EFFECT OF ANTIDIURETIC HORMONE ON RENAL MEDULLARY CYCLIC AMP IN THE NEWBORN

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
LEE-TZE LU
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ABSTRACT

EFFECT OF ANTIDIURETIC HORMONE ON RENAL MEDULLARY CYCLIC AMP IN THE NEWBORN

By

Lee-tze Lu

In the mammalian kidney, the antidiuretic hormone (ADH) prevents the excretion of excess water in a dilute urine and affects conservation of water by permitting full operation of the concentrating mechanism. Cyclic AMP is recognized as the intracellular mediator in the action of ADH. The purpose of this study was to determine the effect of ADH on the concentration of cyclic AMP in renal medullary tissue of new-born and adult animals and to elucidate the possible mechanisms responsible for differences in urinary concentrating capacity between the new-born and the adult.

Cyclic AMP concentration was measured *in vitro* using a kidney slice technique. The method involves incubation of medullary slices with ADH in a buffered medium under a gas phase of 100% oxygen. Cyclic AMP concentration was determined by a method based on competition for protein binding of the nucleotide to a cyclic AMP-dependent protein kinase. Cyclic AMP concentration was expressed as picomoles per mg wet medullary tissue.

Adult Spraque-Dawley rats, New Zealand white rabbits and mongrel dogs were used for verifying the method, elucidating the time course, dose-response relationship and for comparing cyclic AMP concentration to the respective young animals. An increase in cyclic AMP concentration in renal medullary slices from three species supports the hypothesis that the action of ADH on epithelial structures are mediated by cyclic AMP.

Cyclic AMP concentration in renal medullary slices from adult rats, rabbits and dogs increased with increased incubation time up to 30 minutes. This increase was seen, however, only when 10 mM theophylline was included in the incubation mixture. ADH increased the concentration of cyclic AMP in renal medullary slices in a dose-dependent manner in the presence of 10 mM theophylline. In the absence of theophylline cyclic AMP concentration was slightly increased but the effect was not dose-related. All the values obtained in the absence of theophylline were significantly lower than in its presence. This suggested that theophylline potentiated the action of ADH by inhibiting the activity of phosphodiesterase in renal medullary tissues.

A developmental pattern of cyclic AMP concentration in response to ADH was observed in dogs from 1 day to 2 weeks of age. Body weight, kidney weight, protein concentration and Uosm/Posm ratio were low in the new-born period. It is suggested that lower cyclic AMP concentration may play some role in the immature concentrating capacity in the young of this species. Data obtained from rabbits did not show a similar

developmental pattern suggesting that factors limiting the concentrating capacity in young animals vary from species to species.

Treatment of 1 day, 3 day and 5 day old dogs with ADH did not increase cyclic AMP concentration or urine osmolar concentration demonstrating both biochemical and physiological insensitivity of young animals to ADH. This may result from a less receptive adenyl cyclase system, or development of resistance to ADH, or difference in the environment at cyclic AMP generation sites.

These observations elucidated a possible role of cyclic AMP in the development of concentrating capacity in the new-born animals. However, these experiments do not exclude the importance of other limiting factors such as short loops of Henle, low urea excretion rate, permeability characteristics of the nephron or limited capacity of the sodium pump.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pharmacology

1972

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. J. B. Hook for his valuable assistance, interest, encouragement and constructive criticism throughout the course of this investigation. I would also like to thank Dr. T. M. Brody, Dr. J. E. Gibson, Dr. M. D. Bailie, and Dr. L. D. Muschek for their helpful assistance in the preparation of this thesis.

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INTRODUCTION

Cyclic AMP (adenosine 3',5'-monophosphate), is recognized as a versatile regulatory agent acting to control the rate of a number of cellular processes. Sutherland and Rall discovered cyclic AMP (1) during a continuation of studies of the mechanism by which epinephrine and glucagon promoted glucose release in the liver. In earlier studies, Sutherland had demonstrated that the rate-limiting enzyme in the conversion of glycogen to glucose was liver phosphorylase. This enzyme existed in two forms, inactive and active, and both epinephrine and glucagon caused the activation of phosphorylase. They also identified an enzyme, dephosphorylase kinase, which activated phosphorylase in the presence of ATP and Mg++, and a phosphatase that inactivated the enzyme. Although they could elicit effects of epinephrine and glucagon on phosphorylase activation in whole homogenates fortified with ATP and Mg++, supernatant fractions containing the phosphorylase system were entirely unresponsive to the hormones. Experiments in which particulate and supernatant fractions were recombined showed that the hormones acted on a component of the particulate fractions to increase the accumulation of a heat-stable factor-cyclic AMP, which, in turn, accelerated the rate of conversion of inactive to active phosphorylase. The enzyme from the particulate fraction responsible for the formation of cyclic AMP was named adenyl cyclase (2).

Adenyl cyclase is widely distributed in nature and has been identified in every mammalian tissue studied with the exception of the mature mammalian erythrocyte (2). In almost all tissues adenyl cyclase is in the particulate fraction, indicating an association with cell membranes (2,3,4). The interactions of several hormones with their target cells have been shown to result in the activation of adenyl cyclase, which catalyzes the transformation of adenosine triphosphate to cyclic AMP and inorganic pyrophosphate. The increased cyclic AMP, acting intracellularly, then carries out the work of the hormone by affecting the activities of enzymes, permeability processes etc. Subsequent studies have shown that cyclic AMP mimics the actions of many hormones (5,6).

The neurohypophyseal hormone, vasopressin (antidiuretic hormone, ADH) and certain of its analogues enhance the permeability of a number of epithelial membranes to water, sodium and certain small molecules. Considerable effort has been expended in searching for an adequate description of these processes and although progress has been achieved, the goal is not in sight. Orloff and Handler (7) proposed that cyclic AMP is the intracellular mediator of the physiological effects of vasopressin. A number of experimental observations are now available which support this proposal and define an integral role of cyclic AMP in the action of vasopressin.

In the mammalian kidney, the antidiuretic hormone prevents the excretion of excess water in a dilute urine and effects further the conservation of water by permitting full

operation of the urinary concentrating mechanism. There is general agreement on the overall nature of the process involved in the production of urine hypertonic to body fluids. Almost all have accepted the interpretation, originally proposed by Wirz, Hargetay and Kuhn (8), that the urine becomes concentrated as it flows through the medullary collecting ducts by passive outward movement of water into an interstitium that has been rendered hyperosmotic by the accumulation of a high concentration of sodium chloride. Most workers would accept the following account of the events involved in the conversion of a large volume of isosmotic glomerular filtrate to a small volume of hyperosmotic urine: In the proximal tubule, as a consequence of the active removal of salt in a highly water-permeable segment, the volume of fluid is markedly reduced without change in its osmotic pressure. A variable fraction of the glomerular filtrate enters the medulla in the thin-walled descending limb of the loop of Henle. As it flows into the hyperosmotic medulla, the fluid becomes progressively concentrated by: (1) loss of water to its highly concentrated surroundings; (2) inward diffusion of urea from the urea-rich interstitium; and (3) possibly some entry of sodium chloride. Somewhere between the bend of the loop and re-entry of the nephron into the cortex the permeability of the loop to water diminishes and outward transport of sodium and chloride dilutes the fluid that remains in the tubule lumen. Thus, when the fluid arrives in the distal convoluted tubule, it is hypotonic to plasma. The salt that has been removed in this dilution process remains behind to raise the

osmolality of the interstitial fluid and blood in the medulla. In the presence of antidiuretic hormone, the fluid flowing through the distal nephron loses its excess water by passive re-equilibration with its surroundings. Before the tubular fluid re-enters the medulla in the collecting ducts, it gives up water to reach the same hyperosmotic state as the surrounding medullary interstitial fluid. It is certain that the major effect of ADH in the concentrating mechanism is exerted through its effects on the permeability to water of the distal nephron.

The view that vasopressin increases the permeability of the distal nephron to water derives mainly from studies utilizing amphibian skin and bladder. Much of the early information stems from the work of Ussing and his collaborators (9). Their conclusions concerning the action of vasopressin have been confirmed and extended by Bentley (10,11), Sawyer (12) and Leaf (13) and their co-workers on the basis of similar studies on amphibian bladder. Their studies demonstrated two characteristic effects of vasopressin, an increase in the permeability of certain epithelial structures to water, and an associated stimulation of sodium transport.

Tischer, et al. (14) studied the morphological changes of renal medulla of rats with hereditary hypothalamic diabetes insipidus during vasopressin-induced antidiuresis. They found that vasopressin-induced antidiuresis in diabetes insipidus rats was associated with: expansion of the medullary interstitium, and widening of the lateral intercellular spaces of

medullary collecting ducts. These findings were not present in untreated diabetes insipidus rats or in normal rats of the same strain, with or without vasopressin administration. The results suggested that fluid reabsorption from collecting ducts during vasopressin-induced antidiuresis occurs at least in part via lateral intercellular channels. How this occurs or what kind of a process is involved is not understood. There are, however, two hypotheses regarding the cellular mode of action of vasopressin. The first is attributable to Ginetzinsky (15), who suggested that vasopressin stimulates the secretion of a depolymerizing enzyme, hyaluronidase, by the renal tubule. The second, that of Schwartz, Fong, Rasmussen and their associates (16,17,18), involves a mechanical system, not metabolic in nature, in which interaction of vasopressin with specific tissue receptors directly initiates structural changes within the membranes.

