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The role of calcium, cAMP, osmotic forces and pH in controlling renin release from renal cortical slices and isolated renin-containing granules

Sigmon, David Henry, Ph.D.

City University of New York, 1988

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THE ROLE OF CALCIUM, cAMP, OSMOTIC FORCES AND pH IN CONTROLLING RENIN RELEASE FROM RENAL CORTICAL SLICES AND ISOLATED RENIN-CONTAINING GRANULES

By

David H. Sigmon

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

1988

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

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Abstract

THE ROLES OF CALCIUM, cAMP, OSMOTIC FORCES AND pH IN CONTROLLING RENIN SECRETION FROM CORTICAL SLICES AND ISOLATED RENIN-CONTAINING GRANULES

by

David H. Sigmon

Adviser: Professor John C.S. Fray

These studies sought, in part, to elucidate the roles of calcium, cAMP, and chemiosmotic forces in the regulation of renin secretion. Renin was measured as renin activity. A 20-fold decrease in the rate of renin secretion was observed when cytosolic calcium concentrations was raised from 10^{-8}M to 10^{-5}M. These findings support the hypothesis that renin secretion is inversely related to cytosolic free-calcium. However, cAMP stimulated renin secretion by a fixed amount (250 ng Angiotensin I per 100 mg tissue per hr) when cytosolic calcium ranged from 10^{-8}M to 10^{-5}M. The dissociation between cytosolic calcium and cAMP suggests that there are two pathways governing the regulation of renin secretion, one calcium-dependent and the other calcium-independent.

Renal cortical slices and isolated granules were also used to investigate the roles of osmotic forces and pH gradients in the regulation of renin secretion and

granular lysis. Renin secretion from renal cortical slices was inhibited by carbonyl-cyanide m-chlorophenylhydrazone (CCCP) and oligomycin. The effect of CCCP was pH-dependent, with maximal inhibition at alkaline pHs. Hypo-osmolality stimulated renin secretion in both cortical slices and isolated granules. The pH effect was biphasic with greater stimulation under strongly acid and alkaline conditions. In isolated granules, the pH effect was chloride and potassium dependent. Removal of either of these ions from the incubation medium inhibited granule release and lysis. 4-acetamido-4'-isothiocyanatostilbene-2,2 disulfonic acid (SITS) and 4,4-diisothiocyanatostilbene-2,2'- disulfonic acid (DIDS), two anion channel blockers, were without effect. The pH effect on isolated granule was abolished by addition of oligomycin and N,N'-dicyclohexylcarbodiimide (DCCD) at pH 5 but not at pH 8; it was enhanced by NH_4^+. Neither valinomycin nor CCCP was effective when presented alone, but in combination they stimulated at all pHs.

The data suggest that swelling of the renin granule, driven by a proton gradient across the granule membrane provides the driving force for renin secretion in a manner consistent with the chemiosmotic mechanism postulated for exocytotic release in other systems. These findings are discussed in terms of a chemiosmotic mechanism for exocytotic release of renin.

Acknowlegements

I which to extend my gratitude to Dr. John. C.S. Fray, my research sponsor, for his excellent supervision, encouragement, moral and financial support. His help, guidance and scientific philosophy have been a source of inspiration throughout the course of my work. Also, his special understanding of me as an individual enabled him to push me to the limit, both intellectually and emotionally. His personal philosophy on life and the search for truth will always be with me and continue to influence me throughout my life. His patience and commitment will always be remembered.

I would also like to thank Dr. Chun Sik Park for all his help and inspiration on the renal cortical slice experiments. In addition, I would like to extend my gratitude to Dr. Claude Saint-Come, Dr. Shirley Russo and Pam Lucchesi of their friendship, moral support and stimulating scientific dialogue during the course of my work.

Finally, I would like to extend a special thanks to Dr. Jill Macoska, Fran Sigmon, Howard Sigmon, Jo Ann Sigmon and Edith Plishner whose love and encouragement kept me going in times of dispair.

DEDICATION

I DEDICATE THIS THESIS TO MY FAMILY WHO HAVE BEEN A SOURCE OF LOVE AND INSPIRATION THROUGHOUT MY LIFE.

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I. Introduction

Although substantial advances have been made within the last decade in elucidating the mechanisms for renin secretion, details of crucial steps in the secretory cascade are still lacking. The major advances include a better understanding of the roles played by calcium, adenosine, and cyclic AMP (cAMP) in signal transduction through the baroreceptor, macula densa receptor and neurohormonal pathways. Physiological events which stimulate renin secretion may utilize these receptors to lower intracellular calcium and/or increase cAMP. For example, it has been demonstrated that the baroreceptor regulates renin secretion via changes in cytosolic calcium such that a decrease in perfusion pressure increases renin secretion by decreasing cytosolic calcium. The macula densa has also been suggested to control renin secretion through a calcium dependent mechanism. Adenosine has been proposed as the signal from the macula densa to the juxtaglomerular (JG) cells. However, the precise mechanism of stimulation of adenosine release by the macula densa remains unclear. Stimulation of the neurohormonal pathway causes changes in renin secretion by varying cytosolic levels of calcium and/or cAMP.

Although these advances have furthered our understanding of how intrarenal mechanisms control renin secretion (by impinging upon the JG cells) they have

provided virtually no information regarding the secretory cascade within the JG cell itself. For example, confusion continues over (1) whether levels of cytosolic calcium actually correlate with renin secretion since a direct demonstration is lacking; (2) whether cAMP stimulates renin secretion through a calcium dependent or independent process; and (3) whether the renin secretory process employs chemiosmotic forces, as demonstrated for other systems. Finally, the role of the secretory granule has not been examined systematically. The following review examines the literature pertaining to each of these areas and indicates how these data serve as a point of departure for the present series of experiments.

A. Review of the literature

1. Stretch Receptor Pathway

The stretch or baroreceptor is one of the intrarenal factors involved in the control of renin secretion. The stretch receptor hypothesis was first proposed in 1959 by Tobian et al. (387), who observed the importance of renal pressure in controlling renin secretion. The work which initiated the search for such a mechanism was performed by Goormaghtigh (148,149) and Goldblatt et al. (141). On the basis of their observations, Tobian (386) hypothesized that changes in mean renal perfusion pressure lead to changes in renin secretion dependent on the degree of

stretch of the JG cells. Shortly thereafter, Tobian (387) suggested that renin release was inversely related to the degree of stretch of the JG cell. The inverse relationship between perfusion pressure (or stretch) and renin secretion has since been supported by the findings of a number of investigators. In the early experiments by Skinner et al. (346,347) a decrease in perfusion pressure correlated well with an increase in renin secretion. However, little attention was paid to how such decreases in pressure might affect other mechanisms involved in controlling renin secretion. To further investigate Tobian's hypothesis, Blaine et al. (33,34,35) developed an in vivo nonfiltering kidney. In this system, a change in renal perfusion pressure does not affect the amount of sodium chloride delivered to the macula densa. In addition, since the kidneys were denervated and the adrenals were removed at the time the kidney was made nonfiltering, the increase in renin secretion observed after acute hemorrhage or during aortic occlusion could only have resulted from activation of the stretch receptor. Blaine's studies provide strong support for Tobian's hypothesis, but only if the kidney is nonfiltering. Evidence that the glomerular filtration rate was minimal, at best, is three-fold: first, lissamine green dye injected into the aorta did not appear in the renal tubule; second, histological examination revealed blockage of the proximal tubule that prevented flow of

filtrate through the kidney; third, when the ureters were reopened creatinine clearance was almost zero (35,192).

Additional evidence for the stretch receptor hypothesis comes from studies in which changes in renin secretion were correlated with changes in vascular resistance (116,117,144,200). These findings are all in agreement with Tobian's original hypothesis. Further support for the stretch receptor hypothesis comes from studies using papaverine, an agent that relaxes and thereby dilates the renal vasculature (86). Papaverine infusion has been demonstrated to block the renin response to acute hemorrhage (425). Papaverine also decreases renin secretion in dogs with thoracic caval constriction (426) and chronic sodium depletion (144), while having no direct toxic effect on the JG cells (425). Though Tobian (386) suggested that the JG cells themselves are the stretch receptors, direct evidence for this was supplied later by Fray and Lush (123), who demonstrated that direct stretching of the JG cells decreased renin secretion.

To understand further the mechanism by which stretch affects renin secretion, a mathematical model was developed which predicts the secretory responses of the JG cells to changes in the degree of afferent arteriole stretch (117,120). The model predicts that decreasing renal perfusion pressure, increasing renal tissue pressure, or increasing arteriolar constriction (ie. increased vascular resistance) should all result in a

decreased stretch and increased renin secretion, whereas vasodilation, which increases the degree of stretch of the JG cell, should inhibit renin secretion (117). There is good experimental evidence for predictions based on this model. Skinner (347) et al. demonstrated that increasing intrarenal tissue pressure increased renin secretion independent of vascular resistance and of initial perfusion pressure. The increased secretory rate could be reduced to control levels by increasing the renal perfusion pressure. However, the change in perfusion pressure required to decrease renin release to control levels was twice the increase in intrarenal tissue pressure. This observation indicates that the pressure effect is not just a simple alteration in transmural pressure. If this were the case, an equal and opposite pressure would be sufficient to return renin secretion to control levels. Fray (117) also demonstrated that papaverine increased renal blood flow while decreasing renin secretion as compared to control. The inhibition of renin secretion by papaverine was not due to increased sodium delivery to the macula densa, since changing the sodium concentration of the perfusate had no effect on renin secretion at constant pressure. On the other hand, vasoconstriction invoked by infusion of phenylephrine or methoxamine at constant pressure resulted in a substantial increase in renin secretion. Although decreased renal blood flow is indicative of increased renal vascular

resistance, it was not responsible for the increase in renin secretion. Once again, changes in sodium delivery to the macula densa were without effect on renin secretion. In addition, the increased renin response could be reversed by infusion of papaverine or high pressure.

The mathematical model is not without its contradictions. A number of investigators have demonstrated that decreasing and increasing renal perfusion pressure stimulates and inhibits renin secretion, respectively (116,125,155,280,396), as the model predicts. However, lowering renal perfusion pressure should lead to a decrease in vascular resistance due to the autoregulatory nature of the renal vasculature. According to the mathematical model, these two signals are contradictory. The decreased perfusion pressure provides a stimulatory signal, whereas the decrease in renal vascular resistance (ie. vasodilation) provides an inhibitory signal. The stimulatory effect of reducing perfusion pressure can be explained by the work of Folkow and Neil (114). They demonstrated that the change in the ratio of the inner radius to the outer radius is very small in response to vasodilation. Therefore the stimulatory effect of low pressure is greater than the inhibitory effect of vasodilation which results in an increase in renin secretion. It has also been demonstrated that low pressure can stimulate renin

secretion in the absence of vasodilation (120). This dissociation occurs at perfusion pressures below the range of autoregulation. Yet, there are investigators who propose that renal vasodilation itself is the ultimate stimulus for activation of the stretch receptor (97,98,210), while others feel that the diameter of the renal arteriole has no effect (85).

While the evidence is convincing that changes in the degree of stretch of the JG cell affect renin secretion, the signal transduction process remains unclear. Goldman (142) proposed that, in general, stretch receptors transduce changes in stretch into a physiological event by altering membrane potential. In response to stretch, smooth muscle cells have been shown to undergo depolarization (53), upon which voltage-sensitive calcium channels open, resulting in an increase in cytosolic calcium. It is possible that JG cells, which are modified smooth muscle cells, respond to stretch in much the same way. Under these conditions, and in accordance with the above mathematical model, an increase in stretch would cause the JG cell membrane to depolarize, resulting in an increase in cytosolic calcium and inhibition of renin secretion (128). On the other hand, a decrease in stretch would hyperpolarize the JG cell membrane, lower cytosolic calcium and increase renin secretion (128). Hyperpolarization could lower cytosolic calcium by decreasing the calcium permeability of the JG cell

membrane (125) and/or by increasing the activity of the Na^+/Ca^{2+} exchanger (125). In support of this, it has been demonstrated that increasing the degree of stretch on epithelial cells (430), red blood cells (228) and stretch receptors (95,178) leads to an increase in membrane permeability to calcium through activation of voltage-sensitive calcium channels. The proposed mechanism for signal transduction is further supported by the finding that renin secretion is inhibited by depolarizing the JG cell with high potassium (112), in the presence but not in the absence of calcium (65,118,293,296). The inhibition can be blocked by the addition of verapamil (118) and D600 (65), two calcium channel antagonists. Moreover, increasing renal perfusion pressure inhibits renin secretion by depolarizing the JG cell membrane and increasing cytosolic calcium levels (124). Finally, Burhle et al. (54) have recently shown that agents known to inhibit renin secretion do so through JG cell depolarization.

Additional confirmation of the hypothesis that changes in membrane potential alter renin secretion is supplied by the observed effects of agonists and antagonists on membrane potential and renin secretion. It has been demonstrated that beta adrenergic agonists (112) and glucagon (120), which both stimulate renin secretion, do so by hyperpolarizing the JG cell membrane. On the other hand, recent evidence by Burhle et al. (54) suggests

that epinephrine and norepinephrine depolarize renin positive cells. There are three possible explanations for this discrepancy. First, the mechanism by which these agents stimulate renin secretion could be through the activation of adenylate cyclase, since cAMP has been suggested to stimulate renin secretion through a mechanism which is independent of the calcium pathway (127,295). A second possibility is that exogenous and endogenous norepinephrine have different effects on renin secretion (367). A third possibility is that at concentrations used by Buhrle et al. (10^{-5} M), epinephrine and norepinephrine depolarize the JG cells by interacting with alpha-adrenergic receptors. On the other hand, angiotensin and vasopressin have been shown to depolarize JG cell membranes and inhibit renin secretion (54). Depolarization is believed to inhibit renin secretion by raising cytosolic calcium, presumable through activation of voltage sensitive calcium channels (125). If this were the case, the magnitude of inhibition would be dependent on the calcium concentration of the extracellular medium, with the greatest inhibition occurring when extracellular calcium concentrations are elevated (410). The effect of angiotensin can be blocked by addition of verapamil, a calcium channel blocker (296). Evidence for the role of calcium in renin secretion under conditions of varying stretch is provided by Fray et al. (124). Using the isolated perfused kidney, they demonstrated that an

increase in wall tension (ie. increased perfusion pressure), which under normal conditions is associated with a decrease in renin secretion, can stimulate renin secretion upon the removal of calcium or by addition of verapamil to the perfusate (18). Therefore, the data indicate that calcium couples renal perfusion pressure to renin secretion under conditions of varying stretch. Similar results are obtained when systemic blood pressure is raised above 125 mm Hg in the presence of EDTA or verapamil (2). These effects seem to be exerted on the JG cells themselves, because a similar response (ie. calcium-dependent inhibition of renin secretion) is also observed in isolated cells (122,128,223). Also, calcium inhibits renin secretion in the nonfiltering kidney (415) and in isolated glomeruli (22). Despite the abundance of indirect evidence pointing to an inverse relationship between renin secretion and cytosolic calcium, direct measurements have not been provided. This thesis aims to fill the gap by demonstrating the relationship between renin secretion and cytosolic calcium.

2. Macula Densa Pathway

There is considerable evidence that the macula densa plays an important role in the control of renin secretion. The macula densa is that region of the early distal tubule which is juxtaposed to the renal afferent and efferent

arterioles. The location of the macula densa cells, their unique structural features (163,314) and their close apposition to the renin secreting JG cells led to the initial hypothesis by Goormaghtigh (146,147), that macula densa cells are involved in the regulation of glomerular filtration rate (GFR). Specifically, it was proposed that these cells monitor the ionic composition of the tubular fluid and elicit (by some undefined pathway) alterations in renin secretion which, in turn cause local changes in renal blood flow to the glomerulus. Histochemical studies revealed a positive relationship between glucose-6-phosphate dehydrogenase activity in the macula densa and renin secretion (or JG cell granulation) under a number of experimental conditions (59,60,111,145). In addition, studies by Barajas (19) demonstrated that decreased sodium load and tubular volume resulted in decreased contact between the macula densa and JG cells that was correlated with increased renin secretion. Thurau and associates also demonstrated, with retrograde perfusion, that renin secretion was influenced by the amount of sodium in the perfusate (388,389,390,391,392,393,394,395). However, their results were opposite to those of Barajas; that is, they provided evidence that an increased sodium load at the macula densa increases renin secretion. Schnermann et al. (338) likewise found a direct relationship between sodium and renin secretion. They showed that an increase in the

perfusion rate of isotonic Ringer solution decreased single nephron GFR, presumably through an increase in renin secretion. Chloride seems to play some role in this process since sodium sulfate and mannitol were without effect. From these data, Schnermann et al. (338) concluded that sodium passing through the distal tubule enters the cells of the macula densa and initiates a sequence of events which leads to increased renin secretion and decreased GFR, due to the local generation of angiotensin.

It was not until the work by Vander and Miller in 1964 (405) that a clear understanding of the role of the macula densa was formulated. They reported that the increase in renin secretion caused by aortic constriction could be blocked by infusion of either osmotic diuretics (mannitol and urea), or direct diuretics such as chlorothiazide and acetazolamide. Since these agents increase sodium delivery to the macula densa, it is concluded that the normal stimulus for renin secretion is a decrease in sodium delivery to the distal nephron. It was also demonstrated that the sodium load at the macula densa decreased renin secretion in association with an increased filtered load (102,233,399). From these studies, Vander (402) proposed that the macula densa is an ion sensor capable of detecting the sodium (and/or chloride) composition of the tubular fluid. The information is then relayed to the JG cell, with a

resulting change in renin secretion. Others have reported increased plasma renin activity following administration of furosemide or ethacrynic acid (80,264) which inhibit sodium reabsorption in the thick ascending limb of the loop of Henle (342,365) and thereby increase the sodium load to the macula densa. Nash (75) proposed that the sodium signal is mediated by a decreased sodium transport, which under normal conditions is the consequence of a decrease in sodium delivery. Studies by Vander et al. (404) are in agreement with Nash's hypothesis, and the authors use it to explain the conflicting results obtained with furosemide. Vander et al. (404) proposed that furosemide has a direct inhibitory effect on the cells of the macula densa which results in decreased sodium transport and increased renin secretion. Other considerations for the role of the macula densa as an ion sensor are less definitive. For example, if the flow rate of tubular fluid through the nephron is reduced, the amount of sodium reabsorbed by the proximal tubule and ascending lirb of the loop of Henle should increase. Therefore, the amount of sodium reaching and being transported by the macula densa will decrease, causing an increase in renin secretion (405). However, in retrospect, the decrease in the tubular flow rate was accomplished by renal arterial hypotension, and under these conditions renin secretion could have been increased through activation of the stretch receptor. On the other

hand, diuretics which increase urinary flow rates should also increase sodium delivery and sodium transport by the cells of the macula densa (405), and thereby inhibit renin secretion. However this does not hold true for all diuretics. Both furosemide and ethacrynic acid increase urinary flow rates while also stimulating renin secretion. As mentioned above, these diuretics have a direct effect on renin secretion which is not associated with their diuretic activity. It is clear that furosemide and ethacrynic acid decrease ion transport at the macula densa (336). Therefore, in the presence of these diuretics, the macula densa sees a low tubular sodium concentration, to which it responds by stimulating renin secretion.

Other evidence for the role of the macula densa came from the work by Shade et al. (344). They compared the effects of infusing hypertonic sodium chloride into anesthetized thoracic caval-constricted dogs with either a single filtering or nonfiltering kidney. Shade et al. (344) demonstrated a decrease in renin secretion in response to hypertonic sodium chloride infusion from the filtering but not from the nonfiltering kidney. Therefore, the inhibition of renin secretion from the filtering kidney must be the result of an inhibitory signal produced by the macula densa, and not from a direct action of sodium chloride on the JG cells. Churchill et al. (70) also established an inverse relationship between renin secretion and the distal tubular sodium load in

anesthetized rats. They demonstrated that the sodium load to the early distal tubule, which was responsible for the macula densa-mediated changes in renin secretion, was directly related to dietary sodium. Thus, the rate of renin secretion was inversely related to dietary sodium. In support of their earlier finding, Churchill et al. (71) also demonstrated that both saline and mannitol infusion decreased renin secretion. The inhibitory effects of these agents correlate well with an increase in sodium delivery to the early distal tubule.

A number of investigators have tried to test the macula densa hypothesis by using renal cortical slices exposed to various sodium chloride incubation media. The results, however, are conflicting. Weinberger and coworkers (11,421) found no changes in renin secretion related to changes in sodium concentration of the incubation medium, when maintaining iso-osmotic conditions. Other investigators have reported a 3- to 4-fold stimulation in renin secretion on increasing the sodium concentration (from 50 to 160 meq/L) when the osmolality was allowed to vary (282). However, if the osmolality was kept constant, renin secretion increased only 1.5-fold over control levels. On the other hand, Lyons and Churchill (247,248) observed an 8- to 10-fold increase in renin secretion associated with a 50 meq/l increase in sodium. Ouabain decreased the response by 54%. However, if a cortical cell suspension was used, an

inverse relationship, unaffected by ouabain, was observed between sodium and renin secretion. The latter results are in agreement with those of Michelakis (265) and Saruta and Matsuke (332), who also observed an inverse relationship between renin secretion and the sodium concentration of the incubation media, whether or not the osmolality was allowed to increase. In taking the opposite approach, that is decreasing the sodium concentration of the incubation medium, Capponi and Vallotton (58) observed a decrease in renin secretion. In addition, furosemide was ineffective for stimulating renin secretion in their preparation (58). These contradictory results observed in vitro could be due to the dissociation of the cells of the macula densa from the juxtaglomerular and/or Goormaghtigh cells (ie. those cells which are located between the macula densa and JG cells). In trying to answer this question, Itoh et al. (185) developed a microdissection technique in which they were able to isolate afferent arterioles either alone or with the macula densa attached (afferent arteriole-macula densa). In these preparations, basal renin release was greater in the afferent arterioles devoid of macula densa (185). One can speculate that the lower basal release in the afferent arteriole-macula densa preparation was due to the high sodium chloride concentration of the incubation medium, which would quickly equilibrate with the cells of the macula densa. Also, addition of furosemide caused a 390%

increase in renin secretion from the afferent arteriole-macula densa, while having no effect on the afferent arteriole. However, these results are not without contradictions, since lowering the sodium chloride concentration of the incubation medium reduced renin secretion from the afferent arteriole but had no effect on the afferent arteriole-macula densa preparation. These results are not consistent with the macula densa hypothesis proposed by Vander (402,404). It is tempting, however, in terms of Vander's hypothesis, to speculate that furosemide's stimulatory effect on renin secretion from the afferent arteriole-macula densa preparation was the result of a decrease in sodium and/or chloride transport by cells of the macula densa (404). While the reason for the disparities in results using identical systems remains unclear, the inconsistencies observed between different in vitro systems could be related to the degree to which the structural integrity between the JG cells and the macula densa is maintained during preparation.

From the data gathered, it was generally accepted that the ion sensed by cells of the macula densa was sodium, and that changes in tubular sodium signal changes in renin secretion. However, chloride may also be important. In 1976 Kotchen et al. (220) presented evidence that chloride was more important than sodium for modulating renin secretion. This was demonstrated by

differential treatment of groups of rats maintained on a low sodium diet (which increased plasma renin activity). Each group received a drinking solution designed to produce either a negative or positive chloride balance. In rats receiving either distilled water or sodium bicarbonate, (negative chloride balance), plasma renin activity remained elevated. However, in rats given isotonic sodium chloride, (positive chloride balance), plasma renin activity decreased. Similar results were obtained in rats given either potassium bicarbonate or potassium chloride, which left the animals in negative or positive chloride balance, respectively (220). In both series of experiments all animals were in positive sodium balance. This raises the possibility that the regulation of renin secretion may correlate with the delivery and possible transport of chloride by the cells of the macula densa. Additional evidence for the role of chloride comes from a subsequent study by Kotchen (221), in which sodium chloride depleted rats were given supplements of various sodium and choline salts. Of all the salts tested, only sodium chloride, sodium bromide and choline chloride were effective in lowering plasma renin activity, despite the fact that all choline salt-treated animals were in negative sodium balance. In addition, in studies in which animals were selectively chloride depleted by peritoneal dialysis (1) in which chloride was replaced by sodium bicarbonate or sodium nitrate, plasma renin activity

increased. In such studies, although plasma volume increased, the increase in renin secretion could not be attributed to alterations in pressure, glomerular filtration rate or plasma potassium concentration. On the other hand, intrarenal infusion of sodium lactate or potassium lactate in dogs reportedly led to a decrease in renin secretion (362). Under these conditions, sodium excretion was elevated. Chloride excretion, however, decreased during sodium lactate infusion and increased during potassium lactate infusion. Also, Blendstrup et al. (27) demonstrated with superfused glomeruli that increasing the sodium concentration of the superfusate from 100 to 135 meq/l, without altering the osmolality, increased renin secretion by 2.5-fold compared to controls. However, if the osmolality of the superfusate was allowed to increase, the renin secretory response was reversed. It was concluded that neither sodium nor chloride had any direct ionic effect on renin secretion, and the stimulation in renin secretion was attributed to increased water movement driven by the osmotic effects of these ions. Therefore, Blendstrup et al. (27) proposed that the rate(of(renin secretion was related to the volume of the JG cells. The possibility that the macula densa acts as an osmometer is supported by a number of investigators (27,129,221). In particular, it was demonstrated by Kirk et al. (221) that changes in osmotic forces within the renal tubules can modify the

interstitial space between the macula densa and the glomerulus. Therefore, it is possible that one mechanism by which the macula densa transmits its signal to the JG cells is an osmotic or tissue pressure gradient. In contrast to the findings by Blendstrup et al. (27), Holdworth and co-workers (172), using a similar glomerular preparation, reported transient increases in renin secretion caused by decreasing the sodium concentration of the superfusate by 30 meq/l without altering osmolality. On the other hand, there is also support for the involvement of both sodium and chloride in regulating the activity of the macula densa and renin secretion (100).

There are several lines of evidence for the notion that ion transport by the cells of the macula densa is important for the signal transduction process. First, Galla et al. (131), using a micropuncture technique, demonstrated that the inability of sodium bicarbonate infusion to inhibit renin secretion was associated with a decrease in absolute chloride absorption in the loop of Henle. Second, patients with Bartter's syndrome, in which the chloride transport capability of the loop of Henle is impaired, have elevated plasma renin activity (18). Third, Welch et al. (419) when comparing the plasma renin concentration of adrenalectomized rats, dexamethasone-treated adrenalectomized rats, and sham operated rats (controls) found that the adrenalectomized animals had the highest plasma renin activity. They also

observed decreased chloride absorption in the loop of Henle of adrenalectomized rats both before and after saline infusion. Therefore, increasing sodium chloride delivery to this region of the nephron had no effect on chloride transport. However, saline infusion into both control and dexamethasone treated animals resulted in significant decreases in plasma renin activity which were unrelated to changes in arterial pressure or glomerular filtration rates. It is worth noting that there were no differences in plasma renin activity between control and dexamethasone treated animals either before or after saline infusion. Therefore, it appears that the stimulation of renin secretion in adrenalectomized rats is related to impaired transport of chloride in the loop of Henle. Also, inverse relationships between chloride transport and renin secretion were observed in control and dexamethasone treated animals. Therefore, one can conclude that dexamethasone restores the transport ability of the loop of Henle, along with the renin secretory response to sodium chloride. The high plasma renin activity in the adrenalectomized animals could have resulted from JG cell hyperplasia. These studies, however, did not address the question of which ion (sodium or chloride) is more dominant in controlling renin secretion. Although the data on ion effects are contradictory, one consistent finding is the importance of a functional ion transport system in the macula densa.

That is, if the cells of the macula densa have a functional transport system, an inverse relationship between sodium chloride load and renin secretion is observed. The relationship is lost if the ion transport system is rendered nonfunctional.

Although the described findings support involvement of the macula densa in regulating renin secretion, there is also evidence against such a mechanism. Park and Malvin (294), using ouabain to block the Na^+/K^+ pump and thereby raise intracellular sodium, observed inhibition of renin secretion only in the presence of calcium. If calcium was removed from the incubation medium, the inhibitory effect of ouabain was abolished. These results suggest that the intracellular sodium concentration may not be an important factor in the signal transduction process. However, it is possible that transduction of the signal from the macula densa to the JG cells involves changes in the cytosolic calcium levels of the cells comprising the macula densa. Therefore, removing calcium from the incubation medium blocks the signal transduction process. Additional studies by Park (128) and others (36,355) indicate dissociations between sodium excretion and renin secretion. Park found that ouabain infusion into the renal arteries of dogs led to a temporal lag of one hour between the inhibition of renin secretion and the increase in sodium excretion.

Despite considerable evidence for macula densa

involvement in the regulation of renin secretion, very little is known about how the signal is transduced and transferred to the JG cells. Underlying the macula densa is a tightly packed layer of cells known as extraglomerular mesangium, Goormaghtigh cells or lacis cells. It has been suggested that these cells are involved in transmitting the signal to the JG cells (50), and indeed these cells are tightly linked by gap junctions to each other and to at least some of the JG and smooth muscle cells of the afferent arteriolar wall (50). A number of transduction signals have been proposed including adenosine (186,290,357), osmotic forces (176,221,335), changes in JG cell membrane potential (24), changes in the calcium concentration of the cells of the macula densa (23,24) or prostaglandins (115,165,337). Adenosine was first linked with the macula densa pathway in 1964 by Thurau (384). Since then, other investigators have provided further support for its role. It has been proposed that increased sodium chloride delivery and transport by the cells of the macula densa results in increased adenosine production and liberation (357) and that adenosine acts at the level of the JG cell to inhibit renin secretion. It is postulated that adenosine is released at the basolateral side of the macula densa in close proximity to the JG cells (357). Support for this hypothesis comes from the observation that saline infusion increases renal adenosine (290). In addition, exogenous

adenosine and adenosine agonists have been shown to inhibit renin secretion. However, these data do not explain the mechanism of action of adenosine. Churchill et al. (73) and Skott (349) proposed that adenosine inhibits renin secretion by increasing cytosolic calcium. Their hypothesis is supported by the observation that in the presence of EGTA, adenosine is ineffective for inhibiting renin secretion (73,349). However, the mechanism by which intracellular calcium is increased remains unclear, since verapamil and D600 did not block adenosine inhibition of renin secretion. On the other hand, others report that adenosine stimulates renin secretion by activating adenylate cyclase (159).

Other possible mediators of the macula densa pathway are prostaglandins. Since concepts of prostaglandin influences on renin secretion, in terms of the macula densa mechanism are controversial, examination of possible roles played by individual prostaglandins is not productive. However, a general discussion of prostaglandins and the macula densa pathway follows: It has been observed that sodium deprived animals have high rates of renin secretion and prostaglandin production (417,441) that can be blocked with indomethacin (52,369). In agreement with these findings, Linas (235) demonstrated in the isolated perfused kidney that the increase in renin secretion associated with the removal of chloride from the perfusion medium was accomplished by accelerated

prostaglandin production. Both increases were blocked by indomethacin. The most convincing evidence for role of prostaglandins as mediators of the macula densa mechanism comes from a study by Gerber et al. (137) who functionally isolated the macula densa from the other intrarenal mechanisms that regulate renin secretion. In kidneys pretreated with propranolol and papaverine, Gerber et al. (137) found that indomethacin blocked the increase in renin secretion in response to renal hypotension. While these studies support a role of prostaglandins in the macula densa pathway, other studies show no such relationship. Zehr et al. (436) demonstrated that increased renin secretion associated with furosemide infusion was not associated with changes in prostaglandin production. Also, when renin secretion was increased by lowering extracellular calcium, prostaglandin synthesis was inhibited, whereas sodium excretion was unaffected (235). The addition of indomethacin further lowered prostaglandin synthesis without affecting sodium excretion. Finally, Vikse and Kiil (413) also found no association between increased sodium excretion, which inhibited renin secretion, and prostaglandin production. From their experiments, Vikse and Kiil (413) concluded that stimulation of renin secretion by the macula densa pathway is independent of prostaglandin production.

Although the precise mechanism remains unclear, a signal that is transferred from the macula densa to the JG

cell does regulate renin secretion. Ultimately, the change in renin secretion is probably related to changes in cytosolic calcium and/or cyclic AMP levels, since these are two intracellular messengers known to control renin secretion.

3. Neurohormonal Pathway

The third intrarenal mechanism involved in regulation of renin secretion is neurohormonal. In 1952 De Muylder (87) identified nerves traveling from afferent renal arterioles to the region of the JG cells. A decade later Barajas (20) documented the presence of nonmyelinated nerve fibers containing dense-core vesicles, indicative of noradrenergic nerves (427) associated with the afferent and efferent arterioles. These findings were confirmed by other investigators who reported that the JG cells of many species are innervated by the sympathetic nervous system. Wagermark et al. (414), using a fluorohistochemical procedure for biogenic amines and specific stains for the JG apparatus, demonstrated the presence of sympathetic nerve terminals containing norepinephrine near the JG cells of the afferent arteriole. These morphological data support a possible functional relationship between the sympathetic nervous system and renin secretion. Such a relationship was first demonstrated by Vander (403), who showed that sympathetic nerve stimulation increased renin

secretion in anesthetized animals. In addition, Assaykeen and Ganong (17) demonstrated that this increased secretion could be blocked with propranolol, a beta adrenergic antagonist. However, one cannot conclude that this secretory response results from a direct action of the neurotransmitter on the JG cell. A second possibility is that the increase in renin secretion is caused by hemodynamic changes which activate one of the other pathways discussed above. It has been demonstrated that renal nerve stimulation also decreases glomerular filtration rate, renal blood flow, and sodium excretion, all of which could increase renin secretion. Johnson and associates (193) were able to isolate the neurohormonal from the stretch receptor and macula densa pathways by using non-filtering kidneys in conjunction with papaverine infusion. Under these conditions, renal nerve stimulation produced a 3-fold increase in renin secretion which could be blocked by propranolol. Also, Taher et al. (371), Ammons et al. (10) and Thames and DiBona (383) demonstrated that low level stimulation of the renal nerve could increase renin secretion without altering renal blood flow, glomerular filtration rate or sodium excretion. The increased secretion was blocked by l-propranolol but not by d-propranolol (which has only 1% of the beta-adrenergic blocking activity) (371). Since there was no hemodynamic response in these experiments, the increase in renin secretion cannot be attributed to

activation of either the macula densa or the stretch receptor. These results strongly suggest that JG cells contain beta-adrenergic receptors, and that norepinephrine released upon renal nerve stimulation binds to the receptors and stimulates renin secretion.

While the evidence is convincing for renal nerve involvement in direct stimulation of renin secretion, the renal nerve has also been implicated as a modulator of renin secretion. Thames and DiBona (383) observed that low frequency renal nerve stimulation, which has no direct effect on renin secretion or renal hemodynamics, increased the renin secretory response to both aortic constriction and furosemide administration. Aortic constriction, in the absence of renal nerve stimulation, has been shown to decrease renal perfusion pressure, renal blood flow and sodium excretion (383). Therefore the increase in renin secretion observed under these conditions must result from activation of the macula densa and/or the stretch receptor. The possibility that this secretory response was due to an increase in renal nerve activity seems unlikely, since aortic constriction increases brachial artery pressure and decreases renal sympathetic nerve activity (207,277). Also, the renin response to aortic constriction is not affected by propranolol. However, if low level renal nerve stimulation is applied in conjunction with aortic constriction, renin secretion increases 20-fold over control and 3-fold over that seen

with aortic constriction alone. Similar results are observed with furosemide, which stimulates renin secretion through the macula densa pathway (383). Low frequency renal nerve stimulation in conjunction with furosemide infusion increases renin secretion to a greater extent than that which can be achieved by furosemide infusion alone. These findings are consistent with observations by several investigators who found that renin secretion was greater in response to furosemide or aortic constriction in an innervated than in a denervated kidney (150,360,361). This variance suggests that basal renal nerve activity may be sufficient to augment the renin secretory response to such stimuli. Thus, although the renal nerve is not required to stimulate renin secretion, it does enhance the secretory response to non-neural stimuli (361,383).

