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Evidence for an ambidirectional magnesium-activated, sodium-potassium dependent adenosine triphosphatase

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EVIDENCE FOR AN AMBIDIRECTIONAL MAGNESIUM-ACTIVATED, SODIUM-POTASSIUM DEPENDENT ADENOSINE TRIPHOSPHATASE

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ABSTRACT

Controversy exists as to whether the Na-K ATPases of euryhaline fishes are the same enzyme. This study demonstrates that the phosphorylated enzyme from gills of Fundulus heteroclitus adapted to freshwater and salt water migrate to the same position in SDS polyacrylamide gels. Similar behavior of the enzyme from both sources in different ionic media is also shown. Additionally, the possible ability of the enzyme to couple an osmoregulatory influx to an excretory efflux in freshwater fish is demonstrated. The term "ambidirectional" is proposed for this enzyme, and its place in certain membrane models is discussed.

INTRODUCTION

The presence of a Mg^{++} activated, (Na^+-K^+)-dependent adenosine triphosphatase (Na-K ATPase) inhibited by ouabain has been described in the microsomal fraction of tissues from numerous species: rabbit kidney (Post, 1968), cat brain and eel electric organ (Siegel et al., 1969), beef heart (Albers, 1969), human erythrocyte (Avruch and Fairbanks, 1972), and frog skin (Boonkoom and Alvarado, 1971), etc. The presence of this enzyme in the gill of a diverse array of fish is also well documented (Jampol and Epstein, 1970 and Maetz, 1970).

Evidence suggests that this enzyme participates in the active transport of Na^+ and K^+ across cell membranes. The mechanism involves Na^+-dependent phosphorylation of the enzyme by ATP and K^+-dependent dephosphorylation (Skou, 1965). Evidence supporting the conclusion that this enzyme serves an osmoregulatory function in fish has been well summarized by Maetz (1971), Motais and Garcia-Romeu (1972), and Hochachka and Somero (1973).

In salt water, fish passively lose water to the hypertonic environment. The consequences of this are amplified by an inflow of Na^+ and other ions. To recoup

the water loss, fish swallow salt water, absorbing water and ions in the intestine and excrete the excess ion load primarily across the gill membrane by way of the Na-K ATPase (Maetz, 1971).

In freshwater the ionic and osmotic gradients reverse. Usually drinking stops and copious amounts of dilute urine are excreted. The fish must also compensate for passive ion losses. The Na-K ATPase may be responsible for this by reversing the direction of its flux, pumping sodium ions in and releasing potassium ions to the external environment as the exchange ion (Maetz, 1971). As potassium is also in lower concentrations externally, it is to the advantage of the fish to exchange another monovalent cation in place of K^+. The ammonium ion appears to substitute for potassium in in vitro ATPase preparations from gills of Fundulus heteroclitus, the common killifish (Towle, unpublished). Maetz (1971) summarizes in vivo arguments which both support and contradict a Na^+/NH_4^+ exchange in species other than F. heteroclitus. Since fish produce ammonia as a metabolic waste product, this ion is perfectly suited for the freshwater requirements of the fish by coupling an osmoregulatory influx to an excretory efflux.

Euryhaline fish — those that can survive abrupt

changes from salt water to freshwater and vice versa — must suddenly and rapidly reverse these adaptive mechanisms to survive. The specific activity of the gill Na-K ATPase changes when such fish are transferred from one environmental salinity to another (Epstein, Katz and Pickford, 1967). When the euryhaline teleost F. heteroclitus is transferred from freshwater to 17°/oo salt water, the specific activity of the Na-K ATPase declines to one-half the freshwater level within 30 minutes (Gilman, 1973). When transferred to 30°/oo salt water, the specific activity doubles within the same time period (Towle, unpublished).

The question exists as to whether one bifunctional Na-K ATPase capable of exchanging Na^+ for NH_4^+, or two unifunctional Na-K and Na-NH_4 ATPases exist in F. heteroclitus as well as other euryhaline fishes. Hammit (1972) has indicated the appearance of a gill protein common to salt water adapted members of this species, as well as one common to freshwater adapted examples. Evidence from euryhaline Anguilla anguilla (European eel) on the basis of differential inhibition of ATPase by actinomycin D in freshwater and salt water suggests that different enzymes are present in fishes of the two environments (Motais, 1970). Pfeiler and Kirschner (1972) propose on the basis of kinetic data from fresh-

water and salt water Salmo gairdneri (rainbow trout) that separate ATPases exist for the two extreme environments. Conte and Morita (1968) have shown that several new protein components appear in the gills of euryhaline Onchorhynchus tshawytscha (chinook salmon) upon transfer from one environment to the other.

In light of these findings, two explanations for euryhalinity are apparent: 1) a new enzyme is rapidly synthesized de novo upon transfer from freshwater to salt water and vice versa; or 2) the freshwater ATPase is modified to become the salt water enzyme and vice versa. The position of most fish researchers in this area is that Na-K ATPases in freshwater and salt water are different.

The Na-K ATPases from freshwater and salt water F. heteroclitus behave similarly to each other and to other Na-K ATPases from other tissues on the basis of inhibition of activity by ouabain. Phosphorylated peptides from ATPases of many diverse organs and species migrate to the same approximate position in paper electrophoresis (Bader, Post and Bond, 1968). The implied but usually unstated position of most non-fish oriented researchers is that the enzyme is the same regardless of the source.

Inasmuch as this area is occluded by conflicting

data and opinion, this study was conducted to compare the freshwater and salt water Na-K ATPases from gills of F. heteroclitus to determine if they are the same enzyme on the basis of polyacrylamide gel electrophoresis of ^{32}P-labelled intermediates.

MATERIALS AND METHODS

Adult male F. heteroclitus were collected by seine on March 6, 1973 in Mobjack Bay at Severn, Virginia. Fish were maintained in either dechlorinated tap water or 30$^{o}/_{oo}$ Instant Ocean (Aquarium Systems, Inc., East Lake, Ohio) in 300 liter Living Stream tanks (Frigid Units, Toledo, Ohio) at a constant temperature of 18 C. Fish were fed commercially prepared fish food (TetraMin) daily. A twelve hour photoperiod was maintained. Fish were acclimated to handling for several weeks prior to sacrificing.

