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FOR ELECTROLYTE TRANSPORT.

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AVIAN SALT GLAND: A MORPHOLOGICAL AND CYTOCHEMICAL STUDY
OF A TISSUE SPECIALIZED FOR ELECTROLYTE TRANSPORT.

by

Billy Joe Martin

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INTRODUCTION

RATIONAL FOR THE STUDY

The intracellular concentrations of sodium and potassium in vertebrate cells differ from that of the extracellular fluids by a factor of approximately 100 and yet the plasma membranes of these cells are, to a varying degree, ion permeable. It follows therefore that active transport of ions is a fundamental and obligate requirement for the maintenance of cell homeostasis. Cellular fluid movement and secretory phenomena must be linked with active ion transport since there is no evidence for active water transport per se. In view of the fundamental importance of transport activity it is not surprising that efforts to understand ion transport at the cellular level have been both widespread and diverse. Although these efforts have produced convincing evidence for certain parameters of the transport process, specific knowledge of the underlying mechanisms remains quite limited.

Chambers (1940) was among the first to realize that certain cells possessed organic coats superficial to their plasma membranes and he suggested that these extraneous coatings were of physiological significance. Later Brandt (1962) emphasized that these materials must be considered in relation to the regulation of exchanges between the cytoplasm and the environment. Bennett (1963), who coined the term "glycocalyx" to describe polysaccharide-rich materials associated with the cell surface, suggested that these materials may be involved in ion transport phenomena. Specifically he indicated that the glycocalyx may have ion exchange resin properties and could therefore accomplish
directed translocation, filtration, and selective binding of ions. By exhibiting any one of these properties acidic carbohydrates at or near the cell periphery could exert an influence on the ionic composition near the cell plasma membrane.

Since these early suggestion there has been a growing interest in the functional relationships between polyanionic surface materials and ion transport. Farber (1960) showed that acid mucopolysaccharides function as cation exchange resins and Eisenman (1962) advanced this idea by demonstrating that cation selectivity in artificial membranes is dependent on membrane negative charges. It is significant here that numerous biophysical studies on artificial membranes have emphasized the importance of fixed charges in ion permeation (Bean et al., 1969; van Breemen and van Breemen, 1969; Coster et al., 1969; Eisenman, 1968; Moore and Schechter, 1969; Pagano and Thompson, 1968; Papahadjopoulos and Ohki, 1969; Parasegian, 1969; Rosenberg and Pant, 1969; Weinstein and Caplan, 1968). Central to the discussion here is the fact that recent evidence from membrane physiologists support and extend these observations to biological membranes. Diamond and Wright (1968), for example, have convincingly demonstrated that ion permeation and selectivity properties of the gall bladder epithelium are determined by anionic sites associated with this surface. Still other studies support a relationship between the anionic carbohydrate, sialic acid, and the transport of inorganic cations and proteins across cell membranes (Cook, 1968).
In studies of the relationships between ion transport and cell morphology there are obvious experimental advantages to working with a tissue functionally specialized for high level electrolyte transport. The secretory cells of such tissues have certain common morphological features. For example, they have large numbers of mitochondria and a very expanded cell surface (Ellis and Able, 1964; Keynes, 1969). This surface expansion often takes the form of infoldings that provide a large surface area in a relatively small space (Fawcett, 1962). Diamond and Bossert (1967, 1968) have attached particular significance to these common architectural features by advancing the hypothesis that the spaces formed by the infoldings are a vital part of the transport mechanism. The finding of common features of geometry that apparently have physiological meaning suggests that further study of the morphology and biochemistry of these tissues should provide an increased understanding of electrolyte transport. Avian supraorbital nasal gland (salt gland) is one of the most highly specialized tissues in this group and possesses characteristics that make it an excellent and uniquely suitable system for study.

CHARACTERISTICS OF AVIAN SALT GLAND

Physiology

Schmidt-Nielsen et al., (1957) who first demonstrated the true function of the avian salt gland, presented physiological evidence that certain marine birds secrete a NaCl solution that is as much as eight times more concentrated than blood plasma. This efficient
extrarenal mode of salt secretion occurs when the birds are submitted to either natural or experimental salt water regimes and demonstrates that they are capable of maintaining water balance in the complete absence of fresh water. The secretory capabilities of the gland are quite impressive. For example, in the herring gull the gland, which by itself represents about 0.1% of the total body weight, is capable of secreting 0.6 ml of fluid per gram of gland per minute (Schmidt-Nielsen, 1962). This is three times the glomerular filtration rate of the human kidney. While maintaining this volume of flow, the gland must also do about 10 Joules/gram-hour of osmotic work to concentrate NaCl from 150 to 720 mM (Keynes, 1969).

The normal physiological stimulus for secretion is an increase in plasma NaCl concentration, but increases in plasma osmolality resulting from an experimental infusion of either sucrose or mannitol cause at least a transitory response (Ash, 1969; Schmidt-Nielsen et al., 1958). It is interesting that Ash (1969) has shown that the infusion of KCl, urea, and dextrose do not produce this effect. The gland's activity is apparently modulated through the central nervous system since stimulation of cholinergic peripheral nerves to the gland causes secretion (Fange et al., 1958)

The highly specialized nature of this gland is also evident upon examining blood flow characteristics. Blood flow is minimal in the non-secretion gland but, upon initiation of secretion, it rises quickly to a level roughly equal to that of the kidney (Keynes, 1969).
Gross Morphology

The glands are paired crescent-shaped structures resting on the frontal bones superior to the orbits of the eyes. Two ducts from the anterior end of each gland descend closely beneath the nasal bones and open directly into the nasal cavity. Thus the effluent is elaborated from the external nares. The main arterial blood supply is the arteria ophthalmica interna, but other relatively large veins and arteries enter the gland from both its anterior and posterior ends. (Marples, 1932; Schmidt-Nielsen, 1960).

Histological Morphology

Each of the paired glands appears to develop embryologically as two outgrowths from the future nasal cavity (Ellis et al., 1963). These two outgrowths form the medial and lateral ducts connecting the glands to the nasal cavity and within the gland divide and differentiate into the two main ducts characteristic for each gland. The medial duct is the progenitor of the anterior two-thirds of the gland and the lateral one gives rise to the posterior one-third. The main ducts develop lateral branches at irregular intervals and these form the central canals of each lobule. Large numbers of secretory tubules, closed at their distal ends, radiate in a "lampbrush fashion" from the central canals and form the gland lobules. The secretory tubules are the functional units of the gland and are formed from a simple epithelium composed of pyrimidal-shaped cells (Ellis et al., 1963). The secretory tubules often bifurcate near their distal ends. Cells forming the walls of the secretory tubules may be divided into two groups on the basis of their
staining characteristics. Most are termed principal cells but a few at the distal end of the tubules are termed peripheral cells (Ellis et al., 1963).

Arteries follow the course of the main ducts through the gland and break into arterioles that parallel the central canals into the lobules. The arterioles form large numbers of capillaries that radiate in close association with the secretory tubules. These capillaries empty into veins at the lobule periphery, thus the blood flow is parallel and countercurrent to the normal flow of the secretory fluid (Schmidt-Nielsen, 1960; Ellis et al., 1963).

Ultrastructural Morphology

The basic fine structure of this tissue has been studied by a number of investigators (Doyle, 1960; Ernst and Ellis, 1969; Fawcett, 1962; Komnick, 1963a-d, 1964, 1965).

The active salt gland provides an ideal demonstration of the two features that seem to best characterize those cells comprising secretory epithelia which are functionally specialized for electrolyte transport. These features are cell surface amplification and a cytoplasm filled with dense populations of mitochondria. In fact, the secretory cells of the salt gland in marine birds possibly represent the most extreme example of an increased surface area observed in any cell type (Fawcett, 1962). Surface invaginations in these cells subdivide most of the cytoplasmic ground substance into large numbers of long mitochondrial filled compartments.

This extreme degree of specialization seems to occur only in the salt glands of birds taken directly from the marine environment or kept
on experimental salt water regimes (Ernst and Ellis, 1969; Komnick, 1963c). Ernst and Ellis observed the effects of salt water regimes on the morphology of salt glands of developing ducks. Their study showed that the fully specialized cells, typical of marine birds, occurred only in the animals maintained on a salt water regime. The ducks developed on a fresh water regime had secretory cells that showed very little specialization. The relatively short length of the secretory tubules in the immature ducks of this study made possible the observation that a gradation of cell specialization occurred along the length of the tubules. The degree of specialization increased from the relatively unspecialized peripheral cells to fully specialized cells near the proximal end of the secretory tubules.

OBJECTIVES OF THE STUDY

This study of the transport system of avian salt gland was conducted because of the considerable amount of new evidence linking membrane charges to ion permeation and because it is now accepted that negatively charged carbohydrate-protein materials are hallmarks of most, if not all, tissues specialized for electrolyte transport. The selection of avian salt gland seems ideal since electrolyte transport is the gland's only known function and the gland may be maintained experimentally in either an active or inactive state. It is also possible to differentiate between these two states by morphological, physiological, and biochemical means.

The specific objectives of the investigation are: (1) to ascertain to what degree anionic surface materials are present on the secretory
cells of avian salt gland, (2) to identify qualitative and/or quantitative differences in surface associated anionic materials that are characteristic for a given physiological state, (3) to determine the chemical nature of these materials, and (4) to elucidate specific relationships to the transport activity.
MATERIALS AND METHODS

ANIMALS

Animals selected for the investigation were laughing gulls (Larus atericilla), a marine species, and domestic mallard ducks (Anas platyrhynchos), a species domestically maintained in the fresh water environment. However this same species of duck in the wild is exposed to a marine environment during the winter phase of its normal migratory cycle. Thus, in the gulls, the salt gland is normally well developed while in domestic ducks it is poorly developed unless the animals are intentionally maintained on a salt water regime.

Laughing gull nestlings were captured from a rookery on South Deer Island near Galveston, Texas, and maintained on a diet of fish flavored catfood\(^1\). Adult male mallards were purchased from a local game bird farm\(^2\) and fed a diet of milo maize and commercial growing mash\(^3\). All the animals were housed in large, outside, but partially enclosed, holding pens. Both ducks and gulls were divided into two experimental groups. One group was given fresh water ad libitum and the other a 2\% NaCl regime ad libitum. The gulls were maintained under these conditions until they had reached a mature size (at least six months of age) before they were used for experimental purposes. Ducks were kept under controlled fresh or salt water conditions at least 21 days before experimental use. This is sufficient time for complete

\(^1\) Puss'n Boots, The Quaker Oats Company, Chicago, Illinois.
\(^2\) Glenbrook Valley Game Bird Farm, Houston, Texas.
\(^3\) Hendrick Grain Company, Houston, Texas.
adaptation of the gland to either fresh or salt water conditions (Ernst et al., 1967; Fletcher et al., 1967; Fletcher and Holmes, 1968).

PHYSIOLOGICAL CONDITIONS

Three physiological conditions were established for the experiments in the investigation: (1) fresh water (FW) animals which were kept on tap water, (2) acute loaded (AC) animals were taken from the fresh water group and immediately given either 1 gm NaCl per Kg of body weight in a 10% solution via a leg vein at the knee or 2 to 3 gm NaCl per Kg body weight in a 10% solution introduced into the stomach (proventriculus) with a soft rubber tube attached to a syringe, and (3) salt water (SW) animals which were kept on a 2% NaCl regime for at least 21 days prior to their use in an experiment. In all experiments with AC birds the animals were allowed to secrete approximately 1 hour before they were sacrificed.

TISSUE PREPARATION FOR LIGHT MICROSCOPE STUDIES

Tissue was prepared specifically for light microscope study using recent improvements in technique which employ water soluble methacrylates as the embedding matrix (Ashley and Feder, 1966; Feder and O'Brien, 1968). The exact procedure used was a modification of that recommended by Ashley and Feder (1966).

For example, birds were killed by decapitation and the gland was quickly excised and cut into transverse slices about 1 mm thick. These slices were fixed at 0-5° C for 12-24 hours in one of the following aqueous solutions: 5% glutaraldehyde, 10% formalin, or 10% acrolein.
In each case 1% calcium acetate was added to the fixing solution. For dehydration the fixed specimens were placed successively in the following solutions for 12-24 hours at 0-5°C: (1) equal volumes of methanol and 2-methoxyethanol (Methyl Cellosolve), (2) 100% ethanol, and finally (3) n-propanol. The "monomer mixture" used for infiltration and embedding was 90% (v/v) glycol methacrylate (hydroxyethyl methacrylate), 5% (v/v) polyethylene glycol 200, and 5% (v/v) distilled water. To facilitate polymerization 0.4% (v/v) 2,2'-azobis 2-methylpropionitrile was added to this mixture as a catalyst. The tissue was transferred from the n-propanol to the "monomer mixture" for 10-24 hours at 0-5°C. Two or three changes of monomer were made. The infiltrated tissue was then placed in #00 gelatin capsules. The capsules were filled with fresh monomer, placed without caps in a 45°C oven for 24 hours, and then transferred to a 60°C oven for 1 to 2 days to complete polymerization and hardening. Sections 0.5 to 1.5 μm thick were cut with dry glass knives mounted in a model MT-2 Porter-Blum microtome.

For routine histological observations sections were stained in the following manner: they were first immersed for 1-5 minutes in coplin jars filled with 1% aqueous solution of acid fuchsin, washed 1-3 minutes in running tap water, and then immersed for 1-5 minutes in 0.05% toluidine blue prepared in 0.02 M benzoate buffer at pH 4.4 (Ashley and Feder, 1966).