Similar results were obtained by infusing epinephrine, a beta-2 adrenergic stimulant (183,231,420), either intravenously or into the renal artery, at a dose to low to affect plasma renin activity. That is, the renin secretory response to a reduction in renal perfusion pressure or sodium deprivation was greater in animals receiving epinephrine (105). However, in the sodium deprived animal, the enhancement of renin secetion could be due to either an increase in renal nerve activity (92) or an increase in the responsiveness of the JG cells to sodium deprivation (121). Kopp and DiBona (216,217)

demonstrated that lowering perfusion pressure increased renin secretion only in the innervated kidney, and that epinephrine infusion into the denervated kidney did not restore the responsiveness of the JG cells to renal hypotension. The lack of effect of epinephrine in the denervated kidney suggests that the concentration infused was not sufficient to activate the beta-adrenergic receptors on the JG cells. However, if the same concentration of epinephrine was infused into the innervated kidney, one sees enhancement of the renin secretory response to low perfusion pressure (216,217). When metoprolol or ICI 118551, beta-1 and beta-2 adrenergic antagonists respectively, were infused along with epinephrine, the enhancement in renal perfusion pressure was not observed. This indicates a direct involvement of the JG cells and their sympathetic innervation in modulating renin secretion to non-neural stimuli (21,86). These results are in agreement with those of Osborn et al. (288) who demonstrated that both basal renin release and the stimulation of renin secretion in response to non-neural stimuli, decreased upon renal denervation. Although these results implicate the renal nerve as a modulator of renin secretion, the possibility that the decrease in renin secretion in the denervated kidney resulted from decreased renal nerve activity, and vice versa in the innervated kidney, has not been eliminated. However, there are studies in which the

responses to other beta-1 agonists was unaffected by renal denervation (166,215). In addition, there are reports that renal denervation of a nonfiltering kidney does not affect the renin secretory response to renal hypotension (289). These studies indicate that under certain conditions, renin secretion is not affected by the basal activity of the renal nerve. Yet, there are studies which indicate that the beta-adrenergic pathway stimulates renin secretion through activation of extrarenal beta-receptors (195,195). In general, there is compelling evidence for the hypothesis that renal nerves are not required to stimulate renin secretion but do enhance secretory responses to non-neural stimuli.

While the importance of the beta adrenergic receptor in the control of renin secretion is well established (10,193,316,321,371,383), the subtype involved seems to vary among species. Since current available data are inconclusive, only the specific effects of agonists and antagonists will be addressed. In 1967 Lands et al. (225) identified two different types of receptors: the beta-1 receptor, which mediates the cardiac response, and the beta-2 receptor which mediates the peripheral vascular response. In many experiments, isoproterenol, a nonspecific beta-adrenergic agonist, was used in conjunction with specific beta-1- or beta-2-antagonists. In rat renal cortical slices and in conscious dogs, atenolol, a beta-1 specific antagonist, prevented the

isoproterenol stimulatory response (69,170,281). These results are consistent with those obtained when low level renal nerve stimulation was used to stimulate renin secretion in the absence of isoproterenol (215,289). Under these conditions, metoprolol (215) or atenolol (287), two beta-1 adrenergic antagonists, or dl-propranolol (215) severely blunted the renin secretory response. On the other hand, neither d-propranolol (215) nor butoxamine, (a beta-2 antagonist (287)) had any effect. However, the work by Olson et al.(283) suggests that beta adrenergic stimulation of renin secretion is not confined to either the beta-1 or the beta-2 receptor subtype. Their work demonstrated that isoproterenol-stimulated renin secretion could be inhibited by infusion of sotolol, a nonspecific beta adrenergic antagonist; atenolol, a beta-1 specific antagonist; or ISP-339, a beta-2 specific antagonist. However, it is worth noting that while renin secretion was blunted in response to these specific antagonists, isoproterenol still caused a significant increase in renin secretion. In addition, albuterol, a beta-2 specific agonist stimulated renin secretion at either high or low concentrations in the presence or absence of atenolol. These results support an earlier study which demonstrated that ISP-339, a beta-2 antagonist, inhibited isoproterenol stimulated renin secretion by about 75%, indicating the involvement of beta-2 receptors (182). On the other hand,

in unanesthetized rabbits, beta-1 blockade with mithoprolol or practolol only partially blocked the renin secretory response to isoproterenol (316), whereas beta-2 blockade with oxyprenolol or pindolol resulted in a more sucessful inhibition (418). In the cat, the ability of a nonspecific beta adrenergic antagonist to cause greater inhibition of renin secretion than the beta-1 specific antagonist led to the conclusion that beta-2 receptors are more important in mediating renin secretion (191). However, the effect of beta-2 specific antagonists was never investigated. In vitro findings on cat renal cortical cells support this interpretation (190). In contrast, Johns (189) found in anesthetized cats that atenolol was a more effective inhibitor of renin secretion in response to renal nerve stimulation that erythro-DL-(7-methylindam-4-yloxy)-3 -isopropylamine, a beta-2 antagonist. At best, high concentrations of beta-2 antagonist inhibited renin secretion by only 40%. In humans, the beta adrenergic subtype involved in either basal (3,134) or adrenergically stimulated renin secretion (103,84) seems to be of the beta-1 subtype.

Studies of receptor subtypes have also been performed on isolated perfused kidneys. These in vitro systems eliminate compensatory mechanisms which may influence renin secretion. However, the same contradictions concerning receptor types involved in the beta-adrenergic stimulation of renin secretion arise. One study

demonstrated that the beta adrenergic response is nondiscriminatory, involving both the beta-1 and beta-2 subtypes (274). In this study, isoproterenol-stimulated renin secretion was suppressed by propranolol, and by acebutolol and M&B 16942, two beta-1 specific antagonists. Also, salbutamol, a beta-2 agonist, stimulated renin secretion 3.5-fold over control values. In other studies (47), greater reduction in isoporoterenol-stimulated renin secretion was observed in response to ISP-339 and H35/35, two beta-2 antagonists than to metoprolol or atenolol, two beta-1 antagonists. A study investigating antagonist binding demonstrated that the isoproterenol dose-response curve for renin secretion was shifted to the right by addition of pindolol or LK203-030 (267). It was concluded that the shift resulted from a competitive interaction between agonist and antagonist for the beta-1 receptor subtype (267). In support, another study using renal cortical slices demonstrated that isoproterenol-stimulated release was severely reduced by timolol or atenolol, nonselective and beta-1 selective antagonists respectively (72).

In trying to correlate the beta adrenergic data to receptor subtypes found in the kidney, one also finds inconsistencies. Using radioligand binding and autoradiography on a glomerular preparation, Summers and McPherson (366) and Engle et al. (99) found only the beta-1 subtype. However, Insel (184) was able to

demonstrate the presence of both receptor subtypes in the rat renal cortex, while Brodde (51) found only beta-1 in the whole kidney.

It is possible that the inconsistencies relate to species variations, to sympathomimetic activity (101), or to the membrane stabilizing properties of some antagonists (331). If either of the latter two possibilities is correct, it will be difficult to interpret the results in which large doses of beta adrenergic antagonists were used. Another possible explanation for the discrepancies might relate to the experimental model (in vitro or in vivo) and/or the possible activation of other renin regulatory pathways, such as those discussed above.

With the present knowledge of beta-adrenergic influences on renin secretion, it is reasonable to conclude that catecholamines released via renal nerve stimulation have two functions: first, they increase renin secretion by interacting directly with beta-adrenergic receptors; and second, they modulate the renin secretory response to nonneural stimuli. However, the role of alpha-adrenergic receptors is less certain. Due to the conflicting findings on the role of alpha-adrenergic receptors in the regulation of renin secretion, only the specific effects of alpha-adrenergic agonists and antagonists will be discussed. Alpha-adrenergic activation can explain the stimulation of renin secretion by epinephrine. That is, epinephrine, at concentrations

above 10^{-8}M, along with other alpha-adrenergic agonists causes vasoconstriction of the afferent arterioles which activates the stretch receptor (316) and/or macula densa (193). Macula densa activation results from a decreased sodium chloride load to the distal tubule (26,139,435), attributed to decreased glomerular filtration rate and increased sodium chloride reabsorption by the proximal tubular cells. The stimulatory effect of alpha adrenergic activation can explain why dibenamine and phentolamine, two alpha adrenergic antagonists, decrease the renin secretory response to hemorrhage and renal nerve stimulation (31,81).

In contrast, there are studies in which alpha adrenergic agonists decreased renin secretion (88,196,239,257). Phenoxybenzamine and phentolamine, two alpha-adrenergic antagonists, have been shown to potentiate the norepinephrine-invoked increases in renin secretion (279). In agreement with these findings, the dose-dependent inhibition of renin secretion caused by epinephrine and norepinephrine at concentrations of 10^{-8}M or greater (58,88,133,270,271,367) can be converted to dose dependent stimulation in the presence of prazosin (270,367) or phenoxybenzamine (88). In addition, other investigators demonstrated that the inhibitory effect of norepinephrine or epinephrine on renin secretion could be converted to a stimulatory one by the addition of

phenoxybenzamine (40) or phentolamine (58,271). These results imply that both epinephrine and norepinephrine, at concentratons of 10^{-6}M or greater, inhibit renin secretion via the the alpha adrenergic pathway. The dual effects of norepinephrine on renin secretion can be explained in terms of whether it is exogenously or endogenously supplied. Sasaki et al. (367) using renal cortical slices, demonstrated that the norepinephrine-induced inhibition of renin secretion could be converted to a stimulation by addition of prazosin, whereas norepinephrine released neuronally, by veratrine, stimulated renin secretion and could be inhibited by propranolol. On the other hand, Khayat et al. (209), using a JG cell suspension, demonstrated that 10^{-5}M norepinephrine gave the same stimulatory response in the presence or absence of phenoxybenzamine. Weinberger et al. (420) demonstrated that 1.5×10^{-9}M epinephrine or norepinephrine stimulated renin secretion and the effects were not potentiated by phentolamine or methoxamine. The data suggest that norepinephrine and epinephrine have greater affinities for beta-receptors. Therefore, at low concentrations, norepinephrine and epinephrine preferentially bind to the beta-adrenergic receptor and stimulate renin secretion. At high concentrations (10^{-6}M or above) these agents also bind to alpha-receptors and thereby suppress the stimulatory response. One can conclude that the

inhibitory effect of alpha-adrenergic activation, under these conditions, dominates over stimulatory effect of beta-adrenergic activation.

In isolated perfused kidneys containing functional macula densa and stretch receptor pathways, alpha adrenergic stimulation with phenylephrine has been shown to prevent the increased renin secretory response to isoproterenol (363,412). If, however, phenoxybenzamine is infused along with phenylephrine, the response to isoproterenol returns (412,363). It was reported that phenylephrine's inhibitory action resulted from increased vascular resistance. However, if vasoconstriction is blocked by dihydralazine, the inhibitory action of phenylephrine remains. One can therefore, conclude that the phenylephrine effect on renin secretion may result from activation of nonvascular alpha receptors. However, tubular function was not monitored in these studies and the possibility remains that phenylephrine is influencing sodium transport. Also, it was observed that while the infusion of low concentrations of norepinephrine stimulated renin secretion, the stimulatory effect of high concentrations could be seen only in the presence of phenoxybenzamine (409). In contrast, Fray (117) observed that methoxamine and phenylephrine increased renin secretion only in the absence of papaverine, suggesting that the stimulatory effect was due to increased vascular resistance. The reasons for these discrepancies are not

apparent. Another alpha-adrenergic agonist, clonidine, was reported to lower basal release (408) and plasma renin activity (302,422) without altering the renin secretory response to isoproterenol (408), suggesting that clonidine may be inhibiting norepinephrine release (30). Most of these results were obtained from in vitro studies, but in vivo studies have yielded the same inconsistencies regarding the role of the alpha adrenergic pathway. In vivo, addition of alpha adrenergic agonists has been shown to have no effect (197,238,319), to stimulate (40,81,278,298,309,330) or to inhibit (278,302,315) renin secretion.

When considering the involvement of the alpha-adrenergic pathway in renin secretion, it is important to note that there are two possible sites of action for alpha-adrenergic agonists and antagonists. The first is the postsynaptic alpha-1 receptor, on the effector cell (30) (ie. the vasculature); the second is the presynaptic alpha-2 receptor (30). Activation of the alpha-1 receptor could result in decreased perfusion pressure (86) with consequent activation of the macula densa and/or stretch receptor. Also, if the agonists are infused intravenously they could affect systemic pressure and thereby increase or decrease renal nerve activity (83,309). On the other hand, alpha-2 receptor blockade could increase the amount of norepinephrine released under basal conditions or during renal nerve stimulation (440).

However, it has been shown that under conditions of low level renal nerve stimulation, in the absence of any changes in hemodynamics or sodium transport, alpha blockade with phentolamine, prazosin, phenoxybenzamine, or methoxamine had no effect on renin secretion (286). However, high concentrations of phenoxybenzamine inhibited release. One explanation is that this antagonist inhibits neurally-mediated renin secretion by a mechanism other than alpha adrenergic blockade. In contrast, high doses of methoxamine increased renin release by about 4-fold. This could result from the activation of the alpha-1 vascular receptor, since renal blood flow and sodium excretion decreased. Such receptor activation could increase renin secretion through the stretch receptor and/or macula densa pathways. Also, a study in hypertensive patients (231) showed that intrarenal infusion of doxazosin, an alpha-1 specific antagonist, or phentolamine caused an increase in renin release. However, during renal nerve stimulation doxazosin had no effect whereas phentolamine enhanced renin secretion. In these studies phentolamine had no effect on renal blood flow, blood pressure or heart rate, suggesting an inhibitory role for the alpha-2 receptor during renal nerve stimulation. In the absence of renal nerve stimulation, both alpha receptor subtypes were involved in inhibiting renin secretion (231). In contrast, Blair (40) demonstrated that after beta adrenergic blockade with

propranolol, phenoxybenzamine decreased plasma renin activity by 50% in response to renal nerve stimulation. This suggests that the renal nerve stimulates renin secretion, in part, through the alpha adrenergic pathway. In agreement with this is a study in which alpha adrenergic blockade with phenoxybenzamine alone had no effect on either renal perfusion pressure or renin secretion. However, following pretreatment with propranolol, alpha blockade decreased both plasma renin activity and the renin secretory rate (37). In addition, intravenous infusion of prazosin decreased plasma renin activity in l-propranolol treated dogs, whereas in denervated kidneys, following propranolol treatment, phenoxybenzamine had no effect on either of the above parameters (37). Also, it was demonstrated that infusion of methoxamine or phenylephrine into denervated kidneys increased renin secretion without changing renal vascular resistance or sodium excretion (38,39). Under these conditions, the increase in renin secretion could be completely blocked by prazosin, but it was unaffected by propranolol. These results suggest that alpha-adrenergic stimulation can increase renin secretion in the absence of any change in vascular resistance. Therefore, it is possible that alpha-adrenergic agonist acts at the level of the JG cell. However, no data exist to support this possibility. Similar results were obtained if the renal nerve was activated by occluding the carotid artery (309).

If phenoxybenzamine is infused into one kidney prior to carotid artery occlusion, basal renin release decreases in conjunction with increased renal blood flow. Upon activation of the carotid baroreflex during phenoxybenzamine infusion, the renin secretory response was inhibited in the ipsilateral kidney, while the contralateral kidney responded normally. The increased renin secretion by the contralateral kidney could be blocked by propranolol (309), which had no effect on the renal hemodynamics. These experiments demonstrate that under certain conditions carotid sinus occlusion produces alpha-adrenergic changes in the kidney which increase renin secretion. Since propranolol failed to alter renal hemodynamics under conditions in which it inhibited renin secretion, it would appear that the propanolol-sensitive process is distal to the alpha adrenergic mediated changes.

What is the signal transduction mechanism of the neurohormonal pathway? In terms of the alpha-adrenergic pathway the data, are conflicting. It is reasonable to conclude that renin secretion is affected by the alpha-adrenergic pathway. However, the mechanism of action depends on the location of the receptors. If the receptors are located on JG cells (for which there is no direct evidence), then alpha adrenergic agonists would have direct effects on renin secretion. Under these circumstances the signal transduction mechanism could

result from changes in cytosolic cAMP and/or calcium. However, if the receptors are located on the renal vasculature, influences on renin secretion would presumably be mediated by alterations in renal hemodynamics. These hemodynamic changes would activate of the macula densa and stretch receptor pathways. The signal transduction mechanisms under these conditions would include those already presented above.

On the other hand, the signal transduction mechanism of the beta adrenergic pathway is better defined. As stated above, beta-adrenergic stimulation has been shown to directly affect renin secretion (10,193,316,321,371,383). It has been demonstrated in other tissues that beta adrenergic receptors are coupled to adenylate cyclase (32,232). Therefore, the binding of beta-adrenergic agonists to their receptors could result in the activation of adenylate cyclase and elevation of cytosolic cAMP levels (138,321,322,411). There is evidence that such a mechanism is involved in the beta-adrenergic regulation of renin secretion (69,116,127,132,279,420). A direct relationship between increases in cytosolic cAMP and renin secretion has been demonstrated using dibutyryl cAMP, a lipophilic analogue of cyclic AMP that freely crosses the plasma membrane. It has been shown in dogs that renal artery perfusion with dibutyryl cAMP (8), but not cAMP (370) increases renin secretion. In addition, both glucagon and forskolin,

which increase renal cAMP levels through different mechanisms (127,295,322,341), also stimulate renin secretion (108,127,295). With respect to beta adrenergic stimulation, epinephrine causes concomitant increases in renin secretion and the cyclic AMP content of incubated tissue (133). These results are in agreement with the ability of theophylline, an inhibitor of phosphodiesterase, to potentiate the increase in renin release in response to norepinephrine in vitro (279).

The mechanism by which cAMP is translated into an increased secretory response is not fully understood. One possibility is that cAMP, through the activation of a calcium pump, lowers cytosolic calcium and thus stimulates renin secretion. Support for this idea comes from Churchill and Churchill (69), who found that experimental manipulations which presumably increase cytosolic calcium levels of the JG cells block the stimulatory effects of both isoproterenol and cyclic AMP. A second possibility is that cAMP decreases cytosolic calcium by inhibiting calcium influx. Such a hypothesis is supported by the work of Kurtz et al. (223) who demonstrated that isoproterenol and forskolin, a receptor-independent activator of adenylate cyclase (341), decreased cytosolic calcium by inhibiting calcium influx into cultured JG cells. A third possibility is that cAMP stimulates renin secretion through a calcium-independent pathway. This hypothesis is supported by the findings of a number of

investigators who demonstrated that catecholamines stimulate renin secretion in the absence of extracellular calcium (126,410) or in the presence of calcium channel blockers (236,273,285,292). Also, epinephrine can still stimulate renin secretion under conditions which increase cytosolic calcium (294). All these findings are consistent with the inability of calcium ionophores to block cAMP stimulation of renin secretion (46). A recent paper by Park et al. (295) demonstrates that cAMP stimulates renin release in a high potassium medium in the presence or absence of calmodulin antagonists. Therefore, cAMP can stimulate renin secretion regardless of whether the calcium-dependent renin secretory pathway is maximally activated, as would be expected in the presence of calmodulin antagonists; or inhibited, as would be expected in the presence of high calcium. With the above contridictory data, it is difficult to conclude whether calcium is the final signal whereby the neurohormonal pathway controls renin secretion. Thus far little is known regarding the mechanisms by which neurohormonal effectors modulate cytosolic calcium. A minimum expectation is that these effectors may shift the calcium-renin secretion curve to the right or left. This thesis investigates the basic curve and shows how these effectors alter the shape of curve.

4. The Renin Granule

Although the mechanisms controlling renin secretion at the organ and cellular levels have been intensively studied, very little work has focused on the process that regulates renin release from isolated renin-containing granules. For the work that has been done, most findings are inconclusive and/or contradictory. With the use of electron microscopy, many investigators have demonstrated that renin is stored in JG cells granules (57,375,380,381). In addition, examination of purified preparations illustrates that renin is packaged in small membrane bound organelles (0.4-1.7 uM in diameter) (381,205) that can be disrupted by treatment with triton X-100 (104,205,239).

The stages of development of the renin granule have been investigated by Taugner and Metz (380). These authors demonstrated that mature renin granules are formed by the fusion of rhomboid protogranules, followed by coalescence of their paracrystalline contents. Protogranules with a paracrystalline matrix predominate in animals in which the renin synthetic process is turned on. Also, through the use of immunocytochemical studies, it was demonstrated that juvenile secretory granules are the sites of conversion and activation of pro-renin to renin. Therefore, in addition to pro-renin, other enzymes involved in post-translational modification of the

pro-renin molecule must be present in immature granules. In most cell types, the packaging and condensing of the secretory product takes place in the most trans cisternae of the Golgi or in specialized Golgi-dependent condensing vacuoles. Regardless of the developmental pathway, the end result is the formation of secretory granules that release their contents by exocytosis upon stimulation (106). Taugner and Metz (380) suggest that two types of immature granules, one with a homogenous matrix and the other with a paracrystalline matrix, represent two separate, and perhaps overlapping pathways for renin granule formation. The paracrystalline protogranules predominate in JG cells that have been stimulated for long periods of time. For example, in patients with Bartter's syndrome or in animals that have been adrenalectomized or chronically treated with captopril, paracrystalline structures are observed in the rough endoplasmic reticula (158,201). During granule formation, a renin positive matrix is observed in the earliest of the rhomboid protogranules. This finding is in agreement with those of Lacasse et al. (224) who noted a substantial increase in renin activity between the Golgi cisternae and the protogranules during granular formation. Also, as the granules mature they become round, and the paracrystalline matrix, if present, disappears. The enzymes and processes which lead to these morphological changes are unknown.

In the past few years, the most impressive work

characterizing the content of renin granules has been done by Taugner and coworkers (375,380,381,382). Through the use of specific antisera and protein A-gold technique in lowicryl- and glycol methacrylate-embedding tissue, Taugner et.al. (379) demonstrated the coexistance of renin and angiotensin II within the renin granule. The concentrations of these peptides increase in response to adrenalectomy and/or sodium depletion. However, those authors could not detect the presence of angiotensin I, angiotensinogen or converting enzyme, which argues against intragranular or intracellular generation of angiotensin II. It was therefore suggested that angiotensin II is generated extracellularly and is subsequently taken up into the JG cell by pinocytosis and incorporated into the granule by granular-pinocytic vesicle fusion. This type of event has not been demonstrated in any other secretory system, and is one criterion by which renin granules are classified as modified lysosomes (381). The findings are consistent with, and supportive of, observations by other investigators who demonstrated the presence of angiotensin II within JG cells (62,376). Two possibile functions for the intragranular angiotensin II are: (1) constriction of the afferent arteriole upstream from the site of release and (2) inhibition of renin secretion via an "ultra-short feedback mechanism." Further investigation is needed to determine if the concentration of angiotensin II is adequate for accomplishing these local events. Recently,

however, Kawamura et al. (206) demonstrated the presence of angiotensin I within the renin granule at concentrations greater than those of angiotensin II. They suggested that Taugner et al. (279) were unsuccessful in detecting angiotensin I because the antisera used were not sufficiently sensitive for histochemical studies. The presence of renin, angiotensin I and II, and angiotensinogen (269) within the kidney raises the possibility of intracellular generation of angiotensins. Kawamura et al. (206) also investigated the effects of dietary sodium on the concentrations of angiotensin I and II within the granule. They observed that rats maintained on low sodium diets had higher levels of both renin and angiotensin II in their granular fractions, whereas the concentration of angiotensin II in renin granules of sodium loaded rats did not change. On the other hand, the angiotensin I content of the granules increased or did not change in rats fed low and high sodium diets, respectively. This suggests that if angiotensin I and II are generated within the renin granule, the granular renin content is not a regulatory factor for their generation. Also, Taugner and associates have demonstrated that renin granules possess lysosome-like properties (381), and contain cathepsin B (327) and D (235), two lysosomal enzymes. These findings led to the conclusion that the intragranular pH must be similar to that of lysosomes and it enabled Taugner et al. to speculate on the possible

functions of cathepsin B and D. They postulated that conversion of prorenin to renin could be mediated by cathepsin B. Cathepsin B is a cysteine protease thought to be involved in the generation of bioactive products in vivo (202,358). Pro-renin is converted to renin through cleavage of a single -lys-arg- peptide bond at positions p1-p2 (375). Cathepsin B has been shown to be particularly effective at cleaving -arg-arg- at position p1-p2 in synthetic substrates (260) and act, in vivo, as a peptidyldipeptidase with a wide range of specificity on proteins and peptides (13,45,261). It has been implicated in the in vivo conversions of proalbumin to albumin (311) and proinsulin to insulin (359). Therefore, it seems likely that cathepsin B can split pro-renin at the -arg-lys- site, which is sensitive to proteolytic attack because of the conformational arrangement of the molecule. Another protease, cathepsin D (which exhibits nonspecific hydrolytic activity) has also been implicated as a modulator of granule renin content. Within the JG cell there are large stores of renin, which are subjected to a wide variety of changes depending on the level of stimulation (ie. the activity of the renin-angiotensin system). Therefore, a sudden decrease in the activity of this system, resulting in decreased renin secretion, would require some means of reducing the amount of stored renin. Taugner et al. (282) suggest that such down regulation of renin can occur through intragranular breakdown mechanisms

involving cathepsin D. This pathway for lowering intracellular renin has been suggested because the transfer of hydrolytic enzymes into the renin granule has never been observed (380,381,382). Both of Taugner's hypotheses concerning possible roles of cathepsins B and D are based on the assumption that the intragranular pH of the renin granule is the same as, or similar to, that of lysosomes (pH 5.2). However, further investigation is needed to confirm this assumption. This thesis provides support for Taugner's hypothesis.

Many investigators have studied the storage form(s) of renin within the granules. Morris and Johnson (268) reported that rat granular renin contains both inactive and active forms with molecular weights of 44000 and 37000 daltons, respectively. The inactive renin can be activated by acid treatment. On the other hand, Sagnella et al. (330), Kawamura et.al. (204), Matsumura et al. (259) and Takaori et al. (372) found only one form of the renin molecule with a molecular weight of 40000-43000 daltons. The renin activity was not enhanced by acidification, freeze thawing, or triton X-100 treatment. However, Takaori et al. (372) reported that their 43000 dalton renin molecule came from a larger (60000 dalton) molecule, while others found 38000 and 36000 dalton forms of rat renin (109,205). Once again, no changes in renin activity resulting from acid, trypsin, dithiothreitol or 2-mercaptoethanol treatment (205). In human renin

granules, Kawamaura et al. (109) found that 25% of the total 48000 dalton granular renin was in an inactive form and could be activated by trypsin treatment without changing the molecular weight. On the other hand, only 0.3% of the renin in dog renin granules was in an inactive, trypsin activatable form (203). The lower percentage of inactive renin in dog renin granules could reflect an increased rate of conversion of prorenin to renin.

Renin release from isolated renin granules has not been investigated in great detail. Moreover, results from various laboratories are inconsistent. In a study of the temperature dependency of granular renin release, Monnisto and Poisner (252) demonstrated that decreasing the incubation temperature from 37°C to 0°C caused a 2-fold increase in granular renin release when granules were incubated in a MOPS-KCl buffer at pH 6.5. On the other hand, Sagnella and Peart (329) and Fanakowa et al. (104) reported 3-fold and 2-fold decreases in renin release, respectively, when the temperature of the KCl-Tris (pH 6.6) and phosphate buffered sucrose (pH 7) incubation medium was lowered to 0°C. In addition, Funakowa et al. showed a time dependent release at 37°C, which plateaued at 30 minutes and did not significantly increase for up to 180 minutes, while at 0°C renin release remained constant for up to 180 minutes.

Different studies have examined the effects of calcium, magnesium, potassium and sodium on granular renin release. Although increasing cytosolic calcium provides a potent signal for shutting down cellular renin secretion, Monnisto and Poisner (252) found no significant differences in granular renin release when granules were incubated at calcium concentrations ranging from 10^{-6}M to 10^{-2}M for 10 or 30 minutes at pH 6.5. However, if the same experiments were carried out at a calcium concentration of 1 mM at varying pH, a stimulatory effect of calcium above pH 7 was observed. Increasing the pH from 6 to 8 in the absence of calcium was without effect. These results could reflect a calcium-dependent pH effect. However, the response was not observed if the incubation time was increased from 10 to 30 minutes. In all of these studies, the renin granules were incubated in KCl-MOPS-EGTA buffer at 37°C. On the other hand, Sagnella and Peart (329) showed a 3-fold decrease in renin release when granules were incubated in buffered isotonic sucrose solution in the presence of 1 mM $CaCl_2$. In terms of other ions, Mannisto and Poisner (252) found that granular renin release was unaffected by 12 mM or 19 mM sodium chloride, 7 mM potassium chloride or 0, 5, or 10 mM magnesium chloride, during a 10 minute incubation at pH 6.5. A detailed study of the roles ions play in granular renin release has never been performed. This thesis attempts to fill that void.

In studying the effects of pH on granular renin release, Monnisto and Poisner (252) demonstrated that during a 10 minute incubation, renin granules are stable between pHs 6 and 8, releasing about 4.5% of the total sedimentable renin. However, a 30 minute incubation produced a 9% and 21% release of total granular renin at pH 6 and 8, respectively. Yet, Sagnella and Peart (329) showed no significant effects on granular renin release when the pH of the incubation medium was raised from 6.6 to 7.2. Thus, whereas pH has been shown to be a central factor in release from other granular systems, its role in renin secretion remains unclear.

The effects of various nucleotides on granular renin release were studied by a number of investigators. It was demonstrated that addition of 0.5 mM or 5.0 mM Mg-ATP, or 5.0mM GTP to a crude preparation of granules decreased renin release (252). This attenuated release was attributed to increased stability of the renin granules. At the same concentrations, nitrogen analogue (MgIMP-PNP) supplementation or addition of either Mg-AMP or ATP in the absence of magnesium, had no effect. Further purification of the granule preparation, Mg-ATP or Mg-GTP at concentrations of 0.5 mM or 5.0 mM did not change renin release (252). Furthermore, Sagnella and Peart (329) found that Mg-ATP (5.0mM), cyclic AMP (1mM), or cyclic GMP (1mM) had no effect when added to an enriched granular fraction incubated in a KCl buffer pH 6.6 at 37°C for

60 minutes. The results of Yamamoto et al. (432,433) are in agreement with those of Sagnella and Peart's in terms of cAMP. However, they found an increase in granular release in response to ATP. Thus, the effects of various nucleotides on granular renin release remain unclear. The currently available conflicting data appear to be related to the purification state of the granule preparation used to generate the information. Resolution of these contradictory results requires development of a pure preparation of renin granules.

Changes in osmolarity directly affect cellular secretion and granular release from a number of systems, including the renin system. Monnisto and Poisner (252) demonstrated that granules kept in a hyperosmotic medium released their contents when transferred to an isotonic medium. They also observed that the renin granules were more stable in 0.25M sucrose as compared to MOPS-KCl of similar osmolarity. The reason for this is not immediately apparent. In addition, Fanakawa et al. (104) observed that as the relative tonicity of the incubation medium decreased, the percent renin release increased. These results suggest that granular renin release can be correlated to an osmotic gradient across the granular membrane. That is, an increase in the inward movement of water causes an increase in renin release, possibly through granular swelling and osmotic lysis. On the other hand, if the osmotic forces favor outward movement of

water, renin release is inhibited. Thus, while there has been some characterization of the osmotic properties of renin granules, sufficient data have not been obtained for a complete analysis of a chemiosmotic mechanism of renin secretion. This thesis provides additional information on the osmotic properties of the granules.

Studies have shown that biomembranes and subcellular organelles are major sites for lipid peroxidation (143,262,318,373,424). Therefore Matsumura et.al. (256,259) investigated the effects of granular lipid peroxidation on renin release. They demonstrated that increasing lipid peroxidation of the renin granule through addition of ascorbic acid or ferrous ions stimulated renin release in a dose and time-dependent manner. However, the degree to which these agents stimulated release was different. In the presence of 50 uM ascorbic acid, 97% of the total granular renin content was released to the incubation medium. On the other hand, 100 uM ferrous ions caused only 63% of the total renin content to be released. These effects could be suppressed by addition of 100 uM N,N'-diphenyl- p-phenylenediamine (259) and tinoridine (256), two antioxidants. Also, addition of dihydroascorbic acid, ferric ions, indomethacin, hydrocortisone or prednisone had no significant effect on lipid peroxidation or renin release (256,269). These results indicate that lipid peroxidation disrupts granular membrane integrity and causes granular lysis.

In conclusion, the preceding review surveys the advances in the field of renin research and the multidimensional regulation of the renin secretory processs. Without a complete understanding of the mechanisms controlling renin secretion, one cannot appreciate the complexity of the renin secretory pathway. The data put forth by a number of investigators demonstrate that calcium and cAMP are two major cytosolic messengers. Although calcium has been suggested to play an inhibitory role, direct correlations between cytosolic calcium and renin secretion have never been demonstrated. cAMP can stimulate renin secretion by two possible mechanisms: (1) by lowering cytosolic calcium or (2) by a calcium independent pathway. Renal cortical slices were used to probe the nature of the renin secretory response to cytosolic calcium and cAMP. In addition, while studies have shown effects of osmolarity on renin secretion, a systematic study of the role of osmotic forces in the regulation of renin secretion has never been done. Moreover, there have been few investigations into the ionic requirements for granular lysis and release. Finally, a model depicting a possible cascade of events for granular release and lysis, in addition to a possible mechanism for renin secretion, has never been developed. With the use of two in vitro systems, renal cortical slices and isolated renin granules, such issues will be addressed.

B. Thesis Rationale

1) In the mechanism of renin secretion, there are at least two cytosolic signals which are important in the secretory response. One is cytosolic calcium. The mechanism of calcium regulation differs from that of most other systems in that raising intracellular calcium inhibits renin secretion in a calmodulin-dependent manner (22,119,258,293,294,411). However, despite the abundance of evidence pointing to an inverse relationship between renin secretion and cytosolic calcium a study of the renin secretory response over a range of calcium concentrations has never been investigated. To fill this gap, experiments were performed on renal cortical slices to attempt to demonstrate more directly the relationship between renin secretion and cytosolic calcium. In these experiments extracellular calcium was buffered at specified concentrations with the use of EGTA. By adding a depolarizing concentration of potassium (59mM), the extracellular and intracellular calcium concentration could be predicted using a null-point calculation, since depolarization of the JG cell would result in the opening of voltage-sensitive calcium channels and thus allow for the equilibration of calcium across the plasma membrane.

2) A second intracellular message important in renin secretion is cAMP. While a number of investigators have

demonstrated the importance of cAMP in renin secretion (69,116,127,132,279,420), it has not been established whether this cyclic nucleotide functions in conjunction with, or independent of, the calcium-calmodulin pathway. cAMP appears to be the mediator of beta-adrenergic stimulation of renin secretion (28,69,127,132,279,420). The view favored by some investigators is that cAMP stimulates renin secretion indirectly by lowering cytosolic calcium (69,223). However, other evidence suggests that cAMP stimulates renin secretion in a calcium-independent manner (126,236,273,285,292,294,410). If cAMP stimulates renin secretion independently of the calcium-calmodulin pathway, one would expect forskolin to stimulate renin secretion by a given amount regardless of cytosolic calcium levels. Therefore, the effect of forskolin on renin secretion at preset cytosolic calcium levels was investigated.

3) A chemiosmotic hypothesis has been proposed as a possible mechanism of exocytosis in a variety of glandular tissues (61,52,152,297,307,328). This mechanism envisions osmotic swelling of the secretory granule as a prerequisite for fusion and subsequent fission of the secretory granule membrane with the plasma membrane. The swelling of the granule is postulated to occur via influx of water driven by an osmotic gradient. The osmotic gradient is generated by the inward transport of osmotically active ions. Although renin secretion from

isolated perfused glomeruli and afferent arterioles is stimulated by lowering the osmolarity of the incubation medium, the role of osmotic forces has never been systematically evaluated. Therefore, with the use of renal cortical slices and isolated renin granules, the role of osmotic forces and proton gradients will be investigated. In addition, with the use of isolated granules, the ionic requirements and the importance of ion translocation in stimulating granular swelling and lysis will be investigated using specific ionophores.

In the first series of experiments, the energy dependency of renin secretion is studied with the use of metabolic inhibitors. These experiments may help determine the locus at which the secretory pathway requires ATP. In the chemiosmotic mechanism, one would expect ATP to be required for granular swelling, for fusion and for fission at the cellular level.

The second series of experiments examines the importance of a pH gradient in renin secretion. If a chemiosmotic mechanism is involved, one would expect that changing the pH gradient across the membrane would influence the amount of renin released into the incubation medium. Furthermore, the addition of the protonophore CCCP would be expected to affect renin secretion in a pH dependent-manner. One would expect CCCP to inhibit renin secretion under conditions in which it effectively reduces the pH gradient. Under conditions where CCCP is incapable

of affecting proton movement (eg. when a pH gradient does not exist), one would expect the renin secretory rate to be unaffected. Interpretation of such studies requires that CCCP's effect is at the level of the renin granule. In addition, studies using isolated granules investigate the possibility that the granular matrix is acidic. One way of testing for the presence of an acidic granular matrix is presentation of a weak base, such as NH_3. Base accumulation in the renin granule should create an osmotic gradient and an increase granule lysis. Where a proton gradient exists, NH_3 freely crosses granular membranes. Once inside the acidic environment, it becomes protonated and trapped. However, if a proton gradient is lacking, (ie. when the pH of the incubation medium equals that of the granular matrix), NH_3 cannot accumulate, and no osmotic gradient is generated.