Preparation of gill microsomes

Fish were removed from the water and decapitated immediately. Gills were excised and washed in homogenization solution containing 0.25 M sucrose, 6 mM ethylenediamine tetraacetic acid (EDTA), and 20 mM imidazole to remove clotted blood. Gill tissue was surgically separated

over ice from the gill arches. (Approximately one gram of tissue was gathered at a time usually requiring eight fish.) A 5% (w/v) tissue homogenate was prepared in the homogenization solution to which had been added 0.1% deoxycholic acid immediately before use. Homogenization was carried out in a 30 ml Potter-Elvehjem homogenizer with a smooth Teflon pestle. The homogenate was filtered through two layers of cheesecloth and centrifuged at 10,000 x g for 35 min at 0 C to remove mitochondria, nuclei, unbroken cells and other debris. The supernatant was then removed and recentrifuged at 105,000 x g for one hour. The supernatant was discarded and the resulting microsomal pellet resuspended in a volume of homogenization solution (without deoxycholic acid) that was equivalent to 3 x the original weight of gill tissue. It was frozen at -20 C until used. Protein concentrations were determined by the method of Lowry et al. (1951).

Phosphorylation of microsomal proteins

Measurement of phosphorylated enzyme intermediates under various ionic conditions were performed in duplicate by the method of Phang and Weiss (1971). ATP-γ^{32}P (specific activity 21 uCi/mumole, New England Nuclear) was reacted at a concentration of 20 muM in 1.0 ml of a pH 7.5 Tris buffer at 4 C with 100 ul of microsomes. Magnesium was

in a concentration of 3 mM. Sodium, when present, was in a concentration of 20 mM. Potassium and ammonium in concentrations of 0, 0.1, 0.5, 1.0, and 5.0 mM were used. After a 30 sec incubation period the reaction was stopped with 0.2 ml of ice cold 50% trichloroacetic acid (TCA). Unlabelled ATP and KH_2PO_4 were added in a concentration of 0.1 uM as a chase. The entire mixture was passed across a Millipore filter (13mm, type HA, 0.45 u pore diameter) held in a Swinnex syringe adaptor. Two 3 ml aliquots of cold 10% TCA were then passed across the filter. The filter was then placed in a scintillation vial with 10 ml of Aquasol (New England Nuclear, Boston, Mass.). Radioactivity was measured on a Nuclear Chicago Mark II scintillation counter.

Electrophoresis of ^{32}P-labelled gill proteins

Electrophoresis was performed in polyacrylamide gels containing sodium dodecyl sulfate (SDS) by the method of Weber and Osborn (1969). $AT^{32}P$ used in these experiments had a specific activity of 16.3 uCi/mu mole. Potassium and ammonium in concentrations of 1 mM only were used. After incubation of gill microsomal proteins with $AT^{32}P$ under the conditions described above, the ATP/Pi chase was added and the entire volume was centrifuged at 50,000 x g for 30 min. The supernatant

was discarded and excess TCA was removed by flotation on 3.0 ml of 0.5 M sucrose. Each pellet was then incubated in 0.25 ml of gel buffer (7.8 g NaH_2PO_4, 20.4 g Na_2HPO_4, 2 g SDS per liter), pH 7.2, containing 1% SDS and 1% β- mercaptoethanol. To this mixture was added three drops of 0.05% Bromophenol blue in sucrose syrup. Electrophoresis was performed at a constant current of 5 ma per gel with the positive electrode in the lower chamber. Both compartments of the electrophoresis apparatus contained gel buffer diluted 1:1 with water. After migration of the marker dye to near the end of the tube, gels were removed from their tubes by rimming. Two gels representing freshwater and salt water microsomal protein complements were stained and destained by the methods of Weber and Osborn (1969).

Other gels representing various ionic incubation conditions were sliced unfrozen in 1.75 mm thicknesses on a fine-wire slicer. A second run was sliced frozen in 1 mm thicknesses on a commercial razor blade "stack". Each slice was incubated overnight at 55 C with 0.3 ml of Protosol (New England Nuclear) containing 5% water (w/v). After addition of 5 ml of Aquasol, each slice was measured for ^{32}P activity on a Nuclear Chicago Mark II scintillation counter.

RESULTS

Exploratory experiments employing the Millipore technique indicate that Na^+-dependent phosphorylation by $AT^{32}P$ of gill microsomes from F. heteroclitus adapted to freshwater and salt water can be detected by levels of radioactivity present. Comparison of the data obtained with increasing concentrations of K^+ and NH_4^+ reveals that microsomes incubated in the presence of 20 mM Na^- and 1 mM K^+ were dephosphorylated to almost the same extent as those incubated with NH_4 in place of K^+. Freshwater and salt water microsomes showed similar responses in this respect. (Figures 1 & 2). Data from samples prepared for electrophoresis indicate that 1 mM K^+ or NH_4^+ dephosphorylated both freshwater and salt water microsomes almost to the level observed with Mg^{++} alone (Figure 3), corroborating earlier results with the Millipore method.

Electrophoretically, gill microsomal proteins from fish adapted to freshwater band in a pattern similar to those adapted to salt water (Figure 4). A major Na^+-dependent ^{32}P-labelled protein was found in the same position in gels from both freshwater and salt water animals. This usually occurred in in the fourth (from the top) gel slice when the wire slicer

was used. Data from the second run show the phosphorylated peak in the fifth and sixth slices. Negligible radioactivity was found in slices 7 - 32 in all gels. (Williams, 1972, found a phosphorylated protein which migrated faster than Na-K ATPase in electrophoresis of the enzyme from human erythrocyte membranes.) Potassium and ammonium dephosphorylated the same major peak, yielding a peak similar in position and magnitude to the residual peak found with Mg^{++} only. (Figures 5 - 8).

DISCUSSION

Preliminary work with Millipore filters demonstrates that the Na-K ATPase of F. heteroclitus gills behaves similarly to Na-K ATPases of other tissues from other species inasmuch as the phosphorous hydrolyzed from ATP is bound to the enzyme in the presence of Na^{+}. Additionally, these experiments show that the requirement for K^{+} as a counter ion is not absolute. Gilman (1973) has previously demonstrated the ability of Na-K ATPase from this source to bind ouabain. These findings further support the contentions of Hochachka and Somero (1973) that Na-K ATPases are all "functional variants" of each other — relatives differing at the most by minor changes

in the amino acid sequence or by association with different lipid moieties in the cell membrane.