In some instances methacrylate sections were stained according to the periodic acid-Schiff procedure in order to demonstrate carbohydrates (Mowry, 1963). The sections were pretreated with dimedone
to block any Schiff positive groups introduced during fixation (Feder and O'Brien, 1968).

The slides were examined with a Zeiss Ultraphot microscope equipped with a bright field and a Zernike phase optical system.

**TISSUE PREPARATION FOR ELECTRON MICROSCOPE MORPHOLOGY**

The glands were quickly excised and placed in a few drops of the fixative. They were trimmed free of extraneous connective tissue and diced into cubes no larger than one millimeter while immersed in the fixative.

**Glutaraldehyde-osmium procedure** (Sabatini et al., 1962).

The tissue was fixed for 90 minutes at room temperature in 3% glutaraldehyde in phosphate buffer (Millonig, 1961) containing 0.5 mM CaCl₂ (pH 7.4). Following fixation, tissues were twice rinsed for 15 minutes in phosphate buffer. The tissue was post fixed for 90 minutes at room temperature in 1% osmium tetroxide prepared in the same buffer. After two quick rinses in tap water the fixed tissue was rapidly dehydrated through increasing concentrations of ethanol and embedded in Epon 812 (Luft, 1961). In some experiments a 0.1 M cacodylate buffer (Gomori, 1955) was substituted for the phosphate buffer.

**Formaldehyde-glutaraldehyde procedure** (Karnovsky, 1965).

Salt glands were submerged for 90 minutes at room temperature in a fixative consisting of a mixture of 4% formaldehyde and 5% glutaraldehyde prepared at a pH of 7.4 in phosphate buffer. After
four 15-minute rinses in phosphate buffer the tissue was post-fixed for 90 minutes at room temperature in 1% osmium tetroxide buffered with either phosphate (Millonig, 1961) or 0.1 M s-collidine buffer (Bennett and Luft, 1959). The final pH of the fixative was 7.4. Following dehydration in ethanol the tissue was embedded in Epon 812.

Survey sections were cut with a glass knife at 0.5-1 μ and stained in equal volumes of methylene blue and Azure II (Richardson et al., 1960). Thin sections (500-800 A) were cut with a diamond knife, stained for 3-5 minutes with 2% aqueous uranyl acetate adjusted to pH 5 with 1 N NaOH, washed in distilled water, and counterstained with lead citrate (Reynolds, 1963). The sections were examined with an RCA EMU-3F electron microscope operated at 50 kv or a Philips 200 electron microscope at 60 kv.

SPECIAL PROCEDURES FOR PRESERVATION OF CARBOHYDRATES

Inert dehydration (Pease, 1966a).

This technique was designed specifically to avoid the leaching out of materials that often occurs with conventional fixation and dehydration procedures. Cubes of tissue 0.5 mm or smaller were dehydrated within 6 minutes in ethylene glycol without fixation. After overnight storage at 0-5°C the tissue was transferred gradually to hydroxypropyl methacrylate (HPMA, Rohm and Haas) by the dropwise substitution method. Following two changes in pure HPMA to which 1% benzoyl peroxide had been added as a catalyst the tissue was transferred to HPMA that had been prepolymerized to a syrupy consistency.
The prepolymerized HPMA contained 1% benzoyl peroxide and 5% divinyl benzene. Final polymerization was accomplished by placing the infiltrated tissue in a 60°C oven overnight. Thin sections of this material were placed on copper grids and stained by immersion for 30 minutes in a 5% aqueous solution of phosphotungstic acid. **TAPO fixation** (Williams and Luft, 1969).

Commonly used chemical fixatives are relatively unreactive with polysaccharides (Bahr, 1954). In an effort to better preserve these materials Williams and Luft (1969) have recently developed a fixative containing a very polysaccharide-reactive compound, tris-(1-aziridinyl) phosphine oxide (TAPO). In these fixations the same procedure as that outlined for the glutaraldehyde-osmium fixations was used with the exception that 1% TAPO was added to the buffered glutaraldehyde fixative.

**TRACER STUDIES**

**Horseradish peroxidase** (Karnovsky, 1965).

This technique provides a very electron dense reaction product and is an excellent procedure for demonstrating extracellular space. In this study 100 mg of horseradish peroxidase (Sigma, Type II) was added to 10 ml of Ringer's solution and the mixture was injected into the leg vein at the knee. Birds were sacrificed 20 minutes later and their salt glands were fixed for 90 minutes at room temperature in 3% glutaraldehyde in phosphate buffer (pH 7.4) containing 0.5 mM CaCl₂. After several buffer rinses the tissue was left overnight in phosphate buffer to which 0.5 mM CaCl₂ had been added. On the following day
the tissue was incubated for 20 minutes at room temperature in 5 mg
3-3' diaminobenzidine hydrochloride (DAB, Sigma) in 10 ml of Tris-
HCl buffer at pH 7.6 to which 0.1 ml of 1% H₂O₂ had been added (H₂O₂
was prepared fresh from 30% stock). Two controls were run, one with
the incubation medium less H₂O₂, and the other with incubation
medium less DAB. Following incubation the tissue was carried through
several tap water rinses. Subsequently, the tissue was post-fixed
for 90 minutes in 1% osmium tetroxide in phosphate buffer containing
0.5 mM CaCl₂ (pH 7.4). After dehydration in ethanol the tissue was
embedded in Epon 812.

Lanthanum (Revel and Karnovsky, 1967).

In this technique lanthanum salts are added to conventional fixing
solutions to provide an extremely electron dense precipitate that
often completely fills extracellular spaces. Stock solutions used
were: 3% La(NO₃)₃ in distilled water brought carefully to pH 7.8
with 0.1 n NaOH, 1.5% osmium tetroxide in 0.22 M s-collidine buffer
(Bennett and Luft, 1959) at pH 7.4, and 3% glutaraldehyde in
s-collidine buffer at pH 7.4. The tissue was fixed at 0-5°C for 90
minutes in a buffered glutaraldehyde-lanthanum solution (2:1, v/v).
After two 15 minute duration washes in cold collidine buffer-lanthanum
solution (2:1, v/v), the tissue was fixed for 90 minutes in cold
buffered osmium tetroxide-lanthanum solution (2:1, v/v). Following a
quick tap water rinse the tissue was transferred to 50% ethanol and
brought to absolute ethanol in 3-5 minutes. The dehydrated tissue was
embedded in Epon 812.
CYTOCHEMICAL STUDIES

Ruthenium red (Luft, 1964).

Ruthenium red is a cytochemical agent that may be used to demonstrate polyanionic carbohydrate polymers at the electron microscope level. Stock solutions used were: (1) purified ruthenium red (3000 ppm) in distilled water; (2) 0.2 M cacodylate buffer at pH 7.4; (3) 9% glutaraldehyde in distilled water; and (4) 5% osmium tetroxide in distilled water. The tissue was fixed overnight at room temperature in one volume each of the glutaraldehyde, buffer, and ruthenium red stock solutions. Following several rinses in a solution composed of equal volumes of the buffer and ruthenium red stock solutions, the tissue was post-fixed in equal volumes of osmium tetroxide, buffer, and ruthenium red stock solutions. Dehydration and embedment was accomplished in the normal manner.

A modification of the above procedure was employed to facilitate better exposure of the tissue to the fixatives. In this instance, the gland was sectioned at 25-50 μ on a Smith-Farquhar tissue chopper* subsequent to a 30-minute initial exposure to the ruthenium-glutaraldehyde fixative. The chopped sections were then processed as above.

Colloidal iron with neuraminidase digestion.

This technique employs the classical Hale reaction (Hale, 1946) in which electron dense, positively-charged colloidal iron micelles bind with anionic sites in the tissue. Blocks of tissue were fixed

for one hour at 0-5°C in 3% glutaraldehyde in 0.12 M phosphate buffer (Sabatini et al., 1962). The final pH of this fixative was 7.4. After several rinses in cold phosphate buffer the tissue blocks were embedded in warm 5% agar, cooled, and sectioned at 25-50 μ with the tissue chopper. Following a few changes in cold acetate buffer (0.116 M NaCl, 40 mM Na-acetate, 4.6 mM CaCl₂) that had been adjusted to pH 6.5, the sections were incubated for 5-8 hours at 37°C in either: (1) 1 ml of the acetate buffer to which 200 μl of neuraminidase* had been added, or (2) 1 ml of the acetate buffer to which 200 μl of distilled water had been added. Subsequently the sections were carried through two, 10-minute changes of 0.12 M phosphate buffer (Millonig, 1961) at room temperature (pH 7.4). The tissue was then post-fixed for 90 minutes at room temperature in 1% osmium tetroxide in 0.12 M phosphate buffer (pH 7.4). After a rapid rinse in the phosphate buffer at pH 7.4 and two washes in aqueous acetic acid (pH 1.8), the sections were immersed for 5-10 hours at room temperature in a solution of dialyzed iron (Rinehard and Abul-Haj, 1951). The iron solution was adjusted to pH 1.8 with glacial acetic acid. The sections were then rinsed in aqueous acetic acid solution at pH 1.8 until the rinsing solution remained completely clear. They were dehydrated in ethanol and embedded in Epon 812.

In a modification of the above procedure some sections that had not been enzyme digested were stained with a colloidal iron solution at pH 3.8.

* Neuraminidase (Vibrio cholerae, Z4), Specific activity 500 units/ml, Mann Research Laboratories, New York.
Thorium dioxide.

Thorium dioxide* provides another positively-charged colloidal micelle that reacts in essentially the same manner as colloidal iron to demonstrate tissue polyanions. Tissue blocks were fixed for 60 minutes at 0-5°C in 3% glutaraldehyde in 0.12 M phosphate buffer (pH 7.4) and sectioned at 25-50 μ on the tissue chopper. These sections were then treated in exactly the same manner as in the colloidal iron procedure with the exception that staining was accomplished in a 5% solution of thorium dioxide adjusted to pH 1.8 with glacial acetic acid.

NEURAMINIC ACID ASSAYS

The excised glands were quickly trimmed free of extraneous connective tissue, weighed, and homogenized in enough cold 0.04 M acetate buffer (pH 6.5) to make a 50 mg/ml homogenate (wet weight). Two ml aliquots were preincubated for 10 minutes at 37°C, then either 200 μl of neuraminidase (100 units), or 200 μl of additional buffer was added to the aliquots to provide a final volume of 2.2 ml. These samples were then incubated (with periodic agitation) for various time increments ranging from 7.5 to 180 minutes. After incubation the samples were chilled on ice for 5 minutes to stop the reaction and then centrifuged for 5 minutes at one-half maximum setting on an International Clinical Centrifuge. 1.5 ml of supernatant was taken from each sample and 200μl of 100% TCA (trichloracetic acid) was then

* Thorotrust, Fellows-Testagar, Detroit, Michigan.
added. The resulting solution was centrifuged for 10 minutes at three-fourths maximum speed on the centrifuge and three 500 µl samples were removed for assay. Samples were assayed by the thiobarbituric acid assay method (Warren, 1959) and the optical densities were determined with a Beckman model DU spectrophotometer. Readings were corrected for 2-deoxyribose. For determination of total neuraminic acid content, 1 ml samples of the 50 mg/ml homogenate were centrifuged and the supernatant was replaced with 2.2 ml of 0.1 N H₂SO₄. These samples were then heated at 80°C for 1 hour and assayed by the thiobarbituric acid method (Warren, 1959).

In one experiment tissue blocks that had been fixed in formaldehyde-glutaraldehyde were sectioned at 80 microns and digested with neuraminidase as above. The supernatant from this digestion was assayed for neuraminic acid content. Also tissue blocks previously fixed in either osmium tetroxide-glutaraldehyde or formaldehyde-glutaraldehyde were washed in cold acetate buffer, homogenized, and assayed for neuraminic acid content.

The method of Lowry et al., (1951) was used to determine the TCA-precipitable protein content of the salt gland homogenate using bovine serum albumin as a standard.
RESULTS

EFFECTS OF DIFFERENT PHYSIOLOGICAL CONDITIONS

Originally both ducks and gulls were selected for this study because gulls in their normal habitat have a well-developed and functional salt gland while mallards are normally found around fresh or brackish water and therefore have a poorly developed gland. Under controlled laboratory conditions, however, glands from either species are easily and readily turned "on" by maintaining the birds on a salt water regime or turned "off" by placing them on a fresh water regime. This "on-off" mode of operation is excellent for experimental studies because animals with inactive glands serve as controls.

Unfortunately gulls could be captured easily only once a year, that time being when they were nestlings. These animals failed to adapt well to captivity and both years they were collected approximately 50% of them died within a very short time and their rate of mortality remained high throughout the period of captivity. Only gulls that appeared to be in a good state of health were used as experimental animals. Mallards were used for most of the experiments in this study since they were easily obtained, remained in a good state of health, and were much easier to work with experimentally.

Gulls maintained on the 2% NaCl regime grow more slowly than their "fresh water" counterparts. Although the data for gulls is rather limited, it can be seen from Table 1 that the SW birds weighed less than the FW animals. The total weight of the salt glands of SW gulls was approximately twice that observed in the FW gulls and this doubling of weight is also apparent when the gland size is expressed as a relative weight (i.e., mg gland weight per 100 grams body weight).
TABLE 1

Effects of different physiological conditions on body and salt gland weight. Weights given as total weight of both glands of a bird. Values expressed as means ± S.E.

