STUDIES ON THE MEDIATION AND MODULATION OF THE HYDROOSMOTIC ACTION OF ANTIDIURETIC HORMONE IN THE TOAD BLADDER AND MEDIATION OF CATECHOLAMINE ACTION IN THE TURTLE BLADDER

by

NIZA LEICHTMAN-DAVIDSON

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1976

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

11/12/76
date

Chairman of Examining Committee

11/25/76
date

Executive Officer

John D. Glass

Esmail Zanjani

Zia J. Penefsky

Supervisory Committee

The City University of New York

Abstract

STUDIES ON THE MEDIATION AND MODULATION OF THE HYDROOSMOTIC ACTION OF ANTIDIURETIC HORMONE IN THE TOAD BLADDER AND MEDIATION OF CATECHOLAMINE ACTION IN THE TURTLE BLADDER

by

Niza Leichtman-Davidson

Advisor: Professor Irving L. Schwartz

The role of adenylate cyclase, and the possible role of protein kinase/phosphatase systems in the mediation and modulation of hormone action on transepithelial transport processes, were investigated in two model epithelial tissues, the urinary bladder of the toad (Bufo marinus) and the urinary bladder of the turtle (Pseudemys scripta).

Toad bladders exhibiting a diminished hydroosmotic response to the second of two "maximal" challenges with arginine vasopressin ("intrinsic inhibition"), proved to have a sustained level of adenylate cyclase activity. Thus the adenylate cyclase step in neurohypophyseal hormone action does not appear to be the locus at which the phenomenon of "intrinsic inhibition" occurs. In contrast, toad bladders exhibiting a diminished hydroosmotic response to arginine vasopressin in the presence of a natural inhibitory agent extracted from the

bladder tissue ("extrinsic inhibition"), proved to have a decreased level of adenylate cyclase activity, suggesting that the adenylate cyclase step in hormone action is the locus at which the phenomenon of "extrinsic inhibition" occurs.

Toad bladders exhibiting an increased hydroosmotic response to the second of two "submaximal" challenges with arginine vasopressin ("facilitation"), proved to have an increased level of adenylate cyclase activity. Thus the adenylate cyclase step in hormone action appears to be the locus at which the phenomenon of "facilitation" occurs.

The optimal cAMP concentration for stimulation of protein kinase was found to be 5×10^{-8}M cAMP. The supernatant fluids of mitochondria-rich cell homogenates and granular cell homogenates derived from toad bladder epithelium were both found to contain cAMP-dependent protein kinase activity using either histone or protein as phosphate acceptor. When the pellet and supernate are incubated together there is less self-phosphorylation than on incubation of the supernate alone. Basal and cyclic AMP-dependent protein kinase activities were also found to be associated with pellets sedimented from mitochondria-rich and granular cell homogenates. Cyclic AMP-dependent self-phosphorylation was apparent when the

pellets were incubated for periods ranging from 1 to 10-20 minutes; with longer periods of incubation, dephosphorylation appeared to be more markedly stimulated than phosphorylation. $ZnCl_2$, 2.5 mM, induced a parallel increase in the self-phosphorylation of mitochondria-rich and granular cell pellets in the absence and presence of cAMP. These findings are consistent with a model for the mediation of the hydro-osmotic action of neurohypophyseal hormones in the toad bladder which involves membrane phosphorylation as the cyclic AMP-mediated step.

Plasma membranes and microsomes derived from turtle bladder epithelial cells were found to contain norepinephrine-stimulated adenylate cyclase, cAMP-dependent protein kinase and cGMP-dependent protein kinase. Luminal plasma membrane fragments, isolated by preparative free flow electrophoresis, were identified by the presence of marker enzymes, alkaline phosphatase and bicarbonate-stimulated ATPase; adenylate cyclase activity, cAMP-dependent protein kinase activity and cGMP-dependent protein kinase activity were all found to be associated with this luminal membrane fraction. Plasma membranes, prepared from turtle bladders exposed to diisothiocyano disulfonic stilbene (DIDS) or to 4-acetamido-4'-isothiocyanato stilbene-2,2'-disulfonic acid (SITS) on the mucosal

surface, were found to be devoid of cAMP-dependent protein kinase activity. The disulfonic stilbene compounds, when placed in the serosal bathing fluid, are known to block the transepithelial reabsorptive flow of Cl^- and HCO_3^- ions in the turtle bladder.

The finding of norepinephrine-sensitive adenylate cyclase and cyclic nucleotide-dependent protein kinase in turtle bladder plasma membranes raises the possibility that these cyclase-kinase systems are involved in the catecholamine-regulated anion transport function of the bladder epithelium.

DEDICATION

This thesis is dedicated to my father, my mother, and my husband - without whose inspiration and love this work would never have been possible.

ACKNOWLEDGEMENTS

I am indebted to my advisor, Dr. Irving L. Schwartz, for his constant guidance and support thoughout my graduate career. In spite of a grueling schedule and myriad commitments, he has always found the time to steer me toward the relevant scientific lead. I thank him for the confidence he has in me, for the advice he has given me during the course of my research and in the writing of this dissertation, and for allowing me the privilege of being his student and of working with him.

Special thanks are owed to Dr. William A. Brodsky and Dr. Rolf Kinne whose guidance was instrumental in launching the third phase of this work. Dr. Brodsky's advice concerning my experimental approaches and his patient, thoughtful and lengthy reviewing of this manuscript are sincerely appreciated.

I would like also to express my thanks to Dr. Linda Shlatz who taught me most of the methods used herein, who guided me through difficult times in the laboratory, and who has been a stalwart friend throughout.

I wish also to acknowledge the excellent technical assistance of Mr. David Rubin, Mrs. Susan Ehrenspeck, and Mrs. Cristina Matons. I also thank Mrs. Helen Washington for her care and patience in typing this manuscript and Ms. Cassie Schwartz for her fine rendering of the illustrations.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

In the most general sense the studies to be described below are concerned with the mediation of hormone action by the cyclic nucleotide-protein kinase/phosphatase system. These studies have been divided into three sections. The first section is concerned with the role of adenylate cyclase in three types of modulation of the reactivity to the hydroosmotic action of neurohypophyseal hormones of a model target organ, the toad bladder. The second section is concerned with the role of cyclic AMP-dependent phosphorylation and/or dephosphorylation in the hydroosmotic response to neurohypophyseal hormones of the two major cell types of the toad bladder epithelium. The third section is concerned with the role of adenylate cyclase and cyclic nucleotide-dependent protein kinase in an organ, the turtle bladder, which does not respond to antidiuretic hormone.

SECTION I: STUDIES OF FACTORS MODULATING THE HYDROOSMOTIC RESPONSE OF THE TOAD BLADDER TO NEUROHYPOPHYSEAL HORMONES.

INTRODUCTION

The studies presented here were designed to develop further knowledge of the sequence of events in hormone action and of the influences that modulate or terminate the response of a prototypic target tissue, the toad bladder, to a class of fast-acting peptide hormones, namely the neurohypophyseal hormones.

In the primordial sea organisms exchanged nutrients and wastes freely (passively) with their environment. With increasing salinity of the ocean, organisms faced a more complex osmotic problem in the regulation of the hydration and electrolyte composition of their body fluids. Likewise, in fresh water, organisms required a more elaborate mechanism for regulating and maintaining body fluid and electrolyte balance.

Marine teleosts, lacking glomeruli, are presumed to form urine entirely as a result of secretion. The kidney of these aglomerular teleosts is mesonephric in type, with tubules consisting of a brush border segment and collecting ducts. The secretory function of these renal tubules is performed by epithelial cells which have an elaborate structure in their basal cytoplasm, with mitochondria arranged in interdigitating compartments and located close to the plasma mem-

brane. The arrangement is similar to that found in other cells involved in the active transport of salts and other solutes. This architecture may serve to facilitate secretion, known to occur with a high pressure, and to require relatively high energy production. On the other hand, the elaborate structure of the cell may be more particularly related to production of hypotonic urine.

The renal tubules of glomerular teleosts probably function as both resorptive and secretory cells. In marine teleosts, which have low glomerular filtration rates, the most important function of the tubules is probably secretion of divalent cations, most notably Ca^{++} and Mg^{++}. Freshwater fish, like amphibians are to some degree permeable to water and solutes. Osmotic regulation is achieved by elimination of a hypotonic urine combined with active absorption of salts by the gills. The kidney's specific function is to balance water entry with an equivalent volume of urine produced by an efficient filtration - reabsorption mechanism. Marine fish drink sea-water to replace water lost osmotically across the body surface. Most of the Na^{+}, Cl^{-}, and K^{+} swallowed with sea water and absorbed from the gut appears to be excreted by an extra-renal route, via the gills.

The site of active reabsorption of electrolytes in the fish nephron is not known. In mammals and amphibians, the major site of electrolyte reabsorption is the proximal tubule. Solute absorption is accompanied by an osmotically equivalent amount of water, so that no significant change in osmotic pressure occurs. In the distal tubule, Na^+ and other "osmolytes" are reabsorbed against an osmotic gradient. This type of reabsorption is postulated to occur in fresh water fish; However, it is not known exactly where dilution of urine takes place in these animals.

The primary role of the mammalian urinary bladder is to function as a storage unit. It is the kidney of mammals that maintains the organisms water balance -- and, indeed, it is the "reconcentrating" system of the kidney, functioning as a result of the release of antidiuretic hormone, that has made possible the existence of higher organism (metozoa) on land. For this reason alone it is not surprising that much interest has developed in the study of action of the antidiuretic hormones.

Phylogenetically, amphibians represent the first vertebrates that can exist for any prolonged time away from water. Amphibian adaptations to conserve body water include:

(1) Ability to restrict water loss from kidney,

(2) to increase water and sodium uptake through skin, and

(3) to store and reabsorb water and sodium from the urinary bladder.

All of these effects are hormonally controlled.

Brünn (1921) was the first to note that, unlike fish, toads and frogs, when placed in water, showed an increase in weight when injected with neurohypophyseal hormones. This effect, often referred to as the "Brunn" or "water" balance effect is caused by retention of water resulting from the action of neurohypophyseal hormones on amphibian skin, kidney and bladder. Indeed, as early as 1799, it had been described that toads had the power to absorb fluids through skin and a large part of this water was thought to be retained in the urinary bladder. However it was not until 1930, when Steen undertook an experimental study of the function of the amphibian urinary bladder, that it was shown that reabsorption of water from bladders of intact frogs took place when these frogs were dehydrated. In 1952, Ewer demonstrated the same effect in toads (Bufo regularis) and found that the injection of extracts of mammalian

neurohypophysies into these animals led to increased water reabsorption from their urinary bladders.

In 1956, Sawyer and Shisgall showed that water transfer in bullfrog took place directly across the bladder wall and also found that extracts of bullfrog neurohypophysis were effective in stimulating this transfer.

The amphibian bladder is very distensible. The mucosal side is lined with epithelial cells which form a single layer when the bladder is fully distended. Toad bladder epithelial cells are typical of epithelial cells in other tissues and the only presently recognized distinguishing specialization associated with the ability to respond to neurohypophyseal hormones with a change in permeability to water is the aggregation phenomenon reported by Kachedorian et al (see below). The mucosal face of the epithelial cell is microvillus in character. The individual cells are otherwise distinguished by their mitochondrial content (mitochondria-rich cells), granules (granular cells) and vesicles (goblet cells).

It has been shown that the skin of amphibians transfers sodium and water from epithelial (mucosal) to serosal surface when neurohypophyseal hormones are present. However, the skin is a complex structure as compared with the bladder.

It was suggested (Sawyer and Shisgall, 1956), that the increased permeability of frog bladder to water in vivo in response to neurohypophyseal hormone may be analogous to the action of antidiuretic hormone in mammalian renal tubules. Later, it was shown by many investigators that in the toad bladder as well antidiuretic hormone acts to increase water permeability of cells (and also to increase Na^+ transport) in a manner analogous to its action on distal portion of the nephron. For this reason, and because it is a simpler structure than amphibian skin, the isolated amphibian bladder was considered to be an ideal preparation for study of membrane function and hormone action.

In 1958, Bentley developed a method for studying the isolated toad bladder which greatly facilitated the investigation of the effect of neurohypophyseal hormones on this tissue. This technique has been utilized in the present study and is described in detail in the Methods section. In this technique, one lobe of the toad bladder (mucosal side facing inwards) is tied on to a hollow glass rod, filled with diluted Ringer's solution and immersed in a bath containing full-strength Ringer's solution. Water transfer takes place from mucosal to serosal surface and is measured gravimetrically.

Water transfer across toad bladder takes place along an osmotic gradient. Without the presence of neurohypophyseal hormone (for example, Vasopressin) there is little net water movement. The rate of movement across the bladder wall is five times greater than that which takes place through the anuran skin and also requires less (about 1% as much) hormone to show stimulation. The permeability barrier of the bladder was shown by various investigators (Civan and Frazier, 1968; Handler et al, 1972; Dibona, et al, 1969) to be in the mucosal cell face.

It has been shown by Goldberg, Schoessler and Schwartz (1963) that following prolonged exposure of the toad bladder to a dose of arginine vasopressin (2.5×10^{-8}M, i.e. a dose which elicits a maximal hydroosmotic response) in the serosal fluid containing isotonic Ringer's solution, the hormone-induced increase in permeability to water reaches an early maximum and then declines exponentially with a half-time ranging from 100-270 minutes (Figure 1). After an initial challenge with hormone (2.5×10^{-8}M), bladders were washed in hormone-free Ringer's solution until baseline low level permeability was regained and the bladders were rechallenged with an identical dose of hormone; the second response was found to be of significantly less magnitude than the initial response (Figure 2). The second response actually approximated the level to which the previous response had been

Figure 1. Prolonged time course of the response of the isolated toad bladder to arginine-vasopressin (AVP), 2.5×10^{-8}M.

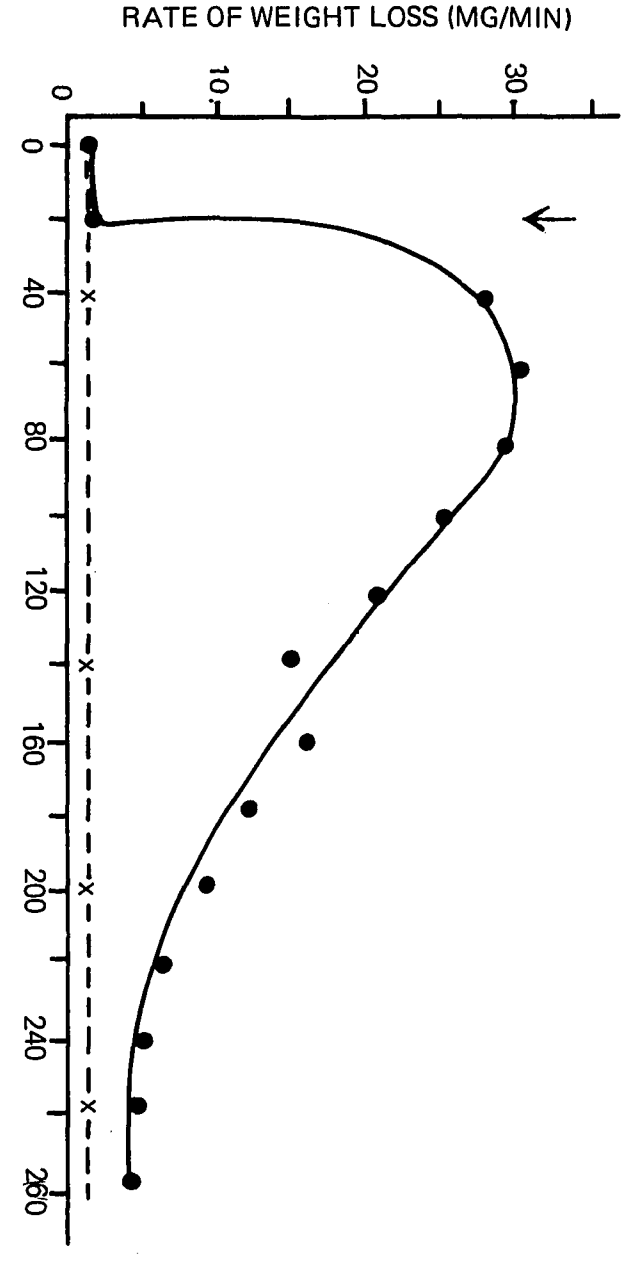

RATE OF WEIGHT LOSS (MG/MIN)
0
10
20
30
0
40
80
120
160
200
240
260
TIME IN MINUTES

FIGURE 1

Figure 2. The phenomenon of "Intrinsic Inhibition". Response of the toad bladder to each of two challenges with arginine-vasopressin, 2.5×10^{-8}M. The bladders were washed in hormone-free Ringer's fluid between challenges.

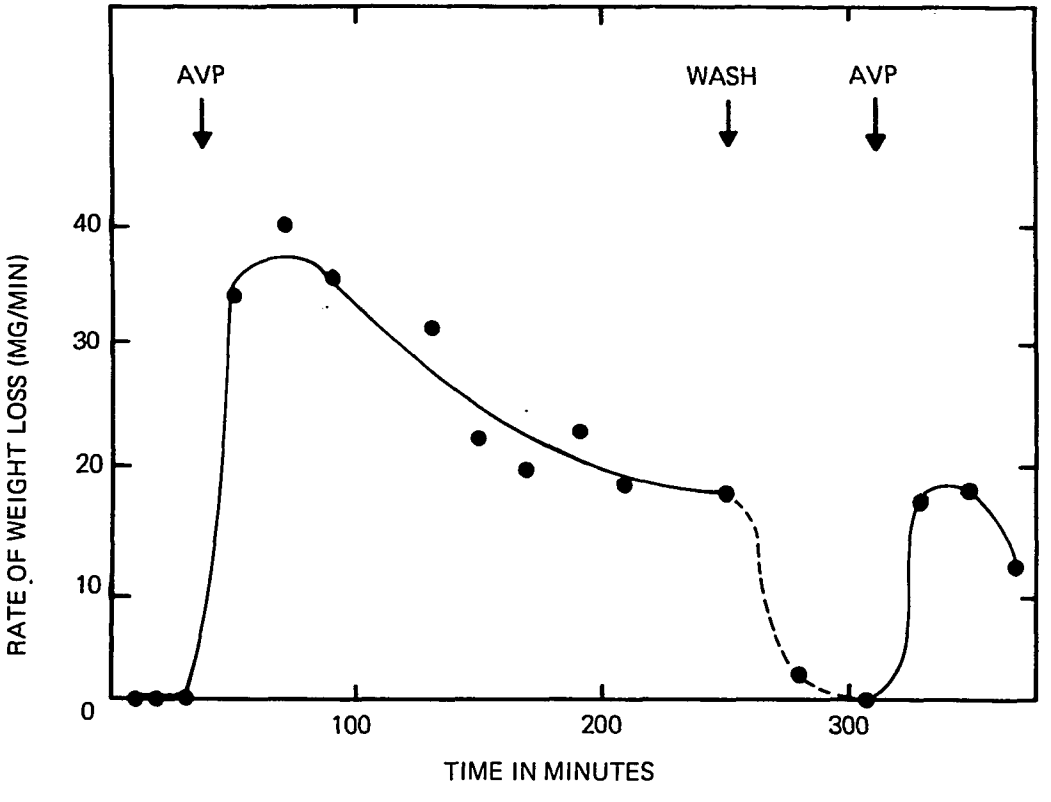

AVP
WASH
AVP
40
30
20
10
0
100
200
300
RATE OF WEIGHT LOSS (MG/MIN)
TIME IN MINUTES

FIGURE 2

allowed to decline before washing the bladders in hormone-free Ringer's solution. Clearly, the reactive capacity of the bladder wall was diminished following each hormonal challenge. This phenomenon was referred to as "intrinsic inhibition".

Edelman et al (1964), after confirming these results, stated that there is "some element of irreversibility in the resistance to vasopressin". They also found that there is a decline in reactivity of the toad bladder to cAMP after repeated challenge. Unlike vasopressin-induced resistance to vasopressin, cAMP-induced resistance to cAMP was completely irreversible. After one or more saturating doses of cAMP, bladders could still respond to vasopressin. Also, after one or more challenges with saturating doses of vasopressin, the bladders were still responsive to cAMP (Figure 3). The conclusion of Edelman and coworkers was that hormone and nucleotide induced diminished reactivity of the target tissue by different mechanisms. In contrast, Schwartz and Walter (1967), found that bladders with self-induced resistance to vasopressin, i.e. bladders exhibiting "intrinsic inhibition" respond submaximally or not at all to cAMP (Figure 4).

Figure 3. Effect of vasopressin (maximal dose) and of cAMP (6.5×10^{-3}M) on rates of osmotic flow after well-developed resistance is interrupted by washing in hormone-free media. Note response to vasopressin of the hemi-bladder resistant to cAMP. [From: Edelman et al, J.C.I., 43:2185, 1964]

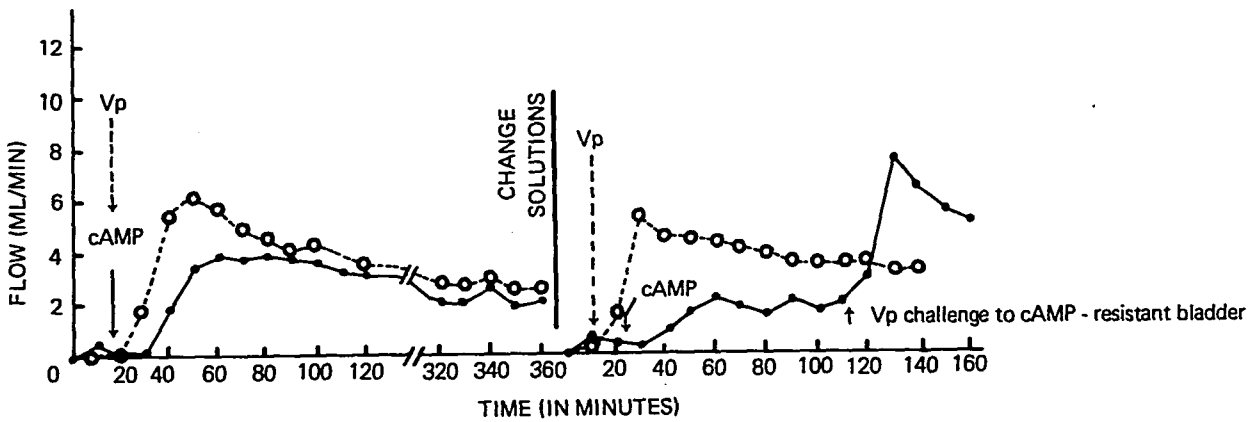

FLOW (ML/MIN)
12
10
8
6
4
2
0
Vp
cAMP
CHANGE SOLUTIONS
Vp
cAMP
Vp challenge to cAMP - resistant bladder
20 40 60 80 100 120 320 340 360 20 40 60 80 100 120 140 160
TIME (IN MINUTES)

FIGURE 3

Figure 4. Response of an experimental hemi-bladder (solid curve) challenged initially with Pitressin and subsequently with cAMP. The paired (control) hemi-bladder was challenged with an identical dose of cAMP (broken curve).
[From: Schwartz and Walter, Amer. J. Med., 42:769, 1967]

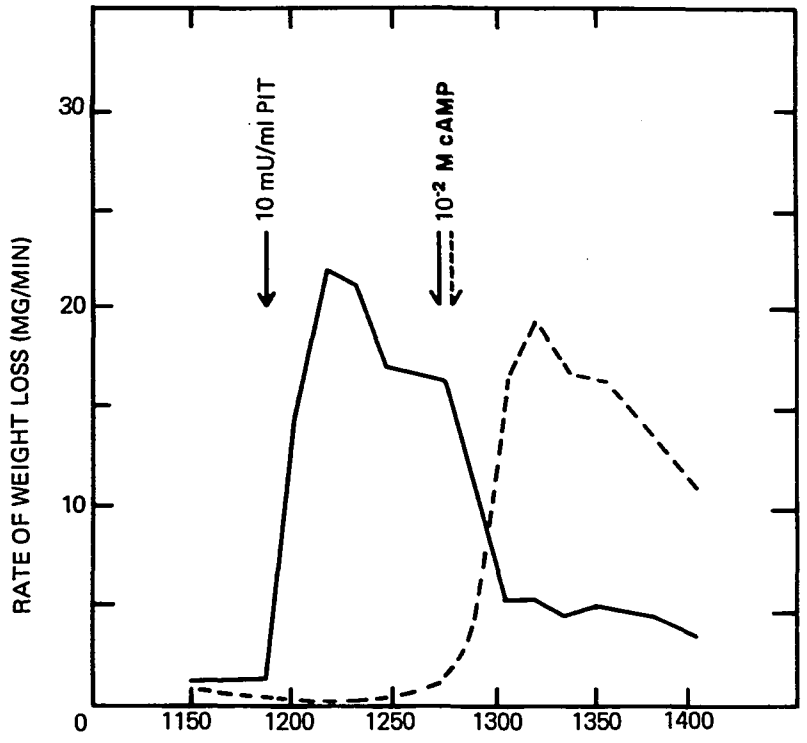

10 mU/ml PIT
10^{-2} M cAMP
RATE OF WEIGHT LOSS (MG/MIN)
30
20
10
0
1150
1200
1250
1300
1350
1400
TIME IN MINUTES

FIGURE 4

In addition to the phenomenon of "intrinsic inhibition", another inhibitory phenomenon identified in toad bladder has been referred to as "extrinsic inhibition". That an extrinsic factor contributes to the decreased response of the toad bladder on prolonged exposure to vasopressin has been demonstrated by the following findings. When bladders are kept in Ringer's solution overnight (with or without hormone in the bath) and, on the next day, fresh bladders are placed in this overnight bath, these fresh bladders show little or no response to hormone even after an additional dose of vasopressin is added (Figure 5). This suggested a release of inhibitor(s) into the serosal bath by the "overnight" bladders (Goldberg et al 1963; Schwartz and Walter, 1967).