According to Ginetzinsky (15), antidiuretic hormone has no direct effect on the permeability of the tubule cells, but instead stimulates the secretion by these cells of hyaluronidase into urine. Subsequent depolymerization of mucopolysaccharide complexes of the intercellular spaces and basement membrane by the enzyme is thought to result in the characteristic increase in the permeability of the collecting duct to water. The following evidence has been presented in support of the theory (15): Hyaluronidase activity in urine is constant and independent of urine flow in osmotic diuresis in the rat at a time when antidiuretic hormone secretion is

assumed to be maximal, but this is not the case in the animal undergoing water diuresis. In the latter situation, when antidiuretic hormone secretion is absent, urinary hyaluronidase activity is inversely related to urine flow. This is as would be expected if the development of antidiuresis requires secretion of the depolymerizing enzymes. Furthermore, on the basis of histochemical studies, Ginetzinsky reported that a marked reduction in mucopolysaccharide material occurs in the papilla of rats in antidiuresis as compared to that in the papilla of animals following 20 to 60 minutes of water diuresis. Additional support for the theory was supplied by Dicker and Eggleton (19) who, in agreement with Ginetzinsky's animal studies, reported that the rate of excretion of hyaluronidase in urine of human subjects undergoing water diuresis varied inversely with urine flow.

The hyaluronidase theory has been severely criticized by Berlyne (20). The reliability and specificity of the hyaluronidase assay used by Ginetzinsky have been questioned, and when Berlyne used an assay which he considered superior he was unable to find any relationship between rate of excretion of the enzyme and urine flow either in water diuresis or in osmotic diuresis. The histochemical observations were not confirmed by Breddy et al. (21), who suggested that the pattern observed by Ginetzinsky in rat papilla may have been the result of sampling at different depths of the medulla in diuretic and non-diuretic animals. Also, Thorn (22) reported that after injection of a large amount of hyaluronidase, the

urinary response was delayed for approximately 20 minutes, whereas that evoked by exogenous hormones was virtually immediate. Rosenfeld et al. (23) were unable to demonstrate any effect of leave in testicular hyaluronidase into the renal artery of the dog which could not be ascribed to concurrent changes in hemodynamics. Finally, neither Leaf (13) nor Bentley (24) observed any effect of hyaluronidase on water permeability of toad or frog urinary bladder. It now seems reasonable to disregard the hyaluronidase thesis since none of the data presented in its support has withstood critical evaluation.

Fong, Rasmussen and Schwartz and their co-workers (16,17,18) proposed that vasopressin binds to toad bladder and kidney at a minimum of two sites, the most important of which involves a covalent linkage between the disulfide bridge of the octapeptide and free sulfhydryl groups on the membrane. Their suggestion was that the hormone-induced increase in permeability is initiated by a series of disulfide-sulfhydryl interchanges which induce separation of fibrillar elements in a protein diffusion barrier (17), or in some other fashion mechanically opens aqueous channels through which water may flow. They initially excluded a metabolic basis for the effect, since they found that none of a series of potent metabolic inhibitors eliminated the water permeability response of the toad bladder to hormone. The thesis is based largely on the experimental observation that tritiated vasopressin binds to kidney and toad bladder, as evidenced by

accumulation of radioactivity with kidney and bladder tissue and its partial release from fixed tissue preparations by cysteine. The latter is a sulfhydryl-containing compound which presumably ruptures the disulfide bond between the hormone and tissue. The extent of binding was said to be related to the physiologic response since the degree of accumulation of radioactivity in kidney protein in antidiuretic rats was greater than in rats excreting larger volumes of urine during recovery from the effects of the hormone. Acidification of the inner bathing solution, which Bentley (10) first demonstrated, eliminates the permeability response of toad bladder to hormone, also reduces the accumulation of radioactivity in the tissue. This is consistent with their hypothesis, since depression of the dissociation of sulfhydryl groups by hydrogen ion should reduce the affinity of the tissue receptor sites for the hormone. They also reported that certain sulfhydryl inhibitors, such as n-ethyl maleimide and p-chlormercuribenzoate, interfere with both the binding of the hormone and its physiological effects.

Although the foregoing hypothesis is attractive, in that it affords an explanation for the cellular action of vasopressin, the results of interest with respect to the possible nature of a linkage between vasopressin and a receptor provide no information concerning the proposed structural alterations within the tissue which are said to be responsible for the hormone induced permeability effects.

The reported effects of n-ethyl maleimide are not necessarily pertinent. Simple incubation of the intact bladder with this agent alone is followed by progressive deterioration of the tissue as evidenced by a decline in net sodium transport and oxygen consumption, as well as unresponsiveness to other agents, such as theophylline and cyclic AMP (7). These latter compounds although not containing disulfide bridges, effectively mimic vasopressin in toad bladder.

In view of all of these arguments, it would seem more reasonable to view the results of Fong, Schwartz and Rasmussen as providing information concerning the nature of a possible linkage between the hormone and its receptor, rather than as evidence for the proposed mechanism for the opening of aqueous channels.

Regardless of the details of the reaction between vasopressin and tissue, there is now a considerable body of evidence in support of the proposal that: (1) this reaction results in an increase in the rate of production of cyclic AMP by pertinent epithelial cells and (2) cyclic AMP is the intracellular mediator of the permeability response to vasopressin.

The two characteristic effects of vasopressin, an increase in the permeability of certain epithelial cells to water, and stimulation of sodium transport have been demonstrated in the amphibian skin and bladder (9,12,13). A similar effect on the permeability to water of isolated rabbit collecting tubule has also been reported (25). If cyclic AMP is the intracellular mediator of the action of vasopressin it

should mimic the hormone in all respects. In toad bladder this has been shown to be the case. The similarity of effects of the two agents, the hormone and its postulated intracellular mediator, on net water flow in isolated rabbit collecting tubule has been reported by Grantham and Burg (25). Their demonstration that cyclic AMP also augments the water permeability of the rabbit collecting tubule and that the biochemical degradation product 5'-AMP does not, firmly establishes the role of the cyclic 3'.5'-AMP system in the renal tubule.

Direct support of the cyclic AMP hypothesis was achieved by the demonstration that the concentration of the nucleotide is markedly increased in renal tissue and in toad bladder following incubation with vasopressin (26,27). In toad bladder Handler et al. (27) were able to show that purified arginine vasopressin and theophylline increase the concentration of cyclic AMP in the tissue whereas no increment in cyclic AMP concentration was effected by either insulin or angiotensin, polypeptides without physiological effects on permeability in this tissue.

The kidneys of most new-born mammals are immature (28,29,30,31). The capacity to concentrate the urine and to conserve water is low due to short loops of Henle and consequent undeveloped countercurrent system in the medulla. Histological investigations of the kidney of the new-born mammal mention the absence of lack of differentiation of the loops of Henle (31,32). The kidney of the new-born rat

resembles that of the foetus of other mammals and man (33). The study of kidney functions of the new-born rat may, therefore, throw some light on renal functions of the foetus of other mammals and man.

Bogomolova (32) studied the cytological and cytochemical change in the rat kidney from birth to old age (30 months). At birth, the rat kidney is not yet fully formed. The tubular system is not fully formed, the loops of Henle are weakly differentiated, the renal papilla is short and the collecting ducts are few in number. Concentrating capacity is low. An increase in urea excretion produces a marked increase in the concentrating capacity. A further possibility contributing to the immature concentrating capacity is a limitation in the capacity of the cells to pump sodium against a gradient. In experiments on rats, Yunibhand and Held (34) were able to demonstrate a significant increase in medullary sodium concentration from 1 to 30 days of age. This may reflect an increase in the length of the loop of Henle or an increased capacity for sodium reabsorption, or both. It is unlikely that reduced urine concentration in the neonate can be attributed to an insufficient amount of antidiuretic hormone. The amount of antidiuretic activity has been estimated in glands of new-born rats (35), puppies (36) and new-born infants (37). And although the amount present is small when compared with that found in adult animals, it is sufficient for physiological measurement.

The neurohypophysis acts as a store, the quantity of vasopressin in it represents the difference between the rate at which it is synthesized and that at which it is released. Increasing evidence is accumulating against the hypothesis that this might constitute a limiting factor in concentrating capacity at that age. The osmoregulatory apparatus in the neonate responds to the stimulation of dehydration and relatively large amounts of antidiuretic hormone are released (38). In contrast to the absence of antidiuretic hormone in the adult, the urine of fully hydrated new-born animals contained a substantial amount of antidiuretic activity, presumably of neurohypophyseal origin (38).

When compared with the effect of similar doses of antidiuretic hormone in adults, it was found that kidneys of new-born humans were highly insensitive to antidiuretic hormone (29). Similar results have been shown in other new-born animals. Though these experiments illustrate the marked insensitivity of the kidney of new-born mammals to the antidiuretic hormone, they fail to indicate the reason for it. This raises the question: Does antidiuretic hormone act on the collecting ducts of new-born mammals in the same way as it acts in the adult?

Studies of enzymes in new-born kidneys have indicated that many enzymes are immature (39,40). Accordingly, adenyl cyclase may be immature in new-born kidneys. Since the kidneys of most neonates are immature, it might be that the protein

receptor of the target site is not yet fully receptive. It was of interest, therefore, to determine the activity of adenyl cyclase and hormone responsiveness in the new-born kidney.

The objective of this investigation originally was to study the adenyl cyclase activity of broken cell preparations. These studies are useful for several reasons, one of which is that the environment in the vicinity of the adenyl cyclase can be greatly simplified and therefore better controlled than in more organized systems (6). In most mammalian tissues which have been studied, using conventional homogenization techniques, adenyl cyclase has been located in the low speed or nuclear fraction, which contains fragments of the cell membranes (2). Often these preparations can be washed repeatedly, thus eliminating many metabolites and soluble enzymes, and in some cases can be taken through additional purification steps with the retention of hormonal sensitivity (2). The ultimate goal here would be to obtain a preparation containing only adenyl cyclase, but to date it has not been possible to purify adenyl cyclase from mammalian sources beyond a certain point without destroying its sensitivity to hormonal stimulation.

