in the reaction mixture although the concentration that activated the enzyme was independent of the protein concentration used (data not shown).

Transition from Induced to Control Levels of
Ouabain Binding on Resuspension in
Normal K⁺ (5.5 mM) Medium

Half-time for reversal was about 5 h. During the reversal a detectable decrease in the number of ouabain binding sites per cell was

evident one h after cells grown in low K^+ medium were resuspended in control medium (Figure 18). By 12 h, the number of sites had returned to the control level.

In Chapter II I demonstrated a 4-4.5 h transit time for the HeLa cell Na,K-ATPase from its cellular locus of synthesis to the surface membrane. If a decrease in the rate of synthesis of the enzyme were responsible for the reversal, a similar lag should be observed before ouabain binding decreased. The data in Figure 18 are taken from 6 repetitions of the experiment in which early (< 4 h) time points during the reversal were taken so as to define any lag that may have been present; none was observed. A model for induction and reversal incorporating these kinetics is given in the Discussion.

Na,K-ATPase Turnover in Induced Cells

The final question to be addressed in this dissertation is whether a decrease in the rate of removal of the Na,K-ATPase from the membrane (turnover) is responsible for the induction. This was examined directly by inducing cells for 45 hours in low K^+ - ^{13}C -substituted amino acid containing medium and then resuspending them in low K^+ - ^{12}C medium (chase). Throughout the chase the enzyme was essentially fully induced; i.e., after 44 hours in low K^+ - ^{13}C -medium, ouabain binding was 192% of controls, halfway through the chase it was 212% of controls, and at its termination it was 208% of controls. The generation time was 33 hours. During the chase, the decrease in density of the ^{13}C -substituted enzyme as it was replaced by enzyme of control density

Figure 18. Reversal of low K^+ -induced increase in ouabain binding per cell following resuspension of induced cells in control (5.5 mM K^+) medium. Cells were suspended in 0.5 mM K^+ or 5.5 mM K^+ for 45 hours and at various times after resuspension in 5.5 mM K^+ , the number of ouabain binding sites per cell was measured as described in Materials and Methods. Pooled data from 6 experiments. Numbers in parentheses describe the number of determinations at that point. Error bars are ± 1 S.E. The line was plotted according to Equation 10, with $k_r = 0.14 \text{ h}^{-1}$.