Karlin and Overweg (1965) showed that the toad bladder contains an inhibitor to oxytocin which, on heat treatment, becomes dialyzable with retention of inhibitory properties. The hydroosmotic response to oxytocin, arginine vasotocin, and theophylline are all inhibited, but the effect of cAMP is unaltered. Because the inhibitor blocked the effects of drugs of different nature, it was concluded (1) that the inhibition probably does not compete with these agents for specific receptor sites and (2) because it did not

Figure 5. The phenomenon of "Extrinsic Inhibition". Lack of response of a "fresh" toad bladder challenged with 1.25×10^{-8}M arginine-vasopressin (open circles, broken line). For explanation, see text.

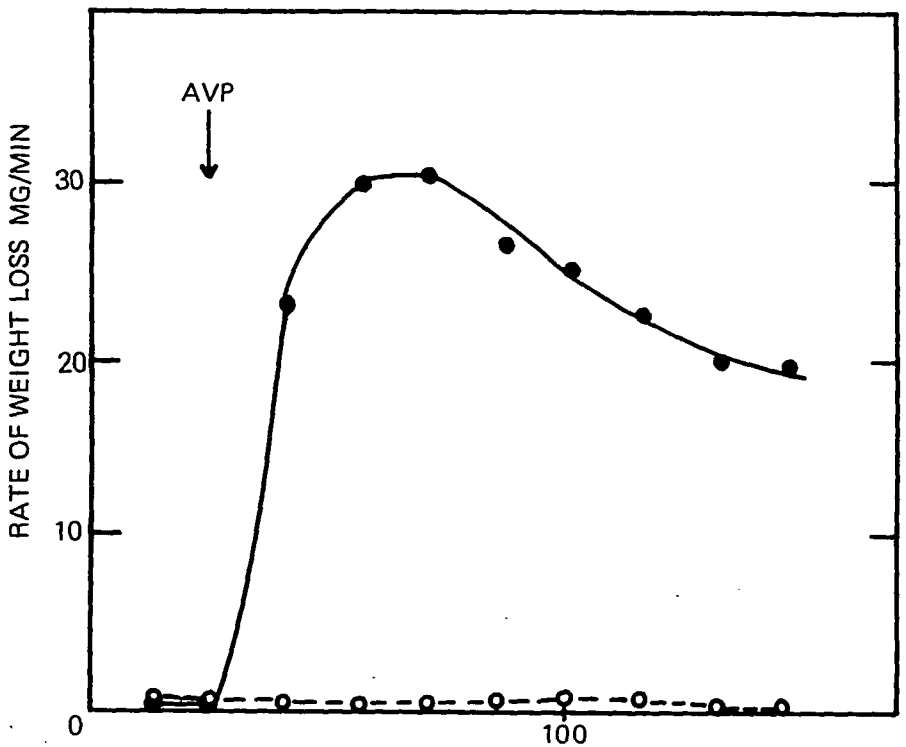


FIGURE 5

affect the bladder's response to cAMP, the inhibitor must act at some point in the reaction sequence prior to the cAMP-dependent step.

In contrast to the phenomena of "intrinsic inhibition", the response of the toad bladder to neurohypophyseal hormones is enhanced under certain circumstances. One of these circumstances, referred to as "facilitation", is illustrated by the following experiment: when bladders are challenged with low doses of hormone (0.5 - 2.5 mU/ml, about 1/5 maximal dose), the response to a second low dose challenge is greater than the response to the first challenge (Figure 6). It can also be seen in Figure 6 that the reactivity of the bladder to hormone can be enhanced by prolonging the period of incubation prior to initial challenge, but the magnitude of this effect is considerably less than the enhancement noted after a second low dose challenge.

With the finding by Sutherland and Rall (1960) that the actions of fast acting peptide and amine hormones are mediated by cAMP, various laboratories began studying the importance of this nucleotide in the action of vasopressin and other hormones. Orloff and Handler (1961) were the first to show that cAMP is involved in the action of vasopressin in toad bladder. This finding provided an important basis for much subsequent work

Figure 6. The phenomenon of "Facilitation". Response of an experimental hemi-bladder (solid curve) challenged with two successive submaximal doses of Pitressin (1.25 mu/ml). The paired control hemi-bladder was challenged only once. For explanation, see text.

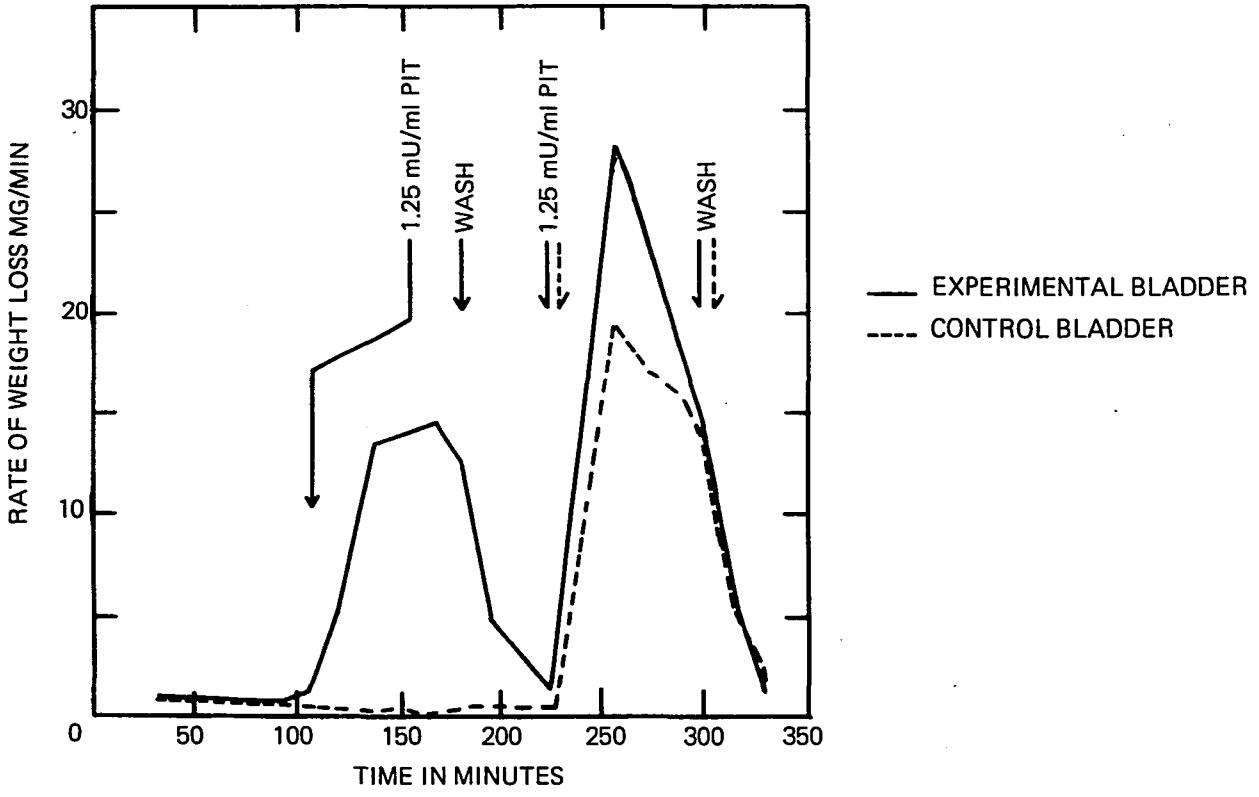

RATE OF WEIGHT LOSS MG/MIN
30
20
10
0
50
100
150
200
250
300
350
TIME IN MINUTES
1.25 mU/ml PIT
WASH
1.25 mU/ml PIT
WASH
EXPERIMENTAL BLADDER
CONTROL BLADDER

FIGURE 6

on the sequence of events that constitutes the membrane action of vasopressin in its amphibian and mammalian target tissues.

This sequence of events, as we how know it, is illustrated in Figure 7:

Step 1: It is now realized that hormone-receptor interaction is the first step in a complex chain of events that ultimately leads to the hormone effect. The hormone is a "first messenger" and binds selectivity to the target cell.

Step 2: The second step appears to be a coupling in which the hormone-receptor complex further interacts with the target cell to generate a biochemical or biophysical signal which stimulates the enzyme, adenylate cyclase.

Step 3: Activation of adenylate cyclase.

Step 4: Production of cyclic 3,5' AMP (adenylate cyclase catalyzes the conversion of ATP to cAMP).

Step 5: A tentative 5th step is stimulation of cAMP-dependent protein phosphokinase (which has been found in plasma membrane fractions of frog bladder, toad bladder, mammalian renal medulla and many other tissues).

Step 6: Phosphorylation of membrane proteins catalyzed by cAMP-dependent protein kinase(s).

It is hypothesized currently that phosphorylation (or perhaps dephosphorylation) of membrane proteins causes a change in membrane structure such that pores or channels through which water can be transported are in some manner created de novo, widened or exposed.

Vasopressin (a nonapeptide, see Figure 8) binds reversibly to receptors on the basal-lateral surface of its target cells (Davidson and Schwartz, unpublished data). Vasopressin is synthesized in hypotholamic nuclei, mainly the supraoptic nucleus, but to some extent also in the periventricular nucleus (Sachs et al 1969). The hormone is in all probability synthesized as part of a macromolecular complex by classical nucleic acid - mediated peptide biosynthesis on ribosomes. The material is packaged, transported along the axon and stored at the axon terminal in neurosecretory granules.

The sum of various excitatory and inhibitory postsynaptic potentials will determine whether or not the neurosecretory cell will fire. As the wave of depolarization spreads along the relevant axon, calcium ions (which move into the axoplasm) trigger the release of vasopressin. By exocytosis the contents of the neurosecretory granule spill into the

Figure 7. Current view of the sequence of events initiated by binding of Neurohypophyseal Hormone to Hydroosmotic Receptor of Amphibian Bladder or Mammalian Kidney. (V_p, vasopressin; R, receptor; AC, adenylate cyclase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; cAMP, adenosine 3',5' monophosphate; P, Protein phosphate; P_i, inorganic phosphate).

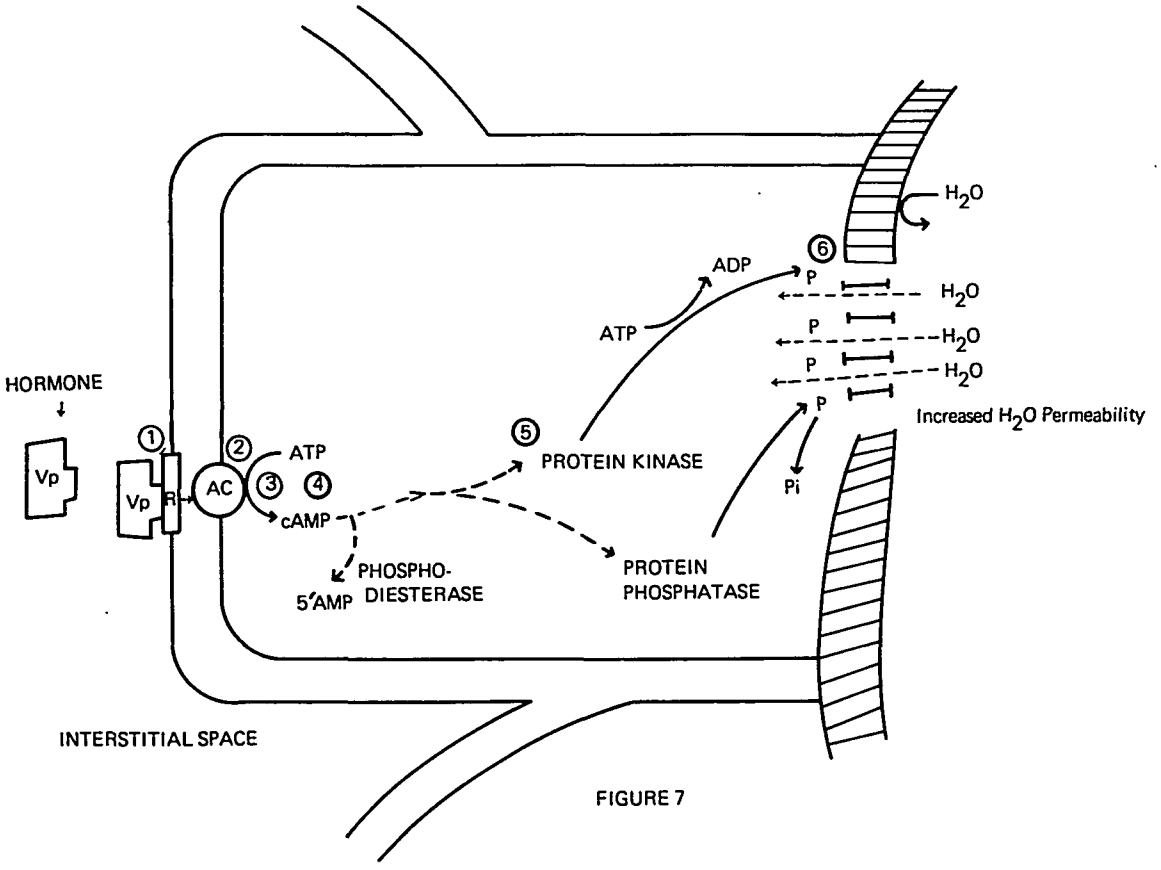
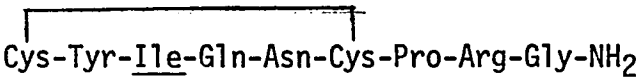

HORMONE
Vp
Vp
R
AC
ATP
cAMP
PHOSPHO-
DIESTERASE
5'AMP
PROTEIN KINASE
ATP
ADP
PROTEIN
PHOSPHATASE
Pi
P
H_2O
Increased H_2O Permeability
INTERSTITIAL SPACE

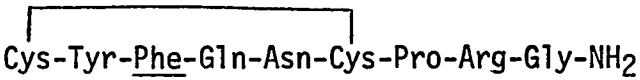
FIGURE 7

Figure 8. Amino acid sequences in arginine vasotocin, arginine vasopressin, and deamino-8-D-arginine vasopressin.

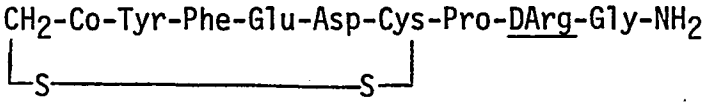
Arginine Vasotocin (AVT) (birds, reptiles, amphibia, fish)



Arginine Vasopressin (AVP) (mammals)



deamino-8-D-arginine vasopressin (DDAVP)



perivascular space and enters the bloodstream.

The release of vasopressin is triggered by two important parameters, small changes in the first being more important than small changes in the second. These parameters are plasma hyperosmolarity (osmotic regulation) and blood volume depletion (volume regulation). With minimal or even moderate perturbation in blood volume, osmotic pressure regulation takes presedence over volume regulation; however with severe perturbation in blood volume, volume regulation predominates over osmotic regulation.

Once vasopressin has been released into the circulation, the response of target tissue can be modulated by a variety of conditions in addition to those associated with the phenomena of "intrinsic inhibition", "extrinsic inhibition" and "facilitation" as noted above. In the toad bladder, it has been shown (Eggena et al, 1970; Handler et al 1970) that adrenal steroids, such as aldosterone, when present in the serosal bathing fluid stimulate or "facilitate" the hydroosmotic response to Vasopressin. Because the osmotic water flow response to cAMP is also increased when bladders are treated with aldosterone, it can be concluded that whatever its mechanism may be -- the steriod enhancement of the hydroosmotic response to vasopressin of the toad bladder occurs at a point beyond the step of adenylate cyclase stimulation.

Eggena et al (1970), Urakabe et al (1970), and Parisi et al (1969) have shown that hypertonicity of the serosal bath can induce the hydroosmotic response of the toad bladder to antidiuretic hormone. Specifically Eggena et al (1970) found that the net rate of water flux from mucosal bath to serosal bath increased from 1 mg/min. to 35 mg/min. when the bladder was exposed on its serosal surface to Ringer's fluid containing an additional 6.5 gm/l of NaCl. This response to the stimulus of serosal hypertonicity resembles the response of the toad bladder to Vasopressin and to cAMP. Both occur in the absence of mucosal sodium indicating that the observed water flux is independent of solute movement, both responses are reversible and the ultrastructure of the bladder epithelium is modified similarly in both types of stimulation. Serosal hypertonicity did not disrupt the integrity of the permeability barrier of the bladder. When similar concentrations of mannitol or potassium were placed in the serosal bathing fluid instead of NaCl, similar results were obtained. When urea replaced NaCl in the serosal fluid, no increase in water transfer was observed suggesting that the extracellular - intracellular osmotic gradient was the critical factor in initiating the

hormone-like response. This osmotic gradient, established by increasing the concentration of a poorly permeant solute in the serosal bath, appears to be capable of eliciting the same response as vasopressin. When bladders were incubated in aldosterone-containing serosal fluid and then challenged with hypertonic Na^+-Ringer's solution, these bladders were more permeable to water than bladders challenged in the same way but which had not been pre-incubated in the steroid solution (Eggena et al, 1970; Handler et al, 1970; and Parisi et al, 1969). Aldosterone appears to facilitate the hydroosmotic action of hypertonicity, not by inducing a subthreshold stimulus which is amplified by hypertonicity, but more probably, the aldosterone-sensitive step is located later in the sequence of events than the step which is influenced by serosal hypertonicity. It is likely that the aldosterone-induced enhancement of the response to serosal hypertonicity involves the same process by which aldosterone enhances the permeability response of the toad bladder to vasopressin and to exogenous cAMP. The steroid-enhanced response is associated with an increase in intracellular cAMP which is the result of a steroid-elicited decrease in cAMP degradation by phosphodiesterase. It

has been suggested that aldosterone may initiate synthesis of a protein inhibitor of phosphodiesterase (Stoff et al, 1973).

In addition to aldosterone, the effect of other agents on permeability changes induced by hypertonicity were also examined. Prostaglandin E_1, a non-competitive inhibitor of neurohypophyseal hormones, (Eggena et al, 1970) and manganese ions which inhibit the response of the toad bladder to vasopressin, (Bentley, 1967) are both known to decrease cAMP formation. However, neither agent was capable of decreasing the permeability changes induced by hypertonicity. When zinc (10^{-5}M) was placed in the serosal bath, or when potassium was omitted, or serosal H^+ concentration was increased, there was a drastic decrease in the hydroosmotic action of hypertonicity (Eggena et al, 1970). It has been shown that the response of the toad bladder to vasopressin and to exogenous cAMP, in isotonic media, is also inhibited by zinc ions (Bentley, 1967), low potassium (Finn et al, 1966), low pH (Gulyassy and Edelman, 1965; Orloff and Handler 1967; Schwartz and Walter 1967), and low temperature (Schwartz and Walter, 1967). In the light of such findings, it may be concluded that the effect of hypertonicity is exerted at a step subsequent to cAMP formation.

It is of interest that prior to the recent studies of the response of the toad bladder to serosal hypertonicity, Bentley (1964) had suggested that during severe dehydration, an increase in plasma tonicity can increase the water permeability of the frog bladder and thereby effect water conservation by a mechanism more direct than that mediated by the neurohypophysis.

A number of other agents have been studied which inhibit the hydroosmotic response of the toad bladder to vasopressin and/or to exogenous cAMP. Those that inferfere with formation of intracellular cAMP or which block the action of cAMP subsequent to its formation are of particular interest. Calcium is one such agent.

Calcium may be involved in the final effector process either by altering the conformation of lipoproteins, by enzymatic effects, by an action on a contractile system, or by an unknown calcium-dependent membrane phenomenon.

Bentley (1959) found that baseline water transfer across toad bladder is increased at low concentrations of calcium ions, but that the effect of the hormone on the water permeability of the bladder is diminished. Bentley's interpretation was that the phenomenon may be based on a calcium ion requirement for maintenance of normal bladder structure.

Schwartz and Walter (1968) observed structural changes in epithelial cells of bladders washed repeatedly with calcium-free solution. The nuclei and mitochondria of bladders treated in this way become swollen and many mitochondria disappeared.

Peachey (1964) and Hays et al (1965) demonstrated that elimination of calcium from the serosal bath led to epithelial cell separation and detachment, and loss of the barrier function of the toad bladder epithelium When strontium or magnesium ions were substituted for calcium ions, the structural integrity of the bladder wall was maintained, but the capacity for repetitive response to hormone was impaired. Thus, it was concluded that calcium is necessary for maintenance of the normal, low permeability of the toad bladder to water in the absence of hormone and that Ca^{++} was also necessary for the elicitation of the hydroosmotic response to hormone.

Either a subnormal or an excessively high concentration of calcium decreases the neurophpophyseal hormone-induced activation of adenylate cyclase (Marumo and Edelman, 1971). Earlier Petersen and Edelman (1964) and Argy et al (1967) had shown that, in the intact toad bladder, 20 mM calcium blocks the hydroosmotic, but not the natriferic

response to neurohypophyseal hormones. However, this concentration of calcium does not block either of these responses to cAMP. These findings served to localize the inhibitory action of calcium at the receptor-cyclase level and also led to the concept that hydroosmotic and natriferic responses are mediated by different cAMP pools within the hormone-sensitive cell. Later, a similar separation of effects was shown with PGE_1 (Lipson et al, 1971) confirming the latter concept.

Bentley (1967) found that manganese ions decreased the hydroosmotic response to neurohypophyseal hormones, but had no effect on the response to cAMP. Manganese is thought to directly inhibit the neurohypophyseal hormone-sensitive adenylate cyclase. Hynie and Sharp (1971) showed a stimulation of adenylate cyclase activity with magnesium ions in concentrations of 1 - 25 mM. Magnesium increases the level of basal enzyme activity and, therefore, there is a decrease in the relative stimulation of the enzyme by neurohypophyseal hormones (Birnbaumer and Yang, 1974).

Monovalent ions also exert an effect on the hydroosmotic response of the toad bladder to neurohypophyseal hormones. The reactivity of neurohypophyseal hormone-sensitive adenylate cyclase of rabbit kidney homogenates is inhibited by sodium or potassium

concentrations exceeding 200 mM (Dousa, 1972; Dousa and Hechter, 1970).

Potassium depletion in the serosal bath, inhibits the responsiveness of the bladder to the action of vasopressin, regardless of the presence of an osmotic gradient (Finn et al 1966). These investigators also showed that removal of potassium ions decreased the response to a submaximal concentration of cAMP. This suggests that the potassium requirement for vasopressin action in membrane permeability occurs at a point which is beyond the step at which vasopressin stimulates formation of cAMP. One of the well established effects of potassium depletion on mammalian renal function is the induction of a vasopressin-resistent defect in the ability to form concentrated urine. This decrease in concentrating ability is due to an insensitivity to vasopressin resulting from a decrease in the concentration of potassium ions in the cell and/or extracellular fluid.

While the absence of potassium ions in the mucosal bath is without effect on the hydroosmotic response to neurohypophyseal hormone, the presence of sodium ions in the mucosal as well as in the serosal bath is necessary for full manifestation of the increased water transfer in response to hormone (Bentley, 1959).

The presence of sodium in the mucosal bath increases water transfer in response to hormone; when 50% of the sodium normally present in the serosal bath is removed, there is a decrease in the response to hormone. Neither lithium, choline, nor potassium when placed in the mucosal bath can substitute for the effect of sodium.*

Although the molecular mechanism involved in the final permeability change induced by vasopressin and cAMP is not known, it has been established at the cellular level that vasopressin stimulates contraction of certain types of smooth muscle as well as promoting transcellular water movement across the epithelium of the distal portion of the mammalian renal tubule and across the amphibian skin and bladder (Sawyer, 1961).

During the past decade a number of studies have suggested that microtubules and microfilaments are associated with several types of intracellular phenomenon (Porter, 1966; Buckley and Porter, 1967; Behnke et al, 1971). Therefore, Taylor et al (1973) investigated the possibility that the action of vasopressin in transcellular movement by a mechanism involving these subcellular structures.

---

*It has been shown that lithium in fact inhibits both neurohypophyseal hormone-sensitive adenylate cyclase and vasopressin-induced antidiuresis (Singer et al 1970).

Colchicine, vinblastine, and podophyllotoxin are antimitotic agents which bind to microtubule subunit protein (tubulin) and prevents the assembly of microtubules. Cytochalasin B disrupts microfilaments. The effects of these agents on osmotic water movement and on sodium transport in response to vasopressin and to cAMP in Bufo Marinus were examined by Taylor et al, (1973) who demonstrated that these agents inhibit the hydroosmotic response but not the natriferic action of neurohypophyseal hormone and cAMP on the toad bladder.

Lumicolchicine, an analog of colchicine that lacks antimitotic activity and does not bind to tubulin, had no inhibitory action on the neurohypophyseal hormone-induced or cyclic AMP-induced hydroosmotic response of the amphibian bladder. This suggests that the inhibitory effect of colchicine is specifically related to its ability to bind to a microtubule subunit protein. In addition, the time-dependence, concentration-dependence, and reversibility was similar for inhibition by these agents of neurohypophyseal hormone-induced water movement in the toad bladder and of the interactions of these agents with microtubular protein <u>in vitro</u> and microtubular systems <u>in vivo</u> (Taylor et al 1973).

Cytochalasin B disrupts actin-like microfilaments in many cells. It inhibits hexose and nucleoside uptake, this action being ascribed to its interaction with plasma membranes (Mizel and Wilson, 1972; Zigmond and Hirsch, 1972; Plagemann and Estensen, 1972). Therefore, the decrease in vasopressin-induced water movement by cytochalasin B may be due to interference with microfilament function, or interaction with actin, or both. It is also consistent with the action of the drug on the plasma membranes of toad bladder epithelial cells.