<table>
<thead>
<tr>
<th>Physiological condition</th>
<th>No. of birds</th>
<th>Body weight (g.)</th>
<th>Salt gland weight (mg.)</th>
<th>Relative gland weight (mg.% B.W.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh water ducks (FW)</td>
<td>17</td>
<td>1187 ±30</td>
<td>174 ± 6</td>
<td>14.9 ±0.7</td>
</tr>
<tr>
<td>Acute loaded ducks (AC)</td>
<td>7</td>
<td>1065* ±47</td>
<td>146 ± 8</td>
<td>13.6* ±0.9</td>
</tr>
<tr>
<td>Salt water ducks (SW)</td>
<td>22</td>
<td>1091* ±37</td>
<td>350** ±20</td>
<td>32.4** ±1.6</td>
</tr>
<tr>
<td>Fresh water gulls (FW)</td>
<td>4</td>
<td>318 ±27</td>
<td>158 ± 33</td>
<td>49.0 ±4.7</td>
</tr>
<tr>
<td>Salt water gulls (SW)</td>
<td>2</td>
<td>288 ±8</td>
<td>310 ±50</td>
<td>106 ±15</td>
</tr>
</tbody>
</table>

*P value > 0.05 when compared to fresh water condition.

**P value < 0.001 when compared to fresh water condition.
It is interesting that gulls seem to develop a "normal" size salt gland without a salt water stimulus. This is apparent since FW gulls in this study had glands with relative weights not greatly different from those observed by Staaland (1967) for several species of gulls taken directly from the marine environment. Also the relative weights of the salt glands of the SW gulls in this study were slightly higher than the highest values Staaland observed in gulls taken directly from the environment.

The mallards initially lost weight when they were placed on the salt water regime but it can be seen from Table 1 that this weight was regained by the end of the adaptation period (21 days). This is apparent since there was no significant difference in the body weights of the FW and SW ducks used in this study.

The total weights of the salt glands of mallards also doubles when the animals are salt water adapted. This doubling of gland weight with salt water adaptation has been observed in a number of species of birds (e.g., Ellis et al., 1962; Ernst et al., 1967; Fletcher et al., 1967; Holmes and Stewart, 1968; Schmidt-Nielsen and Fange, 1958; Stewart et al., 1969). The relative weight of the salt glands of the SW mallards of this study was approximately twice that of the FW group and reached the value reported for domestic (Peking) ducks that have been maintained on a salt water regime. It is of interest here that Schmidt-Nielsen and Kim (1964) found that wild mallards could not maintain water balance on a 1.5% NaCl regime and yet the domestic variety of mallard used in this study was maintained indefinitely on a 2% NaCl regime with no adverse effects.
The change in size and gross morphology of the salt glands of mallards when they become salt water adapted may be observed in Fig. 1.

MORPHOLOGY AT LM LEVEL

The differences in size and shape between the FW and SW adapted intact salt gland that are apparent in Fig. 1 may be seen to better advantage in the transverse histological sections of the glands shown in Figs. 2 and 3. In the latter instance these sections represent mid areas of the respective glands and also serve to illustrate their basic lobular structure. Note that in ducks there are two main ducts (*) from which canals (central canals) radiate at various intervals (arrows) into the glandular lobules. These lobules are incased in a connective tissue sheath and tend to be spherical although their shape may vary considerably.

As was first noted by Marples (1932) this organization is quite different from more marine species of birds such as gulls (Fig. 8), where the lobules form long cylinders that extend nearly the full length of the gland. There are no main ducts in the gull but each lobule contains a central canal (*) into which radially arranged secretory tubules empty (Fig. 9, 10).

In duck salt gland the main ducts are formed by an epithelial lining that is usually two cells in thickness (Fig. 4, 5). These ducts are encircled with a rather thick supporting layer of connective tissue which radiates with reduced thickness to form a connective tissue sheath (interlobular connective tissue) around each lobule.
Figs. 6 and 7 show that each secretory tubule is invested in turn with a very thin connective tissue layer (peritubular connective tissue). In general the relative amount of connective tissue becomes reduced with salt water adaptation. Salt water adaptation also results in an increase in the diameter of the secretory tubules from about 10 μ in glands from control or fresh water adapted animals to about 20 μ in the salt water state (Fig. 6, 7). This hypertrophy of the secretory cells is also apparent from the comparison of the number of nuclei seen in fields of equal magnification of FW (Fig. 6) and SW (Fig. 7) glands. This correlates well with the 58% increase in cell size reported by Holmes and Stewart (1968) as calculated by the relative reduction in the total amount of DNA in salt water adapted tissue. As may be seen in Fig. 7 red blood cells are also more prominent in the SW gland. This relates well to the physiological data indicating that there is a large increase in blood volume when the gland becomes active (Fange, 1963; Keynes, 1969; McFarland, 1966).

With the exceptions of shape and arrangement of the lobules the histological structure of gull salt gland is quite similar to that observed in ducks. The secretory tubules are arranged more orderly in gulls and images frequently permit tracing of a single tubule from the periphery of the lobule to the central canal (Figs. 9, 10). Collectively, the small peripheral cells at the distal ends of the tubules form a zone which is characterized by densely packed nuclei. As the cells become larger and more specialized along the tubule toward the central canal, the nuclei are more widely separated from one another and are more regularly disposed. The doubling of secretory
tubule diameter that resulted from salt water adaptation in ducks also occurs in the gull tissue and this is quite evident when comparisons are made between FW and SW images of equal magnification (Figs. 11, 12). Numerous infoldings of the plasma membrane appear as striations in Fig. 12.

PAS positive striations which appear along the basal and lateral surfaces of the salt water adapted cells are of particular interest in the periodic acid-Schiff (PAS) studies of the methacrylate sections of salt gland (Fig. 13). Comparable regions examined with the aid of the electron microscope reveal that there are extensive lateral and basal invaginations of the surfaces and these in turn are in the form of narrow cytoplasmic compartments. The PAS reaction is specific for vicinal hydroxyl groups and therefore stains such substances as carbohydrates (Nowry, 1963). It would thus appear that significant quantities of these substances are associated with the greatly extended plasma membranes so typical of SW secretory cells.

EM MORPHOLOGY

Initially a number of conventional fixatives were used and it was determined that either the glutaraldehyde (Sabatini et al., 1962) or the formaldehyde-glutaraldehyde (Karnovsky, 1965) fixative provided the best morphological results. Since the glutaraldehyde fixative yielded the best overall preservation and ultrastructure it was the fixative of choice for most of the studies reported here. Observations of the results of other investigators and the experience of this study suggests that the salt water adapted gland may be especially sensitive
to damage during fixation. This difficulty may result from the very high metabolic rate of this tissue or from the possibility that the tissue may have a higher than normal intracellular osmotic pressure (Hokin, 1967). The FW adapted gland is less labile and its morphology is well preserved in either glutaraldehyde or formaldehyde-glutaraldehyde fixative.

Fresh water adapted gland (FW).

The secretory cells of the FW adapted duck exhibit very little specialization (Figs. 14, 15). There are considerable interdigitations of the lateral surfaces of adjacent cells but basal surfaces either completely lack infoldings or possess but a few shallow ones. The nuclei are relatively large and occupy a considerable amount of the total cell volume. Mitochondria are moderate in number and are generally packed in the regions of cytoplasm surrounding the cell nucleus. The cells appear at this magnification to possess typical junctional complexes (Farquhar and Palade, 1965) at their apical surfaces and a few short microvilli project into the tubular lumen. Golgi cisternae and vesicles are usually supranuclear and centrioles often are observed in a more distal region of the apical cytoplasm (Figs. 17-19). Fortuitous sections sometimes show centrioles in the apical cytoplasm of several cells as they converge to form the tubular lumen (Fig. 20). Such images emphasize the orderly disposition of centrioles in these cells.

Fig. 16 is a section cut somewhat oblique with respect to the wall of the secretory tubule, consequently the lateral surfaces of the cells
dominate the micrograph. Note that the leaflets formed by the lateral invaginations of the plasma membrane do not contain mitochondria. The typical morphology of the FW secretory cell may be seen at higher magnification in Fig. 17. The distribution of rough endoplasmic reticulum and free ribosomes are more or less randomly disposed throughout the island of cytoplasm surrounding the nucleus but they are only rarely present in the thin lateral leaflets.

Close examination of the apical cytoplasm (Figs. 18, 19) reveals a variety of vesicular structures, microtubules, and tufts of tonofilaments. Included among the vesicles are coated vesicles (Fig. 19) and multivesicular bodies (Fig. 18). As mentioned above, most junctional complexes appear typical but frequently junctions are observed (Fig. 18) in which the two adjacent membranes do not seem to fuse to form the zonula occludens (tight junction). There is often a complexity of the apical region of these cells that results in the formation of several narrow intercellular canaliculi (Fig. 19).

**Acute loaded gland (AC).**

A close examination of the biopsies removed from acute salt loaded (AC) birds reveals that there are no obvious and consistent morphological differences between FW and AC conditions (Figs. 21-25). **Salt water adapted gland (SW).**

Less differentiated peripheral cells near the blind end of the tubules have much the same morphology as that described for the FW and AC tissue. The cells become increasingly specialized (Fig. 26, 27) toward the central duct and as a result at least two-thirds of the
length of the tubule consists of fully differentiated cells (Figs. 28, 29). The peripheral and hence less specialized cells are somewhat more elongate than typical FW cells. They do, however, exhibit an increased number of lateral invaginations. These in turn form narrow cytoplasmic compartments which may entrap mitochondria (Fig. 26). These cells also begin to develop basal invaginations and the compartments so formed often contain mitochondria.

Fig. 27 illustrates cells that are still more highly specialized. Here the basal infoldings are longer and more numerous and it becomes increasingly difficult to differentiate between basal and lateral infoldings in some areas. In the fully specialized cells much of the cell consists of basal and lateral invaginations in which are trapped large numbers of mitochondria (Figs. 28, 29). These infoldings result in what Fawcett (1962) has termed "basal compartments" that may extend almost the full length of the cell. Often the only significant cytoplasmic space remaining in these cells is a small island of cytoplasm surrounding the nucleus. Virtually all cytoplasm and organelles except mitochondria are excluded from the infoldings and the intracellular space between the two apposing membranes may often be no more than 100A (Fig. 30). The plasma membrane is also closely applied to the external membranes of the mitochondria.

The basal lamina defines the periphery of the tubule and does not follow the invaginations of the cell surface. This is analogous to the organizational pattern of the distal tubule of the kidney. The SW cells characteristically have large numbers of lysosomal structures and numerous mucous-like droplets (Figs. 26-28).
It is of interest here that in contrast to the marked changes that occur in the basal and lateral regions of the SW adapted cells, there are no obvious morphological changes at the apical or luminal surfaces. **Main duct and central canal.**

The epithelial cells that form these structures are not greatly specialized (Figs. 31-33). They possess but moderate specializations of the surface and their cytoplasms contain relatively few mitochondria. It is unlikely therefore that they are directly involved in the active transport of electrolytes. The superficial cells that form the luminal surface of the main ducts have very irregular apical surfaces which often extend into the lumen of the duct (Figs. 32, 33). These protrusions are large enough to be seen at the light level (Figs. 4, 8, 9) and suggest a sloughing off or apocrine-type secretion. The frequent observation of necrotic cells in this region also supports this contention (Fig. 33). **Intralocular arterioles.**

Physiological studies have shown that there is very little blood flow in the nonsecreting salt gland but that during secretion the blood flow rises to a level that parallels that of the kidney (Keynes, 1969). Fange et al., (1958) observed that the arteries to the salt gland are among the largest found in the entire head region. They also noted that, due to an anastomosing arch from the posterior to the anterior branch of the ophthalmic artery, blood flow to the gland could be reduced drastically without affecting flow to the upper beak area.
Komnick (1963) has shown small intralobular arterioles in gull salt gland that may be either contracted with a virtually closed lumen or expanded with a relatively large luminal space. He correlated the open or closed condition with the secretory activity of the gland. Arterioles of this type are frequently observed in duck salt gland (Fig. 34). Longitudinally and transversely oriented smooth muscle cells surround the basement layer which in turn supports the endothelial lining. Nerves that innervate the smooth muscle cells are located just outside the muscularis layer. Obviously such an organizational pattern is consistent with the function of regulation of the diameter of the secretory tubule lumen.

INERT DEHYDRATION

An ever present concern in the preparation of tissue for morphological or cytochemical study is the realization that various materials are leached from the tissue during the fixation and dehydration process (Millonig and Marinuzzi, 1968). This is definitely a problem with respect to carbohydrates which are generally not very reactive with conventional chemical fixatives and the problem becomes even more acute during attempts to visualize extracellular carbohydrates.

In view of these difficulties salt gland was prepared by the Pease (1966a) "inert dehydration" method. This technique theoretically circumvents the extraction problems of chemical fixation and conventional dehydration by the rapid dehydration of unfixed tissue in ethylene glycol followed by embeddment in hydroxypropyl methacrylate. This is essentially a physical method of preservation in which the
water soluble, and freely permeable organic compound, ethylene glycol, is substituted for water in the tissue. Since proteins and some carbohydrate containing substances are less soluble in glycol than in water, these substances are retained in the tissue in substantially their original quantities. Pease (1963) has stained thin sections of tissue prepared in this way with phosphotungstic acid (PTA) and has shown on empirical grounds that the stain has a special affinity for substances known to be PAS-positive and therefore regarded to be, at least in part, polysaccharide. It is important here that Rambourg, et al., (1969) have modified this PTA staining method and claim that they have stained glycoproteins at the surface of a variety of cells.

