1962

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ROCKEFELLER INSTITUTE DOCTORAL DISSERTATION

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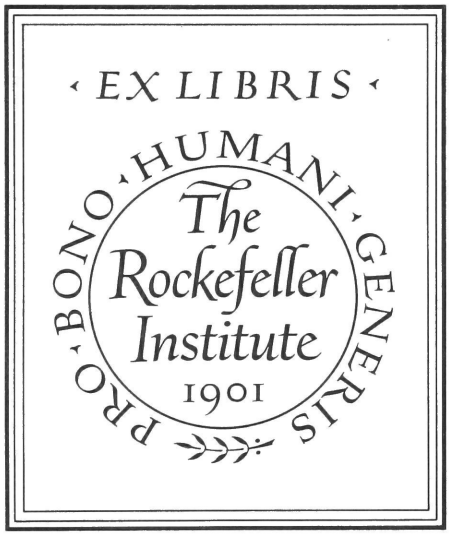

THE INACTIVATION OF NEUROHYPOPHYSEAL HORMONES BY THE TOAD BLADDER

A thesis submitted to the Faculty of The Rockefeller Institute

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

by

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Acceptable for Publication,
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15 April 1962

The Rockefeller Institute

New York, New York

PREFACE

The phenomena of biological transport are well known but poorly understood. Across the plasma membranes of all living cells there is a continuous exchange of material. Some substances move passively, others through the interposition of metabolic processes. In some cases the permeability remains constant in time, but in other important examples the permeability to certain substances can be radically altered. Altered permeability to certain ions accounts for the excitability of nerve and muscle, and altered permeability to water is basic to osmoregulation in land vertebrates. In nerve and muscle, the stimulus producing the alteration in permeability is either an electric current flow or a neurohumor, such as acetylcholine. In the osmoregulatory organs of the land vertebrates, the stimulus to alteration of water permeability is one of the neurohypophyseal hormones.

The work to be described herein was undertaken in an attempt to understand a little better how neurohypophyseal hormones stimulate a change in the water permeability of certain cells, in particular, the epithelial cells of the toad bladder. The mechanism of the permeability change was not uncovered. But something was learned of the ways in which these cells may limit the activity of the neurohypophyseal hormones and, hence, control the effect of the hormones upon them.

I would like to acknowledge the help and encouragement given me by my research advisers, Dr. Philip Siekevitz and Dr. Howard Rasmussen. I would like to thank Dr. Vincent du Vigneaud, Professor of Biochemistry, Cornell Medical School, for his generous gift of oxytocin. Finally, I want to thank Miss Elizabeth Bowman, who typed this thesis.

ABSTRACT

The urinary bladder of the toad, Bufo marinus, was homogenized and the particle-free supernatant fraction prepared. Incubation of this fraction with the hormones, oxytocin, arginine vasopressin, lysine vasopressin, and oxytocin ring amide caused their inactivation. The decrease in hormone activity was roughly exponential with the time of incubation. The hormone inactivating activity of the supernatant fraction was decreased by treatment with N-ethylmaleimide, by heating to 50° C for 5 minutes, and by cold storage at -20° C. The decrease in activity due to cold storage, but not that due to either of the other two treatments, was reversed by the addition of 1 mM cysteine shortly before incubation with hormone. Ammonium sulfate fractions of the supernatant fraction were prepared and tested for oxytocin inactivating activity. The fraction precipitating between 50 and 70% saturation was the most active. In the presence of cysteine, it inactivated 35 mμM oxytocin per mg protein per hour. In the absence of cysteine, the same fraction, after being passed through Sephadex G-25, inactivated 16 mμM oxytocin per mg protein per hour.

The incubation mixtures of the most active ammonium sulfate fraction and oxytocin (no cysteine) was examined chromatographically, following TCA precipitation of the protein. After an incubation of 4 hours at 30° C no oxytocin could be detected on a paper chromatogram. Moreover, there was an increase in ninhydrin positive material as compared with the controls. The same incubation mixture was subjected to performic acid oxidation and then chromatographed. A dense spot corresponding to cysteic acid was obtained. This was interpreted as indicating that the N-terminal half-cystinyl-tyrosine bond of oxytocin had been split.

The ammonium sulfate fractions were also tested for their splitting of the synthetic substrate L-cystine-di-β-naphthylamide (CDNA). This substrate, like oxytocin, contains a N-terminal cystinyl-peptide bond. Again the most active fraction was that precipitating between 50 and 70% saturation. In the presence of 1 mM cysteine, it liberated 70 mμM β-naphthylamine per mg protein per hour. This was about twice its rate of inactivation of oxytocin. This CDNAase activity was increased by cysteine and decreased by N-ethylmaleimide, iodoacetic acid, and oxidized glutathione. It was decreased

also by heating to 50° C for a few minutes. It appeared on the basis of these results that CDNA and oxytocin were being attacked by the same enzyme. The ammonium sulfate fractions were tested for their splitting of L-leucine-β-naphthylamide (LNA). The pattern of the fractionation of the LNAase activity was the same as that of the CDNAase activity. By a number of criteria, it appeared that LNA and CDNA were being split by the same enzyme. On the basis of these results it was concluded that a hormone inactivating enzyme is present in the toad bladder which splits N-terminal cystinyl-peptide bonds and also N-terminal leucinyl-peptide bonds. It was compared with pregnancy serum oxytocinase and leucine aminopeptidase and was found to be different than either of these.

The inactivation of oxytocin by the intact toad bladder was then tested. It was found that the effect of a dose of oxytocin on the water permeability of the bladder decreased with time, but that this decrease was not due simply to inactivation of the hormone. The bladder released into its serosal bath an inhibitor of the action of hormone, and it was inferred that this inhibitor was released in response to the hormone. A theoretical model of hormone action was constructed in which the hormone is inactivated in the epithelial cells of the bladder, and the product of this inactivation acts as a competitive inhibitor of the hormone in its attachment to a receptor site. The solution of the mathematical expressions of this model fit the experimental data of the time course of the action of the hormone, over the entire concentration range. In fitting the theoretical solution to the experimental data, six constants were obtained, four of which are probably characteristic of the hormone species. The implications of this kind of kinetic analysis for the understanding of hormone-structure-function relationships were discussed. It was suggested that a possible physiological role for the inactivating enzyme, found in the first part of this work, is to convert the hormone to an inactive product which acts as an inhibitor of hormone action. In this way, the action of hormone upon a cell could be controlled by the cell itself.

TABLE OF CONTENTS

INTRODUCTION

This thesis is a study of the interaction of neurohypophyseal hormones with the bladder of the toad, Bufo marinus. Two broad fields are involved here, hormone function and biological transport. In the controlled transport of water in certain vertebrate osmoregulatory organs and in the function of neurohypophyseal hormones, these two fields become demonstrably one. We shall begin our discussion with a short history of the function of the neurohypophysis and of its hormones.

A. History of the Neurohypophyseal Hormones

Only the highlights of the advance will be given. Dale (1957) has presented a more complete account of this story and of the confusion and artifacts that plagued early workers in this field. Before 1895 virtually nothing was known of the function of the pituitary gland, or hypophysis.

1895. Oliver and Schäfer discovered the pressor effect of pituitary extracts. They injected extract of whole pituitary intravenously into dogs and observed a rise in blood pressure caused by a pronounced vasoconstriction.

1898. Howell prepared extracts of various parts of the pituitary and found that the pressor substance was limited to the neural lobe.

1906. Dale discovered the oxytocic effect. He found that extracts of the pituitary caused contraction of the pregnant cat uterus in situ.

1909. Dale investigated the pressor substance. He found that it was excreted in the urine, that it was stable to pepsin and to boiling in dilute acid, but that it was readily destroyed by trypsin and hot alkali.

1910. Ott and Scott discovered the milk-ejecting effect of pituitary extracts.

1912. Paton and Watson discovered the blood pressure depressing effect of injected pituitary extracts in birds (opposite to the effect in mammals.

1913. Von der Velden and Faruci, separately, discovered the antidiuretic effect of pituitary extract in a successful application to the treatment of diabetes insipidus, a disease characterized by the excretion of a highly dilute and copious urine.

1919. Dudley achieved a partial separation of the oxytocic and the pressor activities, suggesting that at least two separate substances might be responsible for the effects of the extracts.

1925. Starling and Verney perfused an isolated kidney with a heart-lung preparation. The kidney produced a dilute, copious urine, as in diabetes insipidus. A much smaller volume of concentrated urine was produced instead if either pituitary extract was added to the perfusing blood or an isolated head was added to the preparation and the perfusate passed through it as well as through the kidney. This was good evidence for the physiological function of the antidiuretic substance in pituitary extract.

1928. Kamm and Grote achieved a good separation of oxytocin and vasopressin.

1953. Du Vigneaud and his co-workers and Tuppy, separately, determined the structure of oxytocin. Du Vigneaud and co-workers synthesized oxytocin, and, in the following years, synthesized the other naturally occuring hormones, arginine vasopressin, lysine vasopressin, and arginine vasotocin (Table I) as well as many interesting analogs of these.

B. Natural Distribution of the Neurohypophyseal Hormones

Oxytocin, or some peptide closely resembling oxytocin, is found in all vertebrates examined except in the cyclostomes (Table I). In addition, all non-mammalian vertebrates, except the elasmobranchs, elaborate arginine vasotocin.

Among the mammals, the neurohypophysis of the hog and of the hippopotamus contain lysine vasopressin, and that of all the other mammals examined contains arginine vasopressin.

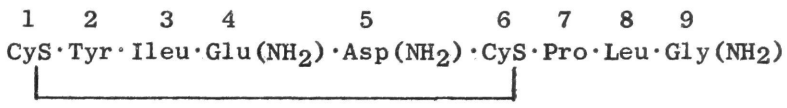
Later we will discuss the physiological functions of these hormones. First let us say a few words about the neurohypophysis itself.

C. Structure and Function of the Neurohypophysis

The principal point we want to make in this section is that the neurohypophyseal hormones are produced in the nerve cells of certain hypothalamic nuclei and are transported down the axons of these cells to their termination in the neurohypophysis where the hormones are stored until released into the circulation (Green and Maxwell, 1959).

TABLE I

The Naturally Occurring Neurohypophyseal Hormones



OXYTOCIN

Analog	Other name	Where found	References
Oxytocin		All vertebrates except cyclostomes.	Heller and Pickering (1961)
Arg^8 - oxytocin	arginine vasotocin	Cyclostomes, fresh water and marine teleosts, reptiles, and birds.	"
Phe^3, Arg^8-oxytocin	arginine vasopressin	Man, ox, horse, sheep, rat, dog, monkey, camel, oppossum, spiny anteater.	Dicker (1961) and Sawyer (1961).
Phe^3, Lys^8-oxytocin	lysine vasopressin	Hog and hippopotamus.	"

In Figure 1 (from Fulton, 1955) we can see the neural connections in the hypophysis of the cat. Note also the vascular connections, especially those between the posterior lobe and the anterior lobe. Table II contains a system of nomenclature for the mammalian hypophysis (Bard, 1956).

TABLE II

Nomenclature of the Mammalian Hypophysis

Adenohypophysis (Lobus glandularis)		1. Pars distalis (Anterior lobe) 2. Pars tuberalis 3. Pars intermedia (Posterior lobe)
Neurohypophysis	Lobus nervosus	1. Infundibular process (Posterior lobe)
	Infundibulum	1. Infundibular stem 2. Median eminence

A hypophysis is highly characteristic of the vertebrates (Green and Maxwell, 1959). It is not, as such, found among the other chordates. On the other hand, all invertebrates above the coelenterates have neurosecretory cells, and structures functionally analogous to the neurosecretory nuclei of the hypothalamus, to the neurohypophysis, and to the adenohypophysis are found in crustaceans and insects (Hanström, 1957).

It is also characteristic of vertebrates that the blood supply to the adenohypophysis first comes in contact with the neurohypophysis (Green and Maxwell, 1959). In the cyclostomes and fish the neurohypophysis is relatively simple, being little more than a thickening of the floor of the diencephalon. The blood supply to the adenohypophysis first passes adjacent to the neurohypophysis. It is thought that in these classes the neurohypophysis functions principally as a link in the central nervous system control over the adenohypophysis (Sawyer, 1961). The neural lobe, as a distinct division of neurohypophysis, appears first in certain lungfish and amphibians. It develops from the caudal portion of the primitive neurohypophysis and is distinguished from the median eminence by its characteristic blood supply. While effluent blood from the median eminence enters the hypophyseal portal vessels, the blood leaving the neural lobe enters the systemic circulation. Hence with the

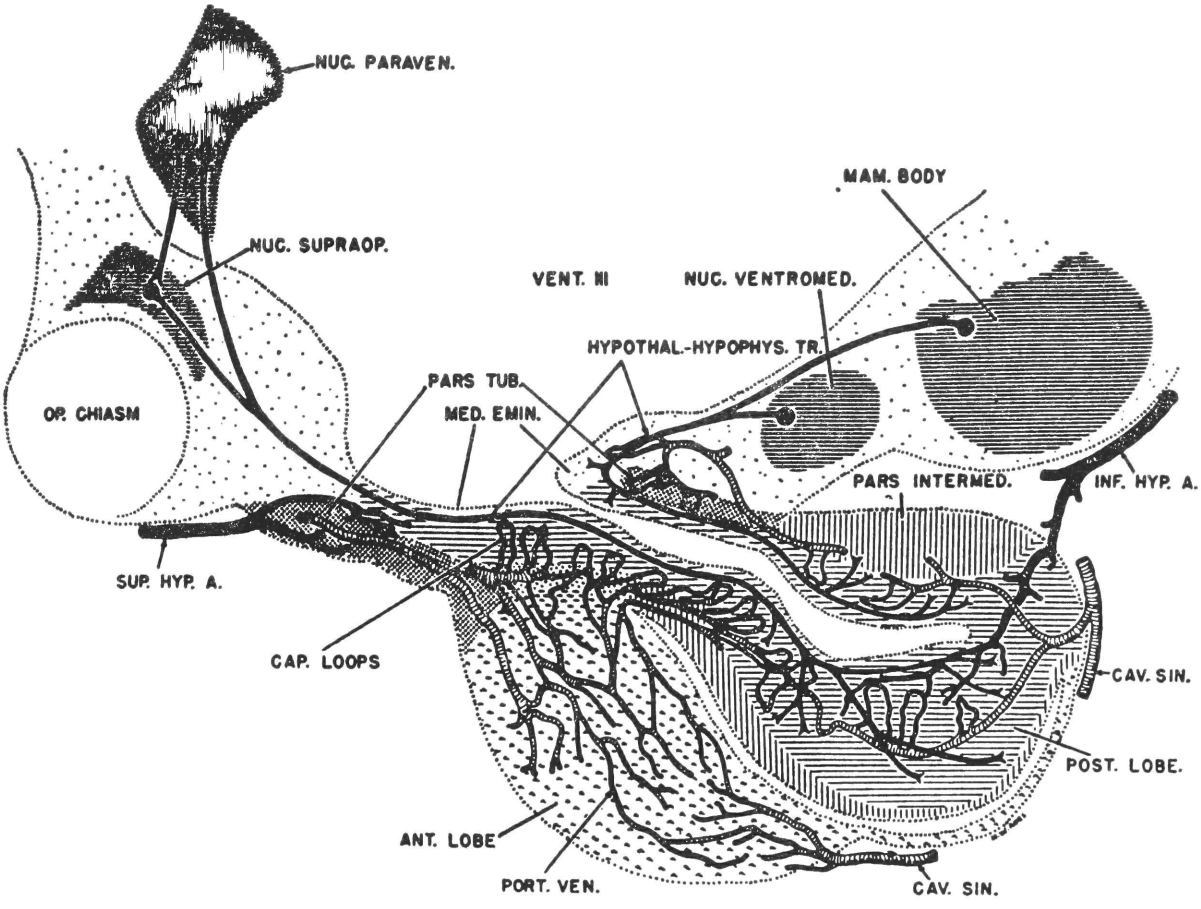


Figure 1. The principal neural and vascular connections of the hypophysis of the cat, in schematic longitudinal section. (From Fulton, 1955)

invasion of land, the primitive neurohypophysis differentiated and the neural lobe secreted into the general circulation substances intimately concerned, as we shall see, with osmoregulation.

In the next section we shall discuss briefly the storage of the hormones and their release from the neurohypophysis. After that we shall discuss the effects of the hormones on their target organs.

D. Storage and Release of the Neurohypophyseal Hormones

The ratios of vasopressor to oxytocic activities, assumed to equal approximately the ratios of oxytocin to vasopressin, are generally different in the hypothalamus and the neurohypophysis. For instance, in the dog's hypothalamus, V/O = 17, and in its neurohypophysis, V/O = 1.5 (Van Dyke, 1957). For many species (man, cat, rat, dog, sheep, pig, and horse), V/O of the neurohypophysis is approximately one (Acher and Fromageot, 1957). However this ratio may be quite different in the immature animal. At birth, in the rat, V/O = 12, while at 40 days, V/O = 1. These studies suggest independent synthesis of oxytocin and of vasopressin in mammals. Moreover, granules centrifuged from homogenates of the neural lobe contain 90% of the hormone activity, and a separation of these granules into a fraction with high pressor activity and one with high oxytocic activity has been reported (La Bella et al., 1962).

There is, incidentally, a great excess of stored hormone compared to the amounts needed to elicit a physiological response. For example, 1 mg of beef posterior pituitary powder contains 1 unit of oxytocic activity and 1 unit of pressor activity, while doses eliciting definite physiological effects are of the order of magnitude of a few milliunits and often less.

Release of hormones from the neurohypophysis can be elicited in many ways. Intracarotid and intravenous injections of hypertonic saline solution, injection of hypertonic saline directly into the hypothalamus, electrical stimulation of the neurohypophysis or of the nucleus supraopticus, injection of acetylcholine into the nucleus supraopticus, hemorrhage or other acute reductions in extra-cellular volume, dehydration, suckling, and coitus are stimuli which release hormone (Dicker, 1961; Share, 1961).

Whatever the stimulus, oxytocin and vasopressin are released together, and, judging from the resultant physiological effects, twenty to one hundred times as much oxytocin as vasopressin is released per stimulus (Dicker, 1961).

E. Antidiuretic and Related Effects of the Neurohypophyseal Hormones

It has already been mentioned that the development of a separate neural lobe, releasing its contents into the systemic circulation, is coincident with terrestrialism. We shall see in this section that the hormones of the neural lobe stimulate water-conservation. Now marine fish conserve water through more or less permanent structural modifications of their kidney such that their urine flow is quite low, and through the excretion of excess salts and of nitrogenous wastes by their gills. Fresh-water fish, on the other hand, put out a voluminous, dilute urine, and reabsorb some salt in kidneys and some salt from the environment through the gills. For these aquatic vertebrates, the environment and its stresses are relatively constant. If the environment does change, as it does for the eels and salmon, the first of which breeds at sea and matures in fresh water, the second of which breeds in fresh water and matures at sea, the changes are gradual and long-lasting. But for the typical land vertebrate, one moment scorched by the sun, the next moment deluged by rain, some mechanism seems necessary to permit rapid osmoregulation. Such a mechanism is the complex of osmoreceptors in the brain, plus hormonal transmitters, plus effector organ or organs, all of which are present in the tetrapods. What these organs are specifically, and how they work, we will discuss next. There are, of course, other important mechanisms of osmoregulation, and we will try to mention some of these as well. An interesting and charming account of the evolution of vertebrate osmoregulation is presented by Homer Smith (1959).

1) Vertebrate osmoregulation: Little is known about the mechanism of water and salt balance of the most primitive living vertebrates, the cyclostomes (Krogh, 1939). The tissues and body fluids of the marine forms are in osmotic equilibrium with the environment. One species, Petromyzon marinus, matures in the sea but migrates to fresh water to breed. Two other species live in fresh water but can penetrate into brackish water. The urine is produced by ultra-filtration, and there is probably some excretion and reabsorption in a short convoluted tubule.

The elasmobranchs maintain themselves by the unique trick of retaining urea. The blood of the marine species is hypertonic to sea water. The electrolyte concentration of their blood, however, is about the same as that of fresh water teleosts. The difference is made up by urea, the concentration of which may reach 2.0 - 2.5%. The urea concentration in the blood of most vertebrates is about 0.01 - 0.03%. The urine of elasmobranchs is hypotonic to their blood. They actually have excess water due to their high internal concentration of urea. They also possess an anal salt gland which secretes a solution isotonic to blood with twice the NaCl concentration and one-twentieth the urea concentration. The fresh water sawfish, Pristes microdin, excretes a very dilute urine and retains far less urea than sea water forms (Smith, 1936).

Fresh water teleosts excrete a copious, dilute urine. The kidney tubules reabsorb salt from the ultra-filtrate. Salt is absorbed by the gills; e.g., the goldfish will reduce the concentration of Cl^- from 1 mM to 0.02 mM (Prosser, 1952). Water enters chiefly through the gills and oral membranes. Fresh water fish do not drink, and their integument is impermeable to water.

Marine fish drink sea water, absorb both salt and water from the intestine, and excrete Mg^{++}, Ca^{++}, and $SO_4^=$ in the urine, and excess Na^+, K^+, and Cl^- through the gills (Prosser, 1950). Urine output is much reduced, and commonly the nephron degenerates, with a shortening of the tubules and even a disappearance of the glomeruli as in the toad fish, Opsanus tau. In this species the urine flow is 2.5 ml per kg per day as compared to 200-400 ml per kg per day in the fresh water gar-pike. In sea water the eel drinks and excretes excess salt through its gills. In fresh water it excretes a dilute urine and replaces salt by feeding.

The African lung-fish, Protopterus aethiopicus, is an interesting water conserver (Smith, 1959). In summer it burrows and secretes a hardening mucous. It aestivates in brick-hard mud and can remain viable for years.

The importance of neurohypophyseal hormones in water balance in fish is uncertain, even though a plentiful amount of arginine vasotocin is present (Sawyer, 1961). Neither mammalian nor teleostean posterior lobe preparatives appear to influence water retention either in marine or in fresh water fish (Pickford, 1959).

"When the Amphibia abandoned the scales of their piscine ancestors in favor of a naked skin they encountered a double hazard, excessive hydration of the body when they were immersed in water and excessive dehydration when they left it" (Smith, 1959). The plasma of a frog (Conway, 1945) or of a toad (Leaf et al., 1958) is isotonic to about 120 mM NaCl. The skin is permeable to water (von Hevesy et al., 1935). In fresh water, then, considerable water flows in and must be eliminated. Again, as in fresh water teleosts, balance is achieved by excretion of a large volume of highly dilute urine (Krogh, 1939). Dilution of the urine occurs in the distal convoluted tubule (Walker et al., 1937).

In air, amphibians lose water rapidly. There is, in this respect, little difference in the rate of loss of water from equally large individuals of fully aquatic species (e.g., Rana clamitans) and almost fully terrestrial species (e.g., Bufo boreas) (Thorson, 1955). Frogs and toads regain lost water by absorbtion through the skin. Tadpoles drink but adults have not been observed to do so (Thorson, 1955). Dehydration and injections of neurohypophyseal hormones increase the rate of uptake of water through the skin (Ewer, 1952).

Many species respond to dehydration or neurohypophyseal hormones by an antidiuresis. Dehydrated Rana pipiens and Bufo bufo almost cease to form urine (Chew, 1961), and dehydration or injection of neurohypophyseal hormones produce an antidiuresis in Bufo marinus (Sawyer, 1957) (Figure 2). Sawyer found that at moderate doses there was a pronounced decrease in urine flow with no change in glomerular filtration rate (Figure 2). At higher doses the filtration rate was decreased somewhat, but he concluded that tubular reabsorbtion of water accounted almost entirely for the antidiuresis. No amphibian, of course, can produce a urine more concentrated than its plasma, and as a rule the urine is hypotonic to plasma.

Charles Darwin considered it "well ascertained, that the bladder of the frog acts as a reservoir for the moisture necessary to its existence...". Injections of Pitressin and Pitocin stimulate the transfer of water from the bladder to the lymph in Bufo carens and Bufo regularis (Ewer, 1952). We will have more to say on the subjects of the bladder and the skin when we discuss their in vitro preparations.

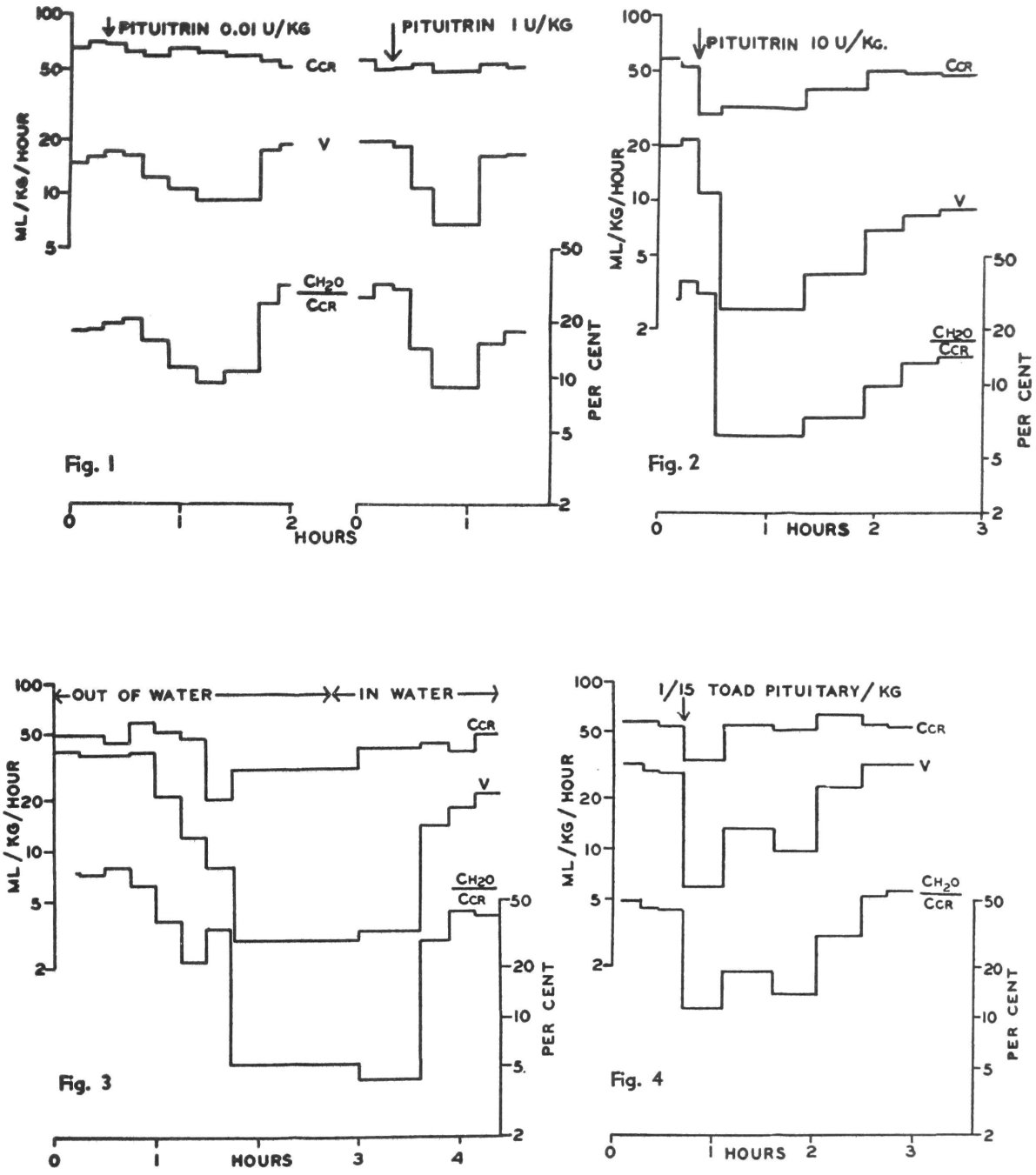


Figure 2. The effect in the toad of mammalian neurohypophyseal extract (Pituitrin), dehydration, and toad pituitary extract on creatinine clearance (C_{cr}), urine flow (V), and free water clearance (C_{H_2O}). (From Sawyer, 1957)

The reptiles are well adapted to land (Prosser, 1950). They all either are viviparous or lay cleidoic eggs. Their skin is impermeable to water. Though, at best, their urine is only isotonic with their plasma, very small quantities are excreted. For example, the lizard, Trachysaurus rugosus, when hydrated forms 0.57 ml per 100 gm per hr and when dehydrated, 0.024 ml per 100 gm per hr, which is only 5% of the rate for a dehydrated rat (Chew, 1961). Two factors contribute to the low urine flow, a low filtration rate and the excretion of uric acid. The renal corpuscles of most land reptiles are poorly developed to begin with, and neurohypophyseal hormones can reduce filtration rates even more. These hormones cause an antidiuresis in T. rugosus, for instance, similar to that observed in dehydration. Moreover, there is osmotic reabsorption of water from the more or less hypotonic urine in the cloaca. As water is withdrawn, uric acid precipitates, freeing more water for reabsorption. The principal avenue of water loss in the land reptiles is evaporation from the lungs. This is diminished by behavioral adjustments such as burrowing and quiescence. Water is replenished in most reptiles by drinking. (Darwin describes the gargantuan drinking of the giant Galapagos tortoise. This beast seems to store fresh water in its bladder for several days). In some species, excess salt is eliminated by nasal secretion of a concentrated saline solution; this occurs in a brackish water terrapin, Malacolemys terrapin, and in the loggerhead turtle, Caretta caretta. The same function is ascribed to the nasal gland of sea snakes and of sea crocodiles (Schmidt-Nielsen and Fange, 1958).

Birds can produce urine hypertonic to their plasma. This ability is associated with the appearance of a thin segment, the loop of Henle, in the bird nephron. The maximum concentration of urine of the house finch, cormorant, and chicken is about twice the concentration of their plasma (Chew, 1961). Neurohypophyseal hormones or dehydration cause an increase in concentration of the urine, and also a decrease in the filtration rate down to 10% of the normal rate. As in reptiles, the renal corpuscles are poorly vascularized. Birds, also, are uricotelic. Evaporative loss from birds is high. They replenish their water by drinking. In response to salt loading, such as the ingestion of sea water, marine birds secrete a concentrated salt solution from their nasal gland; e.g., 900-1,000 mEq. Na^+ per l. is the maximum concentration of the nasal secretion of Leach's Petrel (Schmidt-Nielsen, 1960).