Since results from the above experiments suggested that a pH gradient at the granular level is important in renin secretion, one would expect to find a proton ATPase in the renin granular membrane. This possibility was investigated on isolated granules exposed to oligomycin and DCCD, two proton pump inhibitors.

A third series of experiments was designed to test the possible role of osmotic forces in renin secretion. According to the chemiosmotic hypothesis, varying the osmotic gradient across the granule membrane to facilitate or inhibit the inward movement of water should affect

renin secretion and granular lysis. The osmotic gradient across the granule membrane was varied by changing the osmolarity of the incubation medium. In experiments using renal cortical slices, experiments were performed to ensure that the renin secretory response was not the result of cell damage. Also, the effect of calcium on osmotic responses was investigated, since calcium is known to affect granular swelling in other systems (439) and to be involved in renin secretion.

Given the importance of ion translocation in the chemiosmotic hypothesis, experiments were conducted using the isolated granule to gain insight into the ionic requirements for granular swelling. The ions investigated were potassium, sodium, chloride and magnesium, selected because of their known effects on renin secretion in vivo and in vitro. In addition, magnesium was studied because it is essential for the activity of many ATPases (213,397). In systems in which a chemiosmotic hypothesis has been proposed, granular swelling requires the presence of a permeable anion, usually chloride, to balance the inward transport of protons. In these systems, chloride enters through an anion channel. If chloride plays a similar role in renin secretion, it should be possible to block release from isolated granules with 4-acetamido-4'-isothiocyanatostilbene-2,2 disulfonic acid (SITS) and 4,4-diisothiocyanatostilbene-2,2' disulfonic acid (DIDS), two anion channel blockers.

To explore further the possibility that ion translocation into the renin granule can stimulate granular swelling and lysis, various ionophores were used. In isolated granules the addition of an ion-specific ionophore to the incubation medium should result in the translocation of that particular ion along its concentration gradient, if no other factors capable of inhibiting translocation are present. Due to the observed importance of hydrogen ions and potassium ions in granule lysis, CCCP (a protonophore), valinomycin (a potassium ionophore) and nigericin (a proton-potassium ionophore which transports an equal number of protons and potassium ions in opposite directions) were investigated for their effects on granular lysis.

II. Materials and Methods

A. Renal Cortical Slice Experiments and Experimental Design

Studies of renin secretion from rabbit renal cortical slices were conducted on tissue obtained from rabbits fed a low sodium diet (Bio-Serve Inc., Frenchtown, New Jersey) for at least one week before experiments, and given water ad libitum. Animals maintained on a low sodium diet are known to have an elevated renal renin content (7,154,266). This may involve an increase in the number of renin granules within the JG cells (161,162,356), and possibly

an increase in the quantity of renin stored per granule (249). The renal renin content could also be augmented by increasing the number of JG cells. It is known that low-level stimulation of renin secretion increases the number of renin positive cells within the kidney (378). In addition, the kidneys of sodium-deprived rats release larger quantities and greater fractions of their stored renin than kidneys from sodium loaded animals (121). The increased renin secretion seems to reflect increased responsiveness of the release mechanism to physiological stimulation, and not simply increased renin synthesis (121). Renal cortical slices approximately 0.5 mm in thickness were prepared with a Stadie-Riggs tissue slicer. Cortical slices of this thickness allow for rapid gas and drug exchange between the incubation medium and the cell cytoplasm. The slices were preincubated in 100 ml of standard Krebs-Ringer Biocarbonate (KRB) solution continuously gassed with 95% O_2 and 5% CO_2 at 37°C for 45 to 60 minutes, with two to three washings with prewarmed KRB pre-equilibrated with the gas mixture. The preincubation period allowed for equilibration between the standard KRB and the cells and for removal of renin or subcellular material which might have been released during preparation. Following the preincubation period, 100 to 200 mg (wet weight) of renal cortical slices were incubated in 5 ml of standard or modified KRB. At this weight, the cortical slices were easy to handle, and the

renin released gave results which were well within the limits of the renin assay. The standard KRB has the following composition (mM): NaCl, 118; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.0; $NaHCO_3$, 24; glucose, 1.0; and pH 7.4. Standard KRB was used because it closely resembles the extracellular environment cells are exposed to in vivo. When the pH of the incubation medium was 5.0, 6.0, 7.0, or 8.0, $NaHCO_3$ of the standard KRB was replaced with 10 mM Tris-(hydroxymethyl)aminomethane-acetate (Tris), 2-(N-morpholino)ethanesulfonic acid (MES, pKa 6.15), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, pKa 7.55), or Tris (pKa 8.3) respectively, and gassed with 100% O_2. When testing the effect of medium osmolarity on renin secretion, the sodium concentration of the incubation medium was kept constant at 75 mM. Under these conditions the osmolarity of the incubation medium was 150 mOsm/Kg H_2O. It could be increased to 300 mOsm/Kg H_2O with 150 mM sucrose. The calcium-free incubation medium was prepared by omitting $CaCl_2$ and adding 1-5 mM ethylene glycol bis (B-aminoethyl ester) N,N,N,N,-tetraacetic acid (EGTA) to the incubation medium. The addition of 1-5 mM EGTA ensures that the incubation medium remains calcium-free throughout the experiment.

To establish the basal rate of renin release, tissue slices were incubated under control conditions during the first hour. During subsequent hours the slices were

treated with various agents, and the effects of these agents on the rates of renin secretion were determined. One set of samples was incubated under control conditions throughout the entire experiment and used to detect changes in the rates of renin release that were not attributable to experimental manipulation. Part (1 ml) or all (5 ml) of the incubation medium was removed and immediately centrifuged at 2000xg for 10 minutes at 4°C to remove any cellular debris that could affect the results of the renin assay. The supernatant was frozen for subsequent determination of renin activity. The rate of renin secretion was measured in terms of renin activity and expressed as nanograms of angiotensin I generated per 100 mg wet tissue weight per hour (ng Ang I/100 mg/h), not as renin per se. Other results are expressed as ratios of rates of secretion under experimental conditions to those of controls (E/C). Expressing the results in this manner enables one to clearly see changes in the secretory rates from hour to hour or under different experimental conditions. The data are not expressed as a percentage of the total tissue content due to the difficulty in obtaining accurate values.

B. Renin Granule Preparation

For studies of renin release, granules were isolated

from kidneys of normal male Sprague-Dawley rats obtained from the Charles River Breeding Laboratory, Wilmington, Ma. These animals were maintained on a standard diet of Purina Laboratory rat Chow and given tap water ad libitum for a minimum of three days after arrival. A standard rat diet was chosen because it was observed that the granular renin activity of the renin enriched fraction was well within the limits of the renin assay. Also, we found that granules isolated from rats maintained on a low sodium diet have a higher total renal renin content, but the specific activity of renin in isolated granules is not enriched relative to granules isolated from rats maintained on a standard diet. This finding, however, is not in agreement with reports by other investigators (249).

1. Granule Isolation

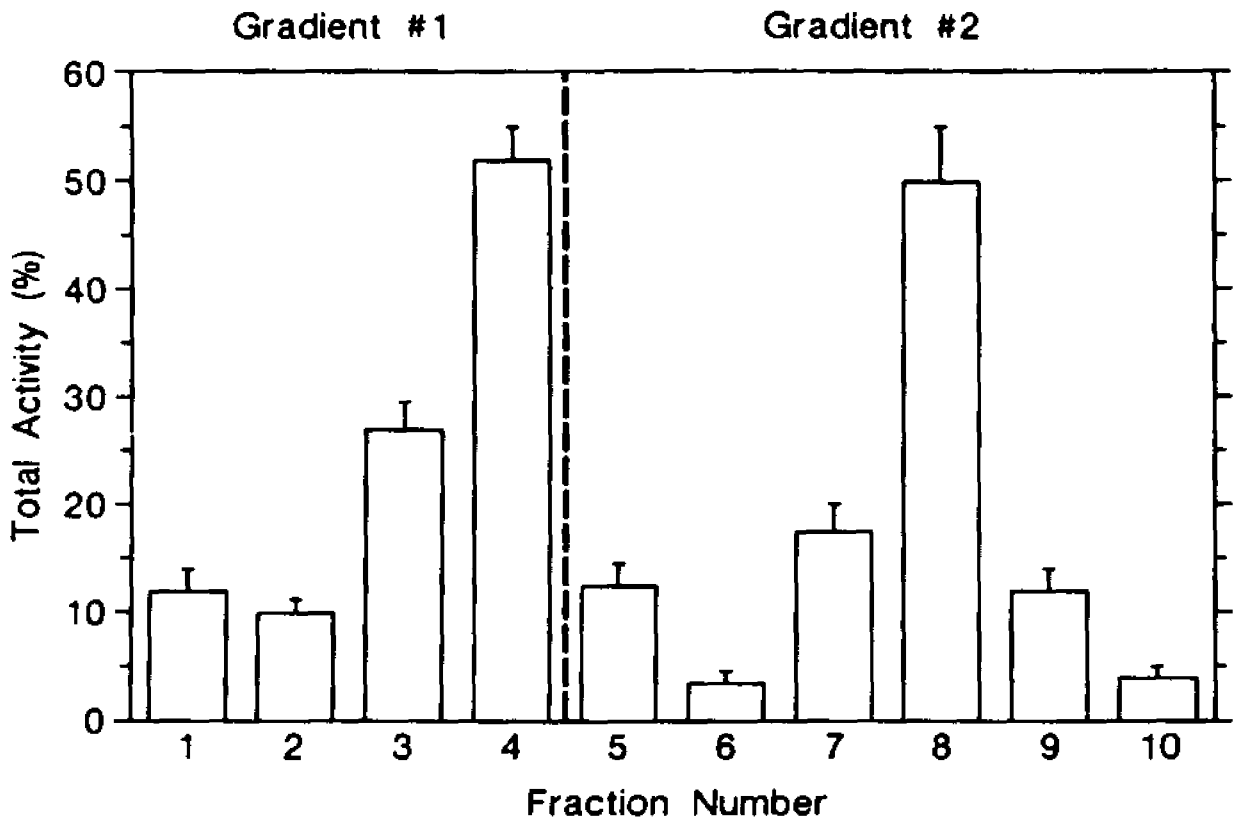
Renin granules were isolated by one of two methods. In the first method, renin granules were isolated from renal cortical homogenates using a discontinuous sucrose density gradient, as described by Sagnella and Peart (229). Four to six male Sprague-Dawley rats weighing 150-250 gm were killed by cervical dislocation. The kidneys were quickly excised and placed in cold (4°C) iso-osmotic sucrose buffered with 3-(N-Morpholino)

propanesulfonic acid (MOPS) at pH 7.0, to reduce protease activity. The renal capsule was carefully removed to avoid damage to the kidney and discarded. The cortex was excised from the underlying medulla in 0.5 mm thick sections, for easy homogenization, using a hand operated-microtome. The cortical slices were weighed and diluted 1:5 (wt:vol) with iso-osmotic sucrose and homogenized in an A189J hand-operated homogenizer obtained from Thomas Scientific. The homogenate was centrifuged in an IEC PR-2 centrifuge at 600xg for 15 minutes. The pellet containing whole cells, nuclei and plasma membrane sheets was discarded, while the supernatant containing subcellular organelles was centrifuged at 2000xg for 15 minutes. The pellet from this centrifugation was discarded and the supernatant containing the renin granules was gently placed over 4.0 ml of 0.6M sucrose and centrifuged at 5000xg for 15 minutes in a Beckman model L5-65 ultracentrifuge using an SW 41 rotor. All fractions except the 0.6M pellet were discarded because of either low renin specific activity or inability to pellet the renin activity. The latter demonstrates that the renin activity in these fractions is in a soluble form. The 0.6M pellet was resuspended in 0.6M sucrose at a volume of 3.0ml/0.5gms of the original tissue. The resuspended pellet was layered onto a discontinuous sucrose gradient containing 3.5 ml of 1.6M sucrose over which was laid 3.5 ml of 1.46M sucrose. The resulting gradient was

centrifuged at 105,000xg for 20 minutes in a Beckman model L5-65 untracentrifuge using an SW-41 rotor. The 1.46-1.6M sucrose interface, which contained 50% of the total renin activity (Figure 1) and the highest level of renin purification (67 fold, Figure 2) was removed and diluted with three volumes of 0.3 M sucrose. Since the renin granules were isolated on a hyperosmotic sucrose gradient, a 30-minute pre-experimental incubation period was allowed for equilibration between the extragranular incubation medium and the intragranular matrix. At the end of each experiment, the renin granules were separated from the incubation media by ultracentrifugation (which released only a small percentage of the total renin in the granules). The pellet was resuspended in 1 ml of 10mM Tris-HCl pH 7 to lyse the granules. Both the lysed pellet and the supernatant were assayed for renin activity by radioimmunoassay. Results were expressed as a percentage of the total renin activity in the incubated samples rather than as absolute values due to the variation of renin activity between samples.

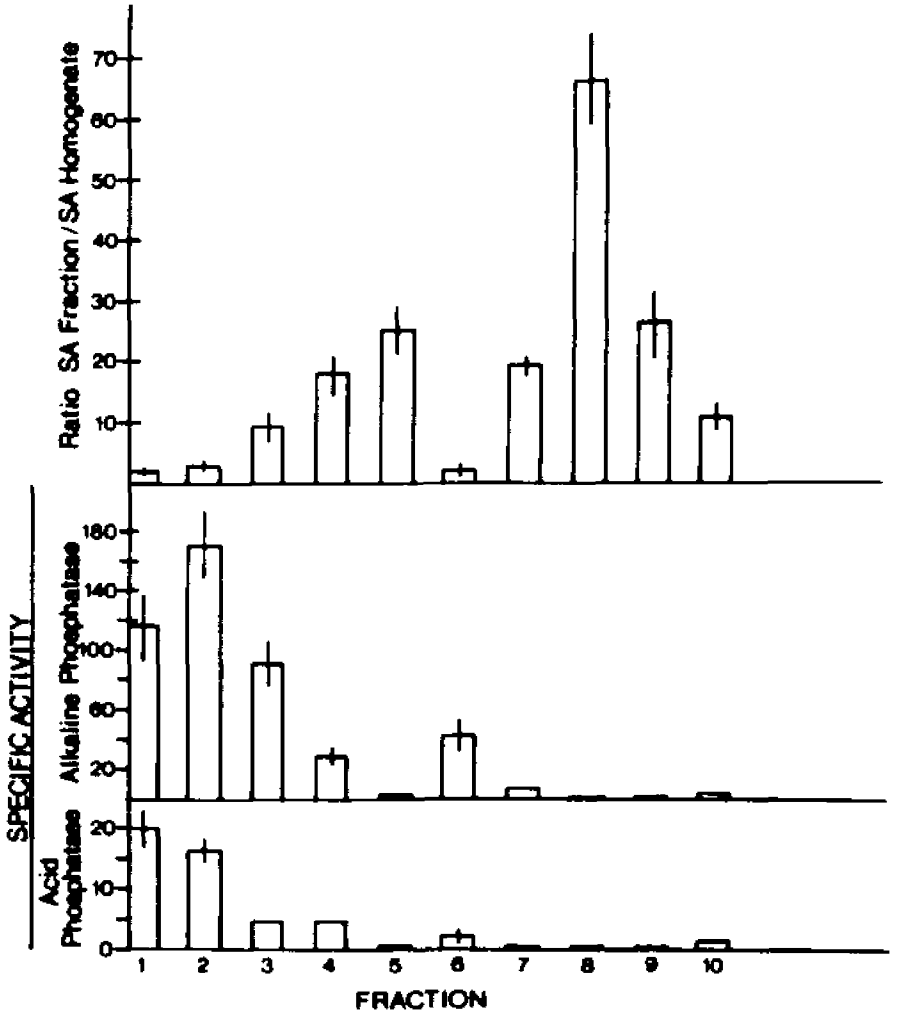
The second method is a percoll isolation procedure based on the method described by Monnisto and Poisner (251). Cortical slices were obtained as stated above, diluted 1:10 (wgt:vol) with 0.25M sucrose, 10mM MOPS, at pH 6.5 and homogenized. The homogenates were centrifuged in a Sorvall RC-3 centrifuge at 1000xg for 10 minutes. The pellet was discarded and the supernatant, from which

Figure 1: Percent Distribution of Renin on Sucrose Gradients.



The percent distribution of renin in each fraction of the sucrose gradient. Values are a mean ±S.E. n=6.

Figure 2: Isolation of Renin Granules by a Discontinuous Sucrose Gradient.



This figure represents the gradient profile of specific activities for three marker enzymes. Fraction 8 has the highest specific activity for renin and the lowest specific activity of either acid or alkaline phosphatase. Values are a mean ± S.E. n=6.

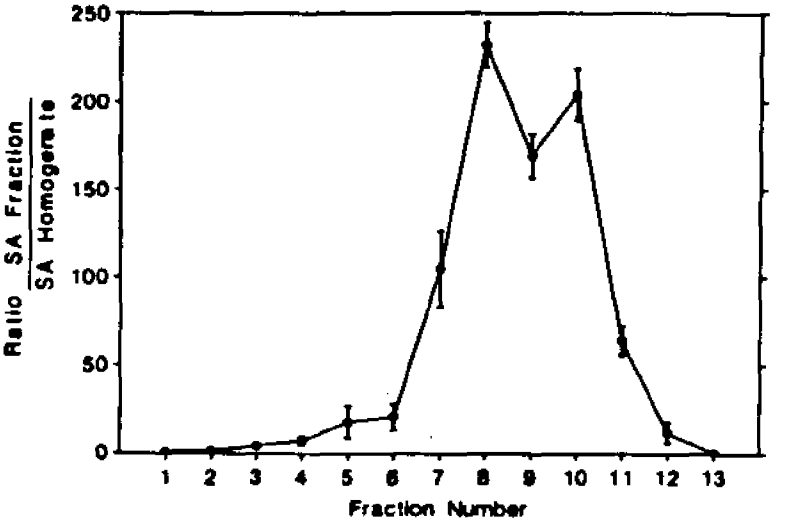
the renin granules were subsequently isolated, was placed on ice. An iso-osmotic percoll stock solution was generated by a 1:10 mixture of percoll (density 1.14 gms/ml) and 2.5M sucrose (density 1.32 gms/ml). This yields a percoll stock solution with a density of 1.15 gms/ml. A continuous percoll gradient ranging in density from 1.03 gms/ml to 1.15 gms/ml was generated with the use of a SG series gradient maker obtained from Hoefer Scientific Instruments. Twelve and one-half ml of the iso-osmotic percoll stock solution (density 1.15 gms/ml) was placed in one chamber of the gradient maker and 12.5 ml 0.25M sucrose (density 1.03) was placed in the other chamber. The less dense, 0.25M sucrose solution slowly mixed with the more dense isotonic percoll solution as it exited the gradient maker. The mixture then passed through a piece of tygon tubing (30 cm long with an inner diameter of 1/32 inch) and into a 25 x 90 mm polycarbonate centrifuge tube. The supernatant from the renal cortical homogenate was then layered carefully onto the gradient (3.5 ml of supernatant containing about 20 mg protein/ml). The gradient was then centrifuged for 15 minutes at 5000xg in a Beckman model 15-65 ultzacentrifuge using an SW-27 rotor. Two ml fractions were collected starting at the top of the gradient. Each fraction was washed three times with 0.25M sucrose, 10mM MOPS pH 6.5, and centrifuged at 100,000xg for 30 minutes to remove the percoll. Washed fractions were then sonicated to lyse the granules and

assayed for renin activity. Additional washes failed to remove more percoll and led to a loss of total renin activity. As can be seen in Figure 3, renin activity in the purified fraction was enriched 232-fold over that in the cortical homogenate. This granule fraction had a low percentage of contamination by lysosomes, mitochondria, and plasma membrane vesicles as judged by the activity of their respective marker enzymes (Figure 5). This represents a substantial improvement over results obtained with sucrose. Figure 3 shows that the highest fold purification was in Fractions 8, 9, and 10, corresponding to a 232-, 167-, and 204-fold purifications, respectively. Figure 4 demonstrates that the highest relative percent peak activity was located in Fractions 7, 8, and 9. It was therefore decided that Fractions 8 and 9 would be used in future experiments, due to their high purity and high relative percent activity.

After each experimental maneuver, the incubation medium in each sample was separated from the granules with the use of a multiwell filtration apparatus obtained from Hoefer Scientific Instruments, using 25mm filter paper with a pore size of 0.45 um. The filtration apparatus consists of 10 wells to which 10 test tubes can be placed to collect the filtrate. The apparatus is attached to a vacuum pump during the filtration process. The pressure within the filtration chamber was monitored by a pressure gauge and did not exceed 25 mm of Hg, a pressure which

Figures 3 and 4: Purification of Renin Granules and relative percent of renin activity using a Percoll Continuous Gradient.

3



4

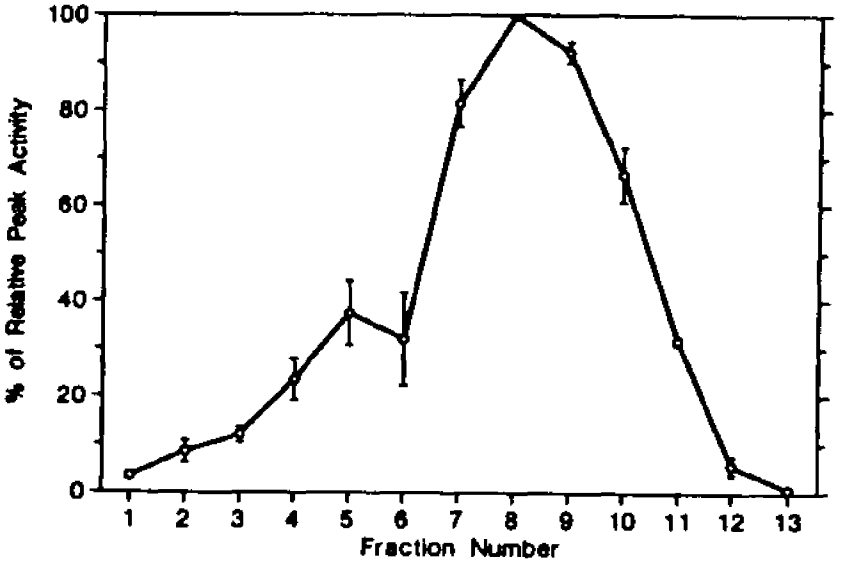
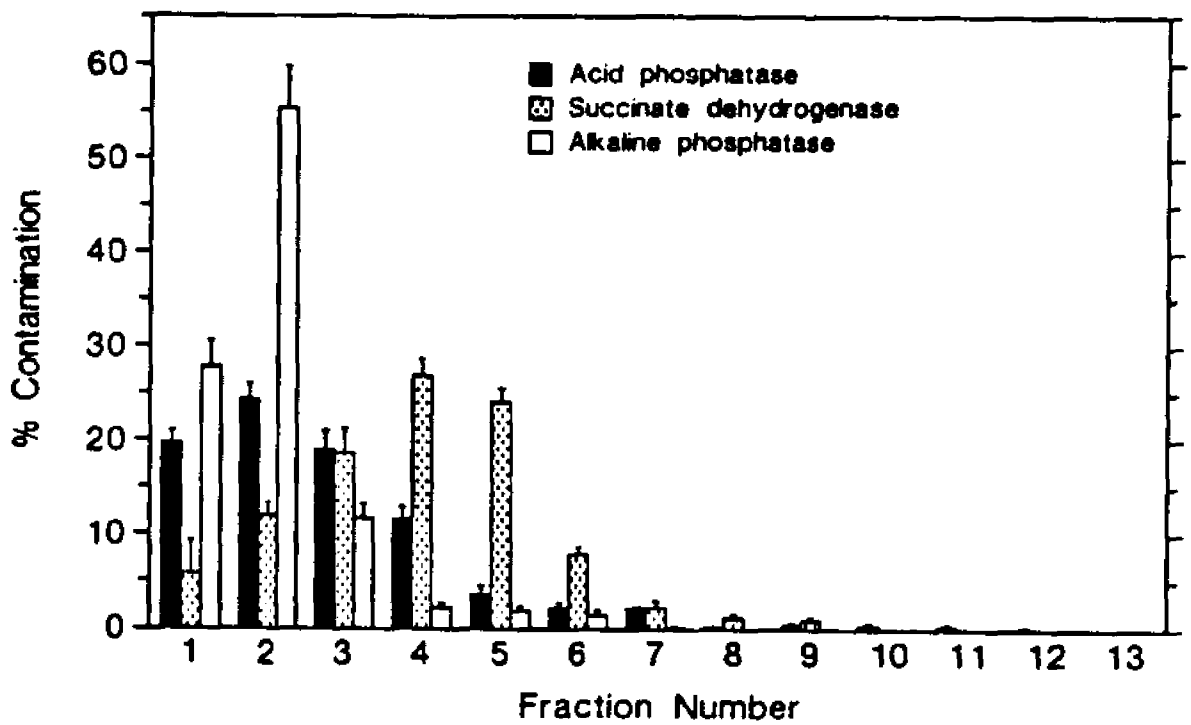


Figure 3. Purification of renin granules using a percoll continuous gradient. The percoll gradient has a density ranging from 1.03 g/ml to 1.149 g/ml, and was centrifuged at 5000xg for 15 minutes. Fractions 8, 9, and 10 represent the renin granules isolated with the highest specific activity. SA = specific activity of renin. Values are a mean ± S.E. n=6.

Figure 4 Relative percentage of renin activity in each fraction collected off the percoll gradient. These relative percentages were generated by dividing the renin activity of each fraction by the fraction with the highest renin activity (Fraction 8).

Figure 5: Purification Profile of Three Marker Enzymes on the Percoll Gradient.



Purification profile of three marker enzymes: acid phosphatase for lysosomes, is measured as the amount of p-Nitrophenol liberated per hour per ml of sample, alkaline phosphatase for plasma membrane vesicles, is measured as the amount of p-Nitrophenol liberated per hour per ml of sample, and succinate dehydrogenase for mitochondria, is expressed as a decrease in absorbance per hour per ml of sample. Fractions were collected from the percoll continuous gradient.

allows for rapid filtration without mechanical lysis of the granules. Preliminary experiments showed that at this filtration pressure, 95.9 ± 2.02% of the granular renin activity and 1.8 ± 0.73% of the soluble renin activity (that renin which is not associated with the granules) was retained on the filter (filter RC55, Table 1). Thus, it appeared that this apparatus was suitable for separating the incubation medium from the renin granules.

2. Renin Granule: Experimental Design

The constituents of the granular incubation medium had the following concentrations (mM): NaCl, 10; KCl or K-glutamate, 137; $MgSO_4$, 5; succinic acid, 5. These concentrations were chosen to approximate the environment to which the granules are exposed in vivo. Depending on the desired pH, 10 mM of one the following buffers was used: Citric acid (pH 5, pKa 3.06), MES (pH 6, pKa 6.15), Imidazole (pH 7, pKa 7.0), and Tris(hydroxymethyl) aminomethane (Tris Base, pH 8 and 9, pKa 8.30). ATP was added at a concentration of 5 mM in all experiments. In studies on the effects of chloride on granular renin release, KCl was replacmd(with varying concentrations of K-glutamate to achieve the desired chloride concentration. In such experiments, the potassium concentrations were held constant at 137 mM. In studies examining the effect of potassium on granular renin release, KCl was replaced

Table 1: The Ability of Different Filters to retain Renin Granule.

ID#	Pore Size (uM)	% Granular retention	Nonspecific binding
RC55	0.45	95.9	1.8
RC57	0.15	66.8	14.7
RC58	0.20	73.0	4.1
OE66	0.20	72.2	11.9
OE67	0.45	77.5	31.5

Renin granules were obtained as described in Materials and Methods. The granules were applied to the filter and filtered at a draw pressure of 15 mM Hg. Soluble renin was obtained by lysing the renin granules in water before applying to the filter.

with choline chloride. In these experiments the chloride concentration was held constant at 137 mM. Samples containing granules from each experiment were sonicated and frozen for later determination of renin activity. Both sonication and freezing have been shown to release granular renin and allow for its detection during the assay.

3. Percoll Gradients vs. Sucrose Gradients: Advantages and Disadvantages

Initially, renin granules were isolated on discontinuous sucrose gradients. This isolation procedure is fast, and it allows one to obtain an enriched renin granular fraction within two hours. One problem with the sucrose technique, however, is the osmotic effect of sucrose on the granules. During the isolation procedure, the granules are exposed to microenvironments of increasing osmolarity. This apparently leads to continous loss of water and/or gain of sucrose by the granular matrix throughout the isolation procedure, as judged by the buoyant density at which the granules are isolated. In sucrose, renin granules are isolated at an interphase with densities of 1.18-1.20 gm/ml (250). On the other hand, granules isolated on percoll gradients have densities of 1.10 and 1.12 gm/ml (205,251,255). This indicates that exposure to the sucrose gradient increases

the density of the renin granules. The sucrose gradients used in these studies could produce a 5-fold increase in the osmolarity of the granular matrix (from 300 mOsm/Kg H_2O to 1600 mOsm/Kg H_2O), an effect that would lead to granular swelling upon transfer to an isotonic (iso-osmotic) incubation medium.

Percoll, which consists of a polyvinylpyrrolidone colloidal silica particles of 15-30 nm in diameter, has a very low osmotic pressure that allows for the generation of gradients which are iso-osmotic throughout. Also, its low viscosity permits very rapid separation of cells and cellular organelles. Renin granules can thus be isolated in less than 90 minutes at greater purity than is achieved with sucrose. One problem with percoll, however, is that it cannot be completely removed from the granular fraction. Consequently, renin granules cannot be easily separated from the incubation medium by centrifugation. Therefore, the filtration mechanism described above was developed. Also, percoll causes formation of a white precipitate when renin granules are incubated at 37°C, pH 5. The white precipitate is not observed when granules isolated on sucrose are incubated under identical conditions. However, this contaminant should not compromise interpretation of the results since percoll is compatible with biological materials, nontoxic, and unable to penetrate biological membranes.

C. Enzyme Assays

1. Renin assay:

It was shown by Skeggs (345) that the primary metabolite of renin activity is angiotensin I (a decapeptide), which is cleaved from the plasma alpha globulin angiotensinogen. Although sometimes characterized as physiologically inactive, angiotensin I can stimulate catecholamine release from the adrenal medulla and prostaglandin release from blood vesicle walls and tracheal smooth muscle (284,301) when present high concentrations. The physiological relevance of the observation is questionable since concentrations of angiotensin I required to stimulate catecholamine release are several orders of magnitude greater than those for angiotensin II (301). The decapeptide is cleaved to biologically active angiotensin II (an octapeptide) by angiotensin converting enzyme. During the assay procedure, the converting enzyme and other proteases are inhibited with 8 mg/ml ethylenediamine tetraacetic acid (EDTA) (77), 0.0056% dimercaprol, and 0.02% 8-hydroxyquinoline (final concentration). The amount of angiotensin I generated is quantified by radioimmunoassay. The principle behind this assay is that radioactive and nonradioactive antigens compete for a fixed number of antibody binding sites (see Yalow and Berson, 431). If a higher concentration of the nonradiolabeled antigen is

added to a tube containing a fixed amount of radiolabeled antigen and antibody, the amount of radiolabled antigen bound to the antibody decreases. The antigen-antibody complex is separated from the free antibody and antigen by differential absorption of the free antibody and antigen onto dextran-activated charcoal. The concentration of the nonradiolabled antigen (that which is generated from the renin activity of the sample) is determined by measuring the radioactivity of the supernatant and obtaining a ratio of counts per minute in the sample supernatant (after charcoal separation) to the total counts added. This ratio is then compared to a standard curve generated from the ratios of samples containing known concentrations of nonradioactive antigen.

In the experiments performed, the assay procedure requires the use of either rat or rabbit renin and dog plasma (substrate). There are limitations in using dog plasma, as opposed to rat or rabbit plasma. It has been shown by Sen et al. (434) that heterologous renin and renin substrate generate less angiotensin than homologous renin and renin substrate. Their results demonstrated that the incubation of an excess amount of rat or rabbit renin with dog substrate results in a 2-fold decrease in the amount of angiotensin generated, compared to thit which is generated using either rat renin with rat substrate or rabbit renin with rabbit substrate. An excess of renin is defined by Sen et al. (434) as the

amount of renin which leads to maximum generation of angiotensin when added to substrate of the same species. Further addition of homologous renin does not increase the amount of angiotensin generated. The relationship between rat or rabbit renin and dog substrate, with respect to angiotensin generation, is linear for up to 60 and 45 minutes, respectively, in the presence of excess renin. During the assay, however, the amount of renin added is never in excess, and the substrate concentration is always too high (40 ng/ml) to become rate limiting. Under these conditions the amount of angiotensin I generated is linear with respect to time and proportional to the renin activity of the sample. Note that these assays measure renin activity, (ie. the amount of angiotensin I generated per hour) as opposed to renin concentration. Thus, the renin being measured is that which is biologically active. At the time these studies were initiated this was the only method available. Assays which directly measure renin concentration have only recently been developed (263).

Renin activity was measured as described previously by Harber et. al. (157) and as modified in our laboratory (117), using nephrectomized dog plasma as substrate. Angiotensin I generated by the catalytic activity of renin on its substrate was quantitated by radioimmunoassay (RIA). The RIA kit for angiotensin I was purchased from New England Nuclear (Boston Ma.).

2. Acid phosphatase assay:

Acid phosphatase activity, a marker enzyme for lysosomes, was determined by the method of Sommer et al. (354) and Jacobsson (188). Acid phosphatase activity was determined for each gradient fraction, and a profile of lysosomal enzyme activity was obtained. Also, acid phosphatase activity was used to determine the amount of lysosomal contamination in the purified renin granular fraction. For this assay an aliquot of sample was incubated with 250 ul of citrate buffer containing 0.09M citric acid and 0.01M chloride, pH 4.8, at 37°C for 30 minutes in the presence of 1 mg p-nitrophenyl phosphate (disodium salt). The final volume of the reaction mixture was 600 ul. At the end of the incubation period the reaction was stopped by addition of 2.5 ml of 0.1 M NaOH. The absorbance of each sample was read at a wavelength of 400 nm against a blank containing assay mixture alone. Acid phosphatase activity was quantitated using p-nitrophenol as a standard.

3. Alkaline Phosphatase:

Alkaline phosphatase, a marker enzyme for plasma membranes, was determined by the method of Lowry et al. (242) and Amador et al (9). The amount of alkaline phosphatase activity was determined for all gradient fractions and was used to construct a separation profile for plasma membrane vesicles. The activity of this enzyme

in the purified granular fraction was used to estimate the degree of contamination by plasma membranes. For this assay samples were incubated at 37°C, for 15 minutes, in an alkaline buffer solution containing 1.5 M 2-Amino-2-methyl-1-propanol buffer, pH 10.3 in the presence of 1 mg p-nitrophenyl phosphate (disodium salt). The final volume of the reaction mixture was 550 ul. At the end of the incubation period, the reaction was stopped by the addition of 5 ml of 0.05 M NaOH. The absorbance of each sample was read at a wavelength of 400 nm, against a blank containing assay mixture alone. The enzyme activity was quantitated using p-nitrophenol as a standard.

4. Succinate dehydrogenase:

Succinate dehydrogenase, a marker enzyme for mitochondria, was determined using the method of Slater and Bonner (351). A separation profile for succinate dehydrogenase was constructed and used as a marker for mitochondrial contamination in the purified renin granular preparation. The reaction was initiated by addition of sample to a reaction mixture consisting of 15 mM sodium succinate, 0.7 mM potassium ferricyanide, 10 mM potassium cyanide, and 0.15M potassium phosphate pH 7.2. The final volume of the reaction mixture was 3 ml. Reactions were run at 20°C in 13 x 75 glass test tubes. Absorbance readings were taken at a wavelength of 400 nm against a blank (containing all the above except potassium

ferricyanide) every 15 minutes for 2-3 hours, or until a constant rate of decline was observed. Enzyme activity was quantitated by comparing the decrease in absorbance of the sample reaction mixture to the blank. The rates measured in this manner are constant with time and proportional to the amount of enzyme activity.

