

Study of Biological Desalination Phenomena: Salt Transport Mechanisms of Selective Avian Salt Glands

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FOREWORD

This is one of a continuing series of reports designed to present accounts of progress in saline water conversion and the economics of its application. Such data are expected to contribute to the long-range development of economical processes applicable to low-cost demineralization of sea and other saline water.

Except for minor editing, the data herein are as contained in a report submitted by the contractor. The data and conclusions given in the report are essentially those of the contractor and are not necessarily endorsed by the Department of the Interior.

ABSTRACT

In Phase I of this program, an analytical study was made of various biological ion transport systems in order to define new approaches for the development of new or improved practical desalination processes. In addition to reviewing the literature (both open and OSW reports) and establishing categories for the classification of the data in terms applicable to engineering applications analysis, a detailed applications analysis of relevant data has been made for a number of reports.

During the second quarter of this program, the applications analysis phase was concentrated on studies involving the application of irreversible thermodynamics to the transport of ions across biological membranes and also studies of synthetic membrane systems. The need for more detailed kinetics information was stressed as well as the need to differentiate between applicable and non-applicable biological systems. Differentiation needs to be made between coupled and uncoupled desalination systems since the former may lose output as efficiency increases.

A comparison of Caplan's mosaic membrane system with conventional reverse osmosis systems, made on a theoretical basis, showed that at a relatively high output level, the present mosaic membrane system requires nearly 100 times as much energy per unit volume of product. To attain comparable performance, the mosaic membranes need considerable improvement.

It is anticipated that the results of the applications analysis study will lead to a few key additional studies of an applied research nature which can establish the validity of hypothesized mechanisms or applications, including feasibility in practical systems.

In Phase II of this program, salient mechanistic and structural characteristics of selective ion transport by avian salt glands were investigated with the overall objective of identifying the mechanisms involved which may have application for the development of new or improved desalination processes. An exhaustive literature study has been completed on the avian salt gland and is included in this report. In preparation for future work, a thorough survey has also been made of tissue electrical stimulation studies.

In the study of the salt gland of the White China goose, some difficulty was experienced in acclimating the geese to high salt concentrations in their diets, but this difficulty was overcome by temporarily replacing the salt water with fresh water for short periods. The glands have been shown to have an active $\text{Na}^{\oplus} - \text{K}^{\oplus}$ ATPase system as shown by inhibition of the enzymatic hydrolysis on the addition of ouabain. Attempts were made to show uptake of $^{22}\text{Na}^{\oplus}$.

The salt glands of White China geese have been studied both in vivo and in vitro. Dr. Donald Douglas has served as consultant in this work and has succeeded in inserting cannulas into the birds so that osmotic loads as well as ^{22}Na could be injected. Systems for separating inorganic ^{22}Na from ^{22}Na attached to an organic moiety have been devised and attempts were made to identify the organic moiety. In the in vitro experiments, there has been minimum incorporation of ^{22}Na into gland slices, even when $\text{Na}^{\oplus} - \text{K}^{\oplus}$ ATPase has been shown to be quite active, leading to the tentative conclusion that this mechanism is not adequate to explain active transport in the avian salt gland.

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1.0 INTRODUCTION

Contract No. 14-01-0001-1674 for a "Study of Biological Desalination Phenomena: Salt Transport Mechanism of Selective Avian Salt Glands" was activated on January 12, 1968. The objectives of this program were to (1) conduct an analytical (paper) study of various biological ion transport systems aimed at defining new approaches and/or concepts for application to the development of a new or improved practical desalination process and (2) investigate and elucidate the salient mechanistic and structural characteristics of selective ion transport by the avian salt glands of White China geese as a specific biological model related to sea gulls.

More specifically, the scope of work for Phase I included the following:

1. Survey literature and OSW biomembrane research program and summarize in terms of a systems analysis relevant to a desalination process. Thus, the various elements or components of each biological ion transport process surveyed shall be identified.
2. Specific and common mechanistic and phenomenological features of each process surveyed shall be described. Special emphasis shall be placed in attempting to define a new or novel separation phenomena.
3. Wherever possible, analogies shall be sought between the mechanism of transport in biological systems with those of practical membrane processes such as electrodialysis and reverse osmosis. Specific differences shall be distinguished as quantitatively as possible.
4. Salt and water permeability data, the permselectivity of the process, and the energy requirements of the process shall be provided where available.
5. Possible new approaches or concepts entailing new or improved membrane configurations, structures or other design features stemming from this analytical study shall be provided.

The scope of work for Phase II included the following:

1. Survey the available information on the mechanism of salt and water transport in avian salt glands and identify the biological structural features responsible for the salt concentrating process.
2. Develop techniques for handling and assaying salt gland membranes from White China geese.
3. Determine and elucidate the effect of electrical stimulation on the uptake and secretion of sodium and chloride ions.

4. Identify the high molecular weight moieties to which ^{22}Na and ^{36}Cl are attached.

5. Identify as best as possible the mechanism of salt and water transport (passive and active) in the avian salt gland of the White China goose, and relate those aspects of the overall mechanism which may have application for the development of a new or improved desalination process.

Thus, the objective of this research program was to determine the salt-concentrating mechanisms of avian salt glands so that the principles could be applied to efficient methods for the desalination of brackish or sea water. It is only in the last ten years, as the result of work by Schmidt-Nielsen, that the function of the salt glands have been known. The efficiency of the process has been studied by McFarland's group. However, at a recent conference sponsored by the Office of Saline Water on "Ion Transport Through Biological Membranes", it was stated many times that the details of transport through membranes (such as the salt gland) were not yet known. Lehninger, for one, referred to the fact that no one had yet arrived at either a charge or material balance for the active transport process.

Schmidt-Nielsen has said "...if there is any organ in which an ionic pump and its metabolic ramifications can be studied in its pure form, the salt gland of marine birds almost seems to be designed for this purpose." Yet there are few laboratories doing research on the salt gland and there is no research on the mechanism of salt concentration by the salt gland which is sponsored by the Office of Saline Water. (The Office is sponsoring the excellent work of Dr. L. E. Hokin on an enzyme which can be obtained from the salt gland and seems to be of great significance in active transport). This program was designed to complement Dr. Hokin's work and to be more oriented to determining the steps in the transport problem for possible application to novel and efficient methods of desalination.

In the Report of the Desalination Research Conference sponsored in 1961 by the Office of Saline Water and the National Science Foundation ("Desalination Research and the Water Problem", Publication 941 of the National Academy of Sciences-National Research Council, 1962), it was pointed out that: "There is good reason to believe that basic research can lead to practical methods of desalting sea water; nearly all of the present processes require more than thirty times the work energy needed for reversible separation." It was further stated that "Living membranes carry on desalination operations with a high degree of efficiency." These conditions are still true for Dr. W. S. Gillam prefixes his report in the 1965 Saline Water Conversion Report with the statement that: "All practical desalination processes presently require many times the theoretical minimum energy, due to inefficiencies resulting from highly complex interactions in the systems concerned."

If we could determine the mechanisms by which avian salt glands concentrate salt, if we could determine how chemical energy is converted to osmotic energy, we should obtain information of fundamental importance for devising a new and potentially highly efficient and useful desalination process.

More specifically, this research was designed to lead to a better understanding of the efficient conversion of one form of energy (metabolic energy) to another (osmotic energy). It is not contemplated that salt glands would be used in any large scale desalination, but rather that the principles which are utilized in this highly efficient system - and with which we now have only fragmentary knowledge - might lead to a practical desalination process completely different from any now being contemplated.

Since the salt gland does consist of membranes, it was believed that the research might also lead to information of value to those processes presently in use for desalination which employ membranes (reverse osmosis, electrodialysis, etc.).

2.0 PHASE I - APPLICATIONS ANALYSIS OF BIOLOGICAL ION-TRANSPORT SYSTEMS

2.1 INTRODUCTION

The objectives of this phase of the program were (1) to examine the possibilities for the present and future application of the principles of biological transport to desalination, (2) to assess the merit of such applications, and (3) to identify areas of needed further research to improve the applicability as deemed desirable because of potential merit. The accomplishment of these objectives was based on an analysis of the information and data obtained from a survey of the pertinent and available literature. The results of this program are described in the following subsections. The procedures and rationale of the literature survey are discussed in Section 2.2. Section 2.3 consists of some general remarks including a description of some of the mechanisms that have been suggested to account for biological, active transport. The results of some experimental studies to characterize the active transport of potassium across cellular membranes are discussed in Section 2.4. Irreversible thermodynamics and its application to coupled transport processes are reviewed in Section 2.5. Sections 2.6 and 2.7 contain discussions of the salt transport across specific, synthetic membrane systems.

In summary some of the more important conclusions are as follows: First, much of the past and current work concerning active transport has dealt only with gross aspects of the process (such as the transport of a material against a concentration gradient, expenditure of metabolic energy, high selectivity for the type of material transported, etc.). Concerted studies to define the mechanism of active transport in living systems have been in progress only during the past 5 to 10 years. As yet, these studies have not yielded sufficient information of the type (chemical kinetics, membrane structure, etc.) required for practical applications.

Second, evidently all active transport systems may not be applicable to desalination problems. Detailed studies seem to show that the transport of potassium across the cell wall of Escherichia coli is an ion exchange process coupled to cell metabolism. Thus, this organism takes up potassium and exudes either potassium or sodium and hydrogen ions. This type of process appears to be general for most animal and plant cells. On the other hand, the secretion of salt by the avian salt gland definitely does not appear to be such an exchange process. Accordingly, this latter type of system should be emphasized in future investigations.

Third, Caplan and others demonstrated a membrane system in which a chemical reaction caused the unidirectional diffusion of an ion. It was found that the coupling between diffusion and the chemical reaction obeyed the Onsager reciprocity theorem. However, the maximum efficiency, based on the conversion of chemical energy to diffusion against a concentration gradient, was only 7 to 25 per cent, depending on the choice of membranes. Although this synthetic system is representative of active transport in overall principle of operation,

it surely is not representative of the details of the process. Undoubtedly its efficiency could be improved by the knowledge of how to construct more suitable membranes. This approach, the study of synthetic active systems, has considerable merit. Such studies may be expected to yield considerable practical knowledge from a relatively smaller effort than must be expended in the study of a biological system, primarily because of relative complexity of the latter.

Fourth, an analysis by Kedem and Caplan has shown that the efficiency of energy conversion for two coupled and linear processes (such as active transport) may not be high, particularly under the conditions desired for desalination practice. This practice would require steady-state operation at fixed concentrations of the chemical reactants for active transport. Under this circumstance, the best efficiency for maximum output is only 50 per cent or less, depending on the degree of coupling between the two processes. Higher efficiencies are possible only with the sacrifice of output, with output vanishing at 100 per cent efficiency. On the other hand, for a direct (no coupling) desalination process, such as reverse osmosis, both high efficiency and output are theoretically possible.

Fifth, the application of mosaic membranes to reverse osmosis desalination processes was examined. Consideration was given to the use of a mosaic membrane, having the properties measured experimentally by Caplan, for the partial desalination of a 0.2M solution of an alkali halide. It was found that even a modest salt reduction, by a factor of 2 to 6, required applied pressures of more than 2500 psig for a maximum output, which was in the range of 1.77×10^{-5} cm³/sec per cm² of membrane area. Compared with a conventional reverse osmosis process at this level of output, the mosaic membrane system requires nearly 100 times as much energy per unit volume of product. However improvement in the relative performance of mosaic membranes may be expected from further research to optimize their properties for desalination. The extent to which these properties must be modified to achieve comparable performance remains to be determined.

2.2 GENERAL SCREENING REVIEW OF PERTINENT LITERATURE

At the beginning of this program, a preliminary analysis was conducted to identify several tentative categories which would be useful for the identification of engineering applications of data and results presented in the available literature pertinent to biological ion transport processes. These preliminary categories included: energy-balance data, chemical kinetics data, thermochemical data, thermal transport information, mass transport data, membrane "maintenance" processes (to maintain viability), momentum (pressure-gradient) transport characteristics, irreversible thermodynamic features, and several others.

A general review of reports and papers covering the more recent research on active membrane transport reveals the basic-research nature of this work, with an emphasis on providing a better understanding of the fundamental

processes involved. Although numerous investigators have reported data for diffusion rates, metabolism, ionic concentration gradients, etc., for living membrane systems, most of these data do not, as yet, provide an adequate basis specifically for engineering analysis that would immediately suggest a practical systems application. Nevertheless, the available data do contribute significantly to an analysis of the physical and chemical phenomena, and to some extent the important subsystem mechanisms, involved in active biological membrane transport, upon which an engineering applications analysis must eventually be based.

Therefore, emphasis was centered on characterizing and interpreting the underlying mechanisms in sufficient detail that hypothesis for analogs of these mechanisms and processes in terms of other physical systems that have already been reduced to practice would be suggested. Then, the engineering applications analysis would follow as a logical consequence. As the physical analogs become identified, they serve as basis for either improvements in present engineering ion transport systems suggested by the biological processes, or new applications to ion transport of engineering systems but which have never before been applied to ion transport per se. For example, devices that can control the electrical field in the vicinity of an ion-transport interface have been considered but have been only marginally beneficial in improving the conversion efficiency; but these may prove to be very beneficial when used to trigger another (interstage) mechanism which serves as the true agent of process improvement (such as an electrically-induced pH change in the pores of a membrane, as an hypothetical example). These were types of suggested approaches that we sought to identify through detailed analysis of the available information on mechanisms associated with biological ion transport. Obviously, these results are expected to lead, as usual, to a few key additional studies, of an applied-research nature, that can then establish the validity of the hypothesized mechanism or application, and the feasibility of its use in a practical system.

The character of the screening review principally involved seeking answers to the following major questions regarding key mechanisms and processes that comprise active transport: (1) the nature of membrane structure and its relationship to the active transport process, (2) the types of chemical reactions that contribute to active transport desalination, their kinetics and thermochemistry, and the identification of those reactions that are controlling; (3) the relationship of diffusion to membrane structure and controlling chemical processes (e.g.: How many sub-processes actually comprise the overall transport process, and how are these to be represented analytically? How does such an analytical coupling of sub-processes improve the application of Onsager's reciprocity relations and other methods of analytical treatment?); (4) the factors that determine throughput capacity, concentration-gradient limitations, energy requirements to the membrane, etc.; and several other questions of this type. Thus, the pertinent available literature was continuously screened to identify those items that potentially could provide at least partial answers to these types of questions. Then, the promising items were carefully scruti-

nized to extract the elements of contributing information which were subsequently combined with other data or results to establish answers to the key questions and provide a basis for mechanism and applicational hypotheses.

2.3 GENERAL CONSIDERATIONS

As is well recognized, the unique feature of active transport across biological membranes is the coupling of absorption and diffusional processes with specific metabolic reactions to force the flow of certain permeants against a gradient of chemical potential (concentration). It has been demonstrated that the energy efficiency of this process can be quite high, at least 60 to 85 per cent, as based on the energetics of the specific reactions involved.²⁰ Further the process can be very specific with regard to the selection of permeant to be transported (e.g., differentiation between sodium and potassium ions).

The coupling between diffusion and chemical reaction is possible only because biological membranes are anisotropic. Thus, a chemical reaction (or reactions) occurs only in specific regions of the membrane, and as a consequence, establishes a gradient of chemical potential between these regions for diffusion. For cell membranes, anisotropy is provided by their protein-lipoid-protein sandwich-like structure. Little is known about the details of the chemical reactions except that the usual energy cycles involving ATP somehow seem to be involved. Specific knowledge about the relationships between diffusion and structure, and about the functional groups, reactants and molecules which permit coupling of diffusion and chemical reactions, and in general, about the mechanism of active transport, are as yet unknown.

Clearly, such knowledge has great potential value for the conception of novel membrane-based desalination processes and for the improvement of existing processes. However, such application will likely be made only within certain limitations. Living membranes are complex organic materials which require continuing maintenance. The energy for transport derives from a sequence of complex chemical reactions involving complex organic fuels. On the other hand the economics of industrial process are such that their energy must be derived from relatively simple physical or chemical processes, and readily available and simple fuels. Thus, in the sense of an exact chemical and physical copy, the direct application of active transport to desalination is unlikely. Instead the value of a thorough knowledge of active transport lies in the possibility of applying its principles of function, using chemical and physical analogues of the actual transport process, to gain an improved desalination process.

2.3.1 Areas of Research and Some Possible Mechanisms

A general survey of the literature relating to both active and non-active transport across membranes has revealed that recent research has been concentrated in four areas: (1) The structure of biological membranes, (2) The thermodynamics of transport and permeation through membranes, (3) Energy Utilization for active transport, and (4) Gross effects such as permeation rate, selectivity and environmental and concentration effects. Katchalsky and Kedem^{21,22} have formulated complete sets of phenomenological coefficients for membrane transport (non-active) through the application of irreversible thermodynamics. Relationships between the coefficients also were established. These will be useful for judging the quality and consistency of permeability data and for estimating the various energy requirements and efficiencies of transport processes. By far, the bulk of the literature deals with membrane structure and the observed, gross aspects of transport across various types of membranes. However, insufficient information and data have been developed, thus far, for delineating in detail the mechanism of active transport, which is crucial for the successful application to desalination technology. This, of course, is reasonable in view of the great experimental difficulties involved. Nevertheless such progress may be expected as new and improved techniques for studying active transport are devised.

In spite of this, many interesting hypotheses for active transport have been proposed. Although not proved they suggest some novel approaches to desalination by membrane-like processes. Three of these hypotheses are reviewed in the following paragraphs, and in addition, the specific information required for practical application is discussed for each, assuming their correctness.

One of the older hypotheses is pinocytosis, a process which involves the engulfment of extracellular fluid by the cell membrane. The hypothesis is suggested by the often observed phagocytosis in which solid matter is ingested into cells. Recently, Gross²³ has proposed a version of pinocytosis which can account for selectivity towards permeants. His model proposes that the cell wall is composed of units of protein-lipid-protein sandwiches bonded together by calcium ions which may be broken, somehow, at will by the cell. The interior protoplasm contains a reserve of these units which may be quickly supplied to repair breaks in the cell wall. (These features have been suggested by the observations of other investigators.) Transport then occurs by adsorption at particular sites on the exterior of these units (which accounts for selectivity), followed by the release of the wall units into the interior of the cell. Simultaneously, a fresh unit from the interior replaces it in the cell wall. One fact which the model does not explain is the desorption of the permeant into a region where its chemical potential is higher than it was where it was adsorbed.

This model of pinocytosis is analogous to a desalination process

using an ion exchange resin. Such a process would be practical provided an economically feasible means could be found to clear the loaded resin of its salt. As pointed out above, this is the same process which is yet to be explained for Gross' model, assuming its correctness.

Another hypothesis utilizes the observation that the lipid layer of the cell wall is pierced by fibrils of protein which are assumed to be hollow and capable of peristaltic action driven by metabolic processes involving ATP. The hypothesis proposes that the permeants are literally pumped through the membrane by the fibrils²⁴. This mechanism is partly analogous to the reverse osmosis process for desalination. On the other hand a unique feature is that the pumping is confined entirely within the membrane. The elucidation and proper application of this mechanism, if it is a correct one, might permit the elimination of pumps and pressure vessels used in the present reverse osmosis process and in general make the process simpler and more efficient.

Another and, up to now, a more popular hypothesis, involves the assumption of a carrier molecule to transport lipophobic ions through the lipid layer of the cell wall. One version of this hypothesis involves the following sequence of events. The first step is the migration of an ion through the outer protein layer of the membrane where it is affixed to a fat-soluble carrier molecule. This simultaneously establishes a concentration gradient for continued migration through this protein layer. At this point the lipid-ion complex can diffuse through the lipid but not the protein layer. Once through the lipid layer the complex is destroyed chemically to maintain a gradient for diffusion. Next, the freed ion diffuses through the inner protein layer into the interior of the cell.

This mechanism has no counterpart in existing desalination methods. Assuming its correctness, application of the principles of this mechanism will require elucidation of the combination of the physico-chemical properties of the membrane and carrier-ion complex which permits the stability and selective diffusion of the latter. In addition knowledge of the details of the chemical reactions involved in the destruction of the complex are required. It should be noted that it is precisely this information that was the objective of Phase II of this program.

2.3.2 Application of the Theory of Absolute Reaction Rates

As pointed out by Katchalsky²⁴, biological membranes, particularly cell membranes, are very thin and their thickness may be no more than the characteristic dimension of a permeant molecule or the carrier molecule for a simple ionic species. In this situation the usual concepts of diffusion and permeation do not apply. Diffusion may be regarded as successive series of random jumps by the diffusing species. In the case of cell membranes, their thickness apparently is of the order of one jump.

On the other hand, this case can be properly treated by the

theory of absolute reaction rates. The central idea of the theory is that all transport processes involve the passage of molecules from a region of high chemical potential to low potential through one or more successive states of intermediate potential. These intermediate states are characterized by having a local minimum of energy. Between successive states are transition states which have local maximum of energy. The rate at which molecules can proceed through the transition state, and therefore the rate at which the transport process occurs, is proportional to the probability that a molecule has sufficient energy to attain the transition state. Thus, the rate constant for the process given by the formula

$$K = \frac{kT}{h} \exp \frac{-\Delta F^\ddagger}{RT} \quad , \quad (1)$$

where k is the Boltzman constant, h is Plank's constant, R is the gas constant, T is the absolute temperature and ΔF^\ddagger is difference in the Gibbs free energy between the transition state and the neighboring, intermediate states, which is often called the free energy of activation. For simple chemical reactions, K is the well known rate constant, and for simple diffusion, K is related to the diffusion coefficient by the formula

$$D = K \lambda^2 \quad (2)$$

where λ is the distance between the intermediate steps.