In mammals, the kidney can produce highly concentrated urine. The maximum ratios of urine concentration to plasma concentration for a few species are (Smith, 1959): man, 4.2; dog, 4.5; seal, 5.6; kangaroo rat, 17. A diagrammatic representation of the mammalian nephron is shown in Figure 3. The renal corpuscle and the tubules are in the cortex of the kidney, and the loops of Henle extend into the medulla, parallel to the collecting ducts. The concentrating ability of the mammalian kidney is directly related to the thickness of the renal medulla (Schmidt-Nielsen and O'Dell, 1961). The thickness of the medulla is an indication of the length of the loops of Henle. Micropuncture and direct cryoscopic examination have indicated that in the concentrating kidney there is a progressive increase in electrolyte and urea concentrations from the cortex to the medullary papilla, and moreover, that the concentrations in the collecting ducts, in the loops of Henle, and in the blood vessels are equal at each level (Wirz, 1957; Wirz, 1961). The mechanism, now accepted, explaining these observations is as follows: First, about 85% of the glomerular filtrate is isotonically reabsorbed in the proximal tubules. The urine then passes through the loops of Henle. In this process Na^+ is actively reabsorbed from the contents of the lumen along the ascending limb of the loop, which is impermeable to water. The descending limb, through which the urine passed first, is permeable to water, or to water and salt. In either case, the concentration of the urine increases by passive diffusion of water out, or water out and salt in, as it flows down the descending limb into the progressively more concentrated regions of the medulla. As the urine turns the corner at the tip of the loop and begins to ascend, a progressive dilution occurs due to the active reabsorbtion of salt into the extra-lumenal fluids. This arrangement is formally analogous to the counter-current multiplication system proposed by Wirz, Hargitay, and Kuhn (1953), and, according to their analysis, would account for the observed concentration profile. When the urine reaches the distal convoluted tubule, in the cortex, it is hypotonic. In the concentrating kidney, the distal tubule is permeable to water and reabsorbtion occurs to isotonicity. Now, the urine flows down again through the collecting ducts. It is here that the final concentrating occurs. In the concentrating kidney, the collecting duct epithelium is permeable to water, and the urine comes into osmotic equilibrium with the progressively more concentrated fluids in passing from the cortex through the medulla. The antidiuretic effect of the neurohypo-

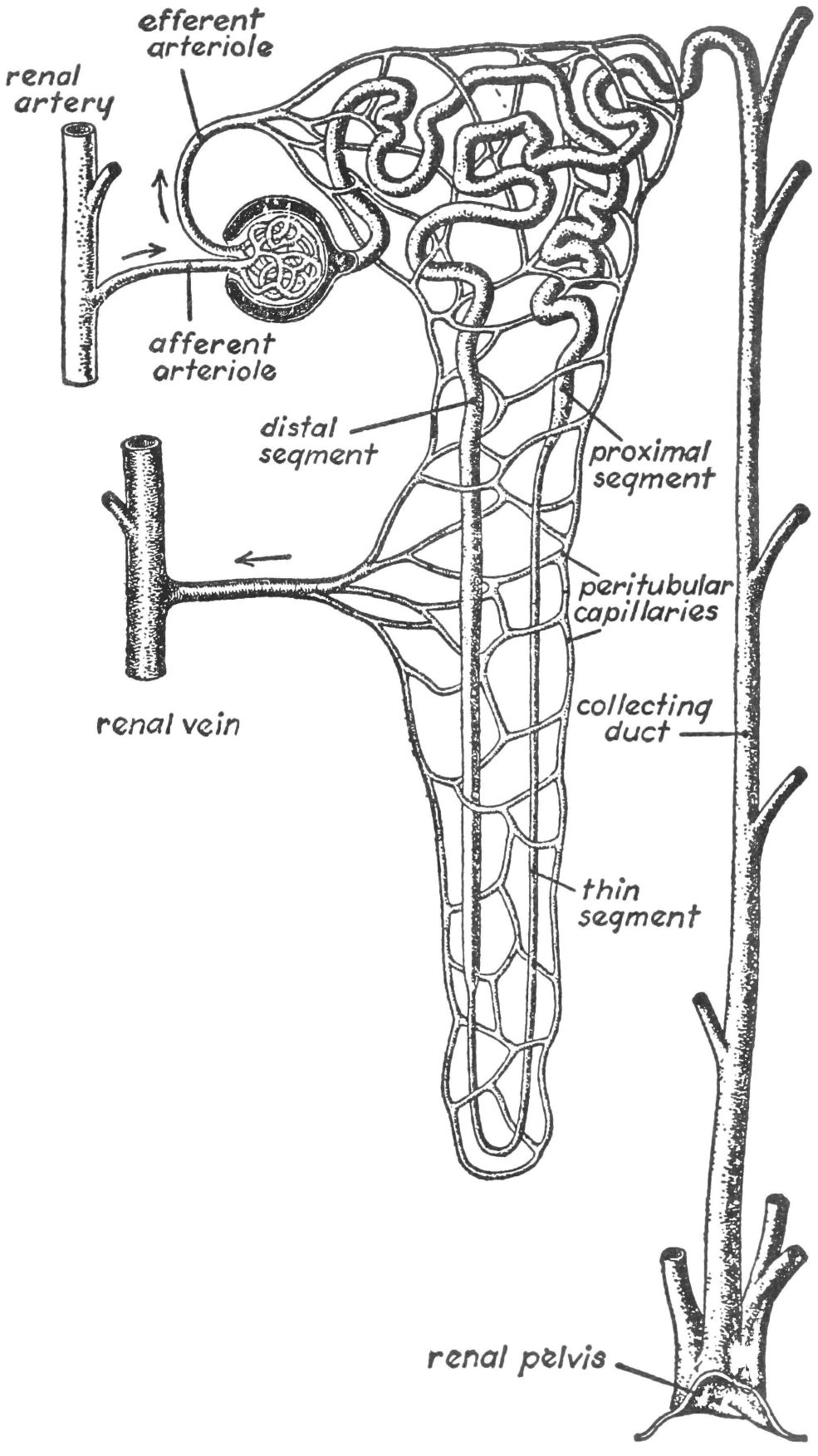


Figure 3. Diagrammatic representation of the mammalian nephron. (From Smith, 1959)

physeal hormones (Figure 4) is explained in terms of this mechanism as increasing the water permeability of the collecting ducts, of the distal convoluted tubules, and, possibly (Wirz, 1961) of the descending limb of the loop of Henle (Figure 5). Antidiuresis is achieved without significant change in the glomerular filtration rate. In diuresis, in the absence of antidiuretic hormones, water is not reabsorbed from the urine in the distal tubule, and urine entering the collecting ducts is hypotonic, and of greater volume than during antidiuresis. In passing through the collecting ducts there is now no change in concentration or in volume, and a dilute, voluminous urine results. The energy requiring step in the concentration of urine is the transport of salt, both in the proximal tubules, in order to reduce the volume entering the loops, and in the loops themselves, in order to produce the necessary concentration profile. Mechanical energy, supplied by the heart, is also necessary, for if the flow is stopped, the mechanism fails (Wirz, 1961). The action of the antidiuretic hormones, then, is to permit passive flow of water down pre-existing concentration gradients. We shall see that these hormones have a similar function in the amphibian skin and bladder. The efficiency of the concentrating mechanism of the mammalian kidney is such that it, together with certain behavioral adjustments, permits certain species to remain in balance on metabolic water solely. The kangaroo rat, Dipodomys merriami, can live without water on air-dried seeds containing 5-10% free water (Chew, 1961).

2) Amphibian skin and bladder: The effects of neurohypophyseal hormones on the isolated skin and bladder have been reviewed by Leaf and Hays (1961). These hormones increase the permeability to water of the skin and of the bladder when added to the solution bathing the serosal side of these membranes. Water moves passively across these tissues in the direction of its concentration gradient, at a rate linearly proportional to this gradient. The bag-shaped bladder can be simply tied to the end of a glass tube, dilute Ringer's placed inside (the mucosal side) and the tube and bladder suspended in Ringer's, in contact with the serosal side, to which hormone can be added. The water loss can be determined by periodic weighings of the tube and attached bladder. The effects of hormones on such preparations of the toad and frog bladder are shown in Figure 6.

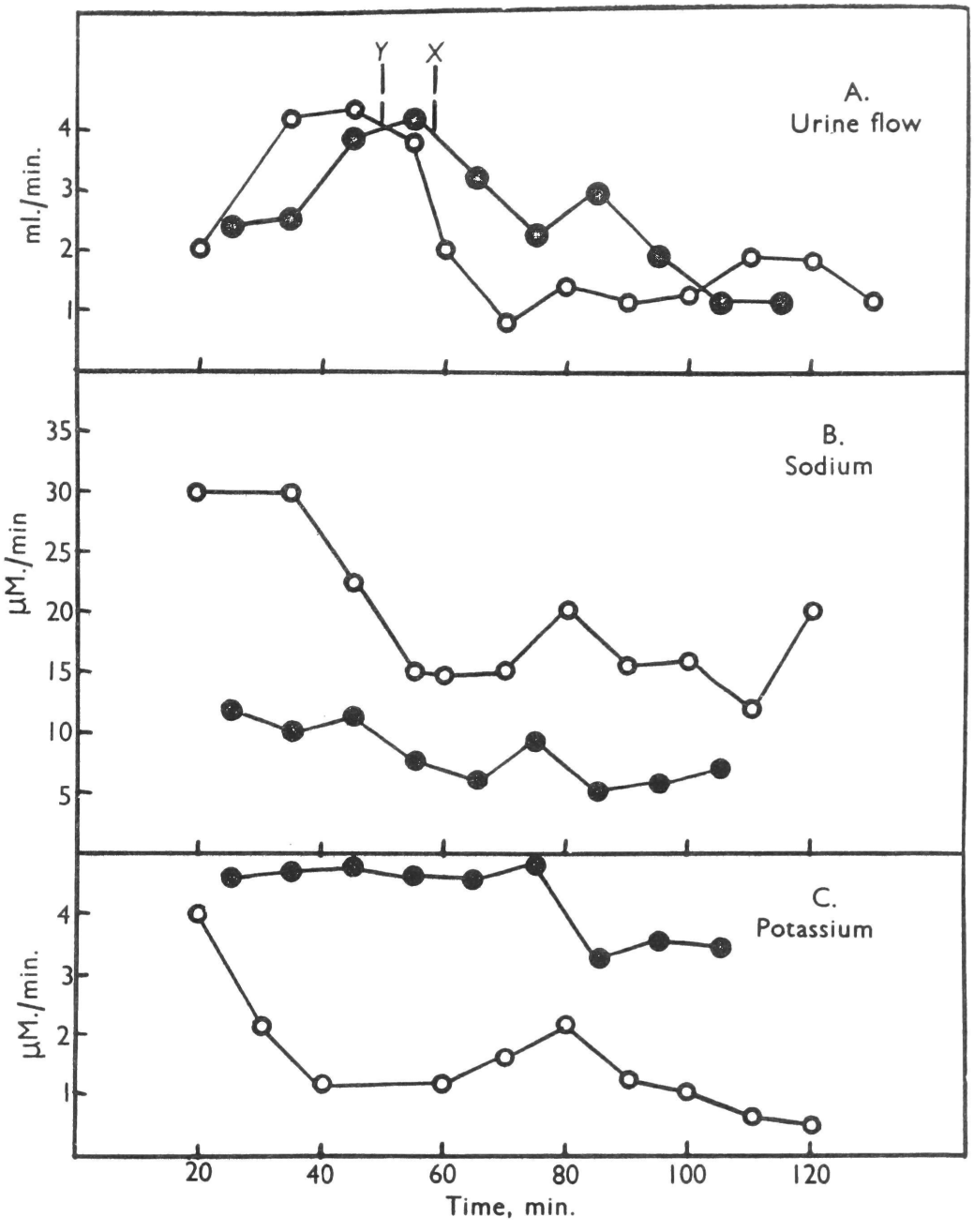


Figure 4. The effect of oxytocin and vasopressin on urine flow, Na, and K excretion during water diuresis in the dog.

The animal was given 300 ml water by mouth at zero time. The open circles represent the results of the intravenous injection of 60 mU oxytocin at X. The filled circles represent the results of the intravenous injection of 4 mU vasopressin at Y. (From Brooks and Pickford, 1957)

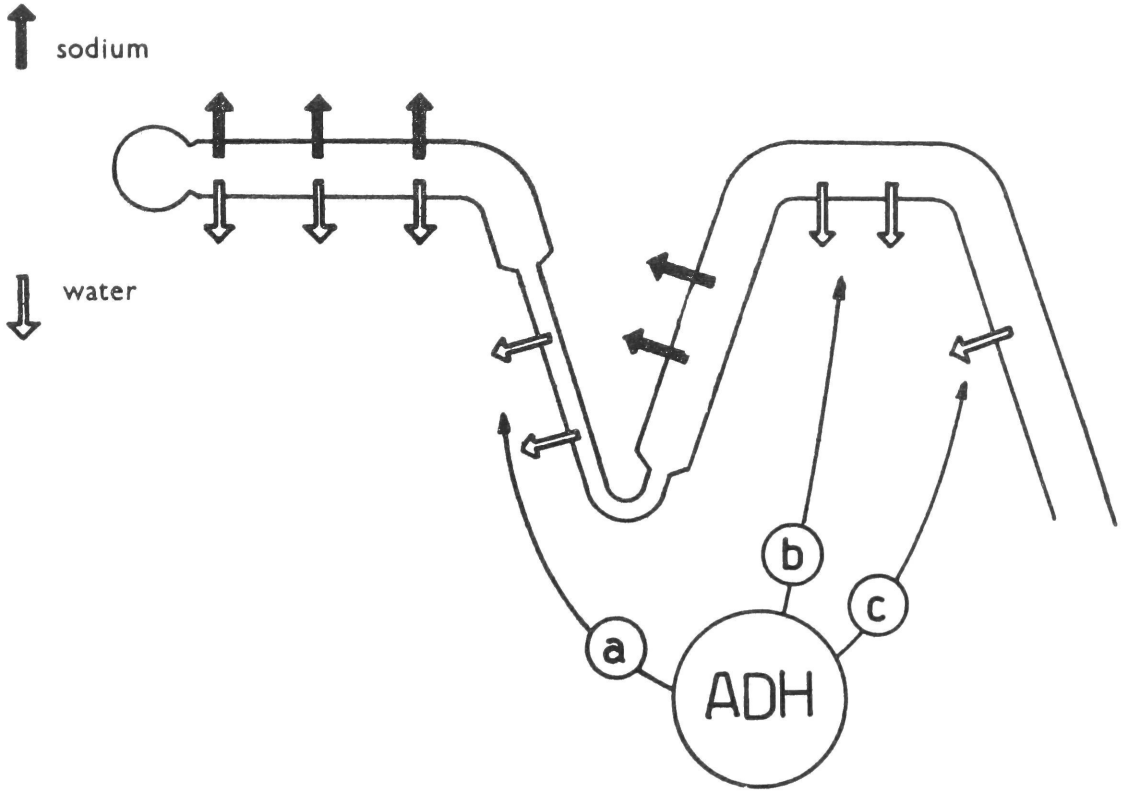


Figure 5. The possible sites of action of the antidiuretic hormone (ADH) in the nephron and the collecting duct. (From Wirz, 1957)

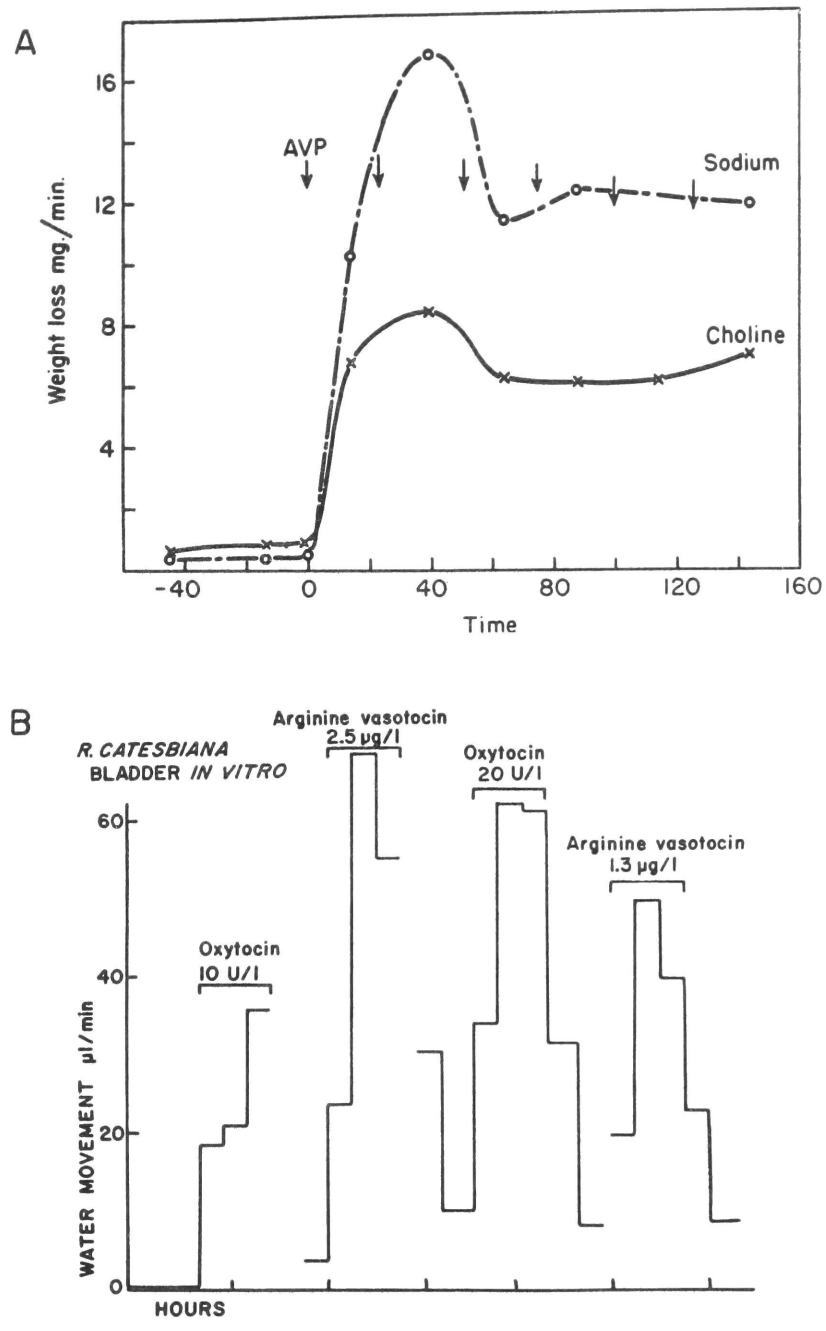


Figure 6. The effect of the neurohypophyseal hormones on the water permeability of the amphibian bladder.

A. The effect of AVP, 0.0125 µg per ml, upon the weight loss of the toad bladder, in ordinary Ringer's and in Ringer's containing choline in place of sodium. The serosal solution and the hormone were renewed at the arrows. The time is in minutes. (From Rasmussen et al., 1960). B. The effects of oxytocin and arginine vasotocin on a single bullfrog bladder. (From Sawyer, 1960).

Now it was early observed that the movement of deuterium-labelled water across the skin or bladder, in the absence of an osmotic gradient, could not account for the net flux of water observed in osmotic flow. In fact, in the absence of hormone, such diffusion flow could account for only one-sixth of the net flux, and, in the presence of hormone, for less than 1% of the net flux. One way of explaining these observations is to assume that water moves through the permeability barriers of the skin and of the bladder in pores, through which an osmotic pressure gradient acts as an equivalent hydrostatic pressure gradient in producing a bulk flow of fluid. Hormone, it is proposed, acts to increase the average radius, r, of these pores. Assuming laminar flow through these pores, the bulk flow increases with r^4, while the diffusion flow only with r^2. According to Leaf's calculations, the average pore radius before hormone is 8 $\mathring{A}$ and after hormone, 40 $\mathring{A}$, a figure larger than one would think practical. Qualitatively, nonetheless, the aqueous channel theory gains support from the observed permeability and flux of small, uncharged, water-soluble molecules, in the presence and absence of hormone and of bulk flow. One modification which has been proposed is that a thin diffusion barrier may be in series with the hormone-effected, porous barrier. If this diffusion barrier were thin enough, and permeable enough to water, the flow of water would have the same character as that through pores while the movements of other molecules, such as thiourea, would be retarded.

In the toad bladder, the permeability barrier effected by the neurohypophyseal hormones is probably at the mucosal surface of the epithelial cells. The epithelium, covering the mucosal surface of the bladder of the toad, Bufo marinus, is 3 to 10 microns thick and consists of squamous epithelial cells, goblet cells, and a third class of cells containing many mitochondria. This epithelium is supported on a lamina propria 30 to several hundred microns thick and containing collagen fibrils, bundles of smooth muscle fibers, and blood vessels (Figure 7 and 8; Peachey and Rasmussen, 1961). The epithelial cells, which greatly outnumber the other types of cells in the epithelium, are tightly held together by a terminal bar apparatus, and are closely apposed near the bladder lumen as well. Phase micrographs (Figure 9) of the bladder, fixed during hormone-induced, osmotic flow of water, with dilute Ringer's on the mucosal side, show swelling of the epithelial cells as compared to the controls without hormone, making it very probable that

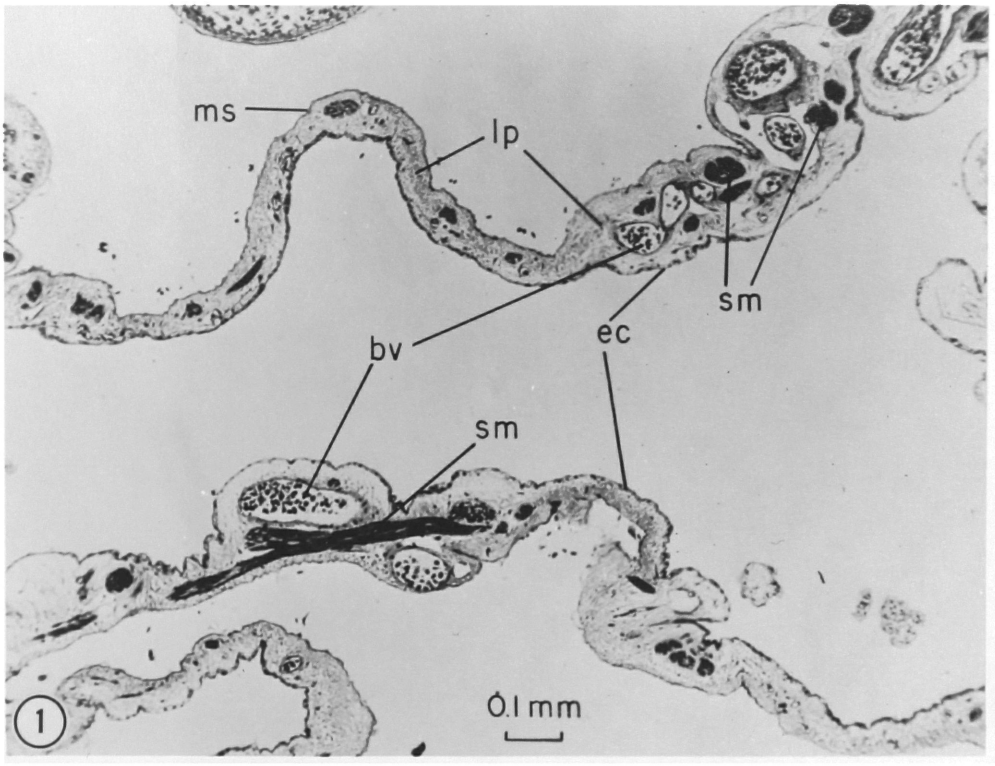


Figure 7. Low magnification light micrograph of a paraffin section of the toad's urinary bladder stained with iron alum-iron hematoxylin and counterstained with eosin. The epithelium (ec) covers the lamina propria (lp) on its mucosal surface. Within the lamina propria are blood vessels (bv) and smooth muscle fibers (sm). An incomplete mesothelium (ms) is found on the serosal side of the bladder wall. Spencer 32 mm objective. X 75. (From Peachey and Rasmussen, 1961).

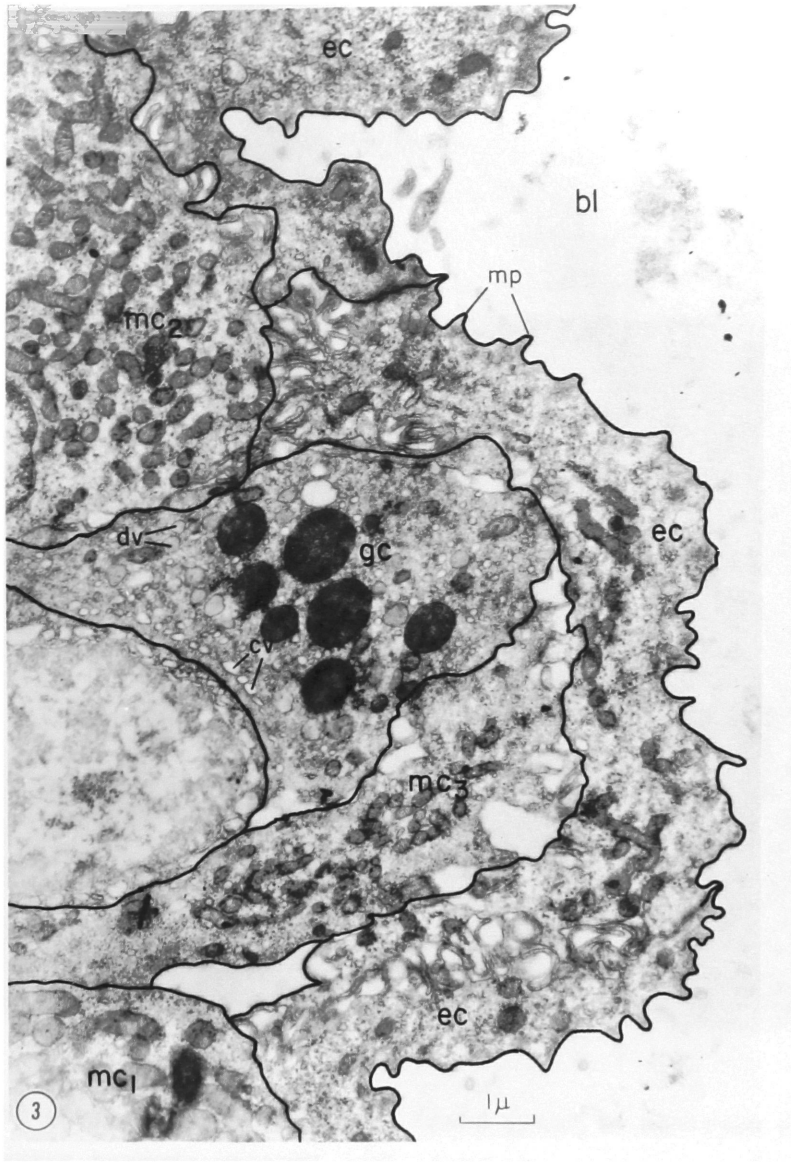


Figure 8. Survey electron micrograph of a normal bladder epithelium illustrating three types of cells found in this cell layer. Three squamous epithelial cells (ec) form the surface bounding the bladder lumen (bl) in this figure. Microprojections (mp) extend from these cells into the lumen. Also indicated are a goblet cell (gc) containing large, dense droplets and vesicles with either clear (cv) or dense (dv) contents. Three cells containing numerous mitochondria are also seen. Within each of these latter cells, the mitochondria are of uniform size, but there is considerable variation in size from one cell to another. The largest mitochondria are found in cell mc_1; cell mc_2 has mitochondria of an intermediate size, and cell mc_3 has the smallest mitochondria in this field. EMU-2B. X 14,000. (From Peachey and Rasmussen, 1961)

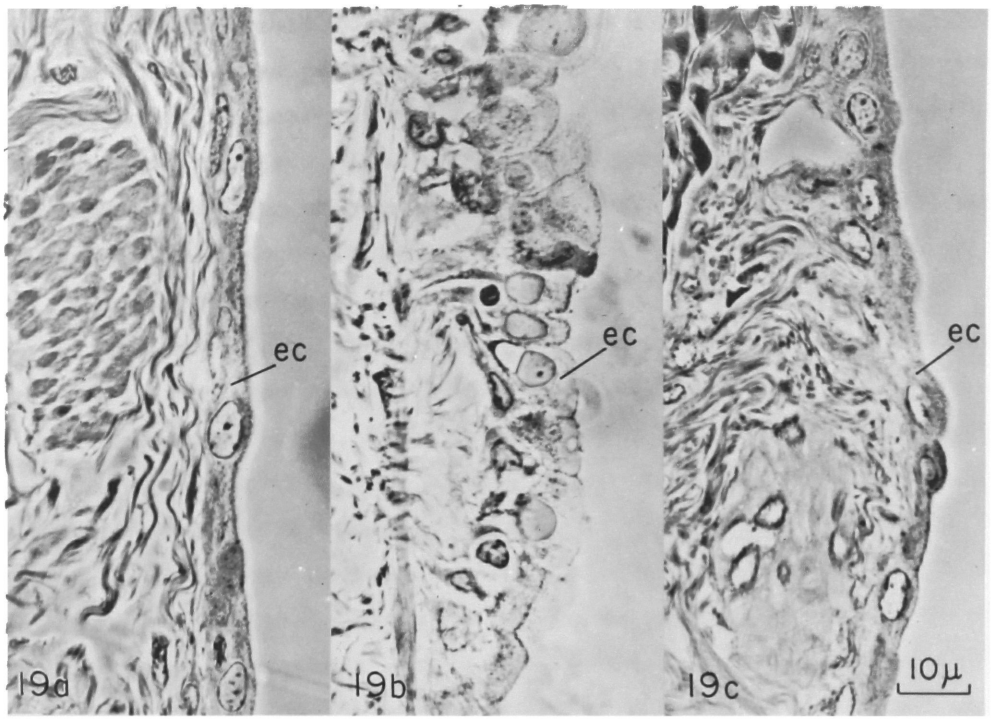


Figure 9. Phase micrographs of bladders fixed prior to addition of hormone (a), during hormone-induced water flow (b), and after removal of the hormone (c).