5. Protein Determination

The protein concentrations of the samples were determined by the method of Bradford (49) as modified by Chiappelli et al.(63) using bovine serum albumin as the standard. This protein assay depends upon the conversion of the Coomassie brillant blue dye from the leuko form to an intensely blue color (317) when the dye anion interacts with the NH_3^+ group on the proteins (107). The interaction appears to be a function of the proton concentration of the assay mixture, with dilute perchloric acid and hydrochloric acid the most effective of the acids tested for assay of proteins in solution. One advantage of the Bradford procedure is that free amino acids and very small peptides do not react. It is therefore possible to determine protein concentrations of undialyzed samples. Also, the Bradford procedure is capable of detecting less than one microgram of protein, as compared to the 5-10 ug range limit of Lowry (333). All samples were read at an absorbance of 595 nm within 10-45 minutes of incubation with the reagent. The dye binding process

is virtually complete within two minutes and the resulting color is stable for approximately 60 minutes (49).

III. RESULTS:

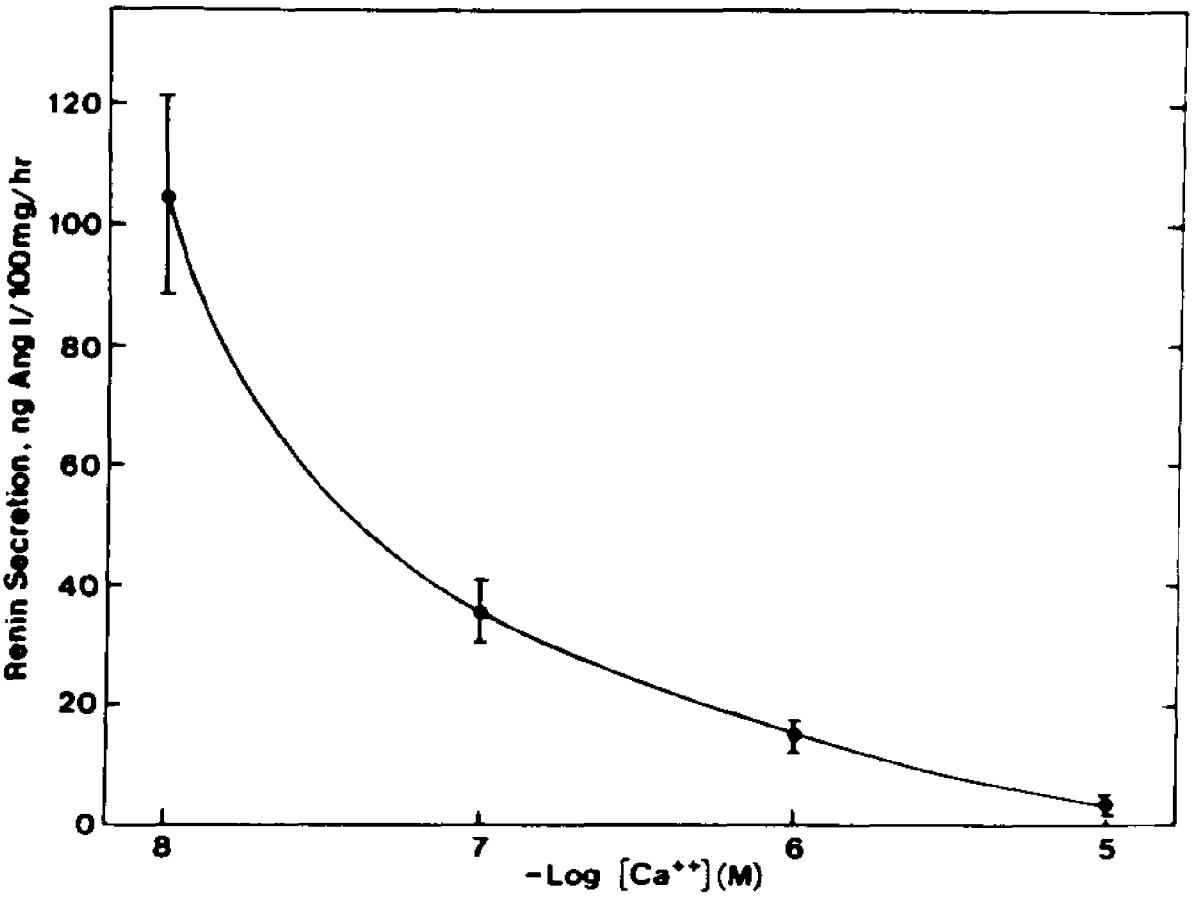
A. Renal Cortical Slice Experiments

The first series of experiments was designed to test the hypothesis that renin secretion is inversely related to cytosolic calcium (22,119,236,294). Renal cortical slices were preincubated under control conditions and washed once in prewarmed calcium-free KRB containing 5mM EGTA to chelate the extracellular calcium in the tissue slices. The washed cortical slices were incubated in 5 ml of modified high potassium KRB containing a calcium concentration which was buffered to 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M with the use of 5mM EGTA. The desired calcium concentration was achieved by addition of appropriate amounts of $CaCl_2$, calculated on the basis of the apparent stability constant of the calcium-EGTA complex of $10^{6.69}$/M (164). The modified high potassium KRB was used to depolarize the JG cells and open voltage-sensitive calcium channels, which results in the equilibration between extracellular and intracellular calcium. Since the calcium concentration of the extracellular compartment was held constant by the EGTA buffer, upon equilibration the intracellular calcium

concentration should approximate the preset calcium concentration of the incubation medium. Figure 6 shows the effects of medium calcium concentrations, in the range of 10^{-8} to 10^{-5} M, on renin secretion in a high potassium KRB. The rate of renin secretion was over 30-fold greater at 10^{-8} M calcium than at 10^{-5} M calcium ($p<0.001$). These results clearly show an inverse relationship between intracellular calcium concentration and renin secretion, thereby confirming the hypothesis.

Another series of experiments was done using canine renal cortical slices. The reason for using canine rather than rabbit renal cortical slices was that in high potassium (59 mM) KRB renin secretion was inhibited by 90% in canine compared to about 50% in rabbit cortical slices. Following the first hour of incubation in which the calcium concentration of the incubation medium was held constant at 10^{-8}, 10^{-6}, or 10^{-5} M and the potassium concentration was maintained at 5.9 mM, the medium potassium concentration was raised to 59 mM. The rate of renin secretion was assessed before and after membrane depolarization with high potassium. When canine renal cortical slices were incubated in normal KRB, the renin secretion rate was independent of extracellular calcium concentrations in the 10^{-8} to 10^{-5} M range. However, when the potassium concentration of the incubation medium was raised to 59mM, the rate of renin secretion was highest at 10^{-8}M and lowest at

Figure 6: Renin Secretion as a Function of Intracellular Calcium Concentration.

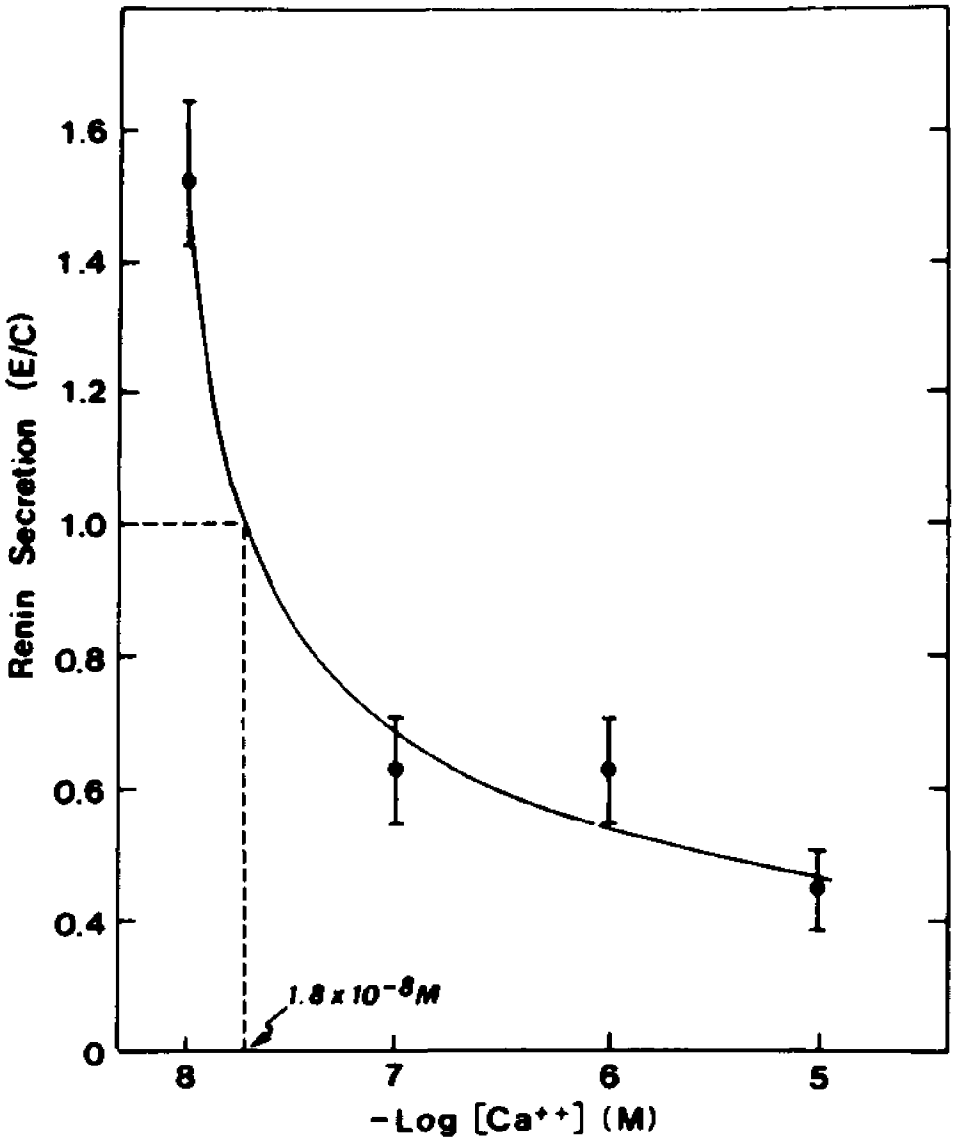


Renal cortical slices were incubated for 1 hour at different calcium concentrations in the presence of depolarizing concentrations of potassium (59 mM). The calcium concentration of the incubation medium was kept constant with EGTA. Under these conditions the intracellular calcium concentration should equal the calcium concentration of the incubation medium.
Each point represents a mean ± S.E. n=5.

10^{-5}M (Figure 7). To correct for possible changes in the cytosolic calcium concentrations at varying extracellular calcium concentrations during the first hour of incubation (control), the results are expressed as the ratio of renin secretion in 59 mM potassium (E) vs. 5.9 mM potassium (C). Renin secretion did not change (E/C = 1) before or after depolarization at an estimated calcium concentration of 1.8×10^{-8} M. At higher calcium levels, renin secretion was inhibited (E/C < 1.0), whereas at lower calcium concentration, renin secretion was stimulated (E/C > 1.0). The value of 1.8×10^{-8}M calcium is probably an underestimate of the resting cytosolic calcium concentration of the JG cell. Kurtz et al. (222) demonstrated with Quin 2 that the cytosolic free-calcium concentration of the unstimulated JG cell is around 3×10^{-7}M, 15 fold higher than the value in Figure 7. These results, however, clearly show an inverse relationship between cytosolic calcium and renin secretion. In Figures 6 and 7, the steepest portions of the curves, that is, the parts of the curves in which small changes in calcium concentrations lead to large changes in renin secretion, are between 10^{-8} and 10^{-7} M. This is within the physiological limits of the cell. The results lend support to the hypothesis that intracellular calcium concentration is an important physiological regulator of renin secretion.

A series of experiments was conducted to investigate

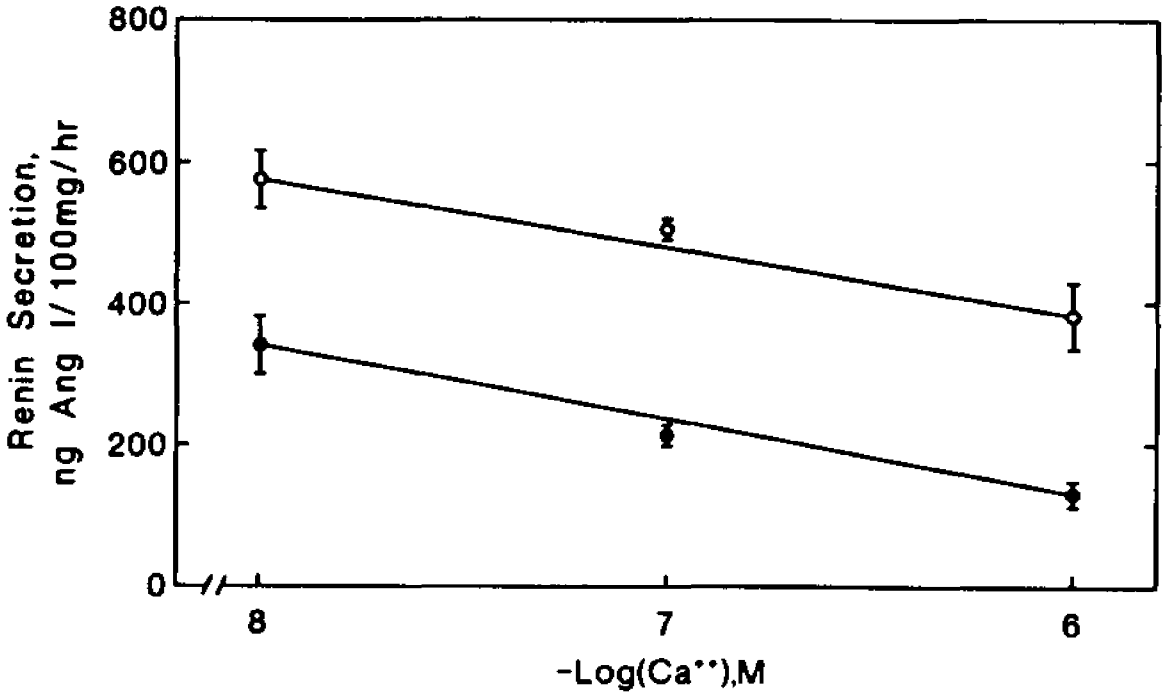
Figure 7: Renin Secretion as a Function of the Calcium Concentration of the Incubation Medium Before and After Potassium Depolarization.



Renal cortical slices were incubated in a standard KRB (5.9 mM potassium) during the first hour of incubation. At the start of the second hour the potassium concentration of the incubation medium was increased to 59 mM. The calcium concentration of the incubation medium was kept constant with EGTA during both incubation periods. Results are expressed as a ratio of renin secretion in high potassium (E) to that in standard (5.9 mM) KRB (C). Values are a mean ± S.E. n=7.

the role of cAMP in renin secretion, specifically, to determine if cAMP stimulates renin secretion by lowering cytosolic calcium. Cytosolic calcium was altered by incubating renal cortical slices in a high potassium (59mM) KRB, in which the extracellular calcium concentration was held constant at 10^{-8}, 10^{-7} or 10^{-6} M with 5 mM EGTA. At the end of the first hour of incubation, forskolin was added to each sample to a final concentration of 10^{-5} M. Forskolin is a direct stimulator of adenylate cyclase that requires no interaction with the beta-adrenergic receptor (341). The effect of forskolin was investigated over the range of calcium concentrations (10^{-8} and 10^{-6}) which were previously demonstrated to exert the greatest effect on renin secretion when incubated in a high potassium (59 mM) KRB (Figure 6). As can be seen in Figure 8, the renin secretion rate was inversely related to cytosolic calcium. Addition of forskolin significantly stimulated renin secretion by a fixed amount, 250 ng Ang. 1/100 mg tissue/hr, regardless of the level of cytosolic calcium. This resulted in an upward shift in the calcium-renin secretory curve (Figure 8). The data in Figure 8 suggest that cAMP's effect on renin secretion is independent of cytosolic calcium. However, one must note that experimental conditions were such that cAMP was unable to influence cytosolic calcium. Therefore, these conditions may not reflect physiological relationships between cAMP

Figure 8: Stimulation of Renin Secretion by Forskolin at Varying Calcium Concentrations in High Potassium KRB.



Renal cortical slices were incubated at a constant calcium concentration of 10^{-8}, 10^{-7}, or 10^{-6}M in high potassium depolarizing KRB to facilitate equilibration between extra- and intracellular calcium (closed circles). Forskolin increased renin secretion by 250 ng Ang 1/100 mg tissue/hr regardless of the medium calcium concentration (open circles). Each value represents a mean ± S.E. n=5.

and cytosolic calcium in vivo.

Another series of experiments was conducted to gain understanding of the energy dependence of renin secretion. Three metabolic inhibitors were used: oligomycin, KCN, and 2-deoxyglucose, each with a different site of action on energy production and utilization. KCN and oligomycin have been shown to inhibit oxidative phosphorylation (312), while 2-deoxyglucose inhibits glycolysis (171). In addition, the mechanisms whereby oligomycin and KCN inhibit oxidative phosphorylation also differ: KCN blocks the last step of electron transport to oxygen (312), while oligomycin inhibits mitochondrial proton ATPase (312,340). Therefore, if renin secretion is tightly coupled to ATP synthesis both oligomycin and KCN would be expected to inhibit renin secretion regardless of the ionic composition of the incubation medium. However, 2-deoxyglucose could be without effect if sufficient concentrations of precursor molecules required for oxidative phosphorylation are present. Table 2 shows the effects of these agents on renin secretion. As can be seen, both KCN and oligomycin inhibited renin secretion by about 65% compared to control ($p<0.001$), while 2-deoxyglucose was without effect. The inhibition of renin secretion by oligomycin and KCN could have several explanations. For one, these inhibitors could cause depletion of cellular ATP required for some energy-dependent step(s) during renin secretion.

Table 2: The Effect of Metabolic Inhibitors on Renin Secretion from Renal Cortical Slices.

Metabolic Inhibitors	Renin Secretion (ng Ang I/100mg tissue/hr.) -Inhibitors (I)	+Inhibitors (II)
Control	62.5 ± 2.7	54.8 ± 4.2
KCN (5 mM)	60.7 ± 9.3	16.1 ± 6.0*
Oligomycin (50ug/ml)	61.1 ± 6.5	20.2 ± 3.0*
2-Deoxyglucose (5 mM)	59.4 ± 8.5	47.9 ± 6.9

Renal cortical slices were incubated in standard KRB for the first 60 minutes in the absence of any inhibitor (-Inhibitor (I)). The metabolic inhibitor was added at the start of the second hour (+Inhibitor (II)). Controls did not receive any inhibitor during the second hour. A ratio of II/I can be calculated if desired. Including the ratios in the table has no added value and the potential for confusion. Values are a mean ± S.E., n=5-6.
* Denotes a significant difference with respect to control, $p<0.005$.

Secondly, ATP depletion could lead to an increase in cytosolic calcium. Third, oligomycin and KCN may inhibit a proton ATPase located in the granular membrane. If the decline in renin secretion is due to ATP depletion, with consequent blocking of a series of energy dependent steps, one would not expect it to be affected by inhibition of the calcium-calmodulin complex. However, if the ATP depletion leads to elevation of cytosolic calcium, one would expect the inhibition to be reversed by incubating the samples in calcium-free media in the presence or absence of a calmodulin antagonist. These possibilities were tested by comparing the effects of KCN and oligomycin on renin secretion from renal cortical slices incubated in calcium-free KRB in the presence and absence of calmidazolium, a potent calmodulin antagonist (400). As can be seen in Tables 3 and 4, KCN had little effect on renin secretion in the absence of calcium or in a calcium-free medium plus calmidazolium. These results suggest that the inhibitory effect of KCN on renin secretion, in a calcium containing medium, is secondary to an increase in cytosolic calcium.

On the other hand, oligomycin inhibited renin secretion equally well in a calcium-free medium in the presence or absence of calmidazolium (Tables 3 and 4) as it did in standard KRB (Table 2). These results suggest that the inhibitory effect of oligomycin was not due to just an increase in cytosolic calcium. Tables 2, 3, and 4

Table 3: The Effect of Metabolic Inhibitors on Renin Secretion from Renal Cortical Slices incubated in a calcium-free medium.

Metabolic Inhibitors	Renin Secretion (ng Ang I/100mg tissue/hr.) -Inhibitors (I)	+Inhibitors (II)
Control	685 ± 158	564 ± 178
KCN (5mM)	571 ± 96.8	470 ± 58.3
Oligomycin (50ug/ml)	671 ± 103	181 ± 42.5*

Renal cortical slices were incubated in a calcium-free medium during the first hour of incubation without inhibitor (-Inhibitor (I)). The inhibitor was added at the start of the second hour of incubation (+Inhibitor (II)). Values are a mean ± S.E., n=6.
* Denotes a significant difference from control of $p < 0.001$.

Table 4: The Effect of Metabolic Inhibitors on Renin Secretion from Renal Cortical Slices Incubated in a Calcium-free Medium Containing Calmidazolium.

Metabolic Inhibitors	Renin Secretion (ng Ang I/100 mg tissue/hr.) -Inhibitor (I)	+Inhibitors (II)
Control	1202 ± 213	953 ± 160
KCN (5mM)	1366 ± 179	1252 ± 177
Oligomycin (50ug/ml)	1228 ± 178	385 ± 76.6*

Renal cortical slices were incubated in a calcium-free medium containing 1 mM EGTA and the calmodulin antagonist calmidazolium ($5x10^{-5}$). During the first hour of incubation the cortical slices were incubated in the absence of any metabolic inhibitor (-Inhibitor (I)). The metabolic inhibitor was added at the start of the second hour (+Inhibitor (II)). The cortical slices were incubated for 60 minutes in the presence of the metabolic inhibitor. Values are a mean ± S.E., n=5-6.
* Denotes a significant difference of $p< 0.0005$ compared to control.

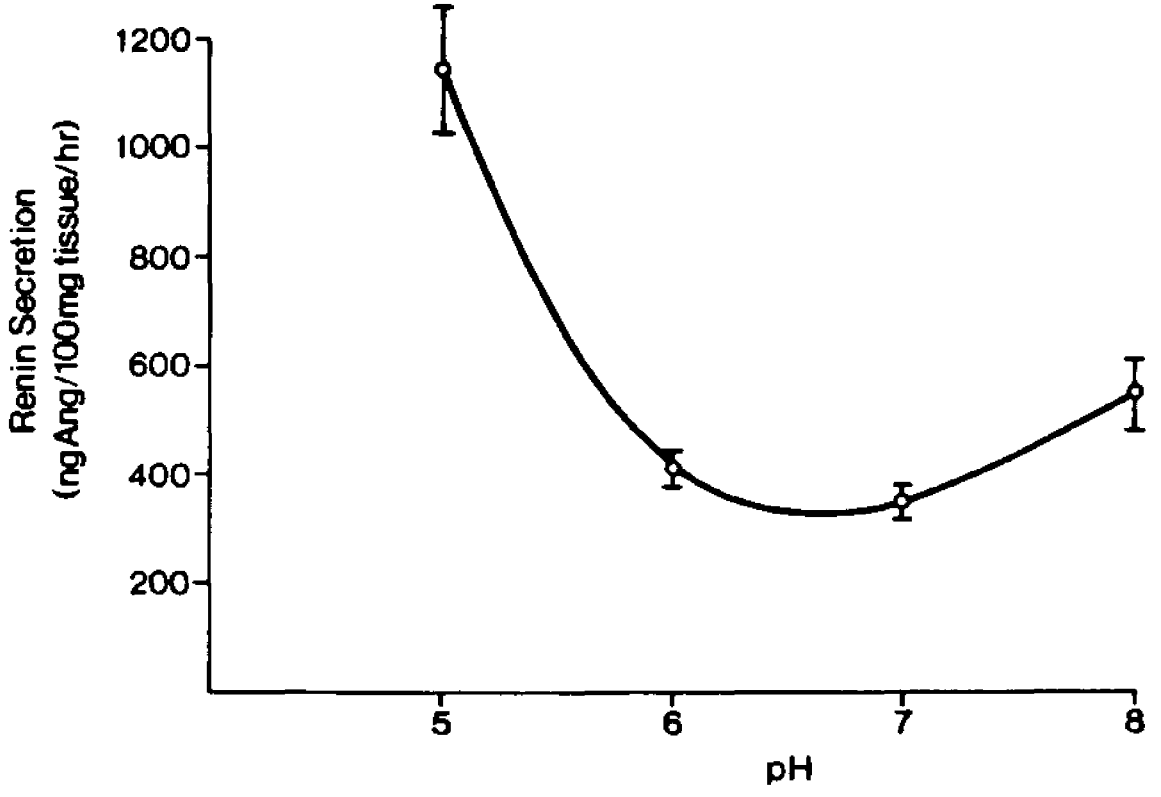
also demonstrate that the degree of inhibition by oligomycin was independent of the control levels of renin secretion. The variability in the control levels of renin secretion in the standard KRB, calcium-free KRB, and calcium-free KRB with calmidazolium may be related to the inhibitory effects of calcium on renin secretion (180,183). These results lend strong support to the possibility that the inhibitory effect of oligomycin results from its action at a site other than the mitochondria, probably a proton ATPase located in the renin granule. Proton ATPases have been demonstrated in all secretory granules studied to date (12,14,16,61,74,152,179,237,328).

The concept of a proton ATPase in the granular membrane naturally brings up the role of pH and the possible importance of a pH gradient. Thus, the effect of altering the pH gradient across the membrane was examined by changing the pH of the incubation medium. These experiments were also motivated by the effect pH has on secretion from other tissues (28,181,313,352). In these experiments, renal cortical slices were incubated in a calcium-free medium at pH 7 during the first hour. At the start of the second hour, the pH of the incubation medium was either held constant or changed to pH 5, 6, or 8. Due to the anticipated effect of pH on renin secretion, CCCP was added to the incubation medium at the beginning of the third hour. However, when testing the reversible effect

of acid pH, the pH of the incubation medium was returned to pH 7 at the start of the third hour.

Figures 9 and 10A and Table 5 show a biphasic effect of pH on renin secretion from renal cortical slices. Renin secretion increased when the pH of the incubation medium was lowered from 7 to 5 and increased again when the pH was raised from 7 to 8. However, no statistically significant change in renin secretion was observed when the pH of the incubation medium was lowered to 6. These results suggest that renin secretion is dependent on the pH gradient across the membrane. If this interpretation is correct, then it should be possible to inhibit renin secretion by abolishing the pH gradient. As can be seen in Figure 10B and Table 5, the ability of CCCP to inhibit renin secretion was dependent on the pH of the incubation medium. The data in Figure 10B are expressed as ratios of the rates of renin secretion at the experimental pH in the presence of CCCP to those in the absence of CCCP. At pH 5, CCCP had no effect on renin secretion ($p>0.05$). However, at pHs 6, 7, and 8, CCCP inhibited renin secretion by $31 \pm 12\%$ ($p<0.05$), $46 \pm 11\%$ ($p<0.01$) and $83 \pm 4.0\%$ ($p<0.0005$), respectively. It is interesting to note that the greater the pH gradient across the membrane, the greater the inhibition by CCCP (ie. CCCP is effective only when it dissipates a pH gradient). (These results illustrate direct involvemmnt of a pH gradient in the renin secretory process.

Figure 9: The Effect of pH on Renin Secretion from Renal Cortical Slices.



Renal cortical slices were incubated in a calcium-free medium containing 1mM EGTA at pH 7 during the first hour of incubation. At the start of the second hour the pH of the incubation medium was changed to pH 5.0, 6.0, or 8.0. This figure represents the amount of renin secreted at each pH at the end of the second hour. Values are a mean ± S.E. n=5

* Denotes a significant difference compared to pH 7 at a level of $p < 0.05$ or lower.

Figures 10A and 10B: The Effect of pH and CCCP on Renin Secretion.

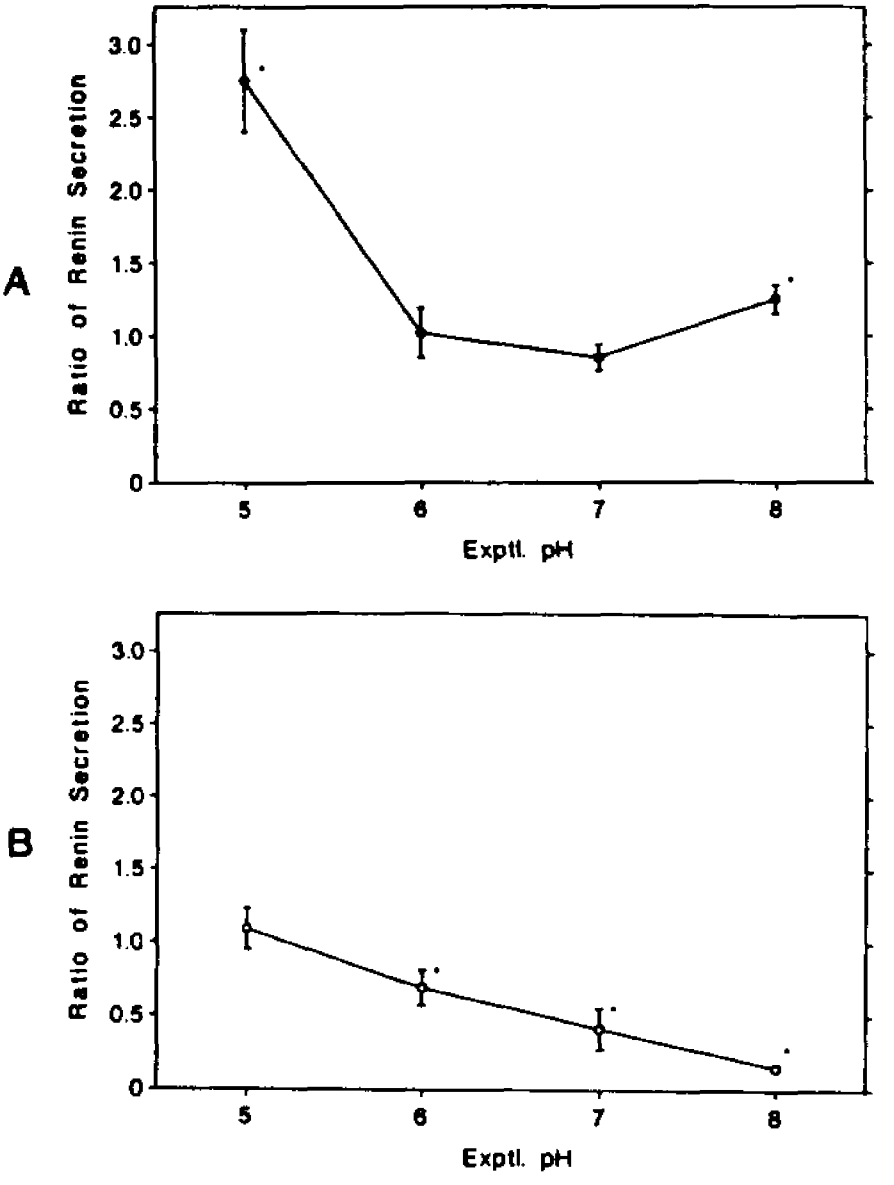


Figure 10 A Experimental conditions are identical to that for Figure 9. The results are expressed as a ratio of renin secretion at experimental pH to that at pH 7. The pH of the incubation medium was changed and maintained at 5.0, 6.0, or 8.0 during the second hour of incubation. Values are a mean ± S.E. n=5.
* Denotes a significant difference of $p < 0.05$ or less from a ratio of 1.0.

B At the start of the third hour CCCP was added to the medium to give a final concentration of 10^{-5}M. The results are expressed as a ratio of renin secretion at experimental pH in the presence of CCCP to that in the absence of CCCP. Values are a mean ± S.E. n=5. * Denotes a significant difference of $p < 0.05$ or lower from a ratio of 1.0.

Table 5: The Effect of pH and CCCP on Renin Secretion from Renal Cortical Slices.

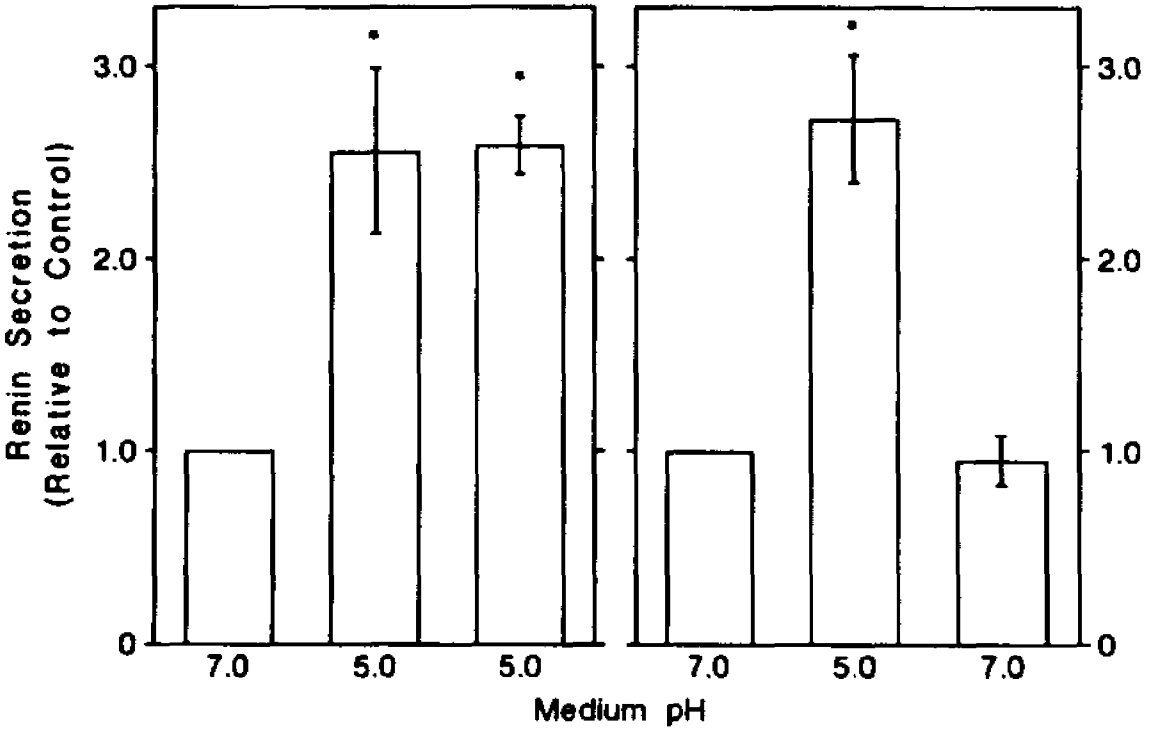
pH Cont.	pH Exp.	Renin Secretion (ng Ang I/100mg tissue/hr.) Cont. pH	Exp. pH	Exp. pH + CCCP
7.0	5.0	419 ± 18.9	1337 ± 121*	1185 ± 123
7.0	6.0	439 ± 51.6	416 ± 36.3	295 ± 56.4*
7.0	7.0	427 ± 57.9	352 ± 35.4	133 ± 41.4*
7.0	8.0	445 ± 45.6	553 ± 64.9*	76.8 ± 6.8*

Renal cortical slices were incubated in a calcium-free medium containing 1 mM EGTA at pH 7 during the first hour. At the start of the second hour, the pH of the incubation medium was changed to either pH 5.0, 6.0, or 8.0. CCCP was added at the start of the third hour of incubation. Therefore, the pH of the incubation medium during the second and third hours of incubation was the same. Values are a mean ± S.E., n=5.
* Denotes a significant change in renin secretion as compared to that during each preceeding incubation period, $p < 0.05$.

Additional experiments were conducted to determine if the pH effect was reversible at acid pH (pH 5). Lowering the pH of the incubation medium from 7 to 5 stimulated renin secretion by 156% above control values ($p<0.01$) (left panel of Figure 11), and this was maintained for two hours. When the pH of the incubation medium was returned to pH 7 at the beginning of the third hour, renin secretion returned to a level that was not significantly different from control (secretion observed at pH 7 during the first hour of incubation, see right panel of Figure 11). These results indicate that the pH effect is reversible and may not be due to cell destruction.

Additional studies were conducted to determine the nature of the effect of CCCP on renin secretion, since it is possible that CCCP exerted its effect through inhibition of oxidative phosphorylation. More specifically, I wanted to examine the possibility that CCCP's inhibitory effect is secondary to a rise in cytosolic calcium. To test this hypothesis, renal cortical slices were incubated in a standard, calcium-free, or calcium-free plus calmidazolium KRB. As can be seen in Table 6, the pattern of responses to CCCP was identical to that observed with oligomycin. Table 6 demonstrates that CCCP inhibits renin secretion by about 70% in standard KRB or calcium-free KRB in the presence or absence of calmidazolium. These results suggest that, in common with oligomycin, CCCP inhibition of renin secretion

Figure 11: The Reversible Effect of Acid pH on Renin Secretion in a Calcium-free Iso-osmotic Medium.