If two separate enzymes were being dephosphorylated, one in the presence of K^+ and the other in the presence of NH_4^+, then the following equation should hold true where $\underline{r}X^+$ denotes the level of radioactivity found in the presence of that cation:

$$(\underline{r}K^+ + \underline{r}NH_4^+) - \underline{r}Mg^{++} = \underline{r}Na^+ \quad (1)$$

(As $\underline{r}Mg^{++}$ is represented in both $\underline{r}K^+$ and $\underline{r}NH_4^+$ it must be corrected for.) I did not find this, rather

$$(\underline{r}K^+ + \underline{r}NH_4^+) - \underline{r}Mg^{++} < \underline{r}Na^+ \quad (2)$$

(Figures 3, 5 - 8)

The electrophoresis work demonstrates more directly that both counter ions dephosphorylate the same enzyme. It would be disadvantageous to the fish to maintain any influx of ammonium in salt water, thus if a different enzyme were present in salt water it would not be expected to be able to use ammonium as a counter ion. Therefore these findings suggest that the same enzyme is at work in both environments. This conclusion is further reinforced by the similarity of stained gels representing freshwater and salt water microsomes, as well as the electrophoretic occurence of the phosphorylated protein in the same position in both types of gels.

It is therefore concluded that the same enzyme

mediates osmoregulation in both freshwater and salt water adapted F. heteroclitus, and that this enzyme has the potential for facilitating excretion of metabolic wastes in freshwater. As this indicates reversal of the ion flux mediating enzyme with relation to the membrane, the term "ambidirectional" is proposed to describe such an enzyme.

Caution must be exercised in making too liberal an interpretation of these conclusions. Polyacrylamide gel electrophoresis is far from the most definitive proof of the identity of an enzyme. A single amino acid substitution of the same charge could leave the protein with the same tertiary structure and thus the same electrophoretic mobility while at the same time promote a subtle change in the physiological quality of the protein. Furthermore, the necessary use of SDS to dissociate the enzyme from membrane fragments masks all charges which causes the enzyme to migrate on the basis of molecular weight alone (Weber and Osborn, 1969). Development of a radioimmunoassay for this enzyme, or purification and sequencing of the protein would provide a far more exact answer to this question.

These experiments provide information which is relevant to membrane models. The reported molecular weight of canine renal medulla Na-K ATPase is of the

order 84,000 daltons (Kyte, 1971). The slow electrophoretic mobility (1.5) found in this experiment is in agreement with an enzyme of this size.(Weber and Osborn, 1969). The commonly accepted width of the cell membrane is 75 - 90 Å (Singer and Nicholson, 1973). Taking these facts into account, the results indicating the ambidirectional nature of this ATPase give further support to the model of a membrane bound protein which protrudes both sides of the lipid bilayer which mediates active transport by a conformational and/or rotational change, as proposed by Racker (1973). Also possible, due to variations in the thickness of the membrane and what the exact three dimensional shape of the enzyme may be, is Racker's second model of a membrane protein lying just within the lipid envelope. A more appealing model for an ambidirectional enzyme (also proposed by Racker) allowing for a protein smaller than the thickness of the membrane which is free to oscillate from surface to surface does not seem applicable in this instance. (Figure 9).

These findings leave one question unanswered. If the Na-K ATPase of F. heteroclitus is the same enzyme found in stenohaline fishes — which seems a reasonable yet unproven hypothesis — then it may reasonably be believed that they too should be euryhaline.

If indeed further research shows Na-K ATPases from euryhaline and stenohaline fishes to be the same, then a further mechanism must be found for the basis of euryhalinity.

Figure 1. Dephosphorylation of freshwater microsomes as a function of counter ion concentrations.