Zwolinski, Eyring and Reese²⁵ applied this theory to several cases of membrane transport (non-active) and derived the corresponding relationships between the permeation constants and the free energy of activation using equation (1). These relationships were then applied to permeability data for the membranes of the eggs of a few marine animals. From Arrhenius-type plots of the data, the energy and entropy of activation ($\Delta F^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$) were computed. They found that the values of energy of activation were approximately the same as those reported for diffusion through solids. However, the entropy values were large positive numbers (15 to 32 e.u.) and never have been observed previously for diffusion. They imply that the diffusion through the membranes involves the breakage of a large number of chemical or strong physical bonds.

The application of this theory and associated analysis would appear to be very beneficial for the job of determining the mechanism of active transport. It is an established technique for assisting the determination of the mechanism of ordinary chemical reactions. Once the chemical species involved in the chemical reaction have been identified, knowledge of the energy and entropy of activation indicate what the controlling chemical or physical processes are. Obviously, the application of this analysis requires permeation data at varying temperatures and for a variety of permeants. Unfortunately, the preliminary survey of the literature indicates that such information may not be generally available.

2.4 SOME EXPERIMENTAL STUDIES OF TRANSPORT ACROSS CELLULAR MEMBRANES

Several items of literature describing the transport of potassium ion across cell membranes have been reviewed. One of these items dealt with the measurement of the rate of transport of ions (isotopically identified) across the membranes of the excised roots from barley seedlings¹⁴. The results of these measurements seem to show the existence of two mechanisms for the transport of potassium and sodium. For media with low concentrations of potassium (0 to 0.2 millimolar) the variance of the rate of transport with concentration in the media was described in the report as following Michaelis-Menton kinetics. (Actually, and more accurately, the kinetics follow that of surface catalysis for which the rate controlling step is the decomposition of the substrate at the active site. It turns out that the rate expression for this is the same as the Michaelis-Menton equation for enzyme catalysis²⁶). Moreover, in this concentration range, the transport mechanism involved is specific for potassium (or rubidium) instead of sodium regardless of the concentration of the latter. At higher concentrations of potassium, a second, but non-specific, transport mechanism was operative. This, too, followed "Michaelis-Menton kinetics." The type of anion, chloride or sulfate, present has no effect on transport via the first mechanism, but does affect transport via the non-specific mechanism. The precise conditions for the measurements were not stated in the report. Presumably, the measurements were for a steady-state exchange process (no growth and constant compositions inside and outside the root tissues). The maximum rate of transport via the first (specific) mechanism was 10 $\mu\text{mols/g}$ of tissue per hour.

A more detailed and complete investigation of potassium transport across the cell wall of a mutant strain of *Escherichia coli* has been made at the Biophysical Laboratory of the Harvard Medical School by S.G. Schultz, A.K. Solomon, W. Epstein, et al. (references 9, 27—32). The following significant features were demonstrated:

(1) During initial growth (logarithmic phase) the intracellular concentration of potassium is greater than in the medium, whereas the intracellular concentration of sodium is less. Upon depletion of the medium and cessation of growth, the intracellular concentrations of potassium and sodium become essentially the same as the concentration in the medium. During growth, the intracellular concentration of potassium can be as much as 10^5 times that of the medium and increases with increasing osmolarity of medium.

(2) Under steady-state conditions (arrested growth and no net transport of ions), the flux (inward and outward) of potassium in a typical medium (pH 7.0) is $1.0 \text{ pmol/cm}^2\text{-sec}$ or $300 \text{ } \mu\text{mols/g}$ of cells-hr, and is insensitive to the extracellular concentration of potassium over wide range. The magnitude is changed by various circumstances including the pH of the medium, temperature and the addition of dinitrophenol (DNP). A decrease in pH or temperature decreases the flux. The fact that both the inward and outward

fluxes of potassium are affected in the identical manner by extracellular concentration, pH, temperature and DNP, is strong evidence that both are part of the same active transport process or are very tightly coupled processes. The inward flux of potassium during the steady state is necessary for the uptake of phosphate by the organism.

(3) Transfer of the resting organisms to a fresh growth medium or the changing of the medium in any of a variety of ways causes a rapid net change in the potassium content of the cells. Raising the pH (which may be accomplished by the addition of alkali or transfer to a fresh medium) or the osmolarity of the medium produces a rapid uptake in potassium. There are at least two mechanisms for this. The first is a saturable process involving the simultaneous extrusion of an equivalent amount of hydrogen ion. The maximum rate by this mechanism is approximately $18 \text{ pmols/cm}^2\text{-sec}$, achieved at an extracellular concentration greater than 5 millimolar. The rate decreases with a decrease of the pH of the medium. The second mechanism is the one-for-one exchange for intracellular sodium. This process is slower than the potassium-hydrogen exchange and occurs upon the transfer of resting cells to fresh media, at which time the concentration of intracellular sodium is high. Both of these mechanisms were believed to occur by separate, but active, carrier-mediated paths.

Results similar to those just described have been reported by other investigators for a variety of cellular membranes. Unfortunately, none of this work (although competently conceived and performed) seems to be directly applicable to practical desalination. The reason for this is that the work is primarily descriptive of the end results (i.e., net effect) of active transport processes. However, as has been pointed out, engineering applications require knowledge of the mechanism of active transport, viz. the specific sequence of chemical and physical processes involved, and the particular structure of the membrane which accomodates these processes.

An important feature of the active transport of potassium for Escherichia coli is that it appears to be a process involving the exchange of intracellular and extracellular cations. This feature also has been observed for other animal cells as well as plant cells. As stated, the evidence is quite conclusive that potassium influx and efflux during steady-state are a part of the same active process. However, for the uptake of potassium in a fresh medium the evidence obtained does not rule out the possibility that hydrogen ion and sodium are eliminated simply by diffusion which is driven by the necessity to maintain electrical neutrality. In general, these results point up the fact that the demonstration of any mechanism for an active transport process must include evidence for either the participation or the absence of effect of all species which move across the membrane.

Another point of interest is the very small rates of transport. For example, the flux of salt representative of the electro dialysis process is

a factor of 10^5 greater than the flux of potassium obtained by active transport in the cell membrane of *E. coli*. For another comparison, the rate constant for potassium transport in the steady-state is 6.7×10^{-9} cm/sec. Since the cell wall is approximately 0.01μ thick, this corresponds to a diffusion coefficient having the apparent value 7×10^{-15} cm²/sec. This value is typical for the diffusion through solid material and is many orders of magnitude less than that expected for diffusion through pores. A partial explanation of the low transport rates may be that a very small fraction of the area of the cell wall is actually involved in the transport process. This is indicated by the fact that the kinetics of the process are that of surface catalysis. A further explanation may arise from the possibility that the transport is tightly coupled to a chemical reaction (metabolism within the cell wall), whose rate must be slow for efficient energy utilization. This point is discussed further in the next sub-section.

2.5 APPLICATIONS OF IRREVERSIBLE THERMODYNAMICS

Kedem and Katchalsky have developed a general set of phenomenological equations for the isothermal transport of solvent, ions and non-ionic materials across single and multiple arrays of membranes (References 33-35). These equations are necessary for the description of transport through mosaic membranes which will be discussed in a subsequent section.

In another important publication³⁶, Kedem and Caplan discuss the degree of coupling and efficiency of energy utilization for two simultaneous processes. Some of the more pertinent ideas which they developed are described below.

Consideration is given to two simultaneous processes for which the phenomenological equations and dissipation function are (using the notation of Kedem and Caplan):

$$\begin{aligned} J_1 &= L_{11}X_1 + L_{12}X_2 \\ J_2 &= L_{21}X_1 + L_{22}X_2 \end{aligned} \tag{3}$$

and

$$\begin{aligned} \phi &= J_1X_1 + J_2X_2 \\ &= L_{11}X_1^2 + 2L_{12}X_1X_2 + L_{22}X_2^2 \end{aligned} \tag{4}$$

respectively. (In equation (4), $L_{12} = L_{21}$ has been assumed.) The two terms in the first form of the dissipation function represent the power expended by each of the two processes, the sum of which must always be greater than or equal to zero. Thus for the case that one of the processes drives the other, as in the case of active transport, the power absorbed by the driven process

cannot exceed that expended by the driving process. These features may be expressed mathematically as follows:

$$J_1 X_1 + J_2 X_2 \geq 0, \quad (5)$$

and if 2 is the driving process,

$$J_2 X_2 \geq - J_1 X_1. \quad (6)$$

The efficiency, η , with which the power expended by process 2 is converted into power for process 1 is given by the ratio of the power expenditure of the processes, and from equation (6):

$$\frac{-J_1 X_1}{J_2 X_2} = \eta \leq 1. \quad (7)$$

The extent to which processes 1 and 2 are coupled together depends on a parameter, q , which is defined by the relation

$$q^2 = \frac{L_{12}^2}{L_{11}L_{22}} \quad (8)$$

The absolute value of q is less than unity, a result which stems from the fact that the dissipation function, for irreversible processes, is a positive-definite quadratic form in the forces, X . The efficiency of conversion may then be written as a function of q and the ratio of the forces, $x = X_1/X_2$:

$$-\eta = \frac{(q + Zx)Zx}{(qZx + 1)}, \quad (9)$$

where $Z = \sqrt{L_{11}/L_{22}}$. From equations (5) and (9) and with $J_2 X_2$ positive, it may easily be shown from equations (7) and (9) that Zx must satisfy the relation

$$-1 \leq \frac{Zx}{q} \leq 0. \quad (10)$$

For values of Zx in this range η must have a maximum which is a unique function of the degree of coupling q . The value of Zx at the maximum is

$$Z_{x_{\max}} = \frac{-(1 - \sqrt{1 - q^2})}{q}, \quad (11)$$

and the corresponding maximum efficiency is

$$\eta_{\max} = \frac{(1 - \sqrt{1 - q^2})^2}{q^2}. \quad (12)$$

Thus, as the degree of coupling approaches unity (tight coupling) the maximum efficiency also approaches unity. However, for these conditions, the rate of the process, the value of J_1 , approaches zero. This is evident from the following argument. From equation (11)

$$\lim_{q \rightarrow 1} \left[Z_{x_{\max}} = \sqrt{\frac{L_{11}}{L_{22}}} \left(\frac{X_1}{X_2} \right)_{\max} \right] = -1,$$

and from equations (7) and (12)

$$\lim_{q \rightarrow 1} \left(\frac{-J_1 X_1}{J_2 X_2} \right)_{\max} = 1.$$

Combining these results gives

$$\lim_{q \rightarrow 1} J_1 = J_2 \sqrt{\frac{L_{11}}{L_{22}}} = X_1 \sqrt{\frac{L_{11}}{L_{22}}} \left(L_{12} - L_{22} \sqrt{\frac{L_{11}}{L_{22}}} \right) = 0,$$

where equations (3) and (8) have been used.

The efficiency of an important mode of operation, applicable to active transport, was considered. For steady-state, process 2 will occur under a constant driving force, X_2 . For this condition a maximum of the output, $-J_1 X_1$, occurs at $Z_x = -q/2$:

$$\frac{d(-J_1 X_1)}{dX_1} = 0 = -(2L_{11}X_1 + L_{12}X_2),$$

$X_2 = \text{constant}$, and therefore

$$x = \frac{X_1}{X_2} = -\frac{L_{12}}{2L_{11}} = \frac{-q}{2Z}.$$

Substitution of this value into equation (9) gives the efficiency at maximum output for a fixed driving force:

$$\eta_{\text{max output}} = \frac{q^2}{2(2-q^2)}. \quad (13)$$

With respect to active transport for this case, process 2 corresponds to the chemical reaction with fixed concentrations of the reactants. Accordingly, equation (13) shows that the maximum efficiency at maximum output is obtained when $q = 1$ and is only 50 percent. On the other hand it is believed that actual biological processes operate at much less than maximum output and their efficiencies are correspondingly greater. As shown above, the output vanishes as the efficiency approaches 100 percent.

2.6 A SYNTHETIC ACTIVE TRANSPORT SYSTEM

In spite of the lack of detailed knowledge of the membrane structure and chemical reactions involved in active transport in biological systems, it nevertheless is possible to construct a system in which a chemical reaction can drive a diffusional process. One such system is described in Reference 37. Anisotropy, a necessary condition for the coupling of a chemical reaction to diffusion, was provided by enclosing the reaction between an anion and a cation membrane. The reaction itself was the enzymatic hydrolysis of an amide* to an ammonium salt. Thus, the hydrolysis caused the flow of the product ions through the corresponding membranes and thereby created a net flow of current or established a potential difference, depending on the conditions maintained outside of the reaction cell.

The results of many experiments showed that this system obeyed linear phenomenological relations. Sets of two different membranes were used;

* N-acetyl-L-glutamic acid diamide

one set had a higher density of fixed charge than the other. The corresponding degrees of coupling obtained were 0.79 and 0.53 respectively, and from equation (12) the values of maximum efficiency were computed to be 25 and 7 percent, respectively.

These results mark the first time that the validity of the phenomenological relations have been demonstrated for the coupling of diffusion with a chemical reaction. The discovery of membranes with less leakage and higher charge density would improve the efficiency of this process. Another reaction system would have to be found in order to produce the transport of sodium. The search for both undoubtedly will be helped by the current studies of biological systems.

2.7 MOSAIC MEMBRANES

2.7.1 General

A mosaic membrane consists of separate patches having fixed positive and negative charges. In effect it is an array of cationic and anionic membranes arranged in parallel to the flow. Although it is not known for certain, it is believed that many biological membranes approximate this configuration.

Such membranes have been studied theoretically by Kedem and Katchalsky³³. A convenient form of the phenomenological equations, which are applicable to transport of salts across such membranes under isothermal conditions, are as follows:

$$\begin{aligned}
 J_V &= L_p (\Delta p - \Delta \Pi_s) + c_s L_p (1 - \sigma) \frac{\Delta \Pi_s}{c_s} - \beta I \\
 J_s &= c_s L_p (1 - \sigma) (\Delta p - \Delta \Pi_s) + c_s \omega' \frac{\Delta \Pi_s}{c_s} - \frac{\tau_1'}{v_1 z_1 F} I \\
 E &= -\beta (\Delta p - \Delta \Pi_s) - \left(\frac{\tau_1'}{v_1 z_1 F} \right) \frac{\Delta \Pi_s}{c_s} - \frac{1}{\kappa} I.
 \end{aligned} \tag{14}$$

(The notation is the same as in References 33 and 34. Further, it has been assumed that there is only one ionized solute.) Kedem and Katchalsky showed that for a parallel array, σ for the composite membrane may be negative, and the permeability of the salt, ω' , for the composite may have values one or two orders of magnitude greater than the value given by simple addition of the separate permeabilities of the two membranes. The negative value of σ for such membranes gives rise to a phenomenon called "anomalous osmosis." The constant, $c_s L_p (1 - \sigma)$, may have large positive values and thus enhance the coupling between the flows of salt, J_s , and water, J_V . For $\Delta p = 0$ and $I = 0$,

the diffusion of salt across the membrane from high to low concentration causes a flow of water in the same direction. The normal osmotic flow of water would be in the reverse direction.

Such a membrane also may be used to perform desalination through the coupling of the movement of salt with the flow of water. Application of hydrostatic pressure causes the flow across the membrane of a solution whose salt concentration is greater than that of the source. Thus, from the equation (14), with $I = 0$ and $\Delta p \gg \Delta \Pi_s$, the flows of water and salt are both in the same direction and the concentration of solute in the flow is

$$\frac{J_s}{J_v} \approx \frac{c_s L_p (1-\sigma)}{L_p} = c_s (1-\sigma),$$

where c_s is an average of the concentration of salt in the solutions on the two sides of the membrane. Thus, the concentration of salt in the flow may be greater than that of the source by approximately the factor of $(1-\sigma)$. For suitably constructed membranes, the value of $(1-\sigma)$ may be as great as 9. Normally, in single membranes, it ranges between 0 and 1.

Caplan and his colleagues have prepared samples of mosaic membranes by mounting beads of ion exchange resins in a silicone rubber³⁸. In keeping with the predictions of Kedem and Katchalsky, increased salt permeability and negative values of σ were measured for these membranes. For one membrane the following values were obtained in one series of measurements:

$$\begin{aligned} L_p &= 0.97 \times 10^{-13} \text{ cm}^3/\text{dyne-sec} \\ \omega' &= 4.9 \times 10^{-15} \text{ moles/dyne-sec} \\ \sigma &= -7.1 \end{aligned}$$

For a mosaic membrane the coupling coefficient between water and salt flows is given by

$$q = \frac{c_s L_p (1-\sigma)}{\sqrt{L_p c_s \omega'}} = (1-\sigma) \sqrt{\frac{c_s L_p}{\omega'}} \quad (15)$$

Utilizing the values given above and with $c_s = 1.25 \times 10^{-4}$ moles/cm³, the average salt concentration of the two solutions used in the experiments, the

coupling coefficient is computed to be $q = 0.4$. From equation (13) the maximum efficiency, $-J_s \frac{\Delta\pi_s}{c_s} / J_v \Delta p$, at maximum output for this membrane is only 4.4 percent.

The physical reason for the increased value of ω' and the negative value of σ for mosaic membranes was discussed by Kedem and Katchalsky.¹⁹ For no net current, equation (14) shows that a voltage develops across the membrane, depending on the hydrostatic and osmotic pressures. This voltage causes a circulation of current between adjacent positive and negative elements (passage of cations and anions in the same direction but in different elements). It is this circulation current which enhances the permeability to salt, and the current together with the "drag force" between the water and the ions gives rise to the negative value of σ .

2.7.2 Desalination with Mosaic Membranes

The desalination system considered is shown in Figure 1. Brine, with a concentration c_1 , enters a well stirred tank, one side of which has the mosaic membrane. For a given applied pressure across the membrane, Δp , steady-state conditions will be reached in which the concentration in the tank reaches c_2 , also the concentration of the product stream, and the concentration of the flow out of the membrane has the value of c_0 . If the values for the inlet flow and applied pressure are properly chosen c_0 will be greater than c_1 , and the product c_2 will have a lower concentration.

The problem of interest is to compute the energy expended per unit flow of product at maximum output and for given values of c_1 and c_2 . The energy utilized per unit area of membrane may be written

$$\phi = J_0 (\Delta p - \Delta\pi_s) + J_{s0} \frac{\Delta\pi_s}{c_s} \quad (16)$$

where equations (4) and (14) have been used.