These findings suggest (Taylor et al, 1973; Dousa and Barnes, 1974; 1974a) that microtubules and perhaps microfilaments play a role in the hydroosmotic action of neurohypophyseal hormones at a step after the generation of cAMP, but that these organelles are not involved in the natriferic action of neurophypophyseal hormones. Neurohypophyseal hormones and cAMP both stimulate exocytosis in the toad bladder epithelium (Mazur et al, 1971; Mazur et al 1972). In other systems the mechanism of exocytosis is known to involve microtubular protein and possibly microfilaments. Dibona et al (1969) reported that the permeability change induced by vasopressin in toad bladder is limited to the granular epithelial cells and Mazur et

al, (1971) suggested that this may be secondary to the release of secretion granules at the apical surface of epithelial cells. Therefore, it is possible that these organelles participate in the hydroosmotic action of neurohypophyseal hormones through their involvement in the mechanism for release of secretion granules from the bladder epithelial cells. The inference here is that the addition to the apical cell surface of secretory material or the incorporation of the secretion granule membrane into the apical membrane of the effector cells may be responsible for the altered membrane permeability (Taylor et al, 1973).

Additional possibilities have been suggested. (1) Microtubules and perhaps microfilaments may play a role in the mechanism of action of neurohypophyseal hormones via an effect of cAMP on a directional cytoplasmic streaming process. (2) Changes in the structure (e.g. by cAMP-dependent phosphorylation) of plasma membrane-associated tubulin or juxtaplasmalemmal components of microtubules, and perhaps microfilaments, might alter the permeability characteristics of the plasma membrane (Dousa and Barnes, 1974). (3) cAMP may affect the state of polymerization and therefore the permeability of a submembrane layer of microtubular protein. (4) There may be no interaction between cAMP

and microtubular protein or microfilaments, the role of these systems being only to provide a structural framework for a functionally effective attachment and orientation of cAMP-dependent enzymes and their substrates and modulators. In this way, by disrupting the framework, the cAMP-protein kinase-phosphoprotein phosphatase, etc. system can be rendered inoperative in the hormonal target cell (Dousa and Barnes 1974).

In the present study, attention has been addressed to some of the factors which modify the activity of neurohypophyseal hormone.target cells to vasopressin and specifically to the site at which these factors exert their effect in the chain of events which constitutes the action of vasopressin on membrane permeability.

MATERIALS

Arginine vasopressin used during this study was synthesized and kindly provided by Drs. J. Roy and J.D. Glass of the Mount Sinai School of Medicine. Pitressin was obtained from Parke-Davis and 1-deamino-8-D-arginine vasopressin (DDAVP) was the gift of Dr. Jan Mulder, Ferring A.B., Malmo, Sweden.

Toads were purchased from National Reagents, Bridgeport, Connecticut.

Calcium chloride, sodium carbonate, lithium chloride, and formic acid were obtained from Fisher Scientific. Potassium chloride, sodium chloride, and magnesium chloride were purchased from Baker. Sucrose was obtained from Mallinkrodt.

EDTA (ethylenediamine tetraacetic acid), EGTA (ethyleneglycol-bis (β-amino ethyl ether) N,N' (tetraacetic acid), tris-HCl [tris (hydroxymethyl) amino methane-HCl], and Bovine serum albumin were purchased from Sigma.

Creatine kinase, creatine phosphate, cyclic adenosine monophosphate (cAMP), adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), were obtained from Boehringer - Mannheim.

[α-^{32}P] ATP was purchased from New England Nuclear. Polygram cel 300 PEI thin layer chromatography plates were obtained from Brinkmann Instruments and Instagel scintillation counting fluid from Packard.

METHODS

Method of Study of Intact Bladders: Gravimetric measurement of hydroosmotic response of the toad bladder to ADH.

Three to six toads were double-pithed and their bladders excised. One hemi-bladder from each toad was used as a control; the other was used as the experimental hemi-bladder. Each bladder was hung on a glass rod (with the mucosal surface facing inwards) and filled with 1/5 strength Ringer's solution (Ringer's solution: 1 mM $CaCl_2$, 2mM KCl, 2.4 mM $NaHCO_3$, 111 mM NaCl, pH 8.0, osmolarity 220-225 mOSM/K_gH_2O) after the method of Bentley (1958). The bathing medium was aerated Ringer's solution. Water transfer, which takes place from mucosal to serosal surface, was measured gravimetrically by weighing the bladders at 20 minute intervals on a Sartorius balance (sensitivity = $\pm$ 0.3 mg).

The physiological response to be tested was the hydroosmotic response of the intact bladder when challenged repeatedly, with a dose of vasopressin (2.5×10^{-8}M) which was capable of eliciting a maximal effect. After a period of equilibration (about one hour, i.e. three weighing periods of 20 minutes each)

in normal hormone-free Ringer's fluid, the baseline level of permeability was established. Then, at a time designated as t=0 min., the experimental bladders were subjected to an initial challenge with hormone by adding vasopressin to the serosal bathing fluid. Controls did not receive hormone. The response, evaluated by measuring the weight loss of the toad bladder assembly, reached a maximum within twenty minutes and then declined.

During the period following the maximum response (starting at t=20 mins.) the control and experimental bladders were washed in fresh, hormone-free Ringer's solution. After the baseline level of impermeability was regained (at t=300 mins.) an identical dose of vasopressin was administered to both the control bladder (1st challenge) and to the experimental bladder (the 2nd challenge). The control bladders showed a hydroosmotic response comparable to that of the experimental bladders following the initial challenge of the latter bladders with hormone. Thus, the former bladders acted as a control for time and also showed that exposure to Ringer's fluid, mechanical handling and changing of solutions did not alter the hydroosmotic response.

Preparation of Toad Bladder Adenylate Cyclase

The method of Bar et al (1970), was used for preparation of toad bladder adenylate cyclase. After challenging the intact bladders with the experimental conditions, they were removed from the bath, weighed and rinsed in fresh Ringer's solution. The epithelial cells were scraped from the mucosal surface by folding the bladder over a glass slide and scraping the cells, with another slide, into a petri dish filled with ice cold STED buffer (.225m sucrose, 0.1 mM EDTA, .01m tris HCl, pH 7.5). Cells were washed and homogenized in the sucrose medium with a tight-fitting glass homogenizer and teflon pestle. This homogenate was spun at 600 x g for 10 minutes and the pellet resuspended in 1.5 ml of sucrose medium. It was then spun again. The second "600 x g pellet" was shown by Bar et al (1970) to exhibit the bulk of adenylate cyclase activity and also the greatest sensitivity to AVP, oxytocin, and LVT. Therefore, this fraction was selected for measurement of the toad bladder adenylate cyclase in this study.

Adenylate Cyclase Assay

Measurement of adenylate cyclase activity was made after the method of Bar (1975), with minor modification. This procedure allows visualization of cAMP during chromatographic workup and follows in principle the method of Bar and Hechter, (1969).

The assay employs a rapid separation of cAMP from other nucleotides with anion-exchange chromoatography using cellulose thin layer sheets impregnated with polyethyleneimine (polymine, PEI plates) washed in H_2O prior to use.

The assay volume is 50 µl, a small volume being favored when using radioactivity in substantial amounts. Each siliconized tube contains a final concentration of (1) cyclase buffer: 50.0 mM Tris/HCl [Tris (hydroxymethyl) amino methane-HCl], pH 8.4; 1 mg/ml Bovine serum albumin; 0.5 mM cAMP (to eliminate the interference of phosphodiesterase -- if this enzyme is present, it will cleave "cold" cAMP which is present in sufficient concentration to greatly minimize the conversion of "hot" cAMP); and 1.0 mM EGTA (to chelate calcium). Creatine kinase and creatine phosphate are provided as an ATP-regenerating system in order to keep ATP levels constant during the experiment. These reagents are present at 1 mg/ml and 25.0 mM, respectively. The above compounds plus 0.2 mM ATP (which acts as a carrier for [α-^{32}P]-ATP) and 10 mM $MgCl_2$ make up the "reaction mixture".

Whenever adenylate cyclase activity was measured the following procedure was carried out: 20 µl of the reaction mixture was placed into test tubes which were in an ice bath. 5 µl water was added and then 5 µl of [α-^{32}P]-ATP (2 x 10^6 cpm/tube). 20 µl of the enzyme was added last (protein concentration 0.1 to 3.0 mg/ml, as determined by Lowry et al (1951) for protein determination). Test tubes were vortexed and placed in a shaking water bath at 37°C for 20 minutes.

The reaction is terminated by addition of 5 µl of "stopping" solution which contains a final concentration of 1.82 mM cAMP, 1.82 mM AMP and 18.2 mM EDTA, pH 7.0. The terminating solution floods the system with "cold" nucleotides. Since the adenylate cyclase reaction is temperature dependent, test tubes are put back into an ice bath after addition of terminating solution.

In the chromatographic polymine (PEI plate) method, samples are spotted directly after the reaction is terminated. PEI plates are washed in water and dried prior to use. Aliquots (approximately 2-5 µl) of sample are applied with capillary tubes as narrow lines (1.5 cm per sample) at the origin (about 1.5 - 2.5 cm from the edge). Seven samples are usually spotted per plate, with 1 cm distance between samples. The plates are allowed

to dry in air and then are run in water until the level of the front is just above the spots. They are then transferred to the chromatographic solvent (0.25 M LiCl) and the nucleotide spots allowed to run to within a few centimeters from the top of the plate (almost the full length of the plate). After drying, the spots are visualized on the plates under UV light (chromato-vue Model cc-20, Ultraviolet Products, Incorporated), marked with pencil and then cut out with a scissors. Figure 9 shows the appearance of the chromatogram under UV light. ATP, ADP, and AMP remain close to the origin. cAMP moves much more rapidly because it has less net charge.

The cAMP spot is cut out separately; the ATP and AMP spots are cut out together. The space between the cAMP spot and the ATP-AMP spot is discarded. The cut pieces are placed in scintillation vials with 10 ml Instagel scintillation counting fluid and counted (10 minutes/sample) in a Beckman Scintillation counter.

cAMP formation is calculated as % of total counts. Absolute rates can be calculated given substrate and protein concentration and incubation time. Rates are expressed as pmoles/mg/minute (pmoles cAMP/mg protein/ minute incubation time).

In this assay it is necessary to test the adequacy of the ATP regenerative system and thus to determine the degree of ATP breakdown. The solvent for this procedure is 2N formic acid + 5 M LiCl. 20 mM ADP carrier is spotted and then a small aliquot of the assay mixture is superimposed on this ADP spot. Figure 10 shows the appearance of this chromatogram when visualized under UV light. ATP migrates only a small distance; ADP migrates further. AMP and cAMP migrate with the solvent front. The plate is cut into two sections (one containing the ATP spot and the other containing all the remaining nucleotides) and then counted as described above. It was necessary for the ratio, $\frac{\text{ATP}}{(\text{ATP}) + (\text{ADP} + \text{AMP} + \text{cAMP})}$, to equal or exceed 85-90% for the regenerating system to be considered adequate. All analyses were carried out in triplicate.

Figure 9. Determination of adenylate cyclase activity: Appearance of the chromatogram under UV light (see text).

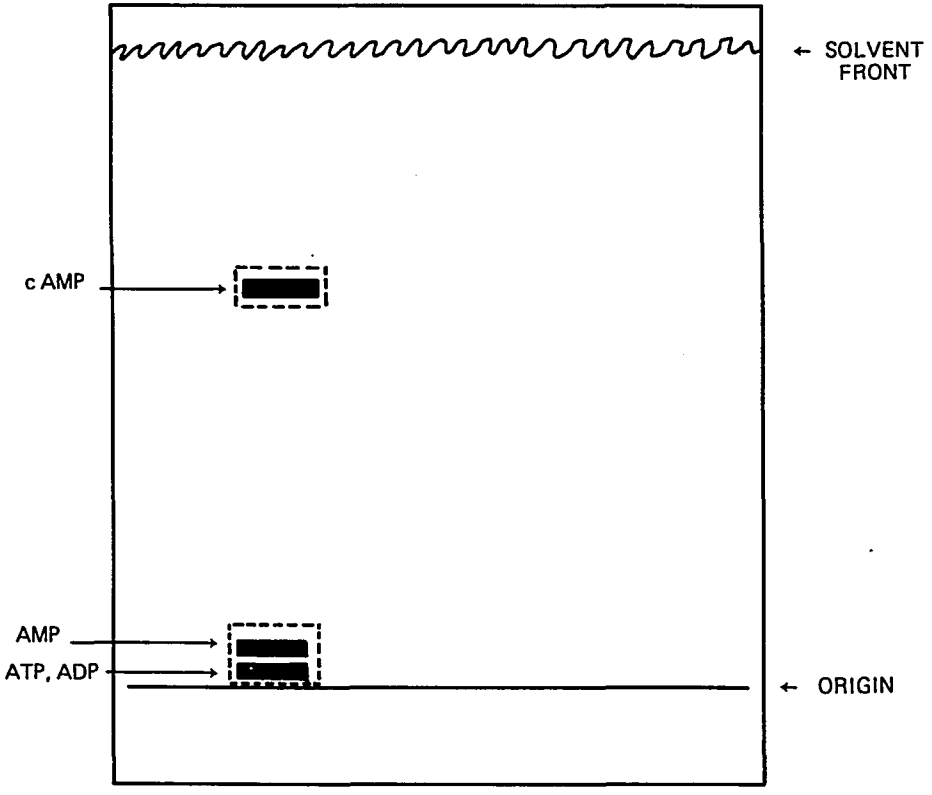


FIGURE 9

Figure 10. Determination of adenylate cyclase activity: Appearance of chromatogram under UV light when testing the adequacy of the ATP regenerating system (see text).

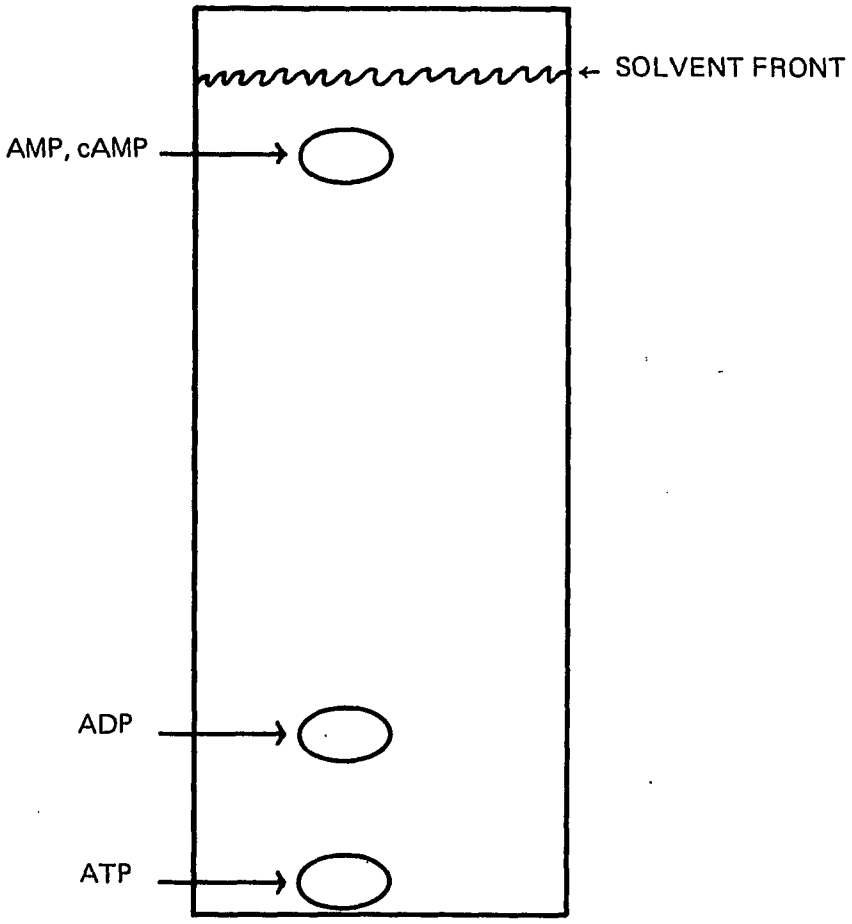


FIGURE 10

Method of Study of the Phenomenon of "Intrinsic Inhibition"

Experimental Design I

To determine whether "intrinsic inhibition" was occurring at the Hormone-Receptor cyclase step, the following experiment was designed.

Intact bladders were allowed to equilibrate in Ringer's solution for one hour. At a time designated t=0, hormone (10^{-6}M AVP) was introduced into the fluid bathing the bladders (i.e. the serosal fluid). At t=10 minutes (response is maximum in the interval from 10-20 minutes, as measured from results of experiments on the hydroosmotic response of intact bladders), half of the hemi-bladders were removed (these are "A" hemi-bladders), weighed, scraped, homogenized, centrifuged at 600 x g and assayed for adenylate cyclase activity. The paired hemi-bladders, designated "B" bladders, were washed and baseline impermeability was allowed to return. At t=300 minutes, these hemi-bladders received a second identical dose of hormone and at t=310 minutes they were removed, weighed, scraped, homogenized, centrifuged at 600 x g and assayed for the adenylate cyclase activity. This experimental protocol is shown schematically in Figure 11.

Figure 11. Experimental protocol used to study the phenomenon of "Intrinsic Inhibition". Experimental Design I.

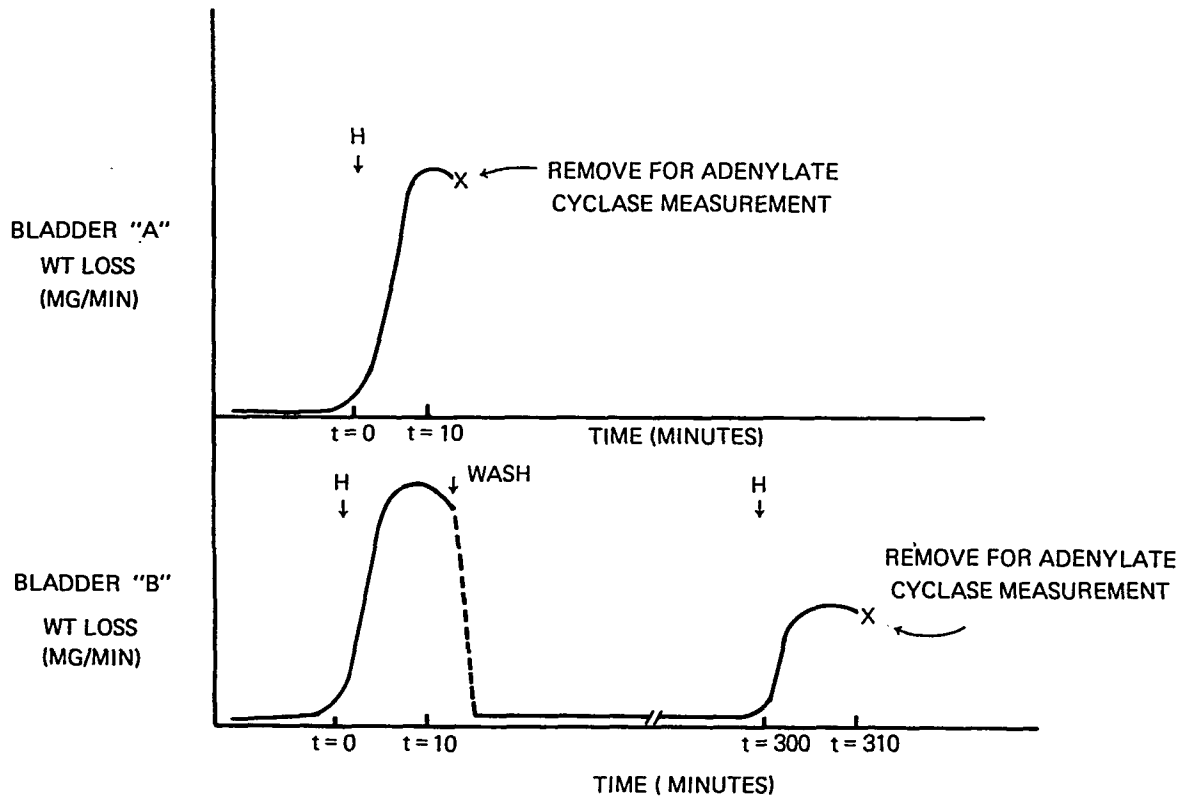

H
X
REMOVE FOR ADENYLATE
CYCLASE MEASUREMENT
BLADDER "A"
WT LOSS
(MG/MIN)
t = 0
t = 10
TIME (MINUTES)
H
WASH
H
REMOVE FOR ADENYLATE
CYCLASE MEASUREMENT
BLADDER "B"
WT LOSS
(MG/MIN)
X
t = 0
t = 10
t = 300
t = 310
TIME (MINUTES)

FIGURE 11

Experimental Design II

In another set of experiments, bladders were set up as before i.e., with an "A" group of hemi-bladders and a "B" group of hemi-bladders. In this protocol the "A" bladders were challenged as before with a maximal dose of vasopressin. At t=10 minutes these bladders were washed and allowed to return to baseline. "B" bladders were not challenged until t=300 minutes, at which time both sets of bladders were challenged with the standard maximal dose of arginine vasopressin (10^{-6}M). At t=310 minutes both sets of bladders were removed and prepared for adenylate cyclase assay. This experimental protocol is shown schematically in Figure 12.

Method of Study of the Phenomenon of "Extrinsic Inhibition"

In order to localize the step which "extrinsic inhibition" is affecting, the following experiment, illustrated in Figure 13, was designed.

Two beakers were set up and left overnight. One beaker, the "control bath" contained 10^{-6}M AVP but no bladders. The other beaker, the experimental bath, contained 10^{-6}M AVP and 6 toad bladders. Both baths were kept overnight at room temperature; the next morning the overnight bladders were removed from the

experimental bath and at t=0 fresh bladders were introduced into both baths along with a dose of AVP identical to that used initially on the previous day. At t=20 minutes both sets of bladders were weighed. The bladders in the control bath showed a response to hormone comparable to that at t=20 minutes from other experiments. The bladders in the "overnight inhibited bath" showed no response to hormones. Mucosal epithelial cells were scraped, homogenized, centrifuged at 600 x g and assayed for adenylate cyclase activity.

Method of Study of the Phenomenon of "Facilitation"

Bladders challenged with a low dose of hormone show a greater response to a second identical low dose challegge than to the initial challenge. Adenylate cyclase assays were carried out on scraped cells obtained from hemi-bladders ten minutes after initial exposure to a low dose (10^{-9}M) of AVP ("A" Bladders) and prepared as described above. Adenylate cyclase assays were also carried out on homogenates prepared from hemi-bladders subjected to two successive challenges with 10^{-9}M AVP, the first at t=10 minutes the second at t=120 minutes ("B" Bladders). These "B" bladders were removed from the serosal bath at t=130 minutes for preparation of mucosal cell homogenates for adenylate cyclase assay.

Figure 12. Experimental protocol used to study the phenomenon of "Intrinsic Inhibition". Experimental Design II.

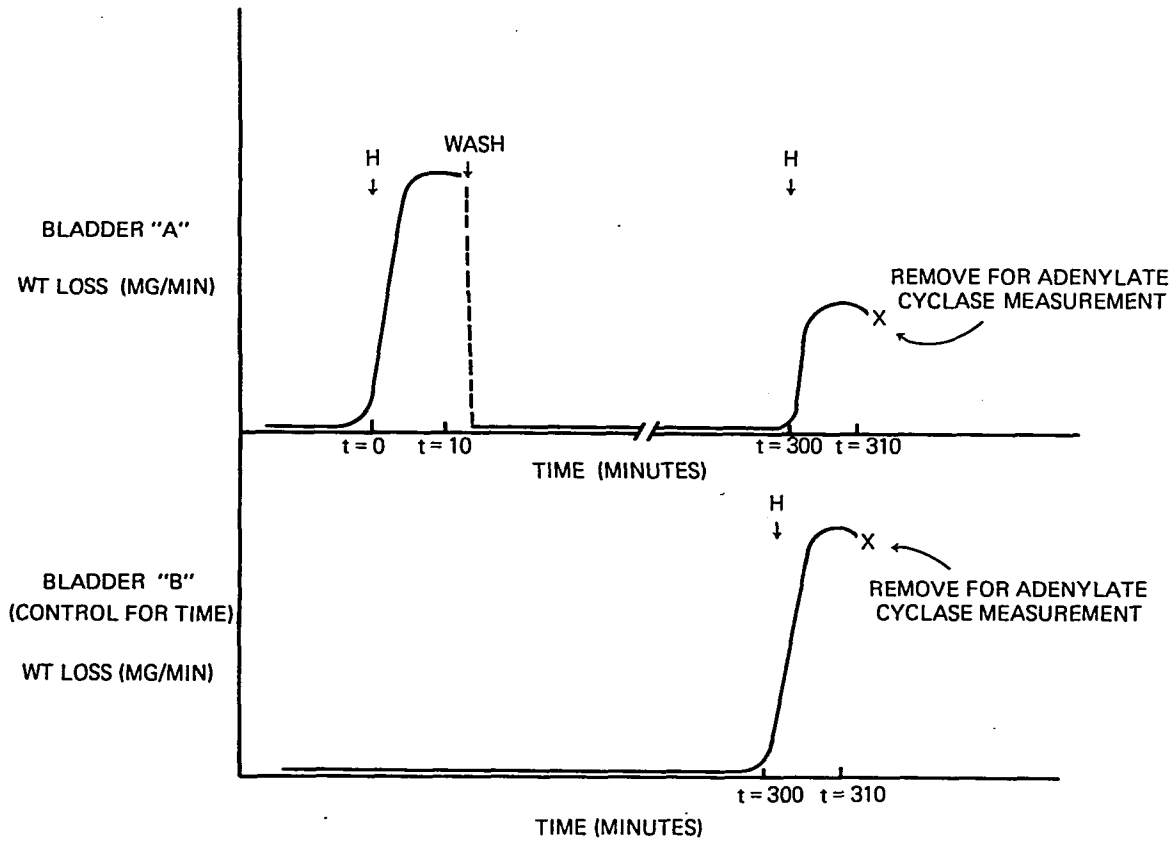
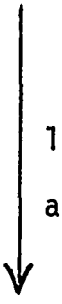

H
WASH
H
BLADDER "A"
WT LOSS (MG/MIN)
REMOVE FOR ADENYLATE CYCLASE MEASUREMENT
X
t = 0
t = 10
t = 300
t = 310
TIME (MINUTES)
H
X
BLADDER "B"
(CONTROL FOR TIME)
WT LOSS (MG/MIN)
REMOVE FOR ADENYLATE CYCLASE MEASUREMENT
t = 300
t = 310
TIME (MINUTES)

FIGURE 12

Figure 13. Experimental design used to study the phenomenon of "Extrinsic Inhibition".