Although this procedure does not provide the characteristic high quality morphology associated with conventional techniques, the integrity of the plasma membranes and the general architecture of the tissue is retained (Figs. 35, 36). Note that the extracellular spaces of the tissue prepared by the PTA technique are intensely stained. This result suggests that in vivo, these spaces may actually contain polysaccharides that become extracted when tissues are prepared by conventional methods.

TAPO FIXATION

Tris-(1-aziridinyl) phosphine oxide (TAPO) is a nitrogen mustard derivative belonging to a group of organic compounds referred to as "alkylating agents." The compound readily reacts with polysaccharides and is employed commercially to give "wet strength" to paper and provide crease-resistant textiles. TAPO has three very reactive
ethylamine groups per molecule and probably forms cross-linkages with
target molecules.

Application of glutaraldehyde-TAP0 fixative to salt gland provided
images characterized by crisp detail and a distinct morphological
appearance (Figs. 37, 38). The matrix materials of mitochondria are
well preserved and therefore appear electron dense. The filamentous
structures (arrow), seen consistently in the apex of these cells when
conventional fixatives are used, are unusually prominent here (Fig.
38). Further the cytoplasmic ground substance is generally less dense
than that observed with conventional procedures. This reduced density
results in an emphasis of membraneous structures. For example,
junctional complexes are well preserved and are suitable for examina-
tion at high magnification. Macula adherens (desmosomes) have a
characteristic morphology (Fig. 39). They have the typical inter-
cellular space (about 250 A) and fine cytoplasmic filaments radiate
from the inner surfaces of the membranes. The zonula adherens appears
typical in every respect. Nearest the lumen there is definitely an
area where the intercellular space is reduced but it is not possible
to see clearly a fusion of the two adjacent membranes to form the
conventional zonula occludens or tight junction. Note that a certain
amount of electron dense material does appear to fill the extracellu-
lar space in this zone.

In Fig. 40, which illustrates a junctional complex between
adjacent cells of the central canal, the fusion of the outer dense
lines of the two cell plasma membranes is more clear.
Numerous droplets, with diameters as great as 0.5 μ and with the morphology of mucous droplets, are observed in the apical regions of the superficial cells of the main ducts (Figs. 41, 42). These droplets were never observed in material prepared with conventional fixatives.

These results are consistent with the observations of Williams and Luft (1969) who used the aldehyde-TAPO combination to fix protozoa. Both studies support the view that TAPO may hold promise as a general fixative that can be used with a variety of tissue types since morphological quality is high and extraction is minimal.

HORSE RADISH PEROXIDASE

Straus (1957) first used the plant enzyme, horseradish peroxidase (HRP), in a procedure which yielded a colored marker or tracer that could be seen with the light microscope. Karnovsky (1965) subsequently extended the usefulness of this procedure to the ultrastructural level by employing a different substrate; i.e., a substrate yielding an electron opaque reaction product.

Horseradish peroxidase is a hemoprotein with a molecular weight of about 40,000 and a molecular diameter of approximately 45 A. The technique provides a noncrystalline insoluble reaction product that is extremely electron dense subsequent to osmium fixation. The procedure is very sensitive because of the amplifying effect of the enzyme; i.e., a large amount of dense reaction product accumulates in the presence of a limited but "recycled" enzyme. For these reasons,
HRP is an excellent tracer for demonstrating extracellular space (Karnovsky, 1965, 1967; Karnovsky and Cotran, 1966; Philpott, 1966; Reese and Karnovsky, 1967; Schneeberger-Keeley and Karnovsky, 1968). The technique was used for this purpose in the present study.

HRP completely fills the matrix area of the peritubular connective tissue and therefore obliterates the loose matrix of the basal lamina (Fig. 43). The tracer enters all the extracellular spaces including the spaces formed by the invaginations of the cell plasma membrane. In areas where these spaces are not completely filled the reaction product adheres to the cell surface (Figs. 43, 44).

The long junctional complexes are quite prominent and have a rather consistent intercellular space of 325 to 375 Å. HRP penetrates the first two elements of the junctional complex and seems in some instances to possibly extend through the zone of the "tight junction." Karnovsky has used this technique to observe what he has termed "gap junctions" in a number of tissue types (Karnovsky, 1966, 1967; Revel and Karnovsky, 1967). It is therefore conceivable that these cells may have maculae occuludens rather than zonulae occuludens. This implies that there may not be a complete physiological "seal" between the luminal and intercellular spaces or that a physiologically tight junction is formed in this zone without the fusion of the adjacent plasma membranes.

LANTHANUM

With the technique of Revel and Karnovsky (1967) which is based on a procedure of Doggenweiler and Frenk (1965) alkaline solutions
of lanthanum salts are employed to demonstrate extracellular spaces in situ.

With this procedure an amorphous and extremely electron dense material, presumably \( \text{La(OH)}_3 \), often completely blocks out the extracellular spaces (Figs. 45-48). The mechanisms underlying lanthanum deposition and retention are poorly understood. Revel offers, however, the explanation that soluble lanthanum nitrate is converted to an unknown ionic complex at physiological pH and subsequently is precipitated by ethanol (Ritch and Philpott, 1969). Both Revel and Karnovsky (1967) and Ritch and Philpott (1969) allow the possibility of direct binding of lanthanum to membranes but suggest, on the basis of their results, that this is rather unlikely. They therefore suggest that the primary mechanism is a general precipitation of the complex into pre-existing spaces.

Other investigators working with chick embryonic cells (Khan and Overton, 1969; Lesseps, 1967; Overton, 1969) suggest that lanthanum binds to these cells and show evidence for reduction of staining by enzyme digestion as support for this idea. It is important to note that all of these latter studies were accomplished with embryonic cells and that the procedures used were different from those employed by Revel and Karnovsky, and by Ritch and Philpott.

Lanthanum procedures are somewhat capricious and problems associated with limited penetration and retention of the complex witnessed by other investigators were also experienced in this study. For example, at favorable locations in the tissue blocks, cells are
completely outlined with lanthanum (Figs. 45, 46); in other areas the tracer is completely missing. Figs. 45 and 46 demonstrate in situ depositions of the lanthanum reaction product along intercellular spaces of cells that are not fully specialized. By contrast, fully specialized cells, characteristic of the salt water adapted gland, are rarely outlined by lanthanum. This supports the contention that there is no direct binding to the plasma membrane. In Fig. 47 the intercellular spaces of a cell with rather well developed lateral invaginations are filled with the lanthanum complex.

The lanthanum reaction product is especially dense in the regions of the junctional complexes (Fig. 46). Close observation of the region suggests that lanthanum penetrates the entire junctional complex (Fig. 48). Revel and Karnovsky (1967) observed "gap" junctions in mouse heart and liver using this lanthanum procedure. It follows, therefore that the junctions of salt gland may also be "gap" junctions.

Following lanthanum staining, a very interesting system of repeating particles associated with tubular extensions of the cell surface were observed in chloride cells of branchial epithelium of Fundulus (Philpott, 1968; Ritch and Philpott, 1969). As these tubules are believed to be the sites of active electrolyte transport, it follows that such a substructure might also be found on the basal and lateral infoldings of avian salt gland secretory cells. Unfortunately the resolution of the micrographs of this study is not sufficiently high to provide a conclusive answer to this question. It is true that there is a suggestion of a substructure of this type in micrographs that have been greatly enlarged.
RUTHENIUM RED

Ruthenium red (RR) is an intensely colored inorganic compound originally used by botanists to stain pectic materials in plant cell walls. This compound seems to have a particular affinity for highly acidic carbohydrate polymers. Luft (1964) has recently developed a method for applying the stain to animal tissues at the EM level.

The exact mechanism of staining is not known but Luft (1965) has conducted in vitro experiments showing that, of a large variety of test substances, a strong reaction occurs only with acidic polysaccharides possessing a high charge density. He demonstrated further that when, and only when, the three components: buffered osmium, chondroitin sulfate, and ruthenium red are mixed, a brown precipitate quickly occurs. These experiments, coupled with the knowledge from the work of Fletcher et al., (1961) that ruthenium red can be easily and reversibly oxidized to ruthenium brown, lead to his proposal of the following staining mechanism. He proposed that (1) ruthenium red first binds to acidic polysaccharides in tissue, (2) ruthenium red is then oxidized to ruthenium brown by osmium tetroxide, (3) and the ruthenium brown then oxidizes the polysaccharide and is thereby reduced to ruthenium red again to complete the cycle. Thus ruthenium red functions as a catalytic agent in a reaction resulting in the oxidation of acidic polysaccharides and, in the same location, a deposition of reduced osmium visible with the electron microscope. Ruthenium red stains structures in tissues known to contain large amounts of acidic polysaccharides and in general exhibits a staining pattern compatible with other cytochemical techniques used to demonstrate this class of compounds.
When the ruthenium red technique is applied to salt gland reasonably good morphological quality is retained (Figs. 49, 50). One limitation of the technique is the poor penetrating characteristics of RR (Luft, 1965, 1966). The RR penetrated salt gland only to a depth of about 30 to 50 μ. Thus it was necessary to trim the embedded tissue blocks so that an exposed surface of the tissue could be examined.

The characteristic RR staining of the basement membrane and underlying connective tissue matrix may be seen in Fig. 49. Note that collagen fibrils do not stain. Of special interest in this study is dense RR staining of the plasma membranes of the secretory cells (Figs. 49-51). The long flocculent or filamentous structures that often extend from the plasma membranes also stain intensely with RR (Figs. 50, 51). These filaments often extend across the space separating adjacent surfaces and their appearance is similar to those seen by Pate and Ordal (1967) between adjacent cells of myxobacteria.

The dense line of RR at the plasma membrane is variable in thickness and in some areas where two membranes are in close apposition, the stain may completely fill the extracellular space (Fig. 51). Ruthenium staining is limited to the outer leaflet of the trilamellar unit membrane. This is demonstrated clearly in Figs. 51 and 52 which are respectively unstained and lead and uranyl "stained" sections.

The intercellular spaces of the junctional complex at the apical ends of these cells are intensely stained by RR (Fig. 53). These junctional complexes, that show no signs of mechanical damage, appear
to stain with RR throughout the region presumably occupied by the "tight junction."

No consistent differences in staining were observed in samples taken from all the different physiological conditions or between duck and gull salt gland.

Since RR does not penetrate intact plasma membranes (Luft, 1966) the chopped section procedure, which allows the staining agent equal access to extra- and intra-cellular sites, was used. A moderate staining of the cytoplasmic ground substance is seen near the exposed edge of the tissue (Fig. 54). Even here RR penetrates only a relatively short distance into the cytoplasm. In Fig. 54 where the outer membrane of a mitochondrion has been broken, the matrix material of the mitochondrion stains rather intensely. It is important to note that even when the stain has equal access to all sites, the sites that stain most intensely are obviously the outer surfaces of the plasma membranes.

COLLOIDAL IRON

Colloidal iron (CI) is another stain that may be employed to localize acidic polysaccharides with the electron microscope. It was originally used at the light level and employed the classical Hale (1946) reaction. In this reaction positively charged colloidal iron particles react electrostatically with acidic sites. It is possible therefore to lower the pH of the staining solution and thereby selectively stain only those strongly acidic sites that would remain dissociated at low pH.
Swift and Adams (1962) used a modification of Hale's procedure to localize extracellular acidic mucosubstances at the EM level and subsequently a number of investigators successfully demonstrated both extra- and intra-cellular acidic carbohydrates with CI (Clark and Curran, 1964; Curran and Clark, 1963; Curran et al., 1965; Gasic and Berwick, 1963; Wetzel et al., 1965).

The specificity of this technique has been well established. When tissues are stained with CI the staining pattern is consistent with light level stains for polysaccharides. Pretreatment of tissues with certain enzymes (e.g., hyaluronidase, neuraminidase) often result in loss or reduction of staining indicating that CI specifically stains the acidic groups located on the substrates for these enzymes (Benedetti and Emmelot, 1967; Gasic et al., 1968). Methylation of tissue carboxyl and/or sulphate groups has been used to block CI staining and therefore demonstrate the selectivity of the procedure (Benedetti and Emmelot, 1967; Gasic et al., 1968; Spicer and Lev, 1967).

This technique may be used for very precise localizations since CI forms a very fine precipitate (as small as 25 Å). This is a distinct advantage in attempts to accurately locate acidic sites on or near plasma membranes (Curran and Clark, 1963; Wetzel et al., 1966).

Initially a procedure basically the same as that used by Wetzel et al., (1966) was employed. Unfortunately this treatment severely damaged salt gland tissue making interpretation difficult. Moreover problems of penetration, experienced by early users of this technique (Curran and Clark, 1963; Curran et al., 1965; Gasic et al., 1968), and
already alluded to in this investigation as a problem with the salt gland in particular, limited the usefulness of the procedure.

Neuraminidase predigestions were combined with CI staining in this study and therefore valid interpretation of the results required that the region of the tissue under observation be completely penetrated with both the enzyme and CI. For example, an area of the predigested tissue not penetrated by the enzyme could contain stainable sites that should have been removed or alternately poor penetration with CI could result in no staining even though acidic sites were present. If either of these conditions occurred the results would be interpreted falsely.

Eventually it was determined that good quality and consistent results could be obtained if the tissue were sectioned with a Smith-Farquhar (1965) tissue chopper rather than with a cryostat. Reliable penetration of the section by both the enzyme and CI was guaranteed by limiting observations to the freshly chopped surface (edge analysis) which was directly exposed to all solutions.