The quantity, c_s , is the average of the salt concentrations on the two sides of the membrane. Dividing by J_2 and using the definition of efficiency, η , for the coupled membrane processes, equation (7), the following formulas

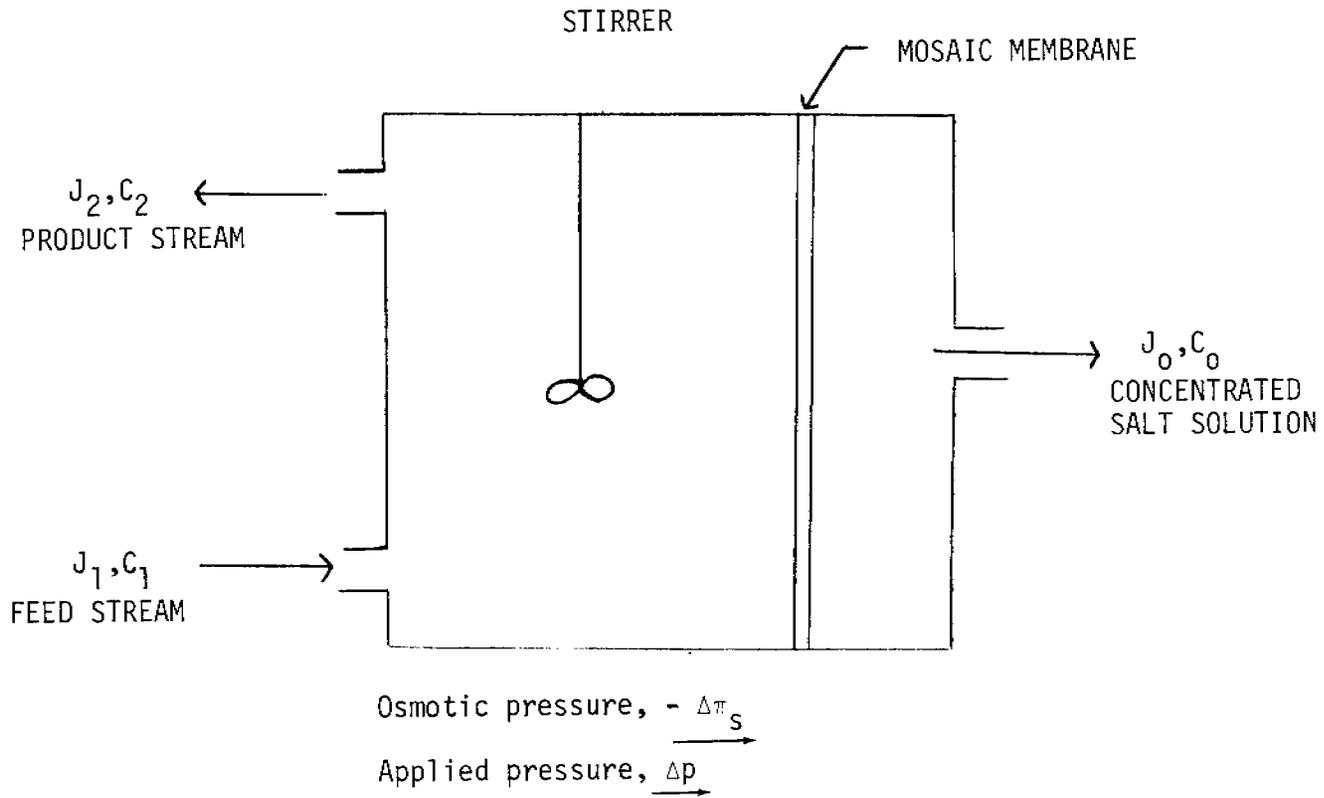


Figure 1. Hypothetical System for Desalination Using Mosaic Membranes

can be obtained after some rearrangement,

$$\frac{\phi}{J_2} = (\alpha - 1) \left(\frac{1}{X} - \frac{\eta}{X} \right) \frac{\Delta\pi_s}{c_s} \quad (17)$$

where

$$\alpha = \frac{J_1}{J_2} \quad (18)$$

and

$$X = \frac{\Delta\pi_s}{(\Delta p - \Delta\pi_s)c_s} \quad (19)$$

The values of η and X may be computed as follows, using the requirement that $J_2/J_1 = 1/\alpha$ shall be a maximum for given values of c_1 and c_2 . Material balances for salt and water may be written as follows:

for water

$$J_2 = J_1 - J_0, \quad (20)$$

and for salt

$$J_{s0} = J_{s1} - J_{s2} = J_1 c_1 - J_2 c_2. \quad (21)$$

Using the definition of η , equation (7), equation (20) and (21) may be combined and rearranged to give the formula

$$\frac{J_2}{J_1} = \frac{1}{\alpha} = \frac{c_1 + \eta/\chi}{c_2 + \eta/\chi}, \quad (22)$$

for which it may be noted

$$\frac{J_{s0}}{J_0} = c_0 = -\eta/\chi. \quad (23)$$

Since applicable values of (η/χ) are both negative and greater than c_1 and c_2 , the maximum value of $1/\alpha$ corresponds to a maximum value of $(-\eta/\chi)$. The quantity $(-\eta/\chi)$ may be computed from the relation,

$$-\eta/\chi = \frac{(1 + \frac{ZX}{q}) \frac{Z}{q}}{\frac{ZX}{q} + \frac{1}{q^2}}, \quad (24)$$

which may be derived from equation (14). From equation (21) and (14), the following supplemental relations may be written

$$q = (1 - \sigma) \sqrt{\frac{c_2 L_p}{\omega'}}, \quad (25)$$

and

$$\frac{Z}{q} = \frac{\omega'}{(1 - \sigma)L_p} \quad (26)$$

Further, c_s may be computed from the relation

$$c_s = \frac{c_0 + c_2}{2} = \frac{c_2 - \eta/\chi}{2}. \quad (27)$$

Substituting equations (25), (26), and (27) into (24) permits computation of $(-\eta/\chi)$ in terms of the parameter, ZX/q ,

$$\frac{\eta}{\chi} = -\frac{b}{2} + \sqrt{\left(\frac{b}{2}\right)^2 + c}, \quad (28)$$

where

$$\frac{b}{2} = \frac{1}{2} \frac{Z}{q} \left[1 + \frac{ZX}{q} - \frac{2}{(1-\sigma)} - \frac{ZX}{q} - \frac{c_2}{(Z/q)} \right], \quad (28a)$$

and

$$c = c_2 \frac{Z}{q} \left[\frac{ZX}{q} + 1 \right]. \quad (28b)$$

Applicable values of $(-\eta/\chi)$ occur only for values of (ZX/q) ranging between -1 and the value prescribed by the condition $c + (b/2)^2 = 0$, for which, it may be shown, $(-\eta/\chi)$ is a maximum.

Values of $(-\eta/\chi)$, the product-to-feed flux ratio, $1/\alpha$, and energy utilization were computed, using equations (28), (22), and (17), respectively, for the reduction of the salinity of an 0.2M salt solution. Selected concentrations ranging from 0.033M to 0.1M were assumed for the product stream. Membrane properties assumed for these calculations were those measured for an experimental membrane operating between salt solutions having concentrations of 0.05M and 0.2M⁽³⁸⁾. The values of the pertinent properties are

$$L_p = 0.97 \times 10^{-13} \text{ cm}^3/\text{dyne-sec}$$

$$\omega' = 4.9 \times 10^{-15} \text{ moles/dyne-sec}$$

$$\sigma = -7.1.$$

Theoretically, it is expected that σ and ω' will vary with the average salt concentration, c_s ⁽³⁴⁾. Consequently the values of the concentration of the feed and product stream were chosen in order that the mean concentration would be close to that used in the experimental measurements.

The results of the calculations are presented in Table I. It is evident that very large applied pressures and expenditures of energy are required for only modest salt reductions. By contrast, a typical reverse osmosis membrane can accomplish the same results using much less energy and much lower applied pressures. Considering the case for which the product salt concentration is 0.1M, the flux of product based on the area of the mosaic membrane is 1.77×10^{-5} cc/cm²-sec, computed using equation (7) and equation (14). For the same operating conditions, a typical reverse osmosis membrane with a filtration coefficient of 1×10^{-5} cc/atm-cm²-sec, requires an applied pressure of only 3.35 atm. The corresponding energy requirement is only 0.09 KWH/1000 Gal.

Obviously, in order to become competitive with conventional membranes for desalination, considerable improvements of the properties of mosaic membranes will be required. What improvements and their extent remain to be defined.

TABLE I

Effectiveness of a Mosaic Membrane for Desalination
of an 0.2M Salt Solution

<u>Product Concentration (moles/liter)</u>	<u>$\frac{\eta}{\chi}$ moles/cc</u>	<u>$\frac{J_2/J_1}{1/\alpha}$</u>	<u>$\frac{\Phi}{J_2}$ KWH/1000 Gal.</u>	<u>Δp (atm)</u>
0.100	3.25×10^{-4}	0.556	15.3	196
0.067	2.84×10^{-4}	0.387	37.0	233
0.050	2.62×10^{-4}	0.292	62.9	257
0.040	2.36×10^{-4}	0.184	-	-
0.033	2.23×10^{-4}	0.120	-	283

3.0 PROGRESS ON PHASE II - MECHANISM OF SALT AND WATER TRANSPORT IN AVIAN SALT GLANDS

3.1 SURVEY OF AVAILABLE INFORMATION ON THE MECHANISM OF SALT AND WATER TRANSPORT IN THE AVIAN SALT GLAND

3.1.1 Function of the Salt Gland

For many years scientists have wondered how marine birds could maintain their salt balance when these birds remained away from land for long periods since the concentration of salt in the blood and body fluids of birds is about 1% and the sea contains over 3% salt. The problem is not so acute with fish-eating birds since fish are approximately 80% H₂O and the salt concentration is appreciably lower in fish than in sea water. However, petrels and penguins subsist to a large extent on invertebrates, and these are in osmotic equilibrium with sea water.

For many years scientists have wondered about the function of the nasal glands (*glandula nasalis*), paired supraorbital structures found in the heads of all birds. In spite of the fact that it had been observed long ago that the size of the glands was much larger in the case of marine birds than in the case of land birds (Technau¹¹²; Holmes *et al.*,⁷³), the only function attributed to these glands was that of secreting fluids analogous to tears which would serve to wash away salt spray. Finally, in 1957, Knut Schmidt-Nielsen solved both problems by some brilliantly simple and clear experiments: Schmidt-Nielsen *et al.*,^{103, 104} administered a measured amount of salt to some captive gulls and then measured the amount of salt excreted both in the urine and from the nasal glands. Only a small part of the ingested salt was excreted in the urine; the majority of the salt was eliminated as a highly concentrated salt solution via the nasal glands - which Schmidt-Nielsen has renamed "the salt glands" (Schmidt-Nielsen⁹⁸). (For a review of the physiology of the salt glands, see Schmidt-Nielsen⁹⁹).

Although these glands are found anatomically in all birds, they have not been found to be functional in all birds. They do not, in any case, function except under conditions of osmotic loading or cholinergic activation. Thus, if birds are kept on a non salt-loaded diet and provided with fresh water to drink, there will be no secretion at all from the salt glands. However, if the fresh water is replaced with water containing 1.5% or more NaCl or/and the food is replaced with high salt content food or/and a sufficient dose of salt is administered to the bird (e.g., by stomach tube or i.v.), then the gland will start secreting. Ellis *et al.*⁴⁷ have stated this more elegantly: "...the genome of the domestic duck contains the necessary information for the development of functional salt-secreting glands, but the full expression of this genetic potential is realized only under conditions of environmental stress."

The volume of secreted fluid, but not the concentration of salt in the fluid, is a function of the salt load given to the bird. The concentration of salt in the secreted fluid varies from species to species, but is fairly constant within one species. The glands may be induced to secrete not only by salt osmotic loading, but also by other osmotic loading (e.g., concentrated sucrose) (Schmidt-Nielsen *et al.*,¹⁰⁴) and by cholinergic activation (by electrical stimulation of the secretory nerve or by the injection of acetylcholine or acetyl- β -methylcholine) (Fänge *et al.*,^{50, 51}). Since the activation is cholinergic, it can be blocked by anesthesia, by atropine, adrenaline, or acetazolamide (Diamox) (Schmidt-Nielsen and Robinson⁵¹). Hokin⁶⁰ has shown that salt gland slices incubated in saline would concentrate Na^{\oplus} if either acetylcholine or ouabain was added to the medium.

The results of Borut and Schmidt-Nielsen⁴¹ demonstrate that although either salt loading or administration of a cholinergic substance will stimulate secretion by the salt gland, the two do not necessarily produce this result by identical mechanisms. Borut and Schmidt-Nielsen studied the respiration of slices of avian salt glands and observed that although the addition of acetyl- β -methylcholine increased the rate of respiration, increasing the concentration of NaCl in the medium decreased the rate of respiration. Hokin⁵⁹ found that the increased respiration did not result in any increase in ADP or in the ATP:ADP ratio.

Lanthier and Sandor⁸⁰ have noted that an equimolar quantity of NaHCO_3 is not as effective as NaCl in stimulating salt gland secretion and NH_4Cl is almost completely non-effective.

Figure 2 shows the location of the salt gland in various birds. In addition to these birds, functioning salt glands have been observed in representatives of each of the major orders of marine birds (penguins, albatrosses, terns, eider ducks, etc.⁹⁷). Under conditions of salt loading, the salt glands of various non-marine birds have been shown to function: geese⁵⁸, White Peking ducks - which are a domesticated variety of the wild mallard duck¹⁰¹, Aylesbury duck¹⁰⁸, etc. Desert birds (the desert partridge and the ostrich) have an unusual secretion pattern¹⁰⁵. When exposed to high temperatures, there is a nasal gland secretion which is unusually high in K^{\oplus} , possibly even 10 times as high as Na^{\oplus} . However, under osmotic stress, (e.g., administration of NaCl), the desert partridge secretes a fluid which is very similar to that obtained from the glands of marine birds (Na^{\oplus} much higher than K^{\oplus}). Even under high salt loads, it has not been possible to observe secretion from the salt glands of pigeons¹⁰⁸, starlings or purple grackles⁵⁵, or chickens^{73,74,101}. Dr. Mabel Hokin has made an extensive study of the differences in salt glands among many avian species (personal communication).

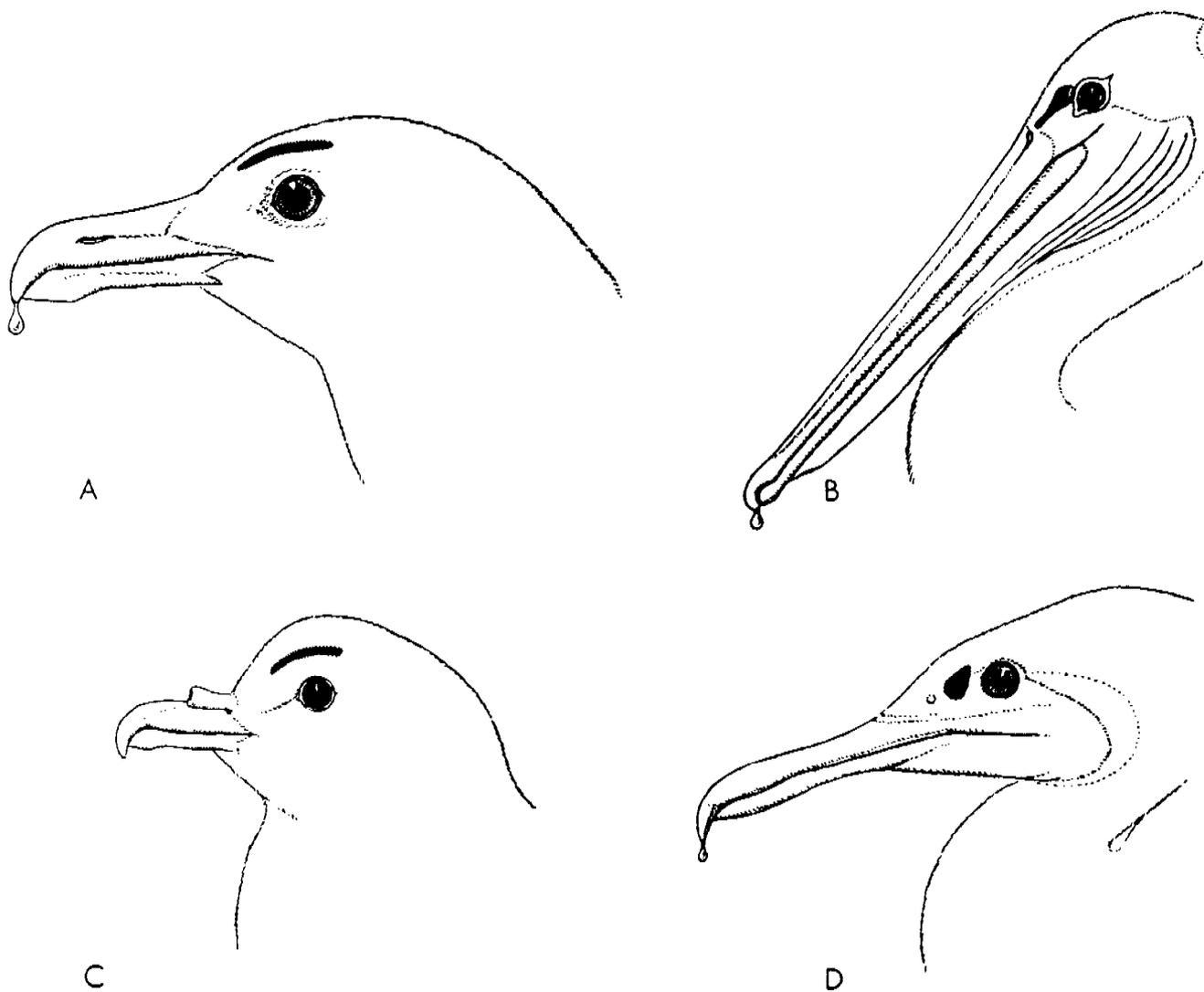


Figure 2. Location of Salt Gland (Hatched Area) in Several Species of Marine Birds. A = Gull; B = Pelican; C = Petrel; D = Cormorant (From Schmidt-Nielsen, 1959).

3.1.2 Structure of the Salt Gland

In Figure 3 is shown the external structure of the salt gland of the gull. When a cross-section of the salt gland is made at the position indicated by "a" in Figure 3, the structures shown in Figure 4 are obtained.

The gland consists of many lobes ("b" in bottom left diagram). When one lobe is enlarged (upper left diagram), it can be seen that each contains thousands of branching tubules radiating from a central duct. Finally when an individual tubule is enlarged (right diagram), it can be seen that each tubule is surrounded by blood capillaries. The flow of blood in the capillaries is counter to the flow in the salt gland tubule. The tubule walls are only one cell thick and consist of rings of wedge-shaped cells. These cells encircle a small hole, or "lumen" through which the salty secretion flows from the tubule into the central canals of the lobe.

The counter-current flow here, and even the general morphology, resemble, to some extent, the kidneys of mammals. There are certain essential differences between salt glands and kidneys: the salt gland is much simpler than the kidney; it has the sole function of excreting salt. The gland fluid contains only H_2O , Na^+ , Cl^- and K^+ . It contains no protein or other constituents.

Together with the simpler function of the salt gland is an increased efficiency of operation: in one minute, the salt gland can produce up to one-half its own weight of concentrated saline. At best, the human kidney can function at 1/10th this rate.

Another major difference between kidney and salt gland is that the kidney functions continuously; the salt gland functions intermittently, only when there is an osmotic or cholinergic demand.

The weight of the salt glands of various birds is given in Table 2. The major effect of the salt content of the diet can be seen in the figures given for the White Peking duck. (This effect had been reported in 1932 by Schildmacher⁹⁵ and in 1926 by Heinroth and Heinroth⁵⁷). The effect of salt in the diet has also been observed in the opposite direction: the weight of herring gull salt glands have decreased to 65% of their initial values when captive gulls are kept on a low salt diet (and controls on a salt water diet maintained the weight of their salt glands)⁴⁰. Staaland¹¹⁰ has correlated the size of the nasal gland of birds with the ecology of the birds. Thus, birds which are rarely seen in marine areas have a low ratio of mg nasal gland/g body weight whereas birds which live in a marine environment, particularly those which feed on planktonic crustaceans have a high ratio of nasal gland/body weight. The larger the nasal gland the higher the concentration of NaCl in the secretion; i.e., there was almost a direct relationship between the length of the secretory tubules and the salt concentration of the nasal gland secretion¹¹⁰. Goertemiller and Ellis⁵⁴ have shown

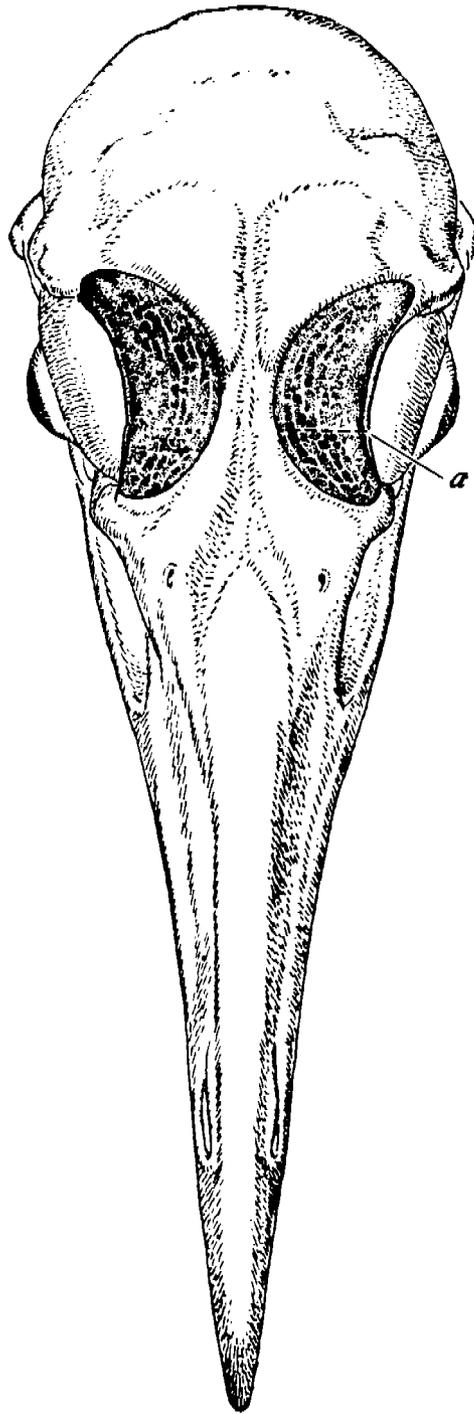


Figure 3. External Structure of the Gull Salt Gland (From Schmidt-Nielsen, 1959).