TRANSITION FROM INDUCED TO CONTROL LEVELS OF OUABAIN BINDING

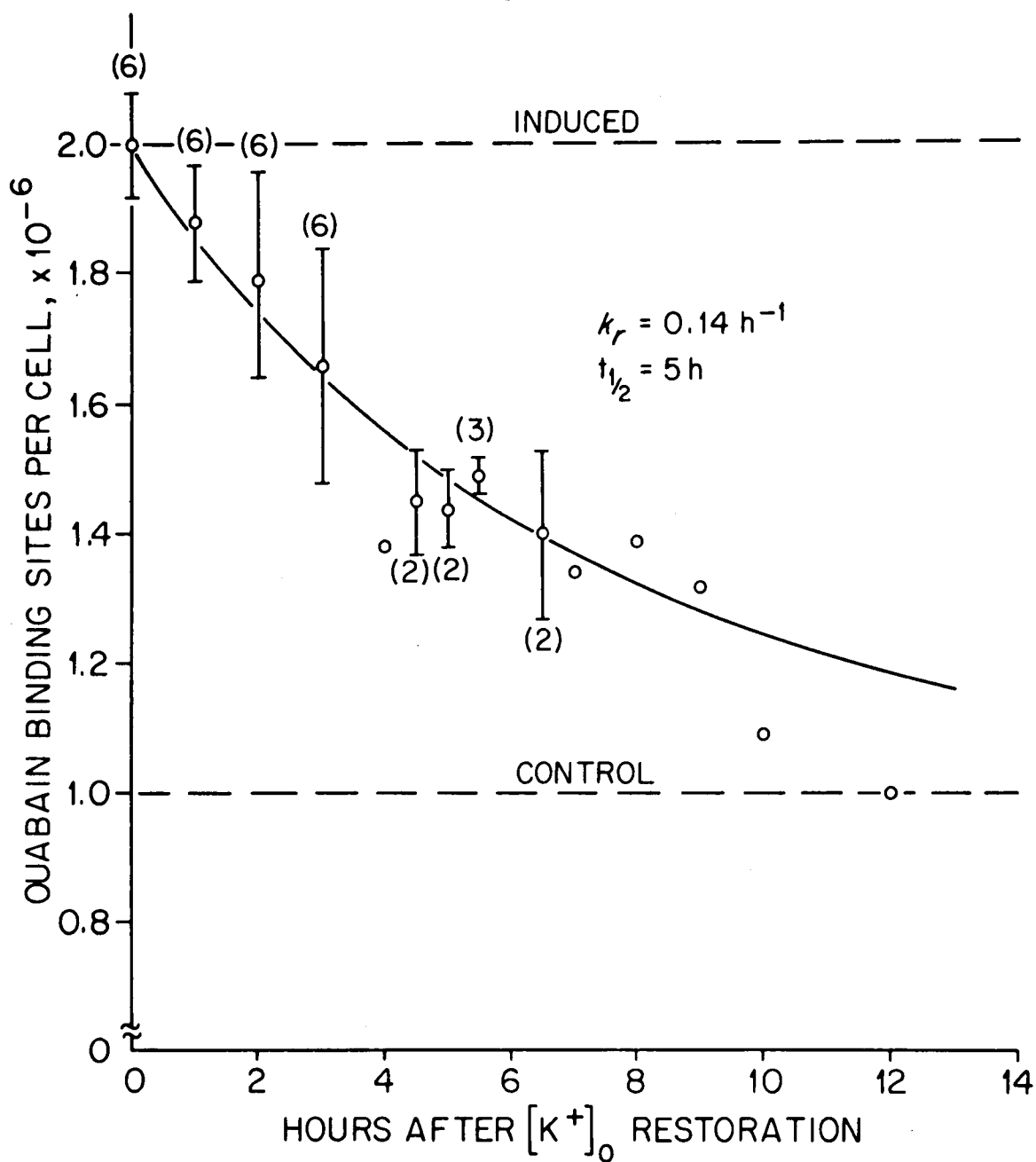


Figure 18.

was followed using the velocity sedimentation analysis described in Chapter II.

In the induced cells, the density shift decreased much more slowly than in controls (Figure 19). After correcting for the dilution of ^{13}C -enzyme due to growth the $t_{1/2}$ for turnover was $0.53 T_G$ (17.5 h) in induced cells as compared with $0.23 T_G$ (5.5 hours) in the faster growing controls. These correspond to rate constants of $1.3/T_G$ and $3.1/T_G$ respectively.

Discussion

The data given above resolve the major ambiguities cited in the Introduction and, in addition, add new information about the response of HeLa cells to the stress of K^+ -deprivation. I have confirmed the observation of Boardman et al. (1974, 1976) that growth of these cells in low- K^+ medium leads to an induction of Na^+, K^+ -transport system. I have now identified the induction by three criteria: (1) ouabain binding to the cell surface, (2) V_{max} of $^{86}\text{Rb}^+$ uptake, and (3) specific Na^+ -dependent, K^+ -sensitive phosphorylation in isolated plasma membrane ghosts. From other data (not shown) it was also found that the ouabain-sensitive Na^+, K^+ -ATPase activity was increased in isolated membranes from the induced cells. The increased K^+ -sensitive phosphorylation and Na^+, K^+ -ATPase activity in the plasma membrane fraction were quantitated in terms of membrane protein. These data exclude the possibility that the larger number of ouabain binding sites per cell is caused by an increase in the amount of plasma membrane per cell, as occurs in the induction of the avian salt gland Na, K -ATPase

Figure 19. Turnover of Na,K-ATPase in low K^+ medium. Cells grown 45 h in low K^+ - ^{13}C -substituted medium were resuspended in low K^+ - ^{12}C medium. At various times after resuspension, plasma membranes were isolated, phosphorylated with $[\gamma\text{-}^{32}P]\text{ATP}$ mixed with control membranes that had been specifically phosphorylated with $[\gamma\text{-}^{33}P]\text{ATP}$ and sedimented in a linear 11-26% metrizamide- D_2O -SDS gradient for 20 h at 50,000 rpm (SW 56 rotor). Both ^{13}C -substituted and control membranes were phosphorylated in the presence of 100 mM NaCl. About 100 μg of ^{13}C -substituted membrane (^{32}P) and 600 μg of control membrane were applied to each gradient. The density shifts for these cells $\blacksquare - \blacksquare$ and for controls ($0 - 0$, taken from Figure 6, page 31) are plotted as functions of their generation times, which were 33 h and 24 h respectively.