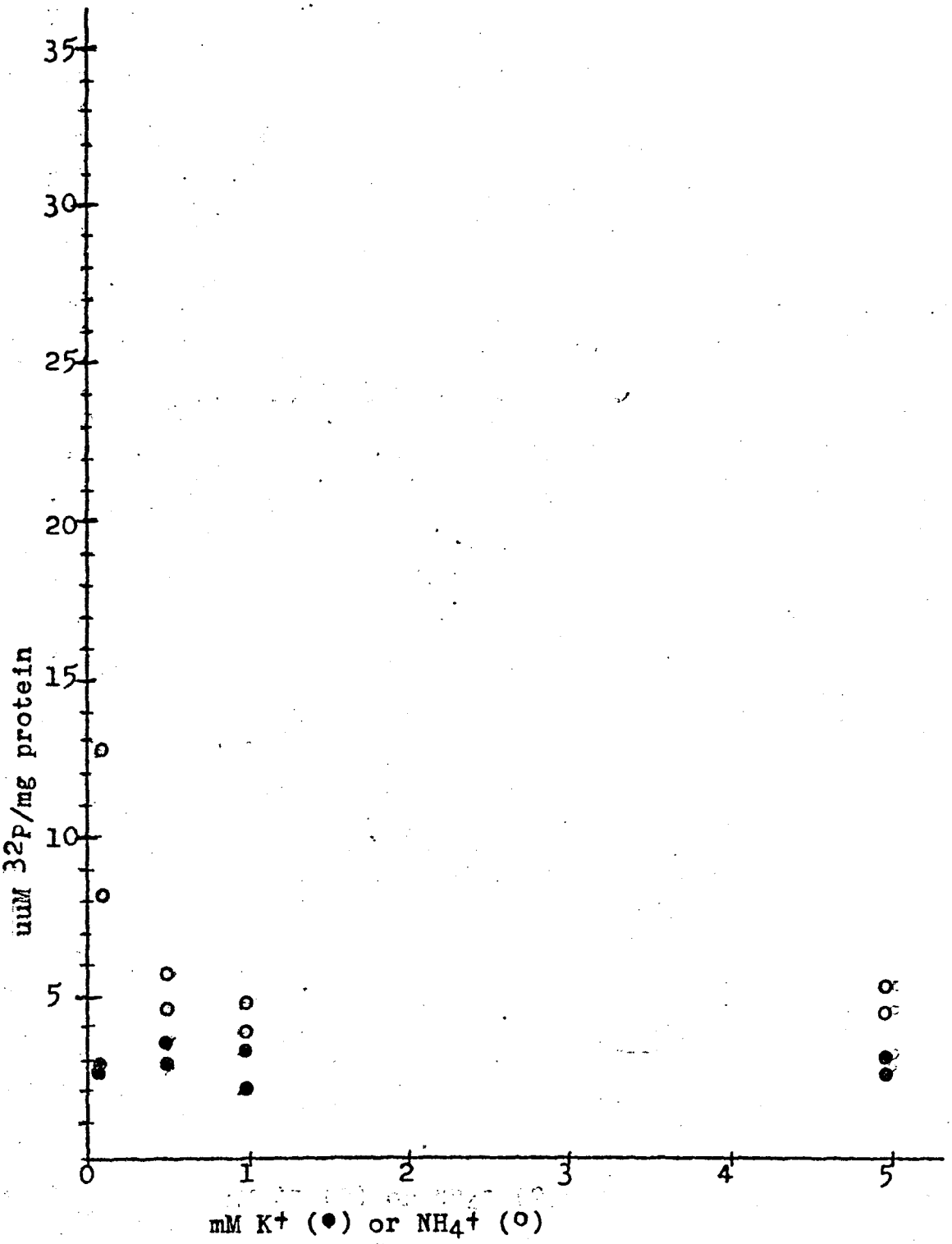

35
30
25
20
15
10
5
0
1
2
3
4
5
uuM 32P/mg protein
mM K+ (●) or NH4+ (o)

Figure 2. Dephosphorylation of salt water microsomes as a function of counter ion concentrations.

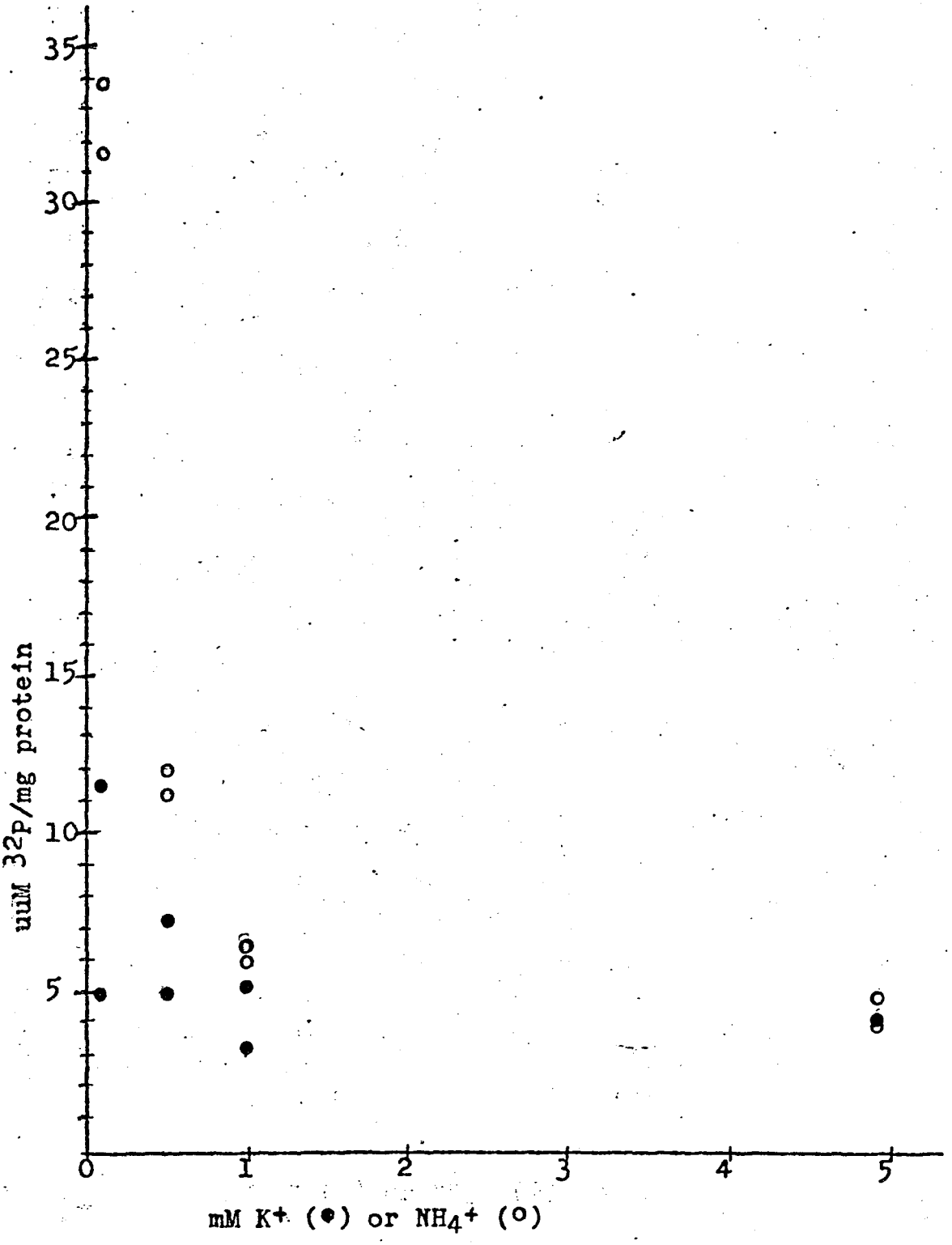

35
30
25
20
15
10
5
0
1
2
3
4
5
uuM 32P/mg protein
mM K+ (•) or NH4+ (o)

Figure 3. Phosphorylation of microsomes prepared for electrophoresis as compared to levels obtained at complete phosphorylation with 20 mM Na^{+} and 3 mM Mg^{++} (100%) ☐ and ⊟ represent data from two separate experiments.