In view of the prolonged treatment of sections with pH 1.8 solutions, the morphology of the tissue at the edge of the section is preserved remarkably well (Figs. 55, 56). Furthermore, there is little evidence of leaching of cytoplasm, mitochondrial matrix, or nuclear ground substances. This is true even in those instances when the integrity of the original body was disrupted at the edge of the chopped sections. A close examination of the chopped edges of the cells where CI has access to all cellular structures reveals that the CI is restricted rather specifically to the plasma membranes. Occasionally, however, a
very slight peppering of CI is seen in the cellular ground substance and this is increased somewhat in the nucleoplasm (Fig. 55). Light staining of the connective tissue matrix also occurs. No difference in intensity of staining was observed between FW and SW tissues.

When sections are predigested with neuraminidase CI staining is greatly reduced (Figs. 57, 58). Note that pieces of material, apparently membranes, extend from the cut edges of the enzyme digested sections (Fig. 58).

The apical surface of the secretory cells stain with CI (Fig. 59) and this staining is likewise reduced by prior enzyme digestion (Fig. 60). Consistent with the results of Benedetti and Emmelot (1967) CI is completely excluded from all parts of the junctional complex (Fig. 59).

Theoretically the specificity of this stain as used here depends on the low pH at which only the very strong acid groups (those with low pKs) will be dissociated and therefore have an affinity for the positively charged colloidal micelle. This mechanism predicts that CI would be less specific at higher pH and there is evidence to support this view (Gasic et al., 1968; Wetzel et al., 1966).

When CI was used at higher pH (pH 3.8) in this study, a distinctly different staining pattern resulted (Fig. 61). The intense staining of the plasma membrane remained but there was an additional thick deposition of stain along the chopped edge of the section. Note also the reticular deposition of CI in the tubular lumen. It is surprising that virtually no penetration of the intercellular spaces occurs at this pH. One possible explanation is that since pH 3.8 is very near the pH at
which this solution begins to precipitate, a shell of CI may quickly form at the surface and greatly lower the permeability of the surface to further immigration of colloidal iron micelles.

In summary, the results of the CI experiments indicate that the plasma membranes of the secretory cells contain a very acidic polysaccharide and the reduced staining resulting from predigestion with neuraminidase indicates that most of the anionic sites are derived from sialic acid.

**THORIUM DIOXIDE**

The thorium dioxide (Thorotrast) procedure also employs the Hale reaction. Brandt and Pappas (1960) demonstrated that thorium micelles bind to the surface coats of amoebae and recently Revel (1964) developed a technique for staining methacrylate thin sections directly with thorium. Revel's technique has been used successfully to demonstrate both extra- and intra-cellular acidic protein-carbohydrate materials (Goldstein and Philpott, 1966; Goldstein, 1969; Ito, 1965; Philpott and Goldstein, 1967). Thorium offers the advantage of a very electron dense particle but the relatively large size (85-200 A) limits precise localization.

Rambourg and Leblond (1967) attempted to demonstrate cell surface coats with thorium dioxide applied to tissue blocks. They observed that the free surfaces of cells stained heavily but the stain penetrated into intercellular spaces only slightly and did not penetrate the plasma membrane at all. As the stain does not penetrate cell surfaces, they point out that it is possible that staining could be attributed to
nonspecific adsorption. Since the staining of chopped sections avoided
the penetration problem encountered with CI it seemed reasonable to try
this procedure with thorium dioxide.

The plasma membranes of secretory cells do indeed stain heavily
with thorium particles (Fig. 62) but a considerable amount of stain is
observed also at the cut edge of the section. A light staining of the
cytoplasm and connective tissue matrix occurs but mitochondria do not
stain. The nucleoplasm is stained except for the areas of chromatin.
Note in Fig. 64 that the tubular lumen, when open to the cut surface,
stains and that the stain is excluded from the entire junctional complex.
Sections predigested with neuraminidase show no reduction in staining
(Fig. 63). It seems likely therefore that the thorium dioxide staining
pattern is more a result of nonspecific adsorption and of poor pene-
trating characteristics of the thorium particles than to specific
staining of anionic sites.

SIALIC ACID ASSAYS

Since the cytochemical studies suggested that sialic acids were a
major contributor to the anionic surface of salt gland secretory cells,
the tissue was assayed for sialic acid content. Table 2 indicates that
prior fixation with aldehyde fixatives has no appreciable effect on the
ability of neuraminidase to release sialic acid from the tissue.
Moreover these data suggest that the enzyme probably penetrates the
tissue sections used in the cytochemical studies. From Figs. 65 and
66 it may be seen that under the conditions used most of the sialic
**TABLE 2**

Effects of fixatives and methods of tissue preparation on the quantity of sialic acid released by neuraminidase digestion. SW adapted gland.

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Method of tissue preparation</th>
<th>μ moles sialic acid released per gram gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaraldehyde-formaldehyde</td>
<td>80 μ cryostat sections</td>
<td>0.98</td>
</tr>
<tr>
<td>Glutaraldehyde-formaldehyde</td>
<td>homogenized</td>
<td>0.87</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>homogenized</td>
<td>1.25</td>
</tr>
<tr>
<td>Glutaraldehyde-osmium tetroxide</td>
<td>homogenized</td>
<td>*</td>
</tr>
<tr>
<td>Unfixed</td>
<td>homogenized</td>
<td>0.85</td>
</tr>
</tbody>
</table>

* Osmium tetroxide prevented assay.
acid is removed from the tissue within about ten minutes and that after 30 minutes essentially all the enzyme reactive material is removed. Thus the data used in Table 3 to calculate neuraminidase-labile sialic acid were taken after either 60 or 120 minute periods of digestion.

The thiobarbituric acid method of assay used in this study is about 12 times more sensitive than other available methods and measures only free sialic acid (Warren, 1959). Thus it is possible to measure the "enzyme-released" sialic acid directly from the digestion media supernatant. Alternately, total sialic acid content may be determined by hydrolizing aliquots of the homogenate and assaying the supernatant (Warren, 1959).

The total sialic acid content (hydrolysis released) of the fresh water adapted salt gland of ducks is 1.58 µmoles per gram gland (wet weight) and this amount was increased with salt water adaptation (Table 3) by about 30% to 2.09 µmoles per gram gland. About 60 to 65% of this sialic acid is removable with neuraminidase. It is interesting that when the sialic acid content is figured on a per unit protein basis the fresh water gland contains 8.08 µmoles per mg. protein, and with salt water adaptation (Table 3) this increases by about 50% to 12.06 µmoles per mg. protein.

From these biochemical data it may then be concluded that neuraminidase does in fact remove significant quantities of sialic acid from salt gland under the conditions used and aldehyde fixatives apparently have no significant inhibitory effect on enzyme activity. Further, there is
TABLE 3

Total and enzyme removable sialic acid content of duck salt gland under different physiological conditions. Values expressed as means ± S.E.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of birds</th>
<th>μmoles sialic acid per g. gland</th>
<th>μmoles sialic acid per g. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh water duck Hydrolysis</td>
<td>8</td>
<td>1.58 ± 0.09</td>
<td>8.08 ± 0.55</td>
</tr>
<tr>
<td>Salt water duck Hydrolysis</td>
<td>6</td>
<td>2.09*± 0.13</td>
<td>12.06*± 0.74</td>
</tr>
<tr>
<td>Fresh water duck Neuraminidase digestion</td>
<td>6</td>
<td>1.01 ± 0.04</td>
<td>5.21 ± 0.22</td>
</tr>
<tr>
<td>Salt water duck Neuraminidase digestion</td>
<td>4</td>
<td>1.25*± 0.04</td>
<td>7.25*± 0.23</td>
</tr>
</tbody>
</table>

* P value < 0.01. In each case salt water conditions are compared to the corresponding freshwater conditions.
an increase in sialic acid content when the gland becomes salt water adapted.
DISCUSSION

GENERAL

The results of this study indicate that domestic mallards are capable of maintaining water balance indefinitely while on a 2% NaCl regime. This is accomplished by a significant increase in the size of the salt gland and in the degree of specialization of the cells comprising the secretory tubules. The histological structure of the tissue is basically the same as that reported by Ellis et al. (1963) in their study of the salt glands of developing Peking ducklings maintained on either fresh or salt water regimes. The relatively large number of dividing cells seen at the blind end of the secretory tubules of developing ducklings were not observed in the mature birds used in the current study.

COMMENTS ON THE SECRETORY CELL MORPHOLOGY

No obvious morphological differences were observed between the salt glands of fresh water and acute salt loaded birds. In both cases a few peripheral cells were seen at the blind end of the secretory tubules and the remainder of the tubule consists of partially specialized cells. The salt water adapted gland contains a few specialized cells at the distal ends of the tubules, but in contrast to the fresh water gland, most of the tubule is comprised of fully specialized cells. Thus, the results of this study are basically consistent with the findings of Ernst and Ellis (1969). Possible exceptions are that very few partially specialized cells were observed in the SW gland, and the fully specialized cells in mature ducks may have deeper basal
infoldings. Indeed, this may be because these were mature, not developing ducks. Further, the ducks used in this study were kept on a salt water regime twice the concentration of that used by Ernst and Ellis.

The most conspicuous characteristic of the fully specialized cells are the lateral and basal infoldings that divide the whole cell into a labyrinth of mitochondria-filled compartments completely surrounded by extracellular spaces. This, or some essentially similar pattern of architecture, is characteristic of a whole array of cells noted for their electrolyte secretory activity or osmoregulatory function. Examples are: choroid plexus (Tennyson, 1960; Pontenagel, 1962), ciliary epithelium (Pappas and Smelser, 1958), elasmobranch rectal gland (Ellis and Abel, 1964), gall bladder epithelium (Diamond and Tormey, 1966), kidney proximal and distal tubules (Pease, 1955; Clark, 1957; Rhodin, 1958), insect Malpighian tubules (Berridge and Oschman, 1969), plant salt glands (Thomson, 1969), rat parotid gland Parks, 1961), insect rectal papillae (Berridge and Gupta, 1967), reptilian salt gland (Bulger, 1963; Philpott and Templeton, 1964), and teleost chloride cell Philpott, 1961; 1968).

The consistent finding of extended surfaces in tissues specialized for transport strongly suggests a general functional significance. Pease (1955, 1956) first recognized that this would provide an increased surface area and Fawcett (1962) suggested that this was a convenient method for "miniaturizing" the metabolic machinery. Diamond (1964) established that water transport in gall
bladder epithelium was probably accomplished by "local osmosis" (i.e., water is coupled to active solute transport and solute transport establishes local areas of high electrolyte concentration that result in the movement of water by osmosis). Since this theoretical model requires that the electrolyte be retained within a localized area until water equilibration occurs, Diamond and Tormey (1966) have proposed that the long intercellular spaces of cells of the gall bladder function in this respect. They have also provided experimental evidence in support of this idea.

This early theory lead to the proposal, by Diamond and Bosset (1967), of the "standing gradient osmotic flow" mechanism. This attractive mathematical model predicts that the volume and osmolarity of a transported fluid will be the direct result of the quantity of solute transported, permeability of the membranes and the geometric parameters of the intercellular spaces. This original model applied only to systems that possessed dead-end channels oriented parallel and with their open ends in the direction of fluid transport. The model could not be applied directly to a variety of other common secretory systems such as those of the choroid plexus, elasmobranch rectal gland, insect Malpighian tubules, avian salt gland, reptile salt gland, and teleost chloride cells. In these systems the only structures that could serve as channels are cellular infoldings whose open ends are in a direction opposite to that described for the gall bladder epithelial cell.
Thus, Diamond and Bossert (1968) modified the basic model so that it could be applied to these systems. The significant change was that the direction of transport through the membranes was reversed. This model is consistent with the observed changes that occur in avian salt gland, which is one of the so-called "backwards" flow systems according to their terminology. In this scheme, since solute is pumped out of the channels, the maximum rate of transport for a given set of parameters is limited by the fact that solute concentration in the channel cannot drop below zero (Diamond and Bossert, 1968). They therefore suggest that epithelia possessing "backwards" channels are likely to have more channels. Moreover in this model secretions of highest osmolarities occur when the membrane water permeabilities are low, a condition which opposes high rates of flow. The scheme would therefore predict that in a system such as avian salt gland, which secretes a very hypertonic fluid at a high flow rate, one would expect to find large numbers of long channels. This is exactly the condition observed. It is also interesting in this respect that marine birds such as gulls, in their high salinity natural environments, secrete a more concentrated fluid than ducks which normally live in less saline environments. And further to this point, the basal invaginations of the secretory cells of the gull extend almost to the apical end of the cells (Komnick, 1963), whereas those of the ducks are much shorter. For example, Ernst and Ellis (1969) noted that in fully specialized cells in developing ducks, the basal invaginations generally reached
only to the level of the nucleus. In this study of mature ducks maintained on a more concentrated salt regime, the invaginations generally extend further toward the cell apex.

As Diamond and Bossert have noted, the standing gradient flow hypothesis also accounts for the unusual observation that salt gland secretes at a relatively constant osmolarity regardless of the rate of secretion (Schmidt-Nielsen, 1960; Schmidt-Nielsen et al., 1958). The hypothesis further implies that the baso-lateral membranes determine the properties of secretion in this tissue and the apical membranes are likely to be highly permeable allowing the salt solution to sweep through passively.