In all cases, the bladder had dilute Ringer's on the mucosal side and Ringer's on the serosal side. The epithelial cells (ec) fixed in the presence of hormone appear swollen (b). Zeiss X 90 phase objective. X 900. (From Peachey and Rasmussen, 1960)

the passage of water is actually through these cells. Dilute Ringer's placed on the serosal side of the bladder, even in the absence of hormone, causes similar swelling of the epithelial cells. The conclusion drawn from these observations is that the serosal surface of the epithelial cells is normally freely permeable to water, while the mucosal surface is relatively impermeable and becomes permeable only in the presence of hormone (added to the serosal side!). Leaf reports similar anatomical evidence from his laboratory (Leaf and Hays, 1961). Actually the first indication of the mucosal barrier was the observation by Leaf that, during anaerobic metabolism, considerably more lactate accumulated on the serosal side of the bladder than on the mucosal side. When the permeability of the two surfaces to lactate was measured, the serosal surface was found to be fifteen times more permeable than the mucosal surface. Similarly, the labelling of tissue water with T_2O or C^{14}-urea, from the mucosal side, was significantly greater in the presence of hormone than in its absence, as would be expected were the hormone to act on the mucosal surface of the epithelium, the serosal surface being more or less unchanged. Lastly, measurements of the inulin space of the bladder with dilute Ringer's on the mucosal side, in the presence and absence of hormone, showed a nearly 50% increase in cell volume, that is, of the non-inulin space, in the presence of hormone. This evidence, of course, supplements the microscopic observations of swelling of the epithelial cells during osmotic passage of water, and is further proof that the water passes through the epithelial cells, not between them, and that the hormone affects a barrier at or near the mucosal surface of these cells.

The toad bladder, as well as the skin, develops a spontaneous potential difference between mucosal and serosal surfaces of the order of 50 millivolts, with the serosal side positive to the mucosal side. A net flux of Na^+ from mucosal to serosal side, counter to its external electrochemical gradient, is observed to take place. An external potential difference can be impressed upon the toad bladder, just cancelling its spontaneous potential difference. In this state, with the potential difference between two points, close to either surface, equal to zero, the current across the bladder is called the "short-circuit current" (Ussing and Zerahn, 1951). This current is carried entirely by Na^+, the net flux of Na^+ being equal to the current flow, and is, therefore, a good measure of the active transport of Na^+. Hormone, added to the serosal surface of the bladder, enhances the short-

circuit current by a factor of 1.5 to 2 (Figure 10). (Hormone incidentally increases water permeability 25-50 times). At the same time, neurohypophyseal hormone significantly increases oxygen consumption and lactate formation, and decreases glycogen content; but these effects of hormone are abolished in sodium-free Ringer's solution, whereas the metabolism, in the absence of hormone, is only slightly less in the absence of Na^+ compared to that in the presence of Na^+. In other words, neurohypophyseal hormone, in the presence of Na^+, increases the metabolism above the level obtained both in the presence and the absence of Na^+. It certainly seems likely that this extra metabolism is coupled with the increased Na transport.

On the other hand, the increase in net water flux, in the presence both of an osmotic gradient and of hormone, does occur in the complete absence of Na^+ and under other conditions during which Na^+ transport does not occur (Figure 6). The absence of Na^+ transport, of course, means the absence of a stimulatory effect of hormone on oxygen consumption. Rasmussen and co-workers (1960) found that arginine vasopressin increased the permeability of the bladder kept at 2.5° C, under nitrogen, in choline Ringer's, and in the presence of 10^{-3} M CN^-. In a separate experiment it was found that 10^{-2} M fluoroacetate, an agent known to inhibit glycolysis, did not inhibit the effect of hormone on the water flux, in choline Ringer's. There was a slight effect in Na Ringer's. On the basis of this evidence it is likely that the increase and maintenance of the water permeability, in the presence of hormone, is not, to any large extent, an energy-requiring process.

Evidence for a site of attachment of the neurohypophyseal hormones, and possible characterization of this site, have been the objects of further experiments with the toad bladder.

Bladders were treated with tritium-labelled arginine vasopressin. These showed a full physiological response. At the peak of the response to hormone, the bladders were rinsed and fixed in ethanol. One set was incubated with 0.1 M glycine in 0.15 M $NaHCO_3$, another in 0.1 M cysteine in 0.15 M $NaHCO_3$. The residual activity of the bladders of each set was determined. The set washed with cysteine had considerably less residual radioactivity (Schwartz et al., 1960). If the same experiment were performed on bladders previously exposed to 10^{-3} M N-ethylmaleimide (NEM), known to prevent the effect of arginine vasopressin on the bladder (Rasmussen et al., 1960), no

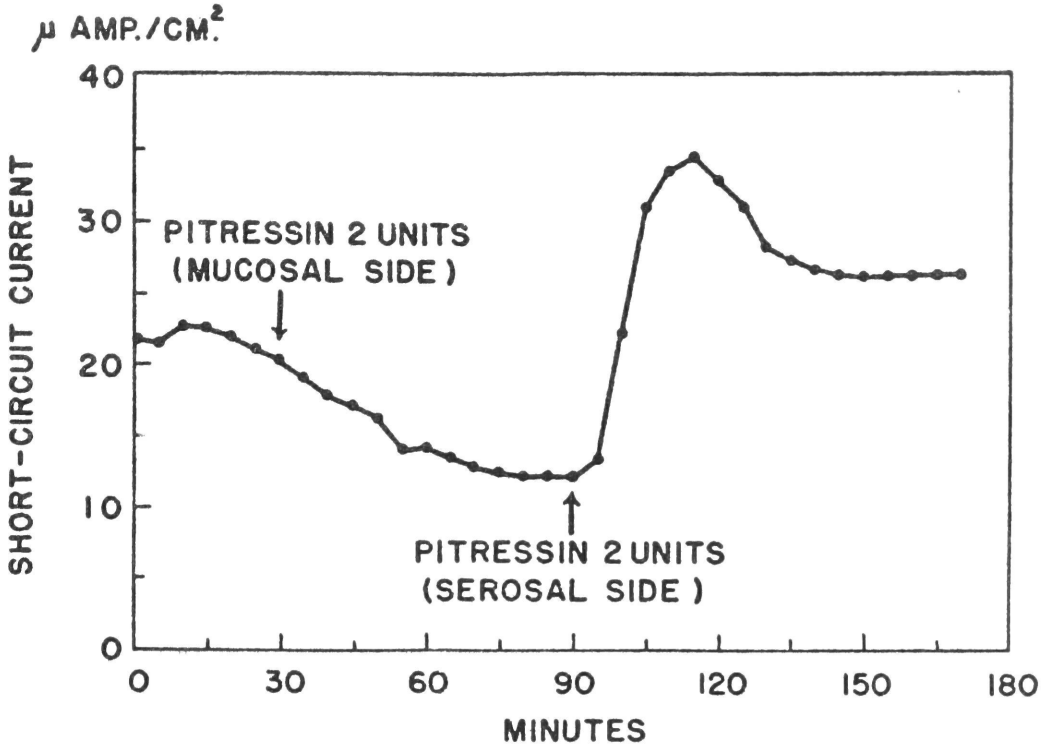


Figure 10. The effect of Pitressin on the short-circuit current across a toad bladder.

Addition of hormone to the mucosal side had no effect. (From Leaf et al., 1958)

significant difference between the residual activities of glycine washed and of cysteine washed bladders was observed. Similar findings were obtained with pre-treatment of the bladders with methylmercuribromide and parachloromercuribenzoate, also known to inhibit the response to arginine vasopressin.

Also, Rasmussen and his co-workers (1960) found that N-ethylmaleimide added before arginine vasopressin inhibited the increase in permeability, but that N-ethylmaleimide added after arginine vasopressin inhibited the decrease in permeability usually observed on the removal of AVP. Altogether, this evidence suggested to these workers that the neurohypophyseal hormones are fixed to their site of action in the bladder by a reversible sulfhydryl-disulfide interchange reaction. Such a mechanism has also been suggested for the attachment of these hormones in the kidney (Fong et al., 1959), and for insulin in muscle (Fong et al., 1962). Insulin, it should be noted, contains, as do the neurohypophyseal hormones, a twenty-membered disulfide ring.

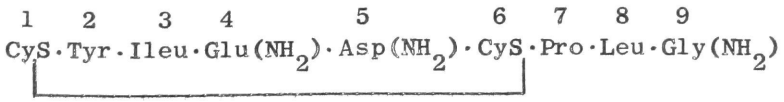
The responses of the bladder to neurohypophyseal hormones are specific. Neither insulin, glucagon, parathormone, epinephrine, nor angiotensin elicit them (Leaf and Hays, 1961). The observation that the excretion of hyaluronidase in the urine increases during antidiuresis (Ginetzinsky, 1958) led to the suggestion that this enzyme might act on a mucopolysaccharide component of the water permeability barrier in the collecting ducts, and thereby increase water reabsorbtion and cause an antidiuresis. However, hyaluronidase had no effect on the permeability of the toad bladder. On the other hand, Rasmussen and his co-workers found that when the mucosal surface of the toad bladder was briefly exposed (1.5 - 5 min) to 10^{-3} M N-ethylmaleimide, a change in permeability, apparently similar to that elicited by hormone on the serosal side, was recorded. At the same time, there was no change in oxygen consumption, even in the presence of Na^{+}. It seems in this case that N-ethylmaleimide is disrupting the mucosal barrier without actually penetrating the cells. Two other substances were found to mimic the action of hormone, when added to the serosal side of the toad bladder. Cyclic 3',5'-AMP is a likely intermediate in the glucogenic action of epinephrine on heart muscle, smooth muscle, and liver, and in the action of ACTH on the adrenal cortex, as well as that of glucagon on liver (Sutherland and Rall, 1960). Theophylline, moreover, is known to prevent the breakdown of cyclic

3',5'-AMP to 5'-AMP. These two substances, cyclic 3',5'-AMP and theophylline, can cause an increase in the water permeability and in the short-circuit current of the toad bladder (Orloff and Handler, 1961). However the concentrations at which they are effective are high: 10^{-3} M cyclic 3',5'-AMP or 10^{-2} M theophylline, as compared with 10^{-8} M arginine vasopressin or 10^{-10} M arginine vasotocin.

When we say that the response of the toad bladder is specific to the neurohypophyseal hormones, we must include not only the natural hormones, all of which have activity, but many of their synthetic analogs as well. In Table III, Column I, are listed a number of analogs of oxytocin. In Column II is the calculated toad bladder activity in units per mg, derived from data of Rasmussen, assuming the value of 360 units per mg for oxytocin, which is its activity in the frog bladder (Heller and Pickering, 1961). These data should be taken as approximations only. However, they do indicate, qualitatively, the relative activities of these various analogs. One can see limited trends in this list. Increased basicity in position 8, other residues remaining unchanged, seems to be correlated with increased toad bladder and rat antidiuretic activity (arginine vasotocin > lysine vasotocin > oxytocin, and arginine vasopressin > lysine vasopressin > leucine vasopressin (phe^3-oxytocin)), and partially correlated (only in the oxytocin series and not in the vasopressin series) with decreased rat uterus activity. But Boissonnas and co-workers (1961) caution against hasty generalizations in this direction and present a counter-example. Obviously, more information than just the total activity is needed. If the assay method is such that the time course of the response is measureable, this data, analyzed in terms of a simple model, would give a number of parameters for each analog and each assay instead of just one. An analysis of the time course of the response of the toad bladder to oxytocin will be given in this thesis. This analysis has not yet been applied to any analogs other than oxytocin, but that different analogs can have different response curves is shown in Figure 11 (Thorn, 1959). Thorn compared the effects of arginine-, lysine-, and leucine-vasopressin on the urine flow and urine osmolality is rats and dogs. He observed that the response to lysine vasopressin was characterized by a fast onset and a rapid decline as compared with the response to arginine vasopressin. The response curve to leucine vasopressin (phe^3-oxytocin) was similar to that of lysine vasopressin. (He

TABLE III

Activities of Analogs of Oxytocin



OXYTOCIN

I	II	III	IV
Analog (as derivative of oxytocin)	Toad bladder (units per mg)[a]	Rat antidiuresis (units per mg)[b]	Rat uterus (units per mg)[b]
ARG^8- (arginine vasotocin)	9000	80 [c]	40[c]
LYS^8- (lysine vasotocin)	2250	24	78
Oxytocin	360	5	450
$Sarcosine^9$-	225		
PHE^3, ARG^8- (arginine vasopressin)	110	400	20
β-MERCAPTOPROPIONIC^1- (desamino oxytocin)	41		active
PHE^3, LYS^8- (lysine vasopressin)	36	250	5
DES-(PRO^7, LEU^8, GLY^9)- (oxytocin ring amide)	26		3
VAL^3-	10	0.8	59
PHE^2-	8.6	0.5	32
PHE^3-	1.1	30	20
PHE^2, PHE^3, LYS^8-	0.7	20	0.3
N-acetyl-	0		1.7

[a] These values were calculated from data of H. Rasmussen giving the half-maximal concentration in moles per liter, assuming a value of 360 units per mg for oxytocin, which is its activity on the frog bladder according to Heller and Pickering (1961).

[b] Boissonnas et al. (1961). All values except those noted c.

[c] Heller and Pickering (1961).

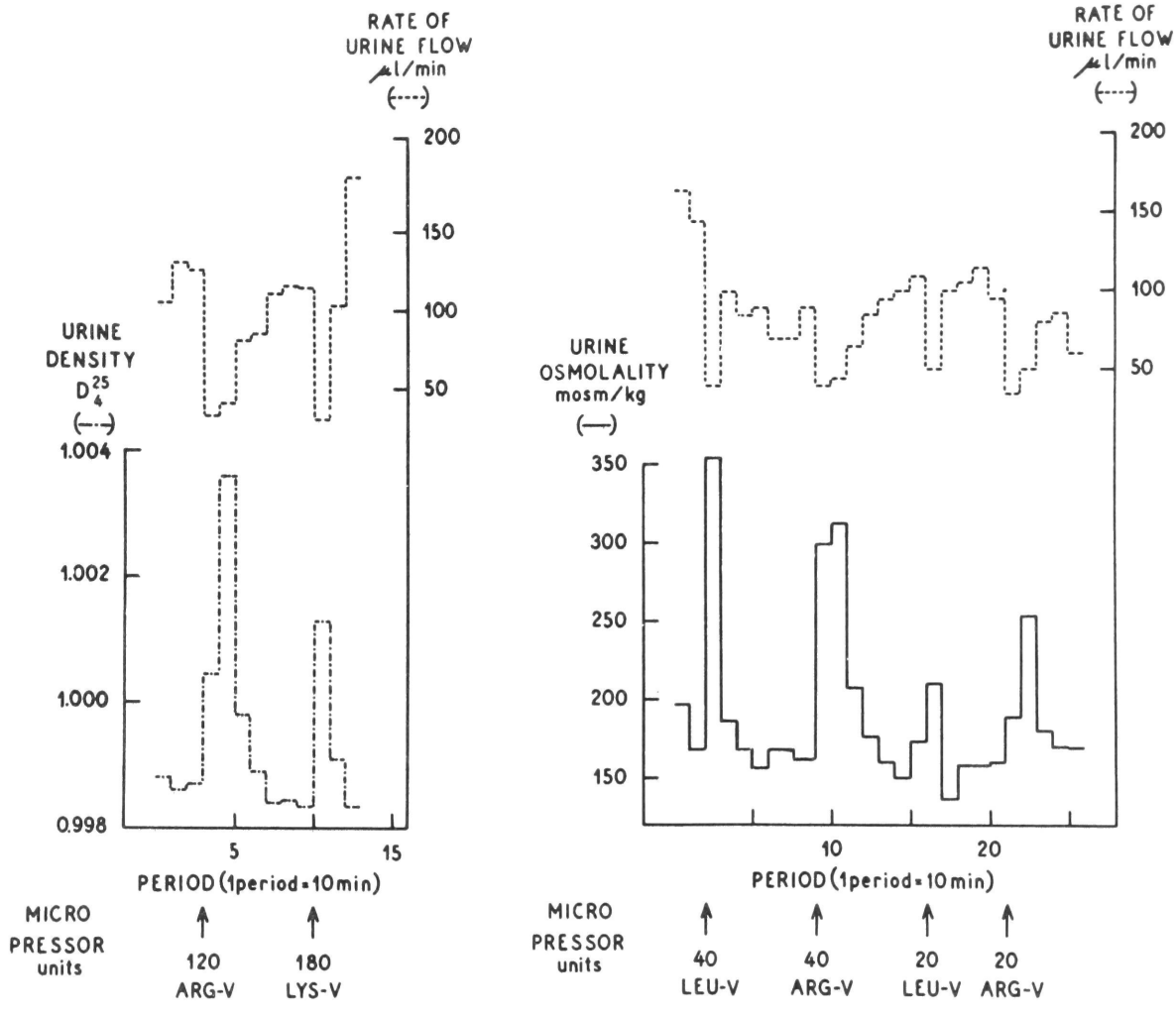


Figure 11. Differences in the response of the rat to intra-venous injections of arginine-, lysine-, and leucine-vasopressin.

The effect of arginine-vasopressin rose more slowly and lasted longer than that of the other two analogs. (From Thorn, 1959)

incidentally found more rat antidiuretic activity with leucine vasopressin than with lysine vasopressin. Compare Table III). The total duration of the increase in urine osmolality was 30-40 min after arginine vasopressin and 20-30 min after the other two preparations.

Some of the synthesized hormone analogs are inhibitory. Table IV (Beránková et al., 1961) summarizes the effects of the more potent of the inhibitory analogs. Tyr $(Me)^2$-oxytocin caused no contraction of the isolated rat uterus at 1,000 times the concentration of the minimum effective dose of oxytocin. However, phe^2-oxytocin contains considerable oxytocic activity. Hence, methylation of the tyrosine hydroxyl group leads to an inhibition while total removal leads to a compound with somewhat decreased activity. The inhibiting effect of Tyr $(Me)^2$-oxytocin in situ lasted about one hour. The potent inhibitor, Gly•Cys'-oxytocin has about 1 unit of avian depressor activity per mg and about the same rat uterus activity (Boissonnas et al., 1961). One could certainly learn a great deal about the function of the neurohypophyseal hormones if the anatomy of these inhibitions could be uncovered. Again, possibly some information would be gained by comparing the kinetics of the response to active analogs in the presence and in the absence of inhibitory analogs.

TABLE IV

Inhibition of the Effects of Oxytocin and Vasopressin (Berankova, 1961).

Inhibitor	Hormone	Effect	Ratio Inhibitor:Hormone	% Inhibition
homo-$Tyr^{2,3}$-oxytocin (decapeptide)	oxytocin	uterus in vitro	300-600:1	75-100%
	oxytocin	avian depressor	300-600:1	75-100%
γ-Glu $(NH_2)^4$-oxytocin	vasopressin	rat pressor	280-720:1	50%
Tyr $(Me)^2$-oxytocin	oxytocin	uterus in vitro	50:1	50%
	oxytocin	uterus in situ	250:1	partial
	vasopressin	rat pressor	1875:1	50%
Gly•Cys^1-oxytocin	oxytocin	avian depressor	17:1	50%

F. Inactivation of the Neurohypophyseal Hormones

After the injection of vasopressin, or following stimulation of the release of endogenous hormone, antidiuretic material is found in the urine. If vasopressin is injected intravenously into a conscious rat, about 10% of the injected activity can be recovered from the urine (Dicker, 1961). Thorn (1959a) obtained urine from rats orally loaded with 5% sodium chloride. Antidiuretic material was found in this urine, and this material had the following properties: the major part of it did not pass an ultrafilter; it was completely inactivated by sodium thioglycolate, and by trypsin and chymotrypsin, while pepsin and pancreatic carboxypeptidase had no effect on the antidiuretic activity. These findings confirm previous results in the dog, that the antidiuretic material is excreted in association with a large molecule. The significance of this is unknown. The inactivation pattern of this material, cited above, is the same as that of lysine- or arginine-vasopressin.

Since only a small fraction of injected or of endogenous antidiuretic hormone activity appears in the urine, the major part must be inactivated by various processes in the tissues and in the blood. The kidneys and the splanchnic area are certainly involved (Dicker, 1961), for removal of the kidneys or the tying off of the coeliac and mesenteric arteries, or both, retarded considerably the rate of disappearance of hormone activity. The blood itself, however, does not seem to be involved in the inactivation (Heller, 1960), except in pregnant primates (see below). Therefore, the rapid decay of injected hormone activity in the circulation, with a half-life of the order of 1 to 2 minutes in the rat and 5 minutes in dog and man is due to its dispersal into the extravascular space. The liver and the kidneys seem to be the main sites of removal, and these organs are thought to rapidly inactivate these peptide hormones (Heller, 1960).

The *in vitro* inactivation of vasopressin and oxytocin has been studied. Vasopressin is inactivated after incubation with slices, or homogenates, of kidney, of liver, of spleen, and of duodenum, but not of muscle (Dicker, 1961). Oxytocin and vasopressin are inactivated by homogenates of several regions of the brain, including the hypothalamus (Hooper, 1961). Homogenates of human placentae inactivate both oxytocin and vasopressin (Hooper, 1960); oxytocin is inactivated mainly by the supernatant fraction, vasopressin by the mitochondria and the microsome fractions. The "oxytocinase" and "vasopressinase"

from this tissue is further differentiated by dissimilar responses to inhibitors and to variation in pH. Smith and Sachs (1961) investigated the inactivation of arginine vasopressin by rat kidney slices. Oxytocin markedly decreased the rate of loss of arginine vasopressin from the incubation mixture; at a ratio of concentrations of oxytocin to vasopressin of 20:1, the rate was inhibited 50%.

The most thoroughly studied of all the inactivating systems is that found exclusively in the plasma of pregnant women and some anthropoid monkeys. An enzyme, which inactivates oxytocin irreversibly, appears in the blood of women after the 16th day of pregnancy, and its activity increases until parturition, when the activity is 28 to 60 times as high as in the first month (Semm, 1961). The origin of pregnancy oxytocinase is thought to be the placenta. Its function is probably the prevention of premature uterine contractions by maintenance of a heavily damped balance between oxytocin production and inactivation. At birth there must be either an increase in uterine sensitivity or a marked increase in oxytocin release, or both. Oxytocinase has been purified and characterized by Tuppy and his associates (Tuppy, 1961, for review). They demonstrated that oxytocinase acted on oxytocin with the splitting of the cystinyl-tyrosine bond in that molecule (see Table I). Synthetic substrates for aminopeptidases were also tested. Among these was L-cystine-di-beta-naphthylamide (CDNA). Oxytocinase preparations split many such substrates, but during purification, the biologically assayed inactivation of oxytocin was always associated with the splitting of CDNA. Moreover, the rise in oxytocinase activity during pregnancy was paralleled by a similar rise in CDNAase activity. Vasopressin is also inactivated to a considerably increased extent by pregnancy sera. However, enzymes other than oxytocinase are probably also involved in this. More will be said about pregnancy oxytocinase in the discussion of my own results on the inactivation of oxytocin.

EXPERIMENTAL

The question we asked was this: We know that the neurohypophyseal hormones have a profound effect on the toad bladder. Can we isolate some component of this tissue which will react with these hormones, this reaction having physiological significance? Of all the possible reactions of extracts with hormones, those that would cause inactivation of the hormones would be the easiest to detect. They would also, because of their generality, be the most difficult to interpret. There could be three modes of inactivation: a change in the structure of the hormone, including denaturation; binding of the hormone to some component of the extract; and the presence in the extract of some factor inhibiting the physiological effect of the hormone. Any or all of these could occur. Needless to say, when cells are disrupted, reactions that might have depended on fine structure will not occur, and reactions that would not normally occur because of structural segregation are likely to occur. Despite these portended difficulties, the inactivation of hormone by bladder homogenates was tested. There was no question that some form of inactivation would take place; but the extent, the specificity, and finally, of course, the significance of such reactions were to be determined or at least pondered.

The work will be presented in sections; the results of each section will be preceded by the pertaining methods.

A. The Inactivation of Hormones by Toad Bladder Fractions

Methods

The toads were a Latin American species, Bufo marinus. They were obtained from the National Reagents Co., and were kept with access to water and without feeding. Males and females were used at first, but later, females, because of their greater size, were used exclusively. Individuals weighed 200 to 400 gm.

The hormones used were prepared by Dr. Rasmussen from natural sources by counter-current distribution and column chromatography. One sample of oxytocin and of oxytocin ring amide was obtained from Dr. du Vigneaud.

1. Preparation of fractions: All operations were carried out at 0-4° C. The general procedure was to pith a number of toads, to excise their bladders into buffer, to mince the bladders, usually with a razor blade on a wax plate, to homogenate, and then to centrifuge the homogenate. The supernatant was fractionated with ammonium sulfate, and the ammonium sulfate fractions were passed through Sephadex G-25 (in lieu of dialysis).

In detail, at first the bladders were carefully homogenated by hand in an all glass homogenizer. The buffer used contained 0.25 M sucrose, 0.05 M tris [tris (hydroxymethyl-) aminomethane], 0.005 M versene [disodium ethylenediamine tetraacetate], and 0.04 M HCl, pH 7.0 (Hall et al., 1960). In all later experiments, homogenization was in a Vir Tis "45" homogenizer at moderate speed (setting 70) for 1 or 2 minutes. The buffer contained 0.05 M tris, 0.005 M versene, and 0.04 M HCl, pH 7.0. The usual proportions were one whole bladder per 4 ml of buffer, which was about 5 gm wet tissue per 100 ml homogenate.

The homogenate was then centrifuged at 700 x G for 10 minutes. This removed a great deal of the connective tissue, whole cells, and nuclei. The supernatant was filtered through sterile absorbent cotton to rid it of the remaining strands of connective tissue, and was then centrifuged at 105,000 x G for 30 minutes. This supernatant fraction we will designate S.

The ammonium sulfate fractionation of S proceeded as follows: about 100 ml of S was stirred slowly as solid ammonium sulfate was gradually added over a 5 minute period. After 30 minutes the solution was centrifuged at

35,000 x G for 15 minutes. The sediment was taken up in tris-versene buffer, and the supernatant was further fractionated by repetition of the above steps. A fraction is designated S(30-50), for instance, if it was precipitated between 30 and 50% saturation. The per cent saturation is actually that at 20° C.

Ammonium sulfate fractions were distributed on a column of Sephadex G-25 (Porath and Flodin, 1959). Sephadex, as received from Pharmacia, was screened between 120 and 200 mesh. The powder was stirred into 50% acetic acid and allowed to swell. The suspension was then degassed by evacuation. The gel was washed first with 40 volumes of water and then with 10 volumes of buffer containing 0.01 M tris and 0.008 M acetic acid, pH 7.5. This buffer, which we will call tris-acetate buffer, was also used to elute the column. The gel was repeatedly stirred into a dilute suspension, allowed to settle, and the fines decanted. A dilute suspension was poured into the column, and after a few centimeters of gel had settled, buffer was permitted to run slowly through. The bed, which had the final dimensions of 48 x 0.9 cm, was thus poured in two stages. The column was washed with 200-300 ml of tris-acetate buffer before use.

As a rule, 5-10 ml of ammonium sulfate fraction, with a protein concentration of about 5 mg per ml, was added carefully to the column. When the fluid reached the level of the top of the bed, elution with tris-acetate buffer was begun. The rate of flow was 15 ml per hour. 1 ml fractions were collected in a Resco Fraction Collector. The optical density of the fractions at 280 mμ was determined in a Beckman DU Spectrophotometer. The void volume of the column was 12 ml. Most of the absorbence at 280 mμ was recovered in tubes 13 through 21 (i.e., from 13 ml to 21 ml). These fractions were pooled and designated by a D following the ammonium sulfate fraction designation; e.g., S(50-70)D. Ammonium ion and chloride ion were found to peak around tube 35. The purpose of this step was to prepare the ammonium sulfate fractions for chromatographic investigation; hence the total salt content was in this way decreased, and chloride ion, which would interfere with a step to be described later (performic acid oxidation), was removed. These fractions as well as the undistributed ammonium sulfate fraction were stored at -20° C.

Three methods were used to determine protein concentration: the turbidimetric method, the Lowry method, and the reading of optical density at 280 mμ directly as mg per ml (Layne, 1957). It was the latter method which was settled on and used routinely.

2. Incubation of S fractions with hormone: The actual composition of the incubation mixtures will be given with the results. In most of the experiments, the hormone concentration in the mixture was about 1 μg per ml. In some experiments, however, the concentration was 100 μg per ml. For oxytocin, with a molecular weight of 1007, this is about 10^{-4} M. When L-cysteine or reduced glutathione (GSH) were added, they were dissolved shortly before use. The incubation was either at room temperature, 24-25° C, or at 30° C. The time of incubation was usually 30 minutes, though at higher hormone concentrations it was generally 1 hour. At the end of the incubation period, 2 ml aliquots of the incubation mixture were added to 3 assay tubes, each containing 25 ml of Ringer's. At the higher hormone concentrations, the incubation mixture was diluted before aliquots were taken. Controls, with buffer replacing the S fraction, were always run simultaneously.

3. Assay of hormones: The response of the toad bladder was used as the basis for the assay (Bentley, 1958). As was described in the Introduction, the neurohypophyseal hormones cause a marked increase in the water permeability of the bladder. This is measured by tying a half-bladder up like a bag, with dilute Ringer's or water inside and with Ringer's outside. Water flows out, and the weight of the half-bladder plus the glass insert to which it is attached plus the inside solution decreases. The decrease is equal to the water loss. The apparatus used is shown in Figure 12.

The actual procedure was as follows: 6 toads were pithed and their bladders removed into Ringer's. The Ringer's contained 110 mM NaCl, 1 mM $NaHCO_3$, 1 mM K_2HPO_4, 0.2 mM KH_2PO_4, and 2 mM $CaCl_2$, pH 7.6. The bladder consists of two bag-like halves, joined at the neck. Each of the halves was separately excised, and they were used in different groups. Each half-bladder was mounted on an insert, tied with surgical silk, and filled with 3 ml of 1/5 Ringer's or water. The insert and half-bladder were then placed in 25 ml of Ringer's in an assay tube. The half-bladders were mounted with the serosal side out, mucosal side in, as in situ. The serosal bath was aerated and mixed by a stream of air entering at the bottom of the tube.