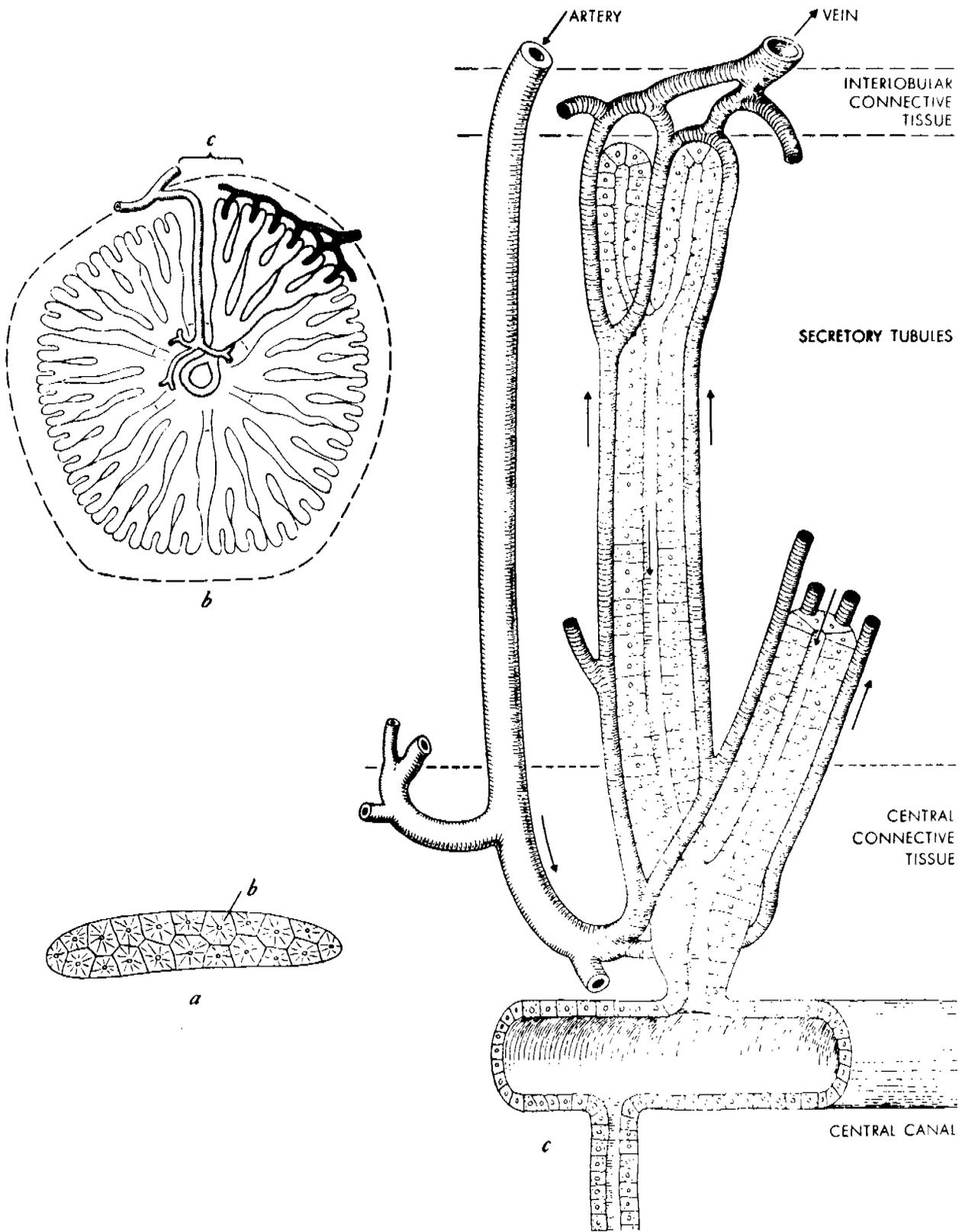


Figure 4. Internal Structure of Gull Salt Gland (From Schmidt-Nielsen, 1959).

<u>BIRD</u>	<u>WEIGHT OF SALT GLANDS</u> <u>(mg)</u>	<u>REFERENCE</u>
Albatross	900	Ellis <u>et al.</u> ⁴⁷
White Peking Duck		
- on normal diet	57	Ellis <u>et al.</u> ⁴⁷
- after salt-loading	151	
White China goose	600	Hokin ⁵⁸
Black-headed gull	50	Fänge <u>et al.</u> ^{50,51}
Common gull	150	Fänge <u>et al.</u> ^{50,51}
Herring gull		Bonting <u>et al.</u> ⁴⁰
- wild	920	
- after 7 weeks on fresh water	600	
Western gull	573-818	McFarland and Sanui ⁸⁸
- after period on 3% NaCl	510-780	McFarland <u>et al.</u> ⁸⁷
Brown pelican	723-1070	Schmidt-Nielsen and Fänge ¹⁰⁰

TABLE 2. WEIGHTS OF SALT GLANDS OF VARIOUS BIRDS

that other salts than NaCl will stimulate the growth of the salt glands of ducklings but that NaCl is the most effective salt for such stimulation.

3.1.3 Salt Concentration

As was mentioned earlier, the salt content of sea water is over 3%, while the salt content of most vertebrate body fluids is less than 1%. (Urine is very hypotonic). Table 3 gives the characteristic salt concentrations of the secretion from the salt glands of various birds. The uniqueness of the salt gland is shown in Table 4 - it is the only known gland in higher vertebrates with a hypertonic secretion.

This secretion contains almost exclusively H_2O , Na^+ , Cl^- and, to a much lesser extent, K^+ . Typical analyses of salt gland secretion are given in Table 5. The concentration of K^+ is much less than 1/15th that of Na^+ in each case. For purposes of comparison, the concentration of the various ions in the plasma of the gull is shown. These concentrations were taken from a secreting gull (one under salt-loading).

Various experimenters have tested the ability of the salt gland to concentrate anions other than Cl^- . I^- was concentrated to ca. 3-4 x the concentration in plasma when NaI was injected into gulls (not quite as high as the concentration of Cl^- ⁴². When an anion with physical chemical properties very similar to Cl^- , SCN^- , was tested, it was secreted by the salt gland at a concentration of 6-7 x that in the plasma, about the same as Cl^- ⁴⁴.

Not only is the concentration of the secreted fluid of the salt gland phenomenal (up to 6-7 times the concentration of the plasma), but so too is the rate of secretion. Thus, when cormorants are given an osmotic load (salt or sucrose), they excrete a 500-600 mN solution at a rate of up to 0.2 ml/min in a 1.5 kg. bird. At this rate, the bird would be depleted of all the NaCl in its body in about 10 hours. In this particular case, Schmidt-Nielsen *et al.*¹⁰⁴ induced secretion in a 1.5 kg bird by administration into the wing vein of 15 ml of 1N NaCl or 15 ml of 2N sucrose. However, McFarland⁸⁴ has shown that even lower amounts of NaCl are sufficient to stimulate salt gland secretion, as low as 0.5g NaCl/kg body weight of gull. Earlier work of McFarland's⁸² had shown salt gland secretion rates for salt-loaded birds ranging from 0.050 ml/min for the American flamingo to 0.685 ml/min for the black-footed albatross.

When 4 g of NaCl (in 50 ml of H_2O) were given orally to a duck¹⁰⁷, secretion started from the salt gland in 45 minutes. By the end of 3-1/2 hours, 0.7 g of Cl^- had been obtained from the nasal secretion and only 0.06 g of Cl^- had been eliminated in the urine. The black-backed gull (*Larus marinus*) is even more efficient⁹⁸: a 1,420 g bird was given 134 ml of sea water via a stomach tube. Within 3 hours, 48 of 54 mEq. of Na^+ which was administered had been excreted: 43.7 mEq via the salt gland and only 4.4 mEq via cloacal excretion.

<u>BIRD</u>	<u>SALT CONCENTRATION (mEq/L) IN SALT GLAND SECRETION</u>	<u>REFERENCE</u>
Cormorant	500-600	Schmidt-Nielsen <u>et al.</u> ¹⁰³
Domestic duck		Schmidt-Nielsen and Kim ¹⁰¹
- on fresh water	270-523 (mean - 435)	
- on salt water	372-599 (mean = 525)	
Mallard duck		Schmidt-Nielsen and Kim ¹⁰¹
- on fresh water	518-561 (mean - 546)	
- on salt water	471-661 (mean = 583)	
Herring gull	700-800	Thesleff & Schmidt-Nielsen ¹¹⁷
Penguin	700-800	Schmidt-Nielsen and Sladen ¹⁰²
Tern	487-508	Hughes ⁷⁵
<hr/>		
Sea Water	500-550	
Isotonic (body fluids)	145	

TABLE 3. SALT CONCENTRATIONS IN THE SECRETION OF SALT GLANDS OF VARIOUS BIRDS

<u>GLAND</u>	<u>SALINITY OF SECRETION</u>
Lacrymal	Isotonic
Digestive	Isotonic
Salivary	Hypotonic
Sweat	Hypotonic
Salt (or Nasal)	Hypertonic

TABLE 4. SALT SECRETION IN GLANDS OF HIGHER VERTEBRATES
(From Schmidt-Nielsen et al.¹⁰⁴)

ELECTROLYTE CONCENTRATION (mEq/L)
IN SALT GLAND SECRETION

SPECIES	Na [⊕]	K [⊕]	Cl [⊖]
Black-footed albatross (<u>Diomedea nigripes</u>)	600-840	17-22	690-942
Laysan albatross (<u>Diomedea immutabilis</u>)	792-900	19-20	818-906
Frigate bird (<u>Fregate minor palmerstoni</u>)	744-792	20-24	818-960
Blue-footed booby (<u>Sula nebouxii</u>)	840	20	817
Red-footed booby (<u>Sula sula</u>)	756-758	17-20	800-883
Flightless cormorant (<u>Nannopterum harrisi</u>)	600	14	659
American flamingo (<u>Phoenicopterus ruber</u>)	600-756	23-37	761-853
Western gull (<u>Larus occidentalis</u>)	792	21	818
Emperor penguin (<u>Aptenodytes forsteri</u>)	588	13	690
King penguin (<u>Aptenodytes patagenicus</u>)	430	12	548
Fairy penguin (<u>Eudypturia minor</u>)	570	22	663
Adelie penguin (<u>Pygoscolis odeliae</u>)	360	11	394
Gentoo penguin (<u>Pygoscolis papua</u>)	708	18	731
Galapagos penguin (<u>Spheniscus mendiculus</u>)	660	17	690

SPECIES	ELECTROLYTE CONCENTRATION (mEq/L) IN SALT GLAND SECRETION		
	Na [⊕]	K [⊕]	Cl [⊖]
Aylesbury duck ^(b)	408	14	495
Brown pelican ^(c) (<u>Pelecanus occidentalis</u> <u>carolinensis</u>)	698	13	722
Cormorant (<u>Phalacrocorax</u> <u>auritus</u>) ^(e)	529	12	517
Sea gull (<u>Larus glaucescens</u> , <u>L. occidentalis</u> , <u>L. argentatus</u> , <u>L. californicus</u>) ^(d)	783	33	690
	ELECTROLYTE CONCENTRATION IN PLASMA		
Sea gull ^(d)	190	4	154

TABLE 5. CONCENTRATIONS OF IONS IN SALT GLAND SECRETIONS^(a)

- (a) From McFarland 182, except where noted
 (b) From Scothorne 107a
 (c) From Schmidt-Nielsen and Fänge 100
 (d) From McFarland 84
 (e) From Schmidt-Nielsen et al. 104

Although the concentration of secreted salt is normally independent of the method used for evoking the response (salt or sugar loading, cholinergic stimulation), the temperature response is dependent on the stimulatory technique¹¹¹. Thus, osmotically-stimulated secretion decreased in volume but not concentration when the salt glands were cooled, but acetyl- β -methylcholine-stimulated salt secretion decreased in concentration during periods of gland cooling. Van Rossum¹¹⁶ suggests that at least, in vitro acetyl- β -methylcholine has two different effects: stimulation of ion transport and stimulation of a reaction leading to the reduction of nicotinamide nucleotides (possibly the reversal of electron transfer). Earlier Van Rossum¹¹⁵ suggested that acetyl- β -methyl choline, as K^{\oplus} , increased permeability to Na^{\oplus} . Mechanistic duality is also suggested by the results of Inoue⁷⁶; he found that mercury poisoning or administration of a carbonic anhydrase inhibitor had little or no effect on the volume secreted by the salt gland but did decrease the salt concentration of the fluid secreted.

3.1.4 Postulated Mechanisms

Since even the most efficient of counter-current processes can only lead to equal concentration of material in the effluent stream as in the affluent stream, and since it has been shown that the salt gland concentrates NaCl as much as 6- or 7-fold, there must be an active transport process involved. Some of the principals of active transport are discussed in the next section. There seems to be a high degree of acceptance of the hypothesis that a $Na^{\oplus} - K^{\oplus}$ dependent, ouabain sensitive ATPase is involved in this cellular active transport (Hokin⁵⁸, Bonting et al.⁴⁰). Fletcher et al.⁵³ have shown that there was a positive correlation between the amount of Na^{\oplus} secreted and the ouabain-sensitive ATPase activity in comparing salt-adapted and fresh water-adapted ducks.

Ernst et al.⁴⁸ have shown that the $Na^{\oplus} - K^{\oplus}$ ATPase activity of salt gland is related to the salt intake of ducks - increasing when the birds are kept on high salt and decreasing after the birds were returned to a fresh water regimen.

McFarland et al.⁸⁶ have measured the activity of many enzymes present in avian salt glands (lactic dehydrogenase, phosphoglucosmutase, aldolase, etc.). Van Rossum¹¹⁴ applied spectrophotometric and fluorometric techniques to the study of the effects of respiratory inhibitors and uncouplers on avian salt gland slices.

More controversial is the involvement of phosphatidic acid as the Na^{\oplus} carrier (pro: Hokin and Hokin^{61-66,69}, con: Bonting et al.⁴⁰ These are discussed in the next Section).

An alternative mechanism for active transport in the salt gland has been proposed by McFarland and Sanui⁸⁸: an elution type mechanism of

ion transport, which Sanui and Pace²²³⁻²²⁵ had originally proposed for liver microsomes and erythrocytes. They postulated that a steady state was produced in which metabolically-produced H^{\oplus} diffused outward through an ion exchange boundary region of the cell and this resulted in the preferential elution of less tightly bound Na^{\oplus} . McFarland and Sanui⁸⁸ called this mechanism into play for the salt gland because they felt that other mechanisms did not account for the cation specificity evinced by the salt gland. At the very recent symposium on "Ion Transport Through Biological Membranes" sponsored by the Office of Saline Water (Bethesda, Md., May 1-2, 1967), the cation selectivity of such membranes was still not unequivocally explained, but there seemed to be some indication that it was related to the pore size of the membrane and the size of the hydrated cation.

Several investigators (e.g. Scothorne¹⁰⁷) have established that active salt gland cells are particularly rich in mitochondria. Since the mitochondria are rich in certain enzymes, various concentrating mechanisms postulated for the salt glands have involved some of these enzymes (e.g., ATPase, succinic dehydrogenase, etc.). Holmes et al.^{73,74} have shown that a salt diet given to various birds results in the increased size of the adrenal glands; the adrenal hormones seem to be involved in eliciting the functioning of the salt glands rather than in the mechanism of concentration of salt by the glands.

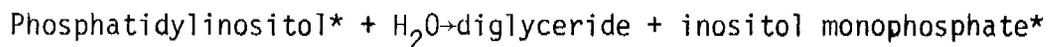
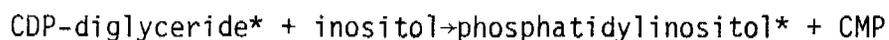
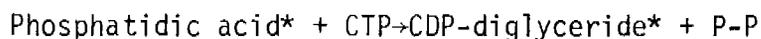
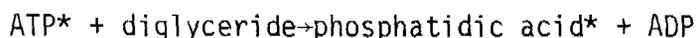
3.1.5 Active Transport

Active transport may be defined in terms of the movement of ions contrary to physical forces resulting in the conversion of metabolic energy to other forms of energy, or active transport may be defined as a movement away from thermodynamic equilibrium. Kedem²¹⁷ has pointed out the applicability of irreversible thermodynamics to active transport and more recently Caplan (1967) reviewed this application, at the symposium on "Ion Transport Through Biological Membranes."

A number of excellent symposia have been held on the general subject of active transport; among these are the following: "Ion Transport Through Biological Membranes", sponsored by the Office of Saline Water, U.S. Department of Interior, May 1-2, 1967; "Sekretion und Exkretion²¹¹", was the theme of the 2nd Conference on the functional and morphological organization of the cell (1964) sponsored by the Gesellschaft Deutscher Naturforscher und Ärzte, the 1963 symposium of the Society of General Physiologists resulted in a book on "The Cellular Functions of Membrane Transport" (ed. by J.F. Hoffman)²¹³ and "Membrane Transport and Metabolism" was the subject of a 1960 Czechoslovakian symposium (ed. by Kleinzeller and Kotyk²¹⁸). Membrane structure and function was the topic of a 1967 conference (Quastel, 1968). Membrane phenomena have been reviewed recently by Rothstein (1968) and the biochemical aspects of active transport have been reviewed by Albers²⁰⁹.

A variety of techniques have led to the conclusion that in the salt gland concentration process there is involved an active Na^{\oplus} transport mechanism and that the Cl^{\ominus} transport is passive. The most direct evidence for this is that of Thesleff and Schmidt-Nielsen¹¹⁷. They found that during secretion of NaCl by the salt gland that the duct of the gland became positive relative to the homolateral jugular vein. Furthermore, when the known sodium transport inhibitor, strophanthin, was administered to the bird, both NaCl secretion and the positive charge (of the duct relative to the jugular vein) were eliminated.

Because of their ability to form lipid-soluble complexes with cations, phosphatides have been suggested as possible cation carriers. One of the strongest items of evidence implicating phosphatidic acid in sodium transport in the avian salt gland is the enhanced incorporation of ^{32}P into phosphatidic acid when albatross salt gland slices were incubated with labeled orthophosphate in the presence of acetylcholine⁶³. (Acetylcholine had been shown by Fänge *et al.*^{50,51} to cause the salt glands to secrete NaCl). In subsequent work (Hokin and Hokin⁶⁶) the Hokins eliminated the possibility that this increased incorporation of ^{32}P into phosphatidic acid in the presence of acetylcholine involved glycerophosphate or other precursors derived from glycerol. They proposed that "phosphatidic acid acts as a transducer for the conversion of the energy of ATP to the osmotic work necessary for Na^+ extrusion" according to a model involving conformational changes effecting the sodium-specific and potassium-specific binding sites (although they recognized the possibility that the results could be interpreted as a response to acetylcholine *per se*). A simplified version of this cycle is illustrated in Figure 5. When the cell secretes salt, some of the phosphatidyl inositol in the membrane is reduced to diglyceride. ATP in the cell provides a phosphate group to produce phosphatidic acid. When the salt secretion stops, the cycle is completed by the conversion of phosphatidic acid to phosphatidyl inositol. Salt secretion may be stopped by eliminating acetylcholine via the addition of cholinesterase or by the addition of atropine. The Hokins⁷¹ propose the following scheme, using known enzymatic reactions:



Not only in the case of the membrane of the avian salt gland, but also in the case of other membranes involved in the active transport of Na^{\oplus}

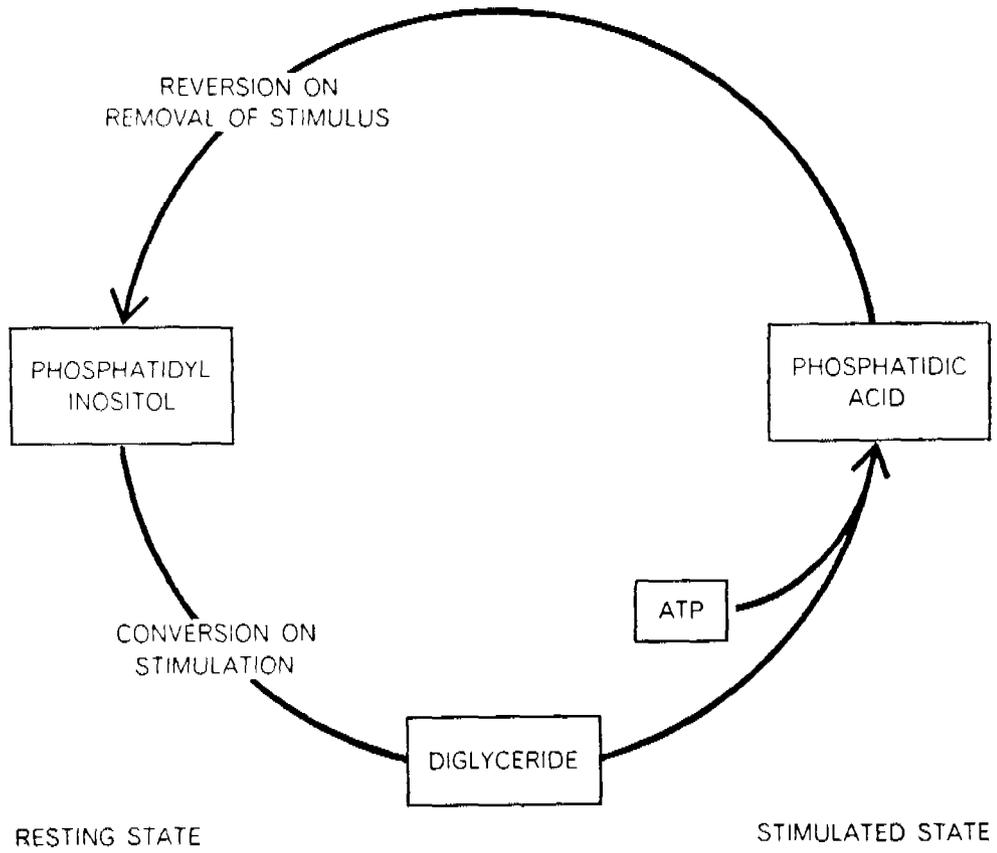


Figure 5. Phosphatidic Acid Cycle (From Hokin and Hokin, 1965a).

and K^{\oplus} , there has been implicated an ATPase (Hokin⁵⁸, Bonting et al.⁴⁰). Although this enzyme has not yet been isolated, many of its characteristics have been determined: 1. It is dependent on both Na^{\oplus} and K^{\oplus} (K_m for Na^{\oplus} = 12.5 m moles/l; K_m for K = 1.5 m moles/l; 2. It is inhibited by ouabain (pI_{50} = 6.30); 3. It has a pH optimum at 7.2 (as opposed to the pH optimum of the Mg-activated ATPase of 8.7); 4. It has a molecular weight of about 480,000; 5. It has many properties in common with other hydrolases, such as sensitivity to DFP, serine in the active site, etc. These last observations (4 and 5) were reported by Hokin at the recent symposium on "Ion Transport Through Biological Membranes." At this symposium there were several reports on this particular enzyme in relation to cation transport. Post gave some preliminary results indicating that mechanistically three processes were involved: 1) combination and dissociation of ions; 2) translocation of ions from one side of the membrane to the other; and 3) phosphorylation and dephosphorylation of the intermediate. Several of the speakers (e.g., Siegel, Post) suggested that there was more than one form of the phosphorylated enzyme, with possibly major differences in the energy of the bond between the enzyme moiety and the terminal phosphorus moiety. The essentiality of ATP to cellular active transport has been reviewed by Whittam²²⁹.