A major factor complicating the quantitative interpretation of most adenyl cyclase measurements is contamination by other enzymes, including ATPase, pyrophosphatase and phosphodiesterase. In this study, cyclic AMP produced from ATP in broken cell preparations was measured by the rate of

conversion of labeled ATP to cyclic AMP (41). Chase and Aurbach (42) found that the medulla was the anatomically specific site at which ADH increase renal adenyl cyclase activity. I was unable to obtain the specific increase in production of cyclic AMP by ADH. Further study of the cyclic AMP fraction by paper chromatography showed that there were contaminations by other nucleotides in this fraction. The validity of the method was then questioned because of failure to separate the pure cyclic AMP from other nucleotides. This also indicated that other enzymes which catalyze the breakdown of ATP or cyclic AMP were not separable from adenyl cyclase in the broken cell preparations. Thus the method was inappropriate.

The results from studies with broken cell preparations are not always directly applicable to events occurring in more highly organized systems. Studies with intact tissues may be especially important from this point of view because they can often be carried out under conditions where the physiological response and the change in cyclic AMP can be measured simultaneously. The information obtained is, therefore, more directly relevant to physiological and clinical situations. Many of the points which can be tested with broken cell preparations can also be studied in intact tissues. In addition, the change in cyclic AMP and the physiological response can be compared as functions of the dose of the hormone needed to elicit the response and the time required for the response to be manifested. The rate or magnitude of some cellular processes

may be directly related to the level of cyclic AMP. Therefore, it was the purpose of this study to compare the change in the concentration of cyclic AMP in the intact tissue of new-born and adult animals in response to antidiuretic hormone and to elucidate the possible mechanisms responsible for the differences in urine concentration capacity between the new-born and the adult.

MATERIALS AND METHODS

1. Materials:

Arginine vasopressin (synthetic) (150 U/ml) was purchased from Nutritional Biochemicals Corporation. Tritiated 3',5'-AMP [8-^{3}H] (20.8 Ci/mMole) was purchased from Schwarz/Mann. Non-radioactive 3',5'-AMP was obtained from P. L. Biochemicals, Inc. Bovine serum albumin (Fraction V) was purchased from General Biochemicals. Other reagents and chemicals were obtained from standard sources.

2. Animals:

Rats of the Sprague-Dawley Strain, New Zealand white rabbits, purebred beagles and mongrel dogs were used. These were housed in the departmental animal quarters under controlled conditions of temperature and humidity appropriate for each species. They received standard laboratory diet and water. Young animals were left with their mothers until sacrificed. One day, 3 day and 5 day old beagles received 0.1 ml (0.5 Unit) ADH, intramuscularly, 2 hrs before sacrifice.

Methods

3. In Vitro Slice Technique

Animals were killed by a blow on the head or by decapitation and the kidneys removed immediately, weighed and placed in ice-cold Krebs-Ringer Tris HCl buffer solution composed of the following: NaCl, 120 mM; KCL, 4.8 mM; $CaCl_2$, 2.6 mM; $MgSo_4$, 1.2 mM; glucose, 2 mg/ml; and Tris-HCl, pH 7.4, 15 mM. Slices of renal medulla were prepared freehand and pooled in ice-cold buffer solution for each experiment. A 40-60 mg quantity was gently blotted and weighed into 10 ml beakers. A 1.0 ml quantity of ice-cold buffer solution was added and the mixture gently swirled until the slices were separated. All beakers were stored in ice until incubations were performed.

4. Incubations and Extractions:

Incubations were carried out in a Dubnoff metabolic shaker at 37° C under a gas phase of 100% oxygen. Arginine vasopressin prepared in 0.25% acetic acid was added to each sample in a volume of 10 µl. Control samples received 10 µl of 0.25% acetic acid. Incubation time varied from 5-60 minutes. After incubation the slices were rapidly removed and homogenized with 1.0 ml of cold 5% trichloroacetic acid in a Dounce ball homogenizer. The homogenizer was rinsed with an additional 1.0 ml of TCA and the rinse added to the original homogenate. The homogenate was centrifuged at 800 x g

for 10 minutes. A 0.1 ml quantity of 1N HCl was added to each supernatant which was then extracted 5 times with 2 volumes of ether. The extracts were lyophilized and redissolved in 0.5 ml of 50 mM sodium acetate at pH 4.0.

5. Measurement of cyclic AMP:

Cyclic AMP was determined by the method of Gilman (43). The assay is based on competition for protein binding of the nucleotide to a cyclic AMP-dependent protein kinase. The nucleotide protein complex is absorbed on a cellulose ester filter.

Procedure:

The cyclic AMP binding reaction was conducted in a total volume of 50 µl in 50 mM sodium acetate, pH 4.0 composed of the following:

H^3-cyclic AMP 20 µl (1.0 pmoles/20 µl) non-
radioactive standard cyclic AMP (1-20 pmole)
10 µl or unknown sample 10 µl
kinase inhibitor 10 µl (1.24 mg/ml)*
protein kinase 10 µl (0.1 mg/ml)*

The reactions were initiated by addition of binding protein-protein kinase and incubated for 1 hr to 2 hrs at 0° C. At equilibrium, the mixtures were diluted to 1 ml with cold 20 mM potassium phosphate, pH 6.0, then passed through a 24 mm cellulose ester (millipore) filter (0.45 µm pore size)

previously rinsed with the same buffer. The filter was then washed with 10 ml of this buffer and placed in a counting vial with 1 ml of Cellosolve^{R}, in which the filter readily dissolves. A scintillation mixture of toluene (750 ml) Cellosolve (ethylene glycol monoethyl-ether) (250 ml), PPO (2,5-Diphenyloxazole) (5.0 gm/l) and dimethyl POPOP-1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-Benzene (100 mg/l) was utilized. Samples were then counted on Beckman LS. 100 liquid scintillation counter.

For the assay of cyclic AMP [^{3}H] C-AMP was utilized at saturating concentration, and the effect of added unknown or standard cyclic AMP solutions could thus be evaluated from a linear decrease in the total bound [^{3}H] cyclic AMP.

*Kinase inhibitor and protein kinase were gifts from Dr. Lawrence Muschek. These were prepared by the method of Miyamoto et al.(65) and Appleman et al.(66).

6. Time course:

Adult rats, rabbits and dogs were used for this study. Incubation times varied from 5-60 minutes. Krebs-Ringer Tris HCl buffer solution containing 10 mM theophylline was used as the incubating medium. ADH concentration used for all incubation times was 100 mU/ml for rats and rabbits and 1 U/ml for dogs. Control samples which received 10 µl of 0.25% acetic acid were used for each incubation time.

7. Dose-response:

The effect of various concentrations of ADH on cyclic AMP concentration in renal medullary slices from adult rats, 1 day old rabbits, adult rabbits, 1 week old, 2 week old, adult mongrel dogs, 1 day old, 3 day old and 5 day old beagles was investigated. Concentrations of ADH ranging from 1 mU/ml to 1U/ml were added to the incubation medium. Experiments were done in the absence and presence of 10 mM theophylline in all species and all ages except 1 day old rabbits. Because of an insufficient amount of medullary tissues in 1 day old rabbit, this study was only carried out in the presence of theophylline. Incubations were carried out at 37° C for 30 minutes under 100% oxygen.

8. Urine osmolality *vs* plasma osmolality (U/P ratio):

Urine and plasma samples were collected from 1 day old, 3 day old, 5 day old, 1 week old, 2 week old and adult dogs. Osmolalities of both urine and plasma of each age were estimated by freezing point depression with an Advanced Osmometer.

9. Protein determination:

Protein concentration in the slice was determined by the method of Lowry *et al*. (44) after digestion of samples of slices for 1 hour with 1 N NaOH.

10. Statistical analyses:

Analysis of variance using a completely randomized design was used (45). Treatment means were compared to control using the least significant difference test. (47). Student's t test for paired data was used where appropriate.

The 0.05 level of probability was used as the criterion of significance in all statistical tests.

RESULTS

Arginine-vasopressin (ADH) increased the concentration of cyclic AMP in rat, rabbit and dog renal medullary slices in a dose-dependent manner in the presence of 10 mM theophylline. In the absence of theophylline cyclic AMP was slightly increased by ADH but this was not dose related. Cyclic AMP concentrations increased with increasing incubation time up to 30 minutes in both control and ADH-stimulated renal medullary tissue in the presence of 10 mM theophylline. A difference in cyclic AMP concentration in tissue between 1 day old and adult rabbits was not detected while the results obtained from 1 week old, 2 week old and adult dogs showed a definite pattern of development in response to ADH.

1. Effect of Incubation Time

The concentration of cyclic AMP in renal medullary slices from adult rats increased with increased incubation time up to 30 minutes (Table 1). This increase was seen, however, only when theophylline (10 mM) was added to the incubation mixture. Optimal incubation time of 30 minutes was observed in both control and ADH-containing beakers. Cyclic AMP concentration in slices in the presence of ADH at each incubation time was greater than the respective

control. At 30 min cyclic AMP concentration increased from 11.7 ± 1.5 to 24.4 ± 2.7 (p moles/mg wet weight ± S.E).

The time course for slices from adult dogs (Fig. 3) and adult rabbits (Fig. 1) were also examined. These tissues displayed the same pattern as was seen with tissue from adult rats. The basal and ADH-stimulated cyclic AMP concentration was lower for both species than that observed in rats. For instance, basal cyclic AMP concentration at optimal incubation time of 30 minutes was 11.7 ± 1.5 p moles/mg wet weight for rats, 2.0 ± 0.4 for rabbits and 2.0 in a single experiment with dog tissue. With ADH stimulation, values increased to 24.4 ± 2.7, 4.3 ± 0.9 and 3.2, respectively.