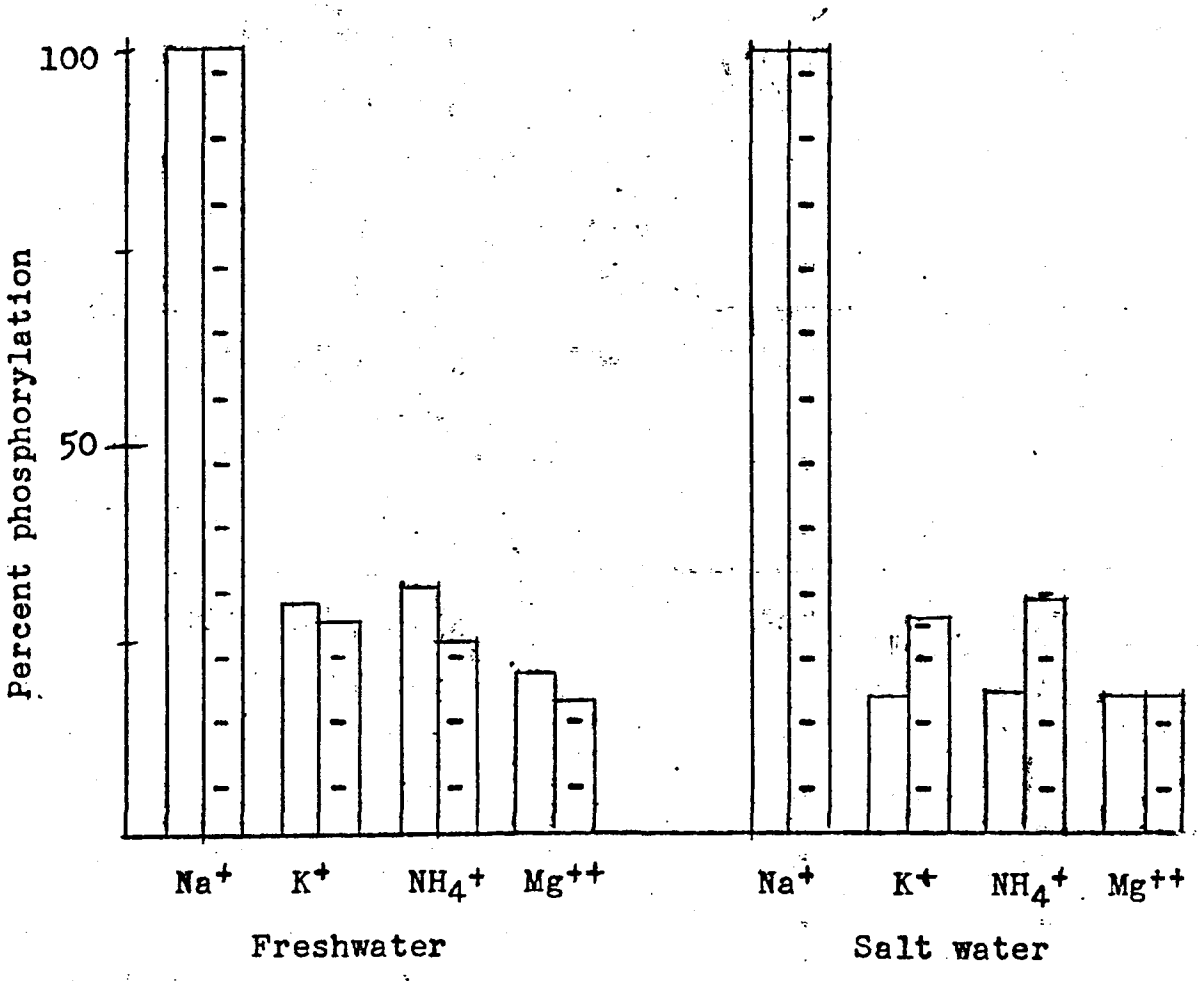

100
50
Percent phosphorylation
Na+
K+
NH4+
Mg++
Freshwater
Na+
K+
NH4+
Mg++
Salt water

Figure 4. Gels from freshwater (left) and salt water (right) microsomes showing similarity of banding patterns. The wire slicer is overlaid in the picture. Note the major protein band occurring in the fourth slice.

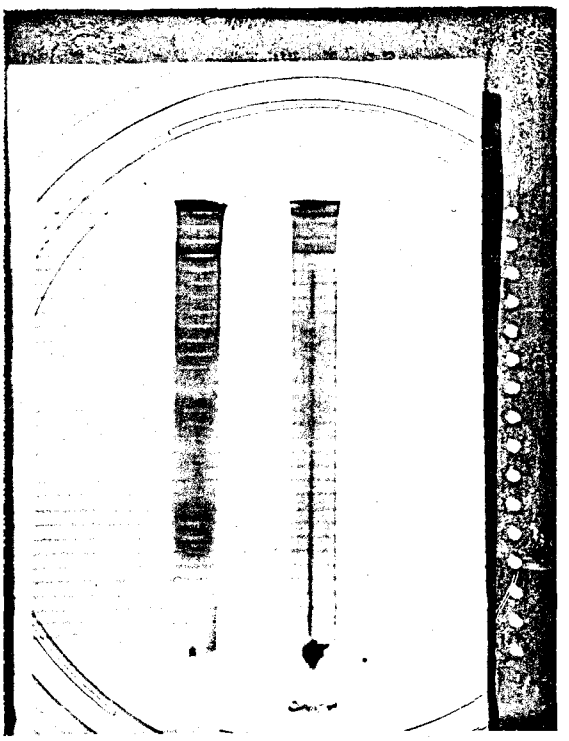


Figure 5. Radioactivity of each gel slice of salt water microsomes incubated with 3 mM Mg^{++} and 20 mM Na^{+}.

Counts per minute x 10^2
1
2
3
4
5
5
10
15
20
25
30
Gel slices

Figure 5A. Same as Figure 5 for the second run.