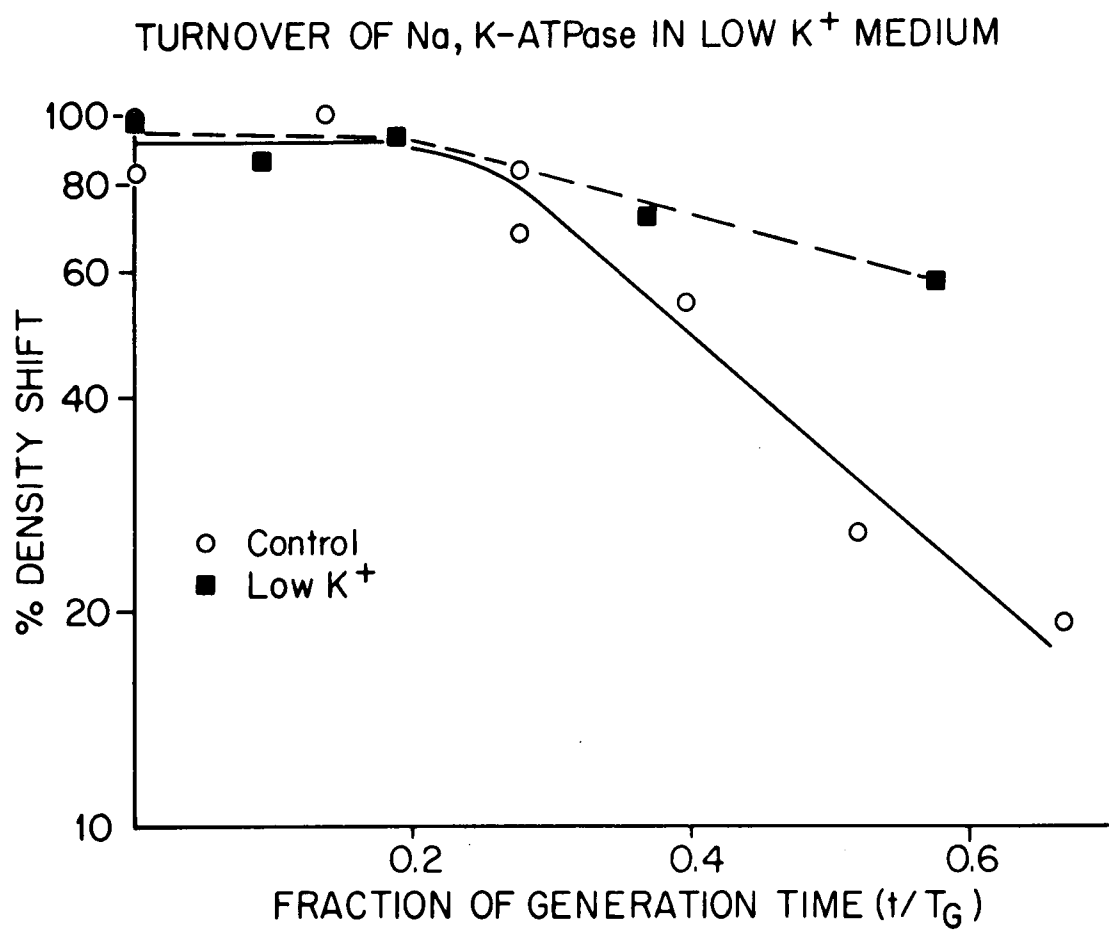


Figure 19.

(Stewart et al., 1976). The specificity of the induction is seen in the unaltered 5'-AMPase activity and K^+ -insensitive phosphorylation.

Transport Capacity of the Induced Ouabain-Binding Sites

A major ambiguity that I sought to resolve was whether the induced sites were capable of the same transport as the control sites. Boardman et al. (1974) calculated that after K^+ -deprivation had led to a doubling of ouabain binding in a pulse label, there was only a 23% increase in transport in their standard assay. Their calculation was based on a model of Na^+, K^+ -ATPase activity by Caldwell (1970) and was an indirect estimate that allowed for the calculated changes in $[Na^+]_i$. I avoided this indirect estimate by cold treatment of the cells which allowed $[Na^+]_i$ to accumulate and stimulate the transport system to V_{max} during the assay. Under these conditions the increase in V_{max} was quantitatively equivalent and temporally parallel to the changes in ouabain binding. The V_{max} per ouabain-binding site remained constant throughout, indicating that the induced binding sites were as capable of transport as those of the controls. This conclusion was strengthened by the observation that Na^+ -dependent, K^+ -sensitive phosphorylation also corresponded quantitatively with ouabain binding.

Nature of the Induction

The constancy of the V_{max} per ouabain binding site indicated that an increase in the amount of functional Na,K-ATPase in the plasma membrane rather than an increase in the maximum activity per transporter

is responsible for the induction. Three other possible causes of the induction are discussed below.

1. Exposure of cryptic transporters: It is known that detergent treatment of membrane-bound Na^+, K^+ -ATPase can increase its activity 2-8-fold (Charnock and Post, 1963; Jørgensen and Skou, 1971; Hansen et al., 1979; inter alia). Hansen et al. (1979) reported that SDS treatment doubled ATP-supported ouabain binding to pig kidney microsomes without affecting P_i - or vanadate-supported binding. They proposed that SDS induced a relaxation of total cooperativity between pairs of nucleotide binding sites on the subunits of Na^+, K^+ -ATPase and suggested that these changes might be duplicated in vivo by some as yet unknown physiological mechanism. It was conceivable that detergent activation might reflect the exposure of cryptic transporters that were already present in the cells, and that the induction observed might reflect the physiological exposure of such transporters. However, the specific phosphorylation of membranes from induced cells was increased by SDS to the same extent as in the controls. Therefore, the induction does not appear to be caused by such a mechanism.

2. Alterations in synthesis: In the course of normal turnover of Na^+, K^+ -ATPase in control cells there is a transit time of 4-4.5 h for the enzyme to traverse from its cellular site of synthesis to the surface membrane (Chapter II). Fully induced cells are similarly in a steady-state of turnover that is maintaining a high level of enzyme in the membrane. If the mechanism of reversal following restoration of

$[K^+]_0$ is the slowing or cessation of synthesis, reversal should not be detectable at the cell surface until the transit time of already synthesized enzyme is complete. This is clearly not the case (Figure 18, p. 88). After $[K^+]_0$ is restored, the number of pulse-labeled ouabain-binding sites begins to fall immediately. This result suggests that either there is no transit time in the induced cells, which seems very unlikely, or that some posttranslational event is the primary factor in regulating the prompt reversal.