The pH of the incubation medium was lowered from 7.0 to 5.0 at the start of the second hour. At the start of the third hour, the pH of the incubation medium remained the same (pH 5, left panel) or was raised back up to pH 7 (right panel). Renin secretion during the second and third hour were corrected for spontaneous changes in renin secretion determined from controls that were maintained at pH 7 throughout the time course of the experiment. Values are a mean ± S.E. n=6.

* Denotes a significant difference of $p < 0.05$ from a ratio of 1.0.

Table 6: The Effect of CCCP on Renin Secretion from Renal Cortical Slices Incubated in Normal, Calcium-free or Calcium-free plus Calmidazolium KRB.

(A) Normal KRB

Metabolic Inhibitor	Renin Secretion (ng Ang I/100 mg tissue/hr. -Inhibitor (I)	+Inhibitor (II)
Control	62.5 ± 2.7	54.8 ± 4.2
CCCP (10^{-5} M)	62.4 ± 10.1	14.6 ± 4.8*

(B) Calcium-Free KRB

Metabolic Inhibitor	Renin Secretion (ng Ang I/100mg tissue/hr. -Inhibitor (I)	+Inhibitor (II)
Control	685 ± 158	546 ± 178
CCCP (10^{-5} M)	755 ± 154	180 ± 26.2*

(C) Calcium-free KRB + calmidazolium

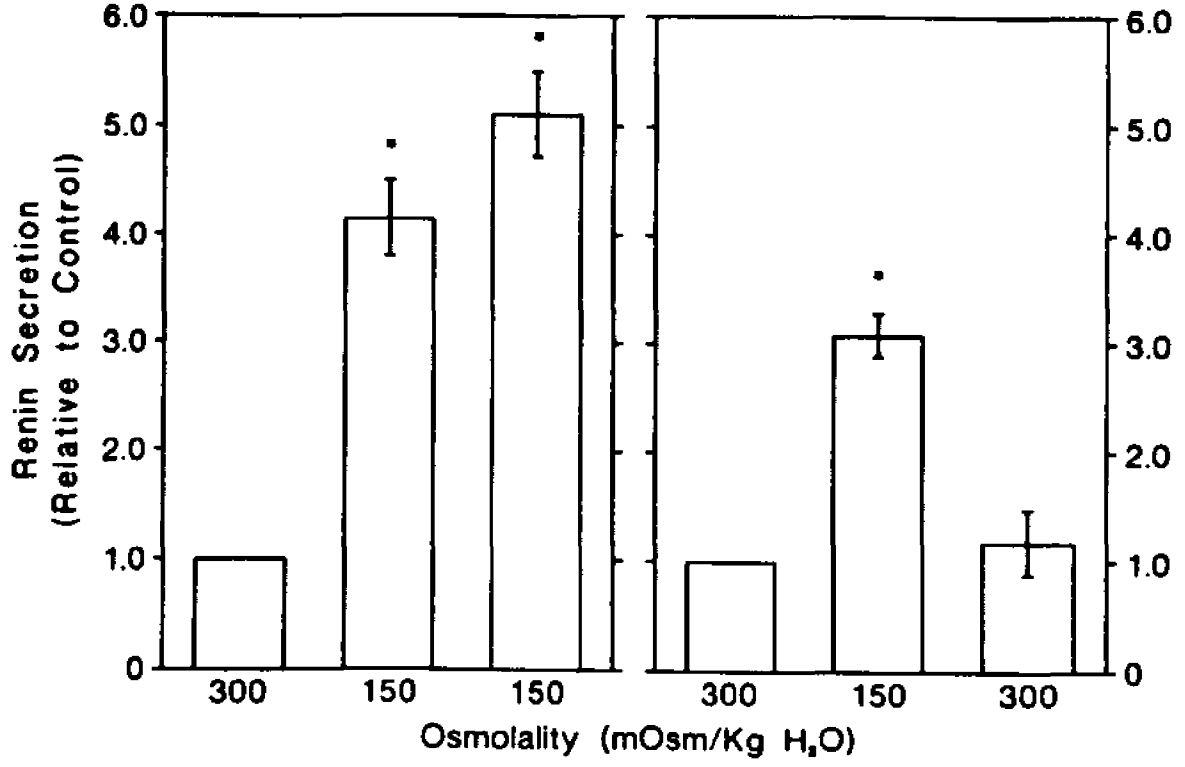
Metabolic Inhibitor	Renin Secretion (ng Ang I/100mg tissue/hr. -Inhibitor (I)	+Inhibtor (II)
Control	1202 ± 213	953 ± 160
CCCP (10^{-5} M)	1582 ± 340	538 ± 119*

Experimental conditions are as described for Tables 1, 2, and 3.
Values are a mean ± S.E., n=5-6.
Asterisks denote a significant difference from control; * $p < 0.005$, ** $p < 0.001$, *** $p < 0.0005$.

was not solely the result of elevation of intracellular calcium levels or activation of the calcium-calmodulin complex. They provide additional support for the view that a proton gradient across the membrane is important in renin secretion.

In other secretory systems in which a pH gradient across the membrane has been shown to be involved in cellular secretion, a chemiosmotic hypothesis has been developed around osmotic forces. In the chemiosmotic mechanism for exocytosis, granular swelling, which is necessary for granular fusion, is driven by an osmotic gradient. To examine the possible role of a chemiosmotic mechanism in renin secretion, the effect of hypo-osmolality on renin secretion in a calcium-free medium was investigated. A calcium-free medium was chosen to maximize the rate of renin secretion. Figure 12 demonstrates the effects of hypo-osmolality on renin secretion. As can be seen, lowering the osmolality of the incubation medium at the start of the second hour significantly increased renin secretion by 414 $\pm$ 35% ($p<0.001$). The stimulatory effect was sustained for at least two hours. However, returning to iso-osmotic conditions (300 mOsm/Kg H_2O) at the start of the third hour of incubation, brought renin secretion to levels which were not significantly different from control. The reversible effects of hypo-osmolality indicate that the increase in renin secretion caused by

Figure 12: The Stimulatory and Reversible Effects of Hypo-osmolality.



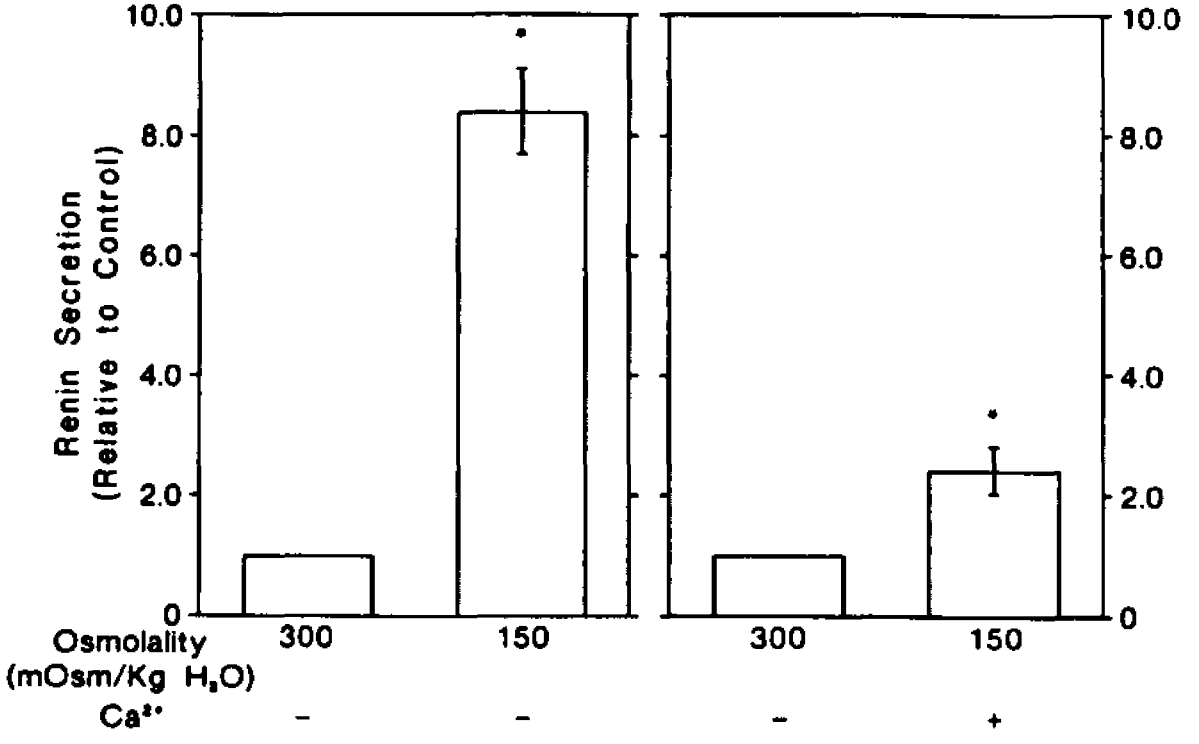
Renal cortical slices were incubated in a calcium-free medium at pH 7.0 during the first hour of incubation. At the start of the second hour, the osmolality of the incubation medium was lowered to 150 mOsm/Kg H_2O. During the third hour of incubation the osmolality was either kept at 150 mOsm/Kg H_2O (left panel) or was returned back to 300 mOsm/Kg H_2O (right panel). Results are expressed as a ratio of renin secretion during the second and third hour to that during the first control period. Renin secretion during the second and third hours are corrected for spontaneous changes in renin secretion observed in control slices that were incubated at 300 mOsm/Kg H_2O throughout the experiment. Values are a mean ± S.E. from n=5 (left panel) or n=6 (right panel).
* Denotes a significant difference from a ratio of 1.0, $p < 0.05$.

increasing the osmotic gradient across the membrane may not have been due to cell damage. In addition, the results indicate that renin secretion can be stimulated by osmotic swelling.

I next examined the effect of calcium on hypoosmotic stimulation of renin secretion. Figure 13 demonstrates once again the stimulatory effect hypo-osmolality has on renin secretion in a calcium-free medium (841% increase compared to control, left panel). However, after lowering the osmolality of the incubation medium to 150 mOsm/Kg H_2O in the presence of 2.5 mM calcium, renin secretion increased by 241% compared to control (Figure 13, right panel). The increase is significantly less than that observed in the absence of calcium (8.41 ± 0.72 vs. 2.41 ± 0.39, $p<0.001$).

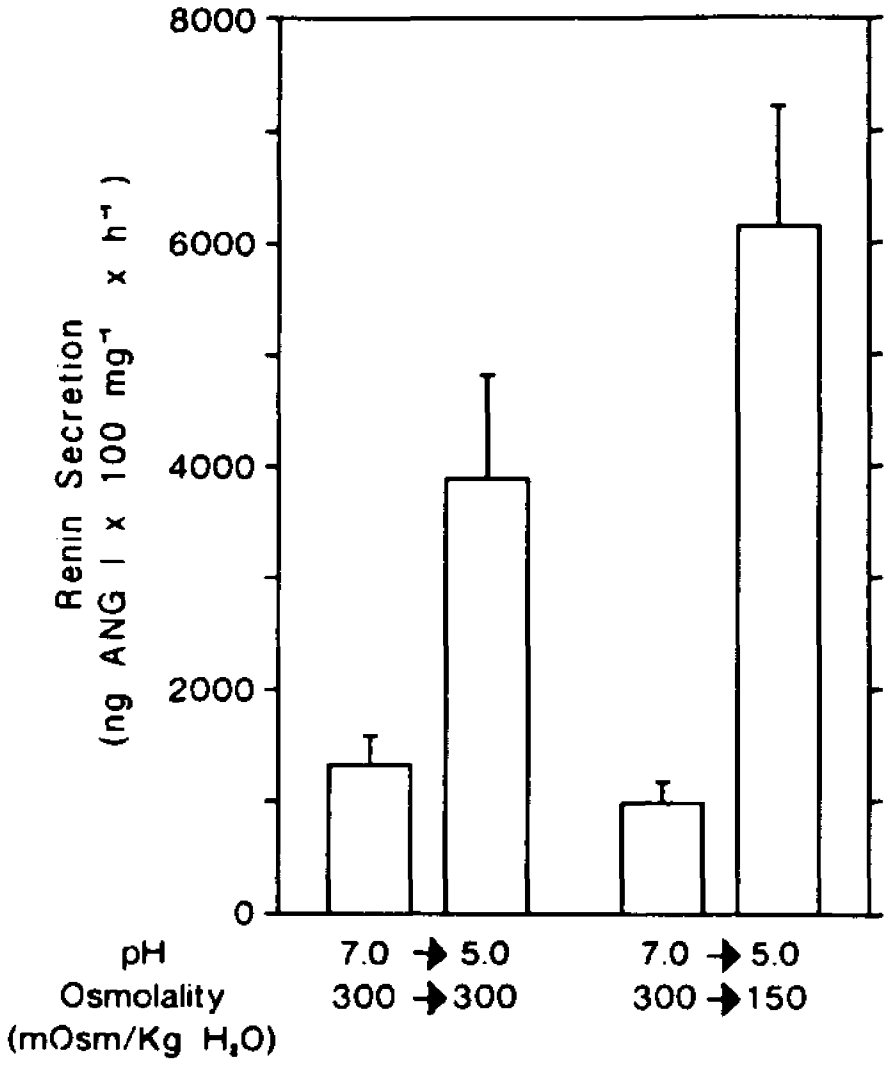
Acidity (pH 5) and hypo-osmolality (150mOsm/Kg H_2O) were combined to determine if their effects are additive. In one series of experiments, renal cortical slices were incubated at an osmolality of 300 mOsm/Kg H_2O, pH 7 during the first hour. At the start of the second hour the pH of the incubation medium was lowered to 5, and the osmolality was either maintained or decreased to 150 mOsm/Kg H_2O. Figure 14 demonstrates that lowering the pH of the incubation medium from 7 to 5 at 300 mOsm/Kg H_2O results in a $187 \pm 31\%$ ($p<0.005$) increase in renin secretion. However, lowering both pH and osmolality increased renin secretion by $530 \pm 22\%$

Figure 13: The Stimulation of Renin Secretion by Hypo-osmolality in the presence and absence of calcium.



Renal cortical slices were incubated in a calcium-free, iso-osmotic medium during the first hour. At the start of the second hour the medium was made hypo-osmotic in the presence (right panel, n=6) or absence (left panel, n=12) of 2 mM calcium.
* Denotes a significant difference between the effect of hypo-osmolarity in the presence and absence of calcium, $p < 0.001$.

Figure 14: The Effect of Acid pH and Hypo-osmolality on Renin Secretion.

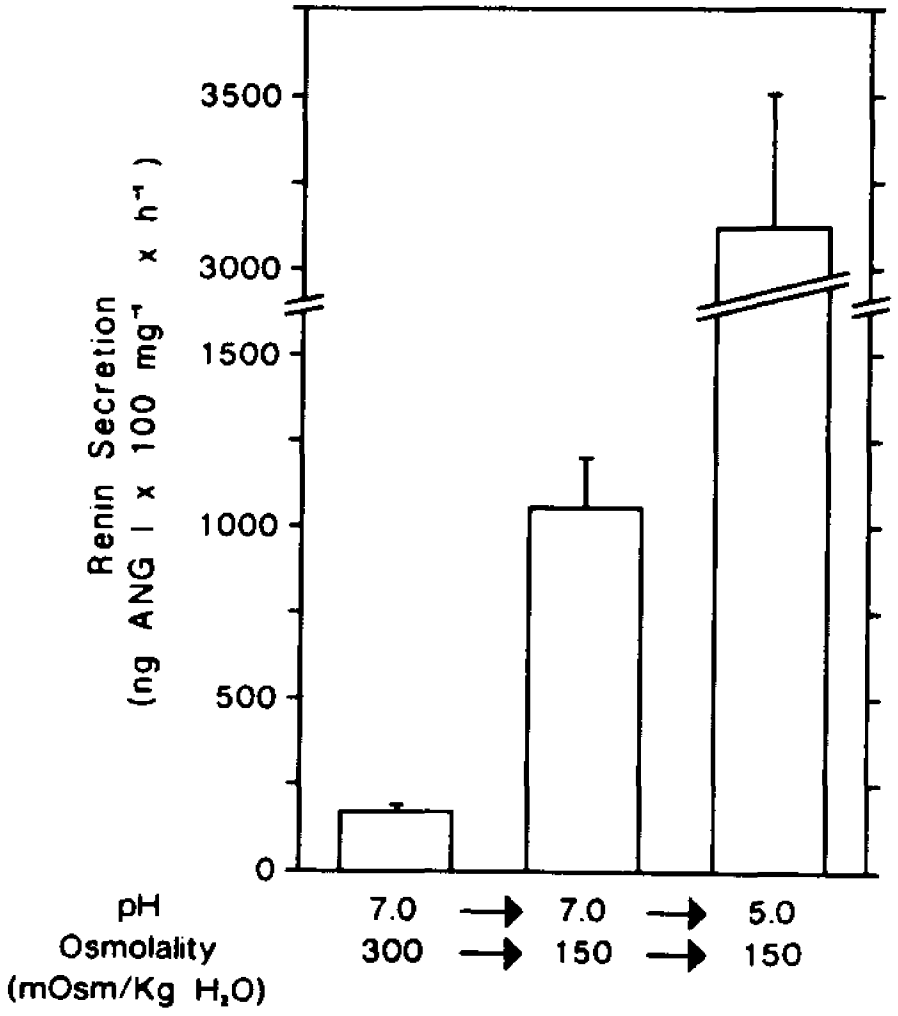


Renal cortical slices were incubated at pH 7 and an osmolality of 300mOsm/Kg H_2O during the first hour of incubation as a control. The pH or osmolality was changed during the subsequent 60 minute incubation period. Lowering the pH from 7.0 to 5.0 under iso-osmotic conditions significantly stimulated renin secretion, $p< 0.005$. Lowering both the pH and osmolality increased renin secretion to an even greater extent, $p< 0.001$, compared to control. Values are a mean ± S.E. n=6.

($p<0.001$). The increase in renin secretion by both combinations was significantly greater than that which was achieved by acid pH alone (5169 $\pm$ 889 vs. 2563 $\pm$ 702 ng Angotensin I/100 mg tissue/hr). A similar pattern was generated when the osmolality of the incubation was lowered to 150 mOsm/Kg H_2O, followed by a decrease in pH (Figure 15). Lowering the osmolality from 300 to 150 mOsm/Kg H_2O at pH 7 increased renin release by 1061 $\pm$ 158 ng Ang I/100 mg tissue/hr ($p<0.005$). Subsequent lowering of the medium pH from 7.0 to 5.0 while maintaining an osmolality of 150 mOsm/Kg H_2O further increased renin secretion by 2004 $\pm$ 349 ng Ang I/100 mg tissue/hr. ($p<0.005$). These results are consistent with the view that incubating renal cortical slices in a hypo-osmotic incubation medium induces swelling of the renin secretory granule. Such an event has been envisioned to be a prerequisite for secretion (153,303) In addition, acid pH may promote granular swelling through activation of a proton pump located in the renin granule membrane. Therefore, the additive effects of hypo-osmolality and acid pH on renin secretion may be explained by a greater increase in granular swelling over that which can be achieved by either hypo-osmolarity or acid pH alone.

Due to the apparent importance of granular swelling in renin secretion the following series of experiments were designed to investigate the effects of various agents

Figure 15: The Effect of a Stepwise Decrease in Medium Osmolality followed by a decrease in pH on Renin Secretion.



Renal cortical slices were incubated at pH 7 and an osmolality of 300 mOsm/Kg H_2O during the first hour of incubation. At the start of the second hour, the osmolality was lowered to 150 mOsm/Kg H_2O followed by a decrease in pH at the start of the third hour. Values are a mean ± S.E. n=6.
* Denotes a significant difference, $p < 0.005$.

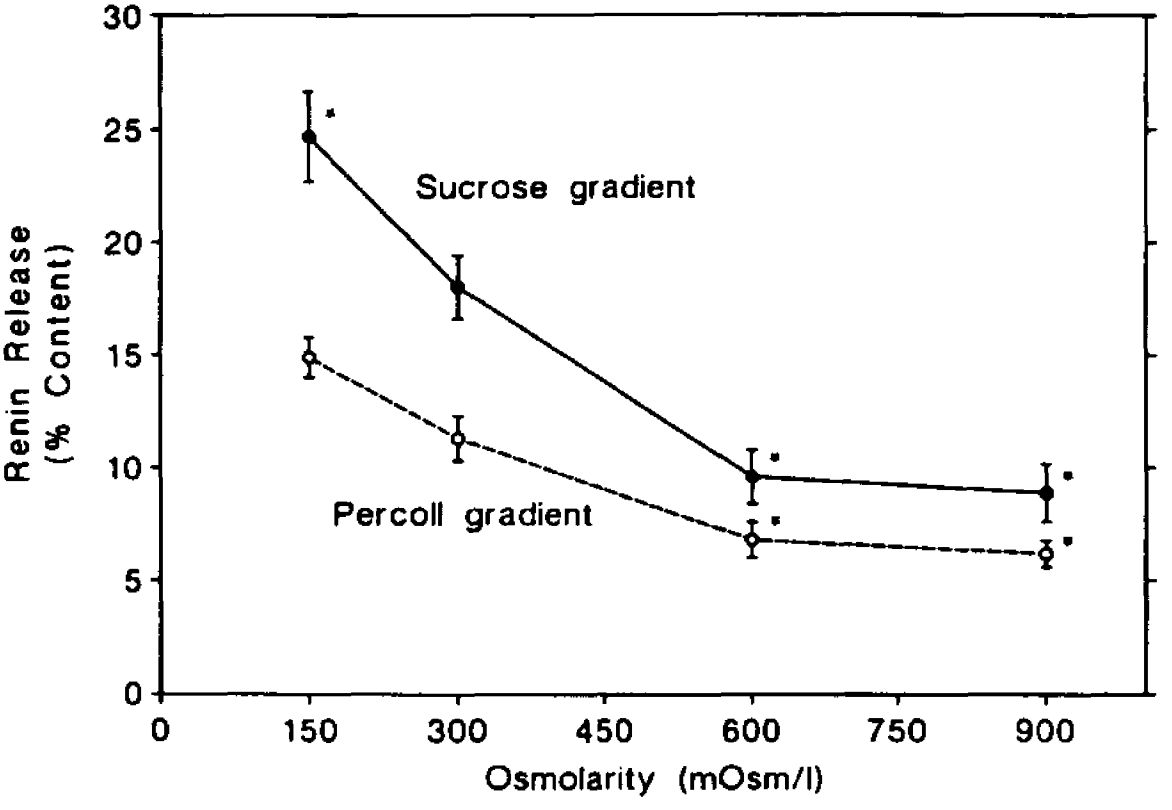
on swelling of the isolated renin granule.

B. Renin Granules

1. Experimental Results

The osmotic properties of the renin granule are important in the context of granular stability as well as in terms of the chemiosmotic mechanism of secretion. That is, the stability of the granules prior to experimental treatment may determine how they respond under varying experimental conditions. Also, the ability of the granule to increase or decrease the release of its contents based on osmotic forces may be important in terms of cellular secretion. Given their osmotic sensitivity, it would be interesting to compare the stability of renin granules isolated on sucrose to those isolated on percoll. Figure 16 shows that renin granules can be manipulated to increase or decrease the release of their contents by exposing them to solutions of varying osmotic strengths. The percentage of renin released upon transfer from the isolation medium to an iso-osmotic (300 mOsm/Kg H_2O) sucrose solution at pH 7.0 depends on the isolation procedure. Granules isolated on sucrose released 18.0 $\pm$ 1.49% of their total renin content compared to only 11.25 $\pm$1.01% released by granules isolated on percoll (n=6, $p<0.01$) . This difference in renin release may reflect

Figure 16: The Effect of Osmolality on Renin Release from Granules Isolated on a Discontinuous Sucrose and Continuous Percoll Gradient.



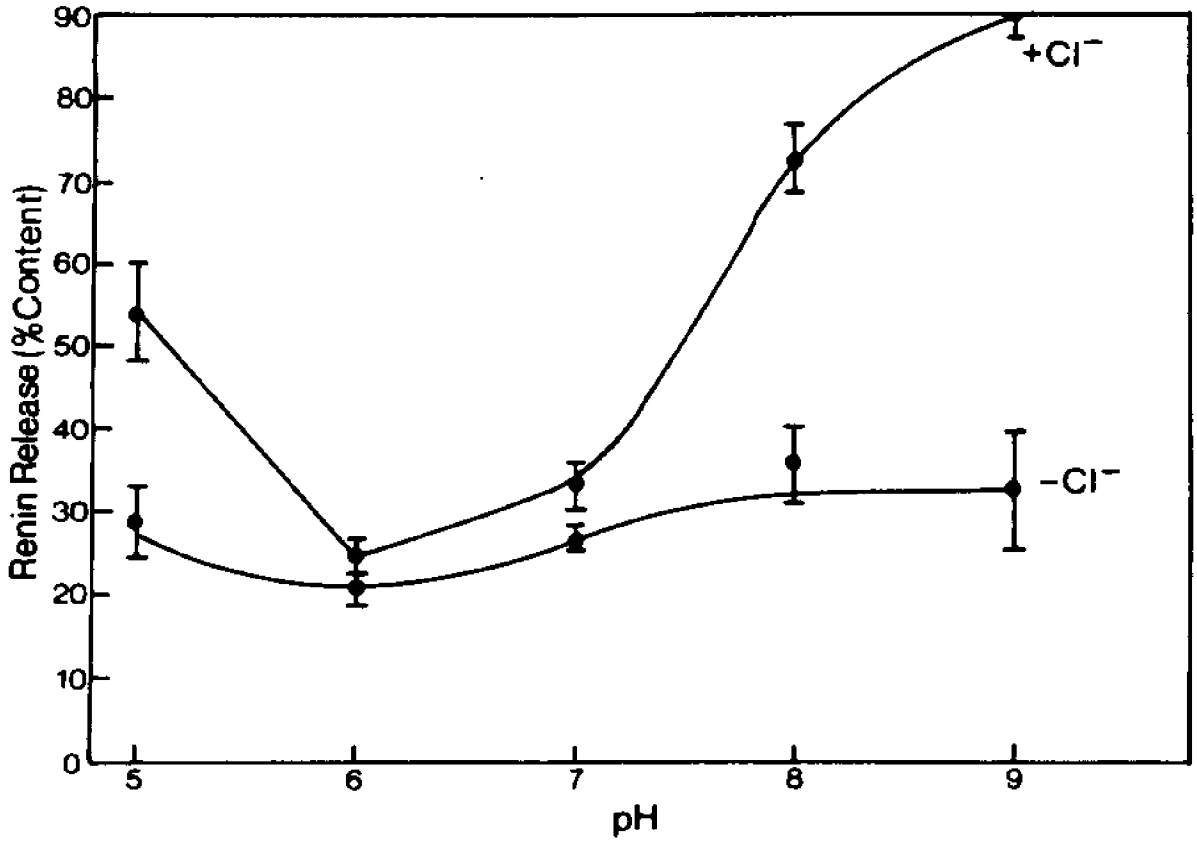
The osmolality of the incubation medium was increased or decreased by varying the sucrose concentration of the incubation medium. Renin released is expressed as a percentage of the total granular content. All samples were incubated at 37^0C for 30 minutes. Values are a mean ± S.E. n=6.
Asterisk denotes a significant difference compared to 300 mOsm/Kg H_2O, * $p< 0.05$ or lower.

differences in granular stability. That is, renin granules isolated on percoll may be more stable with regard to osmotic stress than those isolated on sucrose. The lower stability of the sucrose isolated granules could result(from efflux of water or accumulation of sucrose during the isolation procedure. Water efflux is attributed to the hyperosmotic effect of the sucrose gradient. Therefore, incubating sucrose isolated granules in an iso-osmotic incubation medium could result in greater influx of water, greater granular swelling and a greater percentage of renin released compared to granules isolated on percoll. In addition, Figure 16 demonstrates the inverse relationships between osmolality and renin release. As the osmolality is increased from 300 to 600 mOsm/Kg H_2O, the percentage of renin release decreases from 18.0 $\pm$ 1.49 to 8.77 $\pm$ 0.48 ($p<0.05$) from granules isolated on sucrose and from 11.25 $\pm$ 1.01 to 6.21 $\pm$ 0.58 ($p<0.01$) from granules isolated on percoll. On the other hand, decreasing the osmolality from 300 to 150 mOsm/Kg H_2O increases renin release from 18.0 $\pm$ 1.49 to 23.02 $\pm$ 2.04 ($p<0.05$) from sucrose isolated granules and from 11.25 $\pm$ 1.01 to 14.91 $\pm$ 2.22 from percoll isolated granules. It should be noted that at all osmolalities tested, the percentage renin release from granules isolated on sucrose was consistently greater than for granules isolated on percoll. However, the difference was statistically significant only at

osmolalities of 150 and 300 mOsm/Kg H_2O. Also, the percentages of renin released at each osmolality were significantly different from the percents released at 300 mOsm/Kg H_2O ($p<0.05$ or lower), except for the percoll isolated granules incubated in 150 mOsm/Kg H_2O.

Several series of experiments were performed to test the effect of various conditions on granular renin release. Evidence using renal cortical slices (Figure 9 and Table 5) and in other systems (28,181,276,313, 325,336,352) suggests that the pH of the incubation medium affects release from both tissues and isolated granules. Therefore, I examined renin release from isolated granules as a function of pH. As can be seen in Figure 17, varying the pH of the incubation medium had a biphasic effect on renin release. Renin release increased when the pH of the incubation was raised from 6 to 9 and again when the pH was lowered from 6 to 5. The statistically significant difference was at a level of $p<0.05$ or lower when the effects of one pH was compared with another, except for pHs 6 and 7 for which no level of significance was reached. Since it was possible that the pH of the incubation medium was affecting renin activity, I carried out experiments to test the effect of pH on the activity of soluble renin. Table 7 shows the effects of pH on renin activity. Varying the pH of the incubation medium in the presence of soluble renin obtained from isolated granules had no effect on renin activity. Therefore, the

Figure 17: The Effect of pH and Chloride on Renin Release from Isolated Granules.



Renin granules were incubated in a medium containing (mM): NaCl, 10; KCl, 137; succinic acid, 5; $MgSO_4$, 5; and Na_2-ATP, 5. In the chloride-free medium KCl was replaced with glutamate salt in equimolar concentrations. Depending on the desired pH, 10 mM of one of the following buffers were used: citric acid, pH 5; 2(N-Morpholino) ethanesuflonic acid (MES), pH 6; Imidizole, pH 7; and Tris (hydroxymethyl) aminomethan, pH 8 and pH 9. The amount of renin released is expressed as a percentage of the total granular content. All samples were incubated at 37^0C for 30 minutes. Values are a mean ±S.E. n=6.

* Denotes a significant difference compared to that observed at pH 7 in the presence of chloride, $p< 0.01$ or lower.

** Denotes a significant difference in the amount of renin released in the absence of chloride as compared to that in the presence of chloride, $p< 0.01$ or lower.

Table 7: The Effect of pH on Renin Activity.

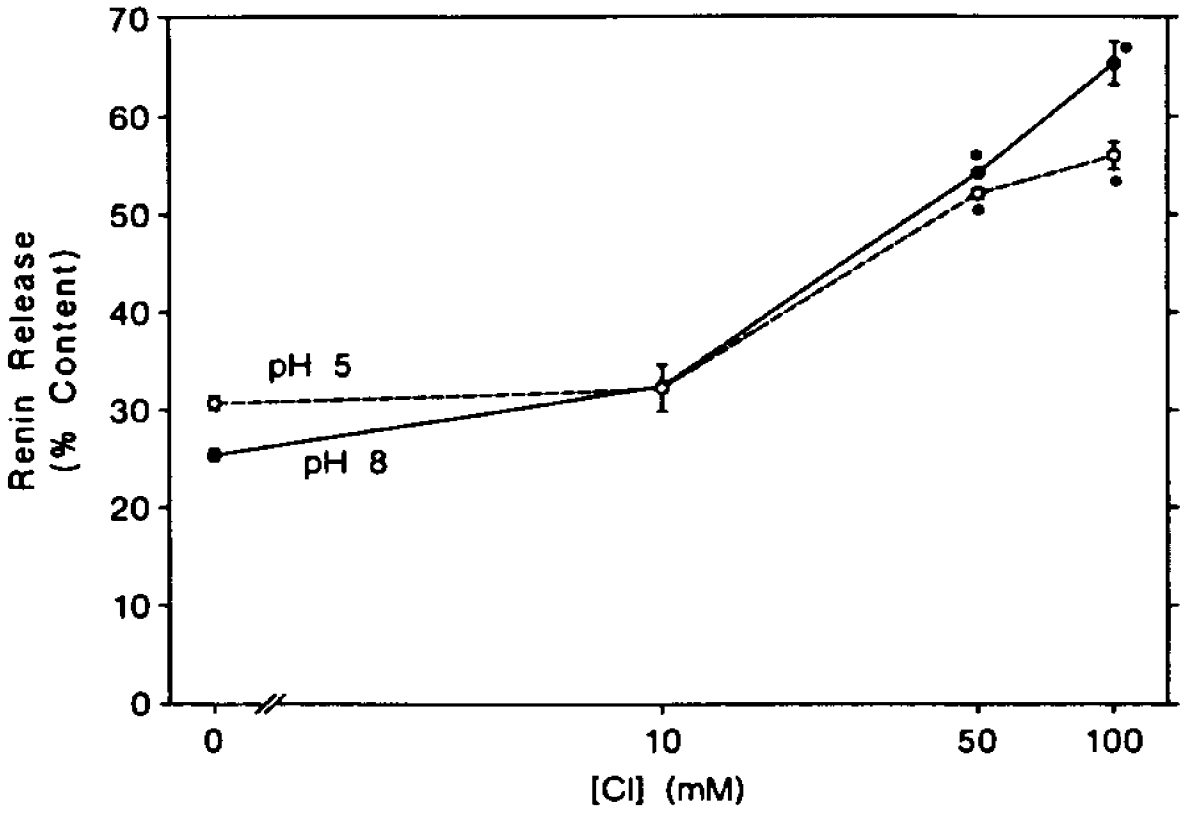
pH	Renin Activity (ng Ang I /hr./ml)
5.0	206.1 ± 2.5
6.0	196.5 ± 0.5
7.0	189.6 ± 6.2
8.0	214.2 ± 2.3
9.0	219.1 ± 8.2

Renin granules were obtained from isolated renin granules by hypo-osmotic shock followed by ultracentrifugation at 100,000xg. The renin was then exposed to pH 5, pH 6, pH 7, pH 8 or pH 9 for 30 minutes before assaying. Results show that the activity of the enzyme is not effected by these pHs. Values are a mean ± E.S. n=4.

differences in renin release observed at different pHs do not appear to be due to effects of pH on activation or inactivation of released renin. Figure 17 also demonstrates that the observed pH effect is chloride dependent ($p<0.01$ or lower), except at pH 6 and 7 in which no statistical significance was reached. Removing chloride from the incubation medium, by replacing KCl with equimolar concentrations of K-glutamate, abolished the pH effect. These results suggest that a pH gradient across the granular membrane provides the driving force needed to stimulate granular renin release in a chloride-dependent manner. In addition, Figure 17 suggests that the greater the pH gradient, the greater the driving force, and the greater the percentage of renin release. Chloride has also been demonstrated to be an important anion for granular release from other systems (15,219,299, 300,304,308).

To investigate further the involvement of chloride in granular renin release, I examined the effects of different chloride concentrations on renin release. In view of the magnitudes of release observed, pHs 5 and 8 were selected. Figure 18 shows that as the chloride concentration of the incubation medium was increased from 0 to 100 mM, renin release increased 1.8- and 2.5-fold at pH 5 and 8, respectively. These results are consistent with those shown in Figure 17 and indicate the important role chloride plays in the regulation of granular renin

Figure 18: The Effect of Varying Concentrations of Chloride on Renin Release from Isolated Granules.