Control bath	Experimental bath
(Ringer's soln. + Hormone)	(Ringer's soln. + Hormone + Toad Bladders)



left overnight

at room temp.

(1) Remove "overnight" bladders from experimental bath and discard.

(2) Add fresh bladders and hormone to control and experimental baths.

(3) Remove bladders at t = 20 minutes and prepare for adenylate cyclase measurement (see text).

RESULTS

Intrinsic Inhibition:

Figure 14 shows the results of a typical experiment in which bladders that were challenged with supra-maximal doses of DDAVP exhibited a reduced response after washout and rechallenge with an identical dose of the hormonal peptide. In eleven other experiments of the same design the findings were similar and the results were statistically significant (P 0.01).

In order to determine at what step in hormone action this modulation was taking place, the adenylate cyclase activity of these bladders was assayed.

The experimental design used to determine whether "intrinsic inhibition" was occurring at the hormone-receptor cyclase step has been detailed in the Methods section but is briefly outlined below (Figure 11).

Intact toad bladders were placed in Ringer's solution for one hour in order to establish their baseline level of permeability. At a time t=0, 10^{-6}M arginine vasopressin (AVP) or deamino-D-arginine-vasopressin (DDAVP) was introduced into the serosal bathing fluid. At t=10 minutes, half the hemi-bladders ("A" group) were removed; the mucosal epithelial cells were scraped, homogenized and the cell free adenylate

Figure 14. Result of experiment showing typical responses of the toad bladder to two successive identical supramaximal doses of hormone. (1 mg DDAVP per ml of bath fluid to establish a final DDAVP conc. of 10^{-6}Molar).

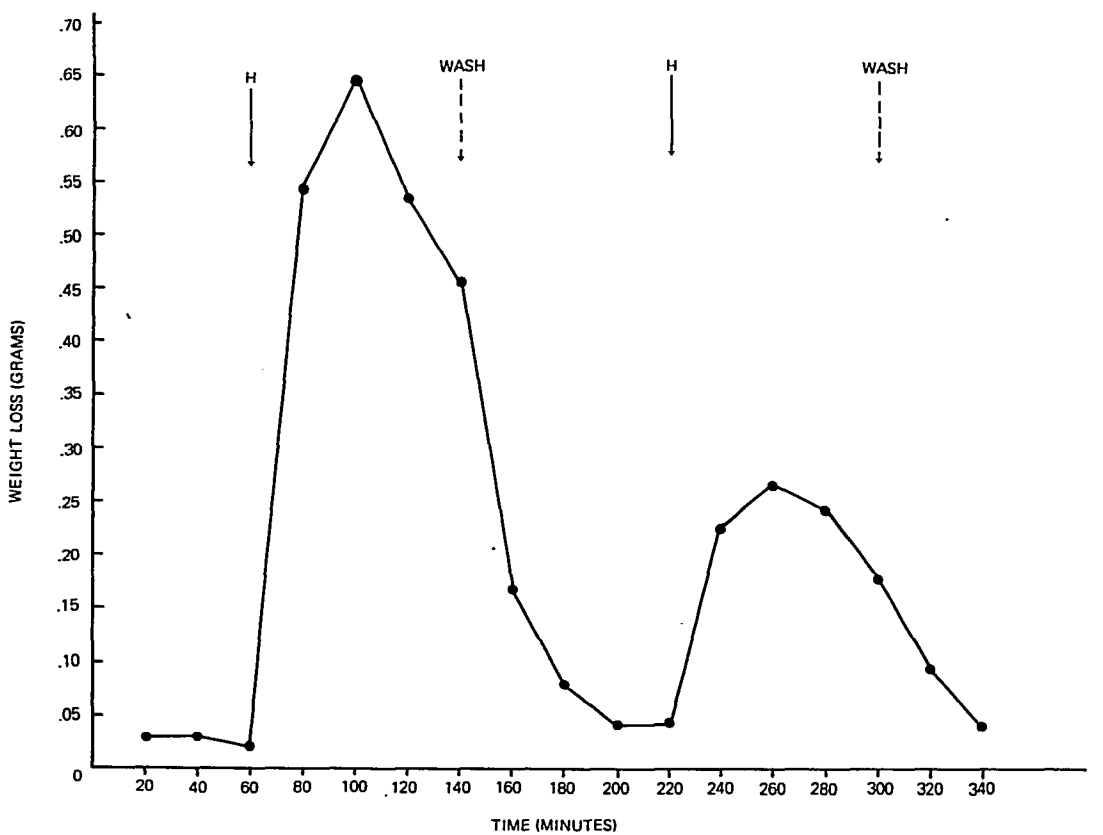

.70
.65
.60
.55
.50
.45
.40
.35
.30
.25
.20
.15
.10
.05
0
H
WASH
H
WASH
WEIGHT LOSS (GRAMS)
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
TIME (MINUTES)

FIGURE 14

cyclase preparation was obtained and assayed for adenylate cyclase activity. The t=10 minutes mark was chosen as the point to remove these bladders because we found that the hydroosmotic response was maximal approximately 10-20 minutes after exposure of the bladder to AVP or DDAVP.

The paired hemi-bladders ("B" bladders), following the initial challenge with hormone, were washed in hormone-free Ringers fluid and their permeability was allowed to return to baseline levels. Then at t=300 minutes the "B" bladders received a second dose of hormone identical to the first. After 10 minutes had elapsed, these bladders were removed, prepared and assayed in the same manner as the "A" group.

In a second set of experiments, the "A" group was treated as described for the "B" group above (Figure 12). The "B" group in this case did not receive any hormonal challenge until t=300 minutes. At this time "B" bladders received a maximal dose of hormone, equal to that given the "A" group. At t=310 minutes both sets of bladders were removed, prepared and assayed for adenylate cyclase activity.

Table 1 shows the results of these experiments. The adenylate cyclase activities 10 minutes after the initial hormonal challenge ("A" bladder of experiment I) is of the same order as the adenylate cyclase activity of the "B" bladder of experiment I (which had received

Table 1. Adenylate cyclase activities of toad bladders which have been "intrinsically inhibited". Six "A" hemibladders and six "B" hemibladders were used in each experiment.

Experimental Design I

	Expt. #1	Expt. #2	Expt. #3
"A" Bladders t = 10 mins.	38	27	41
"B" Bladders t = 310 mins.	34	21	36

Experimental Design II

	Expt. #1	Expt. #2	Expt. #3
"A" Bladders (challenged twice)	41	31	45
"B" Bladders (challenged once, at t = 300 mins.)	37	33	39

Results expressed as pmoles cAMP/mg protein/minute.

Hormonal dose, 10^{-6}M AVP.

two successive hormonal challenges and had been removed for cyclase estimation ten minutes after its second challenge). In contrast to this sustained cyclase response of the cell free bladder preparation, the hydroosmotic response of the intact bladder was significantly inhibited (vide supra). Furthermore, experiment II also showed that when "B" bladders were kept in Ringer's solution for 300 minutes and then challenged for the first time with hormone, they exhibited the same adenylate cyclase activity as "A" bladders which had been challenged twice. Thus, the adenylate cyclase step does not appear to be the locus at which intrinsic inhibition occurs.

Extrinsic Inhibition

In order to localize the step which extrinsic inhibition is affecting, the experiment outlined in Figure 13 was carried out. This experiment is described in detail in the Methods section; it is recapitulated briefly here. One beaker, containing 10^{-6}M AVP and 6 hemi-bladders (experimental bath) and a second beaker containing only 10^{-6}M AVP (control bath) were kept overnight at room temperature (22°C ± 3°C). The next day the bladders were removed from the experimental bath and fresh bladders were challenged in both baths. The fresh bladders incubated in the bath that had contained bladders overnight (experimental bath)

showed no response to hormone while the bladders incubated in the control bath showed the characteristic hormone-induced increase in water permeability. The mucosal epithelial cells of the experimental and control bladders were scraped, homogenized and the homogenate analyzed for adenylate cyclase activity.

Table 2 shows the results of four of these experiments. In three experiments, there is a clear decrease of adenylate cyclase activity in the bladders incubated in the "overnight-inhibited" (experimental) bath. This decrease is in the range of 60-90%. In one experiment there was no difference between the two groups of bladders. However, on the basis of the findings in experiments 1, 2, and 3 and other considerations (see Discussion) we have tentatively concluded that the inhibitor is operative <u>at</u> the adenylate cyclase step.

<u>Facilitation</u>

In order to determine at what step in the AVP-induced sequence of events facilitation is being affected, adenylate cyclase activities were measured on cell-free mucosal epithelial preparations taken from paired hemi-bladders ten minutes after initial exposure to a low dose of hormone (10^{-9}M AVP), and 310 minutes after this challenge (i.e. at ten minutes after the second challenge with the same dose of

Table 2. Adenylate cyclase activities of toad bladders which have been "extrinsically inhibited". Six "A" hemibladders and six "B" hemibladders were used in each experiment.

Expt.	"A" BLADDERS			"B" BLADDERS		
	Basal	$+10^{-6}$M AVP	Δ	Basal	$+10^{-6}$M AVP	Δ
1	11	35	24	15	22	7
2	16	47	31	20	23	3
3	24	62	38	24	46	22
4	15	41	26	19	48	29

Results expressed as pmoles cAMP/mg protein/minute. ("A" Bladders: bladders from control bath removed at peak of physiological response. "B" Bladders: bladders incubated in experimental bath no physiological response. Basal activity was measured in another group of bladders, incubated in Ringer's solution without hormone for the same periods of time as the corresponding "A" and "B" bladders and then removed for adenylate cyclase assay).

hormone). The results of these experiments show that the adenylate cyclase level is higher after a second challenge (Table 3).

Table 3. Adenylate cyclase activities of toad bladders under the condition of "facilitation". Six "A" hemibladders and six "B" hemibladders were used in each experiment.

"A" BLADDERS			"B" BLADDERS		
Basal	Hormone-Stimulated	Δ	Basal	Hormone-Stimulated	Δ
18	35	17	13	41	28
27	49	22	31	67	36

Results expressed as pmoles cAMP/mg protein/minute. "A" Bladders: control; one challenge with 10^{-9}M AVP at t = 0. "B" Bladders: experimental; initial challenge with 10^{-9}M AVP at t = 0; washout at t = 80; repeat challenge with identical dose of AVP at t = 120. (Basal activity measured as in Table 2).

DISCUSSION

The lack of correspondence between the decreased hydroosmotic response of the intact toad bladder (following a repetitive challenge with a supra-maximal dose of neurohypophyseal hormone) and the sustained adenylate cyclase response of a cell free homogenate of the bladder epithelium under the same conditions lead to the conclusion that "intrinsic inhibition" is operative at some locus in the hormone-induced reaction sequence (Figure 7) distal to the adenylate cyclase activation step. Furthermore, it is unlikely that cyclic AMP-dependent phosphorylation (or dephosphorylation) of the plasma membrane of the toad bladder epithelial cell constitutes the locus at which "intrinsic inhibition" is effected because preliminary experiments in our laboratory have shown no difference in the level of phosphorylation of plasma membranes isolated after challenge with a single large dose of hormone as compared with the level of phosphorylation of membranes isolated after repetitive challenges with the same dose of hormone (Schwartz, et al unpublished data). Therefore, the phenomenon of "intrinsic inhibition" is probably established by a

process that acts at a late step in the hormone reaction sequence, viz., after the cyclic AMP-dependent alteration in the state of phosphorylation of a possible pre effector element in the target cell or of the final effector per se (i.e., the luminal diffusion barrier).

In the case of "extrinsic inhibition", it would appear -- on the basis of experiments 1, 2, and 3 of Table 2 we are suggesting that the inhibitor is operative at the adenylate cyclase step.

The degree of cyclase inhibition noted in experiment 2 of Table 2 indicates that the factor of receptor reserve (Eggena et al, 1970; Bar et al 1970; Schwartz, 1971) is not sufficient to prevent manifest inhibition of the hormone-induced hydroosmotic response.

The results noted in experiment 4 may be due to the operation in the control (A) group of bladders of an adventitious inhibitory phenomenon other than "extrinsic inhibition" as defined above. In any case, the decrease in adenylate cyclase activity noted in 3 out of 4 experiments is consistent with Overweg's report that the inhibitory factor did not suppress the cyclic AMP-induced increase in the water permeability of the toad bladder although it did suppress the hydroosmotic response to arginine vasotocin and other neurohypophyseal peptides (Overweg, 1966).

It is possible that an unmasking of receptors and/or an increased efficiency of stimulus-response coupling and/or an increased sensitivity of the enzyme to hormonal stimulation underlies the phenomenon of "facilitation". In any case, it may be concluded that, whatever the mechanism, "facilitation" is manifest at the adenylate cyclase step in hormone action.

When more definitive information is available concerning the nature of the target cell reactions following neurohypophyseal hormone-induced activation of adenylate cyclase, it will be possible then to determine whether the modulating phenomena reported above are operative at multiple loci and, if so, to define such loci. However, it is possible now to extend the experimental approach employed in this study to the many other modes of inhibition (of the toad bladder response to neurohypophyseal hormone) noted in the Introduction to Section I.

SECTION II: STUDIES ON ISOLATED CELL POPULATIONS OF TOAD BLADDER EPITHELIUM (MITOCHONDRIA-RICH AND GRANULAR CELLS).

INTRODUCTION

In this section of the present investigation, the protein kinase activity of isolated cell populations of toad bladder epithelium was studied. Mitochondria-rich and granular cells were prepared by the method of Scott et al, (1974), which utilizes density gradient centrifugation and is described in the Methods section.

Dibona et al, (1969) studied phase and electron micrographs of toad bladder after dilution of the bathing media and/or after the addition of vasopressin. When the mucosal medium alone was diluted (R/5), no morphological changes were observed. However, when vasopressin was added to the serosal side there was an increased cell volume seen in the granular cells, whether the mucosal side was diluted or not (i.e. even without an osmotic gradient). No changes were seen in mitochondria-rich, goblet, or basal cells. Therefore, vasopressin had selectively increased the cell volume of the granular cells of the mucosal epithelium.

When the serosal medium was diluted, all cell types showed increased cell volume. Therefore, increase in cell volume of granular cells alone after vasopressin administration must reflect a hormonal specificity rather than a non-specific limitation in the capacity of other cells, to swell. These findings demonstrated that the action of vasopressin is exerted specifically on the granular cell and at its apical plasma membrane.

The following previous evidence had been adduced in support of the idea that the sodium transport and osmotic (water) effects of vasopressin are mediated at the apical plasma membrane of mucosal cells:

(1) When ^{24}Na, ^{14}C urea, $^{3}H_2O$ was present in the mucosal bath, increased radioactivity within the tissue was seen in response to vasopressin (Frazier, et al, 1962; Leaf, 1960).

(2) With hypotonicity of the mucosal media, no morphological changes were observed. However when mucosal hypotonicity was established and then vasopressin was added to the serosal medium, the mucosal cells swelled (Peachey and Rasmussen, 1961; Dibona, 1969).

Kachadorian et al (1975) have demonstrated, by freeze-fracture electron microscopy, that vasopressin stimulation of isolated toad bladder specifically alters the structure of the luminal membrane of granular cells. They have shown this to be

quantitatively associated with vasopressin-induced osmotic water flow. Thus, the alteration appears to be of functional significance.

It is generally accepted that cAMP mediates the effects of antidiuretic hormone on water and sodium transport in the toad bladder; however the functional significance of cAMP-dependent protein phosphorylation in mediating these actions of ADH remains to be elucidated. DeLorenzo et al, (1973) and Ferguson and Twite (1974) have reported that a cAMP-induced lowering of the level of phosphorylation of a specific protein (protein D) in toad bladder plasma membrane is related to the physiological effects of antidiuretic hormone on Na^+ and water transport, respectively. In most of the studies reported in the literature to date the effect of cAMP on protein kinase(s) has been to increase the activity of the enzyme. In a few instances, however, cAMP appeared to decrease protein kinase activity, that is, the level of substrate phosphorylation was lower in the presence than in the absence of cAMP. This could result either from a cAMP-induced decrease in protein kinase activity and/or from a cAMP-induced increase in phosphoprotein phosphatase activity (perhaps via stimulation of a protein phosphatase kinase).

It was shown by DeLorenzo et al, (1973) that antidiuretic hormone, monobutyrl cAMP, and dibutyrl cAMP each cause a specific decrease in phosphorylation of protein D of the toad bladder. It was further shown that cAMP, when added to bladder homogenates, also caused a decrease in phosphorylation of protein D. This effect was seen only in subcellular fractions containing membrane and was rapid enough to be consistent with a role for protein D in the production of permeability changes leading to increased water and/or sodium transport.

Scott et al, (1974) have shown that after oxytocin challenge, cAMP concentration (as determined by radioimmuno assay) increases only in mitochondria-rich cells. The data also suggested that the mitochondria-rich cell is the locus of H^+ transport (there is greater carbonic anhydrase activity in mitochondria-rich than in granular cells) as well as the initial step in oxytocin - stimulated changes in permeability and transport.

In the scheme shown in Figure 7, Section I Introduction, one of the intermediate steps in hormone action is the activation of a cAMP-dependent protein kinase. If this scheme is correct, in the light of Scott's findings a cAMP-dependent kinase must be present in the granular cells (which respond to neurohypophyseal hormones as noted above) and cAMP must move intercellularly from the mitochondria-rich cells to the granular cells -- and indeed there is a mor-

phological basis for such an intercellular transfer of mediator; namely, a rosette pattern with five granular cells surrounding and making contact with each mitochondria-rich cell (Figure 15). Therefore, to test the scheme given in Figure 15, mitochondria-rich and granular cells were separated and preparations of each cell type were assayed for the presence of cytosolic and membrane-bound cAMP-dependent protein kinase.

Figure 15. Hypothetical Model for Mediation of the Hydroosmotic Action of Neurohypophyseal Hormone in Toad Bladder Epithelial Cells. (MR, Mitochondria-rich cell; G, Granular cells; (R H) , Hormone-Receptor Complex; A.C., adenylate cyclase; P.K. Protein Kinase; Pr PHase, Protein Phosphatase; Pr-P_i, Phosphorylated Protein).

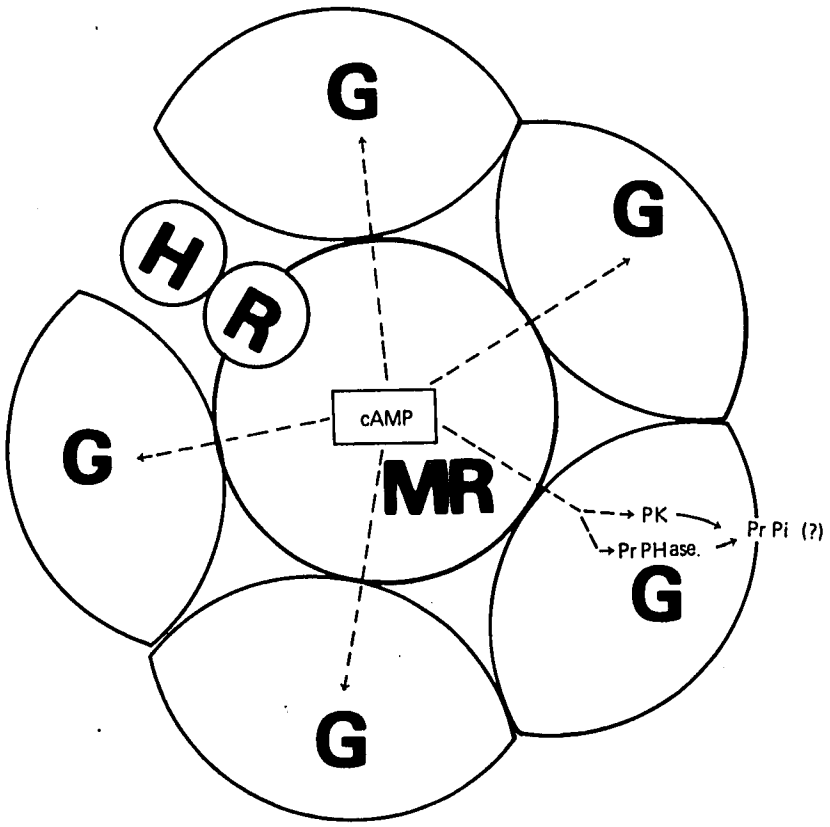


FIGURE 15

MATERIALS

Ficoll was purchased from Pharmacia Fine Chemicals. Histone (type II A, calf thymus) was obtained from Sigma and sodium fluoride and trichloroacetic acid (TCA) from Fisher Scientific. Sodium acetate was purchased from Baker. Theophylline was purchased from Mann Research Laboratories. Millipore filters HA 0.45μ were obtained from Millipore Corporation.

γ-^{32}P-ATP was purchased from New England Nuclear. Otherwise, all materials are the same as described in Section I.

METHODS

Separation of Cell Types

Mitochondria-rich and granular epithelial cells were isolated according to the method of Scott et al, (1974), shown in the flow sheet below (Figure16).

Toads, Bufo marinus, were pithed and their bladders were dissected, excised and rinsed in Ringer's solution. The hemibladders were tied individually to an outlet of a Luer lock syringe, filled and immersed in Calcium-free Ringer solution, containing 2 mM EDTA, and incubated for 45 minutes at room temperature. The intraluminal fluid containing disaggregated mucosal cells was removed and the cells washed twice with EDTA Ringer's at 1935 x g for 15-20 minutes. Sedimented cells were resuspended in 10 ml EDTA Ringer's and layered over a discontinuous gradient of Ficoll in EDTA Ringer solution. (Ficoll is a synthetic copolymer of sucrose and epichlorohydrin, M.W. 400,000, and allows for high density solutions with low osmotic pressure. The large size of the molecule prevents it from penetrating cell membranes and subcellular organelles). The gradient consists of four densities of Ficoll solution: 1.017, 1.035, 1.067, and 1.088gm/cm^3 at 4°C.

Figure 16. Flow diagram for isolation of mitochondria-rich and granular cells of the toad bladder epithelium.
[Ref: Scott et al, 1974]

Bladders

↓

incubated in Ca^{++}-free Ringer's solution containing 2 mM EDTA for 45 min. at room temperature

↓

Remove Intraluminal fluid containing disaggregated mucosal cells

↓

wash cells 2X with EDTA-Ringer's centrifuge at 1935 x g for 15-20 min.

↓

Sedimented cells

↓

Resuspend in 10 ml EDTA Ringer's; layer over a discontinuous Ficoll gradient (1.017. 1.067, and 1.088g/cm^3 at 4°C).

↓

Centrifuge at 27,000 rpm (Beckman SW27 rotor) for 45 min.

↓

4 bands visualized; Band 2, MR cells; Band 3, G cells

↓

Suspend each band in EDTA Ringer's, centrifuge at 27,000 x g for 15-20 min. (at 4°C) and again at 1935 x g for 15-20 min.

↓

Supernatant is poured off.
Pellet is resuspended in 4 mls (not critical) distilled H_2O

↓

Sonicate for two 15 second intervals

↓

Centrifuge sonicate at 35,000 rpm for 30 min. (L5-50 ultracentrifuge)

↓

Supernatant & Pellet

↙ ↘

This is the (MR or G) "supt" used in the protein kinase assays.

Resuspended in 1.5 ml H_2O. This is the "pellet" (MR or G) used in the protein kinase assays.

The cells were centrifuged at 27,000 rpm in a Beckman SW-27 rotor for 45 minutes. Four bands appeared. Mitochondria-rich and granular cells separated into bands 2 and 3 respectively. In order to wash out the Ficoll, each band is suspended in EDTA Ringer's and sedimented in a Sorvall centrifuged at 4°C at 27,000 x g for 15-20 minutes and then again at 1935 x g for 15-20 minutes also at 4°C. The supernatant is poured off and each pellet is resuspended in approximately 4 mls (this is not crucial) of distilled water, and sonicated for two 15 second intervals.

The sonicate is then centrifuged at 35,000 rpm for 30 minutes in an L5-50 ultracentrifuge. The supernatant is removed and saved. This is the granular or mitochondria-rich supernatant used in the assays described here. The pellets are resuspended in 1.5 ml distilled water. This the granular or mitochondria-rich pellet referred to in the results section.