3. Alterations in degradation: All of the observed variations in the amount of Na,K-ATPase during the induction and its reversal can be accounted for by alterations in its degradation rate. Changes in enzyme levels can be described by a simplified relationship between synthesis and degradation (Segal and Kim, 1963; Berlin and Schimke, 1965; Price et al., 1962):

$$\frac{dE}{dt} = k_s - k_d E \quad (8)$$

where E is the amount of enzyme, k_s is a zero-order rate constant of synthesis, and k_d is a first-order rate constant for degradation. The implications of such a model have been discussed by Berlin and Schimke (1965), and this discussion is based on these theoretical considerations. In the steady state, i.e., when $dE/dt = 0$,

$$E = \frac{k_s}{k_d} \quad (9)$$

In steady state control cells there are approximately 10^6 Na,K-ATPase molecules per cell as measured by ouabain binding (Will et al., 1977)

and from the density labeling analysis, $k_d = 0.13h^{-1}$. Therefore, from equation 9, about 2100 Na,K-ATPase molecules are synthesized per cell per minute for turnover. From the generation time it can be calculated that an additional 700 molecules per cell per minute are synthesized for growth.

During growth in low K^+ , there appears to be a lag of several hours before an increase in ouabain binding is observed (Boardman et al., 1974; Cook et al., 1975). The stimulus which triggers the induction may be the slow fall in $[K^+]_i$ (Figure 12a, p. 69). Boardman et al. (1974) have suggested that the concomitant increase in $[Na^+]_i$ is the stimulus. Whether the stimulus is cumulative or requires a threshold to be reached is also unknown. If cumulative, the induction may proceed slowly at first and then accelerate to the maximum rate observed (see Figure 12a, p. 69). Whether or not a step requiring protein synthesis must occur during the lag is also unknown. This lag makes it difficult to precisely estimate the half-time for the transition to the induced state; however, the induction is complete after 24-48 h (Figure 12, p. 69 and data not shown). Assuming a 4-8 h lag (Boardman et al., 1974; Cook et al., 1975; Figure 12, p. 69) I estimate a half-time for induction of 10-20 h.

The time taken for an enzyme to increase or decrease to one-half of the final change to a new steady state amount is equal to its $t_{1/2}$ for degradation (Berlin and Schimke, 1965). Therefore, it is possible experimentally to estimate the half-life of any enzyme by following its time course to a new steady state. By this method, I estimate that during the induction the $t_{1/2}$ for degradation of the Na,K-ATPase is 10-20 h, which corresponds to a $k_d = 0.035-0.059 h^{-1}$. Using this k_d and

assuming that synthesis has not changed, equation 9 predicts that there will be between 1.8×10^6 and 3.6×10^6 Na,K-ATPase molecules per induced cell. This value agrees with the $2-3 \times 10^6$ Na,K-ATPase molecules per induced cell measured by ouabain binding.

I directly measured the steady state rate of removal of Na,K-ATPase from the membrane in both control and induced cells by the density labeling method. The half-times for turnover were $0.53 \text{ } T_G$ or 17.5 h in the induced cells and $0.24 \text{ } T_G$ or 5.5 h in the controls. These correspond to rate constants of $1.3/T_G$ (0.04 h^{-1}) and $3.1/T_G$ (0.13 h^{-1}) respectively. This represents a 2.2-fold decrease in degradation in the induced cells when expressed in terms of the generation time and a 3.2-fold decrease in terms of hours. It is clear from equation 9 that the decrease in the rate constant for degradation in the induced cells, expressed in terms of either the generation time or hours, is sufficient to account for the induction. It is not clear that turnover of the Na,K-ATPase is necessarily a function of the generation time. This point is worth testing since it leaves open the possibility that the decreased $t_{1/2}$ for turnover of the Na,K-ATPase in the induced cells is due to a change in their generation time and is unrelated to the induction.

From the 2.2-fold decrease in terms of the generation time and assuming k_s is constant, equation 9 predicts 2.2×10^6 Na,K-ATPase molecules per cell. Alternatively, assuming 2×10^6 molecules of Na,K-ATPase per cell, one can calculate a k_s of about 2000 molecules per cell per minute, which is very similar to the control value.

A similar analysis using the 3.2-fold decrease in k_d in terms of hours predicts 3.2×10^6 molecules per cell and a k_s of 1300 molecules per cell per minute in the induced cells. This later k_s is less than the control value and suggests that the rate of Na,K-ATPase synthesis may be related to the generation time.

The rapid recovery of $[K^+]_i$ upon warming cells incubated in the cold (Figure 13, p. 73, bottom right) indicates that when induced cells are resuspended in 5.5 mM K^+ medium, $[K^+]_i$ and $[Na^+]_i$ recover quickly. These changes could rapidly trigger the reversal, which occurred without a detectable lag (Figure 18, p. 88).