2. Effect of Varying ADH Concentration

ADH increased the concentration of cyclic AMP in renal medullary slices in a dose-dependent manner. In the absence of theophylline cyclic AMP concentration was slightly increased by ADH over a concentration range of 1 mU/ml to 1 U/ml. When theophylline (10 mM) was included in the incubations higher concentrations of cyclic AMP were detected from all species and all age groups studied (Table 2, 3, 5, Fig. 4, 5). Maximal concentration of cyclic AMP in rat and rabbit tissue was reached in 30 minutes with 100 mU/ml of ADH. Cyclic AMP concentration at 1 U/ml of ADH (22.7 ± 3.1 Picomoles/mg wet weight for rats, 2.91 ± 0.40 Picomoles/mg

wet weight for rabbits) was lower than that at 100 mU/ml (26.1 ± 2.8 Picomoles/mg wet weight for rats, 4.02 ± 0.40 Picomoles/mg wet weight for rabbits). In adult dog tissue maximal concentration was reached at 1 U/ml of ADH (3.93 ± 0.60).

3. Comparison of Cyclic AMP Concentration in Renal Medullary Tissue of Young and Adult Animals

a. 1 Day Old Rabbit vs Adult

Because the quantity of medullary tissue from 1 day old rabbits was limited, 10 mM theophylline was included in all incubations. All rabbits from a single litter were employed to obtain one series of incubation beakers for the 1 day animals. These experiments were conducted to determine the response to ADH under optimal conditions, that is, under maximal inhibition of phosphodiesterase. The results from this study are shown in Figure 2. Neither the basal concentration of cyclic AMP in the slices nor the concentration of cyclic AMP after maximal ADH-stimulation showed any age-related differences. In adult rabbit tissue the optimal incubation time was found to be 30 minutes and the same time was used in the newborn animals.

b. 1 Week old, 2 Week old and Adult Dogs

The concentration of cyclic AMP/mg wet weight in renal medullary slices from dogs at all ages studied was enhanced by ADH in the presence of theophylline but the effect was

most pronounced in the tissue from the adult (Fig. 4). In the absence of theophylline ADH produced a small increase in the concentration of cyclic AMP in the adult (increased from 1.6 ± 0.3 to 2.3 ± 0.3 Picomoles per mg wet weight at maximal response). There appeared to be some small effect in the newborn as well but this was not statistically significant. When theophylline was added to the beakers there was a statistically significant increase in cyclic AMP in tissue from all age animals in the absence of any antidiuretic hormone (Fig. 4). The cyclic AMP concentration increased from 1.3 ± 0.1 to 1.8 ± 0.2 in 1 week old, 1.4 ± 0.1 to 2.3 ± 0.1 in 2 week old, and 1.6 ± 0.3 to 2.3 ± 0.4 in adult. In the presence of theophylline there was a significant increase in cyclic AMP concentration in response to ADH in tissue from all ages though the response in the young animals (increased from 1.8 ± 0.2 to 2.3 ± 0.2 at 1 week; 2.3 ± 0.1 to 2.9 ± 0.3 at 2 weeks) was significantly less than that observed in the adult (increase from 2.3 ± 0.4 to 3.9 ± 0.6). Furthermore, the response in the 1 week animal (1.3 ± 0.2 at 1 U/ml of ADH) was less than that seen in the 2 week animal (1.9 ± 0.3). When the above data were factored by protein concentration rather than wet weight the magnitude of these differences was reduced (Fig. 5) (29 ± 2.5 to 37 ± 0.3, 30.5 ± 2.0 to 38.5 ± 4.0 and 25.5 ± 4.5 to 42 ± 6.5 respectively). Whereas the difference between the control beakers and the theophylline beakers was still apparent. Age related differences in response to antidiuretic hormone were no longer apparent.

4. Comparison of Body Weight, Kidney Weight, Protein Concentration and Uosm/Posm Ratio from 1 Day Old, 3 Day Old, 5 Day Old and Adult Dogs

As shown in Table 4 body weight, kidney weight, protein concentration and U/P ratios were low in the immediate newborn period. The osmolar U/P ratio was less in the 1 day old animals (1.8 ± 0.1) than in the 3 day (2.8 ± 0.6) and 5 day (2.9 ± 0.5) animals. Protein concentration in the medullary tissue was also lower in the newborn animals and was lowest at 1 day. These young animals were all pretreated with ADH 2 hours before sacrifice yet the U/P ratios for osmolality were still relatively low compared to the adult values (6.1 ± 0.6).

5. Comparison of Cyclic AMP Concentration in Response to ADH Among 1 Day Old, 3 Day Old, 5 Day Old and Adult Dogs

When the young animals were pretreated with ADH 2 hours prior to sacrifice cyclic AMP concentration in response to various concentrations of ADH was still below the adult values (Table 5). Though these tissues were capable of responding to theophylline and to antidiuretic hormone the values were still significantly less than that observed in the 1 week animals illustrated in Fig. 4 and 5. For instance, at maximal dose (1 U/ml) of ADH and in the presence of theophylline values obtained for 1 day old, 3

day old, 5 day old and 1 week old animals are 2.55 ± 0.17, 2.27 ± 0.31, 2.59 ± 0.34 and 2.30 ± 0.20 p moles/mg wet weight, respectively.

DISCUSSION

Incubation of renal medullary slices with antidiuretic hormone or with theophylline produced an increase in the concentration of cyclic AMP in the tissue. These results were obtained in tissue from three species: rats (Table 1, 2), rabbits (Figure 1, Table 3) and dogs (Figure 3, 4). A similar effect of ADH has been demonstrated for the toad bladder (7, 27), dog kidney (26) and rabbit collecting tubules (25). The present study is consistent with the hypothesis of Orloff and Handler that the physiological actions of ADH on responsive epithelial structures are mediated by cyclic AMP (7).

The major problem at this time is how cyclic AMP elicits the permeability changes characteristic of the response to ADH. The mechanism is so far still unknown. Cyclic AMP is known to influence a variety of enzymes, including phosphorylase, in other tissues (1). Phosphorylase activity in toad bladder and kidney is increased by ADH and cyclic AMP (46,47). The known metabolic effects of ADH include an increase in oxygen consumption and glycogen breakdown (48). These changes are not evident when the bladder is incubated in a solution free of sodium, although the hormone is still capable of eliciting the characteristic effect

on water movement under these circumstances. It is apparent that stimulation of oxygen consumption and glycogenolysis reflects a metabolic requirement for sodium transport (48). The effects of cyclic AMP on oxygen consumption and glycogenolysis resemble those of ADH and are similarly dependent on the presence of sodium ion in the bathing medium (48). However, an increase in phosphorylase activity is observed in toad bladder incubated in sodium free solution, a situation in which ADH has no observed effect on glycogenolysis. Therefore, phosphorylase activation is not responsible for glycogenolysis in this tissue (48). Although its activation parallels the water permeability response of toad bladder to hormone its function in permeability is also unknown.

Handler et al. (49) observed that the physiological effects of ADH and cyclic AMP on water and sodium permeability of toad bladder are inhibited by certain metabolic inhibitors either in the presence or absence of sodium in the medium. Dinitrophenol, nitrogen, azide, and iodoacetic acid all interfere with the characteristic response to hormone. Apparently interruption of energy production from either glycolysis or oxidative metabolism reduces the capacity of the tissue to alter its permeability. Examination of the reaction of toad bladder to metabolic inhibitors has yielded conflicting results. Rasmussen et al. (17) reported no effect of metabolic inhibitors on the hydro-osmotic response to ADH and inferred from this that the hormone-induced increase in permeability does not require energy. Stimulation of glycogenolysis and

oxygen consumption may reflect increased sodium transport and not represent an integral response to the hormone. It should be apparent that the mechanism of action of ADH is not completely understood. It is likely that the presence of sodium in the media exerts a critical role in elicitating metabolic changes. ADH has two characteristic effects, an increase in permeability of certain epithelial structures to water and associated stimulation of the sodium pump. Possibly different chemical and physical processes are involved in the regulation of these effects, even though each is influenced by cyclic AMP. Petersen and Edelman (50) proposed that ADH stimulates the production of cyclic AMP at 2 separate sites within the cell: one calcium-sensitive, related to the regulation of water movement, the other insensitive to calcium and related to sodium transport. They observed that an increase in the concentration of calcium in the bathing medium reduced the hydro-osmotic effect of ADH without altering its capacity to stimulate sodium transport. This result was later confirmed by Bourguet et al. (51). These reports support the contention that two separate receptors of differing affinity determine the physiological responses to the hormone. Each receptor is involved in the generation of cyclic AMP at an independent site. Orloff et al. (52) favored the proposal that two independent sites of cyclic AMP generation are present in the basal membrane. (It is essential that no exchange of cyclic AMP occurs between the two sites, for without this assumption it would be difficult

to account for the calcium studies.) The view of Orloff et al. (52) was that cyclic AMP in the site related to water movement results in the formation of specific metabolic products which differ from those formed in the site controlling sodium transport. The products may then diffuse to their respective effectors in the apical membrane or in some other fashion induce the characteristic alterations in permeability to water and sodium.

An alternative possibility suggested by Orloff and Handler (52) was that the effect of ADH on sodium transport might be mediated by a different cyclic nucleotide, the production of which is unaffected by calcium. Cyclic AMP could mimic its effect on sodium transport but it could not mimic the effect of cyclic AMP on water permeability. Although it seemed unlikely for a time, the probability that this hypothesis is correct was greatly increased by the demonstration that cyclic GMP shares the ability of cyclic AMP to stimulate sodium transport but has no effect on water permeability (53). It remains to be seen whether ADH can actually stimulate the formation of cyclic GMP in the toad bladder.