Protein Kinase Assay

The protein kinase assay employed in this study is the method of Kuo et al, (1970), slightly modified. The following reagents were used: (concentrations refer to final concentration in incubation; final volume of each sample is .2 ml) 50 mM Na acetate (used as a buffer); 10 mM $MgCl_2$ (the protein kinase reaction is Mg-dependent, since the reaction substrate is Mg-ATP. In this case the ATP is labelled at the γ-phosphate, so that

adenosine-O-P-O-P-O-P* $\xrightarrow{\text{protein}}$ adenosine-O-P-O-P-+-P_i* $\rightarrow$ protein-P_i*; 25 µM ATP (di-Na); 0.3 mM EGTA (EGTA chelates calcium, therefore Ca^{++} levels decrease but Mg^{++}, which is required in this reaction, is less affected). This reaction mixture is buffered at pH 6.5. γ-^{32}P labelled ATP (1×10^7 cpm/ml buffer) is added prior to use. The reaction is started by addition of 50λ of this mixture (solution A) per sample.

cAMP (5×10^{-8}M, unless otherwise stated) is used to stimulate protein kinase activity. NaF, which inhibits ATPase, is employed at a final concentration of 10-20 mM. When studying cAMP dependent protein kinase activity, it is necessary to maintain cAMP levels throughout the run; therefore 2 mM theophylline (an inhibitor of phosphodiesterase) is used. Histone (40 µgm/sample) was sometimes included as a substrate for cytosolic protein kinase. The concentration of membrane protein varied, but fell usually between 20-50 µgm (as determined by the method of Lowry et al (1951). Cold trichloroacetic acid, (a 5% solution) was used to terminate the reaction. Since the phosphorylated substrate is acid-stable this was considered to be a suitable agent.

Samples are kept on ice until the reaction is started. The reaction is carried out in siliconized tubes. NaF, theophylline, cAMP, and membrane protein were added to the test tubes. As noted above the reaction commences on addition of 50λ of solution A per sample. Samples are incubated at 30°C for 5 minutes (unless otherwise specified). The reaction is terminated by addition of 2 ml 5% cold TCA and the samples are kept on ice until filtered through millipore HA 0.45μ filters. Samples are transferred by pipets and washed 3 times with 5 ml of a washing solution containing 5% TCA, 1.25 mM ATP and 25 mM phosphoric acid. The filters are then dried, placed in vials containing 10 ml Instagel scintillation fluid and counted. An aliquot of the radioactive solution is placed on a filter and counted to serve as a standard. Results are expressed as pmoles P_i incorporated/mg protein/minute incubation time.

Calculation is as follows:

$$\frac{(\text{cpm of sample} - \text{cpm of blank}) \times \text{ATP conc.}}{\text{STD cpm}} = \text{x pmoles}$$

x pmoles/mg kinase per sample = pmoles P_i incorp./mg protein.

Dividing by the incubation time one gets:

pmoles P_i incorp./mg protein/minute incubation time.

All analyses were carried out in triplicate.

RESULTS

The optimal concentration for stimulation of protein kinase was found to be 5 x 10^{-8}M cAMP (Figure 17).

The supernatants of the mitochondria-rich cell homogenates and the granular cell homogenates were both found to contain cAMP-dependent protein kinase activity using either histone or protein as phosphate acceptor (figures 18 and 19 and Tables 4 and 5). When the pellet and supernate are incubated together there is less self-phosphorylation than on incubation of the supernate alone.

Basal and cyclic AMP-dependent protein kinase activities, in the absence of histone, were found to be associated with pellets sedimented from mitochondria-rich cell homogenates (Table 6). Similarly, pellets sedimented from granular cell homogenates were found to have basal and cAMP-dependent protein kinase activities (Table 7). The cAMP-dependent self-phosphorylation in the experiments of Table 7 was apparent when the pellets were incubated for periods ranging from 1 to 10-20 minutes; with longer periods of incubation, dephosphorylation appeared to be more markedly stimulated than phosphorylation (Figure 20 and Table 8).

In the presence of 2.5 mM $ZnCl_2$ (added to suppress phosphatase activity) there was a parallel increase in the self-phosphorylation of mitochondria-rich and granular cell pellets in the absence and presence of cAMP (Figure 21).

Figure 17. Effect of cyclic AMP on protein kinase activity of pellets sedimented from homogenates of granular cells isolated from toad bladder epithelium.

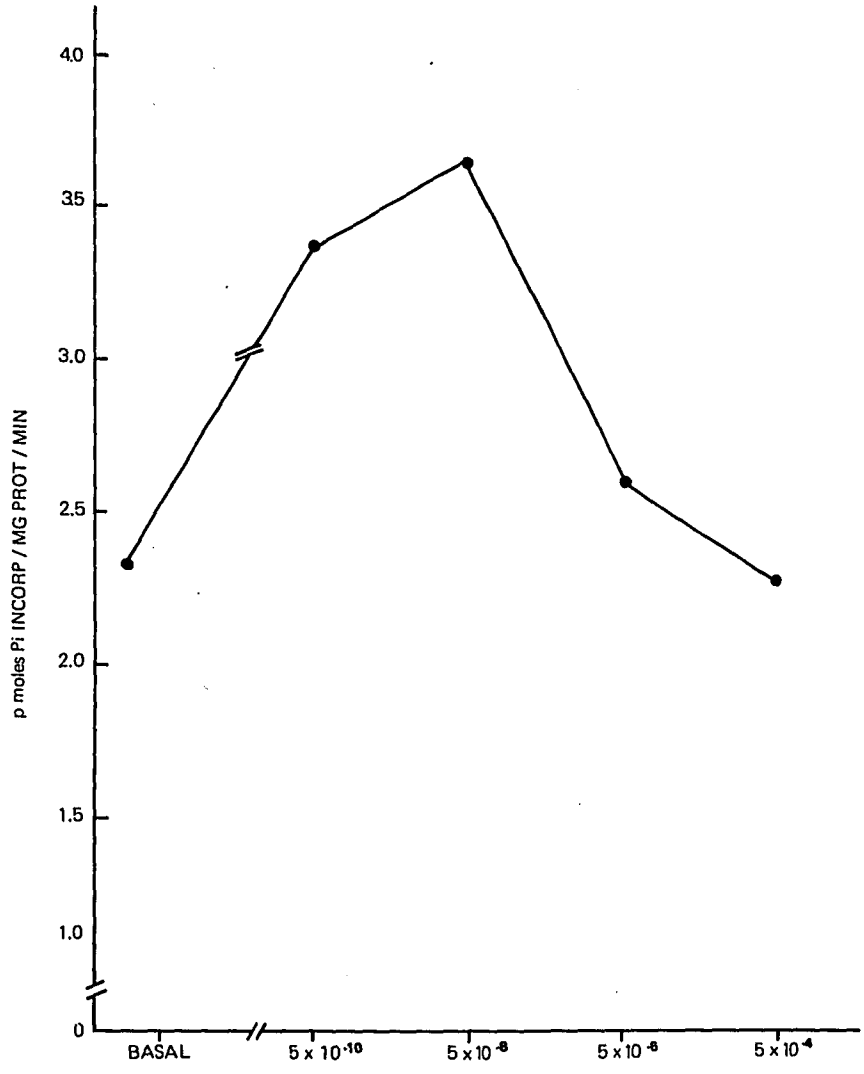

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0
p moles Pi INCORP / MG PROT / MIN
BASAL
5 x 10⁻¹⁰
5 x 10⁻⁸
5 x 10⁻⁶
5 x 10⁻⁴
c AMP CONCENTRATION (MOLAR)

FIGURE 17

Figure 18. Effect of time on protein kinase activity of the cytosol (supernatant fluid) of Mitochondria-rich cells isolated from the toad bladder.

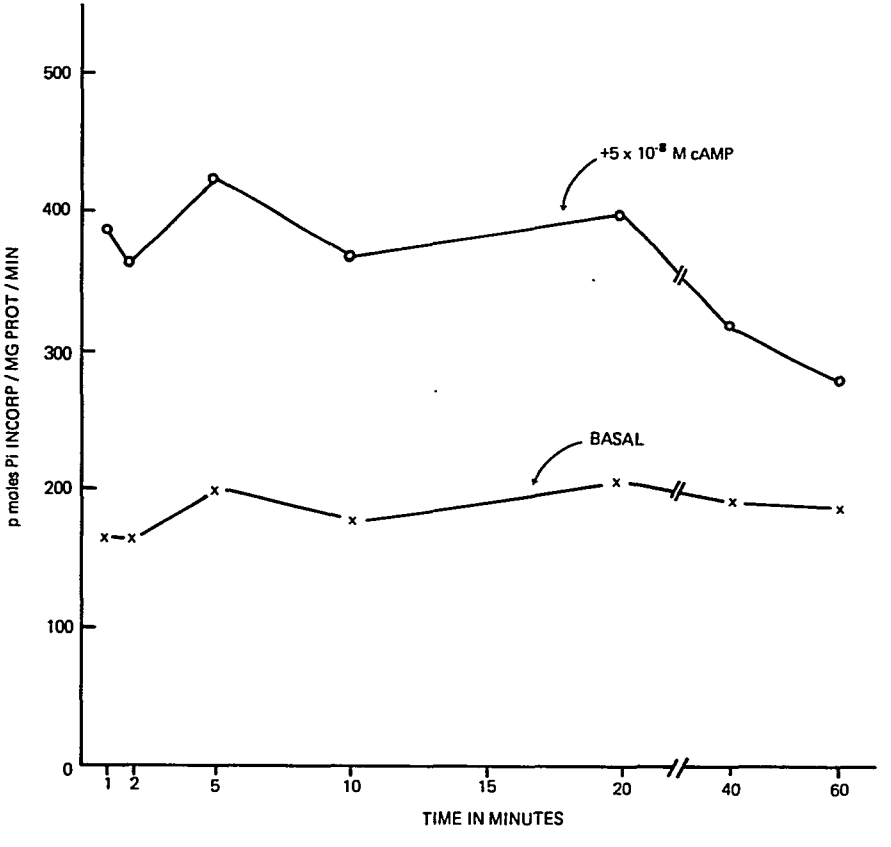

500
400
300
200
100
0
p moles Pi INCORP / MG PROT / MIN
+5 x 10⁻⁸ M cAMP
BASAL
1
2
5
10
15
20
40
60
TIME IN MINUTES

FIGURE 18

Figure 19. Effect of time on protein kinase activity of the cytosol (supernatant fluid) of granular cells isolated from the toad bladder.

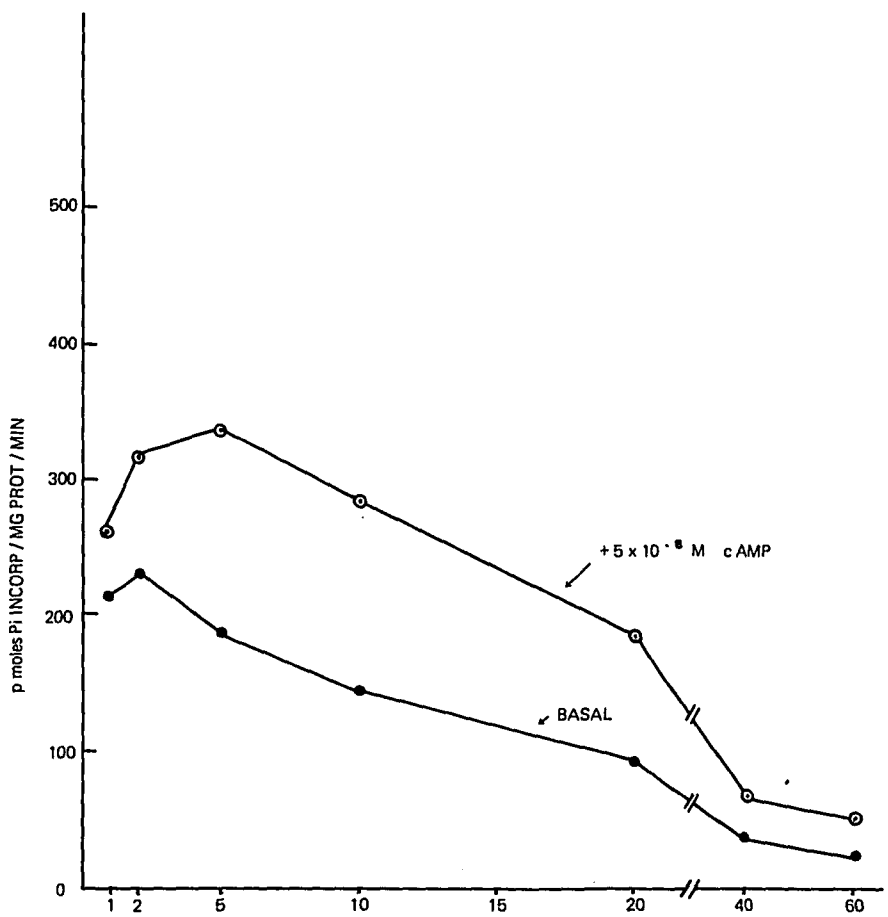

500
400
300
200
100
0
p moles Pi INCORP / MG PROT / MIN
+5 x 10⁻⁶ M c AMP
BASAL
1 2 5 10 15 20 40 60
TIME (MINUTES)

FIGURE 19

Table 4. Time course of protein kinase activity of the cytosol (supernatant fluid) of granular cells and mitochondria-rich cells isolated from the toad bladder.

Time (mins.)	Granular Cell Supernatant		Mitochondria-rich Cell Supernatant	
	Basal	+cAMP	Basal	+cAMP
1	211	262	165	388
2	229	318	162	369
5	167	337	199	426
10	141	282	175	364
20	94	182	202	397
40	39	67	190	315
60	29	52	184	293

Data represents the average of four experiments. Results expressed as pmoles P_i incorp./mg protein/minute. (cAMP conc., 5×10^{-8}M; histone was used as substrate).

Table 5. Experiment to Determine whether Granular and Mitochondria-rich Cell Particulates can serve as phosphate acceptor (substrate) for "supernatant" (cytosolic) protein kinase.

Expt.	Cell Type	Cell Fraction	Basal	+cAMP
21	Granular Cell	Pellet	0.14	—
		Supernatant	27.5	27.34
		Pellet + Supernatant	2.34	2.94
22	Mitochondria-rich Cell	Pellet	3.4	3.4
		Supernatant	12.2	12.2
		Pellet + Supernatant	5.8	7.7

Results expressed as pmoles P_i incorp./mg protein/minute. (cAMP conc., 5×10^{-8}M; no histone was added; incubation time 20 minutes).

Table 6. Basal and cyclic AMP-dependent protein kinase activity of mitochondria-rich cell particulates ("pellets").

Expt.	Protein content (mg)	Basal	+cAMP
7	0.016	.242	1.02
10	0.003	16.6	28.6

Results expressed as pmoles P_i incorp./ mg protein/minute. (cAMP conc., 5×10^{-8}M; no histone was added; incubation time, 20 minutes).

Table 7. Basal and cyclic AMP-dependent protein kinase activity of granular cell particulates ("pellets").

Expt.	Protein content (mg)	Basal	+cAMP
8	.031	2.32	3.6
9	.038	1.16	3.6

Results expressed as pmoles P_i incorp./mg protein/minute. (cAMP conc., 5×10^{-8}M; no histone was added; incubation time: Expt. #8 - 20 min.; Expt. #9 - 10 min.).

Figure 20. Time course of protein kinase activity of particulates ("pellet") from granular cells isolated from toad bladder epithelium.

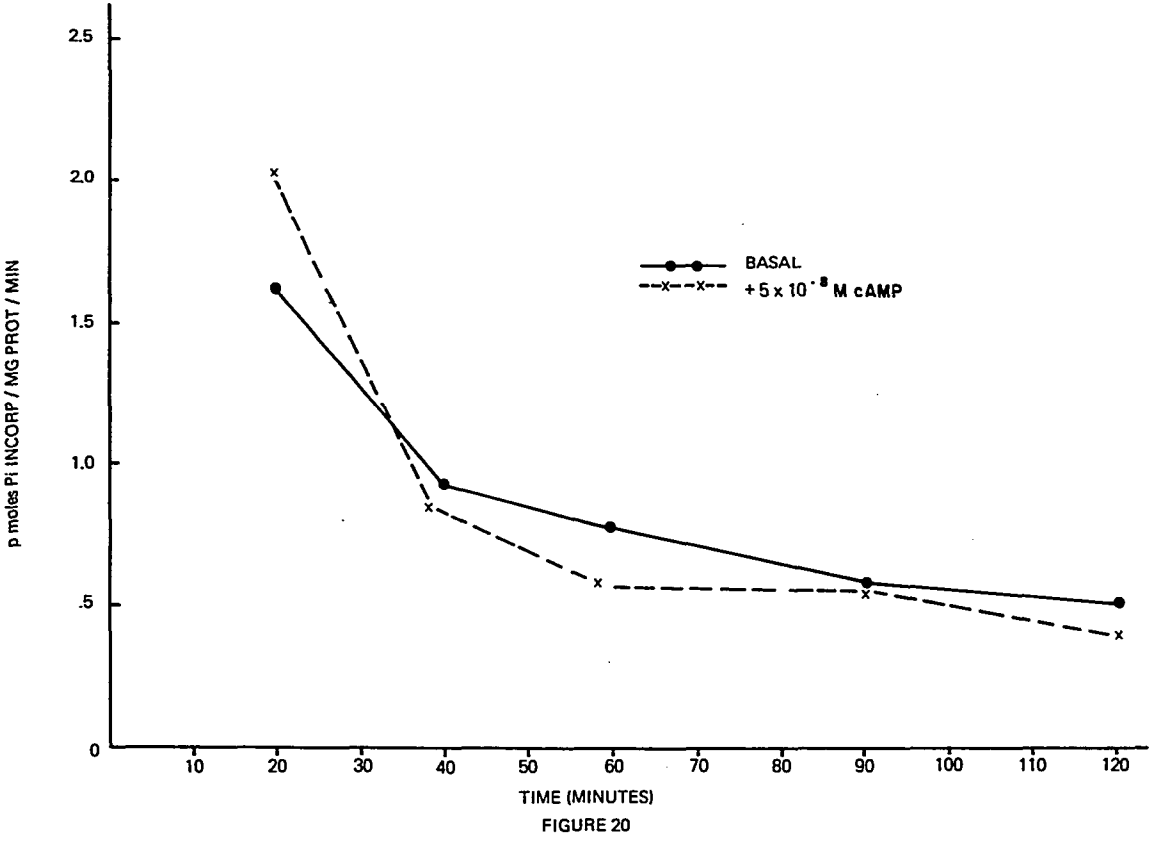

2.5
2.0
1.5
1.0
.5
0
p moles Pi INCORP / MG PROT / MIN
BASAL
+ 5 x 10⁻⁸ M cAMP
10
20
30
40
50
60
70
80
90
100
110
120
TIME (MINUTES)

FIGURE 20

Table 8. Time Course of Protein Kinase Activity of Particulates ("Pellets") from Granular Cells Isolated from Toad Bladder Epithelium.

Time (minutes)	Basal	+cAMP
20	1.65	2.0
40	.93	.82
60	.78	.58
90	.58	.59
120	.53	.42

Results expressed as pmoles P_i incorp./mg protein/minute. (cAMP conc., 5×10^{-8}M; no added histone).

Figure 21. Effect of $ZnCl_2$ on basal and cyclic AMP-dependent protein kinase activity of particulates ("pellets") of Granular and Mitochondria-rich cells isolated from toad bladder epithelium. Cross-hatched bars represent basal protein kinase activity; stippled bars represent cAMP-dependent protein kinase activity. cAMP conc. 5×10^{-8}M; $ZnCl_2$ conc. 2.5 mM.

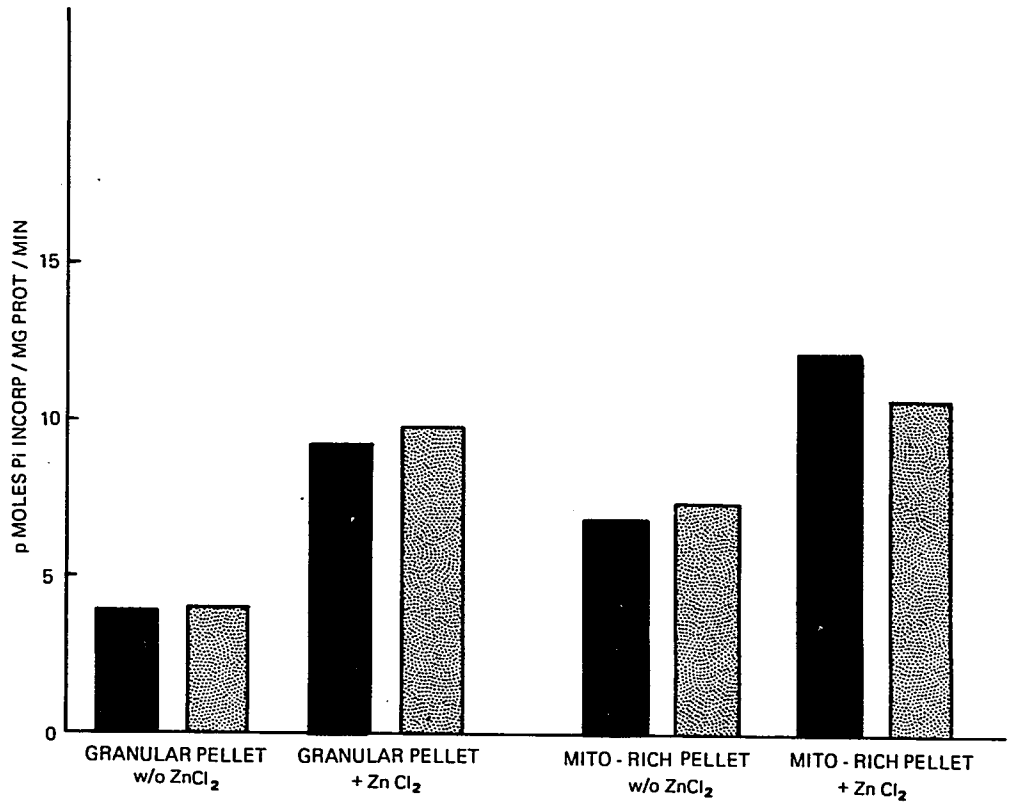

p MOLES Pi INCORP / MG PROT / MIN
15
10
5
0
GRANULAR PELLET w/o $ZnCl_2$
GRANULAR PELLET + $Zn\ Cl_2$
MITO - RICH PELLET w/o $ZnCl_2$
MITO - RICH PELLET + $Zn\ Cl_2$

FIGURE 21

DISCUSSION

The presence of both cytosolic and membrane-bound cAMP-dependent protein kinase(s) in granular cells is consistent with the model for the mediation of hormone action given in Figure 7 in which cAMP-dependent membrane phosphorylation is a step in the neurohypophyseal hormone-induced chain of events leading to increased membrane permeability. The protein kinase(s) in mitohcondria-rich cells may serve to mediate functions other than increased transepithelial water flux, e.g. increased transepithelial sodium flux, glycogenolysis, etc.

The presence of adenylate cyclase activity in both mitochondria-rich and granular cells (Handler and Preston, 1976) suggests that the granular cell can generate cyclic AMP directly in response to hormone and, therefore, that it does not require transfer of this nucleotide mediator from the mitochondria-rich cell.

It is possible that cytosolic and/or membrane-bound cAMP-dependent protein kinase are involved in phosphorylation of the luminal plasma membrane of the responsive cells. The cytosolic kinase is more active than the membrane-bound kinase, but the proximity of the latter enzyme to its substrate might compensate

for the lower activity of the enzyme as well as for its possibly low order of specificity.

Althouth it is our working hypothesis that neurohypophyseal hormones induce an increase in luminal membrane permeability via a cAMP-mediated enhancement of luminal membrane phosphorylation, others (DeLorenzo and Greengard, 1973) have found that cyclic AMP activates a membrane-bound phosphoprotein phosphatase which acts on a specific protein (protein D) in toad bladder membrane preparations. Our observation of an enhanced phosphorylation of mitochondria-rich cell and granular cell pellet preparations during the first 10-20 minutes of incubation (and dephosphorylation only at later intervals) is consistent with a role of phosphorylation in the hydroosmotic action of neurohypophyseal hormone (which characteristically occurs immediately after exposure to hormone and is maximal within 10-20 minutes).

SECTION III: MEDIATION OF CATECHOLAMINE ACTION IN THE TURTLE BLADDER

INTRODUCTION

In a third phase of the present work, protein kinase and adenylate cyclase activities have been studied, using a reptilian model, the turtle urinary bladder which is known to have certain transport functions.

The reptilian bladder is a bilobed sac. Histologically, it has been shown (LeFevre et al, 1971) that the bladder is made up of three layers: (1) serosa, (2) a loose network of connective tissue, smooth muscle and blood vessels, and (3) mucosa.

An ideal membrane for studying transport is a single layer of transporting epithelial cells. Ideally, it should be a membranous sheet without convolutions or non-epithelial cell types. The suitability of the turtle bladder was investigated and it was found to have advantages for transport studies:

(1) It can be obtained in pieces greater than 1 cm^2 (toad bladder epithelial cells yield smaller pieces).

(2) The Na^+ transport system functions in sheets of isolated epithelial layer.

(3) The large size of the cells near the neck of turtle bladders suggests that water and salt transport is more vigorous here.

Isolated urinary bladders from turtles (Pseudemys scripta) transport Na^+, HCO_3 and Cl^- ions against electrical and chemical potential gradients via pumps. These ions move in the same direction, from mucosal to serosal (interstitial) bathing fluid (Brodsky and Schilb, 1966; Gonzalez et al, 1967; Gonzalez et al, 1967a; Schilb and Brodsky, 1966). Movement of water, following Na^+ and Cl^-, is passive (Brodsky and Schilb, 1965).

Acidification of luminal fluid by the turtle bladder has been shown (Brodsky and Schilb, 1966) in isolated urinary bladders of freshwater turtles. Like the mammalian nephron, the turtle bladder actively acidifies luminal fluid to a minimal of pH 4.0. The acidification mechanism may be active reabsorption of HCO_3^- ions. The following results favor this mechanism:

(1) Turtle bladder sacs show that the concentration of luminal CO_2 decreases with the concentrations of luminal HCO_3^- and pH. All three decrease less than in serosal fluid. Solvent flow is directed from mucosal to serosal fluid.