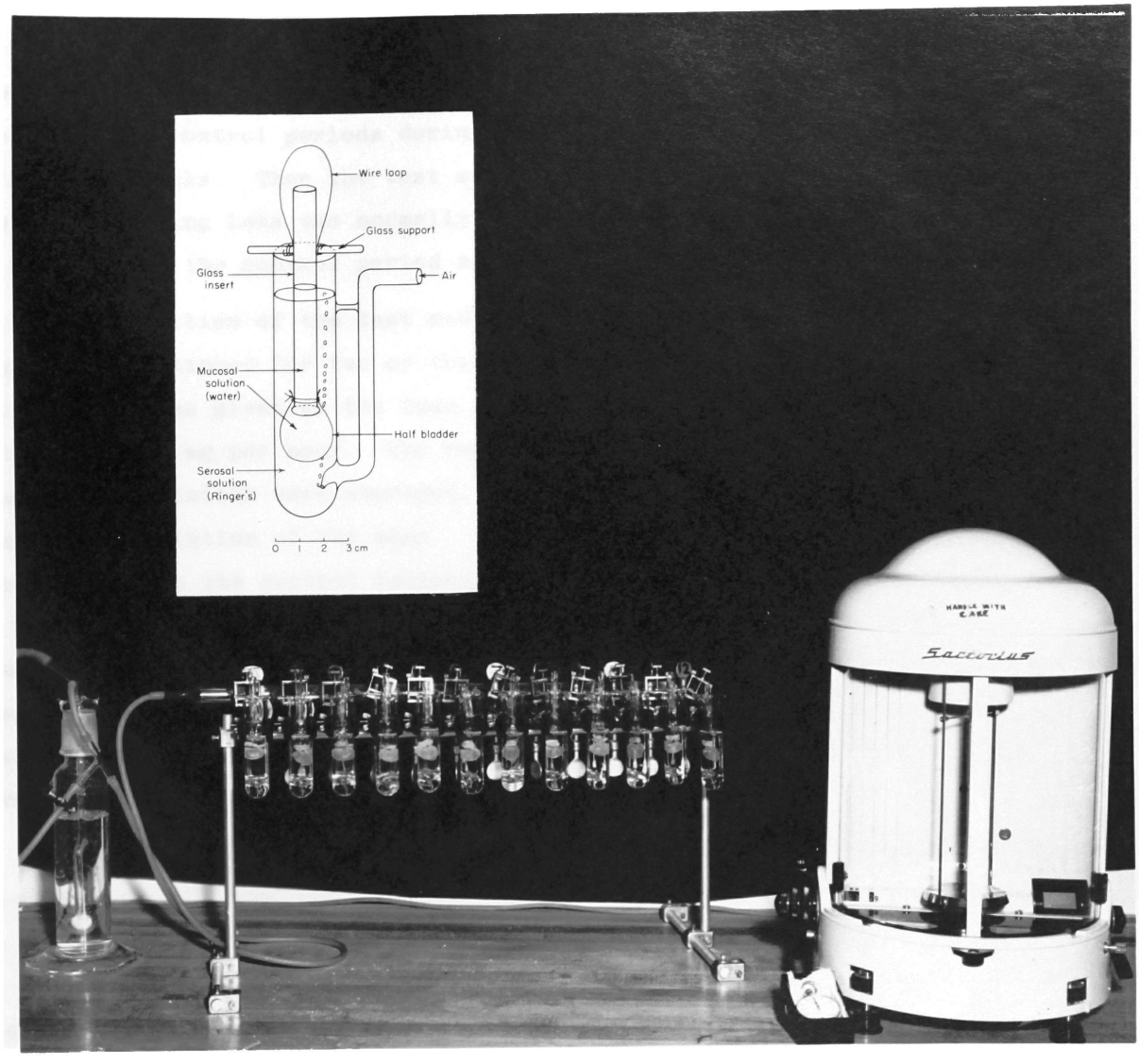


Figure 12. The toad bladder assay apparatus. The insert shows a diagrammatic representation of one assay tube.

The half-bladders were weighed by drawing them carefully up the side of the assay tube so as to remove excess fluid, and by suspending the insert from the beam of a Sartorius balance. The weight was read to 1 mg. The bladders were out of solution 30 seconds or less. There were two or three 20 minute control periods during which the bladders were weighed at 20 minute intervals. Then the test solutions were added to the serosal baths. The resting loss was normally about 1 mg per minute or less. Leaks were detected in the control period as a large loss in weight.

After addition of the test and control solutions to the serosal baths, weighing was continued for two or three 20 minute periods. The response of a half-bladder was given as the loss after addition minus the loss before addition, all in mg per hour. The responses of the half-bladders receiving the same test solution were averaged. The results are given as the mean $\pm$ the standard deviation of the mean. In many experiments the test responses are compared with the control responses directly, without converting them into hormone concentrations. In these, a test response not significantly different than the control response is taken to indicate no inactivation of hormone; a test response not significantly different from zero is taken to indicate complete inactivation, and responses between zero and the control response are taken to indicate partial inactivation.

A more quantitative design was also used. In this, a standard log dose-response curve was determined. The test responses and control responses were compared with this curve, and the difference in concentration of hormone was calculated. Two types of error were involved in these results, the error in the response and the error in the standard curve. The significance of the difference between the control assay and the test assay was calculated only from the error in the response; i.e., it was determined whether the responses were significantly different. But the error in the difference in the log concentration depended on both errors. The standard error of the final result, the difference in log concentration, was estimated as the square root of the sum of the squares of the separate standard errors. In practice, the best design was one in which two dose levels were used for both the test assay and the control assay. Each dose was assayed with three half-bladders. Each individual response was compared with the

standard curve, and the individual log doses were averaged. When the log concentration is converted to the concentration, the standard error becomes a factor. The t-test was used to test significance.

Results

Originally, the ability of bladder extracts to inactivate AVP was tested. It was found that AVP was inactivated by the total bladder homogenate, by the post-nuclei supernatant (i.e., after 700 x G for 10 minutes), by the post-mitochondrial supernatant (i.e., after 10,300 x G for 15 minutes), and by the post-microsomal supernatant (i.e., after 105,000 x G for 30 minutes). These results showed that the particle-free supernatant contained a large part of the inactivating potency of the bladder homogenate, and it was this supernatant, called S, which was regularly used.

Figure 13 shows the inactivation of AVP by S. AVP incubated with S shows almost no activity (I), while AVP incubated with buffer shows full activity (II). An equivalent dose of AVP was finally added to the same half-bladders as (I). It is seen that with this additional dose of AVP, these same bladders showed full activity (III). The two controls, (II) and (III), were not significantly different.

The time course of the inactivation of AVP was determined in two experiments. Different preparations of S were used in these experiments. Figure 14 shows the results of these experiments. We have plotted the actual rate of loss of water. This, as we shall see, is proportional to the log dose of hormone. Hence, a linear decrease in the rate of loss of water with time would correspond to an exponential decrease in hormone activity. Although there is scatter in the data (one of the control values is quite high), a straight line has been drawn. On the basis of this line we can say that the hormone is inactivated under these conditions with a half life of about 10 minutes. After 30 minutes activity is hardly detectable.

There was difficulty in storing S. Storage for a few days at -20° C or overnight at 2-4° C resulted in a loss in inactivating potency. It was found, however, that this loss in potency could be restored with cysteine or reduced glutathione. Figure 15 illustrates the results of an experiment in which S, which had been stored for a few days at -20° C, was incubated with AVP, and with (I) or without (II) added cysteine. With cysteine, there

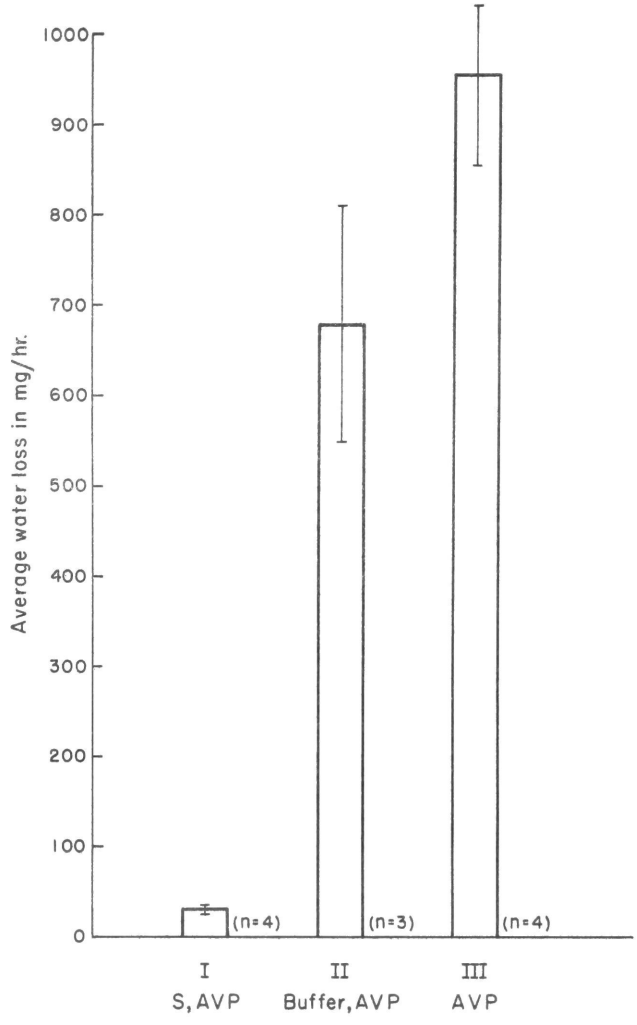


Figure 13. The inactivation of AVP by fraction S.

The incubation mixtures contained: (I) 9 ml S and 1 ml AVP (10 μg/ml); (II) 9 ml buffer and 1 ml AVP. These were incubated for 30 minutes at 25° C. 2 ml was added to each bladder. (III) an equal dose of AVP was added to the same bladders as (I).

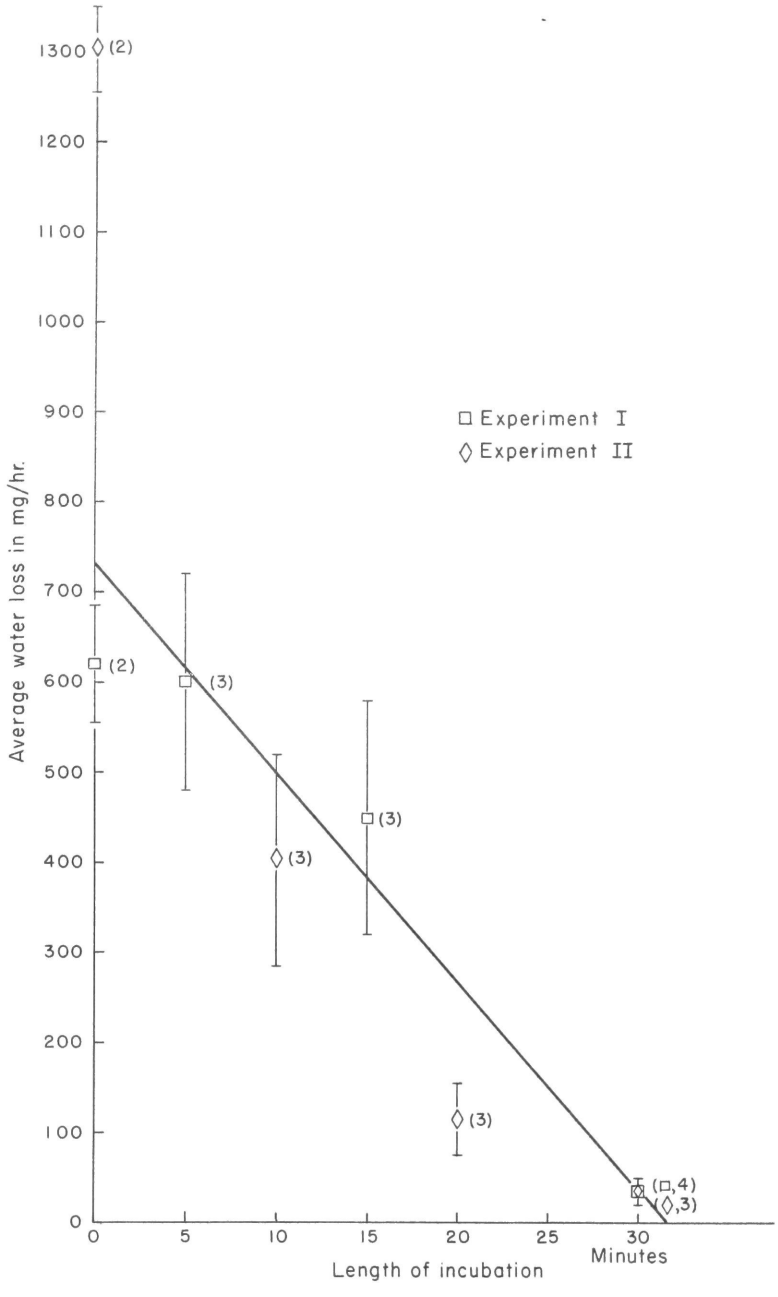


Figure 14. The time course of the inactivation of AVP by S.

The incubation mixtures contained: (I) 9 ml S and 1 ml AVP (10 μg/ml); (II) 6.7 ml S, 0.1 ml 0.1 M cysteine, and 0.2 ml AVP (40 μg/ml). 2 ml was added to each bladder.

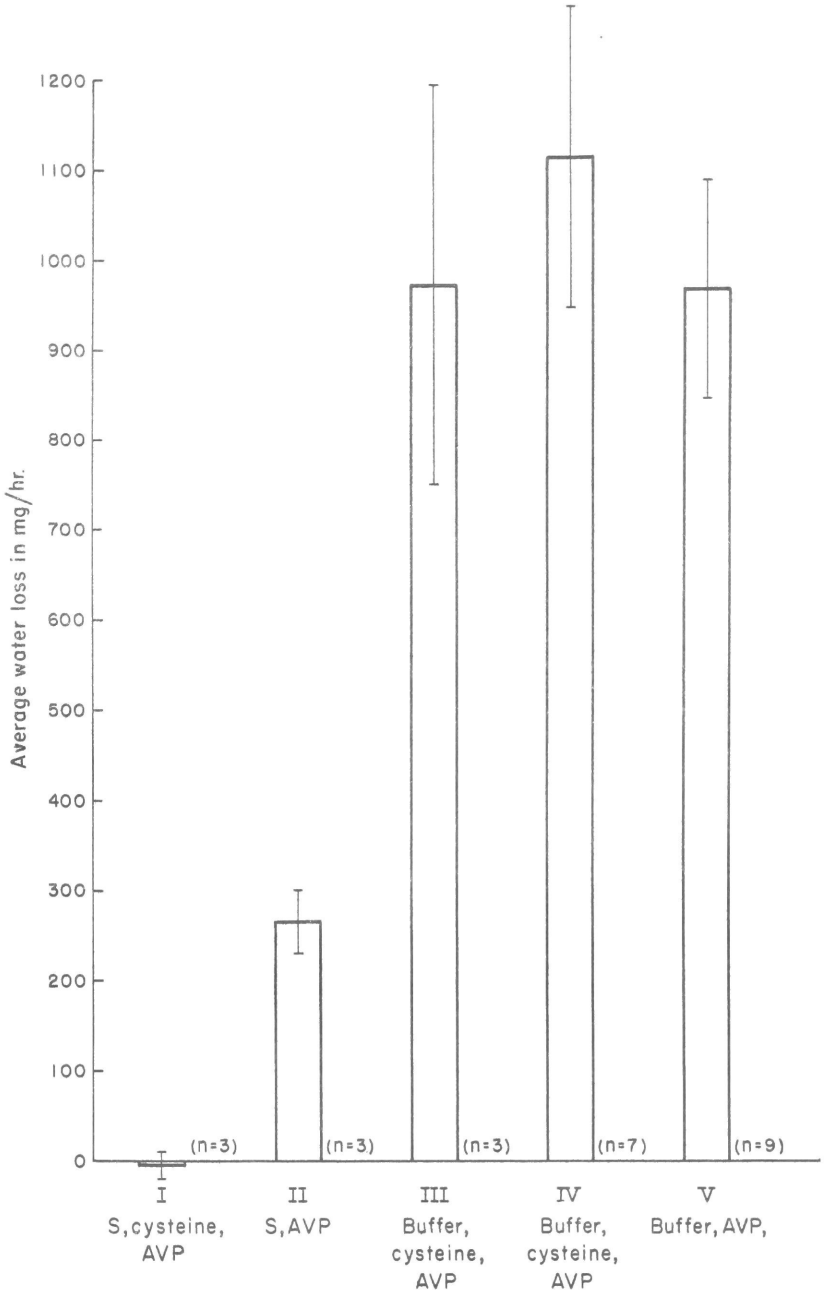


Figure 15. The effect of cysteine on the activity of stored S.

The incubation mixtures contained: (I) 6.3 ml S (stored 2 days at -20° C), 0.7 ml AVP (10 μg/ml), and 0.1 ml 0.1 M cysteine; (II) 6.3 ml S, 0.7 ml AVP; and (III) 6.3 ml buffer, 0.7 ml AVP, and 0.1 ml cysteine. All were incubated at 25° C for 30 minutes. 2 ml was added to each bladder. (IV) and (V) represent the mean of a number of experiments in which AVP was incubated with buffer and with (IV), and without (V); added cysteine.

was complete inactivation of AVP; without cysteine, there was partial inactivation. The control, with buffer, cysteine, and AVP, was fully active (III). In the same figure, a number of controls are compared in which AVP is incubated with (IV) and without (V) cysteine. There is no significant difference ($P > 0.4$) between these responses. Hence, cysteine alone neither inactivates nor potentiates the hormone.

The heat-lability of S was investigated. Aliquots of S were preheated to 50° C and to 70° C for 5 minutes. They were cooled quickly to room temperature and incubated with AVP, and the incubation mixture assayed. Figure 16 summarizes these results. Unheated S completely inactivated the hormone (I). S, heated to 50° C, only partially inactivated the hormone (II); and S, heated to 70° C, did not inactivate the hormone at all (III). The difference between II and III was not quite significant ($0.05 < P < 0.1$). We may conclude that the hormone-inactivating potency of S is increasingly destroyed by temperatures above 50° C. It was found, moreover, that this loss of potency could not be restored with cysteine.

Since the inactivating potency of S was in some cases enhanced by cysteine, it was thought possible that free sulfhydryl groups might be involved in the action of S. For this reason the effect of N-ethylmaleimide (NEM) on S was studied. NEM reacts quickly with sulfhydryl groups. It is not, however, specific for these and reacts also with amino groups, and with imidazole (Smyth et al., 1960). S was treated with 10^{-3} M NEM for 20 minutes at 25° C. Cysteine was added to react with excess NEM, and the treated S was incubated with AVP. Figure 17 summarizes these results. NEM-treated S showed a complete loss of hormone inactivating potency (I). Untreated S, on the other hand, completely inactivated the added hormone (II); i.e., it had full potency. Though not shown in this figure, NEM, after reaction with cysteine, had no effect on the bladder.

The action of S on some of the other hormone analogs was tested. S completely inactivated approximately equally potent doses of arginine vasopressin (AVP), arginine vasotocin (AVT), oxytocin, lysine vasopressin (LVP), and des-(pro^{7}, leu^{8}, gly^{9})-oxytocin (oxytocin ring amide). Figure 18 summarizes these results. The results do not necessarily indicate a lack of specificity of the inactivation. These hormones have quite similar structures (see Table 3), especially around the disulfide bond.

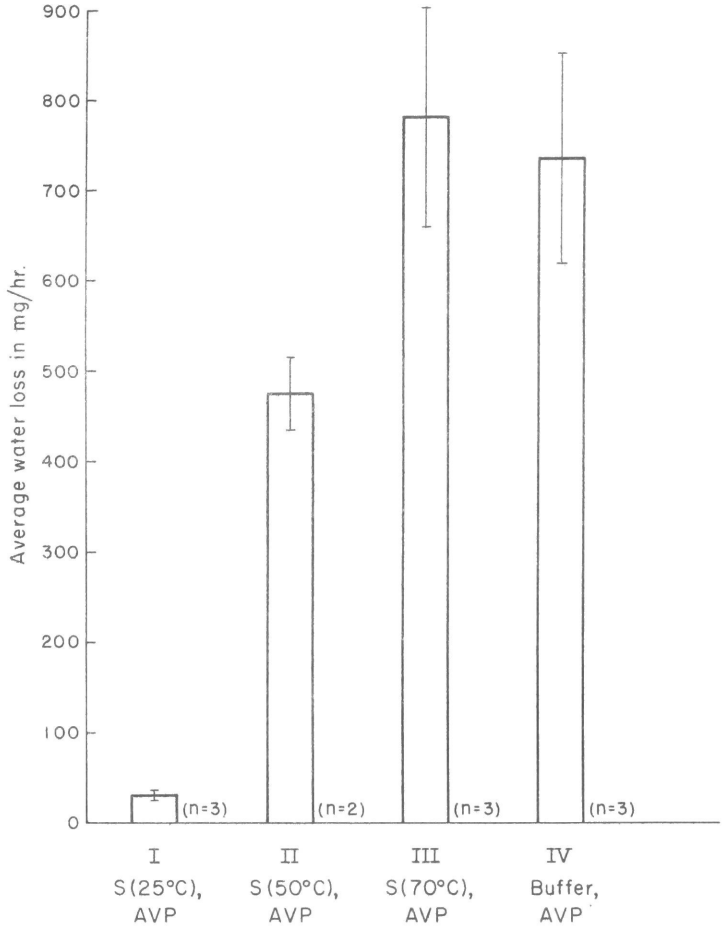


Figure 16. The heat inactivation of S.

The incubation mixtures contained 6.3 ml S, 0.7 ml AVP (10 μg/ml), and 0.1 ml 0.1 M cysteine. S was either untreated (I), or preheated to 50° C for 5 minutes (II), or preheated to 70° C for 5 minutes (III). (IV) contained buffer in place of S. All were incubated at 25° C for 30 minutes. 2 ml was added to each bladder.

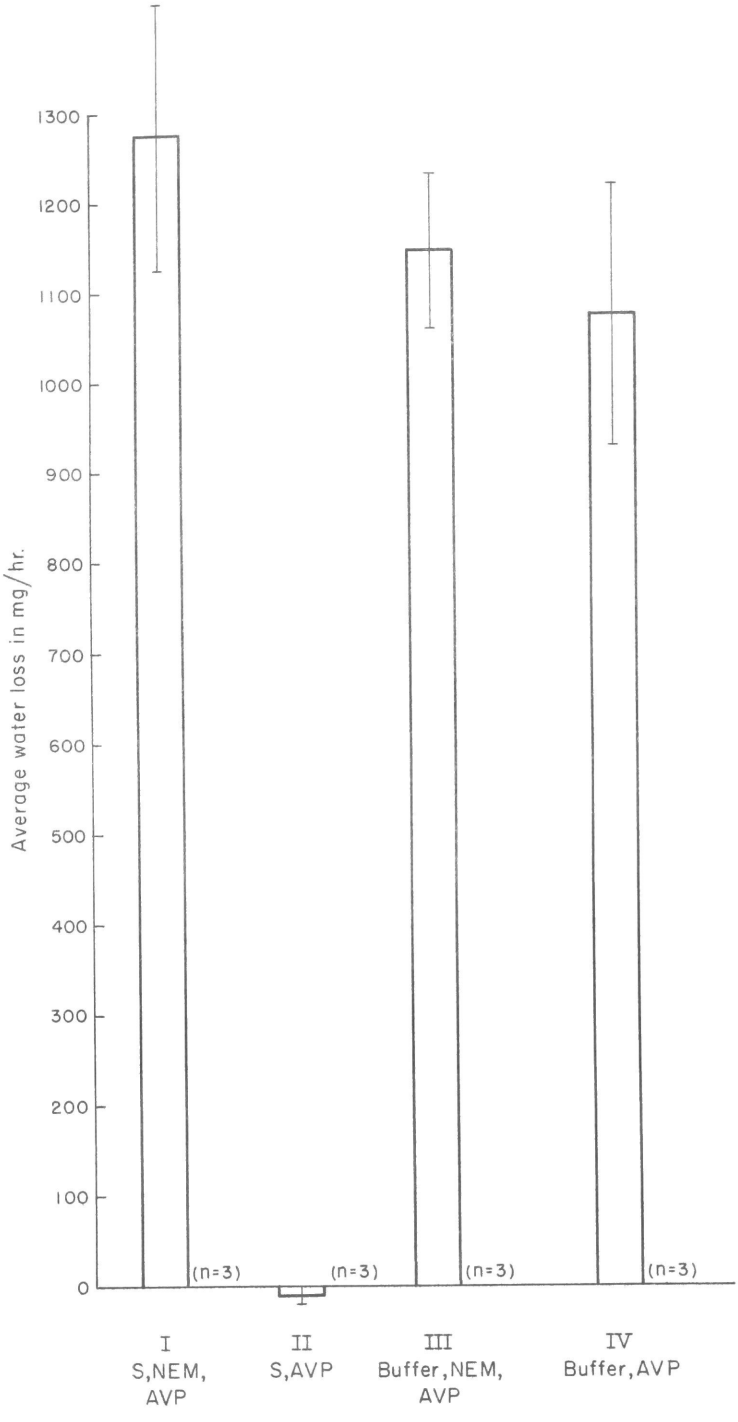


Figure 17. The inactivation of S by N-ethylmaleimide.

The incubation mixtures contained: (I) 6.3 ml S, 0.7 ml AVP, 0.07 ml 0.1 M NEM, and 0.07 ml 0.1 M cysteine; (II) 6.3 ml S and 0.7 ml AVP; (III) 6.3 ml buffer, 0.7 ml AVP, 0.07 ml 0.1 M NEM, and 0.07 ml 0.1 M cysteine; (IV) 6.3 ml buffer and 0.7 ml AVP. The NEM was preincubated with S for 20 minutes. Cysteine was then added. After 10 minutes AVP was added. Incubation was at 25° C for 30 minutes.

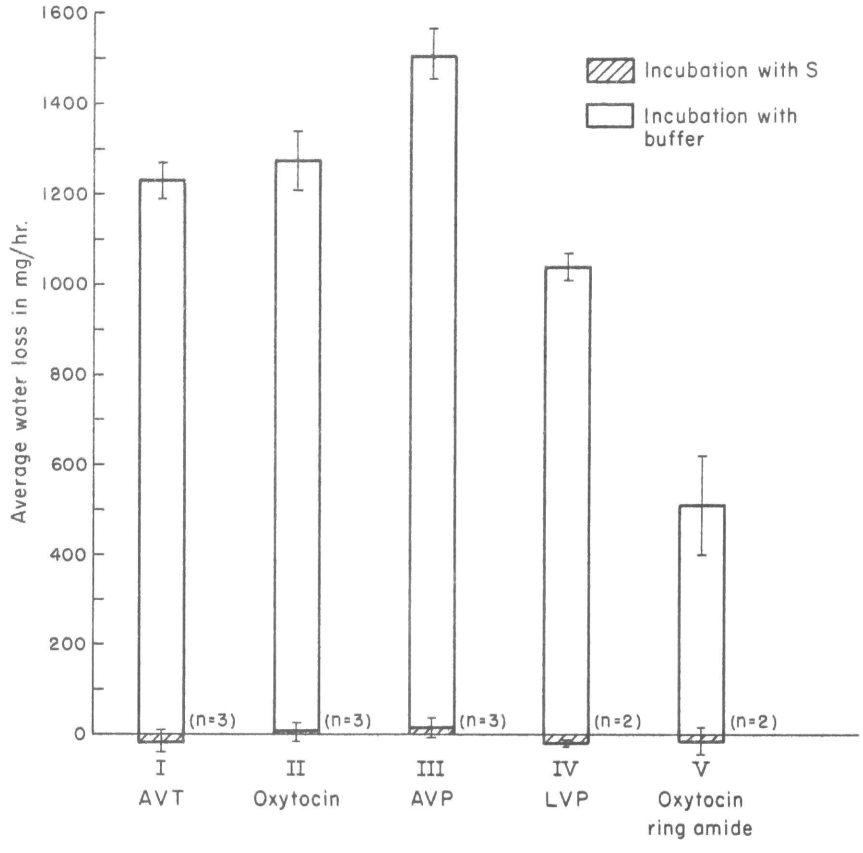


Figure 18. The inactivation of hormone analogs by S.

The incubation mixtures contained 6.3 ml S and 1.4 mM cysteine. The incubation was at 25° C for 30 minutes. The concentrations of the analogs in the control assays were: AVT, 0.00042 μg/ml; oxytocin, 0.042 μg/ml; AVP, 0.084 μg/ml; LVP, 0.17 μg/ml; and oxytocin ring amide, 0.27 μg/ml.

Next, we attempted a partial purification of S by ammonium sulfate fractionation. In these, and all following experiments, oxytocin was used as the substrate. Our first fractionation revealed that among the three fractions, S(0-30), S(30-60), and S(60-85), the first had no detectable inactivating activity, while the latter two were both active. It was also found that activity was considerably reduced in the absence of either cysteine or reduced glutathione, and one of these was always added to the incubation mixture. Three further fractions were then prepared: S(30-50), S(50-70), and S(70-85). They were all found to contain some activity, as was consistent with the previous results; but upon dilution, the fraction S(50-70) was seen to contain the most activity. Figure 19 summarizes these results. Two experiments are represented, one in which 3 ml of each fraction was added to the incubation mixture (final volume 6 ml), and another in which 1.5 ml of each fraction was added. All fractions completely inactivated the hormone at the higher concentration, but when they were diluted two times, the greater inactivating potency of S(50-70) became apparent.

In order to better quantitate the hormone assay, a standard curve, of the loss of water in mg per 40 minutes versus the log of the dose of hormone, was determined (Figure 20). A straight line was fitted for the range 0.004 μg per ml to 0.100 μg per ml. The index of precision was 0.26 (Gaddum, 1953). The results of each assay were compared with the standard curve as described in the Methods.

Two experiments, in which different preparations of S(50-70) were used, gave the rate of inactivation of oxytocin as (I) 30 μg per mg protein per hr and (II) 41 μg per mg protein per hr (1 μg oxytocin equals 10^{-9} moles). The standard error of the log concentration in both these cases was about 0.2; in other words, these results each vary by a factor of about 1.6 (e.g., for (I), from 19 to 48 μg per mg per hr).

The rate of inactivation of oxytocin by the distributed fraction, S(50-70)D, was also determined. This fraction showed considerable activity in the absence of cysteine. With no cysteine present in the incubation mixture, the rate was 16 μg per mg per hr, with an error of a factor of 1.5. The initial substrate concentration in these experiments was 10^{-4} M, or 100 μg per ml, and the final concentration was less than 50 μg per ml. Incubation was at 30° C for 2 hours.