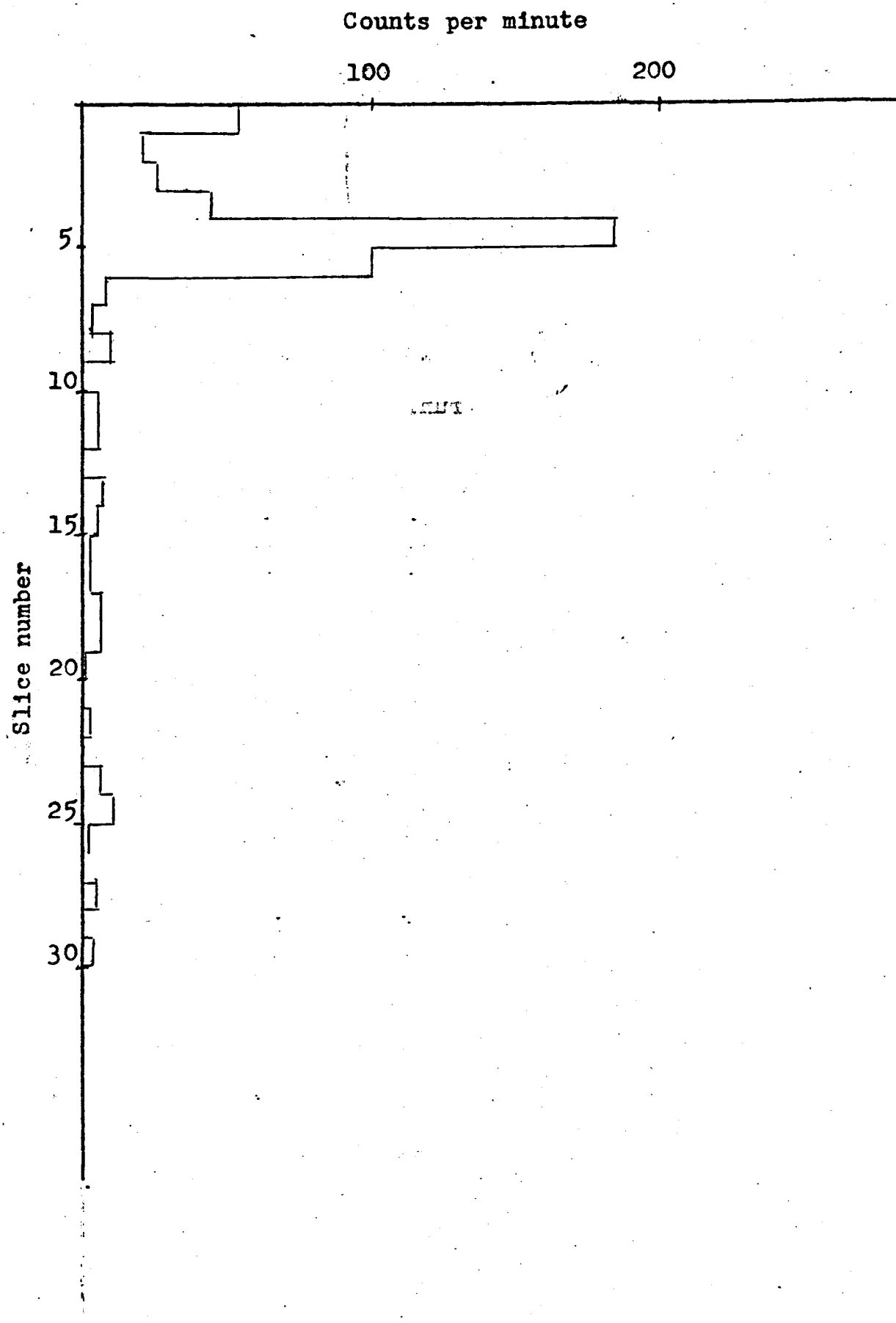

Counts per minute
100
200
5
10
15
20
25
30
Slice number

Figure 6. Radioactivity of each gel slice of freshwater microsomes incubated with 3 mM Mg^{++} and 20 mM Na^{+}.

Counts per minute
200
400
600
800
1000
5
10
15
20
25
30
Gel slice

Figure 6A. Same as Figure 6 for the second run.