(2) Acidification persists in Na^+ free luminal fluid in the turtle bladder (and also in rat proximal tubule). This suggests that little or no H^+-Na^+ exchange is involved in the acidification process.

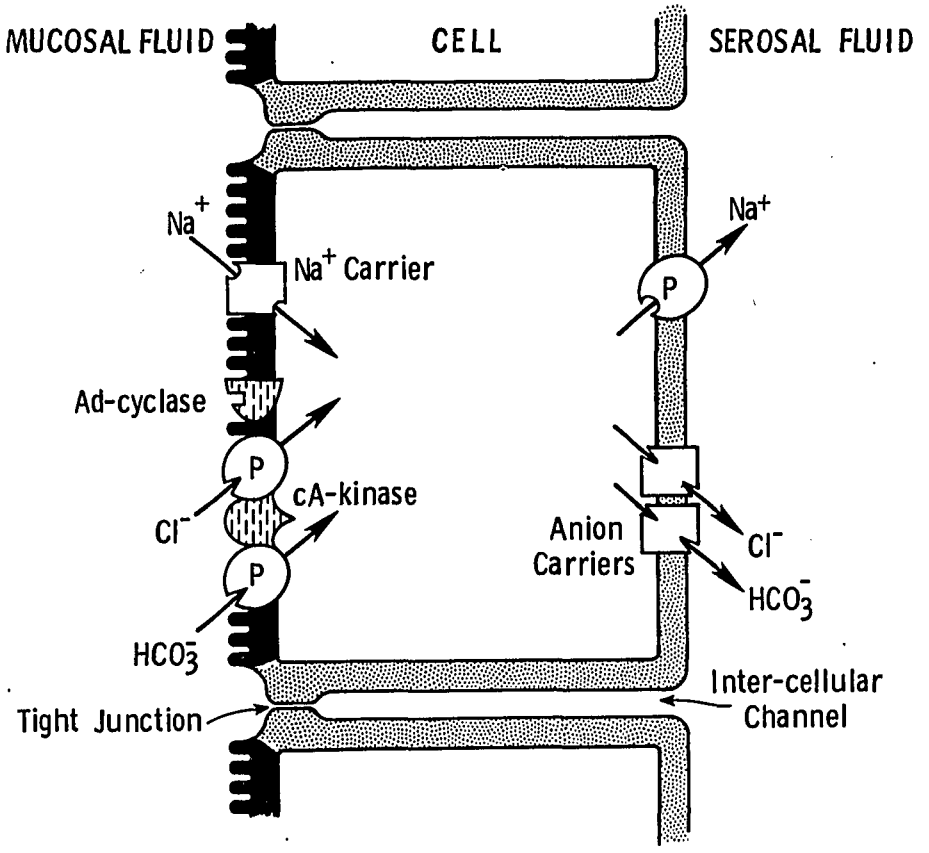
(3) Active HCO_3^--transport as the mechanism of acidification of luminal fluid in turtle bladder would explain the decreasing luminal concentration of CO_2 during acidification, the H^+ accumulation in luminal fluid devoid initially of HCO_3^- and CO_2, and the HCO_3^--sensitive short-circuit current.

The urinary bladder of reptiles is different from both the mammalian kidney and the amphibian bladder in its embryological development, its lesser permeability to water and its lack of a hydroosmotic response to antidiuretic hormone. In view of this ADH insensitivity, norepinephrine was used for the reptilian membrane studies, Brodsky et al (1976) having shown that Cl^- and HCO_3^- transport from mucosal to serosal surface of the turtle bladder is stimulated by norepinphrine, as well as by histamine, histidine, isoproterenol, theophylline, and imidazole but not by epinephrine. These agents exert no effect on Na^+ transport, (although ouabain in the serosal bathing fluid causes a decrease in Na^+ transport (Brodsky et al, 1976). A current working hypothesis of transport in the turtle bladder (Brodsky et al, 1976a) is given in Figure 22

It has become apparent in recent years, that the second messenger for at least some responses to catecholamines is cyclic 3',5'-adenosine monophosphate (Sutherland and Rall, 1960). With this in mind, the studies reported below were undertaken in order to

Figure 22. A current view of the ion selective pathways in the apical and basal-lateral membranes of the turtle bladder epithelial cell.

(Ref: Brodsky and Ehrenspeck, 1976)



Inhibitors of Na^+ transport:

mucosal aspect: amiloride, $[H^+]$

serosal aspect: ouabain, ethacrynic acid.

Inhibitors of anion transport

serosal aspect: diamox, stilbenes.

Stimulators of anion movement:

mucosal aspect: norepinephrine, isoproterenol, theophylline, imidazole, histidine, histamine.

investigate the mechanism of action of catecholamine in mediating anion reabsorption in the turtle bladder.

In addition to catecholamines, cholinergic agents are also known to influence ion transport in the turtle bladder. When added to the serosal bathing fluid, the cholinergic drug acetyl beta-methylcholine (Mecholyl), has been shown to decrease net sodium flux, short-circuit current, electromotive force and transmembrane potential difference (Schilb, 1960). If this agent acts on the active path for sodium transport, it must affect the driving force for sodium transport or the resistance of the active pathway to the flow of sodium ions. It does not appear to act solely by changing the permeability of the bladder.

cGMP has been implicated in mediating the action of cholinergic agents. For example, an increase in cGMP levels has been noted during the action of methacholine (Schultz et al, 1973). Thus, cGMP should be included as a possible candidate for regulating fluid secretion (Berridge, 1975) and its role in the turtle bladder was investigated herein.

Evidence is presented below which demonstrates the presence of a cAMP- and a cGMP-dependent protein kinase in microsomal fractions and, particularly, of a cAMP-dependent protein kinase in the luminal membrane fraction of the mucosal epithelial cells of the turtle

bladder. The latter fraction was obtained by preparative free flow electrophoresis. Also identified in these preparations is a norepinephrine-stimulatable adenylate cyclase. In related aspects of the work presented in this dissertation, $(Na^+ + K^+)$-ATPase has been demonstrated in the basal-lateral plasma membranes of turtle bladder epithelial cells.

MATERIALS

Turtles were purchased from Carolina Biologicals.

Diisothiocyano disulfonic stilbene (DIDS) was synthesized and supplied by Z.I. Cabantchik, Life Sciences Institute, Jerusalem, Israel. 4-Acetamidô-4'-Isothiocyanato stilbene-2,2'-disulfonic acid (SITS) was supplied by BDH Chemical, Ltd., Poole England.

Norepinephrine, histidine, and triethanolamine (TEA) were purchased from Sigma. Cyclic guanine monophosphate (cGMP) and guanine triphosphate were purchased from Boehringer-Mannheim. Otherwise, all materials are the same as described in Sections I and II.

METHODS

Preparation of Turtle Bladder Microsomes.

The turtle bladder microsomes used in this study were prepared by a modification of the method of Solinger et al (1968). The protocol is illustrated in the flow sheet (Figure 23).

Turtles were decapitated and their bladders isolated. Bladders were then incubated as sacs tied to Luer-lock syringe barrels, in "Ca^{++}-free" Na-Ringer's* solution containing 1-2 mM EDTA (220 mOSM/L, pH 7.2 - 7.6), at room temperature for 30-60 minutes. A "Ca-free" Ringer's solution containing EDTA was used because cell organelles have a tendency to form aggregates in the presence of calcium.

After the required incubation time, the bladder sacs were removed from the bath and their walls rubbed gently in order to release the epithelial cells into the mucosal fluid. The suspension is withdrawn with a syringe and rinsed 3 times in Ringer's solution.

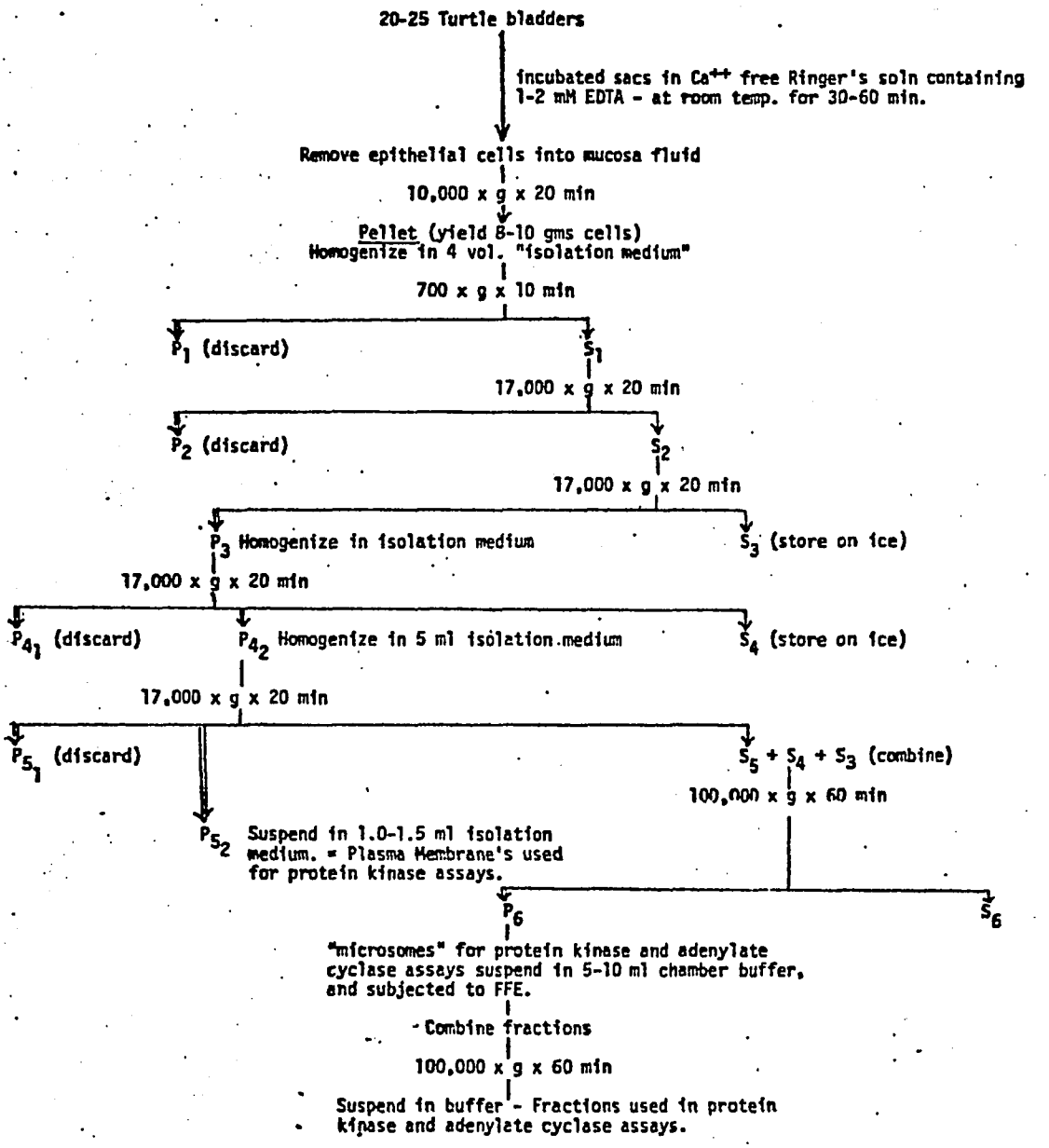
---

* "Ca^{++}-free" means no calcium was added in the preparation of the Ringer's solution.

Figure 23. Flow diagram of Turtle Epithelial Membrane Preparation,

Ref: Solinger et al,(1968)

(with modification).


20-25 Turtle bladders
incubated sacs in Ca++ free Ringer's soln containing 1-2 mM EDTA - at room temp. for 30-60 min.
Remove epithelial cells into mucosa fluid
10,000 x g x 20 min
Pellet (yield 8-10 gms cells)
Homogenize in 4 vol. "isolation medium"
700 x g x 10 min
P1 (discard)
S1
17,000 x g x 20 min
P2 (discard)
S2
17,000 x g x 20 min
P3 Homogenize in isolation medium
S3 (store on ice)
17,000 x g x 20 min
P4 1 (discard)
P4 2 Homogenize in 5 ml isolation medium
S4 (store on ice)
17,000 x g x 20 min
P5 1 (discard)
S5 + S4 + S3 (combine)
100,000 x g x 60 min
P5 2 Suspend in 1.0-1.5 ml isolation medium. = Plasma Membrane's used for protein kinase assays.
P6
S6
"microsomes" for protein kinase and adenylate cyclase assays suspend in 5-10 ml chamber buffer, and subjected to FFE.
Combine fractions
100,000 x g x 60 min
Suspend in buffer - Fractions used in protein kinase and adenylate cyclase assays.

This cell suspension (400 - 700 ml) is collected and all subsequent procedures are carried out on ice or at 4°C during centrifugation. The suspension is centrifuged at 10,000 x g for 20 minutes. This yields 8-10 grams of cells. The pellets (cells) obtained from this centrifugation are homogenized with 20-25 strokes in a Brown-Melsungen homogenizer (with tight fitting pestle) in approximately 4 volumes of isolation medium consisting of 250 mM sucrose and 10 mM triethanolamine (TEA-ST buffer) titrated with HCl to pH 7.6. The homogenate is then centrifuged at 700 x g for 10 minutes. The pellet, which contains nuclei and cell debris, is discarded. The supernatant is centrifuged a second time at 700 x g for 10 minutes, the pellet discarded and the supernatant from this spin is next centrifuged at 17,000 x g for 20 minutes. The resulting supernatant (S_3) is stored on ice. The resulting pellet, which contains mitochondria and membranes is homogenized with 10 strokes in a Dounce homogenizer and centrifuged at 17,000 x g for 20 minutes.

The supernatant of the last centrifugation step (S_4) is added to the previous supernatant (S_3) and kept on ice. The pellet which results is made up of a white fluffy layer on top of a darker layer. The former is made up of plasma membranes, the latter contains mitochondria. The fluffy layer is removed by a light swirl-

ing motion and is resuspended and homogenized with 10 strokes in 5 ml of isolation buffer. It is then centrifuged again at 17,000 x g for 20 minutes. Again two pellets are obtained. The darker pellet, containing mitochondria, is discarded. The white fluffy layer is removed as before and suspended in a final volume of 1.0 - 1.5 ml of isolation buffer. These are the plasma membranes used in the studies of protein kinase and adenylate cyclase referred to in the Results section.

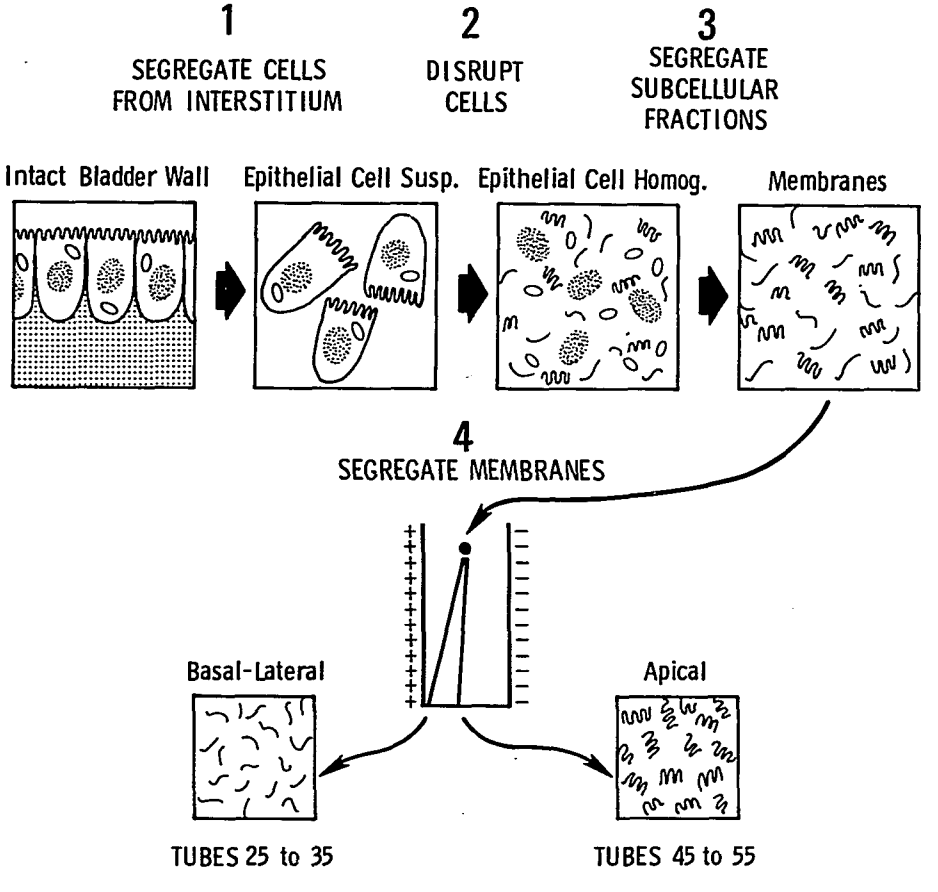
The supernatant resulting from this centrifugation (S_5) is combined with S_4 + S_3 and centrifuged at 100,000 x g for 60 minutes. The resulting pellet is referred to in the Results section as the microsomal preparation; this preparation is also used for protein kinase and adenylate cyclase studies. In addition, this microsomal preparation is also suspended in 5 - 10 ml of chamber buffer (280 mM sucrose, 8.5 mM triethanolamine titrated with 8.5 mM acetic acid and adjusted to pH 7.4 with NaOH) which is used for free flow electrophoresis. Fractions taken from the free flow electrophoresis instrument (Hannig FF5) and assayed for enzymes (marking luminal and contraluminal membranes) are recombined, centrifuged at 100,000 x g for 60 minutes and the pellet is resuspended in buffer or water and then assayed for adenylate cyclase activity and protein kinase activity.

The main features of preparative free flow electrophoresis for dissociating and isolating luminal and contraluminal plasma membranes are as follows.

The separation takes place in a vertical chamber formed by two glass plates between which a buffer flows downward, to be divided into fractions at the bottom of the chamber. An electric field is applied perpendicularly to the direction of buffer flow with the anode on the left and the cathode on the right. The material to be resolved (e.g. turtle bladder microsomes) is injected into a port situated on the right in the upper third of the chamber and the components of the original homogenate are separated in the electric field on the basis of difference in surface charge density, mass, and shape (Figure 24).

The separation of luminal and contraluminal plasma membranes derived from epithelial cells is evaluated by use of marker enzymes(alkaline phosphatase and HCO_3-ATPase for luminal and Na-K-ATPase and Ca-ATPase for contraluminal plasma membrane (Kinne-Saffran and Kinne, 1974; 1974a).

Figure 24. Schematic view of procedures used to separate apical from basal-lateral membranes by homogenization and ultra-centrifugation (steps 1-3) and finally by free flow electrophoresis (step 4).


1
SEGREGATE CELLS FROM INTERSTITIUM
2
DISRUPT CELLS
3
SEGREGATE SUBCELLULAR FRACTIONS
Intact Bladder Wall
Epithelial Cell Susp.
Epithelial Cell Homog.
Membranes
4
SEGREGATE MEMBRANES
Basal-Lateral
Apical
TUBES 25 to 35
TUBES 45 to 55

Preparation of Turtle Bladder Microsomes Incubated with SITS or DIDS

Nine turtle bladders were excised, mounted on Luer lock syringes, rinsed with Ca^{++}-Ringer's solution and filled with same. Three bladders were exposed to DIDS or SITS on their mucosal aspect only, three bladders were exposed to these agents on their serosal aspect only, and three bladders were not exposed to these agents and thus served as controls.

The three bladders chosen for mucosal incubation with SITS (10^{-4}M) were emptied of Ca^{++}-Ringer's solution and filled with enough SITS-containing-Ca^{++}-Ringer's solution to keep the bladders distended (usually 25-30 ml). These bladders were incubated in aerated Ringer's solution containing 0.5% albumin for 10 minutes. Bladders were then removed from the bath, emptied, and rinsed twice with fresh albumin solution. The bladders were then filled with approximately 30 ml of the albumin solution and incubated in a fresh albumin bath for 30-40 minutes. The albumin solution was washed out of the bladders after first dipping them repeatedly in normal Ringer's

solution in order to remove all albumin which might be present on the serosal surface. This is critical to the preparation. The bladders were then filled with Ca^{++}-free Ringer's solution containing EDTA and incubated in this solution with oxygen gassing for a minimum of 45 minutes. The cells were then collected by rubbing the bladders gently, as described earlier.

The three bladders chosen for serosal incubation with SITS were emptied and filled with Ca^{++}-Ringer's solution containing 0.5% albumin. The bladders were incubated for 10 minutes in Ca^{++}-Ringer's solution containing 10^{-4}M SITS. At the end of the incubation time, the bladders were removed from the "SITS bath", dipped in fresh albumin solution and incubated for 30-40 minutes. All further steps were carried out as for those bladders incubated with SITS on the mucosal side.

The three bladders making up the control group were emptied and filled with Ringer's solution containing 0.5% albumin and were incubated in this solution for approximately 40 minutes. Albumin was then washed out and the bladders filled with Ca^{++}-free Ringer's solu-

tion containing EDTA. All remaining procedures were the same as above. It is critical for this preparation that no cross-contamination occurs among the three groups.

The preparation of microsomes taken from these bladders was carried out as described in the previous section.

Adenylate Cyclase Assay

Adenylate cyclase assays carried out on all turtle bladder preparations included: 10 μM GTP in the "reaction mixture"; cyclase buffer pH 7.5; and 10 mM NaF or 10^{-8}M - 10^{-4}M norepinephrine as stimulators of the reaction. Incubation time was ten minutes. Otherwise, same as described in Methods, Section I.

Protein Kinase Assay

Protein kinase activity was assayed just as described in Methods, Section II.

RESULTS

The third phase of this study has been concerned with the protein kinase and adenylate cyclase activities of turtle urinary bladders. Turtle bladder "microsomes" and "plasma membranes", prepared by a modification of the method of Solinger et al (1968) and subfractions subsequently prepared by the free flow electrophoresis method of Hannig (1972) were assayed for cAMP-dependent protein kinase, cGMP-dependent protein kinase, and for adenylate cyclase activities.

These plasma membranes, microsomes and their electrophoretic subfractions were all found to contain a cAMP-dependent protein kinase (Figures 25, 26, and 27). In plasma membranes before the free flow electrophoresis, the basal protein kinase activity was approximately tripled after addition of cAMP (10^{-8} - 10^{-7}M); and approximately doubled after addition of cGMP (10^{-7}M).

In "microsomes" before free flow electrophoresis, basal protein kinase activity, slightly higher than that of the "plasma membranes", was increased two to three fold by cAMP (10^{-8} - 10^{-7}M) and two fold by cGMP (10^{-7}M). As cAMP concentration was either decreased below or increased above 10^{-8}M, protein kinase activity decreased.

Figure 25. cAMP- and cGMP-dependent protein kinase activities of turtle bladder "plasma membranes".

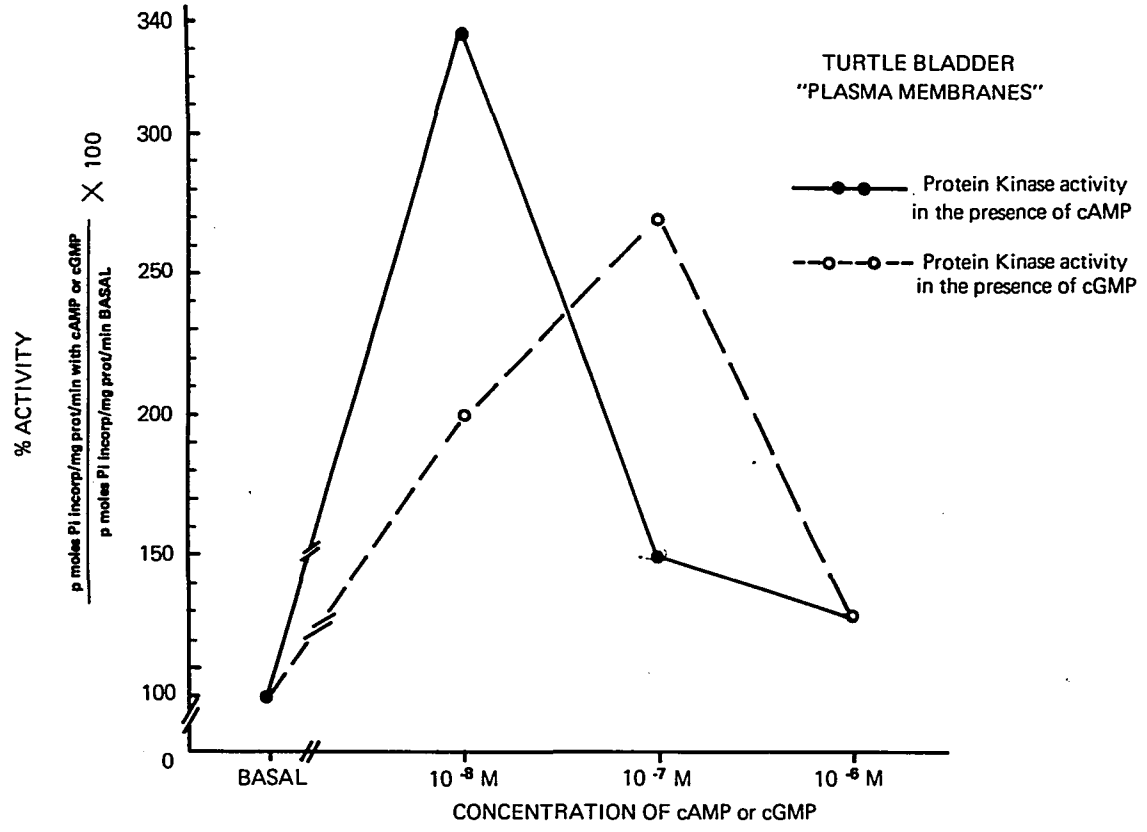

TURTLE BLADDER
"PLASMA MEMBRANES"
Protein Kinase activity in the presence of cAMP
Protein Kinase activity in the presence of cGMP
% ACTIVITY
p moles Pi incorp/mg prot/min with cAMP or cGMP / p moles Pi incorp/mg prot/min BASAL × 100
340
300
250
200
150
100
0
BASAL
10 -8 M
10 -7 M
10 -6 M
CONCENTRATION OF cAMP or cGMP

FIGURE 25

Figure 26. cAMP- and cGMP-dependent protein kinase activities of turtle bladder "microsomes".