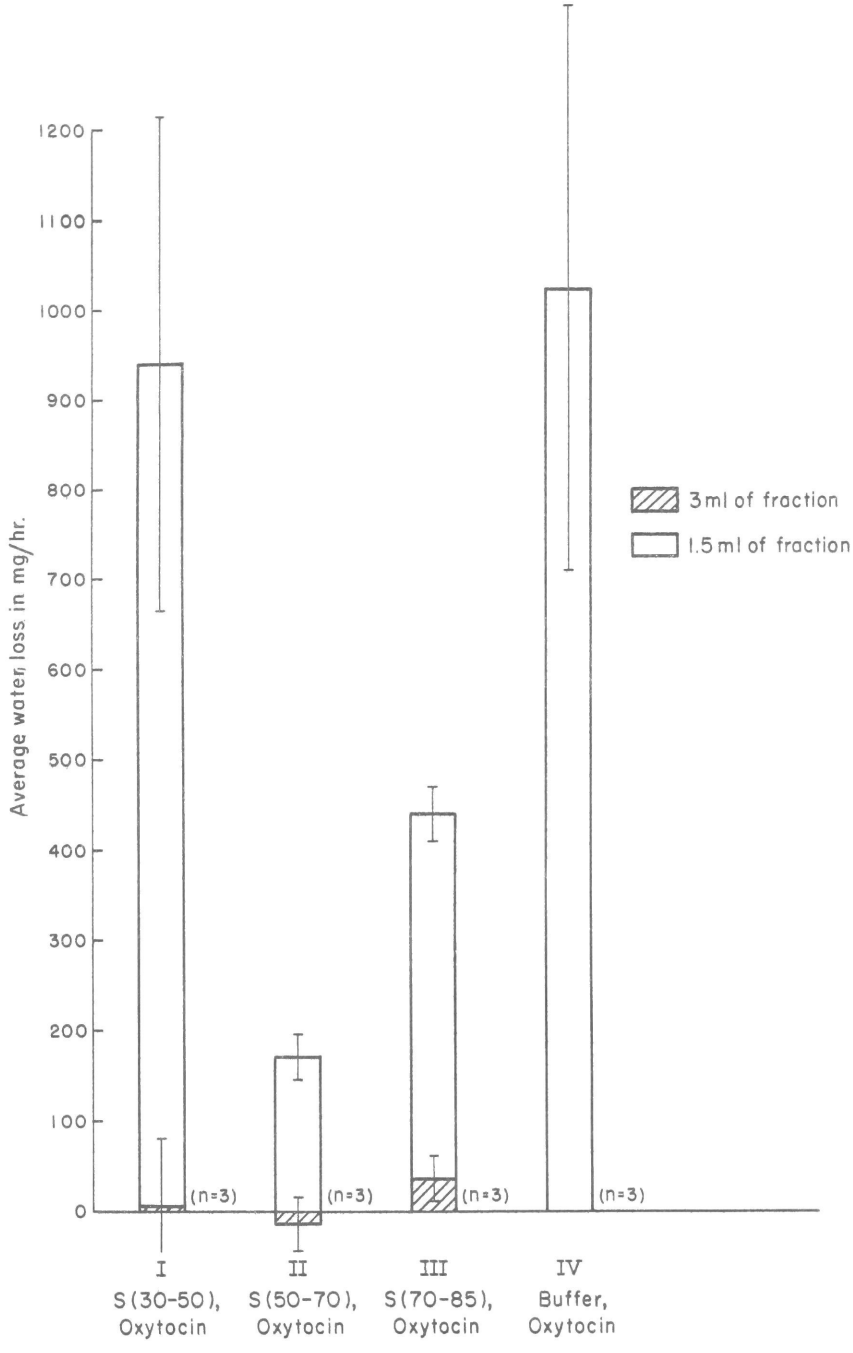


Figure 19. The inactivation of oxytocin by ammonium sulfate fractions of S. The incubation mixtures contained: (I) 3 ml (or 1.5 ml) S(30-50), 3 ml (or 4.5 ml) buffer, 0.2 ml 0.05 M GSH, and 0.8 ml oxytocin (5 μg/ml); (II) 3 ml (or 1.5 ml) S(50-70), 3 ml (or 4.5 ml) buffer, 0.2 ml GSH, and 0.8 ml oxytocin; (III) 3 ml (or 1.5 ml) S(70-85), 3 ml (or 4.5 ml) buffer, 0.2 ml GSH, and 0.8 ml oxytocin; (IV) 6 ml buffer, 0.2 ml GSH, and 0.8 ml oxytocin. Incubation was at 26° C for 30 minutes. 2 ml was added to each bladder.

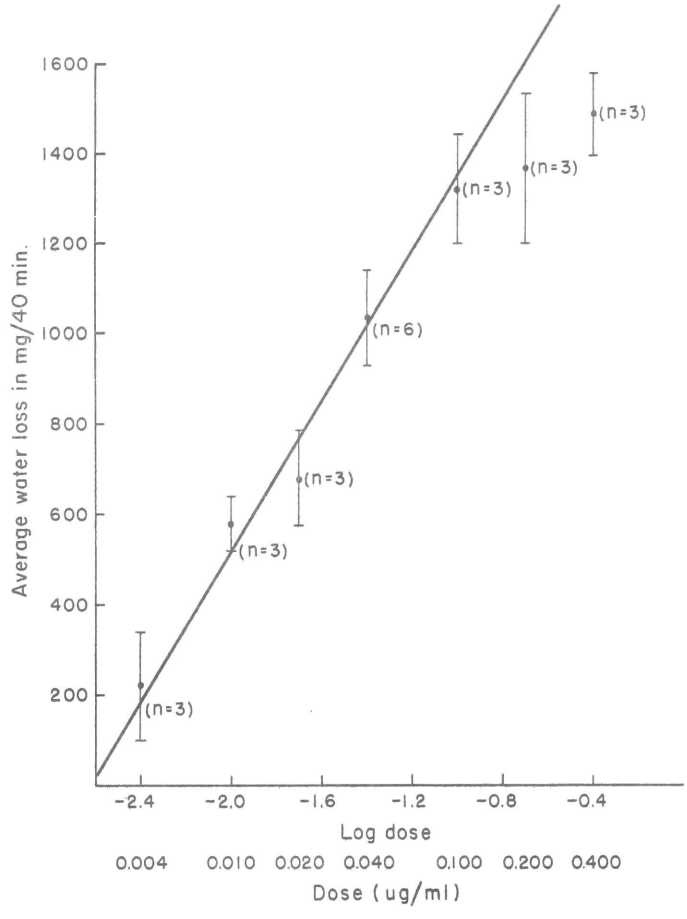


Figure 20. A standard $\log_{10}$ dose-response curve for oxytocin.

S = 219, b = 830, and λ = 0.26, where s is the square root of the sum of the variances of each point divided by the number of degrees of freedom, b is the slope, and λ is the index of precision, equal to s/b.

The results of this section suggested that there might be present in S, and in its ammonium sulfate fractions, some enzyme, or enzymes, which inactivate the neurohypophyseal hormones. To test this hypothesis further we tried to determine whether a particular peptide bond of the hormone molecule was split. If this enzyme acted as an aminopeptidase and split the bond between the N-terminal half-cystinyl residue and the tyrosyl residue, as has been shown to be the case for pregnancy serum oxytocinase (see Introduction), then performic acid oxidation of the disulfide bond of the split product would liberate cysteic acid (Tuppy and Nesvadba, 1957). In the next section are presented the results of such experiments.

B. Chromatographic Examination of Performic Acid Oxidized Incubation Mixtures

Methods

S(50-70) was distributed on Sephadex G-25, as described above, to remove residual ammonium sulfate and to change from tris HCl-versene buffer to tris-acetate buffer. The presence of chloride ion can cause side reactions during performic acid oxidation (Hirs, 1956). The distributed S(50-70) is called S(50-70)D.

The incubation mixtures contained (I) 0.5 ml S(50-70)D and 0.5 mg oxytocin, (II) 0.5 ml S(50-70)D, and (III) 0.5 ml tris-acetate buffer and 0.5 mg oxytocin. These were incubated at 30° C for 4 hours. After incubation, the incubation mixtures were cooled quickly to 0° C, and 30% trichloroacetic acid (0° C) was added to 5% final concentration. The precipitate was spun off, and the supernatant extracted 3 times with 3 volumes of fresh ethyl ether to remove the TCA (Smith and Sachs, 1961). The ether was removed in a stream of nitrogen.

Performic acid was prepared by mixing 9 volumes of 98% formic acid and 1 volume of 30% hydrogen peroxide. This solution was kept at 25° C for 1 hour before it was used. The performic acid solution was pipetted into reaction vessels and cooled to -15° C in a water-ice-ethanol bath. The processed incubation mixtures were cooled to 0° C and added to the performic acid solutions. The volume of the incubation mixtures at this point was about 0.4 ml. This was added to 2 ml of the performic acid solution. The oxidation continued for 90 minutes at -15° C. Then 20 ml of ice cold water was added to each reaction vessel, the contents were quickly frozen in dry ice-acetone, and were lyophilized. The residue was redissolved and relyophilized.

Paper chromatography was performed on Whatmann #1. The solvent system contained n-butanol: acetic acid: water in the volume proportions 100:22:50. This forms a single phase at room temperature. The lyophilized residue was taken up in 0.05 ml water and applied to the paper in 0.005 ml aliquots, with drying in cool air between applications. Chromatography was in the descending direction and for 18 or 19 hours, in which time the solvent front moved about 40 cm. The chromatograms were dried in a hood

for a few hours. When dry, they were sprayed with ninhydrin (0.3% in ethanol, Sigma), and the color was developed overnight at room temperature. The oxytocin moved as a single spot in this solvent system.

Results

Figure 21 is a tracing of a chromatogram of protein-free incubation mixtures before performic acid oxidation. The position of oxytocin in two of the controls, (II) and (IV), is indicated by an arrow. The second spot in (IV) was due either to the buffer or to the treatment with TCA, since it did not appear if oxytocin, in water solution, was applied directly. A spot corresponding to oxytocin did not appear, however, in the incubated mixture of S(50-70)D and oxytocin (I). Moreover, there was a general increase in ninhydrin positive material in (I) which did not occur either in the same mixture kept at 0^{o} C during the incubation period (II), or in S(50-70)D alone, incubated for the same time and at the same temperature as (I). There is a second, faster-moving spot in the incubated mixture of buffer and oxytocin, treated in the same way as (I), but there is no appearance of slower moving spots as in (I). We can conclude that oxytocin is being removed or altered during incubation with S(50-70)D, and that this process coincides with an increase in ninhydrin positive material.

Figure 22 is a tracing of a chromatogram of the protein-free incubation mixtures after performic acid oxidation. The positions of cysteic acid (V) and of performic acid oxidized oxytocin (IV) are shown. It is seen that an oxytocin spot did not appear, and that instead ninhydrin positive material appeared at approximately the position of oxidized oxytocin in (III) and (I). In (I), again, there was an increase in ninhydrin positive material compared to S(50-70)D incubated without oxytocin (II). In particular, a dense spot appeared in (I) at the position of cysteic acid (marked by an arrow). A much lighter spot appeared at the same position in (II). Similar light spots appeared, even before oxidation, in the comparable mixtures (I) and (III) in Figure 21. They could be due to the presence of small amounts of cysteic acid in S(50-70)D. If the dense spot is due to cysteic acid, we may conclude, as explained at the end of section I, that the bond between the N-terminal half-cystinyl residue and

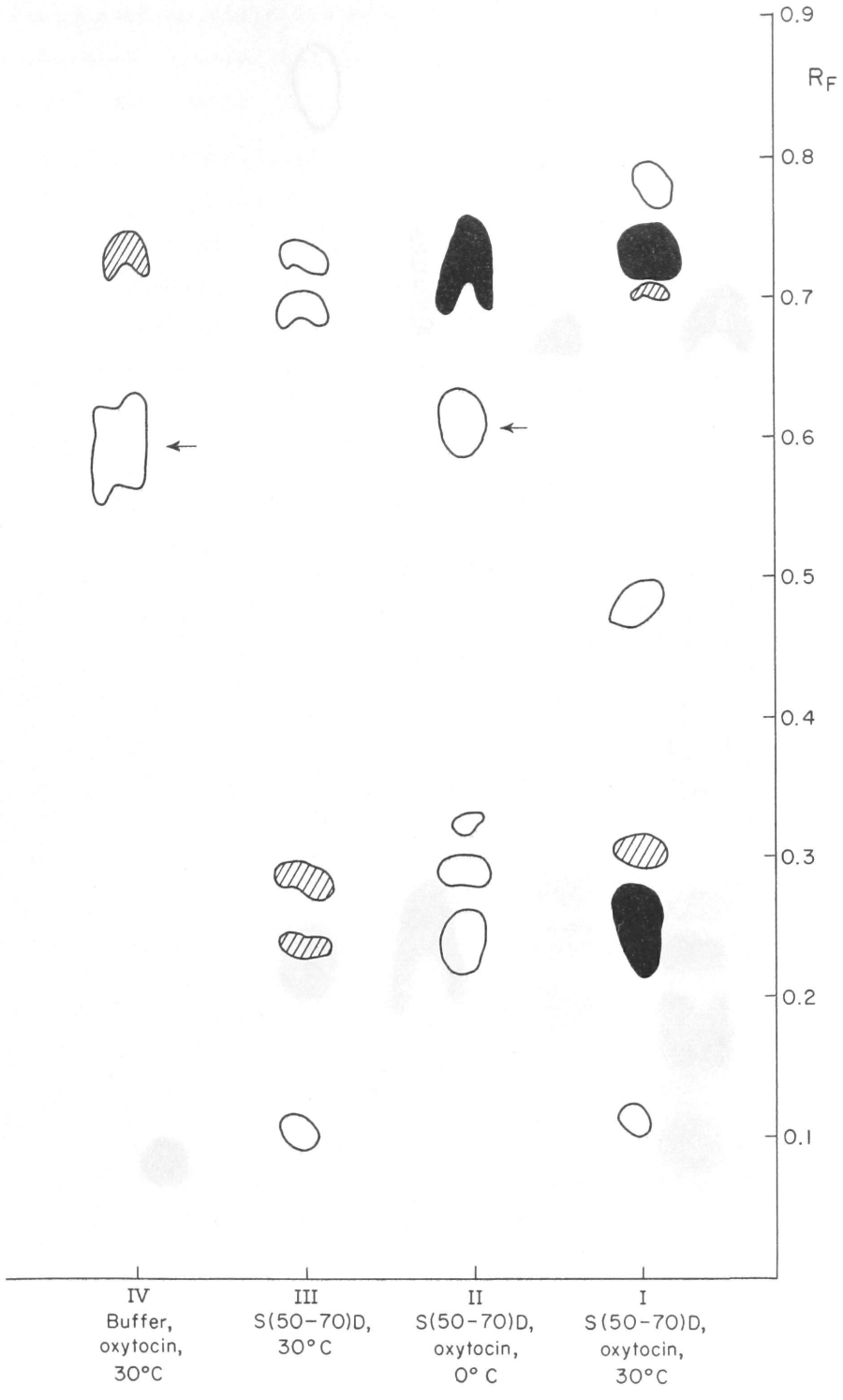


Figure 21. A tracing of a chromatogram of incubation mixtures before performic acid oxidation.

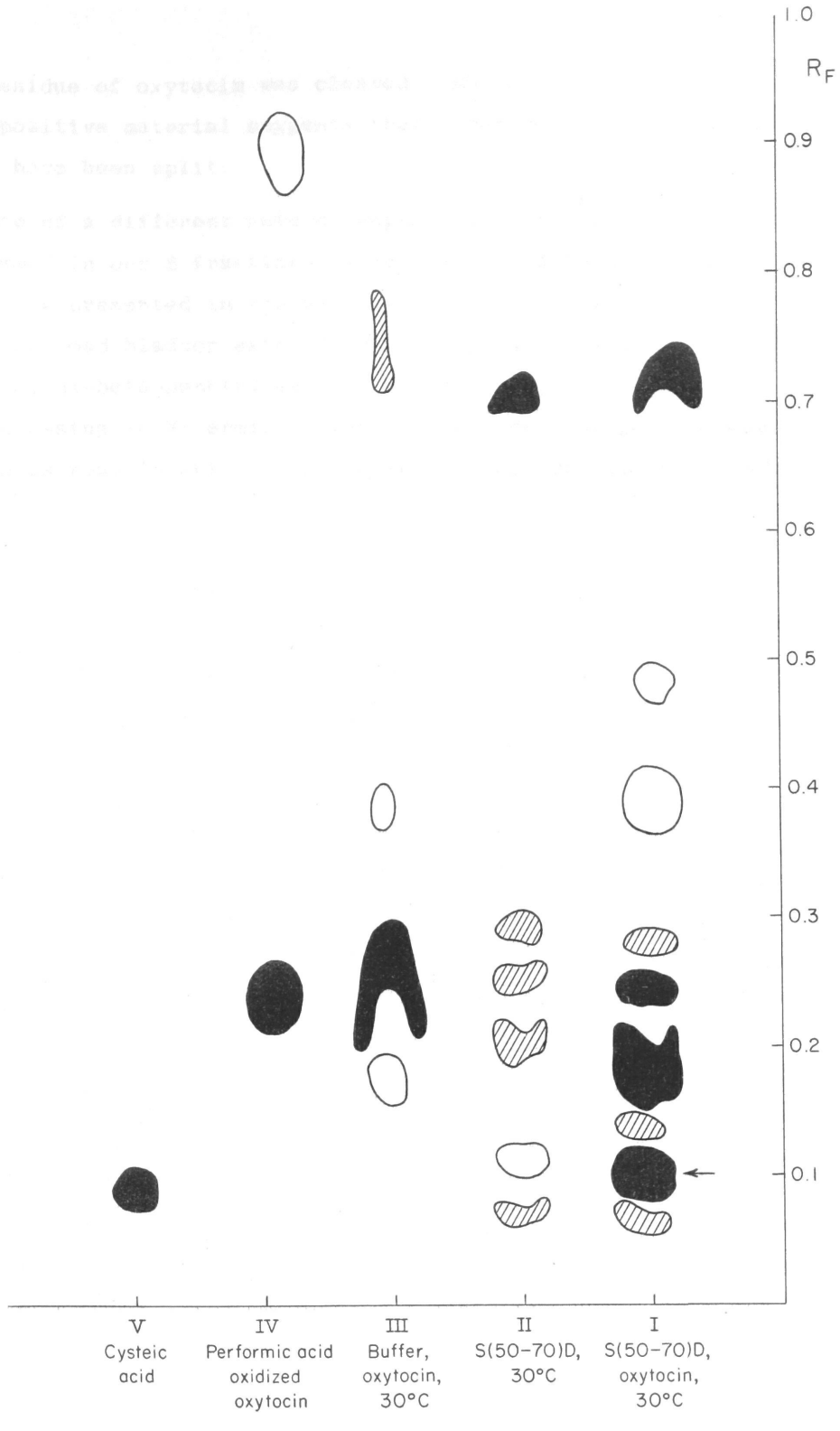


Figure 22. A tracing of a chromatogram of incubation mixtures following oxidation with performic acid.

the tyrosyl residue of oxytocin was cleaved. Moreover, the general increase in ninhydrin positive material suggests that other bonds of the oxytocin molecule also have been split.

Evidence of a different nature, supporting the hypothesis that an enzyme is present in our S fractions, which cleaves N-terminal half-cystinyl-tyrosyl bonds, is presented in the next section. There we review our study of the action of toad bladder extracts on two synthetic substrates. One of these, L-cystine-di-beta-naphthylamide, resembles oxytocin and its hormone analogs in possessing an N-terminal cystinyl-peptide linkage. We shall see that this bond is readily split by our hormone inactivating fractions.

C. The Splitting of Synthetic Substrates

Methods

We are concerned in this section principally with the action of S and its fractions on two substrates, L-cystine-di-beta-naphthylamide (CDNA), and L-leucine-beta-naphthylamide (LNA). The first of these, CDNA, is sparingly soluble at neutral pH, and a saturated solution contains about 6.8×10^{-5} moles per liter. This was, then, the highest concentration of this substrate which we could obtain in our incubation mixtures. In many cases an amount in excess of saturation was added, so that the concentration of substrate remained constant throughout the incubation. CDNA was dissolved either in dilute HCl or in 50% methoxyethyleneglycol (methyl cellosolve). A convenient stock solution was 1 mM CDNA in 2 mM HCl. LNA•HCl was readily soluble in water, and was made up in 1 to 4 mM concentrations. The substrate solutions were stored in amber glass bottles at 2-4° C. Beta-naphthylamine was dissolved in 2 mM HCl at a concentration of 0.4 mM, and was stored in an amber glass bottle at 2-4° C.

The incubation mixtures, made up in duplicate, contained 0.25 ml enzyme fraction, 0.25 ml substrate, and 0.5 ml buffer (the same as that in which the enzyme fraction was dissolved, either tris-acetate or tris-chloride-versene, except in the pH studies, in which phosphate buffer was used). Often smaller volumes were used, but the proportions remained the same. The incubation was usually at 30° C. The pertinent details of the incubation will be given with the results.

Beta-naphthylamine, released by cleavage of the peptide bond, was assayed by the method of Tuppy and Nesvadba (1957), with slight modifications. Following incubation, 1 ml 10% TCA was added to the 1 ml volume of the incubation mixtures. For smaller volume incubation mixtures, all other volumes were proportionally decreased. The TCA-precipitated protein was centrifuged off, and 1 ml of the supernatant was added to 9 ml of a mixture of 2 volumes of 0.36 M HCl and 1 volume of acetone. Then the following additions were made exactly 3 minutes apart: 1 ml 0.1% sodium nitrite, 1 ml 0.5% ammonium sulfamate, and 1 ml 0.1% N(1-naphthyl)-ethylenediaminedihydrochloride. Following addition, the solutions remained in stoppered flasks for 45 minutes at room temperatures. During the addition of the reagents and the development of the color, strong daylight was excluded.

The optical density at 565 mμ was determined in a Coleman Jr. Spectrophotometer, using matched cuvettes. After 45 minutes, the color was still slowly changing, so that each sample was read exactly at the same time following the addition of the last reagent. The blanks were incubation mixtures containing buffer in place of enzyme which were similarly processed as was the standard which contained buffer and beta-naphthylamine. The concentration of beta-naphthylamine in a sample was calculated as the ratio of the optical density of the sample over the optical density of the standard, times the concentration of beta-naphthylamine in the standard. Duplicates were averaged. The average is given ± the average deviation. The standard curve of optical density versus concentration of beta-naphthylamine was linear.

Results

The CDNAase activity of S and of its ammonium sulfate fractions is shown in Table V. There we have given the mean specific activity ± the standard deviation of the mean of a number of different preparations. The pattern of activity is the same as that of the fractions which were obtained from a single preparation of S. S had a specific activity of about 0.2×10^{-7} moles per mg per hr. The ammonium sulfate fraction, S(50-70) had about twice the specific activity of S, about 0.4×10^{-7} moles per mg per hr, while S(0-50) and S(70-85) had each less than 25% of the specific activity of S(50-70), about 0.1×10^{-7} moles per mg per hr. This, of course, represents the same pattern of activity found in the inactivation of oxytocin. Distribution on Sephadex increased the specific activity of S(50-70) about 50% to 0.6×10^{-7} moles per mg per hr, while the specific activity of S(0-50)D and S(70-90)D remained low. The total activity recovered in S(50-70)D was about 50% of that of S.

A less broad ammonium sulfate fractionation resulted in a fraction, S(60-70)D, with a higher specific activity than S(50-70)D. However, the recovery of total activity was not good and S(50-70)D was the best preparation regularly used.

TABLE V

The Rate of Hydrolysis of CDNA by Fractions of S

The values are given ± the standard deviation of the mean. n is the number of different preparations [S(0-50) and S(0-50)D were different preparations]. The incubation mixtures contained S-fraction, buffer, and CDNA in excess of saturation. Incubation was for 30 min or 60 min at 30° C.

Fraction	Mean Rate 10^{-7} moles β-naphthylamine liberated per hour per mg fraction	
S	0.18 ± .07	(n = 3)
S(0-50)	0.07	(n = 1)
S(50-70)	0.35 ± .08	(n = 3)
S(70-85)	0.08 ± .04	(n = 2)
S(0-50)D	0.02	(n = 1)
S(50-70)D	0.60 ± .06	(n = 4)
S(70-90)D	0.01	(n = 1)

S(60-70)D was used however in an attempt to determine approximately the V_{max} and K_m of this preparation. The range of substrate concentrations which could be tested unfortunately was narrow. CDNA precipitated above 6.8×10^{-5}, and below 2×10^{-5} M the initial velocity could not be determined because of the limitations in the sensitivity of the assay. Moreover, the enzyme preparation was not completely stable, and was of course highly impure. Nevertheless, from the data plotted in Figure 23 we can gain some idea of the reaction constants. The plot of the reciprocal initial velocity versus the reciprocal substrate concentration was linear except for one point. On the basis of the line drawn, $V_{max} = 1.7 \times 10^{-7}$ moles per mg per hr and $K_m = 8.5 \times 10^{-6}$ moles per liter.

One other difficulty in the interpretation of these kinetic data should be mentioned. There are two susceptible bonds per molecule of CDNA. The second bond is certainly attacked, since with sufficiently long incubation periods the molar concentration of beta-naphthylamine became greater than the initial molar concentration of CDNA.

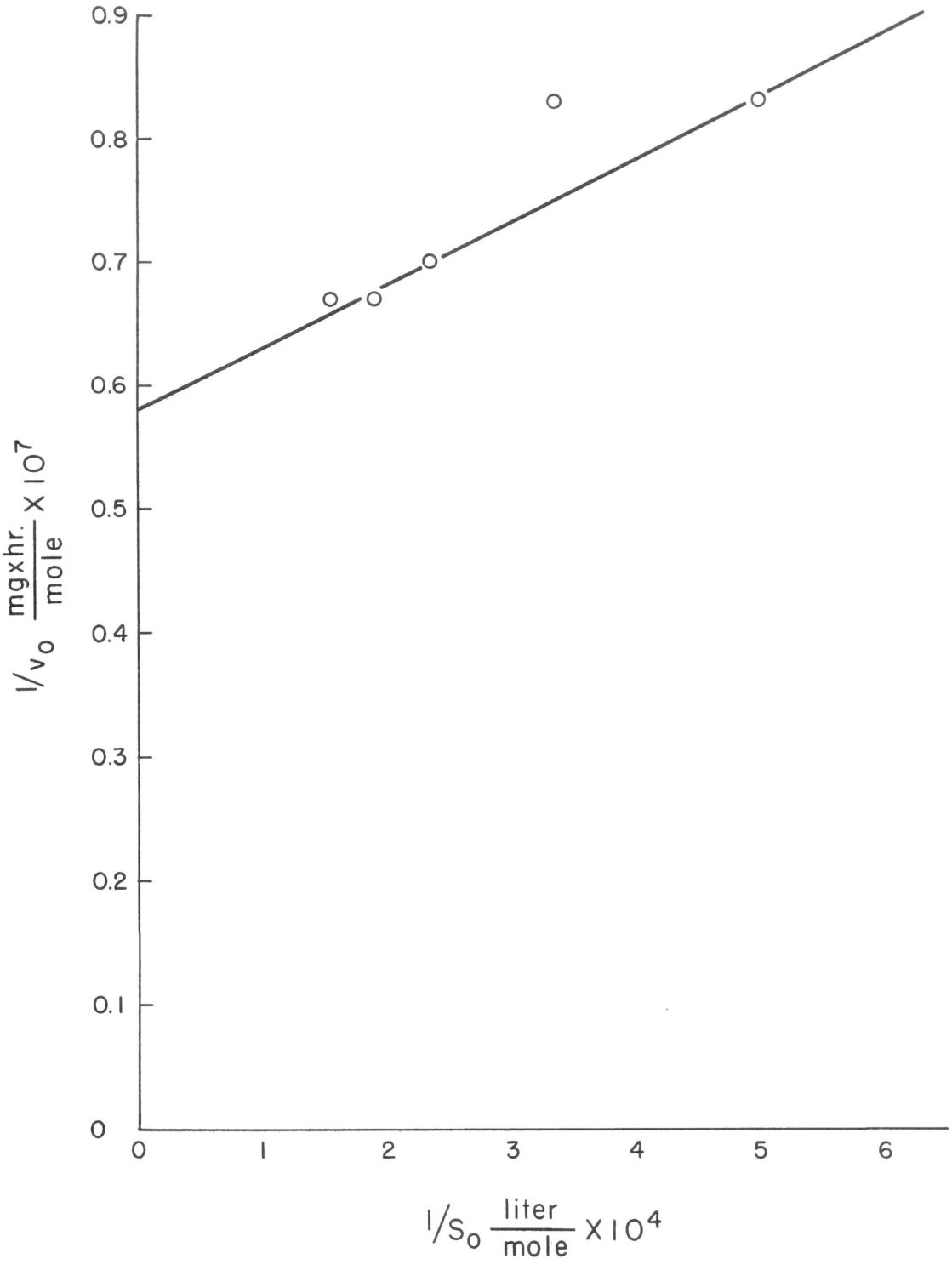


Figure 23. A Lineweaver-Burk plot of the CDNAase activity of S(60-70)D. The incubation mixtures contained 0.25 ml S(60-70)D, 0.5 ml tris-acetate buffer, pH 7.4, and 0.25 ml CDNA of different concentrations. The rates after 5 and 10 minutes were averaged. $V_{max} = 1.7 \times 10^{-7}$ moles per hr, and $K_m = 8.5 \times 10^{-6}$ moles per liter.

We can conclude thus far that CDNA is split by S and its fractions, that the fractionation of CDNAase activity and of oxytocin inactivating activity is similar, and that the actual specific activities are of the same magnitude; e.g., for S(50-70)D, the CDNAase activity is about 0.6×10^{-7} moles per mg per hr and the oxytocin inactivating activity is about 0.2×10^{-7} moles per mg per hr.