Rothstein⁹⁴ discusses several ATPases: 1. $Na^{\oplus} - K^{\oplus}$ ATPase (see above paragraph); 2. Mg ATPase; and 3. Ca ATPase. Each of these enzymes is involved in cation transport, but they act as different enzymes not only in their ionic activation requirements but also with regard to the action of specific inhibitors and other properties. The inhibition of $Na^{\oplus} - K^{\oplus}$ ATPase as well as the simultaneous inhibition of Na^{\oplus} and K^{\oplus} transport has been reviewed by Glynn²¹² and more recently by Ruoho et al.²²². Medzihradsky et al.²²¹ have obtained an apparent molecular weight of 670,000 for $Na^{\oplus} - K^{\oplus}$ ATPase (contrasted to molecular weights of 775,000 and 265,000 for ouabain-insensitive ATPases obtained from the same source - guinea pig brain microsomes).

Van Rossum¹¹³ in studies on fluorescence of salt gland slices has obtained some indications of a possible relationship between ion transport and energy-linked reduction of mitochondrial pyridine nucleotides.

A bibliography on the avian salt gland is included as Section 4.2 on this Report.

3.2 DEVELOPMENT OF TECHNIQUES FOR HANDLING AND ASSAYING SALT GLAND MEMBRANES FROM WHITE CHINA GEESE

It has been shown (Hokin, personal communication) that the White China goose when given a diet containing a sufficient amount of salt develops salt glands of large size and secretes a fluid of high salinity. Although the concentrating of salt secreted from the salt glands of the White China goose is not quite as high as that in several marine species, it is still quite appreciable and has permitted Na^{\oplus} transport studies. The chief

advantages of the goose are the opportunity to obtain reproducible conditions and various practical considerations with regard to securing and holding specimens. However, when results of sufficient interest are obtained, they will be tested on such marine species as sea gulls.

Arrangements were made to obtain White China geese from Truslow Farms at Chestertown, Maryland. Male geese were supplied which had been maintained on 1.5% NaCl as liquid for six weeks prior to delivery. An outdoor cage was built for the birds at the Atlantic Research Principal Laboratories. The laboratory of Dr. Mabel Hokin at the University of Wisconsin was visited during the first quarter. Techniques used in the Wisconsin laboratory for the study of salt glands were observed and our experimental plans were discussed.

At the Atlantic Research Laboratories, the birds were fed Wayne Turkey Maintenance Pellets ad lib. Initially, they were given 2.5% NaCl as drinking water to promote development of the salt glands. After about three weeks, one of the birds died; the salt glands of this bird were quite undeveloped. A normal-appearing goose was sacrificed and although the salt glands were quite small, they were normal in appearance.

On the assumption that the failure of the salt glands to develop the desired extent was the result of failure of the geese to ingest NaCl (the geese tended to minimize their consumption of the saline and to maximize their consumption of water used to wash out their cage), every effort was made to minimize the amount of water available to the birds except for the 2.5% NaCl solution. However, the condition of all of the birds deteriorated under this regime and after one of the geese died, 1.5% NaCl was substituted for the 2.5% NaCl solution. Even this change was not sufficient, and the birds were put on fresh water temporarily after a third bird died. There was a very obvious improvement in the geese when they were placed on fresh water, both in somatic properties, (appearance of eyes, bill, comb, etc.) and in their preening habits. Next the birds were given 1.5% NaCl until negative signs were noted in any of the birds; at that point, the saline was replaced by water for a day or two.

Of the first two geese sacrificed after the above regime was initiated, the first had one normal-appearing salt gland and one abnormal. The latter gland was yellowish-orange in color (as contrasted to the normal bright red), had much connective tissue and fat-like tissue. The abnormal gland was not used in experiments, but the normal gland was used. The second sacrificed bird had two normal-appearing salt glands, although one of these was much smaller than the other.

Salt gland slices were obtained with the aid of a Stadie-Riggs microtome. Typical results from fresh tissue were as follows:

<u>Weight of Slice</u>	<u>Area of Slice</u>	<u>Average Thickness of Slice</u>
30 mg	0.805 cm ²	0.373 mm
34 mg	0.660 cm ²	0.516 mm
33 mg	0.628 cm ²	0.524 mm

Areas were determined with the aid of graph paper squares; average thickness was obtained with the assumption that the slice had a specific gravity = 1.00. Frozen tissue tends to yield somewhat thicker slices, as shown in the following typical data:

<u>Weight of Slice</u>	<u>Area of Slice</u>	<u>Average Thickness of Slice</u>
43 mg	0.53 cm ²	0.81 mm
40 mg	0.56 cm ²	0.71 mm
39 mg	0.57 cm ²	0.67 mm
46 mg	0.58 cm ²	0.79 mm

During the last half of this research project, the White China geese were maintained on 2.0% NaCl solution as sole liquid and fed ad lib. Wayne Turkey Maintenance Pellets. All of the birds remained healthy on this regimen and none of them had to have the saline replaced by unsalted water. All birds showed functioning of the salt glands by the presence of a slight secretion in the external nares. All sacrificed birds had bright red salt glands of proper size and appearance. The glands were smaller than those obtained by Dr. Hokin from her geese. It is suggested that in future series of experiments White China geese breeding stock be obtained from Dr. Hokin's breeder.

Dr. Donald Sterling Douglas has contributed his services as a Consultant to this program during the last half of this program. Dr. Douglas, an Associate Professor of Zoology at George Washington University, is thoroughly familiar with the anatomy and physiology of the avian salt gland since he received his Ph.D. with Prof. Knut Schmidt-Nielsen at Duke University (the "discoverer" of the salt gland) and participated for two years in the U.S. Antarctic Research Program, working primarily on the salt glands of penguins. He has also worked on the salt glands of various birds while Assistant Professor of Physiology at Rutgers as well as currently at George Washington University.

The insertion of a catheter into the brachial vein of a goose has been accomplished many times in this report period by the following technique:

the goose is placed on its back on a restraining board similar to the one described by Hughes in her thesis⁷⁵. After wetting the area where the wing is attached to the body with a Zephiran solution (antiseptic), about 2 ml. of a 20% solution of Lidocaine (local anesthetic) is injected subcutaneously. Next, a cut-down is made to expose the brachial vein. Two pieces of silk thread are placed around the vein. After tying off the vein by tightening the lower thread, an opening is made in the vein wall between the two pieces. A length of 0.05" O.D. polyethylene tubing (which is connected to a syringe) is passed into the vein, and the second thread tightened about it. The syringe is kept filled with isotonic saline at all times except when a syringe containing sucrose, ²²Na, or nembutal replaces it temporarily. To keep the vein open, small amounts of saline are injected frequently. To test for patency, the syringe is extended and one looks for venous blood in the exposed portion of the catheter. Details of individual experiments are given below.

To increase the amount of ²²Na injected into the salt gland, two alternatives have been considered. One of these was to inject the labelled material into an ophthalmic artery since this artery supplies the salt gland⁵³. However, dissection revealed the fact that in the White China goose the ophthalmic artery lies below the salt gland, so that it would be necessary to remove the salt gland before one could get at this ophthalmic artery. Since such removal would 1) entail considerable blood loss (the salt gland bleeds profusely if the head is not decapitated prior to removal of the salt gland) and 2) negate the whole experiment, this approach was dropped. The second alternative considered was retrograde injection into the gland. The technique for cannulation of the salt gland involves an incision into the palate and introduction of tubing into the ducts of the gland. Since it is not possible to check on the accurate placing of these cannulas as in the case of blood vessels, and since the technique is quite difficult, it has been tabled.

3.3 ENZYMATIC ASSAY OF SALT GLAND ACTIVITY

To determine whether or not slices of the White China goose salt gland were metabolically active, they were assayed for Na⁺ - K⁺ ATPase (ATP = adenosine triphosphate) activity. This enzyme has been implicated in the active sodium transport process of the glands. A 43.3 mg slice of fresh goose salt gland was homogenized with 1.0 ml of 0.45M sucrose in a Potter-Elvehjem homogenizer surrounded with ice. After incubating 0.2 ml of this homogenate in Fletcher medium⁵³ (Tris chloride buffer, pH 7.4, 10 m M/l; NaCl, 50 m M/l; KCl, 3.33 m M/l; MgSO₄, 0.8 m M/l; ATP, 1.0 m M/l) at 41° for 15 minutes, the reaction was stopped by the addition of 2.0 ml of 20% trichloroacetic acid to a 0.5 ml aliquot which had 2.5 ml of distilled H₂O added to it. Orthophosphate was then determined by the Fiske and SubbaRow technique as detailed in Sigma Technical Bulletin 670. Thus, the mixture was allowed to stand 5-10 minutes after shaking and was then centrifuged. To 2.0 ml of the centrifugate was added 3.0 ml of distilled water and 1.0 ml of acid molybdate solution. After shaking, 0.25 ml of Fiske-SubbaRow reducer

solution was added, and finally after mixing and then standing for 10 minutes, the solutions were read in a Spectronic 20 at 620 $m\mu$ against a blank in which the homogenate had been omitted. Positive values were obtained in this assay (duplicates: O.D. = 0.062, 0.055); these values were not converted to phosphorus equivalents.

Next, a slice of frozen salt gland (38.2 mg) was homogenized as above; all other steps were similar except that aliquots were removed from the incubation vessel at ten minute intervals. The following results were obtained:

<u>Incubation Time at 41°C</u>	<u>O.D.(620 $m\mu$)</u>
0'	0.048
10'	0.089
20'	0.131
30'	0.152

Thus, inorganic phosphate is progressively being released from ATP, but at a decreasing rate; this is the result one would expect as a result of the catalytic action of $Na^{\oplus} - K^{\oplus}$ ATPase.

Next, one portion of salt gland slice homogenate was boiled for 2' and compared to a sample of homogenate which had not been boiled:

<u>Incubation time at 40°</u>	<u>O.D.-Boiled Homogenate</u>	<u>O.D.-Unboiled Homogenate</u>
1'	0.020	0.040
10'	0.015	0.089
20'	0.016	-
30'	0.012	0.135
60'	-	0.178

This experiment reinforces the conclusion that the observed activity is enzymatic in nature.

As a check that the observed activity resulted from ATPase activity, 0.1 m M/l ouabain was added to one aliquot. (The salt gland slice weighed 34.9 mg). This slice was incubated without prior homogenization.

<u>Incubation Time at 41°</u>	<u>O.D.-Without Ouabain</u>	<u>O.D.-With Ouabain</u>
0'	0.018	0.021
10'	0.052	0.021
30'	0.106	0.031
60'	0.131	0.038

The ouabain almost completely stopped the release of inorganic phosphate.

The salt gland has been shown to possess several ATPases; of these only the $\text{Na}^{\oplus} - \text{K}^{\oplus}$ ATPase is of interest in these active sodium transport studies. Ernst et al.⁴⁸ have shown that it is possible to distinguish between this enzyme and a $\text{Mg}^{\oplus\oplus} - \text{ATPase}$ by the fact that only the former is inhibited by ouabain. Recently, Bakkeren and Bonting²³¹ have published a technique for separating the activity of these two enzymes: pretreatment with 1.5 M urea decreases the activity of the $\text{Mg}^{\oplus\oplus}$ ATPase without affecting the activity of the $\text{Na}^{\oplus} - \text{K}^{\oplus}$ ATPase. $\text{Na}^{\oplus} - \text{K}^{\oplus}$ ATPase may also be inhibited by fluoride²³². (In another recent paper, Bowler and Duncan²³³ have confirmed that $\text{Na}^{\oplus} - \text{K}^{\oplus}$ ATPase and $\text{Mg}^{\oplus\oplus}$ ATPase are separate enzymes).

As another check, a 19.4 mg slice was incubated in medium in which Na^{\oplus} and K^{\oplus} were replaced by $\text{Mg}^{\oplus\oplus}$. This medium contained 10 m M/l Tris Cl buffer, pH 7.4; MgCl_2 , 50 m M/l; MgSO_4 , 0.8 m M/l and ATP, 1.0 m M/l.

<u>Incubation Time</u>	<u>O.D.</u>
0'	0.010
10'	0.031
30'	0.081
60'	0.150

Either the gland contained sufficient Na^{\oplus} and K^{\oplus} to allow $\text{Na}^{\oplus} - \text{K}^{\oplus}$ ATPase to function, or some ouabain-sensitive ATPase other than the $\text{Na}^{\oplus} - \text{K}^{\oplus}$ ATPase is responsible for the previously described results.

3.4 ISOTOPIC ASSAY OF SALT GLAND ACTIVITY

An even more direct assay for the salt gland slices would be to measure radioactive sodium uptake. Furthermore, uptake of the labeled sodium might allow positive identification of the moiety(s) involved in active sodium transport. Work in other laboratories with ^{32}P uptake yields only indirect support for postulated active sodium transport mechanisms.

Ginzburg *et al.*²³⁴ have recently published the first paper which presents direct evidence for the incorporation of Na^{\oplus} into a membrane presumably involved in active salt transport. They use three methods for demonstrating the incorporation of ^{22}Na , and attempts have been made to adapt their techniques to the salt gland problem. In particular their third method seemed to offer great possibility: the membranes are washed in a solution containing $\text{Mg}^{\oplus\oplus}$ at relatively high concentration. Presumably, these ions will be incorporated into all non-specific cation-binding sites. A solution containing dilute $^{22}\text{Na}^{\oplus}$ is then added and after incubation, the tissue is washed and finally ashed with nitric acid. $^{22}\text{Na}^{\oplus}$ incorporation is determined by counting the residue. Inhibition of $\text{Na}^{\oplus} - \text{K}^{\oplus}$ ATPase by ouabain should yield control values.

Slices were incubated in Krebs-Henseleit medium²¹⁹, which had the following composition:

<u>Solution</u>	<u>Parts</u>
0.9% NaCl	100
1.15% KCl	4
0.11 M CaCl_2	3
2.11% KH_2PO_4	1
3.82% $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	1
1.3% NaHCO_3 , gassed for 1 hr. with CO_2	21

A mixture of 5% CO_2 - 95% oxygen was bubbled through the final solution; this same gas mixture was added to each of the incubation systems using the Krebs-Henseleit medium.

A number of slices of frozen goose salt gland were incubated in Krebs-Henseleit medium with ^{22}Na ($1\mu\text{C}$ in each incubation flask). As controls, 10^{-4} M ouabain was added to duplicate systems. After incubation, the flask contents were chilled to 0° and then centrifuged for 10' at 5000 RPM at 0° . After decanting the supernatants, the slices were transferred to a homogenizing tube with 2.0 ml of 0.85% NaCl and homogenized. These homogenates were centrifuged for 15' at 10,000 RPM. Activity was determined on both the final supernatants and the original supernatants (after incubation, but before homogenization) by evaporating them in planchettes and counting in an end-window counter. It was assumed that bound ^{22}Na would show up in the final homogenate as a count greater than that accounted for by trapped solution.

Incubation Time	Weight of Slice	Ouabain	Counts per minute	
			First Supernatant	Second Supernatant
10'	52.1 mg	No	5,410	-
10'	35.3	Yes	5,920	103
30'	29.8	No	5,270	89
30'	28.2	Yes	5,180	77
60'	29.3	No	5,470	93
60'	31.1	Yes	5,180	72

(In these and other isotope experiments in this report, planchettes were counted for 15 minutes or to 10,000 counts).

In the next experiment, H₂O was substituted for the NaCl in the Krebs-Henseleit medium, and incubation times were increased.

Incubation Time	Weight of Slice	Ouabain	Counts per minute	
			First Supernatant	Second Supernatant
30'	15 mg	No	5,500	55
30'	11 mg	Yes	5,170	215
60'	11 mg	No	5,270	49
60'	10 mg	Yes	5,170	44
90'	10 mg	No	5,400	45
90'	10 mg	Yes	5,680	47

The only result which seems to indicate ²²Na uptake is for the slice incubated 30 minutes in the presence of ouabain; this is most likely an erroneous result.

The above isotope experiment was performed with a frozen salt gland. The next experiment was performed with a fresh salt gland (obtained from goose with one undeveloped gland).

<u>Incubation Time</u>	<u>Weight of Slice</u>	<u>Ouabain</u>	<u>Counts per minute</u>	
			<u>First Supernatant</u>	<u>Second Supernatant</u>
30'	21 mg	No	4,930	55
30'	18 mg	Yes	5,320	52
60'	17 mg	No	5,440	52
60'	13 mg	Yes	5,110	38
90'	19 mg	No	5,290	56
90'	25 mg	Yes	5,110	75

Since there was effectively no evidence for ^{22}Na incorporation into the salt gland tissue, in the next experiments, acetylcholine (AcCh) or/and ATP was added. AcCh was added to Krebs-Henseleit medium without NaCl to a final concentration of 10^{-4}M in the experiments indicated; a similar concentration of eserine was added in these experiments in order to inhibit any cholinesterase activity which would hydrolyze the AcCh. ATP was used at the level of 10^{-3}M . After incubating the various mixtures at 41° for one hour and then processing as in the previous experiments, the following results were obtained.

<u>Substrate(s) Added</u>	<u>Counts per minute</u>	
	<u>First Supernatant</u>	<u>Final Supernatant</u>
AcCh	5,760	46
ATP	5,720	51
AcCh & ATP	6,100	49

Since lack of incorporation of ^{22}Na might be attributed to lack of $\text{Na}^\oplus - \text{K}^\oplus$ ATPase activity resulting from the low Na^\oplus concentration in the previous experiment, the experiment was repeated in the presence of complete Krebs-Henseleit medium:

<u>Substrate(s) Added</u>	<u>Counts per minute</u>	
	<u>First Supernatant</u>	<u>Final Supernatant</u>
AcCh	3,870	53
ATP	3,650	61
AcCh & ATP	4,510	43

Next, the experiments were performed on fresh salt gland slices (the last few experiments utilized frozen slices.) Frozen slices had been used since they had shown ATPase activity, and it was found convenient to freeze one of the salt glands at the time of sacrifice and run experiments with fresh slices of the other gland.

<u>Substrate(s) Added</u>	<u>Counts per minute</u>	
	<u>First Supernatant</u>	<u>Final Supernatant</u>
AcCh	3,540	48
ATP	3,720	70
AcCh & ATP	3,910	65

None of the experiments have given evidence for the incorporation of ^{22}Na into the salt gland tissue.

The next experiment in this series involved washing the slices incubated in Krebs-Henseleit (K-H) buffer containing ^{22}Na with a MgCl_2 solution to determine whether such a washing would remove the ^{22}Na label. Since ouabain inhibits the transport of Na out of the cell, experiments were run in the presence and absence of this glycoside. One slice was incubated in 3.0 ml of K-H + $1\mu\text{C } ^{22}\text{Na}$ + 10^{-4}M ouabain. After 1 hr of shaking at 41° , the contents of each flask were centrifuged at 10,000 RPM for 10', supernatants poured off and pellets washed with 2.0 ml of $2.0 \times 10^{-2}\text{M}$ MgCl_2 . After centrifugation, the supernatants were discarded. Finally, the pellets were scraped into glass tubes and dissolved in concentrated HNO_3 . The dissolved contents were dried on a planchette and counted. For comparison, previous results in which the MgCl_2 wash was omitted are included in the following table:

<u>Substrate added</u>	<u>Weight of Slice</u>	<u>MgCl_2 Wash</u>	<u>CPM</u>	<u>CPM/Mg</u>
AcCh	19 mg	No	33	1.7
ATP	21	No	41	1.9
AcCh & ATP	18	No	23	1.3
None	35	Yes	6	0.17
Ouabain	32	Yes	3	0.09

The results seem to indicate that the ^{22}Na is not bound to a moiety in the salt gland slice but can be removed by a MgCl_2 washing.