The morphology of the apical region of the secretory cells provides evidence that the luminal membranes are not involved actively in electrolyte transport. Note that the luminal membranes of the fully specialized secretory cells are not highly amplified. They do contain a few short microvilli but these are also seen in the relatively unspecialized cells of the fresh water adapted gland. Further, there seems to be no immediate source of energy for an active process since relatively few mitochondria are located in the apical region of these cells. This scheme, which delegates a passive role to the luminal membranes, requires that the intracellular Na concentration equal that of the secreted fluid. It is interesting in this regard that Hokin (1967) has indeed provided physiological evidence that this condition exists in avian salt gland.
In gall bladder and reptile kidneys there is evidence that the channels expand as a result of the movement of the transported fluids and that when transport stops the channels collapse. Distended spaces are observed in these tissues when they are fixed during active solute transport but they cannot be seen when transport is stopped prior to fixation (Diamond and Tormey, 1966; Schmidt-Nielsen and Davis, 1968). Experience dictates that it may be difficult to fix tissues rapidly enough to preserve a morphology that relates directly to transport in vivo. In order to overcome this potential difficulty, Diamond and Tormey applied the fixative simultaneously to both sides of the in vitro gall bladder and Schmidt-Nielsen and Davis perfused the reptile kidney with the fixative.

In the experiments of this study it was necessary to kill the animals and excise the gland prior to fixation and no consistent differences were observed between active and inactive glands with respect to the size and distribution of extracellular spaces. This is in contrast to the results of Kornick (1965) who used basically the same fixation procedure to study gull salt gland and reported a correlation between extracellular spaces and secretory activity. The extracellular spaces observed in this study appeared to vary when different fixatives were used and they were quite variable in different areas of the same tissue block. Excluding such obvious differences as those already noted for FW and SW adapted birds, the results of this study suggests that the variability of extracellular spaces observed here may have been more a result of fixation conditions or non-random sampling than the state of activity of the gland at the
time the animal was sacrificed. In this regard, it seems reasonable that in the avian salt gland, a very specialized system developed for the purpose of secreting a fluid of rather constant osmolarity, the secretory cells may develop a morphology with extracellular spaces that are relatively stable. This implies that the spaces would not necessarily collapse with the cessation of secretion.

The quick-freeze techniques that are now available should be very useful in providing valid answers to questions relating to extracellular spaces to either secretory or non-secretory states for the gland.

NATURE OF THE CELL PERIPHERY

Terminology with respect to the cell periphery remains rather confusing. The plasma membrane (i.e., the trilaminar structure seen in electron micrographs) is recognized as a distinct entity but there are still other materials both internal and external to the plasma membrane that are most surely a part of the "functional membrane." For example, such terms as extraneous coat, cell surface, glycocalyx, cell wall, jelly coat, surface coat, cell coat, and fuzz have all been used in reference to materials which are generally believed to consist of either oligosaccharides or polysaccharides linked to protein. Bennett (1963) included under the term "glycocalyx" such diverse structures as amoeba surface coats, basement membranes, plant and bacterial cells walls, gall bladder antennulae microvilliaries, the carbohydrate containing substances responsible for red blood cell immunological characteristics, and the zona pellucida of mammalian eggs.
It seems useful for the purpose of this discussion to divide protein-polysaccharide surface materials into two groups: those that are seen as distinct structures in normal preparations for morphological study and those that can be demonstrated only by special cytochemical techniques. Further it seems conceptually more accurate to consider those surface materials which are closely associated with the plasma membranes as an integral part of the functional membrane rather than as extraneous coatings. Chambers (1940) pointed out that extraneous coats could be removed without apparently destroying the functional integrity of the cell membrane. This is probably not the case for surface materials closely associated with the plasma membrane.

A further complication in discussing the complex heterosaccharides of animal tissues is the problem of nomenclature. Due to the limited, but rapidly increasing, biochemical knowledge of these materials the accepted terminology is continually changing and often confusing (Ginsburg and Neufeld, 1969). This situation is compounded by the fact that cytochemists have developed an alternate set of nomenclature for these same substances. Therefore in this discussion the term "protein-carbohydrate" will include both glycoproteins and mucopolysaccharides; a more detailed classification will generally be avoided.

It is obviously significant that this investigation reveals that the secretory cells of salt gland contain protein-carbohydrate materials at their peripheries (by five independent cytochemical techniques).
The PAS reaction, used classically at the light level to demonstrate complex carbohydrates, indicates areas strongly PAS positive that correspond to the locations of lateral and basal infoldings seen at the EM level.

The PTA stained material is especially interesting since in this experiment the tissue was prepared by the inert dehydration procedure (Pease, 1966), a procedure that retains carbohydrate materials often extracted when more conventional techniques are used. Often the entire extracellular space stains with PTA but in some areas the pattern of staining corresponds closely to that seen with ruthenium red or colloidal iron. Thus this technique also indicates carbohydrate-rich materials restricted to the immediate periphery of the secretory cells.

This correlates well with the results of the TAPO fixations in which there was increased preservation of mitochondrial matrix, tonofilaments, and mucous-like droplets, collectively indicating additional materials were retained by the procedure. Since often the entire extracellular spaces were PTA positive in the tissue prepared by inert dehydration one might expect to observe additional materials in these same spaces with TAPO fixation. The fact that this did not occur suggests that the TAPO procedure does not retain extracellular materials that have no close association with the plasma membranes. Thus, it is likely that these materials were extracted during the ethanol dehydration step that is avoided in the inert dehydration procedure.
The intense staining of the secretory cell plasma membranes observed with ruthenium red is consistent with the preceding findings and, since ruthenium red is specific for carbohydrates that have a high negative charge density, these results indicate that the stained material is strongly anionic.

Although the specificity of thorium dioxide as used in this investigation is somewhat questionable, it is important to note that in areas "open" to the staining solution, the plasma membrane did stain heavily. This result supports the contention that anionic sites are associated with the cell surface.

By contrast, the low pH colloidal iron procedure is apparently very specific. The iron is selective for the plasma membranes and there is a minimum of background staining. Thus the stained material must have anionic sites with quite low pKs. Since pre-digestion with neuraminidase results in a significant reduction in the amount of staining, a major contributor to the anionic nature of the secretory cell periphery is apparently the strongly anionic carboxyl group of sialic acid.

The pK of the carboxyl group of free sialic acid is about 2.6. Thus at the pH of staining (pH 1.8) only about one-tenth of the sites should be dissociated and therefore stainable. The staining should be much more intense at higher pH but unfortunately the stain would be less specific. It is possible also that the sialic acid stained here may have a lower than "normal" pK since Pepper and Jamieson (1969) have found a sialic acid containing glycoprotein in human blood platelets that has a pK in the range of pH 1.8 to
2.2 rather than the higher value generally accepted for the free acid.

It comes as no surprise that there is usually some residual staining because, with the possible exception of red blood cells (Eylar et al., 1962), neuraminidase never removes all the sialic acid present (Benedetti and Emmelot, 1967; Gottschalk, 1958; Hayden and Seaman, 1967; Kraemer, 1966). The enzyme generally removes about 50 to 70% of the acid and this is consistent with that observed in this study.

The incomplete removal of sialic acid is explainable by the fact that certain substitutions of the basic neuraminic acid molecule are enzyme resistant (Paillard et al., 1969; Gibbons, 1963; Pavetto, 1968). It is reasonable also that some sialic acid may be at locations in the cell periphery that are inaccessible to the enzyme. Studies of a variety of cell types indicate that the phosphate groups of phospholipids and sulphate groups may be expected to be at least minor contributors to the charge at the cell periphery (Eylar et al., 1962; Forrester et al., 1962; Hayden and Seaman, 1967; Mehrishi and Grasetti, 1969). It is possible therefore that one or both of these acidic groups may be responsible for the slight residual staining of digested sections.

Success with the use of chopped sections in combination with "edge analysis" in these cytochemical studies suggests that this procedure may be of considerable value in studies where lack of penetration may be a potential problem.
SIGNIFICANCE OF SIALIC ACID ASSAYS

A difficulty in relating the total sialic acid data from this study to that contained in the literature is that much of the latter is derived from studies of either blood cells or cells in culture and the values are usually reported in terms of cells or milliliters of cells (Eylar et al., 1962; Wallach et al., 1964). Recently, extremely high values of sialic acid content have been reported for lactating mammary gland and epididymis (Monis et al., 1969) and it is well known that semen has a very high sialic acid content (Warren, 1959). In these experiments the values likely indicate the sialic acid content of the secreted fluid rather than that actually bound to the parenchymal cell membranes. Further indication that this may be true is the fact that the values reported by Monis et al., are higher than those typically observed for purified plasma membrane fractions (Emmelot, 1964, 1965; Chaudhuri, 1965). It is of interest here that in a list of values for sialic acid content of materials from a variety of sources compiled by Warren (1959), the higher values are for semen or tissues that secrete substances high in protein-carbohydrate content.

If tissues likely to contain extracellular fluids that are high in sialic acid content are excluded from Warren's list, the mean value for the remaining tissues is about 1 μmole per gram of tissue (wet weight). This is approximately one-half the value observed for salt water adapted salt glands of the duck. If one excludes the values for mammary gland and epididymis from the study of Monis et al., (1969) for the reasons discussed above, the mean value for the
remaining tissues of the rat (seminal vesicles, prostate gland, and coagulating gland) is less than 4 μmoles per gram protein. This is less than one-third the value observed for salt water adapted salt gland of the duck (12.06 μmoles per gram protein) and suggests, by comparison, that the salt gland has a high sialic acid content. One must therefore conclude that a substance known to be the major contributor to the electronegativity of the periphery of a number of cell types is present in abundance on the secretory cells of avian salt gland, a tissue functionally specialized for the active transport of NaCl. In this connection, it is of further interest that the gills and pseudobranchs of teleosts fishes, still other examples of tissues functionally specialized for electrolyte transport, also possess a high sialic acid content (Karnaky, unpublished data).

It is somewhat surprising that the difference in sialic acid content between fresh water (8.08 μmoles/g. protein) and salt water (12.06 μmoles/g. protein) adapted salt gland is not greater since the fully specialized cells demonstrate a substantial increase in total surface area. Of course it must be remembered that not all the cells in the tubules are fully specialized and the cells also increase in size. Possibly of even greater importance in this regard is that the fully specialized cells become filled with tremendous numbers of mitochondria and the sialic acid content of this organelle is apparently very low (Patterson and Touster, 1962; Wallach, 1967).

The sialic acid content of salt water adapted salt gland is increased about 1.3 fold per gram gland or about 1.5 fold per gram protein over that observed in the fresh water adapted gland. However,
since the size of the salt gland doubles when ducks are salt water adapted, the total increase in sialic acid per gland would be in the range of 2.6 to 3 fold. This value may be of more significance in relating sialic acid to the increased electrolyte excretory capability of the salt water adapted birds.

JUNCTIONAL COMPLEX

The three elements of the junctional complex as defined by Farquhar and Palade (1963) may be seen in Fig. 18. The area that corresponds to the zonula occludens (tight junction) in the salt gland varies in length from 65 to 835 A and never reaches the 1,000 to 15,000 A range typical for a variety of other cell types (Farquhar and Palade, 1963, 1965; Sedar and Forte, 1964). A second difference is that, at high magnification, it is not possible to see clearly the fusion of the outer dense lines characteristic of typical tight junctions.

If the formula used by Karnovsky (1967) to differentiate between tight and "gap" junctions is applied to these junctions the result is greater than 2, indicating a gap type junction. However the junctions of the central canal cells that clearly exhibit fused outer dense lamina (Fig. 40) provide a result of less than one when the formula of Karnovsky is applied. It is possible that the junctions might be damaged during fixation but tight junctions are the last structures to break when cells are placed under tension (Farquhar and Palade, 1965). For example, when liver cells are dissociated the tight junctions persist even to the extent that a small segment of cytoplasm from the adjacent cell will remain attached to the junction
throughout the experimental procedure (Berry and Friend, 1969). Mechanical damage therefore seems unlikely.

It is interesting that horseradish peroxidase, lanthanum, and ruthenium red penetrate the entire junctional complex, in contrast to colloidal iron and thorium dioxide which are clearly excluded for a distance of 300 to 850 Å. Horseradish peroxidase apparently penetrates endothelial cell junctions (Karnovsky and Cotran, 1966) and lanthanum permeates the "tight" junctions of a number of cell types (Revel and Karnovsky, 1966, 1967). In addition, ruthenium red appears to penetrate some endothelial cell junctions (Luft, 1965). The exclusion of colloidal iron from this area of the junction is consistent with the results of Benedetti and Emmelot (1967) in their study of isolated liver plasma membranes.

It is perhaps significant that all three of the techniques that yield products that apparently penetrate the junctions are accomplished at near neutral pH while the two that did not penetrate involve the Hale reaction at low pH. Since the colloidal iron micelles are smaller (25 to 30 Å in diameter) than the horseradish peroxidase molecules (45 Å in diameter), the iron particles are probably excluded from the junctions for reasons of electrical charge rather than absolute or relative size. Ruthenium red has an affinity for acidic carbohydrates and is used at neutral pH; therefore, it would seem that anionic carbohydrates are present in this area. However, the exclusion of colloidal iron from the junction suggests that strong acids, such as sialic acid, are not present. It is of interest to this discussion that the penetration of tight junctions
by EM tracers and cytochemical agents has been reported for a number of tissues specialized for electrolyte transport (Pease, 1966; Van Lennep, 1968).