The time course for the reversal can be described by an equation for the time course of approach of an enzyme to a new steady state (Berlin and Schimke, 1965):

$$\ln \left(\frac{\frac{E_t}{E_i} - \frac{E_c}{E_c}}{\frac{E_i}{E_i} - \frac{E_c}{E_c}} \right) = -k_r t \quad (10)$$

where E_t is the amount of enzyme during the transition, E_c is the control amount of enzyme, E_i is the induced amount, and k_r is a first-order rate constant for the transition (calculated from the half-time for reversal and from equation 7, p. 33) which is equal to k_d the first-order rate constant for degradation of the enzyme. From Figure 18, page 88, the half-time for reversal and therefore for turnover of the enzyme during the reversal is 5 h.

I conclude that during growth in low K^+ medium degradation of Na,K-ATPase decreases and thereby causes a slow induction of the enzyme. During the reversal, degradation increases to the control rate of 5-6 hours, rapidly decreasing the amount of enzyme to the control level.

The half-times for degradation of the Na,K-ATPase as determined from the time courses of the induction and its reversal agree with, and therefore confirm, those measured directly by the density labeling method. These data also correspond to the half-time for internalization of ouabain in control cells (Will et al., 1977), and therefore leave little doubt that the normal half-time for turnover of the Na,K-ATPase in HeLa cells is 5-6 h.

Short-Term vs Long-Term Regulation

The standard assay for $^{86}\text{Rb}^+$ transport was performed in 5.5 mM $[\text{K}^+]_o$, a concentration that virtually saturates the Na^+, K^+ -ATPase from the extracellular side. The measurement gives the capacity of the cells to accumulate K^+ in any physiological state, and is dependent on both the numbers of transporters and the concentration of $[\text{Na}^+]_i$ which is stimulating the transport system from the cytoplasmic side. The fact that preloading control cells with $[\text{Na}^+]_i$ doubles the uptake with respect to the standard assay suggests that $[\text{Na}^+]_i$ is normally near its K_m value (Figure 14, p. 77).

The short-term response to K^+ -deprivation is a fall in $[\text{K}^+]_i$ and an increase in the standard $^{86}\text{Rb}^+$ flux with very little change in numbers of transporters (Figure 13, p. 73). This response appears to be the result of increased $[\text{Na}^+]_i$, and the system now functions at about 80% of V_{max} . This response is adequate to keep the cells alive and growing in low $[\text{K}^+]_o$, but in a regulatory sense the transport system has less tolerance than before because it is operating near the maximum value

of the intracellular Na^+ site. In the long-term response, due to a decrease in the enzyme's degradation rate during turnover, new functional transporters are induced in the cell surface, $[\text{K}^+]_i$ rises slightly, and the transport per site falls to about 65% of V_{max} , thus restoring some of the flexibility of the system. On reversal of the induced cells by restoration of 5 mM $[\text{K}^+]_o$, the relatively large number of transporters, now maximally stimulated by $[\text{K}^+]_o$, rapidly depletes the cells of $[\text{Na}^+]_i$ to a level below its K_m , transport-per site falls below the control level, and the half-time for turnover of the enzyme returns to the control degradation rate. As the number of transporters falls over the next several hours, the cells return to the control status quo. This interplay of the short-term and long-term responses provides the cells with sufficient K^+ (and Na^+) for maintenance of nearly normal volumes with nearly normal growth rates (Ledbetter and Lubin, 1977).

LITERATURE CITED

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- Addis, J. S., Mazurkiewicz, J. E., and Barnett, R. J. (1979) J. Cell Biol. 83, 273a.
- Akera, T. (1977) Science 198, 569-574.
- Amenta, J. S., Sargus, M. J. and Baccino, F. M. (1977) Biochem. J. 168, 223-227.
- Atkinson, P. H. (1973) in Methods in Cell Biology 7, D. M. Prescott, ed., Academic Press, N. Y., 152-188.
- Atkinson, P. H. (1975) J. Biol. Chem. 250, 2123-2134.
- Atkinson, P. H. (1978) J. Supramol. Struct. 8, 89-109.
- Avruch, J., and Wallach, D. F. H. (1971) Biochem. Biophys. Acta 233, 334-337.
- Baker, P. F. (1972) in Metabolic Transport, Volume VI, L. E. Hokin, ed., Academic Press, N. Y., 243-269.
- Baker, P. F. and Willis, J. S. (1970) Nature 226, 521-523.
- Baker, R. M. (1976) in Biogenesis and Turnover of Membrane Macromolecules, J. S. Cook, ed., Raven Press, N. Y., 93-104.
- Ballard, J. (1977) Essays in Biochem. 13, 1-37.
- Barrett, A. J. (1972) in Lysosomes: A Laboratory Handbook, J. T. Dingle, ed., American Elsevier, New York, 46-135.
- Berlin, C. M. and Schimke, R. T. (1965) Mol. Pharmacol. 1, 149-156.
- Boardman, L., Huett, M., Lamb, J. F., Newton, J. P., and Polson, J. M. (1974) J. Physiol. (Lond.) 241, 771-794.
- Boardman, L., Hume, S. P., Lamb, J. F., McCall, D., Newton, J. P. and Polson, J. M. (1976) in Developmental and Physiological Correlates of Cardiac Muscle, M. Lieberman and T. Sano, eds., Raven Press, New York, 127-138.
- Brake, E. T., Will, P. C., and Cook, J. S. (1978) Membrane Biochem. 2, 17-46.