To eliminate interference from non-specific sites, the experiment was repeated with a pre-incubation in $MgCl_2$ ($2 \times 10^{-2}M$) before adding the K-H, etc. The results were as follows:

<u>Substrate Added</u>	<u>Av. weight of slice</u>	<u>Av.CPM</u>	<u>Av. CPM/mg</u>
None	41.5 mg	1.7	0.04
Ouabain	42.5 mg	1.7	0.04

Thus, almost all incorporation of ^{22}Na has been eliminated.

The experiment (including the pre-incubation with $MgCl_2$) was repeated, but $10^{-3}M$ ATP was added to half of the flasks. The results were as follows:

<u>Substrate(s) Added</u>	<u>Av. weight of slice</u>	<u>Av.CPM</u>	<u>Av. CPM/mg</u>
None	12.5 mg	19	1.5
Ouabain	12.0	17	1.4
ATP	14.5	18	1.2
ATP & ouabain	14.5	15	1.0

The increase over the incorporation observed in the last experiment is not understandable; but the addition of ATP seems to have little effect.

All of the experiments described thus far in this section utilized frozen salt glands. At this point, a fresh gland was obtained and various experiments repeated to determine whether different ^{22}Na incorporation results would be obtained with fresh tissue. Gland slices were incubated in K-H for 1 hr at $41^\circ C$, with or without $10^{-3}M$ ATP, with or without $10^{-4}M$ ouabain, but always with $1\mu C$ of ^{22}Na . The following results were obtained:

<u>Substrate added</u>	<u>Av. weight of slice</u>	<u>Av.CPM</u>	<u>Av.CPM/mg</u>
None	19.0 mg	17	0.89
Ouabain	20.0	17	0.85
ATP	24.0	18	0.75
ATP & Ouabain	25.5	17	0.67

Again, the results are negative.

In the next experiment, the effects of AcCh and ATP were tested. Another modification was made in that slices were pre-incubated for 30 min. at 41° either in $2 \times 10^{-2}M$ $MgCl_2$ or in $2 \times 10^{-2}M$ $MgCl_2$ + $10^{-4}M$ ouabain. In the final incubation, whenever AcCh ($10^{-4}M$) was added, an equal amount of eserine was added (to protect the AcCh from enzymatic hydrolysis). ATP was used at a level of $10^{-3}M$. The following results were obtained:

Pre-incubation	Substrate(s) added to final incubation	Av. weight of slice	Av. CPM	Av. CPM/mg
$MgCl_2$	AcCh	24.0 mg	28	1.2
$MgCl_2$ + ouabain	AcCh	22.5	35	1.6
$MgCl_2$	AcCh + ATP	21.5	31	1.4
$MgCl_2$ + ouabain	AcCh + ATP	25.5	45	1.8

The basic isotope incorporation experiment was repeated, decreasing the incubation time to 20 min. Without the $MgCl_2$ wash, the gland slice had an activity of 145 CPM; this dropped to 57 CPM after the $MgCl_2$ wash. Nevertheless, more activity was observed after a shorter incubation period than used previously.

3.5 ATTEMPTED IDENTIFICATION OF ^{22}Na MOIETY

In a recent paper, Rapoport²³⁰ has stated that "...anisotropic transport...demands for complete analysis a particular knowledge of the system..." This point has been stressed in almost every one of the first phase reports, both formal and informal, and is central to the motivation for much of the research in the second phase: identification of the moieties involved in the active transport of Na^+ .

Fresh salt gland slices (0.262g) were placed in 3 flasks each containing 3.0 ml $2 \times 10^{-2}M$ $MgCl_2$ and shaken for 1 hr at 41°. The slices were then transferred to flasks containing 3.0 ml of K-H + $10^{-4}M$ AcCh, $10^{-4}M$ eserine, $10^{-3}M$ ATP and $1\mu C$ of ^{22}Na . After shaking an additional hour at 41°, the supernatants were replaced by $MgCl_2$ and shaken an additional 30 min. The supernatants were removed after centrifugation and contained 76 CPM (total) of ^{22}Na . The slices were transferred to a Potter-Elvehjem tube, together with 3.0 ml of ethanol (EtOH). After thorough homogenization, the product was centrifuged for 10' at 10,000 RPM. An aliquot of the supernatant was evaporated to dryness and counted; a total count of 20 CPM was indicated in the EtOH supernatant. In spite of this low activity, the material was placed on a Sephadex HP-G-25 column and the column developed with EtOH. No indication of separation of $^{22}NaCl$ from ^{22}Na attached to an organic moiety could be seen.

Next, an effort was made to obtain material with higher activity. Three slices of fresh salt gland were added to 3.0 ml of K-H + 10^{-4} M AcCh, 10^{-4} M eserine, 10^{-3} M ATP and 1μ C of ^{22}Na . After shaking for 2 hours at 41° , the mixture was centrifuged at 10,000 RPM for 10 min. The supernatant was removed; it contained 30,000 CPM of ^{22}Na . The remaining material from each slice was homogenized in 3.0 ml of EtOH. After centrifugation, an aliquot of the supernatant from each slice was evaporated to dryness and counted. The total activity in this supernatant at this stage amounted to about 75 CPM. This material was too weak to show any separation on a column.

Slices from a salt gland were incubated in a similar medium as in the previous paragraph. One slice was oxidized in HNO_3 and yielded 224 CPM. After incubation, another slice was homogenized in 3.0 ml of 90% EtOH. A 0.5 ml aliquot of the supernatant after centrifuging yielded 45 CPM (corresponding to 270 CPM for the entire slice). The remaining 225 CPM were sent through a Sephadex LH-20 column (in EtOH), but no activity peaks were discernible.

In the next experiment, the effect of a final NaCl wash was tested as well as the addition of ouabain to the NaCl wash. Fresh gland slices were individually incubated in 3.0 ml of K-H + 1μ C ^{22}Na for 5 min. at 37° . Then 0.1 ml of 10^{-3} M ouabain was added to each slice and incubation was continued for another 10 min. Then one slice was ashed in HNO_3 and the other slices were washed in 0.9% NaCl or 0.9% NaCl + ouabain before ashing. The final results were as follows:

<u>Final wash</u>	<u>Weight of slice</u>	<u>CPM</u>	<u>CPM/mg</u>
None	30 mg	36	1.2
NaCl + ouabain	27	1.4	0.05
NaCl	28	9.9	0.35

When this experiment was repeated using slices from frozen salt gland and changing the initial incubation period to 20 min. and the second incubation period (i.e., after ouabain) to 15 min., the following results were obtained:

<u>Final Wash</u>	<u>Weight of slice</u>	<u>CPM</u>	<u>CPM/mg</u>
None	88 mg	56.8	6.5
NaCl + ouabain	87	5.6	0.64
NaCl	61	0.1	0

The next series of experiments were concerned with the time factor in incorporation of ^{22}Na . Fresh gland slices were shaken at 37° for different lengths of time in K-H with $0.1\mu\text{C}$ of ^{22}Na added, both with and without added 10^{-3}M ouabain. The following results were obtained:

<u>Incubation period</u>	<u>Added Substrate</u>	<u>Weight of Slice</u>	<u>CPM</u>	<u>CPM/mg</u>
2 min.	None	50 mg	21	0.42
2 min.	Ouabain	64	35	0.54
30 min.	None	34	61	1.8
30 min.	Ouabain	31	42	1.4

This experiment was repeated, using frozen gland slices:

<u>Incubation period</u>	<u>Added Substrate</u>	<u>Weight of Slice</u>	<u>CPM</u>	<u>CPM/mg</u>
2 min.	None	61 mg	51	0.84
2 min.	Ouabain	76	66	0.87
30 min.	None	60	77	1.3
30 min.	Ouabain	49	69	1.4

Next, an intermediate incubation time was inserted:

<u>Incubation period</u>	<u>Added Substrate</u>	<u>Weight of Slice</u>	<u>CPM</u>	<u>CPM/mg</u>
2 min.	None	63 mg	96	1.5
2 min.	Ouabain	46	83	1.8
15 min.	None	74	137	1.9
15 min.	Ouabain	80	109	1.4
30 min.	None	54	93	1.7
30 min.	Ouabain	56	101	1.8

When these results were plotted, no trend was discernible.

The same type of experiment was repeated with very short incubation periods, and also with the use of a 10 sec. wash in K-H after the incubation. The results were as follows:

<u>Incubation period</u>	<u>Weight of Slice</u>	<u>CPM</u>	<u>CPM/mg</u>
10 sec.	43 mg	7	0.16
1 min.	26	11	0.43
3 min.	22	14	0.63
5 min.	17	13	0.77

There is an obvious trend here.

Using this washing technique, gland slices were incubated in K-H + AcCh both with and without ouabain. The following results were obtained.

<u>Incubation period</u>	<u>Added Substrate</u>	<u>Weight of Slice</u>	<u>CPM</u>	<u>CPM/mg</u>
1 min.	None	34 mg	13	0.38
2 min.	None	28	9	0.33
3 min.	None	27	12	0.43
1 min.	Ouabain	32	10	0.33
2 min.	Ouabain	20	10	0.50
3 min.	Ouabain	25	17	0.67

The experiment with added ouabain was repeated, and the same trend noted as above: 1 min. = 0.52 CPM/mg, 2 min. = 0.77 CPM/mg, 3 min. = 0.91 CPM/mg.

3.6 MODEL SYSTEMS

Since it has been postulated⁶¹ that the active transport carrier is phosphatidic acid, an attempt was made to separate ^{22}Na phosphatidate from $^{22}\text{NaCl}$. In order to get 10 mg of phosphatidic acid into solution in 1.0 ml of EtOH, it was necessary to add 0.07 ml of HCl; to this was added $1\mu\text{C}$ of ^{22}Na . An aliquot of 0.5 ml was evaporated to dryness and assayed 2,518 CPM. A similar amount was placed on an LH20 column (0.9 x 30 cm), and this was followed by addition of EtOH. A fraction collector was used which collected 40 drops per fraction and the following assays were obtained on 0.5 ml aliquots from the designated tubes:

<u>Tube</u>	<u>CPM</u>
10	2
15	0
20	3
25	1
30	0
35	3
40	0
45	0
48	0
51	1
54	60
57	244
60	450
61	395
62	238
63	61
64	0
65	0

Of the total of 2,518 CPM placed on the column, 2,411 CPM were recovered, all in one peak.

The experiment was repeated and a similar result obtained:

<u>Tube</u>	<u>CPM</u>
20	0
25	0
30	0
35	0
40	6.5
45	151
50	399
55	1.6
60	0
65	0

Essentially all of the radioactivity was recovered, and it was all in one peak.

3.7 IN VIVO EXPERIMENTS

The general technique for immobilizing the bird and inserting a cannula into a brachial vein have been detailed in Section 3.1. The first goose accorded this treatment (goose #14) was given an i.v. injection of 10 ml of 20% sucrose. After active secretion was noted from the nares, 50 λ (1 μ C) of a ^{22}Na solution in 0.9% saline was injected. The secretion from the nares was collected and saved. The goose was sacrificed by decapitation and the salt glands removed.

For the next bird (goose #15), a thin window counter was set up with lead brick shielding around the bird's neck and the salt gland positioned approximately directly under the window of the counter. The order of injection of sucrose and ^{22}Na was interchanged to investigate the possibility that a slightly higher count than background would be obtained after injection of the ^{22}Na , and that the count would increase even more when the sucrose osmotic load was added since the salt gland would now be functioning at a high rate. However, the counting rate never increased significantly above background. The bird was sacrificed by decapitation and the salt glands removed.

To increase the chances of observing activity in the monitored salt gland, and to obtain gland slices with greater incorporation of ^{22}Na , 200 λ of ^{22}Na (= 4 μ C) was injected into goose #16. After 4 min., 20 ml of 20% sucrose was injected via the cannula. Although the salt gland was monitored for 20 min. after this injection, no appreciable radioactivity was detected. The bird was sacrificed by injecting sodium pentobarbital via the cannula. As opposed to the birds sacrificed by decapitation, there was intense bleeding when the salt glands of this goose were removed.

Slices were obtained from the salt glands of each of these geese. The entire slices were dissolved in HNO_3 , evaporated on planchettes and yielded very low counts: goose #14 - 0.02 CPM/mg; goose #15 - 0.02 CPM/mg; goose #16 = 0.03 CPM/mg. The secretion from goose #14 had an activity of 21 CPM/ml or, assuming S.G. = 1, 0.02 CPM/mg.

The next set of geese were given much larger doses of ^{22}Na . Goose #17 (body weight slightly more than 5 kg) was given i.v. 30 ml of 20% sucrose. Active secretion was observed in less than 5 min. After 2 more min, 2.0 ml of a solution of ^{22}Na in saline (total activity = 25,000 CPM) was injected. The bird was sacrificed after another 5 min. by injection of pentobarbital and the salt glands removed. A slice of one of the glands was dissolved in HNO_3 and plated, and had an activity of 0.07 CPM/mg. Calculation shows that this is ca. 6x the activity one would expect if the ^{22}Na was randomly distributed.

Goose #18 was treated similarly to #17, except that 5x the amount of ^{22}Na was injected and the secretion from the nares was obtained in beakers:

<u>Time Period</u>	<u>Weight of Secretion</u>	<u>Activity of Secretion</u>	
		<u>CPM/ml</u>	<u>Total CPM</u>
0 - 5'	2.09 g	12,700	26,600
5'- 10'	1.77 g	11,900	21,300
10'- 15'	1.34 g	10,300	13,800

The blood had an activity of 250 CPM/ml, at the time of sacrifice: A slice of gland had an activity of 0.68 CPM/mg (this is about twice what would be expected on the basis of the previous goose).

Goose #19 was treated identical to #18. Goose #20 had 2x the amount of ^{22}Na injected, but otherwise was similar to #18 and #19.

Slices were taken from a salt gland from each goose and the activities determined with the following results:

<u>Goose #</u>	<u>Total ^{22}Na injected</u>	<u>CPM/mg gland</u>
17	25,000 CPM	0.07
18	125,000 CPM	0.57
19	125,000 CPM	0.86
20	250,000 CPM	1.34

Since the 2 salt glands from a goose weigh about 1.5 g, one can incorporate about 2,000 CPM under the conditions used for goose #20.

3.8 DETERMINATION AND ELUCIDATION OF THE EFFECTS OF ELECTRICAL STIMULATION ON THE UPTAKE AND SECRETION OF SODIUM AND CHLORIDE IONS

It has been shown by various authors that the results obtained with electrically-stimulated tissues often more closely simulate effects observed *in vivo* than do those obtained with non-stimulated tissues. In particular, stimulation has been successfully applied to the study of brain slices by McIlwain and co-workers and by O'Neill and co-workers (see bibliography on electrical stimulation, Section 4.3.).

An extensive search of the literature has failed to locate any references in which electrical stimulation has been applied to studies of salt glands. However, it is believed that such study would be fruitful, that the salt gland slices would react analagous to brain slices rather than to slices of kidney or liver. (Kidney slices were found to be insensitive to electrical stimulation by Orrego and Lipmann in 1967¹⁸⁹; liver slices were found to be insensitive to electrical stimulation by Hillmann et al. in 1963¹⁴⁰). Baldessarini and Kopin¹²² showed that nervous tissue was not unique in its responsiveness to electrical stimulation; they were able to obtain similar results with regard to increased efflux of tritiated norepinephrine on stimulation of either brain or heart slices. Even earlier, Kratzing¹⁴⁶ had observed the effect of electrical stimulation on guinea pig diaphragm and, to some extent, on guinea pig lung. His results may be summarized as follows:

<u>Sliced Tissue</u>	<u>Effect of Electrical Stimulation</u>	
	<u>On Respiration</u>	<u>On Aerobic Glycolysis</u>
Brain cortex	+51%	+56%
Diaphragm	+39%	+47%
Lung	+19%	0
Kidney	0	0

Among other arguments for similarity between electrical tissue and salt gland tissue are the following facets for stimulated brain slices: (1) the interrelationship between Na⁺ and K⁺ and electrical stimulation (Cummins and McIlwain¹³⁰; Hillman et al.¹⁴⁰); (2) the depression of the respiration of electrically stimulated brain slices by ouabain¹⁹⁷. If electrical stimulation of salt gland slices increases the turnover of Na⁺ 6-fold as observed by Keesey and Wallgren¹⁴³ for brain slices, the technique would be of value.

The electrical stimulator of McIlwain was ordered and was scheduled for delivery at the beginning of the third quarter. The efficacy of the stimulator was first to be tested on brain slices, since the preponderance of work with electrical stimulation has been reported for this tissue. After these control experiments, the stimulator was to be tested on salt gland slices, using ²²Na to test effects on active transport. The reputed effect of tetrodotoxin in countering the promotion of active transport by electrical stimulation was to be tested.

Since the English source for the electrical stimulator failed to deliver the item, plans for the electrical stimulation experiments had to be dropped.

4.0 REFERENCES AND BIBLIOGRAPHIES

4.1 APPLICATIONS ANALYSIS

1. Forte, J.G., Helbock, H. and Saltman, P. (1967). "A Versatile Apparatus for Membrane Transport Studies." *Anal. Biochem.* 20, 545-547.
2. Huebner, G. and Wetzel, K. (1961). "The Mechanism of Water Diffusion Through Living Membranes." *Ber. Deutsch. Bot. Gesel.* 74, 255-256. [in English translation: AD 804903].
3. Van Rossum, G.D.V. (1966). "Movements of Na^+ and K^+ in Slices of Herring-Gull Salt Gland." *Biochim. Biophys. Acta* 126, 338-349.
4. Edelman, I.S. (1968). "Hormones, Nucleic Acids and the Control of Ion Transport." *Biophys. J.* 8, 1-6.
5. Barry, R.J.C. (1967). "Electrical Changes in Relation to Transport." *Brit. Med. Bull.* 23, 266-269.
6. Cohen, S.R. (1967). "A New Quantitative Relation Between Internal and External Steady-State Concentrations in Active Transport." *Experientia* 23, 712-715.
7. Grundfest, H. (1967). "Some Comparative Biological Aspects of Membrane Permeability Control." *Fed. Proc.* 26, 1613-1626.
8. Bresler, E.H. and Wendt, R.P. (1968). "Onsager's Reciprocal Relations in Convective Flow Across Non-Selective Membranes." *Fed. Proc.* 27, 285.
9. Schultz, S.G., Wilson, N.L. and Epstein, W. (1962). "Cation Transport in *Escherichia coli*. II. Intracellular Chloride Concentration." *J. Genl. Physiol.* 46, 159-166.
10. Semenza, G. (1967). "Rate Equations of Some Cases of Enzyme Inhibition and Activation - Their Application to Sodium-Activated Membrane Transport Systems." *J. Theoret. Biol.* 15, 145-176.
11. Gross, L. (1967). "Active Membranes for Active Transport." *J. Theoret. Biol.* 15, 298-306.
12. Shashova, V.E. (1967). "Electrically Active Polyelectrolyte Membranes." *Nature* 215, 846-847.

13. MacGowan, C. I. and Gillam, W.S. (1961). "Part 1. Survey of Physiological Mechanisms of Sodium and Chloride Ion Transport and Design of Experiment for Application to Demineralizing Saline Water. Part 2. Laboratory Evaluation of Use of Algae in Saline Water Conversion. Phase I Final Report." Office of Saline Water, Research and Development Progress Report No. 52.
14. Epstein, E., Di Luzio, F.C., Gillam, W.S. and Kotch, A. (1955). "Salinity and the Pattern of Selective Ion Transport in Plants." Office of Saline Water, Research and Development Progress Report No. 161.
15. Gregor, H.P., Di Luzio, F.C., Gillam, W.S. and Kotch, A. (1966). "Membrane Phenomena." Office of Saline Water, Research and Development Progress Report No. 193.
16. Bricker, N.S., Di Luzio, F.C., Gillam, W.S. and Podall, H.E. (1966). "Solute and Water Transport Across Biologic Membranes." Office of Saline Water, Research and Development Progress Report No. 206.
17. Ussing, H.H., "General Principles and Theories of Membrane Transport," in Metabolic Aspects of Transport Across Cell Membranes, U. Wisconsin Press (1957).
18. Kedem, O. and A. Katchalsky, "Thermodynamic Analysis of the Permeability of Biological Membranes to Non-Electrolytes," *Biochim. Biophys. Acta* 27, 229 (1958).
19. Kedem, O. and A. Katchalsky, "A Physical Interpretation of the Phenomenological Coefficients of Membrane Permeability", *J. Gen. Physiol.* 45, 143 (1961).
20. Ussing, H.H., "Active Transport and Metabolism", Proceedings of the Symposium on Biophysics and Physiology of Biological Transport, Frascati, June 1965.
21. Kedem, O. and A. Katchalsky, "Thermodynamic Analysis of the Permeability of Biological Membranes to Non-Electrolytes", *Biochim. Biophys. Acta* 27, 229 (1958).
22. Kedem, O. and A. Katchalsky, "A Physical Interpretation of the Phenomenological Coefficients of Membrane Permeability", *J. Gen. Physiol.* 45, 143 (1961).
23. Gross, L. "Active Membranes for Active Transport", *J. Theoret. Biol.* 15, 298 (1967).
24. Katchalsky, A., "Living Membranes", *Science and Technology*, December 1967.