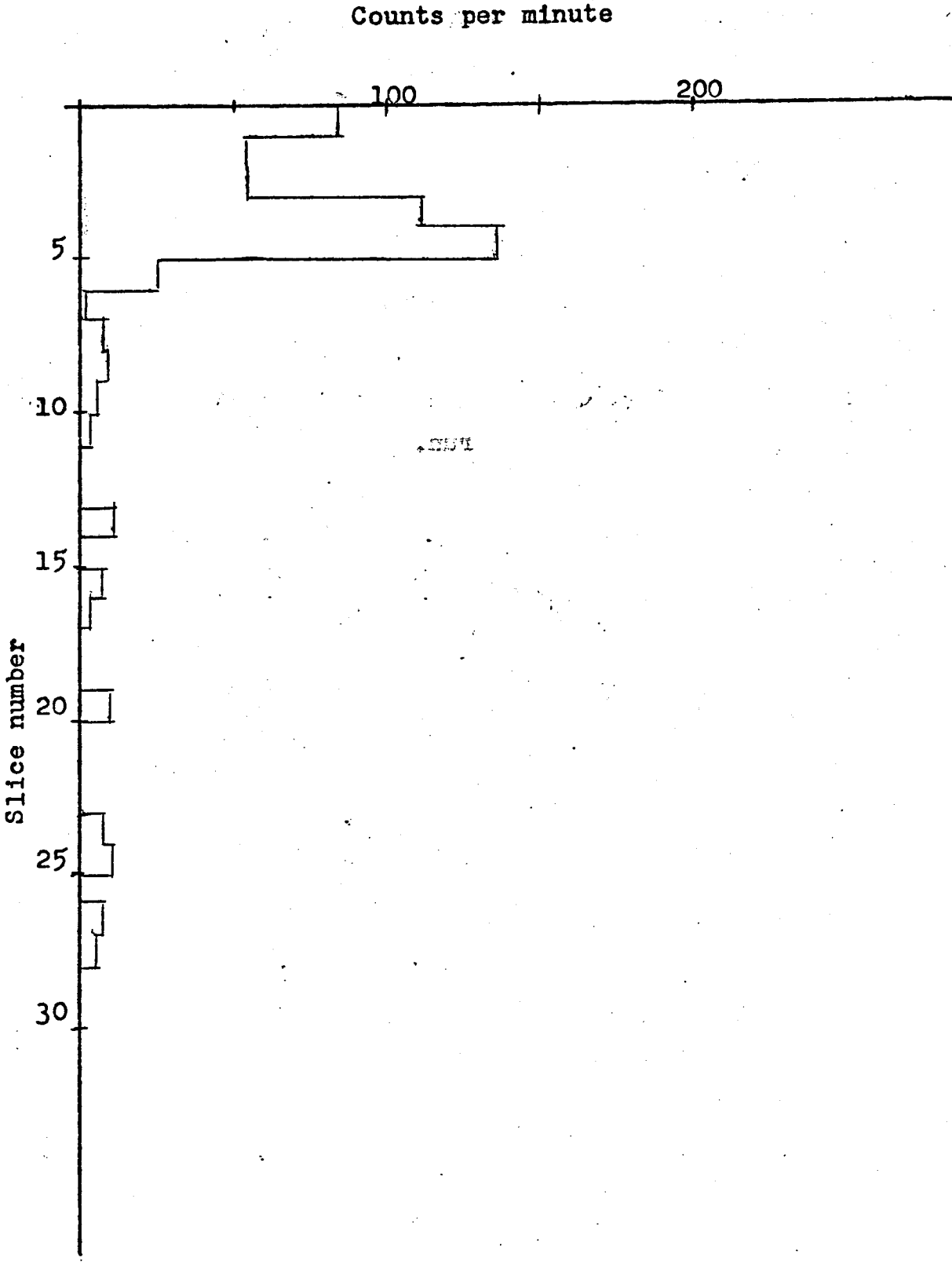

Counts per minute
100
200
5
10
15
20
25
30
Slice number

Figure 7. Salt water gels incubated with 20 mM Na^+, 1 mM K^+ and 3 mM Mg^{++} (I); 20 mM Na^+, 1 mM NH_4^+ and 3 mM Mg^{++} (II); and 3 mM Mg^{++} (III). See Figure 5 for comparison.

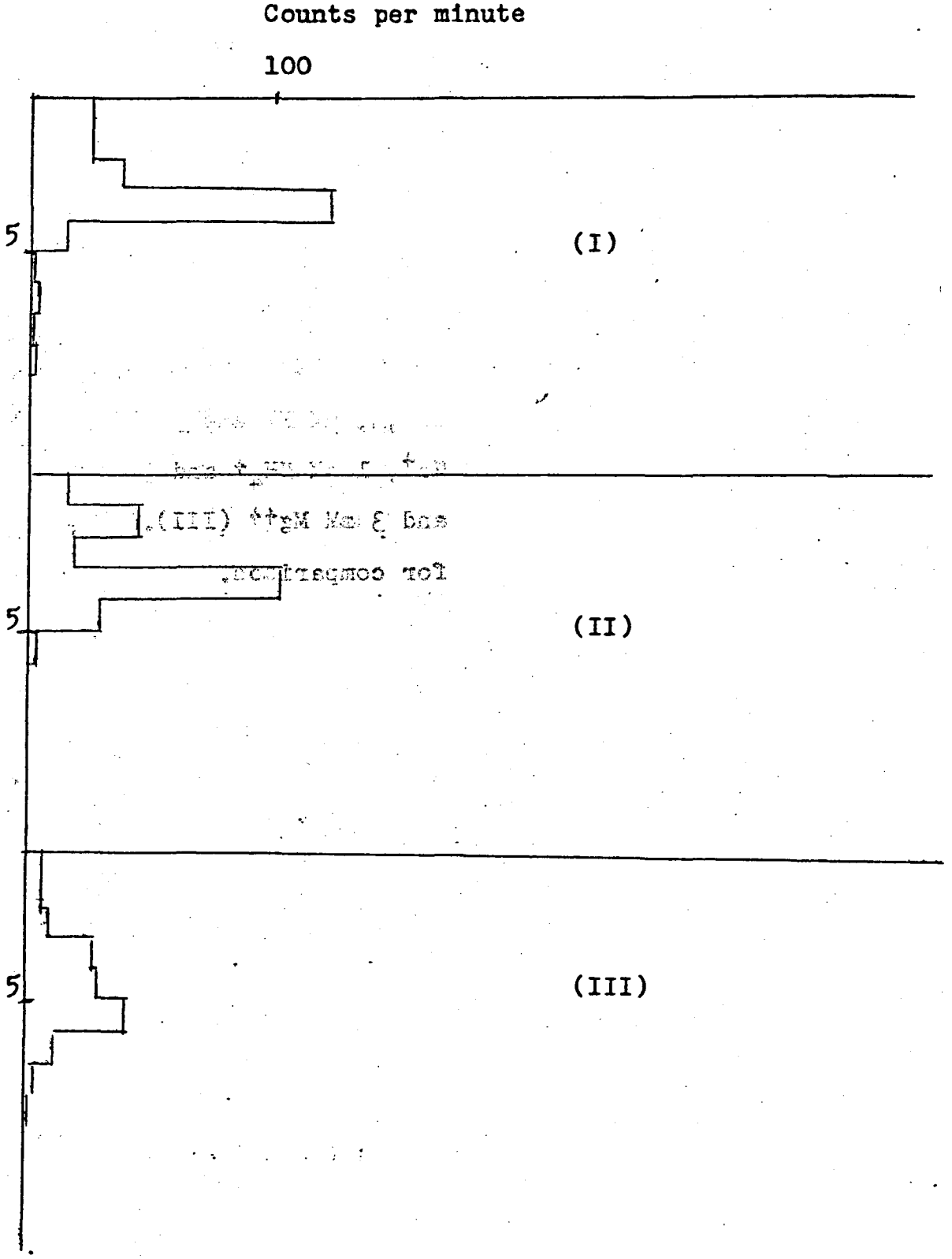

Counts per minute
100
5
(I)
5
(II)
5
(III)

Figure 8. Freshwater gels incubated with 20 mM Na^+, 1 mM K^+ and 3mM Mg^{++} (I); 20 mM Na, 1 mM NH_4^+, and 3 mM Mg^{++} (II); and 3 mM Mg^{++} (III). See Figure 6 for comparison.