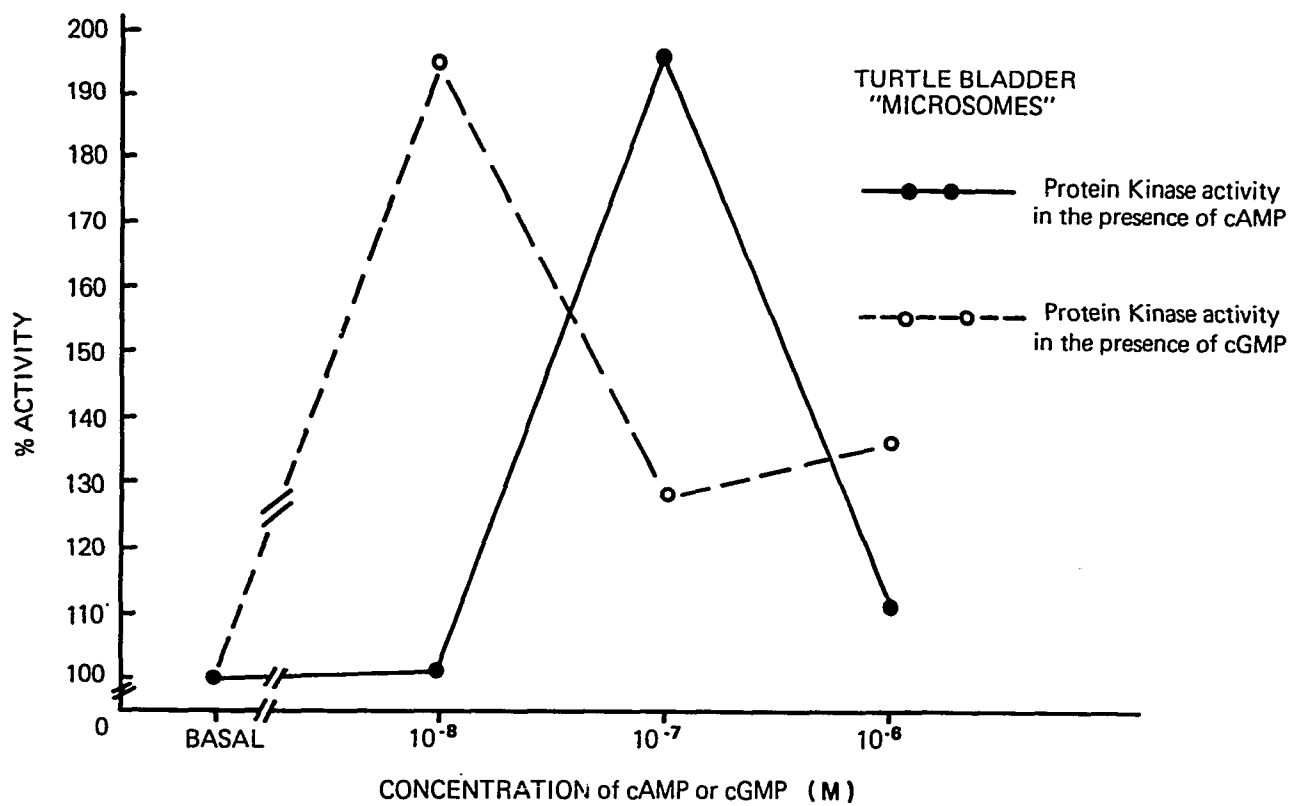
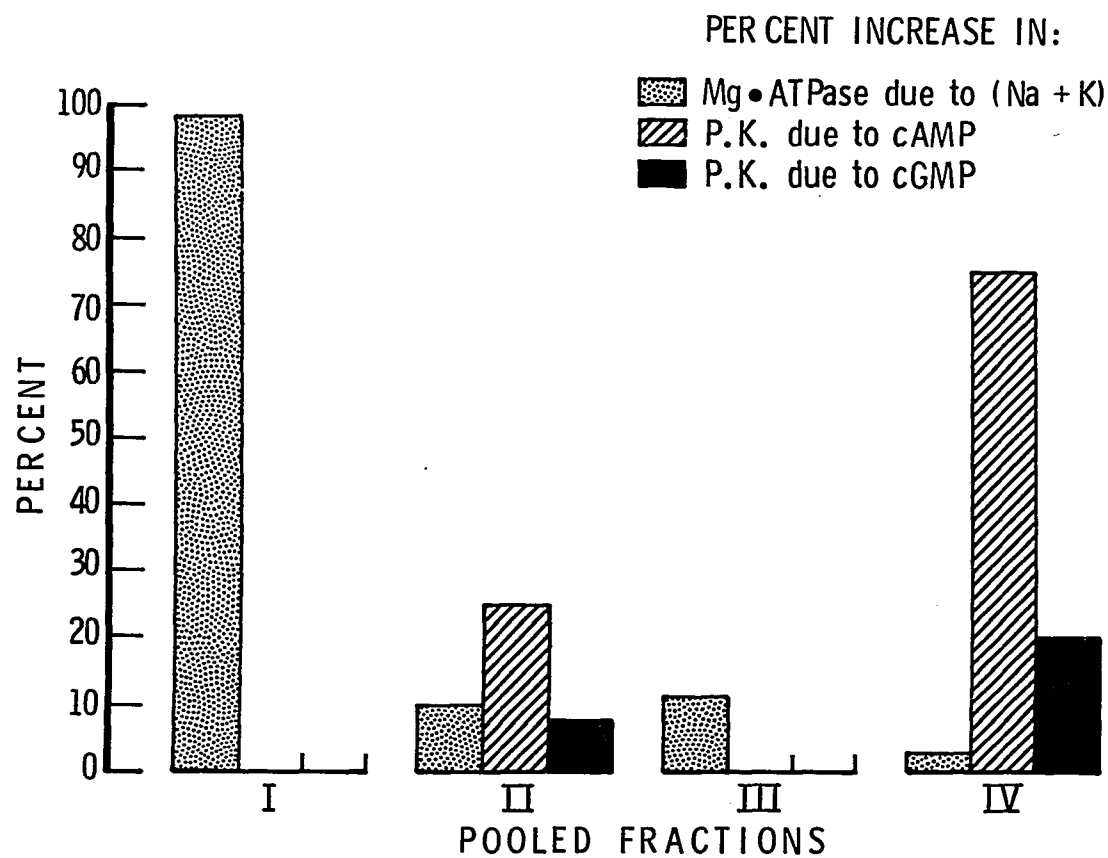

200
190
180
170
160
150
140
130
120
110
100
0
% ACTIVITY
BASAL
10-8
10-7
10-6
CONCENTRATION of cAMP or cGMP (M)
TURTLE BLADDER
"MICROSOMES"
Protein Kinase activity
in the presence of cAMP
Protein Kinase activity
in the presence of cGMP

FIGURE 26

Figure 27. cAMP-dependent and cGMP-dependent protein kinase activities in pooled "fractions" obtained by preparative free-flow electrophoresis.


PER CENT INCREASE IN:
Mg • ATPase due to (Na + K)
P.K. due to cAMP
P.K. due to cGMP
PERCENT
100
90
80
70
60
50
40
30
20
10
0
I
II
III
IV
POOLED FRACTIONS

The protein kinase activity of the turtle bladder microsomes was not a straight-line function of the concentration of microsomal protein (Figure 28) as shown by the fact that the activity reached maximal levels in the presence of a microsomal protein content of approximately twenty micrograms. The protein kinase activity in the plasma membranes as well as in the microsomes (at the twenty microgram level of protein content), represented in Figure 29, reaches a maximal rate at five minutes and subsequently declines.

In an attempt to distinguish this particular protein kinase from the γ-phosphoenzymes of (Na + K)·ATPase in the turtle bladders, the effect of KCl and ouabain were tested. Neither agent alone nor in combination with the other showed any effect on this cAMP-dependent protein kinase activity (Table 9). In other words, cAMP-stimulated protein kinase was apparently distinct from the ATP-induced phosphoenzyme(s) of the (Na + K)·ATPase.

In another set of experiments, microsomes prepared from turtle urinary bladders were exposed to ^{3}H·DIDS or SITS either on the mucosal or on the serosal surface. In microsomes from bladders that were exposed to DIDS or SITS on the mucosal side, no cAMP-stimulated increment of protein kinase activity was detected (Table 10). However, in microsomes from

Figure 28. Effect of "microsome" content on the cAMP-dependent protein kinase activity of the turtle bladder epithelium (% activity denotes:

$$\frac{\text{pmoles } P_i \text{ incorp./mg protein/min + cAMP}}{\text{pmoles } P_i \text{ incorp./mg protein/min Basal}} \times 100.)$$

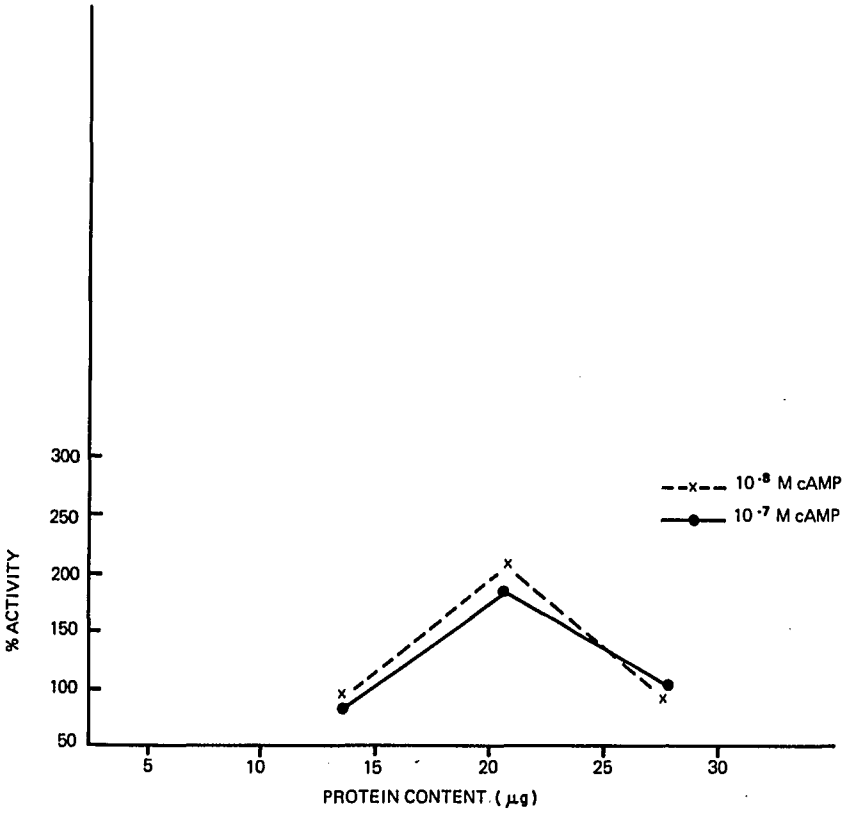

300
250
200
150
100
50
% ACTIVITY
5
10
15
20
25
30
PROTEIN CONTENT (μg)
10⁻⁸ M cAMP
10⁻⁷ M cAMP

FIGURE 28

Figure 29. Effect of time on cAMP-dependent protein kinase activity in "microsomes" prepared from turtle bladder epithelial cells.

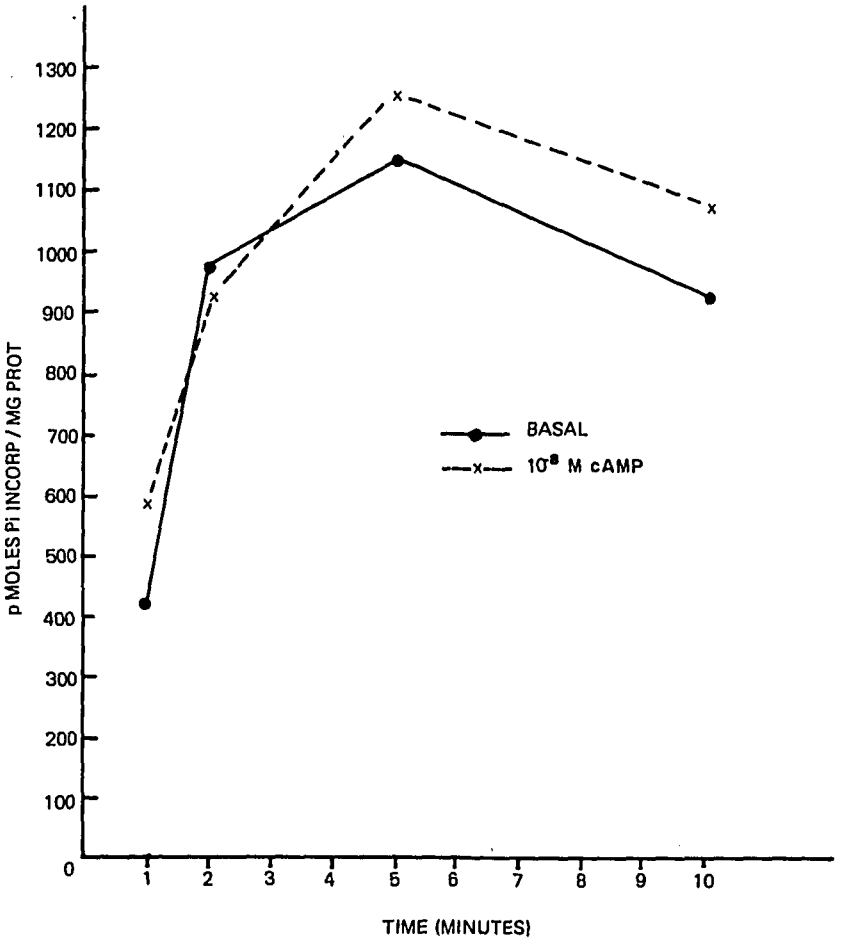

p MOLES Pi INCORP / MG PROT
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10
TIME (MINUTES)
BASAL
10⁻⁸ M cAMP

FIGURE 29

Table 9. Effect of ouabain and/or KCl on the protein kinase activity of "microsomes" isolated from turtle bladder epithelium.

Experimental Condition	Basal	$+10^{-8}$M cAMP
Control (no KCl, no ouabain)	72.71	94.12
+KCl	67.26	81.40
+ouabain	71.77	87.61
+KCl + ouabain	58.65	69.60

Results expressed as pmoles P_i incorp./mg protein/minute. (KCl conc., 10 mM; ouabain conc., 10^{-4}M).

Table 10. Protein kinase activity of "microsomes" isolated from turtle bladders after serosal or mucosal application of SITS (10^{-4}M).

Experimental Condition	Basal	10^{-8}M cAMP	10^{-7}M cAMP
Mucosal SITS	59.73	61.73	62.97
Serosal SITS	42.0	74.6	75.1
Control	47.7	58.3	60.6

Data represents the average of four experiments
Results are expressed as pmoles P_i incorp./mg protein/minute.

bladders exposed to either of these stilbenes on the serosal surface, the cAMP-increment of protein kinase activity was almost twice that of the basal protein kinase (i.e. the same as that found in microsomes from untreated bladders). Thus, the presence of DIDS or SITS on the luminal surface was found to inhibit protein kinase activation.

In addition to protein kinase activity, adenylate cyclase activity was also observed in the plasma membranes and microsomes prepared from turtle urinary bladders. In each of these experiments, adenylate cyclase activity was stimulated by 1.5 to 2.0 fold in the presence of 10^{-2}M NaF or 10^{-4}M norepinephrine (Figure 30). In fact norepinephrine, in concentrations as low as 10^{-8}M, produced the maximal degree of stimulation observed. However, the activity of the same adenylate cyclase was not changed by addition of histidine (10^{-4}M) which, like norepinephrine, has been shown to accelerate anion reabsorption <u>in vivo</u> (Brodsky et al, 1976). The activities of the basal and NaF-stimulated adenylate cyclase in these microsomes remained constant for as long as thirty minutes of incubation time (Figure 31).

Figure 30. NaF- and norepinephrine-stimulatable adenylate cyclase activities of turtle bladder "plasma membranes" and "microsomes".

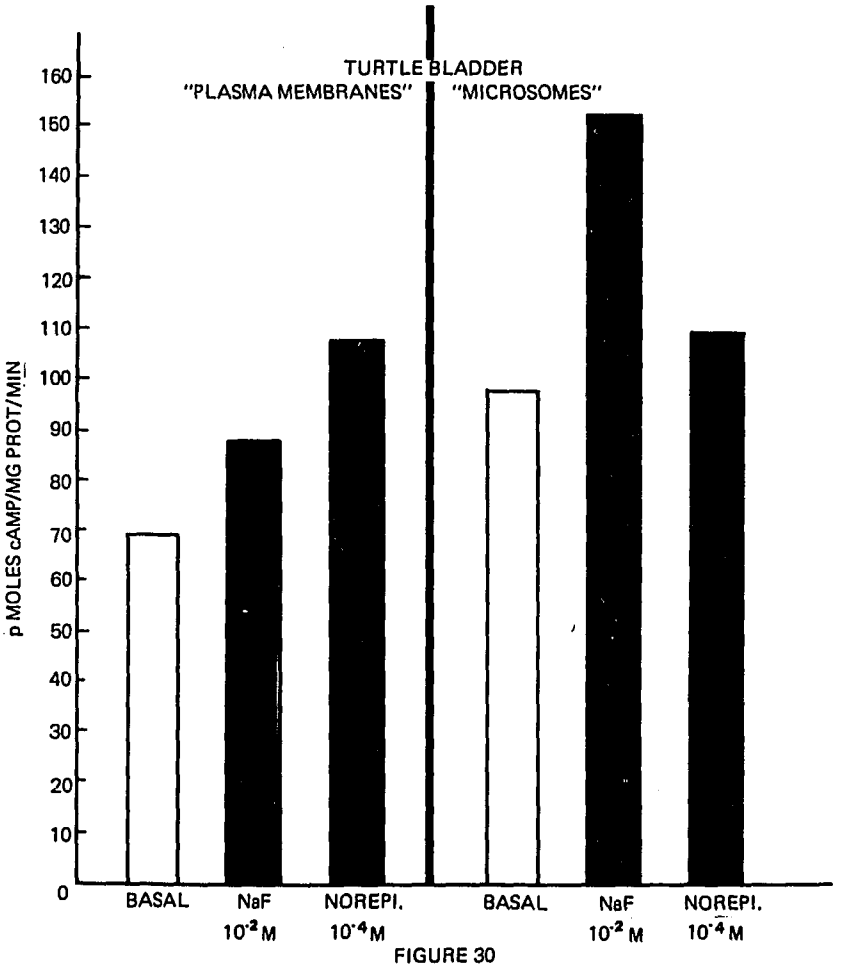

TURTLE BLADDER
"PLASMA MEMBRANES"
"MICROSOMES"
p MOLES cAMP/MG PROT/MIN
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
BASAL
NaF
10^{-2} M
NOREPI.
10^{-4} M
BASAL
NaF
10^{-2} M
NOREPI.
10^{-4} M

FIGURE 30

Figure 31. Effect of time on adenylate cyclase activity of turtle bladder "microsomes". (Open bars, basal activity; hatched bars, with 10^{-2}M NaF.

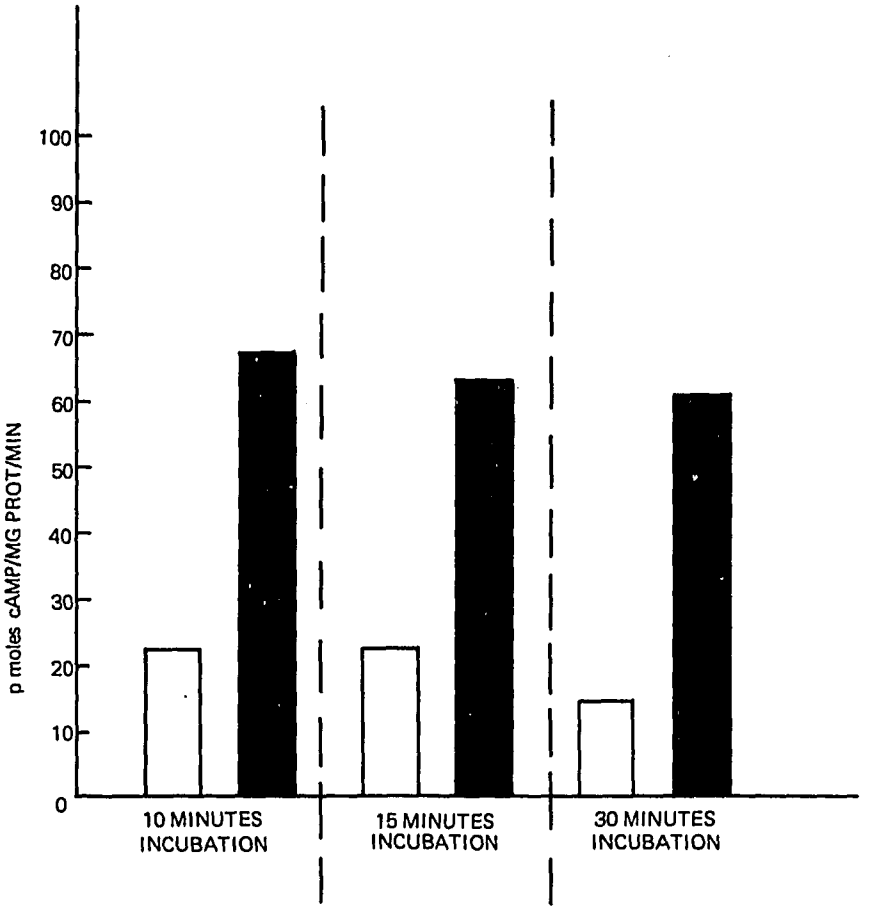
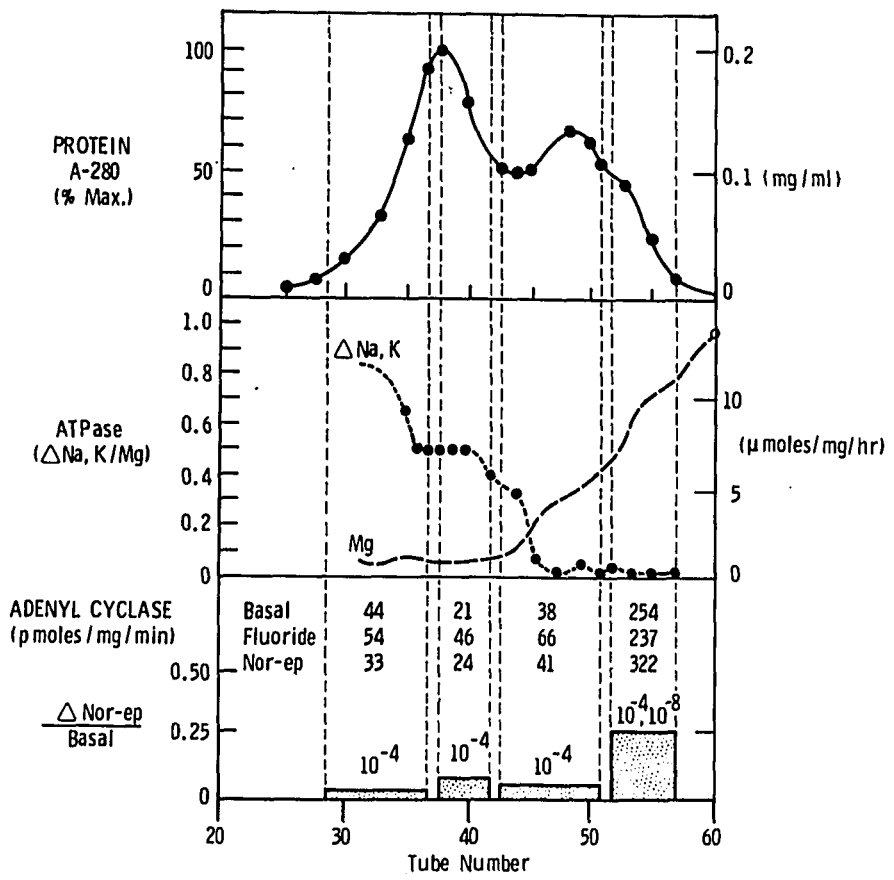

p moles cAMP/MG PROT/MIN
0
10
20
30
40
50
60
70
80
90
100
10 MINUTES INCUBATION
15 MINUTES INCUBATION
30 MINUTES INCUBATION

FIGURE 31

The protein kinase activity of the microsomal fractions migrated toward the negative pole of the free flow electrophoresis system. Fractions collected therefrom revealed enrichment in the cAMP-dependent and cGMP-dependent protein kinase and adenylate cyclase activities of the membrane fragments that migrate toward the electronegative zone. This fraction exhibited approximately a two fold stimulation of protein kinase activity when challenged with 10^{-8} - 10^{-7}M cAMP and a 1.5 fold stimulation with 10^{-7}M cGMP. Norepinephrine (but not NaF) stimulated adenylate cyclase activity in the same fraction. Histidine was found to have no effect (Figure 32).