Cyclic AMP concentration in renal medullary slices was shown to increase with time up to 30 minutes, after which the concentration of nucleotide declined (Table 1, Figure 1, 3). Studies of adenyl cyclase activity with broken cell preparations or cyclic AMP concentration from other tissue in response to hormones such as histamine and norepinephrine often show that the incubation time required to reach maximal response

was shorter than that required in this study. The explanation for this could be that each tissue has a specific affinity for its stimulating hormone. It might take longer for ADH to bind to the receptor in order to change the configuration of the receptor or to elicit the response. Among the studies of cyclic AMP concentrations in the toad bladder the time taken for measurement of cyclic AMP concentration after addition of hormone has ranged from 20-30 minutes (7, 27,54). The decline in concentration of cyclic AMP after 30 minutes can be explained by exhaustion of substrate or by lability of the enzyme. Inactivation of ADH in isolated tissues has been studied by several investigators. Smith and Sachs (55) demonstrated inactivation of ADH in rat kidney slices. Since the enzymes were not released into the medium from the slices, it was concluded that the kidney slices transformed ADH into inactive molecules. Intracellular mechanisms involve reduction of the disulfide bond and enzymatic attack on some groups on the ADH molecule (56).

Cyclic AMP concentration in renal medullary tissue in response to ADH displayed a dose-dependent pattern (Table 2, 3, Figure 2, 4). Cyclic AMP concentrations from rat tissue were much higher than that observed from rabbits and dogs. This may reflect a true species difference. Cyclic AMP concentrations in this study are comparable to those reported by Beck et al. (57) and Senft et al. (58). Slightly lower values found in their work could be due to shorter incubation times (15 minutes). The maximal effect observed in

rat and rabbit tissue was seen with 100 mU/ml of ADH. When the concentration of hormone was increased to 1 unit/ml the response declined. It seems unlikely that pH changes in the media could account for this difference since control samples also received the same volume of acid used for ADH preparation. In many enzyme studies it is not uncommon to find that while the Michaelis law is obeyed at low substrate concentrations the velocity falls off at high concentrations.

In order to explain the inhibitory effect of high concentrations of hormone it is necessary to introduce the receptor concept and consider how receptors and hormones are related to the enzyme adenyl cyclase. The hypothetical model for adenyl cyclase consists of at least two types of subunits, a regulatory subunit, facing the extracellular side, and a catalytic subunit with its active center directed toward the interior of the cell. The receptor is here regarded as part of the regulatory subunit. Interaction with the hormone leads to a conformational change which is extended through the regulatory subunit to the catalytic subunit, thereby altering activity of the latter (6). In the effective receptor-hormone complex one hormone molecule is combined entirely with a receptor. At high hormone concentrations, where the hormone molecules tend to crowd onto the receptor, the chance of formation of ineffective complexes with 2 or more hormone molecules combined with a specific receptor increases. Therefore, high concentrations of the hormone lead to inhibition of enzyme activity. Although hormones might not serve as enzyme

substrates it is logical to suggest that an excess of hormone has inhibited the reaction in a manner analogous to substrate inhibition.

The ability of theophylline to potentiate the effects of ADH on cyclic AMP is demonstrated in this study (Table 2, 3, Figure 2, 4). Theophylline interferes with the action of phosphodiesterase, the enzyme which catalyzes the degradation of cyclic AMP to its physiologically inactive product, 5'-monophosphate (59). In the absence of theophylline cyclic AMP concentrations in the tissue were significantly lower than in its presence. Both ADH and theophylline increased the permeability of tissue to water when added individually to the isolated perfused rabbit collecting tubule (46,25), but the possibility that the two agents might act synergistically in the system has not been tested. It could perhaps be assumed that this would occur if ADH stimulated cyclic AMP formation and theophylline prevented its breakdown. Nevertheless, the finding has been viewed as not necessarily supportive of the hypothesis that cyclic AMP mediates the action of theophylline, since in the mammal organism theophylline is a potent diuretic. It is conceivable, however, that theophylline exerts its diuretic effect on the proximal tubule of the mammalian nephron by interfering with sodium reabsorption to such a degree that any simultaneous effect on water reabsorption elsewhere in the nephron is masked.

Renal medullary tissue from rabbits and dogs did not show similar developmental patterns in terms of cyclic

AMP concentration (Figure 2, 4). In rabbit tissues cyclic AMP concentration was as high in 1 day old tissue as in that of adult. In dogs cyclic AMP concentrations were low at one week and increased to maximal values in adults. It is not known if rabbit kidneys are relatively more mature shortly after birth than are dog kidneys. This finding does not indicate that one day rabbit necessarily has a urinary concentrating capacity equivalent to the adult. Concentrating performance in young animals is limited by several other factors: metabolically low rate of excretion of urea, shortness of the loops of Henle and limitation in the capacity of the cells to pump sodium against a concentration gradient. It is possible that the osmotic gradient between the medulla and the urine is low compared to the adult. Therefore, in this species even if ADH is capable of promoting water movement through the cells of the collecting ducts the final urine concentration could be low. In the case of dogs cyclic AMP concentration was lower in the young animal tissue than in that of adults. Hommes et al. recently studied the development of adenyl cyclase in rat liver, kidney, brain and skeletal muscle. The adenyl cyclase activity in kidney cortex was found to increase gradually to 30 days of age (60). The same developmental pattern may occur in dog kidney. The low activity of adenyl cyclase would well be related to the low tissue concentration of cyclic AMP. Since the action of ADH is mediated by cyclic AMP it might be assumed that the low concentrating capacity in young dogs is partly due to low cyclic AMP

concentration in the tissues. In this study cyclic AMP concentration paralleled the Uosm/Posm between young and adult dogs (Table 4, 5). After injection of ADH in one day old, 3 day old and 5 day old dogs cyclic AMP concentration and urinary osmolar concentration still remained significantly lower than adult values (Table 4, 5). This reflects the insensitivity of the young tissue to ADH administration. There are several possible explanations for this insensitivity. First, based on the developmental pattern of a number of kidney enzymes (39,40,60), adenyl cyclase activity during the period of morphological change of the kidney may be low. Thus, adenyl cyclase could be less receptive to hormone stimulation. Secondly, the environment at the site of cyclic AMP generation, with respect to enzyme, substrate and other constituents may be very different between young and adult animals. If the proposal that cyclic AMP-induced permeability changes occur through metabolic products is valid (52), the difference in environment may determine a difference in metabolic response to cyclic AMP. Thirdly, there is evidence indicating that in the kidneys of mammals ADH must be bound to tissue before it exerts its antidiuretic action (61). Only tissues which bind ADH inactivate it (62). Dicker et al. (62) showed that inactivation of ADH by the particle-free supernatant of kidney homogenates resulted from an enzyme, possibly acting on the disulfide link of the hormone reducing it to sulfhydryl. Since the enzyme would be activated by compounds like cysteine

or glutathione containing an sulfhydryl group, they concluded that the enzyme is an sulfhydryl enzyme. Early studies in newborn dogs demonstrated no limitation in the availability of antidiuretic hormone. Studies in the urine of the newborn animal suggested that these animals contain a substantial amount of antidiuretic activity (38). Martinek and associates (63) suggested that resistance to antidiuretic hormone is demonstrable in early infancy. Possibly in newborn, the affinity of ADH binding sites is reduced. The antidiuretic activity found in newborn urine suggests that in the newborn none or little of the ADH that reaches the kidneys has been inactivated. Following prolonged and repeated exposure to ADH newborn kidneys may eventually become resistant to the hormone. As kidney function increases binding sites may be subjected to new conformational changes and become more receptive and sensitive to the hormone. The hormone could be bound, elicit its effect and then be inactivated by the tissue enzyme. This does not exclude the possibility that the mature kidney cannot develop resistance. Because of rapid inactivation of the hormone after binding to the receptor exposure time of hormone to the receptor could be shorter. Therefore, it may have less opportunity to induce resistance.

Another explanation for the finding that cyclic AMP concentration in medullary slices in young dogs was lower than adult could be related to medullary tissue water content. Horster et al. (64) demonstrated that medullary tissue water content decreases with age in very young dogs. This suggested

that lower cyclic AMP concentration in medullary slices from young animals could be attributed to the higher water content. When cyclic AMP concentration was factored by tissue protein the difference between young and adult was abolished (Fig. 5). This could be interpreted to mean that production of cyclic AMP in tissue from newborn is the same as the adult. Clarification of this would require kinetic analysis of adenyl cyclase activity. However, expressing the data in terms of concentration (i.e., per mg wet weight) more closely represents conditions in the functioning kidney. When expressed in this manner, cyclic AMP concentration (and presumably physiological action) was less in tissue from newborn. Cyclic AMP after formation would be diluted by medullary tissue water. The possibility that young animals are unable to respond to ADH to the same degree as the adult may be due to this diluting effect.

These observations support a role for cyclic AMP in the action of ADH in young and adult animals. They do not exclude, however, the importance of the length of the loops of Henle in the immature concentrating mechanism. It has been shown that a significant portion of the limitation in concentrating capacity in infants results from the low rate of excretion of urea. This is due to the strongly anabolic state that prevails during that period of life. When the infant is fed with urea on a high protein diet a marked increase in concentrating capacity is observed owing entirely to the additional urea. Permeability characteristics of

various segments of the nephron and rate of excretion of electrolytes are all important in the concentrating mechanism. Too little of the renal physiology of the neonate is known to be able to draw conclusions as to which factors are more responsible for the poor ability of the kidney of new-born animals to concentrate their urine.