25. Zwolinski, B.J., H. Eyring and C. E. Reese, "Diffusion and Membrane Permeability. I", J. Phys. Colloid Chem. 53, 1426 (1949).
26. Laidler, K.J., Chemical Kinetics, McGraw-Hill (1950), p. 305.
27. Schultz, S.G. and A.K. Solomon, "Cation Transport in Escherichia coli: I. Intracellular Na and K Concentrations and Net Cation Movement", J. Gen. Physiol. 45, 355 (1961).
28. Schultz, S. G. Epstein, W. and D. A. Goldstein, "Cation Transport in Escherichia coli: III. Potassium Fluxes in the Steady-State", J. Gen. Physiol. 46, 343 (1962).
29. Schultz, S.G., Epstein, W. and A. K. Solomon, "Cation Transport in Escherichia coli: IV. Kinetics of Net K Uptake", J. Gen. Physiol. 47, 329 (1963).
30. Epstein, W. and S. G. Schultz, "Cation Transport in Escherichia coli: V. Regulation of Cation Content", J. Gen. Physiol. 49, 221 (1955).
31. Epstein, W. and S. G. Schultz, "Cation Transport in Escherichia coli: VI. K Exchange", J. Gen. Physiol. 49, 469 (1966).
32. Weiden, P.O., W. Epstein and S. G. Schultz, "Cation Transport in Escherichia coli: VII. Potassium Requirement for Phosphate Uptake", J. Gen. Physiol. 50, 1941 (1967).
33. Kedem, O. and Katchalsky, A., "Permeability of Composite Membranes: Part 1. - Electric Current; Volume Flow and Flow of Solute through Membranes", Trans. Farad. Soc. 59, 1918 (1963).
34. Kedem, O. and A. Katchalsky, "Permeability of Composite Membranes: Part 2, - Parallel Elements", Trans. Farad. Soc. 59, 1931 (1963).
35. Kedem, O. and A. Katchalsky, "Permeability of Composite Membranes, Part 3. - Series Array of Elements", Trans. Farad. Soc. 59, 1941 (1963).
36. Kedem, O. and S.R. Caplan, "Degree of Coupling and Its Relation to Efficiency of Energy Conversion", Trans. Farad. Soc. 61, 1897 (1955).
37. Blumenthal, R., S.R. Caplan, and O. Kedem, "The Coupling of an Enzymatic Reaction to Transmembrane Flow of Electric Current in a Synthetic Active Transport System", Biophys. J. 7, 735 (1967).
38. "Coupling Phenomena in Synthetic Membranes", S. R. Caplan, et al., Quarterly Progress Reports, 1, 2 and 3, Office of Saline Water Grant No. 14-01-0001-977.

4.2 BIBLIOGRAPHY ON AVIAN SALT GLANDS

39. Benson, G.K. and Phillips, J.G. (1964) "Observations on the Histological Structure of the Supraorbital (Nasal) Glands from Saline-Fed and Fresh-water-Fed Domestic Ducks (Anas platyrhynchos)." J. Anatomy 98, 571-578.
40. Bonting, S.L., Caravaggio, L.L., Canady, M.R. and Hawkins, N.M. (1964). "Studies on Sodium-Potassium-Activated Adenosinetriphosphatase. XI. The Salt Gland of the Herring Gull." Arch. Biochem. Biophys. 106, 49-56.
41. Borut, A. and Schmidt-Nielsen, K. (1961). "Respiration of Avian Salt-Secreting Gland in Tissue Slice Experiments." Am. J. Physiol. 204, 573-581.
42. Carey, F.G. and Schmidt-Nielsen, K. (1962). "Secretion of Iodide by the Nasal Gland of Birds." Science 137, 866-867.
43. Chance, B., Lee, C.-P., Oshino, R. and van Rossum, G.D. (1964). "Properties of Mitochondria Isolated from Herring Gull Salt Gland." Am. J. Physiol. 206, 461-468.
44. Douglas, D.S. (1966). "Secretion of Thiocyanate Ion by the Nasal Gland of the Adelie Penguin." Nature 209, 1150-1151.
45. Doyle, W.L. (1960). "The Principal Cells of the Salt-Gland of Marine Birds". Exptl. Cell Res. 21, 386-393.
46. Dulzetto, F. (1965). "The So-Called 'Salt Glands' (Nasal Gland) of Larus ridibundus." Atti Soc. Peloritana Sci. Fis. Mat. Natur. 11, 179-201. [Biol. Ab. 47, 10100 (1966)].
47. Ellis, R.A., Goertemiller, C.C., Jr., De Lellis, R.A. and Kablotsky, Y.H. (1963). "The Effect of a Salt Water Regimen on the Development of the Salt Glands of Domestic Ducklings." Devel. Biol. 8, 286-308.
48. Ernst, S.A., Goertemiller, C.C. Jr. and Ellis, R.A. (1967). "The Effect of Salt Regimens on the Development of (Na⁺ - K⁺)-Dependent ATPase Activity during the Growth of Salt Glands of Ducklings." Biochim. Biophys. Acta 135, 682-692.
49. Fänge, R., Krog, J. and Reite, O. (1963). "Blood Flow in the Avian Salt Gland Studied by Polarographic Oxygen Electrodes." Acta Physiol. Scand. 58, 40-47.
50. Fänge, R., Schmidt-Nielsen, K. and Osaki, H. (1958a). "The Salt Gland of The Herring Gull." Biol. Bull. 115, 162-171.

51. Fänge, R., Schmidt-Nielsen, K. and Robinson, M. (1958b) "Control of Secretion from the Avian Salt Gland." *Am. J. Physiol.* 195, 321-326.
52. Fawcett, D.W. (1962). "Physiologically Significant Specialization of the Cell Surface," *Circulation* 26, 1105-1132.
53. Fletcher, G.L., Stainer, I.M. and Holmes, W.N. (1967). "Sequential Changes in the Adenosinetriphosphatase Activity and the Electrolyte Excretory Capacity of the Nasal Glands of the Duck (*Anas platyrhynchos*) during the Period of Adaptation to Hypertonic Saline." *J. Exptl. Biol.* 47, 375-392.
54. Goertemiller, C.C., Jr. and Ellis, R.A. (1966). "Specificity of Sodium Chloride in the Stimulation of Growth in the Salt Glands of Ducklings." *Zeit. Mik.-Anat. Forsch.* 74, 296-302.
55. Harriman, A.E. and Kare, M.R. (1966a). "Tolerance for Hypertonic Saline Solutions in Herring Gulls, Starlings, and Purple Grackles" *Physiol. Zool.* 39, 117-122.
56. Harriman, A.E. and Kare, M.R. (1966b). "Aversion to Saline Solutions in Starlings, Purple Grackles, and Herring Gulls." *Physiol. Zool.* 39, 123-126.
57. Heinroth, O. and Heinroth, M. (1926-1928). "Die Vögel Mitteleuropas in allen Lebens- und Entwicklungstufen photographisch aufgenommen und in ihrem Seelenleben bei der Aufzucht vom Ei beobachtet." Berlin: H. Bermüller.
58. Hokin, M.R. (1963). "Studies on a $\text{Na}^{\oplus} + \text{K}^{\oplus}$ -Dependent, Ouabain-Sensitive Adenosine Triphosphatase in the Avian Salt Gland." *Biochim. Biophys. Acta* 77, 108-120.
59. Hokin, M.R. (1966). "Respiration and ATP and ADP Levels During Na^{\oplus} Transport in Salt Gland Slices." *Life Sci.* 5, 1829-1837.
60. Hokin, M.R. (1967). "The Na^+ , K^+ and Cl^- Content of Goose Salt Gland Slices and the Effects of Acetylcholine and Ouabain." *J. Genl. Physiol.* 50, 2198-2209.
61. Hokin, L.E. and Hokin, M.R. (1959). "Evidence for Phosphatidic Acid as the Sodium Carrier." *Nature* 184, 1068-1069.
62. Hokin, L.E. and Hokin, M.R. (1960a). "The Role of Phosphatidic Acid and Phosphoinositide in Transmembrane Transport Elicited by Acetylcholine and other Humoral Agents." *Int. Rev. Neurobiol.* 2, 99-136.

63. Hokin, L.E. and Hokin, M.R. (1960b). "Studies on the Carrier Function of Phosphatidic Acid in Sodium Transport. I. The Turnover of Phosphatidic Acid and Phosphoinositide in the Avian Salt Gland on Stimulation of Secretion." J. Gen. Physiol. 44, 61-85.
64. Hokin, L.E. and Hokin, M.R. (1961a) in: Symposium on Membrane Transport, ed. by Kleinzeller, A. and Kotyk, A. Prague: Czechoslovak Academy of Sciences, 204.
65. Hokin, M.R. and Hokin, L.E. (1961b). "Further Evidence for Phosphatidic Acid as the Sodium Carrier." Nature 190, 1016-1017.
66. Hokin, L.E. and Hokin, M.R. (1963a). "Phosphatidic Acid Metabolism and Active Transport of Sodium." Fed. Proc. 22, 8-18.
67. Hokin, L.E. and Hokin, M.R. (1963b) in: Proc. First Intern. Pharmacol. Meeting, 1961 vol 4: Drugs and Membranes. Oxford: Pergamon Press. 23.
68. Hokin, L.E. and Hokin, M.R. (1963d). "On the Lack of Effect of Acetylcholine on Phosphoprotein Metabolism in the Salt Gland of the Sea Gull." Biochim. Biophys. Acta 71, 462-463.
69. Hokin, M.R. and Hokin, L.E. (1964). "The Synthesis of Phosphatidic Acid and Protein-Bound Phosphorylserine in Salt Gland Homogenates." J. Biol. Chem. 239, 2116-2122.
70. Hokin, L.E. and Hokin, M.R. (1965a). "The Chemistry of Cell Membranes." Sci. Am. 213 (4), 78-86.
71. Hokin, M.R. and Hokin, L.E. (1965b). "Interconversions of Phosphatidylinositol and Phosphatidic Acid Involved in the Response to Acetylcholine in the Salt Gland" in: Metabolism and Physiological Significance of Lipids, ed. by Dawson, R.M.C. and Rhodes, D.N. New York: John Wiley & Sons, pp. 423-434.
72. Hokin, M.R. and Hokin, L.E. (1967). "The Formation and Continuous Turnover of a Fraction of Phosphatidic Acid on Stimulation of NaCl Secretion by Acetylcholine in the Salt Gland." J. Genl. Physiol. 50, 793-811.
73. Holmes, W.N., Butler, D.G. and Phillips, J.G. (1961a). "Observations on the Effect of Maintaining Glaucous-Winged Gulls (Larus glaucescens) on Fresh Water and Sea Water for Long Periods." J. Endocrin. 23, 53-61.
74. Holmes, W.N., Phillips, J.G. and Butler, D.G. (1961b). "The Effect of Adrenocortical Steroids on the Renal and Extra-Renal Responses of the Domestic Duck (Anas platyrhynchos) after Hypertonic Saline Loading." Endocrin. 69, 483-495.

75. Hughes, M.R. (1962). "Studies on Renal and Extrarenal Salt Excretion in Gulls and Terns." Ph.D. Thesis, Duke Univ.
76. Inoue, T. (1963). "Nasal Salt Gland: Intedependence of Salt and Water Transport" *Science* 142, 1299-1300.
77. Komnick, H. (1962). "Elektronenmikroskopische Lokalization von Na[⊕] und Cl[⊖] in Zellen and Geweben." *Protoplasma* 55, 414-418.
78. Komnick, H. (1963-1964). "Elektronenmikroskopische Untersuchungen zur Funktionellen Morphologie des Ionentransportes." "...I. Teil: Bau und Feinstruktur der Salzdrüse." *Protoplasma* 56, 274-314 (1963). "...II. Teil. Funktionelle Morphologie der Blütgerässe." *ibid.* 56, 385-419 (1963). "...III. Teil. Funktionelle Morphologie der Tubulsepithelzellen." *ibid.* 56, 605-636, (1963). "...IV. Teil. Funktionelle Morphologie der Epithelzellen des Sammelkanals." *ibid.* 58, 96-127 (1964).
79. Komnick, H. and Komnick, U. (1963). "Elektronenmikroskopische Untersuchungen zur Funktionellen Morphologie des Ionentransportes in der Salzdrüse von Larus argentatus" *Z. Zellforsch.* 60, 163-203.
80. Lanthier, A. and Sandor, T. (1967) "Control of the Salt-Secreting Gland of the Duck. I. Osmotic Regulation." *Canad. J. Physiol. Pharmacol.* 45, 925-936.
81. Marples, B.J. (1932). "The Structure and Development of the Nasal Glands of Birds." *Proc. Zool. Soc. London*, 829-844.
82. McFarland, L.Z. (1959). "Captive Marine Birds Possessing a Functional Lateral Nasal Gland (Salt Gland)" *Nature* 184, 2030.
83. McFarland, L.Z. (1964a). "Static Blood Volume of the Nasal Salt Gland and other Organs of the Sea Gull." *Am. Zool.* 4, (#4), 190.
84. McFarland, L.Z. (1964b). "Minimal Salt Load Required to Induce Secretion from the Nasal Salt Glands of Sea Gulls." *Nature* 204, 1202-1203.
85. McFarland, L.Z. (1965). "Influence of External Stimuli on the Secretary Rate of the Avian Nasal Salt Gland." *Nature* 205, 391-392.
86. McFarland, L.Z., Martin, K.D. and Freedland, R.A. (1964). "The Activities of Selected Enzymes in the Avian Nasal Salt Gland." *Am. Zool.* 4, (#3), 268.
87. McFarland, L.Z., Martin, K.D. and Freedland, R.A. (1965). "The Activity of Selected Soluble Enzymes in the Avian Nasal Salt Gland." *J. Cell. Comp. Physiol.* 65, 237-242.

88. McFarland, L.Z. and Sanui, H. (1963). "Sodium and Potassium Binding by Microsomes from Nasal Salt Gland, Harder's Gland, Kidney and Liver of Sea Gull." *Proc. Soc. Exptl. Biol. Med.* 113, 105-107.
89. McFarland, L.Z. and Warner, R. (1966). "Blood Volume of the Nasal Salt Glands and other Glands and Organs of Sea Gulls" *Nature* 210, 1389-1390.
90. Nechay, B.R., Larimer, J.L. and Maren, T.H. (1960). "Effects of Drugs and Physiological Alterations on Nasal Salt Excretion in Sea Gulls." *J. Pharmacol. Exptl. Ther.* 130, 401-409.
91. Phillips, J.G. and Bellamy, D. (1962). "Aspects of the Hormonal Control of Nasal Gland Secretion in Birds". *J. Endocrin.* 24, VI-VII.
92. Phillips, J.G., Holmes, W.N. and Butler, D.G. (1961). "The Effect of Total and Subtotal Adrenalectomy on the Renal and Extra-Renal Response of the Domestic Duck (Anas platyrhynchos) to Saline Loading." *Endocrin.* 69, 958-969.
93. Quastel, J.H. (1967). "Membrane Structure and Function." *Science* 158, 146-158.
94. Rothstein, A. (1968). "Membrane Phenomena" *Ann. Rev. Physiol.* 30, 15-72.
95. Schildmacher, H. (1932). "Über den Einfluss des Salzwassers auf die Entwicklung der Nasendrüsen." *J. Ornithol.* 80, 293-299.
96. Schmidt-Nielsen, K. (1958). "Salt Excretion in Marine Birds." *Anat. Record* 131, 596-597.
97. Schmidt-Nielsen, K. (1959). "Salt Glands." *Sci. Am.* 200,(1), 109-116.
98. Schmidt-Nielsen, K. (1960). "The Salt-Secreting Gland of Marine Birds." *Circulation* 21, 955-967.
99. Schmidt-Nielsen, K. (1965). "Physiology of Salt Glands." in: Sekretion und Exkretion; Funktionelle und Morphologische Organization der Zelle. Berlin: Springer-Verlag (1965). pp. 269-288.
100. Schmidt-Nielsen, K. and Fänge, R. (1958). "The Function of the Salt Gland in the Brown Pelican." *Auk* 75, 282-289.
101. Schmidt-Nielsen, K. and Kim, Y.T. (1964). "The Effect of Salt Intake on the Size and Function of the Salt Gland of Ducks." *Auk* 81, 160-172.
102. Schmidt-Nielsen, K. and Sladen, W.J.L. (1958). "Nasal Salt Secretion in the Humboldt Penguin." *Nature* 181, 1217-1218.

103. Schmidt-Nielsen, K., Jörgensen, C.B. and Osaki, H. (1957). "Secretion of Hypertonic Solutions in Marine Birds." *Fed. Proc.* 16, 113-114.
104. Schmidt-Nielsen, K., Jörgensen, C.B. and Osaki, H. (1958). "Extrarenal Salt Excretion in Birds." *Amer. J. Physiol.* 193, 101-107.
105. Schmidt-Nielsen, K., Borut, A., Lee, P., and Crawford, E., Jr. (1963). "Nasal Salt Excretion and the Possible Function of the Cloaca in Water Conservation." *Science* 142, 1300-1301.
106. Scothorne, R.J. (1958). "Histochemical Study of the Nasal (Supraorbital) Gland of the Duck." *Nature* 182, 732.
107. Scothorne, R.J. (1959a). "The Nasal Glands of Birds: A Histological and Histochemical Study of the Inactive Gland in the Domestic Duck." *J. Anatomy* 93, 246-256.
108. Scothorne, R.J. (1959b). "On the Response of the Duck and the Pigeon to Intravenous Hypertonic Saline Solutions." *Quart. J. Exptl. Physiol.* 44, 200-207.
109. Scothorne, R.J. (1959c). "Histochemical Study of Succinic Dehydrogenase in the Nasal (Salt-Secreting) Gland of the Aylesbury Duck." *Quart. J. Exptl. Physiol.* 44, 329-332.
110. Staaland, H. (1967a). "Anatomical and Physiological Adaptations of the Nasal Glands in Charadriiformes Birds." *Comp. Biochem. Physiol.* 23, 933-944.
111. Staaland, H. (1967b). "Temperature Sensitivity of the Avian Salt Gland." *Comp. Biochem. Physiol.* 23, 991-993.
112. Technau, G. (1936). "Die Nasendrüse der Vögel" *J. Ornithol.* 84, 511-617.
113. Van Rossum, G.D.V. (1964). "Observations on the Fluorescence Emitted by Slices of Rat Liver and Avian Salt Gland." *Biochim. Biophys. Acta* 88, 507-516.
114. Van Rossum, G.D.V. (1965). "Observations on Respiratory Pigments in Slices of Avian Salt Gland and Rat Liver." "...I. Effects of Inhibitors and Uncouplers." *Biochim. Biophys. Acta* 110, 221-236. "...II. Evidence for Reversal of Electron Transfer." *ibid.* 110, 237-251.
115. Van Rossum, G.D.V. (1966). "Movements of Na⁺ and K⁺ in Slices of Herring-Gull Salt Gland." *Biochim. Biophys. Acta* 126, 338-349.
116. Van Rossum, G.D.V. (1968). "Relation of the Oxidoreduction Level of Electron Carriers to Ion Transport in Slices of Avian Salt Gland." *Biochim. Biophys. Acta* 153, 124-131.
117. Thesleff, S. and Schmidt-Nielsen, K. (1962). "An Electrophysiological Study of the Salt Gland of the Herring Gull." *Am. J. Physiol.* 202, 597-600.

4.3 BIBLIOGRAPHY ON ELECTRICAL STIMULATION OF TISSUE SLICES

118. Ahmed, K., Judah, J.D. and Wallgren, H. (1963). "Phosphoproteins and Ion Transport of Cerebral Cortex Slices." *Biochim. Biophys. Acta* 69, 428-430.
119. Anguiano, G. and McIlwain, H. (1951). "Convulsive Agents and the Phosphates of Brain Examined in Vitro." *Brit. J. Pharmacol.* 6, 448-453.
120. Ayres, P.J.W. and McIlwain, H. (1953). "Techniques in Tissue Metabolism. 2. Application of Electrical Impulses to Separated Tissues in Aqueous Media." *Biochem. J.* 55, 607-617.
121. Bachelard, H.S., Campbell, W.J. and McIlwain, H. (1962). "Sodium and Other Ions of Mammalian Cerebral Tissues, Maintained and Electrically Stimulated in Vitro." *Biochem. J.* 84, 225-232.
122. Baldessarini, R.J. and Kopin, I.J. (1966). "Tritiated Norepinephrine: Release from Brain Slices by Electrical Stimulation." *Science* 152, 1630-1631.
123. Baldessarini, R.J. and Kopin, I.J. (1967). "The Effect of Drugs on the Release of Norepinephrine- H^3 from Central Nervous Tissues by Electrical Stimulation in Vitro." *J. Pharmacol. Exptl. Ther.* 156, 31-38.
124. Bell, J.J. (1958). "Concentration of some Analgesic Compounds and Their Analogues in Tissues Surviving in Vitro." *J. Neurochem.* 2, 265-282.
125. Bollard, B.M. and McIlwain, H. (1959). "Cocaine and Procaine on the Electrically Stimulated Metabolism of Cerebral Tissues."
126. Brierley, J.B. and McIlwain, H. (1956). "Metabolic Properties of Cerebral Tissues Modified by Neoplasia and by Freezing." *J. Neurochem.* 1, 109-118.
127. Burns, B.D. (1958). The Mammalian Cerebral Cortex. London: Arnold.
128. Chan, S.L. and Quastel, J.H. (1967). "Tetrodotoxin: Effects on Brain Metabolism in Vitro." *Science* 156, 1752-1753.
129. Cheng, S.-C. (1961). "Metabolism of Frog Nerve During Activity and Recovery." *J. Neurochem.* 7, 278-288.
130. Cummins, J.T. and McIlwain, H. (1961). "Electrical Pulses and the Potassium and other Ions of Isolated Cerebral Tissues." *Biochem. J.* 79, 330-341.