Much more information is needed to establish the exact nature of the junctional complexes of these cells but certain conclusions seem appropriate. It appears that these cells may not have "typical" tight junctions as the term was originally defined. It is certainly conceivable that the junctions could still function as physiological barriers even though the external dense lines of their membranes do not fuse. Specifically how the presence of physiologically "non-tight" junctions might relate to the mechanism of electrolyte transport in this tissue is a matter that must await further investigation.

SPECULATION CONCERNING THE TRANSPORT MECHANISM

The existing information concerning the physiology, biochemistry, and morphology of avian salt gland, though admittedly incomplete, seem to warrant certain speculations concerning the method of transport. There are obviously only three possibilities with respect to what membranes are involved in active transport; i.e., apical membranes, basolateral membranes, or both. The energy required to transport the volume and concentration of NaCl solution secreted by this gland are tremendous and it follows therefore that the site of active transport would have a large surface area and a ready source of energy. This seems to immediately exclude the apical surface and make the basolateral membranes with their very amplified surface and their close association
with large numbers of mitochondria, the prime candidates. Recall also that the major changes associated with salt water adaptation were an increase in mitochondrial density and basolateral surface area. By contrast there was virtually no change in the apical portion of the cell.

A pinocytotic type of membrane flow in which the NaCl solution would be accumulated at the basal end of the cell and be carried to the cell apex was suggested by Doyle (1960) as a possible transport mechanism in this tissue. This idea was encouraged by the observation in early EM studies of many vacuoles in the active secretory cells (Kommick, 1963). Subsequently, however, more reliable methods for fixation and tissue processing were developed which yielded high quality images that provide no evidence for this method of transport. For example, in this investigation and that of Ernst and Ellis (1969) some few vesicular structures are seen in the cells but they are present in both active and inactive gland cells in near equal numbers. Moreover since this tissue is capable of secreting a volume of fluid equivalent to the weight of the gland per minute, a transport mechanism of this type seems unlikely.

Thus, the most appealing hypothesis at the present time seems to be that NaCl is actively transported into the cell at the basolateral membranes; concentrated here (possibly according to the scheme of Diamond and Bossert (1968), and then moved through the cell and apical membrane by hydrostatic flow. This would of course require that the cells function with an abnormally high osmotic
pressure but since this is known to occur in the vertebrate kidney (Kuhn and Ryffel, 1942; Hargitay and Kuhn, 1951) and in invertebrates (Davson, 1964) such a mechanism does not appear unreasonable.

Since the enzyme Na\(^+\)-K\(^+\) ATPase appears to have a vital role in electrolyte transport (Skou, 1965), and its activity in salt gland has been closely correlated with salt water adaptation (Ernst and Ellis, 1967, 1968; Fletcher et al., 1967), the cytochemical localization of this enzyme should provide an answer to the question of the site of active transport. Unfortunately the validity of the existing procedures for localizing this enzyme have been seriously questioned (Moses and Rosenthal, 1967, 1968; Tice, 1969; Tormey, 1966) and localization of the enzyme must therefore await the development of a valid technique. It is encouraging that successful investigation toward this end is now in progress (Ernst and Philpott, 1970).

POSSIBLE ROLE OF ACIDIC CARBOHYDRATES

Data relating polyanionic carbohydrates to ion permeation and selectivity was mentioned in the introduction of this dissertation. Although their general involvement in these processes seems inevitable it is much more difficult to assess a specific functional role for these highly anionic surfaces as they relate to a particular tissue. The major emphasis of this work has been the qualitative and quantitative determination of anionic protein-carbohydrates in salt gland and their location with respect to the secretory cell morphology. Thus, definitive statements concerning function must remain, at least for the present, tentative and speculative.
Certainly the presence of relatively high values of sialic acid in this very specialized tissue suggests an involvement in the transport activity.

A search of the literature reveals suggestions that sialic acid may be involved in a variety of biological functions. Examples include blood agglutination (Danon et al., 1965; Hirst, 1942), cell aggregation (Kemp, 1968), metastasis (Gasic and Gasic, 1962), neoplasia (Forrester et al., 1962, 1964), protection of cell surface sites (Glaeser and Mell, 1966), protein secretion (Glick et al., 1966), serotonin receptor sites (Carroll and Seveda, 1968; Wesemann and Zilliken, 1968; Wolly and Gommi, 1964, 1965), specificity of cell contact (Glaeser and Mell, 1966), sperm maturation (Bose et al., 1966; Hartree and Srivastava, 1965; Rajalakshmi and Prasad, 1969), structural stability of membranes (Glaeser and Mell, 1966; Weiss, 1961, 1965), and virus recognition sites (Hirst, 1942). Thus, with specialization sialic acid may assume different roles in different cell types. Of particular interest here are the numerous reports implicating sialic acid in cation transport activities (Emmelot and Bos, 1965, 1966; Glick and Githens, 1965; McIlwain, 1963, 1964; Monis et al., 1969; Morard, 1967; Nolte and Ohkuma, 1969; Weiss and Levinson, 1969). Certain of the above experiments (Emmelot and Bos, 1965; McIlwain, 1963, 1964) suggests that sialic acid might in fact be the cation receptor site. For this reason a pilot experiment was conducted with Dr. Ernst in which salt gland homogenate was pre-digested with neuraminidase and then assayed for \( \text{Na}^+ - \text{K}^+ \) ATPase activity. Since no inhibition of activity was observed, it seems
likely that the neuraminidase removable sialic acid is not functioning as the receptor site in this system. This experiment does not eliminate the possibility that the sialic acid not removable with neuraminidase may function as the receptor and, of course, says nothing about the possibility that the acid may serve some vital ancillary function with respect to ion transport in the intact tissue.

It seems appropriate to suggest certain theoretical roles of polyanionic substances in relation to the standing gradient flow hypothesis of Diamond and Bossert (1968). Diamond and Bossert (1967) point out that in channels more narrow than 0.1 μ (which is the case in salt gland) the laws for bulk fluid flow breakdown and the "sliding" of fluid along the wall becomes important. There is evidence that, as a result of charged intracellular macromolecules, cell water may be ice-like; i.e., have a semicrystalline lattice structure (Bernal, 1965; Ling, 1965). Moreover, a layer of water near a surface exhibits the physical properties of bound or structured water and the thickness of this layer depends on the nature of the surface (Derjaguin, 1965). These phenomena account for capillary osmosis and provide an explanation for the movement of fluids through plant micropores. Thus, they must be taken into account in any biological system in which fluids are moved through very small spaces.

These physical data indicate that the electrical charge along the "channels" in salt gland secretory cells should result in the structuring of the fluids contained therein. One effect of this structuring would be to prevent stirring of the fluid in the channels. Diamond and Bossert (1968) have indicated that the existence of the
standing osmotic gradient, vital to their scheme, requires that the fluid contents are effectively unstirred. A potential problem unique to the "backwards" flow system is that all solutes will enter the open end of the channels and those not actively transported may tend to build up opposing gradients that would reduce the efficiency of the system. Diamond and Bossert (1968) suggest that this may be why all known "backwards" systems actively transport NaCl, the major solute of plasma. This makes the nontransported solutes those of negligible importance quantitively. Since the volume of flow in salt gland is so extremely high even these minor solutes could become a problem.

Therefore, it seems reasonable that the negative charges on the membrane infoldings may function to select cations (Na⁺ would be the major one present) and in this way tend to reduce the problem of opposing gradients. In any case, it seems plausible that in an application of the standing gradient flow hypothesis to salt gland, the physiochemical nature of the membranes forming the walls of the channels is an additional parameter that must be considered.

Finally, it is obvious that much more experimental data is needed to establish the exact physiological relationship between anionic protein-carbohydrates and the translocation of electrolyte and water. Further, it seems likely that continued study of the relationships between structure and function in this very interesting transport system will provide information vital to the final solution of this intriguing problem.
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KEY TO ABBREVIATIONS

BL - basal lamina
CT - connective tissue
En - endothelial cell
J - junctional complex
L - lumen
M - mitochondrion
Mt - microtubule
mv - microvilli
N - nucleus
ne - nerve cell
Figure 1.

Note the increased thickness of the salt water adapted gland. The FW gland in this example weighed 90 mg and the weight of the SW gland was 180 mg. The transverse sections at the right of each gland clearly illustrate the change in size that occurs with salt water adaptation.
Figure 2.
A transverse section from the salt gland of a FW adapted duck. A thick shell of connective tissue surrounds the gland and narrows to form sheaths that encase the individual lobules. The two main ducts (*) are apparent and a central canal (arrow) oriented longitudinal to the section connects with the upper central canal. Acid fuchsin and toluidine blue stain. 36X.

Figure 3.
A transverse section from the salt gland of a SW adapted duck. Note the increased size of the section and apparent reduction in the relative amount of connective tissue. Central canals (arrows) connect with both main ducts (*). Acid fuchsin and toluidine blue stain. 36X.
Figure 4.
Fresh water adapted salt gland from duck. The epithelial lining of the main duct appears to be two cells in thickness. Note the projections from some of these cells into the lumen of the duct. The secretory tubules are tortuously arranged within the lobules. Acid fuchsin and toluidine blue stain. 156X.

Figure 5.
Salt water adapted gland from duck. Note the dense homogeneous staining of the cell cytoplasm. Small peripheral cells are indicated by the close placement of nuclei at the distal ends of the tubules (lower portion of figure). Acid fuchsin and toluidine blue stain. 156X.
Figure 6.
Fresh water adapted gland from duck. Numerous secretory tubules are seen in cross section. The tubules average about 10 μ in diameter.
Acid fuchsin and toluidine blue stain. 620X.

Figure 7.
Salt water adapted gland from the duck. The tubules average about 20 μ in diameter. Note the striated appearance of the cytoplasm of many of the cells. The extensive vascularization of this tissue is indicated by the number and location of red blood cells in this figure.
Acid fuchsin and toluidine blue stain. 620X.
Figure 8.
Fresh water adapted gland from the gull. The long somewhat cylindrical lobules are seen in cross section. Secretory tubules extend radially from the central canals. Small relatively unspecialized cells are indicated by the close arrangement of cell nuclei in the distal portion of the tubules. The proximal portion of the tubules contain more specialized cells. Acid fuchsin and toluidine blue stain. 156X.
Figure 9.

Fresh water adapted gland from gull. Note that the epithelial wall of the central canal is 3 or 4 cells thick. A connection between the central canal and the secretory tubules may be observed. Connective tissue forms an incomplete separation between adjacent lobules.

Acid fuchsin and toluidine blue stain. 156X.

Figure 10.

Salt water adapted gland from gull. The wall of the central canal is not as thick as in Figure 9. Three connections between secretory tubules and the central canal may be observed. A greater portion of tubules consist of specialized cells and the tubules appear somewhat longer than in the fresh water adapted gland.

Acid fuchsin and toluidine blue stain. 156X.
Figure 11.
Fresh water adapted gland from gull. Note the gradient of cell specialization along the length of the secretory tubules. The size and degree of specialization of the cells increases from right to left in this figure.
Acid fuchsin and toluidine blue stain. 620X.

Figure 12.
Salt water adapted gland from gull. There is also an increase in the degree of cell specialization from right to left in this figure. The two fold increase in diameter of salt water adapted tubules is obvious when Figures 11 and 12 are compared. Note the striated appearance that results from the mitochondrial filled invaginations of the fully specialized cells.
Acid fuchsin and toluidine blue stain. 620X.
Figure 13.
Salt water adapted gland from duck. The peritubular connective tissue is PAS-positive. Of special interest is the PAS staining of the basal and lateral surfaces of these cells.
Periodic acid-Schiff stain. 1,000X.
Figure 14.

Fresh water adapted gland from duck. A typical secretory tubule in cross section. The tubule consists of 6 pyrimidal shaped cells with a central lumen (L). Nerve cells (ne) may be seen in the upper left and lower right regions of the figure. Portions of capillaries are located in these same two regions (arrow heads). Glutaraldehyde-osmium fixation.

Uranyl acetate and lead citrate stain. 11,200X.
Figure 15.

Fresh water adapted gland from duck. The cells have invaginations at their lateral surfaces but few, if any, are observed at their basal surfaces. A moderate number of mitochondria with well developed cristae are distributed throughout the cytoplasm. Note the supranuclear location of the Golgi complexes and the multi-vesicular bodies (arrow heads) in the apical region of the cells. Glutaraldehyde-osmium fixation.

Uranyl acetate and lead citrate stain. 12,700X.
Figure 16.

Fresh water adapted gland from duck. The plane of this section is oblique to the secretory tubule. Thus the interdigitations of the lateral surfaces of the cells are emphasized. The large nuclei fill most of the cytoplasmic space. Rough endoplasmic reticulum and free ribosomes are distributed randomly through the cytoplasm.

Glutaraldehyde-osmium fixation.

Uranyl acetate and lead citrate stain. 13,800X.
Figure 17.

Fresh water adapted gland from duck.

This cell is typical of the secretory cells of the FW tissue.

Note the junctional complexes (J) at the cell apex.

Glutaraldehyde-osmium fixation.

Uranyl acetate and lead citrate stain. 22,600X.
Figure 18.
Fresh water adapted gland from duck. Short microvilli (mv) project into the lumen. Most junctional complexes appear typical but on occasion they do not appear to have zonulae adherens (*). Note the centriole in cross section (arrow head).
Glutaraldehyde-osmium fixation.
Uranyl acetate and lead citrate stain. 21,000X.