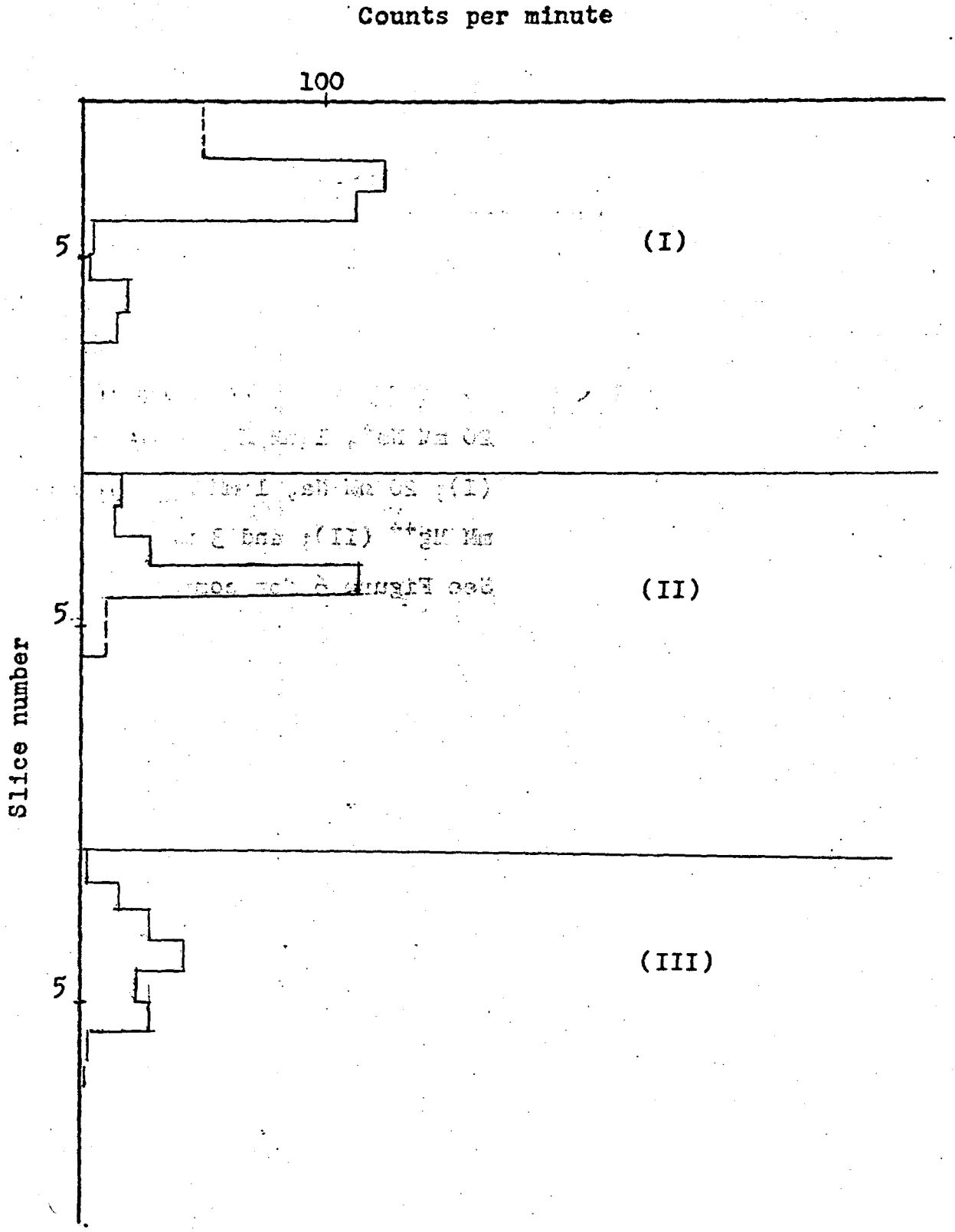
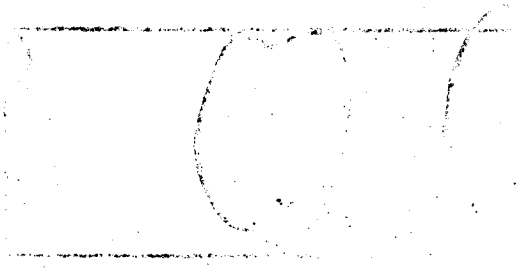
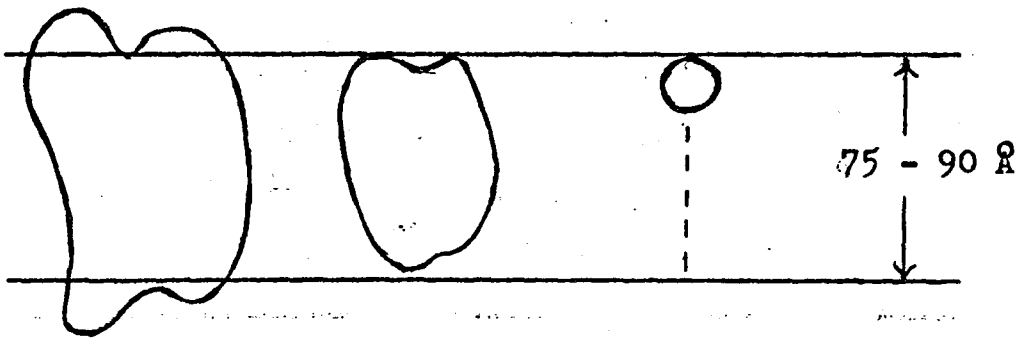

Counts per minute
100
5
(I)
5
(II)
5
(III)
Slice number

Figure 9. Racker's three models of membrane transport protein mechanics.




75 - 90 Å

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VITA

John Dale Hempel was born on December 31, 1949 in Washington D.C. He attended Fairfax County, Virginia Public Schools and was graduated from Thomas Jefferson High School in June, 1967. He received a Bachelor of Science degree from the College of William and Mary in June, 1971 with a major in biology. He entered the University of Richmond Graduate School in February, 1972. While at Richmond he became a member of Beta Beta Beta honorary society. He completed the requirements for the Master of Science degree in August, 1973. Future plans are uncertain.