- Breslow, J. L., Epstein, J., Vayo, M. M., and Fontaine, J. H. (1977) Exp. Cell. Res. 110, 399-407.
- Brostrom, C. O. and Jeffay, H. (1970) J. Biol. Chem. 245, 4001-4008.
- Caldwell, P. C. (1970) in Membranes and Ion Transport, E. E. Bittar, ed., Wiley Interscience, New York, 433-462.
- Carbonetto, S., and Fambrough, D. M. (1979) J. Cell Biol. 81, 555-569.
- Chan, P. C. and Sanslone, W. R. (1969) Arch. Biochem. Biophys. 134, 48-52.
- Charnock, J. S. and Post, R. L. (1963) Austral. J. Exp. Biol. 41, 547-560.
- Christopher, C. W. (1977) J. Supramolec. Struct. 6, 315-324.
- Christopher, C. W., Colby, W. W. and Ullrey, D. (1976a) J. Cell Physiol. 89, 683-692.
- Christopher, C. W., Ullrey, D., Colby, W. and Kalckar, H. (1976b) Proc. Natl. Acad. Sci. USA 73, 2429-2433.
- Churchill, L., and Hokin, L. E. (1979) J. Biol. Chem. 254, 7388-7392.
- Churchill, L., Peterson, G. L. and Hokin, L. E. (1979) Biochem. Biophys. Res. Commun. 90, 488-490.
- Constantino-Ceccarini, E., Novikoff, P. M., Atkinson, P. H., and Novikoff, A. B. (1978) J. Cell Biol. 77, 488-463.
- 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, pp. 343-351.
- Cook, J. S., Vaughan, G. L., Proctor, W. R. and Brake, E. T. (1975) J. Cell Physiol. 86, 59-70.
- 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.
- Cuff, J. M and Lichtman, M. A. (1975) J. Cell Physiol. 85, 217-226.
- Cunningham, D. D. and Pardee, A. B. (1969) Proc. Natl. Acad. Sci. USA 64, 1049-1056.
- Czajka, D. M., Finkel, A. J., Fischer, C. S. and Katz, J. J. (1961) Amer. J. Physiol. 201, 357-362.

- Dehlinger, P. J. and Schimke, R. T. (1971) J. Biol. Chem. 246, 2574-2583.
- Devreotes, P. N. and Fambrough, D. M. (1976) Proc. Natl. Acad. Sci. USA 73, 161-164.
- Devreotes, P. N., Gardner, J. M. and Fambrough, D. M. (1977) Cell 10, 365-373.
- Dixon, J. F. and Hokin, L. E. (1974) Arch. Biochem. Biophys. 163, 749-758.
- Drachman, D. B., Angus, C. W., Adams, R. N. and Kao, I. (1978) Proc. Natl. Acad. Sci. USA 75, 3422-3426.
- Dunn, M. J. (1968) J. Clin. Invest. 48, 674-684.
- Eagle, H. (1955) Science 122, 501-504.
- Edelson, P. J., and Cohn, Z. A. (1976) J. Exp. Med. 144, 1581-1595.
- Eisenman, R. and Vogt, V. M. (1978) Biochim. Biophys. Acta 473, 187-239.
- Epstein, J. and Breslow, J. L. (1977) Proc. Natl. Acad. Sci. USA 74, 1676-1679.
- Ernster, L., Zetterstrom, R. and Lindberg, O. (1950) Acta Chem. Scand. 4, 942-951.
- Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606-2617.
- Filner, P. and Varner, J. E. (1967) Proc. Natl. Acad. Sci. USA 58, 1520-1526.
- Finkel, A. J. (1974) in Proceedings of the First International Conference on Stable Isotopes in Chemistry, Biology, and Medicine. P. D. Klein and S. V. Peterson, eds., CONF-730525, NTIS, Springfield, VA, 187-195.
- Forbush, B. and Hoffman, J. F. (1979) Biochemistry 18, 2308-2315.
- Foster, D. O and Pardee, A. B. (1969) J. Biol. Chem. 244, 2675-2681.
- Frengley, P. A., Peck, W. A. and Lichtman, M. A. (1974) Exp. Cell Res. 88, 442-444.
- Gardner, J. M. and Fambrough, D. M. (1979) Cell 16, 661-674.
- Gargus, J. J., Miller, I. L., Slayman, C. W. and Adelberg, E. (1978) Proc. Natl. Acad. Sci. USA 75, 5589-5593.