The specific CDNAase activity appears to be higher in toad bladder homogenates, and especially homogenates of the mucosal cells of the toad bladder, than in homogenates of other tissues of the toad (Table VI). In all cases the particle-free supernatant fraction of the tissue homogenate was used. Mucosal cells, free of supporting tissue, were obtained by stretching half-bladders, mucosal side out, over a plastic centrifuge tube and by gently scraping the mucosal surface with a clean razor blade. The scrapings were washed off into tris-chloride-versene buffer. They appeared in the phase microscope to consist entirely of sheets of epithelial cells. A supernatant fraction was then prepared from a homogenate of these cells. Supernatant fractions were also prepared of the heart, of the egg mass, and of the liver. The specific activity of the mucosal cell supernatant was almost twice the mean specific activity of the whole bladder supernatant. This clearly suggests that the CDNAase activity is located principally in the epithelium and not in the smooth muscle or blood vessels of the lamina propria (compare Figure 7). Of the other tissues, heart appeared to contain less but comparable specific activity, while that of the egg-mass and of liver was considerably less. Of course, the <u>total</u> activity of the liver would still be greater than that of the bladder. We can conclude that the specific CDNAase activity of the bladder epithelium is considerable and is probably greater than that of other tissues of the toad.

As was the case with the oxytocin inactivating activity, cysteine was found to stimulate CDNAase activity. The effect of 1 mM cysteine on the CDNAase activity of two preparations of S(50-70) and of yet another preparations of S(50-70)D is shown in Table VII. Cysteine, it appears, increased the specific activity of S(50-70) about 80% and of S(50-70)D 60%.

The effects of N-ethylmaleimide (NEM), of iodoacetic acid (IA), and of oxidized glutathione (GSSG) were also determined. NEM had been shown to inhibit the oxytocin inactivating activity of S (see section A). IA will

TABLE VI

The Specific CDNAase Activity of Supernatant Fractions of Various Tissues of the Toad

CDNA was in excess of saturation. Incubation was at 30° C for 2 hours.

S Fraction of	Specific CDNAase Activity 10^{-7} moles β-naphthylamine liberated per hour per mg fraction
Whole bladder	0.18 ± .07
Mucosal cells of bladder	0.33 ± .03
Heart	0.11 ± .01
Egg mass	0.05 ± .01
Liver	0.03 ± .01

TABLE VII

The Effect of Cysteine on the CDNAase Activity of S(50-70) and of S(50-70)D

Cysteine was present in the incubation mixture in the concentrations indicated. CDNA was present in excess of saturation. Incubation was at 30°C for 1 hr.

Fraction*	Cysteine Concentration	Specific Activity 10^{-7} moles β-naphthylamine liberated per hour per mg fraction	Ratio
S(50-70)	0	0.33	1.8
	1 mM	0.60	
S(50-70)	0	0.41 ± .04	1.9
	1 mM	0.78 ± .03	
S(50-70)D	0	0.47 ± .01	1.6
	1 mM	0.74 ± .05	

* The three preparations were from different sources.

react with SH groups with the liberation of hydroiodic acid, and GSSG will form mixed disulfides with free SH groups. These reagents, in 1 mM concentration, caused considerable inactivation of the CDNAase activity of S(50-70). Table VIII summarizes these results. NEM and IA were preincubated with S(50-70) for 20 minutes; then an equivalent amount of cysteine was added to react with excess NEM and IA. 5 minutes later CDNA was added and the incubation begun. GSSG was added at the beginning of the incubation. The activation by cysteine and the inactivation by sulfhydryl reagents suggests the participation of a sulfhydryl enzyme in the splitting of CDNA, as in the inactivation of oxytocin. However, NEM and IA can react with other groups, such as the imidazole of histidine, while GSSG can possibly act as a competitive inhibitor. Nevertheless, that a sulfhydryl enzyme is responsible for both the CDNAase and the oxytocin inactivating activities is an attractive working hypothesis.

TABLE VIII

The Inhibition of the CDNAase Activity of S(50-70) by Sulfhydryl Reagents

1 mM NEM and IA were preincubated with S(50-70) 20 minutes; then cysteine was added to 1 mM. After 5 minutes, CDNA was added in excess of saturation. 1 mM GSSG was added with CDNA. Incubation was at 30° C for 2 hours. NEM = N-ethylmaleimide, IA = iodoacetic acid, and GSSG = oxidized glutathione.

Inhibitor	Specific Activity 10^{-7} moles β-naphthylamine liberated per hour per mg fraction	Per Cent Inhibition
None	0.41 ± .04	-
NEM	0.25 ± .01	40%
IA	0.15 ± .01	65%
GSSG	0.19 ± .01	55%

Further evidence, supporting the view that a single enzyme is responsible for the inactivation of oxytocin and the hydrolysis of CDNA, is that oxytocin inhibited the hydrolysis of CDNA by S(50-70)D. Oxytocin, present in the incubation mixture in the concentration of 0.1 mM, inhibited by 30% the rate of hydrolysis of CDNA present in excess of saturation (i.e., at constant concentration of 6.8×10^{-5} M). The incubation was at 30° C for 30 minutes. In another experiment, 0.75 mM oxytocin inhibited the CDNAase activity of S(50-70)D about 40%. In this case, however, the incubation was longer, 2 hours, during which time the concentration of oxytocin was probably considerably reduced, while the concentration of CDNA, present in excess of saturation, was constant. We will return to this inhibition by oxytocin when we consider its effect on LNAase activity.

The specificity of the hydrolytic activity of the fractions of S was then investigated. Two other substrates were tested, principally LNA, and also D,L-alanine-beta-naphthylamide (ANA). The specific ANAase activity was found to be of the same magnitude as the specific CDNAase activity. It was not investigated further. The LNAase activity was investigated extensively and compared in many experiments with the CDNAase activity. In experiments in which less than 25% of the total substrate was split, the mean specific CDNAase activity of S(50-70)D was $0.6 \pm 0.1 \quad 10^{-7}$ moles per mg per hr, and the mean specific LNAase activity was $5.3 \pm 0.6 \quad 10^{-7}$ moles per mg per hr. The mean ratio of LNAase to CDNAase activity was 8.5. The substrate concentrations were 10^{-3} M LNA, and CDNA in excess of saturation. The incubations were at 30° C for 30 minutes.

Among the fractions S(0-50)D, S(50-70)D and S(70-90)D, all prepared at the same time, S(50-70)D contained the highest specific LNAase activity and, as before, the highest specific CDNAase activity as well (Table IX). In these experiments, the incubation was for 1 hour so that low specific activities could be detected. Also, CDNA was present at saturating concentration, but not in excess. As a result, nearly all the substrate, both LNA and CDNA, was used up in the incubation with the most active fraction S(50-70)D. Hence the specific activity of this fraction as determined was low. Nevertheless, the specific activity of S(50-70)D was still considerably greater than that of the other two fractions. Clearly most of the LNAase activity, together with the CDNAase activity, was in S(50-70)D.

TABLE IX

The LNAase and CDNAase Activities of Fractions of S

The initial substrate concentrations were CDNA, 6.8 x 10^{-5} and LNA, 1.25 x 10^{-4} M. Incubation was at 30° C for 1 hr. In the incubations with S(50-70)D, both with LNA and with CDNA, nearly all the substrate was split.

Fraction*	Protein Recovered in Fraction mg	Substrate	Specific Activity 10^{-7} moles β-naphthylamine liberated per hour per mg fraction
S(0-50)D	97	CDNA	0.02 ± .01
		LNA	0.16 ± .01
S(50-70)D	104	CDNA	>0.24
		LNA	>0.40
S(70-90)D	84	CDNA	< 0.01
		LNA	0.06 ± .01

* These fractions were prepared from a single preparations of S.

The dependence of the CDNAase and of the LNAase activities of S(50-70)D on pH may be compared in Figure 24. The pH curves for these two substrates appear quite similar, with a maximum near pH 7.0. The curve for LNAase activity would have been steeper at higher substrate concentrations, since at the maximum rate nearly all of the available substrate was split.

The heat inactivation of the CDNAase and of the LNAase activity of S(50-70)D was then studied. Aliquots of S(50-70)D were heated to 50° C or 60° C or 70° C for 3 minutes, cooled for 1 minute in an ice-bath, and then incubated at 30° C for 30 minutes with CDNA and with LNA. Inactivation of both activities was slight at 50° C and complete at 70° C. The two activities were then compared after heating aliquots of S(50-70)D to temperatures between 50° C and 60° C (Table X). The results showed a progressive inactivation of both activities, -30% at 52.5° C, -55% at 55° C, and -75% at 57.5° C, in which the ratio of the two activities, however, remained nearly constant.

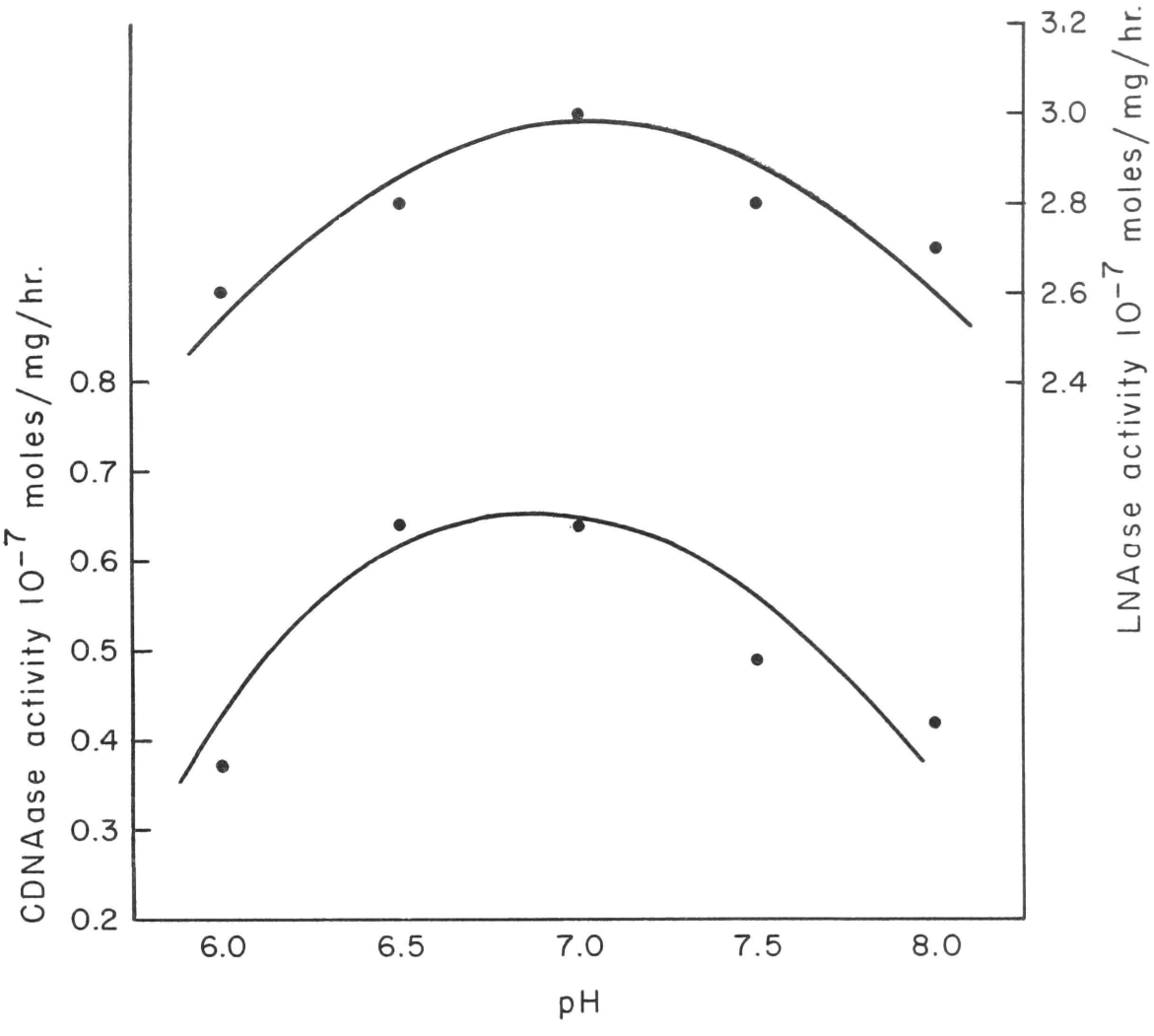


Figure 24. The pH dependence of the CDNAase and of the LNAase activity of S(50-70)D.

The incubation mixtures contained 0.1 ml S(50-70)D, 0.2 ml phosphate buffer, and 0.1 ml substrate. The initial concentration of CDNA was 6.8×10^{-5} M, and of LNA, 1.25×10^{-4} M. Incubation was at 30° C for 30 minutes.

TABLE X

The Relation Rates of Hydrolysis of CDNA and of LNA following Partial Heat Inactivation of S(50-70)D

The incubation mixtures contained 0.1 ml S(50-70)D (treated as indicated), 0.2 ml phosphate buffer, and 0.1 ml substrate; the pH was 7.0; the initial substrate concentrations were: CDNA, in excess of saturation; LNA, 1 mM. Incubation was at 30° C for 30 minutes.

Pre-heat Temperature	Substrate	Specific Activity 10^{-7} moles β-naphthylamine liberated per hour per mg fraction	Ratio $\frac{\text{LNAase}}{\text{CDNAase}}$
-	LNA	4.33	7.6
	CDNA	0.57	
52.5° C	LNA	2.95	7.6
	CDNA	0.39	
55.0° C	LNA	1.90	7.9
	CDNA	0.24	
57.5° C	LNA	1.19	7.9
	CDNA	0.15	

The evidence thus far, from studies on the partial heat inactivation, the pH dependence, and the ammonium sulfate fractionation, tend to support the view that a single enzyme is responsible for both the CDNAase activity and the LNAase activity.

To obtain further evidence either in support or in opposition to this view we tested the effect of a number of agents on the two known activities of S(50-70)D. These results are summarized in Table XI. 0.1 mM oxytocin inhibited LNAase activity twice as much as it did CDNAase activity (-60% compared to -30%). If oxytocin is acting as a competitive inhibitor, we may conclude that it is a better competitor of LNA than of CDNA (the initial concentration of LNA was twice that of CDNA). This is consistent with the view that a single enzyme acts on CDNA and on LNA but that the K_m

of CDNA is less than the K_m of LNA; in other words, the enzyme binds CDNA better than LNA. On the other hand, two enzymes could be present, both of which are inhibited by oxytocin.

TABLE XI

The Effect of Various Agents on the CDNAase and LNAase Activities on S(50-70)D

The incubation mixtures contained CDNAase in excess of saturation and LNA at 10^{-3} M, except for the experiments with oxytocin and with $MgSO_4$ in which the concentration of LNA was 1.25×10^{-4} M. $MnCl_2$ was pre-incubated with S(50-70)D for 75 minutes at 30° C. The other agents were added with substrate at the start of the incubation. Incubations were at 30° C for 30 minutes.

Agent	Per Cent Increase or Decrease of Activity	
	CDNAase	LNAase
0.1 mM oxytocin	-30	-60
1 mM L-cysteine	+60	0
5 mM versene	-10	-35
1 mM L-leucine	0	+15
2 mM $MgSO_4$	0	+20
1 mM $MnCl_2$	-5	-25
1 mM $HgCl_2$	-100	-100

Another large difference in the effect on the two activities was obtained with cysteine. 1 mM cysteine enhanced CDNAase activity by 60%, but did not effect LNAase activity. On the basis of the one enzyme view, this would have to be explained as an effect of cysteine on the substrate, CDNA, perhaps through the formation of the mixed disulfide, L-cystine-mono-beta-naphthylamide, or through reduction to form L-cysteine-beta-naphthylamide. These compounds might be more easily attacked than CDNA.

5 mM versene appeared to have a greater inhibitory effect, and 2 mM $MgSO_4$ a greater stimulatory effect, on LNAase activity than on CDNAase activity. Below, in the Discussion, we will compare these results with those obtained by others with leucine aminopeptidase and with pregnancy serum oxytocinase. In the meantime our working hypothesis is that an enzyme exists in the toad bladder epithelium which inactivates oxytocin, and which acts like an aminopeptidase both in its action on oxytocin and on synthetic substrates. The question we ask now is what possible physiological significance could such an enzyme have. We shall address ourselves to this question in the next section.

D. Interactions of Oxytocin with the Intact Bladder

Methods

The technique of measuring the change in permeability of the bladder in response to oxytocin has been described in Methods, A. A few modifications of the previously described technique were made in the work in this section. The inside of the bladder (i.e., the mucosal solution) was now filled with 5 ml of water rather than 3 ml. The outside (serosal) solution was 25 ml as before, but consisted of Ringer's solution containing 5 mM glucose and 5 mM sodium pyruvate (pH 7.7); the glucose and sodium pyruvate being added shortly before use. In this section we will call this latter solution, simply, Ringer's.

The protocol for these experiments was as follows: Five or six half-bladders, each from a different toad, were used in each experiment. The number of assay tubes which were set up was two, three or four times the number of half-bladders. If six half-bladders were used, then there might be three sets of six tubes each. We will call these sets, tubes 1-6, tubes 7-12, and tubes 13-18. Each tube was filled with 25 ml Ringer's and air bubbled through as previously described (Methods, A). The six half-bladders were mounted on the inserts, filled with 5 ml of water, and placed in tubes 1-6. The half-bladders were then consecutively weighed exactly 2 minutes apart; at 0 min the first, at 2 min the second, etc. After a 20 min interval each half-bladder was weighed again, the first at 20 min, the second at 22 min, etc. The difference between the first and the second weighing corresponded to the loss of water from the inside of the half-bladder during this 20 minute interval. The half-bladders were weighed again after another 20 minute interval. The difference between the second weighing and the third weighing was equal to the loss of water during the second 20 minute interval. All experiments began with two such 20 minute intervals. Together, they constituted a 40 minute control period during which the base line, the rate of loss of water in the absence of hormone, was determined. This rate was always low compared to the rate in the presence of hormone. Shortly before the third, and last, weighing of the control period, an equal dose of oxytocin was added to each of the tubes 7-12; that is, oxytocin was added to tube 7 one minute before the last

weighing of the half-bladder in tube 1, and to tube 8 one minute before the last weighing of the half-bladder in tube 2, etc. The last control weighing of each half-bladder was actually at 19 min 30 sec, so that exactly on the minute each half-bladder was transferred to a tube containing oxytocin, from tube 1 to the balance to tube 7, from tube 2 to the balance to tube 8, etc. The last weighing of the control period was also the first weighing of the first test period. During the test periods the weighing procedure was as follows: After a 19 minute interval, each of the half-bladders (always 2 minutes apart) were weighed and the loss during the past interval quickly calculated. The amount of water lost was then added back to the mucosal solution by the following method. A 2 ml syringe with a short length of polyethylene tubing attached was inserted into the top of the hollow glass insert and a volume of water approximately equivalent to the amount lost was added. During this operation the half-bladder was in its serosal solution. Then the half-bladder was re-weighed and again returned to its assay tube. The entire operation applied to each half-bladder was then to remove from tube, weigh, return to tube, add back loss, remove from tube, re-weigh, and return to tube. This process took 1 minute, so that the first half-bladder was first weighed at 19 minutes and was finally returned at 20 minutes, the second half-bladder was first weighed at 21 minutes and was finally returned at 22 minutes, etc. Hence, during the test periods the actual length of the interval during which the loss was measured was 19 minutes, or in some experiments 9 minutes. In the latter experiments, at most five half-bladders could be accomodated, these being weighed at 9,11,13,15 and 17 min, and next at 19,21,23,25, and 27 min, etc.

Whether the intervals between weighings were 10 minutes or 20 minutes (intervals of actual measured loss of 9 minutes or 19 minutes) the test period ran 60 to 120 minutes. In some experiments, in particular those in which the time course of the rate of loss was determined, the experiment concluded at the end of the test period. In many others, at the end of the test period, the half-bladders were transferred from tubes 7-12 back to tubes 1-6. Weighing continued in tubes 1-6 at 20 minute intervals. By the second 20 minute interval the rate of loss was back to the control values, and the half-bladders were then transferred to a third set of tubes

containing hormone for a second test period. The 40 minute period between two test periods is called a recovery period. An experiment then could run as follows: a 40 minute control period in tubes 1-6, an 80 minute test period in tubes 7-12 (containing oxytocin), a 40 minute recovery period in tubes 1-6, and a 60 minute second test period in tubes 13-18 (containing oxytocin).

The response of a half-bladder during a test period is defined as the total loss of water of that half-bladder during the first 60 minutes of the period. The actually measured loss was for 19 minute intervals. These values were multiplied by 20/19 to correct to 20 minute intervals, and the losses of the first three intervals were added. The response of the first test period is called L_1, and of the second test period, L_2, etc. To compare two test periods, L_1-L_2 was calculated for each half-bladder. The mean difference, $\overline{L_1-L_2} \pm S_m$, the standard deviation of the mean, was calculated. The significance of the mean difference was determined by the t-test. The ratio of the mean hormone concentrations present in the serosal solutions during the first and during the second test periods was estimated from $\overline{L_1-L_2}$ by the following procedure. A standard log concentration-response curve was determined (Figure 26). The regression coefficients of the linear region of this curve were determined. In particular, the slope, b, was determined. Since the response, L, is linearly related to the log concentration of hormone, log C,

$$\overline{L_1-L_2} = b(\overline{\log C_1 - \log C_2}) = b\,\overline{\log \frac{C_1}{C_2}}, \text{ or } \overline{\log \frac{C_1}{C_2}} = \frac{\overline{L_1-L_2}}{b}.$$

The error in $\log \frac{C_1}{C_2}$ is due to the error in $\overline{L_1-L_2}$ and to that in b. The standard deviation of b, however, was only 8% of b and made a negligible contribution to the standard deviation of $\log \frac{C_1}{C_2}$ (less than 10% of total standard deviation). Therefore, the standard deviation of $\overline{\log \frac{C_1}{C_2}}$ was taken as $\frac{S_m}{b}$ (S_m is the standard deviation of the mean of L_1-L_2). $\frac{C_1}{C_2}$ was

estimated by taking the anti-logarithm of $\log \frac{C_1}{C_2}$, and bounds were estimated by taking the anti-logarithm of $\log \frac{C_1}{C_2} + \frac{S_m}{b}$ and of $\log \frac{C_1}{C_2} - \frac{S_m}{b}$.

The osmolal concentration of solutions was determined in a Fiske Osmometer with a KCl standard.

Results

In the previous sections we were concerned with the characterization of certain extracts of the toad bladder which inactivate oxytocin and split N-terminal peptide bonds. We presented evidence that there is at least one enzyme present which splits an N-terminal half-cystinyl peptide bond in the substrate, CDNA, and might inactivate oxytocin by splitting a similar bond. In both of these cases, this enzyme would be acting as a cystine-aminopeptidase. The fact that N-terminal cystinyl bonds are not readily split by the widespread enzyme, leucine aminopeptidase (see the Discussion) encouraged the view that the oxytocin inactivating potency of the bladder extracts could have some specific function with regard to the action of the neurohypophyseal hormones on the intact toad bladder. The simplest idea would be that this enzyme limited the duration of the effect of the hormone through inactivation. It was such an effect then for which we first looked; that is, we observed the time course of the response of the bladder to a single dose of hormone.

The experiments were performed as described in the Methods. There was a 40 minute control period in tubes 1-5; then a 110 minute test period in tubes 6-10 containing oxytocin. The intervals in the test period were 10 minutes. Two experiments, containing 5 half-bladders each, were performed at each hormone concentration. The mean rate of loss in mg per min during each interval was calculated. The results are shown in Figure 25. Characteristically, the maximum rate of loss was reached 10 to 20 minutes after the addition of hormone. Thereafter, the rate of loss declined, and was about 50% maximum 80 to 90 minutes later. At the lowest concentration tested, 2.5 mμM, the effect was barely perceptible; and at the highest concentration, 80 mμM, the rate of loss was only slightly greater than that

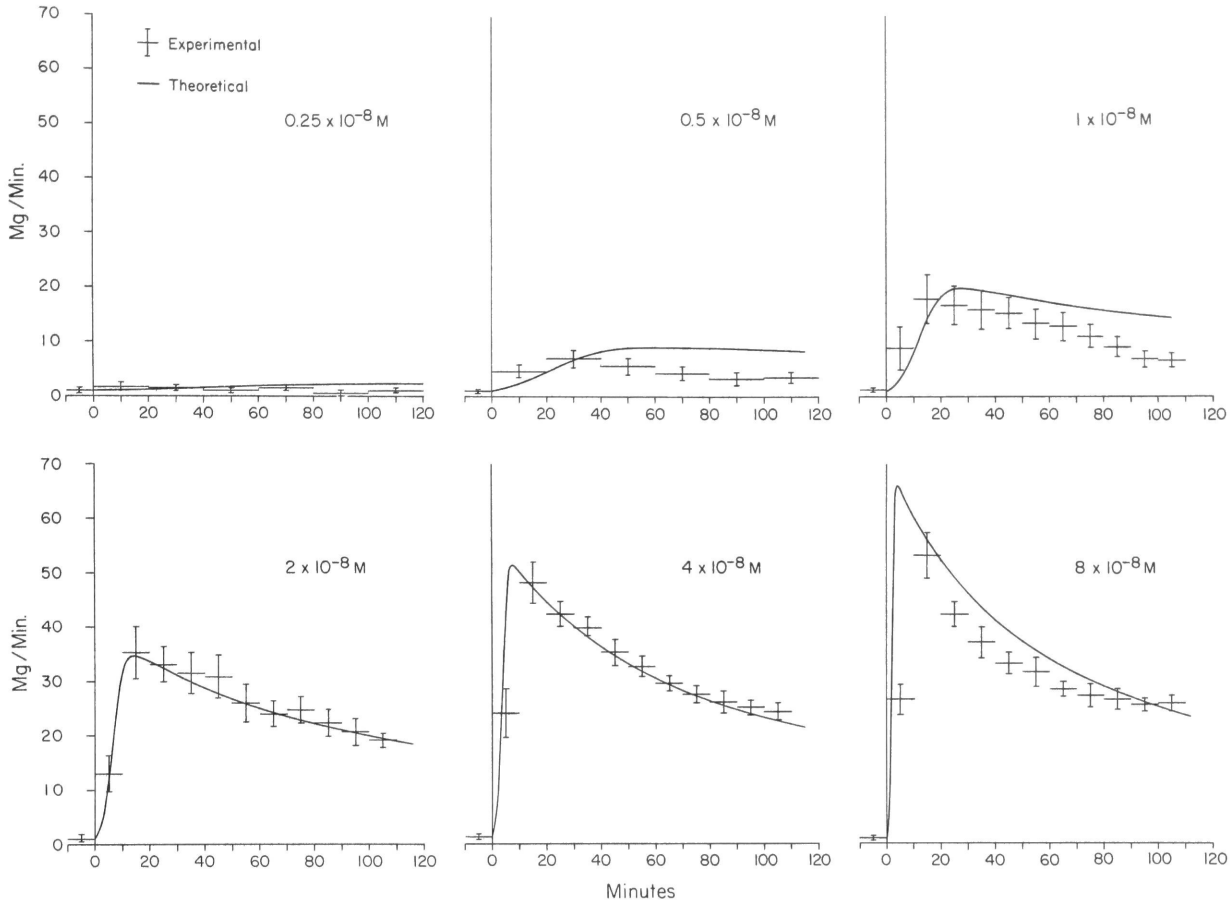


Figure 25. The time course of the effect of various concentrations of oxytocin on the water permeability of the toad bladder.

The experimental values are given as the mean rate of loss in mg per min ± the standard deviation of the mean. The weighing intervals are indicated by the length of the horizontal sections. The half-bladders were placed in Ringer's containing oxytocin at t = 0 min. The mean rate of loss during the 40 min control period is given at t < 0 min. 10 half-bladders were used at each concentration. (The curves were calculated from a theoretical model of hormone action).

at the next lower concentration, 40 mμM (the curves in Figure 25 and Figure 26 have been calculated from a model of hormone action; cf., below). There is no question that the effect of a single dose of oxytocin decreases with time.

The dependence on hormone concentration of the mean response, defined as the mean total loss during the first 60 minutes, is shown in Figure 26. Hormone concentration has been plotted on a log scale. The slope of the regression line fitted to the four central points (5,10,20, and 40 mμM) was calculated to be 2120 ± 170 mg per hour per ten times increase in hormone concentration (i.e., per log unit). The response at 2.5 mμM oxytocin was 80 ± 30 mg per hr. This is not significantly different than the control response which was about 60 mg per hr. Also, the response at 80 mμM oxytocin, 2245 ± 120 mg per hr, is not significantly different than the response at 40 mμM, 2230 ± 130 mg per hr.