SUMMARY

Cyclic AMP concentration was measured in renal medullary tissue from rats, rabbits and dogs. Increased concentration of cyclic AMP in response to ADH supports the hypothesis that the actions of ADH on epithelial structures are mediated by cyclic AMP.

The pattern of development of the concentrating ability was determined in 1 day old, and adult rabbits and dogs from age 1 week to adult by measuring the cyclic AMP concentration in renal medullary slices in response to antidiuretic hormone. Cyclic AMP concentration increased with age in dogs. Body weight, kidney weight, protein concentration and Uosm/Posm ratio from dogs were low in the new-born period. It is suggested that lower cyclic AMP concentration may play some role in the low concentrating capacity in young animals. Data obtained from rabbits did not show a similar developmental pattern, suggesting that other limiting factors in the concentrating mechanism may play major roles in this species.

Treatment of 1 day old, 3 day old and 5 day old dogs with ADH did not increase cyclic AMP concentration nor urine osmolar concentration demonstrating the insensitivity of young animals to ADH. This may result from a less receptive adenyl cyclase system, or development of resistance to ADH,

or difference in the environment at the cyclic AMP generation site.

Cyclic AMP concentration in renal medullary slices from adult rats, rabbits and dogs increased with incubation time up to 30 minutes. This increase was seen only when 10 mM theophylline was added to the incubation mixture. ADH increased the concentration of cyclic AMP in renal medullary slices in the presence of 10 mM theophylline. In the absence of theophylline cyclic AMP concentration was slightly increased but was not dose-related. Values obtained here were also significantly lower than those when theophylline was present.

This study demonstrated the possible role of cyclic AMP in the development of urinary concentrating capacity in new-born animals. However, due to the species difference observed here, cyclic AMP may only play one of several roles in limiting the concentration performance in new-born animals. The importance of other factors such as short loops of Henle, low rate of urea excretion, permeability characteristic of various segments of the nephron, rate of excretion of electrolytes and limitation of capacity for sodium pump should not be overlooked.

Table 1. Relationship between incubation time and cyclic AMP concentration in adult rat renal medulla.[a]

Incubation Time (min)	Cyclic AMP Concentration (picomoles/mg wet wt.) Control	ADH[b]
5	6.6±0.2	11.3±0.7
10	7.3±0.4	13.7±1.0
20	10.5±0.9	17.3±0.7
30	11.7±1.5	24.4±2.7
60	11.6±0.6	17.7±1.1

[a]Each value represents the mean cyclic AMP concentration ± S.E. obtained in 3 experiments. Renal medullary slices from 6 adult rats were pooled for each experiment. Incubations were performed in the presence of 10 mM theophylline.

[b]The concentration of ADH used for each incubation time was 100 mU/ml.

Table 2. Effect of various doses of ADH on cyclic AMP concentration in renal medullary slices from adult rats in the absence and presence of 10 mM theophylline.[a]

ADH Dose (mU/ml)	Cyclic AMP Concentration (picomoles/mg wet wt.) Without Theophylline	With Theophylline
Control	4.9±1.3	9.7±1.6
1	6.5±1.5	14.8±1.4
10	5.8±0.8	17.2±1.3
100	6.8±1.2	26.1±2.8
1000	6.6±0.6	22.7±3.1

[a]Each value represents mean ± S.E. of 3 experiments. In each experiment the medullary slices from 8 adult rats were used and randomly distributed among the incubation beakers.

Table 3. Effect of various doses of ADH on cyclic AMP concentration in renal medullary slices from adult rabbit in the absence and presence of 10 mM theophylline.[a]

ADH Dose (mU/ml)	Cyclic AMP Concentration (picomoles/mg wet wt.) Without Theophylline	With Theophylline
Control	0.98±0.11	2.16±0.24
1	1.11±0.10	2.32±0.23
10	1.15±0.12	2.45±0.20
100	1.62±0.12	4.02±0.40
1000	1.47±0.18	2.91±0.40

[a]Each value represents mean ± S.E. obtained from duplicate determinations in tissue from 4 adult rabbits.

Table 4. Comparison of body weight, kidney weight, protein concentration in the medullary slices and Uosm/Posm ratio from 1 day old, 3 day old, 5 day old and adult dogs.[a]

	Age			
	1 day	3 days	5 days	Adult
Body Weight (gm)	190±26	283±39	272±21	9900[b]
Kidney Weight (gm)	2.59±0.30	4.53±0.74	5.02±0.50	50[b]
Protein Conc. (mg/mg wet wt.)	0.065±0.004	0.074±0.003	0.072±0.003	0.092±0.009
Uosm/Posm	1.8±0.1	2.8±0.6	2.9±0.5	6.1±0.6

[a]Each value represents mean ± S.E. Values for 1 day old, 3 day old and 5 day old were obtained from 5 animals of each age in 4 litters. Four adult animals were used for these determinations.

[b]Typical values for adult female purebred beagles. (67)

Table 5. Comparison of effect of ADH on cyclic AMP concentration (picomoles/mg wet wt.) among 1 day old, 3 day old, 5 day old and adult dogs in the absence and presence of 10 mM theophylline.[a]

ADH Dose (mU/ml)	Age							
	1 day old		3 day old		5 day old		Adult	
	Theophylline		Theophylline		Theophylline		Theophylline	
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
Control	0.81±0.02	1.79±0.06	0.77±0.09	1.54±0.33	1.03±0.06	2.18±0.37	1.62±0.31	2.31±0.40
10	0.79±0.05	2.13±0.17	0.84±0.09	2.13±0.26	1.07±0.04	2.35±0.31	1.71±0.20	3.22±0.40
100	0.83±0.06	2.26±0.21	0.86±0.11	2.23±0.32	1.31±0.08	2.65±0.29	1.92±0.20	3.71±0.60
1000	0.96±0.08	2.55±0.17	0.92±0.07	2.27±0.31	1.23±0.08	2.59±0.34	2.10±0.32	3.93±0.60

[a]Values from young animals were determined from 5 experiments of each age in 4 litters. Animals were pretreated with 0.5 unit ADH 2 hours before sacrifice. Values for adult were obtained from 4 experiments. Each value represents mean ± S.E.

Figure 1. Effect of ADH on cyclic AMP concentration in medullary slices from adult rabbits. Slices were pooled and randomly distributed among the incubation beakers, which were incubated for times ranging from 5 to 60 minutes. Cyclic AMP concentrations per mg wet tissue were measured. Points indicate means ± S.E. for 3 such experiments. The concentration of ADH used for each incubation time was 100 mU/ml. Control samples received 10 µl 0.25% acetic acid were incubated similarly. Incubation were carried out in the presence of 10 mM theophylline.

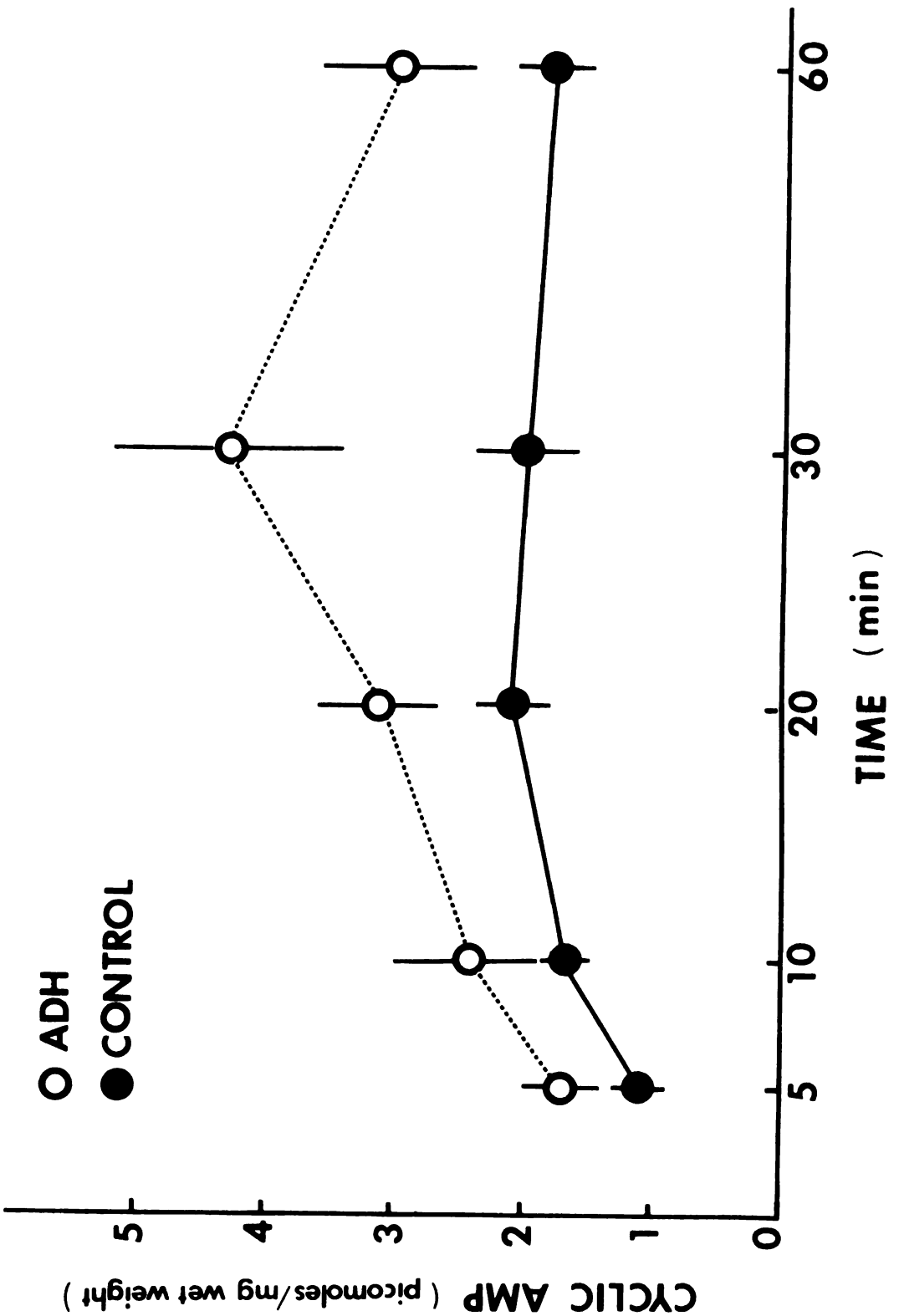

O ADH
● CONTROL
CYCLIC AMP (picomoles/mg wet weight)
0
1
2
3
4
5
5
10
20
30
60
TIME (min)

Figure 2. Cyclic AMP concentrations in renal medullary slices in response to various doses of ADH from 1 day and adult rabbits. Each bar represents the mean ± S.E. from 4 experiments in 4 litters. Incubations were carried out at 37° C for 30 minutes in the presence of 10 mM theophylline.