- Giotta, G. J. (1976) J. Biol. Chem. 251, 1247-1252.
- Glynn, I. M. (1968) Br. Med. Bull. 24, 165-169.
- Glynn, I. M. and Karlsh, S. J. D. (1975) Ann. Rev. Physiol. 37, 13-55.
- Gregg, C. T., Hutson, J. Y., Prine, J. R., Ott, D. G. and Furehner, J. E. (1973) Life Sci. 13, 775-782.
- Hansen, O., Jensen, J., Nørby, J. G. and Ottolenghi, P. (1979) Nature 280, 410-412.
- Hastings, D. F. and Reynolds, J. A. (1979) Biochemistry 18, 817-821.
- Hatanaka, M. (1974) Biochim. Biophys. Acta 355, 77-104.
- Heaton, J. H. and Gelehrter, T. D. (1977) J. Biol. Chem. 252, 2900-2907.
- Hegyvary, C. (1975) Molec. Pharmacol. 11, 588-594.
- Hilden, S. and Hokin, L. E. (1975) J. Biol. Chem. 250, 4022-4024.
- Hokin, L. E. (1974) Ann. New York Acad. Sci. 242, 36-52.
- 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. Chem. 251, 4365-4371.
- Horst, M. N. and Roberts, R. M. (1979) J. Biol. Chem. 254, 5000-5007.
- Huang, C. C., Tgai, C. M. and Canellakis, E. S. (1973) Biochim. Biophys. Acta 332, 59-68.
- Hubbard, A. L., and Cohn, Z. A. (1975) J. Cell Biol. 64, 461-479.
- Hudson, J. E. and Johnson, T. C. (1977) Biochem. J. 166, 217-223.
- Hume, S. and Lamb, J. F. (1974) J. Physiol. (Lond.) 239, 46P-47P.
- Hüttermann, A. and Guntermann, U. (1975) Anal. Biochem. 64, 360-366.
- Hüttermann, A. and Wendlberger, G. (1976) in Methods in Cell Biology, D. M. Prescott, ed., Academic Press, New York, 153-170.
- Ismail-Beigi, F. and Edelman, I. S. (1973) Am. J. Physiol. 225, 1172-1177.
- Isselbacher, K. J. (1972) Proc. Natl. Acad. Sci. USA 69, 585-589.

- Jørgensen, P. L. (1972) J. of Steroid Biochem. 3, 181-191.
- Jørgensen, P. L. (1974) Ann. N. Y. Acad. Sci. 242, 36-52.
- Jørgensen, P. L. and Skou, J. C. (1971) Biochim. Biophys. Acta 233, 366-380.
- Jørgensen, P. L., Skou, J. C. and Solomonson, L. P. (1971) Biochim. Biophys. Acta 233, 381-394.
- Kalckar, H. M. and Ullrey, D. (1973) Proc. Natl. Acad. Sci. USA 70, 2502-2504.
- Kaplan, G., Unkeless, J. C. and Cohn, Z. A. (1979) Proc. Natl. Acad. Sci. USA 76, 3824-3828.
- Kaplan, J. G. (1978) Ann. Rev. Physiol. 40, 19-41.
- Kaplan, J. and Moskowitz, M. (1975) Biochim. Biophys. Acta 389, 306-313.
- Kenney, F. T. (1976) Science 156, 525-528.
- Kletzien, R. F. and Perdue, J. F. (1975) J. Biol. Chem. 250, 593-600.
- Knox, W. H., Perrin, R. G. and Sen, A. K. (1972) J. Neurochem. 19, 2881-2884.
- Kyte, J. (1972) J. Biol. Chem. 247, 7642-7649.
- Laemmli, U. K. (1970) Nature 227, 680-685.
- Ledbetter, M. L. S. and Lubin, M. (1977) Exp. Cell Res. 105, 223-236.
- Lin, M. H. and Akera, T. (1978) J. Biol. Chem. 233, 723-726.
- Lindsay, R. (1974a) J. Int. Med. Res. 5, 331-337.
- Lindsay, R. (1974b) Clin. Sci. Mol. Med. 47, 639-642.
- Lo, C. S., August, T. R., Liberman, U. A. and Edelman, I. S. (1976) J. Biol. Chem. 251, 7826-7833.
- Lo, C. S. and Edelman, I. S. (1976) J. Biol. Chem. 251, 7834-7840.
- Mans, R. J. and Novelli, G. D. (1961) Arch. Biochem. Biophys. 94, 48-53.
- Martineau, R., Kohlbacher, M., Shaw, S. N. and Amos, H. (1972) Proc. Natl. Acad. Sci. USA 69, 3407-3411.

- Merlie, J., Changeux, J. P. and Gros, F. (1978) J. Biol. Chem. 253, 2882-2891.
- Merlie, J., Heinemann, S., Einarson, B. and Lindstrom, J. M. (1979) J. Biol. Chem. 254, 6328-6332.
- Munthe-Kaas, A. C. and Seglen, P. O. (1974) FEBS Lett. 43, 252-256.
- Nakao, M., Nakao, T., Hara, Y., Nagai, F., Yagasaki, S., Koi, M. and Kawai, K. (1974) Ann. N. Y. Acad. Sci. 242, 24-35.
- Nilsen-Hamilton, M. and Hamilton, R. T. (1979) Biochim. Biophys. Acta 588, 322-331.
- Olden, K., Pratt, R. M., Jaworski, C. and Yamada, K. M. (1979) Proc. Natl. Acad. Sci. USA 76, 791-795.
- Olden, K., Pratt, R. M. and Yamada, K. M. (1978) Cell 13, 461-473.
- Peterson, G. L., Ewing, R. D. and Conte, F. P. (1978) Dev. Biol. 67, 90-98.
- Peterson, G. L., Ewing, R. D., Hootman, S. R. and Conte, F. P. (1978) J. Biol. Chem. 253, 4762-4770.
- Pollack, L. R. and Cook, J. S. (1978) J. Gen. Physiol. 72, 6a-7a.
- Post, R. L. (1974) Ann. N. Y. Acad. Sci. 242, 6-11.
- Post, R. L. and Kume, S. (1973) J. Biol. Chem. 248, 6993-7000.
- Post, R. L., Merritt, C. R., Kinsolving, C. R. and Albright, C. D. (1960) J. Biol. Chem. 235, 1796-1802.
- Post, R. L. and Sen, A. K. (1976) Methods Enzymol. 10, 773-776.
- Post, R. L., Sen, A. K. and Rosenthal, A. S. (1965) J. Biol. Chem. 240, 1437-1445.
- Price, V. E., Sterling, W. R., Tarantola, V. A., Hartley, R. W., Jr., and Rechcigl, M., Jr. (1962) J. Biol. Chem. 237, 3468-3475.
- Proctor, W. (1974) Ph.D. Thesis, University of Tennessee.
- Puck, T. T., Marcus, P. I. and Cieciura, J. (1956) J. Exp. Med. 103, 273-284.
- Quastel, M. R., Dow, D. S. and Kaplan, J. G. (1970) in Prog. 5th Leucocyte Culture Conference, J. E. Harris, ed., Academic Press, New York, 94-123.