Now that we see that the effect of a dose of oxytocin declines with time, we must try to answer why. The first question we must ask is whether irreversible, degenerative changes are occurring in the isolated bladder, and whether these changes lead to the decrease in effect. To answer this question the following experiment was performed: There was a 40 minute control period in tubes 1-6, then an 80 minute test period in tubes 7-12 containing 20 mμM oxytocin, then a 40 minute recovery period in tubes 1-6, and then a 60 minute test period in tubes 13-18 containing 20 mμM oxytocin. In other words, after recovery from the first dose, the half-bladders have been exposed to an equal second dose. The time course of the effect of these changes on the rate of water loss is shown in Figure 27. The effect of the first dose of oxytocin was clearly declining by the last 20 minute interval of the first test period. When the half-bladders were removed from Ringer's containing oxytocin and placed in Ringer's not containing oxytocin (at the second arrow), the rate of loss decreased in about 20 minutes to the level of the control. Clearly the effect of oxytocin continues only so long as the hormone is present in the serosal solution. Following the recovery period, the half-bladders responded fully to a second equal dose of oxytocin in new Ringer's. The actual mean difference in response and calculated ratio of hormone concentrations is given in

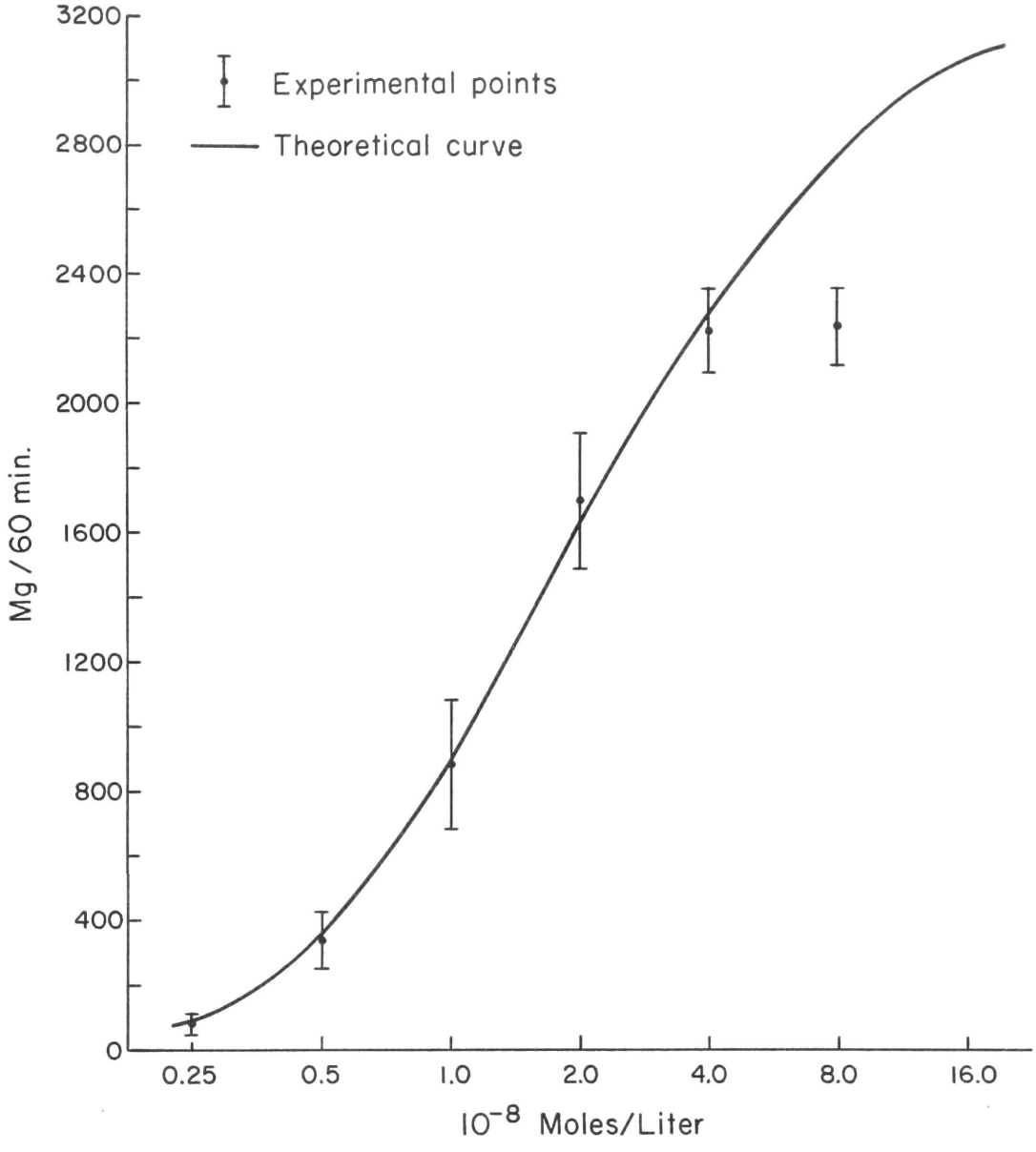


Figure 26. The total loss of water during the first 60 minutes of activity versus the log concentration of oxytocin. The theoretical curve is a plot of the integral from 0 to 60 min of the curves in Figure 25.

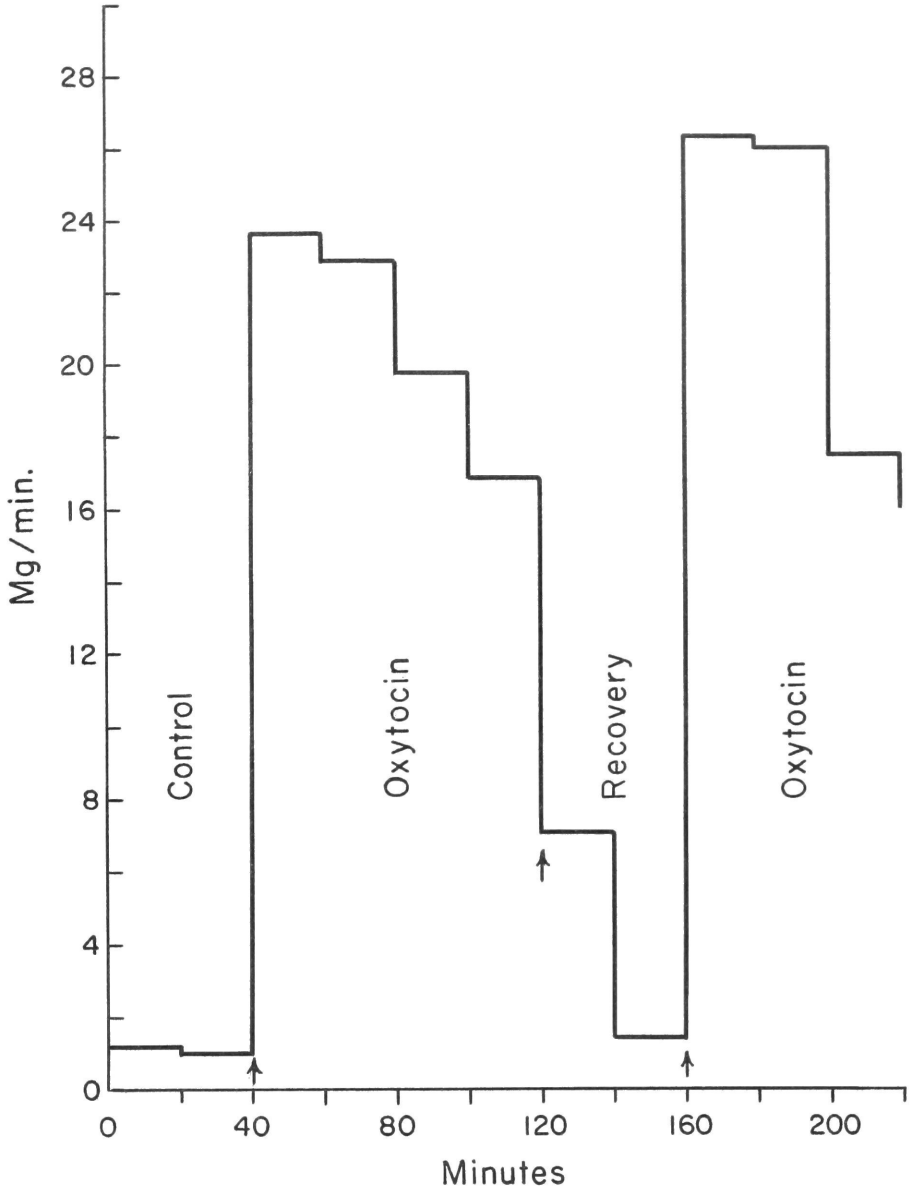


Figure 27. The response of the half-bladders to a second dose of oxytocin following a recovery period.

The mean rate of loss of water in mg per min is plotted against minutes. The 40 min control was in tubes 1-6. The half-bladders were transferred (at the first arrow) to tubes 7-12 containing 20 mμM oxytocin. At 120 min (second arrow) the half-bladders were transferred back to tubes 1-6, and at 160 min (third arrow) to tubes 13-18 containing 20 mμM oxytocin.

Table XII, experiment a. The difference in response was not significant. We can rule out then that the decline in the effect of oxytocin is due to irreversible changes in the isolated bladder.

We might then ask whether the decline is due to the decrease in osmotic gradient caused by the flow of water from the mucosal solution to the serosal solution. This decrease in osmotic gradient can be calculated. From Figure 26, we can see that about 1700 mg, or 1.7 ml, of water enters the serosal solution in one hour at an oxytocin concentration of 20 mμM. The initial volume of the serosal solution was 25 ml. Therefore the concentration of solutes has decreased by a factor of 25/26.7 = 0.94, or by 6%. The concentration of solute in the Ringer's was 224 mOsm. The concentration of solute in the mucosal solution after 60 min was 10 $\pm$ 5 mOsm (most of this solute must have been present from the start in a residual amount of Ringer's in which the bladders were originally washed). Therefore, the decrease in the osmotic gradient was not greater than 10% in the first hour of activity. Moreover, as the rate of water loss declined, the rate of decrease in osmotic gradient would decline also. The following experiment settled this question. The half-bladders were first in tubes 1-6 for 40 minutes, then in tubes 7-12 containing 20 mμM oxytocin for 80 minutes, then in tubes 1-6 for 40 minutes, then for 60 minutes in tubes 13-18 containing 20 mμM oxytocin and to which were added the amount of water lost by each half-bladder during the first 80 minute test period. The amount lost by the first half-bladder was added to tube 13, the amount lost by the second to tube 14, etc. Therefore, if dilution of the serosal solution were an important factor in the decline of the oxytocin effect, the second response should be less than the first. In fact the second response was slightly, though not significantly, greater than the first (Table XII, experiment b). Therefore the dilution of the serosal solution was not an important factor in the decline of the effect of oxytocin.

Another possible explanation for the decline is that the exogenous substrates, glucose and pyruvate, were being depleted, and that in some way the maintenance of high permeability is coupled to the metabolism of these substrates. We tested this with the following experiment. The half-bladders were 40 min in tubes 1-6, 60 min in tubes 7-12 containing 20 mμM oxytocin, 40 min in tubes 1-6, and finally 60 min in tubes 13-18 in which

TABLE XII

Resume of the Inactivation of Oxytocin by the Intact Bladder

Experiment*	Mean Difference in Response between the First and Second Test Periods $\overline{L_1 - L_2}$ mg/hr		Calculated Ratio of Concentrations** $\frac{C_1}{C_2}$
a	-68 ± 52	(P > 0.2)	0.93 ± 0.05
b	-46 ± 72	(P > 0.5)	0.95 ± 0.08
c	-69 ± 91	(P > 0.4)	0.93 ± 0.09
d	723 ± 54	(P < 0.001)	2.2 ± 0.2
e	452 ± 106	(P < 0.01)	1.65 ± 0.2
f	70 ± 94	(P > 0.4)	1.1 ± 0.1
g	372 ± 132	(P < 0.05)	1.5 ± 0.2
h	737 ± 146	(P < 0.01)	2.2 ± 0.4
i	651 ± 140	(P < 0.01)	2.0 ± 0.3
j	-364 ± 109	(P < 0.05)	0.67 ± 0.08

a. 40 min in tubes 1-6; 80 min in tubes 7-12 + 20 mμM oxytocin (L_1); 40 min in tubes 1-6; 60 min in tubes 13-18 + 20 mμM oxytocin (L_2).

b. 40 min in tubes 1-6; 80 min in tubes 7-12 + 20 mμM oxytocin (L_1); 40 min in tubes 1-6; 60 min in tubes 13-18 + 20 mμM oxytocin + the amount of water lost by each half-bladder in the first 80 min test period (L_2).

c. 40 min in tubes 1-6; 60 min in tubes 7-12 + 20 mμM oxytocin (L_1); 40 min in tubes 1-6; 60 min in tubes 13-18 in Ringer's minus glucose and pyruvate + 20 mμM oxytocin (L_2).

d. 40 min in tubes 1-6; 80 min in tubes 7-12 + 20 mμM oxytocin (L_1); 40 min in tubes 1-6; 60 min in tubes 7-12 (L_2).

e. 40 min in tubes 1-6; 80 min in tubes 7-12 + 20 mμM oxytocin (L_1); 40 min in tubes 1-6; 60 min in tubes 7-12 + 10 mμM oxytocin (L_2).

f. 40 min in tubes 1-6; 80 min in tubes 7-12 + 20 mμM oxytocin (L_1); 40 min in tubes 1-6; 60 min in tubes 7-12 + 20 mμM oxytocin (L_2).

g. 40 min in tubes 1-6; 100 min in tubes 7-12 + 10 mμM oxytocin (L_1); 40 min in tubes 1-6; 60 min in tubes 7-12 + 10 mμM oxytocin (L_2).

h. 60 min in tubes 1-6; oxytocin (20 mμM) added to tubes 1-6 just after half bladders transferred out; 60 min in tubes 7-12 + 20 mμM oxytocin (L_1); 40 min in tubes 13-18; 60 min in tubes 1-6 (oxytocin having been in tubes 1-6 for 100 min) (L_2).

i. 60 min in tubes 1-6; 60 min in tubes 7-12 + 20 mμM oxytocin (L_1); 40 min in tubes 13-18; 60 min in tubes 1-6 + 20 mμM oxytocin (L_2).

j. 60 min in tubes 1-6; 60 min in tubes 7-12; 60 min in tubes 1-6 + 20 mμM oxytocin (L_1); 40 min in tubes 13-18; 60 min in tubes 7-12 + 20 mμM oxytocin (L_2).

* The experiments discussed in the text have been described here in a condensed form. In all experiments the six half-bladders are transferred from one set of serosal solutions to another. L_1 is the loss of water during the first hr of the first test period, and L_2 is the loss during the first hr of the second test period.

** $\frac{C_1}{C_2} = 10^{\left[\frac{L_1 - L_2}{2120}\right]}$ (see Methods).

Ringer's containing no glucose and pyruvate was substituted for the usual Ringer's, and containing 20 mμM oxytocin. In other words, the second response was in Ringer's minus glucose and pyruvate. Again the two responses were not significantly different (Table XII, experiment c). We might also mention at this point that the pH of the serosal solutions bathing the half-bladders did not change detectably after 60 min in the presence of 20 mμM oxytocin.

It appears then that we are left with two alternatives: either oxytocin is being gradually inactivated or a reversible change in the bladder epithelium such as the depletion of some critical endogenous substrate is occurring. These alternatives are not mutually exclusive, and both could occur. We tested for inactivation of oxytocin by the following experiment. The half-bladders were 40 min in tubes 1-6, 80 min in tubes 7-12 containing 20 mμM oxytocin, 40 min in tubes 1-6, and then 60 min back in tubes 7-12. If the oxytocin in tubes 7-12 had not been appreciably inactivated during the first test period, the response in the second test period with the half-bladders being placed again in tubes 7-12 should equal the response in the first test period, as would be predicted by the results of experiment a (Table XII). In fact, the second response was significantly less than the first (Table XII, experiment d). The calculated ratio of oxytocin concentrations was 2.2. Hence, since the initial hormone concentration of the first test period was 20 mμM, the initial hormone concentration of the second test period, also the final hormone concentration of the first test period, was apparently 9.1 mμM. It appeared then that during the 80 minute first test period about 50% of the oxytocin had been inactivated. During the same time the mean rate of loss had appreciably declined from 27 mg per min during the first 20 minute interval to 16 mg per min during the fourth, and last, 20 minute interval. Here it seemed we had found the principal factor causing the decline in the effect of a dose of oxytocin.

To verify that the lower second response in the above experiment was due simply to inactivation of the oxytocin, we performed the same experiment, but before the second test period we added to tubes 7-12 the amount of oxytocin that we estimated had been inactivated in the first test period, which in the previous experiment had been 50% of the first dose. Therefore,

we added half of the original dose, which should have brought the concentration of oxytocin in the serosal solutions up to the initial value of 20 mμM. However the results did not support this contention. The second response was considerably less than the first (Table XII, experiment e). The apparent ratio of concentrations was 1.65. Since the initial concentration of oxytocin in the first test period was 20 mμM, that in the second seemed to be 12 mμM. But 10 mμM had been added at the start of the second test period; therefore, the final oxytocin concentration in the first test period apparently was about 2 mμM. During the 80 minute first test period the mean rate of loss of water declined from 29 mg per min during the first 20 minute interval to 22 mg per min during the fourth 20 minute interval. A greater decline in effect than this would be expected if the oxytocin concentration had actually fallen to 2 mμM which is below the lowest concentration in the standard log dose-response curve (Figure 26).

The same experiment was repeated, but instead of the second dose being equal to half the first dose, an equal dose was added the second time. Therefore, the concentration of oxytocin in tubes 7-12 was raised by 20 mμM. Again, if 50% of the original dose of oxytocin remained, then the initial concentration of oxytocin in the second test period would have been about 30 mμM. The first and second responses, however, were not significantly different (Table XII, experiment f). If anything, the second response was actually slightly less than the first. Thus it appeared that the initial oxytocin concentration in the second test period was just 20 mμM. In other words, it appeared that no appreciable amount of oxytocin remained at the end of the first test period. During this test period the mean rate of loss had declined from 19 mg per min to 13 mg per min, the latter rate still being quite substantial compared with the control rate of 1 mg per min.

The above experiment was repeated except that the first test period was extended to 100 minutes (instead of 80 minutes) and the initial concentration of oxytocin reduced to 10 mμM. Again the second dose to tubes 7-12 was equal to the first. In this case the second response was actually significantly less than the first (Table XII, experiment g).

In summary then, when the half-bladders were returned for the second test period to the same serosal solutions in which they had been during the first test period, it appeared that about 50% of the initial dose of oxytocin had been inactivated. When this amount of oxytocin was added to the serosal solutions of the first test period prior to the second test period, it appeared, subtracting the amount added, that about 90% of the original dose had been inactivated. Finally, when a second dose equal to the first was added to the serosal solutions of the first test period prior to the second test period, it appeared that not only was there no oxytocin left from the first period but that the second equal dose had even less effect than the first. But we have already seen that an equal second dose of oxytocin in fresh Ringer's gave a response equal to that produced by the first dose (Table XII, experiment a, b and c). The only possible conclusion is that the second dose of hormone added to the same Ringer's as the first is not having an effect equal to the first, in fact less of an effect, because of some change in the serosal solutions. We have already ruled out the depletion of glucose or of pyruvate as a cause (Table XII, experiment e), and the pH of the serosal solutions does not change (see above). Another possibility, however, is that either an oxytocin inactivating enzyme or an oxytocin inhibitor is accumulating in the serosal solutions. We proceeded to test these possibilities.

The first experiment we tried was as follows: The half-bladders were in tubes 1-6 for 60 min and oxytocin was added to each tube (to 20 mμM) just after each half-bladder was removed, then the half-bladders were 60 minutes in tubes 7-12 containing 20 mμM oxytocin, then 40 minutes in tubes 13-18 (recovery), and then 60 minutes in tubes 1-6. In other words the responses to equal doses of oxytocin were compared; one dose had been added to tubes 7-12 containing fresh Ringer's just before the half-bladders were placed in them, the other dose had been added to tubes 1-6 containing Ringer's in which the half-bladders had remained previous to the addition for 60 minutes, and the oxytocin remained in these tubes for 100 minutes before the half-bladders were placed in them again for the second test period. If an oxytocin inactivating enzyme were leaking out of the bladder into the serosal solution, incubation of oxytocin with that solution for 100 minutes would be expected to lead to considerable inactivation and hence to a decrease in response. In fact this was found to be the case. The second response was considerably less that the first (Table XII, experiment h).

As a control for the above experiment, the identical experiment was done except that the oxytocin was added to the serosal solutions (tubes 1-6) in which the half-bladders had remained for 60 minutes just 30 seconds (instead of 100 minutes) before the half-bladders were placed in these tubes for the second time. If the lower second response in the previous experiment had been due to enzymatic inactivation of oxytocin, surely a 100 minute incubation would cause greater inactivation than a 30 second incubation. In fact, however, the second response was again considerably lower than the first (Table XII, experiment i); moreover, the mean differences of response in this experiment and in the previous experiment (experiment h) were not significantly different ($P > 0.3$). This result clearly supports the view that an inhibitor of the action of oxytocin, not an inactivating enzyme, accumulates in the solution bathing the serosal side of the bladder. We must include the possibility that this inhibitor may be a substance which binds oxytocin, for the accumulation in the serosal solution of a binding substance would explain the results as well as would the accumulation of an inhibitor in the usual sense.

In one further experiment we found that the inhibitory factor could be washed out of the bladder. In this experiment the half-bladders were first in tubes 1-6 for 60 minutes, then in tubes 7-12 for 60 minutes, then back in tubes 1-6 plus 20 mμM oxytocin for 60 minutes, then for 40 minutes recovery in tubes 13-18, and finally for 60 minutes in tubes 7-12 plus 20 mμM oxytocin. In essence we were comparing the amount of inhibiting factor released by the bladders in the first and in the second hour of their remaining in Ringer's. The first response was significantly less than the second (Table XII, experiment j). In other words, more inhibiting factor accumulated during the first hour of soaking than during the second. In fact the second response corresponded to an oxytocin concentration 1.5 times the first. In both cases however, the second as well as the first, the rate of loss declined over the 60 minute test period. In the first the rate decreased from 34 mg per min to 28 mg per min, and in the second from 40 mg per min to 28 mg per min. Hence, if the release of this inhibiting factor into the serosal solution is the cause of the decrease in the rate of loss of water in response to a dose of oxytocin, either there is more of this factor in the bladder than can be washed away in 60 minutes, or more of it is produced in the presence of oxytocin.

In summary, we have first demonstrated that the rate of loss of water in response to a single dose of oxytocin rises to a maximum in 10 to 20 minutes and then decreases to 50% of the maximum in 80 to 90 minutes more. We have inquired into the causes of this decrease in rate and found that it could be accounted for by changes in the hormone activity of the serosal solutions bathing the bladders. It was found that an inhibiting factor was released into the serosal solutions in which half-bladders had been for 60 minutes. This inhibitory factor decreased the subsequent activity of a dose of oxytocin about 50% (Table XII, experiment i). If an approximately equal amount of inhibiting factor accumulated in the serosal solutions in experiments e, f, and g, in which additional doses of oxytocin were added to the serosal solutions, the results of these experiments would be explicable. For instance, in experiment f we added an additional dose of oxytocin equal to the first, but the subsequent response was just equal to the first. If the effective concentration of oxytocin was decreased about 50% by the inhibiting factor accumulated during the first test period such a result would be reasonable. Moreover, we found that the inhibiting factor could be washed out of the bladder, at least in the absence of oxytocin. It should be noted, however, that in all experiments the half-bladders were first soaked for at least 40 minutes (the control period) in different Ringer's than that in which they subsequently responded to oxytocin. In all cases the rate of loss still declined during the test period. One possible explanation for this is that the inhibiting factor is formed in response to oxytocin. In that case, the inhibiting factor washed off the bladders before any exogenous oxytocin had been added to them (experiment h, i, and j) would have to be due to inhibiting factor formed in response to endogenous hormone, probably before the bladders were excised. In the next section we will develop a model of hormone action based on the hypothesis that an inhibiting factor is formed in response to oxytocin, but with the specification that the inhibiting factor is an inactivated product of the hormone itself.

E. A Model for the Control of Hormone Action

In the previous section we found that a likely explanation for the decrease in the effect of a dose of oxytocin is that an inhibitor of the hormone is formed in the bladder, and that the formation of this inhibitor is induced by the hormone itself. We have constructed a model based on this hypothesis. In particular, we assume in this model that the hormone acts by combining reversibly with receptor sites in the bladder epithelium, that the hormone is inactivated in the epithelium by an inactivating enzyme, and that the product of this inactivation can compete with the hormone for the receptor sites. In other words, we hypothesize that the inhibitor that collected in the serosal solutions in the experiments of the previous section was a product of hormone inactivation. The model is schematically represented in Figure 28.

The assumptions we make and their mathematical representations are as follows. We assume that the hormone diffuses from the serosal solution to the receptor sites, which presumably are in the epithelial cells. Furthermore, the permeability of the epithelial cells to the hormone is not constant but is directly proportional to the concentration of hormone already in the cell. The hormone, we know, has a profound effect on the transport of water and salt. We are here postulating that the hormone also effects its own transport. The expression then for the rate of increase of hormone concentration inside the cell is

$$1) \qquad \left[\frac{d(h_i)}{dt}\right]_{dif} = a(h_i)((h_o)-(h_i))$$

where (h_o) is the hormone concentration outside, (h_i) is the hormone concentration inside, and $\underline{a}$ is a constant. This is a diffusion equation in which the permeability is given by $a(h_i)$. This postulate on the hormone permeability has been made so that the theoretical water permeability will increase at low hormone concentrations with the second power of the hormone concentration; that is, so that the predicted dose-response curve will be concave upwards at low hormone concentrations (cf. Figure 26).

We assume next that the hormone combines reversibly with the receptor sites, and that this reaction is at equilibrium. Therefore,

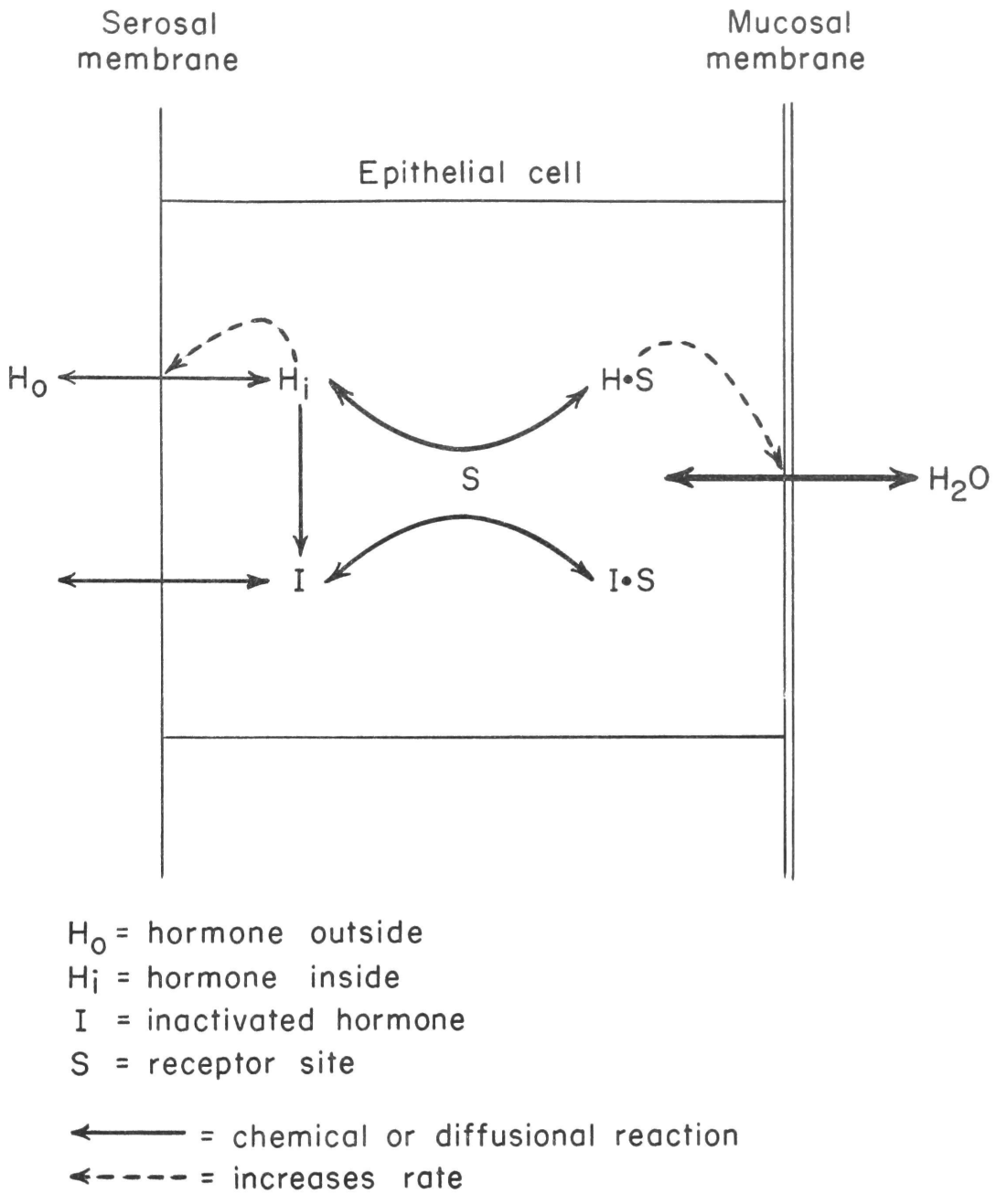


Figure 28. A model of the interaction of hormone with the bladder epithelial cells.

2) $$\frac{(h_i)(s)}{(hs)} = K_H$$

where (s) is the concentration of receptor sites, and (hs) is the concentration of the hormone-receptor complex, and K_H is the equilibrium constant.

We assume that the hormone is inactivated, and that the rate of inactivation is first order (which is not unreasonable considering the low concentrations involved). Therefore,

3) $$\left[\frac{d(h_i)}{dt}\right]_{enz} = -b(h_i)$$

where b is a constant. Furthermore, the rate of change of (i), the concentration of the product of the inactivation is given by

4) $$\frac{d(i)}{dt} = \left[\frac{d(h_i)}{dt}\right]_{enz} = -b(h_i)$$

We are neglecting changes in (i) due to diffusion out of the cell and to further enzymatic degradation.

We assume further that the product of the inactivation combines reversibly with the receptor, and that this reaction is at equilibrium. Therefore,

5) $$\frac{(i)(s)}{(is)} = K_I$$

where (is) is the inactive product-receptor complex, and K_I is the equilibrium constant.

We assume that the permeability of the bladder, w, in mg water lost per half-bladder per min, is directly proportional to the concentration of the hormone-receptor complex. Therefore,

6) $$w = l \cdot (hs)$$

where l is a constant. Equations (1)-(5) must now be solved for (hs) as a function of t, and then (6) will yield w as a function of t. In solving equations (1) and (3), we make one more simplifying assumption and that is

that the outside concentration of hormone, (h_o), remains constant in time. We are assuming essentially that the total amount of hormone inactivated by a half-bladder in 2 hours is small compared to the total amount of hormone in the serosal bath. The validity of this approximation will depend on the rate of inactivation, b, and on the outside hormone concentration, (h_o). Without this assumption, however, the equations could not be solved in closed form.

Let us define the total concentration of receptor sites as (s_t). Then,

$$7) \qquad (s_t) = (s) + (hs) + (is)$$

Combining (7) with (2) and (5), with the elimination of (s) and (is), we get

$$8) \qquad (hs) = \frac{K_I(s_t)(h_i)}{K_H K_I + K_H(i) + K_I(h_i)}$$

Adding (1) and (3), we find the total rate of change of (h_i)

$$9) \qquad \frac{d(h_i)}{dt} = a(h_i)((h_o)-(h_i)) - b(h_i)$$

and we can integrate (9) to get,

$$10) \qquad (h_i) = \frac{(a(h_o)-b)(h_i')}{(a(h_o)-b-a(h_i'))e^{-(a(h_o)-b)t} + a(h_i')}$$

where (h_i') is the initial value of (h_i). (h_i') clearly must be greater than zero; otherwise the initial hormone permeability would be zero. Combining (4) and (10) we can solve for i:

$$11) \qquad (i) = \frac{b}{a}\left[\log \frac{(h_i')}{(h_i)} + (a(h_o)-b)t\right]$$

where the initial value of (i) is taken equal to zero. Finally, then, combining (6), (8), (10), and (11), we have

$$w = \frac{L(h_i)}{K_H + \frac{K_H}{K_I} \cdot \frac{b}{a}\left[(a(h_o)-b)t - \log\frac{(h_i)}{(h_i')}\right] + (h_i)}$$

12)

$$(h_i) = \frac{(a(h_o)-b)(h_i')}{(a(h_o)-b-a(h_i'))e^{-(a(h_o)-b)t} + a(h_i')}$$

where $L = l(s_t)$. We now have an expression for $\underline{w}$ in terms of the independent variables, $\underline{t}$ and (h_o), and of six constants. We must try to evaluate these constants and thereby fit equations (12) to the experimental data represented in Figure 25.