131. FORDA, O. and McILWAIN, H. (1953). "Action convulsants on Electrically Stimulated Metabolism of Separated Mammalian Cerebral Cortex." *Brit. J. Pharmacol.* 8, 225-229.
132. GORE, M.B.R. and McILWAIN, H. (1952). "Effects of some Inorganic Salts on the Metabolic Response of Sections of Mammalian Cerebral Cortex to Electrical Stimulation." *J. Physiol.* 117, 471-483.
133. HÄKKINEN, H.-M., KULONEN, E. and WALLGREN, H. (1963). "The Effect of Ethanol and Electrical Stimulation on the Amino Acid Metabolism of Rat-Brain-Cortex Slices in Vitro." *Biochem. J.* 88, 488-498.
134. HEALD, P.J. (1953). "The Effect of Metabolic Inhibitors on Respiration and Glycolysis in Electrically Stimulated Cerebral-cortex Slices." *Biochem. J.* 55, 625-631.
135. HEALD, P.J. (1956). "Effects of Electrical Pulses on the Distribution of Radioactive Phosphate in Cerebral Tissues." *Biochem. J.* 63, 242-249.
136. HEALD, P.J. (1958). "Phosphorylserine and Cerebral Phosphoprotein." *Biochem. J.* 68, 580-584.
137. HEALD, P.J. (1959). "Studies on the Phosphoproteins of Brain: The Intracellular Localization in Brain of a Phosphoprotein Involved in the Metabolic Response of Cortical Slices to Electrical Stimulation." *Biochem. J.* 73, 132-141.
138. HEALD, P.J. (1962). "Phosphoprotein Metabolism and Ion Transport in Nervous Tissue: A Suggested Connection." *Nature* 193, 451-454.
139. HEALD, P.J. and McILWAIN, H. (1956). "Techniques in Tissue Metabolism. 4. Apparatus for Maintaining and Rapidly Transferring Tissue Sections." *Biochem. J.* 63, 231-235.
140. HILLMAN, H.H., CAMPBELL, W.J. and McILWAIN, H. (1963). "Membrane Potentials in Isolated and Electrically Stimulated Mammalian Cerebral Cortex. Effects of Chlorpromazine, Cocaine, Phenobarbital, and Protamine on the Tissue Electrical and Chemical Responses to Stimulation." *J. Neurochem.* 10, 325-339.
141. HOROWICZ, P. and LARRABEE, M.G. (1958). "Glucose Consumption and Lactate Production in a Mammalian Sympathetic Ganglion at Rest and in Activity." *J. Neurochem.* 2, 102-118.

142. Joanny, P. and Hillman, H.H. (1963). "Substrates and the Potassium and Sodium Levels of Guinea Pig: Cerebral Cortex Slices in Vitro: Effects of Application of Electrical Pulses, of Inhibitors and of Anoxia." J. Neurochem. 10, 655-664.
143. Keeseey, J.C. and Wallgren, H. (1965). "Movements of Radioactive Sodium in Cerebral-Cortex Slices in Response to Electrical Stimulation." Biochem. J. 95, 301-310.
144. Keeseey, J.C., Wallgren, H. and McIlwain, H. (1965). "The Sodium, Potassium and Chloride of Cerebral Tissues: Maintenance, Change on Stimulation and Subsequent Recovery." Biochem. J. 95, 289-300.
145. King, L.J., Schoepfle, G.M., Lowry, O.H., Passonneau, J.V. and Wilson, S. (1967). "Effects of Electrical Stimulation on Metabolites in Brain of Decapitated Mice." J. Neurochem. 14, 613-618.
146. Kratzing, C.C. (1951). "Metabolic Effects of Electrical Stimulation of Mammalian Tissues in Vitro." Biochem. J. 50, 253-257.
147. Kratzing, C.C. (1953). "The Ability of some Carboxylic Acids to Maintain Phosphate Levels and Support Electrical Stimulation in Cerebral Tissues." Biochem. J. 54, 312-317.
148. Kratzing, C.C. (1956). "Metabolic Effects of Azide on Electrically Stimulated Cerebral Cortex." Biochem. J. 62, 127-132.
149. Larrabee, M.G. (1958). "Oxygen Consumption of Excised Sympathetic Ganglia at Rest and in Activity." J. Neurochem. 2, 81-101.
150. Lindbohn, R. and Wallgren, H. (1962). "Changes in Respiration of Rat Brain Cortex Induced by Some Aliphatic Alcohols." Acta Pharmacol. Toxicol. 19, 53-58.
151. Lindbohn, R. and Wallgren, H. (1966). "Oxidation of Acetate by Rat Cerebral Cortex in Vitro and the Effect of Stimulation." J. Neurochem. 13, 573-577.
152. Marks, N. and McIlwain, H. (1959). "Loss of Excitability in Isolated Cerebral Tissues and its Restoration by Naturally Occurring Materials." Biochem. J. 73, 401-410.
153. McIlwain, H. (1951a). "Metabolic Response in Vitro to Electrical Stimulation of Sections of Mammalian Brain." Biochem. J. 49, 382-393.
154. McIlwain, H. (1951b). "A Means of Metabolic Investigation of Small Portions of the Central Nervous System in an Active State." Biochem. J. 50, 132-140.

155. McIlwain, H. (1951c). "Atropine and Related Compounds on the Metabolism of Electrically Stimulated Sections of Mammalian Cerebral Cortex. Brit. J. Pharmacol. 6, 531-539.
156. McIlwain, H. (1951d). "Glutamic Acid and Glucose as Substrates for Mammalian Brain." J.Mental Sci. 97, 674-680.
157. McIlwain, H. (1952). "Phosphates and Nucleotides of the Central Nervous System." Biochem. Soc. Symp. 8, 27-43.
158. McIlwain, H. (1953a). "The Effect of Depressants on the Metabolism of Stimulated Cerebral Tissues." Biochem. J. 53, 403-412.
159. McIlwain, H. (1953b). "Glucose Level, Metabolism, and Response to Electrical Impulses in Cerebral Tissues from Man and Laboratory Animals." Biochem. J. 55, 618-124.
160. McIlwain, H. (1953c). "Substances which Support Respiration and Metabolic Response to Electrical Impulses in Human Cerebral Tissues." J. Neurol. Neurosurg. Psych. 16, 257-266.
161. McIlwain, H. (1954a). "Study of Human Cerebral Biopsy Specimens in an Electrically Excited Condition." AMA Arch. Neurol. Psych. 71, 488-495.
162. McIlwain, H. (1954b). "Characteristics Required in Electrical Pulses for Stimulation of the Respiration of Separated Mammalian Cerebral Tissues." J. Physiol. 124, 117-129.
163. McIlwain, H. (1956a). "Anaerobic Glycolysis of Cerebral Tissues and a Second Electrically-Induced, Metabolic Defect." Biochem. J. 63, 257-263.
164. McIlwain, H. (1956b). "Electrical Influences and Speed of Chemical Changes in the Brain." Physiol. Rev. 36, 355-375.
165. McIlwain, H. (1959a). "Protein Interactions and Metabolic Response to Stimulating Agents in Isolated Cerebral Tissues: Histones as Inhibitors." Biochem. J. 73, 514-521.
166. McIlwain, H. (1959b). Biochemistry and the Central Nervous System. Boston: Little, Brown.
167. McIlwain, H. (1961a). "Neurochemistry: Neural Maintenance and Excitation." Ann. Rep. Chem. Soc. 57, 367-379.
168. McIlwain, H. (1961b). "Apparatus for Applying Electrical Pulses to Isolated Cerebral Tissues During Metabolic Studies." J. Neurochem. 6, 244-252.

169. McIlwain, H. (1962a). "Appraising Enzymic Actions of Central Depressants by Examining Cerebral Tissues." In: Enzymes and Drug Action, ed. by Mongar, J.L., Boston: Little, Brown and Co. pp. 170-205.
170. McIlwain, H. (1962b). "Electrical Pulses and the in Vitro Metabolism of Cerebral Tissues." In: Neurochemistry: The Chemistry of Brain and Nerve, 2nd Edition, ed. by Elliott, K.A.C., Page, I.H. and Quastel, J.H. Springfield, Ill: C.C. Thomas; pp. 212-225.
171. McIlwain, H. (1963). "Metabolic and Electrical Measurements with Isolated Cerebral Tissues: Their Contribution to Study of the Action of Drugs on Cortical Excitability." In: Brain Function, ed. by Brazier, M.A.B. Berkeley: Univ. of Calif. Press.
172. McIlwain, H. (1964). "Polybasic and Polyacidic Substances or Aggregates and the Excitability of Cerebral Tissues, Electrically Stimulated in Vitro," Biochem. J. 90, 442-448.
173. McIlwain, H. and Buddle, H.L. (1953). "Techniques in Tissue Metabolism. 1. A Mechanical Chopper." Biochem. J. 53, 412-420.
174. McIlwain, H. and Gore, M.B.R. (1951). "Actions of Electrical Stimulation and of 2:4-Dinitrophenol on the Phosphates in Sections of Mammalian Brain in Vitro." Biochem. J. 50, 24-28.
175. McIlwain, H. and Gore, M.B.R. (1953). "Induced Loss in Cerebral Tissues of Respiratory Response to Electrical Impulses, and its Partial Restoration by Additional Substrates." Biochem. J. 54, 305-312.
176. McIlwain, H. and Greengard, O. (1957). "Excitants and Depressants of the Central Nervous System, on Isolated Electrically-Stimulated Cerebral Tissues." J. Neurochem. 1, 348-357.
177. McIlwain, H. and Joanny, P. (1963). "Characteristics Required in Electrical Pulses of Rectangular Time - Voltage Relationships for Metabolic Change and Ion Movements in Mammalian Cerebral Tissues." J. Neurochem. 10, 313-323.
178. McIlwain, H. and Rodnight, R. (1962). Practical Neurochemistry. Boston: Little, Brown.
179. McIlwain, H. and Tresize, M.A. (1956). "The Glucose, Glucogen and Aerobic Glycolysis of Isolated Cerebral Tissues." Biochem. J. 63, 250-257.
180. McIlwain, H., Anguano, G. and Cheshire, J.D. (1951). "Electrical Stimulation in Vitro of the Metabolism of Glucose by Mammalian Cerebral Cortex." Biochem. J. 50, 12-18.

181. McIlwain, H., Thomas, J. and Bell, J.L. (1956). "The Composition of Isolated Cerebral Tissues: Ascorbic Acid and Cozymase." *Biochem. J.* 64, 332-335.
182. Narayanaswami, A. and McIlwain, H. (1954). "Electrical Pulses and Metabolism of Cell-free Cerebral Preparations." *Biochem. J.* 57, 663-666.
183. O'Neill, J.J. and Simon, S.H. (1966). "A Technique for the Study of Electrically Stimulated Tissue with ¹⁴C-Labeled Substrates." *Anal. Biochem.* 14, 17-21.
184. O'Neill, J.J., Simon, S.H. and Cummins, J.T. (1963a). "Inhibition of Stimulated Cerebral Cortex Respiration and Glycolysis by Cholinolytic Drugs." *Biochem. Pharmacol.* 12, 809-820.
185. O'Neill, J.J., Simon, S.H. and Shreeve, W.W. (1963b). "Glucose Oxidation in Electrically Stimulated Guinea Pig Slices." *Fed. Proc.* 22, 271.
186. O'Neill, J.J., Simon, S.H. and Shreeve, W.W. (1965). "Alternate Glycolytic Pathways in Brain. A Comparison Between the Action of Artificial Electron Acceptors and Electrical Stimulation." *J. Neurochem.* 12, 797-802.
187. O'Neill, J.J., Duffy, T.E., Simon, S.H. and Shreeve, W.W. (1968). "Influence of Added Pyruvate on Brain Metabolism: A Pasteur-like Effect, Reversible by Electrical Stimulation." In press.
188. Orrego, F. (1967). "Synthesis of RNA in Normal and Electrically Stimulated Brain Cortex Slices in Vitro." *J. Neurochem.* 14, 851-858.
189. Orrego, F. and Lipmann, F. (1967). "Protein Synthesis in Brain Slices. Effects of Electrical Stimulation and Acidic Amino Acids." *J. Biol. Chem.* 242, 665-671.
190. Setchell, B.P. (1959a). "Cerebral Metabolism in the Sheep. 1. Normal Sheep." *Biochem. J.* 72, 265-275.
191. Setchell, B.P. (1959b). "Cerebral Metabolism in the Sheep. 2. Irreversible Hypoglycaemic Coma." *Biochem. J.* 72, 275-281.
192. Thomas, J. (1956). "The Composition of Isolated Cerebral Tissue: Creatine." *Biochem. J.* 64, 335-339.
193. Varon, S. and McIlwain, H. (1961). "Fluid Content and Compartments in Isolated Cerebral Tissues." *J. Neurochem.* 8, 262-275.

194. Wallgren, H. (1960). "Comparison of the Effect of Ethanol and Malonate on the Respiration of Rat Brain Cortex Slices." *Acta Physiol. Scand.* 49, 216-223.
195. Wallgren, H. (1961). "Effects of Acetylcholine Analogues and Ethanol on the Respiration of Brain Cortex Tissue in Vitro." *Biochem. Pharmacol.* 6, 195-204.
196. Wallgren, H. (1963a), "Rapid Changes in Creatine and Adenosine Phosphates of Cerebral Cortex Slices on Electrical Stimulation with Special Reference to the Effect of Ethanol." *J. Neurochem.* 10, 349-362.
197. Wallgren, H. (1963b). "Ouabain-induced Depression of the Respiration of Electrically Stimulated Brain Slices in Presence and Absence of Ethanol." *Ann. Med. Exp. Fenn.* 41, 166-173.
198. Wallgren, H. (1966). "Effects of Alcohol on Biochemical Processes in the Central Nervous System." *Psychosomatic Med.* 28, 431-442.
199. Wallgren, H. and Kulonen, E. (1960). "Effect of Ethanol on Respiration of Rat-Brain-Cortex Slices." *Biochem. J.* 75, 150-158.
200. Yamamoto, C. and McIlwain, H. (1966a), "Electrical Activities in Thin Sections from the Mammalian Brain Maintained in Chemically-Defined Media in Vitro." *J. Neurochem.* 13, 1333-1343.
201. Yamamoto, C. and McIlwain, H. (1966b). "Potentials Evoked in Vitro in Preparations from the Mammalian Brain." *Nature* 210, 1055-1056.
202. Bollard, B.M. and McIlwain, H. (1957). "Metabolism and Metabolic Response to Electrical Pulses in White Matter from the Central Nervous System." *Biochem. J.* 66, 651-655.
203. Greengard, O. and McIlwain, H. (1955). "Anticonvulsants and the Metabolism of Separated Mammalian Cerebral Tissues." *Biochem. J.* 61, 61-68.
204. Heald, P.J. (1954). "Rapid Changes in Creatine Phosphate Level in Cerebral Cortex Slices." *Biochem. J.* 57, 673-679.
205. Heald, P.J. (1957). "Guanosine Di- and Tri-Phosphates in the Phosphate Metabolism of Cerebral Tissues Promoted by Electrical Pulses." *Biochem. J.* 67, 529-536.
206. Lewis, J.L. and McIlwain, H. (1954). "The Action of Some Ergot Derivatives, Mescaline and Dibenamine on the Metabolism of Separated Mammalian Cerebral Tissues." *Biochem. J.* 57, 680-684.

207. Rowell, E.V. (1954). "Applied Electrical Pulses and the Ammonia and Acetylcholine of Isolated Cerebral Cortex Slices." *Biochem. J.* 57, 666-673.
208. Wollenberger, A. (1955). "Action of Protoveratrine on the Metabolism of Cerebral Cortex. 2. Electrically Stimulated Cerebral-Cortex Tissue." *Biochem. J.* 61, 77-80.

4.4 OTHER REFERENCES

209. Albers, R.W. (1967). "Biochemical Aspects of Active Transport." *Ann. Rev. Biochem.* 36, 727-756.
210. Anon. (1962). Desalination Research and the Water Problem. Publication 941. Washington, D.C.: National Academy of Sciences - National Research Council.
211. Anon. (1965). Sekretion und Exkretion; Funktionelle und Morphologische Organization der Zelle. Berlin: Springer-Verlag.
212. Glynn, I.M. (1964). "Action of Cardiac Glycosides on Ion Movements." *Pharmacol. Rev.* 16, 381-407.
213. Hoffman, J.F. (ed.) (1964). The Cellular Functions of Membrane Transport. Englewood Cliffs, N.J.: Prentice-Hall.
214. Hokin, L.E. and Hokin, M.R. (1963c). "Biological Transport." *Ann. Rev. Biochem.* 32, 553-578.
215. Järnefelt, J. and von Stedingk, L.-V. (1963). "Some Properties of Rat Brain Microsomes." *Acta Physiol. Scand.* 57, 328-338.
216. Kao, C.Y. (1966). "Tetrodotoxin, Saxitoxin and Their Significance in the Study of Excitation Phenomena." *Pharm. Rev.* 18, 997-1049.
217. Kedem, O. (1961). "Criteria of Active Transport," pp. 87-93 in Kleinzeller, A. and Kotyk, A. Membrane Transport and Metabolism.
218. Kleinzeller, A. and Kotyk, A. eds. (1961). Membrane Transport and Metabolism. New York. Academic Press.
219. Krebs, H.A. and Henseleit, K. (1932). "Untersuchungen über die Harnstoffbildung im Tierkörper." *Hoppe-Seyler's Z. Physiol. Chem.* 210, 33-66.
220. Marchesi, V.T. and Palade, G.E. (1967). "The Localization of Mg-Na-K-Activated Adenosine Triphosphatase on Red Cell Ghost Membranes." *J. Cell Biol.* 35, 385-404.
221. Medzihradsky, F., Kline, M.H. and Hokin, L.E. (1967). "Studies on the Characterization of the Sodium-Potassium Transport Adenosinetriphosphatase. I. Solubilization, Stabilization and Estimation of Apparent Molecular Weight." *Arch. Biochem. Biophys.* 121, 311-316.

222. Ruoho, A.E., Hokin, L.E., Hemingway, R.J. and Kupchan, S.M. (1968). "Hellebrigenin 3-Haloacetates: Potent Site-Directed Alkylators of Transport Adenosine-triphosphatase." *Science* 159, 1345-1354.
223. Sanui, H. and Pace, N. (1959). "Sodium and Potassium Binding by Rat Liver Microsomes." *J. Gen. Physiol.* 42, 1324-1345.
224. Sanui, H. and Pace, N. (1962a). "A Flow Elution Cation Transport Mechanism." *Fed. Proc.* 21, 144.
225. Sanui, H. and Pace, N. (1962b). "Sodium and Potassium Binding by Human Erythrocyte Ghosts." *J. Cell. Comp. Physiol.* 59, 251-257.
226. Schwartz, A. and Laseter, A.H. (1963). "Observations on the Mechanism of Action of Ouabain on an Active Transport Enzyme System." *Life Sci.* 1, 363-367.
227. Stadie, W.C. and Riggs, B.C. "Microtome for the Preparation of Tissue Slices for Metabolic Studies of Surviving Tissue *in Vitro*." *J. Biol. Chem.* 154, 687-690.
228. Udall, S.L., Hokem, K. and Di Luzio, F.C. "Saline Water Conversion Report for 1965" Washington, D.C.: Superintendent of Documents, U.S. Government Printing Office.
229. Whittam, R. (1964). "The Interdependence of Metabolism and Active Transport" pp. 139-154 in Hoffman, J.F. The Cellular Functions of Membrane Transport.
230. Rapoport, S.I. (1968). "Active Transport in Isotropic and Anisotropic Systems." *J. Theor. Biol.* 19, 247-274.
231. Bakkeren, J.A.J.M. and Bonting, S.L. (1968), "Studies on $(Na^+ - K^+)$ - Activated ATPase. XX. Properties of $(Na^+ - K^+)$ - Activated ATPase in Rat Liver." *Biochim. Biophys. Acta* 150, 460-466.
232. Yoshida, H., Nagai, K., Kamei, M. and Nakagawa, Y. (1968). "Irreversible Inactivation of $(Na^+ - K^+)$ - Dependent ATPase and K^+ - Dependent Phosphatase by Fluoride." *Biochim. Biophys. Acta* 150, 162-164.
233. Bowler, K. and Duncan, C.J. (1968). "The Effect of Temperature on the Mg^{2+} - Dependent and $Na^+ - K^+$ ATPase of a Rat Brain Microsomal Preparation." *Comp. Biochem. Physiol.* 24, 1043-1054.
234. Ginzburg, B.Z., Friedlander, T. and Pouchovsky, E. (1967). "Specific Binding of Sodium and Potassium Ions in Erythrocyte Membranes." *Nature* 216, 1185-1188 (1967).

5.0 PERSONNEL

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