- Reed, B. C. and Lane, M. D. (1980) Proc. Natl. Acad. Sci. USA 77, 285-289.
- Rickwood, D. and Birnie, G. O. (1975) FEBS Letts. 50, 102-110.
- Robinson, J. D. and Flashner, M. S. (1979) Biochim. Biophys. Acta 549, 145-176.
- Rothstein, E. L., Hartzell, R. W., Jr., Manson, L. A. and Kritchevsky, D. (1960) Ann. N. Y. Acad. Sci. 84, 721-726.
- Salter, D. W. and Cook, J. S. (1976) J. Cell Physiol. 89, 143-156.
- Schmidt-Ullrich, R., Wallach, D. F. H. and Ferber, E. (1974) Biochim. Biophys. Acta 356, 288-299.
- Schneider, E. L., Stanbridge, E. J. and Epstein, C. J. (1974) Exp. Cell Res. 84, 311-318.
- Schwartz, A., Lindenmayer, G. E. and Allen, J. C. (1975) Pharmacol. Rev. 27, 3-134.
- Segal, H. L. and Kim, Y. S. (1963) Proc. Natl. Acad. Sci. 50, 912-918.
- Shohet, S. B. (1973) in Erythrocytes, Thrombocytes, Leukocytes, E. Gerlach, K. Moser, E. Deutsche and W. Wilmanns, eds., Georg Thieme, Stuttgart, 115-118.
- Simpson, M. W. (1953) J. Biol. Chem. 201, 143-154.
- Skou, J. C. (1957) Biochim. Biophys. Acta 23, 394-401.
- Smith, E. K. M. and Samuel, P. D. (1970) Clin. Sci. 38, 49-61.
- Smith, L. (1955) Methods Enzymol. 2, 732-740.
- Stewart, O. J., Semple, E. W., Swart, G. T. and Sen, A. K. (1976) Biochim. Biophys. Acta 419, 150-163.
- Strehler, B. L. (1974) in Methods of Enzymatic Analysis, 2nd edition, H. U. Bergmeyer, ed., Academic Press, New York, 2112-2126.
- Sweadner, K. J. and Goldin, S. M. (1980) New Eng. J. Med. 302, 777-783.
- Tanabe, T., Pricer, W. E., Jr., and Ashwell, G. (1979) J. Biol. Chem. 254, 1038-1043.
- Tate, E. T. and Cook, J. S. (1980) Submitted.

- Tell, G. P., Haur, F. and Saez, J. (1978) Metab. Clin. Exp. 27, 1566-1592.
- Tenner, A. J. and Wallace, R. A. (1972) Biochem. Biophys. Acta 276, 416-424.
- Tramacer, M., Borghett, A. F. and Guidotti, G. G. (1977) J. Cell Physiol. 93, 425-434.
- Tweto, J. and Doyle, D. (1976) J. Biol. Chem. 251, 872-882.
- Vaughan, G. L. and Cook, J. S. (1972) Proc. Natl. Acad. Sci. USA 69, 2627-2631.
- Vitetto, E. S. and Uhr, J. W. (1974) J. Exp. Med. 139, 1599-1620.
- Wallace, R. A. (1964) Biochem. Biophys. Acta 86, 286-294.
- Wallick, E. T., Lane, L. K. and Schwartz, A. (1979) Ann. Rev. Physiol. 41, 397-411.
- Welt, L. G., Sachs, J. R. and McManus, T. J. (1964) Trans. Ass. Am. Physns. 77, 169-174.
- Welt, L. G., Smith, E. K. M., Dunn, M., Czerwinski, A. W., Proctor, H., Cole, C., Balfe, J. W. and Gitelman, H. J. (1967) Trans Ass. Am. Physns. 80, 217-226.
- Wiley, J. S. (1969) Nature 221, 1222-1224.
- Will, P. C., Longworth, J. W., Brake, E. T. and Cook, J. S. (1977) Mol. Pharmacol. 13, 161-177.

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