From (12) we derive the following expressions. At $t = 0$, $(h_i) = (h_i')$, and

13) $$w_o = w_{t=0} = \frac{L(h_i')}{K_H + (h_i')}$$

As can be seen in Figure 25, $w_o = 1$ mg per min. By differentiating (10), we find the condition $\left[\frac{d(h_i)}{dt}\right]_{t=0} > 0$ if and only if $(h_o) > \frac{b}{a} + (h_i')$; that is, for $(h_o) \leq \frac{b}{a} + (h_i')$, there is no increase in inside hormone concentration, and hence no effect. Thus we can define a cut-off concentration, (h_c), at or below which the hormone has no effect, and

14) $$(h_c) = \frac{b}{a} + (h_i')$$

We cannot accurately determine (h_c) from our data but we can take it to be a reasonable value consistent with our data. We observed that 2.5×10^{-9} M oxytocin had a barely perceptible effect. We then can reasonably take $(h_c) = 2 \times 10^{-9}$ M.

Quantities that can be approximately determined from the data are the maximum values of $\underline{w}$, w_{max}, and the times of the maxima, t_{max}. Relationships involving these two quantities are derived by setting $\frac{dw}{dt} = 0$:

$$15)\quad \frac{(h_i')}{(a(h_o)-b-a(h_i'))}\, e^{(a(h_o)-b)t_{max}} = \frac{K_I}{b} + \frac{1}{a}\left[(a(h_o)-b)t_{max} - \log \frac{(h_i)}{(h_i')}\, t_{max}\right]$$

and

$$16)\quad \frac{K_H}{K_I} = \frac{(L-w_{max})(a(h_o)-b)(a(h_o)-b-a(h_i'))e^{-(a(h_o)-b)t_{max}}}{w_{max}\, b\left[(a(h_o)-b-a(h_i'))e^{-(a(h_o)-b)t_{max}} + a(h_i')\right]}$$

When $\underline{t}$ is large, equation (10) reduces to $(h_i) = (h_o) - \frac{b}{a}$, and combining this with equation (12), we find

$$17)\quad \frac{L}{w} = \left[\frac{K_H}{(h_o)-\frac{b}{a}}\right]\left[1 - \frac{b}{K_I a}\log \frac{(h_o)-\frac{b}{a}}{(h_i')}\right] + 1 + \frac{K_H}{K_I}bt$$

Equation (17) implies that when $\underline{t}$ is large enough (in practice, when $\underline{t} > t_{max}$), the decline in the permeability, $\underline{w}$, is hyperbolic. At every hormone concentration, (h_o), the plot of $\frac{1}{w}$ versus $\underline{t}$ for $t > t_{max}$ should be linear. The slope, $\underline{m}$, should be equal to

$$18)\quad m = \frac{K_H b}{LK_I}$$

The intercept, $\underline{g}$, of the line depends on (h_o), and is given by

$$19)\quad g = \frac{1}{L}\left[\left(\frac{K_H}{(h_o)-\frac{b}{a}}\right)\left(1 - \frac{b}{K_I a}\log \frac{(h_o)-\frac{b}{a}}{(h_i')}\right) + 1\right]$$

Now using these relationships, (13) through (19), we can determine the six constants. The procedure used was as follows. We calculated from the experimental data $\frac{1}{w}$ for $\underline{t} > t_{max}$ and for (h_o) from 1 to 8 x 10^{-8} M. We then calculated the least-squares line for each (h_o). The slopes were very nearly the same, as predicted, and $\underline{m}$ was taken as the mean slope:

$$20)\quad m = \frac{K_H b}{LK_I} = 2.5 \pm 0.4 \times 10^{-4}$$

Next, equation (15) was used. This equation could be considerably simplified by assuming that the second term on the right was small compared with the first term, an assumption which was later verified, and by neglecting $\underline{b}$ and $a(h_i')$ compared to $a(h_o)$. We were left with the approximation $\frac{(h_i')}{a(h_o)} e^{a(h_o)t_{max}} = \frac{K_I}{b}$. Rearranging and solving for t_{max} gave

$$21) \qquad t_{max} = \frac{1}{a(h_o)} \log \frac{K_I a(h_o)}{b(h_i')}$$

Equation (13), upon rearrangement, yielded $(h_i') = \frac{w_o K_H}{L-w_o}$. But L is an upper bound for $\underline{w}$, and is therefore ≥ 60, while w_o, the initial value of $\underline{w}$, is 1. Therefore,

$$22) \qquad (h_i') = \frac{w_o K_H}{L}$$

to a close approximation. Combining equations (20), (21), and (22), we found

$$23) \qquad t_{max} = \frac{1}{a(h_o)} \log \frac{a(h_o)}{m}$$

which determines t_{max} as a function of (h_o), and of two constants, $\underline{m}$, which we have already evaluated, and $\underline{a}$. We then tried different values of $\underline{a}$ in equation (23) until we found a value which gave values of t_{max} reasonably consistent with the experimental data. The value accepted for $\underline{a}$ was $3 \times 10^{+7}$. Using this value of $\underline{a}$, the error in the approximation of equation (15) by equation (23) was estimated and found to be small.

An approximate relationship involving w_{max} was derived by combining equations (16), (18), and (23), and by again neglecting $\underline{b}$ and $a(h_i')$ compared to $a(h_o)$:

$$24) \qquad \frac{1}{w_{max}} = \frac{1}{(h_o)} \left[\frac{m}{a} + (h_i') \right] + \frac{1}{L}$$

A plot of $\frac{1}{w_{max}}$ (experimental) versus $\frac{1}{(h_o)}$ should yield a straight line. The slope of this line should give an estimate of (h_i'), since both $\underline{m}$ and $\underline{a}$ are known, and the intercept should determine $\underline{L}$. In drawing the line, the points corresponding to the higher concentrations of hormone (20,40, 80 mμM) were weighted more heavily than the points corresponding to the lower concentrations (5 and 10 mμM), because the approximations involved in the derivation of (24) became increasingly inaccurate at the lower values of (h_o). From this line we determined that L = 91 and that $(h_i') = 2.9 \times 10^{-10}$.

From equation (13), and the above values of $\underline{L}$ and (h_i'), K_H was evaluated, and it was found that $K_H = 2.6 \times 10^{-8}$. Substituting the values found for $\underline{a}$ and for (h_i') in equation (14), and taking $(h_c) = 2 \times 10^{-9}$ M, we found that $\underline{b} = 5.1 \times 10^{-2}$. Equation (20) then yielded, $K_I = 5.7 \times 10^{-8}$. Finally we tested our approximations by substituting the estimated values of the constants in the still unused equation (19), and we found that the calculated values of the intercepts agreed well with those of the least-squares lines.

In summary, the constants were estimated as follows:

$$L = 91 \text{ mg} \cdot \text{liter} \cdot \text{min}^{-1} \cdot \text{mole}^{-1}$$

$$a = 3 \times 10^{7} \text{ liter} \cdot \text{mole}^{-1} \text{ min}^{-1}$$

$$b = 5.1 \times 10^{-2} \text{ min}^{-1}$$

$$K_H = 2.6 \times 10^{-8} \text{ mole} \cdot \text{liter}^{-1}$$

$$K_I = 5.7 \times 10^{-8} \text{ mole} \cdot \text{liter}^{-1}$$

$$(h_i') = 2.9 \times 10^{-10} \text{ mole} \cdot \text{liter}^{-1}$$

These values were substituted in equations (12) and $\underline{w}$ was calculated as a function of $\underline{t}$ and of (h_o). The results of these calculations have been plotted in Figure 25, where they may be compared with the experimental data. The closeness of fit suggests that the errors made in the various assumptions must have been compensating. This is certainly true of the error made in assuming that the outside hormone concentration, (h_o), remains constant, and of that made in assuming that the concentration of inhibitor in the cell, $(\underline{i})$, is not decreased by further enzymatic degradation or by diffusion out of the cell. The first error would tend to increase the

theoretical value of $\underline{w}$ and the second to decrease it. We know of course that the inhibitor does diffuse into the serosal bath. Hence in reality both the hormone concentration outside and the inhibitor concentration inside are less for $t > 0$ than is predicted by the model.

The theoretical response (i.e., the loss in mg during the first 60 min of activity) can be calculated at each hormone concentration by numerical integration of the expression

$$L = \int_0^{60} w\,dt$$

$\underline{L}$ has been calculated for values of (h_o) from 2.5 to 160 mμM. The theoretical curve has been plotted in Figure 26 together with the experimental data. There is excellent agreement between theory and experiment except for values of (h_o) exceeding 40 mμM. It is clear from Figure 26 that the experimentally determined response saturates more rapidly than the theory would indicate. We can account for at least part of this discrepancy in an approximation involved in one of the assumptions of the theoretical model. We assumed in equation (6) that the total water permeability, call it w_T, was proportional to the concentration of hormone-receptor complex. However, we previously presented evidence that the hormone acted on a permeability barrier near the mucosal membrane of the epithelial cells (Introduction). Let w_M be the permeability that is affected by the hormone, and let w_S be the permeability of the tissue between the hormone-affected permeability barrier and the serosal side of the bladder. Then since the reciprocals of permeabilities in series are additive (like resistances), we have

$$\frac{1}{w_T} = \frac{1}{w_M} + \frac{1}{w_S}$$

or

$$w_T = \frac{w_M w_S}{w_M + w_S}$$

Therefore, the actually measured permeability, w_T, is proportional to the hormone regulated permeability, w_M, only when w_M is small compared to w_S. Clearly if w_M is approaching an asymptotic limit, w_T will approach a lower asymptotic limit.

In summary, we have analyzed our measurements of the time course of the action of oxytocin in terms of a theoretical model. Six constants were obtained. Two of these, $\underline{L}$ and (h_i'), are probably characteristic of the bladder itself and of the experimental design. The remaining four constants, $\underline{a}$, $\underline{b}$, K_H, and K_I, should be characteristic of the hormone. $\underline{a}$ relates to the permeability of the hormone. $\underline{b}$ is the first order rate constant of the inactivation of the hormone. K_H is the dissociation constant of the hormone-receptor complex, and K_I is the dissociation constant of the inactivated-hormone-receptor complex. A similar analysis must now be applied to the time course of action of other neurohypophyseal hormones to determine whether these constants are really characteristic of the hormone, and whether by this means we can gain insight into the structure-function relationships of these hormones.

DISCUSSION

In our first experimental results we found that the particle-free supernatant fraction of a homogenate of the toad bladder contained hormone inactivating activity (Figure 13). It was found that when hormone, in this case AVP, was incubated with the supernatant fraction, the hormone activity decreased exponentially with time (Figure 14). This evidence supported the view that the inactivation of hormone was enzymatic, and not due to an inhibitor of hormone action already present in the bladder fraction, or to binding of the hormone. The sensitivity of the hormone inactivating activity to cold storage and to heat (Figures 15 and 16) also supported the view that an enzyme was responsible for the inactivation of hormone. Non-specific binding did not seem to be involved; for instance the activity of hormone was not decreased by incubation with bovine serum albumin, even in the presence of cysteine. More directly, we found that when oxytocin was incubated with our best inactivating preparation, and the incubation mixture examined chromatographically, the amount of ninhydrin positive material increased and the amount of oxytocin decreased (Figure 21). Furthermore, when the incubation mixture was treated with performic acid and then chromatographed, a dense spot appeared in the position of cysteic acid (Figure 22). Thus it appeared that oxytocin was being enzymatically degraded, and that at least one of the bonds being split could be identified as the N-terminal half-cystinyl-tyrosine bond.

The results obtained using L-cystine-di-β-naphthylamide (CDNA) as a substrate tend to corroborate the conclusions drawn from the hormone inactivation studies. CDNA contains, like oxytocin, an N-terminal cystinyl-peptide bond. It was found that the ammonium sulfate fraction of the supernatant, precipitating between 50 and 70% saturation (S(50-70)) contained the greatest amount of hormone inactivating activity (Figure 19). This fraction also contained the greatest amount of CDNAase activity (Table V). Moreover the specific activities towards the two substrates were comparable. The rate of inactivation of oxytocin by S(50-70) in the presence of 1 mM cysteine was on the average 35 mμM per mg protein per hr (Results, A). The average rate of splitting of CDNA by S(50-70) in the presence of 1 mM cysteine was 70 mμM per mg protein per hr (Table VII). Clearly the CDNAase

activity was great enough to account for the hormone inactivating activity. Not only were the fractionation patterns of the two activities similar, but these two activities were similarly activated by cysteine and inactivated by N-ethylmaleimide. Stimulation of hormone inactivation by cysteine is shown in Figure 15, and inhibition of the inactivation by NEM in Figure 17. 1 mM cysteine almost doubled the CDNAase activity (Table VII), and 1 mM NEM inhibited this activity 40% (Table VIII). Neither the inactivation of hormone nor the splitting of CDNA, however, had an absolute requirement for cysteine. In both cases there was considerable activity in the absence of cysteine. Another similarity between the two activities is that both were partially inactivated after a few minutes at 50° C (Figure 16 and Table X).

These similarities between the hormone inactivation activity and the CDNAase activity, together with the direct evidence that the N-terminal half-cystinyl-tyrosine bond of oxytocin was split, support the view that an oxytocin inactivating enzyme is present, and that this enzyme splits N-terminal half-cystinyl-peptide bonds, such as are present in all of the neurohypophyseal hormones and in the synthetic substrate CDNA. As a matter of fact, the natural hormones, arginine vasopressin, arginine vasotocin, lysine vasopressin, and oxytocin, as well as the synthetic analog, oxytocin ring amide (see Table III for structures), were all shown to be inactivated by the supernatant fraction, S (Figure 18). The half-cystinyl-tyrosine bond occurs in all of these hormones. A final piece of evidence favorable to the above hypothesis is that oxytocin was an inhibitor of CDNAase activity (Table XI). It would be interesting to test whether CDNA, on the other hand, is an inhibitor of oxytocin inactivation. This we have not yet done.

We have discussed the evidence supporting the hypothesis that a single enzyme is attacking both CDNA and oxytocin. We will now inquire into the specificity of this enzyme. We have already mentioned that the supernatant fraction, S, inactivated all the natural neurohypophyseal hormones and one synthetic hormone. The natural hormones differ from one another in only one or two residues out of nine, and oxytocin ring amide differs from oxytocin in that three residues are removed from the C-terminal end of the molecule. These different molecules contain many similar bonds, among which is, of course, the half-cystinyl-tyrosine bond. Therefore even if the same enzyme were responsible for the inactivation of all of these species, a lack

of specificity would not necessarily be indicated. On the other hand, we have tested our best preparations with the synthetic substrates L-leucine-β-naphthylamide (LNA) and D,L-alanine-β-naphthylamide (ANA). We found that compared to the specific CDNAase activity, there was 8 times as much LNAase activity and about 1.5 times as much ANAase activity. Moreover, by a number of criteria, the CDNAase and LNAase activities belonged to the same enzyme. For instance, both activities precipitated in the same ammonium sulfate fraction (Table IX); they showed similar dependence on pH (Figure 24); and the ratio of the two activities remained constant during progressive inactivation by heat (Table X). But there were also some results differentiating between these two activities. For instance, cysteine (1 mM) was found to increase the CDNAase activity of S(50-70)D 60% while it had no effect on the LNAase activity (Table XI). This might be explained if cysteine acted primarily on the substrate, CDNA, forming a less bulky and possibly more easily attacked mixed disulfide. Also, oxytocin inhibited LNAase more than CDNAase, 60% as compared to 30% (Table XI). Again this could be explained if the enzyme binds cystine peptides better than leucine peptides and hence oxytocin competes more effectively with LNA than with CDNA. There are other minor differences in Table XI between the effect of various agents on these two activities. For these there is no apparent explanation. All in all, it might be that at least a large part of the CDNAase activity and of the LNAase activity resides in a single enzyme. Thus it appears that the hormone inactivating, CDNA splitting enzyme is not absolutely specific for N-terminal cystinyl-peptide bonds. It seems to split also N-terminal leucinyl-peptide bonds.

At the end of the Introduction we mentioned pregnancy serum oxytocinase. Let us see how that enzyme compares with our hormone inactivating enzyme. As we said, pregnancy serum oxytocinase is found in the plasma of pregnant women in increasing amounts until birth. The origin of this enzyme is thought to be the placenta. Here, as with the toad bladder, the inactivating enzyme is associated with the target organ of the hormone, in this case, the uterus. Pregnancy serum oxytocinase has also been shown to split the cystinyl-tyrosine bond of oxytocin. It also splits vasopressin (Wintersberger et al., 1960). The best preparations of pregnancy serum oxytocinase split CDNA at the rate of 2.5×10^{-5} moles of β-naphthylamine liberated per mg protein per hour. This was 4500 times the specific activity of the

starting material (Tuppy and Wintersberger, 1960). Our best preparations, which had been purified 3 or 4 times, had a specific CDNAase activity of about 1/200 of that of purified pregnancy serum oxytocinase (Figure 23). Clearly our starting material had a higher specific CDNAase activity to begin with. The best preparation of pregnancy serum oxytocinase split LNA 11 times faster than CDNA, and with less pure preparations the ratio of activities was even greater. In fact the decrease in this ratio was used by Tuppy and Wintersberger as an indication of purification. Our best preparations split LNA 8 times as fast as CDNA.

We see then that there are basic similarities between the hormone inactivating enzyme from the toad bladder and pregnancy serum oxytocinase. There are however some important differences as well. The rate of hydrolysis of CDNA by pregnancy serum oxytocinase was decreased 30% by 1 mM cysteine and was unaffected by 1 mM NEM or by 1 mM iodoacetic acid (Tuppy and Nesvadba, 1957; Tuppy and Wintersberger, 1960). Clearly these results differ from ours in which the latter two agents were inhibitory (Table VIII), and cysteine increased CDNAase activity 80% (Table VII).

Let us compare our hormone inactivating enzyme now with the ubiquitous leucine aminopeptidase. L-leucineamide and L-leucine-β-naphthylamide are substrates for this enzyme, which appears to be relatively nonspecific except in its requirement for free α-amino group. The enzyme purified from swine kidney is unaffected by glutathione, iodoacetamide, or parahydroxymercuribenzoate (Smith and Speckman, 1955). It is activated by either Mn^{++} or Mg^{++} and is inhibited by versene and pyrophosphate (cf. Table XI). Activity rises from pH 6.0 to pH 7.8 and then remains constant up to pH 9.3 (cf. Figure 24). Finally, it was found that leucine aminopeptidase hydrolysed oxytocin very slowly (Hall and Smith, 1957). N-terminal half-cystinyl-peptide bonds appear to be resistant to the action of this enzyme. In all these characteristics then leucine aminopeptidase differs from the hormone inactivating enzyme of the toad bladder.

As mentioned in the Introduction, AVP has been shown to be inactivated by incubation with rat kidney slices, and this inactivation is inhibited by oxytocin and by parahydroxymercuribenzoate (Smith and Sachs, 1961). Other preparations inactivating the neurohypophyseal hormones have been cited in the Introduction, but none of these, as far as I know, are

well characterized. We may conclude that the hormone inactivating enzyme from the toad bladder is different from pregnancy serum oxytocinase. It differs also from leucine aminopeptidase and from all other well characterized aminopeptidases.

We have found then that extracts of the toad bladder inactivate oxytocin and its analogs. We have presented evidence that this inactivation is due to an enzyme which we have begun to characterize, and which, on the basis of this characterization, appears to be unique. Next, we wanted to show that the intact bladder was capable of inactivating oxytocin; that is, that the inactivating enzyme isolated from the homogenized bladder functions as such in the intact bladder. This hypothesis was consistent with our first observations on the kinetics of hormone action (Figure 25); that is, with the observed decline in the effect of oxytocin on the water permeability of the bladder. However we could not exclude, just on the basis of these observations, other reasonable hypotheses. Neither irreversible changes in the isolated bladder (Table XII, exp. a), nor decreases in the osmotic gradient across the bladder (exp. b), nor depletion of exogenous substrate (exp. c), nor changes in pH were responsible for the observed decline in effect. In fact, it was found that the cause of the decline resided not in a change in the state of the bladder itself, which was capable of responding fully again, but in a change in the state of the serosal bath (exp. d). This then appeared to verify the hypothesis that the decline in effect of hormone was due simply to inactivation of the hormone. However, when the amount of hormone estimated to be inactivated was added as an additional dose, the second response was still lower than expected (exp. e); this was true even when the second dose was equal to the first (exp. f and g). We were thus led to the conclusion that there must be something accumulating in the Ringer's of the serosal bath inhibiting the action of oxytocin on the bladder. We then actually found that an inhibitory substance was washed from the bladders into the serosal bath during the control period, before any oxytocin had been added; and we showed that this inhibitory substance was not enzymatic (exp. h and i). Moreover, the amount of inhibitor that was washed from a bladder could account fully for the observed decline in effect of a dose of hormone. Hence it appeared that there was not significant enzymatic inactivation of oxytocin by the intact bladder. However, we continued to study this inhibitor and found that the amount

that came off in a second washing was considerably less than in the first (exp. j). In all experiments, however, the bladders were washed for at least 40 minutes and often longer in different Ringer's than that in which they received at least one dose of oxytocin. In all of these cases, the effect of the dose declined just as in Figure 25, or Figure 27. Therefore, since we were led to explain the decline in effect as a result of inhibition, and since we actually found an inhibitor in freshly excised bladders, and since this inhibitor was found to be depleted by washing, we were led to the view that new inhibitor must be formed in the presence of hormone.

We now asked how a hormone inhibitor could be formed in response to the hormone itself. One way is that the inhibitor be formed by an enzymatic change in the hormone molecule, such that the changed molecule still combines with the receptor site of the hormone but is nonetheless inactive. We know that some close structural analogs of oxytocin are effective inhibitors of hormone action (Table IV). Here then is a role for a hormone inactivating enzyme, the properties of which could be similar to those described for the hormone inactivating activity of the toad bladder supernatant fraction. This enzyme could inactivate endogenous or exogenous hormone, and the product of the inactivation could compete with the hormone for its receptor sites; i.e., the product of the inactivation would be a competitive inhibitor of the hormone. We would then explain that the inhibitor washed from freshly excised bladders was due to the accumulation in the bladder of the product of the enzymatic inactivation of endogenous hormone. This hypothesis should be subject to direct verification by the isolation of the inhibitor, both from the serosal bath of the intact bladder and from the incubation mixture of hormone and inactivating enzyme.

We next constructed a model of hormone-bladder interaction based partly on the above hypothesis, which combined the observations on the inactivating enzyme with those on the inhibitor released into the serosal bath. The assumptions of that model have already been thoroughly discussed (Experimental, Section E). In translating the model into mathematical equations certain approximations were made, the validity of which we had some reason to doubt. These were, in particular, that the outside hormone concentration, (h_o) remains constant in time (i.e., that the decline in effect is due solely to the accumulation of inhibitor), and that the inhibitor, I, is formed at a rate which is large compared to the rate of its

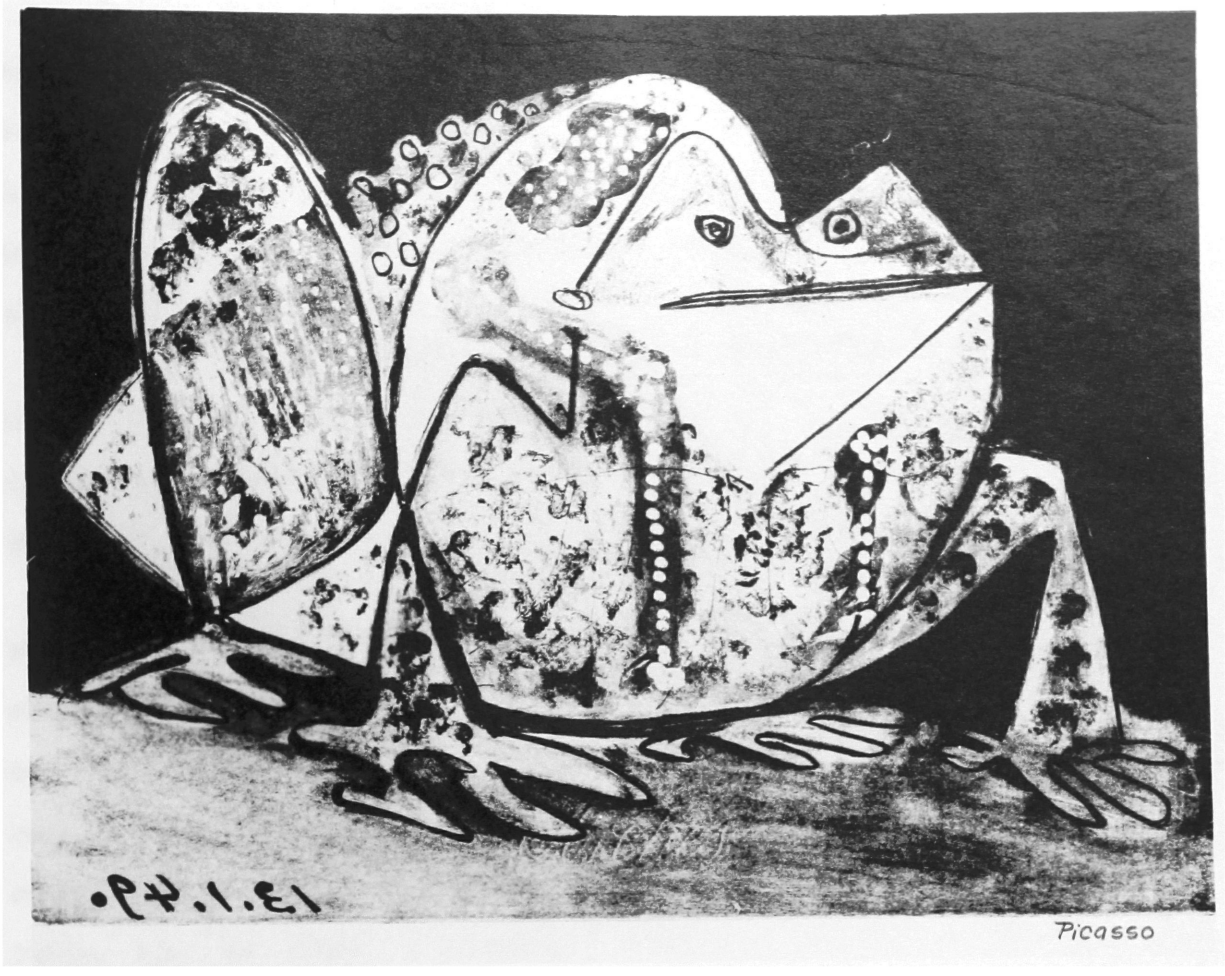
depletion by diffusion and by enzymatic degradation (i.e., that the rate of increase of (i) is simply the rate of inactivation of hormone). It was found however that substantial amounts of inhibitor did appear in the serosal bath. Hence the inhibitor was diffusing out of the cells. Therefore, the actual rate of formation of inhibitor, and hence the rate of inactivation of hormone, must be greater than the theoretical rate in order that the concentration of inhibitor in the cells remain high. In that case, we could no longer ignore the depletion of hormone in the serosal bath. Nevertheless we made these approximations in order to be able to solve the theoretical equations in closed form. The fairly close fit of the theoretical solution to the experimental values (Figures 25 and 26) provides at least some justification for these approximations, that is, assuming that the major assumptions of the model are correct.

Whether or not the assumptions of the model are correct, we have derived an expression which can be made to fit the experimental data with fair accuracy. The six constants of the theoretical expression are evaluated from the data. Four of these, as was pointed out before, are probably characteristic of the hormone. We can expect then that if the different hormone analogs show different kinetics of action, as there is indication that they do (Figure 11), analysis of the data obtained with each analog should provide at least four constants characteristic of that analog. Presently, analogs are compared on the basis of one characteristic constant related to the magnitude of response per mg of hormone (cf. Table III). This comparison has not been particularly fruitful in elucidating structure-function relationships (Boissonnas et al., 1961). Possibly comparison on the basis of four characteristic constants per analog might be more helpful, especially if these constants are not merely empirical but can be related to some model of hormone action. These ideas must be tested by applying the same analysis to a number of analogs of oxytocin.

It might be pointed out that the response of the toad bladder to neurohypophyseal hormones as measured by changes in the short-circuit current looks very similar to the response measured by changes in the water permeability. This can be seen by comparing Figure 10 and Figure 25. The potential difference varies in a similar way. For obvious reasons, it would be more convenient to base our analysis on electrical rather than gravimetric measurements. It must be determined whether the variation, both with

time and with hormone concentration, of the electrical properties of the bladder parallels the variation with these parameters of the water permeability.

We have worked with the isolated toad bladder, a target organ for the neurohypophyseal hormones. Our proposed model for the control of the action of these hormones has been based on observations of this isolated system. We must now ask whether these observations are relevant to the actual time course of hormone action in the animal. The rise and decline of the effect of oxytocin on the isolated bladder occurs in a time interval of a couple of hours. We mentioned in the Introduction that according to some investigators, doses of neurohypophyseal hormones injected into mammals seem to disappear from the circulation with a half-life of a few minutes. On the other hand, in Figure 4 we see that the actual response of the dog to an injection of vasopressin or oxytocin measured as a decrease in urine flow did not decline appreciably after one hour. Similarly, in the toad, the effect of an injected dose of hormone on free water clearance lasted for a matter of hours (Figure 2). Thus the time course of the effect of a dose of hormone injected into the animal is roughly the same as the time course of the effect of a dose of hormone on the isolated bladder. In any case, what we are actually dealing with in the whole animal is a dynamic state in which hormone activity is dependent on the rates of formation and degradation of hormone, and, we suggest, the rates of formation and degradation of hormone inhibitor. From our analysis we suggest further that the effect of the inhibitor would be to limit the duration of the response of target cells; that is, a cell would respond acutely to an increase in hormone concentration, and would then become refractory due to an increase in inhibitor concentration.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