Figure 18 INSET.
Fresh water adapted gland from duck. An "atypical" junctional complex at high magnification. The outer dense lines of the adjacent plasma membranes clearly do not fuse to form the classical tight junction.
Glutaraldehyde-osmium fixation.
Uranyl acetate and lead citrate stain. 71,000X.
Figure 19.

Fresh water adapted gland from duck. The apex of these cells contain a variety of vesicular structures including coated vesicles (arrow heads). Microtubules (Mt) course randomly through the apical cytoplasm. Packets of filamentous material (arrows) are regularly observed in this region. Note the complexity of the cell surface that results in 3 intercellular canaliculi (*). Formaldehyde-glutaraldehyde fixation.

Uranyl acetate and lead citrate stain. 18,200X.
Figure 20.

Fresh water adapted gland from duck. Centrioles may be observed in the apical region of most of the cells. The consistent observation of centrioles in this location indicates their orderly arrangement within these cells.

Formaldehyde-glutaraldehyde fixation.

Uranyl acetate and lead citrate stain. 18,200X.
Figure 21.

Gland from acute loaded duck. A cross section of a typical tubule illustrating that this tissue is not significantly different in morphology from the fresh water condition. This figure should be compared with Figure 14.

Glutaraldehyde-osmium fixation.

Uranyl acetate and lead citrate stain. 8,600X.
Figure 22.
Gland from acute loaded duck. In this section taken longitudinal to the secretory tubule the morphology remains consistent with that observed in the fresh water tissue.
Glutaraldehyde-osmium fixation.
Uranyl acetate and lead citrate stain. 15,900X.
Figure 23.
Gland from acute loaded duck. The plane of this section is oblique to the secretory tubule and should therefore be compared with Figure 16.
Glutaraldehyde-osmium fixation.
Uranyl acetate and lead citrate stain. 14,600X.
Figure 24.

Gland from acute loaded duck. At this magnification the individual secretory cell is emphasized. A comparison with Figure 17 will illustrate that the secretory cells from the two physiological conditions are not significantly different in morphology.

Glutaraldehyde-osmium fixation.

Uranyl acetate and lead citrate stain. 21,000X.
Figure 25.
Gland from acute loaded duck. Note the flocculent material extending from the plasma membranes of these cells. Some of this material is always observed at the surface of the secretory cells but more of it is generally preserved with this fixative.

Formaldehyde-glutaraldehyde fixation.
Uranyl acetate and lead citrate stain. 14,000X.
Figure 26.
Salt water adapted gland from duck. These cells are from near the peripheral or distal end of the tubule and are only partially specialized. They are somewhat more elongate than FW cells and contain an increased number of lateral infoldings some of which have entrapped mitochondria. Note that the cells have begun to develop mitochondrial filled basal infoldings.
Glutaraldehyde-osmium fixation.
Uranyl acetate and lead citrate stain. 10,800X.
Figure 27.
Salt water adapted gland from duck. These cells are somewhat more specialized than those observed in Figure 26. The basal infoldings are more numerous and longer. The infoldings are so extensive that it is difficult in some areas (*) to distinguish between lateral and basal infoldings.

Glutaraldehyde-osmium fixation.

Uranyl acetate and lead citrate stain. 11,200X.
Figure 28.
Salt water adapted gland from duck. The fully specialized cells consist of a tremendously complex network of mitochondrial filled infoldings of the plasma membrane. This provides a highly amplified cell surface and divides the cell into many cytoplasmic compartments. Note that the nucleus is isolated in a small island of cytoplasm. Lipid droplets (arrow heads) are randomly dispersed through the cell.
Glutaraldehyde-osmium fixation.
Uranyl acetate and lead citrate stain. 11,200X.
Figure 29.
Salt water adapted gland from duck. The basal infoldings typically abut against the basal lamina in the fully specialized cells. A thin region of connective tissue (CT) separates the secretory cell basal lamina from the external lamina of the capillary endothelial cell (En).
Glutaraldehyde-osmium fixation.
Uranyl acetate and lead citrate stain. 12,500X.
Figure 30.
Salt water adapted gland from duck. The basal region of a fully specialized cell at high magnification. The plasma membrane is very closely associated with the mitochondria and in regions of the infoldings that do not contain mitochondria the two membranes of an infolding are closely applied (arrow). The basal lamina (BL) and collagen fibrils (arrow head) are prominent in the lower region of the image.

Glutaraldehyde-osmium fixation.
Uranyl acetate and lead citrate stain. 45,200X.
Figure 31.

Fresh water adapted gland from duck. The endothelial wall of a central canal or main duct. The cells exhibit only moderate surface specialization and contain relatively few mitochondria. Glutaraldehyde-osmium fixation.

Uranyl acetate and lead citrate stain. 14,200X.
Figure 32.

Gland from acute loaded duck. These epithelial cells forming the wall of the main duct illustrate the typical morphology in this region. The projections of cytoplasm at the cell apex (arrows) that project into the luminal space are a regular feature of these cells.

Glutaraldehyde-osmium fixation.

Uranyl acetate and lead citrate stain. 11,200X.
Figure 33.

Gland from acute loaded duck. The wall of the main duct consists of relatively small basal cells and large superficial cells. Necrotic cells (*) are frequently observed in the wall of the main duct. Glutaraldehyde-osmium fixation. Uranyl acetate and lead citrate stain. 9,900X.
Figure 34.

Fresh water adapted gland from duck. An intralobular artery in a somewhat contracted state. Smooth muscle cells (Sm) surround the basement layer which, in turn, supports the endothelial lining (En). Nerves (ne) that innervate the smooth muscle cells are located external to the muscularis layer.

Formaldehyde-glutaraldehyde fixation.

Uranyl acetate and lead citrate stain. 16,400X.
Figure 35.
Salt water adapted gland from duck. Tissue was prepared by inert dehydration and stained with 5% phosphotungstic acid. The entire extra-cellular space is PTA-positive.
63,000X.
Figure 36.
Salt water adapted gland from duck. Tissue was prepared by inert dehydration and stained with 5% phosphotungstic acid. The extracellular space between basal infoldings stains intensely with PTA (arrow heads). 94,000X.
Figure 37.
Salt water adapted gland from duck. A cross section taken from near the distal end of the secretory tubule. The cells in this peripheral region of the tubule exhibit very little specialization. TAPO fixation.
Uranyl acetate and lead citrate stain. 10,800X.
Figure 38.
Salt water adapted gland from duck. This peripheral cell is only slightly specialized. Note the extreme density of the filamentous structures (arrows) in the cell apex.
TAPO fixation.
Uranyl acetate and lead citrate stain. 15,400X.
Figure 39.
Salt water adapted gland from duck. The adjacent membranes of the junctional complexes do not appear to fuse to form the tight junction. The intercellular space is reduced in this region and contains an increased amount of electron dense material. TAPO fixation.
Uranyl acetate and lead citrate stain. 94,000X.
Figure 40.
Gland from acute loaded duck. The junctional complex between these epithelial cells from the central canal appears to have the typical fused membranes.
TAPO fixation.
Uranyl acetate and lead citrate stain. 224,000X.
Figure 41.
Salt water adapted gland from duck. The apical region of a superficial cell from the main duct. The mucous droplets (arrows), regularly observed in the apex of these cells when this technique is used, were never observed in the epithelium of the main ducts of tissue prepared by conventional procedures.
TAPO fixation.
Uranyl acetate and lead citrate stain. 21,000X.
Figure 42.
Salt water adapted gland from duck. This image illustrates the very long cytoplasmic projections frequently seen at the luminal surface of superficial cells of the main duct. Note (arrows) that a cell has completely sloughed off except for a small segment retained by the desmosomes.
TAPO fixation.
Uranyl acetate and lead citrate stain. 21,000X.
Figure 43.
Salt water adapted gland from duck. These partially specialized cells are outlined by horseradish peroxidase. The tracer fills the connective tissue matrix and outlines collagen fibrils (arrow). The basal infoldings and intercellular spaces are also penetrated. Note that the tracer penetrates the junctional complexes and, in some instances, appears to penetrate the tight junctions (arrow heads).
14,700X.
Figure 44.
Salt water adapted gland from duck. Note that in intercellular spaces not completely filled with horseradish peroxidase, the tracer adheres to the plasma membranes.
19,100X.
Figure 45.
Gland from acute loaded duck. The lanthanum complex penetrates the intercellular spaces forming a very electron dense outline around the secretory cells. In contrast to horseradish peroxidase (Figure 43), lanthanum is not retained in the connective tissue matrix.
13,900X.
Figure 46.
Gland from acute loaded duck. The lanthanum complex is very concentrated in the intercellular spaces in the regions of the junctional complexes (arrow heads).

11,600X.
Figure 47.
Gland from acute loaded duck. In this region of rather extensive lateral infoldings the extracellular space is completely filled with the lanthanum complex.
26,900X.

Figure 48.
Fresh water adapted gland from duck. The tracer narrows to a fine point in the region of the "tight junction" but seems to penetrate it completely.
25,000X.
Figure 49.
Salt water adapted gland from gull. The basal lamina and connective tissue matrix is ruthenium red positive. Of particular interest is the dense deposition of ruthenium red on the plasma membranes of the secretory cell infoldings.
31,000X.
Figure 50.
Salt water adapted gland from duck. In addition to the deposition
of ruthenium red along the plasma membranes, filamentous structures,
often observed extending from the cell surfaces, are ruthenium red
positive.
72,500X.
Figure 51.
Salt water adapted gland from duck. The ruthenium red deposition along the plasma membranes is variable in thickness and in areas where two membranes are in close apposition RR often extends across the entire extracellular space. 72,500X.
Figure 52.
Salt water adapted gland from gull. This section of tissue prepared by the ruthenium red procedure was also stained with uranyl acetate and lead citrate. After this treatment it becomes clear that RR is limited to the outer leaflet of the trilamellar unit membrane.

99,500X.
Figure 53.
Freshwater adapted gland from duck. Ruthenium red clearly penetrates the junctional complex extending through the zonula occludens region and along the luminal membrane. 70,000X.
Figure 54.
Salt water adapted gland from duck. In this chopped section a moderate amount of ruthenium red may be observed in the cytoplasmic ground substance near the exposed edge of the section. Note the rather intense deposition of RR in the matrix material of the mitochondrion in which the outer membrane has been broken. However, the sites that appear to have the greatest affinity for RR are the outer surfaces of the plasma membranes. 83,200X.
Figure 55.

Fresh water adapted gland from duck. The colloidal iron is rather specific for plasma membranes. In regions where cytoplasm and nucleoplasm are exposed at the edge of the chopped section (arrow heads) very little CI is observed.

22,800X.
Figure 56.
Salt water adapted gland from duck. Note that along the chopped edge of the section only the plasma membranes have dense depositions of colloidal iron.
21,400X.
Figure 57.
Fresh water adapted gland from duck. This chopped section was digested with neuraminidase prior to colloidal iron staining. The significant reduction in CI staining that results from the enzyme digestion becomes obvious when this image is compared with Figures 55 and 56.
21,400X.
Figure 58.
Salt water adapted gland from duck. This image may be compared to Figure 56 in order to observe the reduction in CI that results from digestion with neuraminidase prior to staining. 27,300X.
Figure 59.
Salt water adapted gland from duck. Note that the apical membranes that form the lumen of the secretory tubule stain with CI. Colloidal iron is excluded from the intercellular space in the region of the junctional complex (arrows).
32,800X.
Figure 60.
Fresh water adapted gland from duck. This image illustrates that neuraminidase digestion prior to colloidal iron staining reduces the staining of the luminal membranes. The figure may be compared with Figure 59.
32,800X.
Figure 61.

Fresh water adapted gland from duck. A chopped section stained with colloidal iron at pH 3.8. In addition to the staining of the plasma membranes (arrow), a calyx of CI forms along the edge of the section. Note the reticulum of CI filling the luminal space.

32,800X.
Figure 62.

Fresh water adapted gland from duck. The plasma membranes stain intensely with thorium dioxide. The cytoplasmic ground substances stains lightly and a moderate number of thorium particles are observed in the cell nucleus. In contrast to colloidal iron (see Figure 55), considerable staining of the cytoplasm occurs at the exposed edge of the section (arrow heads). 23,600X.
Figure 63.

Fresh water adapted gland from duck. This chopped section was digested with neuraminidase prior to thorium dioxide staining. Note the intense staining of the exposed edge of the nucleoplasm. This image may be compared with Figure 62 to illustrate that no obvious reduction in the amount of staining resulted from the enzyme digestion.

23,600X.
Figure 64.
Fresh water adapted gland from duck. This image illustrates that the luminal membranes of the secretory cells stain with thorium dioxide when the lumen is open to the exposed edge of the section. Note that thorium dioxide does not penetrate the junctional complexes.
36,500X.
Figure 65.
The graph illustrates the quantity of sialic acid per gram gland (wet weight) removed from duck salt gland by neuraminidase. Note that most of the sialic acid is removed within 10 minutes and after 30 minutes essentially all the enzyme reactive material is removed. Triangles indicate fresh water adapted gland and circles indicate salt water adapted gland. The quantity of neuraminidase was doubled at 60 minutes to obtain the value indicated at 90 minutes. This value indicates that the addition of fresh enzyme results in no significant increase in the amount of sialic acid removed.

Figure 66.
The graph illustrates the quantity of sialic acid per gram protein removed from duck salt gland by neuraminidase digestion. Triangles indicate fresh water adapted gland. Circles indicate salt water adapted gland. The normal quantity of neuraminidase was doubled at 60 minutes to obtain the value shown at 90 minutes.