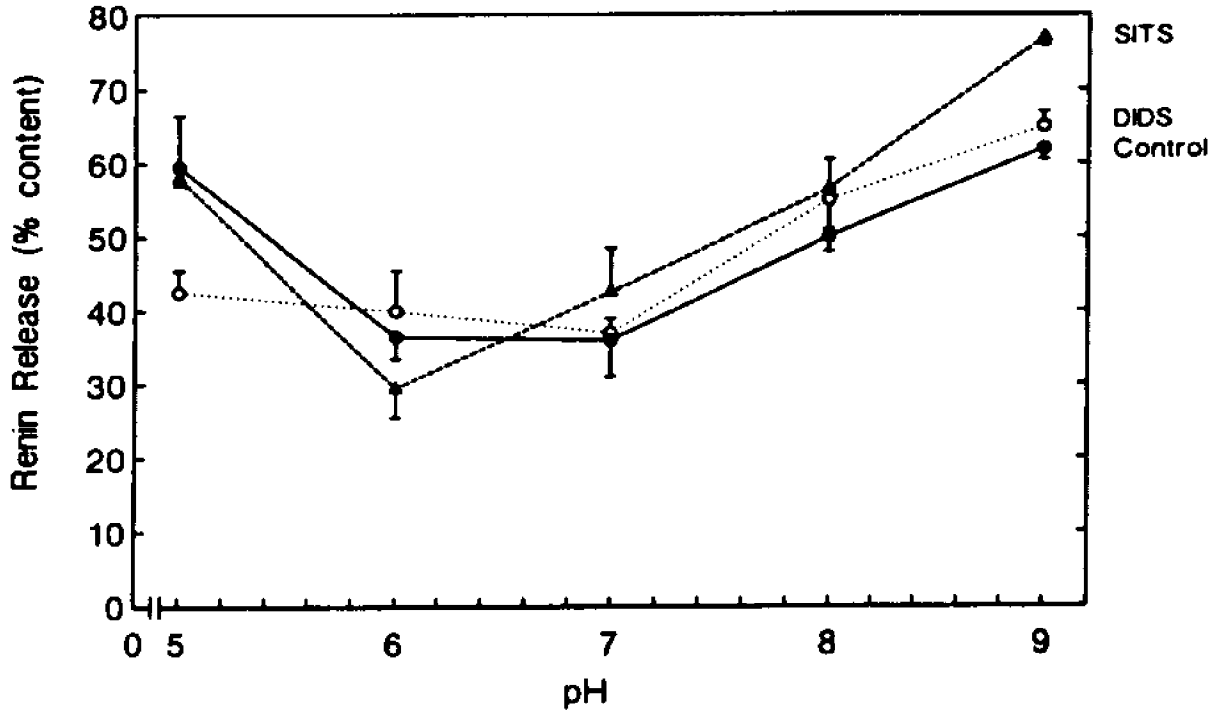
The chloride concentration of the incubation medium was varied by adding KCl and K-glutamate to obtain the desired concentration. The potassium concentration was held constant at 137 mM. Citric acid and Tris base were used to buffer the incubation medium at pH 5 and pH 8, respectively. All samples were incubated at $37^{0}C$ for 30 minutes. Renin release is expressed as a percentage of the total granular content. Composition of the incubation medium was identical to that of Figure 17, except as noted above. Values are a mean ± S.E. n=6.

* Denotes a significant difference compared to zero chloride, $p< 0.01$.

release.

Due to the apparent involvement of chloride in granular release, I assessed the effects of two anion channel blockers, 4-acetamido-4'-isothiocyano-stilbene-2-2'-disulfonic acid (SITS) and 4,4'-diisothiocyanostilbene- 2,2'-disulfonic acid (DIDS), on renin release. Such anion channel blockers have been shown to effectively block chloride transport in other systems at concentrations of 1 mM or less (219,241,254,308). Figure 19 shows that addition of SITS or DIDS to the incubation medium at a concentration of 1 mM had no statistically significant effect on renin release at any pH studied. Thus, the chloride-sensitive component of renin release does not seem to involve anion channels susceptible to these agents. In all granular systems studies to date, in which the presence of a permeant anion (ie. chloride or hydroxyl) was necessary for release, SITS and DIDS have been shown to be potent inhibitors (219,241,308,354). Thus, it is possible that the renin granular membrane is freely permeable to chloride. If this occurs <u>in vivo</u>, then chloride would enter the granule only when a significant concentration gradient exists. This might occur when the granular and plasma membranes are in close contact. However, the data shown in Figures 18 and 20 suggest that this is unlikely. Another possibility is that chloride

Figure 19: The Effect of SITS and DIDS on Renin Release from Isolated Granules.

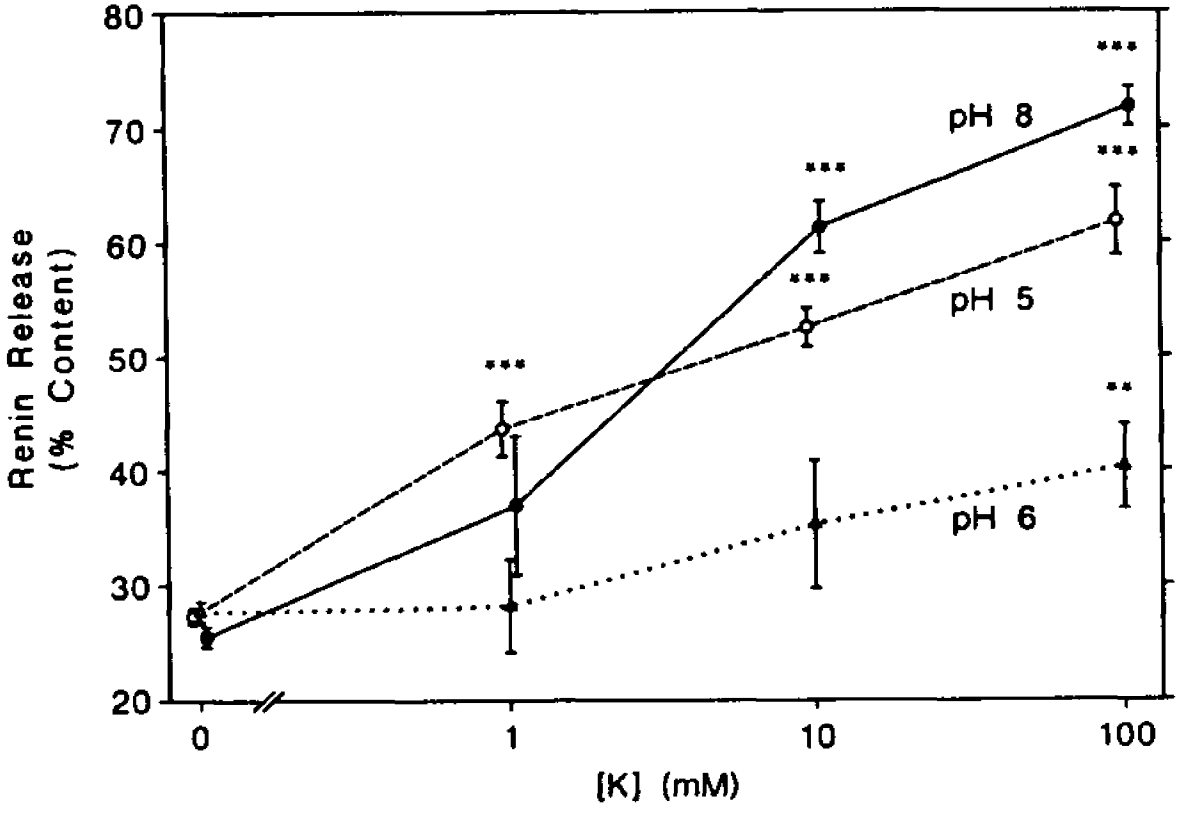


The renin granules were incubated in a medium identical to the chloride containing medium of Figure 17. One set of samples received 1 mM SITS whereas another set received 1 mM DIDS. Methanol, the vehicle for SITS and DIDS did not exceed 0.25% of the total volume. All samples were incubated at $37^{0}C$ for 30 minutes. The amount of renin released is expressed as a percentage of the total granular content. Values are a mean ± S.E. n=3.

functions as part of an antiport exchange mechanism. Under such conditions, anion channel blockers would not be expected to have any effect on renin release. The role of chloride in such an exchange mechanism would be to maintain electrical neutrality and allow for the continuous accumulation of osmotically active ions necessary for granular swelling.

A second mechanism by which osmotically active ions can accumulate is through electroneutral exchange. The ions investigated as possible candidates for such a mechanism were potassium and sodium. The role of potassium in the control of renin release was investigated because it was present in high concentration in the incubation medium; its known to affect renin secretion in vitro; and it is involved as a counter ion in the transport of protons in other systems (eg. the parietal cells of the stomach (253)). Sodium participates in Na^+/H^+ exchange and is known to affect renin secretion in vivo (153). Figure 20 shows the effects of potassium on renin release. In these experiments, the potassium ion concentration was increased over the range of 0 mM to 100 mM, while chloride concentration were maintained at 137 mM by the addition of choline chloride. As can be seen, increasing the potassium ion concentration of the incubation medium resulted in a 2.3-, 1.6-, and 2.8-fold increases in renin release at pHs 5, 6, and 8, respectively. On the other hand, increasing the sodium

Figure 20: The Effect of Increasing Potassium Concentration on Renin Release from Isolated Granules.

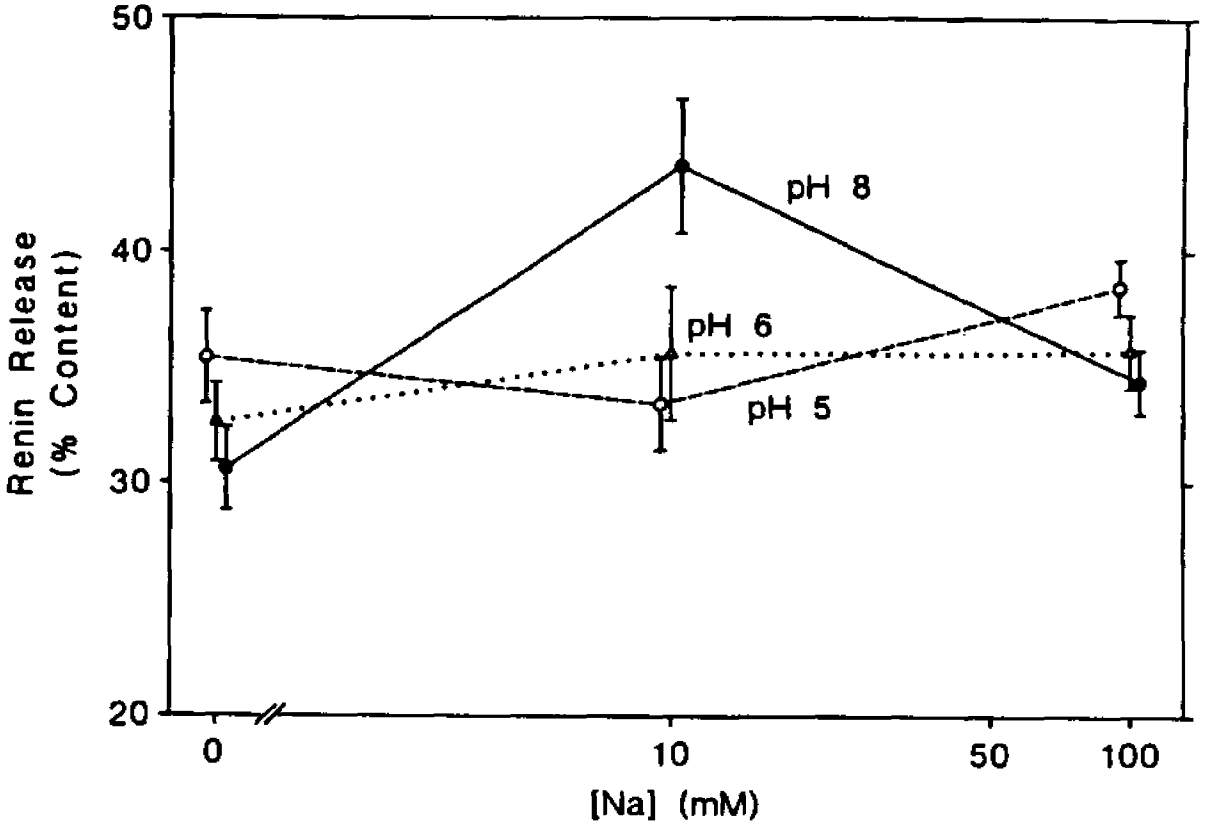


Renin granules were incubated at increasing concentrations of potassium at pH 5, 6, and 8. The ionic composition of the incubation medium was the same as the chloride containing medium of Figure 17 except for the following changes: the chloride concentration was held constant while the potassium ion concentration was varied by the addition of KCl and choline chloride to obtain the desired potassium concentration. The renin granules were incubated at 37^{0}C for 30 minutes. The amount of renin released is expressed as a percentage of the total granular content. Values are a mean ±S.E. n=4.
Asterisks denote a significant difference compared to zero potassium, ** $p < 0.02$, *** $p < 0.001$.

concentration of the incubation medium from 0 to 100 mM was without effect (Figure 21). It should be noted that the low level of renin release in Figure 21 may be due to the absence of potassium from the incubation medium.

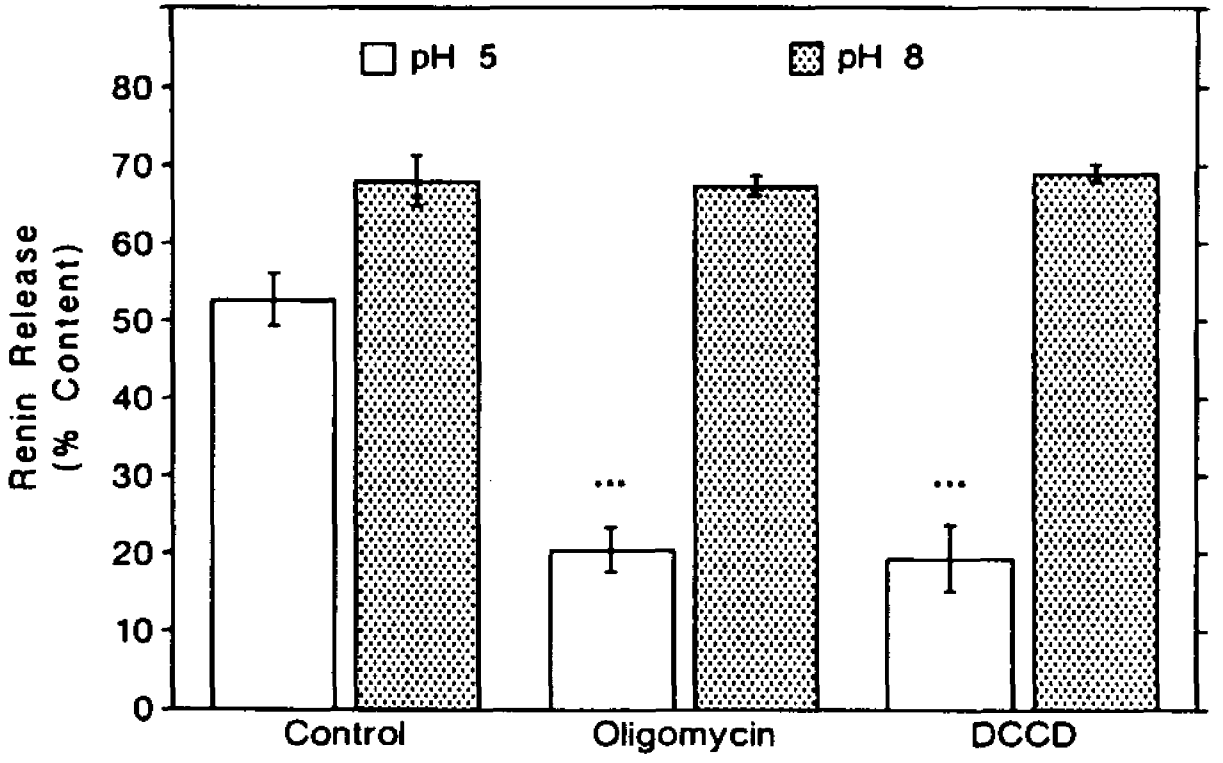
The following studies provide new insight into the role of hydrogen ions in granular renin release. The effect of pH on renin release is interesting and has been shown to occur in other secretory systems (28,181,313,325,336,352). In most systems previously studied, the granular matrix has been shown to be acidic with respect to the cytosol. In these earlier studies, the granular membrane was shown to be impermeable to protons and to contain a proton ATPase. These findings, as well as the results from studies using renal cortical slices, prompted me to examine the effect of two proton ATPase inhibitors, oligomycin (312) and N,N'-dicyclohexylcarbodiimide (DCCD) (12), on granular renin release. Initial studies using granules isolated on sucrose revealed no effect of oligomycin or DCCD on granular release. However, due to technical problems associated with the studies, I decided to reinvestigate the effect of these agents on percoll isolated granules. The experiments were repeated using 50 ug oligomycin/ml (which has been shown to inhibit renin release from renal cortical slices) and 10^{-6}M DCCD. As can be seen in Figure 22, both oligomycin and DCCD inhibited renin release about 2.5-fold compared to control at pH 5

Figure 21: The Effect of Increasing Sodium Concentration on Renin Release from Isolated Granules.



Renin granules were incubated in a medium containing (mM); $MgSO_4$, 5; succinic acid, 5; Na_2-ATP, 5; NaCl, 0 to 100. Choline chloride was added as needed to maintain iso-osmotic conditions. The granules were incubated at 37^0C for 30 minutes. The amount of renin released is expressed as a percentage of the total granular content. Values represent a mean ± S.E. n=4.

Figure 22: The Effect of Oligomycin and DCCD on Renin Release from Isolated Granules.

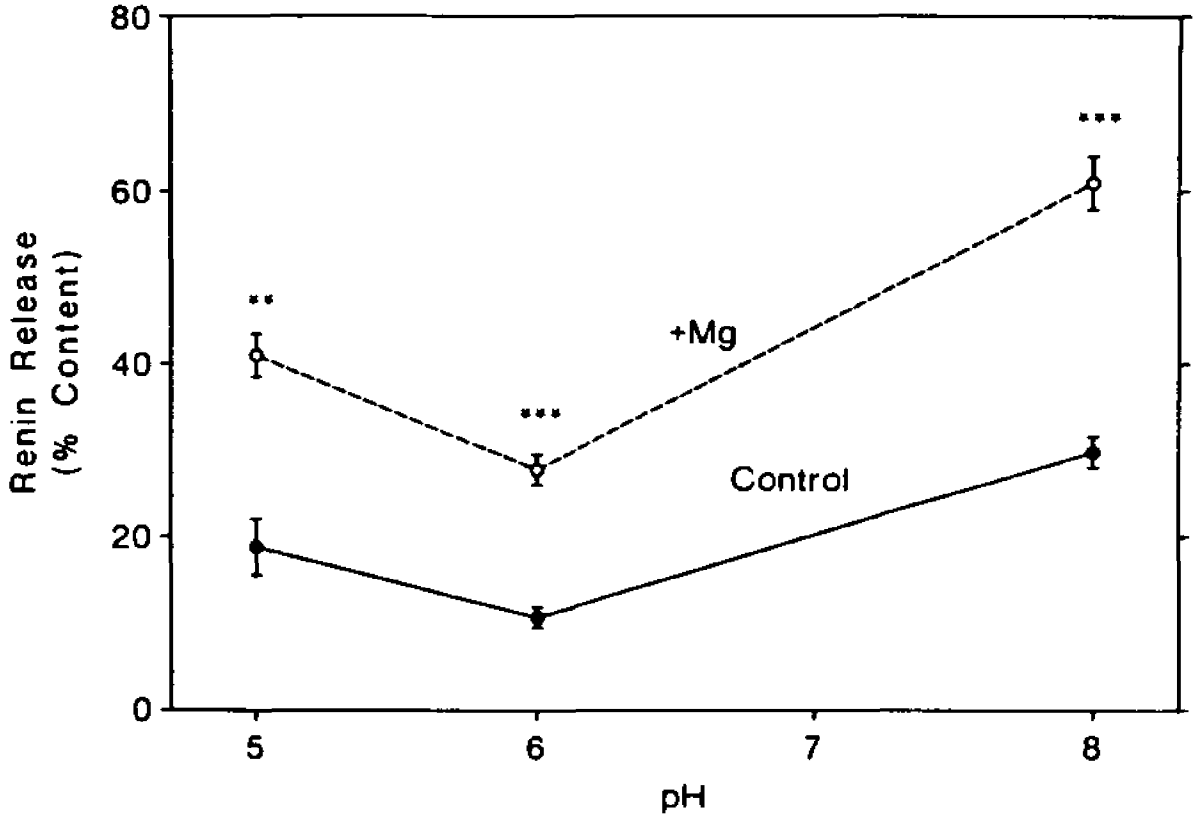


Renin granules were incubated in a medium identical to the chloride containing medium of Figure 17. In addition the medium also contained either 50 ug/ml oligomycin of 10^{-6} M DCCD. The concentration of ethanol, the vehicle for the drug, did not exceed 0.25% of the total volume. The granules were incubated at 37^{o}C for 30 minutes. The amount of renin released is expressed as a percentage of the total content. Values are a mean ± S.E. n=6.
*** Denotes a significant difference compared to control, $p< 0.001$.

($p<0.001$), but had no effect on release at pH 8 ($p>0.05$). These results are consistent with the view that a proton pump exists in the granular membrane. At pH 5 the proton concentration of the incubation medium is high enough and the electrochemical gradient is low enough, to allow the proton ATPase to actively transport protons. The transport of protons creates a gradient which drives the transport of other ions into the granule. The net effect is movement of osmotically active anions into the granule, followed by inward movement of water. The end result is granular swelling and renin release. Therefore, addition of oligomycin or DCCD results in inhibition of proton transport which directly inhibits renin release at this pH. On the other hand, at pH 8, due to a low substrate concentration and hence a potentially large electrochemical (proton) gradient, the activity of the proton ATPase is not required for granular release; therefore, the addition of oligomycin or DCCD would be without effect. The energy required for the accumulation of osmotically active ions would be provided by the pH gradient.

If a proton ATPase exists in the granule membrane one would expect that its activity would be sensitive to the magnesium concentration of the incubation medium, since magnesium is a cofactor for ATPase activity. Therefore, the effects of 1 and 10 mM magnesium on granular renin release were investigated. As can be seen in Figure 23,

Figure 23: The Effect of Increasing Concentrations of Magnesium on Renin Release from Isolated Granules.

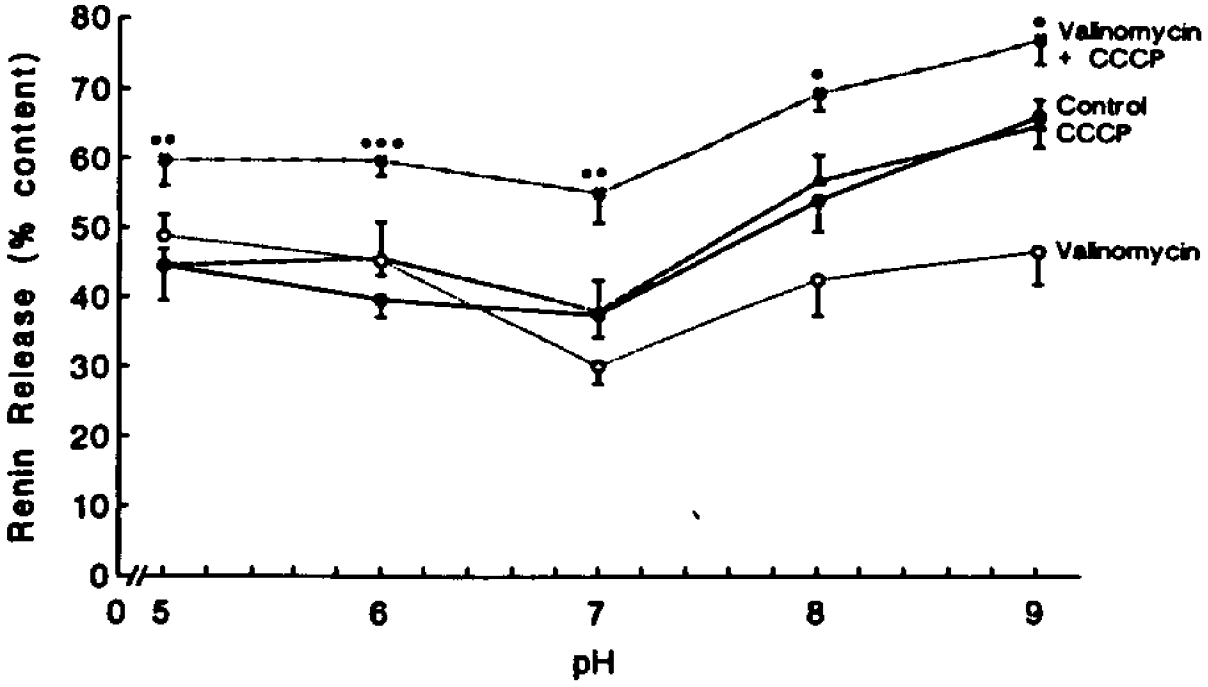


Renin granules were incubated in a medium identical to the chloride containing medium of Figure 17, in the presence or absence of 10 mM magnesium, at varying pH. The granules were incubated at $37^{0}C$ for 30 minutes. The amount of renin released is expressed as a percentage of the total granular content. Values are a mean ± S.E. n=4.
Asterisks denote a significant difference from control, ** $p< 0.02$, *** $p< 0.001$.

granular renin release increased 2.2-, 2.6-, and 2.1-fold at pHs 5, 6 and 8, respectively, upon raising the magnesium concentration. All increases were significant to a level of $p<0.01$ or lower.

Due to the importance of potassium and a proton gradient in granular renin release, the effects of carbonylcyanide,m-chlorophenyl hydrazone (CCCP), a proton ionophore (168), and valinomycin, a potassium ionophore (310), were investigated. As can be seen in Figure 24, the addition of 15 uM CCCP did not change renin release compared to control. A 10-fold increase in CCCP concentration was also without effect. Similarly, the addition of 5 uM valinomycin did not change renin release at pHs 5, 6, and 7, but was inhibitory at pHs 8 and 9 (Figure 24). However, the inhibition reached statistical significance only at pH 9 ($p<0.01$). On the other hand, if 15 uM CCCP and 5 uM valinomycin were added together, a general increase in renin release (between 15% and 35%, $p<0.05$) was observed. While ionophores have been shown to cause granular lysis and release in other systems (93,135,198,136), it is clear from this study that neither valinomycin nor CCCP had any effect when presented alone. The lack of effect by CCCP may be explained by the inability of this agent to equilibrate protons across the granular membrane. Thus, in the presence of CCCP alone transport of a few protons dissipates and possibly reverses the electrical gradient, and inhibits further

Figure 24: The Effect of Valinomycin and CCCP on Renin Release from Isolated Granules.



Renin granules were incubated in a medium of the same composition as the chloride containing medium of Figure 17. CCCP (15 uM) and valinomycin (5 uM) were added separately or together to the isolated renin granule suspension. The concentration of methanol, the vehicle for the drug, never exceeded 0.05%. The granules were incubated at 37^{0}C for 30 minutes. The amount of renin released is expressed as a percentage of the total granular content. Values are a mean ± S.E n=6.
Asterisks denote a significant difference form control, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

proton translocation. Dissipation of the electrical gradient may require the translocation of too few protons to affect the pH gradient. Therefore, under these conditions, renin release is unaffected. If, however, an ion with a similar charge moves in the opposite direction, proton transport continues. This can explain the results obtained by the CCCP/valinomycin combination. The stimulatory effect seen in the presence of both ionophores results from the net gain of osmotically active ions, followed by inward movement of water, with the resultant granular swelling and renin release. The situation, however, may be different at pH 5, a pH at which a proton gradient may not exist. Under these conditions one would not expect a protonophore to have any affect. However, in the presence of both CCCP and valinomycin, electroneutral exchange occurs, resulting in increased granular release. The above possibilities must, however, await direct proof.

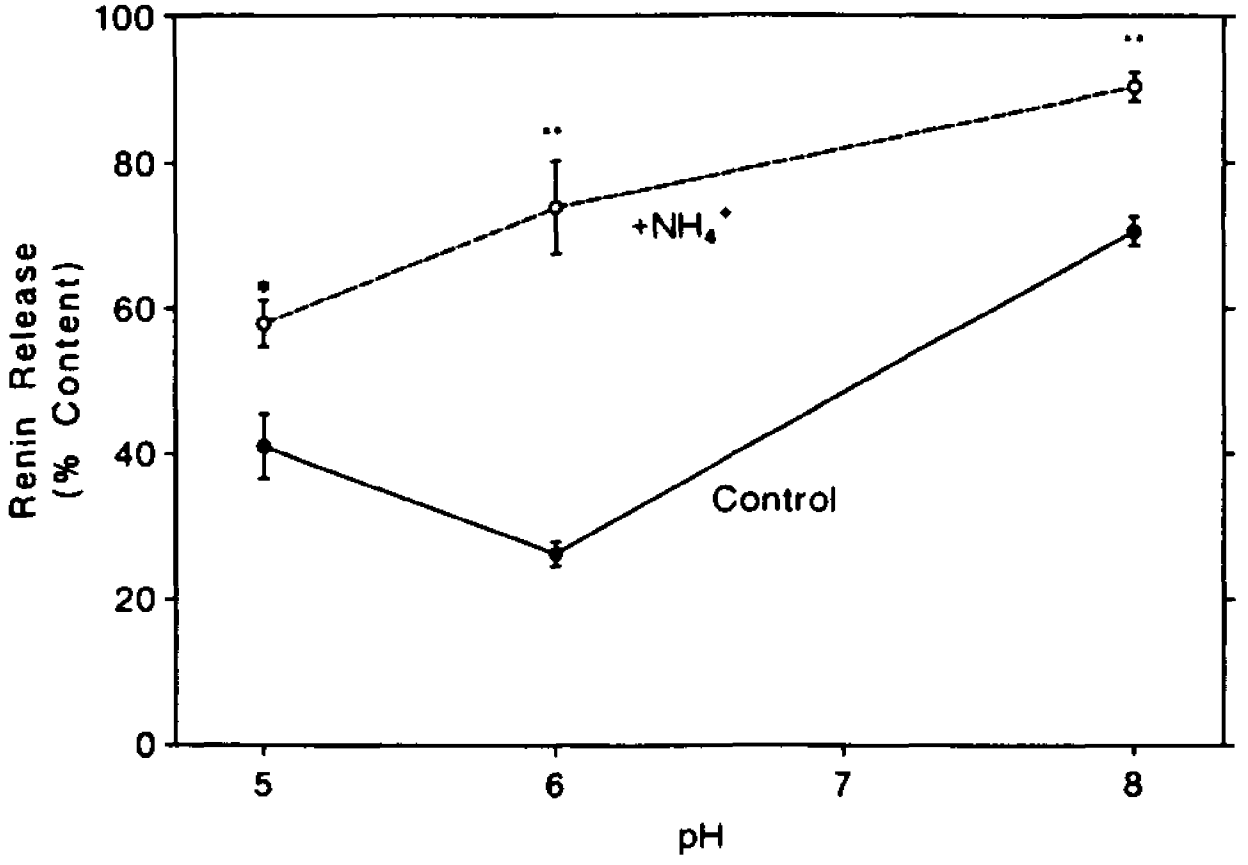
If the granular matrix is acidic with respect to the incubation medium, then exposing the granules to a weak base should increase renin release because a weak base in its uncharged form can freely cross the granular membrane. Upon entering the granular matrix the base would become protonated and therefore trapped inside the granule. The protonated base would cause an increase in the osmotic strength of the granular matrix, resulting in the influx of water, with granular swelling and renin release. The concentration gradient, by which the base enters the

granule, is maintained by its protonation.

Figure 25 shows the results of experiments in which 30 mM NH_4Cl was added to the incubation medium. At this concentration, NH_4Cl has been shown to decrease the proton gradient in chromaffin granules by 35% (198). In these experiments adding 30mM NH_4Cl increased granular renin release by 40%, 185% and 28% over control, at pHs 5, 6 and 8 respectively ($p<0.02$ or lower). The increase in renin release at pH 6 is more than twice that at pH 5. This could be the due to the presence of a greater pH gradient across the membrane. In addition, the increase in medium pH (from 5 to 6) results in a 10-fold increase in the concentration of NH_3 in the incubation medium. This increase in the permeable form of the weak base would result in greater accumulation of its protonated form within the granule matrix; the greater the accumulation (of NH_4^+) the greater the osmotic gradient driving granular swelling and renin release. Therefore, the amount of weak base accumulated depends on the pH of the incubation medium, the proton gradient across the granular membrane, and the pH of the granular matrix.

The above data suggest that potassium and hydrogen ions are very important for the regulation of granular renin release. Therefore, it is possible that renin release is stimulated by activation of a H^+/K^+ antiport exchanger in the presence of chloride, or by an

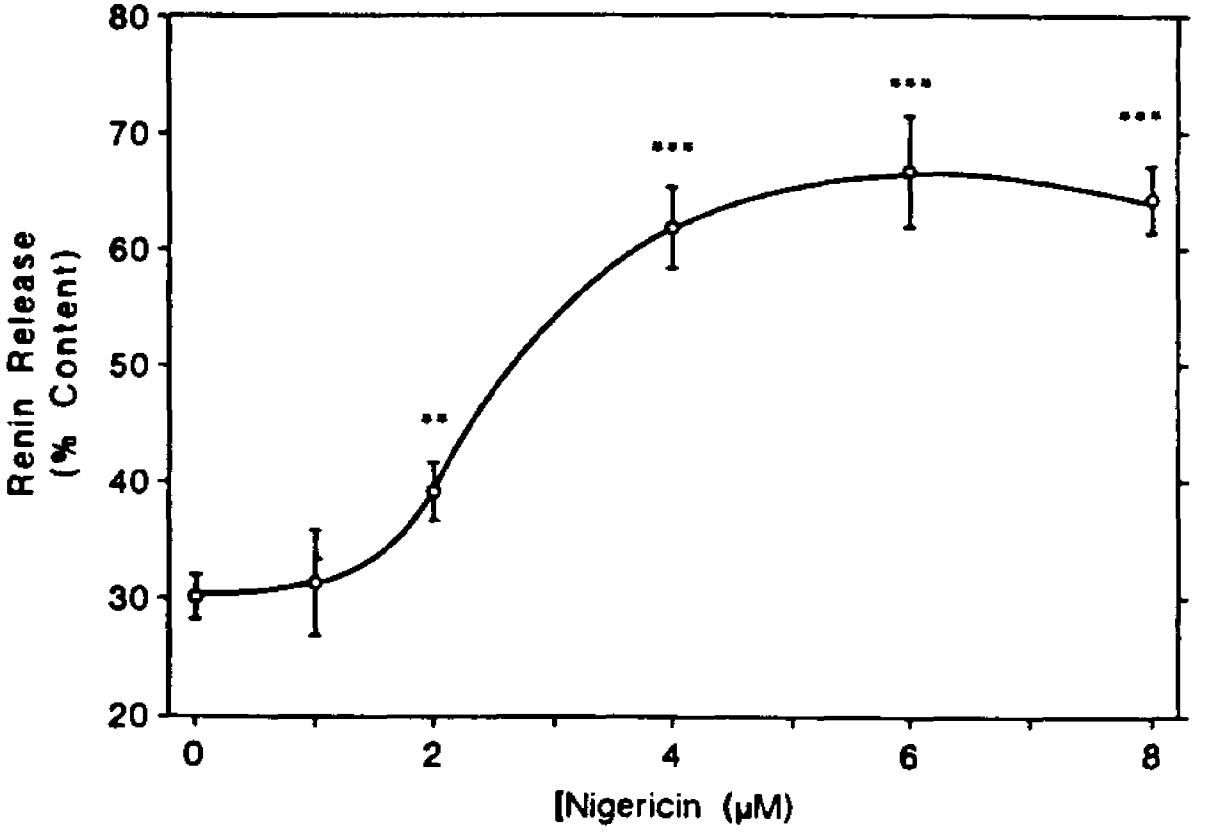
Figure 25: The Effect of NH_4Cl on Renin Release from Isolated Granules.



The renin granules were incubated in a medium identical to the chloride containing medium of Figure 17. Renin granules were incubated at pH 5, pH 6 and pH 8 in the presence and absence of 30 mM NH_4CL at 37^0C for 30 minutes. The amount of renin released is expressed as a percentage of the total granular content. Values are a mean ± S.E. n=6.
Asterisks denote a significant difference from control, * $p< 0.05$, ** $p< 0.001$.