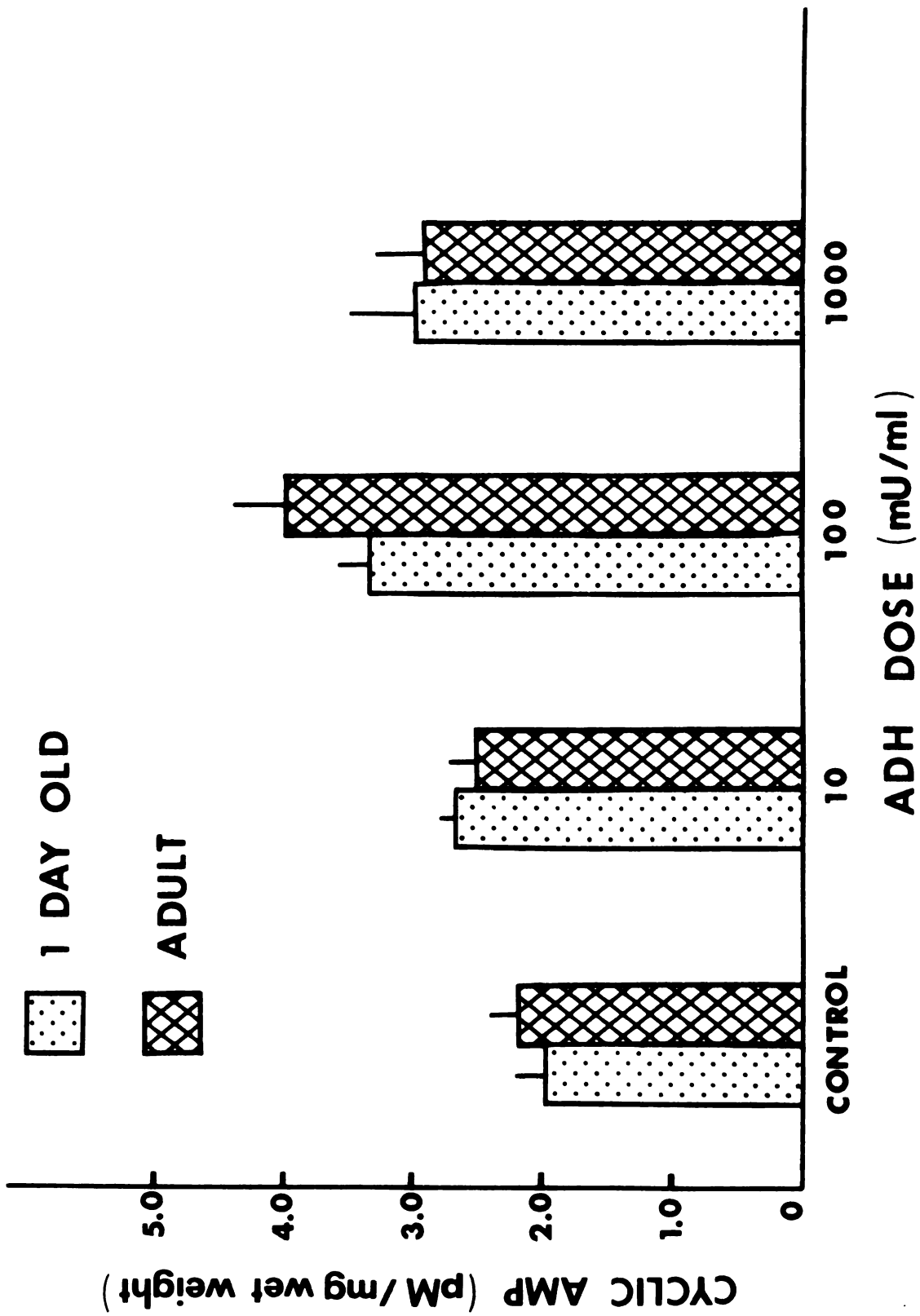

1 DAY OLD
ADULT
CYCLIC AMP (pM/mg wet weight)
5.0
4.0
3.0
2.0
1.0
0
CONTROL
10
100
1000
ADH DOSE (mU/ml)

Figure 3. Effect of varying incubation time on cyclic AMP concentration in renal medullary tissues from adult dog. Points indicate means from duplicate determination in 1 experiment. 1 U/ml of ADH was used for each incubation time. Control samples received 10 µl 0.25% acetic acid. Incubation times ranged from 5 to 60 minutes 10 mM theophylline was included in each incubation.

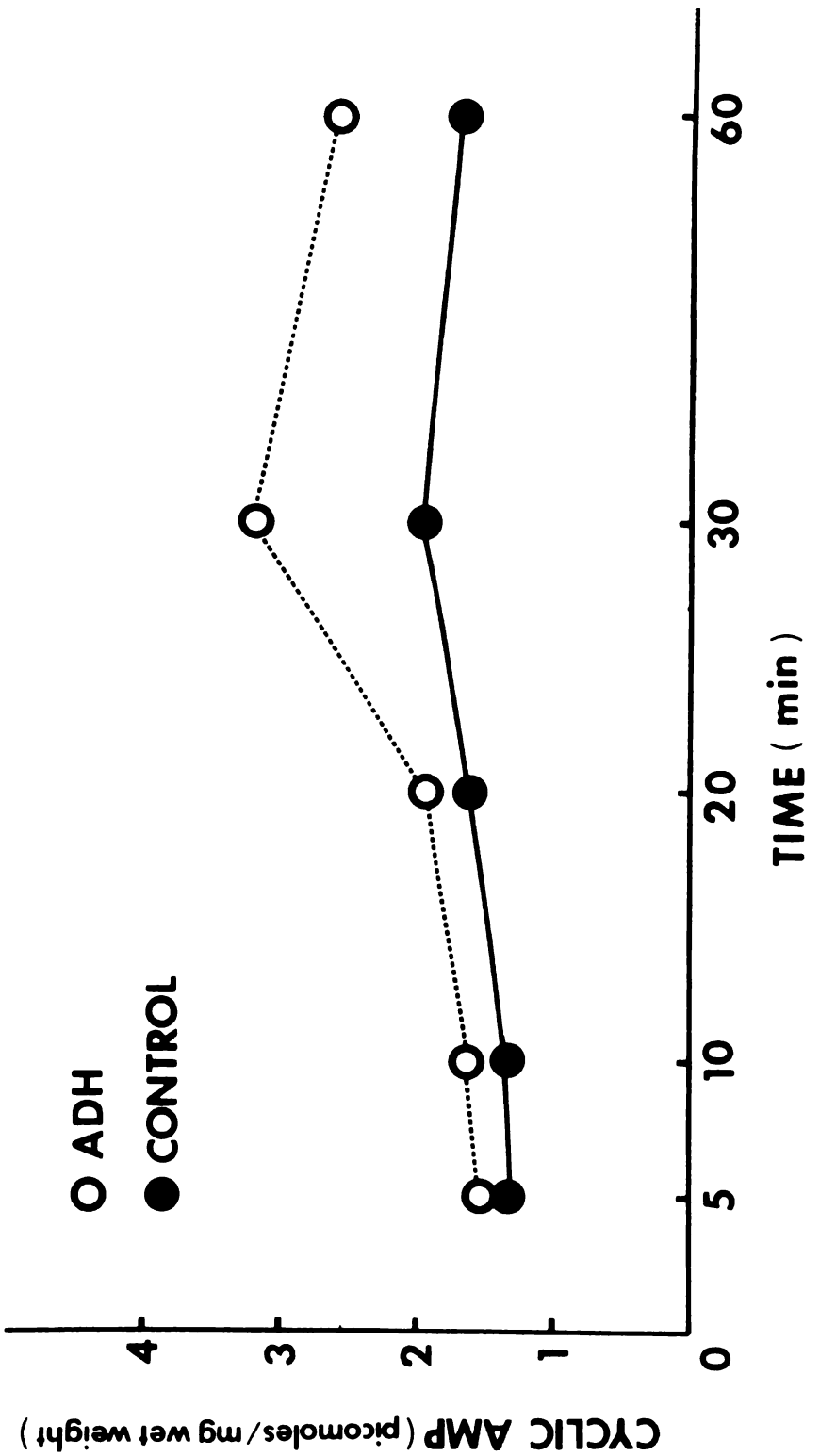

CYCLIC AMP (picomoles/mg wet weight)
0
1
2
3
4
5
10
20
30
60
TIME (min)
O ADH
● CONTROL

Figure 4. Comparison of cyclic AMP concentration in response to various concentrations of ADH in renal medullary slices from 1 week old, 2 week old and adult dogs on the basis of wet medullary tissue. Each bar represents the mean ± S.E. determined in 6 experiments for 1 week old and 2 week old dogs and in 4 experiments for adult dogs. ADH doses ranged from 10 mU/ml to 1 U/ml. In the presence of theophylline cyclic AMP concentrations in tissue from animals of all ages are significantly different from those when theophylline was absent ($p<0.05$). In the adult, ADH (1 U/ml) raised the cyclic AMP concentration significantly above the level observed in the presence of theophylline (10 mM). There was a significant progressive increase in the cyclic AMP concentration produced by ADH (1 U/ml) in tissue obtained from 1 week old, 2 week old and adult animals ($p<0.05$).