Figure 32. Electrophoretic distribution of membrane proteins, Mg^{++}-ATPase and (Na^{+} + K^{+})-stimulated ATPase, and norepinephrine-sensitive adenylate cyclase of turtle bladder "fractions" obtained by preparative free-flow electrophoresis.



Adenylate cyclase activity (basal, $+F^-$, and + norepinephrine) per mg protein is indicated by numbers in the lower panel.

Norepinephrine-stimulated fractional increase in adenylate cyclase activity is indicated by columns.

DISCUSSION

I. Apical Membrane

According to the scheme shown in Figure 22, the apical membrane of the turtle bladder is the site of the anion pump(s). While we have identified an adenylate cyclase and cAMP- and cGMP-dependent protein kinase in this membrane, no identifiable pump-related enzymes(s) or protein(s) are known.

A. In Vitro Protein Kinase Activity

The protein kinase activity observed in turtle bladder "plasma membranes", "microsomes", and fractions obtained via preparative free flow electrophoresis are stimulated by cAMP and cGMP at concentrations within the physiological range. The characteristics of protein kinase from turtle bladder tissue is similar in nature to that from other tissue with respect to k_m, V_{max} and half-time of decay at 30°C.

The fact that cGMP stimulates this protein kinase activity merits comment because there is as yet no known action of this particular nucleotide on the transport function of turtle bladder. For example: (1) cGMP stimulates the Na flux but not water flow in toad

bladders (Bourgoignie et al, 1969). (2) cGMP has been shown to mediate the muscarinic effects of cholinergic agents in smooth muscle; this raises the possibility that the cGMP-dependent protein kinase in turtle bladder membranes may also mediate the effects of cholinergic agents.

It is known that the turtle bladder helps to conserve salts and excretes acid waste. This process is controlled in a positive direction by catecholamine, histidine, etc. There are no known natural inhibitors which function as negative controls.

B. In vitro Adenylate Cyclase Activity.

The location of adenylate cyclase in the apical membrane fragments of the epithelial cells is apparently the first instance of finding this enzyme in other than the basal-lateral membrane of any epithelial cell studied to this date. Whereas histidine has no effect on the adenylate cyclase in membranes extracted from turtle bladder epithelial cells, it has been shown to increase the HCO_3^-- and Cl^--dependent moieties of the short-circuiting current in isolated intact bladders (Brodsky et al, 1976). In this connection, norepinephrine stimulates the adenylate cyclase extracted from broken epithelial cells as well as the anion-related transport

parameters of the intact bladder. This suggests that histidine stimulates transport by a mechanism other than one involving adenylate cyclase, perhaps by a mechanism involving a stimulation of guanylate cyclase with cGMP release and a secondary stimulation of yet another protein kinase with the consequent increase in anion transport.

The localization of the adenylate cyclase-protein kinase system in the apical rather than in the basal-lateral membrane suggests that these enzymes could be part of a "safe-guarding system" for salt conservation, triggered by urinary norepinephrine and/or histidine, and thereby reabsorbing anions (i.e. salts) which might otherwise be lost in the urine.

C. Physiological Implications

What are the physiological implications of the existence of a catecholamine-sensitive adenylate cyclase and a cyclic nucleotide-sensitive protein kinase in the luminal membrane of the turtle bladder epithelial cells?

A tentative answer requires first that the cyclase and kinase activities in isolated sub-cellular particles be extrapolated to the intact, transporting epithelial cell layer. It can be then proposed that:

(1) the attachment of a urinary catecholamine to the adenylate cyclase in the luminal membrane is followed by an increased rate of release of cAMP into the cytoplasm;

(ii) the cAMP then stimulates a neighboring protein kinase in the same membrane; and

(iii) the stimulation of protein kinase is accompanied by a decrease of the "internal resistance" or by an increase of the intrinsic driving force (EMF) of the anion pumps in the apical membrane which accounts for the observed acceleration in the rate of anion transport.

II. Basal-Lateral Membrane

In the basal-lateral membrane of the intact turtle bladder, there appears to be a passive flow of anions via selective paths in the membrane (Figure 22). Certain sites in or near these paths apparently interact with carbonic anhydrase inhibitors and with the disulfonic stilbenes (SITS and DIDS).

A. In Vitro Transport Related Enzymes and Proteins

The means of finding the transport related enzymes in the basal-lateral membrane entail the use of techniques for "membrane localization" (e.g. differential ultracentrifugation, preparative free flow electrophoresis, etc.). Membrane localization also has been studied with the use of DIDS and SITS. Since the serosal addition of ouabain blocks Na^+ transport and since the serosal addition of certain disulfonic stilbenes blocks anion transport, we decided to look for a (Na + K)·ATPase and a disulfonic stilbene binding protein in fragments of the basal-lateral membrane. The disulfonic stilbene compounds, known to block the carrier-mediated exchange of anions across erythrocyte membranes (Cabantchik and Rothstein, 1972), also block the transepithelial reabsorptive flow of Cl^- and HCO_3^- in the turtle bladder (Ehrenspeck and Brodsky, 1976). Since this action involves only anions and is evoked only from the serosal surface (Ehrenspeck and Brodsky, 1976) it can be inferred that these disulfonic stilbenes bind to but do not penetrate the basal-lateral membranes of these epithelial cells. In fact, isothiocyano groups of the stilbenes react with terminal amino groups of the membrane proteins to form a new membrane-thiocarbamate complex, which means that an appropriately

labelled disulfonic stilbene (e.g. ^{3}H·DIDS) can be used as a probe for isolating the anion transport sites in the basal-lateral membrane. Parenthetically, SITS has no effect on sodium transport across the intact bladder, but it inhibits the ($Na^+ + K^+$)·ATPase activity of membranes extracted from turtle bladder cells as well as does ouabain (Solinger et al, 1968; Brodsky and Schilb, 1974). These findings together with the fact that ouabain does block Na^+ transport in-vivo (Solinger et al, 1968) indicate that ouabain itself is a probe for localizing the Na^+ transport sites (i.e. the $Na^+ + K^+$-ATPase) in the same basal-lateral membrane fragments.

Before discussing the details of localizing the basal-lateral membrane fragments with labelled stilbenes, it is pertinent to review the current picture of the anion selective paths and pumps in both membranes of the turtle bladder. It is now thought that there are two discrete pump mechanisms in the apical membrane - one for Cl^- and one for HCO_3^- reabsorption, as well as two discrete carrier systems in the basal-lateral membrane for the passive flow of these anions from the cell to the serosal fluid (Ehrenspeck and Brodsky, 1976). Given that SITS or DIDS bind pre-

ferentially to a site at or near the carrier-containing anion-selective path in the basal-lateral membrane and thereby reduce the net flow of anions across the epithelial cells, it follows that there should be a disulfonic stilbene binding protein in the basal-lateral membrane. This has been confirmed in recent experiments showing that maximal and preferential binding of ^{3}H·DIDS is found in the membranes which also contain maximal amounts of ouabain-sensitive ATPase (Brodsky and Ehrenspeck, 1976). The membranes in which these two activities were localized have been electrophoretically separated from those membranes in which the adenylate cyclase and protein kinase activities were localized (see above). These data suggest that SITS or DIDS cannot gain access to their binding sites on $(Na^+ + K^+)$·ATPase in the intact bladder cell, since neither agent changes the rate of Na^+ transport.

B. Physiological Implications

Although the physiological effects of serosally-applied disulfonic stilbenes (or ouabain) match the findings on isolated membranes, the lack of effect of mucosally-applied stilbenes apparently does not correlate with the findings on isolated luminal membranes. In view of the lack of physiological effect

of disulfonic stilbenes, it is expected that the adenylate cyclase and protein kinase activities in membranes extracted from control bladders should be the same as those from bladders that had been exposed on their mucosal surface to the disulfonic stilbenes. But this is not verified experimentally (Table 10). The protein kinase activity was actually inhibited in these membranes. The implication is not clear. Either the cAMP-stimulated protein kinase activity is not related to the anion transport function, or the manipulations used during the isolation of a stilbene-treated apical membrane somehow alters its in-vitro protein kinase activity. Part of this dilemma might be resolved by additional data on the cGMP-dependent protein kinase which has yet to be assayed under these conditions. Nevertheless, it should be pointed out that the data related to the serosally applied disulfonic stilbenes do match the physiological finding (Table 10).

III. Current View of Anion Transport Regulation

In summary, the current tentative hypothesis of the regulation of anion transport in the turtle bladder is as follows. The effects of norepinephrine suggest that the regulation of anion transport is probably in the apical membrane. For example, the catecholamines (norepinephrine and isoproterenol) increase the rate of transport of Cl^- and HCO_3^- (but not of cation transport) and do so only when added to the mucosal bathing fluid. Moreover, data of the present study have revealed the presence of a norepinephrine-stimulatable adenylate cyclase in the apical membrane fragments extracted from turtle bladder epithelium. In this connection, theophylline, only when present in the mucosal bathing fluid, stimulates the anion transport parameters, thus mimicking the effect of the catecholamines and showing that stimulation of adenylate cyclase by the catecholamines can be by-passed. Given that cAMP is the "second messenger" for accelerating the anion transport, its addition to the bathing fluid should directly increase anion transport. However, cAMP is without effect when present in either bathing fluid. On the other hand, cGMP in the mucosal fluid does accelerate anion transport (Brodsky, personal communication), also mimicking the response to

catecholamines. As yet, we have not tested for the presence of guanylate cyclase in this system.

Recent work, reviewed by Berridge (1975), suggests that calcium (rather than cAMP or cGMP) maybe the second messenger or membrane effector required for the acceleration of anion transport. In this connection, certain calcium ionophores such as A 23187 or X 537A (Caswell and Pressman, 1972; Reed and Lardy, 1972; Scarpa and Inesi, 1972), when applied to one or the other bathing fluid (which must contain calcium), produce a stimulation of anion transport. Without calcium in the mucosal bathing fluid, the ionophore alone produces no effect on anion transport.

The role of cyclic AMP in this scheme may be to facilitate the release, entry or activity of Ca^{++} in some anion transport systems; or these functions may be mediated at a later step by stimulating protein kinase(s).

The interpretation of these data in the turtle bladder is as follows. In the intact cell, stimulated by a catecholamine (first messenger), the first reaction is the activation of adenylate (or guanylate) cyclase with release of cAMP (or cGMP). The cyclic nucleotide then activates a corresponding protein kinase, (i.e. increases the ATP-induced phosphorylation of a membrane-bound protein) which, in turn may cause the release of calcium ions into some intracellular com-

partment. Finally, the calcium ions per se change the character of the specific transport-related element in the membrane, thereby accelerating the anion transport. Within the framework of this hypothesis, the calcium ionophore first provides a Ca-specific pore or carrier in the membrane. Since the calcium concentration of the synthetic Ringer's solution in the mucosal surface is usually 1-2 mM while that of the natural cell fluid is in the micromolar range, the inevitable effect of the ionophore is to induce an influx of calcium ions into the Ca^{++}-poor compartment - which is apparently sufficient to mimic the step following the cyclic nucleotide activation of protein kinase.

IV. Problems Remaining for Future Work

Some of the problems remaining for future work include:

(1) investigation of the effects of calcium ions on in vitro and in vivo bladder preparations (i.e. is there a relation between calcium and either the transport or the adenylate cyclase-protein kinase systems in the turtle bladder?).

(2) measurement of cAMP content of bladder cells after stimulation by norepinephrine.

(3) measurement of guanylate cyclase activity; is this activity stimulated by either histamine or histidine?

(4) characterization of cGMP-dependent protein kinase in untreated and in DIDS or SITS-treated epithelial cell preparations.

(5) direct determination of the action of effectors on the transport system (i.e. by preparation of right side out and inside out vesicles from isolated membranes obtained via free-flow electrophoresis).

(6) reconstitution of a synthetic membrane system and purification of proteins (enzymes) related to the transport functions of the natural membrane in order to determine the specific factor(s) responsible for anion transport in the turtle bladder.

BIBLIOGRAPHY

Argy, W.P., Handler, J.S. and Orloff, J.
Amer. J. Physiol., 213:803, 1967.

Bar, H.P.
In Methods in Pharmacology, E. Daniel and D. Raton, eds.
Plenum Press, N.Y., Vol. 3, p. 593, 1975.

Bar, H.P. and Hechter, O.
Proc. Natl. Acad. Sci., 63:350, 1969.

Bar, H.P. and Hechter, O.
Analytical Biochem., 29:476, 1969.

Bar, H.P., Hechter, O., Schwartz, I.L. and Walter, R.
Proc. Natl. Acad. Sci., 67:7, 1970.

Beck, N.P., Reed, S.W., Murdaugh, H.V. and Davis, B.D.
J. Clin. Invest., 51:939, 1972.

Behnke, O., Forer, A. Emmersen, J.
Nature 234:408, 1971.

Bentley, P.J.
J. Endocrinol., 17:201, 1958.

Bentley, P.J.
J. Endocrinol., 18:327, 1959.

Bentley, P.J.
Comp. Biochem. Physiol., 12:233, 1964.

Bentley, P.J.
J. Endocrinol., 39:493: 1967.

Berridge, M.J.
In Advance in Cyclic Nucleotide Research, Vol. 6,
P. Greengard and G.A. Robison, eds., Raven Press,
N.Y., 1975.

Birnbaumer, L. and Yang P.-C.
J.B.C., 249:7848, 1974.

Bourgoignie, J., Guggenheim, S., Kipnis, D.M. and Klahr, S.
Sciences, 165:1362, 1969.

Brodsky, W.A. and G. Ehrenspeck.
Proc. 9th Ann. Rochester Internat'l. Conf. Environmental Toxicity on "Membrane Toxicity", Plenum Press (In Press), 1976.

Brodsky, W.A., Ehrenspeck, G. and Kinne, R.
Fed. Proc., 35:702, 1976a.

Brodsky, W.A. and Schilb, T.P.
Amer. J. Physiol., 208:46, 1965.

Brodsky, W.A. and Schilb, T.P.
Amer. J. Physiol., 210:987, 1966.

Brodsky, W.A. and Schilb, T.P.
In Current Topics in Membranes and Transport, Vol. 5, Academic Press, Inc., N.Y., 1974.

Brodsky, W.A., Schilb, T.P. and Parkes, J.
In Gastric Hydrogen Ion Secretion, Kasbekar, Sachs and Rehm, eds., Chapter 17, Marcel Dekker Co., N.Y., (In Press), 1976.

Brunn, F.
Z. ges. exp. Med., 25:170, 1921.

Buckley, I.K. and Porter, K.R.
Protoplasma, 64:349, 1967.

Cabantchik, Z.I. and Rothstein, A.
J. Memb. Biol., 10:311, 1972.

Caswell, A.H. and Pressman, B.C.
Biochem. Biophys. Res. Comm., 49:292, 1972.

Civan, M.M. and Frazier, H.S.
J. Gen. Physiol., 51:589, 1968.

DeLorenzo, R.J. and Greengard, P.
Proc. Natl. Acad. Sci., 70:1831, 1973.

DeLorenzo, R.J., Walton, K.G., Curran, P.F. and Greengard, P.
Proc. Natl. Acad. Sci., 70:880, 1973.

Dibona, D.R., Civan, M.M. and Leaf, A.
J. Membrane Biol., 1:79, 1969.

Dousa, T.P.
Amer. J. Physiol., 222:657, 1972.

Dousa, T.P. and Barnes, L.D.
J. Clin. Invest., 54:252, 1974;
Fed. Proc., 33:388 and 1231, 1974a.

Dousa, T.P. and Hechter, O.
Life Sci., 9:765, 1970.

Edelman, I.S., Petersen, M.J. and Gulyassy, P.F.
J. Clin. Invest., 43:2185, 1964.

Eggena, P., Schwartz, I.L. and Walter, R.
In Regulation of Body Fluid Volumes by the Kidney,
J.H. Cort and B. Lichardus, eds., S. Karger, Basel,
Switzerland, p. 182, 1970.

Eggena, P., Schwartz, I.L. and Walter, R.
J. Gen. Physiol., 56:250, 1970a.

Ehrenspeck, G. and Brodsky, W.A.
Biochem. Biophys. Acta, 419:555, 1976.

Ehrenspeck, G., Brodsky, W.A. and Durham, J.
(unpublished data) 1976.

Ewer, R.F.
J. Exp. Biol., 29:173, 1952.

Ferguson, D.R. and Twite, B.R.
J. Endocrinol., 61:501, 1974.

Finn, A.L., Handler, J.S. and Orloff, J.
Amer. J. Physiol., 210:1279, 1966.

Frazier, H.S., Dempsey, E.F. and Leaf, A.
J. Gen. Physiol., 45:529, 1962.

Goldberg, D.C., Schoessler, M.A. and Schwartz, I.L.
The Physiologist, 6:188, 1963.

Gonzalez, C.F., Shamoo, Y.E. and Brodsky, W.A.
Amer. J. Physiol., 212:641, 1967.

Gonzalez, C.F., Shamoo, Y.E., Wyssbrod, H.R., Solinger,
R.E. and Brodsky, W.A.
Amer. J. Physiol., 213:333, 1967.

Grantham, J.J. and Orloff, J.
J. Clin. Invest., 47:154, 1968.

Gulyassy, P.F. and Edelman, I.S.
Biochim. et Biophys. Acta, 102:185, 1965.

Handler, J. and Preston, A.S.
J. Memb. Biol., 26:43, 1976.

Handler, J., Preston, A.S. and Orloff, J.
J. Clin. Invest., 48:823, 1970.

Handler, J.S., Preston, A.S. and Orloff, J.
Amer. J. Physiol., 222:1071, 1972.

Hannig, K.
Handbuch: Modern Separation Methods of Macromolecules and Particles, M. Gerritsen, ed., John Wiley and Sons, Inc., Vol. 2, s. 45, 1969.

Hannig, K.
Handbuch: Techniques of Biochemical and Biophysical Morphology, Glick, D. and Rosenbaum, eds., John Wiley and Sons, Inc., N.Y., 1972.

Hays, R. and Barland, P.
J. Cell Biol., 31:209, 1966.

Hays, R.M. and Leaf, A.
J. Gen. Physiol., 45:905, 1962.

Hays, R.M. and Levine, S.D.
Kidney International, 6:307, 1974.

Hays, R.M., Singer, B. and Malamed, S.
J. Cell Biol., 25:195, 1965.

Heidrich, H.G., Kinne, R., Kinne-Saffran, E. and Hannig, K.
J. Cell Biol., 54:232, 1971.

Hynie, S. and Sharp, G.W.G.
J. Endocrinol., 50:231, 1971.

Kachadorian, W.A., Wade, J.B. and DiScala, V.A.
Science, 190:67, 1975.

Karlin, A. and Overweg, N.I.
Nature, 207:1401, 1965.

Kinne, R. and Kinne-Saffran, E.
PFleugers. Arch., 335, R 42, 1972

Kinne-Saffran, E. and Kinne, R.
Proc. Soc. Expt. Biol., 146:751, 1974.

Kinne-Saffran, E. and Kinne, R.
J. Memb. Biol., 17:263, 1974a.

Kuo, J.F.
J. Biol. Chem., 6:1755, 1974.

Kuo, J.F., Drueger, B.K., Sanes, J.R. and Greengard, P.
Biochim. Biophys. Acta, 212:79, 1970.

Langan, T.A.
In Advances in Cyclic Nucleotide Research, Greengard, P. and Robison, G.A., eds., Raven Press, N.Y., p. 99, 1973.

Leaf, A.
J. Gen. Physiol., 43:175, 1960.

LeFevre, M.E., Cronkite, C. and Brodsky, W.A.
Biochim. Biophys., Acta, 222:212, 1970a.

LeFevre, M.E., Dox, L.J. and Brodsky, W.A.
J. Memb. Biol., 8:205, 1972.

LeFevre, M.E., Gennaro, J.F. and Brodsky, W.A.
Amer. J. Physiol., 219:716, 1970.

LeFevre, M.E., Gennaro, J.F. and Brodsky, W.A.
Anatomical Rev., 171:237, 1971.

Liu, A.Y.-C., and Greengard, P.
Proc. Nat. Acad. Sci., 71:3869, 1974.

Lipson, L., Hynie, S. and Sharp, G.
Ann. N.Y. Acad. Sci., 180:261, 1971.

Lipson, L. and Sharp, G.W.G.
Amer. J. Physiol., 220:1046, 1971.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.
J.B.C., 193:265, 1951.

Marumo, F. and Edelman, I.S.
J. Clin. Invest., 50:1613, 1971.

Mazur, S.K., Holtzman, E., Schwartz, I.L. and Walter, R.
J. Cell Biol., 49:582, 1971.

Mazur, S.K., Holtzman, E. and Walter, R.
J. Cell Biol., 52:211, 1972.

Mizel, S.B. and Wilson, L.
J. Biol. Chem., 247:4102, 1972.

Neer, E.J. and Sukiennik, E.A.
J. Biol. Chem., 250, 7905, 1975.

Omachi, R.S., Robbie, D.E., Handler, J.S. and Orloff, J.
Amer. J. Physiol., 226:1152, 1974.

Orloff, J. and Handler, J. S.
Biochem. Biophys. Res. Com., 5:63, 1961.

Orloff, J. and Handler, J.S.
J. Clin. Invest., 41:702, 1962.

Orloff, J. and Handler, J.S.
Amer. J. Med., 42:757, 1967.

Overweg, N.
J. Pharmacol. & Exper. Therap., 153:314, 1966.

Parisi, M., Ripoche, P. and Bourguet, J.
Internat'l. Congr. Nephrol., 4th, Stockholm, p. 261, 1969.

Peachey, L.D.
J. Cell Biol., 20:95, 1964.

Peachey, L.D. and Rasmussen, H.
J. Biophys. Biochem. Cytol., 10:529, 1961.

Perkins, J.P.
In Advances in Cyclic Nucleotide Research, Vol. 3, P. Greengard and G.A. Robison, eds., Raven Press, N.Y., p. 1, 1973.

Petersen, M.J. and Edelman, I.S.
J. Clin. Invest., 43, 583, 1964.

Plagemann, P.G.W. and Estensen, R.D.
J. Cell Biol., 55:179, 1972.

Porter, K.R.
In Principles of Biomolecular Organization, G.E.W., Wolstenholme and M. O'Connor, eds., Little Brown Co., Boston, p. 308, 1966.

Reed, P.W. and Lardy, H.A.
J. Biol. Chem., 247:6970, 1972.

Rubin, C.S. and Rosen, O.M.
Ann. Biochem., 44:831, 1975.

Sachs, H., Fawcett, P., Takabatke, Y. and Portanova, R.
Rec. Prog. Hormone Res., 25:447, 1969.

Sapirstein, V. and Scott, W.N.
J. Clin. Invest., 52:2379, 1973.

Sawyer, W.H.
Pharmacol. Rev., 13:225, 1961.

Sawyer, W.H. and Schisgall, R.M.
Amer. J. Physiol., 187:312, 1956.

Scarpa, A. and Inesi, G.
FEBS Letters, 22:273, 1972.

Schilb, T.P.
Amer. J. Physiol., 216:514, 1969.

Schilb, T.P. and Brodsky, W.A.
Amer. J. Physiol., 210:997, 1966.

Schilb, T.P. and Brodsky, W.A.
Proc. Internat'l. Biophys. Congr., Viennia, 1966.

Schilb, T.P. and Brodsky, W.A.
Amer. J. Physiol., 222:272, 1972.

Schwartz, I.L., Scott, W.N., Kinne, R., Wyssbrod, H.R., Roth, L.B., and Walter, R.
Proc. 4th American Peptide Symposium, R. Walter and J. Meienhofer, eds., Ann Arbor Science Publishers Inc., Ann Arbor, Mich., p. 691, 1975.

Schwartz, I.L., Shlatz, L.J., Kinne-Saffran, E. and Kinne, R.
Proc. Nat'l. Acad. Sci., 71:2595, 1974.

Schwartz, I.L. and Walter, R.
Amer. J. Med., 42:769, 1967.

Schwartz, I.L. and Walter, R.
Protein and Polypeptide Hormones, Part 1, M. Margoulies, ed., Excerpta Medica Fdn., N.Y., p. 264, 1968.

Schultz, G., Hardman, J.G., Schultz, K., Baird, C.E. and Sutherland, E.W.
Proc. Natl. Acad. Sci., 70:3889, 1973.

Scott, W.N., Sapirstein, V. and Yoder, M.J.
Science, 184:797, 1974.

Scott, W.N., Shamoo, Y.E. and Brodsky, W.A.
Biochim. Biophys. Acta, 219:248, 1970.

Singer, I., Civan, M.M., Baddour, R.F. and Leaf, A.
Amer. J. Physiol., 217:938, 1969.

Singer, I., Dousa, T.P. and Hector, O.
Life Sciences, 9:765, 1970.

Singer, I., Rotenberg, D. and Puschett, J.B.
J. Clin. Invest., 51:1081, 1972.

Solinger, R.E., Gonzalez, C.F., Shamoo, Y.E., Wyssbrod, H.R. and Brodsky, W.A.
Amer. J. Physiol., 215:249, 1968.

Stoff, J.S., Handler, J.S., Preston, A.S. and Orloff, J.
Life Sciences, 13:545, 1973.

Sutherland, E.W. and Rall, T.W.
Pharmacol. Rev., 12:265, 1960.

Sutherland, E.W., Robison, G.A. and Butcher, R.W.
Circulation, 37:279, 1968.

Taylor, A., Mamelak, M., Reaven, E. and Maffly, R.
Science, 181:350, 1973.

Urakabe, S., Handler, J.S. and Orloff, J.
Amer. J. Physiol., 218:1179, 1970.

Wilczewski, T. and Brodsky, W.A.
Amer. J. Physiol., 228:781, 1975.

Zigmond, S.H. and Hirsch, J.G.
Science, 176:1432, 1972.