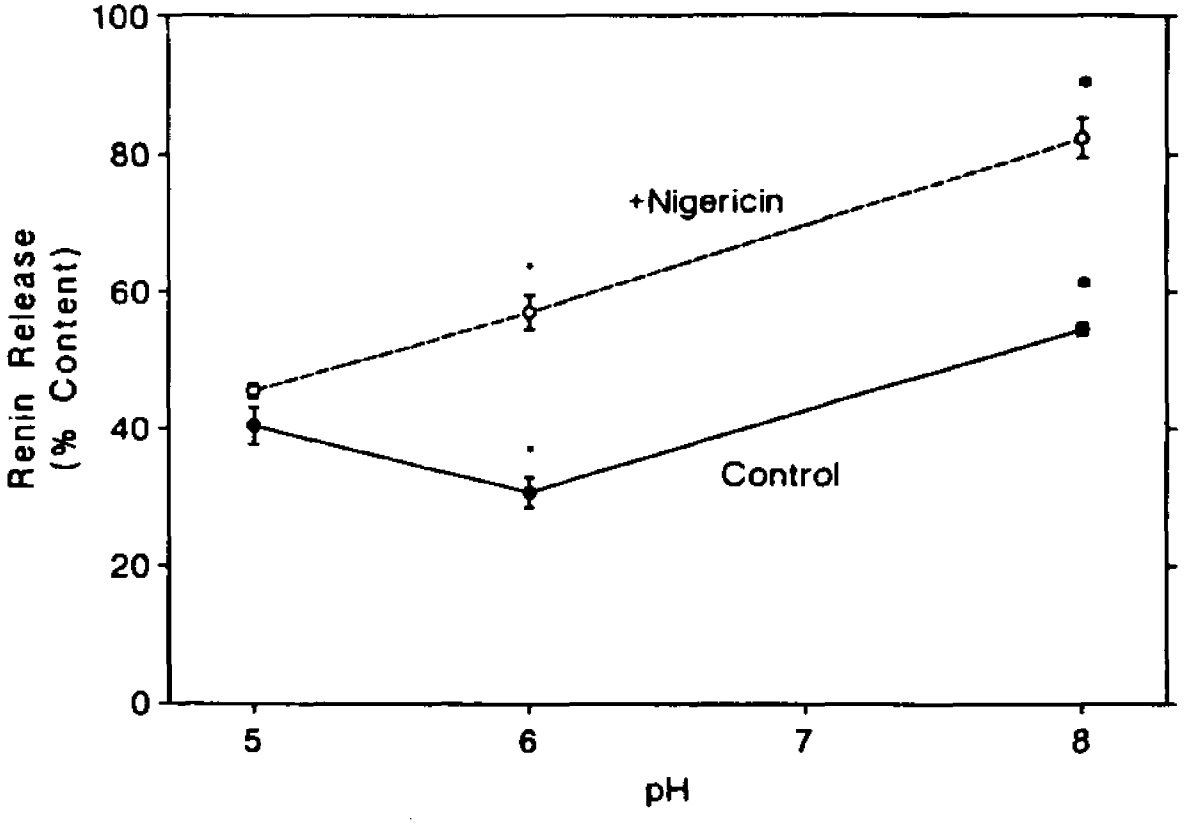
antiport-exchanger-symport complex in which all three ions are involved. If this hypothesis is correct, then one should be able to stimulate renin release with an ionophore that exchanges protons for potassium ions in equimolar concentrations. One such ionophore is nigericin. Figure 26 shows a dose-response curve for nigericin. As can be seen, the increase in granular renin release parallels increasing concentrations of nigericin up to 4uM, at which point the curve plateaus. All subsequent experiments were performed with 4 uM nigericin. Figure 27 shows the effect of nigericin on renin release at different pHs. Nigericin increased granular renin release at all pHs studied. The largest effects were observed at pH 6 and 8 Under these conditions, renin release increased 93% and 51%, respectively ($p<0.001$). The small increase at pH 5 was not significantly different from control and suggests that the proton gradient across the granular membrane is minimal at this pH. This small pH gradient may reduce nigericin's ability to translocate protons and thereby attenuate the increase in renin release. The increase in renin release by nigericin was demonstrated to be potassium-dependent and chloride-independent (Figure 28). These results suggest that a H^+/K^+ exchanger can stimulate renin release in the presence of a proton gradient. The ability of nigericin to increase renin release in the absence of chloride, and the chloride dependency of Figures 17 and 18

Figure 26: The Effect of Nigericin on Renin Release from Isolated Granules.



Renin granules were incubated in a medium identical to the chloride containing medium of Figure 17. The granules were exposed to concentrations of nigericin ranging from 0 and 8 uM for 30 minutes at 37^{0}C. The amount of renin released is expressed as a percentage of the total granular content. Values are a mean ± S.E. n=4.
Asterisks denote a significant difference compared to zero nigericin, ** $p < 0.01$, *** $p < 0.001$.

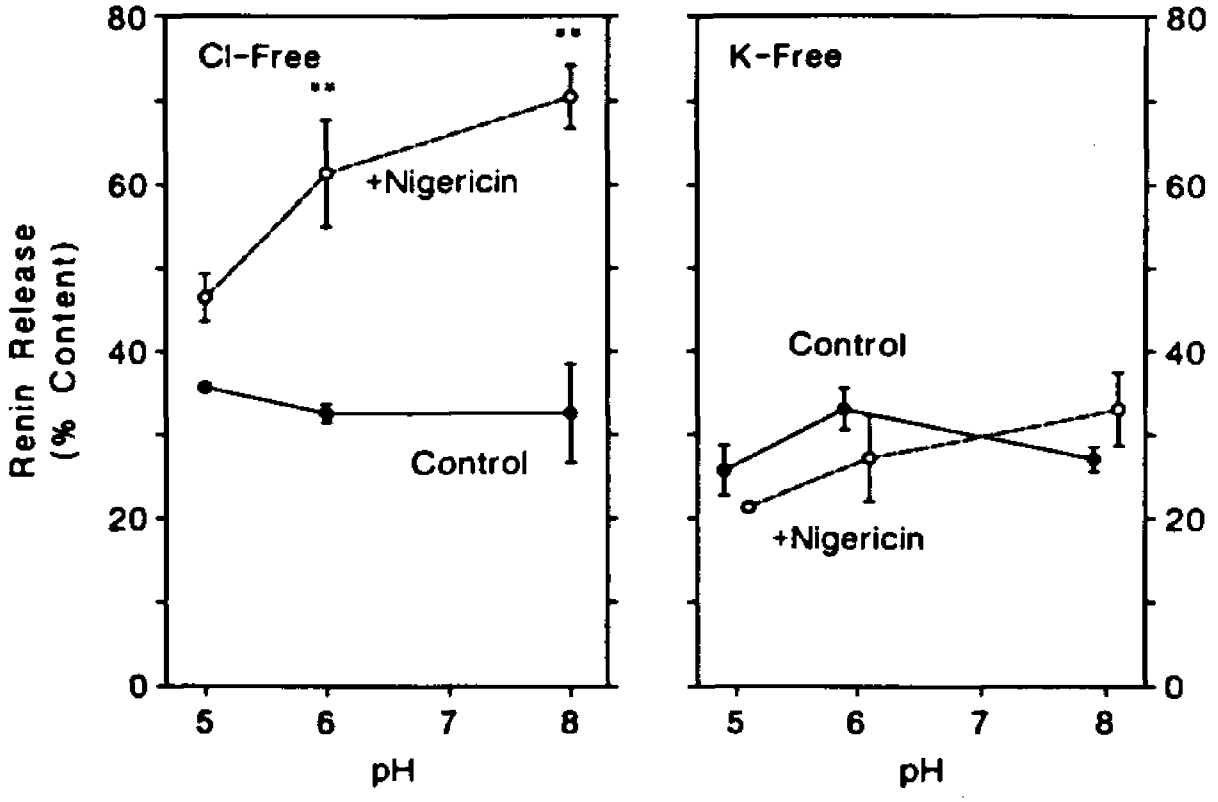
Figure 27: The Effect of Nigericin on Renin Release from Isolated Granules at pH 5, 6, and 8.



Renin granules were incubated in the presence or absence of 4 uM nigericin in an incubation medium identical to the chloride containing medium of Figure 17. The granules were incubated at 37^{0}C for 30 minutes. Tha amount of renin released is expressed as a percentage of the total granular content. Values are a mean ± S.E. n=4.

* Denotes a significnat difference from control, p< 0.001.

Figure 28: The Effect of Nigericin on Renin Release from Isolated Granules Incubated in a Potassium- or Chloride-free Medium.



Renin granules were incubated in a potassium-free and chloride-free incubation mediums containing 4 uM nigericin. The potassium-free medium contained the following components (in mM): Nacl, 10; choline chloride, 137; $MgSO_4$, 5; Na_2-ATP, 5. In the chloride-free medium Na_2SO_4 was substituted for NaCl and K-glutamate replaced choline chloride in equimolar concentration. The granules were incubated at 37^0C for 30 minutes. The amount of renin released is expressed as a percentage of the total granular content. Values are means ± S.E. n=4.

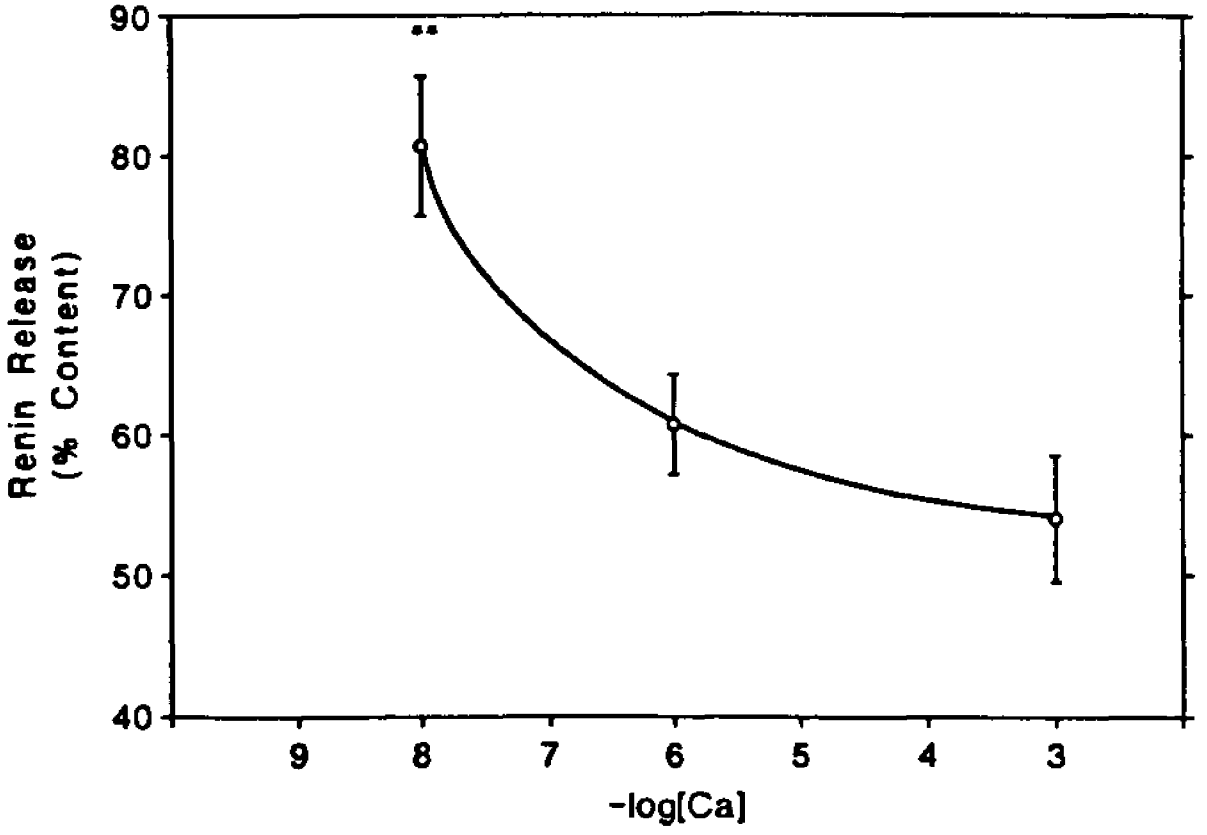
** Denotes a significant difference compared to control, $p< 0.01$.

suggest that the granular antiport exchanger requires the inward transport of chloride. The chloride requirement for granular release could be expressed in one of four ways. First, assuming that the antiport normally operates electrogenically (that is, the proton for potassium exchange ratio is greater that 1), then chloride movement could be required to maintain electroneutrality. Second, even if the exchange of proton for potassium were electroneutral, the extrusion of protons could lead to generation of an electrical gradient if the granules have a high buffering capacity. If such were the case, the loss of a proton would not reduce the free proton concentration within the granule, since protons associated with intragranular proteins would dissociate. Thus chloride might be required to neutralize excess charge within the granules. Third, proton for potassium exchange and the resultant decrease in granular proton concentration might activate the proton ATPase, and perhaps chloride movement accompanies this activation. These three possibilities seem unlikely, however, since SIDS and DIDS do not inhibit renin release. The fourth and most likely hypothesis is that the antiport exchanger involves the transport of all three ions. The stoichiometry, however, must be such that electroneutrality is maintained.

Calcium is an important ion in the regulation of a variety of cellular mechanisms. Its importance in the

regulation of renin secretion was demonstrated above using renal cortical slices. Calcium provides an inhibitory signal for renin secretion in contrast to its role in most glandular cells. In addition to its effects at the cellular level, calcium and other divalent cations have been shown to directly affect granule aggregation. (96,151). For this reason the effect of calcium on granular renin release was investigated. As can be seen in Figure 29, calcium inhibited granular renin release. As the calcium concentration of the incubation medium was lowered from 10^{-3} to 10^{-8}M, granular renin release increased by 16%, a change that was not statistically significant. However, decreasing the calcium concentration to 10^{-8} increased renin release by an additional 27% ($p<0.01$). These results are consistant with the inhibitory effect calcium has on renin secretion from JG cells (see Figures 6 and 7). However, the degree to which increasing calcium concentrations decrease granular renin release is much smaller than that observed in renal cortical slices (Figures 6 and 7). This may indicate that calcium's inhibitory effect on renin release is through the activation of some cytosolic component. In the isolated granule preparation, the isolation procedure could have removed a large portion of the cytosolic component(s) required for calcium's inhibitory action. Under these conditions, calcium's effect would be severely blunted.

Figure 29: The Effect of Calcium on Renin Release from Isolated Granules.



Renin granules were incubated in a medium identical to the chloride containing medium of Figure 17. The calcium concentration of the incubation medium was buffered at either 10^{-8}, 10^{-6}, or 10^{-3} M free-calcium. The desired free-calcium concentration was achieved by the addition of the appropiate amount of $CaCl_2$ which was calculated based on the apparent stability constant of the Ca^{2+}-EGTA complex of $10^{6.69}$ M^{-1} (164). The granules were incubated at $37^{0}C$ for 30 minutes. The amount of renin released is expressed as a percentage of the total granular content. Values are a mean ± S.E. n=7.

*** Denotes a dignificant difference compared to either 10^{-6} or 10^{-3} M free-calcium, $p< 0.01$.

IV. DISCUSSION

These studies provide new insight into the involvement of calcium, cAMP and osmotic forces in the control of renin secretion. The study of calcium has proved timely because it is an important second messenger in the control of renin secretion. The mechanism whereby calcium regulates renin secretion differs from that of most cells in that raising intracellular calcium inhibits renin secretion whereas this ion generally stimulates secretion in other systems. It is widely accepted that stimulation of catecholamine secretion from chromaffin cells (94) or insulin release from beta cells (428,429), requires elevation of cytosolic calcium levels. The general hypothesis of stimulation-secretion coupling shows calcium as the second messenger or cellular signal for the secretory response. Its function is to translate the actions of a diverse number of stimuli into a common exocytotic response. Exocytosis is a ubiquitous cell function that involves a sequence of events including intracellular transport and fusion of membrane-bound cytoplasmic granules with the plasma membranes. The lipid bilayer separating the granular contents from the extracellular space than ruptures- a step Pollard and others (305,307) refer to as fission. Exocytosis serves as a mechanism by which granular contents are secreted into the extracellular space and membrane proteins are

incorporated into the plasma membrane. According to the work by Gumbiner and Kelly (156) there are two pathways for exocytosis: constitutive and regulated. Constitutive exocytosis occurs independently of extracellular stimuli and proceeds at a steady rate. It is responsible for the incorporation of proteins into the plasma membrane and the secretion of the matrix. On the other hand, regulated exocytosis is triggered by an extracellular stimulus and occurs only in the presence of the stimulus. This latter pathway mediates secretion of hormones, neurotransmitters, and enzymes. In addition, it provides a mechanism by which membrane proteins can be rapidly inserted into the plasma membrane (234). As stated above, the current hypothesis in relation to renin secretion is that an increase in cytoplasmic calcium is an inhibitory signal (22,119,294,410). The inverse relationship between renin secretion and cytosolic calcium is supported by the data in Figures 6 and 7. As cytosolic calcium is raised from 10^{-8} to 10^{-5}M, renin secretion is inhibited.

The steepest part of the curve, in which small changes in intracellular calcium lead to large changes in renin secretion, is between 10^{-8} and 10^{-7}M, a range within the physiological limits of the cell as measured by Tsien et al. (398). In addition, Figure 6 suggests that renin secretion is maximally inhibited between 10^{-8} and 10^{-5}M calcium. The critical calcium concentration in Figure 7 seems to be roughly 1.8 x

10^{-8}M. At this concentration renin secretion is neither stimulated nor inhibited, and any deviation from this value results in a change in renin secretion. If calcium levels are raised above 1.8×10^{-8}M, renin secretion is inhibited, whereas if cytosolic calcium falls below this level renin secretion is stimulated. This extrapolated value appears to underestimate the reported value, based on Quin 2 measurements, of 3×10^{-7}M (222). The lower value obtained from Figure 7 could indicate that cytosolic calcium levels at the time of depolarization, in these experiments (measured one hour after the start of the experiment), was lower that the comparable value obtained by Kurtz and coworkers. In Figure 7 a ratio of one indicates that the rate of renin secretion was the same before and after depolarization. This suggests that the net calcium flux under these conditions was zero. The possiblity arises that during the first hour of incubation, in which the renal cortical slices were exposed to the desired calcium concentration in standard KRB, a new 'resting' cytosolic calcium concentration was established. Upon depolarization, (at the start of the second hour of incubation), renin secretion would be stimulated; however, the ratio (E/C) would be less than expected. Under these conditions the curve in Figure 7 would be displaced to the left, resulting in an underestimation of the critical calcium concentration (ie. E/C=1). Also, if one compares the

ratios of renin secretion at 10^{-7} vs. 10^{-6} M calcium (Figure 7) no difference is observed, indicating that renin secretion was not affected by a 10-fold change in calcium. However, in Figure 6, lowering cytosolic calcium from 10^{-6} to 10^{-7} M enhanced renin secretion 2-fold. The discrepancy between Figures 6 and 7 again suggests that the cytosolic calcium concentration extrapolated from Figure 7 is an underestimation. Most stimuli which inhibit renin secretion require extracellular calcium. This has been demonstrated for high perfusion pressure (126), alpha adrenergic agonists (410), angiotensin II (65,410), antidiuretic hormone (66,407), high extracellular potassium (126,294), ouabain (67,294) and vanadate (68). The inhibitory effects of these widely diverse factors can be blocked by organic (65,236,292) and inorganic (236,423) calcium channel blockers. Although such factors were not examined in the present studies, their calcium dependence suggests that they may regulate renin secretion by shifting the curves shown in Figures 6 and 7.

Based on the belief that JG cells are derived from smooth muscle cells, it has been suggested that renin secretion and contraction are coupled by calcium. The present studies may shed some light on this hypothesis. Intracellular calcium concentrations between 10^{-5}M and 10^{-6}M caused maximum inhibition of renin secretion and maximum contraction of vascular smooth

muscle cells (5,208). This may explain why various agents that constrict the renal vasculature also inhibit renin secretion (130,410). However, not all agents which stimulate renin secretion dilate the renal vessels (119,126,236,410). For example, perfusing the isolated kidneys with a calcium-free perfusate results in stimulition of renin secretion with little change in vessel diameters (126,130). This can be explained in terms of Figure 6 and 7. The 10^{-8}M calcium is an order of magnitude lower than the reported threshold of 10^{-7}M needed for muscle contraction (5,208). Therefore, by lowering the intracellular calcium concentration below 10^{-7}M, renin secretion would increase without a change in the contractile state of the JG cell or vascular smooth muscle. It can therefore be concluded that whereas both renin secretion and smooth muscle contraction (including contraction of the JG cell) are regulated by intracellular calcium, the concentrations which control these events are different for the two processes.

cAMP is another intracellular messenger which is involved in the regulation of renin secretion. However, the manner by which cAMP stimulates renin secretion is unknown. The studies using forskolin were designed to investigate the mechanisms of cAMP stimulated renin secretion. Forskolin is a useful tool since it is a potent activator of adenylate cyclase (341) that acts

independently of the beta-adrenergic receptor. Therefore interactions between agonist and receptor need not be considered. As stated in the review of the literature, there are two conflicting views of how cAMP stimulates renin secretion. Some investigators (69,223) postulate that cAMP stimulates renin secretion by lowering cytosolic calcium. Their conclusions are based on the ability of increasing cytosolic calcium to block the stimulatory effect of isoproterenol or cAMP. However, there is also evidence suggesting that cAMP acts independently of cytosolic calcium (126,236,273,285,291,294). The discrepancy in these reports could be due to the relative magnitude of the stimulatory and inhibitory signals. Figure 8 sheds some insight on this problem. It demonstrates the ability of cAMP to stimulate renin secretion by a fixed amount (250 ng Ang. I/100 mg tissue/hr) over the range of calcium concentrations tested. If cAMP stimulates renin secretion by lowering cytosolic calcium, one would expect the stimulatory effect of forskolin to be dependent on cytosolic calcium. This, however, was not observed. Forskolin stimulated renin secretion independently of cytosolic calcium. Similar results have been reported for the stimulatory effects of epinephrine (105) and isoproterenol (227) on renin secretion in vivo. Of particular interest is the ability of cAMP to stimulate renin secretion maximally by a fixed amount when the renal perfusion pressure is at or below

the minimum pressure for autoregulation of the kidney. At low perfusion pressures renin secretion is maximally stimulated by a maximum reduction of cytosolic calcium as predicted by the stretch receptor hypothesis. These results raise the possibility that cAMP stimulates renin secretion without altering cytosolic calcium. Therefore, it is possible that renin secretion maybe regulated by two independent pathways.

The products of secretory cells can be extruded by either exocytosis across the plasma membrane and into the extracellular space or direct transport from their site of synthesis. Secretory products which are released via exocytosis are stored in membrane bound secretory granules. In the presence of agonist, the secretory granule fuses with the plasma membrane and, in terms of a chemiosmotic mechanism, undergoes local osmotic lysis and empties its contents into the extracellular space. While exocytosis is the generally accepted mechanism by which renin is released (374), it has been proposed by some groups (117) that renin is also directly transported across the granular and plasma membranes. Although renin secretion differs in its response to intracellular calcium levels and calmodulin antagonists, there is no evidence that the steps subsequent to these events deviate from those of other systems. Therefore, one can conclude that there are four basic steps involved in renin secretion by exocytosis: (a) Translocation of the secretory granule

from its site of formation to the plasma membrane. (b) Close apposition of the secretory granule and the plasma membrane. (c) Fusion of the secretory granule with the plasma membrane. (d) Fission of the bilayer separating the granular contents from the extracellular space. A final step, which is not part of the exocytotic process but which is important in normal cell function is the retrieval of the secretory granule membrane.

A chemiosmotic model for exocytosis has been advanced as a mechanism by which the contents of secretory vesicles are released from cells (61,152,238,297). This model proposes that a proton gradient across the granule membrane plays a key role in providing the motive force for osmotic swelling (153,303), an obligatory step in exocytosis (153,303). As stated above, the chemiosmotic hypothesis requires the presence of a (granular) transmembrane proton gradient. The gradient is generated by inward transport of protons by a translocating ATPase within the granular membrane (75,113,179,180,328). The end result is a transmembrane gradient with the inside of the secretory granule acidic and positively charged with respect to the cytosol (61,152,237,328). The potential energy derived from this gradient provides the force for accumulating osmotically active ions. Thus, the proton gradient could be coupled to intragranular accumulation of cations such as sodium or potassium via electroneutral H^+/K^+ (253) or H^+/Na^+ (153) exchange.

Also, the positive electrical potential might be coupled to the accumulation of anions such as chloride (303) or hydroxyl (52). Whatever the coupling mechanism, the net effect is the accumulation of osmotically active ions that promote water influx and granular swelling. Therefore, it is the osmotic gradient which ultimately provides the driving force for fusion and fission.

There are a number of concepts which all chemiosmotic models for exocytosis have in common. The first is the presence of a proton ATPase located in the secretory granule membrane. Lucy et al. (243) states that this is the central point to any chemiosmotic model. The importance of the proton gradient is demonstrated by the inhibition of epinephrine (307), serotonin (307) and PTH (52) secretion by FCCP, a proton ionophore which has been shown to dissipate both proton and electrical gradients across membranes (74,199). Proton ATPases have been found in a wide variety of secretory granules (14,16,61,74,152, 237,240,299). The second point common to all chemiosmotic models for exocytosis the involvement of a permeable anion (15,219,199,300,304,308). Addition of Mg^{2+}-ATP, which activates the proton pump, causes osmotic lysis of chromaffin granules only when a permeable anion such as chloride is present (307). Chloride, acting as a counter ion, enters the granule through a channel, and travels down its electrochemical gradient. Anion transport allows for the continued tranport of protons (by the proton

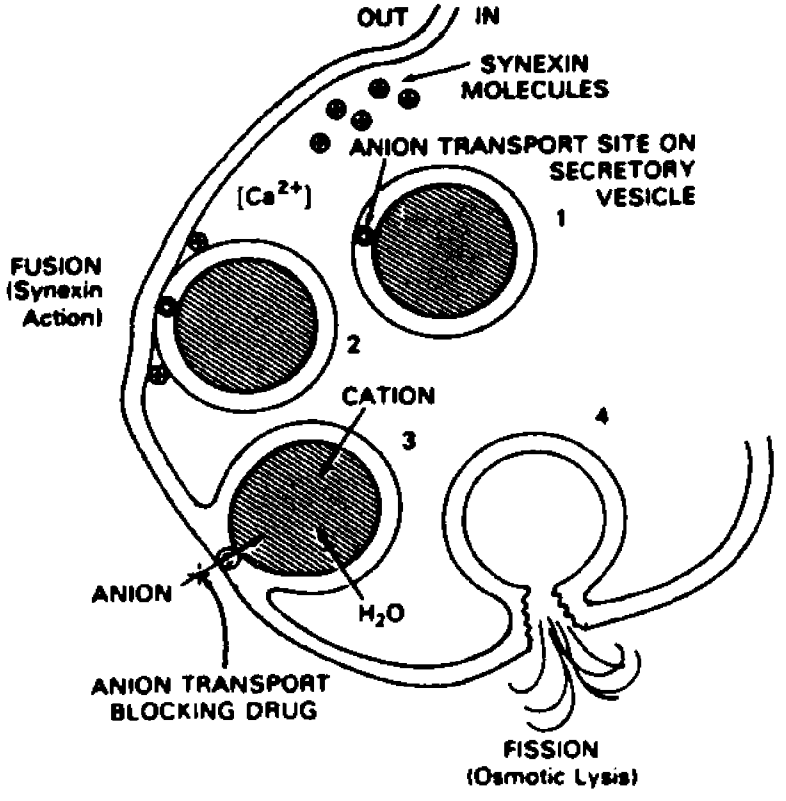
ATPase), resulting in a large osmotic gain by the granule matrix. This increase in the osmotic gradient is directly responsible for the observed granular swelling and osmotic lysis. If chloride is replaced with isethionate (an impermeant anion), granular lysis does not occur. It has also been shown that secretion of serotonin from human platelets (305), parathyroid hormone from dissociated bovine parathyroid (52) and epinephrine from chromaffin cells (305)- all require a permeable anion. The inhibitory effects of SITS, pyridoxal phosphate and probenecid, which are impermeant aromatic anions, demonstrate the presence of anion channels (305,307) and the requirement for permeable anions in both granular lysis and cellular secretion (305,307). Addition of these agents to the incubation media decreases the amount of secretory product released.

The findings with chromaffin granules led to the conclusion that the anion channel is located within the granular membrane (303,307). However, the time during the exocytotic pathway at which anion influx occurs remains unclear. That is, anion influx can occur before or after the secretory granule has fused with the plasma membrane. Due to the presence of a large chloride gradient across the plasma membrane, it seems likely that chloride transport occurs after membrane fusion. In mast cells it has been demonstrated that membrane fusion precedes granular swelling (437). This suggests that if anion

influx is an important component in this cell type it probably occurs after granular fusion. In support of this, Pollard et al. (307) have also proposed that anion translocation in the chromaffin granule occurs after membrane fusion (at the fusion site). These data are contradictory to Lucy et al. (243), who states that the fusion site must be free of both peripheral and integral membrane proteins. The integral membrane proteins would prevent fusion due to steric hindrance, that is, the inability to insert the cytoplasmic, hydrophilic domain of a granular integral membrane protein such as an anion channel, through the hydrophobic lipid bilayer of the plasma membrane. Under these conditions one must consider the alternative, that chloride transport occurs prior to granular fusion and after cell stimulation, when the granule is 'free' in the cytoplasm. The driving force behind anion transport would be the electrochemical gradient across the granular membrane. This would allow for the osmotic gain by the granule matrix. Under such conditions granular swelling may precede and provide the driving force for the fusion process. Further swelling, due to continued transport of ions into the granule results in fission. The anion transport component in other systems, including chromaffin granules, may be structurally and pharmacologically similar to the band III protein of erythrocytes since SITS and DIDS block chloride transport. The third point, which results from the

preceding two, is the ability to alter granular release by varying the osmotic gradient. It has been shown with planar lipid bilayers and lipid vesicles, that fission occurs by osmotic swelling of vesicles which have fused with the planar membrane (78,110,438). Finkelstein et al. (110) have demonstrated that the presence of an osmotic gradient across the planar bilayer resulted in fission rates which were orders of magnitude higher than those in which an osmotic gradient was not present. In vivo, this is accomplished by accumulation of osmotically active ions resulting from activation of the granular proton ATPase.

The following diagrammatic model has been put forth by a number of investigators (52,305,307,308) as a general mechanism of chemiosmotic exocytosis.



The numbers represent the suggested sequence of events leading to the fusion and fission of the granular and plasma membranes. (1) The cytoplasmic granule with its anion transport channel. (2) Upon stimulation, cytosolic calcium rises and the granule fuses with the plasma membrane. Calcium binding proteins such as synexin may facilitate this interaction. (3) A subsequent fusion state follows, in which the anion channel becomes exposed to the extracellular environment and permits the inward movement of a permeable anion. (4) The osmotic strength of the granular matrix increases, resulting in granular swelling and fission. Note, this model depicts the sequence of events postulated for epinephrine secretion from chromaffin granules (305,307).

There are three testable parameters of the chemiosmotic mechanism for exocytosis. First, the effect of osmotic forces can be examined by changing the osmolarity of the incubation medium to induce granular shrinkage or swelling. Second, one can test the effect of a pH gradient across the granular membrane by changing the pH of the incubation medium. Third, one can test for the presence of a proton ATPase with ATPase inhibitors.

The results presented here support a chemiosmotic mechanism for renin secretion. As can be seen in Figure 16, renin granules isolated by either sucrose discontinuous gradients or percoll continuous gradients, could be manipulated to increase or decrease the release

of their contents by varying the osmolality of the incubation medium. The findings imply that renin release from granules can be affected by osmotic gradients and water movement across the granular membrane. As water movement into the granule increases, granular swelling and renin release also increase. On the other hand, as water influx decreases or water efflux increases, renin release is decreased or inhibited. Interestingly, the osmolality of the incubation medium needs to increase only about 2-fold over control (from 300 to 600 mOsm/Kg H_2O) to obtain maximum inhibition of renin release. These results are in agreement with those reported in other systems, in which a chemiosmotic hypothesis has been proposed (15,52,218,291,308,353,406).

Renin secretion from cortical slices was also affected by hypo-osmolarity (Figures 12 and 15). Figure 12 demonstrates the stimulatory effects of hypo-osmotic incubation media on renal cortical slices. As osmolality was lowered from 300 to 150 mOsm/Kg H_2O, renin secretion increased 4-fold. The results can be explained in much the same way as they are explained for renin release from isolated granules. That is, lowering the osmolality of the incubation medium from 300 to 150 mOsm/Kg H_2O results in the formation of an osmotic gradient across the granular membrane, sufficient to drive water influx and granular swelling. The swelling of the secretory granule would ultimately result in its fusion

and fission with the plasma membrane of the JG cell. Also, the osmotic effect is reversible (Figure 12). That is, if the osmolality of the incubation medium is subsequently brought back to 300 mOsm/Kg H_2O, renin secretion returns to control levels. This reversibility demonstrates that the osmotic effect is not due to cellular damage. The interpretation of these results is supported by the findings of Skott and Taugner (350) who demonstrated that a decrease in osmolality caused granular swelling and increased contact between the renin granule and JG cell membrane. The results presented here, in addition to those of Skott and Taugner, (350) suggest that granular swelling is a necessary antecedent for fusion, an interpretation supported by others (152,153,307). Further evidence comes from a second study by Taugner et.al. (274) in which it was suggested that under physiological conditions, an osmotic effect may be essential for initiation of renin secretion. They demonstrated that morphological changes in mature renin granules always occur concurrently with localized and spatially contained changes within the granular matrix. These changes lead to reduction in the electron density of the granular matrix which is consistent with a local increase in volume. It is possible that this volume increment results from an increased water influx caused by the generation of an osmotic gradient. However, there is no direct evidence other than Taugner's morphological data to substantiate

this claim. The results by Taugner and associates lend support to the involvement of a chemiosmotic mechanism in renin secretion and are consistent with the results obtained by other investigators (129,348).

The parallel behavior of isolated granules and renal cortical slices is not unique to the renin system. However, since secretion from cells requires fusion of the secretory granule with the plasma membrane, and not just granular swelling and lysis, there must be a critical point in terms of granular volume, when granular swelling is sufficient to trigger fusion with the plasma membrane. If fusion does not occur, continued swelling would result in intracellular lysis of the granule. It is interesting to note that in studies using planar bilayers and phospholipid vesicles, creation of an osmotic gradient across the planar bilayer leads to fission of phospholipid vesicles juxtaposed to the bilayer. However, vesicles which are not close to or in contact with the bilayer swell, lyse, and release their contents into the bathing medium (78).

As demonstrated above, calcium acts as an inhibitory signal in renin secretion. If 2.5 mM calcium is added to the incubation medium at the time the osmolality is lowered to 150 mOsm/Kg H_2O, the increase in renin secretion is not as great as in a calcium-free medium (Figure 13). One can therefore speculate that under these conditions the inhibitory effects of calcium are at the

level of granular swelling. Calcium may inhibit swelling and block the interaction between the granular and plasma membranes. The mechanism by which calcium inhibits granular swelling is unclear, but the hypothesis for calcium-induced inhibition of renin release is based on the possibility that hypo-osmolality induces granular swelling and renin secretion. Consistent with this is the report by Skott (348) that hypo-osmotic stimulation of renin secretion from isolated glomeruli is much greater if calcium is omitted from the superfusate. Calcium control of granular swelling is supported by a study using sea urchin eggs (439). Fusion of the cortical granules with the egg plasma membranes required an increase in cytosolic calcium. However, the study demonstrated that calcium induced irreversible changes in the cortical granule that were required for granular swelling. In the absence of prior or concurrent exposure to calcium, the granules did not swell and were therefore incapable of interacting with the plasma membrane of the egg (439).

A number of studies have shown that incubation of various cell types (48,61,160,291,339) in hyperosmotic media results in secretion inhibition, as would be predicted by the chemiosmotic mechanism. It has been reported, using isolated perfused kidneys and enriched JG cell suspensions, that addition of albumin (which renders the incubation medium hyperosmotic) stimulates renin secretion (76,122). These findings have been explained by

the chelation of calcium by albumin, with consequent lowering of calcium concentrations and stimulation of renin secretion. Thus, these results do not conflict with the chemiosmotic mechanism of secretion. In further support of the hypothesis, Skott et al. (350) demonstrated that increasing the osmolality of the incubation medium from 330 mOsm to 400 mOsm by the addition of sucrose, decreased renin release to about 15% of control.