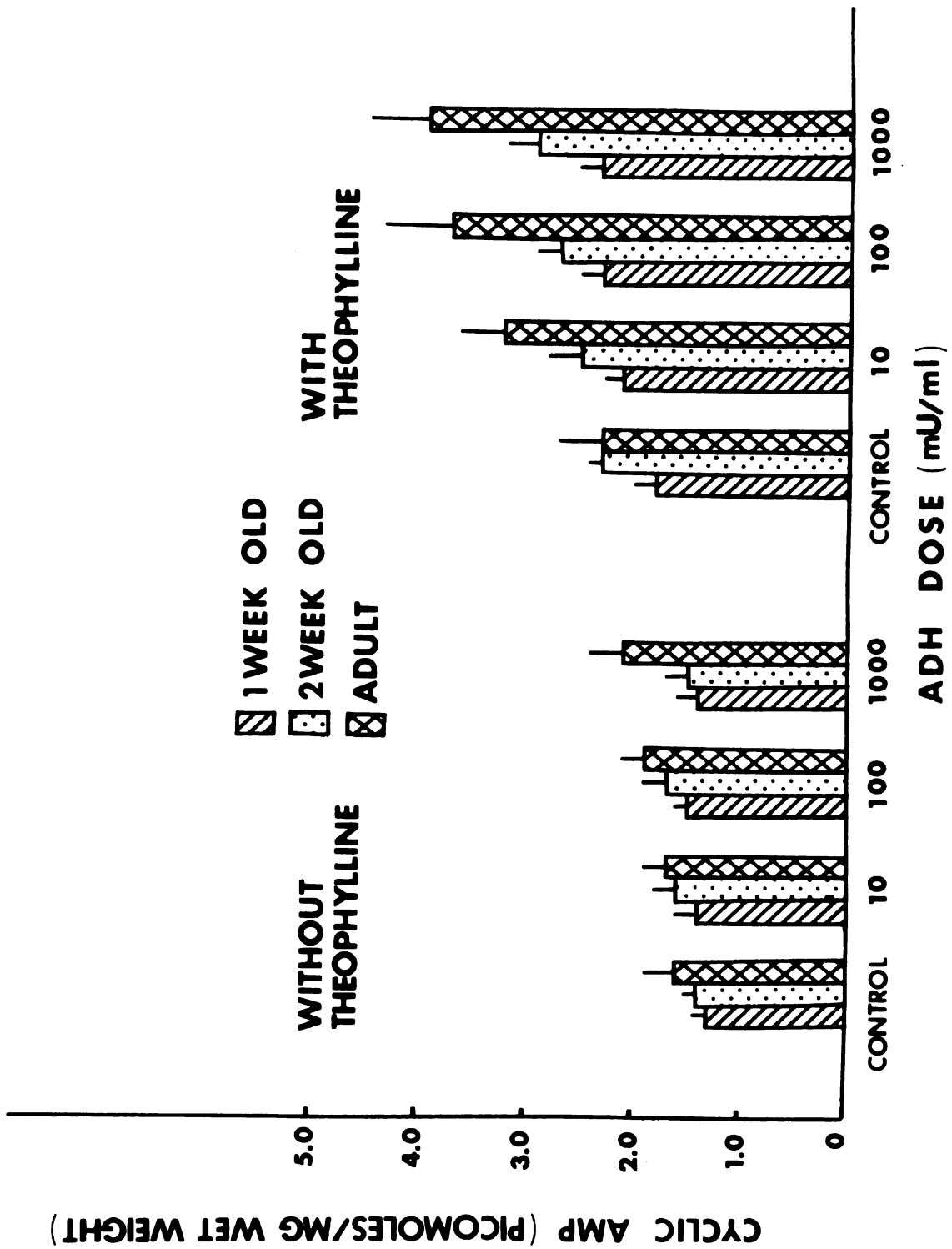
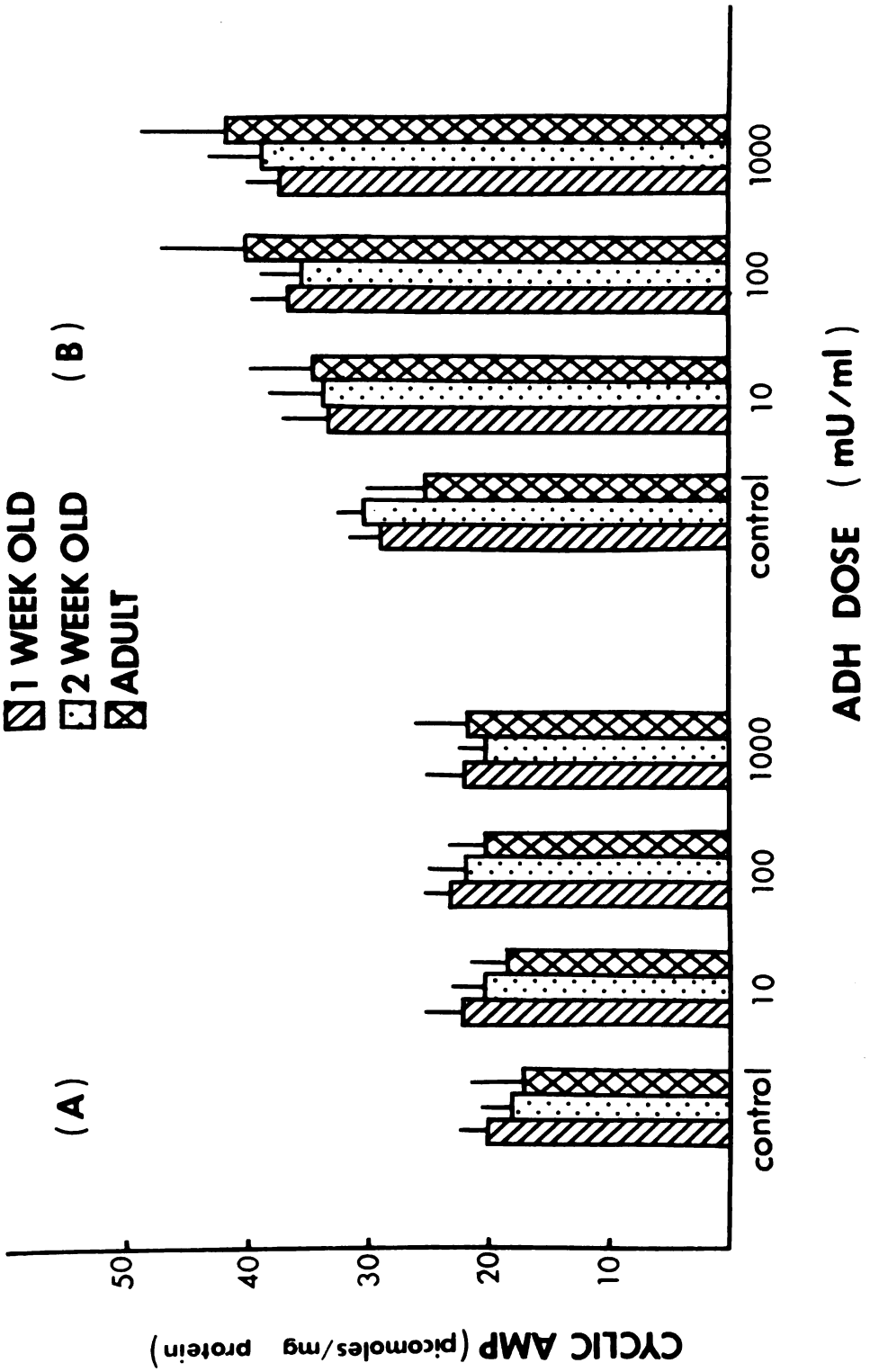

CYCLIC AMP (PICOMOLES/MG WET WEIGHT)
5.0
4.0
3.0
2.0
1.0
0
WITHOUT THEOPHYLLINE
WITH THEOPHYLLINE
1 WEEK OLD
2 WEEK OLD
ADULT
CONTROL
10
100
1000
ADH DOSE (mU/ml)

Figure 5. Comparison of cyclic AMP concentration in response to ADH in renal medullary slices from 1 week old, 2 week old and adult dogs. The data from Figure 4 were converted to cyclic AMP concentration per mg protein. Each bar represents the mean ± S.E.
A) Incubations were carried out in the absence of theophylline.
B) in the presence of 10 mM theophylline.
In the presence of theophylline cyclic AMP concentrations from animals of all ages were significantly different from those when theophylline was absent ($p<0.05$). There were no significant differences among ages in any case.


1 WEEK OLD
2 WEEK OLD
ADULT
(A)
(B)
CYCLIC AMP (picomoles/mg protein)
50
40
30
20
10
control
10
100
1000
control
10
100
1000
ADH DOSE (mU/ml)

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