Some investigators (174,214) have used the effects of hyperosmolarity to both prove and disprove the chemiosmotic hypothesis. To negate the possibility of a chemiosmotic mechanism of secretion on the basis of failure of hyperosmolarity to affect cellular secretion may be symplistic. Hyperosmotic inhibition may depend on whether granular swelling is the driving force for membrane fusion. If this is the case, then inhibition of granular swelling by increasing osmolarity of the incubation medium may inhibit secretion. However, if granular swelling takes place after fusion, then hyperosmolality should have very little effect on exocytosis. This has been observed in mouse mast cells (437). In addition, Holz and Senter demonstrated in digitonin-treated chromaffin cells, that shrunken granules can undergo exocytosis (174). Also, the decrease in cell volume caused by hyperosmolality could result in increased contact between the plasma and granular membranes. This interaction could be sufficient to cause the fusion and

fission of the two interacting membranes. Under the latter condition, granular swelling may not be required.

Under physiological conditions the JG cell granular and plasma membranes may be brought into close apposition by lowering cytosolic calcium to less than 10^{-7}M (Figures 6 and 7)(22,119,294,410) or by inhibiting calmodulin (117,293,258). The low cytosolic calcium could allow the two membranes to overcome short range forces keeping them apart. In systems in which calcium stimulates secretion, this may be accomplished by bridging substances, such as synexin and calcium, or through biochemical modification, (ie. phosphorylation). However, such events have never been demonstrated to be involved in the regulation of renin secretion, and calcium has been shown to inhibit renin release from isolated granules (Figure 29) though the degree of inhibition was not as great as that seen in cortical slices (Figure 7) These investigations show that in renal cortical slices the steepest part of the calcium dose response curve is between 10^{-7} and 10^{-8}M calcium, where the change in renin secretion is about 7-fold. However, if renin granules are exposed to the same calcium concentrations a mere 1.4-fold change in renin release is observed. Although this change is statistically significent, it is 5-fold less than that observed in slices. These results suggest that calcium can act directly on renin granules but the full inhibitory effect probably involves some

cytoplasmic factor as well. Since most of the cytoplasmic factor would be lost during granule isolation, addition of calcium to the granular preparation would be expected to have a lesser effect. On the other hand, one cannot eliminate the possibility that calcium's inhibitory action on the isolated granule is due to its plasma membrane stablizing properties (44,416). Direct actions of calcium on other granular systems have been demonstrated by Edwards et al. (96) and Green et al. (151), who found that high concentrations of calcium or magnesium caused granular aggregation. In 1977, Schober (340) found divalent and trivalent cation binding sites on granule membranes. Based on these observations and the opposite effect calcium has on renin secretion (as compared with most other secretory systems), it is possible that calcium directly inhibits granular renin release.

Additional support for a chemiosmotic mechanism comes from the combined effects of hypo-osmolality and acid pH. The hypo-osmotic effect of 150 mOsm/Kg H_2O can be increased 3-fold by lowering the pH of the incubation medium from 7 to 5 (Figure 15). This increase can be explained in terms of the chemiosmotic hypothesis. That is, at acidic pH the high proton concentration of the incubation medium deminshes the proton gradient across the granular membrane. This reduces electrochemical restraints on proton transport and results in activation of the proton ATPase, thereby facilitating transport of

additional protons from the cytosolic space to the granules. In addition, the augmented ATPase activity generates an electrochemical gradient that can be used to activate an antiport exchanger. This would allow for a greater granular swelling, and a greater renin secretory rate (than can be achieved by incubating in a hypo-osmotic medium alone). The findings suggest that both hypo-osmolarity and acid pH stimulate renin secretion by acting on the JG cells and the renin secretory granules. Also, the additive effects suggest different sites of action. The effects of hypo-osmolality on renin secretion are consistent with those obtained with isolated afferent arterioles (129) rat glomeruli (48,348) and isolated renin granules (Figure 16). Figure 17 demonstrates that the percentage granular renin release was greater at pH 5 than at pH 7. This can be explained on the basis of higher proton ATPase activity at pH 5, resulting in a greater accumulation of osmotically active ions, granular swelling and renin release.

The second testable parameter of the chemiosmotic mechanism is the involvement of a pH gradient. It has been shown in other systems that the secretory pathway is influenced by the pH of the incubation medium. This response is not limited to whole cell preparations. Rothman et al. (325,326,327) demonstrated that the release of enzymes from a pancreatic zymogen granular preparation is affected by the pH of the incubation medium, and that

each enzyme had its own characteristic pH curve. Also, the pH response at the cellular level is not limited to secretion via exocytosis (28,181,313,352). It has been demonstrated that the secretion of insulin from beta cells (181,352), and serotonin from platelets (28) are affected by the pH of the incubation medium.

The effects of pH on renin release from isolated granules and renin secretion from cortical slices are shown in Figures 9, 10 and 17 and in Table 5. The results suggest that as the pH gradient across the granule membrane increases, so does the amount of renin released. This was observed in both isolated renin granules and renal cortical slices (Figure 9, 10 and 17 and Table 5). In addition, if renin secretion from cortical slices is plotted as ratios of the rates at experimental vs. control pHs (E/C), the pHs at which the ratios are equal to 1 are 6.0 and 7.4 (Figure 10). A ratio of one indicates the pH change did not affect renin secretion. These two pHs correspond closely to the pHs which gave the lowest percentages of renin release from isolated granules. This indicates that at pHs 6 and 7 the proton gradient across the granular membrane may be incapable of supporting renin release in either cells or isolated granules. This could be due to the inability of the granule to generate an osmotic gradient of sufficient magnitude to drive granular swelling. Also, stimulation of renin secretion was observed in both isolated granules and cortical slices

when the pH of the incubation medium was lowered to pH 5. This stimulation at acid pH could result from the activation of a proton ATPase located in the granule membrane. This possibility will be discussed below. Figure 11 demonstrates that the pH effect on renin secretion is reversible, and is therefore not caused by cell destruction. The results also show that isolated renin granules and the JG cells behave in a similar manner. The difference in the relative magnitude of release between the two systems could be due to differences in granular stability. It has been suggested that isolated granules are less stable than cellular granules because they become spherical upon isolation, making them more susceptable to osmotic stress (43,173).

The importance of a pH gradient in renin secretion was further demonstrated with CCCP. Dissipation of the proton gradient by this protonophore inhibited renin secretion at all pHs except 5 (Figure 10B). Granular swelling is inhibited because the driving force for accumulating osmotically active ions is lost. Therefore, one might expect that the greater the ability of CCCP to dissipate the proton gradient the greater its inhibitory effect. This phenomenon could depend on the pH of the incubation medium. As the pH of the incubation medium is raised, a greater pH gradient across the granule membrane is generated. Under these conditions CCCP could cause a greater absolute change in the magnitude of the pH

gradient. The reason for this speculation is that CCCP inhibited renin secretion by 83% at pH 8 and by only 30% and 45% at pHs 6 and 7, respectively. However, at pH 5 the inability of CCCP to affect renin secretion is also consistent with the chemiosmotic hypothesis. If the pH of the granular matrix is close to pH 5, then addition of CCCP should have no effect on proton movement. At this pH the rate of renin secretion could be maintained by activating an antiport exchanger or by electroneutral exchange as proposed by Grienstein et al. (153). Both possibilities would result in increased granular swelling. It should be noted, however, that the pH of the renin granular matrix is unknown.

Due to CCCP's effect on oxidative phosphorylation it is possible that the inhibition of renin secretion at pH 6, 7 and 8 results from ATP depletion. However, this possibility seems unlikely when one compares the inhibitory effect of CCCP to that of KCN on renal cortical slices incubated in standard-, calcium-free- and calcium-free with calmidazolium- KRB. One observes that CCCP inhibited renin secretion under all three experimental conditions, whereas inhibition by KCN required calcium (Tables 2, 3, 4 and 6). This suggests that the ability of KCN to inhibit renin secretion is secondary to ATP depletion, with resulting elevations of cytosolic calcium. On the other hand, the ability of CCCP to inhibit renin secretion by the same percentage,

regardless of the experimental conditions, suggests that its site of action is extramitochondrial. Thus, the data suggest that CCCP is acting at the level of the renin granule and is dissipating the pH gradient required for renin secretion at pH 6, 7 and 8. The inhibitory activity of both KCN and CCCP will be discussed in detail below.

In the isolated renin granule, a large proton gradient across the granular membrane could supply the energy needed for osmotic swelling. A chemiosmotic hypothesis proposed by Grinstein et al. (152) predicts that granular swelling is strictly dependent on extragranular pH, with more rapid and extensive swelling observed in the alkaline range. This may explain why the percent renin release from isolated granules is so high at pHs 8 and 9. At alkaline pHs, activation of an antiport exchanger could result in accumulation of osmotically active ions that enhance the inward movement of water and granular swelling. This exchanger could be driven by the proton gradient. If such an exchange does exist in the renin granule, it must be tightly coupled to the inward transport of chloride. It is also possible that the high percentage of renin release at alkaline pH results from accumulation of hydroxyl ions, as demonstrated for parathyroid hormone secretion (52). Hydroxyl ions would enter the granule by moving down their electrochemical gradient. This seems unlikely, however, due to the chloride dependent nature of the pH response. On the

other hand, the evidence obtained at pH 5 suggests that the increased percentage of renin release is attributable to activation of the granular proton ATPase. This enzyme facilitates accumulation of osmotically active ions, in part via activation of antiport exchange mechanisms involving potassium, chloride and hydrogen ions. At acid pH (pH 5), the activity of the proton ATPase supplies the necessary protons required to drive the exchanger. However, at alkaline pHs (8 and 9) the energy for driving the antiport exchanger comes from the large pH gradient. Regardless of the pH of the incubation medium, sodium cannot replace potassium as the cation transported by the antiport exchanger (Figure 21).

The data indicate that at each extreme (pHs 5, 8 and 9), granular swelling results from inward movement of potassium and chloride which is tightly coupled to outward transport of hydrogen ions, through an antiport-exchanger-symport complex mechanism. This conclusion is supported by Figures 17, 18, 20 and 22, which show the importance of chloride and potassium in the pH response. If either of these ions is removed renin release from isolated granules falls to levels which are not significantly different from those observed at pHs 6 or 7 in the presence of these ions. The involvement of potassium and chloride at every pH studied suggests a strong association between the transport of these ions and renin release. The coupling mechanism could involve

inward transport potassium and chloride in exchange for an intragranular proton. Another possibility, however, is that an electroneutral proton/cation exchange occurs. Under such conditions, proton lost during the exchange could be quickly replaced by the buffering capacity of the granular matrix. This would result in net accumulation of positively charged ions and a requirement for chloride as a counter ion which would allow the exchange to continue. Under such conditions one would expect chloride to enter the granule through an anion channel, as has been demonstrated in other systems (305,307). This seems unlikely, however, since neither SITS nor DIDS affected granular release. Also, at pH 5, the effects of potassium and chloride in renin release are identical to those observed at all other pH's. The only difference is that the protons required to drive the antiport exchanger at pH 5 may be supplied by the proton pump.

The ability of an antiport exchanger to support granular renin release was demonstrated with nigericin, an ionophore that exchanges protons for potassium ions in a 1:1 ratio. Figures 27 and 28 show that nigericin supports granular release in a potassium dependent, chloride-independent manner. The chloride independence is not surprising considering the stoichiometry of the exchange. Since the electroneutral exchange does not result in further generation of an electrical gradient, chloride is not required for release. Nigericin thus

increases renin release by exchanging an osmotically inactive ion (hydrogen) for an osmotically active one (potassium). The end result is a net osmotic gain by the granule, leading to increased renin release. Nigericin has been shown to promote release of secretory products via granular lysis in other systems (198).

Figure 24 lends additional support to the possible involvement of an antiport exchanger in granular renin release. Neither CCCP, a proton ionophore (168,312), nor valinomycin, a potassium ionophore (310), alone increased renin release over control values. However, if the two ionophores were presented together, renin release increased at all pHs studied. These results agree with the findings of Johnson and Scarpa (198), and demonstrate that transport of a single ion, with either an ionophore or a transport protein, cannot support release unless a similar charged ion moves in the opposite direction. Under these conditions, the unidirectional transport of a small number of ions leads to development of an electrical gradient which prevents further ion movement and sufficient granular swelling to cause lysis. The results also demonstrate, in the isolated renin granule, the inability of CCCP to completely dissipate the proton gradient across the granular membrane. Therefore, one would expect to see similar results in the presence or absence of CCCP. In the presence of both ionophores, however, the proton gradient is abolished at the expense

of an osmotic gain which is sufficient to cause osmotic swelling and granular lysis.

Mg^{+2}-ATP has been reported to be required for secretion of hormones from a variety of glandular cells (213,397). Its involvement in granular renin release is demonstrated in Figure 23. Here I showed that increasing the concentration of magnesium from 1 to 10 mM leads to a 2.5- and 2.2-fold increase in renin release at pHs 5 and 6, respectively. If, however, the pH gradient across the granular membrane is large enough to drive the antiport exchanger, one would not expect a magnesium-induced activation of the proton-ATPase to have any effect on granular renin release. Nevertheless, release increases 3-fold at pH 8 when the magnesium concentration is raised from 1 to 10 mM. The reasons for such a stimulation remains unclear.

In one respect the present data are difficult to reconcile with a chemiosmotic hypothesis proposed by Pollard et al. (307). In their hypothesis, activation of the proton Mg^{+2}-ATPase results in inward transport of protons and development of an electrical gradient, positive inside. The electrical gradient provides the driving force by which permeable anions, such as chloride, enter the granule. Chloride entry lowers the electrical gradient and thus allows for additional inward transport of protons. The end result is net osmotic gain by the granule, granule swelling and release of granular contents

to the incubation medium. The involvement of chloride as the permeant anion in chromaffin granular lysis, as well as in exocytosis, has been demonstrated by the ability of anion channel blockers SITS, probenecid and pyridoxal phosphate to inhibit both of these processes (305). These agents block chloride transport in erythrocytes by interacting with the integral band III membrane protein (56,212). In the renin granule, however, although chloride is important for renin release, its action cannot be blocked by either SITS or DIDS (Figure 19). These results indicate that chloride entry is not through an anion channel (which can be blocked by these agents), and suggests another mechanism of chloride entry, perhaps an antiport-exchanger-symport complex which couples inward transport of potassium and chloride to outward transport of protons. Another possibility, however, is that the anion transport protein in the renin granular membrane is structurally and pharmacologically different from the band III protein of erythrocytes. However, this seems unlikely because of the importance of chloride in granular release at all pHs studied and the granular responses to oligomycin and DCCD, at pH 8. At pH 8 the proton pump does not seem to play a role in renin release, as suggested by the lack of inhibition by oligomycin and DCCD. In other systems in which chloride moves through an anion channel, the movement depends on the inward transport of protons. However, in the renin granular

system, at pH 8, chloride is still required for renin release.

A key question in any chemiosmotic model concerns the presence of a proton ATPase within the granular membrane. The existence of such an enzyme has been demonstrated in a variety of secretory granules (14,16,68,74,152,240,299,328). This allows for acidification of the granule matrix and provides the energy for either anion transport or H^+/cation exchange. However, the role of the proton ATPase in catecholamine secretion from chromaffin cells has been questioned by Knight and Baker (214). They demonstrated that exocytosis proceeds in the absence of both osmotic and pH gradients. However, one cannot conclude that the proton pumps found in other secretory granules are not necessary for exocytosis. If Knight and Baker are correct, an alternative role for the pH gradient in this system would be for the accumulation of biological amines such as epinephrine, norepinephrine and dopamine. Such a possibility cannot be excluded, but it has never been observed or assigned the function of the pH gradient in other systems.

Although intragranular pH has not been measured directly, Taugner et al. (381) give strong evidence that the granular matrix is acidic with respect to the cytosol. They demonstrated the ability of the granule to accumulate the weak bases chlorphentermine and chloroquine. These

bases accumulate in lysosomes (177,245) whose internal pH had been determined to be approximately pH 5. In addition, the bases cause similar morphological changes in renin granular matrices and lysosomes. Other evidence for an acidic granular matrix comes from the ability of NH_4Cl to cause a greater absolute increase in renin release at pH 6 as compared to pH 5 (experimental - control, Figure 25). These results show the ability of the granular matrix to accumulate NH_4^+. If the granule pH were identical to that of the incubation medium, NH_3 would not be protonated and trapped by the granules, and no accumulation would occur. Under these conditions the percent renin release should be unaffected by NH_4Cl. Using this line of reasoning one would expect a greater increase in renin release at pH 8 (in the presence of NH_4Cl) compared to other pHs. Although the increase in renin release was significant, the high percentage of renin release observed at pH 8, under control conditions, makes it difficult to observe a greater absolute increase in renin release (E-C) in the presence of NH_4Cl. These results suggest that the intragranular pH may be lower than pH 5, and give support to the presence of a proton ATPase in the renin granular membrane.

To further examine this possibility two known proton pump inhibitors, oligomycin (240,312) and DCCD (12) were tested for their effects on granular renin release and

lysis. As shown in Figure 22, both inhibited renin release by 2.5-fold compared to control at pH 5. However, neither agent had any effect at pH 8. The results support the presence of a granular proton ATPase and give credibility to a chemiosmotic mechanism in renin secretion. The ability of proton pump antagonists to inhibit renin release at pH 5 but not at pH 8 is not surprising. At pH 5 the combination of high substrate availability and reduced electrochemical gradient allows for additional transport of the protons required for granular swelling. Therefore, it would be expected that proton pump antagonists would inhibit at this pH. However at pH 8, due to the low concentration of substrate and the large electrical gradient, the proton ATPase would not function. Therefore, one would not expect the addition of these antagonists to have any effect on renin release. The percent release at pH 8, while high, is in accordance with the chemiosmotic hypothesis proposed by Grinstein et al. (153). One can therefore conclude that at alkaline pH the large pH gradient precludes the need for a proton ATPase. However, chloride and potassium are still required. As the pH of the incubation medium falls, the pH gradient deminishes, and the role of the proton ATPase becomes increasingly important.

At the cellular level the role of a proton ATPase in renin secretion was investigated with KCN, oligomycin, CCCP and 2-deoxyglucose. The first three agents are known

inhibitors of ATP synthesis, while the fourth inhibits glycolysis. The three inhibitors of oxidative phosphorylation have different sites of action. KCN inhibits oxidative phosphorylation by blocking the transfer of electrons from cytochrome As to oxygen (364), the final step in the electron transport system. Oligomycin (240,312) inhibits the mitochondrial proton ATPase and CCCP (148,229,312) makes the membrane permeable to protons. Therefore, these two agents act to inhibit oxidative phosphorylation by dissipating the proton gradient across the mitochondrial membrane. Tables 2, 3, and 4 demonstrate that KCN inhibits renin secretion only in the presence of extracellular calcium. In a calcium-free medium in the presence or absence of calmidazolium, KCN had no effect on renin secretion compared to control. These findings are in agreement with those reported by Lyons (246) who also found that KCN inhibited renin secretion only in the presence of calcium. This indicates that KCN's inhibitory effect may result from ATP depletion with consequent elevation of cytosolic calcium (via inactivation of the calcium-ATPase and Na^+/K^+-ATPase). There is no biochemical or physiological evidence that JG cells contain either Na^+/K-ATPase or Ca-ATPase, but both enzymes are present in smooth muscle cells (41,324,327,401). In addition, renin secretion is inhibited by ouabain (67,294), vanadate (68) and low extracellular potassium

(64), all of which are known to inhibit the activity of these pumps. Each of these agents, like KCN, inhibit renin secretion in a calcium-dependent manner. Therefore, it is reasonable to conclude that their actions are mediated by increasing cytosolic calcium. It is for this reason that I postulate KCN's inhibitory action on renin secretion to be mediated by an increase in calcium. That is, ATP depletion could lead to a decreased calcium efflux (from JG cells), caused by decreased activity of calcium pumps and Na^+/Ca^{2+} exchanges. Na^+/Ca^{2+} exchange would be decreased by the low activity of the Na^+/K^+ ATPase that maintains the sodium gradient. Therefore, it may be the rise in intracellular calcium which is responsible for KCN inhibition. It is through this cascade of events, with the exception of ATP depletion, that ouabain, vanadate and low extracelluar potassium are thought to inhibit renin secretion. In agreement with the proposed mechanism of action of KCN, is the ability of cyanide to promote contraction of smooth muscle cells (25,320), presumably by increasing cytosolic calcium. Cyanide's action is dose dependent up to 10^{-5}M, after which its ability to cause contraction declines. The decrease could be due to ATP depletion, since norepinephrine induced contractions are also inhibited by cyanide at concentrations of 10^{-5}M or greater (320). There are, however, two reports in which cyanide failed to inhibit renin secretion

in the presence of extracellular calcium (42,90). The reason for this discrepancy remains unclear.

Unlike KCN, the inhibitory effect of CCCP and oligomycin were independent of calcium or activation of the calcium-calmodulin complex. These results give strong evidence that both CCCP and oligomycin inhibit renin secretion by a process that does not involve the Ca^{2+}-calmodulin pathway, or that these agents act at one or more steps in the renin secretory pathway distal to the site of action of calmidazolium (ie: after the calmodulin regulatory site). It is interesting that CCCP and oligomycin inhibit renin secretion to the same degree regardless of the base line rate established during the first hour of incubation. That is, as seen in Tables 2, 3, 4, and 6, regardless of whether the renin secretory rate was 61 ng Ang I/100mg tissue/hr (in normal KRB) or 10- or 20- fold higher (in a calcium-free or calcium-free plus calmidazolium medium, respectively) renin secretion was inhibited by 60-70% in the presence of these antagonists. One can conclude that the inhibition of renin secretion by CCCP or oligomycin in a calcium-free medium was not solely the result of either ATP depletion or depletion of the renin secretory pool. This is evidenced by the sustained stimulation of renin secretion by KCN in a calcium-free media. Also, the finding that oligomycin and CCCP decrease renin secretion in calcium free media (with or without calmidazolium) to levels that

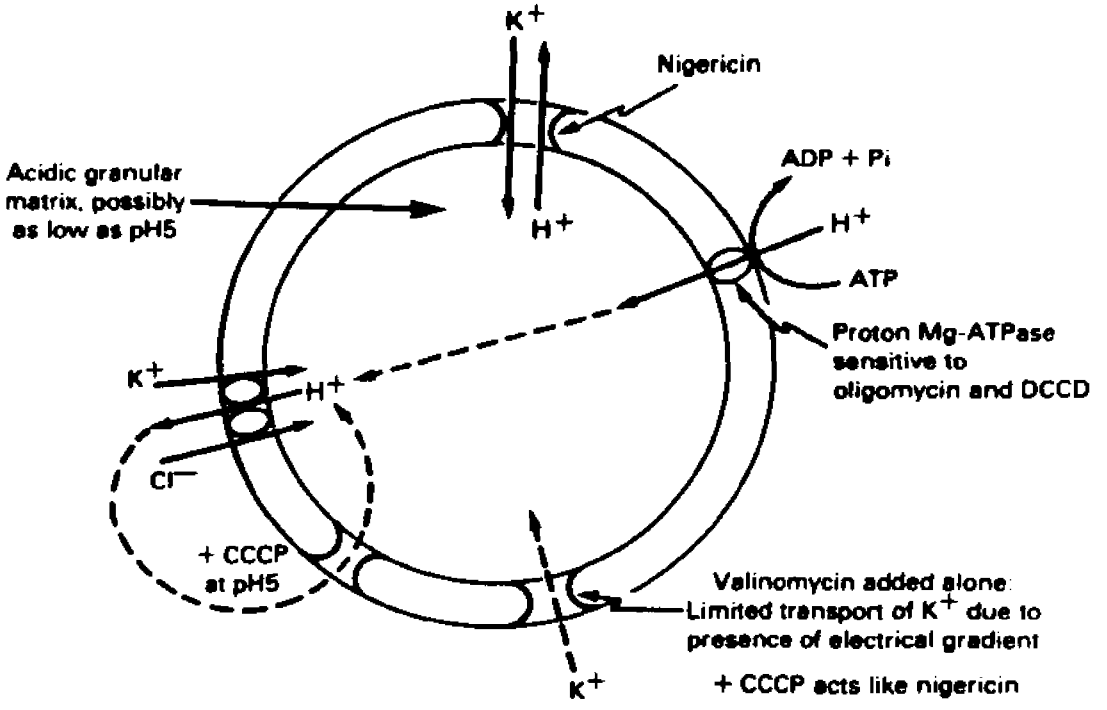
were higher than those observed in normal KRB also indicates that the inhibitory effect was not due to depletion of the secretory pool of active renin, or to inhibition of the conversion of inactive renin to active renin. These results give credibility to the observed pH dependent effect of CCCP (Figure 10B and Table 5).

Comparison of the results obtained with KCN to those obtained with CCCP and oligomycin indicates that the mechanisms of inhibition are different. As stated above, KCN may inhibit renin secretion by raising cytosolic calcium. On the other hand, CCCP and oligomycin may exert their effects via another, possibly similar, mechanism. CCCP and oligomycin dissipate transmembrane proton and/or electrical gradients. In many cell types, a proton gradient across the secretory granule membrane has been postulated to play a key role in exocytosis (61,152,237,328). The results presented in this thesis suggest such a mechanism may also be involved in renin secretion. At the cellular level the chemiosmotic hypothesis proposes that a transmembrane proton gradient and an electrical gradient are generated by a proton ATPase such that the inside of the secretory granule maintains an acidic and positively charged environment over 1-2 pH units (61,152,237,328). The energy derived from the electrochemical gradient is used to accumulate osmotically active ions, resulting in inward movement of water and granular swelling. If similar events are

required for renin secretion, reducing the electrochemical gradient should inhibit the secretory response. It is this mechanism by which CCCP and oligomycin are thought to exert their effects. The inability of either of these agents to reduce renin secretion in calcium-free KRB (with or without calmidazolium) to levels obtained with standard KRB suggests that the stimulatory signal of either low intracellular calcium and/or inhibition of the Ca^{2+}-calmodulin complex may be greater than the inhibitory effect of a dissipated proton gradient. An alternate possibility is that the proton gradient is not completely abolished, but is reduced to levels that can still support renin secretion at a lower rate.

From the data gathered from isolated renin granules and renal cortical slice experiments, the following chemiosmotic model for renin secretion is proposed:

MODEL FOR RENIN RELEASE: A Chemiosmotic Hypothesis



There appear to be two components to the mechanism of renin release from isolated renin granules. The first component is sensitive to both oligomycin and DCCD, since addition of either of these agents inhibits release at acidic pH (ie. pH 5). This implies that a proton ATPase is involved in the release process. The importance of such a transport protein has been demonstrated in other systems in which the proton pump is also inhibited by the addition of oligomycin and DCCD (12,240). Inhibition of the proton ATPase deminishes the numbers of osmotically active ions gained by the granular matrix. This, in turn,

leads to a decreased granular swelling and secretory product release. The second component, which also involves ion transport, is unaffected by either of the antagonists. It is an antiport exchanger for potassium, chloride, and hydrogen. Both transport mechanisms are located in the renin granular membrane. The importance of the antiport exchanger becomes apparent when the granules are incubated at pH 8 or 9.

At pH 5, translocation of hydrogen ions into the granular matrix results from activation of the proton ATPase. The activation is due to reduction or absence of an electrochemical gradient that would normally inhibit proton transport. Under these conditions, the proton pump supplies the hydrogen ions required for activating the antiport exchanger. Thus, the proton ATPase is involved in coupling the two transport mechanisms. Also, activation of the antiport exchanger results in a greater osmotic gain by the granule, such that, the addition of either oligomycin or DCCD inhibits granular swelling by inhibiting the proton ATPase and antiport-exchanger-symport complex. These antagonists inhibit the proton ATPase by interacting directly with transport mechanism. On the other hand, the activity of the antiport-exchanger-symport complex is reduced secondarily because of the inhibition of the proton ATPase. Inactivation of these transport systems prevents formation of the osmotic gradient required for renin

release. These conclusions are supported by results obtained from renal cortical slices in which oligomycin inhibited renin secretion by about 70% at pH 7.4. The inhibition may result from oligomycin's interaction with an extra-mitochondrial ATPase. This interpretation is based on oligomycin's calcium-independent mechanism of action. A possible site of action is the proton ATPase located in the renin granular membrane.

At pH 8, however, the proton gradient across the granular membrane is sufficient to support maximal activity of the antiport exchanger. Therefore, inward transport of protons by the proton ATPase is not required for activation or gain of osmotically active ions by the granular matrix. This may be why oligomycin and DCCD had no effect on granular renin release at this pH. The results imply that the activation of the proton ATPase is not required for granular swelling and renin release at alkaline pHs, a hypothesis supported by Grinstein et al.(153).

Granular renin release can also be inhibited by removing either potassium and/or chloride from the incubation medium. This would prevent the normal functioning of the antiport exchanger. Its inability to function severely limits the activity of the proton ATPase, at pH 5, due to the establishment of an electrical gradient that would limit further proton transport. Under these conditions granular release is reduced to control

levels due to the inability to accumulate a sufficient concentration of osmotically active ions required for granular swelling. This decreased osmotic gain is directly linked to inhibition of the antiport exchanger. Also, this inhibition is not pH dependent in as much as inhibiting the antiport exchanger would cause a decrease in renin release at all pHs.

As diagrammed above, and alluded to in the text, the proposed model shows the importance of chloride in the release process. However, its entry into the granule is not through the conventional anion channel that has been demonstrated in other systems, but as part of an antiport exchanger. This explains why SITS and DIDS, which inhibit release in other systems, had no effect on renin release. Therefore, while chloride is acting as a counter ion in the release of granular renin, its transport is directly coupled to the transport of hydrogen and potassium ions.

In the isolated granule, CCCP at pH 5, could allow short circuiting of the proton ATPase. (That is, protons transported out of the granule by the antiport exchanger could be quickly replaced by inward translocation of protons by CCCP.) Under these conditions CCCP would not be expected to have any effect on granular renin release at acidic pH (ie. pH 5). On the other hand, at more alkaline pHs (i.e. pH 8), CCCP does not completley dissipate the large proton gradient. The remaining gradient is capable of driving the antiport exchanger;

therefore, granular renin release is unaffected. Inability of protonophores to completely dissipate proton gradients has been demonstrated in other granular systems (199). Similar results were observed in the presence of valinomycin alone. A credible explanation for the lack of effect on granular renin release is that valinomycin may be unable to completly dissipate the potassium gradient. Two possibilities arise which could prevent transmembrane equilibration of potassium. First, the presence of an electrical potential across the granular membrane (positive inside) prevents further accumulation of positively charged ions. Second, for pHs at which an electrical gradient does not exist or is severely reduced (ie. pH 5), transport of a few potassium ions generates an electrical potential that prevents further transport of potassium. Such phenomena have been observed in an other system in which valinomycin stimulated catecholamine release from chromaffin granules only in the presence of a permeable anion (i.e. chloride) (93). This finding gives additional support to the hypothesis that chloride entry into the renin granule is not through an anion channel.

However, if both CCCP and valinomycin are added together, more extensive transport of the two ions occurs. This is because each ion, initially transported along its chemical gradient, sets up an electrical gradient that drives the transport of the counter ion (as suggested by Johnson and Scarpa, 198). That is, transport of protons

out of the granule by CCCP would result in establishment of a new electrical gradient, more negative inside, that could drive the inward transport of potassium. The cycle contiunues until accumulation of osmotically active ions is sufficient to cause granular swelling and lysis. Also, proton efflux allows for proton ATPase activation via reduction in the electrochemical gradient. Therefore, the simultaneous presence of both ionophores may produce more extensive granular swelling than can be achieved by either acting alone.

In the JG cell the above model holds except for the mechanism of action of CCCP. In the isolated granule CCCP had no effect on granular release, presumably due to its inability to affect the proton gradient. However, in the intact cell CCCP seems to be capable of dissipating the proton gradient to a level which is incapable of supporting granular swelling at pHs 6, 7 or 8. It is possible that a mechanism within the JG cell counters the electrical effect of CCCP. Under these conditions, CCCP dissipates the proton gradient, and thereby inhibits renin secretion. Such a mechanism is missing from the isolated granules.

In the presence of nigericin, however, granular renin release increased in a potassium-dependent, chloride-independent manner at every pH studied. This increase is due to the electroneutral exchange between intragranular protons and extragranular potassium ions.

The electrical gradient across the granular membrane, if one exists, is not a factor which requires consideration because of the electroneutral nature of the exchange. With nigericin, the ions move along their respective concentration gradients. In addition, proton loss raises intragranular pH and this can, in turn, activate the proton ATPase. The net result, like that observed in the presence of valinomycin and CCCP, is an increased accumulation of osmotically active ions by the granular matrix.

Calcium has been shown to have a direct inhibitory effect on renin release from isolated granules. However, its mechanism of action is obscure. It is possible that in vitro, as well as in vivo, calcium inhibits the osmotic swelling of the renin secretory granule. This interpretation is consistent with the chemiosmotic hypothesis proposed for renin release. In other systems, in which elevation of cytosolic calcium stimulates secretion, it has been suggested that the higher intracellular calcium is required for granular swelling (79,438). Therefore, in the unstimulated cell the low concentration of cytosolic calcium prevents fusion and fission by preventing granular swelling. Also, in chromaffin cells, trifluoperizine (TFP) inhibits steps in the exocytotic pathway subsequent to translocation of the granules to the plasma membrane (55). It is possible that the inhibitory effect of TFP on exocytosis results from

inhibition of granular swelling. On the other hand, in the JG cell, due to the way in which the stimulation-secretion mechanism is coupled to calcium, it is possible that calcium inhibits both granular release and cellular secretion by inhibiting granular swelling. If calcium does inhibit granular swelling the most logical site of action is at the level of the granular membrane. Calcium could prevent granular swelling by interacting with the proton ATPase and/or the antiport exchanger. This would prevent translocation and accumulation of osmotically active ions, resulting in the inhibition of granular swelling and renin release. However, there is no precedent in the literature for calcium acting as a modulator of transport proteins. On the other hand, Scott (348) suggests that calcium affects the intragranular equilibrium between aggregated, osmotically inactive granule contents and the dissolved, osmotically active granular contents. Therefore, calcium removal shifts the equilibrium in the direction of the osmotically active components which stimulate granular swelling and renin release.

VI. Summary and Conclusion

Renal cortical slices were used to investigate the effects of the intracellular messengers calcium and cAMP on renin secretion. Renin secretion was shown to be

inversely related to the cytosolic calcium concentration betwen 10^{-8}M and 10^{-5}M calcium. These results confirm the long-standing hypothesis for this inverse relationship. cAMP has been postulated to mediate beta-adrenergic stimulation of renin secretion. cAMP stimulated renin secretion by a fixed amount over a range of cytosolic calcium concentrations, suggesting a calcium-independent pathway. Thus, cAMP agonists produced an unward but parallel shift in the cytosolic calcium-renin secretion curve. These results indicate the possibility that there are two independent pathways controlling renin secretion. Therefore, it is conceivable that two opposing signals may impinge on the JG cell at the same time. The response of the JG cell is probably determined by the relative magnitudes of the two stimuli.

The second area of investigation was the effects of osmotic forces and pH on renin secretion from both renal cortical slices and isolated granules. The results indicate that both systems responded in much the same way to osmotic forces and to pH changes. Moreover they are consistent with a chemiosmotic mechanism for secretion. The concept suggests that renin secretion from cortical slices and isolated granules should be affected by the pH gradient across the granular membrane. The importance of a pH gradient was demonstrated by the stimulatory effects of both alkaline and acid pHs, the inhibitory effect of CCCP at the cellular level, and the inhibitory effects of

oligomycin and DCCD at the cellular and granular levels. In addition, the concept that the granular matrix is acidic is supported by its ability to accumulate weak bases. All these findings are in agreement with a chemiosmotic mechanism of secretion.

Additional studies on the ionic requirement for renin release from isolated granules suggested that chloride, potassium and protons are transported across the granular membrane by an antiport-exchanger-symport complex. The exchanger transports potassium and chloride into the granule as protons are transported in the opposite direction. The importance of such an exchanger becomes evident when the pH of the incubation incubation medium is increased to pH 8 or 9. In order for ion transport to continue, the exchanger must maintain electroneutrality, suggesting that the transport ratio of protons to potassium ions does not equal 1. The transport differential favors accumulation of osmotically active ions that result in granular swelling, lysis, and renin release.

It is therefore concluded that both renin secretion from renal cortical slices and isolated renin granules follow pathways consistent with a chemiosmotic mechanism of secretion. Under such a mechanism granular swelling is driven by an osmotic gradient generated by ion transport. Ion transport occurs by two independent, but possibly connected, pathways: the proton ATPase and the

antiport-exchanger-symport complex.

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